MONASH University

Defining Novel Targets and Biological Interventions in Human and Experimental Glomerulonephritis

A thesis submitted to Monash University in total fulfilment of the requirements for the degree of *Doctor of Philosophy by*

Kim Maree O'Sullivan

BSc (Anatomy and Structural Biology)

MSc (Anatomy and Structural Biology)

Centre for Inflammatory Diseases

Department of Medicine

Monash University

Monash Medical Centre

Melbourne, Australia

November 2017

CONTENTS

COPYRIGHT NOTICE

ABBREVIATIONS

αSMA	Alpha smooth muscle actin
AAV	ANCA associated vasculitis
ANCA	Anti neutrophil cytoplasmic associated vasculitis
APCs	Antigen presenting Cells
BM	Bone Marrow
cGAS	Cyclic GMP-AMP synthase
COPD	Chronic obstructive pulmonary disease
CSS	Churg Strauss syndrome
CXCL1	Chemokine ligand one
CXCL2	Chemokine ligand two
CYP	Cyclophosphamide
DAPI	4',6-diamidino-2-phenylindole
DCs	Dendritic Cells
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DSCG	Disodium chromoglycate
DTH	Delayed type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
EAV	Experimental autoimmune vasculitis
eGFR	Estimated glomerular filtration rate

EGPA	Eosinophilic Granulamotisis with Polyangiitis
ELISA	Enzyme Linked Immune Absorbent Assay
ERK	Extracellular Signal-Regulated Kinases
ESRD	End Stage Renal Disease
ETs	Extracellular Traps
EUVAS	European Vasculitis Study Group
FCA	Freunds Complete Adjuvant
FCγRIIa	Fragment Crystalline Gamma Riia Receptor
FIA	Freunds Incomplete Adjuvant
FimH	Type 1 Fimbrial Adhesion
FSGS	Focal Segmental Glomerulosclerosis
GBM	Glomerular Basement Membrane
G-CSF	Granulocyte Colony Stimulating Factor
GN	Glomerulonephritis
GPA	Granulamotisis With Polyangiitis
H202	Hydrogen Peroxide
HgCl2	Mercury Chloride
HSCs	Hematopoietic Stem Cells
ICAM-1	Intercellular Adhesion Molecule one
ICAM-2	Intercellular Adhesion Molecule two
IFNγ	Interferon Gamma

IgG	Immunoglobulin G
IL-10	Interleukin 10
IL-12	Interleukin 12
Il-17	Interleukin 17
IL-1β	Interleukin Beta one
IL-6	Interleukin six
LAMP-2	Lysosome-Associated Membrane Protein Two
LDGs	Low Density Granulocytes
LN	Lupus Nephritis
LPS	Lipopolysaccharide
MCs	Mast Cells
mDCs	Myeloid Dendritic Cells
METs	Macrophage Extracellular Traps
MHC	Major Histocompatibility Complex
MMP9	Matrix Metalloproteinase 9
MPA	Microscopic Polyangiitis
MPGN	Membranous Glomerulonephritis
MPO	Myeloperoxidase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCGN	Necrotising Crescentic Glomerulonephritis
NE	Neutrophil Elastase

NETs	Neutrophil Extracellular Traps
NOD	Non-Obese Diabetic
NTN	Nephrotoxic Nephritis
PAD4	Protein Arginine Deiminase 4
PAMPs	Pathogen Associated Molecular Patterns
PMA	Phorbol 12-Myristate 13-Acetate,
PR3	Proteinases 3
PRRS	Pattern Recognition Receptors
RAG2-/-	Recombination Activation Gene 2
RAVE	Rituximab versus Cyclophosphamide for ANCA-Associated Vasculitis
RIPK3	Receptor-interacting serine/threonine-protein kinase 3
ROS	Reactive oxygen species
RPGN	Rapidly Progressive Glomerulonephritis
SCID	Severe Combined Immunodeficiency
SLE	Systemic Lupus Erythematosus
STING	Stimulator of interferon genes
SVV	Small Vessel Vasculitis
TCR	T cell receptor
TGF-β	Tissue Growth Factor Beta
TLR	Toll-like receptors
TNFα	Tumor Necrosis Factor Alpha

TREX1	Three Primer repair exonuclease
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling
UUO	Unilateral Ureteric Obstruction
WKY	Wistar Kyoto

ABSTRACT

Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV), has an unknown aetiology, with a multifactorial pathogenesis. Investigating the underlying pathogenic mechanism is essential for development of effective treatment strategies. Current treatment does not address the primary pathogenesis of AAV, is toxic and contributes to morbidity and mortality rates of AAV. The overall objective of this study was to contribute to the understanding of the pathogenesis of AAV, identify new potential therapeutic targets and assess the benefits of two novel therapeutic strategies in experimental animal models of AAV.

In the second chapter of this thesis, I analyse the extent and nature of myeloperoxidase (MPO) deposition within the glomeruli and the tubulointerstitium of 47 MPO-AAV patient renal biopsies, and sought evidence of the likely cellular source of MPO using confocal microscopy. MPO deposition correlated with glomeruli that had neutrophil extracellular traps (NETs), and infiltration of MPO + Neutrophils and macrophages. This chapter shows that both intracellular and extracellular MPO may contribute to glomerular injury and perpetuation of autoimmunity in MPO-AAV patients.

In chapter three, I examine 44 biopsies of MPO-AAV patients for Mast cells (MCs) and find evidence of an activated mast cell phenotype. Using a model of experimental murine MPO-ANCA glomerulonephritis I demonstrate that administration of a mast cell specific stabilizer, disodium chromoglycate (DSCG), before and after establishment of autoimmunity to MPO stabilises MC degranulation, and attenuates development of GN, mediated by the production of MC derived IL-10. These results suggest that DSCG could be of potential therapeutic benefit in MPO-ANCA GN.

In chapter four, I examine the role of Toll-like Receptors in 38 renal biopsies of AAV patients. This study evaluated aberrant expression of TLR2, TLR4 and TLR9 to test the hypothesis that increased TLR expression would correlate with renal injury. AAV

renal biopsies had significant renal disposition of TLR2, TLR4 and TLR9 compared to control patients. Elevated TLR4 and TLR2 expression correlated with structural injury (histological) and functional injury (eGFR). These observations suggest that TLR4 and TLR2 may be potential therapeutic targets in AAV.

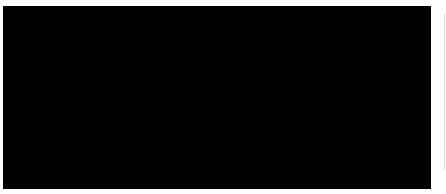
In Chapter five, I test the hypothesis that administration of exogenous DNase I in pharmacological doses will eliminate extracellular DNA released from injured and dying cells (including neutrophils undergoing NETosis) in an experimental model of MPO-ANCA GN. In this model of MPO-ANCA GN autoimmunity to MPO is induced driving the development of focal segmental proliferative GN. I found significant extracellular deposition of DNA in the kidneys of these mice. Administration of DNase I reduced the deposition of DNA and cleared renal DNA when administered to mice with established disease. This was associated with reduced glomerular NETs and MPO deposition, structural injury (segmental glomerulosclerosis) and surprisingly reduced autoimmunity to MPO. DNase I treatment, reduced DC migration to the draining lymph nodes, and increased numbers of CD4 regulatory T cells. These results strongly support a key role for extracellular DNA in this disease by augmentation of anti MPO autoimmunity and pro-inflammatory effects on effector responses in the kidney.

In conclusion, this thesis examines the contribution of glomerular deposition of extracellular MPO, extracellular DNA, effector cells and TLR expression in the pathogenesis of MPO-ANCA GN. These experiments, identified potential therapeutic targets, and explored the use of two targeted therapeutic interventions that may facilitate the development of new treatment strategies for MPO-ANCA GN with decreased toxic side effects.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature :



Print Name: Kim Maree O'Sullivan

Date: 29/11/2017

PUBLICATIONS ARISEN FROM THIS THESIS

1. **O'Sullivan KM**, Lo CY, Summers SA, Elgass KD, McMillan PJ, Longano A, Ford SL, Gan PY, Kerr PG, Kitching AR, Holdsworth SR. Renal participation of myeloperoxidase in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. Kidney Int. 2015 Nov;88(5):1030-46.
2. Gan PY, **O'Sullivan KM***, Ooi JD, Alikhan MA, Odobasic D, Summers SA, Kitching AR, Holdsworth SR. Mast Cell Stabilization Ameliorates Autoimmune Anti-Myeloperoxidase Glomerulonephritis. J Am Soc Nephrol. 2016 May;27(5):1321-33. ***Joint first Author**
3. **O'Sullivan KM***, Ford, SL, Kitching AR, Holdsworth SR.
   Intrarenal Toll-Like Receptor 2 and Toll-Like Receptor 4 Expression correlates with Injury in anti-Neutrophil Cytoplasmic Antibody Vasculitis
   Submtted to Kidney International
4. **O'Sullivan KM***, Dick, J. Alikhan, MA., Kitching AR, Gan, PY, Holdsworth SR.
   Therapeutic use of Deoxyribonuclese I modulates autoimmunity to myeloperoxidase and attenuated glomerular injury in experimental anti- Myeloperoxidase glomerulonephritis
   Submitted as a chapter

OTHER PUBLICATIONS CANDIDATE CONTRIBUTED TO DURING CANDIDATURE NOT INCLUDED IN THESIS

1. King PT, Sharma R, **O'Sullivan KM**, Callaghan J, Dousha L, Thomas B, Ruwanpura S, Lim S, Farmer MW, Jennings BR, Finsterbusch M, Brooks G, Selemidis S, Anderson GP, Holdsworth SR, Bardin PG. Deoxyribonuclease 1 reduces pathogenic effects of cigarette smoke exposure in the lung. Sci Rep. 2017 Sep 21;7(1):12128. doi: 10.1038/s41598-017-12474-5.
2. Gan PY, Fujita T, OOi J., Alikhan MA, Dick J, Shim R, Odobasic D**, O'Sullivan KM,** Kitching AR, Holdsworth SR, Pathogenic role for γδ T cells in Autoimmune Anti-Myeloperoxidase Glomerulonephritis, 2017 In press, Journal of Immunology

3. Sharma R, **O'Sullivan KM,** Holdsworth SR, Bardin PG, King PT, Visualising Macrophage Extracellular Traps Using Confocal Microscopy, 2017 *J. Vis. Exp.* (128), e56459, doi:10.3791/56459 (2017)
4. Ghali JR, **O'Sullivan KM**, Eggenhuizen PJ, Holdsworth SR, Kitching AR. Interleukin-17RA Promotes Humoral Responses and Glomerular Injury in Experimental Rapidly Progressive Glomerulonephritis.Nephron. 2017;135(3):207-223.doi:10.1159/000453059. Epub 2016 Dec 16
5. Westhorpe CL, Bayard JE,**O'Sullivan KM**, Hall P, Cheng Q, Kitching AR, Hickey MJ. In Vivo Imaging of Inflamed Glomeruli Reveals Dynamics of Neutrophil Extracellular Trap Formation in Glomerular Capillaries.Am J Pathol. 2017 Feb;187(2):318-331. doi: 10.1016/j.ajpath.2016.10.008. Epub 2016 Dec 18***Joint first Author**
6. Chang J, Eggenhuizen P, **O'Sullivan KM**, Alikhan MA, Holdsworth SR, Ooi JD, Kitching AR. CD8+ T Cells Effect Glomerular Injury in Experimental Anti-Myeloperoxidase GN. J Am Soc Nephrol. 2016 Jun 10. pii: ASN.2015121356. [Epub ahead of print]
7. Hamour S, Gan PY, Pepper R, Florez Barros F, Wang HH, **O'Sullivan K**, Iwakura Y, Cook T, Pusey C, Holdsworth S, Salama A. Local IL-17 Production Exerts a Protective Role in Murine Experimental Glomerulonephritis. PLoS One. 2015 Aug 28;10(8):e0136238
8. Ghali JR, **O'Sullivan KM**, Eggenhuizen PJ, Holdsworth SR, Kitching AR. FMS-like tyrosine kinase 3 ligand treatment does not ameliorate experimental rapidly progressive glomerulonephritis. PLoS One. 2015 Apr 7;10(4):e0123118..
9. King PT, Sharma R, **O'Sullivan K**, Selemidis S, Lim S, Radhakrishna N, Lo C, Prasad J, Callaghan J, McLaughlin P, Farmer M, Steinfort D, Jennings B, Ngui J, Broughton BR, Thomas B, Essilfie AT, Hickey M, Holmes PW, Hansbro P, Bardin PG, Holdsworth SR. Nontypeable Haemophilus influenzae induces sustained lung oxidative stress and protease expression. PLoS One. 2015 Mar 20;10(3):e0120371.

10. Odobasic D, Muljadi RC, **O'Sullivan KM**, Kettle AJ, Dickerhof N, Summers SA, Kitching AR, Holdsworth SR. Suppression of Autoimmunity and Renal Disease in Pristane-Induced Lupus by Myeloperoxidase. Arthritis Rheumatol. 2015 Jul;67(7):1868-80
11. Odobasic D, Yang Y, Muljadi RC, **O'Sullivan KM**, Kao W, Smith M, Morand EF, Holdsworth SR. Endogenous myeloperoxidase is a mediator of joint inflammation and damage in experimental arthritis. Arthritis Rheumatol. 2014 Apr;66(4):907-17
12. Odobasic D, Ghali JR, **O'Sullivan KM**, Holdsworth SR, Kitching AR Glomerulonephritis Induced by Heterologous Anti-GBM Globulin as a Planted Foreign Antigen. Curr Protoc Immunol. 2014 Aug 1;106:15.26.1-20. doi: 10.1002/0471142735.im1526s106. Review.
13. Odobasic D, Kitching AR, Yang Y, **O'Sullivan KM**, Muljadi RC, Edgtton KL, Tan DS, Summers SA, Morand EF, Holdsworth SR. Neutrophil myeloperoxidase regulates T-cell-driven tissue inflammation in mice by inhibiting dendritic cell function. Blood. 2013 May 16;121(20):4195-204
14. Ooi JD, Chang J, **O'Sullivan KM**, Pedchenko V, Hudson BG, Vandenbark AA, Fugger L, Holdsworth SR, Kitching AR. The HLA-DRB1*15:01-restricted Goodpasture's T cell epitope induces GN. J Am Soc Nephrol. 2013 Feb;24(3):419-31
15. Tan DS, Gan PY, **O'Sullivan KM**, Hammett MV, Summers SA, Ooi JD, Lundgren BA, Boyd RL, Scott HS, Kitching AR, Chidgey AP, Holdsworth SR. Thymic deletion and regulatory T cells prevent antimyeloperoxidase GN. J Am Soc Nephrol. 2013 Mar;24(4):573-85.
16. Gan PY, Summers SA, Ooi JD, **O'Sullivan KM**, Tan DS, Muljadi RC, Odobasic D, Kitching AR, Holdsworth SR. Mast cells contribute to peripheral tolerance and attenuate autoimmune vasculitis. J Am Soc Nephrol. 2012 Dec;23(12):1955-66.
17. Summers SA, Gan PY, Dewage L, Ma FT, Ooi JD, **O'Sullivan KM**, Nikolic-Paterson DJ, Kitching AR, Holdsworth SR. Mast cell activation and degranulation promotes renal fibrosis in experimental unilateral ureteric obstruction.Kidney Int. 2012 Sep;82(6):676-85
18. Ooi JD, Snelgrove SL, Engel DR, Hochheiser K, Ludwig-Portugall I, Nozaki Y, **O'Sullivan KM**, Hickey MJ, Holdsworth SR, Kurts C, Kitching AR. Kidney Int. 2011 May;79(9):977-86

19. Summers SA, Steinmetz OM, Ooi JD, Gan PY, **O'Sullivan KM**, Visvanathan K, Akira S, Kitching AR, Holdsworth SR. Toll-like receptor 9 enhances nephritogenic immunity and glomerular leukocyte recruitment, exacerbating experimental crescentic glomerulonephritis. Am J Pathol. 2010 Nov;177(5):2234-44.
20. Summers SA, van der Veen BS, **O'Sullivan KM**, Gan PY, Ooi JD, Heeringa P, Satchell SC, Mathieson PW, Saleem MA, Visvanathan K, Holdsworth SR, Kitching AR. Intrinsic renal cell and leukocyte-derived TLR4 aggravate experimental anti-MPO glomerulonephritis. Kidney Int. 2010 Dec;78(12):1263-74
21. Summers SA, Hoi A, Steinmetz OM, **O'Sullivan KM**, Ooi JD, Odobasic D, Akira S, Kitching AR, Holdsworth SR. TLR9 and TLR4 are required for the development of autoimmunity and lupus nephritis in pristane nephropathy.J Autoimmun. 2010 Dec;35(4):291-8.
22. Gan PY, Steinmetz OM, Tan DS, **O'Sullivan KM**, Ooi JD, Iwakura Y, Kitching AR, Holdsworth SR. Th17 cells promote autoimmune anti-myeloperoxidase glomerulonephritis. J Am Soc Nephrol. 2010 Jun;21(6):925-31
23. Jones LK, **O'Sullivan KM**, Semple T, Kuligowski MP, Fukami K, Ma FY, Nikolic-Paterson DJ, Holdsworth SR, Kitching AR. Il-1R1 deficiency ameliorated early experimental renal interstitial fibrosis Nephrol Dial Transplant. 2009 Oct;24(10):3024-32. doi: 10.1093/ndt/gfp214. Epub 2009 May 22
24. Li M, **O'Sullivan KM**, Jones LK, Lo C, Semple T, Kumanogoh A, Kikutani H, Holdsworth SR, Kitching R. Endogenous CD100 promotes glomerular injury and macrophage recruitment in experimental crescentic glomerulonephritis. Immunology. 2009 Sep;128(1):114-22.

ABSTRACTS/CONFERENCE PRESENTATIONS DURING CANDIDATURE

1. Oral presentation, 53rd Annual Scientific Meeting, Australian and New Zealand Society of Nephrology, Darwin, Northern Territory, Australia 2017

K.M. O'Sullivan, S.L. Ford, A.R. Kitching, S.R. Holdsworth.

Renal expression of toll-like receptors in patients with crescentic glomerulonephritis

2. Oral presentation, 18th ANCA Vasculitis workshop, Tokyo, Japan 2017

K.M. O'Sullivan, P.Y. Gan, A.R. Kitching and S.R. Holdsworth.

Deoxyribonuclease I reduces glomerular injury and modulated anti- Myeloperoxidase autoimmunity in experimental anti Myeloperoxidase glomerulonephritis

3. Poster presentation, 18th ANCA Vasculitis workshop, Tokyo, Japan 2017

K.M. O'Sullivan, S.F. Ford, A.R. Kitching and S.R. Holdsworth.

Toll-Like Receptor 2, 4 and 9 are associated with histological injury in kidneys from patients with anti neutrophil cytoplasmic antibody vasculitis

4. Oral presentation, Translational Medicine: Innovations in Renal Research, Blenheim, New Zealand, 2016

K.M. O'Sullivan, A.R. Kitching and S.R. Holdsworth.

Toll-like receptor 4, is predominantly expressed over TLR2 and TLR9 in kidneys of patients with anti-neutrophil cytoplasmic antibody vasculitis (AAV)

5. Poster presentation, American Society of Nephrology, Chicago, Illinois, USA 2016

K.M. O'Sullivan, A.R. Kitching and S.R. Holdsworth.

Toll-like receptor 4, is predominantly expressed over TLR2 and TLR9 in kidneys of patients with anti-neutrophil cytoplasmic antibody vasculitis (AAV)

6. Poster presentation, American Society of Nephrology, Chicago, Illinois, USA 2016

K.M. O'Sullivan, P.Y. Gan, A.R. Kitching and S.R. Holdsworth.

Deoxyribonuclease I treatment attenuate neutrophil extracellular trap formation, leukocyte infiltration and inflammation in experimental anti myeloperoxidase glomerulonephritis

7. Oral presentation, Award session Young Investigators for Basic Science, at the Australian New Zealand Society of Nephrology, Perth, WA, Australia 2016

K.M. O'Sullivan, P.Y. Gan, A.R. Kitching and S.R. Holdsworth.

DNase I limits Net Formation and inflammation in murine experimental anti MPO-ANCA glomerulonephritis

8. Oral presentation at the International Congress of Immunology, Melbourne, Australia 2016

K.M. O'Sullivan, P.Y. Gan, A.R. Kitching and S.R. Holdsworth.

DNase I is protective in murine experimental anti- neutrophil cytoplasmic antibody associated glomerulonephritis

9. Poster presentation, Australian Society of Immunology, Canberra, Australia 2015

K.M. O'Sullivan, A.R. Kitching and S.R. Holdsworth.

Toll-like receptor 2, 4 and 9 expression is enhanced in kidneys of patients with anti-neutrophil cytoplasmic antibody vasculitis (AAV)

10. Oral presentation at the American Society of Nephrology Annual meeting, Philadelphia, USA, 2014.

K.M. O'Sullivan, C. Lo, S.A. Summers, P.Y. Gan, S. L. Ford, A.R. Kitching and S.R. Holdsworth.

Neutrophil and Macrophage extracellular traps are prominent in human anti-neutrophil cytoplasmic antibody associated vasculitis

11. Oral Presentation at the Australian and New Zealand Society of Nephrology annual meeting, Melbourne, Australia, 2014.

K.M. O'Sullivan, P.Y. Gan, J.D. Ooi, M. Khouri,, S.A. Summers, A.R Kitching, S.R Holdsworth. Mast cell stabilizers limit pathogenic mast cell degranulation in MPO-ANCA associated glomerulonephritis

12. Oral Presentation at the International Congress of Immunology. Milan Italy,2013

K.M. O'Sullivan, S.L. Ford, C. Lo, S.A. Summers, A.R. Kitching, S.R. Holdsworth.

Glomerular deposition of myeloperoxidase and NETS in human ANCA associated vasculitis correlates with DTH effector cell accumulation.

GENERAL DECLARATION

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy and Research Master's regulations the following declarations are made:I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes original papers published in peer reviewed journals, 1 submitted manuscript and traditional thesis chapters. The core theme of the thesis is MPO-ANCA GN. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Centre for Inflammatory Disease in the Department of Medicine at the Monash Medical Centre under the supervision of Professor Stephen R. Holdsworth, and Professor A. Richard Kitching.
The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.
In the case of chapters 2, 3, 4, 5, and the appendix my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	**Renal participation of myeloperoxidase in anti- neutrophil cytoplasmic antibody (ANCA) associated vasculitis**	Published	Experimental work and design, data analysis and presentation, writing (75%)
3	**Mast Cell stabilization ameliorates autoimmune anti- myeloperoxidase glomerulonephritis**	Chapter	Experimental work and design, data analysis and presentation, writing (45%)
4	**Intrarenal Toll-Like Receptor 2 and Toll-Like Receptor 4 Expression correlates with Injury in anti-Neutrophil Cytoplasmic Antibody Vasculitis**	Submitted	Experimental work and design, data analysis and presentation, writing (80%)
5	**Deoxyribonuclease I modulates autoimmunity to Myeloperoxidase in experimental anti- Myeloperoxidase glomerulonephritis**	Chapter	Experimental work and design, data analysis and presentation, writing (80%)
Appendix	**In vivo Imaging of Inflamed glomeruli reveals dynamics of neutrophil extracellular trap formation in glomerular capillaries**	Chapter	Experimental work and design, data analysis and presentation (25%)

Signed:

[redacted]

Date:29/11/2017

THESIS INCLUDING PUBLISHED WORKS DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes (*1)* original papers published in peer reviewed journals and (*1)* submitted publications. The core theme of the thesis is *MPO-ANCA GN* .The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Medicine under the supervision of *Prof. Stephen Holdsworth* and *Prof. Richard Kitching.* (The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.) In the case of *chapters 2 and 4* my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status (published, in press, accepted or returned for revision, submitted	Nature and % of student of contribution	Co-author name(s) and & of co-authors contribution	Co author(s) Monash Student Y/N
2	***Renal participation of myeloperoxidase in anti- neutrophil cytoplasmic antibody (ANCA) associated vasculitis***	*Published, Kidney International*	*75%* ***First author*** *Experimental design Experimental work, analysis of data and preparation of manuscript*	**Camden Y. Lo** *3% designed macro for measuring extracellular MPO, reviewed manuscript* **Shaun SA Summers** *1% assisted with clinical data* **Kirstin D Elgass** *1% provided imaging assistance* **Paul J. McMillan** *1% assisted in super resolution imaging, reviewed manuscript* **Anthony Longano** *5% (pathologist) scored kidney biopsies for Berden classification and renal injury* **Sharon L. Ford** *2 % constructed human clinical data base* **Poh-Yi Gan** *1% assisted in data analysis and reviewed manuscript* **Peter G. Kerr** *1% renal biopsy procurement and reviewed manuscript* **A.Richard Kitching** *5% Experimental design, review of manuscript* **Stephen R. Holdsworth** *5% Experimental design, review of manuscript*	N N N N N Y N N N N

Thesis Chapter	Publication Title	Status (published, in press, accepted or returned for revision, submitted	Nature and % of student of contribution	Co-author name(s) and & of co-authors contribution	Co author(s) Monash Student Y/N
4	***Intrarenal Toll-Like Receptor 2 and Toll-Like Receptor 4 Expression correlates with Injury in anti-Neutrophil Cytoplasmic Antibody Vasculitis***	*Submitted to Kidney International*	*80%* ***First Author*** *experimental design, Experimental work, analysis of data and preparation of manuscript*	***Sharon L. Ford 1%*** *Construction clinical data base, review of manuscript* ***Anthony Longano 5%*** *Pathologist who scored kidney biopsies for renal injury* ***Richard Kitching 7%*** *Experimental design, review and preparation of manuscript* ***Stephen R. Holdsworth 7%*** *Experimental design, review and preparation of manuscript*	Y N N N

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] Date: 29/11/2017

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] **Date:29/11/2017**

ACKNOWLEDGEMENTS

Foremost I would like to thank my main supervisor Professor Stephen Holdsworth for his encouragement, guidance and support throughout my candidature. I am indebted to you for sharing your passion for research with me. You are truly an inspiring mentor, I am thankful for the patience and understanding you demonstrated whilst I juggled work, a PhD and motherhood. I would also like to acknowledge my co-supervisor Professor Richard Kitching for his meticulous attention to detail. You have taught me to critically appraise my work and how to strive for excellence. I am grateful you allowed me the opportunity to do a PhD as a staff member of your laboratory, and for the support and flexibility you gave me for family commitments.

I am indebted to our Research Assistants past and present for maintaining the laboratory, the work you do does not go unnoticed, in particular I would like to thank:Tim Semple, Alice Wright, Cecilia Lo, and more recently Peter Eggenhuizen, and Virginie Oudin. The camaraderie I share with you all will be treasured for years to come. I would like to acknowledge the support of Dr. Poh-Yi Gan, for guiding me through experimental models of glomerulonephritis and maintaining my sanity, you went beyond the call of duty, and I am truly grateful for your friendship.

I would like to thank Dr. Camden Lo and Dr. Kirstin Elgass, from Monash Micro Imaging for teaching me the finer details of confocal microscopy and image analysis, none of this work would have been possible without your tutorage. I am grateful to Dr. Shaun Summers and Dr. Sharon Ford, for assistance with the clinical data from the patients involved in my work, their support and friendship-I miss our days in the lab together. I am thankful for the moral support and guidance from my colleagues Dr.Sarah Snelgrove, Dr. Belinda Thomas, Dr.Joshua Ooi, Dr. Maliha Alikhan, Dr. Jonathon Dick, Dr.Dragana Odobasic, Cecilia Lo and Megan Huynh.

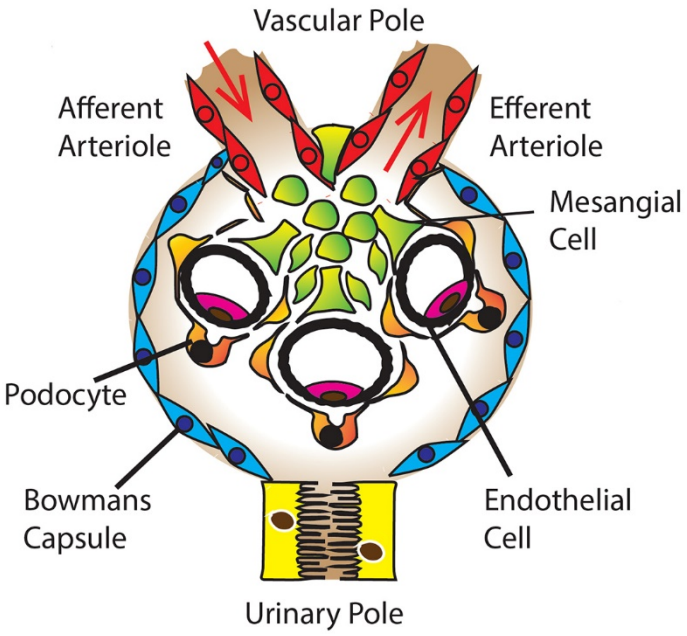
I thank my parents who taught me the value of hard work, perseverance and integrity. You have supported all my endeavours. I would like to acknowledge my parents-in-law Margaret and John, whose unwavering friendship and support of my career made a PhD possible. I will be forever in your debt for the assistance you provided in caring for my children whilst I performed weekend experiments, attended international conferences, and whilst I wrote my Doctoral thesis.

Lastly I dedicate this thesis to my partner Wayne and children Rory, Amelie and Wolf. I thank you for the unconditional acceptance of my absence during chaotic times of my candidature, and allowing me to have the best of two worlds. You are the source of all the greatest moments life has given me.

This research was supported by an Australian Government Research Training Program (RTP) Scholarship.

CHAPTER ONE

The pathogenesis of MPO-ANCA Glomerulonephritis



IMMUNE MEDIATED KIDNEY DISEASE

The anatomical structure of the kidney and function as a filtering unit renders the kidney a vulnerable target of immune mediated disorders, and subject to inflammation as a secondary mechanism. Immune mediated injury which effects the glomerulus in the first instance can lead to many forms of glomerulonephritis. To fully understand the pathogenesis of glomerulonephritis it is essential to understand, basic kidney anatomy and physiology, the different classifications of glomerulonephritis, the pathogenesis of glomerulonephritis, the generation of autoimmunity to the autoantigens, and experimental animal models that allow us to study certain aspects of the pathogenesis of glomerulonephritis.

1.1 Basic kidney anatomy and physiology

A nephron is composed of a glomerulus (capillaries bound by Bowmans Capsule) and a tubule, which acts as the structural and functional filtering unit of the kidney. The glomerulus contains the glomerular capillaries which are supplied blood from the afferent arteriole. The constant flow of blood is filtrated into Bowmans space (the space between Bowmans capsule and the glomerular tuft), where is it exported into the proximal tubule as primary urine. In addition to the glomerular capillaries, the glomerulus consists of Bowmans capsule which is a continuous flat layer of epithelial cells that also envelops the glomerular capillaries with highly specialised epithelial cells termed podocytes. The podocytes form a highly specialised filtration barrier in conjunction with pores (fenestrations) of the glomerular endothelial cells, the glomerular basement membrane (GBM). The glomerulus also contains supporting cells known as the mesangium, which fills the space between the capillaries and has a contractile function which aids in controlling the glomerular filtration rate (Kurts et al., 2013; Scott & Quaggin, 2015).

It is the basic structure of the glomerulus that makes it vulnerable to glomerular immune complex deposition. The fenestrations are susceptible to obstructions by their unusual

porosity and the high pressure and flow through the glomeruli. If the glomerular filtration barrier is damaged, this results in elevated amounts of protein leaking into the glomerular filtrate, causing proteinuria. The amount of protein present in the urine is used clinically to indicate poor kidney function (Scott & Quaggin, 2015).

GLOMERULONEPHRITIS

Glomerulonephritis (GN) is not a single disease entity but a collection of diseases. It is now recognised that most forms of GN result from autoimmunity. The most severe forms of GN have cellular proliferation, segmental necrosis and crescent formation. If left untreated these forms of glomerulonephritis will progress to end stage renal disease and eventually death. There is still an incomplete understanding of the pathogenesis of glomerulonephritis, this review will focus on the recent understanding of the pathogenesis of crescentic glomerulonephritis, the different types of glomerulonephritis, and epidemiology.

Glomerulonephritis (GN) can be divided into two distinct classes, the first known as primary GN is glomerulonephritis which is renal limited (localised to the kidney), such as membranous glomerulonephritis (MPGN), focal segmental glomerulosclerosis (FSGS), immunoglobulin A nephropathy (IgAN), minimal change disease (MCD) membranous glomerulonephritis (MGN), and anti glomerular basement membrane (GBM) GN. Secondary GN refers to GN which occurs in the context of a systemic disease. These include Lupus nephritis (LN) and anti-neutrophil cytoplasmic antibody vasculitis (AAV), rapidly progressive glomerulonephritis (RPGN), AA amyloidosis, AL amyloidosis, acute tubulitis nephritis, diabetes, hypertension, and chronic tubulointerstitial nephritis. In addition, secondary glomerulopathy may also be a result of infection, drugs, and genetic diseases (eg Alport syndrome).

Glomerulonephritis may be either non proliferative or proliferative, characterised by the number of cells within the glomeruli. Non-proliferative GN is defined as a lack of hypercellularity, such as minimal change disease (MCD), membranous GN (MGN), and

focal segmental glomerulosclerosis (FSGS). Proliferative GN is characterised by hypercellularity, and includes IgA nephropathy, membranoproliferative GN (MPGN), post infectious GN, and rapidly progressive crescentic glomerulonephritis (Figure 1). For the purposes of this review I will focus on rapidly progressive glomerulonephritis.

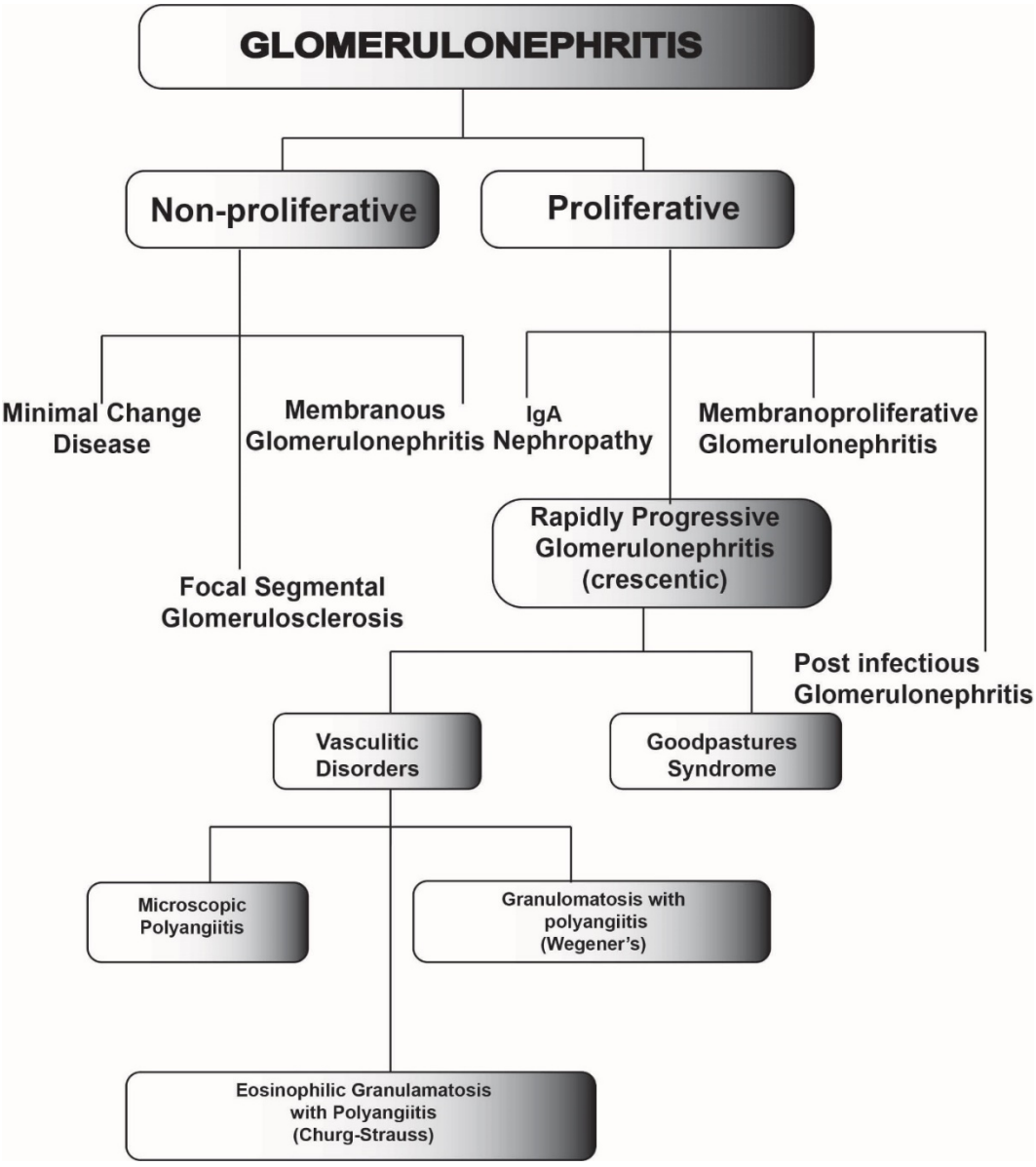


Figure 1. Overview of the types of glomerulonephritis (adapted from Munni, 2016)

1.2 Rapidly progressive glomerulonephritis (RPGN)

Rapidly progressive glomerulonephritis (RPGN) is a clinical syndrome caused by glomerulonephritis. The World Health Organisation defines RPGN as a “rapidly progressive nephritic syndrome as an abrupt or insidious onset of macroscopic haematuria, proteinuria, anaemia and rapidly progressing renal failure”. Patients present with general fatigue, elevated temperature, flu-like symptoms accompanied with weight loss and diminished appetite (Arimura et al., 2016; Greenhall & Salama, 2015). These symptoms occur as a result from systemic inflammation and loss of kidney function. Extra renal symptoms are often present if they have secondary GN. These extra renal features are often characteristic and particular to their specific disease.

In many cases of RPGN standard immunosuppressive treatment is not sufficient (combination therapy of high dose corticosteroids and cytotoxic drugs), and is accompanied with undesirable side effects and a relatively poor prognosis. Teasing out the underlying pathogenic mechanism of RPGN is essential for the development of effective treatment strategies. There is an unmet requirement for therapeutic agents with minimal side effects that improve the quality of life for patients. This review of the literature aims to highlight what is currently known about the pathogenesis of RPGN, where the gaps in our knowledge are and the future direction for targeted treatment strategies.

Based on immunopathologic findings RPGN presents as crescentic glomerulonephritis. Crescentic glomerulonephritis occurs when a pathologic incident causes rupture of the glomerular capillaries allowing trafficking of humoral and cellular inflammatory mediators into Bowmans space. This results in the proliferation of outer epithelial layer of Bowmans capsule forming a cellular crescent (Figure 2, Basic steps of crescentic glomerulonephritis). Glomerular crescent formation is a multi-step process, which may differ in cellular composition dependent on whether Bowman Capsule ruptures during the process, in different diseases. In early crescent formation, the epithelial cells of Bowmans capsule transform from an elongated flat epithelial shape to a cuboidal shape. These cuboidal

shaped epithelial cells, by a mechanism that is still not clear, arrange themselves into a multi-layered structure which fills Bowmans space. In some instances, the crescents contain, both macrophages and fibroblasts (macrophage infiltration increases if Bowmans capsule is ruptured), fibrin and other proteinaceous material As the crescent matures, the epithelial cells flatten, separated by collagen, which eventually replaces the cellular crescents, forming a fibrous crescent (Morita et al., 1973). Podocytes were originally thought not to play a role in crescent formation, but a recent study has demonstrated via electron microscopy, podocytes forming bridges with epithelial cells of Bowmans capsule (Le Hir et al., 2001).

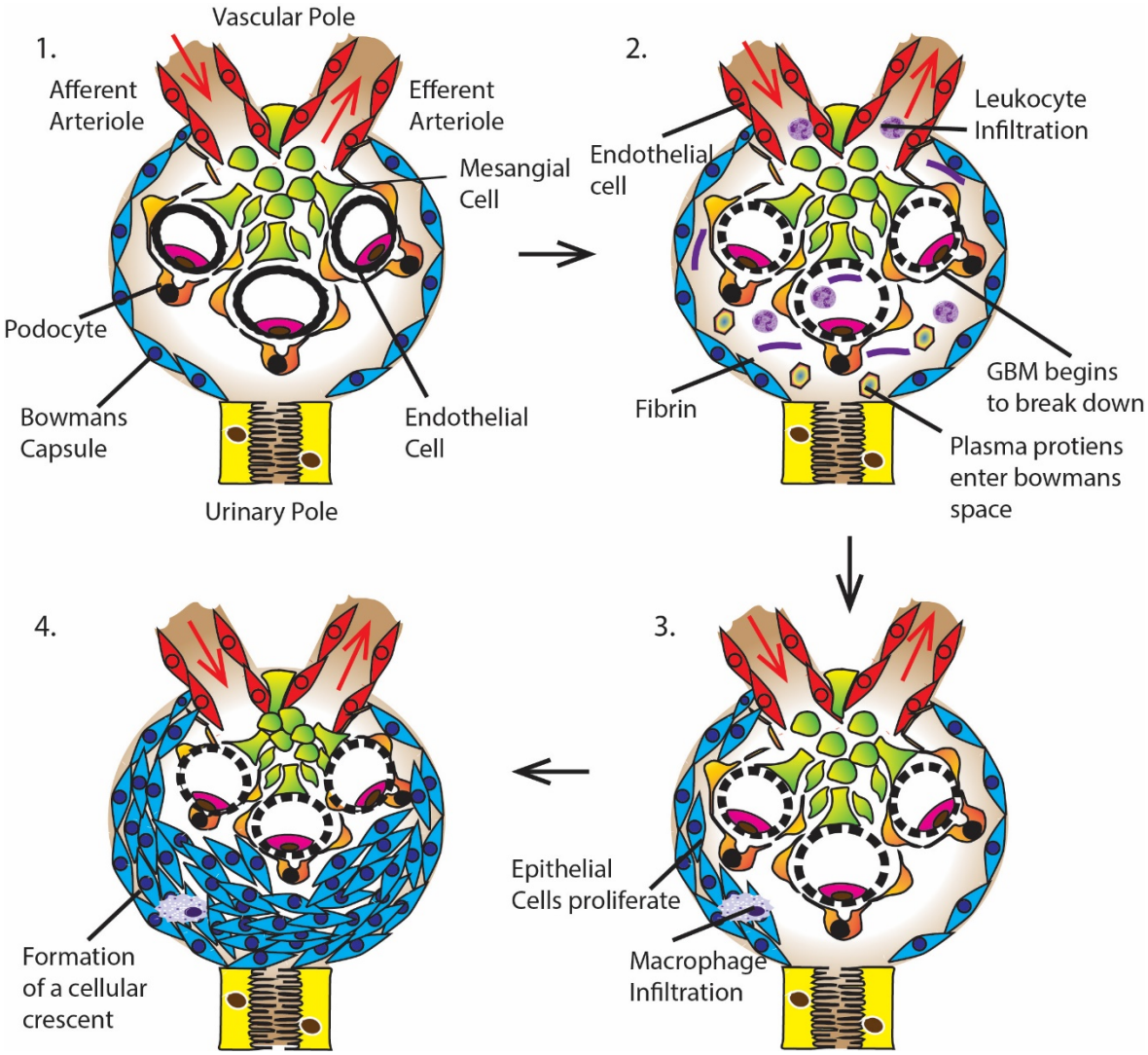


Figure 2. Basic steps of Crescent Formation

(**1**) Normal glomerulus, consisting of Bowmans Capsule, Podocytes (orange), and glomerular basement membrane (GBM), mesangial cells (green) (**2**) Gaps appear in the GBM, and plasma proteins leak into Bowman space, fibrin accumulated, and leukocytes traffic to the glomerulus. (**3**) The epithelial cells of Bowman capsule begin to proliferate, macrophages infiltrate the crescent, (**4**) Cellular crescent is formed, consisting mainly of epithelial cells which full Bowmans space infiltration increases if Bowmans capsule is ruptured), fibrin and other proteinaceous material (Jennette & Hipp, 1986). As the crescent matures, the epithelial cells flatten and take on a fibroblast phenotype, separated by collagen, which eventually replaces the cellular crescents, forming a fibrous crescent (Morita et al., 1973). Podocytes were originally thought not to play a role in crescent formation, but a recent study has demonstrated via electron microscopy, podocytes forming bridges with epithelial cells in Bowmans capsule (Le Hir et al., 2001).

1.3 Classification of Rapidly Progressive Crescentic Glomerulonephritis and Small Vessel Vasculitis

As mentioned in the previous section, the exact initiating mechanism for the rupture of the glomerular capillaries and the resulting cellular crescent formation is unknown. Crescent formation is indicative of severe glomerular injury, and correlates with rapid loss of function. Therefore a rapid diagnosis of crescentic glomerulonephritis is essential for treatment. To date there are two descriptors to aid in both the nomenclature and classification of glomerulonephritis: there is the nomenclature of Small Vessel Vasculitis from the Chapel Hill consensus (2012) and the broad histological classification of crescentic glomerulonephritis, the next section will provide an overview of both these categories (Jennette et al., 2013). Because crescent formation proceeds rapid progression of glomerulonephritis and poor outcome this pattern of disease occurring in renal diseases of quite diverse etiology is often classified as a single entity. Inclusion in the crescentic glomerulonephritis group has no generally agreed pathological definition it generally assumes that the disease has dominant glomerular crescents.

To facilitate accurate diagnosis Crescentic Glomerulonephritis can be divided further into 3 broad categories (Anders, 2000; Jennette, 2003; Jennette & Thomas, 2001; Kitching et al., 2008), characterized on the appearance of the pattern of antibody deposited within the renal biopsy, these three distinct patterns are defined as (see Table 1.):

Type I. Anti-Glomerular Basement Membrane (anti-GBM) glomerulonephritis (20% cases)

Type II. Immune Complex glomerulonephritis (40% of cases)

Type III. Pauci-Immune Glomerulonephritis (40% of Cases)

1.3.1 Type I. Anti-Glomerular Basement Membrane glomerulonephritis

Type I anti-glomerular basement membrane (anti-GBM glomerulonephritis), is also commonly known as Goodpastures Disease. It is characterised immunopathologically via biopsy as having a linear pattern of antibody (IgG) and C3 delineating the glomerular capillaries via immunofluorescence, and circulating anti-GBM autoantibodies. Clinically anti -GBM disease manifests as a rapid onset of RPGN which regularly progresses to end stage renal failure despite treatment (Jennette, 2003). Anti-GBM GN has the highest percentage of crescents at the time of biopsy of all 3 categories. Over 95% of all patients will have crescents in over 50% or more of their glomeruli, and consequently the poorest outcome. Unlike many other autoimmune disease where the auto antigenic target is unknown, the non-collagenous domain of the α3 chain of type IV collagen in anti GBM disease has been identified as the antigenic target (Kalluri et al., 1995).

1.3.2 Type II. Immune complex glomerulonephritis

Type II Immune complex glomerulonephritis is characterised by granular deposits of immune complexes in either the sub endothelial spaces or mesangial matrix. Immune complex glomerulonephritis can arise from several different disease manifestations, but notably include lupus nephritis, IgA nephropathy, cryoglobulinemia and post infectious glomerulonephritis (Jennette, 2003; Kitching et al., 2008). It has been reported that immune complex glomerulonephritis has a lower incidence of crescentic glomerulonephritis than either anti GBM GN or ANCA GN (Jennette, 2003). The 2 major forms of Type II crescentic GN are found in systemic erythematosus (SLE) and IgA disease.

1.3.3 Type III. Pauci-Immune Glomerulonephritis

This category is defined by the paucity of glomerular immunoglobulin and complement which is prominent in Categories I and II. It is now recognised that these patients have anti neutrophil cytoplasmic antibody (ANCA) so are now termed ANCA associated vasculitis (AAV) patients. Although termed “pauci immune” various studies have demonstrated that immune complex deposits are actually present in over fifty percent of patients (Sumida et

al., 2012). AAV develops in response to a loss of tolerance to the neutrophils azurophilic lysosomes myeloperoxidase (MPO), proteinase 3 (PR3) and the subsequent generation of ANCA antibody. Clinically, ANCA testing is used to confirm the antigen specificity for (MPO or PR3) via enzyme-linked immunosorbent assay (ELISA) or indirect immunofluorescence on ethanol fixed neutrophils for P-ANCA (perinuclear staining, associated with MPO-ANCA) or C-ANCA (cytoplasmic staining associated with PR3, and neutrophil granule contents). More recently a new ANCA subtype has been described, lysosomal-associated membrane protein 2 (LAMP-2) has been identified as an additional ANCA target. LAMP-2 autoantibodies active neutrophils *in vitro*, and are prevalent in patients with active pauci-immune focal necrotizing GN (Kain et al., 2008; Kain et al., 1995; Kain et al., 2012). It is important to note approximately 10% of patients, with clinical and histological features of AAV will test negative for ANCA (Eisenberger et al., 2005).

The remainder of this literature review will focus on the current treatment, genetic causes of AAV, and environmental associations but will largely focus on the pathogenesis of AAV. There have been many classifications of crescentic GN over the last 50 years. The nomenclature has been progressively updated as the immunopathologic basis of crescentic GN has become better understood. A major advance is the concept proposed by W. Cousers group and for historical reasons I present the initial classification by this investigator (see Table 1. Immunopathogenic Classification of Crescentic Glomerulonpehritis). Prior to the 1980s all cases of crescentic GN were regarded as being either type I-II. A major advance was the appreciation that most cases of crescentic GN has little or no Ig or complement.

Table 1. Immunopathogenic Classification of Crescentic GN adapted from (Couser, 1988)

I.	**Anti-GBM Antibody**	**20%**
	With lung haemorrhage (Goodpastures Syndrome)	
	Without lung haemorrhage	
	Complicating membranous nephropathy	
II.	**Immune Complex**	**40%**
	Postinfectious	
	Poststreptococcal	
	Visceral abscess	
	Other	
	Collagen-Vascular Disease	
	Lupus Nephritis	
	Henoch-Schonlein purpura	
	Mixed cryoglobulinemia	
	Primary Renal Disease	
	IgA Nephropathy	
	Membranoproliferative glomerulonephritis	
	Idiopathic	
III.	**No immune deposit**	**40%**
	Vasculitis	
	Polyarteritis	
	Wegeners	
	Hypersensitivity vasculidities	
	Idiopathic	

1.3.4 Chapel Hill Consensus Conference (1994 and 2012)

In 1994, the first Chapel Hill Consensus took place to standardise the classification system of vasculitis (see Table 2) (Jennette et al., 1994). In 2012 an updated version was developed with twenty-eight participants from 12 countries with expertise including but not limited to areas of vasculitis, nephrology, and pathology convened to establish disease definitions

and names for the vasculitides (Jennette et al., 2013). The consensus determined the following vessel involvement and nomenclature: Large vessel Vasculitis (Takayasu Arteritis, Giant Cell arteritis), Medium Vessel Vasculitis (Polyarteritis Nodosa, Kawasaki Disease) Small Vessel Vasculitis was further categorised into either Immune complex small vessel Vasculitis (IgA Vasculitis, Cryoglobulinemic vasculitis, anti C1q vasculitis), anti GBM disease, and ANCA associated vasculitis (Microscopic Polyangiitis, Granulomatosis with Polyangiitis, and eosinophilic granulomatosis with polyangiitis) (see table 3.) (Jennette, et al., 2013).

Table.2 Names and definitions of vasculitides adopted by the Chapel Hill Consensus Conference on the nomenclature of systemic vasculitis* adapted from (Jennette et al., 1994)]

Large vessel vasculitis	
Giant cell (temporal) arteritis	Granulomatous arteritis of the aorta and its major branches, with a predilection for the extracranial branches of the carotid artery. Often involves the temporal artery. Usually occurs in patients older than 50 and often is associated with polymyalgia rheumatic
Takayasu arteritis	Granulomatous inflammation of the aorta and its major branches. *Usually occurs in patients younger than 50.*
Medium-sized vessel vasculitis	
Polyarteritis nodosa§ (classic polyarteritis nodosa)	Necrotizing inflammation of medium-sized or small arteries without glomerulonephritis or vasculitis in arterioles capillaries, or venules
Kawasaki disease	Arteritis involving large, medium-sized, and small arteries, and associated with mucocutaneous lymph node syndrome. Coronary arteries are often involved. Aorta

	and veins may be involved. Usually occurs in children.
Small vessel vasculitis	
Wegener's granulomatosis[t]	Granulomatous inflammation involving the respiratory tract, and necrotizing vasculitis affecting small to medium-sized vessels (e.g., capillaries, venules, arterioles, and arteries). Necrotizing glomerulonephritis is common.
Churg-Strauss syndrome[t]	Eosinophil-rich and granulomatous inflammation involving the respiratory tract, and necrotizing vasculitis affecting small to medium-sized vessels, and associated with asthma and eosinophilia
Microscopic polyangiitis[§] (microscopic Polyarteritis)[t]	Necrotizing vasculitis, with few or no immune deposits, affecting small vessels (i.e., capillaries, venules, or arterioles). Necrotizing arteritis involving small and medium sized arteries may be present. Necrotizing glomerulonephritis is very common.
Henoch-Schonlein purpura	Vasculitis, with IgA-dominant immune deposits, affecting small vessels (i.e., capillaries, venules, or arterioles). Typically involves skin, gut, and glomeruli, and is associated with arthralgias or arthritis.
Essential cryoglobulinemic vasculitis	Vasculitis, with cryoglobulin immune deposits, affecting small vessels (i.e., capillaries, venules, or arterioles), and associated with cryoglobulins in serum. Skin and glomeruli are ofien involved.

Cutaneous leukocytoclastic angiitis	Isolated cutaneous leukocytoclastic angiitis without systemic vasculitis or glomerulonephritis.

*Large vessel refers to the aorta and the largest branches directed toward major body regions (e.g., to the extremities and the head and neck); medium-sized vessel refers to the main visceral arteries (e.g., renal, hepatic, coronary, and mesenteric arteries); small vessel refers to venules, capillaries, arterioles, and the intraparenchymal distal arterial radicals that connect with arterioles. Some small and large vessel vasculitides may involve medium-sized arteries, but large and medium-sized vessel vasculitides do not involve vessels smaller than arteries. Essential components are represented by normal type; italicized type represents usual, but not essential, components. § Preferred term.ŧ Strongly associated with antineutrophil cytoplasmic autoantibodies.

The ultimate goal was to make the nomenclature more relevant based on the advances in the knowledge of the disease pathogenesis, so the disease name reflected more accurately the description of the disease. It is important to note that the Chapel Hill Consensus Conference, only determined the nomenclature of the vasculitides, it is not a classification or diagnostic system (Jennette, et al., 2013).

The Chapel Hill Consensus Conference made 4 major changes (which are outlined in tables 3 and 4) to the previous system:

1. Defined a new group, ANCA associated vasculitis
2. Suggested names of individual entities based on pathology and current eponymous titles to be deleted
3. The importance of the pattern of causative autoimmunity was added to the title (eg. MPO-ANCA versus PR3-ANCA
4. Renal limited vasculitis was deleted

Table.3 Names and definitions of vasculitides adopted by the 2012 International Chapel Hill Consensus Conference on the Nomenclature of Vasculitides (Jennette et al., 2013)

Large vessel vasculitis (LVV)
Takayasu arteritis (TAK)
Giant cell arteritis (GCA)
Medium vessel vasculitis (MVV)
Polyarteritis nodosa (PAN)
Kawasaki disease (KD)
Small vessel vasculitis (SVV)
Antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV)
Microscopic polyangiitis (MPA)
Granulomatosis with polyangiitis (Wegener's) (GPA)
Eosinophilic granulomatosis with polyangiitis (Churg-Strauss) (EGPA)
Immune complex SVV
Anti–glomerular basement membrane (anti-GBM) disease
Cryoglobulinemic vasculitis (CV)
IgA vasculitis (Henoch-Scho¨nlein) (IgAV)
Hypocomplementemic urticarial vasculitis (HUV) (anti-C1q vasculitis)
Variable vessel vasculitis (VVV)
Behc¸et's disease (BD)
Cogan's syndrome (CS)
Single-organ vasculitis (SOV)
Cutaneous leukocytoclastic angiitis

Table.3 Names and definitions of vasculitides adopted by the 2012 International Chapel Hill Consensus Conference on the Nomenclature of Vasculitides continued (Jennette, et al., 2013)

Cutaneous arteritis
Primary central nervous system vasculitis
Isolated aortitis
Others
Vasculitis associated with systemic disease
Lupus vasculitis
Rheumatoid vasculitis
Sarcoid vasculitis
Others
Vasculitis associated with probable etiology
Hepatitis C virus–associated cryoglobulinemic vasculitis
Hepatitis B virus–associated vasculitis
Syphilis-associated aortitis
Drug-associated immune complex vasculitis
Drug-associated ANCA-associated vasculitis
Cancer-associated vasculitis
Others

Table 4. Names and definitions of ANCA associated vasculitis subgroups and definitions (adapted from Chapel Hill Consensus 2012)

Small Vessel Vasculitis		Vasculitis predominantly affecting small vessels, defined as small intraparenchymal, arteries, arterioles, capillaries, and venules. Medium arteries and veins may be affected.
	ANCA associated Vasculitis	Necrotizing vasculitis, with few or no immune deposits, predominantly affecting small vessels (i.e., capillaries, venules, arterioles, and small arteries), associated with myeloperoxidase (MPO) ANCA or proteinase 3 (PR3) ANCA. Not all patients have ANCA. Add a prefix indicating ANCA reactivity, e.g.,MPO-ANCA, PR3-ANCA, ANCA-ve
	Micrcrosocopic polyangiitis (MPA)	Necrotizing vasculitis, with few or no immune deposits, predominantly affecting small vessels (i.e., capillaries, venules, or arterioles). Necrotizing arteritis involving small and medium arteries may be present. Necrotizing glomerulonephritis is very common. Pulmonary capillaritis often occurs. Granulomatous inflammation is absent
	Granulomatosis with polyangiitis (Wegener's) GPA	Necrotizing granulomatous inflammation usually involving the upper and lower respiratory tract, and necrotizing vasculitis affecting predominantly small to medium vessels (capillaries, venules, arterioles, arteries and veins).Necrotizing glomerulonephritis is common.

Table 4. Names and definitions of ANCA associated vasculitis subgroups and definitions continued from previous page (adapted from Chapel Hill Consensus 2012)

	Eosinophilic granulamotisis With polyangiitis (Churg-Strauss) (EGPA)	Vasculitis predominantly affecting small vessels, defined as small intraparenchymal, arteries, arterioles, capillaries, and venules. Medium arteries and veins may be affected.

1.4 General overview of Anti Neutrophil Cytoplasmic Antibody Vasculitis

Until the introduction of nitrogen mustard based immunosuppression most noteably cyclophosphamide, treatment was primarily based on high doses of glucocorticoids. Prior to this regime most patients would progress to end stage renal failure within 5 months. (Ball, 2010; Hamour et al., 2010, Jayne, 2009; Morgan et al., 2006). With discovery of ANCA as a diagnostic marker in the 1980s and with current treatment, remission can now be inducted within six months and survival rates have increased to approximately 75% of patients at 5 years. Although these treatment regimens are largely successful, relapse rates remain high at around 50% at 5 years. Despite maintenance therapy, the high percentage of relapses suggest that standard treatment does not actually address the primary pathogenesis of AAV and the toxicity of the treatment itself contributes significantly to the morbidity and mortality rates (Ball, 2010; Hamour et al., 2010 Jayne, 2009; Morgan et al., 2006). Drug-related toxicity and adverse effects will cause difficulty in over 90% of patients. The most common form of death within the first year of contracting the disease will be infection associated with the immunosuppressed state of the patient. Prolonged use of cyclophosphamide is also associated with a high rate of malignancy (Hiemstra & Jayne, 2009).

AAV has an unknown aetiology, with a multifactorial pathogenesis, making it a complex disease to understand. Therefore it essential we further examine the pathogenesis of AAV, so we are able to provide targeted therapy with less side effects. This will need to encompass, the epidemiological and genetic variations of the disease, causes for relapses,

and identification of the key players of the immune system that contribute to the pathogenesis of this disease. The purpose of this review, is to summarise the key findings that have contributed to the wealth of research in the area of AAV, and highlight the areas of deficiency.

Classification of anti-neutrophil cytoplasmic antibody vasculitis

Clinically AAV can be divided into 3 distinct clinical entities based on the 2012 Chapel Hill consensus (see previous tables) (Jennette et al., 2013):

Microscopic polyangiitis (vasculitis with no asthma or granulomas)

Granulomatosis with Polyangiitis (granulomas and no asthma, formally known as Wegener's)

Eosinophilic Granulomatosis with Polyangiitis (eosinophilic granulamotisis with polyangiitis, asthma, formally known as Churg-Strauss)

1.5 Microscopic polyangiitis (vasculitis without asthma or granulomas)

Microscopic Polyangiitis (MPA) is defined as a small vessel vasculitis without asthma or evidence of granulomas, and is classed with systemic vasculitides. MPA is largely associated with a positive clinical test for MPO-ANCA or P-ANCA by ELISA . MPA effects multiple organs, explaining the multitude of symptoms patients can present with such as fever, weight loss, skin lesions (30-60% of patients), including palpable purpura (30-40% patients), pulmonary capillaritis (50% of patients) and gastrointestinal involvement (50% patients). Over 90% of patients will present with glomerulonephritis with proteinuria, and microscopic haematuria. The observed renal lesions are classed as necrotizing crescentic glomerulonephritis, with evidence of rupture of the GBM, fibrinoid necrosis, and both cellular and fibrous crescents. Despite the term pauci immune glomerulonephritis there is evidence of both Ig and C3 deposition in over half of the patients (Haas & Eustace, 2004). Review of

patient survival suggests that patients with MPO-ANCA have a significantly lower survival rate than compared to that of PR3-ANCA, but have similar renal survival (Belmont, 2006; Greco et al., 2015; Hilhorst et al., 2015; Jennette, Falk, Hu, et al., 2013; Mukhtyar et al., 2008; H. Xiao et al., 2016).

1.6 Granulomatosis with polyangiitis (granulomas and no asthma, formally known as Wegeners)

Granulomatosis with polyangiitis (GPA), was formerly known as Wegeners, this term is now disused in favour of a more descriptive term to describe the disease. Although the vasculitis observed in GPA presents identically to MPA it is distinguished by the development of granulamota which is mostly found in the upper and lower respiratory tract, but is also known to affect blood vessels, extravascular tissue and skin. The characteristic granulomatous inflammation is identified by the concentrated infiltration of neutrophils, macrophages and giant cells. Diagnostically GPA presents more often as PR3-ANCA positive by ELIZA and C-ANCA via direct immunofluorescence. Biopsy histopathology is similar to that of MPA, but with less C3d deposition, and no granuloma formation (Belmont, 2006; Greco et al., 2015; Hilhorst et al., 2015; Jennette, Falk, Hu, et al., 2013; Mukhtyar et al., 2008; H. Xiao et al., 2016).

1.7 Eosinophilic Granulomatosis with Polyangiitis (eosinophilic granulamotisis with polyangiitis, asthma, formally known as Churg-Strauss)

Eosinophilic granulomatosis with polyangiitis (EGPA), was formally known as Churg-Strauss syndrome after the 2 pathologists who first described the disease, but EGPA is now favoured due to the descriptive nature of the name. EGPA differs from both MPA and GPA, by the presence of eosinophils within the inflammatory lesions, and clinically by presentation with asthma. In some cases there is presence of eosinophilic pneumonia or eosinophilic gastroenteritis. EGPA is a rare disease, and presents as a prodromal phase consisting of asthma, nasal polyposis, and rhino sinusitis. The second phase is characterised by the

infiltration of eosinophilia into tissue and circulating eosinophilia, followed by the final phase, termed the vasculitic phase. Over half of the patients will also experience cutaneous manifestations including palpable purpura, urticarial rashes and peripheral neuropathy. Like MPA the major ANCA association is with MPO-ANCA, although a minority of patients will also have PR3-ANCA. Like MPA and GPA patients ANCAs are thought to be involved in the pathogenesis of the disease contributing directly to the endothelial vasculitic damage. However, it is thought that the eosinophils may contribute to the endothelial damage directly in the ANCA negative patients with EGPA (Baldini et al., 2010).

RECENT ADVANCEMENT IN TREATMENT FOR ANCA VASCULITIS

The basic principles for the treatment of vasculitis is to reduce the inflammation in the short term and supress the immune system for a longer term benefit. The current regimes of cyclophosphamide and glucocorticoids are both toxic and have adverse affects. Treatment varies according to the current disease state of the patient, current relapse rates are high even when patients are being treated, so treatment has to be tailored to whether patients have exceptionally resisitant disease, mild or moderate disease or are experiencing a relapse. Factors that have led to the development of new therapies for AAV in the last 15-20 years have been attributed to the develoment of randomized trials with both valid and accepted outcome measures, the Standardization for the classification of AAV (Jennette et al., 2013), has been important and the development of ANCA testing via ELISA and the formation of large research consortiums such as VCRC (Vasculitis Clinical Research Consortium) in North America, EUVAS (European Vasculitis Society), Europe and FVSG (French Vasculitis Study Group), France.To discuss the advancements in treatment this section will briefly discuss the evolution of treatment for ANCA vasculitis since the 1970s and some of the sucessful trials for the use of new biologicals such as RITUXIMAB.

Induction treatment, is used to rapidly eliminate destructive inflammatory tissue damage by inducing disease remission. Maintennace therapy is the phase after remission has been

established and the aim is to avoide relapse. Commonly prescribed drugs for vasculitis are prednisone, azathioprine, cyclophosphamide (CYC), methotrexate, mycophenolate and Rituximab. Figure 3 outlines the evolution of treatment for AAV, and lists some of the major trials for new biological therapies in comparison to the current standard treatment of glucocorticoids, CYC and azathioprine for maintenance treatment.

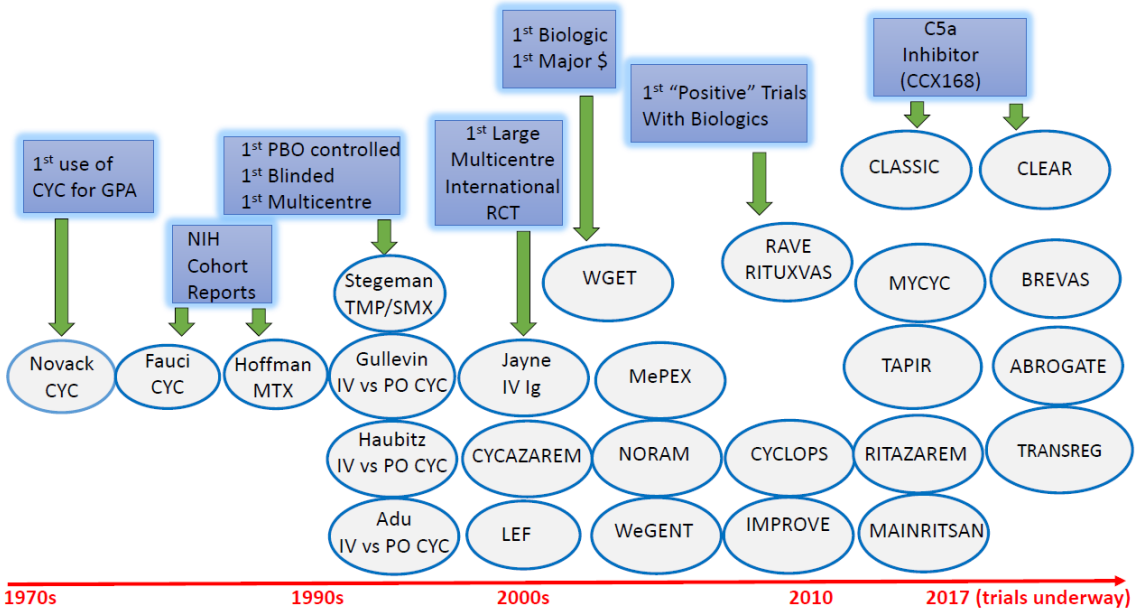


Figure 3. Evolution of treatment for ANCA vasculitis (adapted from Merkel, 2017)

1.8 Cyclophosphamide and Azathioprine

In the 1960s the major disease treatment was glucocorticoids. While inflamation could be attenuated morbidity and death rates remained high. Cyclophosphamide was initially developed as chemotherapy for certain types of cancers, however it also has a supressive effect on the immune system. The use of CYC in treating glomerulonephritis reduced mortality from 80% to 30%. It was not until the 1990s that the first mulitcentred and blinded trials for the use of CYC were conducted for use in AAV. Two studies conducted by (Guillevin et al., 1997; Haubitz et al., 1998) demonstated that intravenous pluse administration of CYC was less toxic, however patient suvival and relapse rates remained

the same between the pulse and oral CYC groups. A third study showed (Adu et al., 1997) that there was not significant differences in infection rates or deaths between patients treated with prednisolone versus CYC. Long term studies demonstrate that decreasing CYC resulted in an increased risk of relapse in the CYCLOPS (pulsed intravenous versus oral cylophosphamide) and CYCAZAREM trials (replacement of CYC with azathioprine) (Harper et al., 2012; Walsh et al., 2014).

1.9 Rituximab

The RAVE (Rituximab versus cyclophosphamide for ANCA assciated vasculits) and RITUVAS (Rictuximab versus cyclophosphamide in ANCA associated renal vasculitis) studies compared the use of Rituximab (anti CD20 antibody) versus CYC.

The RAVE study enrolled 197 patients with severe AAV which were subsequently randomized into a weekly treatment of Rituximab with no accomanying immunosupression or cylophosphamide for induction followed by azathioprine for maintenance therapy. The primary endpoint of this study was to achieve remission at 6 months with out the use of any immune supression with glucocorticoids. The Rituximab group met this endpoint with 64% of patients versus 53% in the CYC and azathioprine cohort (Stone et al., 2010).

The RITUVAS study enrolled 44 newly diagnosed AAV (renal) patients and randomized into either receiving rituximab with 2 pulses of IV CYC or pulsed CYC with azathioprine. The rituximab treated arm of the study achieved remission in 76% of the patients verus 82% in the CYC and azathioprine arm (Jones et al., 2010).

Despite rituximab satisfying criteria for non inferiority when compared to standard treatment with CYC and azathioprine, neither the RAVE or RITUVAS studies reported a decrease in adverse events with the use of rituximab compared to standard care.

1.10 Azathioprine versus Methotrexate for maintenance therapy

Methotrexate (MTX) is commonly used for less severe vasculitis where cyclophosphamide is not required, to decrease inflammation. MTX is often prescribed to control a patients symptoms or prevent the patient from relapsing. In the WEGENT (Wegener's Granulomatosis–Entretien) (Pagnoux et al., 2008) study patients received identical induction therapy consisting of prednisolone and CYC, only patients achieving remission were then assigned treatment groups. One arm of the study receiving MTX and the other arm receiving azathioprine (AZA) after a third pulse of CYC. The study was designed to compare the safety and efficacy of azathioprine compared to methotrexate. MTX was not shown to be safer than AZA but was similar for use as maintenance therapy. The long term outcomes of the WEGENT study (Puechal et al., 2016), showed no differences between either arms in terms of rates of relapse or adverse events. The 10 year remission/survival rate was decreased in those patients with GPA compared to MPA.

1.11 Mycophenolate mofetil

Mycophenolate mofetil (MMF) has been use for both induction treatment and remission maintenance therapy in AAV. It is an immunosuppressant drug which is often used to prevent organ transplant rejection (through inhibiting the expansion of T and B cells). The MYCYC (MMF versus cyclophosphamide for remission induction in AAV) non-inferiority trial demonstrated that MMF is not inferior to CYC, however at the 18 month time point relapses were more prevalent in the MMF group (Jones, 2013). The IMPROVE trial (Hiemstra et al., 2010)(International Mycophenolate Mofetil Protocol to reduce outbreaks of Vasculitides) was designed to compare the effect of MMF versus AZA in the prevention of relapses. One arm of the trial was assigned AZA and the other MMF. MMF was found to be less effective than AZA and adverse events were similar between both treatments.

1.12 C5a inhibitor (CCX168)

The CLEAR and CLASSIC studies investigated the efficacy and safety of the C5a inhibitor CCX168 for the treatment of AAV. The CLEAR trial have just recently published their findings where Avacopan (CCX168) replaced prednisone treatment in newly diagnosed patients (Jayne et al., 2017). The results from the Phase II study (12 weeks) demonstrated that high doses of prednisone can be replaced with Avacopan both effectively and safely in patients with AAV. The Phase III study is ongoing but the estimated completion date is June 2019. The CLASSIC study conducted in the USA and Canada, has used CCX168 in conjunction with either full-dose glucocorticoids, rituximab or CYC, to test the safety profile of CCX168. After 12 weeks CCX168 was found to be safe when used in conjunction with any of the standard of care treatments (Merkel PA, 2016).

1.13 Belimumab

Belimumab is a humanized monoclonal antibody that inhibits BLyS (also known as BAFF) which is a survival factor for B cells therefore a potential therapeutic target in AAV. Belimumab has been shown to be tolerable in trials with patients with SLE. A current trial named BREVAS started in 2012 with results of the phases III study due to be released soon, which examines the efficacy and safety of belimumab in AAV (Clinical trials gov identifier: NCT01663623).

1.14 Abatacept

The safety and efficacy of abatacept (a CTLA4-Ig fusion protein) was determined in a cohort of 20 patients with non-severe GPA. In this small open label trial, abatacept was well tolerated and resulted in 73% of patients reducing prednisone to 0mg, and a high frequency of disease remission (80%)(Langford et al., 2014). ABROGATE a randomized placebo controlled trial will investigate further the potential for abatacept to be therapeutic in the treatment of AAV (Clinical trials.gov identifier NCT02108860).

EPIDEMIOLOGY OF ANCA VASCULITIS

AAV is a relatively rare autoimmune disease with a low incidence rate, therefore epidemiological studies are difficult to interpret when data is obtained from large populations. However with the improvements in the classification of AAV high quality epidemiological studies have been conducted, and data has been collected from Australia, New Zealand, Europe, Japan, USA, China and Europe. What has been established is that AAV differs from other autoimmune diseases in that the gender ratio of those effected is almost balanced, with just a slight increase in male dominance. Of notable significance is that it appears that both the incidence and prevalence of both GPA and MPA differs geographically, which will be discussed in more detail within each geographical location below.

1.15 Australasia

New Zealand is geographically isolated with the nearest neighbour located 2000km south east of (Australia), it is spilt into two main Islands (North and South), with a population of 4,377,944. The population is 67% European, with the remainder comprising of Maori (14.6%), Pacific Island People (12.1%) and 12.1% of other ethnicities (de Zoysa, 2013). The most recent report on vasculitis incidence in New Zealand by de Zoysa et al *(2012),* revealed that although both Maori and Polynesian patients more routinely progress to end stage renal disease, the incidence occurs more frequently in Europeans (60.2 per million vs 34.2 per million Maori or 17.27 per million Pacific Islanders). The prevalence of AAV significantly differs in New Zealand, with a north-south disease gradient. A significant increase in GPA is observed form north to south, however no significant difference in seen for either MPA or EGPA (de Zoysa, 2013). The Incidence rate in New Zealand is higher in autumn versus winter and spring, and is significantly higher in the 70-79 year bracket compared to those under 30 (O'Donnell et al., 2007).

Australia is a large continent with the most recent recorded population at 22,015,576 (2012). Tang et al (2013) studied outcomes in an Australian and New Zealand cohort of patients with end stage renal disease (ESRD), and demonstrated that MPA patients had lower survival rates post-transplant than GPA patients. Of those patients on dialysis, there was no difference between MPA and GPA in terms of survival. Ormerod et al (2008) studied the epidemiology of primary systemic vasculitis in the Australian Capital Territory (ACT) and surrounding rural area. Comparable to other studies, they found that incidence was higher in patients in their 60s and that men and women were equally affected. In the study period from 1995-1999, they found an incidence of 17/million per year, and that during this period there was no significant change in that incidence.

Review of the literature on the epidemiology of AAV in Australia, reveals an unmet need for an Australian wide study, as little can be drawn from the current studies to reliably compare with that of the comprehensive studies conducted in Europe.

1.16 Asia

Japan has a population of 127,368,088 (2012), and is geographically isolated sharing no borders with other countries. Japan has more patients that are MPO-ANCA positive with MPA than patients that are PR3-ANCA positive with GPA. GPA is estimated to be present in less than 2/million patients in Japan (Kobayashi et al., 2010). The Japanese Society of Nephrology conducted a study into the epidemiology of primary renal vasculitis in Japan, and found that the cause of rapidly progressive glomerulonephritis was mostly attributed to renal limited vasculitis (42.1%), followed by MPA (19.4%) and finally anti GBM disease (6.1%). The renal limited vasculitis group were 88.1% positive for MPO-ANCA, and similarly 91.8% of MPA patients were also positive for MPO-ANCA. Of the 1772 patient cohort, only 7.4% of the renal limited vasculitis group were PR3-ANCA positive, compared to the Wegeners granulamotisis group which were 71.1% positive for PR3-ANCA. A small population of patients who had MPA were also PR3-ANCA positive (6.1%). (Koyama et al., 2009). The prevalence of AAV between 2000-2004 in the Miyazakai Prefecture of Japan

found no patients with either WG or CS, and over 91% of the 56 patients were MPO-ANCA positive (Fujimoto et al., 2006). This observation was in concordance with the latitude theory, that suggests that the incidence of WG and MPA correlated with latitude, with WG observed in high latitude areas in Europe and the UK, and MPA with low attitude areas such as the Miyazaki Prefecture of Japan and Bahrain (Kobayashi et al., 2010).This led to studies, comparing the incidence of AAV with that of United Kingdom (UK) and European Systemic Vasculitis Study Group (EUVAS).The UK study demonstrated that the incidence of AAV was similar between countries but the subtypes of AAV were significantly different, with Japan almost solely comprising of MPA patents whereas the UK only had 41% reported patients with MPA (Watts et al., 2008).

China like Japan has a dominant MPA population compromising 80% of the patients with AAV (Chen et al., 2005). GPA in European countries is largely associated with a PR3-ANCA however in China 60% of the GPA patients test positive for MPO-ANCA, have a significantly higher creatinine level at first presentation, accompanied with an increased frequency of chronic lesions via biopsy. Within the Chinese population, AAV was found to effect all ages but was significantly associated with elderly patients (over 40%), with an increased likelihood of having MPA opposed to GPA (Chen et al., 2005).

1.17 UK/Europe

The European Vasculitis Study Group (EUVAS) has allowed detailed data collection of multiple European countries using a standardised system for diagnosis for the different AAV clinical subtypes.

The longest study of (23 years), compared new onset patients (111 GPA and 58 MPA) between 1988-2010 whom presented at the Norfolk and Norwich University Hospital. The overall findings suggested that the incidence of GPA is high compared to other Caucasian populations within the UK (11.3 /million compared to 8.4/ million of the general population of the UK), and that GPA was twice as common than MPA (Watts et al., 2012).

Sweden has recorded the highest prevalence of primary systemic vasculitis (PSV) at 300/million, with WG having 160/million compared to 94/million (reported in a 5 year study conducted between 1998-2002). However the north-south gradient hypothesis was not evident in Sweden with MPA rates comparable to that of southern Europe (Mohammad et al., 2007).

A French study however based in an urban multiethnic area of Seine-St. Dennis Country, a suburb north east of Paris, with a population of 1,382, 928 found MPA (25.1/million) to be more prevalent than GPA (23.7/million) (Mahr et al., 2004). Amongst the ethnic diversity of patients included in this study, Caucasian were twice as likely to develop MPA, WG or CSS. In particular WG was more prevalent in Caucasians than non-Caucasians, suggesting that ethnic difference may be of importance in WG with this geographical area. The higher incidence of MPA suggests like other studies that there may be a decreasing north-south gradient for WG (Mahr et al., 2004).

1.17.1 Conclusions drawn from epidemiological data

Although the relative incidence of ANCA-associated vasculitis appears the same between the east and the west, the clinical subtype MPA appears to be significantly increased in patients in Japan and China compared to Europe and the UK. Furthermore, the number of MPO-ANCA positive patients is significantly higher in Asia compared to Europe and the UK. These results suggest that an individualised approach to treatment of AAV may be warranted which takes into account the geographical difference (including ethnicity), clinical subtype and ANCA classification.

ENVIRONMENTAL INFLUENCES ON AAV

AAV is unusual and dissimilar to other autoimmune diseases in that male and females are effected equally. Studies reporting seasonal variation, and geographical difference (as listed in previous section), suggest that environmental factors may contribute largely to the disease. This section will discuss the evidence suggesting that environmental triggers may contribute to the disease such as, silica, exposure to earthquakes, seasonal variation (infection), and rural versus urban living.

1.18 Silica

Cyrstalline silica has had a known association with other autoimmune disease such as SLE, RA, scleroderma, chronic obstructive pulmonary disease (COPD) and lung cancer (Parks et al., 1999). Silica is found abundantly in rocks, soil and sand. Silica when in its crystalline state exists as quartz, and makes up a small part of the particulate matter of air pollution, which can be inhaled as <5-μm particles. Occupational exposure to silica occurs in many construction processes where silica is used as a raw material, or during the process of mining, road construction, manufacturing of glass, and as the main component of abrasive cleansers (Parks et al., 1999).

A recent meta-analysis and review of the association between silica exposure and AAV demonstrated that, exposure to silica was associated with a doubling in the risk for the development of AAV (Gomez-Puerta et al., 2013). It is thought that this effect is due to the adjuvant effect of silica in enhancing an immune response to a wide range of proteins. Studies on silica exposed individuals peripheral blood mononuclear cells (PBMCs) demonstrated that there is a significant increase in inflammatory cytokines such as interleukin 1 beta (IL1β), Tumour Necrosis Factor alpha (TNFα), and interferon gamma (IFNγ), interleukin 1 (IL-1), interleukin 6 (IL-6) and the anti-inflammatory cytokines such as transforming growth factor beta (TGF-β) and interleukin 10 (IL-10). The production of both pro-inflammatory and anti-inflammatory cytokines suggest that silica induces and increases

hyperactivity of the immune system, by inducing danger signals (Rocha-Parise et al., 2014). Rocha-Parise et al (2014), suggests that silica exposure activates the inflammasome, resulting in T cell activation, and aberrant T regulatory cells causes a loss of tolerance to silica and possibly triggers autoimmunity.

1.19 Earthquakes

In 1995, Kobe, Japan was struck by an earthquake measuring 7.2 on the Richter scale, a catastrophic loss of life was experienced (6336) with a further 41, 521 people injured. The central city of Kobe was completely destroyed, with over 200,000 buildings damaged beyond repair. In the three year period following the earthquake there was a significant increase in the numbers of patients presenting with MPA (14 compared to 15 in the previous 7 year period). There was considerable air pollution after the earthquake containing both asbestos and silica, which are both known to provoke immune responses, suggesting a likely hypothesis for the increased incidence of AAV in the 3 year period after wards (Yashiro et al., 2000).

In New Zealand in 2011 an earthquake struck Christchurch measuring 6.4 on the Richter scale, 185 people were killed and mass destruction of the city occurred. Both air pollutants and ground subsoil liquefaction occurred, resulting in sand and fine dust being produced during the drying process. Farquhar et al (2016), studied the incidence of AAV 3 years prior to the earthquake and 3 years after the earthquake and found that there was no difference in the incidence of AAV or the sub clinical types MPA and GPA. The authors point out that the earthquake severity and population density between the two countries is significantly different, with the poor air quality remaining relatively high in Kobe after the earthquake. Of more interest though is the difference in subtypes of AAV with Japan reporting higher incidence of MPA than Christchurch which has a higher incidence of GPA, suggesting that MPO may be more susceptible to silica enhanced immune stimulation than PR3 patients.

1.20 Infection-cycles

Infections can initiate and exacerbate disease in patients with AAV and provoke disease relapse (Tidman et al., 1998). The incidence of AAV varies between seasons and relapses occur with a peak in winter, which suggests an association with microbial infection. This is further supported with clinical evidence that prophylactic antibiotic therapy in some AAV patients is able to avert relapses (Stegeman et al., 1996; Stegeman et al., 1997).

From 1988-2010 Watts et al., (2012) recorded one hundred and eleven GPA and 58 MPA cases in Norfolk and Norwich University Hospital. The results from this study suggest that GPA may occur in a cyclic pattern. The first peak occurred between 1996-1998, with a second peak 7.6 years later in 2005. This trend was not observed in those patients that were MPO-ANCA positive or MPA patients. Although it has been suggested this observed rise could be due to the new development of ANCA testing, it has been refuted as it would not explain the following decrease before the next incident rice in 2005.

1.21 Molecular mimicry

The observed associations between AAV and infection could also be as a result of molecular mimicry. Molecular mimicry can take place when an immune response generated to eliminate a pathogen, recognises self-proteins, with a similar sequence homology to the immunogenic component of the pathogen. Historical evidence of molecular mimicry came from observations that antibodies specific for Group A Streptococcus, recognised components of mammalian heart muscle (Zabriskie & Freimer, 1966). Further experimental observations in experimental allergic encephalomyelitis (EAE) revealed that the Hepatitis B virus, shared sequence homology with myelin basic protein. Immunisation of rabbits with the Hepatitis B virus peptide, resulted in recognition of self-proteins which shared similar peptide sequence to the viral peptide, and induced (EAE) (Fujinami & Oldstone, 1985). Recently, two studies have investigated the possible role of molecular mimicry in AAV. In the first study, Kain et al., (2008) demonstrated in patients with pauci-immune focal

necrotising glomerulonephritis (FNGN) that an epitope on the lysosome-associated membrane protein 2 (LAMP-2) (recognised by most AAV patients) shared a 100% homology with the bacterial adhesion FimH, and cross react. Studies in rats demonstrated that immunisation with FimH could induce pauci immune FNGN and develop antibodies to both rat and human LAMP-2. The authors propose that this homology between LAMP-2 and FimH, could be the potential mechanism in the triggering of autoimmune disease.

The second study of Roth et al investigated the possible correlation with the production of anti-LAMP2 antibodies and disease activity in patients with AAV (Roth et al., 2012). They however were unable to find a correlation in the human subjects, or able to reproduce the results of Kain et al in rats. The authors do acknowledge the two cohorts of patients do differ between the 2 studies and that the reagents the labs use may differ.

These observations lead to the growing wealth of evidence that GPA may be triggered by an infectious agent adding more weight to the argument that MPA and GPA may be two distinct diseases with differing initiations of disease.

GENETIC STUDIES OF AAV

It is becoming clear with advances in the sensitivity and specificity of MPO and PR3 ELISAs that there is evidence challenging that view that there is a single common disease phenotype for patients (Walsh et al., 2012).

1.22 Associations between disease phenotype and pattern of autoimmunity

Multiple studies have demonstrated that PR3-ANCA positive patients are more frequently prone to relapse than those who are either MPO-ANCA or ANCA negative (de Joode et al., 2013; Franssen et al., 2000; Lionaki et al., 2012). Those patients that are PR3-ANCA positive and have necrotizing crescentic GN also experience a more pronounced decline in renal function than MPO-ANCA positive patients (de Joode et al., 2013). PR3-ANCA positive patients in both the Rituximab for ANCA associated Vasculitis (RAVE) and EUVAS

studies had a higher relapse rate than that of the MPO-ANCA positive patients (Stone et al., 2010; Walsh et al., 2012). Despite the more frequent relapse rate in PR3-ANCA patients, they are less likely to reach end stage renal disease (ESRD). Ultimately it is the MPO-ANCA positive patients that initially present with poorer renal function, and are less likely to recover. It has been proposed that MPO-ANCA patients may respond at a slower rate to treatment than that of the PR3-ANCA patients, accounting for the difference in outcome (Kallenberg, 2014).

1.23 Association between genetic profile and pattern of autoimmunity

MPA is largely associated with a positive result for MPO-ANCA, and GPA is strongly associated with PR3-ANCA. It appears that the dominant ANCA antigen may play an important role in determining the disease phenotype. A genome-Wide Association study in AAV (GWAS) from patients in the UK and northern Europe found that PR3-ANCA positive patients have a genetic association with *HLA-DP*, *SERPINA*1 and *PRTN3* whereas MPO-ANCA positive patients have a genetic association with *HLA-DQ (Lyons et al., 2012).* These gene abnormalities relate to the autoantigen more closely than the clinical phenotype.

PATHOGENESIS OF AAV

1.24 Generation of an immune response in immune renal disease

In 1960 the Nobel Prize in Physiology/Medicine was awarded to Frank Macfarlane Burnett and Peter Medawar for the discovery of what is known today as “acquired immune tolerance”. The Burnett hypothesis was that “if in embryonic life expendable cells from a genetically distinct race are implanted and established, no antibody response should develop against the foreign cell antigen where the animal takes on independent existence” (Bfaf, 1949). Burnett himself was unable to demonstrated his hypothesis experimentally, but instigated a series of experiments in 1953 by Medawar, that demonstrated that early engraftment of donor splenocytes introduced into a mouse inferred tolerance to future grafts

from the donor strain of mice but no other strains (Billingham et al., 2010). Further experiments have gone on to define the role of antibody producing cells, immunological tolerance of both B and T cells, negative selection of B cells and most recently the discovery of T regulatory cells (Chien et al., 1984; Clark et al., 1984; Le Douarin et al., 1996; Nossal & Lederberg, 2009; Nossal & Pike, 1975).

1.24.1 Overview of the immune system

Tolerance refers to the body's capability of distinguishing between self and non-self. Autoimmunity is generated when there is a failure in the immune system to tolerate self-antigens. The immune system can be broadly divided into the innate immune system and the adaptive immune system. Innate immunity is the immune systems first line of defence, it has no memory of contact with previous pathogens and has a quick non-specific response. In contrast the adaptive immune system, generates a specific response to foreign antigen, and generates memory through the expansion of antigen specific lymphocytes. The adaptive immune system can be further divided into cell mediated and humoral immunity.

1.24.2 The innate immune system

The innate immune system recognises a group of conserved molecular patterns known as Pathogen Associated Molecular Patterns (PAMPS) which are recognised by Pattern Recognition Receptors (PRRs). The PAMPs include bacteria, liposaccharides (LPS), lipopetides, peptidoglycans, bacterial flagellin, and viral nucleic acids, these are recognised by PRRs of which Toll-like receptors (TLRs) are the most widely characterised. There are 11 known TLRs in man and 13 in mice. Ligation of TLRs with their specific PAMP instigates cellular signalling via MYD88 (with the exception of TLR3), these TLRs will be discussed in much more detail later in the terms of AAV.

The major non cellular components of the innate immune system are complement (activated by the alternative and Lectin Pathways), the production of cytokines, and the cellular component consisting of dendritic cells, neutrophils, monocytes/macrophages, basophils, eosinophils, natural killer cells, and mast cells. The innate immune system also plays a role

in stimulating the adaptive immune system by producing molecules that activate both naïve T and B cells, and effector responses of adaptive immunity recruits and activates innate leukocytes.

1.24.3 The adaptive immune system

Adaptive immune responses rely on the recognition of antigen by antigen presenting cells. The humoral arm of adaptive immunity is mediated by B cells and the production of antibodies. Whereas the cellular-mediated arm is mediated by T cells. The antigens T cells and B cells recognise differ. T cells can only recognise antigen when it is presented by an antigen presenting cell (APC) in a Major Histocompatibility molecule (MHC) as a peptide. All nucleated cells contain MHC I and CD4 T cells can only recognise antigen presented in the groove of a MHC II molecule expressed by an antigen presenting cell (APC). In contrast the humoral arm of adaptive immunity contains B cells which produce antibody which recognise a wide variety of 3 dimensional molecules and small chemicals that T cells can not recognise. There are 5 classes of antibodies with differing structures and functions (IgG, IgD, IgE, IgG and IgM). The remainder of this section will concentrate on the role of the adaptive immune system in generating autoimmunity and its involvement in the pathogenesis of AAV.

1.24.4 Generation of autoimmunity

Tolerance is maintained by both two groups of immune systems, the primary (central) organs are the Thymus (education of T cells) and Bone marrow which process and selects non-self recognising lymphocytes. The secondary (Peripheral organs) are the lymph nodes, Spleen, the cutaneous immune system and the mucosal immune system (Peyers patches and tonsils). Thymic deletion of auto reactive T cells occurs in the central organs where a lymphocyte that interacts with a MHC molecule containing self-antigen, receives a death signal initiating apoptosis, ensuring the cell dies before becoming functionally competent. This is termed negative selection. There are several mechanisms by which autoreactive T cells are controlled. T cells require two signals to be activated, Signal 1 is from antigen

recognised by an APC MHC, signal 2 comes from co-stimulation by the same APCs. Anergy, occurs when T cells do not receive signal 2 in the form of co-stimulation. Peripheral tolerance is maintained when mature T cells recognise self-antigen by an APC not activated by signal 2, an alternative mechanism if central tolerance fails to eradicate auto reactive cells. This is important as it ensures that there is no T cell response to self-antigen that may have survived elimination in the thymus. Alternatively a self-reactive T cell may be blocked through inhibition, via cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) which ceases the activation of the T cell by reducing co-stimulation via removal of B7 molecules on the cell surface of APCs. Programmed Cell Death 1 (PD-1) also down regulates the activation of both T cells, B cells and macrophages. Finally some lymphoctyes processed in the thymus expressed the transcription factor FoxP3, this induces a regulatory phenotype in cells called thymus derived T regulatory cells (Tregs). In the periphery antigen presentation by inactive APCs induces another family of induced (or antigen specific) T regulatory cells.

Loss of tolerance occurs when these checkpoints fail, primarily it is thought that this occurs due to inheritance of genes that make an individual more likely to be susceptible to autoimmunity and environmental factors that instigate inflammation. An increased genetic susceptibility is inferred on individuals who have polymorphisms in MHC genes. Human leukocyte antigen (HLA) alleles in particular have been shown to be associated with autoimmunity. Polymorphisms in non-HLA genes such as protein tyrosine phosphatase N22 have also been associated with autoimmune diseases such as SLE, RA and diabetes mellitus 1. Environmental factors such as infection may activate self-reactive lymphocytes by providing an environment rich in cytokine production, inducing activated APCs with increased co-stimulation, and the production of microbial peptide antigens with similarities to human self-antigens (molecular mimicry).

GENERATION AND PATHOGENICITY OF ANCA

The likely involvement of ANCA in the pathogenesis of AAV comes from clinical evidence of placental transfer of ANCA inducing foetal vasculitis, in vitro experiments and evidence from animal models (which will be discussed in a later section).

1.25 ANCA titre in AAV patients

ANCA detection either by ELISA or indirect immunofluorescence is used as one of the diagnostic criteria for AAV patients. Many studies have attempted to demonstrate a correlation between ANCA titre and disease activity. One of the first of these studies by van de Woude et al (1985) demonstrated in 41 retrospective patients that ANCA titre correlated closer with disease activity than previous diagnostic laboratory tests used.

More recent studies have also shown a correlation between persistent ANCA titre levels as a predictor of relapse in patients in remission. Sanders et al., (2006) showed in a study of 86 PR3-AAV patients that those patients whom remained PR3-ANCA negative were less likely to relapse compared to those patients who tested positive for PR3-ANCA. Similar findings were also found in a 2 year study of 85 prospective WG patients, where ANCA titres measured every 2 months showed 31 of the 33 patients that relapsed had a rise in ANCA titre prior to relapse (Boomsma et al., 2000). One of the largest recent studies of ANCA in active disease (n=180) in WG patients confirmed previous studies results but found that only 83% of patients with limited disease were positive for ANCA. The authors of this study also suggest that both ELISA and immunofluorescence is required for optimal detection of ANCA, however despite this combination 5 patients in the study with severe active disease remained ANCA negative (Finkielman et al., 2007). Interestingly the same author of this study investigated whether pro PR3-ANCA would have a better predictor value for relapse than mature PR3-ANCA in 156 WG patients within 8 medical centres in the USA. They found that pro PR3-ANCA was only weakly associated with an increase in disease activity determined by the Birmingham Vasculitis Activity score (BVAS), and was no better

than the mature form of PR3-ANCA as a measure of disease activity. The majority of studies in ANCA titre and disease activity have been conducted in PR3-positive patients. Studies in patients whom are MPO-ANCA positive are minimal, but they demonstrate that there is not as a significant correlation in ANCA titre and disease activity as seen in PR3 patients (Hogan et al., 1996; Pagnoux et al., 2008). The accepted consensus is that ANCA titre does not have predicative value in determining relapse, and standard ANCA detection of MPO-ANCA and PR3-ANCA are sufficient enough for diagnosis of AAV.

