The Roles of T helper 17 cells and Regulatory T cells in Experimental Glomerulonephritis.

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Abbreviations

AAV	ANCA-associated vasculitis
Ab	Antibody
ANCA	Anti-neutrophil cytoplasmic antibody
AKI	Acute kidney injury
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ATRA	All trans retinoic acid
BM	Bone marrow
cDC	Conventional dendritic cell
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CXCR	CXC chemokine receptor
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbant assay
EliSpot	Enzyme-linked immunospot
eTregs	Expanded T regulatory cells
FACS	Fluorescence activated cell sorting
FCA	Freund’s complete adjuvant
FCS	Foetal calf serum
FIA	Freund’s incomplete adjuvant
FL	FLT3-ligand
FLT3	Fms-like tyrosine kinase 3
Foxp3	Forkhead box P3
GBM	Glomerular basement membrane
GC	Germinal centre
gcs	Glomerular cross section
GFP	Green fluorescent protein
GN	Glomerulonephritis

GITR	Glucocorticoid-induced tumour necrosis factor receptor family related protein
GVHD	Graft versus host disease
HC	Healthy controls
hpf	High powered field
ICOS	Inducible T cell co-stimulator
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
iTreg	Induced Treg
i.v.	Intravenous
IRI	Ischaemia reperfusion injury
LN	Lupus nephritis (chapter 3 only Lymph node)
LNSC	Lymph node stromal cell
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NETosis	Neutrophil extracellular trap -osis
NFAT	Nuclear factor of activated T cells
nTregs	Natural T regulatory cells
OD	Optical density
OX-40	CD134
PAS	Periodic acid Schiff reagent
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PDCA-1	Plasmacytoid dendritic cell antigen-1
PD-1	Programmed cell death protein-1
PD-L1	Programmed death-ligand 1
PMA	Phorbol 12-myristate 13-acetate
PNA	Peanut agglutinin
PR3	Proteinase 3
pTreg	Peripheral Treg

RAG	Recombinase-activating gene
Rapa	Rapamycin
RPGN	Rapidly progressive glomerulonephritis
RORγt	Retinoid-related orphan receptor yt
RT-PCR	Real time-polymerase chain reaction
SMAD3	SMAD family member 3
TC	Tissue cell
TCR	T cell receptor
Teff	T effector cell
TFH	T follicular helper cell
Th	T helper cell
Treg	T regulatory cell
TNF	Tumor necrosis factor
TGF	Transforming growth factor
TSDR	Treg-specific demethylated region
s.c.	Subcutaneous
SG	Sheep globulin
SLE	Systemic lupus erythematosus
STAT	Signal transducer activator of transcription
WT	Wild type
α3(IV)NC1	Non-collagenous domain of the α3 chain of type IV collagen

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General Declaration

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Declaration for thesis based or partially based on conjointly published or unpublished work

In accordance with Monash University Doctorate Regulation 17.2 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 1 published original paper, 1 original paper 'in press' and 4 unpublished publications in peer reviewed journals.

The core theme of the thesis is explores the roles of two subsets of T helper cells, T helper 17 cells and regulatory T cells, in experimental glomerulonephritis.

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 Diseases, Department of Medicine, Monash University under the supervision of Professor A. Richard Kitching (primary supervisor) and Professor Stephen Holdsworth (secondary supervisor).

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. I declare that certain experiments required the technical expertise of other contributors, including Ms Kim O'Sullivan for fluorescent microscopy, Dr Maliha Alikhan for renal digestion and flow cytometry and Mr Peter Eggenhuizen for technical assistance with experiments. I have collaborated with Dr Dragana Odobasic, Dr Yuan Min Wang and Associate Professor Stephen Alexander in the technical protocol and literature reviews.

In the case of Chapters 1-6, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Publication Status	Nature and extent of Candidate's Contribution
1	Targeting IL-17 and IL-23 in Immune Mediated Renal Disease.	Submitted	Literature review and writing and revising the manuscript (80%)
2	Regulatory T cells in Immune Mediated Renal Disease.	Submitted	Literature review and writing and revising the manuscript (80%)
3	Glomerulonephritis Induced by Heterologous Anti-GBM Globulin as a Planted Foreign Antigen.	Published	Compiling and revising the protocols, writing and revising the manuscript (40%)
4	FMS-like tyrosine kinase 3 ligand treatment does not ameliorate experimental rapidly progressive glomerulonephritis.	In Press	Experimental design, performing experiments, data analysis and writing and revising the manuscript (70%)
5	Induced regulatory T cells are phenotypically unstable and do not protect mice from rapidly progressive glomerulonephritis.	Submitted	Experimental design, performing experiments, data analysis and writing and revising the manuscript (80%)
6	IL-17RA on leukocytes and tissues cells mediates experimental rapidly progressive glomerulonephritis.	Submitted	Experimental design, performing experiments, data analysis and writing and revising the manuscript (70%)

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: .....[redacted].....

Date: 18/3/15

Thesis by Publication

Publications Arising from this Thesis

Odobasic D, Ghali JR, O'Sullivan KM, Holdsworth SR, Kitching AR. Glomerulonephritis Induced by Heterologous Anti-GBM Globulin as a Planted Foreign Antigen. Current Protocols in Immunology, 2014, 106: 15.26.11-15.26.20.

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Papers Submitted for Publication

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Other Papers which the Candidate Contributed to During PhD Candidature

He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, Sun X, Vandenberg K, Rockman S, Ding Y, Zhu L, Wei W, Wang C, Karnowski A, Belz GT, Ghali JR, Cook MC, Riminton DS, Veillette A, Schwartzberg PL, Mackay F, Brink R, Tangye SG, Vinuesa CG, Mackay CR, Li Z, Yu D. Circulating precursor CCR7(lo)PD-1(hi) CXCR5^{+} CD4^{+} T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. Immunity. 2013; 39(4): 770-81.

Summers SA, Alikhan, MA, Chan AJ. Chan, Gan PY, Khouri MB, Ooi JD, Ghali JR, Odobasic D, Hickey MJ, Kitching AR, Holdsworth SR. Endogenous TLR9 regulates acute kidney injury by promoting regulatory T cell recruitment.
Submitted to Journal of the American Society of Nephrology 24 September 2014. Under revision.

Chapter 1: Targeting IL-17 and IL-23 in immune mediated renal disease.

Declaration

Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 1
Declaration by candidate

In the case of Chapter 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Literature review, writing and revising the manuscript	80

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Yuan Min Wang	Review of the manuscript	
Stephen Holdsworth	Review of the manuscript	
Stephen Alexander	Review of the manuscript	
A. Richard Kitching	Writing and revising the manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	Date 18/3/15
Main Supervisor's Signature		Date 18/3/15

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Targeting IL-17 and IL-23 in immune mediated renal disease

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Abstract

T helper (Th) cells belong to the adaptive immune system and provide an effective and antigen-specific means of host protection. Th17 cells are a subset of Th cells, characterized by the production of the inflammatory cytokines interleukin (IL)-17A (IL-17A) and IL-17F, which bind to a receptor complex comprised of IL-17RA and IL-17RC subunits. Th17 cells combat extracellular and fungal infections, but have been implicated in autoimmune diseases. In many autoimmune conditions, the dysregulated immune response involves several parts of the immune system, including autoantibodies, B and T cells. Targeted biological therapies are appealing, as they may prevent unwanted side effects in patients. There is evolving evidence that Th17 cells are important in the kidney, mediating injury in response to vascular or chemical insults to the renal tubules, and in autoimmune diseases of the glomerulus, either through a specific attack on the glomerular basement membrane or as part of a generalized systemic inflammatory disease. Therapies targeting IL-17A, IL-12p40 and IL-17RA are being explored in clinical trials or are being utilized in clinical practice for the treatment of other IL-17 mediated diseases, such as psoriasis. This review explores the current evidence that IL-17A and Th17 cells may be pathogenic in immune kidney disease, including anti-glomerular basement membrane disease, anti-neutrophil cytoplasmic antibody associated vasculitis and lupus nephritis, as well as in acute kidney injury. It will discuss the place that biological agents against IL-17A, IL-12p40 and IL-17RA may have in the treatment of these conditions.

Keywords

Acute kidney injury

Anti-glomerular basement membrane disease

Anti-neutrophil cytoplasmic antibody associated vasculitis

Interleukin 17

Interleukin 12p40

Systemic lupus erythematosus

Th17 cell

Introduction

The innate and adaptive immune systems work in concert, primarily to protect the host from infection. Innate immune cells, which include neutrophils, macrophages and γδ T cells, provide a primary line of defence against infection, in a non-specific manner. CD4+ and CD8+ T cells and B cells mediate the adaptive immune response. T helper (Th) cells recognize specific antigens, which are presented to the T cell receptor (TCR) by MHC II molecules on antigen presenting cells. When appropriate co-stimulation is present, these cells become activated, produce cytokines and growth factors, upregulate chemokine and chemokine receptor expression, and provide help to B cells, enabling antibody class switching.

Th cells were originally divided into Th1 and Th2 subsets by Mosmann and Coffman, based on their effector cytokines, released in response to host infection from intracellular organisms or helminths, respectively [1]. Th1 effectors are defined by their expression of the cytokine interferon γ (IFNγ), whereas Th2 effectors produce interleukin (IL)-4. In 1993, cytokine T lymphocyte antigen 8 (CTLA-8), now called IL-17A (often referred to IL-17), was identified [2]. Subsequently, the related cytokines IL-17B-F were identified, with IL-17F bearing the greatest homology (~50%) to IL-17A [3, 4]. These IL-17A expressing Th cells are known as Th17 cells, with a distinct pathway of differentiation. They are not dependent on the transcription factors T-bet or GATA-3 (required for Th1 and Th2 differentiation, respectively), and IFNγ and IL-4 negatively regulate IL-17A expression by effector or memory T cells [5-7]. The retinoic acid receptor (RAR)-related orphan nuclear receptor, RORγt, is the specific transcription factor for the Th17 lineage [8], but Th17 cells only develop in the presence of the cytokines IL-6, IL-1β, and transforming growth factor β (TGFβ) [9-13]. Th17 cells express the IL-23 receptor (IL-23R), and when IL-23 (from T cells, activated macrophages or dendritic cells [14]) binds to the IL-23R, the terminal differentiation and expansion of these cells occurs [6, 9, 15-19]. IL-23 is composed of 2 subunits, IL-23p19, unique to IL-23, and IL-12p40, shared with IL-12. Th17 cells develop independently of signal transducer and activator of transcription (STAT) 1, STAT4 and STAT6 signalling, required for Th1 or Th2 polarization [7], but require STAT3 signalling in the presence of IL-6 [20]. Th17 cells share many of the same trafficking molecules utilized by Th1 and Th2 cells [21].

The IL-17 receptor family includes 5 receptor subunits; IL-17 Receptor A (IL-17RA)-IL-17RE. IL-17RA is a type 1 transmembrane protein of 864 amino acids, containing an N-terminal signal peptide, extracellular and transmembrane domains with a cytoplasmic tail [22]. IL-17RA is expressed in all major organs (with strong expression in the spleen and kidney) and in many cell lines, including fibroblasts, epithelial cells, mast cells, B and T cell clones, suggesting it has a ubiquitous pattern of expression [22]. IL-17RA forms receptor complexes with other IL-17 receptors [23]. IL-17A and IL-17F signal as homodimers or heterodimers (i.e., IL-17A+IL-17F), through a receptor complex of IL-17RA and IL-17RC [23, 24]. IL-17RC is also widely expressed, predominantly on non-hematopoietic tissues, including vascular endothelium, intestinal, lung and bronchial epithelium, and the kidney, with strong expression identified within glomeruli and medullary tubular epithelium [25, 26].

Cellular sources and actions of IL-17A

Cellular sources of IL-17A

Although Th17 cells are the main adaptive immune cells producing IL-17A, other Th subtypes may also express IL-17A, including regulatory T cells and T follicular helper cells (TFH) [27-29]. It is increasingly recognized that Th phenotype is not fixed; in inflammatory states Th17 cells can change their phenotype and produce IFNγ, behaving as Th1 cells [30, 31]. Innate immune cells, in both mice and humans, also produce IL-17A, including γδT cells, mast cells, natural killer (NK) cells, lymphoid tissue inducer cells and neutrophils [32-38]. Therefore, IL-17A can be considered as a bridge between innate and adaptive immunity.

Actions that promote leukocyte recruitment

IL-17A and IL-17F are similar proinflammatory cytokines, but IL-17A is more potent than IL-17F in inducing inflammation [39]. IL-17A in particular has effects on leukocyte recruitment to inflamed tissues by upregulating endothelial adhesion molecules, cytokines and chemokines. IL-17A acts synergistically with TNF on endothelial cells, upregulating adhesion molecule expression (P-selectin and E-selectin on mouse and human endothelial cells and also vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 on human endothelial cells) and enhancing E-selectin dependent leukocyte rolling on murine endothelial cells [40, 41]. IL-17A, with TNF, promotes neutrophil recruitment by enhancing the expression of

neutrophil attracting chemokines and growth factors by endothelial cells (including CXCL1, CXCL2 [the murine homologues of human IL-8], CXCL5, GM-CSF and G-CSF by murine endothelial cells and CXCL8 from human endothelial cells [40, 41]), thereby permitting neutrophil recruitment and transmigration. IL-17A and TNF enhances CCL5, IL-6 and IL-8 expression from human endothelial cells, but IL-17A (alone or in combination with TNF) does not promote CCL20 expression (for lymphocyte chemotaxis) over TNF alone [42], or CCL5, CXCL9 or CXCL10 expression by mouse endothelial cells [40], suggesting the main action of IL-17A on endothelial cells is to enhance neutrophil, but not T cell, recruitment.

Th17 cells and IL-17A/F signalling promotes protective immunity, particularly at epithelial mucosal surfaces, from fungal, intracellular and Gram negative extracellular pulmonary pathogens [43-46]. IL-17A acts on epithelial cells in models of infection or chemical-induced inflammation. For example, in the lungs, IL-17A signalling increases the expression of the neutrophil attracting chemokines CXCL1, CXCL2 and CXCL5, and other chemokines such as CCL20 (the ligand for CCR6) to attract Th17 cells, and may promote subsequent fibrosis [47-49]. *Il17ra*$^{-/-}$ mice demonstrate impaired dermal delayed type hypersensitivity (DTH), suggesting a role for IL-17A signalling in Th cell recruitment [50].