The existence of ANCA negative patients and the failure of studies to show a correlation between MPO-ANCA and disease activity has made it difficult to fully explain the pathogenicity of ANCA. To add even more confusion naturally occurring anti MPO and PR3 antibodies have been found in 2 studies within sera of healthy individuals (Cui et al., 2010; Olson et al., 2011).There have been many theories put forward to explain why some patients with active disease remain ANCA negative, such as inadequate testing for ANCA (Chen, Kallenberg, et al., 2009). The study of Roth et al (2013), demonstrated that it was the difference in ANCA epitope specificity that explained why standard laboratory assays could not detect ANCA in ANCA negative patient's sera. The author's epitope mapped both human MPO-ANCA and murine sera samples from experimental MPO-ANCA GN and found that the presence of ceruloplasmin within the serum prevented serological detection of ANCA. This study also discovered that it is only epitope specific MPO-ANCA (linear epitope aa 447-459) that elicits an immune response in ANCA negative patients, and it is this epitope that is sensitive to blocking by fragmented ceruloplasmin. The linear epitope aa 447-459 was also found to correlate with active disease in those patients that test positive for MPO-ANCA.

1.26 Placental transfer of ANCA

There are 2 cases of placental transfer of ANCA to a neonate. However, both published papers are reporting the same case, which was unknowingly published by different medical teams involved in the case. For the purposes of this review, I will detail the first published

case report. In 2004 a case report was published detailing the clinical observation whereby MPO-ANCA was transferred from a mother to a neonate via the placenta. A 32 year old women presented 33 weeks pregnant with malaise, dyspnoea, headaches and hypertension. Review of the patient's medical history indicated that she had been diagnosed with Polyarteritis Nodosa 10 years prior, but had been in remission until time of admission. The neonate on day 2 developed respiratory distress and was diagnosed as having pulmonary haemorrhage, a large dose of steroids was administered to the neonate based on the mothers previous medical history of vasculitis, and the belief that maternal ANCA had been transferred to the foetus. The neonate dramatically improved after administration of steroids, and was released from hospital 18 days later in good health (Bansal & Tobin, 2004). Evidence that ANCA in this case was pathogenic comes from the authors data that identical P-ANCA was shown in both the mother and cord blood. As neonates themselves cannot produce IgG, the authors concluded this case represent a human model supporting the premise that ANCA are pathogenic.

1.27 Neutrophil activation by ANCA

The first *in vitro* experiments demonstrating the potential pathogenicity of ANCA came from the laboratory of Falk et al., (1990). In this study Falk et al demonstrated that neutrophils can be activated by ANCA. ANCA sera from patients' with pauci-immune necrotizing and crescentic GN were obtained and the immunoglobulins purified. The authors observed that ANCA when incubated with normal human neutrophils induced a reactive oxygen species burst (ROS), followed by degranulation of the neutrophil. This phenomena was increased in a dose dependent manner. Further flow cytometry studies demonstrated that after priming neutrophils with the cytokine tumour necrosis factor (TNF) neutrophils exteriorised MPO to the cell surface. The surface location of MPO allowed interaction with ANCA. Prior to this study it was not known how ANCA could interact with the target autoantigen MPO when it was contained within cytoplasmic neutrophil azurophil granules.

Brouwer et al., (1994) further demonstrated both *in vitro* and *in vivo* that the numbers of activated neutrophils within kidney biopsies of patients with Wegeners granulomatosis correlated significantly with serum creatinine levels taken at the time of biopsy (22 PR3 positive and 5 MPO positive). All ANCA samples from patients (*n*=19) were capable of activating primed neutrophils, however no correlation was observed between the ANCA titre of patients and the numbers of activated neutrophils within renal biopsies. This was also the first study to demonstrate the presence of neutrophil enzymes MPO, PR3 and elastase (HLE) extracellularly within renal biopsies.

Studies in human ANCA demonstrated that ANCA IgG binds to the neutrophilic antigen and ligates the Fragment Cyrstalline gamma RIIa receptor (FCγRIIa). Porges et al., (1994) demonstrated that blockade of the FCγRIIa with a monoclonal antibody significantly reduced the production of ROS of ANCA activated neutrophils. The authors suggested binding of ANCA to the FCγRIIa receptor may be one mechanism in which human neutrophils activate signal transduction pathways which result in inflammation (Porges et al., 1994).

In addition to FCγRIIa other studies have also shown a role for FcγRIIIb in ANCA activation of neutrophils. Kocher et al., (1998) demonstrated that FCγRIIa requires a high density of ANCA binding whereas FcγRIIIb is expressed at 10x higher density than FcγRIIa on the cell surface of neutrophils. The author suggest these results are indicative of FcγRIIIb being involved in the initial engagement by ANCA and that cross linking of the FcγRIIIb favours adhesion of activated neutrophils to the endothelium.

Further *in vitro* studies demonstrated the role of cytokines in priming neutrophils for ANCA activation. Kettritz et al., (1997) further confirmed the requirement of cytokine priming of neutrophils with TNFα showing the translocation of both MPO and PR3 to the cell surface. The authors also determined which part of the human ANCA molecule was involved in the activation of neutrophils. Studies of both the binding and activating properties of ANCA, indicated that both intact ANCA and the $F(ab)_2$ portion of the Ig could cross link MPO or PR3 on the membrane surface of neutrophils. ANCA binding alone was not sufficient

enough to activate neutrophils, crosslinking of either MPO or PR3 on the cell surface was required to induce a ROS burst.

NEUTROPHIL EXTRACELLULAR TRAPS (NETS)

1.28 Introduction

Neutrophils, also known as polymorphonuclear leukocytes (PMNs) are bone marrow derived granulocytes which are the most abundant leukocyte within the circulation. Neutrophils are a key component of the innate immune system, and the first responder to sites of infection where their fundamental role is to phagocytose infectious bacteria, protozoa and fungi. Neutrophils are released from the bone marrow terminally differentiated where they are considered to have a short life span, within the circulation. Under normal conditions neutrophils are released into the circulation from the bone marrow where they permanently migrate into the circulation.

Until recently the current theory was that neutrophils are short lived, living up to 8-12 hours and an estimated 1-2 days within tissue. However, recently emerging new techniques to observe human neutrophils by labelling *in vivo* with $^{2}H_{2}0$ have revealed that they actually have a half–life of up to 5.4 days. The same study also demonstrated that murine and human neutrophils do not have the same half-life within the circulation, with murine neutrophils only having a half-life of 12.5 hours (Pillay et al., 2010).

During infection neutrophils are rapidly mobilised at a tenfold rate compared to normal conditions. Studies using both mice and human neutrophils have demonstrated the importance of the production of neutrophil chemoattractant chemokines CXCL1 and CXCL2 (in mice) and IL-8 (in humans), which are produced by endothelial cells and megakaryocytes. Granulocyte colony-stimulating factor (G-CSF) is produced in response to infection by many cell types and is influential in stimulating the proliferation and maturation of neutrophils (Gunzer, 2014). Once within the circulation neutrophils are recruited to the sites of infection in a structured manner referred to as neutrophil recruitment.

Through the use of intravital microscopy imaging we know that neutrophil recruitment and transmigration follows 3 basic steps. In the first step neutrophils within the circulation tether and roll on the surface of endothelial cells via selectins, P, E and L which play a role by reducing the speed of the neutrophil in the circulation which allows it to roll, the second signal comes from the neutrophil surface integrins β_1 and β_2 which interact with the ICAM-1 and ICAM-2 ligands on the inflamed endothelium, the rolling neutrophils now stop, and crawl along the endothelium mediated by integrins to the site of emigration, this normally occurs at the tricellular junctions of the endothelial cells, and sites dependent on the composition of the basement membrane, for example areas low in the expression of laminin or collagen will allow easier transmigration of the neutrophil. The bilobed characteristic shape of the neutrophils nucleus is thought to contribute to its pliable form that aids in allowing the neutrophil to squeeze through the endothelium. Once the neutrophils have gained access to the interstitial space they migrate towards the infectious stimulus, where they phagocytose the invading pathogen. Once the neutrophil has eliminated the infectious agent, it undergoes apoptosis through the activation of caspases and the release of reactive oxygen species (ROS) which marks the neutrophil for removal by macrophages to reduce the damage of the surrounding tissue that can be caused from the release of the neutrophil granules (Brinkmann & Zychlinsky, 2007; Gunzer, 2014; Mayadas et al., 2014).

Neutrophils kill bacteria by engulfing microorganisms which are packaged into a phagosome. This phagosome then fuses with lysosomal granules to form a phagolysosome where the microorganisms are killed by exposure to antimicrobial enzymes (i.e. MPO), anti-bacterial peptides and ROS. The formation of the phagolysosome is crucial to prevent the release of potentially damaging enzymes into the surrounding tissue. There are 3 different types of neutrophil granules. Primary granules (also known as azurophilic granules) store the powerful enzymes MPO, neutrophil elastase (NE), PR3, cathepsins, defensins and develop first, followed by the secondary granules (also known as specific granules) which contain lactoferrin, the tertiary granules develop at a later stage of maturation and contain

matric metalloprotease 9 (MMP9) also known as gelatinase B (Brinkmann & Zychlinsky, 2007; Lacy, 2006).

An alternative mechanism by which neutrophils kill microorganisms was discovered by Brinkmann et al and colleagues (Brinkmann et al., 2004). This study demonstrated that activated neutrophils were able to produce extracellular traps containing DNA fibres, coated with neutrophil proteins from the primary granules (MPO, NE, cathepsin G), secondary granules (lactoferrin), tertiary granules (MMP9), and histones (H1, H2A, H2B, H3 and H4 and the H2A-H2B DNA complex). This seminal study suggested that neutrophils produce NETs to amplify the effectiveness of their antimicrobial granules by producing a large net in a concentrated area, which formed both an antimicrobial and physical barrier to prevent the spread of microorganisms. The authors of this study very insightfully suggested that this mechanism may also have a detrimental effect on the host that could stimulate autoimmunity (Brinkmann et al., 2004). This initial work, opened a new field in neutrophil biology that in the last 13 years has expanded to define, the signalling pathways for NET production, identification methods of NETs, implication in autoimmune diseases and a major therapeutic target. The remainder of this section will concentrate on the areas of advancement since Brinkmanns original work, and the role of NETs in AAV.

1.28.1 NETs are an active form of death unique and distinct from apoptosis and necrosis

NET formation is a unique form of neutrophil cell death that it is not instigated by either necrosis or apoptosis. Live cell imaging of NETs by Fuchs et al and colleagues (Fuchs et al., 2007), in the Brinkmann lab revealed the basic steps required for NET formation. PMA stimulated peripheral neutrophils from healthy donors were imaged for 240 minutes, and the steps in NET formation were outlined to follow a pattern: Firstly 60 minutes after stimulation, neutrophil nuclear lobules begin to lose their shape, the nuclear envelope begins to disintegrate into small vessicles and chromatin decondensation begins with segregation of eu- and heterochromatin, 180 minutes after stimulation the nuclear envelop

has completely disintegrated, granular membranes rupture and the decondensed chromatin mixes freely with both the contents of the granules and cytoplasmic material, then the outer cell membrane ruptures and a protrusion of a net like structure comprised of DNA, histones, MPO, and NE is extruded (see Figure 2). The authors through elegant experiments determined that this form of cell death was neither apoptosis nor necrosis as DNA fragmentation a key marker in apoptosis was absent via TUNEL staining in NET formation. Necrotic cells did not make NETs, their nuclear membranes did not rupture, and the nucleus just fused into a homogenous mass with no apparent segregation of eu- and heterochromatin (Fuchs et al., 2007).

1.29 Reactive Oxygen Species, Citrullination of Histones and PAD4 are required for NET formation

1.29.1 ROS

Reactive oxygen species (ROS) is required for the formation of NETs. The most compelling evidence comes from patients whom have genetic deficiencies that cause mutations in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Chronic granulomatous disease (CGD) patients are unable to form ROS and as a result are unable to make NETs (Fuchs et al., 2007). These patients suffer from recurrent bacterial and fungal infections that they are unable to clear efficiently due to the lack of NADPH which is required for phagocytosis by granulocytes. Fuchs et al., (2007) were further able to determine that the addition of H_20_2 to CGD patients neutrophils *in vitro* enables neutrophils to produce NETs, suggesting that the sequence of events that allows NET formation can be restored downstream of NADPH.

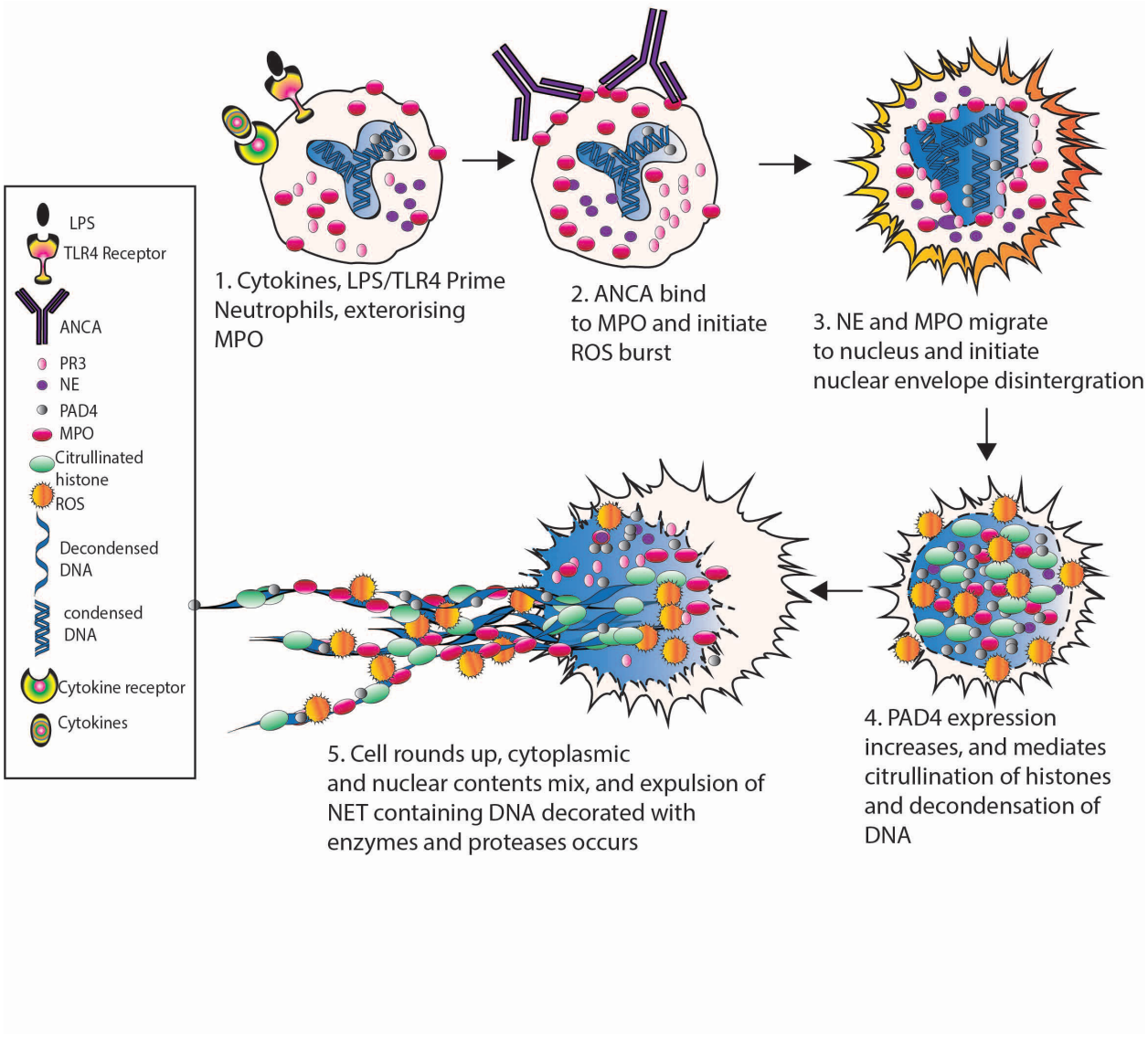


Figure 4. Steps Required for NET formation initiated by ANCA

(**1**) Neutrophils are primed by cytokines (such as TNFα, or LPS), which exteriorises MPO on the cell surface, (**2**) ANCA antibody recognise MPO as their antigen and bind to the surface of the neutrophil which causes a ROS burst (**3**)NE and MPO migrate to the nucleus where they break down the nuclear envelope (**4**) Increased PAD4 expression facilitates citrullination of histones, the contents of the nucleus and the cytoplasm mix as DNA decondenses (**5**) A NET of DNA is released decorated with MPO, NE, PR3, ROS and citrullinated histones.

1.29.2 Neutrophil elastase and Myeloperoxidase

Both NE and MPO are stored in the primary (azurophilic granules) of neutrophils, and are both found abundantly adhered to NET fibres. Experiments by Papayannopoulos et al., (2010) demonstrated that translocation of NE to the nuclear envelope was required for the decondensation of chromatin. After the initial ROS burst NE is released from primary granules where it migrates to the nucleus and digests nucleosomal histones, ultimately instigating the decondensation of the chromatin. MPO was found to also bind to chromatin and enhance decondensation independent of its enzymatic functions. The role of MPO in NET formation was further explored by members of the same lab by Metzler et al., (2011) in subsequent experiments and they found that neutrophils from 100% MPO deficient individuals are unable to form NETs (Metzler et al., 2011). Interestingly neutrophils form individuals with only a partial MPO deficiency were still able to make NETs. MPO contributes to 5% of the dry weight of neutrophils and is the most abundant neutrophil granzyme. MPO generates hypochlorous acid through the oxidation of chloride and halide ions in the presence of H_20_2 ,and is highly antimicrobial. Despite the requirement of MPO to generate NETs and its highly effective antimicrobial properties, a genetic deficiency in MPO does not appear to impact individuals with this defect, they generally live heathy lives.

The importance of MPO in regulating ROS was determined by Bjornsdottir et al., (2015) by demonstrating that MPO inhibitors successfully blocked PMA induced NET formation, and that this process could not be rescued downstream by adding extracellular MPO. Observations by other laboratories suggest the requirement for MPO in NET formation may be dependent on the activating stimulus. Rochael et al and colleagues determined that neither ROS nor MPO was required for NET formation when triggered by *Leishmania* parasites, but NE was required (Rochael et al., 2015). These findings were in agreement with earlier experiments by Parker et al and colleagues who found that MPO was required for NET formation when stimulated by PMA but not by *S.aureus* and *E.coli* (Parker et al., 2012). Overall these experiments do highlight the need for physiological relevant

experiments, although PMA is a potent NET inducer, it is not found physiologically in either humans or mice and caution should probably be used when using it to study signalling pathways, and assessing requirements for enzymes and ROS, as they may not be representative of what happens *in vivo.*

1.29.3 Role of peptidyl arginine deiminase (PAD4) in the citrullination of histones

One of the determining factors that distinguishes between neutrophil NET formation and either apoptosis or necrosis is the citrullination of histones mitigated by PAD4. Initial observations of the role of PAD4 and its association with deimination of histones in neutrophils came from experiments in animal models of rheumatoid arthritis where the expression of PAD4 and citrullinated histones correlated with an increase in disease severity (Lundberg et al., 2005). Neeli et al and colleagues observed in experiments with stimulated human neutrophils that NET formation was associated with deimination of H3, whereas apoptotic cells produced no deimination of H3, making citrullination of H3 a novel marker to distinguish between NET formation and apoptosis (Neeli et al., 2008). To investigate further the requirement of PAD4 in the citrullination of histones in NET formation Wang et al and colleagues demonstrated that the inhibition of PAD4 by the pan PAD inhibitor Cl-amidine failed to produce NETs in HL-60 granulocytes, through the inhibition of H3 citrullination (Wang et al., 2009). The same group followed up these experiments in PAD4-/- mice and demonstrated that PAD4 was required for NET mediated efficient bacterial killing. Neutrophils stimulated to form NETs by LPS, PMA and H_2O_2 *in vitro* all required PAD4. PAD4-/- neutrophils were unable to form NETs in response to any of the stimuli. Using a model of necrotizing fasciitis the authors demonstrated that PAD4-/- mice were unable to produce NETs and had a reduced capacity to clear bacterial infection than PAD4+/+ mice (Li et al., 2010).

1.30 Signalling pathways in NET formation

Several different pathways have been reported in the literature of signalling pathways in NET formation. The first of these pathways is known as the Raf-MEK-ERK pathway. The Raf-MEK-ERK pathway is responsible for controlling the expression of the anti-apoptotic protein Mcl-1. PMA stimulated neutrophils downregulate Mcl-1 immediately after stimulation, which blocks apoptosis and favours NET formation. PKC, cRaf and MEK inhibitors can block this downregulation, suggesting that the raf-MEK-ERK pathway is responsible for controlling NET formation. The authors of this study suggest this pathway could be a potential therapeutic target in diseases where aberrant NET formation occurs (Hakkim et al., 2011).

There has been speculation that NET formation could also be activated by the same pathway that causes necroptosis in neutrophils. Necroptosis is another form of cell death distinct from apoptosis. It is considered to be a programmed form of necrosis. The simplified version of the complex signalling pathway is that once activated a series of signals cause interaction between RIPK3 kinase which phosphorylates MLKL (mixed lineage kinase domain-like), which creates a conformational structural change allowing the oligomerization of MLKL to form a pore, which results in increased osmotic pressure within the cell as ion and water enter the cell resulting in rupture of the cell membrane (Jorgensen et al., 2017). Experiments using *Ripk3-/-* mice and MLKL inhibition by Desai et al and colleagues (Desai et al., 2016) demonstrated that this pathway is used downstream of ROS production. The authors used Nec-1 an inhibitor of necroptosis, which prevents the formation of the necrosome through the modulation of RIPK1 and the subsequent phosphorylation of RIPK3 and MLKL, which prevented the formation of NETs induced by PMA or monosodium urate (MSU) crystals in both human and mouse neutrophils. In direct contrast, Amini et al and colleagues found that NET formation occurred independent of signalling through RIPK3 and MLKL (Amini et al., 2016). Using the neutrophils from the genetically modified mice deficient in *Ripk3* and human neutrophils chemically inhibited with the MLKL inhibitor

necrosulfonamide (NSA), the authors conclude that NET formation is independent of a necroptotic form of death. The notable difference between these 2 studies could be dependent on the different stimuli used to activate and stimulate NET formation. Desai et al used MSU crystals, and PMA whereas Amini et al used GM-CSF primed neutrophils with C5a or LPS, or *E.coli*. As discussed in the previous section NET formation and the required signalling events can differ dependent on stimuli.

1.31 NETs in AAV

The seminal paper indicating an involvement of NETs in AAV came from a series of experiments conducted by Kessenbrock et al., (2009). In this study, the authors demonstrated that ANCA IgG from patients with small vessel vasculitis (SVV) was able to trigger NET formation in isolated neutrophils from healthy individuals primed with TNFα (23% of neutrophils formed NETs), whereas IgG from normal healthy individual was only able to stimulate 11% of neutrophils to form NETs. The authors then investigated whether the auto antigenic targets of this disease MPO and PR3 were present within NETs. They found that both MPO and PR3 were present upon the NET DNA fibres and they were still accessible on the NETs as demonstrated by the ability of purified ANCA IgG to bind to NETs. After establishing that ANCA could activate NET formation and that the auto antigenic targets MPO and PR3 were accessible within the NETs the authors sought evidence of *in vivo* NET formation with the biopsies of SVV patients. Using antibodies to NET components (DNA, histones, MPO, NE, PR3, and LL37), they found that NETs were present in 9/15 biopsies examined.

Further studies of NET involvement in AAV patients include case reports demonstrating NET formation through the use of antibodies against MPO, Citrullinated Histones and PAD4 (Yoshida et al., 2013), or using DAPI in combination with MPO to identify NETs (Nakazawa et al., 2012). A larger study containing 15 AAV patients (for peripheral neutrophil studies) and 6 kidney biopsies in China showed similar results to the Kessenbrock et al study, demonstrating enhanced NET formation in patients with AAV compared to healthy controls.

The authors found evidence of increased NET formation in the biopsies of patients with active crescentic GN but minimal NET evidence in the non-crescentic patients.

Attempts have been made by several research groups to show an association of circulating NET markers and active disease. In a large cohort of 93 AAV patients Soderberg et al., (2015) found that patients with active AAV had significantly more remnants of NETs than healthy controls within their circulation, and no difference between patients in remission and healthy controls. The authors found ANCA negative patients to have higher circulating NET remnants than ANCA positive patients. Of further interest was that in active disease there was a significant negative correlation of NET remnants and MPO-ANCA, and absent when PR3-ANCA patients and NET remnants were correlated. A year later a different group published that the level of circulating Neutrophil extracellular traps did not correlate with disease activity. In this cohort there were 34 patients with active AAV and 63 patients with AAV in remission (Wang et al., 2016). The authors found a significant difference in the numbers of circulating NETs between AAV patients and healthy controls using circulating cell free DNA (cfDNA) as a marker of NETs. They were unable to find any correlation between NET formation in the circulation of patients with active disease when compared to patient in remission. The authors however did find a significant correlation between the number of Cit-H3-DNA complexes in the circulation and in renal biopsies using H3Cit as a marker of NETs. This study also investigated the levels of DNase I an enzyme that specifically degrades DNA and found that patients with AAV had significantly higher circulating levels of DNase I than healthy controls whereas there was no observed difference between the active AAV and remission group. Although both these studies found a difference between healthy controls and active AAV, the difference in results suggests that circulating NETs is not a reliable marker of disease in AAV. The difference between studies could be attributed to the composition of PR3-ANCA patients compared to MPO-ANCA patients, with the latter study having considerably less PR3-ANCA patients.

1.31.1 MPO is biologically active in NETs

The bactericidal capacity of MPO in NETs has been shown *in vivo* to be biologically active when adhered to DNA remnants from NET formation. Parker et al measured the peroxidase activity of MPO release from NETs, and found that over 80% of the MPO released from the neutrophil came from NET formation, and was still biologically active (Parker, et al., 2012). The authors of this study also examined whether the bound MPO was able to kill *staphylococcus aureus* directly but found that the addition of H_20_2 to the media with NETs was required to kill the bacteria. The findings of these experiments have two implications. Firstly MPO adhered to NETs may aid in the bactericidal capacity of NETs and increase the Neutrophils role in reducing the spread of bacteria at sites of infection. The second implication, is that the MPO that is deposited is biologically active and may cause direct injury to the surrounding tissues and also become available as an autoantigen in autoimmunity, such as AAV.

1.32 NETs in other diseases

NETs have not only been implicated in AAV, but are also considered to have a non-redundant role in other autoimmune diseases such as systemic lupus erythematosus SLE, rheumatoid arthritis, gout, ulcerative colitis, Type I diabetes, and non-immune disorders such as sepsis, trauma, deep vein thrombosis, cystic fibrosis, preeclampsia, Ischemia/reperfusion induced acute kidney injury, coagulation, transfusion-related acute lung injury (TRALI), and periodontitis (Barnado et al., 2016; Brinkmann & Zychlinsky, 2012, Grayson & Kaplan, 2016; Malachowa et al., 2016).

The role of NETs in systemic lupus erythematosus (SLE) has probably been the most widely investigated disease in relation to NETs. There is more than one autoantibody implicated in SLE most of which are nuclear in origin, including double stranded DNA, histones, anti-nuclear antibodies (ANA), ribonucleoproteins and Smith antigen, several of which are present in NETs (DNA and histones). Neutrophil presence within skin lesions and glomerular neutrophil accumulation has been reported in the literature in renal

manifestations of SLE (lupus nephritis). Circulating peripheral neutrophils from SLE patients function abnormally, they have a reduced phagocytic ability, over express adhesion molecules and are predisposed to NET formation (Kaplan, 2011).

Peripheral circulating neutrophils from SLE patients can be divided into normal density neutrophils and low density neutrophils (LDGs) These LDGs are found in the blood of all adult SLE patients examined and are considered to be pathogenic due to their increased ability to produce type 1 interferons (IFNs), when stimulated with specific stimuli such as G-CSF, and their increased capacity to cause endothelial cell damage through NET formation. The presence of NETs within glomeruli of SLE renal biopsies is accompanied with an increased amount of anti-double stranded DNA within sera from the same patients (Villanueva et al., 2011). It has been proposed that there is an imbalance between the production of NETs and the ability to degrade NET remnants in SLE. This has been supported by further research demonstrating that some patients with SLE have a reduction in DNase I through an inherited mutation in the DNase I gene. A mutation in the DNase I gene which results in a decrease in the enzymatic activity of DNase I, and an increase in the amount of IgG against nuclear antigens (Yasutomo et al., 2001). It should be pointed out though, that this study examined 20 SLE patients of which only 2 had the DNase I mutation.

Reduced DNase I activity has also been observed in other studies of SLE patients (Martinez-Valle et al., 2009; Sallai et al., 2005; Tew et al., 2001). Studies in the 1990s in lupus prone mice (NZB/WF_1), found administration of recombinant murine DNase I was able to reduce proteinuria, and renal histopathology. These studies encouraged clinical trials in the therapeutic use of recombinant human DNase I in SLE, however treatment was unsuccessful probably due to the heterogeneous nature of the pathogenesis of SLE (Davis et al., 1999).

1.33 NETs transfer neutrophil antigens

Although the role of NETs in enhancing host defence against bacterial infection has been well established, the production of NETs also has potential to expose the immune system to potential self-antigens, and therefore may play a role in perpetuating inflammation by triggering autoimmunity.

The strongest evidence of the role of NETs instigating autoimmunity in AAV comes from animal models of the disease. Sangaletti et al.,(2012) demonstrated that neutrophil antigens from NETs can be transferred to dendritic cells (DCs). Live cell imaging of stimulated pro inflammatory neutrophils co-cultured with myeloid DCs (mDCs) showed that NET forming neutrophils formed stable connection with mDCs, whilst apoptotic neutrophils only formed infrequent contact with mDCs. To examine the possibility of the transfer of the antigens MPO and PR3, from dying neutrophils to mDCs, co- cultures of neutrophils and mDCs were observed and the efficacy of neutrophil antigen transfer characterised. Necrotic cells failed to transfer either MPO or PR3 at all, apoptotic neutrophils transferred both MPO and PR3, the antigens were internalised into the DCs within apoptotic bodies but less frequently than antigen from NET forming neutrophils. Addition of DNase I to the co-culture media prevented the transfer of NET associated antigens, by disintegrating the DNA back bone of the NETs, indicating that the intact DNA structure is required for successful transfer of the autoantigens. Based on these findings the authors of this study transferred the mDCs cultured with either NETs or apoptotic neutrophils into mice via intraperitoneal injection, weekly for a period of 6 weeks, collected serum and examined both the kidneys and lungs for pathology at the termination of the experiment 4 months later. Results demonstrated that the mDCs co-cultured with the NET forming neutrophils had an increased production of circulating ANCA, IgG and C3 deposition, and an increased kidney pathology score compared to the apoptotic neutrophils co-cultured with mDCs, which had a reduced amount of ANCA production and no evidence of vasculitis in either the lungs or kidneys.

This study demonstrated the pro-inflammatory effects of NET forming Neutrophils, and a minimal role for the induction of vasculitis via auto-antigens from apoptotic cells. During apoptosis the neutrophil antigens MPO and PR3 are translocated to the cell surface, so do provide a likely source for interaction with the immune system. The authors of this study conclude that apoptotic cells may induce the production of non-pathogenic ANCA, and that a pathogenic mechanism in AAV skews neutrophil cell death toward NETosis rather that apoptosis. The authors do not discuss the possibility that apoptotic neutrophils co-cultured with mDCs may actually have a protective effect as seen in other diseases such as type 1 diabetes (Marin-Gallen et al., 2010). DCs co-cultured with apoptotic bodies from β cells and injected into mice have been shown to induce a reduction in co-stimulatory molecules and production of cytokines in NOD diabetic mice (Marin-Gallen et al., 2010).

NETs not only exposed the autoantigens MPO and PR3 but also make histones available for interaction with the immune system. Histones themselves elicit strong responses from the immune system by activating DAMPS, recognised in particular by TLR2, TLR4, and NOD. Histones have been implicated in many other forms of disease such as sepsis (Xu et al., 2009), trauma associated lung injury (Abrams et al., 2013), sterile liver injury (Ammollo et al., 2011; H. Huang et al., 2013) and kidney injury (Allam et al., 2012).

A recent study by Kumar et al., (2015) found that targeting the histone disposition from neutrophil cell death via inhibition with the pad inhibitor Cl-amide or histone antibody depletion, reduced glomerulonephritis in a mouse model of anti GBM. A reduction in glomerular crescents, and leukocyte infiltration was significant, indicating that targeting extracellular histones could be an effective treatment in glomerulonephritis.

1.34 ETs formed by other cell types

The formation of extracellular traps is not specific for neutrophils, there are now numerous published studies reporting other leukocyte extracellular traps. Since the first report on NETs by Brinkmann et al., (2004), Mast cells, Eosinophils, and monocytes/macrophages

have been reported to also form extracellular traps. What is common between all cell types is the requirement for tight regulation of the process to ensure collateral tissue damage does not occur during the ET forming process.

Mast cells which are typically associated with allergy and hypersensitivity also form ETs (MCETs), (Branitzki-Heinemann et al., 2012; Scheb-Wetzel et al., 2014; von Kockritz-Blickwede et al., 2008). The extracellular traps are reliant on a ROS burst like neutrophils, and produce DNA web structures consisting of DNA, histones and granular contents from mast cell specific granules such as tryptase. The formation of the MCETs results in the death of the cell, but also increases its bactericidal and fungicidal capacity. MCETs have been shown to be effective in host response against *Candida albicans,* by physically containing the fungi within its DNA fibres (Lopes et al., 2015). MCETs have also been identified in autoimmunity, in psoriasis where both tryptase, chymase and IL-17 are exposed via ETosis rather than degranulation (Scheb-Wetzel et al., 2014).

Eosinophils are traditionally known to play a role in allergy and have been implicated in the pathogenesis of asthma. Eosinophils produce extracellular traps EETs and have been reported in defence against helminths through providing a physical barrier in which the larvae are immobilised to contain the spread of the infestation (Scheb-Wetzel et al., 2014). EETs have been implicated in triggering chronic obstructive pulmonary disease (COPD), which results in chronic inflammation of the airways. Echevarria et al., (2017), found that COPD patients had evidence of both NET and ET formation during different stages of COPD development.

Both monocytes and mature macrophages have been reported to produce macrophage/monocyte extracellular traps (METs). Like neutrophils, METs also can be identified to contain histone-DNA complexes, MPO and citrullinated histones (Chow et al., 2010; King et al., 2015; Wong & Jacobs, 2013). Where METs differ from NETs is the requirement of PAD4 for ET formation. In METs it appears that PAD2 plays a more significant role in the production of METs than that of PAD4 (Mohanan et al., 2013). MET

production has also been reported in COPD. Lung infections of nontypeable *Haemophilus influenza (NTHi)* are common in COPD patients. Activation of METs by NTHi expose protease metalloproteinase 12 (MMP12) and neutrophil elastase (NE), which exacerbate emphysema (King et al., 2015). .

1.35 NETs interactions with T cells

NETs have an established role in innate immunity, but less is known about their interactions with the adaptive immune system. *In vitro* studies of human NETs and T cells have indicated that NETs can prime CD4+ T cells by decreasing their activation threshold (Tillack et al., 2012). Co-culture of T cells and NETs alone increases the upregulation of the T cell activation markers CD25 and CD69, but does not increase T cell proliferation. Co-cultures of DCs, T cells and NETs, results in an increase in the activation of T cells, measured by the increased production of T cell proliferation cytokines IFNγ, IL-17A, IL-4, IL-10 and IL-2. This effect however is not seen when NETs alone are cultured with T cells, or in transwell plates where the DCs and NETs are separated from the T cells, indicating that T cell direct contact with both DCs and NETs is required for T cell activation (Tillack et al., 2012).

In vivo studies of NETs indicate they can activate Th17 cells, indirectly by priming macrophages in human cells and a murine model of atherosclerosis (Warnatsch et al., 2015). Co-cultures of monocytes with NETs activated by cholesterol cyrstals resulted in an increased production of monocyte cytokines (IL-1β). In a model of murine atherosclerosis, NETs were found associated with atherosclerotic lesions, which could be reduced when disease was triggered in NET deficient mice (APoE, PR3, and NE deficient) to prevent NET formation. The production of IL1β by macrophages increased the secretion of T cell derived IL17, and subsequent neutrophil recruitment to vascular endothelium (Warnatsch et al., 2015).

1.36 Extracellular DNA is pro-inflammatory

One of the major components of NETs is the backbone DNA structure. Within NETs this backbone of DNA provides the substrate that the neutrophil enzymes, proteases and histones adhere to. Although the production of NETs helps to fight infection by containing the spread of infectious bacteria, the DNA itself can cause inflammation through activation of DNA sensors, and histone exposure (as discussed in previous section). DNA is released from dying cells through several different mechanisms including apoptosis, necrosis, necroptosis and pyroptosis. Besides the different signalling pathways involved and pathological appearance of these types of cell deaths, the size of the DNA released during death differs also.

DNA derived from NETs can activate the immune system by acting as a danger signal. The nuclear location of DNA offers protection from exposure to the immune system under normal healthy conditions. Self-DNA which is released extracellularly during either pathogenic processes or autoimmunity is seen by the immune system as a DAMP where as non-host DNA is recognised by PRRs. DNA which escapes the nucleus, through non homeostatic mechanisms is detected by several different pathways, due to the location of the particular DNA sensor. TLR9 recognition of DNA occurs within endosomes. DNA sensors via TLR-9 in endosomes, within the cytoplasm where it is detected by three prime repair exonuclease 1 (TREX1), or via the cyclic GMP-AMP synthase pathway (cGAS) which activates the adapter protein Stimulator of Interferon Genes (STING). The different DNA sensors also can control which pathway of cell death may occur. For instance extracellular DNA activation of TLR9 will induce apoptosis, activation of RIP3 will activate necrosis and NETosis, caspase 9 will trigger apoptosis, and activation of AIM2 will induce pyroptosis (Jorgensen et al., 2017).

Apoptosis, is a type of programmed cell death which is caspase dependent. The nucleus during apoptosis blebs and fragments with considerable chromatin condensation, and nucleases cleave and degrade DNA, which results in the death of the cell. The subsequent

DNA released is of low molecular weight and size, presumably to enable efficient phagocytosis (Wyllie et al., 1980). “Eat me” signals displayed on apoptotic cells ensure quick removal, before a dying cell can progress to a secondary necrotic cell, which can instigate a pro-inflammatory reaction. As apoptotic cells are cleared quickly, there is minimal DAMP release and therefore they are considered to be immunologically inert. Considering programmed cell death occurs as part of normal cell turn over, it is important auto reactivity to host cell death does not occur (Sauter et al., 2000).

In contrast to apoptosis, necrotic cell death results in large intact extracellular DNA release, and is caused by direct injury/trauma to the cell or pathological mechanisms. The release of extracellular DNA from necrotic cell, signals danger in the form of DAMPS such as histones and DNA. Necrosis is characterised by rupture of the cell plasma membrane, swelling of cytoplasmic contents, and irreversible damage to intracellular organelles, and release of condensed DNA (Fiers et al., 1999). As discussed in the previous section necroptosis is a regulated form of necrosis, that is mediated by caspase 8 and RIPK3 signalling. Necroptosis activated inflammation causes a mass release of DAMPs into the extracellular environment such as High Mobility Group Box 1 (HMGB1) and pro-inflammatory cytokines such as TNFα, IL-1α and IL-33 (Pasparakis & Vandenabeele, 2015).

Pyroptosis, is a form of programmed cell death that is characterised by caspase I or 11 dependent formation of pores in the cellular membrane, which results is osmotic swelling of the cell, nuclear condensation and eventual cell lysis which releases the cellular contents into the extracellular environment (Fink & Cookson, 2006). Pyroptosis in triggered by activation of the inflammasome, cytosolic sensors which caspase 1 cleaves into pro-IL1β and pro-IL-18. Double stranded DNA released in the cytosol is recognised by absent in melanoma 2 (AIM2), which activates the adapter protein Apoptosis Associated Speck Like Protein Containing Card (ASC), followed by Caspase I cleavage of pro-IL1β and pro-IL-18.

A dysregulation in the clearance of dead or dying cells in any of these types of cell deaths will result in an inflammatory response, or possible instigation of auto- reactivity against self-proteins.

1.37 Vital and mitochondrial NETosis

Neutrophils are also able to produce NETs which do not ultimately end in death of the neutrophil. This has been termed “vital NETosis”, and uses some of the same pathways as traditional “suicidal NETosis”, such as the translocation of NE to the nucleus, activation of PAD4 and decondensation of chromatin. Instead of extruding all the nuclear contents as in suicidal NETosis, in vital NETosis, the DNA is released within vessicles, and the neutrophils phagocytic functions remain intact (Yipp et al., 2012). *In vivo* imaging of neutrophils during gram-positive skin infections, have demonstrated that NET forming neutrophils can still effectively crawl despite being anuclear, and still have the ability to kill bacteria via phagocytosis (Yipp et al., 2012).

Mitochondrial NETosis has also been observed, like normal NETosis it is ROS dependent. Mitochondrial nets have been formed *in vitro* in response to stimulation by GM-CSF and C5a (Yousefi et al., 2009). Recently mitochondrial NETosis has been demonstrated in low density neutrophils from lupus nephritis patients (Lood et al., 2016). The mitochondrial formed NETs were pro-inflammatory and were able to induce production of type 1 interferons. Using MRL/*lpr (*lupus prone mice), it was shown that the *in vivo* administration of MitoTEMPO an inhibitor of mitochondrial ROS was able to reduce NETosis, and ameliorate autoimmunity in the MRL/*lpr* mice (Lood et al., 2016).

1.38 Targeting NETs as therapy in inflammatory diseases

There are 2 potential candidates for targeting NETs as therapy in autoimmune diseases, Cl-amidine (and the second generation BB-Cl amidine), a pan PAD inhibitor and DNase I which specifically targets and clears DNA. Multiple studies have shown a protective effect in autoimmune diseases when PAD4-/- animals have been used, so it would be likely that

a PAD inhibitor would have some effect in ameliorating autoimmunity in diseases in which NETs are likely to play a role such as lupus (Knight et al., 2015) , atherosclerosis (Knight et al., 2014) arthritis (Ghari et al., 2016; Kawalkowska et al., 2016), diabetes (Wong et al., 2015) and AAV (Kusunoki et al., 2016). Although Cl-amidine and BB-Cl amidine which has a longer half-life *in vivo* has been beneficial in animal models, there are concerns that in human diseases that the inhibition of all the PADs not just PAD4 which is implicated in NET formation could have a detrimental effect in patients by increasing their susceptibility to infections.

DNase I, alternatively has been shown to be safe and tolerable in a clinical trial in lupus, although not effective in treating the disease possibly due to the heterogeneity of the disease pathogenesis in Lupus, and the disease progression not reliant on immunoreactivity to DNA alone. However valuable information on the safety and side effects can be taken from the trial, and applied to other forms of autoimmunity where extracellular DNA may play more of a pivotal role. (Davis et al., 1999). DNase I is an ideal treatment as it would not only target NET DNA but DNA from other types of cell death where DNA is released into the extracellular environment such as apoptosis, necrosis and pyroptosis. DNase I is limited as treatment, as it only dismantles the NETs after they have formed, and there has been reports that some NET remnants are left behind in vessel walls such as histones and proteases (Kolaczkowska et al., 2015). However DNase I as part of a combination therapy in autoimmune diseases with a lesser dose of steroids may be effective in decreasing the unwanted side effect from long term high dose steroids.

1.39 NETs as a source of host DNA in adjuvants

Aluminium hydroxide (Alum), is commonly used as an adjuvant in human vaccines. Although it is well recognised that Alum elicits a Th2 response through the production of the Th2 specific cytokine IL-4, the actual mechanism of Alums adjuvant property is not fully understood (Brewer et al., 1999). The pro-inflammatory properties of extracellular DNA act as DAMPS which mediates Alum based adjuvant activity. The role of extracellular DNA

behaving as an adjuvant in Alum vaccination was confirmed by giving DNase I which reduced antigen specific CD4 T cell specific responses and humoral immunity (Marichal et al., 2011). Recent studies on the role of host extracellular DNA behaving as an adjuvant in alum immunisation have highlighted it is DNA from neutrophil swarming and deposition of DNA from NETs at the site of injection, that account for the source of DNA (Stephen et al., 2017) To further confirm the role of NET DNA as an adjuvant, Pad4-/- mice (NET deficient mice), were immunised with alum, but failed to produce DNA from NETs at the immunisation sites, and had a significant reduction in antigen specific T cells and humoral immunity through a reduction in the production of IgG_1. Without NET deposited extracellular DNA, PAD4-/- mice were unable to produce pro inflammatory DNA to act as an adjuvant in Alum immunisations (Stephen et al., 2017).

EXPERIMENTAL MODELS OF GN AND AAV

Human studies of AAV allow us to make observations and clinical correlations. However to isolate the effects of different components of the pathogenesis of disease, requires the use of animal models of glomerulonephritis and AAV. By using animal models, we can look at different stages of disease progression, the initiating factors, progression of disease, and identify potential therapeutic targets. These models allow the conduction of experiments where chimeric animals can be produced to isolate the effects of certain molecules or cell populations. Of great clinical and translational significance, is the use of animal models of disease to test potential therapeutic agents and biologicals. In this review, I will cover the standard accepted models for crescentic glomerulonephritis and ANCA vasculitis, highlighting their individual strengths and potential weaknesses.

1.40 Experimental anti-glomerular basement disease glomerulonephritis

Experimental anti glomerular basement disease glomerulonephritis (anti GBM GN), is often referred to in literature as nephrotoxic nephritis (NTN). It has been used in many different experimental species (mice, rats, and rabbits). This model relies on the production of an antibody raised against GBM proteins. In our laboratory we use an antibody raised in sheep against mouse renal cortex, but it is not uncommon to also use rabbits or horse immunised with GBM proteins. The disease induced in animals exist in two distinct phases, termed the heterologous and autologous phase of injury. The firsts phase, the heterologous phase occurs when the foreign antibody binds to the GBM, initiating glomerular injury and inflammation. Neutrophil recruitment occurs within 2-4 hours of immunisation causing a transient peak in proteinuria which subsides after 24 hours. The resulting immune response to the heterologous anti GBM globulin is dose dependent, the amount of antibody administered will determine if the second autologous phase is initiated. The autologous phase of injury, happens a week after the induction of the heterologous phase, and involves the adaptive immune response. This is characterised by the development of an immune response to the anti GBM antibody. Autologous antibody is deposited in the glomerulus and the characteristics of a Delayed Type Hypersensitivity (DTH) immune reaction occur with the recruitment of macrophages and T cells, which cause a severe form of crescentic glomerulonephritis (Hammer & Dixon, 1963; Odobasic et al., 2014).

Another form of this model, referred to as the accelerated model of anti-GBM GN, relies on a pre-immunisation with the heterologous globulin antigen. For example if the anti GBM antibody was raised in sheep, mice are immunised subcutaneously in adjuvant with normal sheep Ig several days prior to anti GBM administration (4 days in our laboratory). This step sensitizes the mice, prior to receiving the anti GBM antibody. The advantage of the accelerated form of the model, compared to the previously described non accelerated model, is a rapid induction of GN, allowing less anti GBM antibody to be used, and an immediate autologous phase which results in a shorter experimental model. Disadvantages

of both models, is the variability between batches of anti GBM antibody. Dose testing of each new batch has to be carried out to ensure, premature death of the animal does not occur. There is a fine line between establishing anti GBM GN and death. In the accelerated model, there is also an increased risk of anaphylaxis after administration of the anti GBM antibody (Odobasic et al., 2014). Caution must also be used with genetic backgrounds of the mice used in the model. C57/Bl6 mice are more susceptible to GN disease induction and is characterised by recruitment DTH effector cells (Huang et al., 1994). Where GN induction in BALB/c mice is caused by autologous antibody, complement and neutrophils (Huang, et al., 1997).

1.41 Experimental autoimmune glomerulonephritis

This model occurs by immunisation with isologous GBM in CFA to induce autoimmunity to the GBM. The most widely characterised version of this model uses human collagenase solubilised GBM in Freunds Complete Adjuvant (FCA) (Steblay, 1962) or recombinant α3 chain of type IV collagen (α3(IV)NC1 (Abbate et al., 1998). Induction of disease with either, results in a linear deposition of anti GBM antibody, similar to what is seen in the human form of the disease (Hopfer et al., 2003). Disadvantages of this model, is the lack of reproducibility in mice, where disease develops at a slower rate, (7-8 weeks) (Hopfer et al., 2003).

1.42 Experimental models MPO-ANCA-GN

The majority of experimental models of MPO-ANCA GN are induced in either rats or mice. The first animal model described in literature was a model of experimental vasculitis by Mathieson et al (1991). This model used subcutaneous injections of mercury chloride ($HgCl_2$) to rats over a 10 day period. This resulted in injury within 24 hours which became more severe as time points increased to termination of the experiment at 2 weeks. Leukocyte infiltration, and inflammatory changes were seen, in the lungs, liver duodenum and caecum. The same group went on to describe the development of antibodies to MPO in this model

(Esnault et al., 1992). Further experiments with this model, showed that depletion of neutrophils with a monoclonal antibody, reduced the tissue injury, indicative of neutrophils playing a major role in the development of vasculitis induced by $HgCl_2$ (Qasim et al., 1996). Unfortunately this model not only induced the production of anti MPO antibodies, but a wide spectrum of antibodies making it difficult to attribute disease manifestations directly to the production of anti MPO antibodies.

Brouwer et al generated a model of MPO-ANCA-GN by perfusing rat kidneys with rat anti MPO Ig (generated by immunizing rats with human MPO) which cross react with rat MPO (Brouwer et al., 1993). This was followed with unilateral perfusion of the kidney with neutrophil antigens MPO and PR3, neutrophil elastase and hydrogen peroxide. Rats immunised with anti MPO antibody, developed severe necrotising crescentic glomerulonephritis (NCGN) with glomerular crescent formation, disruption in Bowmans Capsule accompanied with interstitial leukocyte infiltration. Although, MPO-ANCA GN in the human disease is termed “pauci immune”, this model of the disease demonstrated transient MPO, C3 and IgG deposition lining the GBM at a 24 hour time point, which subsided on day 4 and 10 after perfusion. This lead the authors to conclude that ANCA associated GN is possibly initiated by immune complex deposition in the initial stages of diseases, these immune complexes are then degraded by the recruitment of activated neutrophils, accounting for the “pauci immune” effect we see in human AAV. The same group further demonstrated that inducing clamp renal ischemia/reperfusion injury in anti MPO immunised rats instead of perfusing with hydrogen peroxide contributed to renal damage in the same model. This effect was thought to occur due to the production of reactive oxygen species by endothelial cells (Brouwer et al., 1995). These experiments were repeated by a different group (Yang et al., 1994), in spontaneously hypertensive rats, which developed severe lesions characterised by segmental necrosis, glomerular crescent formation, mesangial expansion, consistent with a proliferative form of glomerulonephritis. Unlike Bouwer et al these mice still had MPO, IgG and C3 deposition evident on day 4 and 10.

Heeringa et al., (1997), extended the findings of Brouwer et al, and immunised rats with MPO in FCA supplemented with mycobacterium tuberculosis (H37RA) subcutaneously, to induce an immune response to MPO. Two weeks later rats, were administered human neutrophil lysosomal extract, through the jugular vein. Rats developed antibodies to MPO, and granuloma formation and evidence of vasculitis in both the lungs and small intestines, whereas no other organs were effected. Although this model did not cause vasculitis in the kidney it demonstrate that MPO antibodies in the presence of neutrophil contents may contribute to the disease pathogenesis.

Kobayashi et al., (1995) raised rabbit anti Rat MPO antibodies and immunised rats prior to administering nephrotoxic serum to induce anti GBM GN, mice were culled at different time point (3 and 15 hours and 14 days after administration of nephrotoxic serum). Although there were an increased infiltration of neutrophils and fibrin deposition in these mice, glomerular crescent formation or segmental necrosis failed to develop. This is possibly due to the strain of experimental rats used (Wistar rats) which are less susceptible to crescent formation. What the results from these experiments did demonstrate was the effect of anti MPO antibodies and nephrotoxic serum in recruitment of neutrophils to the glomerulus.

Heeringa et al., (1996) followed up their previous experimental models, by first giving MPO subcutaneously in FCA (and H37A) followed by a subsequent sub-nephritogenic dose of rabbit anti rat GBM antibody intravenously 2 weeks later, instead of neutrophil extracts used in the groups previous model. The control group received only anti GBM serum, and control immunisation (containing acetate solution). This model resulted in significant renal injury in the group that had received MPO immunisation, evident by increased proteinuria, glomerular crescent formation, segmental necrosis, and tubulointerstitial damage. These experiments gave the first evidence that antibodies to MPO, were able to exacerbate anti GBM disease, indicative of a potential pathogenic role for ANCA antibodies.

Xiao et al., (2002) immunised MPO-/- with MPO, to generate an immune response to MPO. Splenocytes were removed from these mice and passively transferred intravenously into

recombinase-activating gene 2-deficient (RAG2$^{-/-}$) mice, which lack functioning B and T cells. Mice receiving either 1x10^{8} or 5x10^{7} splenocytes developed severe necrotizing, crescentic GN. This model however does not isolate the effect of MPO antibody alone as pathogenic anti MPO T cells are likely to of been generated and transferred with the splenocytes, into recipient mice. To isolate the effect of anti MPO antibody alone, purified anti MPO-IgG was administered to RAG2-/- mice, resulting in a mild pauci immune type of glomerulonephritis. This demonstrated that MPO antibody alone was sufficient to induce glomerulonephritis, without an adaptive immune response indicative of a pathogenic role for ANCA. Further studies using the same model of anti MPO GN, demonstrated that neutrophils are the key cell in the development of anti MPO GN. Xiao et al., (2005) prevented the development of renal injury through administration of a neutrophil depleting antibody.

Infections are known to trigger and exacerbate ANCA vasculitis (Tidman et al., 1998), based on this premise Huugen et al., (2005) administered an intraperitoneal dose of LPS as a proinflammatory stimulus, one hour after injection of the anti MPO antibody. Mice culled 6 days later had a significant increase in anti MPO titre when administered 5µg/g LPS and anti MPO antibody, compared to mice given anti MPO antibody alone. At 6 days significant haematuria, crescent formation, necrosis, and glomerular infiltrates of macrophages and CD45+lecukocytes, was evident when given a LPS dose of 0.5µg/g or 5µg/g and anti MPO antibody compared to control mice given anti MPO antibody alone. Mice culled 1 day after disease induction demonstrated significant number of glomerular neutrophils when given anti MPO antibody and LPS (0.5µg/g). In a separate experiment blood sample were taken 1 week prior to administration of LPS, 1 hour after and 6 days later, to measure the effect of LPS on MPO and TNFα levels. Serum TNFα were increased 1 hour after administration of LPS, and undetectable at the latter time points. Whereas serum MPO peaked on day 1 after the administration of LPS. Pre-treatment of mice at day -1 with TNFα neutralising antibodies resulted in a significant reduction in glomerular crescent formation, but had no effect on the percentage of glomerular necrosis, haematuria. leukocyturia, or creatinine

levels. Although a mild protective effect was observed by neutralising TNFα, these results are indicative of TNFα independent mechanisms occurring in anti MPO GN.

The studies of both Xiao et al and Huugen et al draw attention to the variability in the administration of anti MPO antibodies to induce MPO-GN in mice. Although both studies induce crescentic glomerulonephritis, there was a discrepancy in the degree of severity between the two studies. Different levels of crescent formation and ANCA titre were observed between the two studies, with the latter Huugen study showing less severe GN. These difference could be due to the route of administration chosen for delivery of the anti MPO GN (intravenously versus intraperitoneal) or the variability in the batches of MPO antibody used to induce disease.

Little et al., (2005) utilised a glomerulonephritis susceptible Wistar Kyoto (WKY) rat model of experimental autoimmune vasculitis (EAV) developed within their laboratory, to demonstrate *in vivo* evidence of the role of ANCA in anti MPO GN. WKY rats are immunised intramuscularly with human MPO in FCA. Six weeks post immunisation high titre of serum ANCA and pauci immune necrotizing crescentic glomerulonephritis are observed, clinical features that are consistent with human AAV. Anti hMPO antibody generated in the sera of hMPO immunised mice, was pooled and the Ig fraction purified using ammonium sulphate precipitation. Intra vital microscopy on mesenteric vasculature at the 6 week time point demonstrated that there was no difference in leukocyte adhesion or transmigration between the hMPO immunised rats and the controls. Administration of topical CXCL1 (a neutrophil chemoattractant) in the hMPO immunised mice demonstrated a significant increase in the numbers of leukocyte adhesion and migration. This provided evidence that ANCA in an inflammatory environment could potentiate leukocyte transmigration and therefore be instrumental in participating in the pathogenesis of AAV. Intravital microscopy of the anti hMPO administered rats demonstrated a significant increase in leukocyte adhesion but not transmigration. Administration of topical CXCL1, demonstrated a similar effect to what was observed in the hMPO immunised mice, with both leucocyte adhesion and transmigration

significantly advanced. This provided *in vivo* evidence of the potential of ANCA in a proinflammatory environment to instigate the pathogenesis of AAV.

Schreiber et al., (2006) demonstrated the crucial role of neutrophil MPO expression in a mouse model of AAV. This model uses MPO-/- mice, immunised with murine MPO which are irradiated to deplete endogenous bone marrow cells. $MPO^{-/-}$ mice, or $MPO^{+/+}$ mice are then reconstituted with either $MPO^{+/+}$ bone marrow cells or $MPO^{-/-}$ bone marrow cells. Chimeric mice consisting of $MPO^{+/+}$ BM into $MPO^{-/-}$ mice immunised with MPO induced a necrotising crescentic GN, whereas irradiated WT mice reconstituted with $MPO^{-/-}$ BM, did not develop NCGN, due to the lack of the auto antigenic target MPO. These experiments provided evidence that MPO is required for the establishment of NCGN, and that ANCA are likely to play a pathogenic role. This experimental model of anti MPO GN, also has increased numbers of glomeruli with crescents, high MPO titre, and a paucity of antibody deposition in glomeruli, which mimics the human manifestation of AAV. Disadvantages of this model, would be the high degree of technical expertise and reproducibility.

None of these previous models, had addressed the contribution of cell mediated immunity. Ruth et al., (2006) in our laboratory developed a model to assess the role of MPO specific CD4 T cells. Mice were immunised with 10µg of hMPO subcutaneously in FCA to establish autoimmunity to MPO, as this immunisation does not result in the development of glomerulonephritis alone, a sub-nephritogenic dose of anti GBM is administered intravenously 10 days later, to recruit neutrophils to the glomeruli where they deposit MPO, and trigger disease. Four different time points were assessed (day 4, 5, 7 and day 10 after anti GBM delivery) to determine which time point would generate a sufficient number of crescents in the hMPO immunised mice compared to the control BSA immunised mice. Day four was considered to be the optimal time point to terminate the experiment as at this time point, minimal IgG deposition was evident with in glomeruli indicative of a reduced autologous immune response to the anti GBM serum administered to trigger disease. The hMPO immunised mice in this model had significant development of glomerular crescents,

and recruitment of CD4+ T cells, macrophages and neutrophils, compared to MPO-/- mice who had similar immune responses but a reduced recruitment of effector cells and crescent formation. This was indicative of planted glomerular MPO in this model acting as the auto-antigenic target in this disease. Further experiments in the same experimental model using a depleting CD4 antibody, demonstrated a role for MPO specific CD4 T cells in the disease pathogenesis. Mice that were depleted of CD4 T cells, had reduced crescent and effector cell glomerular recruitment. Whereas the same model in B cell depleted mice developed crescents and recruited effector cells, despite the lack of antibody. This model, establishes autoimmunity to MPO using sub-nephritogenic doses of anti GBM, which allows initiation of glomerulonephritis through the glomerular recruitment of neutrophils which deposits MPO that is recognised by MPO specific CD4 T cells, which initiate recruitment of DTH effector cells and the subsequent GN. In this model, both cellular and humoral responses to MPO are generated, however the generated anti MPO antibody is not sufficient enough to recruit glomerular neutrophils to initiate disease. The establishment of autoimmunity followed by administration of the anti GBM sera in this model allows cell mediated immunity to be isolated without the requirement for ANCA antibody. The limitation of this model is that experimental mice cannot be studied beyond 4 days due to the generation of the host's immune response to the foreign anti GBM sera, which will create another subsequent phase of glomerular injury, not initiated by anti MPO induced responses.

EXPERIMENTAL MODELS OF PR3 ANCA-GN

The role of MPO-ANCA in the pathogenesis of AAV has been established, whereas there is a lack of evidence for PR3-ANCA pathogenicity in animal models of the disease. The largest confounding factor for establishing animal models of experimental PR3-ANCA-GN, is the dissimilarity between human PR3 and rodent PR3, and the failure of human PR3 to cross react with murine PR3. Experimental attempts at immunising mice with either human or murine PR3 have failed to generate ANCA associated vasculitis in animal models. In recent years some laboratories have had some success in generating models of PR3-ANCA. Primo et al (2010), have shown that transfer of splenocytes from rmPR3 immunised C57/BL/6 mice to RAG1-/- mice (lacking functioning B and T lymphocytes) are unable to generate antibody to PR3. However, the authors of this study were able to break tolerance to PR3 in non-obese diabetic (NOD) mice, which did not generate disease. Splenocyte transfer for rmPR3-immunised mice into NOD severe combined immunodeficiency (SCID) mice, did generate glomerulonephritis characterised by glomerular crescent formation, fibrinoid necrosis and protein casts. The disadvantages of this model, is the significant difference between mouse and human PR3. Murine neutrophils do not express PR3 on their cell surface, and therefore unable to provide a cell surface antigenic target for circulating ANCA. This dissimilarity potentially renders experiments in murine models redundant due to the difference in pathophysiology of PR3-ANCA disease between human and mice.

Little et al (2012) circumvented the lack of murine neutrophil cell surface PR3 expression by the generation of mice with a humanised immune system in order to study the potential pathogenicity of anti PR3 antibodies *in vivo.* Irradiated immunodeficient NOD-SCIDIL12Rγ-/- , were reconstituted with human hematopoietic stem cells (HSCs). After 6 weeks flow cytometry analysis of the chimerism demonstrated evidence of successful reconstitution with human lymphocyte, neutrophils and monocytes. Purified human PR3 IgG was passively transferred into the chimeric mice, which resulted in glomerulonephritis 6 days

after injection, and lung haemorrhage. This provided convincing proof of concept that anti PR3 antibody plays a pathogenic role in potentiating disease in AAV.

IMMUNE MEDIATORS OF GN

1.43 T cells

T cells play a pivotal role in cell mediated immunity, and can be divided into different subsets based on the cell surface markers and effector molecules each subset produces. T cells are not terminally differentiated but exhibit plasticity in response to different microenvironments. Broadly speaking the subsets can be divided into CD4 T cells, CD8 T cells, NKT cells, and γδT cells. For the purpose of this review I will briefly explain the function of each subset, and the known associations with ANCA vasculitis.

1.43.1 Characterisation of CD4 T cells

CD4 T cells can be further polarised into several subtypes based on their cell surface markers and effector functions. T_H1 CD4+ T cells, are controlled by the nuclear transcription factors T-bet, STAT4, and STAT1. T_H1 CD4+ T cells secrete IFNγ, IL-2 and LTα, and are induced by IL-12. The primary function of T_H1 CD4+ T cells is to provide protection against intracellular pathogens through the production of the effector molecule IFNγ, which recruits and activates macrophages, to phagocytose and destroy the pathogenic micro-organisms (Zhu et al., 2010).

T_H2 CD4+ T cells, are regulated by the nuclear transcription factors GATA3, STAT6, DEC2, and MAF. The maintenance and regulation of T_H2 CD4+ T cells is dependent on IL-4, IL-33 and IL-25. T_H2 CD4+ T cells produce the effector cytokines IL-4, IL-5, IL-13, and IL-10. The primary role of T_H2 CD4+ T cells is to facilitate the elimination of extracellular parasites (Nakayama et al., 2017).

T_H17 CD4+ T cells are controlled by the transcription factors RORγt, STAT3, RORα. T_H17 CD4+ T cells are generated from naïve T cells in the presence of TGFβ and IL-6, and require

both IL-23 and IL-21 for cell maintenance. T_H17 CD4+ T cells are primarily involved in defence against fungi and extracellular bacteria (Burkett et al., 2015).

CD4 +T regulatory cells can be divided into 2 further sets, Natural T_{reg} Cell and Inducible T_{reg} cells. Natural T_{reg} cells are characterised by the cell surface markers αβ TCR, CD4, CD3, CD25 , GITR ,CTLA4, and the nuclear transcription factors FOXP3, STAT5, FOXO1, FOXO3. Natural T_{reg} cells produce the effector cytokines IL-10, TGFβ, IL-35 and are generated in the thymus, where they are involved in tolerogenic responses. Inducible T_{reg} cells are characterised by the same cell surface markers as Natural T_{reg} cells markers (αβ TCR, CD4, CD3, CD25, GITR, CTLA4) but can be controlled by some additional transcription factors FOXP3, FOXO1, FOXO3, STAT5, SMAD2, SMAD3, and SMAD4. Inducible T_{reg} cells produce the cytokines IL-10 and TGFβ, These cells are found in the periphery where they are produced from naïve CD+ T cells in response to TGFβ and IL-2 (Ohkura et al., 2013).

$T_{FH}22$ CD4+ T cells are characterised by the cell surface markers αβ TCR, CD4, CD3, CXCR5, PD1, , OX40L, CD40L, ICOS, IL-21R, SLAM and the transcription factors BCL-6, STAT3. $T_{FH}22$ CD4+ T cells produce the cytokine IL-21. $T_{FH}22$ CD4+ T cells are involved in the selection of high affinity B cells with in germinal centres of lymph nodes (Ma & Deenick, 2014).

T_H9 CD4+ T cells are characterised by the cell surface markers αβ TCR, CD4, CD3 and the transcription factor PU.1. T_H9 CD4+ T cells produce the effector cytokines IL-9, IL-10. The primary function of T_H9 CD4+ T cells is defences against extracellular parasites (Kaplan et al., 2015).

For the purposes of this review I will concentrate on the role of the T_H effector cells T_H1 and T_H17 and T regulatory cells.

1.43.2 The role of CD4 T_H1 cells in experimental crescentic glomerulonephritis

An overreaction of the immune system to an antigen the host has been pre-sensitized to can provoke a hypersensitivity reaction. Each type of hypersensitivity reaction can be categorised according to the type of immune response provoked by exposure to the antigen. Type I is Allergy and is mediated by IgE (eg. anaphylaxis), Type II is Cytotoxic and antibody dependent, and is mediated by IgM, IgG or complement (eg.Good pastures syndrome and Graves' disease), Type III Immune Complex disease is mediated by IgG, Complement and neutrophils, (eg. rheumatoid arthritis, membranous nephropathy, SLE), Type IV delayed type hypersensitive (DTH), is cell mediated, and antibody independent, is mediated by CD4 T_H1 cells in response to re-exposure to an antigen and results in recruitment of macrophages and production of the cytokines (IFNγ, IL-2), which induces an inflammatory response, Type V is autoimmune and is mediated by IgM, IgG and complement (eg. Graves' disease) (Gell, 1963).

The experimental model of crescentic GN, is used to replicate human crescentic GN. The murine model of anti GBM GN is induced by administration of antibodies raised against the glomerular basement membrane (GBM), and is commonly referred to as nephrotoxic serum nephritis (NTN). The heterologous phase of injury is induced by administration of a heterologous antibody commonly raised in either sheep (sheep anti mouse GBM) or rabbit (rabbit anti mouse GBM) which results in linear deposition of antibody to the GBM accompanied with transient neutrophil accumulation and degranulation, and proteinuria (which resolves within 24 hours). The autologous phase of injury, occurs a week later in response to the planted foreign antigen (heterologous anti GBM), driven by a CD4 T_H1 DTH response with macrophage recruitment, and induction of inflammation which results in crescentic glomerulonephritis (Odobasic et al., 2014; Odobasic et al., 2005).

The murine model of anti GBM GN allows experiments where the effects of T cells can be isolated. Results from these *in vivo* manipulations indicate that different subsets of CD4 T

helper cells may play a different role according to the stage of disease activity. Early studies indicating a functional role for CD4 T_H1cells come from animal models using an anti GBM antibody to induce crescentic GN in rats. Administrating a monoclonal antibody to CD4, resulted in a reduction in the number of accumulating CD4 T cells and proteinuria indicating a functional role of CD4 T cells in crescentic GN (Huang et al., 1994). Using genetically deficient CD4-/- mice in a model of crescentic anti GBM showed similar results with attenuation of crescentic GN, with decreased crescent formation and proteinuria (Li et al., 1998). Using the same anti GBM GN model in experimental mice deficient in CD4 T_H1 cell cytokines IFNγ, or blocked using an IL-12 neutralising antibody resulted in attenuation of disease in C57Bl/6 mice. (Kitching, et al., 1999a; Kitching, et al., 1999b). BALB/c mice in comparison do not develop crescentic GN in response to anti GBM antibody due to the regulatory effects of IL-4, or a lack of sensitivity or production of IL-12. Administration of IL-12 to BALB/c mice, does induce CD4 T_H1 cell driven response, further demonstrating a functional role for IL-12 in crescentic GN (Kitching, et al., 1999b).

1.43.3 Role of CD4 T cells in human crescentic GN and AAV

A role for aberrant T cell proliferation and polarisation has been established through histological studies in human kidney biopsies from AAV patients, clinical studies of peripheral leukocytes in AAV patients and the relative contribution of CD4+ T cells to the disease explored in animal models of AAV.

Early studies in human tissue with crescentic GN demonstrated that there is an increased accumulation of T cells in renal biopsies compared to control tissue. The T cells observed were predominantly CD4 T cells, with 80% of the total leukocyte tubulointerstitial infiltrate comprised of T cells, and 30% within glomeruli. (Stachura et al., 1984) The CD4 T helper T set was predominantly present over cytotoxic T cells in the 16 patients observed. Whereas studies in pauci immune glomerulonephritis demonstrated that CD4 T helper cells and CD8 T suppressor cells (*n*=15 patients) were present in equal numbers within the glomerulus and were associated with delayed type hypersensitivity (DTH) mediators macrophages and

fibrin (Cunningham et al., 1999). Observations in a later study in AAV renal biopsies (*n*=65) found an increased number of CD4+ T cells and CD8+ T cells, with a slight increase in the number of CD8+ T cells compared to CD4+ T cells within glomeruli, however the largest sub group of leukocyte observed were CD3+T cells within the tubulointerstitium. The presence of the T cells did not correlate with histological injury or with initial serum creatinine taken at the time of the biopsy, however their presence suggests a non-redundant role.

1.43.4 The role of CD4 T_H17 cells in autoimmunity

The role of CD4 T_H1 cells in driving crescentic GN had already been well established, when experiments in an autoimmune model of murine encephalomyelitis (EAE) for multiple sclerosis showed that neither IFNγ deficient mice nor IFNγR mice were protected from disease (Chu et al., 2000; Willenborg et al., 1996). This observation was revealed in other multiple T_h1-IFNγ pathway deficient mice, where targeting components of IFNγ or IL12 (receptors and subunits of the molecules), were not protective but induced high susceptibility to autoimmunity in models of EAE (Becher et al., 2002; Gran et al., 2002; Zhang et al., 2003) . This challenged the premise that aberrant CD4 T_H1 responses alone caused organ specific immunity. The identification of another set of CD4+T cell with a pathogenic role in autoimmunity was revealed in a central nervous system CNS autoimmunity model (Langrish et al., 2005). Langrish et al found that this new subset of CD4+T cells required IL-23 for proliferation and were highly pathogenic. IL-23 shares a common subunit p40 with IL-12, but has different effector functions, it promotes IL-17 secretion from CD4+ T cells (Aggarwal et al., 2003). Subsequent experiments in EAE revealed this new subset of CD4 +T_H17 Cells, were required to induce disease (Komiyama et al., 2006). IL17-/- mice failed to develop EAE due to lack of T cell sensitisation, demonstrating a role for CD4 T_H17 cells in the early initiation of disease.

The idea of CD4+T_H1 dominance in anti GBM disease was challenged with the realisation that IFNγ-/- mice developed severe disease, whilst experiments with IL12-/- mice offered

protection due to the shared sub unit p40 with IL-23, essentially eliminating immune driven CD4+T_H1 and CD4+T_H17 responses (Kitching et al., 2005).

Summers et al examined the role of CD4+T_H1versus CD4+T_H17 in inducing GN. Polarised T_H1 or T_H17 cells to ovalbumin were transferred into RAG-/- mice. Anti GBM disease was induced through conjugation of ovalbumin to a non-nephritogenic anti GBM antibody, which localises to the anti GBM, to plant ovalbumin (the antigen), but does not induce disease itself. These experiments demonstrated that both CD4+T_H1 and CD4+T_H17 cells contribute to the pathogenesis of GN. CD4+T_H17 responses played a role in early disease through production of IL-17 which recruits neutrophils to the glomerulus, whereas CD4+T_H17 cells induced a DTH type of reaction which recruited macrophages and initiated glomerular lesions consistent with GN (Summers et al., 2009). Further experiments in murine anti-GBM disease, using IL-17, IL-12 and IL23 (p19) demonstrated that there is an intricate relationship between T_H1 and T_H17 cells in the development of crescentic GN (Odobasic et al., 2011). Mice deficient in IL17A, were protected from renal disease in the initial phase of injury (day 6), however IL-17A deficiency augmented disease at day 21. These observations established that T_H17 cells promote early crescent formation, but offers protection through the production of IL-17A, at later stages of disease which inhibits pathogenic T_H1 responses.

1.43.5 T regulatory cells in glomerulonephritis

There are two distinct subsets of T regulatory cells: 1) naturally occurring regulatory T cells (nT_{reg}) are produced and differentiated in the thymus and constitutively express CD4, CD25, and FoxP3, and secrete IL-10 and TGFβ.; 2) induced iT_{reg} in contrast are generated from naïve T cells by MHC II presentation of antigens in the periphery but also express CD4, CD25, and FoxP3, and secrete IL-10 and TGFβ (Abbas et al., 2013).The primary function of Tregs is to maintain tolerance through suppression of effector immune cells. This effect can be antigen presenting cell (APC), dependent requiring direct contact between the APC and T regulatory cell via their TCR, or indirect non APC-dependent via the production of IL-10 and TGFβ, which inhibits the effector function of dendritic cells (DCs) and supresses T_H1

and T_H17 responses. Tregs also supress the function of effector CD8+ T cells through inhibition of IL-2 production limiting CD8+T cell proliferation (Piccirillo & Shevach, 2001). T regulatory cells require both IL-2 and TGFβ, for proliferation. Tregs require an exogenous source of IL-2, to proliferate and upregulate CD25 expression. Experimental evidence suggests that the source of IL-2 is T_H effector cells, and that the frequency of IL-25 (receptor for IL-2) is so great on Tregs that surrounding effector cells become starved of IL-2. Treg consumption of IL-2 may be one of the mechanisms in which Tregs supress T effector cells, by removing IL-2 from the environment which suppresses proliferation of T effector cells (de la Rosa et al., 2004).

Defective T regulatory cells contribute to the progression of autoimmune diseases. In human patients with anti-GBM disease, there is a significant disproportionate amount of T effector cells to T regulatory cells in the peripheral blood in the acute phase of disease, compared to patients in remission (Salama et al., 2003). In murine models of anti-GBM disease, the transfer of CD4+CD25+T_{regs} 24 hours prior to triggering disease, is able to significantly reduce functional injury through a reduction in proteinuria, and histological injury with reduced numbers of leukocyte infiltration and glomerular damage (Wolf et al., 2005). In a model of accelerated anti GBM disease where mice are sensitised 4 days before administration of anti-GBM antibody to induce disease, tracking T regulatory cells in foxP3 (GFP) reporter mice provided evidence of Foxp3+ cell infiltration within kidneys. Deletion of FoxP3 cells using $Foxp3^{DTR}$ after disease was initiated resulted in increased proteinuria, glomerular leukocyte infiltration and glomerular crescents and necrosis, highlighting the importance of competent Tregs in autoimmunity (Ooi et al., 2011). Additional experimental evidence in murine nephrotoxic nephritis (NTN) demonstrated that T regulatory cell depletion, increased number of crescentic glomeruli, through enhanced T_H1 responses. Deletion of FoxP3 cells in the heterologous phase of injury did not affect crescent formation, and the Th17 response was unaltered. IFNγ T cell responses were increased both

systemically and locally in kidney tissue indicating that T regulatory cells play a significant role in reducing Th1 immune responses in NTN (Paust et al., 2011).

The evidence of a dysregulation in T regulatory cells in AAV is less clear, with many conflicting studies demonstrating either an increased proportion of Tegs in the peripheral PBMCs (Free et al., 2013) or a decreased proportion of Tregs (Abdulahad et al., 2007; Rimbert et al., 2011). Regardless of the inconsistency in these reports, further studies from *in vitro* experiments have shown that the Tregs from patients with acute disease and those in remission have impaired suppressor function, with a reduced ability to inhibit T effector cell proliferation *in vitro* (Abdulahad et al., 2007; Morgan et al., 2010).

Experiments in murine MPO-ANCA GN, show that T regulatory cells are required for the tolerance to MPO. Depletion of T regulatory cells prior to disease induction, results in signification proliferation of anti MPO specific T cells, increased humoral immunity with severe glomerulonephritis (Tan et al., 2013).

1.43.6 CD8+ T cells in AAV

CD8+ T cells are generated in the thymus and express CD8 receptors consisting of 1 alpha chain (CD8α) and 1 beta chain (CD8β), and a TCR. The function of CD8+ T cells is to kill cells infected by viruses or intracellular bacteria. CD8+ T cells can only recognise antigen when it is presents via the MHC class I molecule on APCs. Naïve CD8+ T cells can recognise antigen but are incapable of killing infected cells until they differentiate into a cytotoxic CD8 + cell CTL. Differentiation into a CTL cells requires: 1) antigenic stimulation via the TCR; 2) co-stimulation (i.e. CD40, CD28) and 3) cytokine receptor stimulation (IFNα, IL-12) (Mescher et al., 2006; Z. Xiao et al., 2009). Activated CTLs will proliferate, generate effector cytokines IFNγ, TNFα, and the effector molecules granzyme and perforin. These activated and clonally expanded CTLs will migrate into tissue, where they will kill the antigen specific infected cells (Obar & Lefrancois, 2010). The majority of effector CTLs will die via

apoptosis (95%), leaving a small percentage of memory CD8+ T cells, within multiple tissues of the body, that will recognise that specific antigen again with re exposure.

Human AAV kidney biopsies show an increased number of CD8+ T cells within glomeruli and the tubulointerstitium, which correlate with increased creatinine levels, and histopathological parameters, demonstrating a likely participation in the pathogenesis of AAV (Cunningham et al., 1999; Weidner et al., 2004). There is a dysregulation in the proportion of CD4 T cells to CD8 T cells with an increase in CD8 T cells in Wegeners granulomatosis within the circulation (Ikeda et al., 1992). AAV patients have an increased proliferation of peripheral circulating CD8+CD28+CD11b+ T cells which secrete IFNγ, show an activated phenotype and are resistant to apoptosis. The production of IFNγ by CD8+CD28+CD11b+ T cells may contribute to the ongoing autoimmunity by prolonging the life of neutrophils in AAV (Iking-Konert et al., 2008).Further proof of a pathogenic role for CD8+ T cells came from gene expression assays from peripheral CD8+ cells in AAV patients. Poor prognosis in this group of AAV patients was associated with an expanded population of CD8+ T memory cells, which have increased expression of the interleukin 7 receptor (IL7R) (McKinney et al., 2010).

Murine studies of experimental MPO-ANCA GN show that depletion of CD8+ T cells in the effector phase of disease significantly decreases glomerular and functional injury. Transfer of MPO specific CD8 + T cells ($MPO_{431-439}$) without the contaminating CD4+ T cell population, are capable of inducing glomerular injury, further supporting that pathogenic role of CD8+ T cells in AAV (Chang et al., 2017).

1.44 B cells

B cells are an essential component of the humoral arm of the adaptive immune response. B cells produce antibody, can present antigen, and secrete immunomodulating cytokines independent of antibody production. Activated B cells clonally expand and turn into plasma cells in response to antigens. Plasma cells produce antibodies against specific antigens,

and secrete either (IgG, IgA, IgG, or IgE) dependent on the pathogen, which neutralises micro-organisms by binding to them or coating them for opsonisation and subsequent phagocytosis.

In AAV it is the production of ANCA by B cells that contribute to the pathogenesis of AAV. The most convincing evidence of the involvement of B cells in AAV comes from large clinical trials using the B cell depleting anti CD20 monoclonal antibody Rituximab. Successful remission in refractive AAV patients has been achieved using rituximab (Stone et al., 2010) (Rituximab versus cyclophosphamide for ANCA associated vasculitis, RAVE study), in direct comparison with cyclophosphamide in a randomised trial demonstrated similar results between both arms of the trial (randomised trial of rituximab versus cyclophosphamide in ANCA associated vasculitis RITUXVAS) (Jones et al., 2010). The outcomes of these two trials concluded that rituximab was safe and effective in treatment of AAV, with less side effects than cyclophosphamide treatments.

B regulatory cells (Bregs) are characterized by cell surface expression of CD5 and IL-10 production, and inhibit T effector cell proliferation. Assessment of the B cell subsets in AAV has demonstrated that there is a reduction in the numbers of circulating Bregs in disease remission of AAV patients, compared to healthy controls. In active disease PR3-ANCA+ patients (but not MPO-ANCA+ patients) had a reduction in Bregs compared to healthy controls (Todd et al., 2014).

1.45 Monocytes/Macrophages in glomerulonephritis

Monocytes are generated from hematopoietic stem cells in the bone marrow. Monocytes migrate from the circulating blood stream into the tissues where they differentiate into macrophages. The primary function of macrophages is host defence, through phagocytosis of micro-organisms, clear cell debris from cell death (apoptosis, NETs, Necrotic cells etc.) and initiate tissue repair and healing. Macrophages are also able to present antigen to T helper cells via MHC class II molecules. In inflammation macrophages can be divided into

either M1 or M2 macrophages based on their phenotype. M1 macrophages (also known as classically activated macrophages) are generated in response to IFNγ and LPS and are considered to be pro-inflammatory as they secrete IL-12, and IL-23. M2 macrophages (alternative macrophage activation) are induced by IL-4 and IL-13, and preferentially secrete IL-10 over IL-12.

The role of macrophages in crescentic glomerulonephritis has been well known since the 1970s when large infiltration of macrophages were identified within injured glomeruli of patients with glomerulonephritis. Subsequent culture of these glomerular cells showed that the predominant cell type was that of macrophages opposed to epithelial cells within glomerular crescents (Atkins et al., 1976).

Animal models of anti GBM disease provided further evidence of macrophage recruitment to sites of glomerular injury (Bagchus et al., 1990; Hara et al., 1991; Ikezumi et al., 2003), and human studies in AAV have demonstrated a correlation of glomerular macrophage accumulation with disease severity (judged by histopathology and functional injury), and evidence of activated macrophages (Brouwer et al., 1994; Cunningham et al., 1999; Rastaldi et al., 2000; Weidner et al., 2004) . It's not surprising then that depletion of macrophages would have a therapeutic effect in glomerulonephritis. Using a wide range of methods to deplete macrophages different studies have demonstrated a functional role for macrophages in renal disease (Duffield et al., 2005; Huang, et al., 1997; Ikezumi et al., 2003; Van Rooijen & Sanders, 1994). Clodronate liposomes injected directly into the tissue, or administered systemically are ingested by macrophages inducing apoptosis and subsequent depletion of macrophages from both the tissue and draining lymph nodes. Using this method, depletion of macrophages in animal models of renal disease, have shown a reduction in renal injury (Huang, et al., 1997). Targeting macrophage chemoattractants (i.e. monocyte chemoattractant protein) and adhesion molecules (i.e. intercellular adhesion molecule 1, ICAM-1) to prevent glomerular recruitment of macrophages has also had a protective effect in glomerulonephritis (Lloyd et al., 1997).

Macrophages have been found present in human atherosclerosis plaques as either MPO positive or MPO negative cell populations (Sugiyama et al., 2001), and circulating monocytes demonstrate similar subtypes with MPO high and MPO low cell populations. Activated monocytes are able to generate and store MPO. It is possible the MPO positivity observed in tissue macrophages could be as a result of phagocytosis of apoptotic neutrophils or NET remnants (Farrera & Fadeel, 2013), which accounts for the MPO positivity. In inflammation the abundant numbers of degranulating neutrophils, or netting neutrophils do provide a likely source of MPO for ingestion through phagocytosis for macrophages. In inflammation however it is also possible that activated tissue macrophages respond to cytokines within the local microenvironment such as GM-CSF and produce their own source of MPO.

The role of MPO positive MPO macrophages has not been fully explored in AAV. Considering the heterogeneity of macrophages, it is possible that MPO positive macrophages are Type 1 macrophages and are responsible for perpetuating inflammation in autoimmunity, whereas the Type 2 macrophages are considered to be the healing type of macrophage. Studies thus far have only shown the presence of M2 macrophages within glomerular lesions in AAV (Zhao et al., 2015) or crescentic glomerulonephritis (Li et al., 2017) .This could be for 2 reasons, the first reason is patients quite often present with symptoms after the initiation of renal disease, it is highly likely that the M1 type of macrophage has already infiltrated and caused damage, and what is observed in biopsies at the time of presentation is the M2 macrophages. The second reason, is the lack of tools currently available to assess the 2 populations of macrophages within tissue due to the lack of specific markers for type 1 and type 2 Macrophages that work in formalin fixed paraffin tissue for renal biopsies. The contribution of M1 macrophages or MPO positive macrophages in AAV still remains unclear.

1.46 Dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells (APCs), derived from hematopoietic bone marrow progenitor cells. Their primary function is to present antigen proteins from micro-organisms to T cells. Immature DCs reside in the epithelia such as skin, whereas mature DCs reside in the lymph nodes and spleen. DCs can be divided further into two subsets, known as classical and plasmacytoid. Classical DCs are present within epithelia, peripheral tissue, and lymphoid organs. Plasmacytoid DCs are present both in the tissue and blood and they are the primary producers of type 1 Interferons.

Studies in biopsies of AAV patients have shown that immature and mature DCs are present in glomeruli in significant numbers compared to healthy controls (Wilde et al., 2009). This observation is not consistent with other forms of renal diseases. Studies in lupus nephritis have shown that CD68 macrophages preferentially infiltrate glomeruli, whereas DCs (DC-sign positive) infiltrate the tubulointerstitium and correlate with renal injury (Segerer et al., 2008). Murine models of NTN, show similar results to the Lupus human studies, with DCs only found within the tubulointerstitium of both the control and inflamed kidneys, and notably absent from the glomeruli (Kruger et al., 2004; Soos et al., 2006). Observations in human biopsies of AAV are confounded due to the lack of DC markers available that work on formalin fixed paraffin embedded tissue, and work relies on the availability of frozen biopsies to perform studies. These observations from a limited study though could suggest a differential role of DCs in AAV compared to other forms of GN and should be explored further.

MAST CELLS

Paul Ehrlich and Elie Mitchnikoff received the Nobel Prize over a century ago in recognition of their contribution to the field of immunology. Mast cells (MCs) were first described in Ehrlichs doctoral thesis who named them “Mastzellen” cells, “mast” in German means fattening, and Ehrlich thought the role of mast cells was to nourish the surrounding tissue

through release of the large granules. Elrichs discovered that mast cell granules specifically reacted metochromatically to aniline dyes, one of the methods still used to identify mast cells to day. Elrich, observed the presence of mast cells in chronic inflammation and tumours, and concluded they were there to nourish the tissue (Beaven, 2009). This we now know to be incorrect, but the actual function of mast cells eluded many scientists after Elrich, and to this day mast cells have been shown to be multi-faceted. They play a traditional role in innate immunity (helminth parasites), a role in allergy, and more recently have been shown to have immunomodulatory functions.

MCs are derived from $CD34^{+}$/c-kit^{+}/Fc$\varepsilon$$RI^{-}$ pluripotent progenitor cells. MCs have a long life, and are found in many organs throughout the body, but are most commonly found in skin and mucosal surfaces. MCs only mature once they are recruited into tissue, where they have the capacity to develop into a heterogeneous population. MC plasticity, has recently been attributed to the diverse transcriptional properties of mast cells, which are distinct from other lymphoid and myeloid cell populations. Expression profiling of MCs, demonstrated that MCs are enriched in transcriptions for genes for a wide variety of proteases, sensing genes, and genes involved in metabolic pathways. Genes between the different tissue types of MCs differed widely, evidence that MCs can respond to their environment (Dwyer et al., 2016).Once recruited from circulating blood into the tissues , MCs differentiate into different types of mast cells based on their location and granule content. In humans MCs develop into 3 different subtypes of mast cells, Mast cells that are positive for tryptase (MC_T), chymase (MC_c) and double positive for both tryptase and chymase (MC_{TC}) (Pejler et al., 2007). In mice, MCs are subtyped according to their location and heparin content within their granules. Connective tissue MCs (CTMC) which contain ample heparin containing granules and mucosal mast cells (MMCs) where heparin is either absent or minimal (Wernersson & Pejler, 2014).

1.47 Mast cells in allergy

The role of MCs in allergy has been well studied, especially in IgE mediated allergic disease. Mast cell activation in the context of allergy, is regulated through FcεRI a high affinity receptor for IgE (Kraft & Kinet, 2007; Okayama & Kawakami, 2006). Antigen specific IgE is produced by B cells in response to antigen presentation by DCs. IgE binds to FcεRI on the MC surface, activating the cell to degranulate, which releases a multitude of different proteases (chymase, tryptase), histamine, serotonin, heparin and secrete cytokines, all within minutes of exposure to the antigen (Galli & Tsai, 2012).

1.48 Mast cells in immunomodulation

MCs are also now been recognised to play a role in adaptive immune responses. *In vitro* experiments on bone marrow derived mast cells BMMC, demonstrated that TLR4 engagement of MCs induces the secretion of cytokines and chemokines cytokines such as IFNγ, IL-6, IL-4, IL13, TNFα, IL-5, instead of the release of histamine as TLR2 elicits (Supajatura et al., 2002). These MC derived cytokines can influence the polarisation and activation of T cell subsets. CD4+ T cell effector functions can be regulated by MCs. IFNγ primed MCs under inflammatory conditions in human psoriatic skin can present antigen to CD4+ memory T cells, and skew the T cell response to an IL-22 response. MCs are often observed in close contact with T cells in psoriatic skin, where they are well placed to perpetuate inflammation (Gaudenzio et al., 2013). MCs are also able to present antigen to CD8+ T cells via MHCI (Malaviya et al., 1996), and MC and CD8+ T cell contacts have been observed in both healthy skin and patients with alopecia areata (AA). Increased contact between MCs and CD8+ T cells is observed in animal models of AA, indicating that MCs may play a role in the disease by enhancing CD8 T cell effector functions. MCs stimulated by LPS and IFNγ have also been observed to transiently express MHCII and present antigen to T cells, and instigate proliferation of antigen specific T regulatory cell populations (Kambayashi et al., 2009).

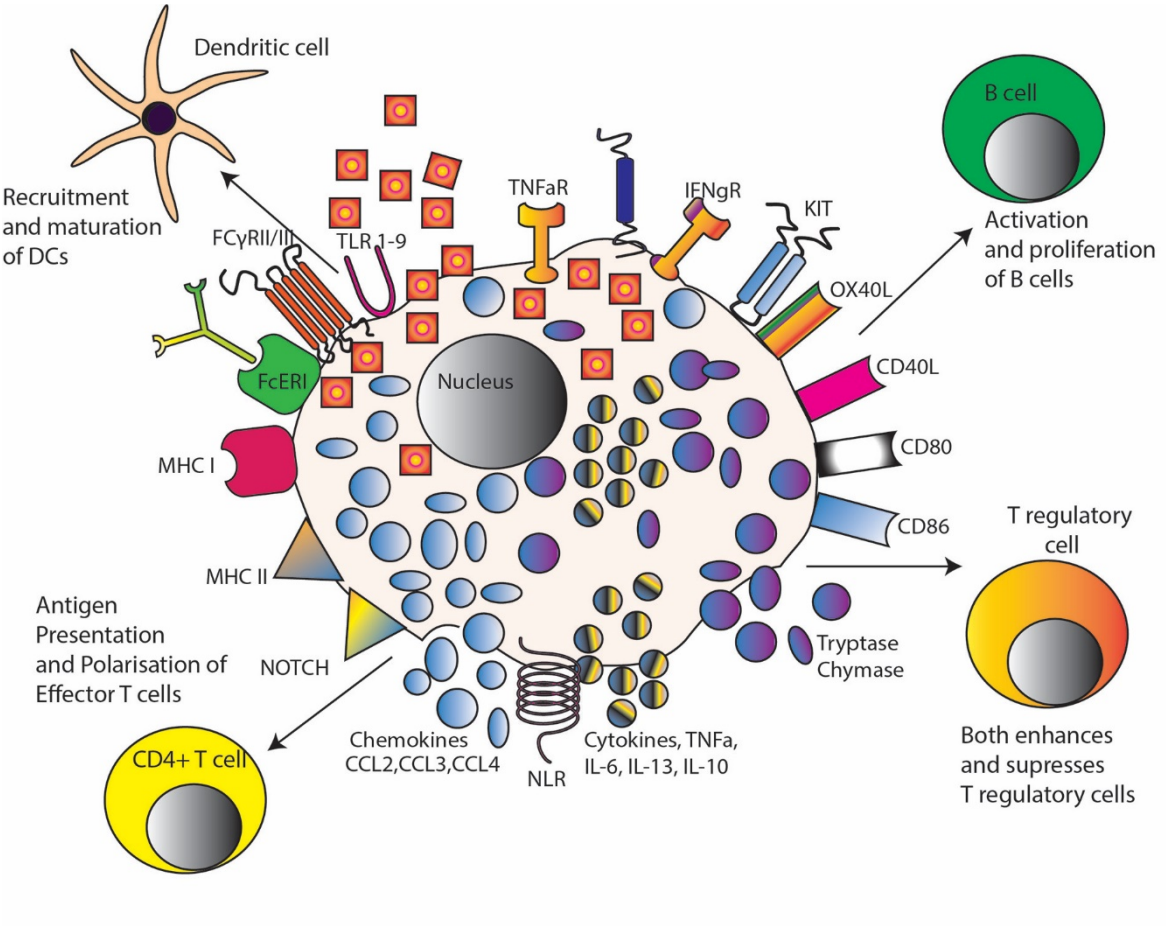


Figure 5. Mast cell action on Immune cells, Receptors, Chemokines and Cytokines

Figure demonstrates receptors known to be expressed by mast cells, Chemokines, and cytokines synthesised by mast cells. Mast cell derived factors can enhance T regulatory cell function, polarise CD4 effector T cells, promote activation and proliferation of B cells, and enhance DC maturation and recruitment.

1.49 Mast cells in autoimmunity

To examine and isolate the role of MCs, strains of MC deficient mice have been generated. C-Kit (also known as CD117 and Mast/stem cell growth factor receptor) is a receptor expressed by MCs which is crucial for the development, activation and survival of MCs (Gilfillan & Rivera, 2009). C-kit is not specific to MCs, it's also expressed in high levels in common myeloid progenitors, hematopoietic stem cells, germ cells and interstitial cells of Cajal. The first generation of MC deficient mice were generated by disrupting components of the KIT signalling pathway. $Kit^{W/Wv}$ mice are deficient in MCs, but the genetic mutation also causes other abnormalities, such as sterility, anaemia, bile reflux, idiopathic dermatitis, deletion of interstitial cells of Cajal and a reduced number of TcR$\gamma\delta$ intraepithelial lymphocytes, neutrophils and basophils (Grimbaldeston et al., 2005). The $Kit^{wsh/wsh}$ mice have a mutation in the kit gene, which regulates KIT signalling and therefore deletes MCs. $Kit^{wsh/wsh}$ mice unlike $Kit^{W/Wv}$ mice, are not sterile and are spared from anaemia, and reduced number of TcR$\gamma\delta$ intraepithelial lymphocytes. However, $Kit^{wsh/wsh}$ mice do have a pro-inflammatory phenotype, which results in splenomegaly (Grimbaldeston et al., 2005). For this reason, experiments dependent on MC deletion as a proof of concept are performed in a 2 stage process. In the first stage, experiments are performed in the $Kit^{wsh/wsh}$ mice, then to confirm the results, in the second stage a second set of experiments are conducted where $Kit^{wsh/wsh}$ mice are reconstituted with WT bone marrow derived MCs which have been differentiated *in vitro,* to demonstrate the role of MCs (Galli et al., 1992). These steps, confirm that it is the absence of MCs that contribute to the disease process being studied, and not caused by a disruption in Kit signalling.

The role of MCs has been investigated in a multitude of autoimmune disease using experiment MC deficient mouse models. Interestingly, in models of experimental arthritis, protection from the development of arthritis was only apparent when $Kit^{W/Wv}$ mice were used. The same experimental model of arthritis in $Kit^{wsh/wsh}$ mice did not offer protection, potentially due to the increased numbers of neutrophils in $Kit^{wsh/wsh}$ mice, one of the key effector cells

in the development of arthritis (Zhou et al., 2007). Models of experimental autoimmune encephalomyelitis (EAE) using the $Kit^{W/Wv}$ mice, have shown contradicting results with some authors demonstrating protection (Robbie-Ryan et al., 2003, Sayed et al., 2011), and others demonstrating enhanced disease (Piconese et al., 2011).

Mast cells have been proven to be pivotal in maintaining tolerance of skin allografts. Lu et al demonstrated that $Kit^{wsh/wsh}$ mice are unable to maintain tolerance in a skin transplant model. Histological analysis demonstrated MCs and T regulatory cells in close contact within the tolerant skin grafts. T regulatory cells through the production of IL-9, were shown to be crucial in the recruitment of MCs to the allografts (Lu et al., 2006).

1.50 Mast cells in kidney disease

Mast cells are minimally present in the normal kidney, but increase in number in different kidney diseases (Blank et al., 2007). Mast cells have been implicated in both the pathogenesis of kidneys diseases and involved in healing and tissue repair in fibrosis. In human crescentic glomerulonephritis a significant numbers of infiltrating MCs cells are found in the tubulointerstitium and glomeruli compared to controls, and correlate with tubulointerstitial fibrosis (El-Koraie et al., 2001; Hiromura et al., 1998; Toth et al., 1999). MCs are implicated in chronic rejection of human renal allografts, where MC infiltration correlates with fibrosis and tubular atrophy (Pardo et al., 2000). In lupus nephritis, renal infiltrating MCs correlate with fibrosis, and initial serum creatinine, and expression of α smooth muscle actin (αSMA), an indicator of fibrosis (Danilewicz & Wagrowska-Danilewicz, 2001). High levels of IgE have also been associated with lupus nephritis, elevated levels of IgE are associated with periods of active disease and subside during remission (Elkayam et al., 1995).

Histological observation of MCs in human diseases indicate an association with MC numbers and disease severity. To determine a functional role for MCs, experimental models of disease are required to define the pathogenic or protective mechanism. $Kit^{W/Wv}$ mice are

protected from the development of experimental anti GBM disease, which is associated with a decreased glomerular expression of adhesion molecule 1 (ICAM-1) and P selectin, and reduced leukocyte recruitment. This indicates a role for MC mediated adhesion molecule expression in GN (Timoshanko et al., 2006). $Kit^{wsh/wsh}$ mice are protected in an experimental model of renal fibrosis using unilateral ureteric obstruction (UUO). Collagen deposition, macrophage and CD4 + T cell recruitment is significantly reduced in $Kit^{wsh/wsh}$ mice. Reconstitution of $Kit^{wsh/wsh}$ mice with WT bone marrow derived MCs, restored disease with MC recruitment and degranulation evident 6 hours after UUO (Summers et al., 2012). Eller et al., 2011) described Treg/MC interactions essential in a model of anti GBM disease in $Kit^{W/Wv}$ mice. WT Treg reconstituted into WT recipients, results in protection from GN, accompanied with enhanced MC recruitment to the draining lymph nodes. In contrasts, transfer of WT Tregs into $Kit^{W/Wv}$ mice, resulted in enhances susceptibility to GN. This mechanism was dependent on T regulatory cell derived IL-9, for the effective recruitment of MCs, indicative of requirement for MCs for effective protection from GN provided by T regulatory cells. The role of MCs for establishing peripheral tolerance in anti MPO GN, was explored by Gan et al., (2012). $Kit^{wsh/wsh}$ mice, developed more severe anti MPO-GN than WT mice, with a reduction in numbers of recruited T regulatory cells to the draining lymph nodes, and decreased levels of IL-10. Reconstitution of $Kit^{wsh/wsh}$ mice with WT bone marrow derived MCs, increased the numbers of MCs and Regulatory cells in the lymph nodes and diminished disease severity. *In vitro* co–cultures of MCs and T-regulatory cells determined it was MC derived lL-10 which enhanced regulatory responses.

Studies on the role of MCs in human or experimental AAV are limited. Otsubo et al found a significant correlation between tubulointerstitial MC recruitment and fibrosis in a small cohort (*n*=13) of AAV patients (Otsubo et al., 2003). A study in a larger cohort of renal biopsies (*n*=22) from patients with AAV conducted to determine the cellular source of IL-17A, reported IL17+/MC tryptase+ cell infiltration within the tubulointerstitial compartment of biopsies, but not within glomeruli (Velden et al., 2012).

INTRINSIC GLOMERULAR CELLS

1.51 Endothelial cells

Endothelial cells play an important role in mediating injury to the glomeruli. It is through the endothelium that neutrophils and other leukocytes migrate. The transmigration of leukocytes is mediated by the expression of chemokine, cytokines, and the expression of adhesion molecules, a dysregulation in any of these factors will affect the role of endothelial cells in maintaining entry to tissues (Alon & Feigelson, 2002; Kang et al., 2002; Muller, 2003; Yadav et al., 2003).

Neutrophils are the first cells to transmigrate through the glomerular endothelium in response to inflammation, and are the sentinel cell responsible for the damage occurred in ANCA glomerulonephritis (Xiao et al., 2002). In murine models of AAV, glomerular endothelial expression of TLR4 (neutrophil recruitment), and the neutrophil chemokine attractants CXCL1 and CXCL2 (the murine homolog of human IL-8) is significantly increased compared to healthy controls (Summers, et al., 2010). Cultured human glomerular endothelial cells produce CXCL8 significantly in response to stimulation with LPS, in significant amounts relative to podocytes, mesangial cells and healthy control glomerular endothelial cells (Summers et al., 2010).

The basic hypothesis on the cause of endothelial cell damage in glomerulonephritis, is due to the loss of homeostasis which is necessary to maintain a constant eGFR. A decrease in eGFR results in release of cyclooxygenase-2 from the macular densa and subsequent production of prostaglandins, which causes dilation of the afferent arteriole, and constriction of the afferent arteriole. As the glomerular hydrostatic pressure increases the endothelial cells, podocytes and mesangial cells are all damaged, and the induction of proteinuria occurs (Brenner et al., 1982; Kang et al., 2002).

Human glomerular endothelial cells express MHCII at relatively levels in normal human kidneys whereas MHCII is only expressed on murine endothelial cells during inflammation

(Muczynski et al., 2003). This is important in the progression of MPO-AAV, as it has been shown that MPO can be internalised by endothelial cells, identifying them as a potential glomerular endogenous source of antigen presentation (MPO) in AAV (Jerke et al., 2013; Yang et al., 2001).

Endothelial cell markers CD31, CD34 and Von Willebrand Factor are all lost in response to glomerular injury in human glomerulonephritis (Gluhovschi et al., 2010). In regions of glomerular scarring all 3 markers are lost, even when conventional histology shows the presence of endothelium. This indicates that damage to the endothelium can occur early on in terms of glomerular damage. Attempts to correlate endothelial damage with functional injury and histology is difficult as the markers are lost in injury, therefore only correlations with healthy intact endothelium can be made (Gluhovschi et al., 2010).

1.52 Podocytes

Podocytes are specialised epithelial cells with long foot processes termed pedicels with small fenestrations, these pedicels wrap around the glomerular endothelium. The primary function of the podocyte is to prevent the loss of large macromolecules into the bloodstream, such as albumin. The composition of cellular crescents in crescentic glomerulonephritis is heterogeneous, and contains the proliferating epithelial cells, macrophages and podocytes. The composition of the crescent changes according to which stage of disease progression the glomerulus is in. For example, animal models of anti GBM disease in rats, demonstrate that in early crescent formation, crescents consist of epithelial cells whilst the basement membrane is intact, however once rupture of the basement membrane occurs, an increased number of macrophages are observed within the crescent (Lan et al., 1992). However, crescent formation in animal anti GBM disease is species dependent, in both mice and rabbits, macrophages are only rarely observed within crescent formation, and parietal epithelial cells dominate the crescent (Cattell & Jamieson, 1978; Sterzel & Pabst, 1982; Wheeler et al., 1993). Evidence of the involvement of podocytes came from an experimental model of anti GBM disease in mice, where it was demonstrated that podocytes form bridges

between the glomerular basement membrane (GBM) and the parietal basement membrane (PBM). Podocyte “bridges” occurred prior to crescent formation, and podocytes were found within the crescent at late stages of crescent formation, generally in clusters. This phenomena was a regular occurrence, observed in over 80 glomeruli by light microscopy and transmission electron microscopy (Le Hir et al., 2001).

Glomerular podocytes express MHCII under inflammatory conditions in experimental models of renal disease (Coers et al., 1994; Goldwich et al., 2013). Therefore, like endothelial cells if MPO can be internalised by podocytes it is possible, that podocytes could act as professional APCs within the glomeruli and present antigen to MPO specific CD4 T cells and perpetuate autoimmunity. There is little data or evidence of MPO internalisation by podocytes, but it is investigated in human MPO-AAV in chapter 2 of this thesis.

The loss of podocytes is a characteristic feature of progressive glomerulonephritis (Kim et al., 2001; Kriz & LeHir, 2005). A recent study in AAV patients revealed using multivariate statistical methods that podocyte density per glomerulus is more of a predictive value for outcome in end stage renal disease than the percentage of normal glomeruli (Zou et al., 2016).

Like endothelial cell injury, conventional markers for immunohistological detection of podocytes are lost with injury [nephrin, synaptopodin (SNY), neprilysin (NEP), Wilms tumour protein-1 (WTP-1)], making *in situ* identification within renal biopsies problematic (Kubiak-Wlekly et al., 2009).

1.53 Mesangial cells

Mesangial cells together with podocytes and endothelial cells form the filtering component of the glomerulus. Mesangial cells support the glomerular endothelium, generate their own extracellular matrix, and contribute to glomerular filtration by regulating capillary flow. Mesangial cells produce their own extracellular matrix, containing type IV collagen, laminin and fibronectin to name a few, the production of this matrix it tightly regulated in health but

can become dysregulated during disease. Mesangial cells require both podocytes and endothelial cells for proliferation and growth. Mesangial cells are co-dependent on the production of PDGF-β by endothelial cells, deletion of PDGF-β in the endothelium , prevents mesangial cell growth (Soriano, 1994). Likewise endothelial cells require mesangial cells for vascular growth. Without the production of factor ephrin B2 results in glomeruli with a decreased number of endocapillary loops (Schlondorff, 1987; Schlondorff & Banas, 2009).

The role of mesangial cells in glomerulonephritis is not well defined. However, animal experiments have given some indication of what role they may play. Stimulated mesangial cells express MHCII, whereas unstimulated mesangial cells do not express MHCII. They can express MHCII when stimulated with IFNγ alone or in combination with TNFα and IL-1β. Our laboratory has demonstrated that the deletion of the MHCII gene in mice confers protection from experimental anti GBM disease, with a reduction in crescent formation and accumulation of CD4+ T cells (Li et al., 1998). The crucial role of glomerular intrinsic cell production of MHCII was further demonstrated by reconstituting MHCII deficient mice with bone marrow cells from wild type mice. These mice were still protected from GN, suggesting that intrinsic glomerular cells provide antigenic presentation through MHCII in experimental GN (Li et al., 1998). Further experiments from our laboratory demonstrated that mesangial cell expression of CD40 a co-stimulator molecule plays a role in experimental anti GBM disease. Genetic deletion of CD40 from mice, prevents the development of autoimmunity to the sheep Ig used to induce disease. Reconstitution of bone marrow from wild type animals with intact CD40 restored antigen specific autoimmunity, but mice were still protected due to a reduction in DTH cell effector recruitment to the glomerulus. This indicated that CD40 expression by mesangial cells play a non-redundant role in the pathogenesis of GN (Ruth et al., 2003).

Mesangial cells in experimental anti GBM have been shown to produce pro inflammatory cytokines such as IL12, IFNγ and TNFα. IL12-/- deficient mice are significantly protected from GN, reconstituting mice with wild type bone marrow offered protection but not to the

same extent in the IL12-/- mice (Timoshanko et al., 2001). IFNγ-/- mice are protected from experimental anti GBM disease. Reconstitution of irradiated IFNγ-/- mice with WT bone marrow allowed the isolation of the effect of depleting IFNγ from intrinsic glomerular cells alone. The chimeric IFNγ mice, also had a significant reduction in GN similar to the IFNγ-/- mice. The characteristic DTH response seen in challenging the foot pad with antigen was only reduced in the IFNγ-/-, indicative of a requirement for both intrinsic glomerular IFNγ production and bone marrow derived cell production (Timoshanko et al., 2002).

TOLL-LIKE RECEPTORS (TLRS)

1.54 Introduction to TLRs

The first model of self-versus non self was proposed by Burnett (as described in an earlier section of this review), and later modified by Janeway who developed a model to explain the recognition of infectious non self. Janeways model is based on the premise that antigen presenting cells (APCs) have a built in recognition of self-versus infectious non-self based which can recognise evolutionary distant PAMPS. Activated APCs recognise these PAMPs through their PRRs, upregulate costimulatory molecules and present antigen to T cells. It is the PRRs that allow the recognition of “infectious-non-self” and non-infectious-self ”(Janeway, 1989). This model however did not offer an explanation as to why autoimmunity was triggered. Matzinger, (2002) proposed the “danger model” to expand on the previous models of self versus non self. The danger model proposed that APC activation can occur through the release of danger signals from cell death. In programmed cell death such as apoptosis, cells are cleared by macrophages due to the “eat me” signals displayed on their cell surface. In other forms of death such as necrosis, the cellular contents are expelled into the extracellular environment where they potentially act as DAMPs. This model introduced the concept that APCs could respond to both endogenous (DAMPs) and exogenous (PAMPs) signals. This model explained why in the absence of infection autoimmunity could still occur.

Toll-like receptors are one of the largest families of innate pattern recognition receptors (PRRs) which recognise pathogen associated molecular patterns (PAMPS) on microbes, and danger associated molecular patterns (DAMPs), and play a key role in host defence from infection (Jimenez-Dalmaroni et al., 2016). There are 11 members in human and 13 in mice. The primary role of TLRs is to detect danger signals in both the extracellular and intracellular compartments of cells. TLRs are located on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, TLR11, TLR12 and TLR13), where they recognise PAMPS and

DAMPs or localise within endosomes such as TLRs 3, 7, 8, and 9, where they detect self DNA and nucleic acids or viral and bacterial nucleic acids (Baccala et al., 2009; Jimenez-Dalmaroni et al., 2016). The different compartmental cellular expression of TLRs, ensures widespread detection of potential danger signals. TLRs are likely to play a pathogenic role in autoimmunity, firstly in initiation of inflammation through recognition of PAMPS and subsequent activation of TLRs in infection, or DAMPS in sterile inflammation instigating autoimmunity.

Toll-like receptor signalling occurs through several signalling pathways though adaptor proteins. The first is the myeloid differentiation factor 88 (MyD88) dependent pathway, through which TLR1, TLR2, TLR7 and TLR9 signalling occurs. The second adaptor protein is a MYD88 like (MAL), through which TLR2 and TLR4 signalling occurs indirectly through MAL which in turn recruits MYD88. TLR3 signalling occurs through the TIR domain containing adaptor protein- inducing IFN-β (TRIF). TLR4 can also access TRIF through a TRIF related adaptor molecule (TRAM). Signalling through the MYD88 pathway results in NF-κB production, which induces the production of inflammatory cytokine and expression of chemokines. Activation of the TRIF dependent pathway, through NF-κB resulting in signalling through interferon regulatory protein 3 (IRF3) which induces production of IFN-β and the induction of IFN inducible genes which produce type 1 interferons. TLR1, TLR2 and TLR5 are also able to activate NF-κB production independent of MYD88 through phosphatidylinositol 3-kinase (Valles et al., 2014).

1.55 TLRs in renal disease

TLR expression has been studied in many forms of renal disease and normal kidneys. Evidence from these experiments demonstrate that there is a discrepancy in TLR expression dependent on the disease type or in some cases the method of TLR detection (mRNA versus immunohistochemistry). TLRs are expressed in normal kidneys, TLR1, TLR2, TLR3, TLR4 and TLR6 mRNA are expressed in normal mouse cultured tubular epithelia cells, whereas TLR5 and TLR9 where not expressed (Tsuboi et al., 2002). The

most widely studied TLR in normal human and mouse kidneys is TLR2. Shigeoka et al demonstrated that TLR2 is expressed in murine kidneys, and found constitutive staining within endothelial cells (shown by colocalization with CD31, an endothelial cell marker), mesangial cells, Bowmans capsule, the basolateral membrane of renal tubules, and within the endothelium of blood vessels (Shigeoka et al., 2007). In comparison, when renal ischemia injury (IR) was induced in mice, the distribution of TLR altered significantly where TLR2 expression translocated from a lateral membrane distribution in tubular epithelial cells to a cytoplasmic location after injury. This study also examined the effect of genetic deletion of TLR2 from mice and compared it to genetic deletion of the downstream signalling adaptor proteins MYD88 and TRIF. TLR2-/- mice had enhanced protection to IR compared to mice deficient in the adaptor proteins. These findings indicate that there is a potential alternative signalling pathway independent of MYD88 and TRIF for TLR2, and importantly that TLR2 plays a significant role in the pathogenesis of IR. Interestingly, previous studies of IR by Wolfs et al., (2002) using *in situ* hybridization for TLR detection, found no TLR2 or TLR4 expression within glomeruli. The difference could be explained by either the different methods for mRNA expression, induction of IR or the genetic background of mice, (C57/Bl6 in Shigeoka et al study, versus Swiss mice in Wolfs study).

Glomerulonephritis induced by nephrotoxic antibody, has demonstrated a crucial role for the involvement of TLR2, on both immune cells and intrinsic cells of the glomerulus. Brown et al (Brown et al., 2006) generated bone marrow chimeras to isolate the effect of intrinsic TLR2. Results from this study revealed that the contribution of TLR2 on intrinsic renal cells from chimeric mice (deficient in Bone marrow TLR2) had significantly more renal injury judged by albuminuria compared to complete TLR2-/- depletion. Using the known TLR2 agonist Pam_3CysK_4, supernatant from cultured TLR2 intact and TLR2-/- mesangial cells were measured by ELISA for the expression of CXCL1 and CXCL2 neutrophil chemokines. The TLR2-/- mesangial cells did not produce either CXCL1 or CXCL2, indicative of TLR2 requirement for production of both chemokines. Of significant interest is that TLR2

expression on neutrophils was required for inducement of GN more so than intrinsic renal TLR2 expression (Brown et al., 2006).

Cryoglobulinemic membranoproliferative glomerulonephritis (MPGN) is a systemic disease which results in the deposition of cyroglobulins (circulating complexes of immunoglobulins) in the glomerular capillary walls and mesangium. Histologically it is characterised by glomerular infiltration of macrophages and immune complexes. In murine models of MPGN, TLR4 has been shown to be expressed within podocytes (Banas et al., 2008). Cultured podocytes incubated with TLR4 ligands (LPS, and Lipid A) show significantly increased chemokine expression. Using TLR4 specific small interfering (siRNA), enabled the experimental inhibition of TLR4, to demonstrate inhibition of TLR4 rendered podocytes unresponsive to Lipid A, but not LPS. This indicates that in addition to TLR4 as a ligand for LPS another TLR4 independent mechanism for LPS stimulation exists in podocytes. Fibrinogen, another TLR4 ligand, which is abundantly present in many forms of GN, was also demonstrated to induce TLR4 dependent expression of chemokines (Banas et al., 2008). A further study by Motojima et al., (2010) in a model of glomerular sclerosis using similar techniques (podocyte culture and siRNA) demonstrated a role for podocyte fibrinogen, which increased the mRNA expression of both TLR4 and TLR2. TLR2 and TLR4 have also been demonstrated to play a role in acute kidney injury (AKI), which is a sterile inflammatory response, in which there is widespread cellular death. The death of tubular epithelial cells results in histone release into the extracellular space, where they have the potential to act as DAMPS. Allam et al., (2012) demonstrated *in vitro* that histone release from dying tubular epithelial cells increased TLR2 and TLR4 expression. Genetic deletion of TLR2 and TLR4 decreased renal injury through the inhibition of TLR dependent cytokine production. These findings indicate the cell death and the release of histones is recognised by the immune system as danger signals and induces inflammation.

TLR2 and TLR4 have also been implicated in diabetic nephropathy (DN). In experimental diabetic nephropathy induced by streptoztocin, TLR2-/- mice were protected from the

development of DN. *In vitro* podocyte and tubular cell culture with high glucose induced TLR expression, and production of TGF-β (Mudaliar et al., 2014). Examination of TLR4 expression in renal biopsies from type 2 diabetes (T2D) patients, show increased expression of the TLR4 gene and protein expression. The downstream TLR4 signalling pathway through NFκB was also significantly enhanced in T2D. Of particular significance is that in a 6 year follow up of patients, the increased expression of the TLR4 gene within glomeruli from the first presentation biopsy, was able to predict a decrease in eGFR using multiple regression analysis (Verzola et al., 2014).

A role for TLR9 has been implicated in the pathogenesis of lupus in humans. A small study of childhood onset lupus nephritis (LN) (*n*=4) comparing active LN versus non active LN has shown that TLR9 is present within podocytes in active LN but absent in non-active LN. The increased expression of TLR9 in active disease also correlated with increased titre to anti double stranded DNA and proteinuria, indicating a functional role for TLR9 in the pathogenesis of lupus (Machida et al., 2010). A larger study of patients with LN (n=26), examined the expression of TLR3, TLR7, TLR8 and TLR9 and correlated them with clinical and histological parameters. Compared to controls there was a significant increase in tubulointerstitial TLR expression in all examined TLRs, only a significant increase in glomerular expression was observed for TLR9. TLR9 correlated with renal SLE activity index (SLEDAI), a measure of disease activity based on clinical observations and tests (Conti et al., 2016).

Our laboratory has shown that TLR9 is required for the full progression of kidney injury and development of autoimmunity in a model of anti-GBM disease (Summers, et al., 2010). Administration of a TLR9 ligand (synthetic oligodeoxynucleotides (ODNs), containing unmethylated deoxycytidyl-deoxyguanosine (CpG) motifs, known as CpG-ODN), in a model of anti GBM resulted in GN. Genetic deficiency in TLR9 attenuated the resulting GN. Using chimeric mice, (TLR9-/-, reconstituted with WT CD45.1 bone marrow) demonstrated that it was the bone marrow derived TLR9 that played a major role in the induction of the disease.

Interestingly in animal models of lupus using the MRL/Mp$^{lpr/lpr}$ mouse strain that develop lupus like symptoms spontaneously, TLR9-/- increases kidney injury, and likelihood of death (Christensen et al., 2006). This is indicative of endogenous TLR9 having an important role in regulating immunomodulation.

1.56 Toll like receptors in AAV

Toll- like receptors may be the essential link between infection and autoimmunity. Infections can initiate and exacerbate disease in patients with AAV and provoke disease relapse (Tidman et al., 1998). Seasonal variation of the incidence of AAV and relapse occurring with a peak in winter suggests an association with microbial infection. This is further supported with clinical evidence that prophylactic antibiotic therapy in some AAV patients is able to avert relapses (Stegeman et al., 1996; Stegeman et al., 1997). Studies have demonstrated a pathogenic role for toll like receptors (TLRs) in mouse models of MPO-ANCA-GN (Summers, et al., 2010b; Summers et al., 2011; Summers, et al., 2010c; Summers, et al., 2010d). TLR2, TLR4 and TLR9 were chosen in this thesis due to their known association in disease models of MPO-ANCA GN and studies of peripheral leukocytes in patients with AAV (Summers et al., 2011; Summers, et al., 2010c; Tadema et al., 2011).

TLR2 is a cell surface receptor which recognises the widest range of ligands due to its shared structure with TLR1/6. Known exogenous ligands for TLR2 are lipomannan (mycobacterium), lipotechoic acids (gram-positive bacteria), liposaccharides (LPS, gram-negative bacteria) zymosan, and pepitdylyglycans (Jimenez-Dalmaroni et al., 2016). TLR2 is constitutively expressed in normal human kidneys, in both glomeruli and the interstitium (Shigeoka et al., 2007). Potential endogenous ligands for TLR2 include, high mobility group box 1 (HMGB1), fibrinogen, fibronectin, biglycan, monosodium urate crystals and heat shock proteins (Eleftheriadis et al., 2012; Jimenez-Dalmaroni et al., 2016). AAV patients have aberrant TLR2 expression on circulating monocytes compared to that of healthy controls, and mouse studies have shown that TLR2 ligand (pamityol-3-cysteine-serine-lysine -4) can direct autoimmunity in an anti-MPO mouse model by inducing Th17 CD4 T

cells, suggesting a potential role for TLR2 in AAV (Summers et al., 2011; Tadema et al., 2011).

TLR4 is a cell surface receptor that together with its coreceptor MD-2 recognises the exogenous ligands LPS, taxol, viral glycoproteins and MMTV envelop proteins(Richez et al., 2011). Many endogenous components can bind to TLR4, including extracellular matrix components fibronectin, fibrinogen and hyaluronic acid oligiosaccharides from necrotic cells, and the nuclear protein HMGB1. Immune cells stimulated with pro-inflammatory cytokine will export HMGB1 to the cytoplasm which can be released extracellularly by necrotic cells (Dumitriu et al., 2005; Scaffidi et al., 2002) Fibrinogen in particular in known to be recognized by TLR4 and induces chemokine production by macrophages (Richez et al., 2011). Evidence of the crucial role of TLR4 involvement in AAV comes from animal studies which have demonstrated that functional and histological renal injury in a model of MPO-ANCA is TLR4 dependent and expression is wide spread in tissue. *In vivo* studies with human glomerular cell line studies also demonstrate an increase in TLR4 expression after LPS stimulation (Summers, et al., 2010c).

Intracellular TLRs such as TLR9 are located within endosomes, and it has been hypothesised that they play a crucial role in the discrimination between the recognition of self-DNA and viral DNA. TLR9 recognises un-methylated CpG motifs from bacterial DNA or its synthetic analogue synthetic oligonucleotides containing a CpG motif (CpG-ODNs) (Ivanov et al., 2007). TLR9 endogenous ligands include self- DNA and HMGB1. There is an association between the formation of neutrophil extracellular traps (NETs) which extrude extracellular DNA in the form of histones and chromatin, and AAV (Kessenbrock et al., 2009; O'Sullivan et al., 2015). TLR9 is activated by extracellular DNA, and studies have shown that NETs can activate TLR9 in DCs (Garcia-Romo et al., 2011; Lande et al., 2011; Lande et al., 2007).

COMPLEMENT

The role of complement in AAV was mostly ignored due to the pauci immune description of the renal component of the disease. The complement system consists of multiple membrane bound and plasma proteins designed to interact with each other to exert effector functions to eliminate pathogens. The complement system, is activated by 3 different pathways, but they all ultimately form C3 convertase. This produces C3a and C3b, which leads to C5 complex.

Evidence for a role of complement in AAV comes from animal studies. Xiao et al., (2007) demonstrated a role for the alternative complement pathway in a murine model of anti MPO GN described previously [anti MPO IgG and anti MPO splenocytes models, Xiao et al., 2002). Experimental anti MPO GN was induced in mice deficient in various components of the complement system. C4-/- mice developed anti MPO GN, whereas both C5-/- and factor B-/- mice were protected from disease. The authors tested the hypothesis that it was factors released by ANCA activation of neutrophils that activated the complement system in human AAV. Normal human neutrophils were activated with TNFα prior to incubation with anti MPO IgG and PR3 IgG, which resulted in significant production of C3a compared to normal IgG controls. Subsequent studies, demonstrated that inhibition of C5 with a depleting antibody also protected mice from disease (Huugen et al., 2007). Schreiber et al., (2009) showed a role for the C5a receptor in neutrophil activation in their model of anti MPO GN [described previously, Schreiber et al., 2006). Blocking the C5aR, with a C5aR blocking antibody prevented the neutrophil respiratory burst induced by ANCA Ig. MPO immunised MPO-/- deficient mice were irradiated and reconstituted with bone marrow from C5aR-/- mice, and compared to controls that were reconstituted with WT bone marrow. Mice receiving the WT BM developed glomerular crescents and necrosis, haematuria and proteinuria whereas the C5aR reconstituted mice were protected. These experiments determined that C5a is required for ANCA activation of neutrophils, whereas C3 is only required for the generation of C5a. Xiao et al., (2014) confirmed in their murine model of anti MPO GN that blocking

the C5aR *in vivo,* attenuated disease. Results from these experiments collectively suggest that the C5aR inhibition may be a potential therapeutic in AAV.

Recent exploration of human renal biopsies of AAV, suggest that all though the disease is considered to be “pauci immune”, immune complexes can be present in glomerular lesions. Haas et al, demonstrated that in an electron microscopy study, that over half of the 126 patient cohort had evidence of either Ig or complement deposition within their glomeruli, and those with immune complex deposits had an increased level of proteinuria and glomerular crescent formation (Haas & Eustace, 2004),. A smaller Japanese study demonstrated similar results, which could stratify patients into those with or without immune complexes. The group with immune complexes had increased proteinuria compared to those patients without immune complex deposition (Sumida et al., 2012). In a Chinese cohort of patients (*n*=112), renal C3c deposition in patients with AAV (*n*=44), correlated with poor renal function, glomerular lesions, and high levels of urinary protein compared to C3c negative patients *n*= 68)(Chen, et al., 2009).

MYELOPEROXIDASE

1.57 Introduction

Verdoperoxidase, was the first term used to describe Myeloperoxidase (MPO) in reference to its green colour. It was first described in the pus discharge of patients with tuberculous empyema (Agner et al., 1948). In the next 30 years the term verdoperoxidase, was changed to myeloperoxidase to reflect its origin from myeloid lineage, but its exact function was still unknown. Klebanoff, (1967) was the first to determine the anti-bactericidal function of MPO against *Escherichia coli*, and documented that both iodide ions and H_20_2 were required for effective bacterial killing. These seminal studies also demonstrated that the iodide ions could be replaced with chloride (Cl). In a further study Klebanoff, (1968), found that MPO in the presence of endogenous H_20_2 provided by the bacteria itself (*Lactobacillus acidophilus,)* was effective in killing the bacteria without any requirement for the addition of exogenous

H_20_2.The efficiency in which MPO could kill bacteria was enhanced at pH 5, with the addition of halides and H_20_2. All the subsequent work on the MPO-H_20_2 system, in bacterial killing is based on this original work of Klebanoff.

The purpose of this review is to highlight the importance of MPO in innate immunity, its role in adaptive immunity, to discuss it cellular origin, and its potential direct injurious role and through autoimmunity.

1.58 MPO origin and MPO-H_20_2 system

MPO is synthesised in the bone marrow during myelopoiesis. The common progenitor Myeloblast through which both neutrophils and monocytes/macrophages are generated, has the highest concentration of MPO mRNA, as each neutrophil and monocyte/macrophage terminally differentiates in to mature cells, MPO mRNA is no longer detected, indicating the mature cell no longer generates any new MPO upon maturation (Cowland & Borregaard, 1999). The MPO mRNA, is detected in low levels in differentiated monocytes, and completely absent from mature macrophages (Cowland & Borregaard, 1999). It has been documented that mature macrophages positive for MPO have been found infiltrating atherosclerotic plaques in both mice and humans, indicating that activated macrophages may be stimulated in an inflammatory environment to instigate new synthesis of MPO (McMillen et al., 2005; Sugiyama et al., 2001).

Schultz & Kaminker, (1962) demonstrated that MPO is greater than 5% of the dry weight of neutrophils in human blood. MPO is contained within the acidic primary granules of the neutrophil, bound to an extracellular matrix composed of sulphated proteoglycans which bear a negative charge. As MPO is highly cationic, it binds to the negatively charged granule extracellular matrix, which combined with the low pH of the granules renders the MPO inactive. Once the neutrophil is activated, primary granules are dispensed to the phagosome, combine with activated NADPH oxidase which produces 0_2 to ultimately generated H_20_2, and hence the MPO-H_20_2 system originally described by Klebanoff (Babior,

2004; Babior et al., 2002; Klebanoff, 1967, 1968). Dependent on the extracellular environment the MPO- H_20_2 system is capable of generating a multitude of varying oxidant species. In particular HOCl, which in physiological conditions exists as HOCl +OCl^-, whereas in low pH conditions if can form Cl_2, all of which have the potential to damage surrounding extracellular structures (Davies et al., 2008).

Although the MPO-H_20_2 system, has been demonstrated to be effective in microbicide killing of bacterial pathogens, the health of MPO genetically deficient humans is largely unaffected. Hereditary MPO deficiencies vary according to country. A study of 100 MPO deficient and partially deficient individuals, demonstrated that as a group complete MPO deficient people have a higher rate of infection compared to healthy controls with normal levels of MPO (Kutter et al., 2000) .

1.59 Immunomodulatory properties of MPO

In addition to the micro bactericidal properties of MPO, studies have reported experimental evidence that MPO can enhance immune responses. The major phylogenetic difference between human MPO and murine MPO, is the amount of MPO in murine neutrophils is less than 20% of what human neutrophils contain (Noguchi et al., 2000; Rausch & Moore, 1975). Early studies used recombinant human MPO (rhMPO), as human neutrophils had more MPO for purification purposes. Lefkowitz et al., (1993) used recombinant human MPO *in vitro* to measure macrophage response in murine macrophages. Stimulation of macrophages with rhMPO *in vitro* resulted in an increased generation of TNFα and IFNα/β in a dose dependent manner, and an increased rate of phagocytosis with high doses of rhMPO. *In vivo* administration of rhMPO mice demonstrated a peak of TNFα and IFNα/β rapidly compared to invitro (3h peak *in vitro* versus a 90 minute peak *in vivo*). This study demonstrated that rhMPO can enhance the proinflammatory cytokine (TNFα and IFNα/β) secretion of murine macrophages. In arthritis the production of TNFα by macrophages plays a pivotal role in perpetuating rheumatoid arthritis by inducing a cascade of cytokines that results in inflammation (Burmester et al., 1997). In a model of experimental arthritis,

Gelderman et al., (1998), demonstrated that both enzymatically and inactive MPO aggravated rheumatoid arthritis. Mono-articular arthritis, was induced in female Lewis rats by administration of purified streptococcal cell wall (PG-APS), into one ankle joint, and saline into the other. The resulting arthritis was allowed to subside to gauge a base line joint swelling. Administration of either enzymatic porcine MPO (pMPO) or partially inactive MPO (iMPO) induced an arthritic flare based on increased joint swelling 24-48 hours after the injection. Administration of mannans a mannose receptor inhibitor simultaneously with MPO, did not result in an arthritic flare. This data provided evidence to support the hypothesis that MPO binds to macrophages via the mannose receptor.

Recently in our laboratory we have shown in antigen induced arthritis (AIA) that enzymatic MPO deposited by MPO in lymph nodes can inhibit adaptive immunity, by decreasing DC activation (Odobasic et al., 2013). AIA a T cell driven form of rheumatoid arthritis, was induced by intra-articular administration of either methylated bovine serum albumin [(mBSA), BSA-AIA] or ova (OVA-AIA) in either WT animals or MPO-/- mice. The effect of MPO-/- deficiency resulted in increased CD4+ T cell activation, and production of IFNγ, in both the BSA-AIA and OVA-AIA models. Peak infiltration of neutrophils to the draining lymph node occurred at 4 hours, and was associated with increased MPO activity. Confocal microscopy determined evidence of both intracellular and extracellular MPO in the lymph nodes, and cellular contact between dendritic cells and MPO of extracellular origin. *In vivo* observations of dendritic cell (DCs) and neutrophil co-cultures provided evidence that the presence of DCs resulted in an increased production of MPO by neutrophils. *In vivo* inhibition of MPO with 4 aminobenzoic acid hydrazide (ABAH) in mice that had received OVA/LPS reduced LN MPO activity whilst neutrophil recruitment and numbers were not reduced. At both 4 hour and 18 hour time points MPO deletion and inhibition decreased DC activation, evident by a significant decrease in the expression of DC activation markers MHC-II and CD86. These studies support the hypothesis that MPO can have an immunomodulatory effect on DCs.

Evidence from these studies warrants further investigation for several reasons. Firstly, these experiments have been carried out in murine models. Mice not only have reduced neutrophil MPO content, they also have a significant reduction in circulating neutrophils compared with humans and therefore are physiologically different (Rausch & Moore, 1975). Secondly, the role of MPO may be organ or disease specific, having a protective immunomodulatory effect in peripheral lymph nodes in AIA, but a deleterious effect in renal disease where MPO acts as an antigen. Dissecting the multifaceted role of MPO is crucial to our understanding of its contribution in both health and disease. This is especially important if we target MPO therapeutically, considering MPO is required for efficient neutrophil bactericidal properties, and in some situations has an immunomodulatory effect, inhibition may be deleterious. Effective inhibition would have to protect the enzymatic role and immunomodulatory role of MPO whilst inhibiting its deleterious effect.

CLINICAL SIGNIFICANCE OF THE PROJECT

AAV has an unknown aetiology, with a multifactorial pathogenesis, making it a complex disease to understand. Dissecting the underlying pathogenic mechanisms is essential to aid in the development off effective treatment strategies with minimal side effects that will improve the quality of life for patients. Despite maintenance therapy, the high percentage of relapses suggest that standard treatment does not actually address the primary pathogenesis of AAV and the toxicity of the treatment itself contributes to the morbidity and mortality rates.

Long term management of AAV patients is a continuous balancing act, trying to reduce the severity of disease without contributing to morbidity and mortality through the use of immunosuppression. The objective of this thesis is to add to our understanding of the pathogenesis of AAV, and introduce potential therapeutic targets. In chapter 2, I demonstrate the extent of extracellular MPO deposition within glomeruli of patients with AAV, which supports the hypothesis that MPO derived from degranulating or netting neutrophils contribute to this disease. In chapter 3, I introduce the use of disodium chromoglycate a drug that targets mast cell specific calcium channels, which blocks mast cell degranulation whilst protecting its immunomodulatory effects. In chapter 4 I identify that Toll-like receptor 2, Toll-like receptor 4 and Toll-like receptor 9 have an increased expression within renal biopsies of AAV patients. This suggests that TLRs contribute to the injury and possibly perpetuate inflammation in this disease, this identifies them as possible potential therapeutic targets. Finally in chapter 5, I use DNase I which specifically clears extracellular DNA, and reduces disease in 2 different animal models of anti MPO-GN. This is of particular interest as DNase I has been shown to be well tolerated by patients in clinical trials of SLE (although not useful as treatment in SLE), and could possibly be use to lower the burden of immunosuppression in standard treatment regimes (Davis et al., 1999).

HYPOTHESES AND AIMS

The overall objective of this study was to contribute to the understanding of the pathogenesis of AAV, identify potential new therapeutic targets and assess the benefits of 2 alternative therapeutics to current standard treatments in animal models of AAV.

Chapter 2: Renal participation of myeloperoxidase in anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis

Hypothesis: Extracellular MPO derived from neutrophil degranulation or leukocyte extracellular traps participates in inducing glomerular and interstitial injury.

Aims:

1. To semi-quantitate the prominence of NET formation within renal biopsies
2. To determine the cellular sources of MPO
3. Semi-quantitate extracellular and intracellular MPO
4. To determine the presence of Macrophage extracellular traps (METS)
5. Correlate glomerular leukocytes and MPO with pathology and functional injury

Chapter 3: Mast Cell stabilization ameliorates autoimmune anti-myeloperoxidase glomerulonephritis

Hypothesis: Stabilisation of Mast cells by disodium chromoglycate (DSCG) will prevent mast cell degranulation and prevent injury in a model of anti-myeloperoxidase glomerulonephritis.

Aims:

1. To assess participation and activation state of MCs in biopsies of AAV patients
2. Demonstrate the protective effect of DSCGs is specific to its effect on MCs
3. To determine if DSCG can prevent establishment of anti-MPO autoimmunity
4. To determine if DSCG is therapeutic in mice with established autoimmunity

Chapter 4. Intrarenal Toll-Like receptor 4 and Toll-Like receptor 2 expression correlates with kidney injury in anti-neutrophil cytoplasmic antibody associated vasculitis

Hypothesis: Toll-like Receptor 2, Toll-like Receptor 4 and Toll-Like receptor 9 will be dysregulated in patients with AAV and contribute to the injury and perpetuation of inflammation.

Aims:

1. To investigate the cellular distribution of TLR2, TLR4 and TLR9 in human kidneys
2. Determine a correlation between TLR expression and glomerular pathology
3. Determine if there is a correlation between TLR expression and functional injury
4. Determine if there is an association between TLR expression and potential ligands

Chapter 5. Therapeutic use of Deoxyribonuclease I modulates autoimmunity to myeloperoxidase and attenuates glomerular injury in experimental anti myeloperoxidase glomerulonephritis

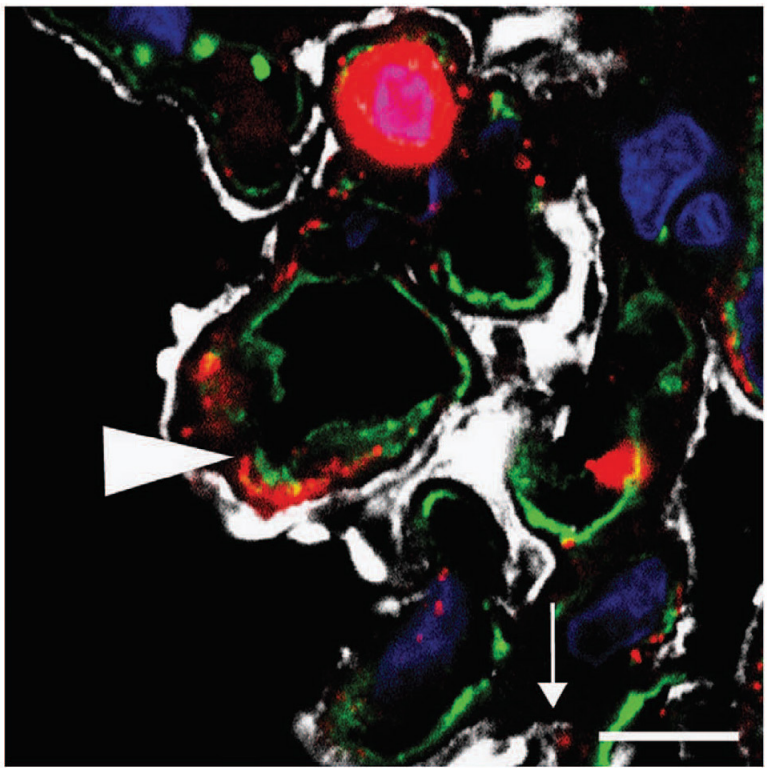
Hypothesis: DNase I will eliminate extracellular DNA in a model of MPO-ANCA GN and effectively treat both GN and the underlying autoimmunity

Aims:

1. Determine if extracellular DNA is present within glomeruli of patients with AAV
2. Determine if DNase I treatment can reduce extracellular DNA in a mouse model of anti-MPO GN with established autoimmunity to MPO
3. Determine if DNase I can prevent the development of autoimmunity to MPO
4. Determine the effect of DNase I on DC migration to the lymph nodes after immunisation with MPO
5. Determine the effect of DNase I in a humoral model of ANCA-GN

CHAPTER 2: MPO IN AAV

Renal Participation Of Myeloperoxidase In Anti-Neutrophil Cytoplasmic Antibody (ANCA)-Associated Glomerulonephritis



http://www.kidney-international.org basic research

Renal participation of myeloperoxidase in antineutrophil cytoplasmic antibody (ANCA)–associated glomerulonephritis

Kim M. O'Sullivan[1], Camden Y. Lo[2], Shaun A. Summers[1,3,✠], Kirstin D. Elgass[2], Paul J. McMillan[4,5], Anthony Longano[6], Sharon L. Ford[1,3], Poh-Yi Gan[1], Peter G. Kerr[3], A. Richard Kitching[1,3] and Stephen R. Holdsworth[1,3]

[1]Centre for Inflammatory Diseases, Monash University Department of Medicine, Clayton, Victoria, Australia; [2]Monash Micro Imaging, Monash University, Clayton, Victoria, Australia; [3]Department of Nephrology, Monash Health, Clayton Victoria, Australia; [4]Department of Biochemistry and Molecular Biology, BIO21 Institute, University of Melbourne, Parkville, Victoria, Australia; [5]Biological Optical Microscopy Platform, University of Melbourne, Parkville, Victoria, Australia and [6]Department of Pathology, Monash Health, Clayton, Victoria, Australia

Myeloperoxidase (MPO) is an important neutrophil lysosomal enzyme, a major autoantigen, and a potential mediator of tissue injury in MPO-ANCA-associated vasculitis (MPO-AAV) and glomerulonephritis. Here we examined MPO deposition in kidney biopsies from 47 patients with MPO-AAV. Leukocyte accumulation and fibrin deposition consistent with cell-mediated immunity was a major feature. Tubulointerstitial macrophage, CD4+ and CD8+ T-cell, and neutrophil numbers correlated with low presenting eGFR. MPO was not detected in kidneys from patients with minimal change or thin basement membrane disease, but was prominent in glomerular, periglomerular, and tubulointerstitial regions in MPO-AAV. Extracellular MPO released from leukocytes was pronounced in all MPO-AAV patients. Similar numbers of neutrophils and macrophages expressed MPO in the kidneys, but colocalization studies identified neutrophils as the major source of extracellular MPO. Extraleukocyte MPO was prominent in neutrophil extracellular traps in the majority of patients; most of which had traps in half or more glomeruli. These traps were associated with more neutrophils and more MPO within glomeruli. Glomerular MPO-containing macrophages generated extracellular trap-like structures. MPO also localized to endothelial cells and podocytes. The presence of the most active glomerular lesions (both segmental necrosis and cellular crescents) correlated with intraglomerular CD4+ cells and MPO+ macrophages. Thus, cellular and extracellular MPO may cause glomerular and interstitial injury.

Kidney International advance online publication, 15 July 2015; doi:10.1038/ki.2015.202

Correspondence: *Stephen R. Holdsworth, Centre for Inflammatory Diseases, Monash University Department of Medicine, Monash Medical Centre,* [redacted]

✠Deceased.

Received 21 August 2014; revised 7 May 2015; accepted 14 May 2015

KEYWORDS: ANCA; glomerulonephritis; inflammation; macrophages; renal biopsy; vasculitis

Proteinase 3 (PR3) and myeloperoxidase (MPO) are the major autoantigens in antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV). This form of vasculitis is strongly associated with glomerulonephritis. Although autoimmunity to either PR3 or MPO can be found in each of these forms of vasculitis, their clinical features, natural history, and genetic associations suggest that MPO-ANCA- and PR3-ANCA-associated vasculitis are different but related autoimmune diseases.[1] In the case of MPO, evidence from several animal models shows that autoimmunity to MPO directs the development of focal, segmental necrotizing pauci-immune crescentic glomerulonephritis.[2]

MPO is an unusual autoantigen as it is itself a regulator of the extent of developing autoimmunity[3] as well as having the potential to be directly injurious to the major target organs. MPO is a key enzyme in host defense through its ability to oxidize hydrogen peroxide in the presence of chloride ions to hypochlorous acid, a major bactericide. Along with its intracellular phagolysomal role, MPO is released into the surrounding tissue by both neutrophils and macrophages in response to proinflammatory stimuli, in association with phagocytosis and by forming neutrophil extracellular traps (NETs).[4–6] Monocyte/macrophages also have the ability to produce extracellular traps,[7–11] but little is known about the production of METs in human AAV. Extracellular MPO is biologically active and induces oxidant injury to surrounding cells and extracellular proteins.[12] There is good evidence that the release of MPO by leukocytes accumulating in the kidney is a major source of the associated injury in AAV.[13]

Recent evidence shows that neutrophil-derived MPO attenuates the development of adaptive immunity and autoimmunity by its dampening effects on antigen-presenting

cells.[3] MPO-deficient ($Mpo^{-/-}$) mice show enhanced autoimmunity. In animal models of passive ANCA-induced glomerular injury, $Mpo^{-/-}$ mice show significantly reduced glomerular injury as the target autoantigen (MPO) is not present to bind ANCA.[14,15] Thus, MPO could potentially modulate or mediate glomerular injury in glomerulonephritis.

MPO-ANCA-associated glomerulonephritis is an unusual autoimmune disease in that the major target organ does not normally contain the target autoantigen, MPO. When ANCA bind to neutrophils, they accumulate in glomeruli where they release MPO into the extracellular environment by degranulation and NET formation.[5,13] This is likely to both mediate injury directly and to plant the autoantigen into the major target organ, initiating a second significant phase of injury as the effectors of anti-MPO autoimmunity (ANCA and anti-MPO CD4+ cells) target this planted antigen.[16,17] Although it is not possible to assess the role of intrarenal MPO in AAV by these manipulations in humans, to gain insights into the possible role of renal MPO we defined its deposition and its pathological and clinical associations in kidneys of a cohort of AAV patients.

RESULTS

Patient cohort and renal pathology

A total of 47 renal biopsies from people with a first presentation of MPO-AAV were included in this study. The population studied was mainly men (68%) with a mean age of 67 ± 2 years. They had severe disease (mean estimated glomerular filtration rate (eGFR) 22 ± 2 ml/min per 1.73 m^2) and exhibited elevated inflammatory markers (Table 1). Glomerular lesions were categorized according to the histological classification scheme of Berden *et al.*[18] The distribution was focal (28%), crescentic (23%), mixed (37%), and sclerotic (11%). Tubulointerstitial disease was graded by the extent of interstitial fibrosis and atrophy,[19] with the majority being grade 2.

Table 1 | Clinical and histological features of patients with MPO-ANCA-associated glomerulonephritis

Patient characteristics	
Patient number	47
Age at biopsy (years)[a]	67 ± 2
Sex (F/M)	15/32
Number of glomeruli[b]	19.4 ± 1.5
Laboratory values	
MPO-ANCA titer (U/ml)[a]	136 ± 15
Serum creatinine (μmol/l)[a]	287 ± 49
eGFR (ml/min per 1.73 m^2)	22 ± 2
ESR[a] (mm/h)	63 ± 5
CRP[a] (mmol/l)	33.9 ± 12.5
UTP-24 h[c] (g/day)	1.85 ± 0.04
Red blood cells (cells/HPF)[d]	137 ± 682
Extrarenal involvement	
Lung, URT, skin, arthralgia	16/47
Biopsy classification[e]	
Focal	13
Crescentic	11
Mixed	17
Sclerotic	5
Interstitial fibrosis/atrophy[f]	
Grade 1: 0–30%	11
Grade 2: 31–60%	34
Grade 3: >60%	2

Abbreviations: ANCA, antineutrophil cytoplasmic antibody; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; F, female; HPF, high-powered field; M, male; MPO, myeloperoxidase; URT, upper respiratory tract; UTP, urinary total protein.
[a]Expressed as median ± s.e.
[b]Expressed as mean ± s.e.
[c]The 24-h urinary protein excretion (38 patients).
[d]Median ± s.d.
[e]Berden *et al.*[18]
[f]Ford *et al.*[19]

Intrarenal leukocyte accumulation in patients with MPO-ANCA-associated glomerulonephritis

As in previously published studies,[20–22] all biopsies demonstrated accumulation of leukocytes in glomerular, periglomerular, and interstitial compartments. Enumeration of glomerular leukocytes confirmed the presence of macrophages, neutrophils, and T cells in all kidneys of patients with MPO-ANCA-associated glomerulonephritis. The predominant glomerular leukocyte was CD68+ monocyte/macrophages (Figure 1a), followed by CD15+ neutrophils and T cells. Intraglomerular CD8+ cells were more frequent that CD4+ T cells, whereas FoxP3+ cells were uncommon but still more frequently found than in control biopsies (minimal change or thin basement membrane disease, $n = 10$). Periglomerular leukocytes were most commonly monocyte/macrophages, followed by CD4+ and CD8+ T cells in equal numbers. Neutrophils and FoxP3 cells, although present to a lesser degree, were more common than in control biopsies (Figure 1b). In the tubulointerstitium, leukocytes were found in focal aggregates and were also present diffusely through the interstitium. T cells were more common than macrophages within the tubulointerstitium (Figure 1c). PR3-AAV patients exhibited similar leukocyte numbers (Supplementary Figure S1 online).

We sought to determine whether intrarenal leukocyte numbers inversely correlated with presenting eGFR. Correlations between eGFR and CD68+ cells, CD15+ cells, and T cells within glomeruli did not reach statistical significance (data not shown). However, the extent of interstitial infiltration of CD4+ T cells, CD8+ T cells, CD68+ macrophages, CD15+ cells, and FoxP3+ T cells all correlated with lower presenting eGFR (Figure 1d–h). Patients with MPO-ANCA glomerulonephritis had significant fibrin deposition in glomerular lesions (Figure 1i), associated with the presence of T cells and macrophage accumulation, consistent with delayed-type hypersensitivity responses.

Extracellular MPO is prominent in kidneys of patients with MPO-ANCA-associated glomerulonephritis

Extracellular ('extraleukocyte') MPO was defined as positive MPO staining that was not colocalized with CD45+

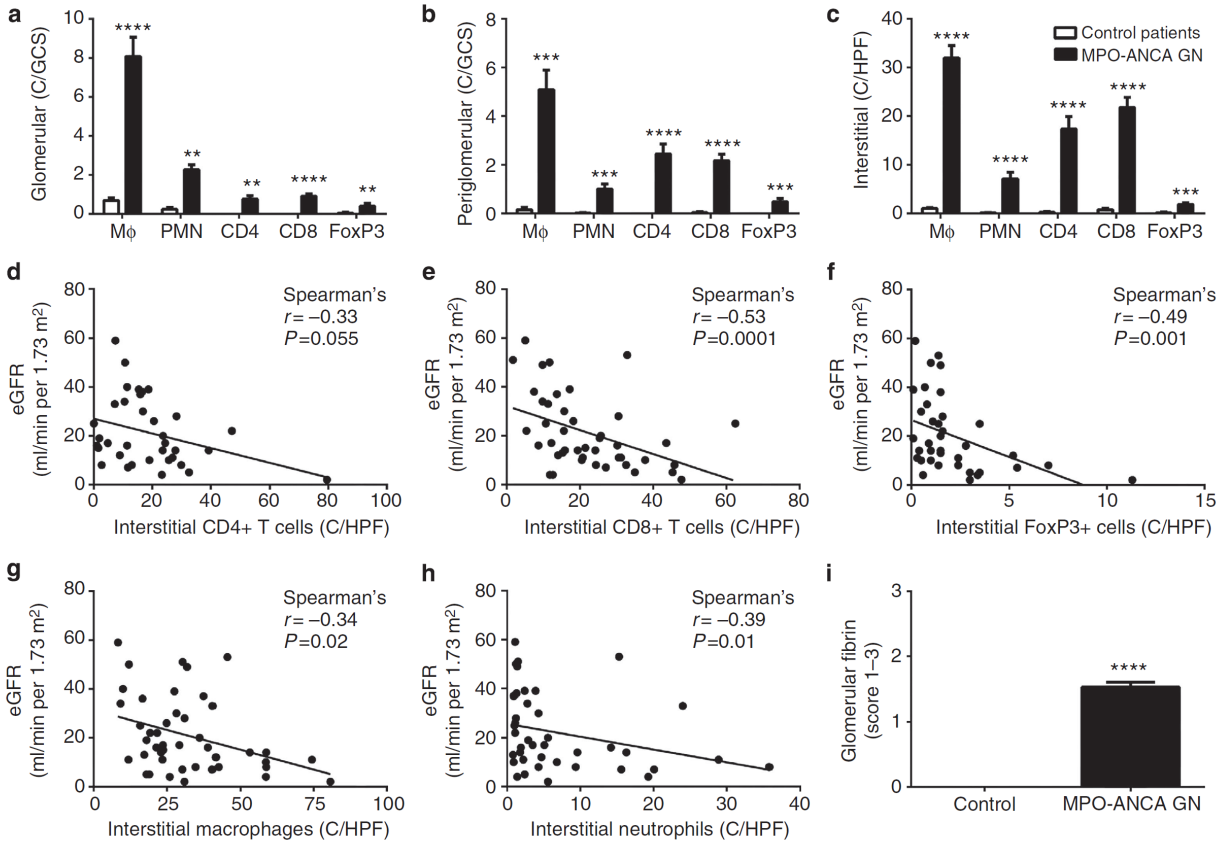


Figure 1 | Assessment of leukocytes and fibrin in MPO-ANCA GN biopsies. (**a–c**) Frequencies of macrophages, neutrophils, and T cells were assessed by immunostaining in biopsies of MPO-ANCA GN patients ($n = 47$) and controls (minimal change and thin basement membrane disease, $n = 10$), shown as the mean cells per glomerular cross-section (C/GCS, **a**, **b**) or cells per high-powered field (C/HPF, **c**). (**a**) MPO-ANCA GN patients had significantly more glomerular macrophages (Mϕ), neutrophils (PMNs), and CD4+ and CD8+ cells than those of the control cohort. (**b**) The predominant periglomerular leukocyte in the MPO-ANCA GN cohort were macrophages, followed by T cells, with CD4+ and CD8+ cells being present in equal numbers and then neutrophils. (**c**) Interstitial T cells were the predominant cell type within the interstitium, with CD4+ and CD8+ cells being detected in similar numbers. (**d–h**) Correlation of leukocytes with eGFR demonstrated that (**d**) interstitial CD4+ T cells, (**e**) CD8+ T cells, (**f**) macrophages, and (**g**) neutrophils were correlated with a low eGFR at biopsy. (**i**) MPO-ANCA GN patients had significantly more fibrin deposition in glomerular lesions than control patients. ANCA, antineutrophil cytoplasmic antibody; eGFR estimated glomerular filtration rate; GN, glomerulonephritis; MPO, myeloperoxidase; PMN, polymorphonuclear leukocyte. $^{**}P < 0.005$, $^{***}P < 0.0005$, $^{****}P < 0.0001$.

leukocytes. Extracellular MPO was found in all biopsies of MPO-ANCA glomerulonephritis and in a mean of 93% of all glomeruli examined (glomeruli with extracellular MPO present in individual biopsies ranged between 37 and 100%). Extracellular MPO was associated with intraglomerular aggregates of CD45+ cells but was also found in glomeruli with few CD45+ cells, within areas of sclerosis (Figure 2a–d). In the tubulointerstitium, deposits of extracellular MPO were found within tubular epithelial cells, among large areas of aggregated CD45+ cells and within tubular casts (Figure 2e and Supplementary Figure S2 online). Periglomerular and interstitial areas were evaluated as a single area and interstitial MPO was expressed as median ± s.e.m. from each of 217 × 172 µm fields of view (×400). The extent of extracellular MPO deposition was significantly greater within the glomeruli of MPO-ANCA patients than those of the control group that was minimal and comparable to background levels (Figure 3a). Similarly, periglomerular and interstitial areas exhibited non-CD45+ cell–associated extracellular MPO (Figure 3b). In patients with MPO-AAV, extracellular MPO made up 24.7% of all MPO within glomeruli and 36.5% of all MPO in the tubulointerstitium (Figure 3c and d). Examination of extracellular MPO in renal biopsies of patients with PR3-AAV ($n = 11$) demonstrated amounts and patterns of extracellular MPO deposition similar to that seen in MPO-ANCA patient biopsies in both glomeruli and interstitium (Figure 3e and Supplementary Table S1 online). Examination of glomeruli of patients with ISN/RPS (International Society of Nephrology/Renal Pathology Society) class IV lupus nephritis who had a similar degree of crescentic glomeruli to that of the MPO-ANCA glomerulonephritis patients (21% vs. 19%)

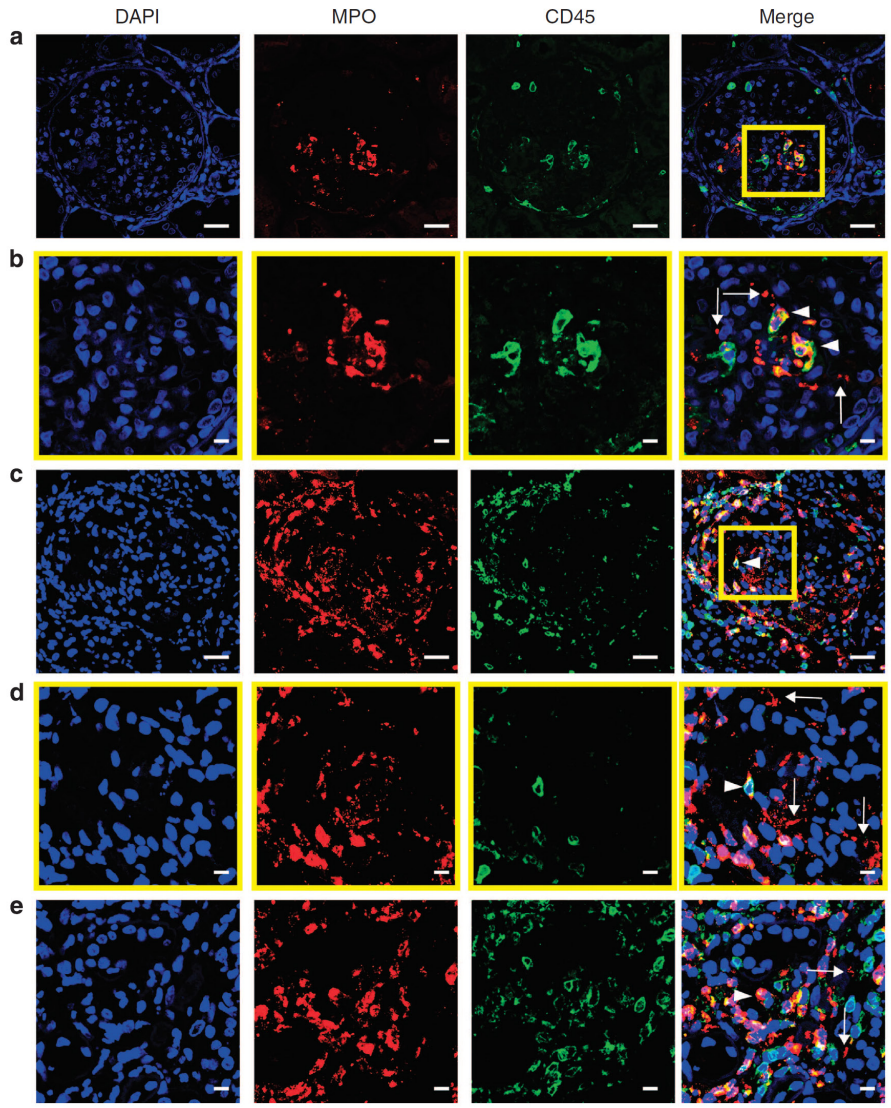


Figure 2 | Extracellular MPO is detected in glomeruli and the interstitium of MPO-ANCA GN patients. An anti-CD45 antibody (a pan-leukocyte marker, shown in green) and an anti-MPO antibody (shown in red) were used to define the expression of intracellular and extracellular MPO in MPO-ANCA GN kidneys. Nuclei were stained blue with DAPI. (**a**) Low-powered image (×600) reveals MPO within CD45+ leukocytes (yellow in the merged image) and extracellular (red in the merged image) in a mildly affected glomerulus. (**b**) High-powered images (yellow bordered inset from **a**) demonstrating intracellular (yellow, arrowheads) and extracellular MPO (red, arrows). (**c**) Extracellular MPO (red) in a segmentally damaged glomerulus (segmental lesion with loss of DAPI+ cells) with few CD45+ cells (green, large arrowhead) is shown. (**d**) High-powered images (yellow bordered inset from **c**) showing multiple deposits of extracellular MPO (arrows), with several MPO+CD45+ cells (one indicated by arrowhead). (**e**) High-powered images of the tubulointerstitium demonstrating extracellular MPO (arrows) and CD45+MPO+ cells. Large-scale bars = 50 μm, small-scale bars = 10 μm. ANCA, antineutrophil cytoplasmic antibody; DAPI, 4',6-diamidino-2-phenylindole; GN, glomerulonephritis; MPO, myeloperoxidase.

demonstrated significantly lower amounts of extracellular MPO deposition in both glomerulus and interstitium, with fewer neutrophils in the interstitium. Immunologically mediated primary membranous nephropathy also demonstrated significantly lower amounts of both glomerular and interstitial MPO deposition (Supplementary Table S1 online).

Leukocyte/MPO colocalization shows neutrophils are the major source of extracellular MPO

To determine which cell produces the majority of extracellular MPO in glomeruli, serial sections from 10 randomly selected samples were probed for CD15, CD68, and MPO. Leukocyte-associated extracellular MPO was assessed by measuring MPO fluorescence intensity adjacent to the cell

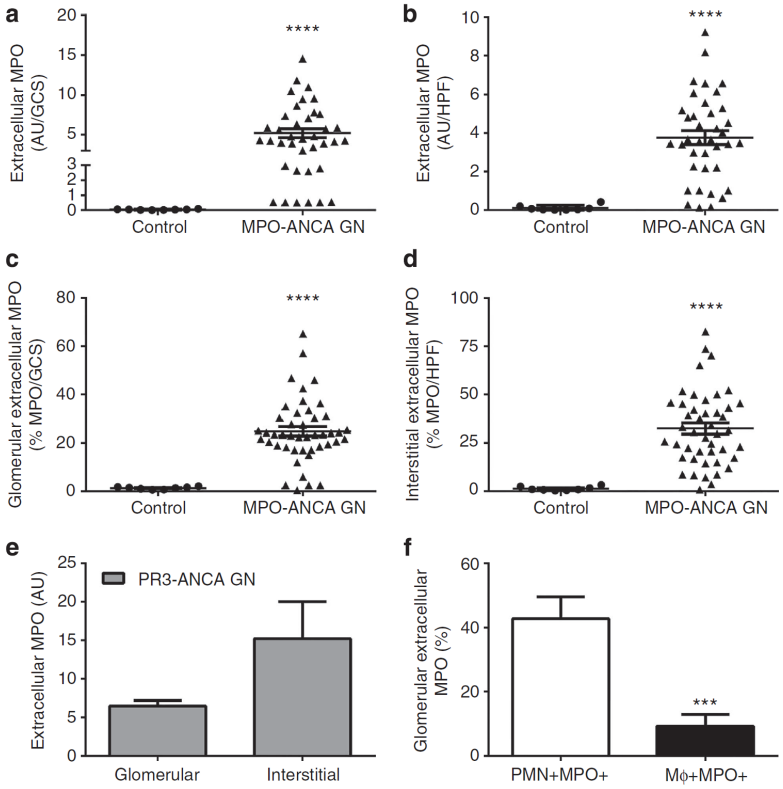


Figure 3 | Substantial amounts of MPO are deposited in kidneys of patients with MPO-ANCA GN. (**a**) Image analysis demonstrates that intracellular MPO and deposited extracellular ('extraleukocyte') MPO are higher in glomeruli of MPO-ANCA GN patients ($n = 47$, shown as arbitrary units per glomerular cross-section (AU/GCS)) than that of control disease group where extracellular MPO is virtually absent ($n = 10$). (**b**) A similar result is shown for the tubulointerstitial/periglomerular regions where significant amounts of extracellular MPO were deposited per high-powered field (HPF). The proportion (expressed as a percentage) of MPO that was deposited (extracellularly) within glomeruli (**c**) and the tubulointerstitial/periglomerular regions (**d**) was higher than that of the control disease group. (**e**) Extracellular MPO was deposited in a similar manner in PR3-ANCA GN patients ($n = 11$). (**f**) Serial sections of MPO-ANCA GN patients ($n = 10$) were stained for CD15 (neutrophils) and MPO and CD68 (macrophages). CD15+ neutrophils were associated with a greater proportion of deposited MPO than CD68+ macrophages. ANCA, antineutrophil cytoplasmic antibody; GN, glomerulonephritis; MPO, myeloperoxidase; PR3, proteinase 3. $^{***}P < 0.0005$, $^{****}P < 0.0001$.

periphery of each CD15+MPO+ and CD68+MPO+ cell and expressed as a proportion of the total MPO present within the glomerulus. More extracellular MPO was in direct juxtaposition to neutrophils than macrophages (Figure 3f), consistent with neutrophils being the major cellular source of extracellular MPO.

MPO is found in all neutrophils, with some renal macrophages also expressing MPO

All neutrophils were MPO+, whereas only 20–30% of macrophages were positive for MPO (Figure 4a and b). Periglomerular MPO+ neutrophil accumulation was similar to that of MPO+ macrophages. Within the interstitium, MPO+ macrophages were less frequent than MPO-negative macrophages, but were higher than those of the control group (Figure 4c–e). Glomerular MPO+ cells correlated with low presenting eGFR (Table 2). However, there was no correlation with MPO+CD15+ or MPO+CD68+ cells individually. Interstitial MPO+ macrophages, neutrophils, and all MPO+ cells correlated with low eGFR, but there was no correlation with extracellular MPO deposited in glomeruli or in the tubulointerstitium.

NETs are associated with more neutrophils and extracellular MPO in MPO-ANCA biopsies

We sought to define the extent and nature of MPO-containing extracellular traps (ETs) in glomeruli of patients with MPO-ANCA-associated glomerulonephritis. Sections were probed for MPO, histones, and chromatin as previously published.[4,7] ETs were frequently detectable in kidneys of patients with MPO-AAV (Figure 5a and b). Whereas macrophages have recently been shown to also be capable of forming ETs,[9,10,23] ETs are classically derived from neutrophils (NETs).[4,5] Therefore, we examined the potential neutrophil origin of these MPO-containing ETs using serial tissue sections. One section was stained for MPO/chromatin/

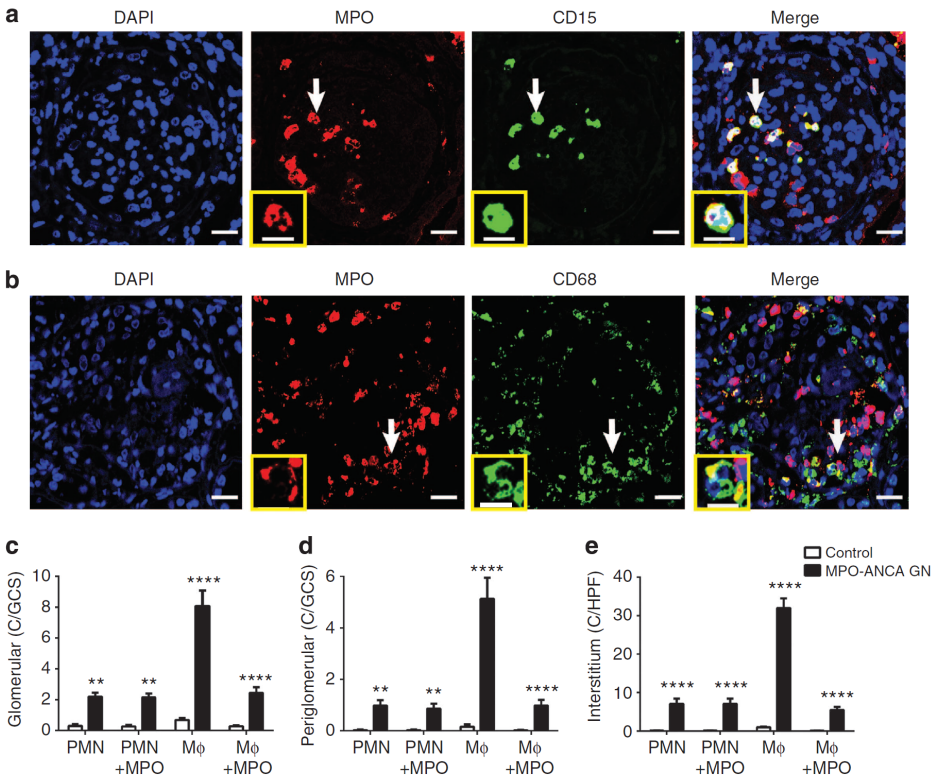


Figure 4 | Numbers and proportions of MPO-expressing neutrophils and macrophages. (**a**) Kidney sections were double labeled for MPO (red) and CD15 (neutrophils (PMN), green). All neutrophils were MPO+ (yellow, arrow). (**b**) Kidney sections labeled for MPO (red) and CD68 (macrophage/monocytes (Mϕ), green) showed that approximately one-third of all macrophages were MPO+. (**c–e**) The frequencies of MPO+ intrarenal neutrophils and macrophages were significantly higher in the MPO-ANCA GN group ($n = 48$) than those of the control disease group ($n = 10$). All neutrophils and a proportion of macrophages were MPO+, with each cell type contributing approximately one-half of MPO+ leukocytes. Patterns in the glomerular, periglomerular, and interstitial areas were similar. Scale bars in insets = 10 μm, large pictures = 50 μm. ANCA, antineutrophil cytoplasmic antibody; DAPI, 4',6-diamidino-2-phenylindole; GN, glomerulonephritis; MPO, myeloperoxidase; PMN, polymorphonuclear leukocyte. $^{**}P < 0.005$, $^{****}P = 0.0001$.

Table 2 | Correlation of intrarenal MPO and MPO+ leukocytes with eGFR

	Glomerulus[a]		Tubulointerstitium[b]	
	r	*P*-value	*r*	*P*-value
All MPO	0.035	0.833	0.068	0.685
MPO+ cells	−0.301[c]	0.047	−0.383[c]	0.015
CD68+ MPO+	−0.177	0.250	−0.419[c]	0.005
CD15+ MPO+	−0.210	0.192	−0.390[c]	0.013
Extracellular MPO	−0.067	0.688	−0.103	0.537

Abbreviations: eGFR, estimated glomerular filtration rate; MPO, myeloperoxidase.
[a]Cells per glomerular cross-section.
[b]Cells per high-powered field.
[c]Indicates a significant correlation.

histones, and the next for neutrophil elastase (a product of neutrophils but not macrophages), chromatin, and histones. NETs were present in glomeruli and the interstitium, undergoing different stages of NET formation (Figure 5c–f). In all, 61% of MPO-ANCA biopsies had NETs present within their glomeruli, but NETs were not found in controls. Of MPO-ANCA glomerulonephritis patients with NETs, over half (59%) exhibited NETs in ⩾50% of glomeruli, showing that NETosis was a frequent event (Figure 6a). There were more neutrophils in the glomeruli of patients with the presence of NETs (Figure 6b). Semiquantification of MPO (extracellular and cellular) within glomeruli confirmed a significantly higher extracellular and total MPO expression in patients with NETs (Figure 6c and d). There were no significant differences between the numbers of other leukocyte subtypes within the glomeruli and the presence of NETs (data not shown). Although NETs were found mainly within aggregates of neutrophils, single neutrophils undergoing NET formation within glomeruli were also observed.

To further validate the identification of NET-forming cells, 10 of the biopsies where glomeruli were exhibiting NET formation were further probed in serial sections for MPO, 4',6-diamidino-2-phenylindole (DAPI), and other known NET constituents. Peptidyl arginine 4 deiminase (PAD4) is a nuclear enzyme that can prevent the induction of neutrophil

apoptosis[24] and is essential for the production of NETs.[25–27] Citrullinated histones (histone H3Cit) correlate with extensive chromatin decondensation, as occurs in NET formation.[28–30] Figure 7a demonstrates that the percentage of glomeruli with NETs, identified with H2A-H2B, MPO, and DAPI, was identical to the proportion of glomeruli with the

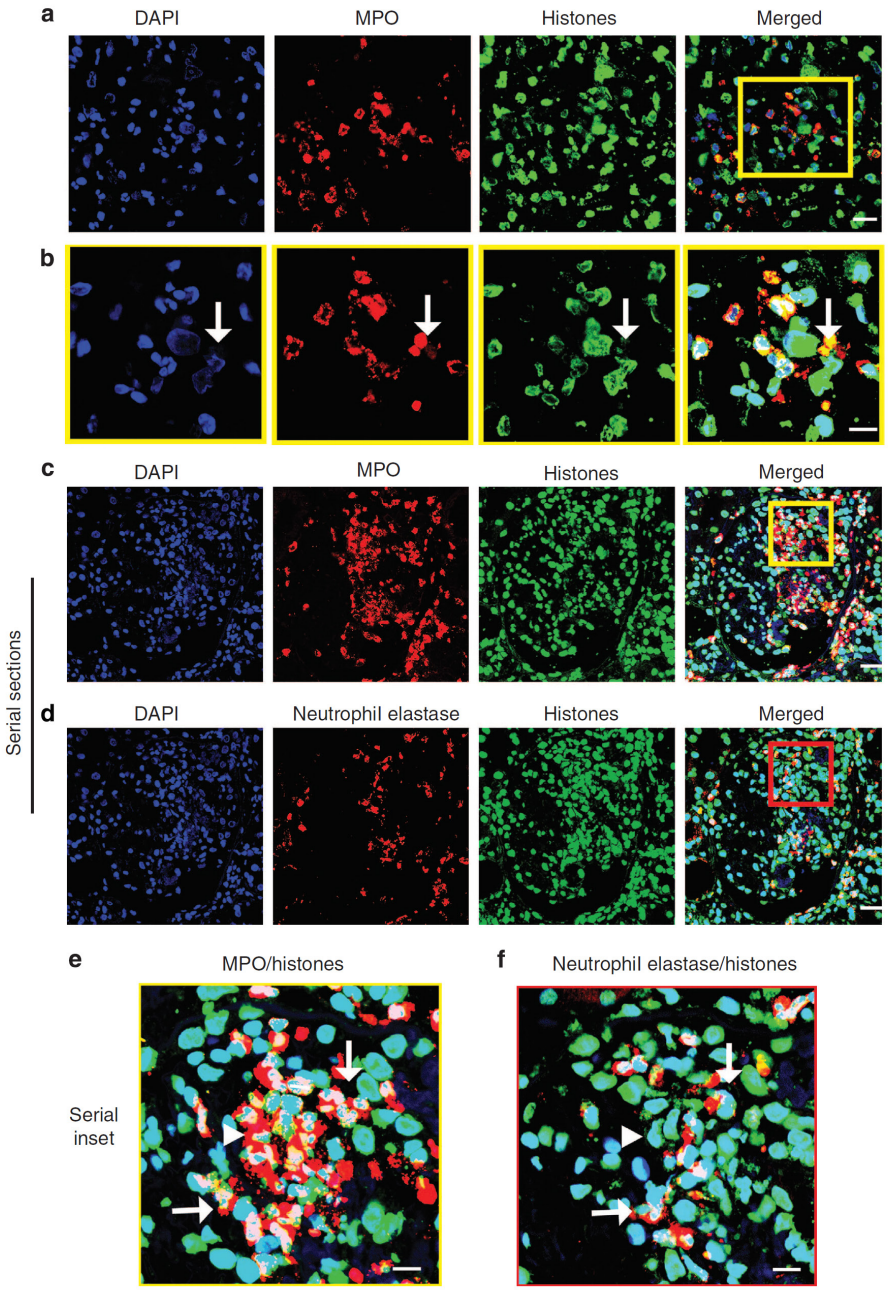


Figure 5 | Neutrophil extracellular traps (NETs) are found in the glomeruli of patients with MPO-ANCA GN. (**a**) Extracellular traps were identified as the colocalization of MPO (red), H2A-H2B histones (green), and chromatin (DAPI, blue). (**b**) High-magnification image of yellow inset from **a** (scale bar = 5 μm). To demonstrate that neutrophils produce extracellular traps within glomeruli, (**c**) sections were stained for H2A-H2B (histones, green), DAPI (chromatin, blue), and MPO (red) and compared with (**d**) serial sections stained for H2A-H2B, DAPI, and neutrophil elastase (red, neutrophils). NETs were identified by colocalization of all three markers (white) in both sections (scale bar = 50 μm). (**e**, **f**) High-powered images of inset areas of **c** and **d** demonstrate neutrophils producing NETs (arrows) and some extracellular trap–producing cells that are negative for neutrophil elastase (arrowhead). Scale bar = 10 μm. ANCA, antineutrophil cytoplasmic antibody; DAPI, 4',6-diamidino-2-phenylindole; GN, glomerulonephritis; MPO, myeloperoxidase.

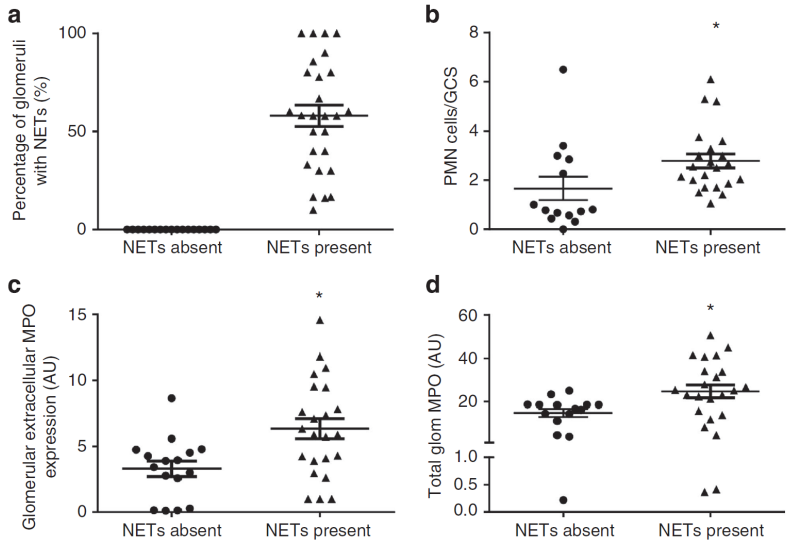


Figure 6 | Quantitation of glomerular NETs by serial sections and their association with extracellular MPO and neutrophils. (**a**) Of the 27 (of 44) patients in which intraglomerular NETs were detected, >59% demonstrated NETs in over half their glomeruli (by analysis of serial sections probed for DAPI, H2A-H2B, and either MPO or neutrophil elastase). (**b**) Intraglomerular neutrophil (PMN) numbers were significantly higher in patients with NETs compared with those of glomeruli with no NETs. (**c**) Quantitative image analysis demonstrates significantly higher amounts of extracellular MPO in NET-positive patients. (**d**) Compared with patients who had no NETs in glomeruli, those with intraglomerular NETs showed increased total glomerular MPO. AU, arbitrary unit; DAPI, 4',6-diamidino-2-phenylindole; GCS, glomerular cross-section; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PMN, polymorphonuclear leukocyte.*$P<0.05$.

NET constituents, MPO, PAD4, and H3Cit (Figures 7b–d). Using three-dimensional structural illumination (3D-SIM) super-resolution microscopy, a further five biopsies with NETs demonstrate the morphological and biochemical differences between a resting neutrophil, an apoptotic cell, and a NET-forming cell *in vivo* (Figure 8a–d). Definitive colocalization of NET constituents MPO, PAD4, and H3Cit is evident, as is H2A-H2B, MPO, and H3Cit colocalization (Figure 8c and d).

Glomerular macrophages are prominent producers of ET-like structures in MPO-ANCA-associated glomerulonephritis

As not all ETs were neutrophil elastase positive, and as many macrophages express MPO in these lesions (Figure 4c), a randomly selected cohort of 10 biopsies in which ETs had been detected were examined. Serial sections were assessed for macrophage extracellular traps (METs). In 6/10 biopsies, there were cells extruding nuclear material and MPO that were not neutrophils (Figure 9a–c). These cells were CD68+, indicating that glomerular MPO+ macrophages may be another source of MPO-containing ETs. However, monocytes/macrophage-derived METs were less frequently seen than NETs. Because of a lack of known markers for MET production, detailed analysis was not possible.

Associations between macrophages and extracellular MPO accumulation with histological classes of MPO-ANCA glomerulonephritis

The recent classification scheme demonstrated correlations between histopathology and disease outcome,[18] with a sclerotic pattern having the worst outcome and focal (⩾50% normal glomeruli) the best. MPO and MPO-containing leukocytes were examined in the different histological categories (Table 3). Although numbers of patients within groups limited definitive statistical outcomes, in crescentic (⩾50% cellular crescents) and mixed biopsies, macrophages were more common compared with patients with focal disease. There were consistent numerical increases in MPO containing cells (neutrophils and MPO+ macrophages) and extracellular MPO that did not reach statistical significance. Similar trends existed when crescentic biopsies were compared with sclerotic biopsies, except for extracellular MPO deposition. Neither the frequency of NET formation in NET+ patients nor numbers of patients with NETs differed significantly between groups, although there were more patients with NETs in the mixed class than any other class (data not shown). Correlations between MPO or inflammatory cells and indicators of acute and severe glomerular lesions were examined, using the percentages of glomeruli affected as a continuous variable. The percentage of glomeruli with the most active lesions, that is, with both segmental necrosis and cellular crescents, correlated strongly with the number of MPO+ CD68 cells, cellular MPO, the proportion of extracellular MPO, and CD4+ T cells (Table 4).

Endothelial cells, podocytes, and dendritic cells were positive for MPO

MPO is highly cationic and after release from cells could adhere to glomerular structures, including endothelial cells.

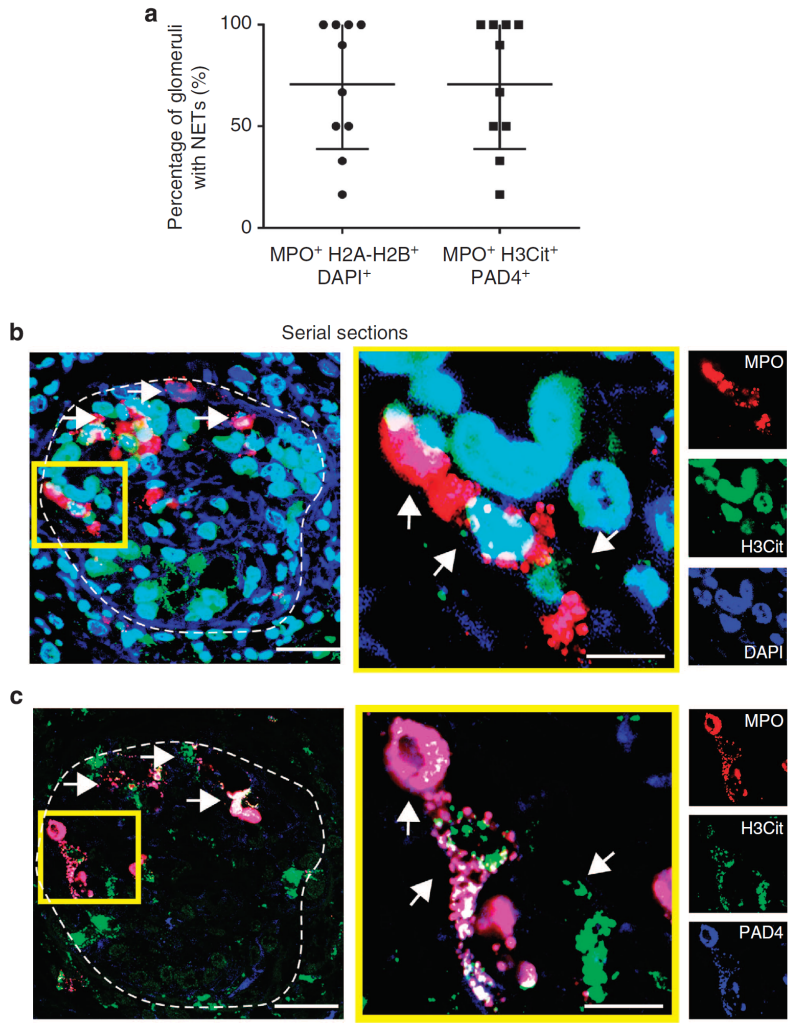
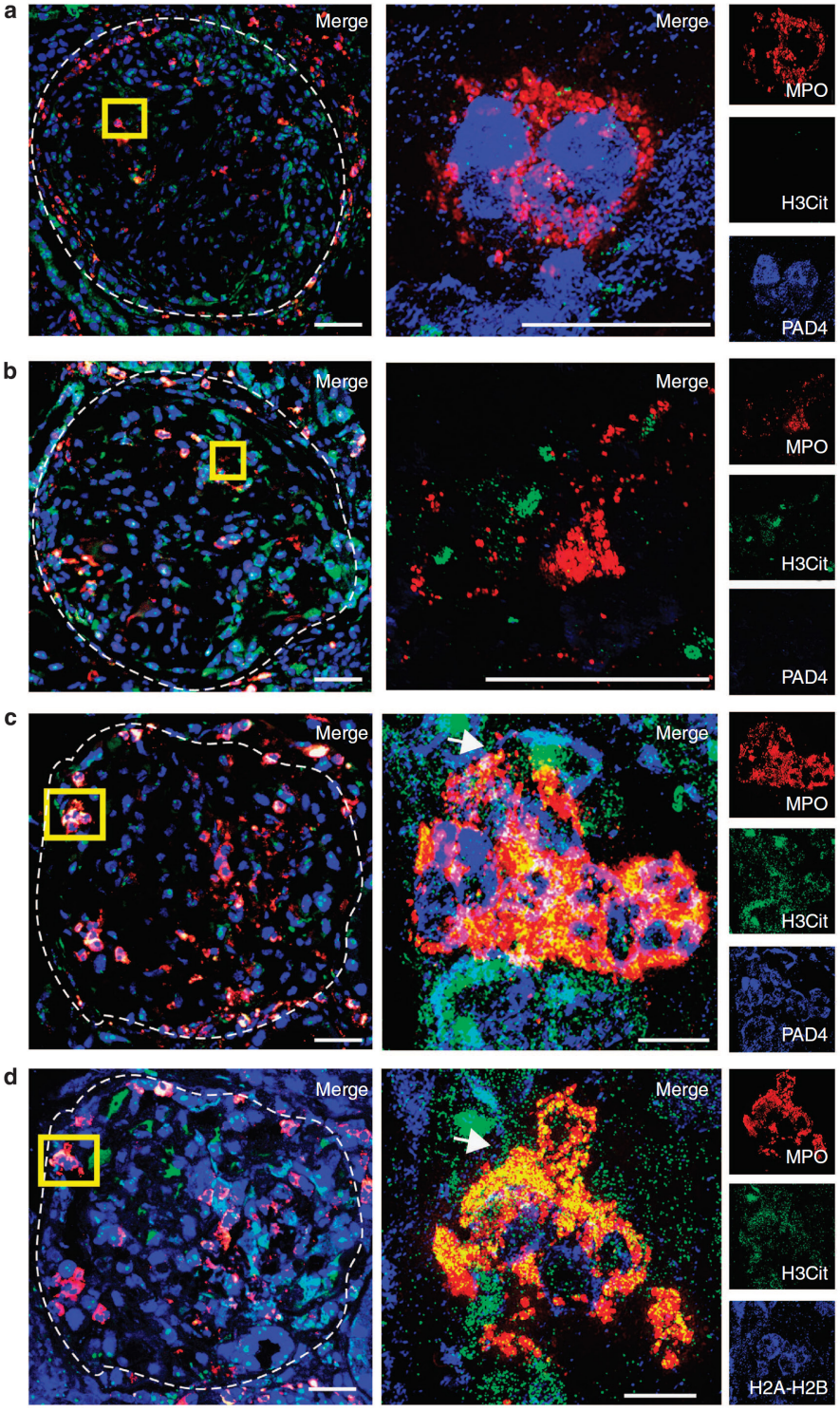


Figure 7 | Serial sections of glomerular NETs are positive for PAD4 and H3Cit. (**a**) Ten serial sections from patients with NETs within glomeruli identified with H2A-H2B, MPO, and DAPI demonstrate the percentage of glomeruli with NETs is identical by staining with the NET constituents MPO, H3Cit, and PAD4. (**b**) Sections stained for H2A-H2B (histones, green), DAPI (chromatin, blue), and MPO (red) and compared with (**c**) serial sections stained for H3Cit (green) MPO (red), and PAD4 (blue); arrows indicate areas of NET production within glomeruli (scale bar = 50 μm). NETs were identified by colocalization of all three markers in each serial section. In the high-magnification cellular inset, arrows indicate the same cell in each serial section (scale bar = 5 μm). H3Cit, citrullinated histone H3; DAPI, 4′,6-diamidino-2-phenylindole; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PAD4, peptidyl arginine 4 deiminase.

To determine whether endothelial cells were positive for MPO, CD34 was used to identify endothelial cells. CD34 staining is intact in control biopsies (Figure 10a), with no MPO staining. In MPO-ANCA-associated glomerulonephritis, as glomerular injury becomes more severe, CD34 is lost (CD31 and von Willebrand's factor showed a similar pattern, data not shown). However, CD34+MPO+ double-positive staining is present in some areas (Figure 10b and c). In some glomeruli, endothelial cell markers are lost, but MPO appears to decorate the glomerular capillaries (Figure 8d). Colocalization with MPO, CD34, and nephrin showed MPO located in capillary loops of endothelial cells, and potentially subendothelially. To a lesser extent, MPO was found adherent to podocyte cell membranes (Figure 10d and e). Although the CD34 endothelial marker is lost in active lesions, we did observe based on morphology and pattern of MPO distribution CD4+ T cells (arrow) in close proximity to MPO on the endothelial surface of the capillary wall (Supplementary Figure S2 online). As dendritic cells (DCs) have been known to take up MPO, glomeruli were examined for DCs, using DC-SIGN (Dendritic Cell–Specific Intercellular adhesion molecule-3-Grabbing Non-integrin). DCs were found primarily in the periglomerular and interstitial regions, and only occasionally within glomeruli. Supplementary Figure S2c online shows a rare glomerular DC adjacent to MPO, and a periglomerular DC positive for MPO (arrows).