Actions on neutrophils

IL-23 and IL-17A are important for neutrophil homeostasis. Mice deficient in IL-12p40 have reduced circulating neutrophils and serum IL-17A concentrations, with restoration of neutrophils being dependent on IL-23 but not IL-12, indicating that IL-23 regulates granulopoiesis and acts upstream of IL-17A [51]. IL-17A acts with, and upstream of, granulocyte colony stimulating factor (G-CSF), promoting G-CSF and stem-cell factor (SCF) release from bone marrow stromal cells, thereby stimulating hematopoietic colony formation (in the bone marrow and spleen), increasing circulating neutrophil levels [52, 53]. Once apoptotic neutrophils are phagocytosed by macrophages, IL-23 production is down-regulated and IL-17A levels are reduced, curtailing granulopoiesis [54]. There are conflicting reports on whether *Il17ra*$^{-/-}$ mice have lower [38, 54] or normal peripheral blood neutrophil numbers [55, 56] compared to WT mice. IL-17A and IL-17F do not directly activate neutrophils [57] and resistance to these cytokines may be due to neutrophils not expressing IL-17RC [57], despite expressing IL-17RA [58]. In humans, activated neutrophils can attract Th17

and Th1 cells, by producing the chemokines CCL2, CCL20 and CXCL10, and Th17 cells recruit neutrophils directly through release of CXCL8, and can modulate neutrophil function through IL-17-independent mechanisms (through production of granulocyte macrophage [GM]-CSF, TNF, IL-6 and IFNγ) [57].

Actions on intrinsic kidney cells

In the kidney, IL-17A signalling, particularly in an inflammatory environment, promotes neutrophil and T cell attracting chemokine production. Both mouse mesangial cells and tubular epithelial cells (TECs) express IL-17RA and IL-17RC [59-62]. IL-17A acts on mouse mesangial cells synergistically with TNF to enhance expression of neutrophil attracting chemokines and CCL2, CCL3 and CCL20 [59, 60]. TECs respond to IL-17A signalling by increasing expression of CXCL5 and (in combination with CD40 ligand) CCL2, CCL5, CXCL8, IL-6 and complement factor 3 (C3) [61, 63, 64]. With TNF or IL-1β , IL-17A enhances G-CSF production by human renal proximal TECs, but IL-17F does not [62]. IL-17A also upregulates the expression of chemokines (including CCL2, CCL7, CXCL1, CCL20) from cultured fibroblasts [5].

IL-17A and Th17 cells in immune mediated kidney diseases

Th17 cells have been implicated in experimental models and human studies of a number of autoimmune diseases, including multiple sclerosis, psoriasis, and inflammatory bowel disease (reviewed in [65-67]). In autoimmune diseases affecting the kidney causing glomerulonephritis (GN), glomeruli are infiltrated with immune cells, mesangial cells proliferate, and in severely inflamed glomeruli, evidence of cellular crescents and segmental necrosis may be present. In humans, these diseases can cause rapid loss of renal function, which may be permanent; the most common causes of rapidly progressive GN are systemic lupus erythematosus, anti-glomerular basement membrane (GBM) disease and anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV). Other insults to the kidney, such as profound renal ischemia and subsequent reperfusion (as occurs in transplantation) also cause inflammation, but primarily involve the tubulointerstitium rather than the glomerulus. The role of IL-17A and Th17 cells in inflammatory renal disease is an

evolving field of research, and will be discussed in more detail. Figure 1 provides an overview of the roles of IL-17A and IL-23 in immune mediated kidney diseases.

Systemic Lupus Erythematosus (SLE)

SLE is the prototypic systemic autoimmune disease characterized by the development of multiple autoantibodies, especially to nuclear antigens, for example anti-double stranded (ds) DNA antibodies. This disease classically affects females of child bearing age, and can be organ and life-threatening [68]. Approximately 30-40% of patients with SLE develop lupus nephritis (LN), particularly in those of an African-American background [69-71]. Many factors have been implicated in disease pathogenesis, including genetic polymorphisms, epigenetic modifications of DNA, hormonal influences, and loss of tolerance through dysregulation of immune cells, including B and T cells [68].

IL-17 related cytokines are elevated in the sera of SLE patients, although studies aiming to correlate these with clinical outcomes provide conflicting results. IL-17A is found at higher concentrations in the sera of SLE patients compared to healthy controls (HC), and has been found in some studies to correlate with disease severity scores and anti-dsDNA titers [72-74], with IL-17A and IL-23 levels being elevated in patients with Class IV LN, the most severe class of LN [75]. However, other studies have found that while IL-17A levels in LN patients were increased compared to HC and patients without LN, levels did not correlate with disease severity scores or proteinuria [76]. Similarly, IL-17A levels in SLE patients did not correlate with disease activity scores or anti-dsDNA antibodies, but were elevated in patients with central nervous system involvement [77].

Th17 cells are a major source of IL-17A in SLE. Compared to peripheral blood mononuclear cells (PBMCs) from HC, patients with SLE have increased IL-17-expressing PBMCs, with greater IL-17 production from both CD4+ and double negative (DN) T cells [78, 79], and increased peripheral blood Th17 cell proportion in SLE patients has been correlated with proteinuria, LN and disease severity [75, 80, 81]. The balance of circulating Th cells in SLE appears skewed towards an increased Th17/Th1 cell ratio [79, 81] and LN patients have fewer regulatory T cells (Tregs) but

more Th17 cells in peripheral blood than HC or SLE patients without LN, with an elevated Th17/Treg ratio correlating with worse disease activity scores [76]. Neutrophils from both SLE patients and HC express IL-17A mRNA and externalize IL-17A during the formation of neutrophil extracellular traps (NET), potentially an important process in LN [82].

A number of studies have examined intrarenal leukocytes in human LN to investigate whether Th17 cells and/or IL-17A contribute to the development or severity of GN. The presence of Th17 cells and other IL-17A producing cells within nephritic kidneys, and correlation with poorer renal outcomes and disease activity scores, suggests these cells are pathogenic in SLE. In renal biopsies of patients with LN, IL-17A and IL-23 expression has been identified in DN, CD4+ and CD8+ T cells [78]. Glomerular IL-17A and IL-23 expression correlated with renal disease activity in patients with LN [75]. T cells in glomeruli and the interstitium of patients with severe (Class IV) LN produce IL-17A but not IFNγ [83], and there were more glomerular and interstitial infiltrating IL-17A+ T cells in severe (Class IV or V) LN, compared to milder renal disease (Class III) [83]. While the expression of IL-17A positively correlated with certain clinical endpoints and disease activity, it negatively correlated with chronicity index scores on renal biopsy, suggesting that IL-17A may be involved in inflammation during the acute phase of disease [83]. A study of biopsies from pediatric LN patients identified both IFNγ+ and IL-17A+ cells in glomeruli and interstitial regions; interstitial IFNγ+ and IL-17A+ cells were CD4+ T cells, whereas those in the glomeruli were not. As in adult studies, IL-17A correlated positively with disease activity, but negatively with glomerular filtration rate in this study [84]. Th17-related cytokine and RORγt mRNA expression is increased in the urinary sediment of patients with LN, but the level of expression was reduced in patients with active compared to quiescent disease, was inversely proportional to disease activity and expression of Th1-related cytokines and transcription factors [85]. Although this seems contradictory, this study highlights the importance of evaluating inflammatory cell populations at a renal tissue level in disease.

Several murine models of SLE exist (characterized by the spontaneous development of autoantibodies, arthritis and GN as mice age; Table 1). Studies in these mice indicate that IL-23, IL-17A and Th17 cells contribute to disease pathogenesis.

Stimulated T cells from lupus-prone mice express more IL-17A and IL-23R mRNA than lymphocytes from controls, particularly with increasing disease progression [86]. IL-23 signalling is important for Th17 cell development in these lupus-prone mice. IL-23R deficiency resulted in fewer DN T cells and IL-17A+ cells in lymph nodes, with reduced anti-dsDNA antibodies and improved clinical disease in mice [87]. IL-23 enhanced IL-17A production by CD4+ and DN T cells and transfer of IL-23-expanded lymphocytes into *Rag1*$^{-/-}$ mice induced proteinuria and GN, increased IgM, IgG and C3 deposition within glomeruli, and increased IL-17A+ cells in the kidneys, suggesting the Th17-polarized lymphocytes were pathogenic [86].

However, the exact role Th17 cells play in the development of LN in lupus-prone mice remains poorly defined. In MRL/lpr mice, CD3+IL-17A+ cells peak within nephritic kidneys at week 19, while CD3+IFNγ+ cells increase from week 15 onwards, however the majority of CD4+, CD8+ and DN T cells were IFNγ+ compared to IL-17A+ by week 24 [88]. *Il17ra*$^{-/-}$ MRL/lpr mice were not protected from nephritis compared to MRL/lpr mice, and did not have any differences in renal leukocyte infiltration, renal CD4+IFNγ+ T cells, serum IFNγ or auto-antibody levels [88]. Similarly, in mature NZB/NZW mice, proportions of renal IL-17A+ T cells (predominantly DN T cells) were lower than IFNγ+ T cells (mostly CD4+ T cells); when anti-IFNγ or anti-IL-17A was given to NZB/NZW mice after 23 weeks old, nephritis was reduced in the anti-IFNγ group but not the anti-IL-17A group compared to control mice [88]. Taken together, these studies suggest Th17 and Th1 cells are involved in GN in murine SLE; Th17 cells may have early and Th1 cells later involvement in perpetuating autoimmunity, however, the degree to which they are pathogenic may be influenced by genetic factors inherent in these mice.

Signalling through IL-17RA is important in a number of other SLE models (Table 1). In pristane-induced lupus, *Il17a*$^{-/-}$ mice were protected from lupus and lupus nephritis, potentially through the absence of early IL-17A production from innate cells, while *Il17ra*$^{-/-}$ mice were protected from GN and autoantibody development compared to WT mice [89, 90], with expansion of Tregs and reduction in DN T cells in pristine-treated *Il17ra*$^{-/-}$ mice [90]. In mice deficient in the inhibitory Fcγ receptor, FcγRIIb, IL-17A signalling accelerates nephritis and increases mortality, demonstrated by the use of *Il17ra*$^{-/-}$ *Fcgr2b*$^{-/-}$ mice and CIKS deficient (*Traf3ip2*$^{-/-}$) *Fcgr2b*$^{-/-}$ mice (deficient

in the adaptor protein Act1, required for IL-17 cytokine signalling) [91]. The role of IL-17A has also been assessed in mice developing a lupus-like disease through treatment with activated lymphocyte derived (ALD)-DNA (Table 1); mice concurrently receiving an adenovirus vector for overexpression of IL-17A had greater proteinuria and worse GN than controls, while *Il17ra*$^{-/-}$ mice given ALD-DNA had attenuated renal injury, as did WT mice treated with ALD-DNA and a neutralizing antibody to IL-17A [92]. These alternative models of SLE reinforce that IL-17 signalling through IL-17RA contributes to autoimmunity, with subsequent development of LN.

Anti-GBM disease (also known as Goodpasture's disease)

Anti-GBM disease is a rapidly progressive form of GN characterized by segmental or global necrosis of capillary tufts, disruption of the GBM and Bowman capsule with crescent formation, and linear deposition of IgG on the GBM. It has a bimodal age distribution, affecting males in their 20s and females in their 6-8th decade of life [93]. While this disease is rare, end stage kidney disease is a frequent outcome [94]. Human anti-GBM disease develops in response to autoantibodies to the non-collagenous (NCI) domain of the α3 (and also the α5) chain of type IV collagen, located in the kidney, lung, testis and eye [95-98], and often also presents with pulmonary haemorrhage (referred to as Goodpasture's syndrome). The α3, α4 and α5 subunits interact to form a triple helix (referred to as a protomer). Two protomers cross-linked together form a hexamer, and this hexameric structure effectively sequesters these epitopes. However, if the structure of the collagen is disrupted, these epitopes are exposed and available for binding by autoantibodies [95].

While human anti-GBM disease is characterized by the presence of anti-α3(IV)NC1 autoantibodies, several studies point to a pathogenic role for T cells. CD4+ T cells recognizing α3(IV)NCI are found at greater frequency in PBMCs from patients with acute anti-GBM disease compared to those in the convalescent phase of disease [99], with a CD4+CD25+ regulatory T cell population being increased in convalescent patients, suggesting that peripheral regulation of effector T cell responses are important [100]. α3(IV)NCI is expressed in the normal human thymus, therefore central T cell tolerance may also be important in preventing auto-reactivity [99] and the disease exhibits strong positive and negative MHC Class II associations [101]. At present, there are no published studies examining the role of IL-17A or Th17 cells in

humans with anti-GBM disease. This disease can be modeled by immunizing mice with α3(IV)NCI or α3/α5 dimers. Although tolerance is readily broken, renal injury is mild [102]. IFNγ deficient mice develop worse GN than *Il12p40*-/- mice, and *Il23p19*$^{-/-}$ mice (deficient in IL-23, but not IL-12) have diminished autoimmunity to the Goodpasture antigen and less disease, with reduced cellular and humoral immune responses and diminished inflammatory cytokine production [103, 104].

However, the most commonly used models of rapidly progressive GN are not autoimmune, but are induced by the injection of heterologous globulin (usually sheep in origin) directed against renal basement membranes (Table 1). This results in a short period of heterologous injury in response to the antibody, followed by an autologous phase of disease, as adaptive immunity develops in response to the foreign antigen (sheep globulin; SG) planted along the GBM. SG-specific CD4+ T cells and mouse anti-sheep IgG antibodies are responsible for this autologous phase (known as 'non-accelerated anti-GBM disease') [105]. Mice may also be sensitized to SG several days prior to anti-GBM globulin administration, permitting adaptive immunity to develop to the nephritogenic antigen before administration of the globulin, resulting in rapid development of GN ('accelerated anti-GBM disease').

Prior to the discovery of Th17 cells, the Th1 immune response was shown to be critical for the development of GN in these murine models [106-108]. Specifically, renal injury was exacerbated through the administration of recombinant IL-12 [109], diminished in mice treated with anti-IFNγ antibodies [106] and GN was attenuated in mice genetically deficient in T-bet, IFNγ and IL-12p40 [110-112]. More recent studies have shown that IL-23, IL-17A, Th17 cells and innate cellular sources of IL-17A are also pathogenic in similar models of "anti-GBM" disease. *Rorc*$^{-/-}$ mice (lacking the gene for RORγt), *Il23p19*$^{-/-}$ and *Il17a*$^{-/-}$ nephritic mice demonstrate reduced renal injury, [56, 60, 113]. γδT cells, an innate CD3+ cell population, are an important source of IL-17A within the kidney at an early stage (3 days) after anti-GBM disease induction, and deficiency of γδT cells resulted in reduced GN with fewer neutrophils, macrophages and Th17 cells in the kidney at day 6 [114].

As both Th1 and Th17 cells (and their effector cytokines) contribute to GN and systemic inflammatory immune responses in these models, there has been increasing focus on understanding the relationship between these Th subsets and the differences in local inflammatory responses induced by either Th1 or Th17 cells. Summers et al. showed definitively that both Th1 and Th17 antigen-specific cells could induce GN, in a T cell transfer system using Th1 or Th17 cells from TCR transgenic mice (Table 1) [115]. In this model, Th17 recipients had increased early glomerular neutrophil infiltrates, and albuminuria, while Th1 recipients had early macrophage recruitment, with later development of albuminuria [115].