DISCUSSION

The current studies define the pattern and extent of MPO expression in the kidneys of patients with MPO-ANCA-associated glomerulonephritis and the association of MPO with features of disease severity. Most of the MPO, present throughout diseased kidneys, was within the leukocyte populations known to express MPO, neutrophils, and monocyte/macrophages, although a substantial proportion was detected



outside of leukocytes ('extracellularly') in glomeruli, periglomerular regions, and the tubulointerstitium. Leukocyte infiltration was a major feature of the pathology seen in these patients' biopsies. Leukocytes were prominent in almost all glomeruli and were associated with fibrin deposition. The leukocyte accumulation and fibrin deposition is consistent with a delayed-type hypersensitivity-like lesion directed by antigen-specific CD4+ cells, and confirms previous reports in this disease.[13,20,21,31,32] The presence of intrarenal CD8+ T cells also confirms previous studies[20,21] and are consistent with data showing CD8+ cell gene transcription signatures correlated with poor prognosis in AAV.[33]

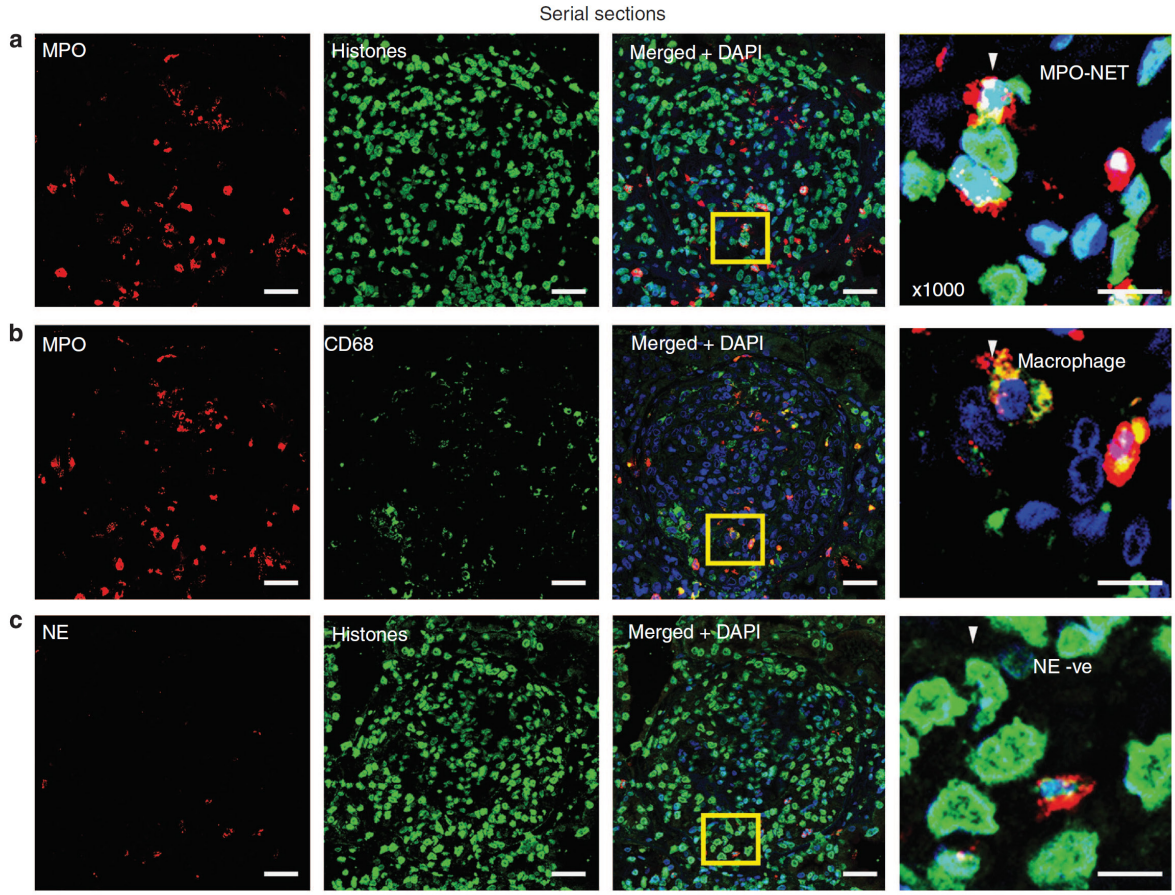


Figure 9 | Glomerular MPO+ macrophages produce extracellular trap–like structures (METs). To determine whether neutrophil elastase-negative extracellular traps (that were positive for MPO/histones/DAPI) were derived from MPO+ macrophages, serial sections from 10 MPO-ANCA patients with glomerular extracellular trap formation were probed for (**a**) MPO, (red)/H2A-H2B histones (green)/DAPI (blue), (**b**) MPO (red)/CD68 (green)/DAPI (blue). and (**c**) neutrophil elastase (red)/H2A-H2B histones (green)/DAPI (blue). In addition to neutrophils producing extracellular traps, MPO+ macrophages in glomeruli also can form extracellular traps. Arrowheads indicate the same cell in three panels being positive for MPO/histones/DAPI and CD68 (macrophage marker), but negative for neutrophil elastase, confirming it is a macrophage extruding extracellular trap–like structures. Low-powered images scale bars = 50 µm. High-powered images, scale bar = 10 µm. ANCA, antineutrophil cytoplasmic antibody; DAPI, 4',6-diamidino-2-phenylindole; MPO, myeloperoxidase; NE, neutrophil elastase.

Figure 8 | Super-resolution microscopy images of NETs. Three-dimensional structural illumination (3D-SIM) super-resolution microscopy of a further five samples from patients with NETs in glomeruli demonstrates the morphological difference between intact neutrophils, cell debris from an apoptotic or necrotic cell, and NET-forming cells. (**a**) Intact neutrophil, with MPO (red), PAD4 (blue, dense nuclear localization), and no H3Cit staining (green). (**b**) Cellular debris from an apoptotic or necrotic MPO+ cell, with MPO (red), minimal PAD4 (blue), H3Cit (green), and no colocalization evident. (**c**) NET-forming cell or cells *in vivo* with definitive colocalization of the NET constituents MPO (red), H3Cit (green), and PAD4 (blue) and in a serial section (**d**) stained (arrows) for MPO (red), H3Cit (green), and H2A-H2B (blue). Cells were also positive for neutrophil elastase by a further serial section (not shown). Lower-powered image magnification, scale bar = 50 µm. Super-resolution images, scale bar = 10 µm. H3Cit, citrullinated histone H3; DAPI, 4',6-diamidino-2-phenylindole; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PAD4, peptidyl arginine 4 deiminase.

Our data on the distribution of extracellular and intracellular MPO within kidneys of MPO-ANCA patients confirm and extend previous observations.[4,13,32,34] MPO was found within glomerular-infiltrating leukocytes, endothelial cells, adherent to areas of sclerosed tissue within glomeruli and the interstitium, tubular epithelial cells, and tubular casts. As endothelial cells lose their specific endothelial cell markers when injured,[35] severely damaged endothelial cells were not

Table 3 | Frequency of MPO-producing cells and extracellular MPO in MPO GN patients in the different Berden classifications[1]

	Focal[a] $n=13$	Crescentic[b] $n=11$	Mixed[c] $n=17$	Sclerotic[d] $n=5$	Significance[2]
PMN					
Glomerular	1.5 ± 0.6	2.5 ± 0.5	2.5 ± 0.4	1.7 ± 0.6	NS
Periglomerular	0.15 ± 0.1	0.9 ± 0.4	1.4 ± 0.4	0.85 ± 0.5	a–c*, a–b**
Interstitial	3.2 ± 1.4	10 ± 3.4	8.2 ± 2.3	5.3 ± 0.96	NS
Mϕ					
Glomerular	4.7 ± 1.1	11 ± 1.5	10 ± 2.2	5.1 ± 2.1	a–b*, a–c*, b–d**
Periglomerular	3.1 ± 1.2	6.6 ± 1.5	6.7 ± 1.6	6.2 ± 2.8	NS
Interstitial	21.6 ± 3.2	41.4 ± 6.4	33.4 ± 4.0	34.6 ± 6.5	a–b*, a–c*
MPO+ Mϕ					
Glomerular	1.8 ± 0.6	3.1 ± 0.5	3.1 ± 0.9	1.2 ± 0.6	a–b*, c–d*
Periglomerular	0.6 ± 0.3	1.8 ± 0.9	1.2 ± 0.9	0.2 ± 0.1	a–c*, a–b*, b–d*, c–d**
Interstitial	2.4 ± 0.5	8.4 ± 1.7	5.5 ± 1.4	7.0 ± 4.1	NS
MPO+ cells					
Glomerular	4.5 ± 1	10 ± 1.5	9.9 ± 2.5	4.7 ± 1.2	a–c*, b–d*
Periglomerular	1.4 ± 0.5	4.1 ± 1.5	3.4 ± .7	1.6 ± 0.5	NS
Interstitial	11 ± 2.1	19 ± 4.1	14 ± 2.6	12 ± 3.5	NS
Extracellular MPO					
Glomerular	4.4 ± 1.0	6.3 ± 1.6	4.7 ± 1.0	5.3 ± 0.9	NS
Interstitial	14 ± 3.9	20 ± 5.2	7.9 ± 1.6	19 ± 6.0	a–c*, b–c**

Abbreviations: GN, glomerulonephritis; Mϕ, macrophage; MPO, myeloperoxidase; NS, not significant; PMN, polymorphonuclear leukocyte.
*$P<0.05$, **$P<0.005$.
[1]Berden *et al.*[18] classification.
[2]Results from Kruskal–Wallis test, reported as mean ± s.e.m.

Table 4 | Correlation of glomerular leukocytes and MPO with glomeruli affected by crescent formation and segmental necrosis

	Glomerular cells				
	MPO+CD68 *r*	MPO+CD15 *r*	Cellular MPO *r*	% EC[a] MPO *r*	CD4+ *r*
% Normal glomeruli	0.004	−0.266	−0.281	−0.295	−0.104
% Cellular crescents	0.242	0.127	0.231	−0.224	−0.033
% Segmental necrosis	0.186	0.288	0.298	0.000	0.136
% Segmental necrosis and cellular crescents	0.558[b]	0.203	0.462[b]	0.406[b]	0.369[c]

Abbreviations: EC, extracellular; MPO, myeloperoxidase.
[a]Percentage extracellular MPO.
[b]Correlation is significant at 0.01 level (two tailed).
[c]Correlation is significant at the 0.05 level (two tailed).

Figure 10 | Endothelial cells and podocytes demonstrated positive staining for MPO. Endothelial cells and podocytes shed their identifying cell surface markers CD34 (endothelial cells) and nephrin (podocytes) as glomerular injury becomes more severe. (**a**) Normal glomerulus from minimal change disease demonstrated intact CD34+ endothelial cells (green) with no intraglomerular MPO (no red staining). Scale bar = 50 µm. (**b**) CD34+ intact capillary loops positive for MPO (yellow; inset ×1200 scale bar = 20 µm). (**c**) Injured glomerulus with less CD34+ endothelial cells staining (green) and with MPO+ areas (red). Scale bar = 50 µm (inset, scale bar = 20 µm). (**d**) More severe glomerular injury with no CD34+ endothelial cells, but capillaries lined with MPO deposits (inset scale bar = 20 µm). (**e**) To further characterize cells positive for MPO, quadruple staining for endothelial cells (CD34, green), podocytes (nephrin, white), MPO (red), and DAPI (blue) was performed. In some glomeruli, there are areas of CD34+ capillary loops still intact that colocalize with MPO (scale bar = 50 µm). Inset shows MPO is close to capillary walls (arrowhead) and near podocytes (arrow) (scale bar = 10 µm). DAPI, 4',6-diamidino-2-phenylindole; MPO, myeloperoxidase.

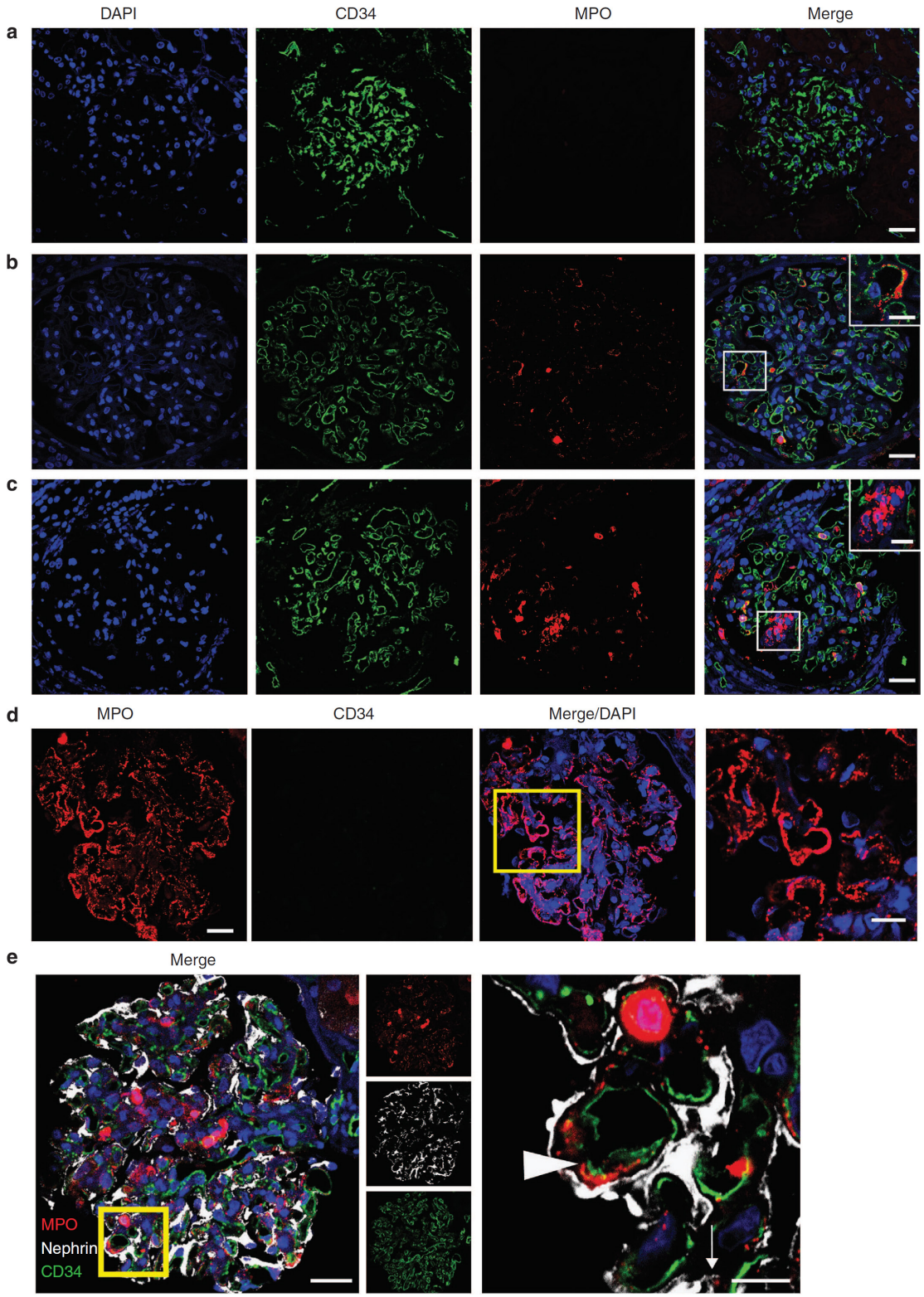

DAPI
CD34
MPO
Merge
a
b
c
MPO
CD34
Merge/DAPI
d
Merge
e
MPO
Nephrin
CD34

Table 5 | Primary antibodies used for immunohistochemistry and immunofluorescence

Antibody	Specificity	Dilution	Source
Mouse anti-human CD4	T helper cells	1:20	Novocastra[b]
Mouse anti-human CD8	Cytotoxic T cells	0.11111	DAKO[a]
Mouse anti-human FoxP3	Regulatory T cells	5 μg/ml	EBioscience[c]
Mouse anti-human CD15	Neutrophils	1:25	Vector[d]
Mouse anti-human CD68	Macrophages	1:40	Vector[d]
Mouse anti-human CD45	All leukocytes	1:20	DAKO[a]
Mouse anti-mouse H2A-H2B	Histones	1 μg/ml	In house(5)
Mouse anti-human CD34	Endothelial cells	1:50	DAKO[a]
Sheep anti-human nephrin	Podocytes	5 μg/ml	LifeSpan Biosciences[e]
Rabbit anti-human MPO	Myeloperoxidase	1:50	Thermofisher[f]
Goat-anti-human MPO	Myeloperoxidase	10 μg/ml	R&D Systems[g]
Mouse anti-human PAD4	PAD4	1:50	Abcam[h]
Rabbit anti-human H3Cit	Citrullinated histones	1:100	Abcam[h]
Sheep anti-human NE	Neutrophil elastase	1:100	LifeSpan Biosciences[e]
Mouse anti-human DC-SIGN	Dendritic cells	1:100	Abcam[h]
Rabbit anti-human NE	Neutrophil elastase	1:100	Abcam[h]

Abbreviations: DC-SIGN, dendritic cell–specific intercellular adhesion molecule-3–grabbing non-integrin; H3Cit, citrullinated histone H3; MPO, myeloperoxidase; NE, neutrophil elastase; PAD4, peptidyl arginine 4 deiminase.
[a]Glostrup, Denmark.
[b]Leica Microsystems, Newcastle Upon Tyne, UK.
[c]San Diego, CA.
[d]Peterborough, UK.
[e]Seattle, WA.
[f]Waltham, MA.
[g]Minneapolis, MN.
[h]Cambridge, UK.

positive for other endothelial cell markers. However, based on observations in less damaged areas (where CD34+ endothelial cells were still present) and morphological observations in our studies, it is likely that many damaged endothelial cells are MPO positive.

That MPO adheres to glomerular endothelial cells *in vivo* is important as it extends the current understanding derived from *in vitro* experiments.[36,37] There are two distinct mechanisms previously published by which MPO may be internalized by endothelial cells. *In vitro*, extracellular MPO can be translocated and internalized into endothelial cells and epithelial cells.[36] Alternatively, MPO can be directly transferred from neutrophils to endothelial cells in a β2-integrin–mediated, cell–cell contact manner that may occur with little or no release of extracellular MPO.[37] As human glomerular endothelial cells and podocytes express major histocompatibility complex class II in inflammatory states,[38–40] internalized MPO may be processed and presented as peptides to autoreactive MPO-specific CD4+ T cells by intrinsic kidney cells.[41] Mesangial cells can also express major histocompatibility complex class II, but because of a lack of specific mesangial cell marker, we could not determine whether MPO localized to mesangial cells.[42] Definitive identification of the candidate glomerular cells presenting MPO to antigen-specific CD4+ and CD8+ T cells is an area for future study. Although whereas the role of 'professional' antigen-presenting cells, such as DCs, in glomeruli is not clear,[43] there are abundant mononuclear phagocytes, including DCs in the tubulointerstitium, that could present MPO peptides to antigen-specific T cells.

Neutrophils and MPO+ macrophages were detected in equal numbers within glomeruli of MPO-ANCA patients, but neutrophils contained more MPO than macrophages, implying that neutrophils, crucial in the pathogenesis of ANCA-associated glomerulonephritis,[14,22,44] contribute more to renal MPO deposition than macrophages. Extracellular MPO was deposited either through NET formation or by degranulation, as patients whose biopsies were negative for NETs still demonstrated prominent extracellular MPO. To determine whether MPO presence in glomeruli could be related to autoimmunity to MPO, we studied PR3-ANCA patients with similar patterns of glomerular injury but no evidence of anti-MPO autoimmunity. The fact that similar patterns and extent of MPO deposition was observed suggests that MPO deposition is related to leukocyte influx and activation in AAV, and is not specific for autoimmunity to MPO. However, in patients with class IV lupus nephritis we found less MPO present in kidneys, when taking into account neutrophil numbers. This is consistent with AAV inducing a higher level of neutrophil activation, and consequently a higher number of glomeruli affected by segmental necrosis.

Herein, we have defined and quantitated substantial glomerular deposition of NETs in a large series of MPO-AAV patients. We used a number of markers. The more conventional technique is to demonstrate colocalization of nuclear (DAPI and the H2A-H2B histone complex) and cytoplasmic components (MPO). We validated this technique using the colocalization of PAD4 (a nuclear enzyme that is vital for NET production), H3Cit (a marker of decondensed chromatin required for NET formation), and MPO. We

distinguished NET-forming cells from either resting or apoptotic/necrotic cells using these markers in 3D-SIM super-resolution microscopy that provided markedly increased subcellular resolution. As over half the MPO-AAV patients had NETs within their glomeruli, potentially inducing injury as well as depositing the autoantigen (MPO), NETs may be a potential therapeutic target, and warrants further investigation.

Our findings report for the first time the presence of MET-like structures in MPO-ANCA glomerulonephritis. Macrophages have been shown to produce MPO[45] and METs have been detected in a limited number of other conditions.[7,8] Macrophages are also known to engulf NET remnants[46] and ingest apoptotic MPO-containing neutrophils,[45,47] these features together with the lack of specific MET markers together, make infallible MET detection difficult *in vivo*. There are two subtypes of macrophages infiltrating biopsies: MPO+ macrophages, capable of producing extracellular traps, and MPO− macrophages. MPO+ macrophages, some of which generate METs, are likely to be M1 macrophages. M1 macrophages are proinflammatory and injurious, producing proinflammatory cytokines and high levels of reactive intermediates, whereas M2 macrophages are more likely to be protective/tissue-repairing macrophages.[48] Macrophage and extracellular MPO accumulation was observed in glomeruli of biopsies falling within the crescentic category, possible markers of active disease.[21] Intracellular MPO (in neutrophils and macrophages) and extraleukocyte MPO (from degranulation and NET formation) were numerically higher in crescentic biopsies, suggesting that intrarenal MPO might also indicate disease activity (although numbers of patients limited the power of this part of the study). Correlation with eGFR at biopsy revealed that interstitial accumulation of each leukocyte subpopulation (macrophages, neutrophils, and T cells) correlated with poor renal function, highlighting the potential importance of delayed-type hypersensitivity-like injury in this disease. The role of T cells in the pathogenesis of AAV has been studied in our laboratories in animal studies showing that autoreactive MPO-specific CD4+ T cells[15–17,49] and interleukin (IL)-17A-producing MPO-specific CD4+ T cells contribute to disease.[49] Human studies also support an injurious role for IL-17A-producing T cells, as well as IL-17A production by neutrophils and mast cells.[50] There are limited studies of the cytokines in human AAV. Elevated levels of IL-17A and IL-23 have been reported in the serum from patients with AAV.[51] Assessment of renal biopsies from AAV patients have shown that IL-17A is expressed by neutrophils and mast cells in diseased kidneys.[50]

MPO may exert an influence on the progression of ANCA vasculitis in several ways. First, in the current studies, MPO was found within intrinsic glomerular cells and macrophages, both of which can present MPO to antigen specific T cells via major histocompatibility complex class II and may contribute to cell-mediated effector injury. Second, MPO has a direct effect on glomerular cells by binding to both endothelial and epithelial cells. The resulting internalization of MPO may result in oxidative damage, reacting with hydrogen peroxide (found in basal levels in endothelial cells) to form injurious hypochlorous acid.[36,52,53] Third, MPO may be deposited in either a biologically active or inactive manner. MPO is deposited in both enzymatically active and inactive forms at site of inflammation.[12,54] Once MPO is released into the environment, changes in pH and proteases can render MPO inactive.[12] Within glomeruli, deposited extracellular MPO may become both inactive and alter its conformation, potentially rendering it less recognizable by ANCA. [55] This could contribute to the paucity of glomerular immunoglobulin deposition, despite the prominent presence of MPO.

In conclusion, the current studies comprehensively demonstrate the extent of extracellular deposition of MPO, with and without NETs, and prominent leukocyte intracellular MPO presence in MPO-ANCA-associated glomerulonephritis. It reveals the participation of METs containing MPO in this disease, for the first time. It also demonstrates an association between MPO, effector cells, and fibrin deposition, consistent with extracellular MPO acting as a planted antigen in the kidney directing a delayed-type hypersensitivity-like response. These results provide evidence to support the hypothesis that extracellular MPO derived from neutrophil degranulation or leukocyte ETs participates in inducing glomerular and interstitial injury.

MATERIALS AND METHODS

Patient cohort and biopsy specimens

A total of 58 patients presenting with AAV and glomerulonephritis (47 MPO-ANCA+ and 11 PR3-ANCA+) from 2001 to 2011 at Monash Medical Centre, Clayton, Victoria, Australia, were included in this study. Clinical and laboratory data were obtained from hospital records and pathology archives. In all cases the diagnosis was confirmed by renal biopsy with a positive ANCA result by both direct immunofluorescence and antigen-specific enzyme-linked immunosorbent assay at presentation. Biopsies from 10 patients with minimal change disease or thin membrane disease, 6 patients with lupus nephritis ISN/RPS class IV, and 8 patients with primary membranous nephropathy served as control tissues. Three independent pathologists examined and classified biopsies according to the classification scheme of Berden *et al.*[18] and one (AL) assessed each biopsy for the proportions of normal glomeruli, glomeruli exhibiting segmental necrosis, cellular crescents, or both segmental necrosis and cellular crescents.

Immunohistochemistry and immunofluorescence

Sections (2 μm) of formalin-fixed, paraffin-embedded tissue specimens were mounted on superfrost plus slides (Menzel, Braunschweig, Germany), dewaxed, rehydrated, and pretreated with antigen retrieval solution Tris-EDTA pH 9 in a pressure cooker for 10 min, blocked (30 min) in either 10% chicken sera in 5% bovine serum albumin/phosphate-buffered saline (immunofluorescence) or horse serum (immunohistochemistry), and probed with antibodies against CD4, CD8, FoxP3 CD68, CD15, MPO, H2A-H2B, CD34, CD45, neutrophil elastase, PAD4, H3Cit, DC-SIGN, and nephrin (Table 5) in 1% bovine serum albumin/phosphate-buffered saline for 16 h (4 °C). For CD4, CD8 T cells, and FoxP3 cell staining, antigen

retrieval was performed and a universal VECTORSTAIN ABC-AP kit (Vector, Peterborough, UK) was used, visualized with a VECTOR Red alkaline phosphatase substrate kit (Vector), and counterstained with hematoxylin. For DC-SIGN+ DCs, a tyramide signaling kit (Molecular Probes, Eugene, OR) was used to enhance sensitivity (see Supplementary Methods online). Fluorescent detection was achieved by incubation with either Alexa Fluor 594-conjugated chicken anti-rabbit IgG or Alexa Fluor 488-conjugated chicken anti-mouse IgG (both from Molecular Probes 1:200, 40 min, room temperature). To quench tissue autofluorescence, slides were incubated with Sudan Black (Sigma-Aldrich St Louis, MO, 0.1% in 70% ethanol, 30 min), washed in phosphate-buffered saline, and coverslipped in DAPI prolong gold (Molecular Probes). Fluorescent images were acquired using a NIKON C1 confocal laser scanning head attached to Nikon Ti-E inverted microscope (Coherent Scientific, SA, Australia); 405, 488, and 561 nm 647nm lasers were used to specifically excite DAPI, Alexa 488, Alexa 594, and Alexa 647. Single plane $512 \times 512 \times 12$bit images were captured in a line-sequential manner (4 line averaging) using a $20\times$, $40\times$, or $60\times$ objective. Sections for 3D-SIM super-resolution microscopy were mounted on number 1.5 coverslips (HD, Scientific, NSW, Australia), and stained for PAD4, MPO, H3Cit, H2A-H2B, and neutrophil elastase (see Supplementary Methods online for staining procedures and image acquisition).

Analysis and quantification of leukocytes and MPO

The glomerular frequencies of leukocytes and MPO+ cells were determined as the sum of expressing cells in all nonsclerosed glomeruli divided by the number of nonsclerosed glomerular cross-sections (except for data in Supplementary Table S1 online where globally sclerosed glomeruli were included). Periglomerular cells were found between Bowman's capsule and adjacent tubules. Tubulointerstitial leukocytes were counted in 10 high-powered ($400\times$) fields and expressed as cells per high-powered field. Extracellular and Intracellular MPO was measured by a macro using ImageJ analysis software (NIH, Bethesda, MD). Intracellular (leukocyte-associated) MPO was defined being associated with CD45 (CD45+MPO+ cells). Extracellular MPO was measured as MPO+CD45− staining. The macro evaluated both the area and intensity and expressed the results as arbitrary units (See Supplementary Methods online for details).

Statistical analyses

Statistical differences between groups were analyzed using the Mann–Whitney *U*-test (two groups) and Kruskal–Wallis test (three or more groups). Nonparametric Spearman's rank was used to test correlations between two independent variables. Significance was set at $P<0.05$. SPSS 20 (IBM, Armonk, NY) or GraphPad (La Jolla, CA) software was used.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This study was funded by a Project Grant from the National Health and Medical Research Council of Australia (606683). The 3D-SIM super-resolution microscopy imaging was performed at the Biological Optical Microscopy Platform, The University of Melbourne (www.microscopy.unimelb.edu.au). We thank Kevan Polkinghorne for statistical advice.

SUPPLEMENTARY MATERIAL

Table S1. Comparison of MPO expression in kidney of patients with MPO-AAV, Class IV lupus nephritis, membranous nephropathy and PR3-AAV.

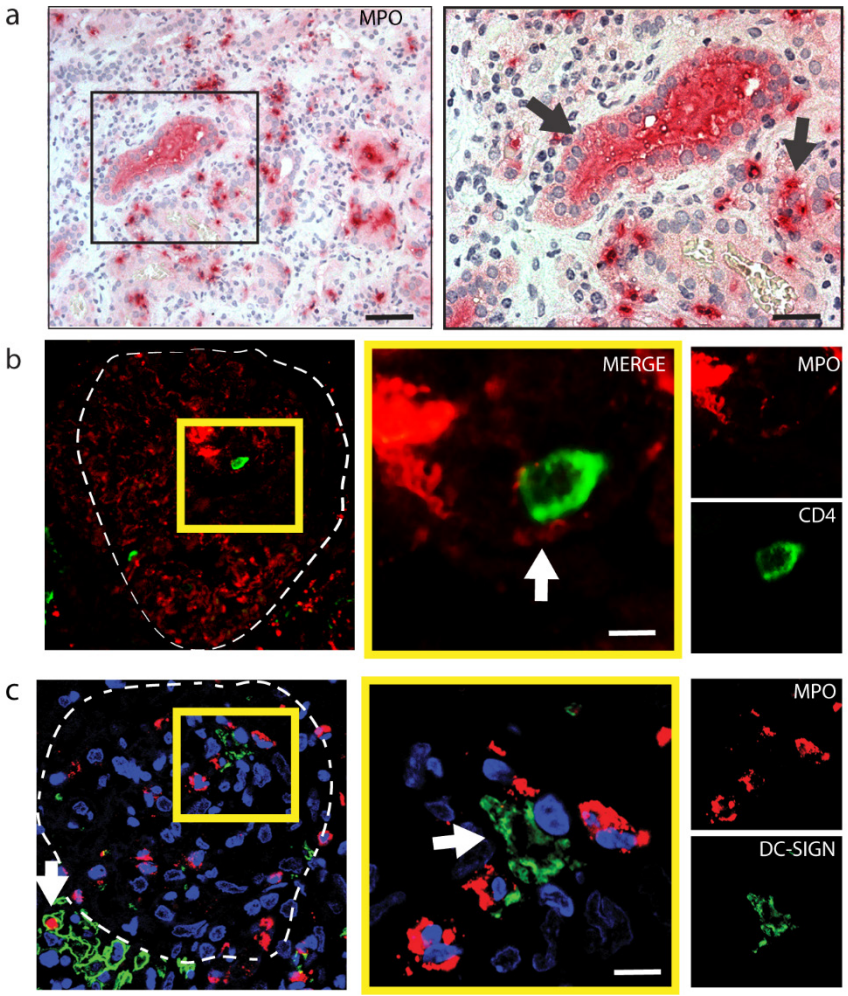
Supplementary Methods. Assessing and Quantitating MPO Deposition in Kidneys.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Gatenby PA. Anti-neutrophil cytoplasmic antibody-associated systemic vasculitis: nature or nurture? *Intern Med J* 2012; **42**: 1066–1067.
2. Coughlan AM, Freeley SJ, Robson MG. Animal models of anti-neutrophil cytoplasmic antibody-associated vasculitis. *Clin Exp Immunol* 2012; **169**: 229–237.
3. Odobasic D, Kitching AR, Yang Y *et al.* Neutrophil myeloperoxidase regulates T-cell-driven tissue inflammation in mice by inhibiting dendritic cell function. *Blood* 2013; **121**: 4195–4204.
4. Kessenbrock K, Krumbholz M, Schonermarck U *et al.* Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med* 2009; **15**: 623–625.
5. Brinkmann V, Reichard U, Goosmann C *et al.* Neutrophil extracellular traps kill bacteria. *Science* 2004; **303**: 1532–1535.
6. Nakazawa D, Shida H, Tomaru U *et al.* Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. *J Am Soc Nephrol* 2014; **25**: 990–997.
7. Liu P, Wu X, Liao C *et al.* Escherichia coli and Candida albicans induced macrophage extracellular trap-like structures with limited microbicidal activity. *PLoS One* 2014; **9**: e90042.
8. Wong KW, Jacobs WR Jr. Mycobacterium tuberculosis exploits human interferon gamma to stimulate macrophage extracellular trap formation and necrosis. *J Infect Dis* 2013; **208**: 109–119.
9. Mohanan S, Horibata S, McElwee JL *et al.* Identification of macrophage extracellular trap-like structures in mammary gland adipose tissue: a preliminary study. *Front Immunol* 2013; **4**: 67.
10. Hellenbrand KM, Forsythe KM, Rivera-Rivas JJ *et al.* Histophilus somni causes extracellular trap formation by bovine neutrophils and macrophages. *Microb Pathog* 2013; **54**: 67–75.
11. Aulik NA, Hellenbrand KM, Kisiela D *et al.* Mannheimia haemolytica leukotoxin binds cyclophilin D on bovine neutrophil mitochondria. *Microb Pathog* 2011; **50**: 168–178.
12. Grattendick K, Stuart R, Roberts E *et al.* Alveolar macrophage activation by myeloperoxidase: a model for exacerbation of lung inflammation. *Am J Respir Cell Mol Biol* 2002; **26**: 716–722.
13. Brouwer E, Huitema MG, Mulder AH *et al.* Neutrophil activation in vitro and in vivo in Wegener's granulomatosis. *Kidney Int* 1994; **45**: 1120–1131.
14. Xiao H, Heeringa P, Hu P *et al.* Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest* 2002; **110**: 955–963.
15. Ruth AJ, Kitching AR, Kwan RY *et al.* Anti-neutrophil cytoplasmic antibodies and effector CD4+ cells play nonredundant roles in anti-myeloperoxidase crescentic glomerulonephritis. *J Am Soc Nephrol* 2006; **17**: 1940–1949.
16. Gan PY, Holdsworth SR, Kitching AR *et al.* Myeloperoxidase (MPO)-specific CD4+ T cells contribute to MPO-anti-neutrophil cytoplasmic antibody (ANCA) associated glomerulonephritis. *Cell Immunol* 2013; **282**: 21–27.
17. Ooi JD, Chang J, Hickey MJ *et al.* The immunodominant myeloperoxidase T-cell epitope induces local cell-mediated injury in antimyeloperoxidase glomerulonephritis. *Proc Natl Acad Sci USA* 2012; **109**: E2615–E2624.
18. Berden AE, Ferrario F, Hagen EC *et al.* Histopathologic classification of ANCA-associated glomerulonephritis. *J Am Soc Nephrol* 2010; **21**: 1628–1636.
19. Ford SL, Polkinghorne KR, Longano A *et al.* Histopathologic and clinical predictors of kidney outcomes in ANCA-associated vasculitis. *Am J Kidney Dis* 2014; **63**: 227–235.
20. Weidner S, Carl M, Riess R *et al.* Histologic analysis of renal leukocyte infiltration in antineutrophil cytoplasmic antibody-associated vasculitis: importance of monocyte and neutrophil infiltration in tissue damage. *Arthritis Rheum* 2004; **50**: 3651–3657.
21. Cunningham MA, Huang XR, Dowling JP *et al.* Prominence of cell-mediated immunity effectors in "pauci-immune" glomerulonephritis. *J Am Soc Nephrol* 1999; **10**: 499–506.
22. Jennette JC, Falk RJ, Hu P *et al.* Pathogenesis of antineutrophil cytoplasmic autoantibody-associated small-vessel vasculitis. *Annu Rev Pathol* 2013; **8**: 139–160.

23. Aulik NA, Hellenbrand KM, Czuprynski CJ. Mannheimia haemolytica and its leukotoxin cause macrophage extracellular trap formation by bovine macrophages. *Infect Immun* 2012; **80**: 1923–1933.
24. Neeli I, Khan SN, Radic M. Histone deimination as a response to inflammatory stimuli in neutrophils. *J Immunol* 2008; **180**: 1895–1902.
25. Rohrbach AS, Slade DJ, Thompson PR *et al.* Activation of PAD4 in NET formation. *Front Immunol* 2012; **3**: 360.
26. Li P, Li M, Lindberg MR *et al.* PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med* 2010; **207**: 1853–1862.
27. Leshner M, Wang S, Lewis C *et al.* PAD4 mediated histone hypercitrullination induces heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures. *Front Immunol* 2012; **3**: 307.
28. Wang Y, Li M, Stadler S *et al.* Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J Cell Biol* 2009; **184**: 205–213.
29. Dwivedi N, Radic M. Citrullination of autoantigens implicates NETosis in the induction of autoimmunity. *Ann Rheum Dis* 2014; **73**: 483–491.
30. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A *et al.* NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med* 2013; **5**: 178ra140.
31. Couser WG. Glomerulonephritis. *Lancet* 1999; **353**: 1509–1515.
32. Kawashima S, Arimura Y, Sano K *et al.* Immunopathologic co-localization of MPO, IgG, and C3 in glomeruli in human MPO-ANCA-associated glomerulonephritis. *Clin Nephrol* 2013; **79**: 292–301.
33. McKinney EF, Lyons PA, Carr EJ *et al.* A CD8+ T cell transcription signature predicts prognosis in autoimmune disease. *Nat Med* 2010; **16**: 586–591 581p following 591.
34. Saeki T, Kuroda T, Morita T *et al.* Significance of myeloperoxidase in rapidly progressive glomerulonephritis. *Am J Kidney Dis* 1995; **26**: 13–21.
35. Niemir ZI, Kubiak A, Olejniczak P *et al.* Can von Willebrand factor, platelet-endothelial cell adhesion molecule-1 and thrombomodulin be used as alternative markers of endothelial cell injury in human glomerulonephritis? *Rocz Akad Med Bialymst* 2004; **49**: 213–218.
36. Yang JJ, Preston GA, Pendergraft WF *et al.* Internalization of proteinase 3 is concomitant with endothelial cell apoptosis and internalization of myeloperoxidase with generation of intracellular oxidants. *Am J Pathol* 2001; **158**: 581–592.
37. Jerke U, Rolle S, Purfurst B *et al.* beta2 integrin-mediated cell-cell contact transfers active myeloperoxidase from neutrophils to endothelial cells. *J Biol Chem* 2013; **288**: 12910–12919.
38. Goldwich A, Burkard M, Olke M *et al.* Podocytes are nonhematopoietic professional antigen-presenting cells. *J Am Soc Nephrol* 2013; **24**: 906–916.
39. Coers W, Brouwer E, Vos JT *et al.* Podocyte expression of MHC class I and II and intercellular adhesion molecule-1 (ICAM-1) in experimental pauci-immune crescentic glomerulonephritis. *Clin Exp Immunol* 1994; **98**: 279–286.
40. Eiserich JP, Baldus S, Brennan ML *et al.* Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science* 2002; **296**: 2391–2394.
41. Muczynski KA, Ekle DM, Coder DM *et al.* Normal human kidney HLA-DR-expressing renal microvascular endothelial cells: characterization, isolation, and regulation of MHC class II expression. *J Am Soc Nephrol* 2003; **14**: 1336–1348.
42. Chadban SJ, Tesch GH, Foti R *et al.* Interleukin-10 differentially modulates MHC class II expression by mesangial cells and macrophages in vitro and in vivo. *Immunology* 1998; **94**: 72–78.
43. Kitching AR. Dendritic cells in progressive renal disease: some answers, many questions. *Nephrol Dial Transplant* 2014; **29**: 2185–2193.
44. Rastaldi MP, Ferrario F, Crippa A *et al.* Glomerular monocyte-macrophage features in ANCA-positive renal vasculitis and cryoglobulinemic nephritis. *J Am Soc Nephrol* 2000; **11**: 2036–2043.
45. McMillen TS, Heinecke JW, LeBoeuf RC. Expression of human myeloperoxidase by macrophages promotes atherosclerosis in mice. *Circulation* 2005; **111**: 2798–2804.
46. Farrera C, Fadeel B. Macrophage clearance of neutrophil extracellular traps is a silent process. *J Immunol* 2013; **191**: 2647–2656.
47. Silva MT. Macrophage phagocytosis of neutrophils at inflammatory/infectious foci: a cooperative mechanism in the control of infection and infectious inflammation. *J Leukoc Biol* 2011; **89**: 675–683.
48. Locati M, Mantovani A, Sica A. Macrophage activation and polarization as an adaptive component of innate immunity. *Adv Immunol* 2013; **120**: 163–184.
49. Gan PY, Steinmetz OM, Tan DS *et al.* Th17 cells promote autoimmune anti-myeloperoxidase glomerulonephritis. *J Am Soc Nephrol* 2010; **21**: 925–931.
50. Velden J, Paust HJ, Hoxha E *et al.* Renal IL-17 expression in human ANCA-associated glomerulonephritis. *Am J Physiol Renal Physiol* 2012; **302**: F1663–F1673.
51. Nogueira E, Hamour S, Sawant D *et al.* Serum IL-17 and IL-23 levels and autoantigen-specific Th17 cells are elevated in patients with ANCA-associated vasculitis. *Nephrol Dial Transplant* 2010; **25**: 2209–2217.
52. Heeringa P, Brouwer E, Klok PA *et al.* Autoantibodies to myeloperoxidase aggravate mild anti-glomerular-basement-membrane-mediated glomerular injury in the rat. *Am J Pathol* 1996; **149**: 1695–1706.
53. Johnson RJ, Couser WG, Chi EY *et al.* New mechanism for glomerular injury. Myeloperoxidase-hydrogen peroxide-halide system. *J Clin Invest* 1987; **79**: 1379–1387.
54. Bradley PP, Christensen RD, Rothstein G. Cellular and extracellular myeloperoxidase in pyogenic inflammation. *Blood* 1982; **60**: 618–622.
55. Xu PC, Hao J, Chen M *et al.* Influence of myeloperoxidase-catalyzing reaction on the binding between myeloperoxidase and anti-myeloperoxidase antibodies. *Hum Immunol* 2012; **73**: 364–369.



Supplementary Figure Legend 1. Tubule cell, DC-SIGN+ DCs and CD4 T cell association with extracellular MPO (a) Low power figure demonstrates Tubular cells, and a Tubular cast positive for MPO (red) counterstained with Hematoxylin (Blue), 200x (scale bar 20μm) higher power insert shows Tubular cells surrounding a tubular cast are positive for MPO, as is adjacent cells in the tubular interstitium (400x, scale bar =10μm). (**b**) CD4 T cells (green)

Supplementary Methods

Tyramide signalling detection of DC-SIGN+ DCs and CD4 T cells with MPO

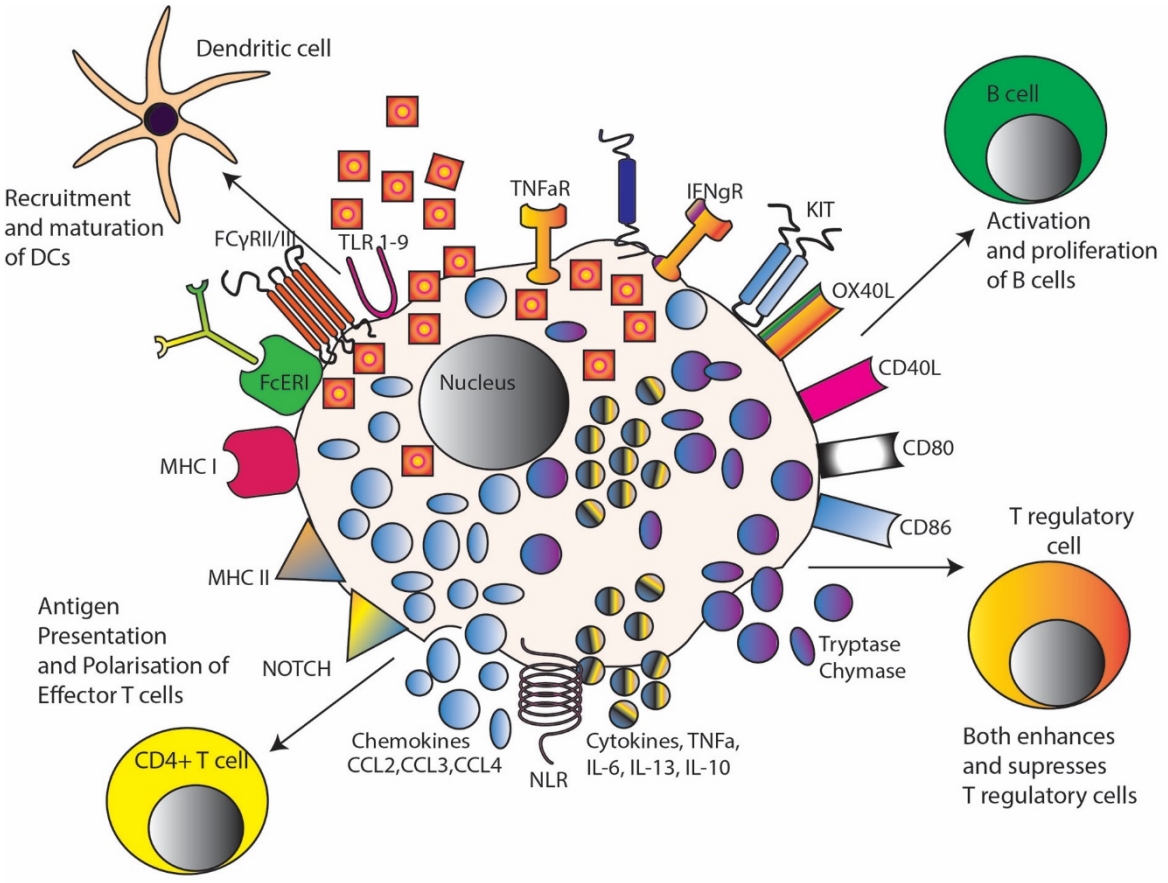
Two-micron sections of formalin fixed paraffin embedded tissue specimens were mounted on superfrost plus slides (Menzel, Braunschweig, Germany), dewaxed, rehydrated, endogenous peroxidase blocked with 0.3% hydrogen peroxide in Methanol for 10 minutes, and pre-treated with antigen retrieval solution Tris-EDTA pH 9 in a pressure cooker for 10 minutes, blocked (30 minutes) in either 10% chicken sera in 5% bovine serum albumin (BSA)/phosphate buffered. Tissue was probed with antibodies sequentially, first with either Mouse anti DC-SIGN (ABCAM), or mouse anti CD4 (Novascastra) at a 1:20 dilution, avidin and biotin blocked for 10 minutes each (avidin/ biotin blocking kit, Vector laboratories), secondary detection with horse anti mouse Biotinylated secondary antibody at 1:100 for 40 minutes at RT,(Vector Laboratories), Streptavidin- Horse Radish Peroxidase (HRP) (Molecular Probes TSA Kit, Life Technologies) was used to detect the biotinlylated secondary, TSA labelling was achieved with with ALEXA 488 dye diluted in amplification buffer 1:100 (provided in TSA kit, Moleculr Probes). Sections were washed and sequentially stained for MPO with rabbit anti mouse MPO (Thermofisher) 1:50, for 1 hour, washed and incubated for 40 minutes with secondary antibody chicken anti rabbit Alexa 594, washed in PBS, and counter stained in Sudan black 0.3% in 70% ethanol, 30 minutes (SIGMA-ALDRICH), washed in PBS and viewed). Fluorescent images were acquired using a NIKON C1 confocal laser scanning head attached to Nikon Ti-E inverted microscope; 405nm, 488nm and 561nm 647nm lasers were used to specifically excite DAPI, Alexa 488, Alexa 594 and Alexa 647. Single plane 512x512x12bit images were captured in a line sequential manner (4 line averaging) using a 20x, 40x or 60x objective. Images were processed in Image J software (NIH).

3D- Structured Illumination Microscopy

Formalin fixed paraffin embedded kidney biopsy samples were cut at 2µm and mounted on poly-L-Lysine (Sigma) coated #1.5 Coverslips (HD Scientific), oven dried for 1 hour at 60°C, cleared in Histosol, rehydrated in graded alcohols, and subjected to antigen retrieval as above, staining, and quenching of autofluorescence in Sudan Black as above. Sections were mounted onto glass slides (HD Scientific), with DAKO fluorescent antifade mounting media (DAKO). Low power tile scans were captured on a Delta Vision Wide field microscope (Applied Precision GE Healthcare, Rydalmere, Australia) using a 20x objective lense (0.75NA), and OAI (Optical Axis Integration) scans to compensate for focus change across large areas of the sample. Samples were excited using 485/20, 560/25, or 650/13 excitation filters and imaged using emission band pass filters at 531/22, 607/36, or 684/24 nm. Low resolution tile scans were used to locate and image the cell of interest on a Delta Vision OMX V4 Blaze™ (Applied Precision). For super resolution imagine samples were excited using 488, 568 or 642 nm lasers and imaged using band pass filters at 528/48, 609/37 and 683/40nm with a 60x Oil immersion lens (1.42 NA). Image reconstruction was performed with SOFTWORX software (Applied Precision). Images are presented as projections of whole tissue z-stacks taken at 0.2µm intervals. All images were processed using Image J software (http://rsweb.nih.gov./ij/).

CHAPTER 3: MCS IN AAV

Mast Cell Stabilization Ameliorates Autoimmune Anti-Myeloperoxidase Glomerulonephritis



BASIC RESEARCH www.jasn.org

Mast Cell Stabilization Ameliorates Autoimmune Anti-Myeloperoxidase Glomerulonephritis

Poh-Yi Gan,* Kim M. O'Sullivan,* Joshua D. Ooi,* Maliha A. Alikhan,* Dragana Odobasic,* Shaun A. Summers,*[a] A. Richard Kitching,*[†] and Stephen R. Holdsworth*[†]

*Centre for Inflammatory Diseases, Monash University Department of Medicine, Clayton, VIC, Australia; [†]Department of Nephrology, Monash Health, [redacted]

ABSTRACT

Observations in experimental murine myeloperoxidase (MPO)-ANCA-associated vasculitis (AAV) show mast cells degranulate, thus enhancing injury as well as producing immunomodulatory IL-10. Here we report that, compared with biopsy specimens from control patients, renal biopsy specimens from 44 patients with acute AAV had more mast cells in the interstitium, which correlated with the severity of tubulointerstitial injury. Furthermore, most of the mast cells were degranulated and spindle-shaped in patients with acute AAV, indicating an activated phenotype. We hypothesized that the mast cell stabilizer disodium cromoglycate would attenuate mast cell degranulation without affecting IL-10 production. We induced anti-MPO GN by immunizing mice with MPO and a low dose of anti-glomerular basement membrane antibody. When administered before or after induction of MPO autoimmunity in these mice, disodium cromoglycate attenuated mast cell degranulation, development of autoimmunity, and development of GN, without diminishing IL-10 production. In contrast, administration of disodium cromoglycate to mast cell-deficient mice had no effect on the development of MPO autoimmunity or GN. MPO-specific $CD4^+$ effector T cell proliferation was enhanced by co-culture with mast cells, but in the presence of disodium cromoglycate, proliferation was inhibited and IL-10 production was enhanced. These results indicate that disodium cromoglycate blocks injurious mast cell degranulation specifically without affecting the immunomodulatory role of these cells. Thus as a therapeutic, disodium cromoglycate may substantially enhance the regulatory role of mast cells in MPO-AAV.

J Am Soc Nephrol 27: 1321–1333, 2016. doi: 10.1681/ASN.2014090906

BASIC RESEARCH

Mast cells (MCs) are best characterized in pathology by their effector roles in IgE-dependent degranulation and by their release of pro-inflammatory mediators in allergy and anaphylaxis.[1] However, it is now recognized that MCs also play important roles in host defense and also in non-allergic inflammatory diseases, particularly those initiated by autoimmunity. The functional diversity of MC phenotypes allows for their participation in the generation of adaptive immune responses, playing either injurious or modulatory roles in many chronic human diseases and animal models of these diseases.[2]

A functional role for MCs in a particular human disease can be suspected by confirming MC presence in diseased target organs and demonstrating a correlation between MC activation status and disease outcome. This potential cause and effect association can be strengthened by studies in relevant murine models of the particular diseases comparing disease patterns and outcomes between MC-deficient ($Kit^{Wsh/Wsh}$) mice and $Kit^{Wsh/Wsh}$ mice reconstituted with MCs.[2–5] The mechanistic basis of

Received September 18, 2014. Accepted July 21, 2015.

P.Y.G. and K.M.O'S. contributed equally to this work.

[a]Deceased.

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Professor Holdsworth, Centre for Inflammatory Diseases, Monash University, Department of Medicine, Monash Medical Centre, 246 Clayton Road, Clayton, VIC 3168, Australia. Email: Stephen.holdsworth@monash.edu

MC-enhanced injury is by MC degranulation, which promotes injurious inflammation and enhances the capacity of dendritic cells (DCs) to drive autoimmunity.[6] Using these techniques, MCs have been demonstrated to be pathogenic in many diseases, including experimental autoimmune encephalomyelitis,[7] collagen induced arthritis,[8] type 1 diabetes mellitus (in non-obese diabetic mice),[9] bullous pemphigus[10] and systemic sclerosis.[11]

The somewhat simplistic concept that MCs are only pro-inflammatory has been complicated by evidence demonstrating an essential role for MCs in the induction and maintenance of tolerance. The list of diseases in which the net effect of MCs is immunomodulatory is growing and includes studies in ultraviolet-B light[12] or chemical induced suppression of contact hypersensitivity,[13] mosquito bite induced suppression of delayed type hypersensitivity (DTH),[14] induced peripheral tolerance to skin allograft transplants,[15] protection from anti-glomerular basement membrane (GBM),[16,17] and anti-myeloperoxidase glomerulonephritis (anti-MPO GN).[18] The mechanistic basis of these effects is also becoming better understood and includes MC synthesis of anti-inflammatory molecules (TGF-β and IL-10), the expression of surface molecules (OX40L and PD-L1) that may facilitate immunoregulation following direct contact with regulatory T cells (Tregs)[19] and reciprocally, Treg-derived IL-9 to enhance MC immunomodulation.[17]

In this current study, we investigated possible associations between infiltrating renal MCs and kidney function in patients with GN, a key feature of MPO-ANCA-associated vasculitis (MPO-AAV). This is an autoimmune disease that, despite current best practice, has a 5-year mortality of 30% and for which current treatments are non-specific and have considerable toxicities.[20] The disease is characterized by its strong association with circulating autoantibodies (ANCA) that recognize auto-antigens[21] found in neutrophil lysosomal azurophilic granules,[22] typically proteinase-3 and MPO. The renal lesion of MPO-AAV has a unique pathology characterized by focal and segmental necrotizing crescentic GN with little or no immunoglobulin deposition in glomeruli (thereby being designated as 'pauci-immune'). While immunoglobulin deposits are absent or rare in active ANCA-associated crescentic GN, kidney biopsies demonstrate DTH effectors; $CD4^+$ T cells, macrophages, and fibrin.[23] Several studies have shown that MCs are present in renal lesions in this disease but the functional role of these cells remains to be defined.[24,25] In this current study, we show that MCs are prominent in MPO-AAV GN, displaying an activated degranulating phenotype and greater numbers in patients with the most severe tubulointerstitial injury.

We have established an experimental autoimmune murine model of anti-MPO GN that exhibits the pathognomonic features observed in patients with MPO-AAV and found that MCs are immunomodulatory via MC IL-10 production enhancing immunosuppressive functions of Tregs.[18] Other studies in skin transplantation have shown that MCs closely interact with Tregs in the transplanted skin to maintain tolerance. However, induced degranulation of MCs leads to acute inflammation and graft rejection.[15] We hypothesize that in the autoimmune anti-MPO GN model, MC degranulation would similarly be pro-inflammatory and injurious in the induction of MPO autoimmunity by promoting the loss of tolerance to MPO. Therefore MCs could play opposing roles in MPO-AAV. Within the lymph nodes (LNs), IL-10 secreted by MCs is immunomodulatory and favors tolerance, while degranulating MCs may be pro-inflammatory in the induction of autoimmunity and also enhance effector responses in the major target organ, the kidney. Several MC stabilizing drugs have been developed. Among the best known is disodium cromoglycate (DSCG), a calcium channel targeting drug[26] that blocks MC degranulation and has been used in the treatment of anaphylaxis and allergic diseases including asthma and mastocytosis for over 30 years.[27,28] Its predominant effect is on preventing degranulation, while *de novo* synthesis and release of cytokines is not significantly affected.[29] Given that both degranulation and synthesis of immunomodulatory IL-10 may occur in MPO-AAV, selective enhancement of MC modulatory function by DSCG administration may represent a new therapeutic strategy.

RESULTS

Human Studies of MC Phenotype and Prominence in MPO-AAV and GN

The study population comprised 44 patients who satisfied study inclusion requirements (first presentation with renal biopsy with proven focal segmental, immune negative, necrotizing crescentic GN together with circulating MPO-ANCA, the absence of granulomata and a renal biopsy with at least six glomeruli). The mean age of the patients was 67 ± 2 years, 68% were male and 34% had extra renal disease (one or more of respiratory, joint or skin involvement). The patients had evidence of systemic inflammation with elevated C-reactive protein (CRP) (70 ± 13.9 mmol/l) and positive enzyme-linked immunosorbent assay (ELISA) for MPO-ANCA (mean 134 ± 16 U/ml). The cohort had significant renal functional impairment (eGFR mean 21.6 ± 2.4 ml/min per 1.73 m^2), serum creatinine (mean 395 ± 53 μmol/l), proteinuria (mean 1.8 ± 0.4 g/24 hrs), and hematuria (547 ± 682 red cells/l) (Table 1).

To assess the participation and significance of MCs in the GN of AAV, we analyzed the extent of renal MC infiltration in kidney biopsies from patients with MPO-AAV compared with biopsies from control patients with minimal glomerular damage, the non-inflammatory conditions; minimal change disease and thin basement membrane diseases (n=9). MCs were seen throughout the kidneys of patients with MPO-AAV. Although glomerular MC presence was rarely observed in control patients, MCs were detected in the glomeruli of MPO-AAV patients at low levels (0.03 ± 0.01 MCs/gcs, $P<0.05$) (Figure 1A). MCs were

Table 1. Clinical, demographic and immunologic data and indices of renal disease features among MPO-AAV patients

Demographic data		
	Patient number	44
	Sex (f/m)	14/30
	Age[a] (years)	67±2
Renal involvement		
	Serum creatinine[a] (μmol/l)	395±53
	eGFR[a] (ml/min per 1.73 m^2)	21.6±2.4
	Proteinuria[a,b] (g/day)	1.8±0.4
	RBC[a,c] (urine cells/hpf)	547±682
Extra renal involvement		
	Lung or upper respiratory tract or skin or arthralgia presenting creatinine	15/44
Immunologic data		
	ANCA (MPO) titer[a] (U/ml)	134±16
	CRP[d] (mmol/l)	70±13.9

[a]Reported as mean±SEM.
[b]Urinary total protein over 24 hours.
[c]Red blood cell excretion.
[d]CRP on admission.

predominantly found in the interstitium (Figure 1, B and C) and their frequency correlated with severe tubulointerstitial injury (Figure 1D). There was no significant correlation between the numbers of interstitial MCs and eGFR at presentation in patients with MPO-AAV (Spearman's rank-order correlation; $r = -0.27$, $P=0.07$). Furthermore, we assessed MC phenotypic markers indicative of an activated phenotype and more than 50% of MCs found in MPO-AAV patients were degranulated (Figure 1, E and F; red arrow and intact MC [black arrow]) and >70% were spindle shaped significantly higher than among controls (Figure 1G and H; green arrow). Therefore, MCs are found in kidney biopsies of patients with MPO-AAV and GN; showing an activated phenotype suggesting they play a pro-inflammatory, pathogenic role in disease.

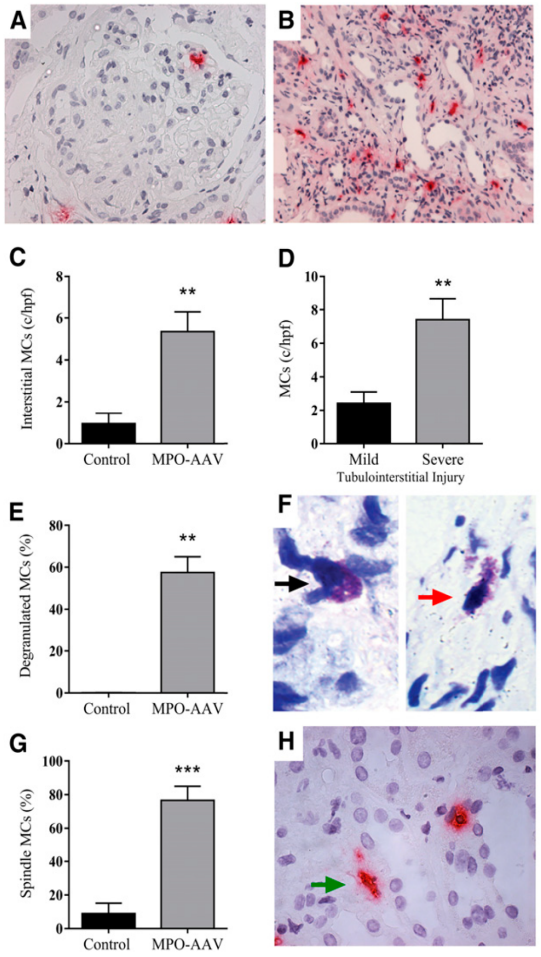


Figure 1. MC presence and activation status in renal biopsies from patients with MPO-AAV. Frequency of kidney MCs was determined by immunohistochemical staining for MC tryptase (A and B). Prominent interstitial MC accumulation was observed in patients with MPO-AAV (n=44 patients biopsies) compared with patients with minimal change GN (n=9 patient biopsies) (C). Patients with severe tubulointerstitial injury had greater numbers of infiltrating MCs (D). Most MCs in MPO-AAV biopsies (n=25) were degranulated (E and F; intact MC, black arrow and degranulated MC, red arrow) and spindle shaped (G and H; green arrow). Error bars represent mean±SEM with statistical analysis by Mann–Whitney t-test. ***$P<0.001$, **$P<0.01$.

DSCG Prevents the Development of Systemic Anti-MPO T Cell Autoimmunity

To assess the effects of DSCG in experimental autoimmune anti-MPO GN, C57BL/6 mice received DSCG (intraperitoneally, daily) while controls received saline. We induced a T cell mediated model of autoimmune anti-MPO GN by immunizing mice with native mouse MPO on day 0, then boosting the response on day 7. On day 16, disease was triggered by the passive transfer of low-dose anti-GBM globulin that recruits neutrophils to glomeruli and deposits MPO, the autoantigen, in the kidney.[30,31] Autoimmunity and renal injury were assessed at the end of the experiment after a further 4 days. DSCG did not alter anti-MPO antibody titers (Figure 2A) but did attenuate systemic anti-MPO T cell responses. MPO-specific dermal footpad DTH responses were reduced compared with saline-treated controls (Figure 2B). Effects on the anti-MPO autoimmune response were determined by isolating draining LNs from MPO immunization sites and restimulating with MPO *ex vivo*. T cell proliferation was reduced in mice treated with DSCG, associated with diminished IFN-γ and IL-17A production (Figure 2, C–E). However, the anti-inflammatory cytokine, IL-10, was significantly elevated in DSCG-treated mice (Figure 2F). These results highlight that DSCG is an immunomodulatory therapeutic capable of attenuating the development of anti-MPO autoimmunity.

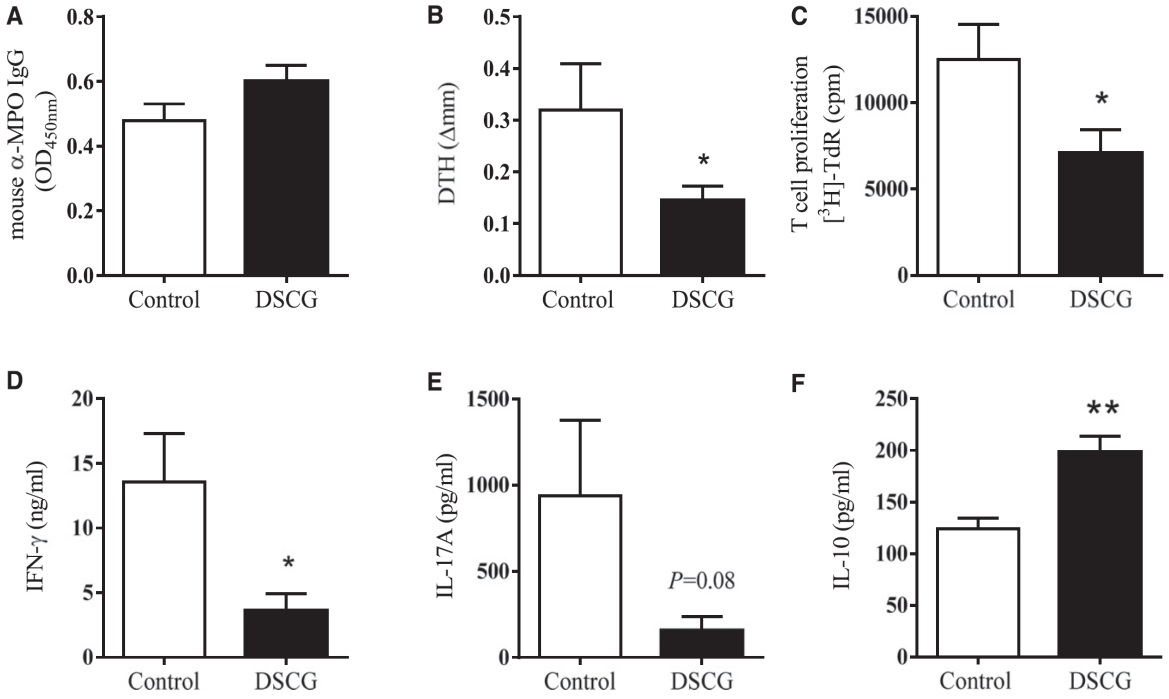


Figure 2. DSCG prevents the development of anti-MPO autoimmunity. ANCA levels were unaffected (A), MPO induced T cell recall responses assessed by DTH (B), proliferation (C), IFN-γ (D), and IL-17A (E) were all reduced while IL-10 responses were increased (F) by DSCG treatment (DSCG n=8, controls n=7). Error bars represent mean±SEM with statistical analysis by Mann–Whitney t-test. $^{**}P<0.01$, $^{*}P<0.05$.

Preventative Treatment of DSCG Protects from the Development of GN

DSCG administered prior to establishment of anti-MPO autoimmunity attenuated functional kidney injury as measured by albuminuria (Figure 3A) and the proportion of glomeruli with segmental necrosis (Figure 3, B and C) compared with controls. Reduction in functional kidney injury was associated with decreased numbers of glomerular leukocytes, including $CD4^+$ T cells and macrophages, while no difference in glomerular neutrophils was observed (Figure 3D). Furthermore, DSCG as a preventative treatment significantly reduces kidney MC infiltration to a similar level observed in naïve C57BL/6 mice (dotted line) (Figure 3, E and F).

DSCG Effects are Mast Cell Specific

The selectivity of DSCG as an MC stabilizer is controversial as any generalized mechanisms of action, independent of MCs, have not been well defined. DSCG may inhibit the activation of human neutrophils, eosinophils and monocytes, and stimulate activated B cells to produce immunoglobulin *in vitro*.[32,33] However, in over 30 years of safe clinical use, major effects on immunity have not been evident. To verify that the protective effects of DSCG in the development of experimental autoimmune anti-MPO GN were due only to its effects on MCs, DSCG was administered to MC-deficient ($Kit^{Wsh/Wsh}$) mice prior to induction of autoimmune anti-MPO GN (control $Kit^{Wsh/Wsh}$ mice received saline). $Kit^{Wsh/Wsh}$ mice treated with DSCG exhibited similar auto-reactivity and disease to control treated $Kit^{Wsh/Wsh}$ mice, with no significant differences in anti-MPO autoimmunity (dermal DTH, T cell cytokine production and anti-MPO antibodies; Figure 4A–D). Renal injury was similarly (albuminuria and glomerular leukocyte accumulation; Figure 4, E and F) unaffected. These results demonstrate that in experimental autoimmune anti-MPO GN, DSCG acts specifically through MCs.

DSCG Effects on MC–Dendritic Cell Interactions

We hypothesized that the major effects of DSCG in this disease model would be mediated by preventing MC degranulation. This was assessed in the LN draining the sites of MPO immunization, the maneuver that provokes loss of tolerance and development of nephritogenic autoimmunity. While MC numbers in draining LNs were similar (Figure 5A), we observed significant attenuation of MC degranulation as determined by quantitating the number of degranulating MCs (Figure 5B; red arrow and Figure 5C) and by measuring extracellular tryptase released from MCs in LNs draining sites of MPO immunization (Figure 5D) following DSCG administration. This was associated with fewer DCs (Figure 5E) that were less activated ($CD11c^+MHCII^+$ and $CD11c^+CD40^+$; Figure 5, F and G), and more apoptotic (Figure 5H) compared with saline treated controls. These data support our hypothesis that DSCG acts by reducing MC degranulation,

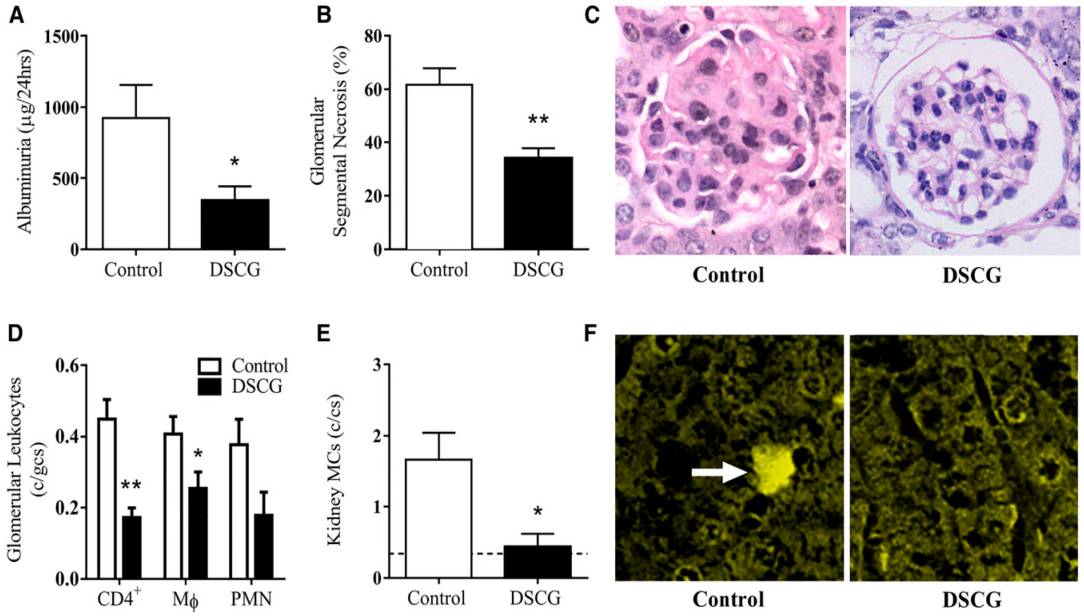


Figure 3. DSCG administration prior to MPO immunization, as a preventative, protected from the development of GN. Renal injury assessed by albuminuria (A), glomerular segmental necrosis (B and C) and glomerular leukocyte accumulation (D) was attenuated in mice that received DSCG (n=8), compared with saline treated controls (n=7). Kidney MCs were identifiable in controls and significantly reduced by DSCG to numbers similar to those observed in naïve C57BL/6 mice (dotted line; n=5) (E). Kidney MCs were identified and quantified following methyl Carnoy's fixation and Berberine sulfate staining, white arrow (F). Error bars represent mean±SEM with statistical analysis by Mann–Whitney t-test. $^{**}P<0.01$, $^{*}P<0.05$.

mitigating against the development and maintenance of injurious anti-MPO autoimmunity.

Therapeutic Potential of DSCG to Dampen Anti-MPO Autoimmunity and GN

To determine whether DSCG is therapeutic in mice with established anti-MPO autoimmunity, daily DSCG administration was commenced on day 10 (after the completion of the MPO immunization schedule). Compared with saline-treated controls, DSCG treatment attenuated systemic anti-MPO T cell responses measured by dermal DTH (Figure 6A) as well as diminished IFN-γ and IL-17A by lymph node cells (Figure 6, B and C). The diminished anti-MPO autoimmunity in DSCG-treated mice was associated with less glomerular segmental necrosis (Figure 6D), fewer glomerular $CD4^+$ T cells, macrophages, neutrophils (Figure 6E), and less albuminuria (Figure 6F). Collectively, DSCG, a low clinical toxicity MC stabilizer, has the capacity to limit established anti-MPO autoimmunity as well as preventing the development of GN.

Effects of DSCG on Mast Cells to Inhibit T Cell Proliferation *Ex Vivo*

$CD4^+Foxp3^-$ T effector cells (anti-MPO $CD4^+$ Teff) from MPO-immunized Foxp3-GFP mice were stimulated *ex vivo* with MPO and compared with anti-MPO Teffs stimulated with MPO, but co-cultured with bone marrow derived mast cells (BMMCs) in the presence or absence of DSCG. The MPO triggered anti-MPO Teff proliferation increased 3-fold in the presence of BMMCs. The addition of DSCG to co-cultures significantly restricted Teff proliferation (Figure 7A), while no difference was observed in IFN-γ concentration in cultured supernatants (Figure 7B). Interestingly, DSCG was able to significantly enhance IL-10 production by co-cultured BMMCs and Teffs compared with untreated BMMCs and Teffs (Figure 7C). No IL-10 was detectable when Teff or MCs were cultured alone. These studies demonstrate a direct immunomodulatory effect of DSCG on BMMCs and Teffs co-cultures.

The Protection Afforded by DSCG is IL-10 Dependent

MCs are immunomodulatory by their capacity to synthesize and secrete IL-10. To determine whether DSCG's protective effects are IL-10 dependent, anti-MPO GN was induced in $Kit^{Wsh/Wsh}$ mice that were either reconstituted with WT or IL-10 deficient ($^{-/-}$) BMMCs. Both groups were then treated with DSCG. DSCG treated IL-$10^{-/-}$MC→$Kit^{Wsh/Wsh}$ mice developed significant MPO specific dermal DTH footpad swelling that was markedly reduced in WTMC→$Kit^{Wsh/Wsh}$ mice treated with DSCG (Figure 8A). Comparing MPO-specific T cell production of pro-inflammatory cytokines by *ex vivo* MPO restimulation of cells from LN draining MPO immunization sites between WT and IL-$10^{-/-}$MC reconstituted $Kit^{Wsh/Wsh}$

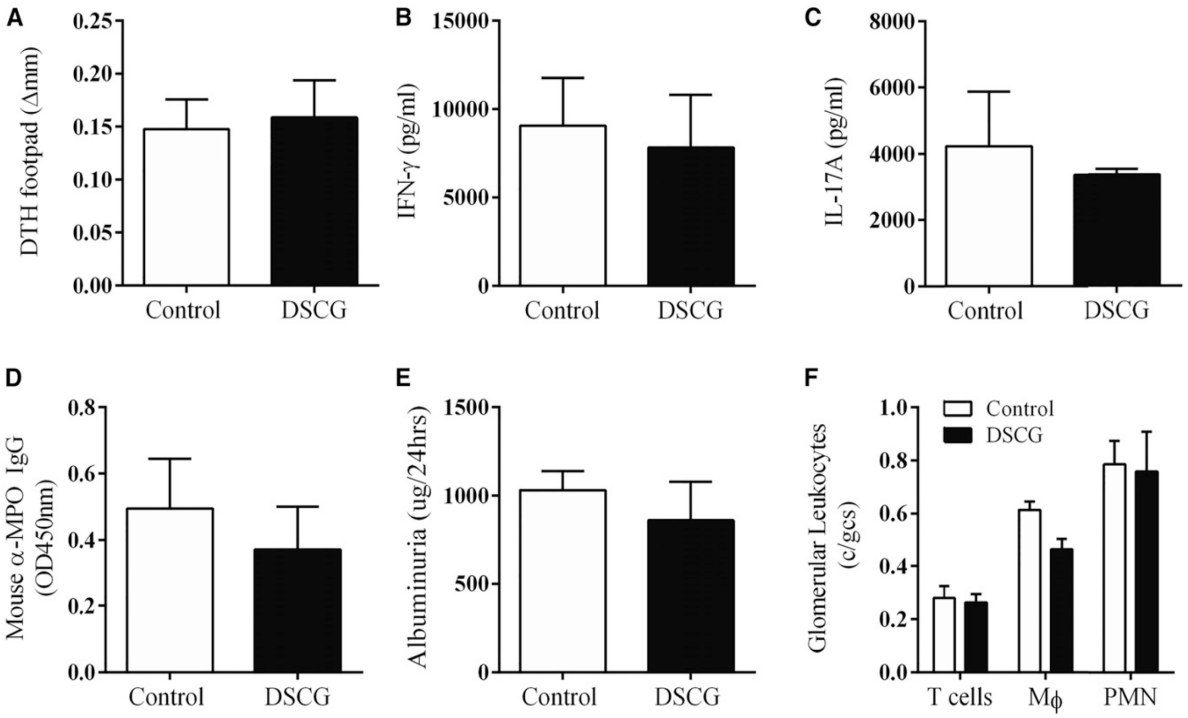


Figure 4. DSCG effects are mast cell specific. MPO immunized $Kit^{Wsh/Wsh}$ mice treated with DSCG (n=9) in a preventative protocol developed similar systemic MPO T cell (DTH, IFN-γ and IL-17A; A–C) and humoral responses (mouse anti-MPO IgG, D) compared with saline treated MPO immunized $Kit^{Wsh/Wsh}$ mice (n=9). No difference in renal injury (albuminuria and glomerular leukocyte accumulation; E and F) was observed between groups. Error bars represent mean±SEM with statistical analysis by Mann–Whitney t-test.

mice receiving DSCG shows that IFN-γ production was significantly higher in IL-$10^{-/-}$MC→$Kit^{Wsh/Wsh}$ mice (Figure 8B). No difference in IL-17A production was observed between groups (Figure 8C). Additionally, DSCG treatment did not limit numbers of infiltrating glomerular leukocytes in IL-$10^{-/-}$MC→$Kit^{Wsh/Wsh}$ mice, compared with WTMC→$Kit^{Wsh/Wsh}$ mice (Figure 8, D–F). Glomerular segmental necrosis was assessed histologically on PAS-stained kidney sections, demonstrating significantly more structural glomerular injury in IL-$10^{-/-}$MC→$Kit^{Wsh/Wsh}$ mice (Figure 8, G and H). There was no difference in 24-hour albuminuria (Figure 8I). These results reiterate the importance of MC IL-10 to modulate the extent of MPO autoimmunity and GN, demonstrating that daily DSCG administration works by blocking MC degranulation while requiring IL-10 production.

DISCUSSION

The current studies, using observations in human MPO-AAV and intervention studies in an experimental model of MPO-ANCA associated GN, suggest MC degranulation enhances the development of renal vasculitis and that MC stabilizers such as DSCG might be employed in limiting autoimmunity and disease. MCs have emerged as potential key players in the induction, amplification, and paradoxically immunomodulation of autoimmunity in general and rapidly progressive forms of GN in particular.[2,17,34,35] In MPO-AAV, MCs are a major leukocyte population seen in infiltrating damaged kidneys. Although the functional role of MCs in patients with MPO-AAV is uncertain, the patients with the highest MC density had the most severe tubulointerstitial damage. Furthermore, little is known about the phenotype of renal MCs in human MPO-AAV. The current study confirms that MCs in the kidney of patients with this disease degranulate and adopt a spindle shape, thus demonstrating a pro-inflammatory phenotype. One recent paper reports that MCs are prominent producers of IL-17 in ANCA-associated GN.[36] These findings are consistent with MCs having an acute injurious role.

In addition, our current experimental studies suggest that MCs can enhance the development of autoimmunity by interacting with DCs in LNs. We have previously shown in this model that MCs have a net protective effect, resulting from their interactions with Tregs which enhances immunomodulation.[18] MC-deficient mice develop more severe disease than WT mice and this enhanced autoimmunity and glomerular injury can be attenuated by MC reconstitution. Those results together with the current studies confirm that MCs play two distinct roles – pro-inflammatory and immunomodulatory. MC degranulation occurs within seconds of MC activation and preformed mediators are released within hours. While

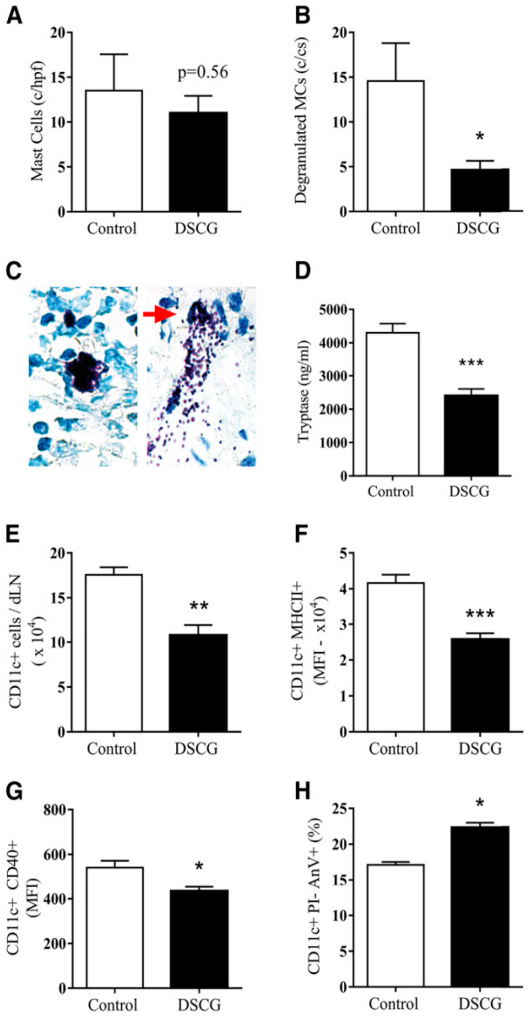


Figure 5. Blocking MC degranulation limits dendritic cell activation. In draining LNs 18 hours post-MPO immunization, DSCG treatment (n=5) does not affect MC numbers (A) but reduces the proportion of degranulated MCs (B) as indicated by the red arrow (C) compared with controls (n=5). Extracellular tryptase protein obtained from LNs draining MPO immunization sites was significantly reduced in mice that received DSCG compared with saline treated controls (D; DSCG n=8, control n=8). DSCG treatment decreased DC numbers (E), activation markers, $CD11c^{+}MHC\text{-}II^{+}$, $CD11c^{+}CD40^{+}$ expression (F and G) and enhanced DC apoptosis (H). Error bars represent mean±SEM with statistical analysis by Mann–Whitney t-test. ***P<0.001, **P<0.01, *P<0.05.

independent of degranulation, MCs can synthesize and release an array of cytokines *de novo*, depending on the activating stimulus. MC activation through TLR4 induces synthesis and release of TNF-α, IL-6, IL-1, and IL-13,[37] which can potentially activate DCs. However, MC activation through vitamin D receptors results in the release of IL-10, without inducing MC degranulation.[38] In experimental anti-MPO GN, MCs were observed to be degranulating in LNs, where tolerance to MPO was being lost. We therefore used DSCG, a drug whose major MC effect was to prevent degranulation. DSCG attenuated MC degranulation and this was associated with reduced DC activation and increased apoptosis. At the same time, DSCG administration was also associated with enhanced IL-10 generation. These events were accompanied by significant reduction in anti-MPO autoimmunity and glomerular injury.

Cromolyns have been used for over 30 years as standard treatment for stabilizing (*i.e.*, preventing degranulation) MCs in allergy and mastocytosis. These drugs have also been widely used in experimental animal models. There is some controversy as to whether DSCG can effectively attenuate MC degranulation in mice.[39] However, there are a number of reports showing that this drug successfully stabilizes MCs by attenuating MC degranulation in a number of murine models.[40–42] We also demonstrated that DSCG could significantly attenuate MC degranulation in a model of passive cutaneous anaphylaxis in C57BL/6 mice (Supplemental Figure 1). To demonstrate that DSCG's mechanism of action did not extend beyond stabilizing MCs in experimental anti-MPO GN, the same experiments were performed in MC-deficient ($Kit^{Wsh/Wsh}$) mice. In the absence of MCs, DSCG administration had no effect on the generation of anti-MPO autoimmunity (DTH responses to MPO, MPO-specific cytokine production of IFN-γ or IL-17A and serum anti-MPO titers), glomerular leukocyte accumulation or glomerular histologic and functional injury.

While the therapeutic potential of DSCG as a preventative therapy in the development of experimental anti-MPO GN is interesting, clinically, MPO-AAV patients undergo treatment only after autoimmunity is established. Therefore, we sought to determine whether DSCG treatment after established anti-MPO autoimmunity will attenuate GN. DSCG treatment diminished the severity of GN. This attenuation was associated with reduced DTH and MPO specific Th1 and Th17 cell directed responses. Together, these studies strongly support consideration of a clinical trial of DSCG as a potentially safe and effective-treatment for anti-MPO GN.

To further explore the capacity of DSCG to block MC enhancement of anti-MPO $CD4^{+}$ effector T cell MPO recall responses, *ex vivo* T cell/MC co-cultures were studied. Recall responses of $CD4^{+}$ T cells to MPO were enhanced by co-culture with MCs. However this enhancement was significantly less when effector cells were co-cultured with DSCG treated MCs. In previous studies of *ex vivo* co-culture of MCs, anti-MPO Teff and Tregs, we were able to show that MC IL-10 augments Treg immunosuppression.[18] These current experiments show that DSCG could stimulate IL-10 production from co-culture of MCs and Teff cells in the absence of $Foxp3^{+}$ Tregs. These results support an interpretation that DSCG's effects on preventing MC degranulation result in the modulation of MC interactions on Teff cells, with the resultant attenuation of anti-MPO autoimmunity and GN.

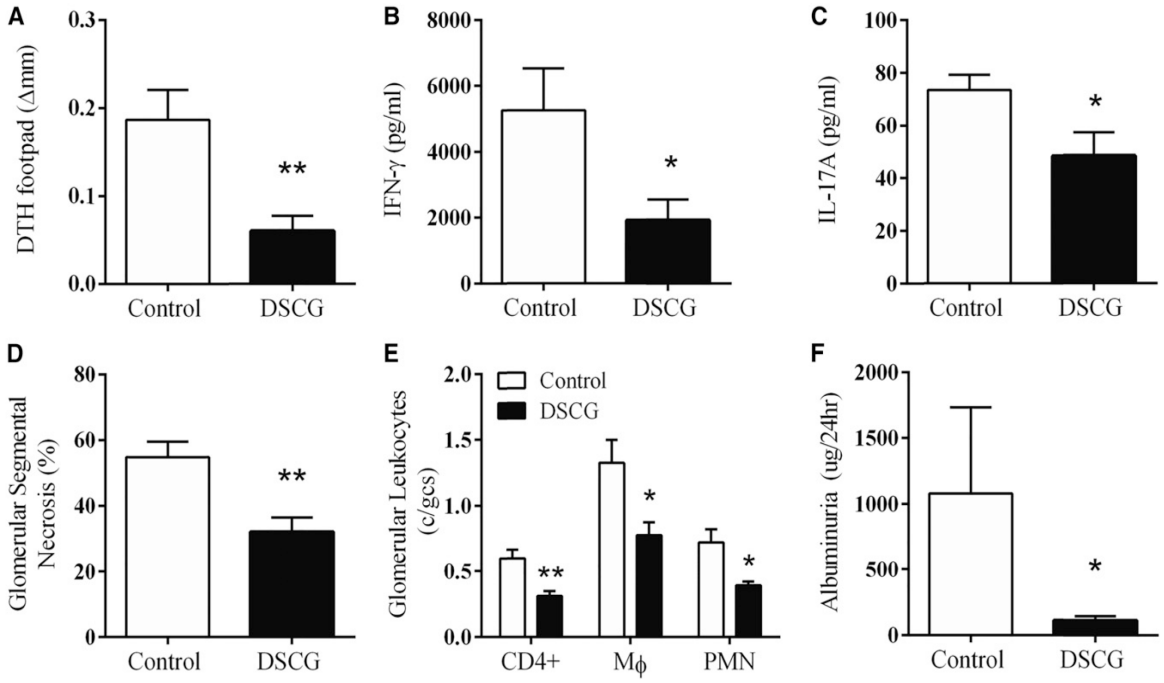


Figure 6. DSCG treatment of mice with established MPO autoimmunity (day 10) diminishes the extent of effector T cell responses assessed by MPO T cell recall responses including skin DTH (A) and LN IFN-γ and IL-17A responses (B and C). Renal injury, segmental glomerular necrosis (D), glomerular leukocyte accumulation (E), and functional injury (albuminuria, F) were also reduced by DSCG treatment (DSCG n=6, control n=6). Error bars represent mean±SEM with statistical analysis by Mann–Whitney t-test. $^{**}P<0.01$, $^{*}P<0.05$.

Taking the results from the human biopsy observations and the animal experiments, we believe MCs play both injurious and protective roles. MC degranulation is directly injurious in the major affected organ, the kidney and in the immune system, degranulating MCs drive the induction and severity of anti-MPO autoimmunity. However, MCs also play a protective role in modulating the intensity of effector anti-MPO T cell responses via IL-10 production in the immune system. DSCG acts only on the injurious contribution of MCs (degranulation) without effecting MC mediated immunomodulation. These findings suggest a new therapeutic use of this drug in treating autoimmune anti-MPO GN.

CONCISE METHODS

Patient Cohort

Forty-four patients admitted to Monash Medical Centre (MMC) were categorized as having MPO-AAV by clinical, serological and histologic features. Patients with first presentation of vasculitis, a

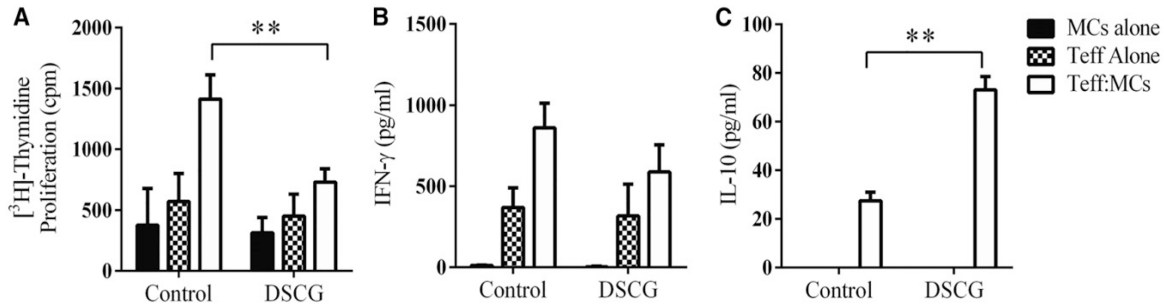


Figure 7. DSCG attenuates MC enhancement of MPO induced recall responses of anti-MPO Teffs. Controls demonstrate that DSCG does not alter the proliferative response and IFN-γ production of either Teff alone or MCs alone (A and B). Co-culture of Teff and MCs induced IL-10 production which was significantly enhanced in the presence of DSCG (C) while the addition of DSCG did not induce IL-10 production by Teff or MCs alone in this assay. Error bars represent mean±SEM with statistical analysis by two-tailed paired t-test. $^{**}P<0.01$, $^{*}P<0.05$.

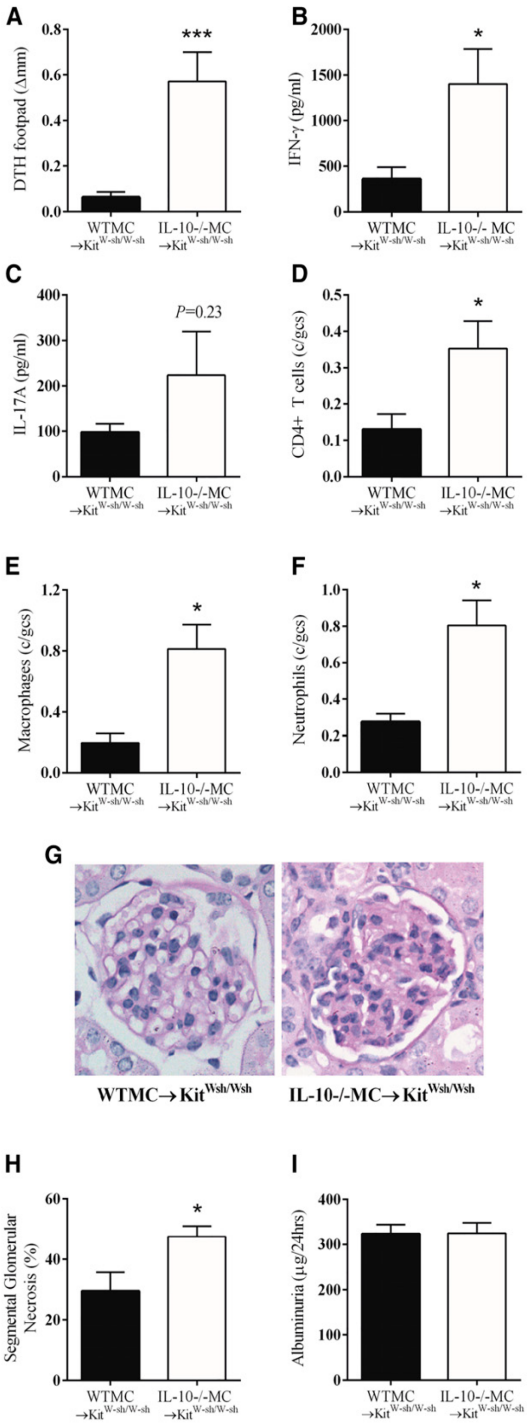


Figure 8. MC IL-10 attenuates anti-MPO GN and is not inhibited by DSCG. DSCG treated IL-10$^{-/-}$MC→Kit$^{Wsh/Wsh}$ mice (n=7) did not attenuate MPO specific DTH footpad swelling (A), anti-MPO T cells, IFN-γ (B) and IL-17A production (C). Accumulation of glomerular T cells, macrophages, neutrophils and segmental necrosis was greater in DSCG treated IL-10$^{-/-}$MC→Kit$^{Wsh/Wsh}$ versus WTMC→Kit$^{Wsh/Wsh}$ mice (n=7) (D–H). Albuminuria was NS between groups (I). Error bars represent mean±SEM with statistical analysis by Mann–Whitney t-test. ***P<0.001, *P<0.05.

positive MPO-ANCA result by both direct immunofluorescence and antigen-specific ELISA was obtained along with clinical evidence of significant renal impairment (serum creatinine >200 μmol/L) and active urinary sediment with the presence of a systemic inflammation (raised CRP and erythrocyte sedimentation rate), together with focal and segmental necrotizing crescentic GN with little or no immunostaining for immunoglobulin or complement were retrospectively sequentially collected. Biopsies were assessed if they had a minimum of six glomeruli (range 6–37 glomeruli). Tubulointerstitial disease was graded by the extent of interstitial fibrosis and atrophy as Grade 1 (mild) or Grade 2 (severe).[43] Renal function was assessed by eGFR (ml/min per 1.73 m^2) assessed at the time of renal biopsy. Nine patients with thin basement membrane disease or adult minimal change GN were used as 'normal' controls. Studies were approved by the Monash University Human Research Ethics Committee.

Mast Cell Staining in Human Biopsies

Mast Cell Tryptase

Formalin fixed paraffin embedded 2 μm tissue specimens (n=44 MPO-AAV, n=9 Control patients), were mounted, dewaxed, and rehydrated in graded alcohols, and pretreated with antigen retrieval solution tris-EDTA (pH 9) in a pressure cooker for 10 minutes, blocked (30 minutes) in 5% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) (immunofluorescence) with 10% horse serum (immunohistochemistry) and probed with mouse anti-human mast cell tryptase 1/1000 (DAKO, Glostrup, Denmark) in 1% BSA/PBS for 16 hours (4°C). A universal VECTORSTAIN ABC-AP kit (Vector, Peterborough, UK) was used and visualized with a VECTOR red alkaline phosphatase substrate kit (Vector) and counterstained with hematoxylin. Sections were assessed for the total numbers of spindle MCs and expressed as a percentage of the total MC population, within the glomerulus (cells/gcs), peri-glomerular regions (cells/gcs), and the interstitium (expressed as the average of 10 hpf).

Toluidine Blue

The kidney biopsies of 25 MPO-AAV patients with adequate, available sample material were used to analyze MC degranulation. Their clinical, demographic, and immunologic data and indices of renal disease characteristic were representative of the total MPO-ANCA patient population studied (Supplemental Table 1). Formalin-fixed paraffin embedded 2 μm sections of tissue specimens were mounted on Superfrost Plus slides (Menzel, Braunschweig, Germany), dewaxed, rehydrated in graded alcohols, and stained with 0.5% potassium permanganate (Sigma-Aldrich, St Louis, MO) for 2 minutes, washed and stained for 1 minute with a 2% aqueous solution of potassium metabisulphite (Sigma-Aldrich), washed and stained with acidified 0.1% toluidine blue O (Sigma-Aldrich) for 5 minutes, washed, dehydrated, cleared and cover slipped. MCs were identified by characteristic metachromatic granules. Degranulated MCs were identified as those with metachromatic granules close to

the cell membrane or staining less than half of the cytoplasm as previously described.[44] MCs were quantified in 10 random non-overlapping high-powered fields (100×) of view and expressed as percentage of degranulating MCs/mm^2 per total mast cells per mm^2 for human biopsies, and the same procedure was repeated for mouse LN.