Early IL-17A signalling and Th17 cells may influence the development of a later Th1 and IFNγ response, although the exact relationship between the two responses remains a little unclear. *Il17a*$^{-/-}$ mice, but not *Ifng*$^{-/-}$ mice, were protected from early renal injury [116, 117]. *Ifng*$^{-/-}$ mice or anti-IFNγ mAb treatment protects mice from GN at later timepoints [117, 118]. However, the nature of the cross-regulatory roles of IL-17A and IFNγ is less clear. At a later phase of disease, *Il17a*$^{-/-}$ mice had worse GN with more CD4+ T cell infiltration, and greater systemic IFNγ production, suggesting IL-17A regulated pathogenic Th1 responses [116]. However, in a model where *Rag1*$^{-/-}$ mice were reconstituted with splenocytes from *Il17a*$^{-/-}$ or *Ifng*$^{-/-}$ mice and GN was induced, mice given *Il17a*$^{-/-}$ splenocytes had fewer renal Th1 cells and reduced renal expression of IFNγ [117]. Furthermore, recipients of *Ifng*$^{-/-}$ splenocytes had increased renal Th17 cells and IL-17A. Notwithstanding the potential for different rates of lymphocyte proliferation in the lymphopenic host (IFNγ has anti-proliferative effects), these studies suggest that IFNγ regulates early IL-17A responses [117]. Overall, these data support a model in which renal injury in experimental rapidly progressive GN can be mediated by either or both Th1 and Th17 subsets, with early IL-17A signalling from innate immune cells, such as γδT cells, enhancing neutrophil recruitment, and as adaptive immunity develops, Th17 immune responses being regulated by IFNγ and the subsequent Th1 response.

ANCA-Associated Vasculitis

Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV) is a disease characterized by inflammation of small-sized blood vessels, located within the kidney and lung, and clinically includes the disease syndromes granulomatosis with

polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA). These autoantibodies target constituents of neutrophil cytoplasmic granules, namely myeloperoxidase (MPO), proteinase 3 (PR3) and LAMP-2 (lysosomal-associated membrane protein 2). It had been postulated that these enzymes could be externalized by neutrophils during inflammation, permitting the generation of autoantibodies; ANCA interacts with primed neutrophils (even in the absence of infection), may localize them to glomeruli, inducing NETosis, with the subsequent externalization of PR3 and MPO [119, 120].

Limited human studies exploring IL-17A in AAV have identified that IL-17A may underlie disease and may play a role in disease relapse. Neutrophils, mast cells and T cells expressing IL-17A have been identified in the glomeruli and interstitium of renal biopsies from patients with AAV GN [33]. Patients with acute AAV have elevated circulating IL-17 and IL-23 levels (with IL-23 levels correlating with disease activity scores), which remain elevated in a proportion of patients whose disease becomes quiescent [121]. Conventional immunosuppressive therapy was not effective in suppressing IL-23 and IL-17A in all patients [121]. Patients with GPA in remission may have circulating effector Th subsets skewed towards Th17 and Th2, rather than Th1 cells [122], and a reduction in Treg:effector Th ratio (along with abnormal Treg function) has also been found in AAV patients [123, 124].

Rodent studies of AAV developing to the autoantigen PR3 are hampered by the lack of a robust animal model mimicking human disease, despite recent attempts [125, 126]. However, MPO-induced models do exist, in part because of the strong homology between human and mouse MPO. One murine model of MPO-AAV involves sensitization of mice to MPO to generate anti-MPO humoral and cellular autoimmunity, before GN is induced by planting MPO within glomeruli (Table 1). Using this model, Gan et al. found that IL-17A signalling and Th17 cells were pathogenic. Compared to WT controls, *Il17a*$^{-/-}$ mice had reduced GN and dermal DTH, with fewer renal CD4+ T cells, neutrophils and macrophages [127]. To overcome any reduction in MPO deposition in the kidneys through impaired neutrophil recruitment in *Il17a*$^{-/-}$ mice, MPO was directly injected into the kidneys of sensitized WT and *Il17a*$^{-/-}$ mice, with *Il17a*$^{-/-}$ mice subsequently having fewer macrophages in the kidney. Even when neutrophils were depleted prior to the intra-

renal MPO injections, *Il17a*$^{-/-}$mice still had reduced macrophage recruitment, indicating the importance of IL-17A signalling in the cellular DTH response [127].

Acute Kidney Injury (AKI)

AKI is a common event in patients admitted to hospital, occurring the setting of insults to the kidney following shock, surgery, sepsis, or the administration of nephrotoxic drugs and radio-contrast. AKI, even mild renal injury that does not require temporary renal replacement therapy (i.e. dialysis), has been associated with increased length of stay in hospital, increased patient mortality 90 days after discharge and on long-term follow-up [128, 129]. The contribution of the IL-23 and IL-17 in human AKI is yet to be clearly defined. Murine models of AKI include Ischemia Reperfusion Injury (IRI), as a model of temporary renal ischemia, as may occur in the setting of renal transplantation, cardiopulmonary bypass or hypovolemic shock (Table 2). Another model of AKI induces tubulointerstitial injury by administration of cisplatin, a chemotherapeutic agent (Table 2).

In murine IRI, IL-17A is an important early mediator of injury; plasma and renal IL-17A levels increase and peak at 12-16 hrs post-IRI, with high levels being sustained until 24hrs [130]. Administration of anti-IL-17A antibodies prior to, or following, IRI reduced renal injury [34, 130], with attenuated renal injury also being seen when IRI was induced in mice with genetic deficiencies of IL-17A, IL-17RA, IL-12p40 or IL-23p19 compared to WT [34]. The beneficial effects of anti-IL-17A antibodies prior to IRI were mediated by reduced renal and plasma levels of pro-inflammatory cytokines and chemokines (including TNF, IL-6, IL-1β, CCL2, and CCL3) [130]. It appears that innate cells are an important source of IL-17A in response to tissue injury in AKI. *Rag1*$^{-/-}$ (T and B cell deficient mice) are protected from renal injury in IRI upon treatment with anti-IL-17A mAb, suggesting IL-17A-producing innate cells are the predominant mediator of injury in IRI, with assessment of renal leukocytes by flow cytometry, along with adoptive transfer studies of *Il17a*$^{-/-}$ vs WT neutrophils into *Il17a*$^{-/-}$ recipients subjected to IRI, confirming neutrophils as the major innate IL-17A producing cell type contributing to early IRI [34].

In cisplatin induced AKI, renal IL-17A mRNA expression peaks 24 hours after cisplatin, returning to baseline by 96 hours [131]. Cisplatin-treated *Il17a*$^{-/-}$ and *Rorc*$^{-/-}$ mice are protected from AKI and administration of anti-IL-17A mAb to WT mice prior to cisplatin also attenuated renal injury. The renal leukocytes producing IL-17A in cisplatin-induced AKI include neutrophils and CD4+ T cells; *Il17a*$^{-/-}$ mice have fewer neutrophils within the kidney at 24 hours, but similar CD4+ T cell numbers, suggesting IL-17A is important for neutrophil recruitment in this model [131]. Although neutrophil depletion prior to cisplatin reduced AKI compared to controls, treatment of neutrophil-depleted mice with anti-IL-17A mAb further reduced AKI, with NK cells (but not γδT cells), being found to be the innate IL-17A-producing cell type contributing to early renal injury in this model of AKI [131].

Experiences with monoclonal antibodies against IL-12p40, IL-17A and IL-17RA in immune disease

There have been no trials of anti-IL-23 or IL-17 therapies in glomerulonephritis or AKI. However, a number of humanized mAbs against IL-12p40, IL-17A and IL-17RA have been tested in published Phase II and III clinical trials in the treatment of autoimmune diseases where these cytokines have been implicated in disease pathogenesis. Tables 3 and 4 describe the results from the published experience with biological agents acting against IL-12p40 or IL-17A and IL-17RA, respectively. Disappointingly, studies of these therapies in patients with Crohn's disease or multiple sclerosis have not found them to be efficacious, raising questions over the applicability of animal studies to human disease, whether IL-17 and IL-23 signalling is pathogenic in these diseases, the completeness of blockade with these agents, and the most appropriate time for initiating therapy in these chronic diseases.

A number of adverse effects have been noted with these agents, with minor effects, such as injection site reactions and nasopharyngitis, being most commonly reported. Neutropenia can also occur, although severe neutropenia requiring drug cessation is reported infrequently. As with all immunomodulatory therapies, concern about the risks of infection and malignancy require ongoing evaluation, as such risks may not be able to be assessed adequately in short-term Phase II-III trials. Experience with ustekinumab (anti-IL-12p40 mAb) therapy for psoriasis over 4 years has been

reported, with rates of adverse events (serious infections, non-melanoma skin cancer and other malignancies) being stable over the follow-up period [132]. There was no relationship to the dose of ustekinumab used, and the cumulative rates of these adverse events were consistent with those in the general and psoriasis populations [132-134]. Ustekinumab therapy did not increase the risk of tuberculosis reactivation in patients treated with latent tuberculosis infection [135]. Of possible concern is a disputed potential increase in rates of major cardiovascular events (myocardial infarctions, cerebrovascular accidents or cardiovascular deaths) that has been reported with anti-IL-12p40 antibodies (compared to placebo) [136-138]. Identification of an increase in cardiovascular events may depend on the statistical methods used to assess these events [139, 140] and if this association exists, it is not clear if this is a class effect.

While an increase in the future risk of malignancy has not been identified as a problem in Phase II-III trials, case reports regarding ustekinumab describe the rapid development of squamous cell carcinomas following ustekinumab therapy [141] and malignant melanoma [142]. Other cutaneous complications have been reported, including lymphomatoid drug reaction [143], cutaneous infarction [144], and multi-dermatomal Herpes zoster [145], linear IgA bullous dermatosis [146], erythema annulare centrifugum [147] and alopecia areata [148]. Reversible posterior leukoencephalopathy after ustekinumab therapy has occurred [149]. Development of anti-drug antibodies to ustekinumab, ixekizumab and sekizumab (both anti-IL-17A mAbs) has been reported, although no patient was found to have experienced a clinical adverse event due to these antibodies [150-153]. However, the concern is that such antibodies may reduce drug efficacy or heighten risk of allergic reactions [150, 153]. Therefore, as these therapies become integrated into clinical practice, ongoing surveillance of recipients of these therapies for long-term consequences is needed.

Targeting the IL-17A and IL-12p40 is efficacious in the treatment of psoriasis, but whether this form of therapy will be successful in inflammatory or autoimmune renal disease is less clear. While research suggests that IL-17A is elevated early in disease/renal injury, it might also be important in regulating later inflammatory responses. Further understanding of the coordination of the immune response throughout the evolution of early to established disease is required, but this will be

challenging to evaluate in humans, as anti-GBM disease, severe LN and AAV are rapidly progressive diseases, meaning patients tend to present with established rather than early disease. At present, biomarkers do not exist to accurate assess the chronicity of disease or adequately predict disease relapse. While there are some similarities, these diseases probably have differences in elements of disease pathogeneses, so IL-17A/IL-23 blockade may have different effects in each. The place of IL-17A/IL-23 blockade in renal autoimmune disease may be as an adjunct to conventional treatment or in disease maintenance/relapse prevention in the first instance. Clinical trials are required to evaluate this and will require multi-center studies, due to the fact these conditions are uncommon. In terms of ischemic insults to the kidney, IL-17A/IL-23 blockers may be easier to evaluate, particularly in procedures where an insult is predictable, such as in cardiopulmonary bypass for cardiac surgery or renal transplantation.

Conclusion

Human studies and functional studies in experimental models point to an important role for IL-17A, and potentially IL-23, in inflammatory and immune mediated kidney disease. There is much to be learnt, as it is increasingly evident that IL-17A produced by CD4+ T cells and innate immune cells contributes to early injury in inflammatory disease states and may coordinate the subsequent immune response generated by Th1 cells. Agents targeting IL-12p40, IL-17A and IL-17RA are relatively safe and efficacious in psoriasis, but are not effective in multiple sclerosis or inflammatory bowel disease. Clinical trials will be required to assess their efficacy in AKI and autoimmune renal diseases.

Conflict of interest

The authors declare no conflict of interest.

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Tables

Table 1

Models of murine immune mediated kidney diseases involving T helper cells.

Model	Description of Model	Renal Manifestations	References
Systemic Lupus Erythematosus			
MRL/lpr mice	Lupus-prone mouse strain	Glomerular endothelial and mesangial cell proliferation, occasional crescents and basement membrane thickening IgG and C3 deposition in granular pattern in glomerular capillary walls, mesangium and tubulointerstitium.	[154]
NZBxNZW F1 (NZB/NZW)	Lupus-prone mouse strain	Glomerular mesangial and intravascular proteinaceous deposits, proliferation and crescent formation. Mouse IgG and C3 in granular pattern in glomerular capillary walls, mesangium and tubulointerstitium.	[154]
Pristane-induced SLE	Mice are administered 2,6,10,14-tetramethylpentadecane i.p., with development of autoimmunity. GN develops after 6 months.	Dependent on genetic background. Increased glomerular size, mesangial cell proliferation, mononuclear cell, infiltration increased mesangial extracellular matrix and thickened GBM. Glomerular deposition of IgG, IgM and C3.	[155]
FcγRIIb$^{-/-}$.B6	Lupus-prone mouse strain	Spontaneously develop glomerulosclerosis with hypercellularity, mesangial thickening and IgG deposition.	[156]
Activated lymphocyte derived DNA	BALB/c mice injected with genomic DNA (from Con A activated splenocytes) s.c. in FCA on week 0, boosted weeks 2, 4 (FIA), euthanized at 6 months.	Glomerular cell proliferation and tubular dilatation, with mesangial cell proliferation and podocyte foot process fusion. Mesangial IgG and C3 deposition, with C3 also seen in glomerular capillary walls.	[157]

Anti-glomerular basement membrane disease			
Non-accelerated anti-GBM disease (aka nephrotoxic nephritis [NTN] or nephrotoxic serum nephritis)	Sheep are immunised with murine renal cortices, permitting the generation of sheep anti-mouse GBM antibodies. Mice are injected with sheep anti-mouse GBM IgG, planting it in the glomerulus.	Two phases of renal injury: (1) Heterologous injury, dependent on the antibody binding to the GBM, with neutrophil recruitment and degranulation within the glomerulus within the first 24 hrs. (2) Autologous injury, where an adaptive immune response develops to sheep Ig on the GBM by day 6-7. Glomerular crescents and segmental necrosis by day 21. IgG and C3 on the GBM.	[105, 158, 159]
Accelerated anti-GBM disease	Mice are sensitized to normal sheep globulin (administered subcutaneously with FCA), then receive sheep anti-mouse GBM IgG.	As mice have been sensitized to sheep IgG, an adaptive immune response occurs immediately. Glomerular necrosis and crescents 10 days after sheep-anti-mouse GBM administration.	[105]
Experimental autoimmune GN	Mice are sensitized subcutaneously to α3(IV)NC1 s.c. in adjuvant.	Renal injury develops in the following weeks immunization. Can produce severe GN in naïve rats, with the generation of T cells specific for this peptide.	[102, 103, 160]
Planting of Ovalbumin (OVA) conjugated to a non-nephritogenic IgG within glomerulus	Ovalbumin (OVA) conjugated to a non-nephritogenic IgG is administered i.v. then activated T cells from OVA-specific TCR transgenic mice are transferred.	Glomerular cellular proliferation, segmental necrosis and occasional crescent formation.	[115, 161]
MPO-ANCA Associated Vasculitis			
Cellular and humoral immunity to MPO	Mice are sensitized and boosted to MPO (FCA/FIA) then given a low dose of sheep anti-mouse GBM antibody to cause neutrophils to externalise MPO in the glomerulus.	Glomerular necrosis and crescents.	[102, 120]

Abbreviations: ANCA, anti-neutrophil cytoplasmic antibody; con A, concanavalin A; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; GBM, glomerular basement membrane; GN, glomerulonephritis; OVA, ovalbumin; IgG, immunoglobulin G; IgM, immunoglobulin M; i.v., intravenous; MPO, myeloperoxidase; s.c., subcutaneous; TCR, T cell receptor; α3(IV)NCI, α3 chain of the noncollagenous (NCI) domain of type IV collagen.