Mice

C57BL/6 (wild-type), Kit$^{Wsh/Wsh}$ and Foxp3-GFP male mice (n=6–10 per group) were bred at Monash Medical Centre Animal Facilities, Monash University, Australia. IL-10$^{-/-}$ C57BL/6 male mice purchased from the University of Adelaide (South Australia, Australia) and originally from Jackson Laboratories, were used as donors to obtain BMMCs. All mice were housed in specific pathogen-free conditions at Monash Medical Centre, and studies were approved by Monash University Animal Ethics Committee in accordance with the Australian National Health and Medical Research Council animal experimentation guidelines.

Experimental Design

WT mice were immunized intraperitoneally with 20 μg murine MPO in Freund's complete adjuvant (FCA; Sigma-Aldrich) and boosted subcutaneously with 10 μg murine MPO in Freund's incomplete adjuvant (FIA; Sigma-Aldrich) on day 7. Murine MPO was purified from differentiated 32Dcl3 cells as described previously.[45] Disease was initiated ('triggered') by intravenous injection of 1.5 mg anti-GBM globulin consecutively on days 16 and 17. Anti-MPO autoimmunity and GN was assessed 3 days later (day 20).

Mast Cell Reconstitution

Kit$^{Wsh/Wsh}$ was reconstituted with 5×10^6 MCs (intravenously) from 6- to 8-week-old wild-type or IL-10$^{-/-}$ mice using a standard *in vitro* differentiation of MC technique.[5,18] Following MC reconstitution (WTMC→Kit$^{Wsh/Wsh}$ and IL-10$^{-/-}$MC→Kit$^{Wsh/Wsh}$), mice were administered daily with DSCG beginning a day prior to the induction of MPO autoimmunity.

Disodium Cromoglycate (DSCG) Treatment

We chose a protocol of administration that has been shown to attenuate MC degranulation in several mouse models of disease *in vivo*.[40–42] In all experiments, to stabilize MC degranulation, 10 mg/kg DSCG (Sigma-Aldrich) was reconstituted in saline and injected intraperitoneally.

Passive Cutaneous Anaphylaxis

To assess the capacity of DSCG to attenuate MC degranulation in C57BL/6 WT mice, we confirmed in an established model of MC mediated injury (passive cutaneous anaphylaxis) that DSCG (10 mg/kg) could attenuate this well studied model of MC degranulation. WT mice were passively sensitized with α-DNP IgE (10 ng subcutaneous footpad; Sigma-Aldrich) and challenged 24 hours later with DNP-BSA (100 μg intravenously, Life Technologies). Two hours prior to challenge, WT mice received either DSCG (n=10) or saline (n=10) intraperitoneally. Six hours post-challenge, footpads were harvested, fixed in methyl Carnoy's and toluidine blue stained for degranulated MCs (as described above). Results are expressed as percentage degranulated MCs.

DSCG Administration in Anti-MPO Autoimmunity and GN

DSCG was administered daily intraperitoneally beginning a day prior to MPO immunization (day −1). When DSCG was used to treat mice with established anti-MPO autoimmunity, DSCG was administered daily intraperitoneally post-MPO immunization schedule (day 10). Non-treated control mice received the same volume of saline daily.

Assessment of Renal Disease and Immune Cell Infiltration

Histologic assessment of renal injury was performed on 3 μm thick, formalin-fixed, paraffin-embedded, periodic acid-Schiff-stained kidney sections. A minimum of 30 consecutive glomeruli/mouse were examined and results expressed as percentage of segmental glomerular necrosis per glomerular cross-section (gcs). Glomerular CD4$^+$ T cells, macrophages, and neutrophils were assessed by an immunoperoxidase-staining technique on 6-μm thick, periodate lysine paraformaldehyde fixed, OCT frozen kidney sections. The primary antibodies used were GK1.5 for CD4$^+$ T cells (anti-mouse CD4$^+$; American Type Culture Collection, Manassas, VA), FA/11 for macrophages (anti-mouse CD68 from Dr. Gordon L. Koch, Cambridge, England), and RB6–8CS for neutrophils (anti-GR-1; DNAX, Palo Alto, CA). A minimum of 30 glomeruli were assessed and results expressed as cells/gcs (c/gcs).

Kidney MCs were detected by staining with MC heparin. Acidified toluidine blue, while it is effective for the detection of LN MCs, fails to detect kidney MCs. Mouse kidneys were removed, halved, and fixed in Methyl Carnoy's fixative (60% methanol, 30% chloroform, and 10% glacial acetic acid) for 4 hours at room temperature, subjected to routine tissue processing and embedded in wax. Non-serial 3 μm cross-sections were cut and every 4th kidney section was mounted on glass slides, cleared, immersed in ethanol/acetic acid (3:1) for 15 minutes, rinsed in 100% ethanol for a further 15 minutes, washed in distilled water for 10 minutes and stained with 0.02% (w/v) berberine sulfate (Sigma-Aldrich Pty Ltd) diluted in acidic distilled water (pH 4, with 1% citric acid), washed in acidic distilled water (pH 4), air dried and mounted with glycerol. Slides were examined under a fluorescent microscope using the FITC/488 filter set (Leica-Microsystems, North Ryde, Australia). A minimum of six kidney cross-sections were examined for berberine sulfate positive cells as determined by bright yellow fluorescence counted using 400× magnification. Results are expressed as MCs per kidney cross-section (c/cs).

Urine was collected by housing mice in individual metabolic cages over the final 24 hours of experiment. Albuminuria was assessed by ELISA (Bethyl Laboratories, Montgomery, TX) and expressed as μg/24 hr.

Systemic Immune Responses to MPO

ELISA was used to detect circulating serum anti-MPO IgG titers using 100 μl/well, 1 μg/ml murine MPO and horseradish peroxidase conjugated sheep anti-mouse IgG (1:1000; Amersham Biosciences, Rydalmere, Australia).

To assess MPO-specific dermal DTH, mice were challenged by intradermal injection of 10 μg murine MPO in 30 μl saline in the right hind footpad (the contralateral footpad received saline). DTH

was quantified 24 hours later by measuring the difference between footpad thickness (Δmm) using a micrometer.

For assessment of cytokine production, lymphocytes were cultured for 72 hours (37°C, 5% CO_2) at 4×10^6 cells/ml per well with or without MPO (10 μg/ml) in supplemented RPMI (10% FCS, 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 0.1 mg/ml streptomycin; Sigma). IFN-γ, IL-17A, and IL-10 were measured by ELISA.[46] For IFN-γ, the mAbs were rat anti-mouse IFN-γ (R4–6A2; BD Pharmingen) and biotinylated rat anti-mouse IFN-γ (XMG1.2; BD Pharmingen). IL-17A was measured by using paired antibodies (DuoSet; eBioscience, San Diego, CA), IL-10 by using rat anti-mouse IL-10 capture antibody (18141D; BD Pharmingen), and biotinylated rat anti-mouse IL-10 detection antibody (BD Pharmingen).

MPO-specific cell proliferation was measured by culturing lymphocytes at 5×10^5 cells/well in 96-well flat-bottom plates (Sarstedt, Newton, NC), re-stimulated with or without 10 μg/ml MPO and incubated for 72 hours. During the last 16 hours of culture, 0.5 μCi of [^{3}H]-thymidine (PerkinElmer, Waltham, MA) was added. [^{3}H]-Thymidine incorporation was measured as previously described.[18]

Assessment of MC Degranulation and DC Status in Experimental Anti-MPO GN

DSCG was administered intraperitoneally to WT mice a day before 15 μg MPO/FCA immunization (subcutaneously). Control MPO/FCA immunized WT mice were administered with saline. Eighteen hours post MP/FCA immunization, LNs draining MPO immunization sites were harvested. Histologic assessment of MC prominence and MC degranulation was determined by staining LN sections using the toluidine blue method as described above.

A standard method of quantifying MC degranulation is by measuring extracellular tryptase. A MC degranulation ELISA kit determines the amount of tryptase protein by measuring tryptase activity (IMM001; EMD Millipore, Billerica, MA). The assay was performed according to the manufacturer's recommendations. Briefly, draining LNs were harvested (controls, n=8 and DSCG, n=8), placed in 1× assay buffer (IMM001; Part No. 90570), and pushed through a 35 μm cell strainer cap (Falcon, Mexico). Cell suspensions were centrifuged at 700 ***g*** and supernatant collected, discarding the cell pellet. Tryptase positive control standards (IMM001; Part No. 90572) and experimental samples were incubated at 37°C for 90 minutes with Tryptase Substrate (IMM001; Part No. 90569). The absorbance values were read at 405 nm using a microplate reader (Tecan, Infinite M1000 PRO, Austria) and results expressed as ng/ml.

DC activation (identified as CD11c^{hi} cells) on isolated LNs was measured by flow cytometry. LN cells were stained for 20 minutes at 4°C with the following directly conjugated antibodies: hamster anti-mouse CD11c PE (HL3; BD Pharmingen), rat anti-mouse CD40 FITC (3/32; BD Pharmingen), and anti-mouse MHC-II PE (M5/114, gift from K. Shortman, Walter and Eliza Hall Institute, Parkville, Australia). For the detection of apoptotic cells, isolated LN cells were resuspended in 100 μl of Annexin-V labelling solution (Roche, Mannheim, Germany), which contained 10 μg/ml of propidium iodine and incubated for 15 minutes at room temperature. Cells were analyzed on the FACSCanto II machine using FACSDiva software (BD Biosciences) and data analyzed using FlowJo (TreeStar, Palo Alto, CA).

Assessment of DSCG to Inhibit *in vitro* MPO CD4 T Effector Cell Recall Response

To obtain MPO-CD4$^+$ Teff cells, Foxp3-GFP mice (n=6) were immunized with MPO and FCA subcutaneously. Ten days later, draining LNs from MPO immunization sites were harvested, and CD4$^+$ T cells isolated by magnetic separation using mouse CD4 (L3T4) microbeads (Miltenyi Biotec, Bergisch, Gladbach) and then sorted based on GFP expression on the Mo-Flo XDP cell sorter (Beckman Coulter, Lane Cove, NSW, Australia). CD4$^+$Foxp3$^-$ MPO Teff cells (1×10^5) were co-cultured with WT BMMCs (1×10^5) together with erythrocyte-lysed, MACS CD4 depleted, mitomycin C-treated (50 μg/ml for 30 mins at 37°C) Kit$^{Wsh/Wsh}$ splenocytes (2×10^5 cells). Cells were added in a 96-well round-bottom plate with 10 μg/ml MPO for 72 hours, and proliferation was measured by adding [^{3}H]-thymidine for the last 16 hours of culture. Results were expressed as percentage anti-MPO Teff proliferation. IFN-γ in cultured supernatants was measured using BD Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences) and IL-10 was measured using a BD Cytometric Bead Array Mouse IL-10 Enhanced Sensitivity Flex Set with Enhanced Sensitivity Master Buffer Kit (BD Biosciences) as per manufacturer's instructions.

Statistical Methods

Results are expressed as the mean±SEM. Mann–Whitney t-test was used for non-parametric data and paired t-test for comparison in *ex vivo* co-culture experiments. All data were analyzed with Graph Pad Version 6 (GraphPad Prism; GraphPad Software Inc., San Diego, CA). Differences were considered to be statistically significant if $P<0.05$.

ACKNOWLEDGMENTS

These studies were supported by Grant No. 1011724 from the National Health and Medical Research Council of Australia.

DISCLOSURES

None.

REFERENCES

1. Kalesnikoff J, Galli SJ: New developments in mast cell biology. *Nat Immunol* 9: 1215–1223, 2008
2. Walker ME, Hatfield JK, Brown MA: New insights into the role of mast cells in autoimmunity: evidence for a common mechanism of action? *Biochim Biophys Acta* 1822: 57–65, 2012
3. Brown MA, Hatfield JK: Mast cells are important modifiers of autoimmune disease: With so much evidence, why is there still controversy? *Front Immunol* 3: 147, 2012
4. Galli SJ, Kitamura Y: Genetically mast-cell-deficient W/Wv and Sl/Sld mice. Their value for the analysis of the roles of mast cells in biologic responses in vivo. *Am J Pathol* 127: 191–198, 1987

5. Grimbaldeston MA, Chen CC, Piliponsky AM, Tsai M, Tam SY, Galli SJ: Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. *Am J Pathol* 167: 835–848, 2005
6. Shelburne CP, Nakano H, St John AL, Chan C, McLachlan JB, Gunn MD, Staats HF, Abraham SN: Mast cells augment adaptive immunity by orchestrating dendritic cell trafficking through infected tissues. *Cell Host Microbe* 6: 331–342, 2009
7. Secor VH, Secor WE, Gutekunst CA, Brown MA: Mast cells are essential for early onset and severe disease in a murine model of multiple sclerosis. *J Exp Med* 191: 813–822, 2000
8. Pimentel TA, Sampaio AL, D'Acquisto F, Perretti M, Oliani SM: An essential role for mast cells as modulators of neutrophils influx in collagen-induced arthritis in the mouse. *Lab Invest* 91: 33–42, 2011
9. Hübner MP, Larson D, Torrero MN, Mueller E, Shi Y, Killoran KE, Mitre E: Anti-FcεR1 antibody injections activate basophils and mast cells and delay Type 1 diabetes onset in NOD mice. *Clin Immunol* 141: 205–217, 2011
10. Heimbach L, Li Z, Berkowitz P, Zhao M, Li N, Rubenstein DS, Diaz LA, Liu Z: The C5a receptor on mast cells is critical for the autoimmune skin-blistering disease bullous pemphigoid. *J Biol Chem* 286: 15003–15009, 2011
11. Seibold JR, Giorno RC, Claman HN: Dermal mast cell degranulation in systemic sclerosis. *Arthritis Rheum* 33: 1702–1709, 1990
12. Grimbaldeston MA, Nakae S, Kalesnikoff J, Tsai M, Galli SJ: Mast cell-derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. *Nat Immunol* 8: 1095–1104, 2007
13. Limón-Flores AY, Chacón-Salinas R, Ramos G, Ullrich SE: Mast cells mediate the immune suppression induced by dermal exposure to JP-8 jet fuel. *Toxicol Sci* 112: 144–152, 2009
14. Depinay N, Hacini F, Beghdadi W, Peronet R, Mécheri S: Mast cell-dependent down-regulation of antigen-specific immune responses by mosquito bites. *J Immunol* 176: 4141–4146, 2006
15. Lu LF, Lind EF, Gondek DC, Bennett KA, Gleeson MW, Pino-Lagos K, Scott ZA, Coyle AJ, Reed JL, Van Snick J, Strom TB, Zheng XX, Noelle RJ: Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* 442: 997–1002, 2006
16. Hochegger K, Siebenhaar F, Vielhauer V, Heininger D, Mayadas TN, Mayer G, Maurer M, Rosenkranz AR: Role of mast cells in experimental anti-glomerular basement membrane glomerulonephritis. *Eur J Immunol* 35: 3074–3082, 2005
17. Eller K, Wolf D, Huber JM, Metz M, Mayer G, McKenzie AN, Maurer M, Rosenkranz AR, Wolf AM: IL-9 production by regulatory T cells recruits mast cells that are essential for regulatory T cell-induced immune suppression. *J Immunol* 186: 83–91, 2011
18. Gan PY, Summers SA, Ooi JD, O'Sullivan KM, Tan DS, Muljadi RC, Odobasic D, Kitching AR, Holdsworth SR: Mast cells contribute to peripheral tolerance and attenuate autoimmune vasculitis. *J Am Soc Nephrol* 23: 1955–1966, 2012
19. Frossi B, Gri G, Tripodo C, Pucillo C: Exploring a regulatory role for mast cells: 'MCregs'? *Trends Immunol* 31: 97–102, 2010
20. Little MA, Pusey CD: Glomerulonephritis due to antineutrophil cytoplasm antibody-associated vasculitis: an update on approaches to management. *Nephrology (Carlton)* 10: 368–376, 2005
21. Davies DJ, Moran JE, Niall JF, Ryan GB: Segmental necrotising glomerulonephritis with antineutrophil antibody: possible arbovirus aetiology? *Br Med J (Clin Res Ed)* 285: 606, 1982
22. Falk RJ, Jennette JC: Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N Engl J Med* 318: 1651–1657, 1988
23. Cunningham MA, Huang XR, Dowling JP, Tipping PG, Holdsworth SR: Prominence of cell-mediated immunity effectors in "pauci-immune" glomerulonephritis. *J Am Soc Nephrol* 10: 499–506, 1999
24. Otsubo S, Nitta K, Uchida K, Yumura W, Nihei H: Mast cells and tubulointerstitial fibrosis in patients with ANCA-associated glomerulonephritis. *Clin Exp Nephrol* 7: 41–47, 2003
25. Holdsworth SR, Summers SA: Role of mast cells in progressive renal diseases. *J Am Soc Nephrol* 19: 2254–2261, 2008
26. Mazurek N, Berger G, Pecht I: A binding site on mast cells and basophils for the anti-allergic drug cromolyn. *Nature* 286: 722–723, 1980
27. Kuzemko, JA: Twenty years of sodium cromoglycate treatment: a short review. Respir Med 83 Suppl A: 11–14; discussion 15–16, 1989
28. Horan RF, Sheffer AL, Austen KF: Cromolyn sodium in the management of systemic mastocytosis. *J Allergy Clin Immunol* 85: 852–855, 1990
29. Theoharides TC, Kempuraj D, Tagen M, Conti P, Kalogeromitros D: Differential release of mast cell mediators and the pathogenesis of inflammation. *Immunol Rev* 217: 65–78, 2007
30. Gan PY, Steinmetz OM, Tan DS, O'Sullivan KM, Ooi JD, Iwakura Y, Kitching AR, Holdsworth SR: Th17 cells promote autoimmune anti-myeloperoxidase glomerulonephritis. *J Am Soc Nephrol* 21: 925–931, 2010
31. Ruth AJ, Kitching AR, Kwan RY, Odobasic D, Ooi JD, Timoshanko JR, Hickey MJ, Holdsworth SR: Anti-neutrophil cytoplasmic antibodies and effector CD4+ cells play nonredundant roles in anti-myeloperoxidase crescentic glomerulonephritis. *J Am Soc Nephrol* 17: 1940–1949, 2006
32. Kay AB, Walsh GM, Moqbel R, MacDonald AJ, Nagakura T, Carroll MP, Richerson HB: Disodium cromoglycate inhibits activation of human inflammatory cells in vitro. *J Allergy Clin Immunol* 80: 1–8, 1987
33. Kimata H, Yoshida A, Ishioka C, Mikawa H: Disodium cromoglycate enhances ongoing immunoglobulin production in vitro in human B cells. *Immunology* 73: 31–35, 1991
34. Timoshanko JR, Kitching AR, Semple TJ, Tipping PG, Holdsworth SR: A pathogenetic role for mast cells in experimental crescentic glomerulonephritis. *J Am Soc Nephrol* 17: 150–159, 2006
35. Scandiuzzi L, Beghdadi W, Daugas E, Abrink M, Tiwari N, Brochetta C, Claver J, Arouche N, Zang X, Pretolani M, Monteiro RC, Pejler G, Blank U: Mouse mast cell protease-4 deteriorates renal function by contributing to inflammation and fibrosis in immune complex-mediated glomerulonephritis. *J Immunol* 185: 624–633, 2010
36. Velden J, Paust HJ, Hoxha E, Turner JE, Steinmetz OM, Wolf G, Jabs WJ, Özcan F, Beige J, Heering PJ, Schröder S, Kneißler U, Disteldorf E, Mittrücker HW, Stahl RA, Helmchen U, Panzer U: Renal IL-17 expression in human ANCA-associated glomerulonephritis. *Am J Physiol Renal Physiol* 302: F1663–F1673, 2012
37. Supajatura V, Ushio H, Nakao A, Akira S, Okumura K, Ra C, Ogawa H: Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *J Clin Invest* 109: 1351–1359, 2002
38. Biggs L, Yu C, Fedoric B, Lopez AF, Galli SJ, Grimbaldeston MA: Evidence that vitamin D(3) promotes mast cell-dependent reduction of chronic UVB-induced skin pathology in mice. *J Exp Med* 207: 455–463, 2010
39. Oka T, Kalesnikoff J, Starkl P, Tsai M, Galli SJ: Evidence questioning cromolyn's effectiveness and selectivity as a 'mast cell stabilizer' in mice. *Lab Invest* 92: 1472–1482, 2012
40. de Vries VC, Wasiuk A, Bennett KA, Benson MJ, Elgueta R, Waldschmidt TJ, Noelle RJ: Mast cell degranulation breaks peripheral tolerance. *Am J Transplant* 9: 2270–2280, 2009
41. Summers SA, Gan PY, Dewage L, Ma FT, Ooi JD, O'Sullivan KM, Nikolic-Paterson DJ, Kitching AR, Holdsworth SR: Mast cell activation and degranulation promotes renal fibrosis in experimental unilateral ureteric obstruction. *Kidney Int* 82: 676–685, 2012
42. Summers SA, Chan J, Gan PY, Dewage L, Nozaki Y, Steinmetz OM, Nikolic-Paterson DJ, Kitching AR, Holdsworth SR: Mast cells mediate acute kidney injury through the production of TNF. *J Am Soc Nephrol* 22: 2226–2236, 2011
43. Ford SL, Polkinghorne KR, Longano A, Dowling J, Dayan S, Kerr PG, Holdsworth SR, Kitching AR, Summers SA: Histopathologic and clinical predictors of kidney outcomes in ANCA-associated vasculitis. *Am J Kidney Dis* 63: 227–235, 2014
44. Huang M, Pang X, Letourneau R, Boucher W, Theoharides TC: Acute stress induces cardiac mast cell activation and histamine release, effects

that are increased in Apolipoprotein E knockout mice. *Cardiovasc Res* 55: 150–160, 2002
45. Apostolopoulos J, Ooi JD, Odobasic D, Holdsworth SR, Kitching AR: The isolation and purification of biologically active recombinant and native autoantigens for the study of autoimmune disease. *J Immunol Methods* 308: 167–178, 2006
46. Kitching AR, Turner AL, Wilson GR, Semple T, Odobasic D, Timoshanko JR, O'Sullivan KM, Tipping PG, Takeda K, Akira S, Holdsworth SR: IL-12p40 and IL-18 in crescentic glomerulonephritis: IL-12p40 is the key Th1-defining cytokine chain, whereas IL-18 promotes local inflammation and leukocyte recruitment. *J Am Soc Nephrol* 16: 2023–2033, 2005

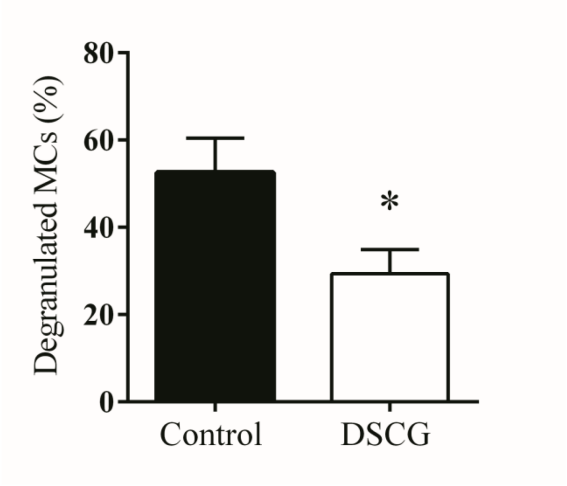
This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2014090906/-/DCSupplemental.

Supplementary Table 1. Comparisons of clinical, demographic and immunological data and indices of renal disease characteristics between total MPO-AAV patient population and subset population used for MC degranulation analysis.

		Total MPO-ANCA patient population	Subset for MC degranulation analysis
Demographic data			
	Patient Number	44	25
	Sex (f/m)	14/30	9/16
	Age[1] (years)	67±2	66±3
Renal involvement			
	Serum creatinine[1] (µmol/L)	395±53	355±55
	eGFR[1] (mL/min/1.73m^2)	21.6±2.4	23.8±3.5
	Proteinuria[1,2] (g/day)	1.8±0.4	1.2±4.2
	RBC[1,3] (urine cells/hpf)	547±682	474±122
Extra renal involvement			
	Lung or upper respiratory tract or skin or arthralagia presenting creatinine	15/44	10/25
Immunological data			
	ANCA (MPO) titer[1] (U/ml)	134±16	120±21
	CRP[4](mmol/L)	70±13.9	75±0.4

[1]Reported as mean ± SEM, [2]urinary total protein over 24 hr, [3]Red Blood Cell excretion, [4]C reactive protein on admission.

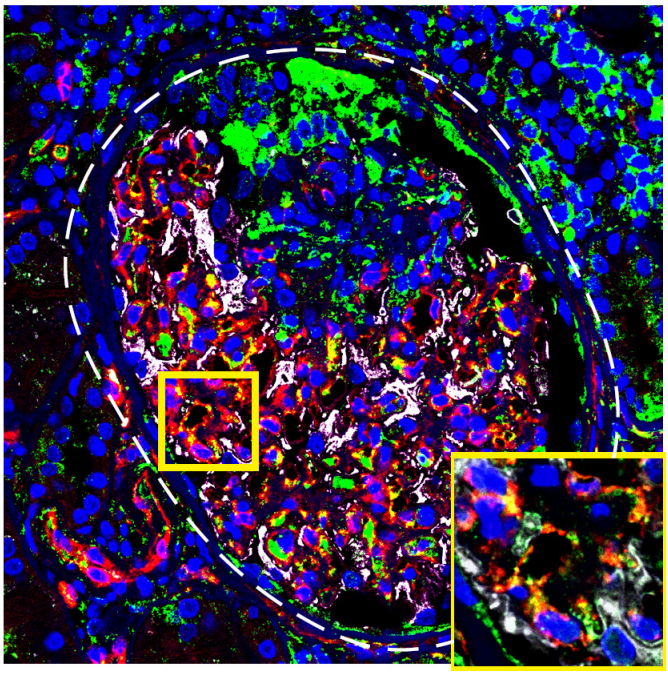
Supplementary Figure 1



Supplementary Figure 1: DSCG significantly reduces MC degranulation in a model of passive cutaneous anaphylaxis. C57BL/6 mice were sensitized subcutaneously (footpad) with 10ng α-DNP IgE and challenged 24hrs later with 100µg DNP-BSA intravenously. Two hours prior challenge (triggering MC degranulation), mice were injected intraperitoneally with either saline (control; n=10) or DSCG (n=10). Footpads were obtained and stained with toluidine blue to assess MC degranulation. Error bars represent mean ± SEM with statistical analysis by Mann-Whitney t-test *P<0.05.

CHAPTER 4:TLRS IN AAV

Intrarenal Toll-Like Receptor 2 and Toll-Like Receptor 4 Expression correlates with Kidney Injury in anti-Neutrophil Cytoplasmic Antibody Vasculitis



Intrarenal Toll-like receptor 4 and Toll-like receptor 2 expression correlates with injury in anti-neutrophil cytoplasmic antibody associated vasculitis

Kim M O'Sullivan MSc[1]
Sharon L Ford, MBBS[1]
Anthony Longano, MBBS[2 †]
A. Richard Kitching, MBChB, PhD[1, 3, 4 *]
Stephen R Holdsworth, MBBS, PhD[1, 3 *]

[1] Centre for Inflammatory Diseases, Monash University Department of Medicine, 246 Clayton Rd, Clayton, VIC 3168, Australia
[2]Department of Pathology, Monash Health, 246 Clayton Rd, Clayton VIC 3168, Australia
[3] Department of Nephrology, Monash Health, 246 Clayton Rd, Clayton VIC 3168, Australia
[4] Department of Paediatric Nephrology, Monash Health, 246 Clayton Rd, Clayton VIC 3168, Australia

[*] These authors equally contributed to this manuscript and are co-corresponding authors
[†] Current address: Department of Anatomical Pathology, Eastern Health, 5 Arnold St, Box Hill, VIC 3128, Australia

Running title: TLR2, TLR4 and TLR9 in AAV

Correspondence: Professor Stephen Holdsworth, Centre for Inflammatory Diseases, Monash University, Department of Medicine, Monash Medical Centre, 246 Clayton Road, Clayton, VIC 3168, Australia
Professor Richard Kitching Centre for Inflammatory Diseases, Monash University, Department of Medicine, Monash Medical Centre, 246 Clayton Road, Clayton, VIC 3168, Australia

Phone: [redacted] **Fax:** [redacted]
Email: [redacted]

Words: 4000 (4000), Abstract: 208 (1488 characters) References: 40

Disclosure: Nothing to disclose

ABSTRACT

In anti-neutrophil cytoplasmic antibody associated vasculitis (AAV), toll like receptors (TLRs) may be engaged by infection associated patterns and by endogenous danger signals, linking infection and innate inflammation with this autoimmune disease. This study examined intrarenal TLR2, TLR4 and TLR9 expression and renal injury in AAV, testing the hypothesis that increased TLR expression correlates with renal injury. Patients with AAV exhibited both glomerular and tubulointerstitial expression of TLR2, TLR4 and TLR9, with TLR4 being the most prominent in both compartments. Glomerular TLR4 expression correlated with glomerular segmental necrosis and cellular crescents, with TLR2 expression correlating with glomerular segmental necrosis. The extent and intensity of glomerular and tubulointerstitial TLR4 expression, and the intensity of glomerular TLR2 expression inversely correlated with the presenting eGFR. While myeloid cells within the kidney expressed TLR2, TLR4 and TLR9, TLR2 and TLR4 co-localized with endothelial cells and podocytes, whereas TLR9 was expressed predominantly by podocytes. The relevance of intrarenal TLR expression was further supported by the co-localization of TLRs with their endogenous ligands high mobility group box 1 and fibrinogen. Therefore, in AAV, the extent of intrarenal TLR4 and TLR2 expression and their correlation with renal injury indicates that TLR4, and to a lesser degree TLR2, may be potential therapeutic targets in this disease.

INTRODUCTION

Anti-neutrophil cytoplasmic antibody (ANCA) -associated vasculitis (AAV) often involves the kidney, with necrotising and crescentic glomerulonephritis (GN) that can result in progressive renal failure. Proteinase 3 (PR3) and myeloperoxidase (MPO) are the recognised dominant autoantigens in this disease. While the pathogenesis of AAV is not yet fully understood, ANCA have been shown to be pathogenic *in vitro* and *in vivo* (1, 2). Like many autoimmune diseases, AAV is likely to result from a combination of genetic susceptibility elements and multiple environmental influences, with infections being linked both to disease onset and to relapse (3-5). Toll-like receptors are innate pattern recognition receptors (PRRs), located on the cell surface or intracellularly within endosomes (6) that recognise pathogen associated molecular patterns (PAMPS) on microbes, and also endogenous danger associated molecular patterns (DAMPs). While TLRs play a key role in host defence, they may also link infection, danger and autoimmunity (6).

Several lines of evidence implicate TLRs in the pathogenesis of AAV. Seasonal variations in the incidence of AAV and relapses, with a peak in winter suggest an association with microbial infection (3), with prophylactic antibiotic therapy having the capacity to avert relapses in some patients (7). *In vitro* studies using human ANCA and leukocytes have demonstrated the capacity of TLRs to prime neutrophils and to promote ANCA production (8, 9), while in mouse models of anti-MPO GN, TLR engagement is pathogenic (10, 11). In the current studies, we chose TLR2, TLR4 and TLR9 due to their relevance in studies of peripheral leukocytes in human AAV and their pathogenicity in experimental anti-MPO GN (8-10, 12, 13). TLR2 and TLR4 are expressed on the cell surface and recognise a range of PAMPs. These include, for TLR2, recognising lipotechoic acids, peptidoglycans, zymosan and lipomannan (6) and for TLR4 lipopolysaccharides (LPS) and viral glycoproteins (14). In contrast, TLR9 is intracellularly located within endosomes, where it recognises hypo-methylated CpG motifs derived from microbial and from self DNA (15, 16). TLRs have a

number of endogenous ligands, including extracellular matrix proteins and heat shock proteins, and self- and altered self-nucleic acids. The nuclear protein high mobility group box 1 (HMGB1) can be released extracellularly (including from necrotic cells) and can bind to TLR2, TLR4 and TLR9 to induce the release of pro-inflammatory cytokines (17). Fibrinogen is another well-described endogenous ligand that binds to both TLR4 and TLR2. When recognized by TLR4, fibrinogen induces chemokine production by macrophages (14), while TLR9 endogenous ligands include endogenous hypomethylated DNA, self DNA and HMGB1 (15, 18).

TLR2, TLR4 and TLR9 have each been linked to AAV in human and experimental studies. AAV patients exhibit aberrant TLR2 and TLR9 expression on circulating monocytes and TLR2 and TLR9 ligands direct autoimmunity to MPO in a mouse model by inducing Th17 and Th1 cells respectively (10, 13). Extracellular DNA released as histones and chromatin via neutrophil extracellular traps (NETs) that are found intrarenally in AAV, activates TLR9 (16, 19-21). Neutrophils from AAV patients stimulated with TLR4 and TLR9 ligands release MPO (8), human glomerular cells express TLR4 and secrete CXCL8 (IL-8) after TLR4 ligand stimulation *in vitro* (12) and *in vivo* studies in murine MPO-ANCA induced GN demonstrate the functional relevance of this expression (12).

While several human AAV studies have examined the expression of TLRs in circulating leukocytes (8, 13, 22), only one has assessed the distribution of TLR2, TLR4 and TLR9 in kidneys from patients with AAV (23). Somewhat counterintuitively, Wang et al. found that intrarenal TLR4 (and to some degree TLR2) expression negatively correlated with histological and functional renal injury, while significant intrarenal TLR9 expression was not detected. The current studies aimed to characterize the cellular distribution and extent of TLR2, TLR4 and TLR9 expression in human ANCA-associated GN, to establish whether intrarenal TLRs co-localised with the endogenous ligands HMGB1 and fibrinogen, and to determine whether TLR expression correlated with histological and functional injury.

RESULTS

Patient characteristics

A total of 38 renal biopsies from patients with a first presentation of AAV were used in this study (30 MPO-AAV and 8 PR3-AAV, Table 1). Both cohorts presented with significant renal disease, with an estimated glomerular filtration rate (eGFR) of 21±3 ml/min/1.73^2 in MPO-AAV and 13±3 ml/min/1.73^2 in PR3-AAV patients (*P*=0.253).

TLR4 is more prominently expressed in glomeruli compared to TLR2 and TLR9

To examine the relative expression of TLR2, TLR4 and TLR9, serial sections from AAV patient biopsies were probed with antibodies (Ab) for each TLR. Two methods were used, in separate analyses, to control for differences that may exist between the Ab used for each TLR. Both the mean intensity of staining (Figure 1a) and the proportion of each glomerular cross section (GCS) staining positive (Figure 1b) were assessed for each TLR, using the Image J software programme. TLR2, TLR4 and TLR9 were each present in glomeruli of patients with AAV (Figure 1c). However, the predominant TLR expressed within glomeruli was TLR4, with both the mean signal intensity (Figure 1a) and extent of staining (Figure 1b) being approximately six times that of TLR2 and threefold that of TLR9 (Figure 1a-c). Isotype controls demonstrating minimal staining (Supplementary Figure 1a) served both to assist in determining the optimal dilution of the primary Ab, and to set a threshold for the background for analyses. To demonstrate the specificity and fidelity of the anti-TLR9 Ab, we validated the presence of TLR9 using three different Ab raised in three different species (Supplementary Figure 1b).

In AAV, TLR2 and TLR4 associate with both endothelial cells and podocytes, whereas TLR9 is more strongly podocyte associated

To determine whether TLRs were differentially associated with glomerular endothelial cells or podocytes, serial sections stained for each TLR were then probed for CD34 (endothelial

cells) and nephrin (podocytes). TLR4 expression was frequently associated with both endothelial cells and podocytes (Figure 2). Expression was more prominent in areas where CD34 and nephrin had been shed (a sign of cellular damage and/or progression), and in cellular crescents (19), but less prominent in more preserved glomeruli. Like TLR4, TLR2 was also present on both endothelial cells and podocytes (Figure 2) and was more prominent in segmental areas lacking CD34 or nephrin staining, but TLR2 was also found in glomeruli with minimal histological pathology (intact CD34 and nephrin staining, and without crescent formation). TLR9, although expressed on endothelial cells was more prominent on podocytes and was also observed in areas of segmental necrosis and cellular crescents (Figure 2).

Glomerular TLR4 expression correlates with severe glomerular lesions and inversely correlates with presenting eGFR

To determine the relationship between glomerular TLR expression and glomerular injury, correlations between the percentage of glomeruli affected with acute and severe glomerular lesions were examined as a continuous variable (Figure 3, Table 2). TLR4 expression reflected histological and functional injury. The intensity and extent of glomerular TLR4 expression correlated with glomeruli exhibiting both segmental necrosis and cellular crescent formation (Figure 3a and b). Concordant with these findings, a significant negative correlation with presenting eGFR was observed for both the intensity and the proportion of glomerular cross sections positive for TLR4 staining (Figure 3c and d). TLR2 expression also correlated with some parameters of disease severity (Table 2 and Supplementary Table 1). Glomerular expression of TLR2 correlated with the proportion of glomeruli affected by segmental necrosis, with both the intensity of staining and relative proportion of the glomerulus. The intensity of TLR2 staining inversely correlated with eGFR. TLR9 expression did not correlate with glomerular lesions or presenting eGFR (Table 2 and Supplementary Table 1). Glomerular TLR expression was evaluated in the context of the Berden classification of glomerular histopathology in AAV (24) that treats lesion type as a

categorical variable. In these analyses, the relatively low numbers of biopsies in each category did not permit conclusive statistical results (Supplementary Figure 2).

TLR2, TLR4 and TLR9 are expressed by glomerular infiltrating cells

Based on studies implicating monocyte/macrophage and neutrophil TLRs in AAV, we sought evidence of TLR expression *in vivo* on intraglomerular infiltrating leukocytes. A subset of MPO-AAV biopsies (*n*=10) were stained for TLR expression on myeloid cells, using CD68 as a marker for macrophages and neutrophil elastase (NE) as a marker for neutrophils (Figure 4). TLR expression was prevalent in glomerular macrophages and neutrophils: TLR2 staining was evident in 41% of macrophages and 31% of neutrophils, TLR4 in 54% of macrophages and 58% of neutrophils, and TLR9 in 53% of macrophages and 81% of neutrophils. Thus, although there were infiltrating cells positive for TLRs within glomeruli, most TLR2, TLR4 and TLR9 expression related to intrinsic glomerular cells (endothelial cells and podocytes, Figures 2 and 4).

HMGB1 and fibrinogen, endogenous TLR ligands, co-localise with glomerular TLRs

As the endogenous TLR ligands, HMGB1 and fibrinogen have been implicated in AAV (25, 26), we sought evidence of co-localization of these endogenous ligands with TLRs. HMGB1, a ligand for TLR2, TLR4 and TLR9, was present in most affected glomeruli (Figure 5a) and co-localised most prominently with TLR4, and to a lesser degree with TLR2 and TLR9. Thus, although HMGB1 is a ligand for each of TLR2, TLR4 and TLR9, TLR4 may act as the dominant TLR for HMGB1. Fibrinogen, known to be present in segmental lesions and in glomerular crescents in AAV, and a known ligand for TLR2 and TLR4, was also present in diseased glomeruli (Figure 5b). As for HMGB1, co-localization was most prominent in the case of TLR4. In some instances HMGB1, fibrinogen and a TLR could be co-localized in the same cell (Supplementary Figure 3). Neither HMGB1 nor fibrinogen was present in

biopsies of patients with the non-proliferative glomerular lesion, minimal change disease (data not shown).

Tubulointerstitial TLR4 expression is prominent and correlates inversely with renal function

TLR2, TLR4 and TLR9 expression was also assessed in the tubulointerstitium in AAV. Similar to the approach employed in assessing glomeruli, TLR staining in serial sections (Figure 6a) was assessed both for mean fluorescence intensity (Figure 6b) and the percentage area of tubulointerstitial staining in 10 high powered fields (Figure 6c). As for glomerular TLR expression, TLR4 was overall the most highly expressed TLR. While there was no correlation between presenting eGFR and tubulointerstitial TLR2 or TLR9 (Supplementary Table 1), there was, similar to glomerular TLR4, an inverse correlation between both TLR4 staining intensity and TLR4 positive tubulointerstitial area and the presenting eGFR (Figure 6d, e).

Intrarenal TLR expression patterns are similar in MPO-AAV and PR3-AAV

As MPO-AAV and PR3-AAV have differing genetic associations and may be distinct diseases with syndromic overlap (4), we analysed TLR staining according to ANCA specificity (MPO-AAV, *n*=30; PR3-AAV *n*=8, Table 1). The same TLR4 dominant pattern of staining was observed in both MPO-AAV and PR3-AAV groups, with TLR4 being the dominant TLR within glomeruli and the tubulointerstitium (Table 3).

Intrarenal TLR expression in AAV compared with minimal change disease and lupus nephritis ISN/RPS Class IV

Renal biopsies were assessed from subjects with control diseases, to determine whether TLR expression differed between different renal diseases (Figure 7, Supplementary Figure 4). The control diseases assessed were minimal change disease (*n*=8) as a form of non-proliferative GN with preserved renal structure, and ISN/RPS (International Society of

Nephrology/Renal Pathology Society) Class IV lupus nephritis (LN, *n*=8, with the majority classed as active, global). Intrarenal TLR2 expression in AAV was significantly more than in either minimal change disease or Class IV LN (Figure 7a, d, g, j). In both glomerular and tubulointerstitial compartments, TLR4 expression in AAV was more than in minimal change disease but similar to that seen in Class IV LN (Figure 7b, e, h, k). Similar findings were evident for TLR9, though there was a trend in most expression parameters towards increased expression in AAV (Figure 7c, f, i, l).

DISCUSSION

These studies demonstrate substantial intrarenal expression of TLR2, TLR4 and TLR9, in patients with AAV. The major findings of the studies are 1) the dominance of TLR4, its inverse correlation with presenting eGFR and correlation with severe glomerular injury 2) the presence, albeit at a lower level, of TLR2 that was also associated with injury, 3) the clear presence of intrarenal TLR9 without a significant association with injury, 4) the co-localization of the TLR ligands HMGB1 and fibrinogen with TLRs, 5) similar patterns in both MPO-AAV and PR3-AAV and 6) in class IV LN, the presence of similar degrees of intrarenal TLR4 and TLR9, but a paucity of TLR2 compared with AAV.

Compared to TLR2 and TLR9, TLR4 was more extensively and more strongly expressed in kidneys of AAV patients. For both glomerular and tubulointerstitial TLR expression, two analyses were performed. After determining the optimal titration of the three detecting antibodies, both intensity of staining and the proportion of each glomerulus or tubulointerstitial field that was TLR positive were assessed. Results from these two parameters were largely concordant throughout the study. TLR4 expression in glomeruli correlated with proportions of glomeruli exhibiting both segmental necrosis and cellular crescent formation, and inversely correlated with eGFR at biopsy. TLR4 was expressed relatively prominently in both endothelial cells and podocytes, consistent with findings in experimental MPO-ANCA associated GN (12). TLR4 was also prominent in areas within glomeruli lacking endothelial and podocyte markers: these markers are lost in significant injury (19). Human glomerular endothelial cells and podocytes express the prototypic neutrophil chemoattractant CXCL8 after TLR4 engagement, linking TLR4 expression in AAV to a key mechanism of injury (12, 27). TLR2 was also present in AAV patients' kidneys, to a lesser degree than TLR4, but significantly more than in MCD or class IV LN. TLR2 expression correlated with the proportion of glomeruli exhibiting acute segmental lesions. The significant correlations of the percentage of glomeruli affected by segmental necrosis

and/or cellular crescents with TLR2 and TLR4 suggests that these TLRs relate to the most active of glomerular lesions. Although TLR9 did not correlate with renal injury, the widespread intrarenal expression of TLR9 does not exclude a local role for TLR in amplifying inflammatory responses.

TLRs can influence AAV at multiple stages of disease pathogenesis. There is evidence from human studies and animal models of TLR involvement in loss of tolerance, in influencing the direction of autoimmunity, in the priming of neutrophils (and potentially monocytes) and in triggering disease (8-13). The presence of TLRs within the kidney in AAV with, for TLR4 and TLR2, expression being linked to severe acute lesions, implies a further role for local TLRs in promoting tissue inflammation in AAV. While a significant proportion of neutrophils and macrophages expressed TLRs, which is consistent with TLRs contributing to their inflammatory phenotype, the majority of TLR expression was on and within intrinsic renal cells. Mechanistically, intrinsic kidneys cells express TLRs and respond to TLR ligands by producing a variety of pro-inflammatory mediators. Functionally, in experimental AAV, intrinsic cell TLR expression is important in MPO-ANCA induced neutrophil localization and injury, and in models of other renal diseases intrinsic kidney cell expression of TLR2, TLR4 and TLR9 is also important (28-32). Thus, when combined with *in vitro* and *in vivo* functional studies, the current studies implicate intrarenal expression of TLRs in promoting leukocyte recruitment, local inflammation and perpetuating local renal tissue injury.

Wang et al examined TLR expression in AAV kidneys and demonstrated minimal intrarenal TLR9. They reported that TLR4 was inversely associated with histological injury and the presenting serum creatinine, with TLR2 being inversely associated with serum creatinine. The premise that proinflammatory TLRs would be less prominent in more severe injury is counterintuitive as the correlation of renal TLR expression with less inflammation and better kidney function would imply that TLR2 and TLR4 play regulatory roles in AAV, a scenario not substantiated by *in vitro* and *in vivo* functional studies. In these two critical areas, our

studies demonstrate not only the opposite, but the more logical and anticipated result. We were able to prove substantial intrarenal TLR9 expression using three different primary anti-TLR9 Abs. However, while TLR9 is definitely expressed in kidneys in AAV and may have a pathogenic role in disease, there was no clear correlation between intrarenal expression and injury. In the case of TLR2 and TLR4 we found that these proinflammatory molecules did correlate with histological and functional injury. Our current results are concordant with experimental studies and support strategies aimed at limiting TLR's functions and their consequences.

Although it is possible that in patients, the ligands for these intrarenal TLRs are PAMPs derived from microbial triggers, it is more plausible that in AAV endogenous DAMPs serve as TLR ligands within the kidney. In the current studies we have shown that HMGB1 and fibrinogen, DAMPs are present in glomeruli of patients with AAV and co-localize with TLRs. HMGB1, a known ligand for TLR2, TL4 and TLR9 (15, 33), is released from both activated and necrotic cells. HMGB1 can mediate glomerular cell proliferation via TLR2 (34), signal through TLR4 to promote inflammation (33) and promote TLR9 activation by enhancing the recognition of CpG-ODN (15). While not all studies are concordant as to whether serum HMGB1 levels reflect disease activity, circulating HMGB1 levels are elevated in AAV and also may prime neutrophils (35-37). Intraglomerular fibrinogen has long been known to be present in AAV and other forms of rapidly progressive GN (26, 38), as a product of activation of the coagulation system. However, fibrinogen is also a ligand for both TLR2 and TLR4, where it can promote chemokine expression and leukocyte recruitment (32). NETs are present *in situ* within glomeruli from patients with AAV (19, 20). In addition to other known endogenous TLR ligands, histones released through NETosis or dying intrinsic kidney cells act as DAMPs for TLR2, TLR4 and TLR9 and induce proinflammatory cytokine production (39). In particular, TLR9 senses extracellular DNA from necrotic and NETotic cells, which may amplify neutrophil recruitment and cytokine release (16, 18, 40).

While there are differences between MPO-AAV and PR3-AAV as diseases (4), the current studies do not demonstrate differences in intrarenal TLR expression between patients who have lost tolerance to either MPO or PR3 (albeit with relatively low numbers of PR3-AAV subjects). The extent of TLR2, TLR4 and TLR9 expression in AAV was compared to two other glomerular diseases. In MCD, where although patients are proteinuric, glomerular and tubulointerstitial structure is preserved (aside from podocyte foot process fusion), TLRs were expressed only at a low level. The degree of TLR4 and TLR9 expression was similar in AAV and Class IV LN, but TLR2 was significantly increased in AAV. This relatively selective increase in TLR2 expression may imply a pathogenic role for TLR2 in AAV.

Accumulating evidence from human observations and *in vitro* studies, together with animal models of AAV suggest that TLRs play a major role in this disease. The current studies comprehensively demonstrate the pattern and extent of intrarenal TLR2, TLR4 and TLR9 expression in AAV. The key finding was the dominance of TLR4, which not only was the most prominently expressed TLR in this disease, but also correlated with important indices of histological and functional injury. TLR2 expression also correlated with some indices of severity, while TLR9, though definitively present in the kidneys of patients with AAV, did not clearly relate to the degree of renal injury. Based on these findings, TLR4 and potentially TLR2 may be therapeutic targets in AAV.

MATERIALS AND METHODS

Patient cohort and biopsy specimens

A total of 38 patients presenting with AAV and GN (30 MPO-AAV and 8 PR3-AAV) were included in this study. Patients from this cohort were a subset of the cohort described in a previous report (19). For disease comparisons, eight biopsies from patients with minimal change disease (MCD), and eight with LN ISN/RPS Class IV served as control tissue (two biopsies were Class IV-G (A), three Class IV-G (A/C), one Class IV-S (A), one Class IV S A/C, and one Class IV/V-S (A/C)). Biopsies were collected between 2001 and 2013 at Monash Medical Centre, Clayton, Victoria, Australia. Three independent pathologists examined and classified the AAV biopsies according to the classification scheme of Berden et al. (24). Of the MPO-AAV cohort, 23% were focal, 37% crescentic, 30% mixed and 10% exhibited a sclerotic pattern. The patterns of the PR3-AAV cohort were 25% focal, 25% crescentic, 40% mixed and 13% sclerotic. One pathologist scored the biopsies according to the proportions of normal glomeruli and those exhibiting segmental necrosis, cellular crescents or both segmental necrosis and cellular crescents. Clinical and laboratory data were obtained from hospital records. ANCA testing was performed by both indirect immunofluorescence and enzyme linked immunosorbent assay at presentation.

Confocal microscopy

Sections (2 μm) of formalin-fixed, paraffin-embedded tissue specimens were mounted on Superfrost Plus slides (Menzel ,Braunschweig, Germany), dewaxed, rehydrated, and pre-treated with antigen retrieval solution Tris-EDTA pH 9 in a pressure cooker for 10 min, blocked (30 min) in 10% chicken sera (or donkey serum) in 5% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) and probed with Ab against TLR2, TLR4, TLR9, CD34 (endothelial cell marker), nephrin (podocyte marker), neutrophil elastase (neutrophil marker), CD68 (macrophage marker), HMGB1 and fibrinogen (Table 4) in 1% bovine serum albumin/phosphate-buffered saline for 16 h (4°C). Fluorescent detection was achieved by

incubation with either Alexa Fluor 594-conjugated chicken anti-rabbit IgG ,Alexa Fluor 488-conjugated chicken anti-mouse IgG, Alexa Fluor 647 conjugated donkey anti sheep IgG or Alexa fluor 488-conjugated chicken anti goat IgG (all from Molecular Probes 1:200, 40 min, room temperature). To quench tissue auto-fluorescence, slides were incubated with Sudan Black (Sigma-Aldrich St Louis, MO, 0.1% in 70% ethanol, 30 min), washed in phosphate-buffered saline, and cover slipped in DAPI prolong gold (Molecular Probes). Fluorescent images were acquired using a NIKON C1 confocal laser scanning head attached to Nikon Ti-E inverted microscope (Coherent Scientific, SA, Australia); 405, 488, and 561 nm 647nm lasers were used to specifically excite DAPI, Alexa 488, Alexa 594, and Alexa 647. Single plane 512 × 512 × 12 bit images with 4 line averaging were captured in a line sequential manner, and used for analysis.

Analysis of TLR expression

TLR expression was measured using Image J (NIH, Bethesda, USA). Relative levels of TLR expression were quantitated as a product between TLR signal area multiplied by the TLR signal intensity. Analyses utilised the following formula applied to every single pixel in an image Signal $(TLR)_{Total}$ –Signal $(TLR)_{background}$ = Signal(TLR) expressed in AU. For the measurement of glomerular TLR signal a Region of Interest (ROI) was drawn using the pencil tool and the pixels were processed using the formula above. Tubulointerstitial TLR expression was assessed in 10 high powered fields (400x), measured in Image J, and expressed as the mean per high powered field, in AU and % area.

Statistical Analyses

Statistical analysis for non-parametric data was performed using Mann-Whitney *U* Test for 2 groups and the Kruskal-Wallis test for three or more groups. Correlation between two independent continuous variables was analysed using the non-parametric Spearman's rho, Significance was set at $P<0.05$, using SPSS (IBM, Armonk, NY) or GraphPad (La Jolla, CA) statistical software.

REFERENCES

1. Xiao H, Heeringa P, Hu P, Liu Z, Zhao M, Aratani Y, et al. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. J Clin Invest. 2002;110:955-63.

2. Rarok AA, Limburg PC, Kallenberg CG. Neutrophil-activating potential of antineutrophil cytoplasm autoantibodies. J Leukoc Biol. 2003;74:3-15.

3. Tidman M, Olander R, Svalander C, Danielsson D. Patients hospitalized because of small vessel vasculitides with renal involvement in the period 1975-95: organ involvement, anti-neutrophil cytoplasmic antibodies patterns, seasonal attack rates and fluctuation of annual frequencies. J Intern Med. 1998;244:133-41.

4. Lyons PA, Rayner TF, Trivedi S, Holle JU, Watts RA, Jayne DR, et al. Genetically distinct subsets within ANCA-associated vasculitis. N Engl J Med. 2012;367:214-23

5. Stegeman CA, Tervaert JW, Sluiter WJ, Manson WL, de Jong PE, Kallenberg CG. Association of chronic nasal carriage of Staphylococcus aureus and higher relapse rates in Wegener granulomatosis. Ann Intern Med. 1994;120:12-7.

6. Jimenez-Dalmaroni MJ, Gerswhin ME, Adamopoulos IE. The critical role of toll-like receptors--From microbial recognition to autoimmunity: A comprehensive review. Autoimmun Rev. 2016;15:1-8.

7. Stegeman CA, Tervaert JW, de Jong PE, Kallenberg CG. Trimethoprim-sulfamethoxazole (co-trimoxazole) for the prevention of relapses of Wegener's granulomatosis. Dutch Co-Trimoxazole Wegener Study Group. N Engl J Med. 1996;335:16-20.

8. Holle JU, Windmoller M, Lange C, Gross WL, Herlyn K, Csernok E. Toll-like receptor TLR2 and TLR9 ligation triggers neutrophil activation in granulomatosis with polyangiitis. Rheumatology (Oxford). 2013;52:1183-9.

9. Hurtado PR, Jeffs L, Nitschke J, Patel M, Sarvestani G, Cassidy J, et al. CpG oligodeoxynucleotide stimulates production of anti-neutrophil cytoplasmic antibodies in ANCA associated vasculitis. BMC Immunol. 2008;9:34.

10. Summers SA, Steinmetz OM, Gan PY, Ooi JD, Odobasic D, Kitching AR, et al. Toll-like receptor 2 induces Th17 myeloperoxidase autoimmunity while Toll-like receptor 9 drives Th1 autoimmunity in murine vasculitis. Arthritis & Rheumatism. 2011;63:1124-35.

11. Huugen D, Xiao H, van Esch A, Falk RJ, Peutz-Kootsstra CJ, Buurman WA, Tevaert JW, Jennette JC, Heeringa P. Aggravation of anti-myeloperosidase antibody-induced glomerulonpehritis by bacterial lipopolysaccharide:role of tumor necrosis factor-alpha.Am J Pathol 2005 Jul;167:47-58

12. Summers SA, van der Veen BS, O'Sullivan KM, Gan PY, Ooi JD, Heeringa P, et al. Intrinsic renal cell and leukocyte-derived TLR4 aggravate experimental anti-MPO glomerulonephritis. Kidney Int. 2010;78:1263-74.

13. Tadema H, Abdulahad WH, Stegeman CA, Kallenberg CG, Heeringa P. Increased expression of Toll-like receptors by monocytes and natural killer cells in ANCA-associated vasculitis. PLoS One. 2011;6:e24315.

14. Richez C, Blanco P, Rifkin I, Moreau JF, Schaeverbeke T. Role for toll-like receptors in autoimmune disease: the example of systemic lupus erythematosus. Joint Bone Spine. 2011;78:124-30..

15. Ivanov S, Dragoi AM, Wang X, Dallacosta C, Louten J, Musco G, et al. A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. Blood. 2007;110:1970-81.

16. Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, et al. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. Nature. 2007;449:564-9.

17. Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, et al. High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J Physiol Cell Physiol. 2006;290:C917-24.

18. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature. 2002;416:603-7.

19. O'Sullivan KM, Lo CY, Summers SA, Elgass KD, McMillan PJ, Longano A, et al. Renal participation of myeloperoxidase in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. Kidney Int. 2015;88:1030-46.

20. Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, Werb Z, et al. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med. 2009;15:623-5.

21. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. Sci Transl Med. 2011;3:73ra19.

22. Lai Y, Xue C, Liao Y, Huang L, Peng Q, Huang B, et al. Differential Expression of Toll-Like Receptor Signaling Pathway Is Associated with Microscopic Polyangiitis in Peripheral Blood Neutrophils. Immunol Invest. 2017;46:375-84.

23. Wang H, Gou SJ, Zhao MH, Chen M. The expression of Toll-like receptors 2, 4 and 9 in kidneys of patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis. Clin Exp Immunol. 2014;177:603-10.

24. Berden AE, Ferrario F, Hagen EC, Jayne DR, Jennette JC, Joh K, et al. Histopathologic classification of ANCA-associated glomerulonephritis. J Am Soc Nephrol. 2010;21:1628-36.

25. Ma YH, Ma TT, Wang C, Wang H, Chang DY, Chen M, et al. High-mobility group box 1 potentiates antineutrophil cytoplasmic antibody-inducing neutrophil extracellular traps formation. Arthritis Res Ther. 2016;18:2.

26. Cunningham MA, Huang XR, Dowling JP, Tipping PG, Holdsworth SR. Prominence of cell-mediated immunity effectors in "pauci-immune" glomerulonephritis. J Am Soc Nephrol. 1999;10:499-506.

27. Cockwell P,, Brooks CJ, Adu D, Savage CO. Interleukin-8: A pathogenic role in anti neutrophil cytoplasmic autoantibody-associated glomerulonephritis. Kindey Int. 1999 Mar:55:852-63.

28. Brown HJ, Lock HR, Sacks SH, Robson MG. TLR2 stimulation of intrinsic renal cells in the induction of immune-mediated glomerulonephritis. J Immunol. 2006;177:1925-31.

29. Wolfs TG, Buurman WA, van Schadewijk A, de Vries B, Daemen MA, Hiemstra PS, et al. In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation. J Immunol. 2002;168:1286-93.

30. Banas MC, Banas B, Hudkins KL, Wietecha TA, Iyoda M, Bock E, et al. TLR4 links podocytes with the innate immune system to mediate glomerular injury. J Am Soc Nephrol. 2008;19:704-13.

31. Verzola D, Cappuccino L, D'Amato E, Villaggio B, Gianiorio F, Mij M, et al. Enhanced glomerular Toll-like receptor 4 expression and signaling in patients with type 2 diabetic nephropathy and microalbuminuria. Kidney Int. 2014;86:1229-43.

32. Motojima M, Matsusaka T, Kon V, Ichikawa I. Fibrinogen that appears in Bowman's space of proteinuric kidneys in vivo activates podocyte Toll-like receptors 2 and 4 in vitro. Nephron Exp Nephrol. 2010;114:e39-47.

33. Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, et al. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. Shock. 2006;26:174-9.

34. Feng XJ, Wu C, Yang GF, Liu QJ, Liu JX, Hao J, et al. TLR2 Plays a Critical Role in HMGB1-Induced Glomeruli Cell Proliferation Through the FoxO1 Signaling Pathway in Lupus Nephritis. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 2016;36:258-66.

35. de Souza A, Westra J, Bijzet J, Limburg PC, Stegeman CA, Bijl M, et al. Is serum HMGB1 a biomarker in ANCA-associated vasculitis? Arthritis Res Ther. 2013;15:R104.

36. Wibisono D, Csernok E, Lamprecht P, Holle JU, Gross WL, Moosig F. Serum HMGB1 levels are increased in active Wegener's granulomatosis and differentiate between active forms of ANCA-associated vasculitis. Ann Rheum Dis. 2010;69:1888-9.

37. Wang C, Wang H, Chang DY, Hao J, Zhao MH, Chen M. High mobility group box 1 contributes to anti-neutrophil cytoplasmic antibody-induced neutrophils activation through receptor for advanced glycation end products (RAGE) and Toll-like receptor 4. Arthritis Res Ther. 2015;17:64.

38. Neale TJ, Tipping PG, Carson SD, Holdsworth SR. Participation of cell-mediated immunity in deposition of fibrin in glomerulonephritis. Lancet. 1988;2:421-4.

39. Allam R, Scherbaum CR, Darisipudi MN, Mulay SR, Hagele H, Lichtnekert J, et al. Histones from dying renal cells aggravate kidney injury via TLR2 and TLR4. J Am Soc Nephrol. 2012;23:1375-88.

40. El Kebir D, Jozsef L, Pan W, Wang L, Filep JG. Bacterial DNA activates endothelial cells and promotes neutrophil adherence through TLR9 signaling. J Immunol. 2009;182:4386-94.

Table 1. Clinical and histological features of patients with ANCA associated glomerulonephritis

Patient Characteristics	**All AAV**	**MPO-AAV**	**PR3-AAV**
Patient number	38	30	8
Age at biopsy	65±3	66±3	59±3
Sex (F/M)	17/29	9/21	4/4
Number of glomeruli	19±1	21±2	14±4
Laboratory Values			
ANCA titre (U/ml)	n/a	128±17	142±29
eGFR (ml/min/1.73 m^2)	19±3	21±3	13±3
ESR (mm/h)	80±7	72±8	101±7
CRP (mmol/l)	94±20	77±22	150±49
UTP-24h (g/day)	1.72±0.4	1.60±0.6 (*n*=13)	1.88±1.0 (*n*=5)
Red blood cells (cells/HPF)	698±131	636±146	899±301
Extrarenal involvement	13/38	10/30	3/8
Biopsy histology			
% Normal glomeruli	34±5	31±5	46±10
% Cellular crescents	27±2	26±5	30±8
% Segmental necrosis	11±4	12±3	8.±3
% Cellular crescents and segmental necrosis	7±1	7±2	4±3

Table 1. Abbreviations, ANCA, anti neutrophil cytoplasmic antibody; CRP, C reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; MPO, myeloperoxidase; n/a not applicable; PR3, proteinase 3; UTP, 24 hour urinary protein excretion.

Table 2. Correlation of glomerular expression of TLR2, TLR4 and TLR9 with glomeruli injury and renal function

	TLR2 [1]Intensity	TLR2 % Area	TLR4 Intensity	TLR4 % Area	TLR9 Intensity	TLR9 % Area
Spearman's	r	r	r	r	r	r
% Normal glomeruli	-0.115	-0.155	-0.254	0.141	-0.239	-0.278
% Cellular crescents	0.287	0.160	0.374*	0.229	-0.059	0.032
% Segmental necrosis	0.354*	0.343*	0.453**	0.200	0.280	0.174
% Segmental necrosis and cellular crescents	0.258	0.241	0.572**	0.420*	0.363	0.206

* Correlation is significant at 0.05 level (two tailed)
** Correlation is significant at 0.01 level (two tailed)
[1] Intensity refers to the intensity of fluorescent staining measured in Image J
Abbreviations, TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; TLR9, Toll-like receptor 9.

Table 3. Comparison of intrarenal TLR expression in MPO-AAV and PR3-AAV patients

	Glomerulus		**Tubulointerstitium**	
	Intensity (AU/GCS)	% Area (AU/GCS)	Intensity (AU/HPF)	% Area (AU/HPF)
MPO-AAV (*n*=30)				
TLR2[a]	1.6±1.2	4.8±2.7	3.8±1.5	9.3±2.5
TLR4[b]	9.3±2.5	26.9±4.4	13.6±2.5	27.4±4.1
TLR9[c]	3.9±0.8	14.4±2.6	10.5±2.1	18.2±3.0
***Significance*[1]**	a-b****, b-c****, a-c^{ns}	a-b****, b-c*, a-c****	a-b***,b-c**, a-c**	a-b***,b-c^{ns}, a-c^{ns}
PR3-AAV (*n*=8)				
TLR2[d]	2.2±1.1	9.0±2.9	5.6±1.3	11.5±3.0
TLR4[e]	15.7±2.3	51.0±6.1	26±5.6	41.7±4.6
TLR9[f]	3.9±1.3	12.9±3.8	3.9±1.3	16.0±4.5
***Significance*[1]**	a-b***,b-c***, a-c^{ns}	a-b***,b-c***, a-c^{ns}	a-b*,b-c*,a-c^{ns}	a-b***,b-c*,a-c^{ns}

Abbreviations: AU, arbitrary units; GCS, glomerular cross section; HPF, high power field; MPO-AAV, myeloperoxidase-anti neutrophil cytoplasmic antibody associated vasculitis; ns, not significant; PR3, proteinase 3; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; TLR9, Toll-like receptor 9.
[1] Kruskal-Wallis test, reported as mean ± s.e.m; [2] % Area refers to the percentage area of the glomerulus with staining. *P<0.05, **P<0.005, ***P<0.001, ****P<0.0001

Table 4. Primary antibodies used for immunofluorescence and co-localisation studies

Antibody	Specificity	Dilution	Source
Mouse anti-human TLR2	TLR2	5µg/ml	ABCAM[1]
Mouse anti-human TLR4	TLR4	5µg/ml	ABCAM
Mouse anti-human TLR9	TLR9	5µg/ml	ABCAM
Rabbit anti-human TLR2	TLR2	5µg/ml	Santa Cruz[2]
Rabbit anti-human TLR4	TLR4	1/50	ABCAM
Rabbit anti human TLR9	TLR9	5µg/ml	ABCAM
Goat anti-human TLR9	TLR9	1/100	ABCAM
Rabbit anti-human CD34	Endothelial Cells	1/50	ABCAM
Mouse anti-human CD34	Endothelial Cells	1/50	DAKO[3]
Sheep anti-human nephrin	Podocytes	5µg/ml	LifeSpan Bioscience[4]
Sheep anti-human neutrophil elastase	Neutrophils	1/100	LifeSpan Biosciences
Mouse anti-human CD68	Macrophages	1/40	Dako
Rabbit anti-human HMGB1	HMGB1	1/100	ABCAM
Goat anti-human fibrinogen	fibrinogen	1/10000	ABCAM

Table 4. Abbreviations: HMGB1, high mobility group box 1; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; TLR9, Toll-like receptor 9.

[1] Cambridge, UK; [2] Dallas, TX, USA; [3] Glostrup, Denmark; [4] Seattle, MA

Supplementary Table 1. Correlations between glomerular and tubulointerstitial TLR expression and presenting eGFR

eGFR	TLR2 [1]Intensity	TLR2 [2]% Area	TLR4 Intensity	TLR4 % Area	TLR9 Intensity	TLR9 % Area
Spearman's r	r	r	r	r	r	r
Glomerular	-0.389*	-0.235	-0.392*	-0.443**	-0.131	-0.188
Tubulointerstitial	-0.144	-0.012	-0.372*	-0.468**	-0.074	-0.061

*Correlation is significant at 0.05 level (two tailed)
**Correlation is significant at 0.005 level (two tailed)
[1]Intenisty refers to the intensity of fluorescent staining measured in Image J
[2]% Area refers to the percentage area of the glomerulus with staining
Abbreviations:TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; TLR9, Toll-like receptor 9.

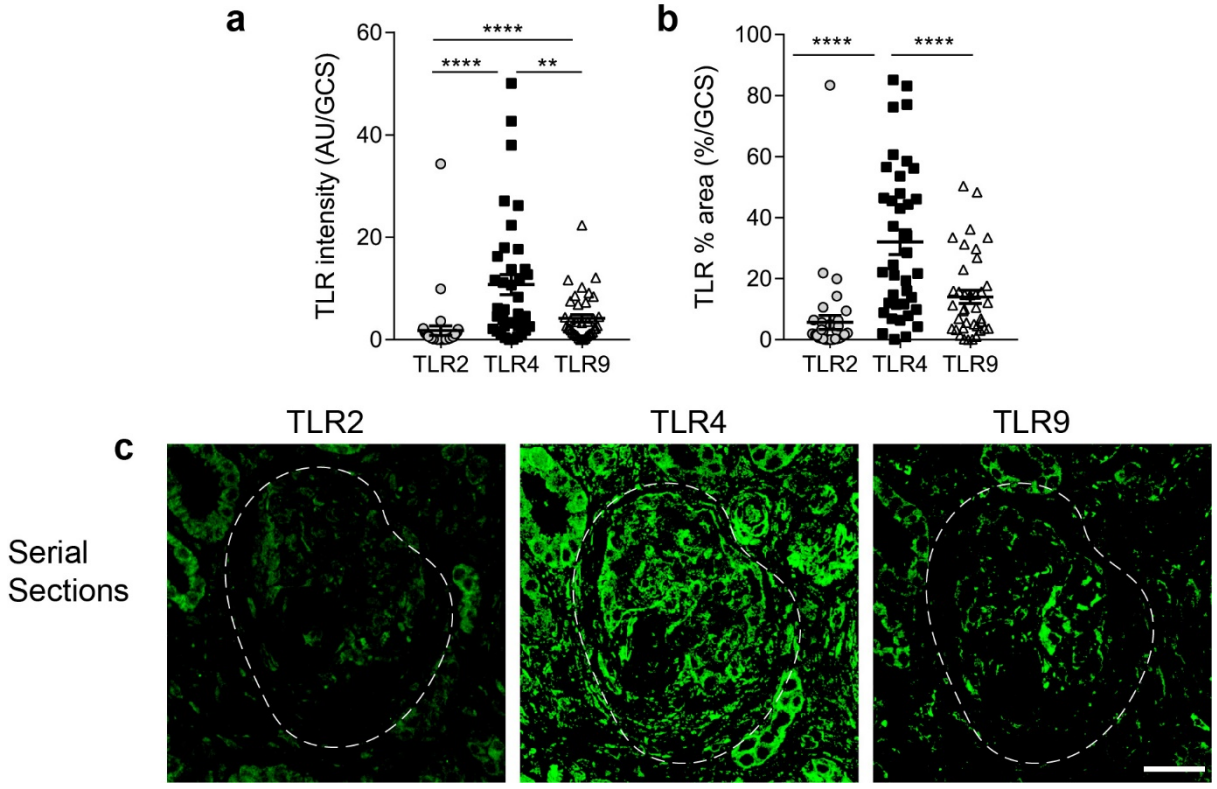


Figure 1. TLR4 is the predominant TLR expressed within glomeruli of patients with ANCA-associated glomerulonephritis. (**a**) Semi-quantitative assessment and comparison of the intensity of intraglomerular TLR2, TLR4 and TLR9 expression in AAV patients demonstrates that compared to TLR2 and TLR9, TLR4 is most highly expressed in glomeruli. (**b**) Semi-quantitative assessment and comparison of the proportion of the glomerulus positive for TLR2, TLR4 or TLR9 shows a similar TLR4 dominant pattern. (**c**) Representative images, showing serial sections stained with anti-TLR2, TLR4 and TLR9 antibodies illustrating that TLR4 is prominently expressed within glomeruli of AAV patients. Data are expressed as the mean ±SEM from 38 biopsies, analysed by the Kruskal Wallis test. ***P*<0.005, *****P*<0.0001. Original magnification 600x, scale bar = 20μm. TLR, Toll like Receptor.

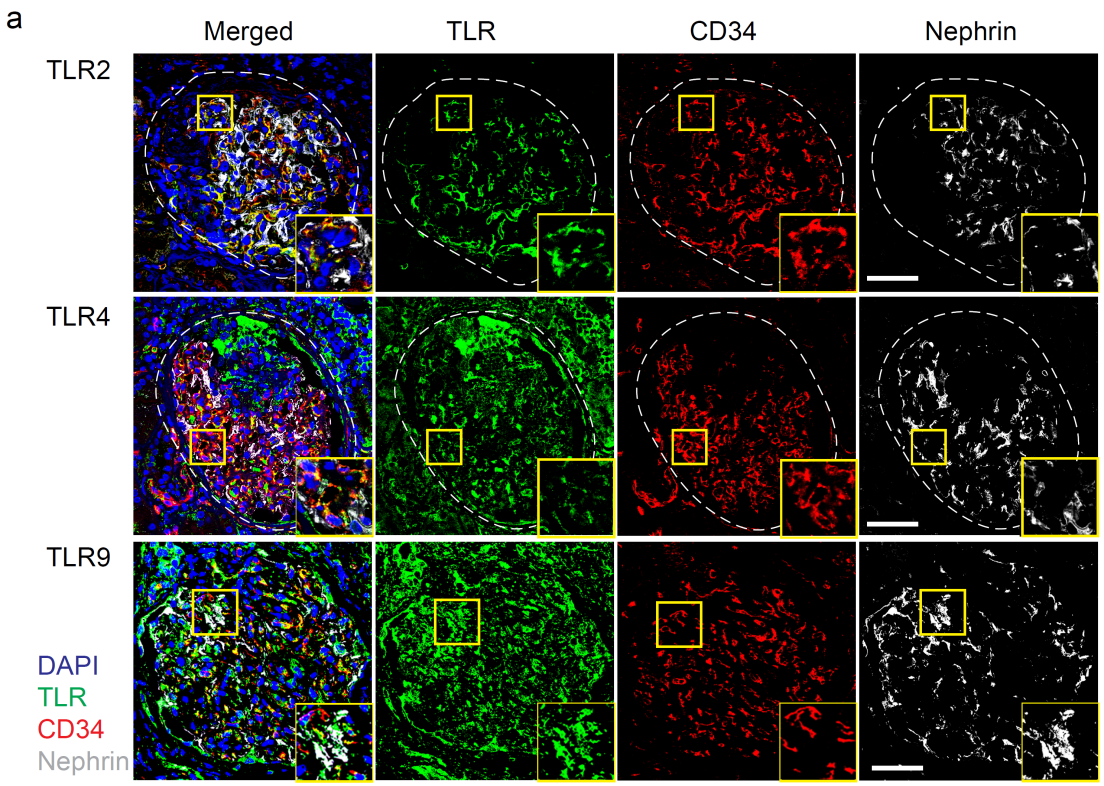


Figure 2. In ANCA-associated glomerulonephritis, TLR2 and TLR4 associate with endothelial cells and podocytes, whereas TLR9 associates largely with podocytes. Representative examples of the pattern of TLR expression with reference to glomerular endothelial cells and podocytes, with staining on the same section for each TLR (green), CD34 (red, denoting endothelial cells), nephrin (denoting podocytes, grey) and DAPI (blue, nuclear marker). Original magnification 800x, scale bar = 20μm. DAPI, 4',6-diamidino-2-phenylindole; TLRs, Toll Like Receptors.

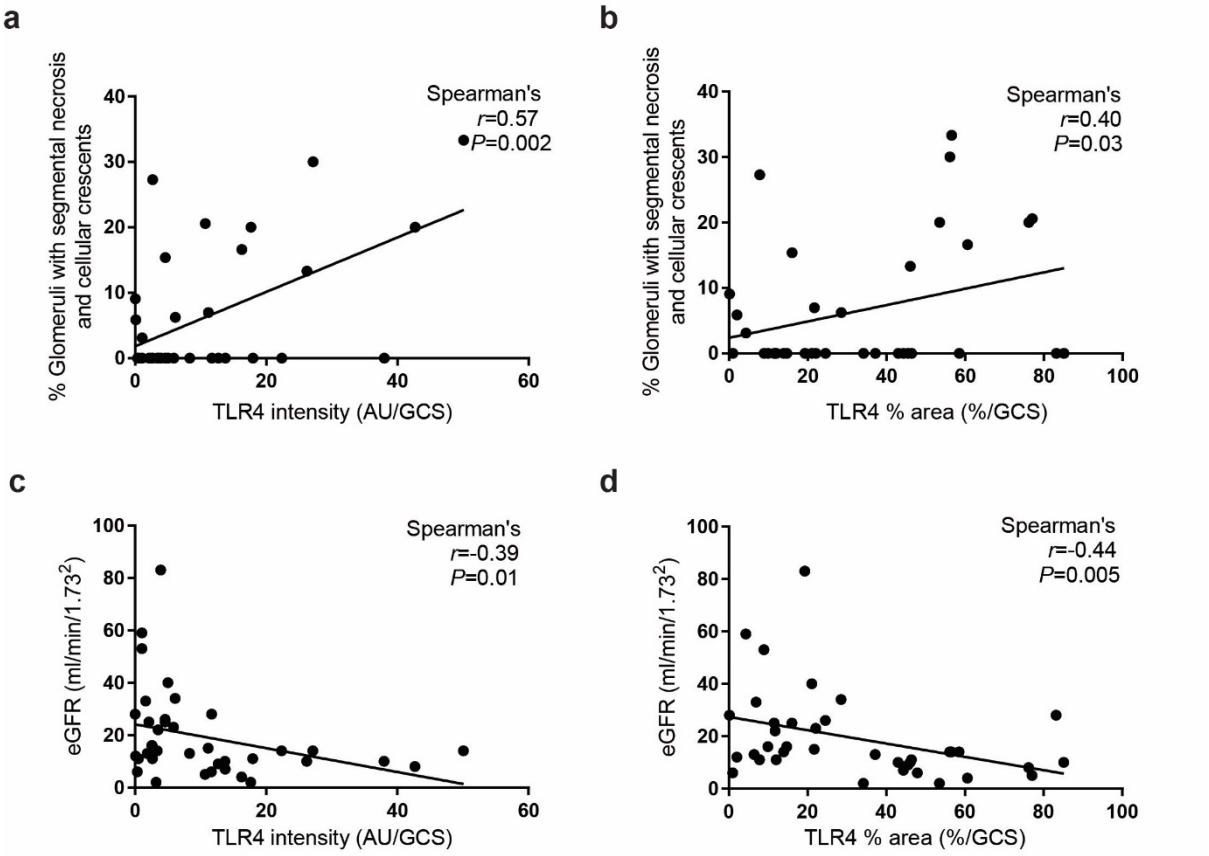


Figure 3. Glomerular TLR4 expression is associated with more severe glomerular disease. The proportion of glomeruli with the most active glomerular lesions containing both cellular crescents and segmental necrosis correlates with both the intensity of TLR4 staining (**a**) and the percentage area of the glomeruli positive for staining (**b**). Both the intensity (**c**) and area (**d**) of TLR4 staining correlate inversely with eGFR at presentation. (**a-b**) Data are means ±SEM from the 38 AAV patients' biopsies analysed by the non-parametric Spearman's correlation (**a-d**). eGFR, estimated glomerular filtration rate; TLR4, Toll like receptor 4.

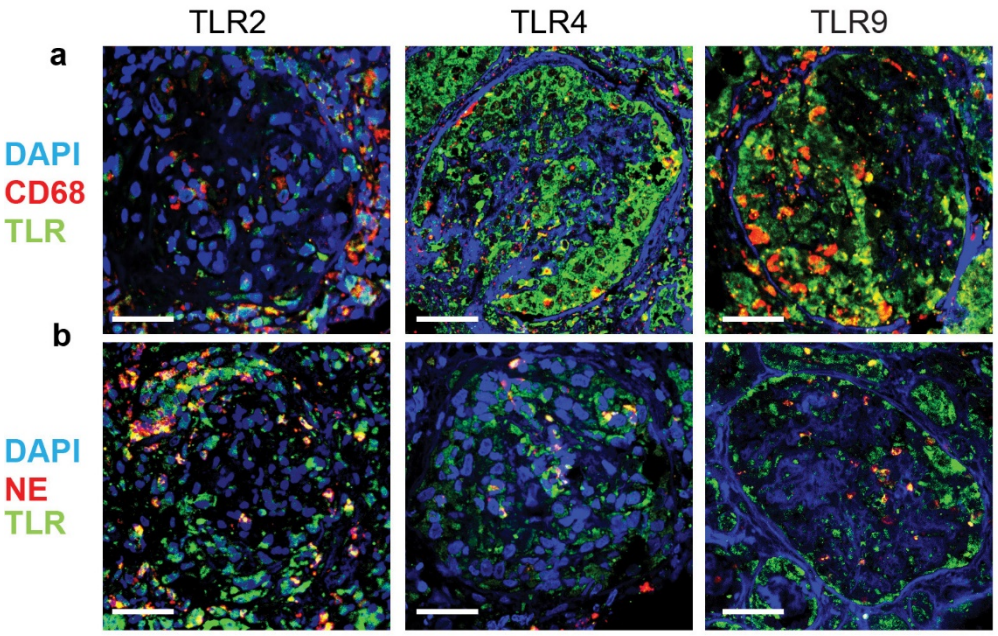


Figure 4. Glomerular infiltrating myeloid cells express TLR2, TLR4 and TLR9. Kidney biopsies from *n*=10 patients, stained for TLR2, TLR4 or TLR9 (green), DAPI (blue, nuclear marker) and (**a**) CD68 (macrophages, red) or (**b**) neutrophil elastase (neutrophils, red). Original magnification 600x, scale bar = 20 μm. DAPI, 4',6-diamidino-2-phenylindole; TLR, Toll like receptor.

Figure 5

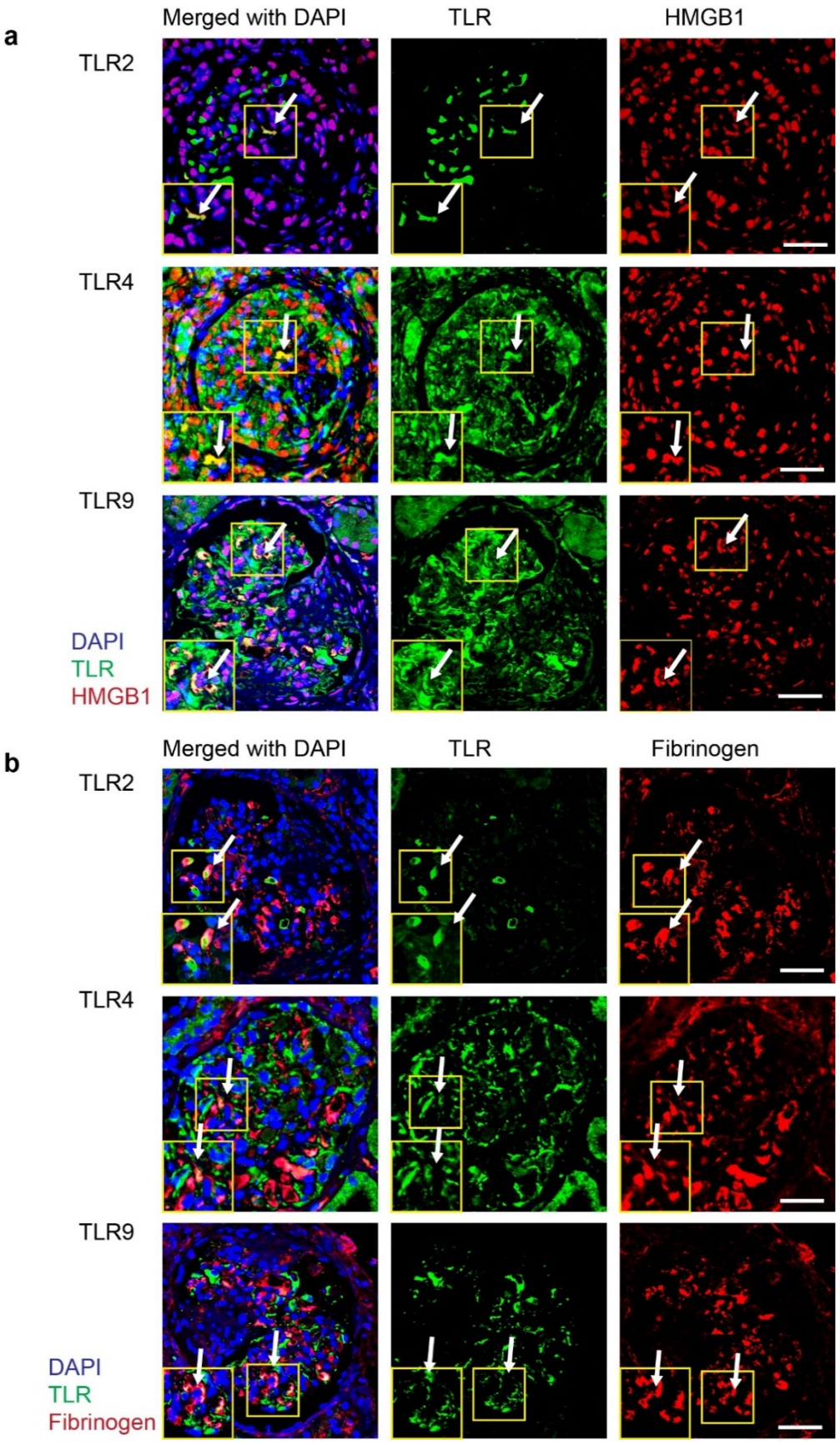

a
Merged with DAPI
TLR
HMGB1
TLR2
TLR4
TLR9
DAPI
TLR
HMGB1
b
Merged with DAPI
TLR
Fibrinogen
TLR2
TLR4
TLR9
DAPI
TLR
Fibrinogen

Figure 5 Legend. Co-localization of the endogenous TLR ligands HMGB1 and fibrinogen with Toll like receptors in glomeruli of patients with AAV. (a) TLR2, TLR4 or TLR9 (green) is co-localized with the endogenous TLR ligand HMGB1 (red) within glomeruli of patient biopsies with AAV. Inset shows higher power magnification of cells positive for both HMGB1 and the relevant TLR. (**b**) TLR2, TLR4 or TLR9 (green) is co-localized with the endogenous TLR2 and TLR4 ligand fibrinogen (red) within glomeruli of patient biopsies with AAV. Inset shows higher power magnification of co-localization of fibrinogen and the relevant TLR. Original magnification 600x, scale bar = 20 μm. DAPI, 4',6-diamidino-2-phenylindole; HMGB1, High mobility group box 1; TLR, Toll like receptor.

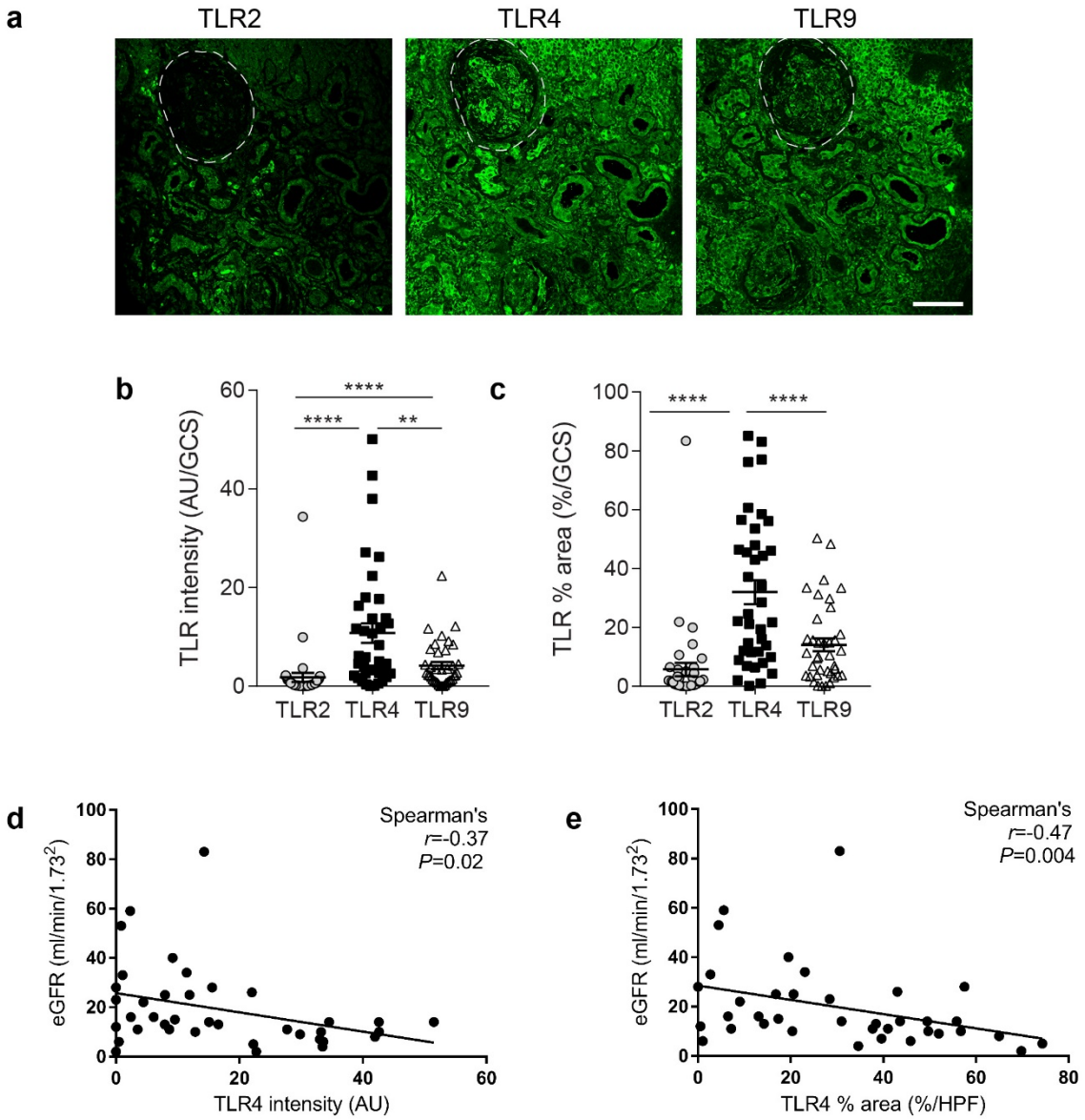


Figure 6. Tubulointerstitial TLR2, TLR4 and TLR9 expression in AAV patients. (**a**) shows representative TLR2, TLR4 and TLR9 staining often in and around the same tubules and infiltrating cells. Semi-quantitative assessment and comparison of the intensity (**b**) and the proportion (**c**) of tubulointerstitial high powered field (mean of 10) positive for TLR2, TLR4 or TLR9 expression in AAV patients demonstrating a TLR4 dominant pattern. Tubulointerstitial TLR4 intensity (**d**) and extent (**e**) are negatively correlated with presenting eGFR. Original magnification 200x, scale bar = 50μm. *P<0.05, **P<0.005, ****P<0.0001. Data are means ±SEM from 38 biopsies in each group analysed by the Kruskal Wallis test. AU, arbitrary units; eGFR, estimated glomerular filtration rate; TLR, Toll- like Receptors.

Figure 7

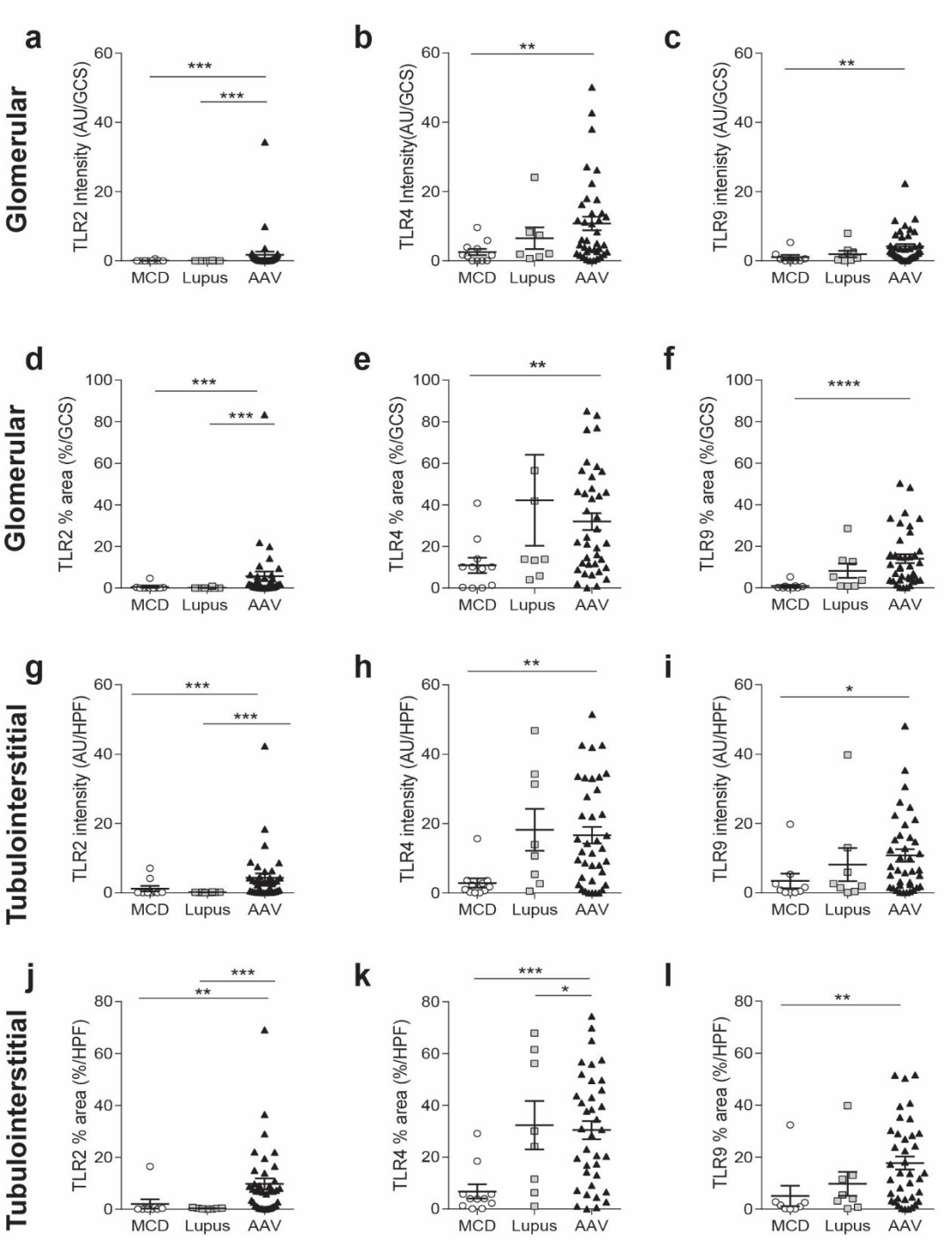
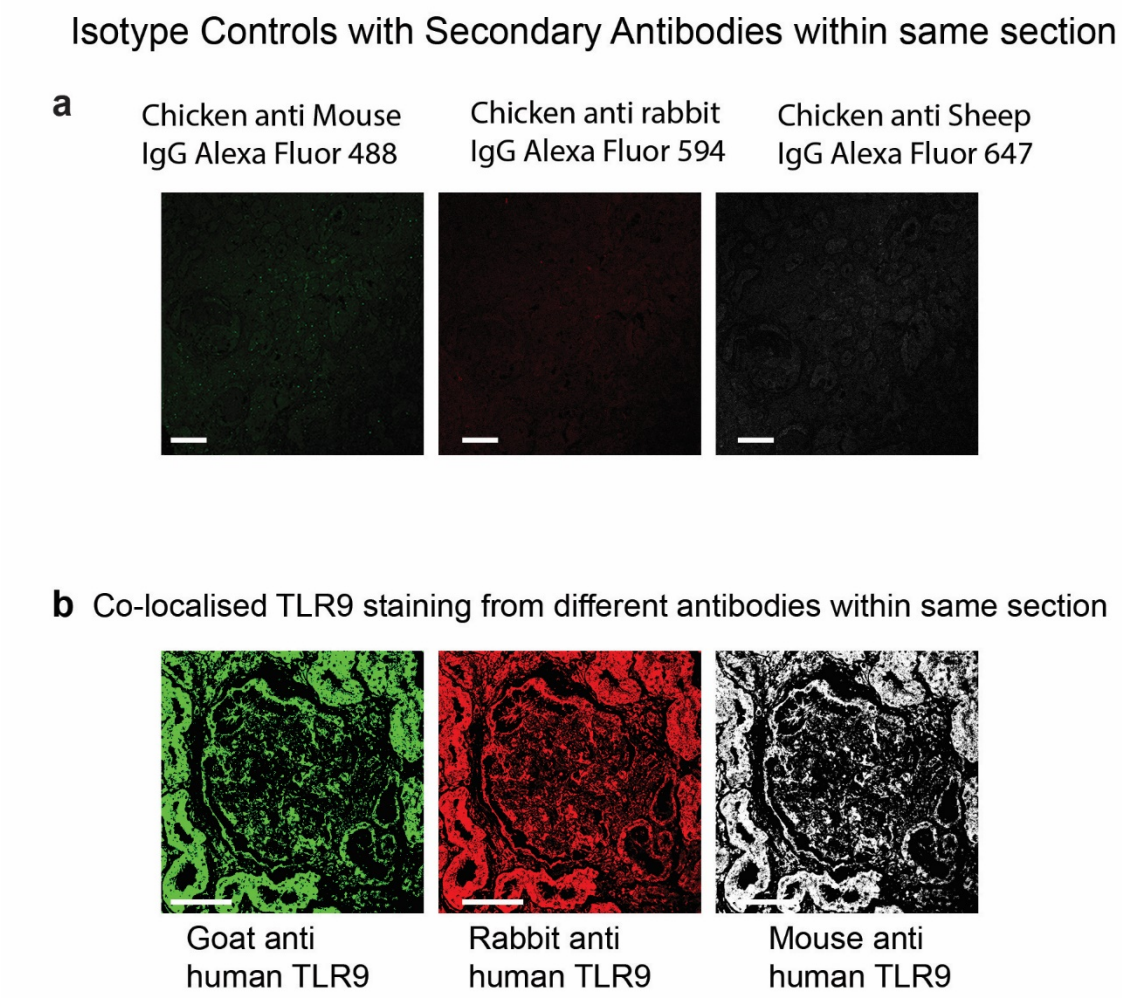
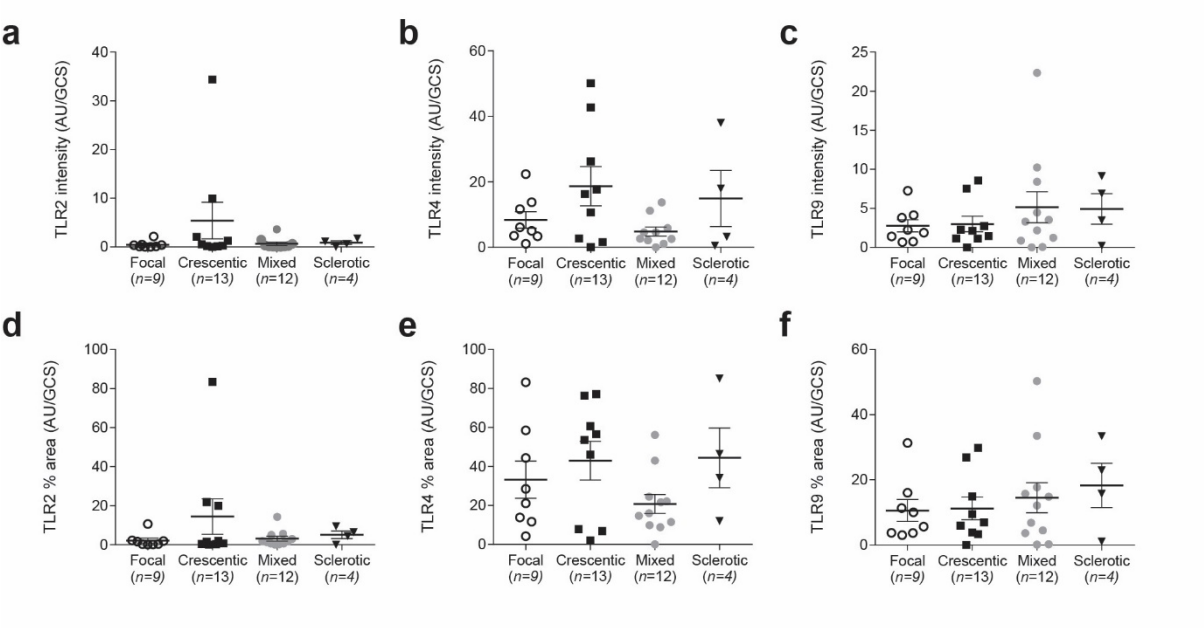

a
Glomerular
TLR2 Intensity (AU/GCS)
b
TLR4 Intensity(AU/GCS)
c
TLR9 intenisty (AU/GCS)
d
Glomerular
TLR2 % area (%/GCS)
e
TLR4 % area (%/GCS)
f
TLR9 % area (%/GCS)
g
Tubulointerstitial
TLR2 intensity (AU/HPF)
h
TLR4 intensity (AU/HPF)
i
TLR9 intensity (AU/HPF)
j
Tubulointerstitial
TLR2 % area (%/HPF)
k
TLR4 % area (%/HPF)
l
TLR9 % area (%/HPF)
MCD
Lupus
AAV

Figure 7 Legend. Comparison of TLR expression in ANCA-associated glomerulonephritis and control disease tissues, minimal change disease and Class IV ISN/RPS lupus nephritis. TLR2, TLR4 and TLR9 expression both in the intensity of the signal (**a-c**) and percentage area covered (**d-f**) are significantly increased within glomeruli of AAV patients when compared to those with minimal change disease. The intensity and extent of TLR2 expression in glomeruli were significantly increased compared to those with lupus nephritis. Similar findings were evident in the tubulointerstitial compartment, with TLR2, TLR4 and TLR9 expression being significantly increased both in intensity of signal (**g-i**) and the proportion of the tubulointerstitium expressing the relevant TLR (**j-l**), in the AAV patient cohort compared to minimal change disease (g-l), TLR2 was also significantly increased when compared to the cohort of patients with lupus nephritis. **P*<0.05, ***P*<0.05, ****P*<0.0005. Data are means ±SEM from eight minimal change disease, 10 class IV lupus nephritis and 38 AAV biopsies, in each group analysed by the Kruskal Wallis test. AU, arbitrary units; GCS, glomerular cross section; HPF, high powered field; TLR, Toll- like Receptors.