Table 2

Commonly used models of murine acute kidney injury.

Model	Description of Model	Renal injury induced	References
Ischemia reperfusion injury (IRI)	Bilateral renal artery clamping for a period of time (ischemia; usually 22-30 minutes), then released (reperfusion).	Tubulointerstitial injury and inflammation in cortical and medullary regions of the kidney. Vascular/endothelial damage, oxygen free radical production, innate and adaptive immune cell infiltration and inflammatory cytokine release.	[162, 163]
Cisplatin Nephrotoxicity	Mice are administered cisplatin i.p.; model lasts 72-96 hours before mouse death from renal failure.	Tubulointerstitial injury and inflammation in cortical and medullary regions of the kidney.	[164]

Abbreviations: i.p., intraperitoneal.

Table 3

Phase II and III trials of agents targeting IL-12p40.

Agent and Description	Indication	Trial Details	Primary Endpoint	Primary endpoint achieved	Reference
Ustekinumab (CNTO 1275) Centocor Humanized anti-IL-12p40 mAb	Moderate-severe plaque psoriasis	PHOENIX I; Ustekinumab 45 or 90mg wks 0, 4 then 12 wkly vs placebo. Crossover to ustekinumab at wk 12 for placebo group; follow-up until wk 28.	PASI75 by wk 12	Yes	[165]
	Moderate-severe plaque psoriasis	PHOENIX I (extension study): Responders re-randomized to 12 wkly ustekinumab or withdrawal; partial responders continued ustekinmab 8 wkly.	PASI75, DLQI and PGA	Yes	[166]
	Moderate-severe plaque psoriasis	PHEONIX II: 52 wk trial. Ustekinumb 45/90mg wks 0, 4, then every 12 wk or placebo; partial responders wk 28 re-randomized to 8/12 wkly ustekinumab.	PASI75 by wk 12	Yes	[167]
	Moderate-severe plaque psoriasis	ACCEPT: Ustekinumab 45 or 90mg (weeks 0 and 4) vs etanercept (50mg twice wkly for 12 weeks)	PASI75 by wk 12	Yes	[168]
	Moderate-severe plaque psoriasis	PEARL: Ustekinumab 45mg at weeks 0, 4 and 16 vs placebo at wks 0 and 4 with ustekinumab wks 12 and 16.	PASI75 by wk 12	Yes	[169]
	Moderate-severe plaque psoriasis	Japanese Ustekinumab Study Group: ustekinumab 45 or 90mg wks 0, 4 and 12 wks vs placebo at wks 0 and 4 and ustekinumab at wk 12. 72 wk study.	PASI75 by wk 12	Yes	[170]

Psoriatic arthritis/ plaque psoriasis	Ustekinumab 63 or 90mg wkly, wks 0-3, then placebo at wks 12 and 16 vs placebo wks 0-3 then ustekinumab 63mg wks 12 and 16. 36 wk study.	ACR20 response at wk 12	Yes	[171]
Psoriatic arthritis	PSUMMIT I: Ustekinumab 45 or 90mg or placebo at wks 0, 4 and 12 weekly. Follow-up to 52 weeks.	ACR20 response at wk 24	Yes	[172]
Psoriatic arthritis	PSUMMIT II: Ustekinumab in patients with psoriatic arthritis despite conventional non-biological and anti-TNF therapy	ACR20 response at wk 24	Yes	[173]
Moderate-severe Crohn's disease	Population 1: Four arms, early placebo s.c. then ustekinumab at 8 wks and vice versa, or i.v. early ustekinumab or placebo then ustekinumab at 8 wks. Population 2: 16 wks study; s.c 90mg ustekinumab wkly for 4 wks or one i.v. 45mg dose.	Clinical response with >25% and >70 points in CDAI score at wk 8 for population 1	No	[174]
Moderate-severe Crohn's disease	CERTIFI study group: Disease resistant to anti-TNF therapy. 1, 3 or 6mg/kg ustekinumab or placebo at wk 0. Re-randomization wk 8 depending on clinical response to maintenance placebo or ustekinumab. Follow-up 22 wks.	≥ 100 point reduction in baseline CDAI score at wk 6	Yes 6mg/kg group.	[175]
Relapsing remitting Multiple Sclerosis	Placebo vs 45, 90mg every 8 wks. 90 or 180mg s.c. wks 0, 1, 2, 3, 7, 11, 15, 19. 90mg group had placebo on weeks 7 and 15. 37 wks follow-up.	New lesions on serial MRI over 23 weeks.	No	[176]

	Trials underway (see clinicaltrials.gov): Acute graft versus host disease, moderate-severe active Crohn's disease, psoriasis vulgaris, vascular inflammation in psoriasis, SLE, Type I diabetes mellitus, chronic atopic dermatitis, common variable immunodeficiency. NCT01713400, NCT01369355, NCT01999868, NCT02349061, NCT02204397, NCT02187172, NCT02117765, NCT01806662, NCT02199496				
Briakinumab (ABT-874) Abbott Laboratories Anti-IL-12/23p40 mAb	Moderate-severe plaque psoriasis	Briakinumab (s/c 200mg week 0 then 100mg every 2nd week or 200mg weekly for 4 wks then 2 wkly or 200mg wkly) vs placebo. Follow-up 12 wks.	PASI75 by wk 12	Yes	[177]
	Moderate-severe plaque psoriasis	Briakinumab (200mg wks 0, 4, then 100mg wk 8) or placebo; re-randomized at 12 wks to briakinumab 100mg wkly every 4 wks vs briakinumab 100mg every 12 wks vs placebo until wk 52.	PASI75 by wk 12 and PGA of 0/1 at wks 12 and 52.	Yes	[178]
	Moderate-severe plaque psoriasis	Phase III, 12 wk study. Briakinumab (200mg wks 0 and 4, then 100mg wk 8), etanercept (50mg twice wkly 3-4 days apart between wks 0-11) or placebo.	PASI75 by wk 12 and PGA of 0/1	Yes	[179]
	Moderate-severe plaque psoriasis	Briakinumab (200mg wks 0 and 4, then 100mg wk 8), etanercept (50mg twice wkly 3-4 days apart between wks 0-11) or placebo. Follow-up 12 wks.	PASI75 by wk 12 and PGA of 0/1	Yes	[180]
	Moderate-severe psoriasis	Briakinumab (200mg wks 0 and 4, 100mg wk 8 and 4 wkly thereafter) vs methotrexate (5-25mg wkly).	PASI75 by wk 24 and 52 and PGA of 0/1	Yes	[181]
	Multiple Sclerosis	Briakinumab 200 mg every other wk (EOW), ABT-874 200 mg every wk (EW), or placebo. Follow-up 24 wks.	Cumulative number of MRI lesions at wk 24.	Yes 200mg EOW	[182]

Apilimod Mesylate (STA-5326) Synta Inhibits c-Rel translocation, suppresses IL-12p35/IL-12p40 transcription	Moderate-severe Crohn's disease	Apilimod mesylate 50mg or 100mg p.o. daily vs placebo.	Reduction in CDAI of ≥10 points at day 29.	No	[183]
	Rheumatoid Arthritis	Apilimod + MTX. 29 patients. 100mg/day Apilimod vs placebo for 4 weeks (stage 1), or 8 weeks (stage 2), then enrolled in stage 3 with 100mg BD or placebo for 8 weeks, with optional 4 week extension.	DAS28 and ACR20	No	[184]
Tildrakizumab (MK-3222) Merck	Humanized anti-IL-23 mAb. Trials underway (see clinicaltrials.gov): Moderate-severe chronic plaque psoriasis. NCT01722331				
Guselkumab (CNTO 1959) Janssen	Human Anti-IL-23 mAb. Trials underway (see clinicaltrials.gov): Moderate-severe plaque type psoriasis, psoriatic arthritis, pustular or erythrodermic psoriasis. NCT02207231, NCT02207244, NCT02343744, NCT02319759, NCT02203032, NCT02325219				

Abbreviations: ACR20, American College of Rheumatology 20% response; CDAI, Crohn's disease activity index, DAS28, Disease Activity Score in 28 joints; mAb, monoclonal antibody; MTX, methotrexate; PASI75, at least 75% improvement in Psoriasis Area and Severity Index; PGA, physician's global assessment; s.c., subcutaneous; i.v., intravenous.

Table 4

Phase II and III trials of anti IL-17A and anti-IL17RA antibodies.

Agent and Description	Indication	Trial Details	Primary Endpoint	Primary endpoint achieved	Reference
Ixekizumab (LY2439821) Eli Lilly Humanized IgG4 anti IL-17A antibody	Moderate-severe plaque psoriasis	s.c. 10, 25, 75, 150mg ixekizumab vs placebo at 0,2,4,8,12 wks.	PASI75 by week 12	Yes, except 10mg	[185]
	Moderate-severe plaque psoriasis	Open label extension study. Patients treated with s.c. 10, 25, 75, 150mg ixekizumab vs placebo at 0,2,4,8,12 wks who failed to reach PASI <75% by wk 20 were given ixekizumab 120mg every 4 wks If PASI75 reached, treatment free period between wk 20-32, eligible for further ixekizumab if PASI<75 or at wk 32 if PASI75 maintained. Follow-up 52 wks.	Ongoing monitoring of PASI75 and adverse events	77% PASI75, 68%, PASI90, 48% PASI100.	[186]
	Rheumatoid arthritis	Part A: Ixekizumab i.v. 0.06, 0.2, 0.6 or 2.0 mg/kg (escalating) or placebo followed for 8 wks. Part B: 0.2, 0.6 or 2.0mg/kg ixekizumab or placebo administered every 2 wks for 5 doses, follow-up16 wks.	Part A: safety and tolerability. Part B: safety and DAS28 at wk 10.	Yes.	[151]

	Rheumatoid arthritis	Placebo or Ixekizumab 3, 10, 30, 80 or 180mg s.c. at wks 0, 2, 4, 6, 8 and 10. Placebo or ixekizumab 80 or 180mg in patients with inadequate response to anti-TNF therapy.	ACR20 at week 12 in biologic naïve patients.	Yes	[187]
Secukinumab (AIN-457) Novartis Pharmaceuticals Fully human IgG1κ anti-IL-17A monoclonal antibody	Moderate-severe Crohn's disease	2 x 10mg/kg i.v. secukinumab vs placebo.	Reduction in CDAI by ≥50points at 6 wks.	No	[188]
	Rheumatoid arthritis	Monthly s.c. secukinumab at 25, 75, 150 or 300mg vs placebo.	ACR20 at wk 16	No	[189]
	Uveitis (non-infectious)	Secukinumab 300 mg s.c. or 10 mg/kg i.v. every 2 wks, 4 doses, or secukinumab 30 mg/kg i.v. every 4 wks, 2 doses vs placebo. Follow-up 57 days.	≥2 grade reduction in vitreous haze score or trace/ absent vitreous haze	Yes (i.v. compared with s.c.)	[190]
	Uveitis	SHEILD: Behçet's uveitis. s.c. loading phase then maintenance s.c. secukinumab 300 mg every 2 or 4 wks or placebo. INSURE: Active, noninfectious, non-Behçet's uveitis. Secukinumab 300 mg 2 or 4 wkly or 150 mg 4 wkly or placebo. ENDURE: quiescent, non-infectious, non-Behçet's uveitis. Secukinumab 300 mg 2 4 wkly 150 mg 4 wkly, or placebo.	Reduction of uveitis recurrence or vitreous haze score during withdrawal of concomitant immunosuppressive medication.	No for all 3 studies	[191]

Moderate-severe plaque psoriasis	s.c. placebo or secukinumab (1×25 mg, 3×25 mg, 3×75 mg or 3×150 mg) at wks 0, 4 and 8. After12 wk treatment period, patients entered a follow-up of 24 wks.	PASI75 at wk 12	Yes, 3 × 75 mg and 3 × 150 mg groups.	[192]
Moderate-severe plaque psoriasis	s.c. placebo or one of three secukinumab 150 mg induction regimens: single (wk 0), early (wks 0, 1, 2, 4) and monthly (wks 0, 4, 8). PASI 75 responders at wk 12 were re-randomized to either secukinumab 150 mg at wks 12 and 24) or a treatment-at-start-of-relapse regimen	PASI75 at wk 12	Yes for both induction regimens .	[193]
Active psoriatic arthritis	Phase II randomized controlled trial.. Two i.v. doses 10 mg/kg secukinumab or placebo 3 wks apart. Follow-up 24 wks.	ACR20 at wk 6	No.	[194]
Moderate-severe plaque psoriasis	ERASURE: s.c. secukinumab 300 mg or 150 mg wkly for 5 weeks, then 4 wkly vs placebo. FIXTURE: s.c. secukinumab 300 mg or 150 mg weekly for 5 weeks, then every 4 weeks vs etanercept 50 mg (twice weekly for 12 weeks, then once weekly). Follow-up 52 weeks.	PASI75 and the response of 0 or 1 on the modified investigator's global assessment at wk 12.	Yes, both doses in both trials	[152]

	Moderate-severe psoriasis, rheumatoid arthritis and chronic non-infectious uveitis	Psoriasis: 3mg/kg i.v. AIN457 vs placebo. Follow-up 12 wks. RA: 10mg/kg AIN457 vs placebo, wks 0, 3 followed for 16 wks. Uveitis: open label, 2 infusions AIN457 (10mg/kg) at wks, 0, 3 compared to patients in an open-label trial of infliximab.	Psoriasis: PASI75/PGA wk 4. RA: ACR20 wk 6. Uveitis: drug safety and tolerability.	Yes (psoriasis and RA). No (uveitis)	[195]
	Trials underway (see clinicaltrials.gov): Psoriasis with inadequate response to anti-TNF Ab, ankylosing spondylitis, rheumatoid arthritis, rheumatoid arthritis with inadequate response to anti-TNF, psoriatic arthritis, scalp psoriasis, uncontrolled asthma. NCT01961609, NCT02159053, NCT01640938, NCT01377012, NCT0177037, NCT01901900, NCT01989468, NCT02267135, NCT02294227, NCT01478360				
Brodalumab (AMG 827) Amgen Humanized IgG2 anti IL-17RA antibody	Moderate-severe plaque psoriasis	70, 140, 210mg at day 1 and wks 1,2,4,6,10 or 280mg monthly of brodalumab vs placebo. Follow-up 12 wks	PASI75 by wk 12	Yes	[196]
	Psoriatic arthritis	140 or 280mg brodalumab or placebo day 1, wks 1, 2, 4, 6, 8, 10. At wk 12, offered open label brodalumab. Follow-up 52 wks.	ACR20 at wk 12	Yes	[197]
	Moderate-severe asthma	140, 210 or 280mg brodalumab or placebo s.c. on day 1, weeks 1, 2, 4, 6, 8, 10. Follow-up 12 wks.	ACQ total score change (reduction ≥0.5) at wk 12	No	[198]
	Trials underway (see clinicaltrials.gov): asthma, psoriatic arthritis. NCT01902290, NCT02029495, NCT02024646				

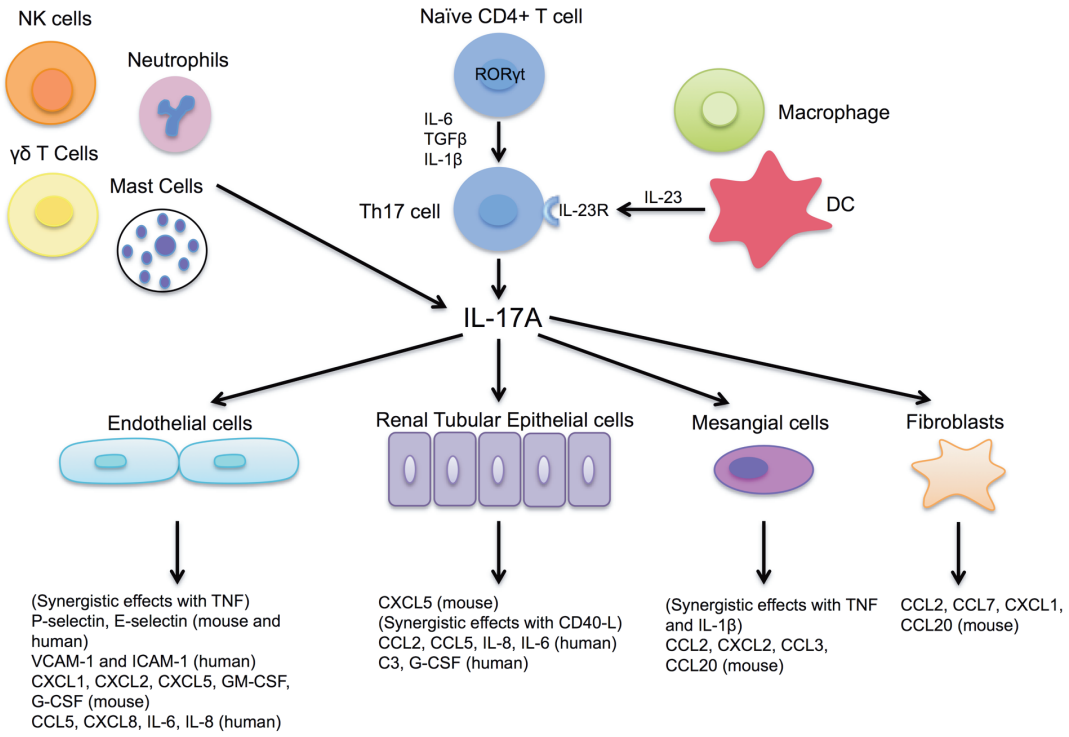
Abbreviations: ACQ, asthma control questionnaire; ACR20, American College of Rheumatology 20% response; CDAI, Crohn's disease activity index, DAS28, disease activity score 28; i.v., intravenous; MTX, methotrexate; PASI75, at least 75%

improvement in Psoriasis Area and Severity Index; PASI90, at least 90% improvement in Psoriasis Area and Severity Index; PASI100, 100% improvement in Psoriasis Area and Severity PGA, physician's global assessment; RA, Rheumatoid Arthritis; s.c, subcutaneous; TNF, tumor necrosis factor.

Figures

Figure 1

Cellular sources of IL-17A and its effects on intrinsic renal cells.



Abbreviations: CCL, chemokine (c-c motif) ligand; CXCL, chemokine (c-x-c motif) ligand; C3, complement factor 3; DC, dendritic cell; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; IL-23R, IL-23 receptor; NK, Natural Killer; Th17, T helper 17; TGF, transforming growth factor; VCAM-1, vascular cell adhesion molecule 1.

Chapter 2: Introduction to regulatory T cells and regulatory T cells in immune mediated renal disease.

Declaration

PART B: Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Literature review, writing and revising the manuscript	80

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Stephen Holdsworth	Review of manuscript	
A. Richard Kitching	Writing and review of manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	Date 18/3/15
Main Supervisor's Signature	[redacted]	Date 18/3/15

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Introduction to regulatory T cells

Regulatory T cells and pathway of differentiation

In 1995, Sakaguchi et al. identified that CD4+ T cells expressing the IL-2 receptor α chain (IL-2Rα; known as CD25) were immunosuppressive; transfer of CD4+CD25+ depleted T cells into mice conferred the spontaneous development of widespread autoimmune disease, including glomerulonephritis (Sakaguchi et al. 1995), which have since been called regulatory T cells (Tregs). CD4+CD25+ cells account for ~10% of all naïve CD4+ T cells (Thornton et al. 1998). Tregs can be generated and matured in the thymus (thymic Tregs, tTregs or natural Tregs, nTregs) or induced from naïve CD4+ T cells in the periphery (adaptive or peripheral Tregs, pTregs) (Abbas et al. 2013). nTregs constitutively express high levels of CD25 and Forkhead box protein 3 (Foxp3), a nuclear transcription factor which is the 'master regulator' defining Tregs in mice (Sakaguchi et al. 1996, Lahl et al. 2007). Tregs have T cell receptors (TCRs) that may be specific for self-antigens (Romagnoli et al. 2002); *Aire* (autoimmune regulator) gene expression in thymic medullary epithelial cells permits the presentation of tissue-restricted peptides to CD4+CD8+ (double positive) T cells, enabling the development of central tolerance to self-antigens (Mathis et al. 2009, Lee et al. 2011). pTregs are generated from naïve CD4+ T cells in response to antigen presented via major histocompatibility complex class II (MHCII) to their TCR, co-stimulation by CD28 and in the presence of IL-2 and TGFβ during infection or tissue injury (Povoleri et al. 2013). Natural and peripheral Tregs differ in the complementarity-determining region 3 (CDR3) amino acid sequences within their TCRs, without overlap between the antigens recognised by these TCRs, therefore both types of Tregs are believed to be necessary for the control of autoimmunity (Haribhai et al. 2011).

Tregs are relatively anergic cells (Thornton and Shevach 1998, Fontenot et al. 2005). Tregs do not express IL-2 upon stimulation, therefore they are dependent on IL-2 from other sources for proliferation, such as Teff (Williams et al. 2007). IL-2 acts on CD25, with downstream signalling permitting interaction of the IL-2 receptor with the adaptor protein signal transducer activator of transcription (STAT) 5, leading to further upregulation of CD25 and expression of Foxp3 (Lan et al. 2008).

Transforming growth factor β (TGFβ) is also required for the induction and maintenance of Tregs in the periphery (Chen et al. 2003, Fantini et al. 2004, Marie et al. 2005, Li et al. 2006, Luo et al. 2007), potentially through inducing the transcription factors NFAT and Smad3, which bind to the Foxp3 enhancer element of the Foxp3 gene, promoting Foxp3 expression (Tone et al. 2008). Endogenously, dendritic cells (DCs), particularly within gut associated mesenteric lymph nodes, produce TGFβ, inducing Foxp3 expression in naive CD4+ T cells (Coombes et al. 2007, Yamazaki et al. 2007, Yamazaki et al. 2013). TGFβ can also induce Foxp3 expression in Teff, converting them to another type of 'induced' regulatory T cell (Chen, Jin et al. 2003, Marie, Letterio et al. 2005).

Foxp3

Foxp3 contains a forkhead domain at its C-terminus, which permits its translocation to the nucleus, where it forms homodimers (dependent on a leucine zipper domain) and binds to gene promoter regions on DNA, repressing gene transcription (Schubert et al. 2001, Lopes et al. 2006). Foxp3 expression is mainly restricted to CD4+ T cells (Fontenot et al. 2005). Although some Foxp3 expressing CD4+ T cells do not express CD25, it is Foxp3 expression that confers the Treg immunosuppressive phenotype (Fontenot, Rasmussen et al. 2005). Continued Foxp3 expression by mature Tregs is required for their ongoing suppressive phenotype. Loss of Foxp3 expression results in loss of expression of CD25, GITR and CTLA-4, impaired ability to suppress the proliferation of CD4+CD25- T cells in co-culture, and acquisition of the ability to produce cytokines characteristic of Teff (including IFNγ, TNF and IL-17A) (Wan et al. 2007, Williams and Rudensky 2007, Komatsu et al. 2009, Rudensky 2011). Mutations in the Foxp3 gene in both humans and mice results in lymphoproliferation and autoimmunity; mice develop the *scurfy* mouse phenotype characterised by multi-organ autoimmune disease, splenomegaly, lymphadenopathy, insulitis and severe skin inflammation, which is lethal a few weeks after birth, while humans develop IPEX syndrome, manifesting as immune dysregulation, polyendocrinopthy, enteropathy, which is X-linked, with polyclonal CD4+CD8- T lymphocyte infiltration of affected organs (Blair et al. 1994, Bennett et al. 2001, Brunkow et al. 2001, Wildin et al. 2001, Lahl, Loddenkemper et al. 2007, Ramsdell et al. 2014).

Human Tregs

In mice, Tregs are characterised by the expression of the Foxp3 and CD25. In humans, Foxp3 expression can be transient, reflecting recent T cell activation rather than a suppressive phenotype (Gavin et al. 2006). There is also less discrete CD25 expression by human T cells; CD4+CD25^{high} and the CD25$^{low\text{-}intermediate}$ populations account for ~1-2% and 16% of CD4+ cells, respectively, with the remainder being CD4+CD25- effector T cells (Teff) (Baecher-Allan et al. 2001). Human Tregs are presently defined based on the expression of surface markers, including CD25^{high}, CD127-, CD39+ and CTLA-4, GITR and HLA-DR; the absence of a single marker denoting a human Treg is a challenge in the study of their role in human disease.

Mechanisms of Treg-mediated suppression

Human and murine Tregs are able to suppress the proliferation and attenuate the function many cell types, including CD4+ and CD8+ T cells, B cells, natural killer (NK) and NK T cells, DCs and monocytes/macrophages (Shevach et al. 2001, Azuma et al. 2003, Fallarino et al. 2003, Fontenot et al. 2005, Lim et al. 2005, Taams et al. 2005, Ralainirina et al. 2007). The suppressive effect of human and murine Tregs is mediated by a number of mechanisms. Direct cell contact between the Treg and cell being regulated is required, and Tregs must be activated via their TCR (Thornton and Shevach 1998, Dieckmann et al. 2001, Jonuleit et al. 2002). It has been suggested that Tregs may act as an 'IL-2 sink', removing IL-2 from the microenvironment of Teff, thereby restricting Teff proliferation and survival (de la Rosa et al. 2004, Fontenot, Rasmussen et al. 2005). Another suppressive mechanism is the production of inhibitory cytokines, including IL-10, TGFβ and IL-35 (Dieckmann, Plottner et al. 2001, Levings et al. 2002, Collison et al. 2007, Collison et al. 2009, Shalev et al. 2011); some studies suggest these cytokines may be dispensable for Treg suppressive function, however these findings may be influenced by the type of Treg in question, ie, nTreg vs pTreg (Shalev, Schmelzle et al. 2011).

Tregs express a number of suppressive molecules on their cell surface, thereby down-regulating proliferation in responder cells. These include CTLA-4, GITR, OX-40

and membrane bound TGFβ (Takahashi et al. 2000, Chen et al. 2003, Yong et al. 2007). Tregs can alter metabolic pathways in target cells, through expression of CD39 and CD73 on the Treg cell surface, catalysing extracellular ATP to adenosine, thereby inhibiting signals promoting inflammasome-mediated inflammation, and by transferring cyclic AMP to responder cells (Salcido-Ochoa et al. 2010, Shalev, Schmelzle et al. 2011). Tregs can also have cytotoxic effects on effector cells, through the release of the cytolytic enzymes granzyme B and perforin (Grossman et al. 2004, Gondek et al. 2008, Shalev, Schmelzle et al. 2011). Tregs utilise similar chemokine receptors to Teff to enable them to migrate to secondary lymphoid organs (including CCR7, CD62L and CXCR5) and into tissues (Forster et al. 2008, Mailloux et al. 2010, Turner et al. 2010).

Regulatory T cells in immune mediated renal disease

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Short Running Title: Regulatory T cells in Renal Disease

Abstract

Regulatory T cells (Tregs) are CD4+ T cells which can suppress immune responses by effector T cells, B cells and innate immune cells. This review discusses the role that Tregs play in murine models of immune mediated renal diseases and acute kidney injury and in human autoimmune kidney disease (such as systemic lupus erythematosus, anti-glomerular basement membrane disease, anti-neutrophil cytoplasmic antibody associated vasculitis). Current research suggests that Tregs may be reduced in number and/or have impaired regulatory function in these diseases. Tregs possess several mechanisms by which they can limit renal and systemic inflammatory immune responses. Although Treg therapy may be a way of treating immune renal disease in the future, the best way to utilise Tregs as a cellular therapy in humans is as yet not clearly established.