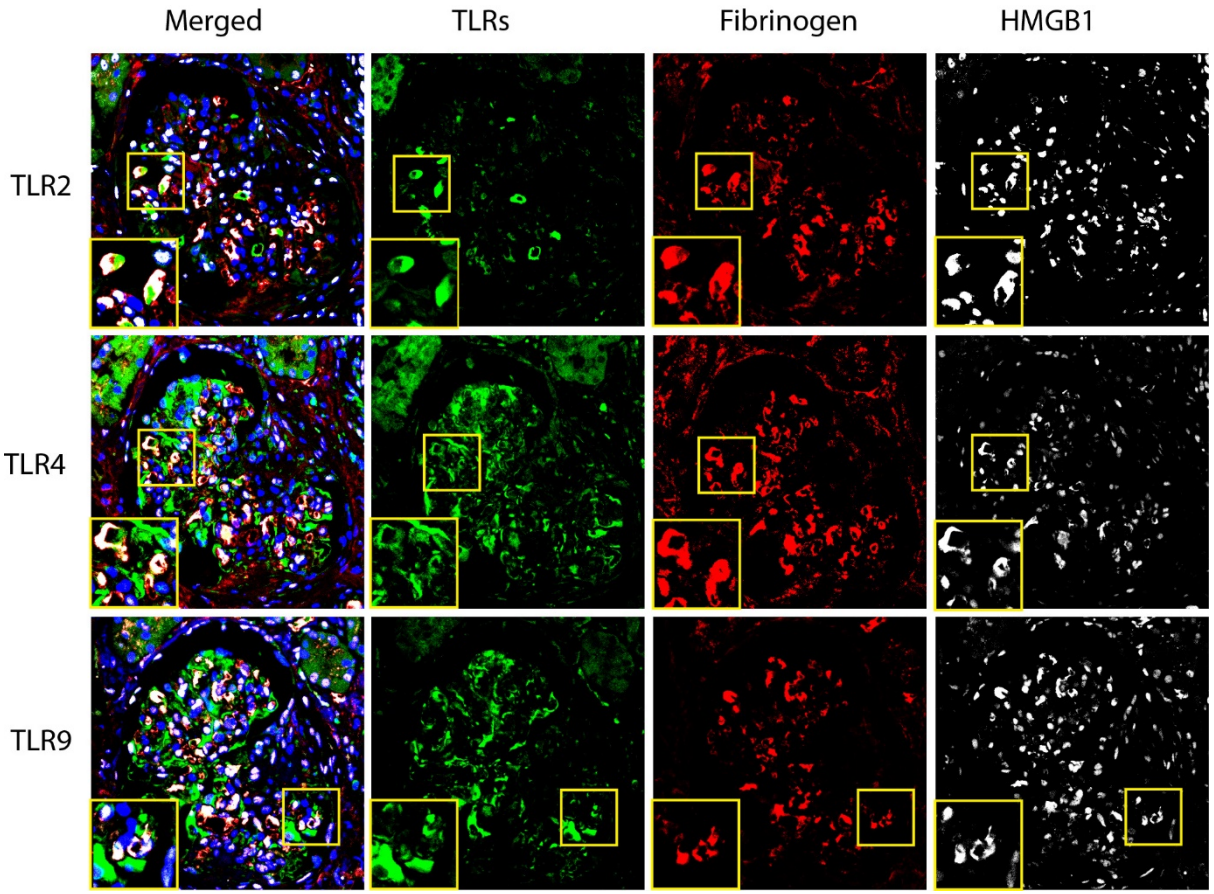
Supplementary Figure 1.



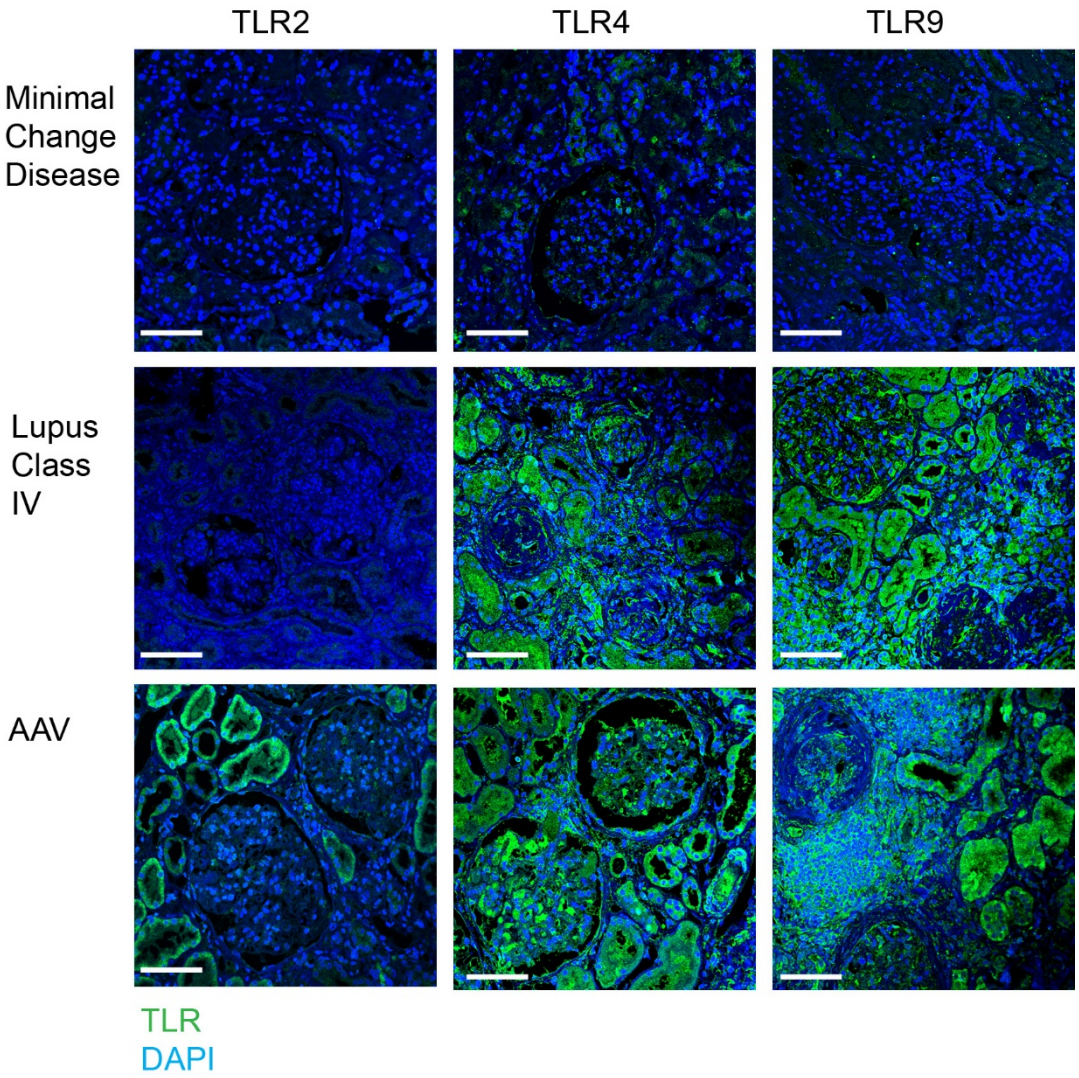
Supplementary Figure 1. Negative controls for TLR staining and TLR9 antibody validation (**a**) Negative controls, stained with secondary antibodies in the absence of specific primary antibodies, show minimal positivity above background fluorescence. The use of Sudan black results in minimal non-specific auto fluorescent staining. Original magnification 200x, scale bar = 30μm. (**b**) To ensure the specificity of the anti-TLR9 antibodies used, three different antibodies raised in three different species were applied to the same section (triple staining) This panel demonstrates that all three anti-TLR9 antibodies stain the same cells within glomeruli indicating the same specificity, and therefor validity, in the study. The antibodies used were goat anti-human TLR9 (green), rabbit anti-human TLR9 (red), mouse anti-human TLR9 (grey/white). Original magnification 600x, scale bar = 20μm.



Supplementary Figure 2. TLR2, TLR4 and TLR9 expression according to the AAV classification scheme of Berden et al. With the possible exception of the intensity and extent of the TLR2 signal being numerically but non-significantly higher in the crescentic classification compared to that of the focal, mixed and sclerotic categories, there was no association between category of glomerular disease and TLR expression.



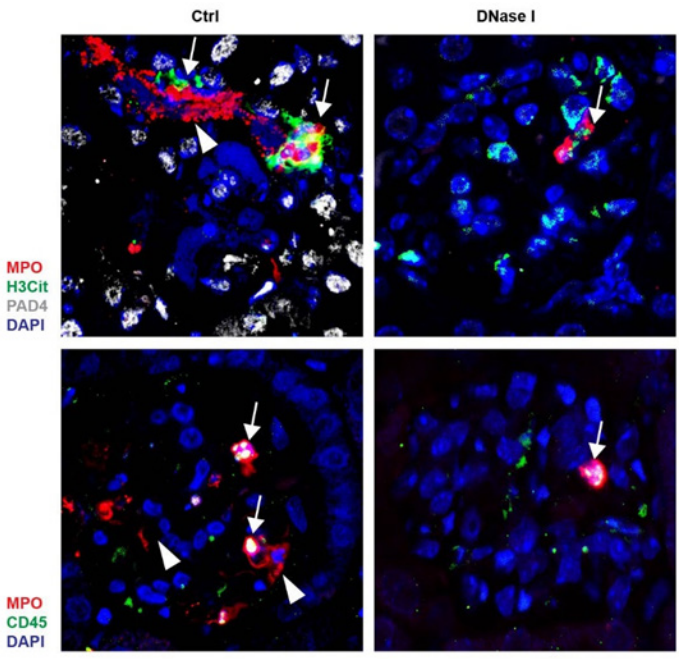
Supplementary Figure 3. Co-localization of both TLR ligands HMGB1 and fibrinogen, with TLRs. Serial sections stained for TLR2, TLR4 and TLR9 (green) in the same glomerulus co-localised with known TLR endogenous ligands fibrinogen (red) and HMGB1 (white). Yellow inset shows high powered image of the relevant TLR col-localising with both Fibrinogen and HMGB1 within the same cells. Original magnification 600x DAPI, 4',6-diamidino-2-phenylindole; HMGB1, High Mobility Group Box 1; TLR, Toll like receptor. Scale bar =20μm.



Supplementary Figure 4. TLR expression in kidneys from patients with minimal change disease, class IV lupus nephritis and ANCA-associated vasculitis. Representative images of TLR2, TLR4 and TLR9 expression (green) with DAPI (blue) of each biopsies of the different kidney diseases. Minimal change disease patients (MCD) have negligible TLR staining in both the glomeruli and tubulointerstitium. TLR2 staining was notably minimal in the lupus nephritis patients compared to TLR4 and TLR9, where moderate staining was present. AAV patients in comparison have considerably more TLR2 staining than either the MCD or lupus nephritis patients. Original magnification 200x, scale bar = 50μm. DAPI, 4',6-diamidino-2-phenylindole; TLR, Toll Like Receptor, MCD Minimal change disease

CHAPTER 5: DNASE I TREATS ANTI MPO GN

Therapeutic use of Deoxyribonuclease I Modulates Autoimmunity to Myeloperoxidase and Attenuates Glomerular Injury in Experimental anti-Myeloperoxidase Glomerulonephritis



Introduction

A variety of proteins are released from dying and injured cells and act as “danger signals” or danger associated molecular pattern (DAMP) proteins. DNA is a prominent DAMP and is highly pro-inflammatory. It is recognised by a family of receptors including Toll-like Receptors (TLRs), specifically TLR7 and TLR9. DAMP receptor signalling generates inflammation and is critical in host defence but also drives the generation of injurious autoimmunity.

Extracellular DNA can be released by several forms of death including necrosis and pyroptosis. Neutrophil extracellular traps (NETs), formed by neutrophils is termed NETosis and is recognised as a significant mechanism of injury in autoimmune disease. NETs were first described by Brinkmann et al., (2004), as a form of neutrophil cell death distinct from both apoptosis and necrosis. Activated neutrophils (via a plethora of stimuli, including ANCA, LPS, and various cytokines) go through a process whereby a reactive oxygen species (ROS) burst precedes chromatin decondensation and the nuclear envelope disintegration allows the cytoplasmic contents to mix with nuclear material which is then expelled from the cell membrane forming a net like structure composed of chromatin (including DNA), MPO, PR3, neutrophil elastase, ROS and histones. This process is thought to occur to enhance neutrophil bactericidal killing capacity, however in sterile inflammation, they are potentially injurious to bystander tissue. The prominence of NETs in glomeruli and their known capacity to induce injurious inflammation makes blockade of NET formation a potential therapeutic approach. Extracellular DNA has the capacity to drive autoimmunity by enhancing pathological leukocyte mediated injury and is an essential component in the formation of NETs. Animal studies have shown that dendritic cells loaded with remnants of NETotic neutrophils containing the immunogenic proteins PR3 and MPO are enough to induce the development of anti-neutrophil cytoplasmic antibody (ANCA) and renal injury (Sangaletti et al., 2012). Our previous studies have shown that over 60% of MPO-ANCA Glomerulonephritis (GN) patients have glomeruli with NETs, accompanied with an increase in the deposition of glomerular extracellular MPO (O'Sullivan et al., 2015). Therefore, clearing extracellular DNA by therapeutic DNase I

treatment is likely to be effective in treating anti-MPO GN. Deoxyribonuclease I (DNase I) is a normal constituent of human plasma and is produced by the salivary glands and pancreas, and specifically targets and clears extracellular DNA. DNase I can be used at therapeutic levels, and is already use safely in patients with Cystic Fibrosis (Pressler et al., 2009).

Extracellular DNA fragments are normally cleared by extracellular DNase I and excess DNA is taken up by cells through scavenger receptors inducing DEC205 on dendritic cells. Intracellular sensors recognising DNA and RNA include endosomol located Toll-like receptors (TLRs) and cGAS which signal the expression of acute phase inflammatory cytokines as well as AIM2 that induces inflammasome formation and the generation and release of IL-1 and IL-18 (Jorgensen et al., 2017).

Another pathway of injury unique to this disease is the ability of ANCA to deliver activated neutrophils to the glomerular capillary networks where they can be directly injurious but also plant their disease initiating auto-antigen MPO extracellularly in glomeruli where it is not normally expressed. This translocation of target auto-antigen from circulating neutrophils to glomeruli is the reason that glomeruli become the secondary passive target in anti MPO GN. Systemic CD4 T cell driven anti MPO cellular autoimmunity is thus able to induce DTH type injury involving effector CD4 and CD8 T cells, macrophages and fibrin deposition, prominent participants in the glomerular pathology of this disease. The current study assessed the role of extracellular DNA in a well established model of autoimmune MPO-ANCA associated glomerulonephritis (anti MPO GN) by demonstration that therapeutic elimination of extracellular DNA by therapeutic doses of intravenous (IV) DNase I is able to effectively treat both GN and the underlying autoimmunity.

Materials and Methods

Patient Cohort

A total of 7 renal biopsies from AAV patients presenting with AAV and glomerulonephritis form 2001-2016, at Monash Medical Centre, Clayton, Victoria Australia, were used in this study. Patients from this cohort were a small subset of a cohort studied previously (used in chapter 2 of this thesis).Biopsies from 7 minimal change disease (MCD) patients were used as control tissue.

Mice

C57BL/6 mice were bred at Monash Medical Centre Animal Facilities and used at 8-10 weeks old. FcγRIIB-/-mice (C57BL/6J background: B6; 129S-Fcgr2b^{tm1Ttk}/J) were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred at Monash Medical Centre Animal Facilities, Monash University, Australia. The phenotype of FcγRIIB-/- mice was confirmed by flow cytometry with anti-B220 and anti-CD16/CD32Ab (BD Biosciences, North Ryde, NSW, Australia), as previously described (Takai, Ono, Hikida, Ohmori, & Ravetch, 1996). Mice aged 8-10 weeks were used for experiments approved by the Monash University Animal Ethics committee, under pathogen-free conditions.

Experimental Design

To assess cell mediated injury, WT mice were immunized intraperitoneally with 20 µg recombinant murine MPO in Freund's complete adjuvant (FCA; Sigma-Aldrich) and boosted subcutaneously with 10 µg murine MPO in Freund's incomplete adjuvant (FIA; Sigma-Aldrich) on day 7. Recombinant murine MPO was isolated and purified as described previously (Apostolopoulos et al., 2006). Disease was initiated ('triggered') by intravenous injection of 1mg anti-GBM globulin followed by 2mg anti-GBM globulin 2 hours later on day 17. Anti-MPO autoimmunity and GN was assessed 3 days later (day 20). Non treated mice received vehicle

injections of saline whereas control mice received an irrelevant antigen, OVA instead of MPO. Treated mice received DNase I 10mg/kg intravenously 4 hours after the last anti-GBM globulin to ensure recruited neutrophils had deposited MPO to initiate disease, and twice daily until termination of the experiment on day 20.

To isolate the effect of DNase I on humoral immunity, FcγRIIB-/- mice which are susceptible to vasculitis were given a passive transfer of anti MPO-IgG (generated by immunizing MPO-/- mice as previously described (Ooi et al., 2012). Disease was initiated by intraperitoneal injection of LPS (0.5μg/g) 2 hours prior to intravenous transfer of anti-MPO IgG, which recruits neutrophils to glomeruli without causing significant injury. DNase I was given intravenous at day -1, and daily until termination of the experiment on day 6.

Extracellular DNA, Neutrophil extracellular Traps and extracellular MPO detection

For measurement of extracellular DNA, 3μm formalin fixed paraffin embedded (FFPE) kidney sections were cleared in xylene, rehydrated in graded alcohols, and immersed in antigen retrieval solution (10mM Tris, 1mM EDTA, pH 9.0) and boiled in a pressure cooker for 10 minutes. Sections were blocked in 10% Chicken sera in 5% Bovine serum albumin (BSA)/ phosphate buffered saline solution (PBS) for 30 minutes and stained with a rabbit anti mouse Beta actin antibody (ABCAM) at 1μg/ml in 1%BSA/PBS, as a cell marker (and to identify glomerular regions of tissue for measurement) overnight at 4°C. For secondary detection a chicken anti rabbit Alexa Fluor 488 antibody at 1:200 was used for 40 minutes at RT (Molecular Probes, Thermofisher). Auto fluorescence was quenched in Sudan Black (Sigma-Aldrich St Louis, MO, 0.1% in 70% ethanol, 30 min) and mounted with DAPI prolong gold mounting media to detect dsDNA. NETs and extracellular MPO were detected as published previously (O'Sullivan et al., 2015). Briefly, Sections (3 μm) of formalin-fixed, paraffin-embedded tissue specimens were mounted on superfrost plus slides (Menzel, Braunschweig, Germany), dewaxed, rehydrated, and pre-treated with antigen retrieval solution Tris-EDTA pH 9 (10mM

TRIS, 1mM EDTA) In a pressure cooker for 10 min, blocked (30 min) in either 10% chicken sera in 5% bovine serum albumin/phosphate-buffered saline (immunofluorescence) and probed with antibodies against goat anti human MPO (detects Mouse MPO) rat anti mouse CD45 for the detection of extracellular MPO, and mouse anti human PAD4 (detects mouse PAD4), rabbit anti human H3Cit (detects mouse H3Cit), and goat anti human MPO (detects mouse MPO) in 1% bovine serum for the detection of NETs, diluted in albumin/phosphate-buffered saline for 16h (4 °C). Secondary detection was with either Alexa Fluor 594-conjugated chicken anti goat IgG or Alexa Fluor 488-conjugated chicken anti-rabbit IgG, or donkey anti mouse 647 IgG (all, from Molecular Probes 1:200, 40 min, room temperature). To quench tissue auto fluorescence, slides were incubated with Sudan Black, washed in phosphate-buffered saline, and cover slipped in DAPI prolong gold (Molecular Probes). Fluorescent images were acquired using a NIKON C1 confocal laser scanning head attached to Nikon Ti-E inverted microscope (Coherent Scientific, SA, Australia); 405, 488, and 561 nm 647nm lasers were used to specifically excite DAPI, Alexa 488, Alexa 594, and Alexa 647. Single plane 512 × 512 × 12 bit images were captured in a line-sequential manner (4 line averaging) using a 20 ×, 40 ×, or 60 × objective.

Assessment of ecDNA, extracellular MPO and DNase I

To measure the ecDNA in both human renal biopsies and murine kidneys, “analyse particle” in Image J was used and circularity set to include nuclear staining, the nuclear stain was thresholded, marked and excluded from analysis using the watershed function in Image J, leaving only the extra nuclear stain to be measured (NIH, Bethesda, MD). The average integrated intensity/density was used and expressed in arbitrary units per glomerular cross section. Extracellular MPO was measured by a macro using Image J analysis software (NIH, Bethesda, MD). Intracellular (leukocyte-associated) MPO was defined being associated with CD45 (CD45+MPO+ cells). Extracellular MPO was measured as MPO+CD45-ve staining. The macro evaluated both the area and intensity and expressed the results as arbitrary units (AU).

Assessment of renal human and mouse DNase I

Histologic assessment of renal injury was performed on 3 μm thick, formalin-fixed, paraffin-embedded human renal biopsies from AAV patients and MCD patients, and murine models of anti MPO-GN and GN induced by passive ANCA. Sections were cleared in Histosol, rehydrated in graded alcohols and then blocked with 10% Horse serum in 5% BSA/PBS for 30 minutes at RT and incubated with a mouse anti human DNase I antibody (1/100, Santa Cruz) over night at 4 degrees. Sections were washed, and endogenous peroxidase blocked with 1% Hydrogen Peroxide (H_20_2) in Methanol for 20 minutes, followed by blocking with avidin and biotin using a commercially available blocking kit per manufacturer's instructions (Vector Laboratories). Secondary antibody detection was performed with a horse anti mouse biotinylated antibody at 1/60 (Vector Laboratories) for 40 minutes at RT, and detected with an avidin biotin complex conjugated to HRP (ABC-HRP) for 40 minutes (Vector Laboratories), and detected with 3,3′-Diaminobenzidine (DAB brown, SIGMA), dehydrated, cleared in Histosol and mounted in vector shield permanent mounting media. Five low powered (20x objective lens) fields of view of the tubulointerstitium were analysed and graded 1-3 according to the amount of stain present within tubulointerstitial epithelial cells in the renal biopsies and 10 fields of view were analyzed for mouse kidney tubulointerstitium.

Assessment of renal injury and leukocyte infiltration

Histologic assessment of renal injury was performed on 3 μm thick, formalin-fixed, paraffin-embedded, periodic acid-Schiff-stained kidney sections. A minimum of 30 consecutive glomeruli/mouse were examined and results expressed as percentage of segmental glomerular necrosis per glomerular cross-section (gcs). Glomerular CD4+ T cells, macrophages, and neutrophils were assessed by an immunoperoxidase-staining technique described in the previous section, on 6-μm thick, periodate lysine paraformaldehyde (PLP) fixed, OCT frozen kidney sections. The primary antibodies used were GK1.5 for CD4+ T cells (anti-mouse CD4+; American Type Culture Collection, Manassas, VA), FA/11 for macrophages (anti-mouse CD68 from Dr. Gordon L. Koch, Cambridge, England), and RB6–

8CS for neutrophils (anti-GR-1; DNAX, Palo Alto, CA). A minimum of 30 glomeruli were assessed and results expressed as cells/gcs (c/gcs).

Systemic Immune Responses to MPO

Lymph nodes were harvested aseptically from mice and placed in media containing: 5%FCS, 2 mM L-glutamine, 50 mM 2-ME, 100 U/ml penicillin, and 0.1 mg/ml streptomycin; (Sigma). Single cell suspensions were achieved through gently mashing lymph nodes through a 70µm mesh filter (BD, Falcon, NJ,USA). Lymph node cell concentration was calculated via assessing viability with trypan blue and enumerated using a haemocytometer. Lymph nodes were then centrifuged and resuspended in the appropriate cell concentration for *in vitro* cultures, flow cytometry and ELISPOTS in media containing:10%FCS, 2 mM L-glutamine, 50 mM 2-ME, 100 U/ml penicillin, and 0.1 mg/ml streptomycin; (Sigma). IFN-γ and IL-17A production was assessed by ELISPOT (BD Biosciences, Mouse IFN-γ ELISPOT kit and Mouse IL-17A ELISPOT kit) with draining LN cells seeded at $5x10^5$ cells/well restimulated with 10µg/ml of recombinant MPO for 18 hours. IFN-γ and IL-17A producing cells were enumerated with an automated ELISPOT reader system. To assess MPO-specific dermal DTH, mice were challenged by intradermal injection of 10 µg murine MPO in 30 µl saline in the right hind footpad (the contralateral footpad received saline). DTH was quantified 24 hours later by measuring the difference between footpad thicknesses (Δmm) using a micrometer.

Flow cytometry and intracellular staining

DCs were identified as $CD11c^{hi}$ cells on isolated draining LN cells as measured by flow cytometry. LN cells were stained for 30 minutes at 4˚C with the following directly conjugated antibodies: hamster anti-mouse CD11c PE (HL3), hamster anti-mouse CD11c PerCP/Cy5.5 (N418, BioLegend), rat anti-mouse CD40 FITC (3/32, BD Biosciences), rat anti-mouse MHC-II APC/Cy7 (M5/114.15.2, BioLegend), rat anti-mouse ICOS Ligand PE (HK5.3, BioLegend), mouse anti-mouse OX40L PE (8F4, BioLegend), hamster anti-mouse CD80 FITC (16-10A1, eBioscience), rat anti-mouse CD86 APC/Cy7 (GL-1, BioLegend). CD4 (GK1.5, BD),CD69

(H1.2F3, BD). For analysis of Treg response 5 x 10^5 LN cells were cultured for 72 hours with 10µg/ml MPO. Cell trace violet (CTV, Thermofisher), as per manufacturer's instructions was added at 0 hours and cells stained using anti- (Foxp3FJK-16s, EBioscience) and Foxp3 fixperm kit (EBioscience) Cells were analysed on the Beckman Coulter Navios platform and data analysed using FlowJo software (TreeStar, Palo Alto, CA).

Statistical Analyses

Results are expressed as the mean ± SEM. Mann–Whitney t-test was used for non-parametric data or Kruskal-Wallis for 3 or more groups. All data were analyzed with GraphPad Version 6 (GraphPad Prism; GraphPad Software Inc., San Diego, CA). Differences were considered to be statistically significant if $P<0.05$.

Results

Patient Characteristics

Kidney biopsies from a small cohort of MPO-AAV GN patients (*n*=7, patient clinical data, Table 1) and patients with minimal change disease served as control tissues (*n*=7), were used to assess the presence of extracellular fragments of DNA. As a cohort the MPO-ANCA patients presented with severe renal disease with an average estimated glomerular filtration rate (eGFR) of 31.29 ml/min/1.73^2.

Table 1. Clinical Data from patients with ANCA associated Vasculitis

Patient Characteristics	
Patient number	7
Sex F/M	2/5
Number of glomeruli	16.7 (±9)
Laboratory values	
MPO-ANCA titre (U/ml)	116.8 (±32)
eGFR (ml/min per 1.73 m^2)	31.29 (±8)
ESR (mm/h)	80.6 (±9)
CRP (mmol/l)	28.5 (±12)
Red blood cells (cells/HPF)	988 (±613)

Abbreviations: ANCA, anti-neutrophil cytoplasmic antibody; CRP, C reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; MPO, myeloperoxidase

Extracellular DNA is prominent in human renal biopsies from patients with MPO-ANCA vasculitis

Our research group has previously examined renal biopsies from MPO-ANCA GN patients for neutrophil extracellular traps and found that over 60% of patients had NETs within their glomeruli (O'Sullivan et al., 2015). We now sought evidence of alternative forms of extracellular DNA deposition from other types of cell death.

To determine the amount of ecDNA in the renal biopsies, we used a method that would pick up the majority of glomerular deposited ecDNA regardless of the type of cell death (i.e. apoptotic, necrotic or NETotic) Extracellular DNA was assessed by using colocalization studies using DAPI as a marker for dsDNA and Beta actin as a structural component of all cells to delineate glomerular and cell structures. Using Image J, fragmented DNA from outside the nucleus of cells was identified and measured, and expressed in arbitrary units (see methods section for details). There was a significant amount of dsDNA found within the glomeruli of MPO-ANCA GN patients compared to that of the control, minimal change disease patients which had minimal dsDNA staining present within glomeruli (Figures 1, A, B, and D). It has been reported that patients with MPO-ANCA GN have lower levels of circulating DNase I (Nakazawa et al., 2014), which may reduce the digestion of NETs and prevent removal of apoptotic bodies. As kidneys are one of the largest producers of endogenous DNase I we sought evidence of DNase I depletion in MPO-ANCA patients. Staining with DNase I antibody demonstrated that AAV patients have significantly less DNase I expression within the tubulointerstitium when compared to those from patients with minimal change disease (MCD) (Figures 1,C and E).

Figure 1. Extracellular DNA is a prominent feature in glomeruli in patients with MPO-ANCA associated vasculitis (AAV)

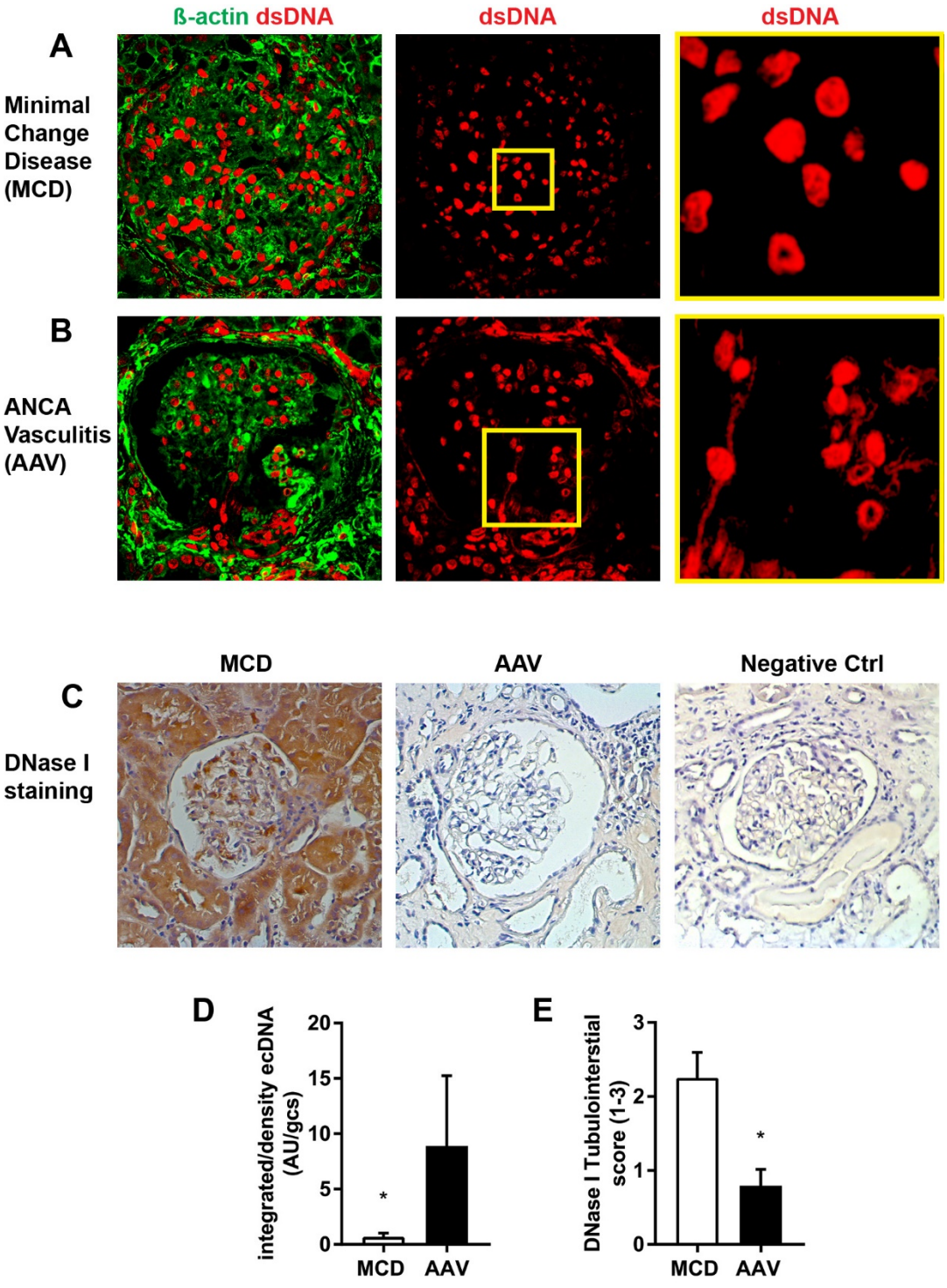


Figure 1 Legend. Extracellular DNA is a prominent feature in glomeruli in patients with ANCA associated vasculitis (AAV) Renal biopsies stained for dsDNA (red) and counterstained with β-actin (green) to aid in identification of glomeruli and cells demonstrate patients with minimal change disease (MCD), have minimal ecDNA deposited within glomeruli (**A**), inset box shows higher power field of view with minimal ecDNA compared with patients with MPO-ANCA vasculitis (**B**) with extensive ecDNA. (**C**) MCD patients have significant tubulointerstitial expression of DNase I compared to the tubulointerstitium of patients with AAV. (**D**) Semi quantitation in Image J of ecDNA in MCD and AAV patients and expressed as arbitrary units, (**E**) *P<0.05 Data are means ± SEM Human Data is from n=7 in each group and analyzed by Mann-Whitney U. MPO, myeloperoxidase, ANCA anti neutrophil cytoplasmic antibody, GN, glomerulonephritis, Ctrl, Control; DNase I, Deoxyribonuclease I; dsDNA, double stranded deoxyribonucleic acid, ecDNA, extracellular deoxyribonucleic acid. Original magnification 400x, HP inset 3000x.

Therapeutic DNase I diminishes the prominent glomerular extracellular DNA deposition found in murine anti MPO GN

To define the role of DNase 1 in autoimmune anti MPO-GN, we used a well characterised murine model (Ooi et al .,2014). In this model anti MPO autoimmunity is induced in C57Bl/6 (WT) mice by subcutaneous immunisation with recombinant MPO in Freunds Complete Adjuvant (FCA) on day 0 and boosted again on day 7 in Freunds Incomplete Adjuvant (FIA). Immunisation alone is not enough alone to induce disease in this model therefore a subnephritgenic dose of anti -GBM Ig is administered on day 16 which results in a 2 hour transient accumulation of glomerular neutrophils which deposit MPO the auto-antigen and initiates Delayed Type Hypersensitivity (DTH) mediated disease (Figure 2A). To confirm that the anti GBM immunoglobulin (Ig) did not itself cause renal injury at the dose used, we administered anti GBM to mice immunised with an irrelevant protein, ovalbumin OVA (*n*= 4). In the absence of induced anti MPO autoimmunity, this dose of anti GBM Ig, did not induce significant DTH, MPO specific immune responses, or histological focal segmental glomerulosclerosis (Figure 3 A-D). Therapeutic DNase I (*n*=6, or saline to control groups, *n*=6) administration began 4 hours after anti-GBM administration. Renal injury and autoimmunity to MPO was assessed at the end of the experiment 4 days later. To determine the amount of ecDNA in this mouse model, we used a method that would pick up the majority of glomerular deposited ecDNA regardless of the type of cell death (i.e. apoptotic, necrotic or NETotic) Extracellular DNA in this mouse model of anti MPO GN was assessed by using colocalization studies using DAPI as a nuclear marker/detection of dsDNA and Beta actin a structural component of all cells to delineate glomerular and cell structures. Using Image J, fragmented DNA from outside the nucleus of cells was identified and measured, and expressed in arbitrary units (see methods section for details). There was a significant reduction in the amount of extracellular DNA deposited within the glomeruli of mice treated with DNase I compared to the vehicle treated mice (Figures 2, B, C and E). Anti MPO-GN mice had significantly less DNase I expression within the tubulointerstitium compared to ova control mice, and was therapeutically restored with DNase I treatment (Figure 2D, 2F).

Figure 2. Extracellular DNA is a prominent feature in vehicle treated experimental MPO-AAV GN

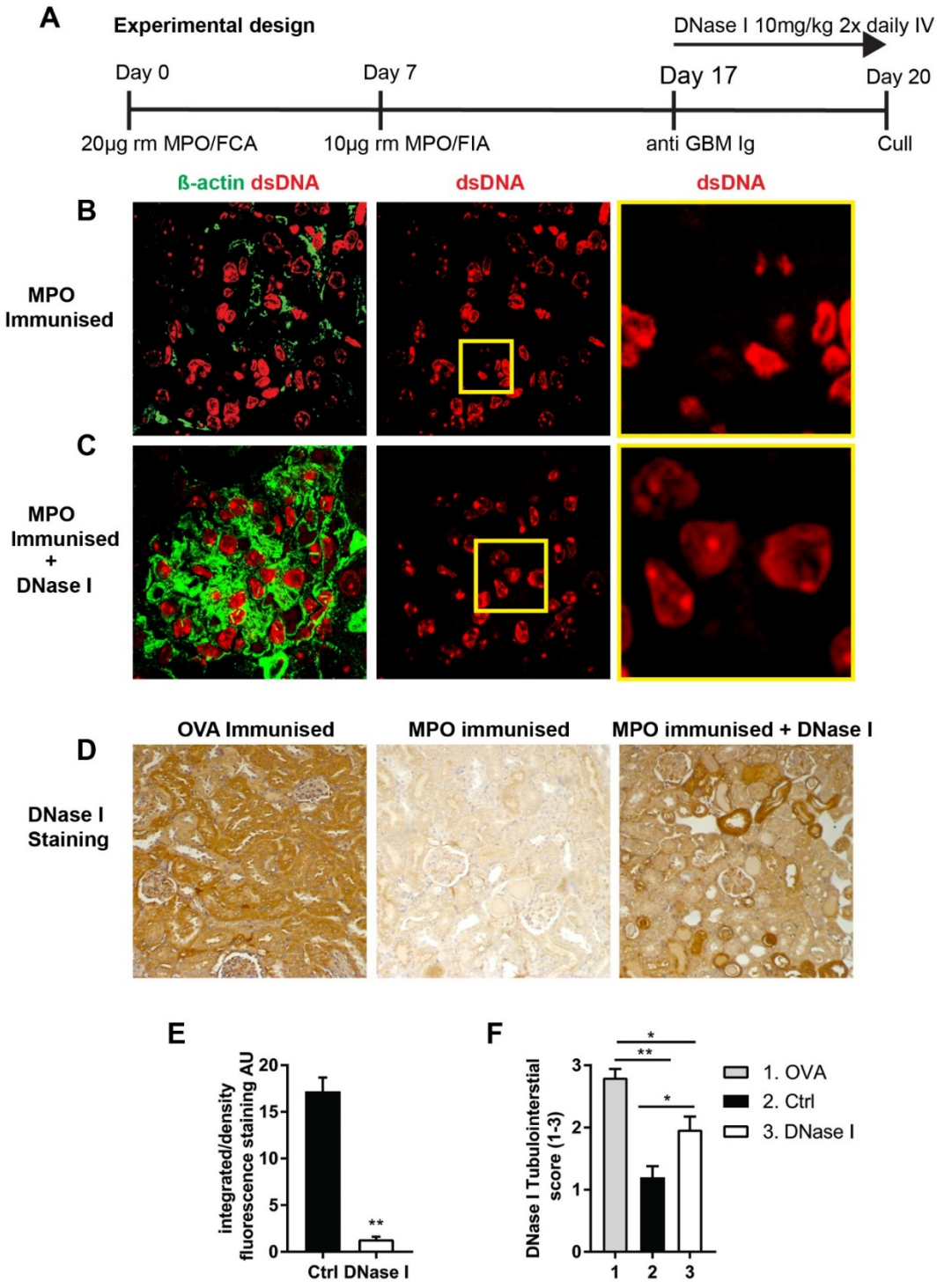


Figure 2 Legend. Extracellular DNA is a prominent feature in vehicle treated experimental MPO-ANCA GN. (**A**) Experimental murine MPO-ANCA GN vehicle treated mice have significant deposits of ecDNA (red) as determined by measuring extra nuclear DNA and counter stained with β actin (green)(**B**), compared to mice therapeutically administered DNase I (**C**). Administration of DNase I partially replenishes level of DNase I (**D**). Semi quantitation in Image J of ecDNA expressed as arbitrary units (**E**). Histological scoring of the tubulointerstitium of mouse kidneys for DNase I (**F**) *P<0.05, **P<0.01. Data are means ± SEM from six mice in each group analyzed by Mann-Whitney U or Kruskal-Wallis for 3 or more groups. Abbreviations :MPO, myeloperoxidase, ANCA anti neutrophil cytoplasmic antibody, GN, glomerulonephritis, Ctrl, Control; DNase I, Deoxyribonuclease I; ecDNA, extracellular deoxyribonucleic acid. Original magnification 400x

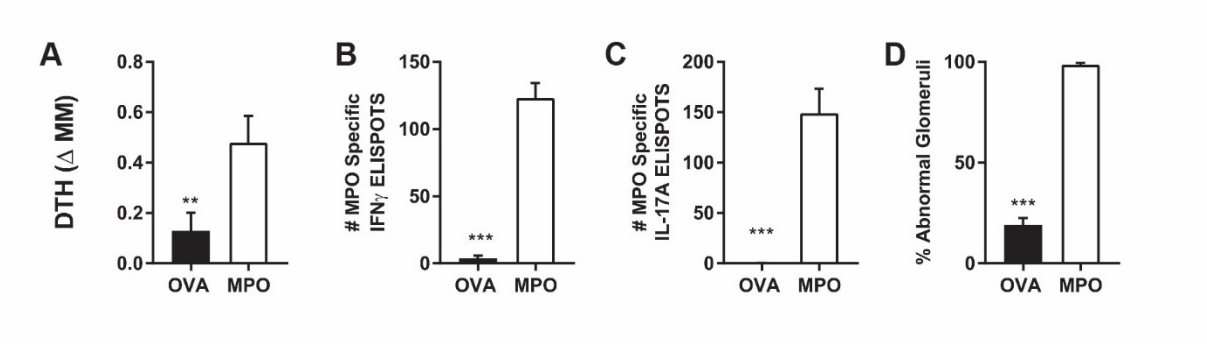


Figure 3. Mice immunised with an irrelevant antigen OVA do not develop autoimmunity to MPO shown by lack of development of humoral and cellular responses to MPO. OVA immunised mice do not develop significant DTH in the foot pad when challenged for 24 hours when immunised with OVA compared to mice immunised with MPO, nor do cells from the draining lymph nodes produce MPO specific IFNγ or IL-17A ELISPOTS in response to MPO (A). **P<0.01, ***P<0.001 OVA, Data are means ± SEM from six mice in the MPO group, and 4 mice in the OVA immunised group analyzed by Mann-Whitney U. Ovalbumin; MPO, myeloperoxidase.

DNase I treatment diminishes glomerular injury and autoimmunity to MPO

We sought evidence of NET deposition using the widely published and defined method of co-localization of citrullinated histones (H3Cit), a marker of decondensed DNA, DAPI to mark extracellular chromatin, MPO, and PAD4 an enzyme required for the citrullination of histones a crucial step in NETosis (Brinkmann et al., 2004; Kessenbrock et al., 2009; O'Sullivan et al., 2015). NETs were found in 80% of glomeruli in the saline group, while DNase I treatment significantly reduced the number of glomeruli with NETs to less than 10% (Figure 4, A and B). The significant reduction in the number of NETs was accompanied with a significant reduction in the amount of extracellular MPO deposited within glomeruli of the treated mice (extracellular MPO was defined as non- colocalised CD45 and MPO, and measured using a macro in Image J, (Figures 4 C and D). DNase I treated mice had a significant reduction in dermal DTH, measured by a 24 hour footpad challenge compared with saline treated control group (Figure 5 A). Assessment of the numbers of MPO-specific IFNγ and IL17A producing cells from lymph nodes draining sites of MPO immunisation demonstrated a significant reduction in the frequency of both these cell populations in the DNase I treated group compared to the saline treated control group (Figures 5 B and C). DNase I treatment significant reduced glomerular

injury with a reduction in segmental necrosis, a reduction in the accumulation of glomerular DTH effector cells, macrophages, neutrophils and CD4 T cells and this was associated with a less fibrin deposition when compared to the control group (Figures 5, D-H). Albuminuria (24hr) was reduced in the DNase I treated group , indicating that as well as a reduction in NET production, subsequent MPO deposition and glomerular DTH effector cells and glomerular injury, DNase I also has an effect on functional injury (P=0.06, Figure 5 I).

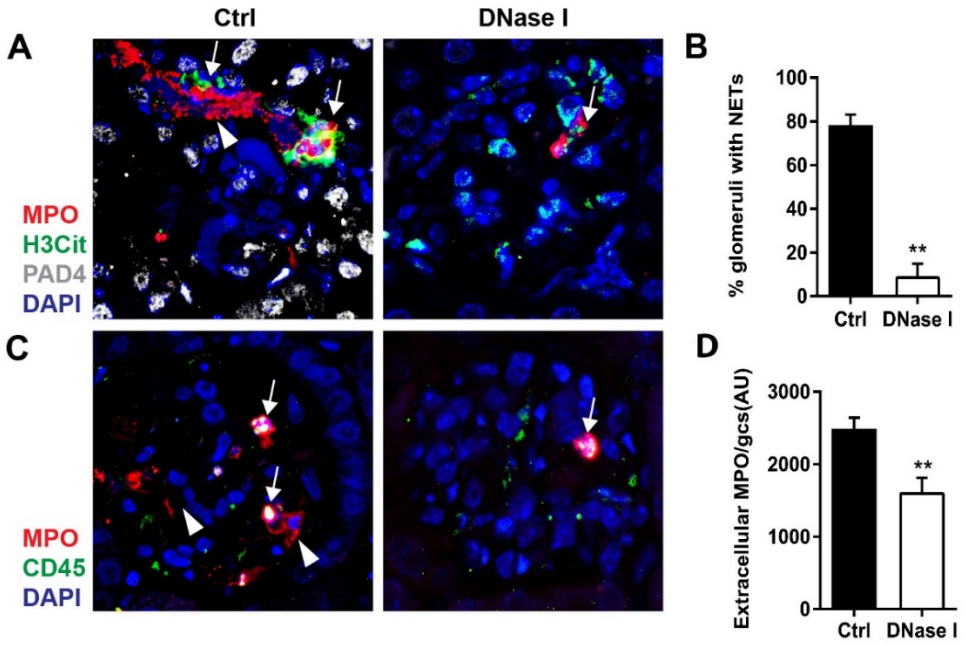


Figure 4. DNase I treatment significantly reduces NET formation when initiated after the establishment of autoimmunity to MPO and induction of experimental anti MPO-glomerulonephritis (**A**). Glomeruli from non- treated mice have significant numbers of NETs within glomeruli seen by co-localisation of MPO (red), H3Cit (green), PAD4 (white) and DAPI (Blue) indicated by arrows, and deposits of MPO indicated by arrow head, whereas DNase I treated mice have a significant reduction in the number of NETs (arrow)(**B**). Semi-quantification of the numbers of glomeruli with NETs show a significant reduction of NET formation in those mice treated with DNase I (**C**). Non-treated mice show glomeruli with significantly greater deposits of extracellular MPO determined as non-colocalised CD45 (green) and MPO (red) (arrows) or intracellular MPO indicated by co-localisation of CD45 and MPO (arrow head), compared to glomeruli from mice which received DNase I treatment (**D**) Semi-quantification of extra cellular MPO using a macro in Image J demonstrates a significant reduction in the amount of extracellular MPO in the DNase I treatment group (**E**). ** P<0.01. Data are means ± SEM from six mice in each group analyzed by Mann-Whitney U. MPO, myeloperoxidase: PAD4, peptidylarginine deiminase; H3Cit, Citrullinated Histone 3; DAPI, 4', 6-diamidino-2-phenylindole, Ctrl, Control; DNase I, Deoxyribonuclease I; CD45, pan leukocyte marker C45. Original Magnification 600

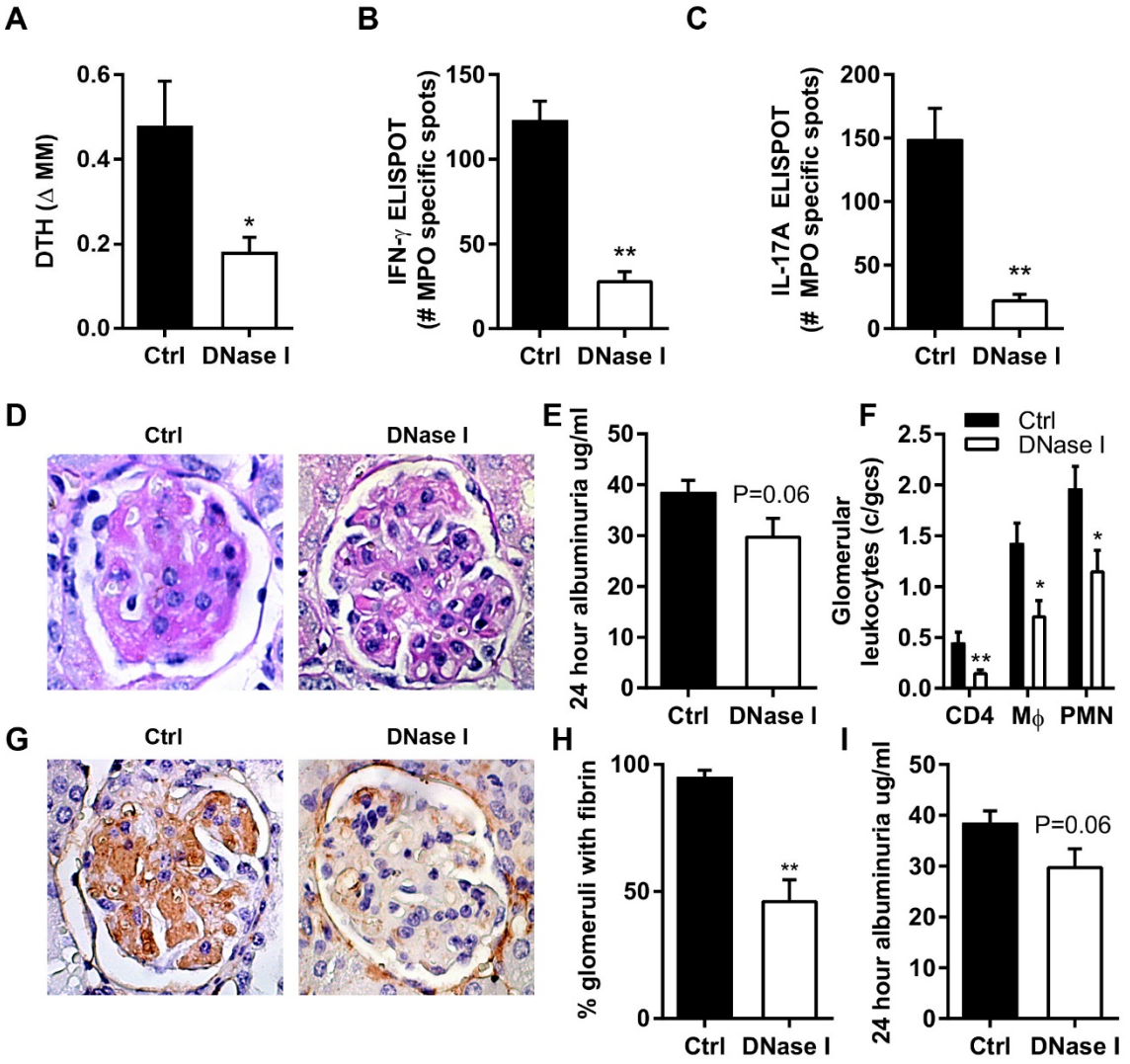


Figure 5. **DNase I treatment reduces systemic autoimmunity to MPO and glomerular injury in established anti MPO –GN.** DNase I treatment reduces foot pad swelling when challenged with MPO (**A**). MPO specific T cell IFNγ and IL-17A ELISPOTs from single cells isolated from lymph nodes from draining sites of MPO immunisation are reduced with DNase I treatment (**B** and **C**). Glomerular necrosis is reduced with an associated significant reduction in glomerular leukocyte recruitment (macrophages, Neutrophils and CD4 T cells), with DNase I treatment (**D-H**). Functional injury as assessed by albuminuria is also less in DNase I treated mice (**I**). *P<0.05, **P<0.01. Data are means ± SEM from six mice in each group analyzed by Mann-Whitney U. Anti MPO-GN, Anti Myeloperoxidase Glomerulonephritis; MPO, myeloperoxidase; IFNγ, Interferon gamma; IL-17A, Interleukin 17A, ELISPOT, Enzyme-Linked ImmunoSpot; DNase I, Deoxyribonuclease I; Ctrl, Control

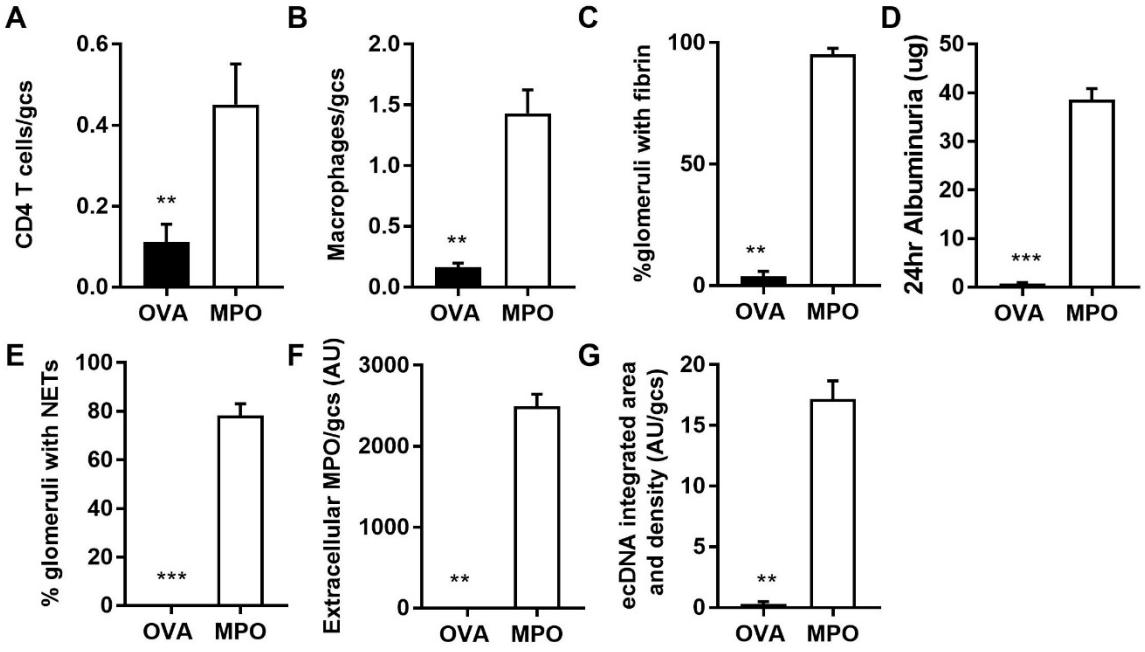


Figure 6. **Subnephritigenic anti GBM Ig does not induce injury alone**. Glomerular injury in anti MPO GN results from effector responses to MPO and cannot be attributed to direct glomerular injury from the GN inducing anti GBM antibodies administered to induce glomerular MPO deposition. In the absence of systemic autoimmunity to MPO (as shown in control mice give OVA in FCA) administration of a subnephritigenic dose of anti GBM antibody does not induce systemic leukocyte influx (**A-C**) albuminuria (**D**) , NET deposition (**E**), extracellular MPO deposition (**F**), or ecDNA deposition (**G**) *P<0.05,**P<0.01, ***P<0.001. Data are means± SEM from six mice in each group analyzed by Mann-Whitney U. Ctrl, Control; DNase I, Deoxyribonuclease I. MPO, myeloperoxidase, OVA, ovalbumin, ecDNA extracellular deoxyribonucleic acid, NETs, neutrophil extracellular traps.

DNase I attenuates the establishment of autoimmunity to MPO

We compared the development of anti MPO autoimmunity in C57/BL6 mice treated with DNase I and control mice given saline. To assess the effect of DNase I on the establishment of MPO autoimmunity, DNase I was given to C57/Bl6 WT mice (*n*=10) for 11 days, 1 day prior to sub cutaneous immunisation with recombinant MPO and CFA, culled 10 days later, and compared to MPO/CFA immunised C57/Bl6 (*n*=10) saline-treated controls (Figure 8 A). A third group of animals were immunised with OVA an irrelevant protein to confirm antigen specificity of induced autoimmunity to MPO. OVA immunised animal did not develop MPO specific autoimmunity (Figures 7, A-D). DNase I treated mice had a significant reduction in dermal

DTH, measured by a 24 hour footpad challenge with MPO (Figure 8 B). Cells from the draining lymph nodes from MPO immunisation sites, were restimulated with MPO ex vivo for 16 hours. Assessment of MPO-specific proliferation of IFNγ producing cells from the draining lymph nodes demonstrated a significant reduction in the numbers of cells via ELISPOT in the DNase I treated group whereas there was no significant differences in proliferation of MPO-specific IL-17A cells. (Figures 8, C and D). Interestingly, there was a significant upregulation in the percentage of MPO specific CD4+FoxP3+CTV+ T regulatory cells from the draining lymph nodes of the mice treated with DNase I (Figure 8, E). This data strongly suggests that DNase I inhibits the development of anti MPO autoimmunity by reducing the numbers of generated effector cells and increasing the number of regulatory cells.

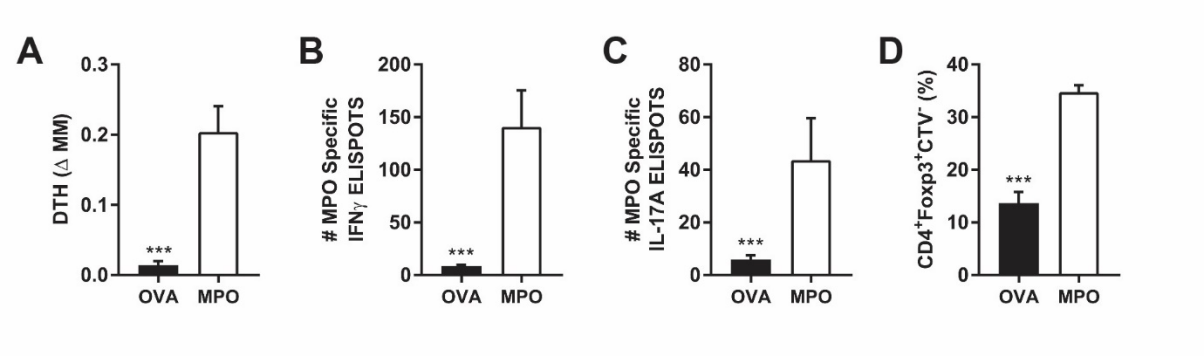


Figure 7. Mice immunised with OVA do not develop autoimmunity to MPO. Mice immunised with OVA, and culled 10 days later do not develop DTH to OVA (**A**), when challenged for 24 hours in the footpad, or produce significant MPO specific IFNγ (**B**) or IL-17A ELISPOTS (**C**) or significant T regulatory cells (**D**) in response to MPO, from cells from the draining lymph nodes.

DNase I inhibits DC migration and activation in lymph node draining MPO immunisation sites

To test the hypothesis that DNase I can modulated DC activation and induce T regulatory cells the effect of DNase I was examined at the time of antigen presentation. DNase I was administered IV to C57/BL6 mice 24 hours before immunisation with MPO in FCA, and mice culled 18 hours later (Figure 9, A). Draining lymph nodes were removed and cells examined for CD11c+ and their co-expression of activation markers by FACs analysis. Eighteen hours after immunisation with MPO the mice treated with DNase I had a significant reduction in the proportion of CD11c +ve (Figure 9, B) DCs migrating to the draining lymph nodes had a statistically significant reduction in all the activation markers including CD80, CD86, CD40, OX40L, ICOS, and MHCII (Figures 9, C-H). The DNase I treated group also had significant reduction in the proportion of activated T cells (CD4+CD69+) (Figure 9, I). These findings are consistent with DNase I inhibiting DC activation and migration to nodes. Therefore, it is likely that DNase I induced reduction in DC antigen presentation is the mechanism by which this enzyme reduces anti MPO autoimmunity.

DNase I attenuates establishment of disease in an ANCA mediated model of anti MPO GN

To isolate the effect of DNase I on ANCA induced glomerular injury, we used a well characterised model of glomerular injury induced by passive transfer of high titre ANCA. The injection of purified anti MPO IgG induces pauci-immune necrotizing GN, and isolates the effect of antibody alone without the contribution of anti MPO T cell autoimmunity. We used the widely studied protocol of injecting LPS and high titre ANCA (Xiao et al., 2002). Mice were culled 6 days later to assess glomerular and functional injury. DNase I (or control saline) was given 24 hours prior to disease induction and 12 hourly until the end of the experiment (Figure 10, A). The amount of ecDNA was examined using the same method described in the previous sections. Results demonstrated a significant reduction in the amount of extracellular DNA deposited within the glomeruli of mice treated with DNase I compared to the vehicle treated

mice (Figures 10 B, C and E). DNase I expression within the tubulointerstitium of anti MPO GN mice was significantly less compared to those anti MPO-GN mice therapeutically treated with DNase I (Figures 10, D and F).

The saline treated group had prominent glomerular NET formation, whereas the DNase I treated mice had no glomeruli with NETs. Furthermore, the non-treated mice had significant deposits of extracellular MPO (as defined previously) whilst those that received DNase I had significantly less MPO deposition (Figures 11, A-D). Examination of glomerular injury assessed histologically on PAS-stained kidneys sections demonstrated a significant reduction in abnormal glomeruli in the DNase I treated group as well as significant reductions in the numbers of infiltrating glomerular neutrophils, macrophages and CD4 T cells. This was accompanied by a reduction in functional injury measured by albuminuria (Figures 12, A-I). Together these results indicated that DNase I is effective in attenuating ANCA dependent NETosis and consequent glomerular injury.

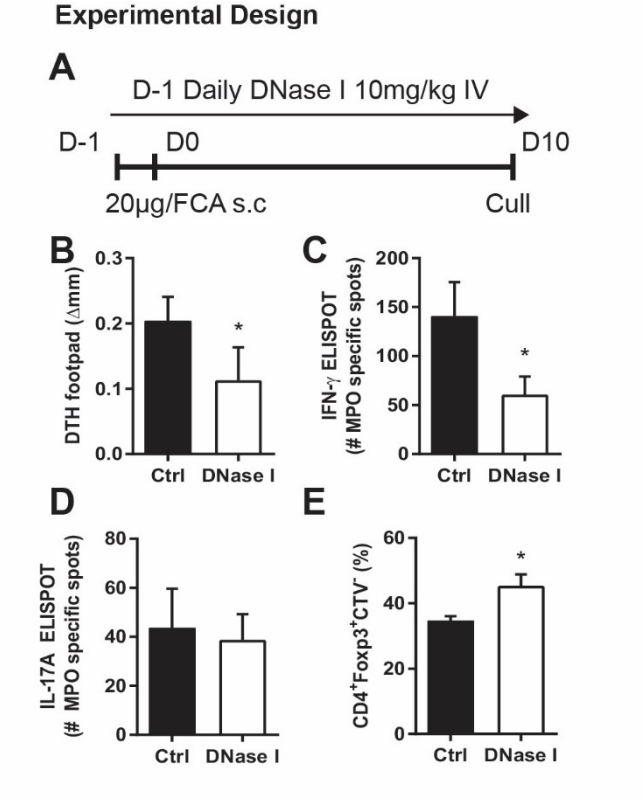


Figure 8. DNase I treatment given during establishment of autoimmunity to MPO reduces systemic anti MPO autoimmunity and enhances the percentage of MPO reactive Tregs (**A**). DNase I reduces foot pad swelling in response to challenge with MPO (**B**). DNase I treatment reduces IFNγ MPO specific ELISPOTS whereas IL-17A MPO ELISPOTS are unaltered (**C** and **D**). The proportion of CD4+FoxP3+CTV+ cells from the draining lymph nodes are significantly increased (**E**). *P<0.05. Data are means ± SEM from ten mice in each group analyzed by Mann-Whitney U. D-1, day minus 1; FCA, Freunds Complete Adjuvant; µg, micrograms; kg, Kilograms; IV, intravenous tail injection; DTH, Delayed Type Hypersensitivity MPO, myeloperoxidase; IFNγ, Interferon gamma; IL-17A, Interleukin 17A, ELISPOT, Enzyme-Linked ImmunoSpot; DNase I, Deoxyribonuclease I; Ctrl, Control.

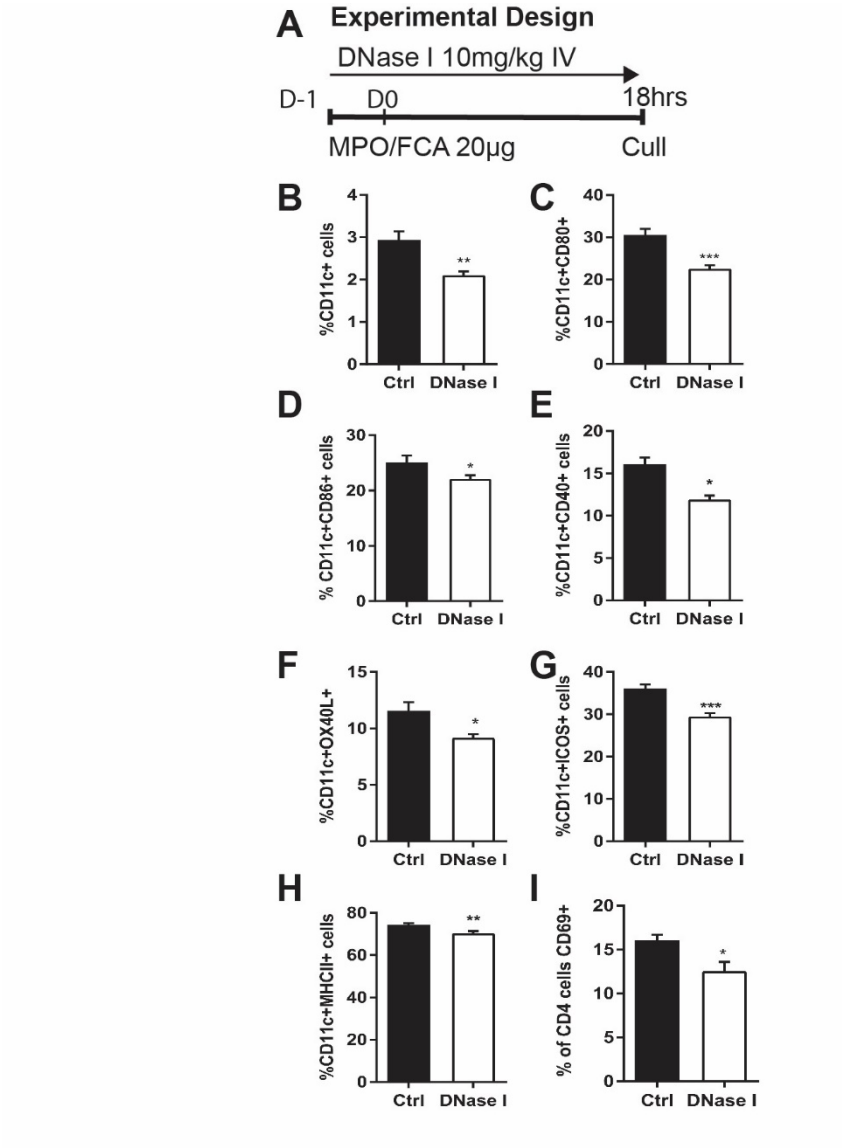


Figure 9. FACS analysis of CD11c+ Dendritic cells from mice given DNase I at the time of antigen presentation (**A**) DNase I given prior to 18 hour immunisation with MPO in FCA, reduces the numbers of CD11c+ Dendritic Cells migrating to the draining lymph nodes and reduces CD11c+ co-stimulatory molecules CD80, CD86 and CD40 expression (**B-D**), CD11c+OX40L and CD11c+ICOS+ cells (**E-F**) and CD11c+MHCII+, with an increase in the proportion of activated CD4+CD69+ T cells. *P<0.05, **P<0.01, ***P<0.001. Data are means ± SEM from ten mice in each group analyzed by Mann-Whitney U. FACS, Fluorescence-activated cell sorting; CD80, Cluster of differentiation; CD86, Cluster of differentiation 86; CD40, Cluster of differentiation 40(co-stimulatory molecules); OX40L, CD252 (marker of activation);ICOS, Inducible T-cell co-Stimulator; MHCII, major histocompatibility complex; CD69, Cluster of differentiation 69 (marker of T cell antigen activation). DNase I, Deoxyribonuclease I; Ctrl, Control.

Figure 10. Extracellular DNA is prominent in ANCA dependent MPO-GN

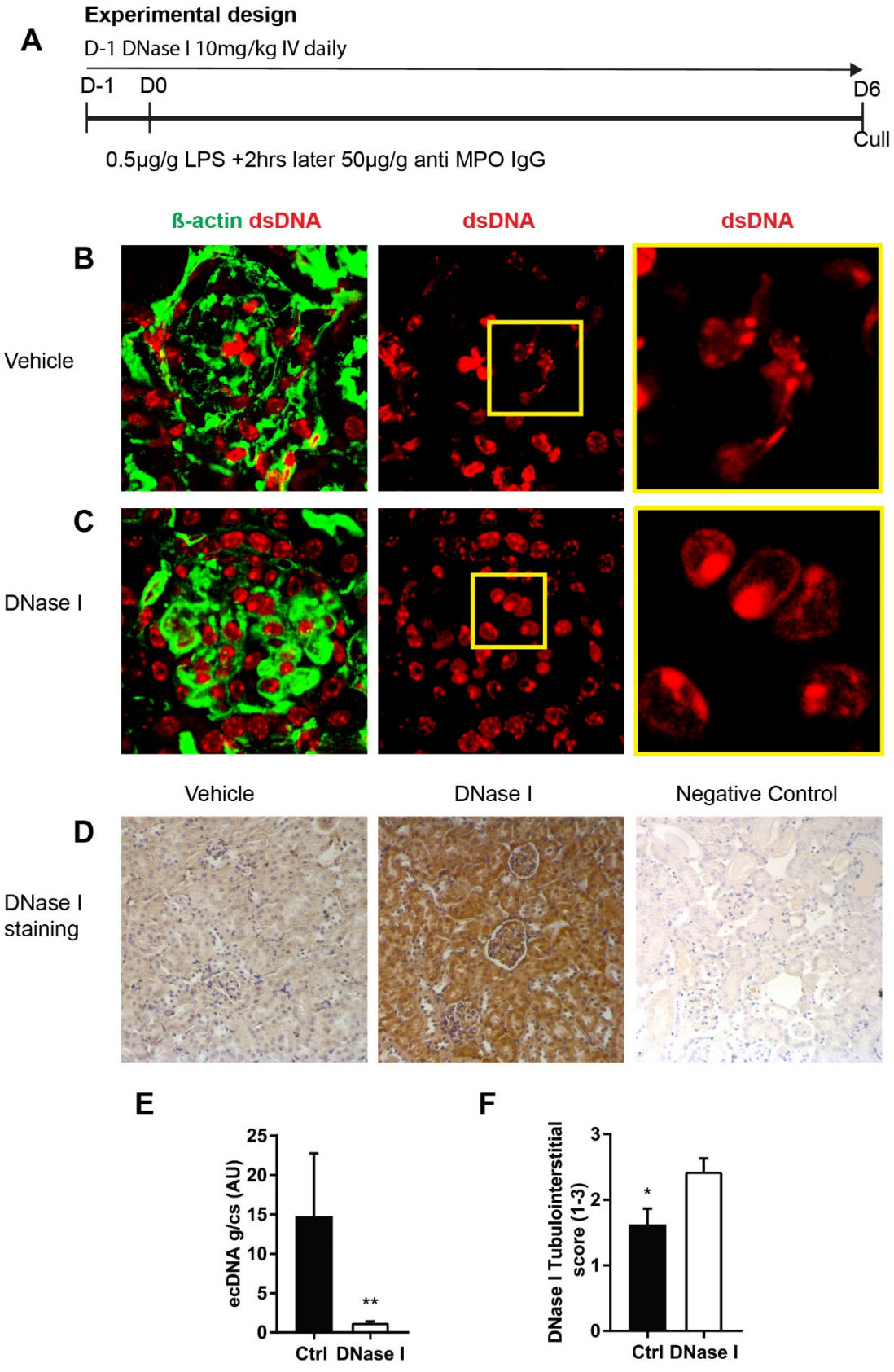


Figure 10 Legend. Extracellular DNA is a prominent feature in in vehicle treated ANCA dependent anti MPO GN. (**A**) Experimental murine anti- MPO GN vehicle treated mice have significant deposits of ecDNA (red) as determined by measuring extra nuclear DNA and counter stained with β actin (green)(**B**), compared to mice therapeutically administered DNase I (**C**). Administration of DNase I partially replenishes level of DNase I (**D**). Semi quantitation in Image J of ecDNA expressed as arbitrary units (**E**). Histological scoring of the tubulointerstitium of mouse kidneys for DNase I (**F**) *P<0.05, **P<0.01. Data are means ± SEM from six mice in each group analyzed by Mann-Whitney U or Kruskal-wallis for 3 or more groups. Abbreviations: MPO, myeloperoxidase, ANCA anti neutrophil cytoplasmic antibody, GN, glomerulonephritis, Ctrl, Control; DNase I, Deoxyribonuclease I; ecDNA, extracellular deoxyribonucleic acid. Original magnification 400x.

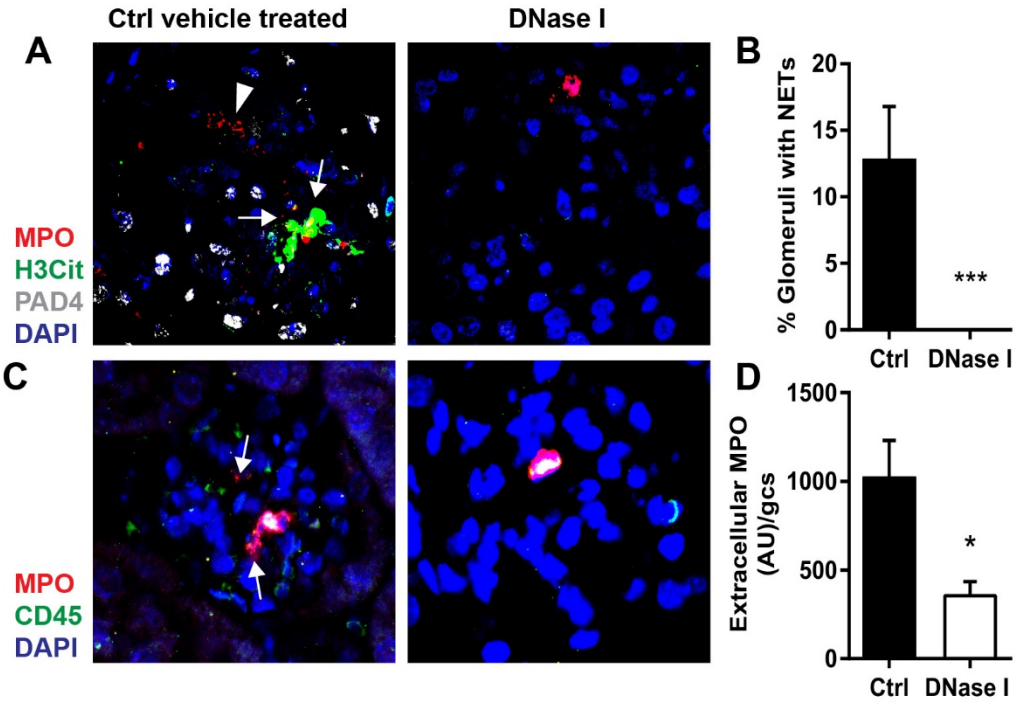


Figure 11. NET formation is abolished in an ANCA dependent model of anti MPO GN. (**A**)Non-treated mice developing a passive MPO ANCA induced model of GN demonstrate significant glomerular NET formation, detected by MPO (red) PAD4 (white) H3Cit (green) and DAPI blue (arrow) compared to DNase I treated mice where NET formation was abolished (**B**) Semi-quantification of NETs demonstrated a significant reduction in NET formation with DNase I treatment. (**C**) Non-treated Mice have significant deposits of extracellular MPO measured using a macro in Image J, DNase I treatment significantly reduces the extent of extracellular MPO accumulation (**D**). *P<0.05, ***P<0.001. Data are means ± SEM from six mice in each group analyzed by Mann-Whitney U. MPO, myeloperoxidase: PAD4, peptidylarginine deiminase; H3Cit, Citrullinated Histone 3; DAPI, 4', 6-diamidino-2-phenylindole, Ctrl, Control; DNase I, Deoxyribonuclease I; CD45, pan leukocyte marker C45. Original Magnification 600x.

Figure 12. DNase I treatment reduces glomerular injury in a ANCA dependent model of anti MPO GN

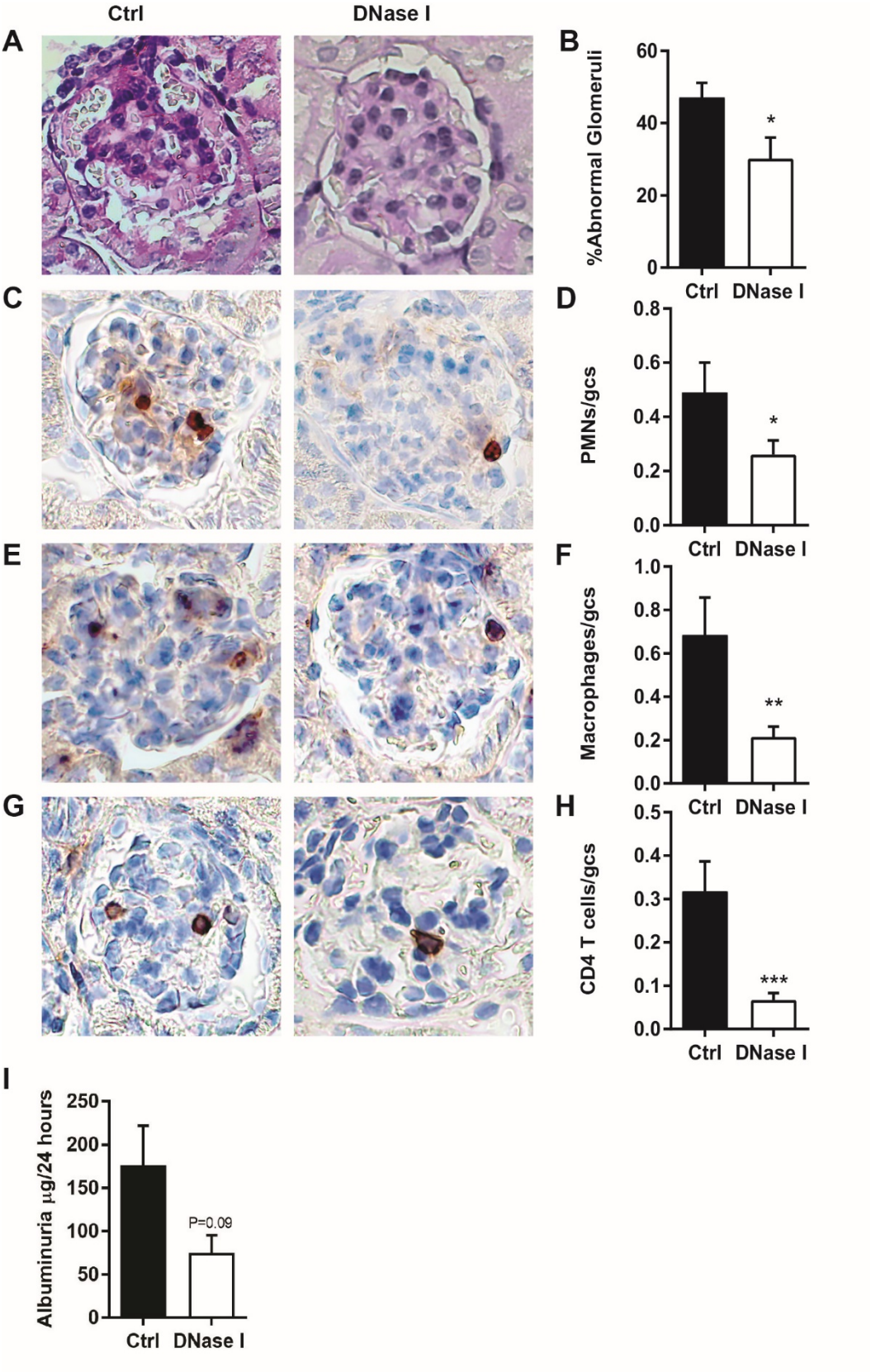


Figure 12 Legend. DNase I treatment reduces glomerular injury in an ANCA mediated model of anti MPO-GN (**A-B**) Abnormal glomeruli are reduced in mice given DNase I treatment, with a significant reduction in the recruitment of glomerular leukocytes (neutrophils, macrophages, CD4 T cells) (**C-H**) and a reduction in 24 hour albuminuria (**I**). *P<0.05, ** P<0.01, ***P<0.001. Data are means± SEM from six mice in each group analyzed by Mann-Whitney U. Ctrl, Control; DNase I, Deoxyribonuclease I. Original Magnification 400x.

Discussion

The current studies demonstrate that therapeutic administration of DNase I to mice with established anti MPO autoimmunity and GN was able to significantly attenuate both autoimmunity and GN. This supports the potential use of this therapy as treatment for human disease (human anti MPO GN) or MPO ANCA microscopic polyangiitis. These results are consistent with effective treatment of disease resulting from administration of exogenous DNase I which reduced the extracellular deposited DNA burden. DNase I may also act upon DNA produced from other types of cell death such as necroptosis a form of regulated necrosis which is also associated with NET like DNA, or apoptosis (Desai et al., 2016). DNase I has been shown to be protective in a rat model of ischemia/reperfusion-induced acute kidney injury. DNase I treated rats demonstrated an increased clearance of DNA from renal apoptotic cells with an enhancement of renal cell proliferation (Peer et al., 2016).

DNase I administration significantly attenuated the generation of anti MPO autoimmunity consistent with the role of DNA in driving the generation of autoimmunity. Surprisingly, even in mice with well-established anti-MPO autoimmunity, DNase I treatment was able to significantly attenuate autoimmunity within 4 days, an important characteristic for treating rapidly progressive GN. These results suggest that DNase I therapy is likely to induce early clinical responses. As well as attenuation of T effector cell responses, we observed an increase in T regulatory cells and a significant increase in the frequency of MPO specific Tregs. It is likely that this suppression may account for the rapidity of the diminution of established anti-MPO autoimmunity.

The only way that anti MPO autoimmunity can be generated in animals is by active immunisation with MPO and adjuvant. While this develops nephritigenic cell mediated immunity and ANCA, the ANCA is not of sufficient titre to initiate GN. In this model, humoral autoimmunity is by-passed by planting MPO in glomeruli allowing GN to develop and the role

of DNase I to be evaluated. In this active model of cell mediated MPO ANCA GN, DTH effector responses induce glomerular focal necrosis, macrophage recruitment, activation and fibrin deposition the key elements of crescent formation. DNase I administered after induction of autoimmunity and initiation of glomerular injury, reduced the recruitment of glomerular neutrophils, decreased NET formation and subsequent deposited extracellular MPO, therefore reducing the amount available to maintain effector responses. This was accompanied by an increase in the proportion of antigen specific T regulatory cells in the draining lymph nodes (CD4+FoxP3+) and decreased numbers of MPO specific IFNγ cells.

Pre-emptive DNase I administration attenuated the development of autoimmunity to MPO resulting in reduced MPO specific DTH response and the frequency of MPO specific IFNγ producing cells while increasing the frequency of MPO specific CD4+FoxP3+ Tregs (activating in recall response to MPO). These results confirm a role for DNA in the induction of anti-MPO autoimmunity suggesting that either the adjuvant contains significant DNA or attracts local inflammation depositing endogenous DNA. Direct proof for a role for DNA comes from the capacity for DNA subcomponents (e.g. CpG, bacterial DNA component) alone to induce anti-MPO autoimmunity and GN (Summers et al., 2011).

Furthermore, DNase I given at the time of antigen presentation results in a reduction in the proportion of DCs migrating to the draining lymph nodes from MPO immunisation sites. The migrating DCs also show a reduction in DC activation markers resulting in decreasing the frequency of activated CD4 effector T cells. DNA provides a danger signal to both macrophages and DCs which increases levels of MHCII expression (Ishii et al., 2001; Suzuki et al., 1999). The mechanism of this activation involves selective DC uptake by the scavenger receptor DEC-205 followed by binding to the DNA cytosolic receptor TLR9 inducing upregulation of co-stimulatory molecules (CD40 and CD80/86). Therefore, it is likely that DNase I acts by degrading the amount of extracellular DNA available to act as DAMPs. DNase I has been shown to inhibit the induction of DC MHCI and MHCII. Furthermore, evidence for a homeostatic/protective role for DNase I or DNase II is the observation that mice deficient in

DNase I or DNase II will develop autoimmunity spontaneously with outcomes that include glomerulonephritis (Napirei et al., 2000; Yoshida et al., 2005).

Assessment of humoral autoimmunity can be studied by immunizing mice and pooling high-titre MPO-ANCA sera and assessing the effects of these antibodies by passively transferring MPO-ANCA IgG to naïve WT recipients. Passive MPO-ANCA IgG produces relatively weak disease and most investigators co-inject an adjuvant, TLR ligand or a proinflammatory cytokine. We demonstrated using the humoral model of passive ANCA induced GN that ANCA recruits neutrophils to glomeruli and directs their NETosis and deposition of NET associated and cell free extracellular DNA and MPO resulting in glomerular injury. Our study demonstrated that administration of DNase I reduced the amount of extracellular MPO able to cause injury either directly or as an autoantigen by recruiting MPO-specific CD4 T cells. DNase I treatment of *in vivo* neutrophils has shown that DNase I is associated with a decrease in ROS levels and does not directly inhibit MPO, but disintegrates the extra-cellular matrix of DNA that the MPO is associated with, which decreases the biological activity of the MPO (Munafo et al., 2009). These modulating effects on neutrophils in the circulation are likely to account for the significant reduction of ANCA inducing neutrophil accumulation in glomeruli. DNase I given at therapeutic doses abrogates NET formation, DNA deposition and significantly reduces glomerular neutrophil accumulation. This results in significant improvement in glomerular function (assessed by albuminuria) and pathological injury (assessed by focal segmental necrosis). In the passive ANCA induced GN model of injury, T cell autoimmunity is absent therefore permitting direct assessment for the role of DNase I on humoral immunity. Both these models are of insufficient duration to effectively assess the effects of DNase I on the long term development of ANCA.

DNase I is already used effectively in treatment of cystic fibrosis to hydrolyze the DNA present in mucous from lungs largely containing dead neutrophils (Pressler, 2008). A randomized clinical trial treating patients with SLE has demonstrated that administration of recombinant human DNase I is safe and tolerated well by patients, but clinically ineffective against SLE.

The study was limited by small patient numbers and limited exploration of routes of delivery of DNase I, however it demonstrated previously unknown pharmacokinetics of DNase I in humans and no significant adverse events following treatment (Davis et al., 1999).

It is important to note that the critical steps in the immunopathogenesis of anti-MPO GN include neutrophil accumulation in glomeruli, NET formation, deposition of glomerular MPO as well as the prominence in the target organ of DTH effector T cell, macrophages and fibrin which was observed in this model in mice, in a manner exactly the same as seen in human anti MPO GN. This disease is driven by autoimmunity to the dominant MPO epitope recognised by sera of patients with MPO which has strong homology with the immunodominant nephritic T cell epitope in mice (Ooi et al., 2012). The fact that all of these pathways are immunomodulated by DNase I suggests a symbiosis of therapeutic relevance. It also strongly suggest that this treatment could be effective in man. Moreover, the low incidence of adverse effects and high tolerance in patients suggests that DNase I is likely to be a substitute for harmful anti-inflammatory drugs like corticosteroids. This current work gives strength to the proposition that a head-to-head evaluation of standard therapy plus DNase I substituted for corticosteroids versus standard therapy plus corticosteroids should be considered.

Acknowledgments

We acknowledge Monash Micro Imaging for the provision of the Nikon, Confocal Microscope, and the Australian Government for providing a Research Training Program Scholarship.

REFERENCES

Apostolopoulos, J., Ooi, J. D., Odobasic, D., Holdsworth, S. R., & Kitching, A. R. (2006). The isolation and purification of biologically active recombinant and native autoantigens for the study of autoimmune disease. *J Immunol Methods, 308*(1-2), 167-178. doi:10.1016/j.jim.2005.10.011

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., .Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science, 303*(5663), 1532-1535. doi:10.1126/science.1092385

Davis, J. C., Jr., Manzi, S., Yarboro, C., Rairie, J., McInnes, I., Averthelyi, D.,Klippel, J. H. (1999). Recombinant human Dnase I (rhDNase) in patients with lupus nephritis. *Lupus, 8*(1), 68-76.

Desai, J., Mulay, S. R., Nakazawa, D., & Anders, H. J. (2016). Matters of life and death. How neutrophils die or survive along NET release and is "NETosis" = necroptosis? *Cell Mol Life Sci, 73*(11-12), 2211-2219. doi:10.1007/s00018-016-2195-0

Ishii, K. J., Suzuki, K., Coban, C., Takeshita, F., Itoh, Y., Matoba, H.,Klinman, D. M. (2001). Genomic DNA released by dying cells induces the maturation of APCs. *J Immunol, 167*(5), 2602-2607.

Kessenbrock, K., Krumbholz, M., Schonermarck, U., Back, W., Gross, W. L., Werb, Z., .Jenne, D. E. (2009). Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med, 15*(6), 623-625. doi:10.1038/nm.1959

Munafo, D. B., Johnson, J. L., Brzezinska, A. A., Ellis, B. A., Wood, M. R., & Catz, S. D. (2009). DNase I inhibits a late phase of reactive oxygen species production in neutrophils. *J Innate Immun, 1*(6), 527-542. doi:10.1159/000235860

Nakazawa, D., Shida, H., Tomaru, U., Yoshida, M., Nishio, S., Atsumi, T., & Ishizu, A. (2014). Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. *J Am Soc Nephrol, 25*(5), 990-997. doi:10.1681/ASN.2013060606

Napirei, M., Karsunky, H., Zevnik, B., Stephan, H., Mannherz, H. G., & Moroy, T. (2000). Features of systemic lupus erythematosus in Dnase1-deficient mice. *Nat Genet, 25*(2), 177-181. doi:10.1038/76032

O'Sullivan, K. M., Lo, C. Y., Summers, S. A., Elgass, K. D., McMillan, P. J., Longano, A., Holdsworth, S. R. (2015). Renal participation of myeloperoxidase in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. *Kidney Int, 88*(5), 1030-1046. doi:10.1038/ki.2015.202

Ooi, J. D., Chang, J., Hickey, M. J., Borza, D. B., Fugger, L., Holdsworth, S. R., & Kitching, A. R. (2012). The immunodominant myeloperoxidase T-cell epitope induces local cell-mediated injury in antimyeloperoxidase glomerulonephritis. *Proc Natl Acad Sci U S A, 109*(39), E2615-2624. doi:10.1073/pnas.1210147109

Ooi, J. D., Gan, P. Y., Odobasic, D., Holdsworth, S. R., & Kitching, A. R. (2014). T cell mediated autoimmune glomerular disease in mice. *Curr Protoc Immunol, 107*, 15 27 11-19. doi:10.1002/0471142735.im1527s107

Peer, V., Abu Hamad, R., Berman, S., & Efrati, S. (2016). Renoprotective Effects of DNAse-I Treatment in a Rat Model of Ischemia/Reperfusion-Induced Acute Kidney Injury. *Am J Nephrol, 43*(3), 195-205. doi:10.1159/000445546

Pressler, T. (2008). Review of recombinant human deoxyribonuclease (rhDNase) in the management of patients with cystic fibrosis. *Biologics, 2*(4), 611-617.

Pressler, T., Karpati, F., Granstrom, M., Knudsen, P. K., Lindblad, A., Hjelte, L., Scandinavian, C. F. S. C. (2009). Diagnostic significance of measurements of specific IgG antibodies to Pseudomonas aeruginosa by three different serological methods. *J Cyst Fibros, 8*(1), 37-42. doi:10.1016/j.jcf.2008.08.002

Sangaletti, S., Tripodo, C., Chiodoni, C., Guarnotta, C., Cappetti, B., Casalini, P.,Colombo, M. P. (2012). Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity. *Blood, 120*(15), 3007-3018. doi:10.1182/blood-2012-03-416156

Summers SA, Steinmetz OM, Gan PY, Ooi JD, Odobasic D, Kitching AR, et al. Toll-like receptor 2 induces Th17 myeloperoxidase autoimmunity while Toll-like receptor 9 drives Th1 autoimmunity in murine vasculitis. Arthritis & Rheumatism. 2011;63(4):1124-35.

Suzuki, K., Mori, A., Ishii, K. J., Saito, J., Singer, D. S., Klinman, D. M.,Kohn, L. D. (1999). Activation of target-tissue immune-recognition molecules by double-stranded polynucleotides. *Proc Natl Acad Sci U S A, 96*(5), 2285-2290.

Takai, T., Ono, M., Hikida, M., Ohmori, H., & Ravetch, J. V. (1996). Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. *Nature, 379*(6563), 346-349. doi:10.1038/379346a0

Xiao, H., Heeringa, P., Hu, P., Liu, Z., Zhao, M., Aratani, Y.,Jennette, J. C. (2002). Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest, 110*(7), 955-963. doi:10.1172/JCI15918

Yoshida, H., Okabe, Y., Kawane, K., Fukuyama, H., & Nagata, S. (2005). Lethal anemia caused by interferon-beta produced in mouse embryos carrying undigested DNA. *Nat Immunol, 6*(1), 49-56. doi:10.1038/ni1146

CHAPTER 6: DISCUSSION AND CONCLUSION

DISCUSSION AND CONCLUSION

ANCA associated glomerulonephritis is a unique autoimmune disease as the target autoantigens MPO, PR3 and LAMP2 are not constitutively expressed by the kidney. Crescentic glomerulonephritis, without the deposition of immunoglobulins was first described by Stilmant et al in 1979 (Stilmant et al., 1979) and further characterised by Falk and Jennette in 1988 (Falk & Jennette, 1988), to be associated with circulating Anti Neutrophil Cytoplasmic Antibody (ANCA), directed against myeloperoxidase (MPO) and (PR3), constituents of neutrophil cytoplasmic lysosomes. In AAV activated neutrophils are recruited to the glomerulus where neutrophil MPO is exteriorised to the cell surface, providing an antigenic target for ANCA, which binds to the exposed MPO on the cell surface and triggers the release of MPO via neutrophil degranulation or NETosis. The role of MPO once deposited within the glomeruli is not completely understood, however it has been hypothesised to play different roles.

Firstly, the release of MPO can cause direct injury through the release and activation of the MPO-H_20_2 system. Extracellular MPO is highly cationic and will bind to endothelial cells within the capillary loops of glomeruli, subsequent halogenation of the tissue can occur when MPO facilitates the catalytic conversion of H_20_2 to $HOCl^-$ resulting in extensive damage to glomeruli (Johnson et al., 1987). This paradigm would support a non-immunologic role for MPO, and could offer an explanation as to why in the absence of antibody deposition injury occurs in AAV. Secondly, activated glomerular neutrophils, can be triggered by bound ANCA to release NETs containing DNA, citrullinated histones, proteases and MPO (Kessenbrock et al., 2009; Wang et al., 2016). MPO when bound to DNA is proinflammatory with increased biological activity compared to extracellular MPO, and has the potential to cause direct injury (Parker, et al., 2012). Lastly, the glomerular deposition of MPO, provides an autoantigenic target for CD4 T effector cells generated specifically due to the loss of tolerance to MPO, independent of the humoral response generated by ANCA. Glomerular crescent formation, macrophage recruitment and fibrin

deposition, with the accumulation of pathogenic CD4 T cells (Th1 and Th17), are indicative of a delayed type hypersensitivity reaction playing a role in the pathogenesis of AAV (Gan et al., 2010; Ruth et al., 2006).

In this thesis, I hypothesised that an initial loss of tolerance to MPO results in the production of MPO-ANCA and the generation of MPO specific CD4 T cells within the circulation. ANCA activated neutrophils are recruited to the glomerulus (via upregulation of TLRs and chemokines on intrinsic glomerular cells) where they degranulate or produce NETs of DNA decorated with MPO. This process occurs normally to enhance neutrophils bactericidal killing capacity, however in sterile inflammation they are potentially injurious to bystander tissue, depositing proteases, enzymes, and reactive oxygen species (ROS). Importantly in this disease the MPO is exteriorized and has enhance biological activity when adhered to DNA. After the MPO is deposited DTH effectors (CD4 T cells, Macrophages and fibrin) are recruited subsequently bringing in more neutrophils, resulting in a vicious cycle of glomerular insult which develops in focal segmental glomerulonephritis.

In chapter two, I hypothesised that extracellular MPO derived from neutrophil degranulation or leukocyte extracellular traps participates in inducing glomerular and interstitial injury. To test this hypothesis, I assessed the renal deposition of MPO within kidney biopsies of 47 MPO-AAV patients and 11 PR3-AAV patients and correlated it with the degree of histological and functional injury. MPO was associated with MPO+ Neutrophils and macrophages, and the glomerular intrinsic cells podocytes and endothelial cells. I defined the identification of renal NETs and enumerated the number of glomeruli containing NETs, the largest of this type of study in AAV done to date. A unique finding of this study was the identification of MPO+ macrophage extracellular traps (METs), which have not previously been described in AAV. This observation suggests that macrophages which are numerically significantly greater in number within the glomeruli of AAV patients than neutrophils, may play more of a role in the pathogenesis of AAV than previously thought. It is likely these MPO+ cells represent proinflammatory M1 macrophages, and warrants further investigation.

The numbers of CD4 and CD8 positive T cells correlated with a decrease in functional injury determined by eGFR, indicative of a pathogenic role. This supports observations in murine models of MPO-ANCA GN our laboratory has established, where a role for pathogenic CD4 and CD8 T cells has been demonstrated (Chang et al., 2017; Ruth et al., 2006). This work extends current knowledge derived from *in vitro* experiments and experimental models of MPO-ANCA GN and determines a likely pathogenic role for MPO in the progression of AAV.

Studies on the role of MCs in human or experimental AAV are limited. Histological observation of MCs in glomerulonephritis have been observed with an association between MC numbers and disease severity, indicating a potential injurious role in the pathogenesis of GN (Otsubo et al., 2003; Velden et al., 2012). To date, there is no published work indicating the phenotype of the mast cells infiltrating the kidneys of patients with AAV. Mast cells can have both an injurious pro-inflammatory role as well as immune regulatory functions through the secretion of IL-10. In chapter three, I hypothesised that the stabilisation of mast cells by disodium chromoglycate (DSCG) will prevent mast cell degranulation and prevent injury in a model of anti- myeloperoxidase glomerulonephritis. To test this hypothesis, I collaborated with a colleague within our laboratory Dr. Poh-Yi Gan and determined that an activated mast cell phenotype is present in 44 MPO-AAV patient renal biopsies. Secondly, using a model of experimental murine MPO-ANCA glomerulonephritis we demonstrate that administration of a mast cell specific stabilizer disodium chromoglycate (DSCG) before and after establishment of autoimmunity to MPO stabilises MC degranulation, and attenuates development of GN, mediated by the production of MC derived IL-10. These results suggest that DSCG can prevent the pro-inflammatory injurious function of MCs and enhance the immunomodulatory role of MCs, and therefore could be a potential therapeutic in AAV.

Infections can initiate and exacerbate disease in patients with AAV and provoke disease relapse (Tidman et al., 1998). Toll- like receptors may be the essential link between infection

and autoimmunity. In chapter 4, I hypothesised that Toll-like Receptor 2 (TLR2), Toll-like Receptor 4 (TLR4) and Toll-Like receptor 9 (TLR9) will be dysregulated in patients with AAV and contribute to the injury and perpetuation of inflammation. To test this hypothesis I examine the role of Toll-like Receptors in 38 renal biopsies of AAV patients. This study evaluated aberrant expression of TLR2, TLR4 and TLR9 to test the hypothesis that increased TLR expression would correlate with renal injury. AAV renal biopsies had significant renal disposition of TLR2, TLR4 and TLR9 compared to control patients. Elevated TLR4 and TLR2 expression correlated with structural injury (histological) and functional injury (eGFR). These observations suggest that TLR4 and TLR2 may be potential therapeutic targets in AAV.

In chapter two, I found evidence of considerable NET deposition within the glomeruli of patients with AAV. The deposition of NETs correlated strongly with the amount of glomerular deposited MPO, indicative of NETs providing a substrate for MPO. The key component of NETs is DNA which is critical for NETosis and is a powerful immune stimulant, which enhances the biological activity of MPO (Parker, et al., 2012). Therefore specifically targeting and disrupting extracellular DNA accumulation, eliminates the pro-inflammatory effect of extracellular DNA, and eliminates the support network for the adhered MPO. DNase I specifically targets and clears extracellular DNA and is already used therapeutically to treat cystic fibrosis patients to clear mucous and neutrophil nuclear remnants (Suri et al., 2002). In Chapter 5, I test the hypothesis that DNase I will eliminate extracellular DNA released from injured and dying cells (including neutrophils undergoing NETosis) in an experimental model of MPO-ANCA GN. DNase I, reduced NET formation, glomerular MPO deposition, structural injury (segmental glomerulosclerosis) and surprisingly reduced autoimmunity to MPO. DNase I treatment, reduced DC migration to the draining lymph nodes, and significantly reduced the DC expression of activation markers (CD80, CD86, MHCII, CD40, and significantly reduced the number of activated T cells (defined by the numbers of CD4 T cells expressing CD69). Surprisingly, administrating DNase I also

modulated the immune response resulting in an increased number of CD4 T regulatory T cells. These results strongly support a key role for extracellular DNA in this disease by augmentation of anti MPO autoimmunity and proinflammatory effects on effector responses in the kidney.

An additional study included in this thesis within the appendix demonstrates that NETs may play a role in other forms of glomerulonephritis. Collaboration with colleagues within our laboratory using intra vital microscopy demonstrated that NETs are present within glomeruli in an experimental mouse model of acute anti GBM. NETs were present transiently within glomeruli in this model, however DNase I was able to moderately reduce injury, indicating that in this alternative form of GN, NETs may also play a role in injury.

Future Directions

It has been projected in Australia that that the cost of treating end stage renal disease (ESRD), will accumulate to 12 billion dollars from the year 2009-2020. Glomerulonephritis, contributes to 20% of the total listed Australian Chronic Kidney Disease (CKDs) and is the second most frequent cause of end stage renal failure in Australia. Current treatment consists of non-targeted immunosupression which is toxic and contributes to morbidity and mortality rates. The average wait time for a kidney transplant in Australia is 3-4 years, it is projected that 0.5% of those on the waiting list will die before receiving a transplant (Kidney Health, Australia 2017). There is an unmet requirement for the development of therapeutics which will target pro inflammatory immune responses, whilst enhancing immunoregulatory mechanisms, without causing adverse effects.

I have demonstrated in this thesis, several potential therapeutic targets in AAV. In Chapter 2, I demonstrate an association with extracellular MPO and disease severity. This suggests that MPO could be a potential target. Questions remain, regarding the nature of the deposited MPO. If the deposited MPO is biologically active and causing direct injury, blocking or eliminating MPO with MPO inhibitors maybe a therapeutic avenue. However, if

the role of MPO is in providing antigen for CD4 T cells, inhibitors may not be as effective. What this chapter highlights is the significant amount of extracellular MPO deposited in the glomeruli of AAV patients, available for binding with ANCAs, yet the disease is characterised as being pauci immune. Further investigations are required to see if the deposited MPO is in a conformation not recognised by ANCA.

In chapter 3, I investigate the role of pro-inflammatory Mast Cells in AAV. Disodium chromoglycate proved to be of therapeutic benefit in an animal model of MPO-ANCA GN. It is unlikely that MCs act alone in the pathogenesis of AAV, but participate in the overall pathogenesis that also involves ANCAs, MPO and T cells. Given the immunomodulatory function of MCs it would be interesting to investigate the role of DSCG in combination with other standard treatment, to see if immunosuppression can be reduced to a level with minimal side effects, in animal models of MPO-ANCA GN.

In chapter 4, I demonstrate that both TLR2 and TLR4 are significantly increased in patients with AAV. Investigation of TLR inhibitors in animal models of the MPO-ANCA GN could reveal new therapeutics, which could be used alone or in combination with standard treatments.

In Chapter 5, I demonstrate the successful attenuation of MPO-ANCA GN in 2 different animal models, using DNase I. This suggests that DNase I is able to modulate both cell mediated pathogenesis and humoral mechanisms. Given that in our model, treatment was given after establishment of MPO autoimmunity, this suggests that DNase I may be a powerful therapeutic in AAV. Further investigations into the pro-inflammatory nature of DNA, in this disease both NET derived and from other sources would be of great value.

Bibliography

BIBLIOGRAPHY

Abbas, A. K., Benoist, C., Bluestone, J. A., Campbell, D. J., Ghosh, S., Hori, S., et al (2013). Regulatory T cells: recommendations to simplify the nomenclature. *Nat Immunol, 14*(4), 307-308. doi:10.1038/ni.2554

Abbate, M., Kalluri, R., Corna, D., Yamaguchi, N., McCluskey, R. T., Hudson, B. G., et al (1998). Experimental Goodpasture's syndrome in Wistar-Kyoto rats immunized with alpha3 chain of type IV collagen. *Kidney Int, 54*(5), 1550-1561. doi:10.1046/j.1523-1755.1998.00153.x

Abdulahad, W. H., Stegeman, C. A., van der Geld, Y. M., Doornbos-van der Meer, B., Limburg, P. C., & Kallenberg, C. G. (2007). Functional defect of circulating regulatory CD4+ T cells in patients with Wegener's granulomatosis in remission. *Arthritis Rheum, 56*(6), 2080-2091. doi:10.1002/art.22692

Abrams, S. T., Zhang, N., Manson, J., Liu, T., Dart, C., Baluwa, F., et al (2013). Circulating histones are mediators of trauma-associated lung injury. *Am J Respir Crit Care Med, 187*(2), 160-169. doi:10.1164/rccm.201206-1037OC

Adu, D., Pall, A., Luqmani, R. A., Richards, N. T., Howie, A. J., Emery, P., et al (1997). Controlled trial of pulse versus continuous prednisolone and cyclophosphamide in the treatment of systemic vasculitis. *QJM, 90*(6), 401-409.

Aggarwal, S., Ghilardi, N., Xie, M. H., de Sauvage, F. J., & Gurney, A. L. (2003). Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem, 278*(3), 1910-1914. doi:10.1074/jbc.M207577200

Agner, K., Andersson, N. S., & Nordenson, N. G. (1948). Verdoperoxidase: a ferement isolated from leucocytes. *Acta Haematol, 1*(3), 193-211.

Allam, R., Scherbaum, C. R., Darisipudi, M. N., Mulay, S. R., Hagele, H., Lichtnekert, J., et al (2012). Histones from dying renal cells aggravate kidney injury via TLR2 and TLR4. *J Am Soc Nephrol, 23*(8), 1375-1388. doi:10.1681/ASN.2011111077

Alon, R., & Feigelson, S. (2002). From rolling to arrest on blood vessels: leukocyte tap dancing on endothelial integrin ligands and chemokines at sub-second contacts. *Semin Immunol, 14*(2), 93-104. doi:10.1006/smim.2001.0346

Amini, P., Stojkov, D., Wang, X., Wicki, S., Kaufmann, T., Wong, W. W., et al (2016). NET formation can occur independently of RIPK3 and MLKL signaling. *Eur J Immunol, 46*(1), 178-184. doi:10.1002/eji.201545615

Ammollo, C. T., Semeraro, F., Xu, J., Esmon, N. L., & Esmon, C. T. (2011). Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein C activation. *J Thromb Haemost, 9*(9), 1795-1803. doi:10.1111/j.1538-7836.2011.04422.x

Anders, H. J. (2000). Diagnosis and management of crescentic glomerulonephritis: state of the art. *Saudi J Kidney Dis Transpl, 11*(3), 353-361.

Arimura, Y., Muso, E., Fujimoto, S., Hasegawa, M., Kaname, S., Usui, J., et al (2016). Evidence-based clinical practice guidelines for rapidly progressive glomerulonephritis 2014. *Clin Exp Nephrol, 20*(3), 322-341. doi:10.1007/s10157-015-1218-8

Atkins, R. C., Holdsworth, S. R., Glasgow, E. F., & Matthews, F. E. (1976). The macrophagen in human rapidly progressive glomerulonephritis. *Lancet, 1*(7964), 830-832.

Babior, B. M. (2004). NADPH oxidase. *Curr Opin Immunol, 16*(1), 42-47.

Babior, B. M., Lambeth, J. D., & Nauseef, W. (2002). The neutrophil NADPH oxidase. *Arch Biochem Biophys, 397*(2), 342-344. doi:10.1006/abbi.2001.2642

Baccala, R., Gonzalez-Quintial, R., Lawson, B. R., Stern, M. E., Kono, D. H., Beutler, B., & Theofilopoulos, A. N. (2009). Sensors of the innate immune system: their mode of action. *Nat Rev Rheumatol, 5*(8), 448-456. doi:10.1038/nrrheum.2009.136

Bagchus, W. M., Jeunink, M. F., & Elema, J. D. (1990). The mesangium in anti-Thy-1 nephritis. Influx of macrophages, mesangial cell hypercellularity, and macromolecular accumulation. *Am J Pathol, 137*(1), 215-223.

Baldini, C., Talarico, R., Della Rossa, A., & Bombardieri, S. (2010). Clinical manifestations and treatment of Churg-Strauss syndrome. *Rheum Dis Clin North Am, 36*(3), 527-543. doi:10.1016/j.rdc.2010.05.003

Ball, G. V. (2010). The history of ANCA-associated vasculitis. *Rheum Dis Clin North Am, 36*(3), 439-446. doi:10.1016/j.rdc.2010.05.004

Banas, M. C., Banas, B., Hudkins, K. L., Wietecha, T. A., Iyoda, M., Bock, E., et al (2008). TLR4 links podocytes with the innate immune system to mediate glomerular injury. *J Am Soc Nephrol, 19*(4), 704-713. doi:10.1681/ASN.2007040395

Bansal, P. J., & Tobin, M. C. (2004). Neonatal microscopic polyangiitis secondary to transfer of maternal myeloperoxidase-antineutrophil cytoplasmic antibody resulting in neonatal

pulmonary hemorrhage and renal involvement. *Ann Allergy Asthma Immunol, 93*(4), 398-401. doi:10.1016/S1081-1206(10)61400-7

Barnado, A., Crofford, L. J., & Oates, J. C. (2016). At the Bedside: Neutrophil extracellular traps (NETs) as targets for biomarkers and therapies in autoimmune diseases. *J Leukoc Biol, 99*(2), 265-278. doi:10.1189/jlb.5BT0615-234R

Beaven, M. A. (2009). Our perception of the mast cell from Paul Ehrlich to now. *Eur J Immunol, 39*(1), 11-25. doi:10.1002/eji.200838899

Becher, B., Durell, B. G., & Noelle, R. J. (2002). Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *J Clin Invest, 110*(4), 493-497. doi:10.1172/JCI15751

Belmont, H. M. (2006). Treatment of ANCA-associated systemic vasculitis. *Bull NYU Hosp Jt Dis, 64*(1-2), 60-66.

BFaF, F. The production of antibodies. 2nd ed. Melbourne: Mac Millan; 1949.

Billingham, R. E., Brent, L., & Medawar, P. B. (2010). 'Actively acquired tolerance' of foreign cells. 1953. *J Immunol, 184*(1), 5-8.

Bjornsdottir, H., Welin, A., Michaelsson, E., Osla, V., Berg, S., Christenson, K., et al (2015). Neutrophil NET formation is regulated from the inside by myeloperoxidase-processed reactive oxygen species. *Free Radic Biol Med, 89*, 1024-1035. doi:10.1016/j.freeradbiomed.2015.10.398

Blank, U., Essig, M., Scandiuzzi, L., Benhamou, M., & Kanamaru, Y. (2007). Mast cells and inflammatory kidney disease. *Immunol Rev, 217*, 79-95. doi:10.1111/j.1600-065X.2007.00503.x

Boomsma, M. M., Stegeman, C. A., van der Leij, M. J., Oost, W., Hermans, J., Kallenberg, C. G., et al (2000). Prediction of relapses in Wegener's granulomatosis by measurement of antineutrophil cytoplasmic antibody levels: a prospective study. *Arthritis Rheum, 43*(9), 2025-2033. doi:10.1002/1529-0131(200009)43:9<2025::AID-ANR13>3.0.CO;2-O

Branitzki-Heinemann, K., Okumura, C. Y., Vollger, L., Kawakami, Y., Kawakami, T., Naim, H. Y., et al (2012). A novel role for the transcription factor HIF-1alpha in the formation of mast cell extracellular traps. *Biochem J, 446*(1), 159-163. doi:10.1042/BJ20120658

Brenner, B. M., Meyer, T. W., & Hostetter, T. H. (1982). Dietary protein intake and the progressive nature of kidney disease: the role of hemodynamically mediated glomerular injury in the pathogenesis of progressive glomerular sclerosis in aging, renal ablation, and intrinsic renal disease. *N Engl J Med, 307*(11), 652-659. doi:10.1056/NEJM198209093071104

Brewer, J. M., Conacher, M., Hunter, C. A., Mohrs, M., Brombacher, F., & Alexander, J. (1999). Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. *J Immunol, 163*(12), 6448-6454.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., et al (2004). Neutrophil extracellular traps kill bacteria. *Science, 303*(5663), 1532-1535. doi:10.1126/science.1092385

Brinkmann, V., & Zychlinsky, A. (2007). Beneficial suicide: why neutrophils die to make NETs. *Nat Rev Microbiol, 5*(8), 577-582. doi:10.1038/nrmicro1710

Brinkmann, V., & Zychlinsky, A. (2012). Neutrophil extracellular traps: is immunity the second function of chromatin? *J Cell Biol, 198*(5), 773-783. doi:10.1083/jcb.201203170

Brouwer, E., Huitema, M. G., Klok, P. A., de Weerd, H., Tervaert, J. W., Weening, J. J., & Kallenberg, C. G. (1993). Antimyeloperoxidase-associated proliferative glomerulonephritis: an animal model. *J Exp Med, 177*(4), 905-914.

Brouwer, E., Huitema, M. G., Mulder, A. H., Heeringa, P., van Goor, H., Tervaert, J. W., et al (1994). Neutrophil activation in vitro and in vivo in Wegener's granulomatosis. *Kidney Int, 45*(4), 1120-1131.

Brouwer, E., Klok, P. A., Huitema, M. G., Weening, J. J., & Kallenberg, C. G. (1995). Renal ischemia/reperfusion injury contributes to renal damage in experimental anti-myeloperoxidase-associated proliferative glomerulonephritis. *Kidney Int, 47*(4), 1121-1129.

Brown, H. J., Lock, H. R., Sacks, S. H., & Robson, M. G. (2006). TLR2 stimulation of intrinsic renal cells in the induction of immune-mediated glomerulonephritis. *J Immunol, 177*(3), 1925-1931.

Burkett, P. R., Meyer zu Horste, G., & Kuchroo, V. K. (2015). Pouring fuel on the fire: Th17 cells, the environment, and autoimmunity. *J Clin Invest, 125*(6), 2211-2219. doi:10.1172/JCI78085

Burmester, G. R., Stuhlmuller, B., Keyszer, G., & Kinne, R. W. (1997). Mononuclear phagocytes and rheumatoid synovitis. Mastermind or workhorse in arthritis? *Arthritis Rheum, 40*(1), 5-18.

Cattell, V., & Jamieson, S. W. (1978). The origin of glomerular crescents in experimental nephrotoxic serum nephritis in the rabbit. *Lab Invest, 39*(6), 584-590.

Chang, J., Eggenhuizen, P., O'Sullivan, K. M., Alikhan, M. A., Holdsworth, S. R., Ooi, J. D., & Kitching, A. R. (2017). CD8+ T Cells Effect Glomerular Injury in Experimental Anti-Myeloperoxidase GN. *J Am Soc Nephrol, 28*(1), 47-55. doi:10.1681/ASN.2015121356

Chen, M., Kallenberg, C. G., & Zhao, M. H. (2009). ANCA-negative pauci-immune crescentic glomerulonephritis. *Nat Rev Nephrol, 5*(6), 313-318. doi:10.1038/nrneph.2009.67

Chen, M., Xing, G. Q., Yu, F., Liu, G., & Zhao, M. H. (2009). Complement deposition in renal histopathology of patients with ANCA-associated pauci-immune glomerulonephritis. *Nephrol Dial Transplant, 24*(4), 1247-1252. doi:10.1093/ndt/gfn586

Chen, M., Yu, F., Zhang, Y., & Zhao, M. H. (2005). [An analysis of multi-system involvement and renal pathology of anti-neutrophil cytoplasmic autoantibodies-related vasculitis]. *Zhonghua Nei Ke Za Zhi, 44*(11), 828-831.

Chien, Y. H., Gascoigne, N. R., Kavaler, J., Lee, N. E., & Davis, M. M. (1984). Somatic recombination in a murine T-cell receptor gene. *Nature, 309*(5966), 322-326.

Chow, O. A., von Kockritz-Blickwede, M., Bright, A. T., Hensler, M. E., Zinkernagel, A. S., Cogen, A. L., . . . Nizet, V. (2010). Statins enhance formation of phagocyte extracellular traps. *Cell Host Microbe, 8*(5), 445-454. doi:10.1016/j.chom.2010.10.005

Christensen, S. R., Shupe, J., Nickerson, K., Kashgarian, M., Flavell, R. A., & Shlomchik, M. J. (2006). Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity, 25*(3), 417-428. doi:10.1016/j.immuni.2006.07.013

Chu, C. Q., Wittmer, S., & Dalton, D. K. (2000). Failure to suppress the expansion of the activated CD4 T cell population in interferon gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. *J Exp Med, 192*(1), 123-128.

Clark, S. P., Yoshikai, Y., Taylor, S., Siu, G., Hood, L., & Mak, T. W. (1984). Identification of a diversity segment of human T-cell receptor beta-chain, and comparison with the analogous murine element. *Nature, 311*(5984), 387-389.

Coers, W., Brouwer, E., Vos, J. T., Chand, A., Huitema, S., Heeringa, P., et al (1994). Podocyte expression of MHC class I and II and intercellular adhesion molecule-1 (ICAM-1) in experimental pauci-immune crescentic glomerulonephritis. *Clin Exp Immunol, 98*(2), 279-286.

Conti, F., Spinelli, F. R., Truglia, S., Miranda, F., Alessandri, C., Ceccarelli, F., .et al (2016). Kidney Expression of Toll Like Receptors in Lupus Nephritis: Quantification and Clinicopathological Correlations. *Mediators Inflamm, 2016*, 7697592. doi:10.1155/2016/7697592

Couser, W. G. (1988). Rapidly progressive glomerulonephritis: classification, pathogenetic mechanisms, and therapy. *Am J Kidney Dis, 11*(6), 449-464.

Cowland, J. B., & Borregaard, N. (1999). The individual regulation of granule protein mRNA levels during neutrophil maturation explains the heterogeneity of neutrophil granules. *J Leukoc Biol, 66*(6), 989-995.

Cui, Z., Zhao, M. H., Segelmark, M., & Hellmark, T. (2010). Natural autoantibodies to myeloperoxidase, proteinase 3, and the glomerular basement membrane are present in normal individuals. *Kidney Int, 78*(6), 590-597. doi:10.1038/ki.2010.198

Cunningham, M. A., Huang, X. R., Dowling, J. P., Tipping, P. G., & Holdsworth, S. R. (1999). Prominence of cell-mediated immunity effectors in "pauci-immune" glomerulonephritis. *J Am Soc Nephrol, 10*(3), 499-506.

Danilewicz, M., & Wagrowska-Danilewicz, M. (2001). Quantitative analysis of interstitial mast cells in lupus and non-lupus membranous glomerulopathy. *Pol J Pathol, 52*(4), 211-217.

Davies, M. J., Hawkins, C. L., Pattison, D. I., & Rees, M. D. (2008). Mammalian heme peroxidases: from molecular mechanisms to health implications. *Antioxid Redox Signal, 10*(7), 1199-1234. doi:10.1089/ars.2007.1927

Davis, J. C., Jr., Manzi, S., Yarboro, C., Rairie, J., McInnes, I., Averthelyi, D., et al (1999). Recombinant human Dnase I (rhDNase) in patients with lupus nephritis. *Lupus, 8*(1), 68-76.

de Joode, A. A., Sanders, J. S., & Stegeman, C. A. (2013). Renal survival in proteinase 3 and myeloperoxidase ANCA-associated systemic vasculitis. *Clin J Am Soc Nephrol, 8*(10), 1709-1717. doi:10.2215/CJN.01020113

de la Rosa, M., Rutz, S., Dorninger, H., & Scheffold, A. (2004). Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur J Immunol, 34*(9), 2480-2488. doi:10.1002/eji.200425274

de Zoysa, J. R. (2013). Vasculitis in New Zealand. *Clin Exp Nephrol, 17*(5), 712-714. doi:10.1007/s10157-012-0703-6

Desai, J., Kumar, S. V., Mulay, S. R., Konrad, L., Romoli, S., Schauer, C., et al (2016). PMA and crystal-induced neutrophil extracellular trap formation involves RIPK1-RIPK3-MLKL signaling. *Eur J Immunol, 46*(1), 223-229. doi:10.1002/eji.201545605

Duffield, J. S., Tipping, P. G., Kipari, T., Cailhier, J. F., Clay, S., Lang, R.,et al (2005). Conditional ablation of macrophages halts progression of crescentic glomerulonephritis. *Am J Pathol, 167*(5), 1207-1219. doi:10.1016/S0002-9440(10)61209-6

Dumitriu, I. E., Baruah, P., Manfredi, A. A., Bianchi, M. E., & Rovere-Querini, P. (2005). HMGB1: guiding immunity from within. *Trends Immunol, 26*(7), 381-387. doi:10.1016/j.it.2005.04.009

Dwyer, D. F., Barrett, N. A., Austen, K. F., & Immunological Genome Project, C. (2016). Expression profiling of constitutive mast cells reveals a unique identity within the immune system. *Nat Immunol, 17*(7), 878-887. doi:10.1038/ni.3445

Eisenberger, U., Fakhouri, F., Vanhille, P., Beaufils, H., Mahr, A., Guillevin, L., et al (2005). ANCA-negative pauci-immune renal vasculitis: histology and outcome. *Nephrol Dial Transplant, 20*(7), 1392-1399. doi:10.1093/ndt/gfh830

El-Koraie, A. F., Baddour, N. M., Adam, A. G., El Kashef, E. H., & El Nahas, A. M. (2001). Role of stem cell factor and mast cells in the progression of chronic glomerulonephritides. *Kidney Int, 60*(1), 167-172. doi:10.1046/j.1523-1755.2001.00783.x

Eleftheriadis, T., Pissas, G., Liakopoulos, V., Stefanidis, I., & Lawson, B. R. (2012). Toll-like receptors and their role in renal pathologies. *Inflamm Allergy Drug Targets, 11*(6), 464-477.

Elkayam, O., Tamir, R., Pick, A. I., & Wysenbeek, A. (1995). Serum IgE concentrations, disease activity, and atopic disorders in systemic lupus erythematosus. *Allergy, 50*(1), 94-96.

Eller, K., Wolf, D., Huber, J. M., Metz, M., Mayer, G., McKenzie, A. N., et al (2011). IL-9 production by regulatory T cells recruits mast cells that are essential for regulatory T cell-induced immune suppression. *J Immunol, 186*(1), 83-91. doi:10.4049/jimmunol.1001183

Esnault, V. L., Mathieson, P. W., Thiru, S., Oliveira, D. B., & Martin-Lockwood, C. (1992). Autoantibodies to myeloperoxidase in brown Norway rats treated with mercuric chloride. *Lab Invest, 67*(1), 114-120.

F., B. F. a. F. (1949). *The production of antibodies* (2nd ed.). Melbourne: Mac Millan.

Falk, R. J., & Jennette, J. C. (1988). Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N Engl J Med, 318*(25), 1651-1657. doi:10.1056/NEJM198806233182504

Falk, R. J., Terrell, R. S., Charles, L. A., & Jennette, J. C. (1990). Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. *Proc Natl Acad Sci U S A, 87*(11), 4115-4119.

Farquhar, H. J., McGettigan, B., Chapman, P. T., O'Donnell, J. L., Frampton, C., & Stamp, L. K. (2017). Incidence of anti-neutrophil cytoplasmic antibody-associated vasculitis before and after the February 2011 Christchurch Earthquake. *Intern Med J, 47*(1), 57-61. doi:10.1111/imj.13246

Farrera, C., & Fadeel, B. (2013). Macrophage clearance of neutrophil extracellular traps is a silent process. *J Immunol, 191*(5), 2647-2656. doi:10.4049/jimmunol.1300436

Faurschou, M., Westman, K., Rasmussen, N., de Groot, K., Flossmann, O., Hoglund, P., et al European Vasculitis Study, G. (2012). Brief Report: long-term outcome of a randomized clinical trial comparing methotrexate to cyclophosphamide for remission induction in early systemic antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheum, 64*(10), 3472-3477. doi:10.1002/art.34547

Fiers, W., Beyaert, R., Declercq, W., & Vandenabeele, P. (1999). More than one way to die: apoptosis, necrosis and reactive oxygen damage. *Oncogene, 18*(54), 7719-7730. doi:10.1038/sj.onc.1203249

Fink, S. L., & Cookson, B. T. (2006). Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cell Microbiol, 8*(11), 1812-1825. doi:10.1111/j.1462-5822.2006.00751.x

Finkielman, J. D., Lee, A. S., Hummel, A. M., Viss, M. A., Jacob, G. L., Homburger, H. A., . . . Group, W. R. (2007). ANCA are detectable in nearly all patients with active severe

Wegener's granulomatosis. *Am J Med, 120*(7), 643 e649-614. doi:10.1016/j.amjmed.2006.08.016

Franssen, C. F., Stegeman, C. A., Kallenberg, C. G., Gans, R. O., De Jong, P. E., Hoorntje, S. J., & Tervaert, J. W. (2000). Antiproteinase 3- and antimyeloperoxidase-associated vasculitis. *Kidney Int, 57*(6), 2195-2206. doi:10.1046/j.1523-1755.2000.00080.x

Free, M. E., Bunch, D. O., McGregor, J. A., Jones, B. E., Berg, E. A., Hogan, S. L., et al (2013). Patients with antineutrophil cytoplasmic antibody-associated vasculitis have defective Treg cell function exacerbated by the presence of a suppression-resistant effector cell population. *Arthritis Rheum, 65*(7), 1922-1933. doi:10.1002/art.37959

Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., et al (2007). Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol, 176*(2), 231-241. doi:10.1083/jcb.200606027

Fujimoto, S., Uezono, S., Hisanaga, S., Fukudome, K., Kobayashi, S., Suzuki, K., et al (2006). Incidence of ANCA-associated primary renal vasculitis in the Miyazaki Prefecture: the first population-based, retrospective, epidemiologic survey in Japan. *Clin J Am Soc Nephrol, 1*(5), 1016-1022. doi:10.2215/CJN.01461005

Fujinami, R. S., & Oldstone, M. B. (1985). Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science, 230*(4729), 1043-1045.

Galli, S. J., & Tsai, M. (2012). IgE and mast cells in allergic disease. *Nat Med, 18*(5), 693-704. doi:10.1038/nm.2755

Galli, S. J., Tsai, M., Gordon, J. R., Geissler, E. N., & Wershil, B. K. (1992). Analyzing mast cell development and function using mice carrying mutations at W/c-kit or Sl/MGF (SCF) loci. *Ann N Y Acad Sci, 664*, 69-88.

Gan, P. Y., Steinmetz, O. M., Tan, D. S., O'Sullivan, K. M., Ooi, J. D., Iwakura, Y., et al (2010). Th17 cells promote autoimmune anti-myeloperoxidase glomerulonephritis. *J Am Soc Nephrol, 21*(6), 925-931. doi:10.1681/ASN.2009070763

Gan, P. Y., Summers, S. A., Ooi, J. D., O'Sullivan, K. M., Tan, D. S., Muljadi, R. C., et al (2012). Mast cells contribute to peripheral tolerance and attenuate autoimmune vasculitis. *J Am Soc Nephrol, 23*(12), 1955-1966. doi:10.1681/ASN.2012060572

Garcia-Romo, G. S., Caielli, S., Vega, B., Connolly, J., Allantaz, F., Xu, Z., et al (2011). Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med, 3*(73), 73ra20. doi:10.1126/scitranslmed.3001201

Gaudenzio, N., Laurent, C., Valitutti, S., & Espinosa, E. (2013). Human mast cells drive memory CD4+ T cells toward an inflammatory IL-22+ phenotype. *J Allergy Clin Immunol, 131*(5), 1400-1407 e1411. doi:10.1016/j.jaci.2013.01.029

Gelderman, M. P., Stuart, R., Vigerust, D., Fuhrmann, S., Lefkowitz, D. L., Allen, R. C., et al (1998). Perpetuation of inflammation associated with experimental arthritis: the role of macrophage activation by neutrophilic myeloperoxidase. *Mediators Inflamm, 7*(6), 381-389. doi:10.1080/09629359890758

Gell, P. G. H., Coombs R.R.A. (1963). The classification of allergic reactions underlying disease. *Clinical Aspects of Immunology, 1st Edition*(Oxford, England), Section IV.

Ghari, F., Quirke, A. M., Munro, S., Kawalkowska, J., Picaud, S., McGouran, J., et al (2016). Citrullination-acetylation interplay guides E2F-1 activity during the inflammatory response. *Sci Adv, 2*(2), e1501257. doi:10.1126/sciadv.1501257

Gilfillan, A. M., & Rivera, J. (2009). The tyrosine kinase network regulating mast cell activation. *Immunol Rev, 228*(1), 149-169. doi:10.1111/j.1600-065X.2008.00742.x

Gluhovschi, C., Gluhovschi, G., Potencz, E., Herman, D., Trandafirescu, V., Petrica, L., et al (2010). The endothelial cell markers von Willebrand Factor (vWF), CD31 and CD34 are lost in glomerulonephritis and no longer correlate with the morphological indices of glomerular sclerosis, interstitial fibrosis, activity and chronicity. *Folia Histochem Cytobiol, 48*(2), 230-236. doi:10.2478/v10042-010-0004-4

Goldwich, A., Burkard, M., Olke, M., Daniel, C., Amann, K., Hugo, C., et al (2013). Podocytes are nonhematopoietic professional antigen-presenting cells. *J Am Soc Nephrol, 24*(6), 906-916. doi:10.1681/ASN.2012020133

Gomez-Puerta, J. A., Gedmintas, L., & Costenbader, K. H. (2013). The association between silica exposure and development of ANCA-associated vasculitis: systematic review and meta-analysis. *Autoimmun Rev, 12*(12), 1129-1135. doi:10.1016/j.autrev.2013.06.016

Gran, B., Zhang, G. X., Yu, S., Li, J., Chen, X. H., Ventura, E. S., et al (2002). IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. *J Immunol, 169*(12), 7104-7110.

Grayson, P. C., & Kaplan, M. J. (2016). At the Bench: Neutrophil extracellular traps (NETs) highlight novel aspects of innate immune system involvement in autoimmune diseases. *J Leukoc Biol, 99*(2), 253-264. doi:10.1189/jlb.5BT0615-247R

Greco, A., De Virgilio, A., Rizzo, M. I., Gallo, A., Magliulo, G., Fusconi, M., et al (2015). Microscopic polyangiitis: Advances in diagnostic and therapeutic approaches. *Autoimmun Rev, 14*(9), 837-844. doi:10.1016/j.autrev.2015.05.005

Greenhall, G. H., & Salama, A. D. (2015). What is new in the management of rapidly progressive glomerulonephritis? *Clin Kidney J, 8*(2), 143-150. doi:10.1093/ckj/sfv008

Grimbaldeston, M. A., Chen, C. C., Piliponsky, A. M., Tsai, M., Tam, S. Y., & Galli, S. J. (2005). Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. *Am J Pathol, 167*(3), 835-848. doi:10.1016/S0002-9440(10)62055-X

Guillevin, L., Cordier, J. F., Lhote, F., Cohen, P., Jarrousse, B., Royer, I., et al (1997). A prospective, multicenter, randomized trial comparing steroids and pulse cyclophosphamide versus steroids and oral cyclophosphamide in the treatment of generalized Wegener's granulomatosis. *Arthritis Rheum, 40*(12), 2187-2198. doi:10.1002/1529-0131(199712)40:12<2187::AID-ART12>3.0.CO;2-H

Gunzer, M. (2014). Traps and hyper inflammation - new ways that neutrophils promote or hinder survival. *Br J Haematol, 164*(2), 189-199. doi:10.1111/bjh.12608

Haas, M., & Eustace, J. A. (2004). Immune complex deposits in ANCA-associated crescentic glomerulonephritis: a study of 126 cases. *Kidney Int, 65*(6), 2145-2152. doi:10.1111/j.1523-1755.2004.00632.x

Hakkim, A., Fuchs, T. A., Martinez, N. E., Hess, S., Prinz, H., Zychlinsky, A., & Waldmann, H. (2011). Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nat Chem Biol, 7*(2), 75-77. doi:10.1038/nchembio.496

Hammer, D. K., & Dixon, F. J. (1963). Experimental glomerulonephritis. II. Immunologic events in the pathogenesis of nephrotoxic serum nephritis in the rat. *J Exp Med, 117*, 1019-1034.

Hamour, S., Salama, A. D., & Pusey, C. D. (2010). Management of ANCA-associated vasculitis: Current trends and future prospects. *Ther Clin Risk Manag, 6*, 253-264.

Hara, M., Batsford, S. R., Mihatsch, M. J., Bitter-Suermann, D., & Vogt, A. (1991). Complement and monocytes are essential for provoking glomerular injury in passive Heymann nephritis in rats. Terminal complement components are not the sole mediators of proteinuria. *Lab Invest, 65*(2), 168-179.

Harper, L., Morgan, M. D., Walsh, M., Hoglund, P., Westman, K., Flossmann, O., et al (2012). Pulse versus daily oral cyclophosphamide for induction of remission in ANCA-associated vasculitis: long-term follow-up. *Ann Rheum Dis, 71*(6), 955-960. doi:10.1136/annrheumdis-2011-200477

Haubitz, M., Schellong, S., Gobel, U., Schurek, H. J., Schaumann, D., Koch, K. M., & Brunkhorst, R. (1998). Intravenous pulse administration of cyclophosphamide versus daily oral treatment in patients with antineutrophil cytoplasmic antibody-associated vasculitis and renal involvement: a prospective, randomized study. *Arthritis Rheum, 41*(10), 1835-1844. doi:10.1002/1529-0131(199810)41:10<1835::AID-ART16>3.0.CO;2-Q

Heeringa, P., Brouwer, E., Klok, P. A., Huitema, M. G., van den Born, J., Weening, J. J., & Kallenberg, C. G. (1996). Autoantibodies to myeloperoxidase aggravate mild anti-glomerular-basement-membrane-mediated glomerular injury in the rat. *Am J Pathol, 149*(5), 1695-1706.

Heeringa, P., Foucher, P., Klok, P. A., Huitema, M. G., Tervaert, J. W., Weening, J. J., & Kallenberg, C. G. (1997). Systemic injection of products of activated neutrophils and H2O2 in myeloperoxidase-immunized rats leads to necrotizing vasculitis in the lungs and gut. *Am J Pathol, 151*(1), 131-140.

Hiemstra, T. F., & Jayne, D. (2009). Newer therapies for vasculitis. *Best Pract Res Clin Rheumatol, 23*(3), 379-389. doi:10.1016/j.berh.2009.01.005

Hiemstra, T. F., Walsh, M., Mahr, A., Savage, C. O., de Groot, K., Harper, L., European Vasculitis Study, G. (2010). Mycophenolate mofetil vs azathioprine for remission maintenance in antineutrophil cytoplasmic antibody-associated vasculitis: a randomized controlled trial. *JAMA, 304*(21), 2381-2388. doi:10.1001/jama.2010.1658

Hilhorst, M., van Paassen, P., Tervaert, J. W., & Limburg Renal, R. (2015). Proteinase 3-ANCA Vasculitis versus Myeloperoxidase-ANCA Vasculitis. *J Am Soc Nephrol, 26*(10), 2314-2327. doi:10.1681/ASN.2014090903

Hiromura, K., Kurosawa, M., Yano, S., & Naruse, T. (1998). Tubulointerstitial mast cell infiltration in glomerulonephritis. *Am J Kidney Dis, 32*(4), 593-599.

Hogan, S. L., Nachman, P. H., Wilkman, A. S., Jennette, J. C., & Falk, R. J. (1996). Prognostic markers in patients with antineutrophil cytoplasmic autoantibody-associated microscopic polyangiitis and glomerulonephritis. *J Am Soc Nephrol, 7*(1), 23-32.

Hopfer, H., Maron, R., Butzmann, U., Helmchen, U., Weiner, H. L., & Kalluri, R. (2003). The importance of cell-mediated immunity in the course and severity of autoimmune anti-glomerular basement membrane disease in mice. *FASEB J, 17*(8), 860-868. doi:10.1096/fj.02-0746com

Huang, H., Chen, H. W., Evankovich, J., Yan, W., Rosborough, B. R., Nace, G. W., . .et al (2013). Histones activate the NLRP3 inflammasome in Kupffer cells during sterile inflammatory liver injury. *J Immunol, 191*(5), 2665-2679. doi:10.4049/jimmunol.1202733

Huang, X. R., Holdsworth, S. R., & Tipping, P. G. (1994). Evidence for delayed-type hypersensitivity mechanisms in glomerular crescent formation. *Kidney Int, 46*(1), 69-78.

Huang, X. R., Holdsworth, S. R., & Tipping, P. G. (1997). Th2 responses induce humorally mediated injury in experimental anti-glomerular basement membrane glomerulonephritis. *J Am Soc Nephrol, 8*(7), 1101-1108.

Huang, X. R., Tipping, P. G., Apostolopoulos, J., Oettinger, C., D'Souza, M., Milton, G., & Holdsworth, S. R. (1997). Mechanisms of T cell-induced glomerular injury in anti-glomerular basement membrane (GBM) glomerulonephritis in rats. *Clin Exp Immunol, 109*(1), 134-142.

Huugen, D., van Esch, A., Xiao, H., Peutz-Kootstra, C. J., Buurman, W. A., Tervaert, J. W., . . . Heeringa, P. (2007). Inhibition of complement factor C5 protects against anti-myeloperoxidase antibody-mediated glomerulonephritis in mice. *Kidney Int, 71*(7), 646-654. doi:10.1038/sj.ki.5002103

Huugen, D., Xiao, H., van Esch, A., Falk, R. J., Peutz-Kootstra, C. J., Buurman, W. A., . . . Heeringa, P. (2005). Aggravation of anti-myeloperoxidase antibody-induced glomerulonephritis by bacterial lipopolysaccharide: role of tumor necrosis factor-alpha. *Am J Pathol, 167*(1), 47-58.

Ikeda, M., Tsuru, S., Watanabe, Y., Kitahara, S., & Inouye, T. (1992). Reduced CD4-CD8 T cell ratios in patients with Wegener's granulomatosis. *J Clin Lab Immunol, 38*(3), 103-109.

Ikezumi, Y., Hurst, L. A., Masaki, T., Atkins, R. C., & Nikolic-Paterson, D. J. (2003). Adoptive transfer studies demonstrate that macrophages can induce proteinuria and mesangial cell proliferation. *Kidney Int, 63*(1), 83-95. doi:10.1046/j.1523-1755.2003.00717.x

Iking-Konert, C., Vogl, T., Prior, B., Wagner, C., Sander, O., Bleck, E., et al , Hansch, G. M. (2008). T lymphocytes in patients with primary vasculitis: expansion of CD8+ T cells with the propensity to activate polymorphonuclear neutrophils. *Rheumatology (Oxford), 47*(5), 609-616. doi:10.1093/rheumatology/ken028

Ivanov, S., Dragoi, A. M., Wang, X., Dallacosta, C., Louten, J., Musco, G., et al (2007). A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. *Blood, 110*(6), 1970-1981. doi:10.1182/blood-2006-09-044776

Janeway, C. A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol, 54 Pt 1*, 1-13.

Jayne, D. (2009). Review article: Progress of treatment in ANCA-associated vasculitis. *Nephrology (Carlton), 14*(1), 42-48. doi:10.1111/j.1440-1797.2009.01101.x

Jayne, D. R. W., Bruchfeld, A. N., Harper, L., Schaier, M., Venning, M. C., Hamilton, P., .et al Group, C. S. (2017). Randomized Trial of C5a Receptor Inhibitor Avacopan in ANCA-Associated Vasculitis. *J Am Soc Nephrol, 28*(9), 2756-2767. doi:10.1681/ASN.2016111179

Jennette, J. C. (2003). Rapidly progressive crescentic glomerulonephritis. *Kidney Int, 63*(3), 1164-1177. doi:10.1046/j.1523-1755.2003.00843.x

Jennette, J. C., Falk, R. J., Andrassy, K., Bacon, P. A., Churg, J., Gross, W. L., et al. (1994). Nomenclature of systemic vasculitides. Proposal of an international consensus conference. *Arthritis Rheum, 37*(2), 187-192.

Jennette, J. C., Falk, R. J., Bacon, P. A., Basu, N., Cid, M. C., Ferrario, F., et al Watts, R. A. (2013). 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheum, 65*(1), 1-11. doi:10.1002/art.37715

Jennette, J. C., Falk, R. J., Hu, P., & Xiao, H. (2013). Pathogenesis of antineutrophil cytoplasmic autoantibody-associated small-vessel vasculitis. *Annu Rev Pathol, 8*, 139-160. doi:10.1146/annurev-pathol-011811-132453

Jennette, J. C., & Hipp, C. G. (1986). The epithelial antigen phenotype of glomerular crescent cells. *Am J Clin Pathol, 86*(3), 274-280.

Jennette, J. C., & Thomas, D. B. (2001). Crescentic glomerulonephritis. *Nephrol Dial Transplant, 16 Suppl 6*, 80-82.

Jerke, U., Rolle, S., Purfurst, B., Luft, F. C., Nauseef, W. M., & Kettritz, R. (2013). beta2 integrin-mediated cell-cell contact transfers active myeloperoxidase from neutrophils to endothelial cells. *J Biol Chem, 288*(18), 12910-12919. doi:10.1074/jbc.M112.434613

Jimenez-Dalmaroni, M. J., Gerswhin, M. E., & Adamopoulos, I. E. (2016). The critical role of toll-like receptors--From microbial recognition to autoimmunity: A comprehensive review. *Autoimmun Rev, 15*(1), 1-8. doi:10.1016/j.autrev.2015.08.009

Johnson, R. J., Couser, W. G., Chi, E. Y., Adler, S., & Klebanoff, S. J. (1987). New mechanism for glomerular injury. Myeloperoxidase-hydrogen peroxide-halide system. *J Clin Invest, 79*(5), 1379-1387. doi:10.1172/JCI112965

Jones R, H. L., Ballarin J,Blcokmans D, Brogan P, Bruchfeld A et al. (2013). A randomized trial of mycophenolate mofetil versus cylophosphamide for remission induction of ANCA-associated vasculitis "MYCYC". *Presse Med, 42*, 678-679.

Jones, R. B., Tervaert, J. W., Hauser, T., Luqmani, R., Morgan, M. D., Peh, C. A.,et al European Vasculitis Study, G. (2010). Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis. *N Engl J Med, 363*(3), 211-220. doi:10.1056/NEJMoa0909169

Jorgensen, I., Rayamajhi, M., & Miao, E. A. (2017). Programmed cell death as a defence against infection. *Nat Rev Immunol, 17*(3), 151-164. doi:10.1038/nri.2016.147

Kain, R., Exner, M., Brandes, R., Ziebermayr, R., Cunningham, D., Alderson, C. A., et al Kerjaschki, D. (2008). Molecular mimicry in pauci-immune focal necrotizing glomerulonephritis. *Nat Med, 14*(10), 1088-1096. doi:10.1038/nm.1874

Kain, R., Matsui, K., Exner, M., Binder, S., Schaffner, G., Sommer, E. M., & Kerjaschki, D. (1995). A novel class of autoantigens of anti-neutrophil cytoplasmic antibodies in necrotizing and crescentic glomerulonephritis: the lysosomal membrane glycoprotein h-lamp-2 in neutrophil granulocytes and a related membrane protein in glomerular endothelial cells. *J Exp Med, 181*(2), 585-597.

Kain, R., Tadema, H., McKinney, E. F., Benharkou, A., Brandes, R., Peschel, A., et al Rees, A. J. (2012). High prevalence of autoantibodies to hLAMP-2 in anti-neutrophil

cytoplasmic antibody-associated vasculitis. *J Am Soc Nephrol, 23*(3), 556-566. doi:10.1681/ASN.2011090920

Kallenberg, C. G. (2014). Advances in pathogenesis and treatment of ANCA-associated vasculitis. *Discov Med, 18*(99), 195-201.

Kalluri, R., Wilson, C. B., Weber, M., Gunwar, S., Chonko, A. M., Neilson, E. G., & Hudson, B. G. (1995). Identification of the alpha 3 chain of type IV collagen as the common autoantigen in antibasement membrane disease and Goodpasture syndrome. *J Am Soc Nephrol, 6*(4), 1178-1185.

Kambayashi, T., Allenspach, E. J., Chang, J. T., Zou, T., Shoag, J. E., Reiner, S. L.,et al Koretzky, G. A. (2009). Inducible MHC class II expression by mast cells supports effector and regulatory T cell activation. *J Immunol, 182*(8), 4686-4695. doi:10.4049/jimmunol.0803180

Kang, D. H., Kanellis, J., Hugo, C., Truong, L., Anderson, S., Kerjaschki, D., et al Johnson, R. J. (2002). Role of the microvascular endothelium in progressive renal disease. *J Am Soc Nephrol, 13*(3), 806-816.

Kaplan, M. H., Hufford, M. M., & Olson, M. R. (2015). The development and in vivo function of T helper 9 cells. *Nat Rev Immunol, 15*(5), 295-307. doi:10.1038/nri3824

Kaplan, M. J. (2011). Neutrophils in the pathogenesis and manifestations of SLE. *Nat Rev Rheumatol, 7*(12), 691-699. doi:10.1038/nrrheum.2011.132

Kawalkowska, J., Quirke, A. M., Ghari, F., Davis, S., Subramanian, V., Thompson, P. R., Venables, P. J. (2016). Abrogation of collagen-induced arthritis by a peptidyl arginine

deiminase inhibitor is associated with modulation of T cell-mediated immune responses. *Sci Rep, 6*, 26430. doi:10.1038/srep26430

Kessenbrock, K., Krumbholz, M., Schonermarck, U., Back, W., Gross, W. L., Werb, Z., et al Jenne, D. E. (2009). Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med, 15*(6), 623-625. doi:10.1038/nm.1959

Kettritz, R., Jennette, J. C., & Falk, R. J. (1997). Crosslinking of ANCA-antigens stimulates superoxide release by human neutrophils. *J Am Soc Nephrol, 8*(3), 386-394.

Kidney Health Australia (2017), Australia accessed July 2017. www.kidney.org.au

Kim, Y. H., Goyal, M., Kurnit, D., Wharram, B., Wiggins, J., Holzman, L., . . . Wiggins, R. (2001). Podocyte depletion and glomerulosclerosis have a direct relationship in the PAN-treated rat. *Kidney Int, 60*(3), 957-968. doi:10.1046/j.1523-1755.2001.060003957.x

King, P. T., Sharma, R., O'Sullivan, K., Selemidis, S., Lim, S., Radhakrishna, N., . . . Holdsworth, S. R. (2015). Nontypeable Haemophilus influenzae induces sustained lung oxidative stress and protease expression. *PLoS One, 10*(3), e0120371. doi:10.1371/journal.pone.0120371

Kitching, A. R., Holdsworth, S. R., & Hickey, M. J. (2008). Targeting leukocytes in immune glomerular diseases. *Curr Med Chem, 15*(5), 448-458.

Kitching, A. R., Holdsworth, S. R., & Tipping, P. G. (1999a). IFN-gamma mediates crescent formation and cell-mediated immune injury in murine glomerulonephritis. *J Am Soc Nephrol, 10*(4), 752-759.

Kitching, A. R., Tipping, P. G., & Holdsworth, S. R. (1999b). IL-12 directs severe renal injury, crescent formation and Th1 responses in murine glomerulonephritis. *Eur J Immunol, 29*(1), 1-10. doi:10.1002/(SICI)1521-4141(199901)29:01<1::AID-IMMU1>3.0.CO;2-R

Kitching, A. R., Turner, A. L., Wilson, G. R., Semple, T., Odobasic, D., Timoshanko, J. R., Holdsworth, S. R. (2005). IL-12p40 and IL-18 in crescentic glomerulonephritis: IL-12p40 is the key Th1-defining cytokine chain, whereas IL-18 promotes local inflammation and leukocyte recruitment. *J Am Soc Nephrol, 16*(7), 2023-2033. doi:10.1681/ASN.2004121075

Klebanoff, S. J. (1967). Iodination of bacteria: a bactericidal mechanism. *J Exp Med, 126*(6), 1063-1078.

Klebanoff, S. J. (1968). Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *J Bacteriol, 95*(6), 2131-2138.

Knight, J. S., Luo, W., O'Dell, A. A., Yalavarthi, S., Zhao, W., Subramanian, V., Kaplan, M. J. (2014). Peptidylarginine deiminase inhibition reduces vascular damage and modulates innate immune responses in murine models of atherosclerosis. *Circ Res, 114*(6), 947-956. doi:10.1161/CIRCRESAHA.114.303312

Knight, J. S., Subramanian, V., O'Dell, A. A., Yalavarthi, S., Zhao, W., Smith, C. K., Kaplan, M. J. (2015). Peptidylarginine deiminase inhibition disrupts NET formation and protects against kidney, skin and vascular disease in lupus-prone MRL/lpr mice. *Ann Rheum Dis, 74*(12), 2199-2206. doi:10.1136/annrheumdis-2014-205365

Kobayashi, K., Shibata, T., & Sugisaki, T. (1995). Aggravation of rat nephrotoxic serum nephritis by anti-myeloperoxidase antibodies. *Kidney Int, 47*(2), 454-463.

Kobayashi, S., Fujimoto, S., Takahashi, K., & Suzuki, K. (2010). Anti-neutrophil cytoplasmic antibody-associated vasculitis, large vessel vasculitis and Kawasaki disease in Japan. *Kidney Blood Press Res, 33*(6), 442-455. doi:10.1159/000320383

Kocher, M., Edberg, J. C., Fleit, H. B., & Kimberly, R. P. (1998). Antineutrophil cytoplasmic antibodies preferentially engage Fc gammaRIIIb on human neutrophils. *J Immunol, 161*(12), 6909-6914.

Kolaczkowska, E., Jenne, C. N., Surewaard, B. G., Thanabalasuriar, A., Lee, W. Y., Sanz, M. J., Kubes, P. (2015). Molecular mechanisms of NET formation and degradation revealed by intravital imaging in the liver vasculature. *Nat Commun, 6*, 6673. doi:10.1038/ncomms7673

Komiyama, Y., Nakae, S., Matsuki, T., Nambu, A., Ishigame, H., Kakuta, S., Iwakura, Y. (2006). IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol, 177*(1), 566-573.

Koyama, A., Yamagata, K., Makino, H., Arimura, Y., Wada, T., Nitta, K., Japan, R. R. G. (2009). A nationwide survey of rapidly progressive glomerulonephritis in Japan: etiology, prognosis and treatment diversity. *Clin Exp Nephrol, 13*(6), 633-650. doi:10.1007/s10157-009-0201-7

Kraft, S., & Kinet, J. P. (2007). New developments in FcepsilonRI regulation, function and inhibition. *Nat Rev Immunol, 7*(5), 365-378. doi:10.1038/nri2072

Kriz, W., & LeHir, M. (2005). Pathways to nephron loss starting from glomerular diseases-insights from animal models. *Kidney Int, 67*(2), 404-419. doi:10.1111/j.1523-1755.2005.67097.x

Kruger, T., Benke, D., Eitner, F., Lang, A., Wirtz, M., Hamilton-Williams, E. E., . . . Kurts, C. (2004). Identification and functional characterization of dendritic cells in the healthy murine kidney and in experimental glomerulonephritis. *J Am Soc Nephrol, 15*(3), 613-621.

Kubiak-Wlekly, A., Perkowska-Ptasinska, A., Olejniczak, P., Rochowiak, A., Kaczmarek, E., Durlik, M., . . . Niemir, Z. I. (2009). The Comparison of the Podocyte Expression of Synaptopodin, CR1 and Neprilysin in Human Glomerulonephritis: Could the Expression of CR1 be Clinically Relevant? *Int J Biomed Sci, 5*(1), 28-36.

Kumar, S. V., Kulkarni, O. P., Mulay, S. R., Darisipudi, M. N., Romoli, S., Thomasova, D., Anders, H. J. (2015). Neutrophil Extracellular Trap-Related Extracellular Histones Cause Vascular Necrosis in Severe GN. *J Am Soc Nephrol, 26*(10), 2399-2413. doi:10.1681/ASN.2014070673

Kurts, C., Panzer, U., Anders, H. J., & Rees, A. J. (2013). The immune system and kidney disease: basic concepts and clinical implications. *Nat Rev Immunol, 13*(10), 738-753. doi:10.1038/nri3523

Kusunoki, Y., Nakazawa, D., Shida, H., Hattanda, F., Miyoshi, A., Masuda, S., Ishizu, A. (2016). Peptidylarginine Deiminase Inhibitor Suppresses Neutrophil Extracellular Trap Formation and MPO-ANCA Production. *Front Immunol, 7*, 227. doi:10.3389/fimmu.2016.00227

Kutter, D., Devaquet, P., Vanderstocken, G., Paulus, J. M., Marchal, V., & Gothot, A. (2000). Consequences of total and subtotal myeloperoxidase deficiency: risk or benefit ? *Acta Haematol, 104*(1), 10-15. doi:41062

Lacy, P. (2006). Mechanisms of degranulation in neutrophils. *Allergy Asthma Clin Immunol, 2*(3), 98-108. doi:10.1186/1710-1492-2-3-98

Lan, H. Y., Nikolic-Paterson, D. J., & Atkins, R. C. (1992). Involvement of activated periglomerular leukocytes in the rupture of Bowman's capsule and glomerular crescent progression in experimental glomerulonephritis. *Lab Invest, 67*(6), 743-751.

Lande, R., Ganguly, D., Facchinetti, V., Frasca, L., Conrad, C., Gregorio, J., Gilliet, M. (2011). Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med, 3*(73), 73ra19. doi:10.1126/scitranslmed.3001180

Lande, R., Gregorio, J., Facchinetti, V., Chatterjee, B., Wang, Y. H., Homey, B.,Gilliet, M. (2007). Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature, 449*(7162), 564-569. doi:10.1038/nature06116

Langford, C. A., Monach, P. A., Specks, U., Seo, P., Cuthbertson, D., McAlear, C. A., Vasculitis Clinical Research, C. (2014). An open-label trial of abatacept (CTLA4-IG) in non-severe relapsing granulomatosis with polyangiitis (Wegener's). *Ann Rheum Dis, 73*(7), 1376-1379. doi:10.1136/annrheumdis-2013-204164

Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., Cua, D. J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med, 201*(2), 233-240. doi:10.1084/jem.20041257

Le Douarin, N., Corbel, C., Bandeira, A., Thomas-Vaslin, V., Modigliani, Y., Coutinho, A., & Salaun, J. (1996). Evidence for a thymus-dependent form of tolerance that is not based on elimination or anergy of reactive T cells. *Immunol Rev, 149*, 35-53.

Le Hir, M., Keller, C., Eschmann, V., Hahnel, B., Hosser, H., & Kriz, W. (2001). Podocyte bridges between the tuft and Bowman's capsule: an early event in experimental crescentic glomerulonephritis. *J Am Soc Nephrol, 12*(10), 2060-2071.

Lefkowitz, D. L., Mills, K. C., Moguilevsky, N., Bollen, A., Vaz, A., & Lefkowitz, S. S. (1993). Regulation of macrophage function by human recombinant myeloperoxidase. *Immunol Lett, 36*(1), 43-49.

Li, J., Yu, Y. F., Liu, C. H., & Wang, C. M. (2017). Significance of M2 macrophages in glomerulonephritis with crescents. *Pathol Res Pract*. doi:10.1016/j.prp.2017.04.011

Li, P., Li, M., Lindberg, M. R., Kennett, M. J., Xiong, N., & Wang, Y. (2010). PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med, 207*(9), 1853-1862. doi:10.1084/jem.20100239

Li, S., Kurts, C., Kontgen, F., Holdsworth, S. R., & Tipping, P. G. (1998). Major histocompatibility complex class II expression by intrinsic renal cells is required for crescentic glomerulonephritis. *J Exp Med, 188*(3), 597-602.

Lionaki, S., Blyth, E. R., Hogan, S. L., Hu, Y., Senior, B. A., Jennette, C. E., Falk, R. J. (2012). Classification of antineutrophil cytoplasmic autoantibody vasculitides: the role of antineutrophil cytoplasmic autoantibody specificity for myeloperoxidase or proteinase 3 in disease recognition and prognosis. *Arthritis Rheum, 64*(10), 3452-3462. doi:10.1002/art.34562

Little, M. A., Smyth, C. L., Yadav, R., Ambrose, L., Cook, H. T., Nourshargh, S., & Pusey, C. D. (2005). Antineutrophil cytoplasm antibodies directed against myeloperoxidase

augment leukocyte-microvascular interactions in vivo. *Blood, 106*(6), 2050-2058. doi:10.1182/blood-2005-03-0921

Little, M.A.,Al-Ani B., Ren,S. Al-Nuaimi, Leite, M. Alpers, C.E., Savage, C.O., Duffield J.S. (2012). Autoantibodies racaptiualte systemic vasculitis in mice with a humanized immune system. PLOS One 7; (1) e28626

Lloyd, C. M., Minto, A. W., Dorf, M. E., Proudfoot, A., Wells, T. N., Salant, D. J., & Gutierrez-Ramos, J. C. (1997). RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 is involved in crescent formation and interstitial fibrosis. *J Exp Med, 185*(7), 1371-1380.

Lood, C., Blanco, L. P., Purmalek, M. M., Carmona-Rivera, C., De Ravin, S. S., Smith, C. K., . . . Kaplan, M. J. (2016). Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. *Nat Med, 22*(2), 146-153. doi:10.1038/nm.4027

Lopes, J. P., Stylianou, M., Nilsson, G., & Urban, C. F. (2015). Opportunistic pathogen Candida albicans elicits a temporal response in primary human mast cells. *Sci Rep, 5*, 12287. doi:10.1038/srep12287

Lu, L. F., Lind, E. F., Gondek, D. C., Bennett, K. A., Gleeson, M. W., Pino-Lagos, K., Noelle, R. J. (2006). Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature, 442*(7106), 997-1002. doi:10.1038/nature05010

Lundberg, K., Nijenhuis, S., Vossenaar, E. R., Palmblad, K., van Venrooij, W. J., Klareskog, L., Harris, H. E. (2005). Citrullinated proteins have increased immunogenicity and

arthritogenicity and their presence in arthritic joints correlates with disease severity. *Arthritis Res Ther, 7*(3), R458-467. doi:10.1186/ar1697

Lyons, P. A., Rayner, T. F., Trivedi, S., Holle, J. U., Watts, R. A., Jayne, D. R., Smith, K. G. (2012). Genetically distinct subsets within ANCA-associated vasculitis. *N Engl J Med, 367*(3), 214-223. doi:10.1056/NEJMoa1108735

Ma, C. S., & Deenick, E. K. (2014). Human T follicular helper (Tfh) cells and disease. *Immunol Cell Biol, 92*(1), 64-71. doi:10.1038/icb.2013.55

Machida, H., Ito, S., Hirose, T., Takeshita, F., Oshiro, H., Nakamura, T., Yokota, S. (2010). Expression of Toll-like receptor 9 in renal podocytes in childhood-onset active and inactive lupus nephritis. *Nephrol Dial Transplant, 25*(8), 2530-2537. doi:10.1093/ndt/gfq058

Mahr, A., Guillevin, L., Poissonnet, M., & Ayme, S. (2004). Prevalences of polyarteritis nodosa, microscopic polyangiitis, Wegener's granulomatosis, and Churg-Strauss syndrome in a French urban multiethnic population in 2000: a capture-recapture estimate. *Arthritis Rheum, 51*(1), 92-99. doi:10.1002/art.20077

Malachowa, N., Kobayashi, S. D., Quinn, M. T., & DeLeo, F. R. (2016). NET Confusion. *Front Immunol, 7*, 259. doi:10.3389/fimmu.2016.00259

Malaviya, R., Twesten, N. J., Ross, E. A., Abraham, S. N., & Pfeifer, J. D. (1996). Mast cells process bacterial Ags through a phagocytic route for class I MHC presentation to T cells. *J Immunol, 156*(4), 1490-1496.

Marichal, T., Ohata, K., Bedoret, D., Mesnil, C., Sabatel, C., Kobiyama, K., Desmet, C. J. (2011). DNA released from dying host cells mediates aluminum adjuvant activity. *Nat Med, 17*(8), 996-1002. doi:10.1038/nm.2403

Marin-Gallen, S., Clemente-Casares, X., Planas, R., Pujol-Autonell, I., Carrascal, J., Carrillo, J., Vives-Pi, M. (2010). Dendritic cells pulsed with antigen-specific apoptotic bodies prevent experimental type 1 diabetes. *Clin Exp Immunol, 160*(2), 207-214. doi:10.1111/j.1365-2249.2009.04082.x

Mathieson, P.W., Stapelton, K.J., Oliveira, D.B.G., Lockwood,M.C. (1991). Immunoreguatlion of meruric choride-induced autoimmunity in Brown Norway rats:A role for CD8+ T cells revealed by *in vivo* depletion studies. European Journal of Immunology 21(9) 2105-2109

Martinez-Valle, F., Balada, E., Ordi-Ros, J., Bujan-Rivas, S., Sellas-Fernandez, A., & Vilardell-Tarres, M. (2009). DNase 1 activity in patients with systemic lupus erythematosus: relationship with epidemiological, clinical, immunological and therapeutical features. *Lupus, 18*(5), 418-423. doi:10.1177/0961203308098189

Matzinger, P. (2002). The danger model: a renewed sense of self. *Science, 296*(5566), 301-305. doi:10.1126/science.1071059

Mayadas, T. N., Cullere, X., & Lowell, C. A. (2014). The multifaceted functions of neutrophils. *Annu Rev Pathol, 9*, 181-218. doi:10.1146/annurev-pathol-020712-164023

McKinney, E. F., Lyons, P. A., Carr, E. J., Hollis, J. L., Jayne, D. R., Willcocks, L. C., Smith, K. G. (2010). A CD8+ T cell transcription signature predicts prognosis in autoimmune disease. *Nat Med, 16*(5), 586-591, 581p following 591. doi:10.1038/nm.2130

McMillen, T. S., Heinecke, J. W., & LeBoeuf, R. C. (2005). Expression of human myeloperoxidase by macrophages promotes atherosclerosis in mice. *Circulation, 111*(21), 2798-2804. doi:10.1161/CIRCULATIONAHA.104.516278

Merkel PA, N. J., Jimenez R, Spiera RF, Rovin BH, Bomback A, Pagnoux C, Potarca A, Schall TJ, Bekker P. A (2016). A Randomized Clinical Trial of CCX168, an Orally Administered C5aR Inhibitor for Treatment of Patients with ANCA-Associated Vasculitis [abstract]. *Arthritis Rheumatol., 68 (suppl 10).*

Merkel, PA (2017). “Current and Future Cinical and Translational Research Studies in Vasculitis”. The 18th International Vascultiis and ANCA Workshop, Tokyo, Japan.

Mescher, M. F., Curtsinger, J. M., Agarwal, P., Casey, K. A., Gerner, M., Hammerbeck, C. D., Xiao, Z. (2006). Signals required for programming effector and memory development by CD8+ T cells. *Immunol Rev, 211*, 81-92. doi:10.1111/j.0105-2896.2006.00382.x

Metzler, K. D., Fuchs, T. A., Nauseef, W. M., Reumaux, D., Roesler, J., Schulze, I., Zychlinsky, A. (2011). Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood, 117*(3), 953-959. doi:10.1182/blood-2010-06-290171

Mohammad, A. J., Jacobsson, L. T., Mahr, A. D., Sturfelt, G., & Segelmark, M. (2007). Prevalence of Wegener's granulomatosis, microscopic polyangiitis, polyarteritis nodosa and Churg-Strauss syndrome within a defined population in southern Sweden. *Rheumatology (Oxford), 46*(8), 1329-1337. doi:10.1093/rheumatology/kem107

Mohanan, S., Horibata, S., McElwee, J. L., Dannenberg, A. J., & Coonrod, S. A. (2013). Identification of macrophage extracellular trap-like structures in mammary gland adipose tissue: a preliminary study. *Front Immunol, 4*, 67. doi:10.3389/fimmu.2013.00067

Morgan, M. D., Day, C. J., Piper, K. P., Khan, N., Harper, L., Moss, P. A., & Savage, C. O. (2010). Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional impairment of T-regulatory cells. *Immunology, 130*(1), 64-73. doi:10.1111/j.1365-2567.2009.03213.x

Morgan, M. D., Harper, L., Williams, J., & Savage, C. (2006). Anti-neutrophil cytoplasm-associated glomerulonephritis. *J Am Soc Nephrol, 17*(5), 1224-1234. doi:10.1681/ASN.2005080882

Morita, T., Suzuki, Y., & Churg, J. (1973). Structure and development of the glomerular crescent. *Am J Pathol, 72*(3), 349-368.

Motojima, M., Matsusaka, T., Kon, V., & Ichikawa, I. (2010). Fibrinogen that appears in Bowman's space of proteinuric kidneys in vivo activates podocyte Toll-like receptors 2 and 4 in vitro. *Nephron Exp Nephrol, 114*(2), e39-47. doi:10.1159/000254390

Muczynski, K. A., Ekle, D. M., Coder, D. M., & Anderson, S. K. (2003). Normal human kidney HLA-DR-expressing renal microvascular endothelial cells: characterization, isolation, and regulation of MHC class II expression. *J Am Soc Nephrol, 14*(5), 1336-1348.

Mudaliar, H., Pollock, C., Ma, J., Wu, H., Chadban, S., & Panchapakesan, U. (2014). The role of TLR2 and 4-mediated inflammatory pathways in endothelial cells exposed to high glucose. *PLoS One, 9*(10), e108844. doi:10.1371/journal.pone.0108844

Mukhtyar, C., Flossmann, O., Hellmich, B., Bacon, P., Cid, M., Cohen-Tervaert, J. W., European Vasculitis Study, G. (2008). Outcomes from studies of antineutrophil cytoplasm antibody associated vasculitis: a systematic review by the European League Against Rheumatism systemic vasculitis task force. *Ann Rheum Dis, 67*(7), 1004-1010. doi:10.1136/ard.2007.071936

Muller, W. A. (2003). Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol, 24*(6), 327-334.

Munni, A. (2016). Production and Characterization of Recombinant Rat Non-Collagen Domain of <i>¦Á</i>3 Chain of Type IV Collagen <i>¦Á</i>3 (IV) NC1 Antigen. *CellBio, Vol.05No.03*, 12. doi:10.4236/cellbio.2016.53003

Nakayama, T., Hirahara, K., Onodera, A., Endo, Y., Hosokawa, H., Shinoda, K., Okamoto, Y. (2017). Th2 Cells in Health and Disease. *Annu Rev Immunol, 35*, 53-84. doi:10.1146/annurev-immunol-051116-052350

Nakazawa, D., Tomaru, U., Yamamoto, C., Jodo, S., & Ishizu, A. (2012). Abundant neutrophil extracellular traps in thrombus of patient with microscopic polyangiitis. *Front Immunol, 3*, 333. doi:10.3389/fimmu.2012.00333

Neeli, I., Khan, S. N., & Radic, M. (2008). Histone deimination as a response to inflammatory stimuli in neutrophils. *J Immunol, 180*(3), 1895-1902.

Noguchi, N., Nakano, K., Aratani, Y., Koyama, H., Kodama, T., & Niki, E. (2000). Role of myeloperoxidase in the neutrophil-induced oxidation of low density lipoprotein as studied by myeloperoxidase-knockout mouse. *J Biochem, 127*(6), 971-976.

Nossal, G. J., & Lederberg, J. (2009). Pillars article: antibody production by single cells. 1958. Nature 181: 1419-1420. *J Immunol, 182*(3), 1231-1232.

Nossal, G. J., & Pike, B. L. (1975). Evidence for the clonal abortion theory of B-lymphocyte tolerance. *J Exp Med, 141*(4), 904-917.

O'Donnell, J. L., Stevanovic, V. R., Frampton, C., Stamp, L. K., & Chapman, P. T. (2007). Wegener's granulomatosis in New Zealand: evidence for a latitude-dependent incidence gradient. *Intern Med J, 37*(4), 242-246. doi:10.1111/j.1445-5994.2006.01297.x

O'Sullivan, K. M., Lo, C. Y., Summers, S. A., Elgass, K. D., McMillan, P. J., Longano, A., Holdsworth, S. R. (2015). Renal participation of myeloperoxidase in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. *Kidney Int, 88*(5), 1030-1046. doi:10.1038/ki.2015.202

Obar, J. J., & Lefrancois, L. (2010). Memory CD8+ T cell differentiation. *Ann N Y Acad Sci, 1183*, 251-266. doi:10.1111/j.1749-6632.2009.05126.x

Odobasic, D., Gan, P. Y., Summers, S. A., Semple, T. J., Muljadi, R. C., Iwakura, Y., Holdsworth, S. R. (2011). Interleukin-17A promotes early but attenuates established disease in crescentic glomerulonephritis in mice. *Am J Pathol, 179*(3), 1188-1198. doi:10.1016/j.ajpath.2011.05.039

Odobasic, D., Ghali, J. R., O'Sullivan, K. M., Holdsworth, S. R., & Kitching, A. R. (2014). Glomerulonephritis Induced by Heterologous Anti-GBM Globulin as a Planted Foreign Antigen. *Curr Protoc Immunol, 106*, 15 26 11-20. doi:10.1002/0471142735.im1526s106

Odobasic, D., Kitching, A. R., Semple, T. J., Timoshanko, J. R., Tipping, P. G., & Holdsworth, S. R. (2005). Glomerular expression of CD80 and CD86 is required for leukocyte accumulation and injury in crescentic glomerulonephritis. *J Am Soc Nephrol, 16*(7), 2012-2022. doi:10.1681/ASN.2004060437

Odobasic, D., Kitching, A. R., Yang, Y., O'Sullivan, K. M., Muljadi, R. C., Edgtton, K. L., Holdsworth, S. R. (2013). Neutrophil myeloperoxidase regulates T-cell-driven tissue inflammation in mice by inhibiting dendritic cell function. *Blood, 121*(20), 4195-4204. doi:10.1182/blood-2012-09-456483

Ohkura, N., Kitagawa, Y., & Sakaguchi, S. (2013). Development and maintenance of regulatory T cells. *Immunity, 38*(3), 414-423. doi:10.1016/j.immuni.2013.03.002

Okayama, Y., & Kawakami, T. (2006). Development, migration, and survival of mast cells. *Immunol Res, 34*(2), 97-115. doi:10.1385/IR:34:2:97

Olson, S. W., Arbogast, C. B., Baker, T. P., Owshalimpur, D., Oliver, D. K., Abbott, K. C., & Yuan, C. M. (2011). Asymptomatic autoantibodies associate with future anti-glomerular basement membrane disease. *J Am Soc Nephrol, 22*(10), 1946-1952. doi:10.1681/ASN.2010090928

Ooi, J. D., Snelgrove, S. L., Engel, D. R., Hochheiser, K., Ludwig-Portugall, I., Nozaki, Y., Kitching, A. R. (2011). Endogenous foxp3(+) T-regulatory cells suppress anti-glomerular basement membrane nephritis. *Kidney Int, 79*(9), 977-986. doi:10.1038/ki.2010.541

Ormerod, A. S., & Cook, M. C. (2008). Epidemiology of primary systemic vasculitis in the Australian Capital Territory and south-eastern New South Wales. *Intern Med J, 38*(11), 816-823. doi:10.1111/j.1445-5994.2008.01672.x

Otsubo, S., Nitta, K., Uchida, K., Yumura, W., & Nihei, H. (2003). Mast cells and tubulointerstitial fibrosis in patients with ANCA-associated glomerulonephritis. *Clin Exp Nephrol, 7*(1), 41-47. doi:10.1007/s101570300005

Pagnoux, C., Hogan, S. L., Chin, H., Jennette, J. C., Falk, R. J., Guillevin, L., & Nachman, P. H. (2008). Predictors of treatment resistance and relapse in antineutrophil cytoplasmic antibody-associated small-vessel vasculitis: comparison of two independent cohorts. *Arthritis Rheum, 58*(9), 2908-2918. doi:10.1002/art.23800

Pagnoux, C., Mahr, A., Hamidou, M. A., Boffa, J. J., Ruivard, M., Ducroix, J. P.,French Vasculitis Study, G. (2008). Azathioprine or methotrexate maintenance for ANCA-associated vasculitis. *N Engl J Med, 359*(26), 2790-2803. doi:10.1056/NEJMoa0802311

Papayannopoulos, V., Metzler, K. D., Hakkim, A., & Zychlinsky, A. (2010). Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol, 191*(3), 677-691. doi:10.1083/jcb.201006052

Pardo, J., Diaz, L., Errasti, P., Idoate, M., de Alava, E., Sola, I., Panizo, A. (2000). Mast cells in chronic rejection of human renal allografts. *Virchows Arch, 437*(2), 167-172.

Parker, H., Albrett, A. M., Kettle, A. J., & Winterbourn, C. C. (2012). Myeloperoxidase associated with neutrophil extracellular traps is active and mediates bacterial killing in the presence of hydrogen peroxide. *J Leukoc Biol, 91*(3), 369-376. doi:10.1189/jlb.0711387

Parker, H., Dragunow, M., Hampton, M. B., Kettle, A. J., & Winterbourn, C. C. (2012). Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *J Leukoc Biol, 92*(4), 841-849. doi:10.1189/jlb.1211601

Parks, C. G., Conrad, K., & Cooper, G. S. (1999). Occupational exposure to crystalline silica and autoimmune disease. *Environ Health Perspect, 107 Suppl 5*, 793-802.

Pasparakis, M., & Vandenabeele, P. (2015). Necroptosis and its role in inflammation. *Nature, 517*(7534), 311-320. doi:10.1038/nature14191

Paust, H. J., Ostmann, A., Erhardt, A., Turner, J. E., Velden, J., Mittrucker, H. W., Tiegs, G. (2011). Regulatory T cells control the Th1 immune response in murine crescentic glomerulonephritis. *Kidney Int, 80*(2), 154-164. doi:10.1038/ki.2011.108

Pejler, G., Abrink, M., Ringvall, M., & Wernersson, S. (2007). Mast cell proteases. *Adv Immunol, 95*, 167-255. doi:10.1016/S0065-2776(07)95006-3

Piccirillo, C. A., & Shevach, E. M. (2001). Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. *J Immunol, 167*(3), 1137-1140.

Piconese, S., Costanza, M., Musio, S., Tripodo, C., Poliani, P. L., Gri, G., Pedotti, R. (2011). Exacerbated experimental autoimmune encephalomyelitis in mast-cell-deficient Kit W-sh/W-sh mice. *Lab Invest, 91*(4), 627-641. doi:10.1038/labinvest.2011.3

Pillay, J., den Braber, I., Vrisekoop, N., Kwast, L. M., de Boer, R. J., Borghans, J. A., Koenderman, L. (2010). In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood, 116*(4), 625-627. doi:10.1182/blood-2010-01-259028

Porges, A. J., Redecha, P. B., Kimberly, W. T., Csernok, E., Gross, W. L., & Kimberly, R. P. (1994). Anti-neutrophil cytoplasmic antibodies engage and activate human neutrophils via Fc gamma RIIa. *J Immunol, 153*(3), 1271-1280.

Primo, V.C., Marusic S., Franklin C.C., goldman, W.H., Achaval, C.G., et al (2010) Anti-PR3 immune responses induce segmetnal and necrotising glomerulonephrtiis. Clin Exp Immunol 159:327-37

Puechal, X., Pagnoux, C., Perrodeau, E., Hamidou, M., Boffa, J. J., Kyndt, X., French Vasculitis Study, G. (2016). Long-Term Outcomes Among Participants in the WEGENT Trial of Remission-Maintenance Therapy for Granulomatosis With Polyangiitis (Wegener's) or Microscopic Polyangiitis. *Arthritis Rheumatol, 68*(3), 690-701. doi:10.1002/art.39450

Qasim, F. J., Mathieson, P. W., Sendo, F., Thiru, S., & Oliveira, D. B. (1996). Role of neutrophils in the pathogenesis of experimental vasculitis. *Am J Pathol, 149*(1), 81-89.

Rastaldi, M. P., Ferrario, F., Crippa, A., Dell'Antonio, G., Casartelli, D., Grillo, C., & D'Amico, G. (2000). Glomerular monocyte-macrophage features in ANCA-positive renal vasculitis and cryoglobulinemic nephritis. *J Am Soc Nephrol, 11*(11), 2036-2043.

Rausch, P. G., & Moore, T. G. (1975). Granule enzymes of polymorphonuclear neutrophils: A phylogenetic comparison. *Blood, 46*(6), 913-919.

Richez, C., Blanco, P., Rifkin, I., Moreau, J. F., & Schaeverbeke, T. (2011). Role for toll-like receptors in autoimmune disease: the example of systemic lupus erythematosus. *Joint Bone Spine, 78*(2), 124-130. doi:10.1016/j.jbspin.2010.09.005

Rimbert, M., Hamidou, M., Braudeau, C., Puechal, X., Teixeira, L., Caillon, H., Josien, R. (2011). Decreased numbers of blood dendritic cells and defective function of regulatory T cells in antineutrophil cytoplasmic antibody-associated vasculitis. *PLoS One, 6*(4), e18734. doi:10.1371/journal.pone.0018734

Robbie-Ryan, M., Tanzola, M. B., Secor, V. H., & Brown, M. A. (2003). Cutting edge: both activating and inhibitory Fc receptors expressed on mast cells regulate experimental allergic encephalomyelitis disease severity. *J Immunol, 170*(4), 1630-1634.

Rocha-Parise, M., Santos, L. M., Damoiseaux, J. G., Bagatin, E., Lido, A. V., Torello, C. O., Queiroz, M. L. (2014). Lymphocyte activation in silica-exposed workers. *Int J Hyg Environ Health, 217*(4-5), 586-591. doi:10.1016/j.ijheh.2013.11.002

Rochael, N. C., Guimaraes-Costa, A. B., Nascimento, M. T., DeSouza-Vieira, T. S., Oliveira, M. P., Garcia e Souza, L. F., Saraiva, E. M. (2015). Classical ROS-dependent and early/rapid ROS-independent release of Neutrophil Extracellular Traps triggered by Leishmania parasites. *Sci Rep, 5*, 18302. doi:10.1038/srep18302

Roth, A. J., Brown, M. C., Smith, R. N., Badhwar, A. K., Parente, O., Chung, H., Falk, R. J. (2012). Anti-LAMP-2 antibodies are not prevalent in patients with antineutrophil cytoplasmic autoantibody glomerulonephritis. *J Am Soc Nephrol, 23*(3), 545-555. doi:10.1681/ASN.2011030273

Roth, A. J., Ooi, J. D., Hess, J. J., van Timmeren, M. M., Berg, E. A., Poulton, C. E.,Falk, R. J. (2013). Epitope specificity determines pathogenicity and detectability in ANCA-associated vasculitis. *J Clin Invest, 123*(4), 1773-1783. doi:10.1172/JCI65292

Ruth, A. J., Kitching, A. R., Kwan, R. Y., Odobasic, D., Ooi, J. D., Timoshanko, J. R., Holdsworth, S. R. (2006). Anti-neutrophil cytoplasmic antibodies and effector CD4+ cells play nonredundant roles in anti-myeloperoxidase crescentic glomerulonephritis. *J Am Soc Nephrol, 17*(7), 1940-1949. doi:10.1681/ASN.2006020108

Ruth, A. J., Kitching, A. R., Semple, T. J., Tipping, P. G., & Holdsworth, S. R. (2003). Intrinsic renal cell expression of CD40 directs Th1 effectors inducing experimental crescentic glomerulonephritis. *J Am Soc Nephrol, 14*(11), 2813-2822.

Salama, A. D., Chaudhry, A. N., Holthaus, K. A., Mosley, K., Kalluri, R., Sayegh, M. H., Lightstone, L. (2003). Regulation by CD25+ lymphocytes of autoantigen-specific T-cell responses in Goodpasture's (anti-GBM) disease. *Kidney Int, 64*(5), 1685-1694. doi:10.1046/j.1523-1755.2003.00259.x

Sallai, K., Nagy, E., Derfalvy, B., Muzes, G., & Gergely, P. (2005). Antinucleosome antibodies and decreased deoxyribonuclease activity in sera of patients with systemic lupus erythematosus. *Clin Diagn Lab Immunol, 12*(1), 56-59. doi:10.1128/CDLI.12.1.56-59.2005

Sanders, J. S., Huitma, M. G., Kallenberg, C. G., & Stegeman, C. A. (2006). Prediction of relapses in PR3-ANCA-associated vasculitis by assessing responses of ANCA titres to treatment. *Rheumatology (Oxford), 45*(6), 724-729. doi:10.1093/rheumatology/kei272

Sangaletti, S., Tripodo, C., Chiodoni, C., Guarnotta, C., Cappetti, B., Casalini, P., Colombo, M. P. (2012). Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity. *Blood, 120*(15), 3007-3018. doi:10.1182/blood-2012-03-416156

Sauter, B., Albert, M. L., Francisco, L., Larsson, M., Somersan, S., & Bhardwaj, N. (2000). Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med, 191*(3), 423-434.

Sayed, B. A., Walker, M. E., & Brown, M. A. (2011). Cutting edge: mast cells regulate disease severity in a relapsing-remitting model of multiple sclerosis. *J Immunol, 186*(6), 3294-3298. doi:10.4049/jimmunol.1003574

Scaffidi, P., Misteli, T., & Bianchi, M. E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature, 418*(6894), 191-195. doi:10.1038/nature00858

Scheb-Wetzel, M., Rohde, M., Bravo, A., & Goldmann, O. (2014). New insights into the antimicrobial effect of mast cells against Enterococcus faecalis. *Infect Immun, 82*(11), 4496-4507. doi:10.1128/IAI.02114-14

Schlondorff, D. (1987). The glomerular mesangial cell: an expanding role for a specialized pericyte. *FASEB J, 1*(4), 272-281.

Schlondorff, D., & Banas, B. (2009). The mesangial cell revisited: no cell is an island. *J Am Soc Nephrol, 20*(6), 1179-1187. doi:10.1681/ASN.2008050549

Schreiber, A., Xiao, H., Falk, R. J., & Jennette, J. C. (2006). Bone marrow-derived cells are sufficient and necessary targets to mediate glomerulonephritis and vasculitis induced by anti-myeloperoxidase antibodies. *J Am Soc Nephrol, 17*(12), 3355-3364. doi:10.1681/ASN.2006070718

Schreiber, A., Xiao, H., Jennette, J. C., Schneider, W., Luft, F. C., & Kettritz, R. (2009). C5a receptor mediates neutrophil activation and ANCA-induced glomerulonephritis. *J Am Soc Nephrol, 20*(2), 289-298. doi:10.1681/ASN.2008050497

Schultz, J., & Kaminker, K. (1962). Myeloperoxidase of the leucocyte of normal human blood. I. Content and localization. *Arch Biochem Biophys, 96*, 465-467.

Scott, R. P., & Quaggin, S. E. (2015). Review series: The cell biology of renal filtration. *J Cell Biol, 209*(2), 199-210. doi:10.1083/jcb.201410017

Segerer, S., Heller, F., Lindenmeyer, M. T., Schmid, H., Cohen, C. D., Draganovici, D., Schlondorff, D. (2008). Compartment specific expression of dendritic cell markers in human glomerulonephritis. *Kidney Int, 74*(1), 37-46. doi:10.1038/ki.2008.99

Shigeoka, A. A., Holscher, T. D., King, A. J., Hall, F. W., Kiosses, W. B., Tobias, P. S., McKay, D. B. (2007). TLR2 is constitutively expressed within the kidney and participates in ischemic renal injury through both MyD88-dependent and -independent pathways. *J Immunol, 178*(10), 6252-6258.

Soderberg, D., Kurz, T., Motamedi, A., Hellmark, T., Eriksson, P., & Segelmark, M. (2015). Increased levels of neutrophil extracellular trap remnants in the circulation of patients with small vessel vasculitis, but an inverse correlation to anti-neutrophil cytoplasmic antibodies during remission. *Rheumatology (Oxford), 54*(11), 2085-2094. doi:10.1093/rheumatology/kev217

Soos, T. J., Sims, T. N., Barisoni, L., Lin, K., Littman, D. R., Dustin, M. L., & Nelson, P. J. (2006). CX3CR1+ interstitial dendritic cells form a contiguous network throughout the entire kidney. *Kidney Int, 70*(3), 591-596. doi:10.1038/sj.ki.5001567

Soriano, P. (1994). Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev, 8*(16), 1888-1896.

Stachura, I., Si, L., & Whiteside, T. L. (1984). Mononuclear-cell subsets in human idiopathic crescentic glomerulonephritis (ICGN): analysis in tissue sections with monoclonal antibodies. *J Clin Immunol, 4*(3), 202-208.

Steblay, R. W. (1962). Glomerulonephritis induced in sheep by injections of heterologous glomerular basement membrane and Freund's complete adjuvant. *J Exp Med, 116*, 253-272.

Stegeman, C. A., Tervaert, J. W., de Jong, P. E., & Kallenberg, C. G. (1996). Trimethoprim-sulfamethoxazole (co-trimoxazole) for the prevention of relapses of Wegener's granulomatosis. Dutch Co-Trimoxazole Wegener Study Group. *N Engl J Med, 335*(1), 16-20. doi:10.1056/NEJM199607043350103

Stegeman, C. A., Tervaert, J. W., & Kallenberg, C. G. (1997). Co-trimoxazole and Wegener's granulomatosis: more than a coincidence? *Nephrol Dial Transplant, 12*(4), 652-655.