Key Words

Acute Kidney Injury

Anti-Cytoplasmic Neutrophil Antibody-Associated vasculitis

Anti-Glomerular Basement Membrane Disease

Lupus erythematosus, Systemic

Regulatory T cell

Introduction

Regulatory T cells (Tregs) are CD4+ T helper (Th) cells that function to suppress inflammatory responses (see review article Wang, Nephrology 2015). This review will summarise what is understood about the role of Tregs in immune-mediated renal diseases, including anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV), anti-glomerular basement membrane (anti-GBM) disease and systemic lupus erythematosus (SLE), proteinuric renal diseases and acute kidney injury (AKI) induced by ischaemia reperfusion injury (IRI) or cisplatin. Accurately defining the role of immune cells in humans with these diseases is challenging for a number of reasons. Patients have heterogeneity not only in the presence or absence or organ involvement but also the pattern of renal injury detected in some diseases (eg in SLE). Most studies are based on assessment of peripheral blood mononuclear cells (PBMCs), which may not accurately reflect the immune cell infiltrates found within the kidney, which themselves vary with disease activity, severity and progression and be modified by treatment with immunomodulatory therapies. Furthermore, defining Tregs in humans is not entirely straightforward, as the surface marker CD25 and the nuclear transcription factor Foxp3 (which can only be assessed by fixation and permeabilisation of cells) which define Tregs in mice, can be upregulated on activated human T effector cells (Teff).[1] This combination of potential lack of specificity and technical challenges means other surface markers, such as $CD127^{negtive/low}$, are employed to identify Tregs;[2] variability in the definition of a human Treg may restrict study comparability. Although many advances in understanding have come from animal models of immune disease, animal models are imperfect. Disease is often induced by immunisation with self or foreign antigens or by exposure to drugs (rather than evolving spontaneously), models may lack some of the pathological features of human diseases, and do not tend to spontaneously remit or relapse. Tregs can modify immune renal disease by acting within lymphoid organs, where primary antigen presentation occurs and immune responses are established, or at sites of inflammation within tissues, ie, the kidney (Fig. 1). Both sites of regulation are relevant in kidney diseases and need to be considered when studying the phenotype and roles of Tregs in renal disease.

Regulatory T cells in Anti-Glomerular Basement Membrane (GBM) Disease

Humans

Although anti-GBM disease is uncommon, several studies have assessed the role of central and peripheral tolerance in this disease. The auto-antigen in this disease, the non-collagenous domain of the α3 chain of Type IV collagen (α3(IV)NC1), is expressed in the human thymus, suggesting not only that central tolerance is important in maintaining tolerance to this auto-antigen, but also that α3(IV)NC1-specific Tregs might be present.[3] Patients with acute anti-GBM disease have a higher ratio of antigen-specific Teff:Treg cells in the peripheral blood than convalescent patients, with increased IFNγ production by these Teff when CD25+ cells were depleted from cultures *ex vivo*, suggesting regulatory properties in the CD25+ population.[4] PBMCs from patients with acute anti-GBM disease proliferate and produce IFNγ in response to α3(IV)NC1 peptide stimulation, whereas PBMCs from patients in remission do not proliferate, but secrete IL-10,[5] indicating the emergence of a regulatory population that is likely to be important in the restoration of tolerance in this disease.

Mice

Most studies involving regulatory T cells in mice have used models of "anti-GBM disease" (also known as nephrotoxic serum nephritis or Masugi nephritis). These models are induced by adaptive immunity to an exogenous antigen (a foreign globulin) planted in glomeruli.[6] In this regard, there is no autoimmunity, meaning that Treg behaviour is driven by the immune response to a foreign antigen. Kidney injury, while having a number of the features of anti-GBM disease, is not directed against Type IV collagen in the GBM. Nonetheless, studies in these models have been informative in the roles of Tregs in severe, rapidly progressive glomerulonephritis (GN). Both endogenous and exogenous Tregs suppress local and systemic inflammatory responses. Endogenous Tregs accumulate within nephritic kidneys in these disease models, particularly within periglomerular and tubulointerstitial regions [7, 8]. Depletion of endogenous Tregs after disease induction increased glomerular crescents, segmental necrosis and proteinuria, with increased pro-inflammatory cytokine production by splenocytes.[7] Endogenous Treg depletion before induction of nephritis resulted in more glomerular crescents, increased renal leukocyte infiltration

and enhanced Th1 responses, changes that were IFNγ dependent.[8] Adoptive transfer of Tregs to mice (during the sensitisation phase of the "accelerated" anti-GBM disease model) protected recipients from GN, reduced macrophage, CD4+ and CD8+ T cell accumulation within glomeruli, without altering humoral immune responses; transferred Tregs were identified within secondary lymphoid organs, but not the kidney.[9] Tregs induced in the context of immunisation with sheep globulin (ie, activated Tregs including antigen-specific Tregs) produced more IL-10 and were more suppressive of Teff proliferation in *in vitro* co-cultures than Tregs from naïve mice,[7] suggesting that antigen specificity and/or the activation status of Tregs enhances their regulatory function.

Mediators of Treg protection have been studied in this model. They include CCR7 (a chemokine receptor permitting CD4+ T cells and dendritic cells [DCs] to home to the T cell zones in lymph nodes),[10] CCR6 (which promotes localisation of Tregs to kidneys)[11] and IL-10. Along with their capacity to suppress IFNγ production by Teff in *in vitro* co-cultures, Tregs produce IL-10.[8, 12] IL-10 deficient mice developed more severe GN in anti-GBM models, with increased systemic and local pro-inflammatory cytokine production.[12, 13] Although cells other than Tregs produce IL-10, specific deletion of IL-10 from Tregs enhanced glomerular injury and systemic immune responses.[12] Recent work by Kluger et al. has shown that Treg expression of transcription factors utilised by specific Th subsets are necessary for Tregs to control the local and systemic pro-inflammatory responses induced by these Th lineages.[14] Signal transducer and activator 3 (STAT3)-expressing Th17 cells are pathogenic in this group's model of GN.[15, 16] When anti-GBM GN was induced in mice with Foxp3+ cell specific STAT3 deletion, renal injury was enhanced, renal Treg recruitment was impaired (through loss of CCR6 expression) and greater renal Th17 cell recruitment was observed, with heightened systemic Th17 responses.[14]

Regulatory T cells in ANCA-Associated Vasculitis (AAV)

Humans

While there are conflicting reports as to whether circulating Tregs are increased or reduced in patients with AAV, there is convincing evidence that Foxp3+ Tregs are functionally deficient, though the published literature suggests a complex scenario

exists.[17-21] Rimbert et al. assessed CD4+CD25+CD127$^{low/-}$ T cells in PBMCs between untreated AAV patients and healthy controls (HC), identifying decreased Treg number in AAV patients, but a similar proportion of Tregs among CD4+ T cells, as the patients were lymphopaenic.[17] Other studies have found an increased proportion of Tregs (CD4+CD25^{high} or CD4+CD25^{high}Foxp3+) in acute AAV patients compared to HC or AAV patients in remission,[18, 20] or an increased proportion of CD4+CD25^{high} Tregs, but reduced CD4+CD25^{high}Foxp3+ proportion.[19, 21] Foxp3 expression amongst PBMCs from AAV patients in remission was reduced compared to HC, but Tregs from both groups were equally suppressive, suggesting a deficiency in Treg number in the AAV group.[22]

Despite the inconclusive data on peripheral Treg numbers in AAV, there is evidence that Tregs from acute or convalescent AAV patients are functionally impaired, with diminished ability to suppress Teff proliferation (using Teff from AAV patients or HC) in *in vitro* suppression assays;[17-21] the mechanism for this functional defect is not clearly defined, but does not appear to be due to reduced Treg Foxp3 or CD39 expression,[17] or due to enhanced Treg apoptosis.[20] Free et al. have shown a greater proportion of Tregs from AAV patients have a mutation in exon 2 of the FOXP3 gene compared to HC, which may contribute to their impaired function.[18] CD4+CD25^{high} Tregs from patients had reduced expression of CCR4 compared to HC Tregs, suggesting impaired Treg trafficking to peripheral tissues or to sites of Teff proliferation.[19] T cells from myeloperoxidase (MPO)+ AAV patients with acute disease produced more IFNy after MPO stimulation than those from MPO+ AAV patients in remission, but Treg depletion did not augment proliferative responses, suggesting ineffective Tregs or an escape from regulation by Teff cells.[22] Tregs from AAV patients may themselves possess an effector, rather than regulatory, phenotype; proliferative responses in HC and ANCA- AAV patient PBMCs were enhanced after depletion of CD25^{high} cells, whereas PBMC proliferation was reduced when CD25^{high} cells were depleted from ANCA+ AAV patients.[21] In addition to variant Tregs, certain Teffs (CD4+CD127^{high}CD25^{int} cells) in AAV patients are more resistant to suppression by Tregs,[18] and AAV patients in remission demonstrate an expanded Teff memory compared to naïve CD4+ T cell population, suggesting ongoing antigenic stimulation.[23] This suggests that both altered Treg function and Teff resistance to suppression contributes to disease in AAV.

Tregs are present in kidneys of patients with AAV, though the functional relevance of this to histological outcomes or disease activity is currently unclear. When comparing Foxp3:T cell ratios in the kidney, no difference in tubulointerstitial damage score or glomerulosclerosis was identified, but higher Foxp3:CD3 ratios within the tubulointerstitium, but not in glomeruli, were present in dialysis free AAV patients compared to AAV patients requiring dialysis.[24]

Mice

Studies of Tregs in AAV using murine models are somewhat constrained by the lack of a model that spontaneously develops disease comparable to humans, and in the case of proteinase 3 (PR3), by the lack of homology between mouse and human PR3. However, there is some evidence that Tregs participate in the maintenance of tolerance to MPO, conferring protection against GN. The autoimmune regulator (AIRE) transcription factor is responsible for expression of tissue-restricted antigens in the thymus, and functions not only to facilitate central tolerance but also to assist in development of antigen-specific thymic Tregs.[25-27] MPO is highly Aire regulated in the murine thymus and Aire deficient mice have enhanced autoimmunity to MPO after immunisation.[28] One murine model of AAV generates autoimmunity to MPO by sensitising mice to MPO (permitting the development of MPO-specific CD4+ Th cells) before planting MPO in glomerular vessels, inducing GN.[29, 30] Treg depletion prior to sensitisation with MPO exacerbated nephritis with more neutrophil, macrophage and CD4+T cell infiltrates in glomeruli, and enhanced anti-MPO autoimmunity.[28] Female SCG/Kj mice, derived from lupus prone mouse strains, spontaneously develop serum perinuclear ANCA and crescentic GN (with mesangial immune deposits).[31] Recent experiments in this strain showed that eicosapentaenoic acid treatment limited disease, coincident with more Foxp3+ T cells within lymph nodes and upregulated intrarenal expression of Foxp3 and CTLA-4, implicating Tregs in its protective effects.[32]

Regulatory T cells in Systemic Lupus Erythematosus (SLE)

Humans

A number of lines of evidence implicate abnormal Treg number and function in SLE. Peripheral blood Treg frequencies are reduced in people with SLE,[33-35] with reduced Foxp3 expression and impaired suppressive activity *ex vivo* by CD4+CD25^{high} cells in active disease.[36] Treg proportions negatively correlate with circulating Th17 cells and disease activity.[35] Specific polymorphisms in CTLA-4 and TGFβ genes are associated with reduced frequency of CD4+CD25+CD45RO+ T cells in patients.[37] CD4+CD25^{bright} Tregs from SLE patients express high levels of Fas (CD95) and stimulated Tregs were more susceptible to Fas-dependent apoptosis, suggesting that enhanced Treg apoptosis may be a mechanism for Treg dysfunction in SLE.[38] While the majority of reports support abnormal Treg function in SLE, Yates et al found that Tregs (CD4+CD25+), co-cultured with anti-CD3/CD28 or nucleosome-stimulated effector T cells had intact suppressive function.[39] There are reports that following therapy for SLE, an increase in the proportion of Tregs occurs.[35]

Urinary Foxp3 mRNA levels are elevated in patients with active lupus nephritis (LN) compared to those with quiescent LN or HC, and correlated with global and renal disease activity, proteinuria, anti-ds DNA titres and acute histological activity index on renal biopsy.[40] The elevated urinary Foxp3 mRNA levels in active LN could reflect increased inflammation in active renal disease, defective suppression of renal inflammation by Tregs or conceivably that cells other than Tregs are expressing Foxp3 (ie, activated CD4+ T cells).

In vitro co-cultures using patients' Tregs and B cells found that Tregs could reduce IgG production and enhance B cell apoptosis, suggesting SLE patients may have hyperactive B cells, rather than a defect in Treg function.[41] Administration of the B cell depleting therapy, Rituximab (an anti-CD20 monoclonal antibody), increased expression of Foxp3, CD25, GITR, CTLA-4 and TGFβ1 mRNA in PBMCs of patients with SLE one month later, although the mechanism underpinning these changes is unclear.[42] Rituximab was also associated with a sustained reduction in expression of the co-stimulatory molecule CD40L (CD154) on CD4+ T cells.[43] This suggests that expression of Treg related transcription factors, surface markers and cytokines can

be induced with Rituximab and may be relevant to responses to therapy. Another method of promoting Treg proliferation and survival is the administration of low dose IL-2, currently being explored in a clinical trial (https://clinicaltrials.gov/ct2/show/NCT02084238).

Mice

There are a number of lines of evidence both implicating T cells in the pathogenesis of SLE and suggesting that manipulating Tregs might be of therapeutic value. The most studied murine models of SLE use genetically predisposed mice, including the NZBxNZW F1 (NZB/NZW), MRL/lpr and MRL/Mp strains, which spontaneously develop autoantibodies, proteinuria, haematuria and GN with age.[44] As these mice age, Tregs increase in frequency and number in lymphoid organs, despite autoimmunity developing.[45, 46]

Transfer of *ex vivo* expanded Tregs from NZB/NZW mice into recipients without clinical disease delayed the development of overt renal disease and prolonged survival,[47] with similar protective findings in a regimen that included glucocorticoids and cyclophosphamide.[48] While Tregs in MRL/Mp mice have a regulatory phenotype, Teff may be more resistant to suppression by Tregs from either MRL/Mp or control mice.[49] Tregs also regulate B cells in murine lupus; T cell depletion in NZB/NZW mice, followed by adoptive transfer of Tregs limited humoral autoimmunity, by induction of B cell apoptosis and anergy, mediated by Treg release of perforin and granzyme, expression of CTLA-4 and prevention of CD86 upregulation on B cells.[41, 50]

Manipulating immune responses to promote tolerance, through the administration of a peptide (from the V_H region of mouse anti-DNA antibodies) in a tolerogenic manner to NZB/NZW mice, improved survival and delayed the onset of nephritis.[51, 52] This was associated with reduced cellular and humoral autoimmunity and increased proportions of antigen-specific Tregs whose effects on B cells were dependent on cell-cell contact and the suppressive cell surface markers TGFβ-latency associated protein and GITR.[52-54] This suggests that tolerance can be induced in lupus-prone mice, mediated by enhanced regulatory function of Tregs. The mechanisms involved

in loss of tolerance, and endogenous mechanisms to restore tolerance in SLE, are yet to be elucidated.