Stephen, J., Scales, H. E., Benson, R. A., Erben, D., Garside, P., & Brewer, J. M. (2017). Neutrophil swarming and extracellular trap formation play a significant role in Alum adjuvant activity. *npj Vaccines, 2*(1), 1. doi:10.1038/s41541-016-0001-5

Sterzel, R. B., & Pabst, R. (1982). The temporal relationship between glomerular cell proliferation and monocyte infiltration in experimental glomerulonephritis. *Virchows Arch B Cell Pathol Incl Mol Pathol, 38*(3), 337-350.

Stilmant, M. M., Bolton, W. K., Sturgill, B. C., Schmitt, G. W., & Couser, W. G. (1979). Crescentic glomerulonephritis without immune deposits: clinicopathologic features. *Kidney Int, 15*(2), 184-195.

Stone, J. H., Merkel, P. A., Spiera, R., Seo, P., Langford, C. A., Hoffman, G. S., Group, R.-I. R. (2010). Rituximab versus cyclophosphamide for ANCA-associated vasculitis. *N Engl J Med, 363*(3), 221-232. doi:10.1056/NEJMoa0909905

Sugiyama, S., Okada, Y., Sukhova, G. K., Virmani, R., Heinecke, J. W., & Libby, P. (2001). Macrophage myeloperoxidase regulation by granulocyte macrophage colony-stimulating factor in human atherosclerosis and implications in acute coronary syndromes. *Am J Pathol, 158*(3), 879-891. doi:10.1016/S0002-9440(10)64036-9

Sumida, K., Ubara, Y., Nomura, K., Hoshino, J., Suwabe, T., Hiramatsu, R., Ohashi, K. (2012). ANCA-associated crescentic glomerulonephritis with immune complex deposits. *Clin Nephrol, 77*(6), 454-460.

Summers, S. A., Gan, P. Y., Dewage, L., Ma, F. T., Ooi, J. D., O'Sullivan, K. M., Holdsworth, S. R. (2012). Mast cell activation and degranulation promotes renal fibrosis in experimental unilateral ureteric obstruction. *Kidney Int, 82*(6), 676-685. doi:10.1038/ki.2012.211

Summers, S. A., Steinmetz, O. M., Li, M., Kausman, J. Y., Semple, T., Edgtton, K. L., Kitching, A. R. (2009). Th1 and Th17 cells induce proliferative glomerulonephritis. *J Am Soc Nephrol, 20*(12), 2518-2524. doi:10.1681/ASN.2009030337

Summers, S. A., Hoi, A., Steinmetz, O. M., O'Sullivan, K. M., Ooi, J. D., Odobasic, D., Holdsworth, S. R. (2010a). TLR9 and TLR4 are required for the development of autoimmunity and lupus nephritis in pristane nephropathy. *J Autoimmun, 35*(4), 291-298. doi:10.1016/j.jaut.2010.05.004

Summers, S. A., Steinmetz, O. M., Ooi, J. D., Gan, P. Y., O'Sullivan, K. M., Visvanathan, K., Holdsworth, S. R. (2010b). Toll-like receptor 9 enhances nephritogenic immunity and glomerular leukocyte recruitment, exacerbating experimental crescentic glomerulonephritis. *Am J Pathol, 177*(5), 2234-2244. doi:10.2353/ajpath.2010.100153

Summers, S. A., van der Veen, B. S., O'Sullivan, K. M., Gan, P. Y., Ooi, J. D., Heeringa, P., Kitching, A. R. (2010c). Intrinsic renal cell and leukocyte-derived TLR4 aggravate experimental anti-MPO glomerulonephritis. *Kidney Int, 78*(12), 1263-1274. doi:10.1038/ki.2010.327

Summers, S. A., Steinmetz, O. M., Gan, P. Y., Ooi, J. D., Odobasic, D., Kitching, A. R., & Holdsworth, S. R. (2011). Toll-like receptor 2 induces Th17 myeloperoxidase autoimmunity while Toll-like receptor 9 drives Th1 autoimmunity in murine vasculitis. *Arthritis & Rheumatism, 63*(4), 1124-1135.

Supajatura, V., Ushio, H., Nakao, A., Akira, S., Okumura, K., Ra, C., & Ogawa, H. (2002). Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *J Clin Invest, 109*(10), 1351-1359. doi:10.1172/JCI14704

Suri, R., Marshall, L. J., Wallis, C., Metcalfe, C., Bush, A., & Shute, J. K. (2002). Effects of recombinant human DNase and hypertonic saline on airway inflammation in children with cystic fibrosis. *Am J Respir Crit Care Med, 166*(3), 352-355. doi:10.1164/rccm.2110015

Tadema, H., Abdulahad, W. H., Stegeman, C. A., Kallenberg, C. G., & Heeringa, P. (2011). Increased expression of Toll-like receptors by monocytes and natural killer cells in ANCA-associated vasculitis. *PLoS One, 6*(9), e24315. doi:10.1371/journal.pone.0024315

Tan, D. S., Gan, P. Y., O'Sullivan, K. M., Hammett, M. V., Summers, S. A., Ooi, J. D., Holdsworth, S. R. (2013). Thymic deletion and regulatory T cells prevent antimyeloperoxidase GN. *J Am Soc Nephrol, 24*(4), 573-585. doi:10.1681/ASN.2012090898

Tang, W., Bose, B., McDonald, S. P., Hawley, C. M., Badve, S. V., Boudville, N., Johnson, D. W. (2013). The outcomes of patients with ESRD and ANCA-associated vasculitis in Australia and New Zealand. *Clin J Am Soc Nephrol, 8*(5), 773-780. doi:10.2215/CJN.08770812

Tew, M. B., Johnson, R. W., Reveille, J. D., & Tan, F. K. (2001). A molecular analysis of the low serum deoxyribonuclease activity in lupus patients. *Arthritis Rheum, 44*(10), 2446-2447.

Tidman, M., Olander, R., Svalander, C., & Danielsson, D. (1998). Patients hospitalized because of small vessel vasculitides with renal involvement in the period 1975-95: organ involvement, anti-neutrophil cytoplasmic antibodies patterns, seasonal attack rates and fluctuation of annual frequencies. *J Intern Med, 244*(2), 133-141.

Tillack, K., Breiden, P., Martin, R., & Sospedra, M. (2012). T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses. *J Immunol, 188*(7), 3150-3159. doi:10.4049/jimmunol.1103414

Timoshanko, J. R., Holdsworth, S. R., Kitching, A. R., & Tipping, P. G. (2002). IFN-gamma production by intrinsic renal cells and bone marrow-derived cells is required for full expression of crescentic glomerulonephritis in mice. *J Immunol, 168*(8), 4135-4141.

Timoshanko, J. R., Kitching, A. R., Holdsworth, S. R., & Tipping, P. G. (2001). Interleukin-12 from intrinsic cells is an effector of renal injury in crescentic glomerulonephritis. *J Am Soc Nephrol, 12*(3), 464-471.

Timoshanko, J. R., Kitching, A. R., Semple, T. J., Tipping, P. G., & Holdsworth, S. R. (2006). A pathogenetic role for mast cells in experimental crescentic glomerulonephritis. *J Am Soc Nephrol, 17*(1), 150-159. doi:10.1681/ASN.2005080799

Todd, S. K., Pepper, R. J., Draibe, J., Tanna, A., Pusey, C. D., Mauri, C., & Salama, A. D. (2014). Regulatory B cells are numerically but not functionally deficient in anti-neutrophil cytoplasm antibody-associated vasculitis. *Rheumatology (Oxford), 53*(9), 1693-1703. doi:10.1093/rheumatology/keu136

Toth, T., Toth-Jakatics, R., Jimi, S., Ihara, M., Urata, H., & Takebayashi, S. (1999). Mast cells in rapidly progressive glomerulonephritis. *J Am Soc Nephrol, 10*(7), 1498-1505.

Tsuboi, N., Yoshikai, Y., Matsuo, S., Kikuchi, T., Iwami, K., Nagai, Y., Matsuguchi, T. (2002). Roles of toll-like receptors in C-C chemokine production by renal tubular epithelial cells. *J Immunol, 169*(4), 2026-2033.

Uribe Echevarria, L., Leimgruber, C., Garcia Gonzalez, J., Nevado, A., Alvarez, R., Garcia, L. N., Maldonado, C. A. (2017). Evidence of eosinophil extracellular trap cell death in COPD: does it represent the trigger that switches on the disease? *Int J Chron Obstruct Pulmon Dis, 12*, 885-896. doi:10.2147/COPD.S115969

Valles, P. G., Lorenzo, A. G., Bocanegra, V., & Valles, R. (2014). Acute kidney injury: what part do toll-like receptors play? *Int J Nephrol Renovasc Dis, 7*, 241-251. doi:10.2147/IJNRD.S37891

van der Woude, F. J., Rasmussen, N., Lobatto, S., Wiik, A., Permin, H., van Es, L. A.,The, T. H. (1985). Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet, 1*(8426), 425-429.

Van Rooijen, N., & Sanders, A. (1994). Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods, 174*(1-2), 83-93.

Velden, J., Paust, H. J., Hoxha, E., Turner, J. E., Steinmetz, O. M., Wolf, G., Panzer, U. (2012). Renal IL-17 expression in human ANCA-associated glomerulonephritis. *Am J Physiol Renal Physiol, 302*(12), F1663-1673. doi:10.1152/ajprenal.00683.2011

Verzola, D., Cappuccino, L., D'Amato, E., Villaggio, B., Gianiorio, F., Mij, M., Garibotto, G. (2014). Enhanced glomerular Toll-like receptor 4 expression and signaling in patients with type 2 diabetic nephropathy and microalbuminuria. *Kidney Int, 86*(6), 1229-1243. doi:10.1038/ki.2014.116

Villanueva, E., Yalavarthi, S., Berthier, C. C., Hodgin, J. B., Khandpur, R., Lin, A. M., Kaplan, M. J. (2011). Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *J Immunol, 187*(1), 538-552. doi:10.4049/jimmunol.1100450

von Kockritz-Blickwede, M., Goldmann, O., Thulin, P., Heinemann, K., Norrby-Teglund, A., Rohde, M., & Medina, E. (2008). Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood, 111*(6), 3070-3080. doi:10.1182/blood-2007-07-104018

Walsh, M., Faurschou, M., Berden, A., Flossmann, O., Bajema, I., Hoglund, P., European Vasculitis Study, G. (2014). Long-term follow-up of cyclophosphamide compared with azathioprine for initial maintenance therapy in ANCA-associated vasculitis. *Clin J Am Soc Nephrol, 9*(9), 1571-1576. doi:10.2215/CJN.00100114

Walsh, M., Flossmann, O., Berden, A., Westman, K., Hoglund, P., Stegeman, C., European Vasculitis Study, G. (2012). Risk factors for relapse of antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheum, 64*(2), 542-548. doi:10.1002/art.33361

Wang, H., Sha, L. L., Ma, T. T., Zhang, L. X., Chen, M., & Zhao, M. H. (2016). Circulating Level of Neutrophil Extracellular Traps Is Not a Useful Biomarker for Assessing Disease Activity in Antineutrophil Cytoplasmic Antibody-Associated Vasculitis. *PLoS One, 11*(2), e0148197. doi:10.1371/journal.pone.0148197

Wang, Y., Li, M., Stadler, S., Correll, S., Li, P., Wang, D., Coonrod, S. A. (2009). Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J Cell Biol, 184*(2), 205-213. doi:10.1083/jcb.200806072

Warnatsch, A., Ioannou, M., Wang, Q., & Papayannopoulos, V. (2015). Inflammation. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. *Science, 349*(6245), 316-320. doi:10.1126/science.aaa8064

Watts, R. A., Mooney, J., Skinner, J., Scott, D. G., & Macgregor, A. J. (2012). The contrasting epidemiology of granulomatosis with polyangiitis (Wegener's) and microscopic polyangiitis. *Rheumatology (Oxford), 51*(5), 926-931. doi:10.1093/rheumatology/ker454

Watts, R. A., Scott, D. G., Jayne, D. R., Ito-Ihara, T., Muso, E., Fujimoto, S., Hashimoto, H. (2008). Renal vasculitis in Japan and the UK--are there differences in epidemiology and clinical phenotype? *Nephrol Dial Transplant, 23*(12), 3928-3931. doi:10.1093/ndt/gfn354

Weidner, S., Carl, M., Riess, R., & Rupprecht, H. D. (2004). Histologic analysis of renal leukocyte infiltration in antineutrophil cytoplasmic antibody-associated vasculitis: importance of monocyte and neutrophil infiltration in tissue damage. *Arthritis Rheum, 50*(11), 3651-3657. doi:10.1002/art.20607

Wernersson, S., & Pejler, G. (2014). Mast cell secretory granules: armed for battle. *Nat Rev Immunol, 14*(7), 478-494. doi:10.1038/nri3690

Wheeler, J., Robertson, H., Morley, A. R., & Appleton, D. R. (1993). Anti-glomerular basement membrane glomerulonephritis (anti-GBM GN) in the mouse: BrdU-labelling indices and histological damage. *Int J Exp Pathol, 74*(1), 9-19.

Wilde, B., van Paassen, P., Damoiseaux, J., Heerings-Rewinkel, P., van Rie, H., Witzke, O., & Tervaert, J. W. (2009). Dendritic cells in renal biopsies of patients with ANCA-associated vasculitis. *Nephrol Dial Transplant, 24*(7), 2151-2156. doi:10.1093/ndt/gfp019

Willenborg, D. O., Fordham, S., Bernard, C. C., Cowden, W. B., & Ramshaw, I. A. (1996). IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *J Immunol, 157*(8), 3223-3227.

Wolf, D., Hochegger, K., Wolf, A. M., Rumpold, H. F., Gastl, G., Tilg, H., Rosenkranz, A. R. (2005). CD4+CD25+ regulatory T cells inhibit experimental anti-glomerular basement membrane glomerulonephritis in mice. *J Am Soc Nephrol, 16*(5), 1360-1370. doi:10.1681/ASN.2004100837

Wolfs, T. G., Buurman, W. A., van Schadewijk, A., de Vries, B., Daemen, M. A., Hiemstra, P. S., & van 't Veer, C. (2002). In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation. *J Immunol, 168*(3), 1286-1293.

Wong, K. W., & Jacobs, W. R., Jr. (2013). Mycobacterium tuberculosis exploits human interferon gamma to stimulate macrophage extracellular trap formation and necrosis. *J Infect Dis, 208*(1), 109-119. doi:10.1093/infdis/jit097

Wong, S.L, Demers M., Martinoid, K. Gallant M., Wang,Y.,Goldfine, A.B., Kahn, C.R., Wagner, D.D. (2015). Diabetes primes neutrophils to undergo NETosis which impairs wound healing. Nature Medicine 21, 815-819 doi:10.1038/nm.3887

Wyllie, A. H., Kerr, J. F., & Currie, A. R. (1980). Cell death: the significance of apoptosis. *Int Rev Cytol, 68*, 251-306.

Xiao, H., Dairaghi, D. J., Powers, J. P., Ertl, L. S., Baumgart, T., Wang, Y., Jennette, J. C. (2014). C5a receptor (CD88) blockade protects against MPO-ANCA GN. *J Am Soc Nephrol, 25*(2), 225-231. doi:10.1681/ASN.2013020143

Xiao, H., Heeringa, P., Hu, P., Liu, Z., Zhao, M., Aratani, Y., Jennette, J. C. (2002). Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest, 110*(7), 955-963. doi:10.1172/JCI15918

Xiao, H., Heeringa, P., Liu, Z., Huugen, D., Hu, P., Maeda, N., Jennette, J. C. (2005). The role of neutrophils in the induction of glomerulonephritis by anti-myeloperoxidase antibodies. *Am J Pathol, 167*(1), 39-45. doi:10.1016/S0002-9440(10)62951-3

Xiao, H., Hu, P., Falk, R. J., & Jennette, J. C. (2016). Overview of the Pathogenesis of ANCA-Associated Vasculitis. *Kidney Dis (Basel), 1*(4), 205-215. doi:10.1159/000442323

Xiao, H., Schreiber, A., Heeringa, P., Falk, R. J., & Jennette, J. C. (2007). Alternative complement pathway in the pathogenesis of disease mediated by anti-neutrophil cytoplasmic autoantibodies. *Am J Pathol, 170*(1), 52-64. doi:10.2353/ajpath.2007.060573

Xiao, Z., Casey, K. A., Jameson, S. C., Curtsinger, J. M., & Mescher, M. F. (2009). Programming for CD8 T cell memory development requires IL-12 or type I IFN. *J Immunol, 182*(5), 2786-2794. doi:10.4049/jimmunol.0803484

Xu, J., Zhang, X., Pelayo, R., Monestier, M., Ammollo, C. T., Semeraro, F., Esmon, C. T. (2009). Extracellular histones are major mediators of death in sepsis. *Nat Med, 15*(11), 1318-1321. doi:10.1038/nm.2053

Yadav, R., Larbi, K. Y., Young, R. E., & Nourshargh, S. (2003). Migration of leukocytes through the vessel wall and beyond. *Thromb Haemost, 90*(4), 598-606. doi:10.1160/TH03-04-0220

Yang, J. J., Jennette, J. C., & Falk, R. J. (1994). Immune complex glomerulonephritis is induced in rats immunized with heterologous myeloperoxidase. *Clin Exp Immunol, 97*(3), 466-473.

Yang, J. J., Preston, G. A., Pendergraft, W. F., Segelmark, M., Heeringa, P., Hogan, S. L., Falk, R. J. (2001). Internalization of proteinase 3 is concomitant with endothelial cell apoptosis and internalization of myeloperoxidase with generation of intracellular oxidants. *Am J Pathol, 158*(2), 581-592. doi:10.1016/S0002-9440(10)64000-X

Yashiro, M., Muso, E., Itoh-Ihara, T., Oyama, A., Hashimoto, K., Kawamura, T., Sasayama, S. (2000). Significantly high regional morbidity of MPO-ANCA-related angitis and/or nephritis with respiratory tract involvement after the 1995 great earthquake in Kobe (Japan). *Am J Kidney Dis, 35*(5), 889-895.

Yasutomo, K., Horiuchi, T., Kagami, S., Tsukamoto, H., Hashimura, C., Urushihara, M., & Kuroda, Y. (2001). Mutation of DNASE1 in people with systemic lupus erythematosus. *Nat Genet, 28*(4), 313-314. doi:10.1038/91070

Yipp, B. G., Petri, B., Salina, D., Jenne, C. N., Scott, B. N., Zbytnuik, L. D., Kubes, P. (2012). Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med, 18*(9), 1386-1393. doi:10.1038/nm.2847

Yoshida, M., Sasaki, M., Sugisaki, K., Yamaguchi, Y., & Yamada, M. (2013). Neutrophil extracellular trap components in fibrinoid necrosis of the kidney with myeloperoxidase-ANCA-associated vasculitis. *Clin Kidney J, 6*(3), 308-312. doi:10.1093/ckj/sft048

Yousefi, S., Mihalache, C., Kozlowski, E., Schmid, I., & Simon, H. U. (2009). Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ, 16*(11), 1438-1444. doi:10.1038/cdd.2009.96

Zabriskie, J. B., & Freimer, E. H. (1966). An immunological relationship between the group. A streptococcus and mammalian muscle. *J Exp Med, 124*(4), 661-678.

Zhang, G. X., Gran, B., Yu, S., Li, J., Siglienti, I., Chen, X., Rostami, A. (2003). Induction of experimental autoimmune encephalomyelitis in IL-12 receptor-beta 2-deficient mice: IL-12 responsiveness is not required in the pathogenesis of inflammatory demyelination in the central nervous system. *J Immunol, 170*(4), 2153-2160.

Zhao, L., David, M. Z., Hyjek, E., Chang, A., & Meehan, S. M. (2015). M2 macrophage infiltrates in the early stages of ANCA-associated pauci-immune necrotizing GN. *Clin J Am Soc Nephrol, 10*(1), 54-62. doi:10.2215/CJN.03230314

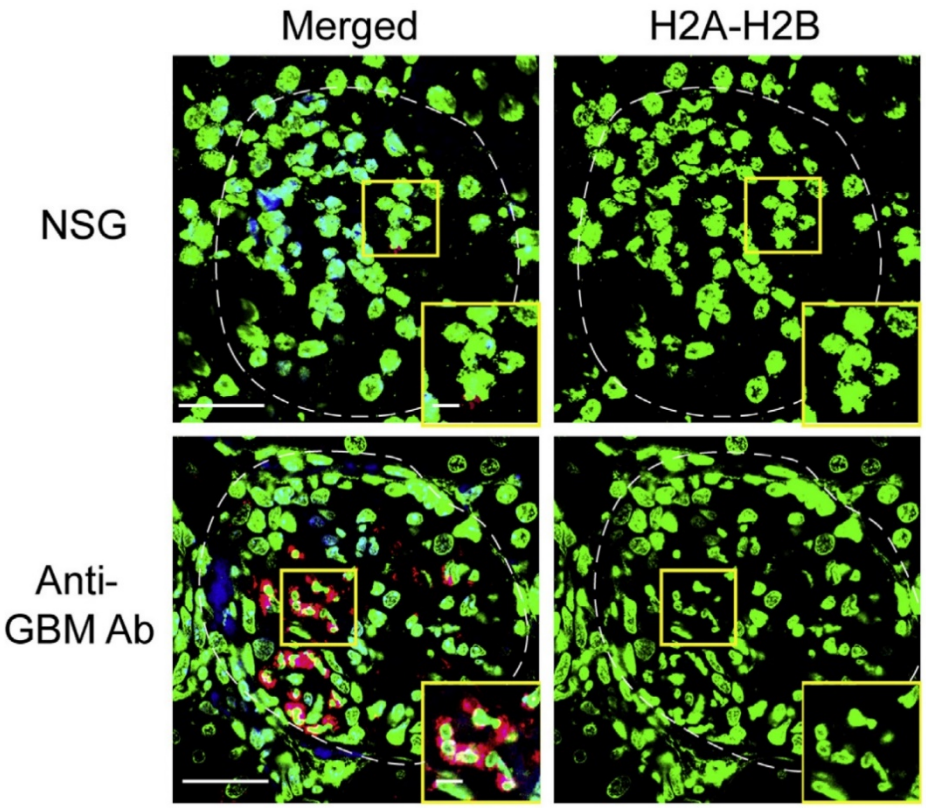
Zhou, J. S., Xing, W., Friend, D. S., Austen, K. F., & Katz, H. R. (2007). Mast cell deficiency in Kit(W-sh) mice does not impair antibody-mediated arthritis. *J Exp Med, 204*(12), 2797-2802. doi:10.1084/jem.20071391

Zhu, J., Yamane, H., & Paul, W. E. (2010). Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol, 28*, 445-489. doi:10.1146/annurev-immunol-030409-101212

Zou, R., Wang, S. X., Liu, G., Yu, F., Chen, M., & Zhao, M. H. (2016). Podocyte Detachment Is Associated with Renal Prognosis in ANCA-Associated Glomerulonephritis: A Retrospective Cohort Study. *Medicine (Baltimore), 95*(15), e3294. doi:10.1097/MD.0000000000003294

APPENDIX:

In Vivo Imaging Of Inflammed Glomeruli Reveals Dynamics Of Neutrophil Extracellular Trap Formation In Glomerular Capillaries



The American Journal of Pathology, Vol. 187, No. 2, February 2017

ELSEVIER

The American Journal of PATHOLOGY

ajp.amjpathol.org

CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

In Vivo Imaging of Inflamed Glomeruli Reveals Dynamics of Neutrophil Extracellular Trap Formation in Glomerular Capillaries

Clare L.V. Westhorpe,* James E. Bayard,* Kim M. O'Sullivan,* Pam Hall,* Qiang Cheng,* A. Richard Kitching,*†‡ and Michael J. Hickey*

From the Centre for Inflammatory Diseases, Monash University Department of Medicine, and the Departments of Nephrology† and Pediatric Nephrology,‡ Monash Medical Centre, Clayton, Victoria, Australia*

Accepted for publication October 6, 2016.

Address correspondence to Michael J. Hickey, Ph.D., Centre for Inflammatory Diseases, Monash University Department of Medicine, Monash Medical Centre, [redacted]

Neutrophil extracellular traps (NETs) have been documented in glomeruli of patients with glomerulonephritis. However, the dynamics of NET formation in the glomerulus and their functional contribution to acute glomerular injury are poorly understood. Herein, we used *in vivo* multiphoton microscopy to investigate NET formation in the acutely inflamed glomerulus. Glomerular inflammation was induced using an antibody against the glomerular basement membrane. After induction of inflammation, multiphoton microscopy revealed that approximately 20% of glomeruli contained structures composed of extracellular DNA within the capillaries. These structures were not seen in mice depleted of neutrophils, consistent with them being NETs. Most contained myeloperoxidase, as seen in NETs in other tissues, whereas intraglomerular NETs did not contain significant levels of the histone H2Ax or neutrophil elastase. *In vivo* imaging revealed that intraglomerular NETs were present only transiently, suggesting that NETs were susceptible to disruption under the high shear conditions in glomerular capillaries. Investigation of NETs under flow conditions *in vitro* supported this concept. Dissolution of NETs via DNase I did not alter anti–glomerular basement membrane antibody-induced glomerular injury, as assessed via albuminuria, although the degree of microscopic hematuria was reduced by this intervention. These data indicate that in this model of acute, neutrophil-dependent glomerulonephritis, NETs are generated in the glomerular capillaries, where they are short lived and make a modest contribution to glomerular injury. *(Am J Pathol 2017, 187: 318–331; http://dx.doi.org/10.1016/j.ajpath.2016.10.008)*

Glomerulonephritis is a key cause of end-stage renal failure, and in many forms of glomerulonephritis, leukocytes play critical roles in glomerular injury.[1,2] In acute glomerulonephritis, neutrophils accumulate in glomeruli and depletion of neutrophils or inhibition of their recruitment to the glomerulus reduces the level of injury, indicating a central role for neutrophils in this form of glomerular inflammation.[1,3–5] Despite these studies, the mechanisms whereby neutrophils are recruited to the unique microvasculature of the glomerulus, and their behavior after their recruitment, had remained poorly understood. To overcome this, we recently used *in vivo* multiphoton microscopy to examine neutrophil recruitment to acutely inflamed glomeruli of mice.[1] These studies demonstrated that in response to acute inflammatory stimulation, neutrophils are retained within the glomerular capillary lumen for prolonged periods, in some cases migrating extensively, and mediate glomerular injury without leaving the vasculature.[1] These and other studies have demonstrated that the effector mechanisms by which neutrophils mediate glomerular injury include generation of reactive oxygen species and release of proteases, including myeloperoxidase (MPO), neutrophil elastase, and

Supported by the National Health and Medical Research Council, Australia, project grants 1045165 and 1064112 (M.J.H. and A.R.K.) and Fellowship 1042775 (M.J.H.).

C.L.V.W., J.E.B., and K.M.O. contributed equally to this work.

Disclosures: None declared.

http://dx.doi.org/10.1016/j.ajpath.2016.10.008

cathepsin G.[1,6–9] However, during the past decade, an additional neutrophil-derived effector mechanism has been identified—generation of neutrophil extracellular traps (NETs).[10–14] Little is known about the role of NETs in glomerular injury in acute glomerulonephritis.

NETs are webs of DNA and intracellular proteins extruded by highly activated neutrophils.[15] These structures are coated with histones and proteolytic granule proteins, including MPO and neutrophil elastase, and are capable of capturing and killing microbes.[10,16] Although NETs were initially associated with the response to bacterial infection, more recently they have been implicated in various forms of sterile inflammation, including anti–neutrophil cytoplasmic antibody–positive glomerulonephritis and lupus nephritis.[14,17–23] Components of NETs are capable of injuring endothelial cells, raising the possibility that the presence of these structures in glomeruli contributes to injury to the glomerular endothelium and other glomerular cells.[24–26] This possibility is strengthened by reports that removal of NETs and histone neutralization reduce tissue injury and inflammation.[12,18,26] Despite this, little is known about the dynamics of the generation of these structures in the glomerular microvasculature.

The initial description of the dynamics of NET formation was based on *in vitro* analyses of neutrophils exposed to stimuli, including phorbol myristate acetate and live bacteria, and demonstrated that NET formation is a form of cell death (NETosis) in which neutrophils extrude DNA several hours after stimulation.[10] However, recent imaging-based analyses of NET formation have shed interesting new light on the nature of this process *in vivo*.[12,13] Studies of skin infections revealed that neutrophils in the skin can release NETs within minutes of encountering Gram-positive bacteria.[13] Moreover, neutrophils remained alive and retained the ability to migrate after NET generation, enabling wider spread of NETs within the skin. NETs can also be generated by neutrophils within the microvasculature. In a model of systemic endotoxemia, NETs were generated in the lumen of the hepatic sinusoids, where evidence indicated that they mediated hepatic injury.[12] Our recent studies indicate that neutrophils can mediate functional glomerular injury without leaving the glomerular capillaries, indicating that effector mechanisms generated within the microvasculature are critical to glomerular injury. However, the dynamics of NET formation in the glomerular microvasculature and the contribution of NETs to acute glomerular injury remain unclear. Therefore, the aim of this study was to investigate NET formation in the acutely inflamed glomerulus, using *in vivo* imaging to study the dynamics and nature of this process in the glomerulus. The results indicate that, in a model of *in situ* immune complex deposition, NETs are generated in glomerular capillaries, where they persist only briefly. In this model, removal of NETs does not affect albuminuria, the key readout of glomerular injury, although it does reduce the degree of microscopic hematuria.

Materials and Methods

Animals

Male C57BL/6 mice were used for intravital microscopy. All experimental procedures were approved by the Monash University Animal Ethics Committee.

Antibodies and Reagents

Sheep anti-mouse glomerular basement membrane antibody (anti-GBM Ab) was prepared as described.[5,27] As control Ab, normal sheep globulin (NSG) was prepared from nonimmune sheep serum using an identical protocol. Suppliers for reagents were as follows: Sytox Orange and DyLight conjugation kits (Life Technologies, Clayton, VIC, Australia); fluorescein isothiocyanate dextran (Sigma Aldrich, Castle Hill, NSW, Australia); anti–Gr-1 (RB6-8C5) conjugated to e450 or phycoerythrin (eBioscience, San Diego, CA); goat anti-mouse histone H2Ax and goat anti-mouse neutrophil elastase (Santa Cruz Biotechnology, Dallas, TX); mouse anti-mouse H2A-H2B (in house)[10,23]; DNase I (Roche Diagnostics, Dee Why, NSW, Australia); and Cl-amidine (Cayman Chemicals, Ann Arbor, MI). Anti-MPO used in *in vivo* imaging studies and mouse anti-mouse H2A-H2B were generated in house.[10,28]

NET Detection via Immunohistochemistry

NETs were detected via immunohistochemistry of kidney sections using a recently published technique.[23] Images were acquired with a Nikon C1 confocal microscope (Nikon Imaging, Sydney, NSW, Australia), and NETs were defined as areas of colocalization of citrullinated histone H3 (a NET marker)[29], MPO, and peptidyl arginine deiminase-4 (PAD4) associated with extracellular DNA (stained via DAPI). At least 20 glomeruli were assessed per section, and the data were displayed as NETs/glomerular cross section. In parallel experiments, sections were costained for H2A-H2B (using mouse anti-mouse H2A-H2B, 1 μg/mL) and MPO, and NETs were defined as areas of colocalization of H2A-H2B, MPO, and DNA.

Unilateral Ureteric Ligation

To prepare the kidney for glomerular intravital microscopy, mice underwent unilateral ureteric ligation, and imaging experiments were performed after 12 weeks, as previously described.[5,27,28]

Renal Intravital Multiphoton Microscopy

The protocol for multiphoton microscopy imaging of the hydronephrotic kidney was as described previously, with minor modifications.[5] The kidney was visualized with a Leica SP5 microscope (Leica Microsystems, Heidelberg, Germany) with a 20× 1.0 numerical aperture objective lens and a

SpectraPhysics MaiTai pulsed infrared laser (NewSpec, Myrtle Bank, SA, Australia) tuned to 810 nm. Images were collected using a 3- to 6-μm Z-step size with a Z-depth of approximately 150 μm. Image stacks were acquired every 30 seconds. Recordings were analyzed using *Imaris* (Bitplane, Zurich, Switzerland). In some experiments, imaging of intact kidneys was performed, using 3-week-old mice weighing 10 to 12 g, as previously described.[1] In these experiments, the dose of anti-GBM Ab was adjusted according to the weight of the mice, whereas imaging was performed as described above.

Experimental Protocol

Mice were injected i.v. with either 15 or 20 mg of anti-GBM Ab, or NSG as control. For imaging experiments, mice received anti−Gr-1 (2 μg) and fluorescein isothiocyanate dextran (250 kDa) i.v. just before imaging. For assessment of NET protein markers, anti-MPO (5 μg), anti-H2Ax (5 μg), anti−H2A-H2B (10 μg), or anti−neutrophil elastase (2 μg), all conjugated to DyLight 650, were administered at the same time.[12] Kidneys were imaged over the subsequent 60 minutes, recording 10 separate fields containing three to five glomeruli each, for a 5-minute duration. As a result, 30 to 40 glomeruli were examined in each mouse, for a period of 5 minutes. To examine extracellular DNA release, at the end of 60 minutes of imaging, Sytox Orange (1 nmol/mouse i.v.) was administered and Z-stacks of approximately 10 fields containing two to three glomeruli each were recorded over the next 10 minutes. For neutrophil depletion, anti−Gr-1 (160 μg i.p.) was injected 24 hours before experiments.[5] To examine the functional role of NETs, mice received either the PAD4 inhibitor Cl-amidine (10 mg/kg i.p.) or DNase I (2000 U i.v.) 10 to 15 minutes before induction of the anti-GBM Ab model.[12,30−32]

Assessment of Renal Injury

Albuminuria was measured on 24-hour urine collections using a mouse albumin enzyme-linked immunosorbent assay quantification kit (Bethyl Laboratories, Montgomery, TX). Urine creatinine measurements were determined using an autoanalyzer-based alkaline picric acid method. Hematuria was assessed via dipstick.

Assessment of Resistance of NETs to High Shear Conditions

Mouse neutrophils were isolated from bone marrow via magnetic-based negative selection (Miltenyi-Biotec, Macquarie Park, NSW, Australia), and loaded into intercellular adhesion molecule-1−coated microfluidic devices (Microfluidic ChipShop, Jena, Germany) at 5×10^5/mL in Ca^{2+}/Mg^{2+}-containing Hanks' balanced salt solution. Neutrophils were allowed to adhere for 45 minutes, then the chambers were loaded with lipopolysaccharide (LPS; 1 μg/mL) and incubated at 37°C for 3 hours to stimulate NET formation.[33] At the end of the incubation, Sytox Orange (1 μmol/L) and Alexa 488−conjugated anti−Gr-1 (1 μg/mL) were loaded into the chambers at low shear to stain the NETs and neutrophils. Chambers were examined using an API Deltavision imaging system (GE Healthcare Australia Pty. Ltd., Parramatta, NSW, Australia) on an IX71 Olympus microscope (Olympus Australia, Notting Hill, VIC, Australia), and images were recorded using a $CoolSnap_{HQ}$ camera (Photometrics, Tucson, AZ). Images of NETs were recorded while Hanks' balanced salt solution was perfused through the chamber either at 1 dyn/cm^2 (sinusoidal shear) for 6 minutes or progressively at 1, 10, and 20 dyn/cm^2 for 2 minutes each, with the latter two rates representing glomerular capillary shear. Intensity of NET staining (Sytox Orange) was quantitated using ImageJ software version 1.51g (NIH, Bethesda, MD; *http://imagej.nih.gov/ij*).

In some experiments, NET generation by neutrophils adherent to endothelial cells was examined. The MS1 mouse endothelial cell line (CRL-2279; ATCC, Manassas, VA) was grown in Dulbecco's modified Eagle's medium with 5% fetal calf serum, and plated onto fibronectin-coated 35-mm culture dishes the day before experiments. Bone marrow cells (2×10^6/mL) were allowed to adhere to endothelial monolayers for 30 minutes, then cells were exposed to LPS (1 μg/mL) in Hanks' balanced salt solution under either static conditions or constant shear in a Glycotech flow chamber system, as previously described.[34] Preparations were subsequently stained with anti−Gr-1−Alexa Fluor 488 and Sytox Orange, to detect neutrophils and NETs, respectively, and examined via the Deltavision imaging system described above.

Statistical Analysis

For statistical comparisons, either a one-way analysis of variance with a post hoc test (Dunnet's) or a *t*-test was used. For nonnormally distributed data, either Kruskal-Wallis or Mann-Whitney tests were used. $P < 0.05$ was defined as statistically significant. Data are displayed as means ± SEM.

Results

NETs Are Detectable via Immunohistochemistry in Anti-GBM Ab-Induced Glomerulonephritis

We first used immunohistochemistry to examine the incidence of NETs in the anti-GBM Ab model of neutrophil-dependent glomerular inflammation, using methods we have recently used to characterize NETs in biopsy specimens from glomerulonephritis patients.[23] Kidney sections were stained for citrullinated histone H3, MPO, PAD4, and DNA and examined by confocal microscopy. NETs were defined as areas of colocalization of citrullinated histone H3, MPO, and PAD4 associated with extracellular DNA. NETs were extremely rare in NSG-treated mice (0.04 ± 0.04 NETs/glomerular cross section, $n = 4$) but were readily detectable 2 hours after anti-GBM Ab administration (1.61 ± 0.05

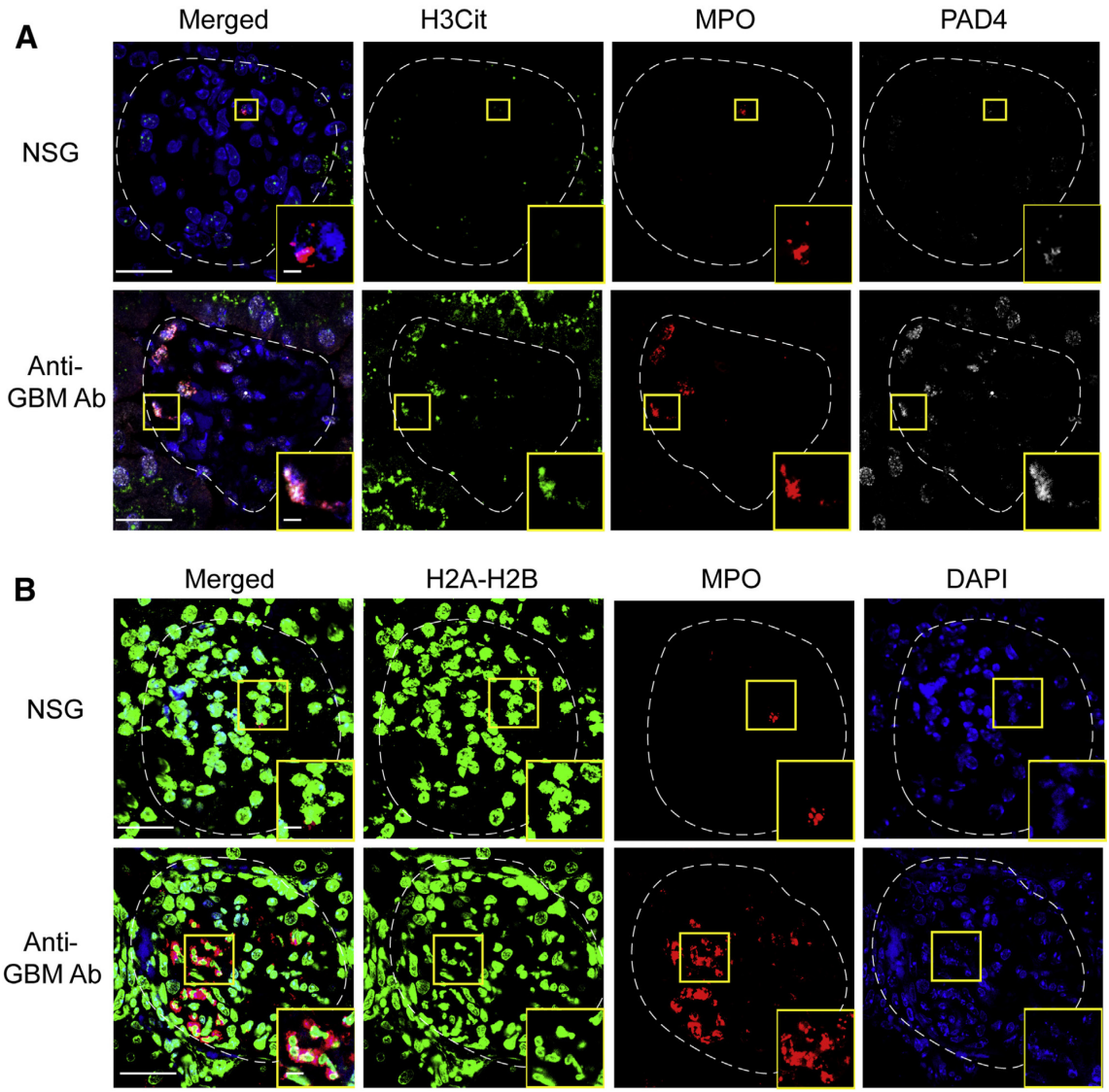


Figure 1 NETs are generated in glomeruli after anti-GBM Ab administration. Kidneys of mice were examined for NET formation via immunohistochemistry 2 hours after induction of anti-GBM Ab-induced glomerulonephritis. **A:** Staining is shown for citrullinated histone H3 (H3Cit; green), myeloperoxidase (MPO; red), and peptidyl arginine deiminase 4 (PAD4; white) in kidneys of mice treated with either normal sheep globulin (NSG) or anti-GBM Ab (glomeruli defined by **dotted line**). **Left column** shows merged images, which also include DNA staining via DAPI (blue). **Boxed areas** are shown at higher magnification in the **insets**. In NSG example, **insets** display magnified image of a non-NETing neutrophil (ie, lacking extracellular DNA). In anti-GBM Ab example, **insets** display magnified image of a NETing neutrophil (ie, positive for all three markers and extracellular DNA). **B:** Staining for H2A-H2B (green), MPO (red), and DNA (DAPI; blue) in kidney of mouse treated with either NSG or anti-GBM Ab (glomeruli defined by **dotted line**). **Left column** shows merged image. In NSG example, insets display magnified image of a non-NETing neutrophil. In anti-GBM Ab example, **insets** display magnified image of a NETing neutrophil (ie, positive for H2A-H2B, MPO, and extracellular DNA). Scale bars: 20 μm (**A** and **B**, **main images**); 5 μm (**A** and **B**, **insets**).

NETs/glomerular cross section, $n = 4$) (Figure 1A). As an alternative approach, we stained for H2A-H2B colocalized with MPO and extracellular DNA and also observed NETs meeting this definition in inflamed, but not noninflamed, glomeruli (Figure 1B). We therefore proceeded to use *in vivo* microscopy of mice undergoing this model to characterize intraglomerular NET formation more fully.

Assessment of Glomerular NETs via Multiphoton Microscopy

Using multiphoton microscopy, we observed that anti-GBM Ab (both 15 and 20 mg) induced an increase in neutrophil dwell time without an increase in the number of neutrophils that underwent adhesion (30 seconds or longer) in the

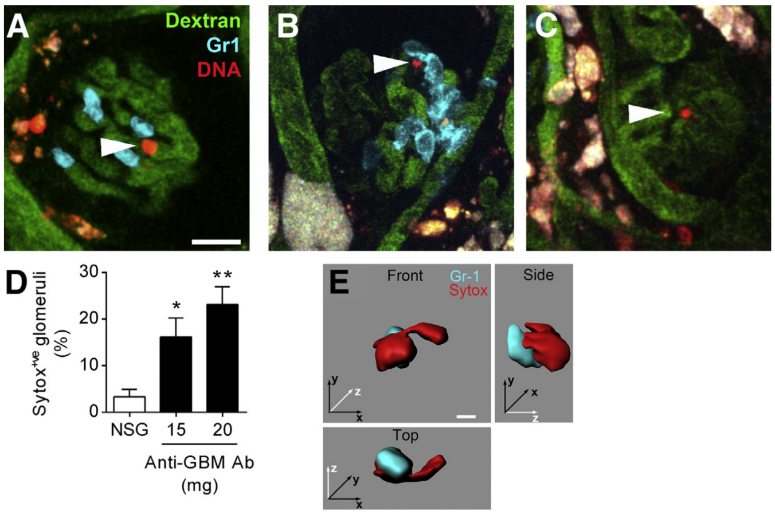


Figure 2 Extracellular DNA is detectable in inflamed glomeruli via multiphoton microscopy. **A**–**C:** Examples of the morphology of Sytox Orange–stained structures (red; **arrowheads**) in glomerular capillaries 2 hours after administration of anti-GBM Ab. Neutrophils are stained via anti–Gr-1 (cyan), and the vasculature is labeled using fluorescein isothiocyanate dextran (green). **D:** Quantitation of Sytox Orange–stained structures in glomeruli of mice treated with either normal sheep globulin (NSG) or anti-GBM Ab (15 or 20 mg). **E:** Three-dimensional surface rendering of a neutrophil (anti–Gr-1; cyan) with an adjacent extracellular Sytox Orange–stained structure (red), consistent with NET generation. See also Supplemental Video S1. Data are shown as means ± SEM (**D**). $n = 6$ per group (**D**). $^{*}P < 0.05$, $^{**}P < 0.01$ versus NSG. Scale bars: 20 μm (**A**–**C**); 5 μm (**E**).

glomerular capillaries (data not shown), as we have previously observed.[1] Previous imaging studies have used extracellular DNA dyes, such as Sytox Orange, to label DNA released from activated neutrophils.[12–14,35] Sytox Orange was administered i.v. 2 hours after anti-GBM Ab, and glomeruli were examined in the subsequent 10 minutes. Extracellular Sytox Orange–labeled structures were detectable within glomerular capillaries (Figure 2). These structures were variable in morphology, occurring on some occasions as discrete, compact regions of staining approximately 2 to 3 μm in diameter, or alternatively as larger structures occupying much of the capillary lumen (Figure 2, A–C).

The percentage of glomeruli positive for Sytox Orange–stained structures was markedly elevated in mice treated with anti-GBM Ab, at both 15 and 20 mg, relative to that in NSG-treated controls (Figure 2D). Detailed three-dimensional analysis of multiphoton images provided evidence that DNA stained by Sytox Orange was located outside the neutrophil cell membrane, labeled by anti–Gr-1 monoclonal Ab (Figure 2E and Supplemental Video S1), consistent with NET formation. These findings indicate that NETs generated in glomerular capillaries in response to anti-GBM Ab can be detected *in vivo* via multiphoton microscopy. It was notable that the structure of these intravascular NETs was markedly different from those previously observed in the sinusoids of the endotoxemic liver, where NETs formed extensive webs throughout the sinusoidal network (Supplemental Figure S1A).[12] In the glomerulus, NETs were predominantly much smaller and mostly present immediately adjacent to a neutrophil.

Expression of NET Markers in Anti-GBM Ab-Induced Extracellular Traps

The aim of the next series of experiments was to characterize the expression of protein markers normally associated with NET formation. NETs have been shown to include a wide range of nuclear and granular neutrophil proteins.[23] Furthermore, antibodies against histones, MPO, and neutrophil elastase have been used to detect NET formation in *in vivo* imaging studies.[12–14] In initial experiments, we focused on MPO, examining the colocalization of MPO and Sytox-stained structures along with anti–Gr-1–stained leukocytes. In NSG-treated mice, MPO^{+} structures were rarely detected in glomerular capillaries, despite the presence of neutrophils undergoing brief periods of retention (Figure 3, A and B). In contrast, 1 to 2 hours after administration of anti-GBM Ab, extracellular MPO was detectable in glomerular capillaries (Figure 3, A and B). Coadministration of Sytox Orange in these mice demonstrated that the frequency of MPO^{+} structures was comparable to that of $Sytox^{+}$ structures. Moreover, most Sytox Orange–stained structures also stained positive for MPO (Figure 3, A and B). Generation of these structures was neutrophil dependent in that they were almost completely absent in neutrophil-depleted mice (Figure 3B). Together, these observations provide evidence for the formation of MPO-bearing NETs within the glomerular microvasculature during the response to anti-GBM Ab.

MPO^{+} structures were observed continuously throughout the second hour of the anti-GBM Ab response (Figure 3C), indicating that intraglomerular NETs were generated in an ongoing manner throughout this phase of the response. We next examined whether the MPO staining was $Gr\text{-}1^{+}$ cell-associated or occurred in the absence of neutrophils. The frequency of cell-associated MPO increased significantly in anti-GBM Ab–treated mice relative to mice that received NSG (Figure 3D). The level of cell-free MPO did not show a significant increase under inflammatory conditions (Figure 3D). Together, these observations indicate that during the response to anti-GBM Ab, neutrophils undergo combined release of DNA and MPO in 15% to 20% of glomeruli.

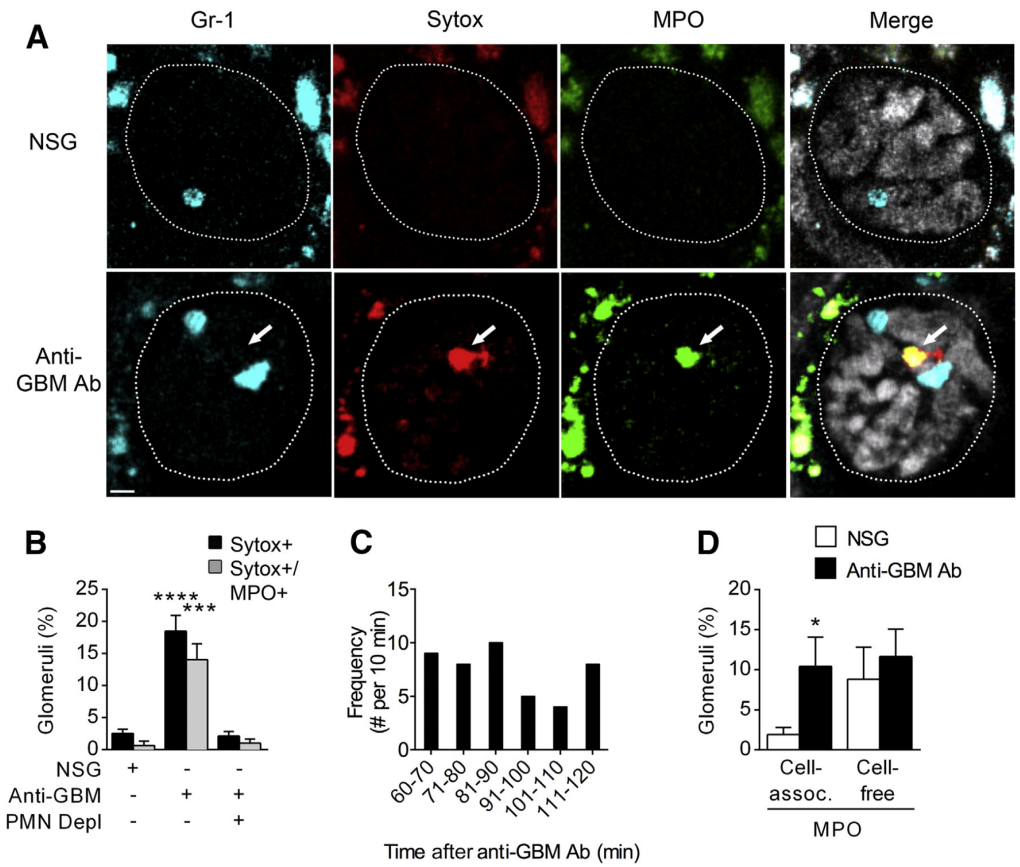


Figure 3 Extracellular myeloperoxidase (MPO) is detectable in inflamed glomeruli via multiphoton microscopy and is colocated with DNA. **A:** Multiphoton images of glomeruli from mice treated with either normal sheep globulin (NSG; **top row**) or anti-GBM Ab (**bottom row**), showing individual staining for neutrophils (anti–Gr-1; cyan), extracellular DNA (Sytox Orange; red), extracellular MPO (green), and the merged image with the vasculature shown in gray (glomerulus defined by **dotted lines**). An area of colocalization of DNA and MPO (**arrows**) is present in the anti-GBM Ab-treated mouse, but not the NSG-treated mouse. **B:** Percentage of glomeruli showing either Sytox-stained structures or structures positive for both Sytox and MPO staining, in NSG-treated mice, anti-GBM Ab-treated mice, and anti-GBM Ab-treated mice after neutrophil depletion. **C:** Histogram of timing of occurrence of MPO^+ structures during the second hour after anti-GBM Ab treatment, segregated into 10-minute windows. **D:** Percentage of glomeruli showing either cell-associated (cell-assoc.) or cell-free MPO in the second hour after either NSG or anti-GBM Ab treatment. Data are shown as means ± SEM (**B**–**D**). $n = 6$ to 9 mice per group (**B**–**D**). $^*P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ versus NSG. Scale bar = 10 μm (**A**). PMN Depl, neutrophil depletion.

We next examined the release of H2Ax and neutrophil elastase, additional intracellular proteins shown previously to be associated with NET formation *in vivo*.[12,14] Although in this series of experiments, 15% to 20% of glomeruli displayed detectable Sytox Orange–positive structures, on average <5% of glomeruli displayed anti-H2Ax staining, with some mice showing no anti–H2Ax-labeled structures (Figure 4A). To confirm that we could detect NETs using this antibody, we examined the hepatic microcirculation in the systemic endotoxemia model used by McDonald et al.[12] In this model, we readily detected web-like $H2Ax^+$ structures in the hepatic sinusoids of endotoxemic mice (Supplemental Figure S1A), consistent with previous observations,[12] demonstrating that this antibody was functional in our hands. To determine whether intraglomerular NETs showed other differences in their histone content, we used intravital microscopy to assess the presence of H2A-H2B–positive structures in inflamed glomeruli, using the same antibody as used in the immunohistochemical studies (Figure 1). However, unexpectedly, no positive staining for H2A-H2B was detectable via *in vivo* microscopy in either intraglomerular NETs (data not shown) or NETs in hepatic sinusoids in the systemic endotoxemia model (Supplemental Figure S1A). In contrast, immunohistochemical analysis was able to detect structures positive for H2A-H2B, as well as H3Cit and PAD4, in sections of inflamed glomeruli (Figure 1) and livers of endotoxemic mice (Supplemental Figure S1, B and C). The reason why $H2A\text{-}H2B^+$ structures were detectable via immunohistochemistry (Figure 1) but not via *in vivo* microscopy using the same antibody is unclear, but may reflect difficulties in antibody binding under shear flow in the microvasculature.

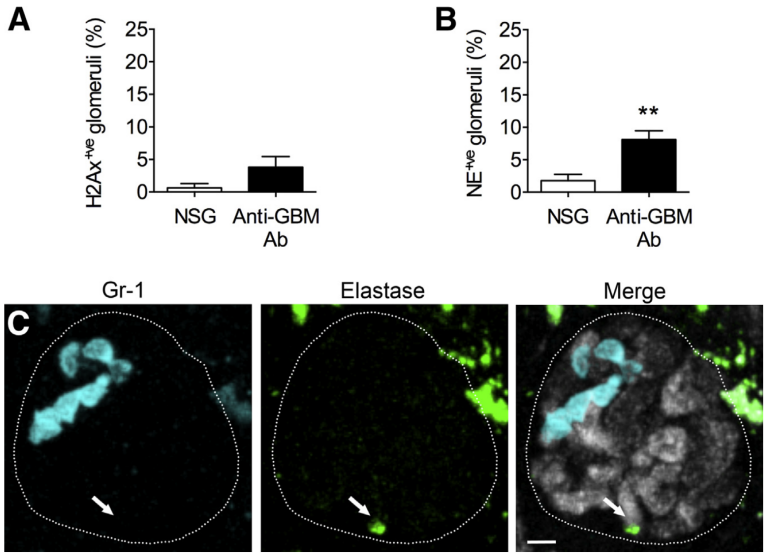


Figure 4 Extracellular H2Ax and neutrophil elastase are less frequent in inflamed glomeruli. **A** and **B:** Percentage of glomeruli showing either anti–H2Ax-stained structures (**A**) or anti–neutrophil elastase (NE)–positive structures (**B**) in mice treated with either normal sheep globulin (NSG) or anti-GBM Ab. **C:** Multiphoton images of a glomerulus from an anti-GBM Ab–treated mouse, showing individual staining for neutrophils (anti–Gr-1; cyan), neutrophil elastase (green), and the merged image with the vasculature shown in gray (glomerulus defined by **dotted line**). **Arrows** denote position of positive elastase staining. Data are shown as means ± SEM (**A** and **B**). $n = 6$ mice per group (**A** and **B**). $^{**}P < 0.01$ versus NSG. Scale bar = 10 μm (**C**).

Investigation of expression of extracellular neutrophil elastase in glomerular capillaries demonstrated that anti–neutrophil elastase–positive structures were rare in uninflamed mice, being detected in <2% of glomeruli (Figure 4B). During the second hour after anti-GBM Ab administration, there was a significant increase in the frequency of anti–elastase-stained structures within glomeruli (Figure 4B). These structures were smaller than those detected with Sytox Orange, being typically <3 μm in diameter. Interestingly, these structures were not colocalized with neutrophils (Figure 4C) or with DNA detectable via Sytox Orange staining (data not shown), observations consistent with neutrophil elastase being deposited by neutrophils in glomeruli independently of NET generation. In summary, taking together the differences between NETs formed in the glomerular capillaries and liver sinusoids both in morphology, and in staining for H2Ax and elastase, these findings indicate that intraglomerular NETs have structural differences from those formed in other vascular beds under other inflammatory conditions.

Intraglomerular NETs Are Predominantly Short-Lived Structures

Previous studies of intravascular NET generation in systemic endotoxemia indicated that NETs were prevalent and stable in the hepatic sinusoids, where blood flow and hence shear rate are relatively low (approximately 1 dyn/cm^2), whereas they were not detected in venules immediately downstream of the sinusoids, where shear rate is substantially higher.[12,36] This indicated that the formation and/or persistence of these structures can be influenced by the forces associated with flowing blood. Therefore, we next examined the stability of NETs formed in the glomerular capillaries, using MPO staining to detect NETs during the second hour of the anti-GBM Ab model. In many cases, MPO staining could be observed adjacent to neutrophils in one imaging frame, but was subsequently no longer detectable 30 or 60 seconds later (Figure 5, A–C, and Supplemental Video S2). Analysis of the duration of MPO$^+$ structures revealed that most of these structures were present for <30 seconds, with only rare instances where they persisted for >2.5 minutes (Figure 5D). To assess whether this response was associated with induction of hydronephrosis, we performed similar experiments examining glomeruli in intact kidneys, making use of the smaller kidneys in young mice. In these experiments, the durations of MPO$^+$ structures were similar to that seen in glomeruli in post-hydronephrotic kidneys, being predominantly of <30 seconds (Figure 5D). Together, these data indicate that under the acute inflammatory conditions examined herein, NETs generated in the glomerular microvasculature are predominantly short-lived structures, and that this response is not because of possible changes associated with hydronephrosis.

Given that shear rate in the glomerular microvasculature is high, typically between 5 and 20 dyn/cm^2,[37,38] we hypothesized that the transient nature of NETs in the glomerular microvasculature stemmed from the high shear rate in these vessels. To test this hypothesis, we used an *in vitro* microfluidic flow chamber system to compare the stability of NETs under shear equivalent to that in hepatic sinusoids with that existing in the glomerulus. Mouse neutrophils were loaded into chambers, stimulated with LPS to induce NET formation, and then NETs were stained using Sytox Orange. NETs were then exposed to either constant shear equivalent to that in hepatic sinusoids (1 dyn/cm^2) or increasing shear in a stepwise manner to that in glomerular capillaries (10 and 20 dyn/cm^2). At 1 dyn/cm^2, NETs were

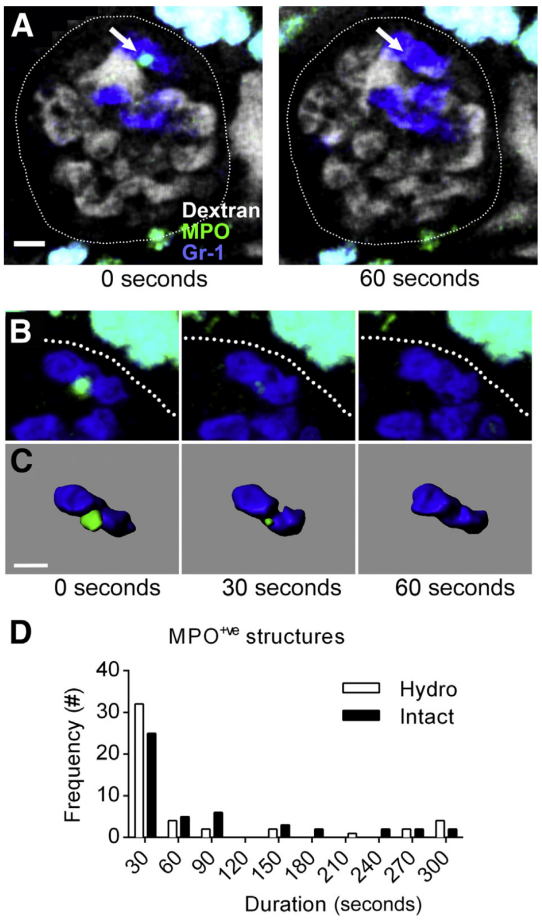


Figure 5 Myeloperoxidase $(MPO)^{+}$ structures present in glomerular capillaries are transient. **A:** Multiphoton images of the same glomerulus taken over a 60-second interval, showing neutrophils (anti–Gr-1; blue), MPO (green), and vasculature (gray). An MPO^{+} structure present in the first image (**arrows**) is absent in the second. **B** and **C:** High-power image sequence (**B**) and three-dimensional surface rendering (**C**) of neutrophils and MPO from **A**, taken at 30-second intervals, illustrating disappearance of MPO from the neutrophil surface. **Dotted lines** define perimeter of glomerulus (**A** and **B**). **D:** Histogram showing the duration of MPO^{+} structures in the second hour of the anti-GBM Ab model, with data derived from either post-hydronephrotic (hydro) kidneys or intact (intact) kidneys from young mice. See also Supplemental Video S2. $n = 7$ mice per group (**D**, hydro kidneys); $n = 6$ mice per group (**D**, intact kidneys). Scale bars = 10 μm (**A** and **C**). Original magnification, ×60 (**B**).

relatively stable during a 6-minute observation period (Figure 6, A and C). However, on increasing shear to 10 dyn/cm^2, NETs underwent rapid disruption, with DNA staining being significantly reduced within 30 seconds, and subsequently remaining significantly lower than NETs exposed to constant low shear (Figure 6, B and C). In parallel experiments, we examined generation of NETs by neutrophils adherent to endothelial cells. When neutrophils were allowed to adhere to endothelial cells and then stimulated with LPS under static conditions ($n = 5$ individual experiments), NETs were readily detectable (Supplemental Figure S2), as seen when adherent on intercellular adhesion molecule-1. In contrast, if LPS was applied under constant shear and Sytox Orange staining applied at the end of the LPS stimulation ($n = 4$ experiments), NETs were extremely infrequent. These experiments demonstrated that the inhibitory effect of shear on NET persistence observed with neutrophils adherent on an intercellular adhesion molecule-1 substrate also applied when NETs were adherent to endothelial cells. Together, these findings support the contention that the high shear rate in glomerular capillaries is a key factor underlying the transient nature of glomerular NETs.

Functional Contribution of NETs to Glomerular Injury in Anti-GBM Ab-Induced Glomerulonephritis

Finally, we addressed whether NETs contribute to glomerular injury in the anti-GBM Ab-induced model of glomerulonephritis. This model results in neutrophil-mediated functional glomerular injury, as detected by albuminuria in the 24 hours after antibody administration.[1,5] Herein, we observed a similar response, with a significant increase in urinary albumin/creatinine ratio as well as increased hematuria in anti-GBM Ab-treated mice, relative to untreated mice (Figure 7, B and C). Two approaches were used to examine the functional contribution of NETs to glomerular injury in this model. To inhibit NET formation, mice were treated with the PAD4 inhibitor Cl-amidine, an agent that has been shown to inhibit NET generation and reduce NET-related pathology in models of inflammatory disease.[30–32] Alternatively, mice were treated with DNase I to remove NETs after their generation, as previously described.[12,14] To confirm that these treatments affected the extent of NET generation in the glomerulus, intraglomerular NET formation was assessed using immunohistochemistry (Figure 7A). In mice administered anti-GBM Ab, pretreatment with Cl-amidine reduced NET formation by >50%, whereas DNase I treatment was more effective, reducing the rate of NET formation by approximately 80% relative to mice receiving anti-GBM Ab alone (Figure 7B). To assess the contribution of NETs to functional glomerular injury in this model, we examined albuminuria, the key readout of glomerular barrier dysfunction. In mice treated with either DNase I or Cl-amidine, albuminuria was not significantly reduced relative to that in mice treated with anti-GBM Ab alone (Figure 7C). As an additional assessment of glomerular injury, we also examined hematuria by dipstick. Herein, we observed that only DNase I, which was more effective in reducing the prevalence of NETs than PAD4 inhibition, attenuated the development and extent of microscopic hematuria seen in untreated anti–GBM Ab-treated mice (Figure 7D). Together, these data indicate that in this acute model of glomerulonephritis, NETs formed in glomerular capillaries do not modulate changes in albumin leakage, but

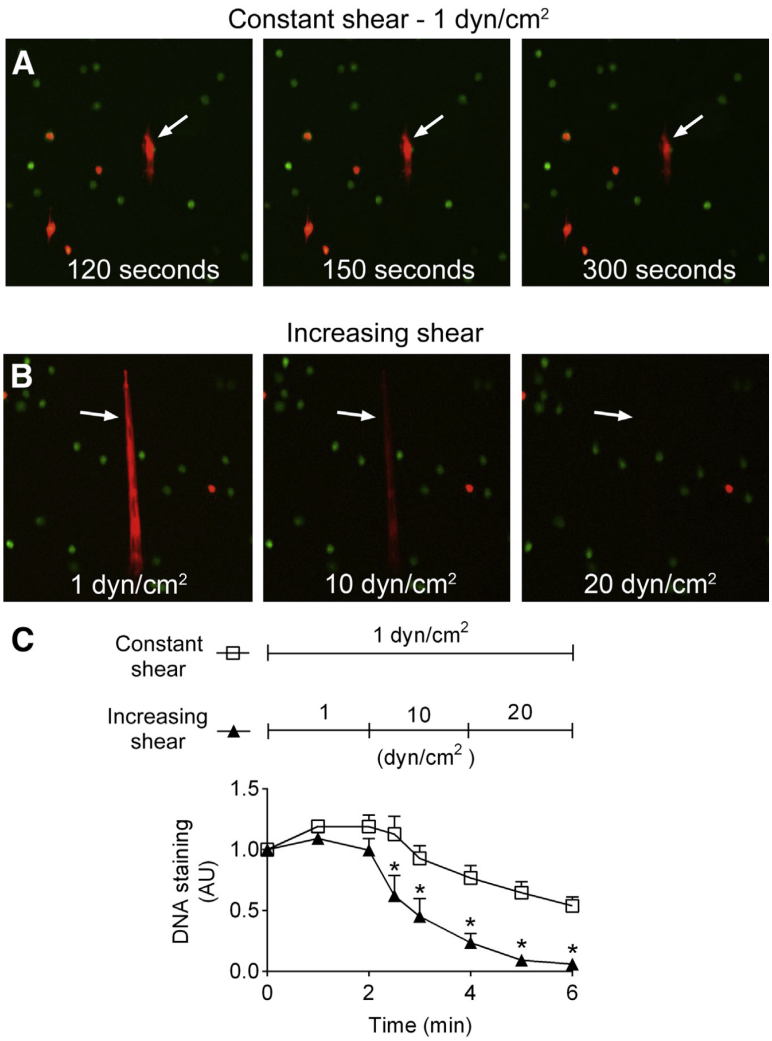


Figure 6 NETs are unstable under glomerular shear conditions. NETs were generated in a microfluidic flow chamber under static conditions, stained with Sytox Orange and Alexa 488—anti—Gr-1 (green), and then imaged (**A** and **B**) while being exposed to either constant low shear equivalent to that in the hepatic sinusoids (1 dyn/cm^2; **A**) or increasing to shear equivalent to that in glomerular capillaries (10 and 20 dyn/cm^2) at 2-minute intervals (**B**). (Experimental timelines indicated at the top of the graph in **C**). **A:** NET (**arrows**) in constant shear shown after 2-, 2.5-, and 5-minute exposure to 1 dyn/cm^2. **B:** NET (**arrows**) under increasing shear shown after exposure to 1 dyn/cm^2 (2 minutes), 10 dyn/cm^2 (30 seconds), and 20 dyn/cm^2 (1 minute). **C:** DNA staining intensity normalized to that at the start of the experiment. Data are shown as means ± SEM (**C**). $n = 4$ (**C**, constant shear); $n = 6$ (**C**, increasing shear). $^*P < 0.05$ versus constant shear group. Original magnification, ×20 (**A** and **B**). AU, arbitrary unit.

do contribute to glomerular dysfunction revealed by assessment of hematuria.

Discussion

Although immunohistochemical studies have demonstrated that NETs are present in glomeruli of patients with anti—neutrophil cytoplasmic antibody—positive glomerulonephritis and lupus nephritis,[21–23] the dynamics of NET formation in this unique microvascular bed have not been investigated. Herein, we demonstrate that in the anti-GBM Ab model of acute glomerulonephritis, NETs are generated in the glomerular capillaries. Via direct visualization of the inflamed glomerular microvasculature, we show, however, that these structures are predominantly short-lived, being unable to persist under the high shear conditions present in glomerular capillaries. Using this approach, we observed that NET formation occurs in 15% to 20% of glomeruli examined. In these experiments, glomeruli were observed for a maximum of only 5 minutes each. Given the short duration of most of the MPO$^+$ structures observed, it is likely that this approach led to an underestimate of the proportion of glomeruli affected by NETs, and that during the course of this model, NETs develop in >20% of glomeruli. In addition, these experiments reveal that intraglomerular NETs are structurally distinct from NETs formed in other intravascular locations, in terms of their size and protein content. Consistent with their limited duration of persistence in the glomerulus, removal of NETs from the microvasculature did not affect the key readout of glomerular injury of albuminuria, although degradation of NETs via DNase did reduce microscopic hematuria. These findings indicate that in addition to the proinflammatory roles of NETs previously described in various organs and inflammatory

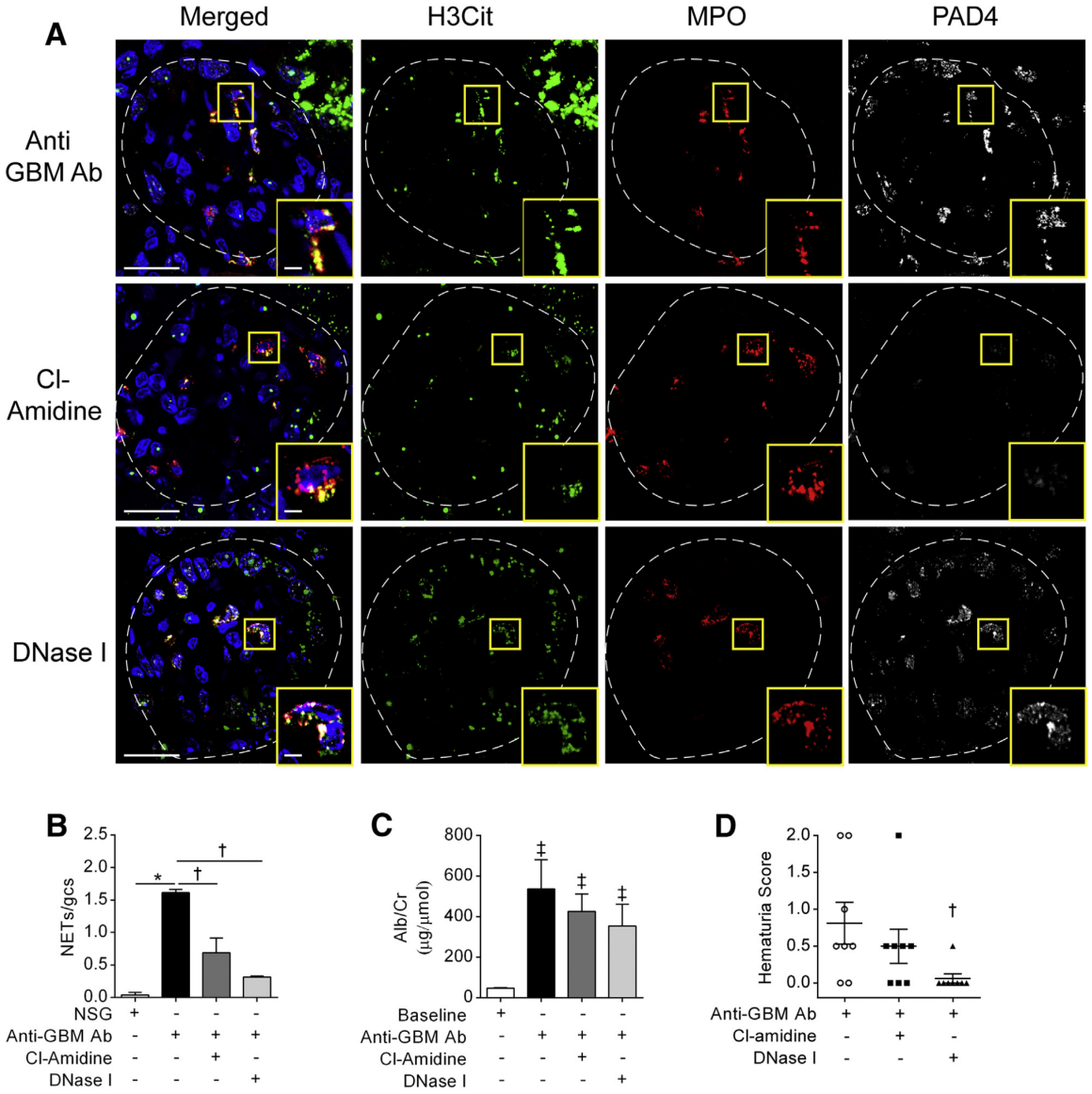


Figure 7 Effects of inhibition of NET generation or DNase I treatment on NET generation, albuminuria, and hematuria in anti-GBM Ab model. **A** and **B:** Effect of peptidyl arginine deiminase-4 (PAD4) inhibition via Cl-amidine and DNase I treatment on intraglomerular NET formation, as determined via immunohistochemistry 2 hours after induction of anti-GBM Ab-induced glomerulonephritis. Staining is shown for citrullinated histone H3 (H3Cit; green), myeloperoxidase (MPO; red), and PAD4 (white) in kidneys of mice treated with anti-GBM Ab alone or with anti-GBM Ab plus either Cl-amidine or DNase I (glomeruli defined by **dotted line**). **Left column** shows merged images, which also include DNA staining via DAPI (blue). **Boxed areas** are shown at higher magnification in the **insets**. In anti-GBM Ab example, **inset** displays a magnified image of a NETing neutrophil, defined as for Figure 1. In Cl-amidine and DNase I examples, **insets** display magnified images of non-NETing neutrophils (ie, lacking extracellular DNA). **B:** Quantitative analysis of NET frequency in corresponding mice. Data are shown for mice treated with normal sheep globulin (NSG), anti-GBM Ab alone, and anti-GBM Ab plus either Cl-amidine or DNase I. **C** and **D:** Overnight urinary albumin/creatinine ratio (Alb/Cr; **C**) and hematuria score (**D**) were measured in untreated mice (albumin/creatinine only), anti-GBM Ab-treated mice, and anti-GBM Ab-treated mice pretreated with either Cl-amidine or DNase I. In **D**, data for individual animals are shown, and Kruskal-Wallis analysis was used. Data are means ± SEM (**B**–**D**). $n = 4$ mice per group (**B**); $n = 8$ mice per group (**C** and **D**). $^{*}P < 0.05$ versus NSG; $^{\dagger}P < 0.05$ versus anti-GBM Ab; $^{\ddagger}P < 0.05$ versus baseline. Scale bars: 20 μm (**A**, **main images**); 5 μm (**A**, **insets**). gcs, glomerular cross section.

conditions,[18–20,22] they also contribute to the development of hematuria in the acutely inflamed glomerulus.

In systemic endotoxemia and models of deep vein thrombosis and acute lung injury, intravenous treatment with DNase has been shown to rapidly dissolve NETs and reduce their injurious effects.[12,14,18] Similarly, inhibition of NET generation via blocking the PAD4 pathway has been found to be highly protective in models of colitis, lupus, and

sepsis, and to reduce renal injury in a 7-day model of glomerulonephritis.[26,31,32,39] These results provide strong evidence that interfering with NET activity could be a useful therapeutic strategy in inflammatory disease. Herein, we observed that interfering with NET formation or persistence did not reduce albuminuria, although NET dissolution via DNase I did protect against hematuria. This finding indicates that NETs do contribute to some degree to the breakdown of the glomerular barrier in this acute *in situ* complex model of glomerulonephritis, although removing NETs does not protect against the development of pathological albuminuria.

In these experiments, NET generation was inhibited using the PAD4 inhibitor, Cl-amidine, whereas NETs were disrupted using DNase I. Although complementary, these approaches are subtly different. Inhibition of NET generation prevents all components of NETs, including DNA, histones, and proteolytic enzymes, from being released by neutrophils. In contrast, DNase I is only able to affect NETs after their release, rapidly dissolving extracellular DNA.[40] Recent work has demonstrated that under some circumstances, NET-associated histones and elastase remain attached to the vascular lining after removal of the DNA backbone via DNase treatment.[40] As such, we elected to use both approaches to examine the role of NETs in glomerular injury. Despite the different modes of actions of these agents, both reduced the frequency of NETs in acutely inflamed glomeruli. Neither approach affected albuminuria, although NET dissolution via DNase I did protect against hematuria, indicating that NETs promote breakdown of the glomerular barrier to some degree in this acute *in situ* complex model of glomerulonephritis. The reason for the observed different effects on hematuria of inhibition of NET generation via Cl-amidine and NET dissolution via DNase I is unclear, but may reflect the less extensive degree of inhibition of NET formation seen in Cl-amidine–treated mice versus those treated with DNase I.

Given that the toxic and proteolytic components of NETs, including histones, MPO, and elastase, were either absent or failed to persist within the glomerular capillaries after NET release, it is not surprising that inhibition of NET generation or NET disruption was not completely protective in this model. We hypothesized that the fragile nature of NETs may underlie their inability to persist in the high shear environment of the glomerular capillaries, and this hypothesis was supported by *in vitro* flow chamber experiments in which glomerular shear levels caused rapid disruption of NETs. These findings indicate that the high shear conditions within the glomerular microvasculature underlie the rapid disappearance of NETs in the glomerulus. Along with the lack of effect of NET dissolution on anti-GBM Ab-induced albuminuria, these findings raise the possibility that the hydrodynamic conditions within the acutely inflamed glomerular microvasculature alone provide a degree of protection against the toxic effects of NETs.

Immunohistochemical comparison of NETs in glomeruli versus those in the hepatic microcirculation showed that NETs in both locations contained citrullinated H3 histone, H2A-H2B, and MPO. In contrast, analysis via *in vivo* microscopy revealed that intraglomerular NETs differ from hepatic NETs in other protein constituents. Via *in vivo* microscopy, H2Ax, MPO, and neutrophil elastase are present on intravascular NETs in hepatic sinusoids, and similar findings have been made for intravascular NETs in a model of deep vein thrombosis induced by blood flow restriction.[12,14] In contrast, in the glomerulus, we found that MPO, but not neutrophil elastase or H2Ax, was associated with the extracellular DNA released by neutrophils. The reasons for these differences are not clear. It is possible that the attachment of these proteins to the DNA is insufficiently strong to resist disruption under the high shear conditions in the glomerular capillaries. In addition, it is notable that elastase was detectable in inflamed glomeruli, but was not associated with extracellular DNA. This finding provides evidence that elastase present in the glomerulus in this model of glomerular inflammation was released by neutrophils via a conventional degranulation pathway, rather than as part of NET release. Previous studies have demonstrated a role for neutrophil elastase in glomerular injury during acute anti-GBM Ab-induced glomerulonephritis.[9] The present findings suggest that this role for elastase occurs independently of NET generation.

In these studies, we used an acute model of neutrophil-dependent glomerulonephritis in which the neutrophil recruitment response lasts for only 4 to 8 hours, after which leukocyte accumulation and proteinuria subside.[1,4] As such, there is minimal ongoing disruption to the capillary structure, and glomerular blood flow persists. However, in the more severe forms of glomerulonephritis seen clinically, many glomeruli undergo marked structural changes, including glomerular hypercellularity because of leukocyte recruitment and proliferation of intrinsic glomerular cells, and deposition of fibrin and extracellular matrix.[2] This is associated with endothelial damage, as demonstrated by loss of endothelial proteins von Willebrand factor, platelet endothelial cell adhesion molecule-1, and CD34.[23] It is conceivable that under these conditions, the perturbations to the endothelium and glomerular structure lead to disruption of the normal glomerular blood flow and reduced shear conditions, which would favor persistence of NETs. Moreover, in highly damaged glomeruli, neutrophils are present in the extravascular compartment of the glomerulus, where they are no longer exposed to the shear forces of flowing blood. Supporting this concept, we recently comprehensively examined the existence of NETs in glomeruli of renal biopsy specimens from patients with anti–neutrophil cytoplasmic antibody–associated glomerulonephritis. Many of these patients had severe glomerular pathology, as indicated by a high rate of crescent formation or segmental necrosis. On average, NETs were present in >60% of glomeruli, and in 100% of glomeruli in some patients, a rate of NET formation substantially higher than in the model examined herein.[23] These findings raise the possibility that in severely inflamed

glomeruli, conditions are more favorable for NET persistence than during an acute short-lived model of glomerulonephritis, and that therefore NET removal as a therapeutic modality might be more effective. This possibility is supported by the results of Kumar et al,[26] who recently reported a protective effect of PAD4 inhibition in a 7-day model of anti-GBM Ab-induced glomerulonephritis associated with severe glomerular injury.

Neutrophils have been shown capable of incorporating mitochondrial DNA in NETs under certain circumstances.[41,42] Moreover, NETs composed of mitochondrial DNA can be generated in approximately 30 minutes,[41] whereas generation of NETs composed of nuclear DNA is thought to take several hours because of the requirement for extensive nuclear remodeling.[43] The present finding that neutrophils retained in inflamed glomeruli for, on average, 20 to 25 minutes[1] generated NETs raised the possibility that these structures were derived from mitochondrial DNA. However, the components of intraglomerular NETs detectable via immunohistochemistry argue against this idea. Intraglomerular NETs were observed by immunohistochemistry to incorporate both citrullinated histone H3 and H2A-H2B (Figure 1). Because mitochondrial DNA is free of histones,[44] this evidence indicates that nuclear DNA is an important component of the NETs observed in this study, while not excluding the possibility that mitochondrial DNA does contribute to some extent. Finally, exposure of neutrophils to *Staphylococcus aureus* has been shown to result in release of nuclear DNA in 5 to 60 minutes,[45] indicating that DNA incorporated in NETs generated rapidly is not exclusively of mitochondrial origin.

In these experiments, MPO was detected in glomeruli both associated with neutrophils and in a cell-free form (ie, not associated with neutrophils). Activated neutrophils can release MPO rapidly via conventional degranulation, as well as in association with NET generation. MPO released via degranulation is thought to contribute to MPO deposition and retention in tissues.[23,46,47] Although cell-associated MPO will only persist locally for as long as the neutrophil does, cell-free MPO can be retained in tissues for much longer periods. Under these conditions, it could be damaging directly, via generation of hypochlorous acid, as well as acting as a local autoantigen in patients with autoimmunity to MPO.[48] However, in the present study, much of the cell-free MPO detected was only present for brief periods, suggesting that it was unlikely to contribute to injury significantly.

In conclusion, these experiments demonstrate that NETs are generated in the glomerulus in the anti-GBM Ab model of glomerulonephritis, the prototypical and most widely used model of acute neutrophil–mediated glomerular inflammation.[5,6,8] However, these intravascular NETs have different temporal and protein characteristics from those observed in other settings. Despite their relatively short intravascular lifespan, strategies to promote NET removal reduced the level of glomerular injury to a modest degree, indicating that NETs can contribute to glomerular injury. These studies provide further evidence of the unique nature of the glomerulus and its response to inflammatory stimulation, and demonstrate the value of direct *in vivo* visualization of the glomerulus in aiding our understanding of the nature of this response.

Acknowledgments

We thank Drs. Kirstin Elgass, Camden Lo, and Sarah Creed (Monash Micro Imaging) for assistance with optical microscopy; Dr. Connie Wong (Monash University) for liver imaging; and Dr. Bryan Yipp (University of Calgary) for helpful discussions.

M.J.H., J.E.B., and A.R.K. conceived the experiments; J.E.B., P.H., K.M.O., Q.C., C.L.V.W., and M.J.H. performed the experiments; J.E.B., P.H., C.L.V.W., and M.J.H. analyzed the data; M.J.H., C.L.V.W., and A.R.K. wrote the manuscript.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2016.10.008*.

References

1. Devi S, Li A, Westhorpe CL, Lo CY, Abeynaike LD, Snelgrove SL, Hall P, Ooi JD, Sobey CG, Kitching AR, Hickey MJ: Multiphoton imaging reveals a novel leukocyte recruitment paradigm in the glomerulus. Nat Med 2013, 19:107–112
2. Kitching AR, Holdsworth SR, Hickey MJ: Targeting leukocytes in immune glomerular diseases. Curr Med Chem 2008, 15:448–458
3. Tipping PG, Huang XR, Berndt MC, Holdsworth SR: A role for P-selectin in complement-independent neutrophil-mediated glomerular injury. Kidney Int 1994, 46:79–88
4. Tang T, Rosenkranz A, Assmann KJ, Goodman MJ, Gutierrez-Ramos JC, Carroll MC, Cotran RS, Mayadas TN: A role for Mac-1 (CDIIb/CD18) in immune complex-stimulated neutrophil function in vivo: Mac-1 deficiency abrogates sustained Fcgamma receptor-dependent neutrophil adhesion and complement-dependent proteinuria in acute glomerulonephritis. J Exp Med 1997, 186:1853–1863
5. Kuligowski MP, Kitching AR, Hickey MJ: Leukocyte recruitment to the inflamed glomerulus: a critical role for platelet-derived P-selectin in the absence of rolling. J Immunol 2006, 176:6991–6999
6. Boyce NW, Holdsworth SR: Hydroxyl radical mediation of immune renal injury by desferrioxamine. Kidney Int 1986, 30:813–817
7. Tipping PG, Boyce NW, Holdsworth SR: Relative contributions of chemo-attractant and terminal components of complement to anti-glomerular basement membrane (GBM) glomerulonephritis. Clin Exp Immunol 1989, 78:444–448
8. Odobasic D, Kitching AR, Semple TJ, Holdsworth SR: Endogenous myeloperoxidase promotes neutrophil-mediated renal injury, but attenuates T cell immunity inducing crescentic glomerulonephritis. J Am Soc Nephrol 2007, 18:760–770
9. Schrijver G, Schalkwijk J, Robben JC, Assmann KJ, Koene RA: Antiglomerular basement membrane nephritis in beige mice: deficiency of leukocytic neutral proteinases prevents the induction of albuminuria in the heterologous phase. J Exp Med 1989, 169:1435–1448

10. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A: Neutrophil extracellular traps kill bacteria. Science 2004, 303:1532–1535
11. Brinkmann V, Zychlinsky A: Beneficial suicide: why neutrophils die to make NETs. Nat Rev Microbiol 2007, 5:577–582
12. McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P: Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. Cell Host Microbe 2012, 12:324–333
13. Yipp BG, Petri B, Salina D, Jenne CN, Scott BN, Zbytnuik LD, Pittman K, Asaduzzaman M, Wu K, Meijndert HC, Malawista SE, de Boisfleury Chevance A, Zhang K, Conly J, Kubes P: Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. Nat Med 2012, 18:1386–1393
14. von Bruhl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, Khandoga A, Tirniceriu A, Coletti R, Kollnberger M, Byrne RA, Laitinen I, Walch A, Brill A, Pfeiler S, Manukyan D, Braun S, Lange P, Riegger J, Ware J, Eckart A, Haidari S, Rudelius M, Schulz C, Echtler K, Brinkmann V, Schwaiger M, Preissner KT, Wagner DD, Mackman N, Engelmann B, Massberg S: Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med 2012, 209:819–835
15. Brinkmann V, Zychlinsky A: Neutrophil extracellular traps: is immunity the second function of chromatin? J Cell Biol 2012, 198: 773–783
16. Urban CF, Reichard U, Brinkmann V, Zychlinsky A: Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. Cell Microbiol 2006, 8:668–676
17. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, Punaro M, Baisch J, Guiducci C, Coffman RL, Barrat FJ, Banchereau J, Pascual V: Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med 2011, 3:73ra20
18. Rossaint J, Herter JM, Van Aken H, Napirei M, Doring Y, Weber C, Soehnlein O, Zarbock A: Synchronized integrin engagement and chemokine activation is crucial in neutrophil extracellular trap-mediated sterile inflammation. Blood 2014, 123:2573–2584
19. Kaplan MJ, Radic M: Neutrophil extracellular traps: double-edged swords of innate immunity. J Immunol 2012, 189:2689–2695
20. Chen G, Zhang D, Fuchs TA, Manwani D, Wagner DD, Frenette PS: Heme-induced neutrophil extracellular traps contribute to the pathogenesis of sickle cell disease. Blood 2014, 123:3818–3827
21. Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, Werb Z, Grone HJ, Brinkmann V, Jenne DE: Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med 2009, 15:623–625
22. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, Rubin CJ, Zhao W, Olsen SH, Klinker M, Shealy D, Denny MF, Plumas J, Chaperot L, Kretzler M, Bruce AT, Kaplan MJ: Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. J Immunol 2011, 187:538–552
23. O'Sullivan KM, Lo CY, Summers SA, Elglass KD, McMillan PJ, Longano A, Ford SL, Gan PY, Kerr PG, Kitching AR, Holdsworth SR: Renal participation of myeloperoxidase in antineutrophil cytoplsamic antibody (ANCA)-associated glomerulonephritis. Kidney Int 2015, 88: 1030–1046
24. Xu J, Zhang X, Pelayo R, Monestier M, Ammollo CT, Semeraro F, Taylor FB, Esmon NL, Lupu F, Esmon CT: Extracellular histones are major mediators of death in sepsis. Nat Med 2009, 15:1318–1321
25. Saffarzadeh M, Juenemann C, Queisser MA, Lochnit G, Barreto G, Galuska SP, Lohmeyer J, Preissner KT: Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. PLoS One 2012, 7:e32366
26. Kumar SV, Kulkarni OP, Mulay SR, Darisipudi MN, Romoli S, Thomasova D, Scherbaum CR, Hohenstein B, Hugo C, Muller S, Liapis H, Anders HJ: Neutrophil extracellular trap-related extracellular histones cause vascular necrosis in severe GN. J Am Soc Nephrol 2015, 26:2399–2413
27. Devi S, Kuligowski MP, Kwan RY, Westein E, Jackson SP, Kitching AR, Hickey MJ: Platelet recruitment to the inflamed glomerulus occurs via an alphaIIbbeta3/GPVI-dependent pathway. Am J Pathol 2010, 177:1131–1142
28. Kuligowski MP, Kwan RY, Lo C, Wong C, James WG, Bourges D, Ooi JD, Abeynaike LD, Hall P, Kitching AR, Hickey MJ: Anti-myeloperoxidase antibodies rapidly induce alpha-4-integrin-dependent glomerular neutrophil adhesion. Blood 2009, 113:6485–6494
29. Wong SL, Demers M, Martinod K, Gallant M, Wang Y, Goldfine AB, Kahn CR, Wagner DD: Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. Nat Med 2015, 21:815–819
30. Willis VC, Gizinski AM, Banda NK, Causey CP, Knuckley B, Cordova KN, Luo Y, Levitt B, Glogowska M, Chandra P, Kulik L, Robinson WH, Arend WP, Thompson PR, Holers VM: N-alpha-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide, a protein arginine deiminase inhibitor, reduces the severity of murine collagen-induced arthritis. J Immunol 2011, 186:4396–4404
31. Chumanevich AA, Causey CP, Knuckley BA, Jones JE, Poudyal D, Chumanevich AP, Davis T, Matesic LE, Thompson PR, Hofseth LJ: Suppression of colitis in mice by Cl-amidine: a novel peptidylarginine deiminase inhibitor. Am J Physiol Gastrointest Liver Physiol 2011, 300:G929–G938
32. Knight JS, Zhao W, Luo W, Subramanian V, O'Dell AA, Yalavarthi S, Hodgin JB, Eitzman DT, Thompson PR, Kaplan MJ: Peptidylarginine deiminase inhibition is immunomodulatory and vasculoprotective in murine lupus. J Clin Invest 2013, 123:2981–2993
33. Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y: PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. J Exp Med 2010, 207:1853–1862
34. Cheng Q, Fan H, Ngo D, Beaulieu E, Leung P, Lo CY, Burgess R, van der Zwan YG, White SJ, Khachigian LM, Hickey MJ, Morand EF: GILZ overexpression inhibits endothelial cell adhesive function through regulation of NF-kappaB and MAPK activity. J Immunol 2013, 191:424–433
35. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, Patel KD, Chakrabarti S, McAvoy E, Sinclair GD, Keys EM, Allen-Vercoe E, Devinney R, Doig CJ, Green FH, Kubes P: Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat Med 2007, 13:463–469
36. Lalor PF, Herbert J, Bicknell R, Adams DH: Hepatic sinusoidal endothelium avidly binds platelets in an integrin-dependent manner, leading to platelet and endothelial activation and leukocyte recruitment. Am J Physiol Gastrointest Liver Physiol 2013, 304:G469–G478
37. Remuzzi A, Brenner BM, Pata V, Tebaldi G, Mariano R, Belloro A, Remuzzi G: Three-dimensional reconstructed glomerular capillary network: blood flow distribution and local filtration. Am J Physiol 1992, 263:F562–F572
38. Ballermann BJ, Dardik A, Eng E, Liu A: Shear stress and the endothelium. Kidney Int 1998, 54:S100–S108
39. Martinod K, Fuchs TA, Zitomersky NL, Wong SL, Demers M, Gallant M, Wang Y, Wagner DD: PAD4-deficiency does not affect bacteremia in polymicrobial sepsis and ameliorates endotoxemic shock. Blood 2015, 125:1948–1956
40. Kolaczkowska E, Jenne CN, Surewaard BG, Thanabalasuriar A, Lee WY, Sanz MJ, Mowen K, Opdenakker G, Kubes P: Molecular mechanisms of NET formation and degradation revealed by intravital imaging in the liver vasculature. Nat Commun 2015, 6:6673
41. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU: Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell Death Differ 2009, 16:1438–1444
42. Wang H, Li T, Chen S, Gu Y, Ye S: Neutrophil extracellular trap mitochondrial DNA and its autoantibody in systemic lupus erythematosus and a proof-of-concept trial of metformin. Arthritis Rheumatol 2015, 67:3190–3200
43. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V, Zychlinsky A: Novel cell death program leads to neutrophil extracellular traps. J Cell Biol 2007, 176:231–241

44. Bogenhagen DF: Mitochondrial DNA nucleoid structure. Biochim Biophys Acta 2012, 1819:914–920
45. Pilsczek FH, Salina D, Poon KK, Fahey C, Yipp BG, Sibley CD, Robbins SM, Green FH, Surette MG, Sugai M, Bowden MG, Hussain M, Zhang K, Kubes P: A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. J Immunol 2010, 185:7413–7425
46. Eckle I, Kolb G, Heiser C, Havemann K: Stimulation of neutrophil elastase and myeloperoxidase release by IgG fragments. Clin Exp Immunol 1990, 81:352–356
47. Baldus S, Eiserich JP, Mani A, Castro L, Figueroa M, Chumley P, Ma W, Tousson A, White CR, Bullard DC, Brennan ML, Lusis AJ, Moore KP, Freeman BA: Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. J Clin Invest 2001, 108:1759–1770
48. Ooi JD, Chang J, Hickey MJ, Borza DB, Fugger L, Holdsworth SR, Kitching AR: The immunodominant myeloperoxidase T-cell epitope induces local cell-mediated injury in anti-myeloperoxidase glomerulonephritis. Proc Natl Acad Sci U S A 2012, 109:E2615–E2624