Regulatory T cells in Acute Kidney Injury (AKI)

At present, data about Tregs in human AKI is limited. However murine studies have shown a protective role for Tregs in AKI (Fig. 2) and suggest that they have therapeutic potential in human AKI. CD4+ T cells promote injury in ischaemia reperfusion injury (IRI).[55] However, transfer of kidney mononuclear cells from mice 24 hours after IRI into T cell deficient mice with IRI protected the recipients from tubular injury, suggesting a protective effect of one or more leukocyte subsets.[56] Following IRI, the proportion of both renal Teffs and Tregs (CD4+CD25+Foxp3+) are increased.[57] The use of commonly used immunosuppressive agents to deplete or restrict T cell proliferation in transplantation (such as anti-thymocyte globulin or mycophenolate mofetil) do not protect against experimental IRI, as they reduce Treg accumulation in the kidney along with Teffs.[58, 59]

Tregs confer protection during the 'ischaemia phase' of IRI, suggesting they regulate innate immune responses to tissue injury. Depleting Tregs prior to IRI increased renal functional and histological injury,[60, 61] with increased neutrophil and macrophage accumulation,[62] while transfer of Tregs into immunodeficient mice before IRI limited injury.[62] Tregs also limit injury in the 'healing phase' of IRI by supressing pro-inflammatory responses from innate and adaptive immune cells. Depleting Tregs 24hrs post IRI exacerbated tubular damage, reduced tubular proliferation at day 10, and increased renal inflammatory cell infiltrates, whereas transfer of Tregs into mice after IRI ameliorated tubular injury, improved tubular regeneration, and reduced the accumulation of proinflammatory T cells and DCs.[57]

Tregs suppress injury in IRI through multiple mechanisms including the production of IL-10,[62] signalling through surface receptors, including GITR,[60] CTLA-4[63] and programmed death (PD)-1[64], and Treg homing to the inflamed kidney in response to upregulated chemokine expression, including CXCL9, CCL5, CXCL10.[63] New methods that exploit the suppressive capacity of Tregs are being explored as therapy

for IRI. Sphingosine-1-phosphate receptor agonists and sphingosine kinase inhibitors protect against IRI by increasing Treg proportions in the spleen and kidney.[63, 65] Transfer of bone marrow derived murine mesenchymal stem cells into mice shortly after IRI improved injury and reduced renal IFNγ producing CD4+ and CD8+ T cells by inducing splenic Tregs that could traffic to the kidney.[66] Administration of JES6-1, a monoclonal antibody to the IL-2Rβ chain (preventing IL-2 binding to the IL-2Rβ chain, but not the IL-2Rα chain, ie CD25), along with IL-2 (creating an IL-2/anti-IL-2 complex), prior to IRI increased Tregs within the spleen and kidney post-IRI, protecting against early renal injury, neutrophil and macrophage infiltration, independently of TGFβ or IL-10 production.[67]

AKI can be induced by administering the chemotherapeutic drug, cisplatin, to mice, with injury mediated by CD4+ T cells.[68] Early studies showed that exogenous IL-10 protected against cisplatin and IRI induced AKI.[69] Transfer of Tregs prior to cisplatin administration improved mouse survival and reduced renal injury, while depletion of endogenous Tregs prior to cisplatin worsened renal injury.[70] When naïve CD4+CD25+ (Tregs) or CD4+CD25- (Teff) were transferred into T cell deficient mice before cisplatin injection, mice receiving Tregs had improved survival, with protection against renal injury, reduced renal macrophage recruitment and pro-inflammatory cytokine expression, suggesting the Tregs suppress pro-inflammatory responses by innate immune cells.[70] Bee venom increases splenic Treg populations, and administration to mice before cisplatin conferred protection from AKI with reduced intrarenal inflammation, dependent on Tregs.[71]

Regulatory T cells in Proteinuric Renal Diseases

Humans

Defects in Treg number and function may be important in minimal change disease. Children with active steroid sensitive nephrotic syndrome had fewer peripheral blood Tregs than those in remission.[72] Another study including adults and children with minimal change disease showed a similar frequency of Tregs (CD4+CD25+) compared to HC, but Tregs from patients with active disease were less effective in supressing Teff compared to Tregs from HC or patients in remission, potentially via impaired IL-10 production.[73]

Rodents

Tregs have been studied in models of proteinuric renal diseases, where renal injury is induced by adriamycin or doxorubicin. These models have two phases, an acute phase and a more chronic stage, where adriamycin nephropathy (AN) produces injury similar to focal segmental glomerulosclerosis (FSGS). Tregs play a protective role in AN. Transfer of CD4+CD25+ Tregs or Foxp3-transduced CD4+ T cells protected mice against functional and histological renal damage, with reduced monocyte and macrophage infiltration.[74, 75] Tregs suppressed macrophage, TNF and IL-12 production in co-culture, via TGFβ.[74] Tregs probably recognise renal antigens in AN, as transfer of Tregs with a restricted TCR failed to affect renal injury.[76] In addition to Foxp3-transduced cells, other manoeuvres to improve Treg number or function have reduced renal injury, including transferring Tregs that overexpress CD39[77] and expanding Tregs *in vivo* by administering IL-2/anti-IL-2 complexes, a strategy that was protective even after the onset of renal injury.[78]

Another model of FSGS is the Buffalo/Mna rat model, which spontaneously develops nephrotic syndrome at 3 months, with FSGS by 4 months of age. A deoxyspergualin derivative (LF15-0195) could prevent and treat the development of proteinuria in this model.[79] LF15-0195 increased the proportion of CD4+CD25+ T cells in blood and spleen of treated rats (without changing the overall frequency of T, B or NK cells), with adoptive transfer of CD4+CD25+ T cells from LF15-0195 treated rats, but not from control rats, reducing proteinuria in recipients after transfer.[79]

Conclusion

Tregs play an important to role in modulating innate and adaptive immune responses in a number of models of renal disease. There is evolving evidence that they are important in human renal diseases, both for maintaining tolerance to auto-antigens and in the regulation and repair of established renal injury. However, there are still many unanswered questions as to how Tregs suppress immune responses in autoimmune renal diseases. The issue of how best to harness their therapeutic potential for the treatment of disease is also unclear, but may include utilising *in vitro* expanded Tregs as a cellular therapy or developing agents that enhance the suppressive mechanisms utilised by Tregs.

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Conflict of Interest

The authors declare no conflict of interest.

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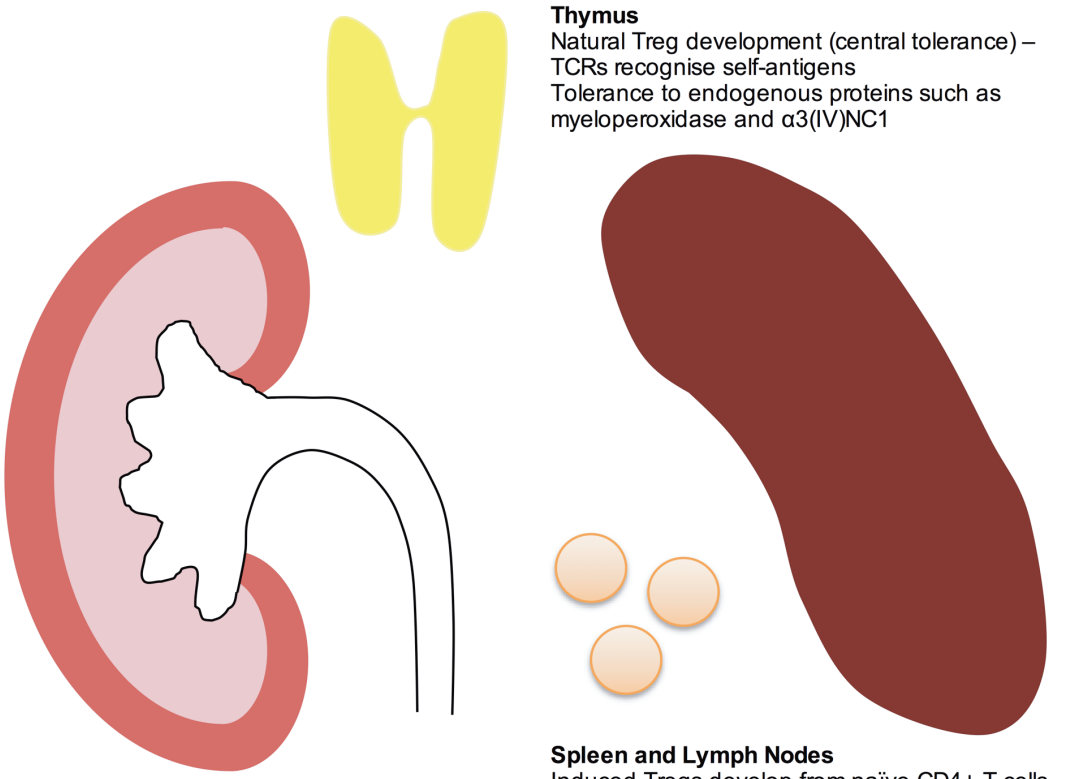
Figures

Figure 1

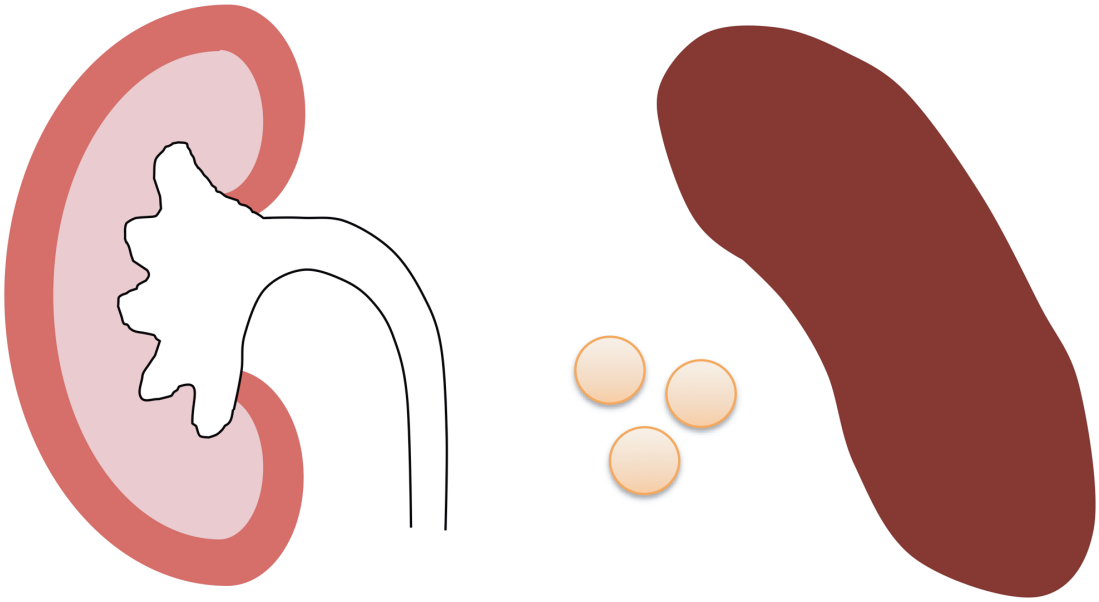
Location of Treg development and action in models of autoimmune renal diseases.



Abbreviations: α3(IV)NC1, non-collagenous domain of the α3 chain of Type IV collagen; IgG, immunoglobulin G; Teff, T effector cell; TCR, T cell receptor; Th, T helper cell; Treg, regulatory T cell.

Figure 2

Location of Treg action in models of acute kidney injury.



Kidney
Tregs home to kidney in response to chemokine (CXCL9, CCL5, CXCL10) expression
Regulate innate immune cell:
•Recruitment (neutrophils, macrophages, CD4+ and CD8+ T cells [acting in an innate capacity])
•Cytokine production – TNF, IL-1β

Spleen and Lymph Nodes
Modulate Teff by mechanisms including:
•Suppressive cytokine production – IL-10
•Suppressive surface markers on Tregs – GITR, PD-1, CTLA-4

Abbreviations: Teff, T effector cell; TCR, T cell receptor; Treg, regulatory T cell.

Chapter 3: Glomerulonephritis induced by heterologous anti-GBM globulin as a planted foreign antigen.

Declaration

Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Compiling and revising protocols Writing the manuscript with revisions	40

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Dragana Odobasic	Compiling and revising protocols, writing the manuscript with revisions	
Kim O'Sullivan	Revising protocols, review of manuscript	10
Stephen Holdsworth	Review of the manuscript	
A. Richard Kitching	Writing and revising the manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 18/3/15
Main Supervisor's Signature	[redacted]	**Date** 18/3/15

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Glomerulonephritis Induced by Heterologous Anti-GBM Globulin as a Planted Foreign Antigen

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ABSTRACT

The glomerulonephritides are diseases characterized by immune-mediated glomerular inflammation. Most severe and rapidly progressive forms of glomerulonephritis feature the participation of injurious leukocytes that localize to glomeruli. This unit describes classical models of rapidly progressive glomerulonephritis in mice, induced by injecting heterologous globulin (raised in sheep) that binds to the glomerular basement membrane. These models have been particularly useful in defining the participation of effector leukocytes in severe glomerular disease. In these models, injury typically occurs in two phases. In the initial, heterologous phase, injury is mediated by the globulin bound within the glomerulus acting as an antibody. The later, autologous phase of injury is mediated by the host's adaptive immunity to the heterologous globulin now functioning as a planted foreign antigen within glomeruli. As autologous phase injury is driven by immunity to sheep globulin, assessment of antigen-specific systemic immunity to sheep globulin is critical when using this model. *Curr. Protoc. Immunol.* 106:15.26.1-15.26.20. © 2014 by John Wiley & Sons, Inc.

Keywords: glomerulonephritis • cell-mediated immunity • planted antigen • T lymphocytes

INTRODUCTION

The kidney is vulnerable to a variety of types of immune-mediated injury. When this injury primarily involves the glomerulus, it is known as glomerulonephritis (GN). The glomerulonephritides are a collection of diseases, most of which are caused by dysregulated immunity, often autoimmunity. Both humoral and cellular effectors participate in different forms of GN.

Effector responses in proliferative and rapidly progressive forms of GN have been modeled for many years in rodents by injecting polyclonal antibodies targeting renal basement membranes (Masugi, 1934). These antibodies are raised in other species, particularly sheep and rabbits. After intravenous (i.v.) injection into naïve mice (see Basic Protocol 1), a small proportion of these heterologous antibodies bind in a linear fashion to the glomerular basement membrane (GBM) and, to a much lesser degree, the tubular basement membrane. They rapidly engage innate effectors and classically induce dose-dependent glomerular injury within 24 hr. This initial, "heterologous phase" injury, induced by the transferred antibody acting as an antibody, is characterized by transient leukocyte accumulation within glomeruli that results in proteinuria.

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The large dose of foreign antigen delivered systemically induces an adaptive immune response to the heterologous globulin (often raised in sheep). The antibody now acts as a foreign antigen. After several days, a second and classically more severe phase of injury occurs, mediated by adaptive (host) immunity to the heterologous globulin bound to the kidney (Unanue and Dixon, 1965). Both humoral and cellular effectors have the capacity to play a role in effecting glomerular injury. This model can be "accelerated" (see Basic Protocol 2) by sensitizing mice to normal sheep globulin (NSG; usually subcutaneously, s.c., in adjuvant) 4 to 10 days prior to challenging mice by injecting them with sheep anti-mouse GBM antibodies. This allows the immediate induction of an autologous phase response, as an adaptive T and B cell response to the injected heterologous globulin present on the GBM.

Several Support Protocols are included in this unit. Support Protocol 1 describes how to prepare a fraction of mouse kidney cortex, which is used to immunize sheep to generate anti-mouse GBM. Support Protocol 2 describes a method for absorbing and precipitating the globulin fraction of sheep serum containing antibodies against mouse basement membrane proteins. Support Protocol 3 describes how to determine the optimal doses of sheep anti-mouse GBM for induction of glomerulonephritis. Support Protocols 4 and 5 describe ways to assess intrarenal leukocyte accumulation. Finally, Support Protocol 6 provides steps used to detect mouse IgG or C3 deposition in glomeruli using immunofluorescence.

These models are particularly useful to study effector mechanisms and how they lead to renal injury. In the heterologous phase, they model in situ immune complex disease. In the autologous phase, they model rapidly progressive forms of GN induced by cellular (and in some cases humoral) effector responses. It is important to understand that in the autologous phase, the injurious adaptive immune response is to a foreign antigen and not a self-antigen (Unanue et al., 1965).

Nomenclature is a significant factor in these disease models that often leads to significant confusion. None of the names are ideal. These models can be known as experimental anti-GBM GN, nephrotoxic serum nephritis, or Masugi nephritis. Each name fails to clearly and accurately describe the model. Avoiding the term nephrotoxic serum nephritis and using terms like experimental anti-GBM GN, Masugi nephritis, or planted antigen nephritis with clarification of heterologous or autologous and accelerated or non-accelerated is recommended. Further, more detailed comments on nomenclature issues in these models can be found in the Commentary section.

As experiments involve working with animals, researchers should ensure that all experiments are approved by the appropriate institutional and/or national review boards.

BASIC PROTOCOL 1

INDUCTION OF NON-ACCELERATED AUTOLOGOUS PHASE ANTI-GBM GN

"Non-accelerated" anti-GBM GN is induced in mice by administration of anti-GBM globulin raised in other species (e.g., sheep, rabbit). As discussed above, the development of renal injury occurs in two distinct phases. The heterologous phase is induced by passive transfer of the heterologous antibodies and is immediate, but transient. The second, or autologous, phase is mediated by the adaptive immune response that develops against the planted antigen in glomeruli (sheep globulin acting as an antigen). Proliferative GN develops 6 to 7 days after anti-GBM globulin injection. More severe (necrotising and crescentic) GN starts to develop around day 10 and is established by day 21. With the globulin preparation used in C57BL/6 mice studies, severe glomerular injury in this phase of GN is mediated predominantly by delayed type hypersensitivity (DTH) effectors

($CD4^+$ T cells, macrophages, and fibrin), which are directed to the heterologous globulin as a planted foreign antigen in the glomerulus.

Materials

Sheep anti-mouse GBM globulin (see Support Protocols 1 and 2)
Male C57BL/6 mice, aged 6 to 12 weeks
Paraformaldehyde-periodate-lysine solution (PLP, see recipe)
20% sucrose solution, dissolved in PBS
Optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura)
10% formalin
70% ethanol solution in distilled water
Paraffin
Liquid nitrogen

30-, 26-, or 23-G needles
1-ml syringes
Heat lamp or heat pad
Mouse retaining cone (or similar apparatus to facilitate i.v. injection)
Metabolic cages
5-ml tubes
Sterile 1.5- and 0.5-ml microcentrifuge tubes (Eppendorf)
Cryomoulds (Tissue-Tek, Sakura)

Additional reagents and equipment for parenteral injections (see *UNIT 1.6*), blood collection (see *UNIT 1.7*), euthanasia of mice (see *UNIT 1.8*), and immunohistochemistry (see *UNIT 21.4*)

Inject mice

1. Inject anti-GBM globulin i.v., using a 26- or 30-G needle and 1-ml syringe, into mice via the tail vein. To determine the correct dose of anti-GBM globulin to be used, see Support Protocol 3. After anti-GBM globulin injection, monitor mice daily.

It is possible to inject anti-GBM globulin in two doses 2 to 4 hr apart, if required.

Assess disease

Assess heterologous phase disease

2a. Euthanize mice and collect kidneys 2 hr after anti-GBM globulin injection to assess neutrophil infiltration by immunohistochemistry (see Support Protocol 4), *or*

2b. Place mice in metabolic cages 15 to 30 min after anti-GBM globulin injection to collect urine in 5-ml tubes over the next 24 hr to measure proteinuria.

Constant access to water is required for mice while in metabolic cages, but food is not desirable due to potential for contamination of urine (the risk of contamination may depend on the design of the caging apparatus). If mice look unwell, they should not be placed in metabolic cages as their condition can deteriorate.

Assess autologous phase anti-GBM GN (7, 14, or 21 days)

2c. Place mice into metabolic cages to collect urine for measurement of proteinuria 24 hr before the end of experiments.

3c. Obtain blood (e.g., by cardiac puncture with a 26-G needle and 1-ml syringe) to measure circulating mouse anti-sheep IgG antibodies (by ELISA) as well as serum urea.

4c. Euthanize mice (see *UNIT 1.8*) and remove kidneys after 7 to 21 days for assessment of various parameters.

Renal perfusion may be beneficial in some situations, but is not routinely required.

5c. In this systemic process, both kidneys will be diseased in the same manner, so remove and halve both kidneys in the coronal plane to maximize the area of renal cortex that will be seen on renal histology sections. Then, perform the following on each of the four halves:

i. Fix one half of a kidney (along with a small piece of spleen as a positive control) in a 5-ml tube containing 3 to 4 ml PLP 4 hr at 4°C, then remove PLP and replace with 5 ml of 20% sucrose. Replace with fresh 20% sucrose solution three times every 2 to 3 hr on the following day before embedding the kidney in OCT. Freeze gently on dry ice and store at −80°C.
PLP fixation will be used for determination of renal leukocyte accumulation by immunohistochemistry (see Support Protocol 4).

ii. Fix one half in a 5-ml tube containing 3 to 5 ml 10% formalin for 24 hr at room temperature. Then, wash in 70% ethanol solution, process tissue for a 6-hr cycle in a standard tissue processor, and embed in paraffin for assessment of kidney histology, e.g., by periodic acid Schiff's (PAS) or hematoxylin/eosin (H&E) staining.

iii. Immediately freeze one half in a 1.5-ml microcentrifuge tube in liquid nitrogen and store at −80°C for measurement of renal inflammatory mediators (e.g., cytokines, chemokines) by real-time polyemerase chain reaction (see *UNIT 10.23*).

iv. Gently freeze (on dry ice) one half in a cryomold containing OCT and store at −80°C for assessment of glomerular deposition of mouse Ig and complement by immunofluorescence (see Support Protocol 5).

6c. Collect spleen aseptically, lyse red blood cells (RBC), and prepare a single cell suspension for assessment of systemic immunity to sheep globulin.

Lymph nodes can also be collected, but since in the non-accelerated model the antigen (sheep globulin) is injected i.v., the spleen is a relevant secondary lymphoid organ where the adaptive nephritogenic immune response is generated. The following are some of the more commonly assessed parameters: (1) T and B cell activation by expression of surface activation markers (flow cytometry, see Chapter 5), (2) T and B cell proliferation (see UNITS 3.10 & 3.12), (3) cytokine production (see Chapter 6), and (4) measuring the frequency of CD4+CD25+foxp3+ regulatory T cells by flow cytometry.

BASIC PROTOCOL 2

INDUCING ACCELERATED AUTOLOGOUS PHASE ANTI-GBM GN

In the accelerated anti-GBM model, prior sensitization of mice with NSG several days prior to anti-mouse GBM antibody administration permits the generation of antigen-specific T cells as well as mouse anti-sheep globulin antibodies. This is achieved through the s.c. administration of NSG in adjuvant 4 days prior to the induction of nephritis. Extending the period of sensitization to ≥7 days should be done cautiously, as this will increase the chance of a lethal anaphylactoid reaction after injection of sheep-anti-mouse GBM globulin. Following administration of anti-mouse GBM antibodies, glomerular autologous phase injury is cell mediated (part of the adaptive immune response) from the outset.

There are some advantages of an accelerated anti-GBM model compared to the non-accelerated model, including the rapid development of GN, a shorter duration, a reduced dose of anti-GBM antibody that can usually be used to induce nephritis, and the immediate onset of autologous phase of glomerular injury, which is mediated by T cell infiltration. As there can be significant variation among sheep anti-mouse GBM sera between batches and between the sheep, it is important that every batch be dose tested in this model to establish the correct dose of globulin required to induce significant GN without death.

Materials

5 mg/ml normal sheep globulin (NSG) in PBS
Male C57BL/6 mice (6 to 12 weeks old)
Complete Freund's adjuvant (CFA; *UNIT 2.4*)
Sheep anti-mouse GBM globulin (see Support Protocols 1 and 2)

Sonicator
1-ml syringes
30-, 26-, and 23-G needles

Additional reagents and equipment for production of polyclonal antibodies using complete Freund's adjuvant (see *UNIT 2.4*), and assessing disease (see Basic Protocol 1)

Sensitize mice to NSG

Sensitize mice 4 days prior to the induction of nephritis.

1. Combine 5 mg/ml NSG diluted in PBS with an equal volume of FCA and mix gently. Each mouse will require 200 μl.
2. Emulsify using a sonicator, keeping solution on ice. Pulse the solution intermittently, checking the consistency and viscosity every 10 pulses until a continuous colloidal suspension is formed.

   It is important that the resulting emulsion does not solidify, which prevents the emulsion from being pushed through a 26-G needle.

   Emulsification can also be performed by mixing the liquid by repeated transfer between two glass syringes (UNIT 2.4).

3. Keep the tube containing the emulsion on ice and sensitize mice immediately.
4. Gently shake the tube to ensure the emulsion is well mixed, then draw up the emulsion into a 1-ml syringe. Attach a 26-G needle to the syringe.

   This emulsion is moderately thick and can be difficult to draw up with a 26-G needle; therefore, draw up the emulsion with the 1-ml syringe before applying the needle to prime it.

   If the volume injected is >100 μl or if the material is injected too superficially, mice can develop ulcerating lesions over the site of the subcutaneous injections.

5. Inject each mouse s.c. with 200 μl of the emulsion (containing a total of 0.5 mg of NSG) divided equally between two injection sites.

   The site of injection can be at the neck (single injection of 200 μl) or at the base of the tail (100 μl to both right and left sides).

Inject anti-GBM globulin

6. Four days after NSG/CFA injection, induce nephritis by i.v. injection of anti-GBM globulin, using a 26- or 30-G needle and 1-ml syringe, into the tail vein.

   If the same sheep anti-mouse GBM antibody is to be used for the accelerated and non-accelerated models, the dose of antibody required in the accelerated model is often much lower and may be around the nephritogenic threshold, i.e., the dose in the heterologous phase that induces abnormal proteinuria in ~50% of mice. After nephritis is induced, mice should be monitored daily.

7. Assess disease and immune responses 10 days after injection of anti-GBM antibodies as outlined in Basic Protocol 1.

As mice have been sensitized s.c., removing and assessing cells from the lymph nodes that drain the immunization site is relevant.

SUPPORT PROTOCOL 1

PREPARATION OF A MOUSE KIDNEY CORTEX FRACTION

This protocol describes the preparation of mouse glomeruli-rich kidney cortex, which can be used (with an adjuvant such as CFA) to immunize sheep. Circulating antibodies that develop against mouse glomeruli (more specifically, to the GBM) can be obtained from the sheep serum (see Support Protocol 2).

Materials

Normal mice of any strain
Phosphate buffered saline (PBS), ice cold

Sterile 50-ml tubes
Petri dishes
Forceps
Scissors
Scalpel blade
Tissue homogenizer
Refrigerated centrifuge
Microscope slides and coverslips
Microscope
Sonicator
Scale
5-ml tubes

Additional reagents and equipment for euthanasia of mice (see *UNIT 1.8*) and blood collection (see *UNIT 1.7*)

1. Euthanize mice (see *UNIT 1.8*). Collect whole kidneys from normal (i.e., untreated) mice into a sterile 50-ml tube and store at −80°C until needed.

   Approximately 50 kidneys should be enough to immunize one sheep.

   Perform subsequent steps on ice.

2. Thaw kidneys in a clean petri dish on ice and remove the capsule from kidneys by peeling the thin outside layer from each kidney.

3. Cut kidneys in half coronally and remove the medulla from each half of the kidney. Discard the medulla and keep the kidney cortex in a petri dish on ice.

   Some laboratories have isolated glomeruli from the kidney cortex using differential sieving (Salant and Cybulsky, 1988) to prepare a membrane fraction, but this is not strictly necessary and is more difficult to perform for mouse kidneys than for rat or rabbit kidneys.

4. Dice the cortices from all kidneys into small pieces (2- to 3-mm pieces) using a scalpel blade and place into a 50-ml tube containing 20 ml cold PBS.

5. Homogenize using a tissue homogenizer at a setting of ~40% to 60% of maximum, until there are no visible pieces of tissue remaining.

6. Wash two times in 45 ml cold PBS by centrifuging 5 min at 700 × *g*, 4°C, between washes.

   After each wash, the supernatant will appear cloudy caused by smaller particles that are discarded. Glomeruli are denser and are found in the pellet; therefore, discard the supernatant and keep the pellet.

7. After the second centrifugation, resuspend the pellet in sterile water to lyse cells and facilitate sonication.
8. Place a sample of the homogenized solution onto a microscope slide and apply a coverslip, then view under a microscope.

   There will be a few tubules, but the majority of structures visible will be glomeruli.

9. Sonicate the solution (in pulses) on ice until glomeruli are disrupted (verified by microscopy).

   This may take some time, but continue until no intact glomeruli are visible.

10. Wash three times in cold PBS by centrifuging 5 min at 1750 × *g*, 4°C, between washes.
11. After the third centrifugation, remove the supernatant and weigh the pellet. Resuspend the pellet in cold PBS to 60 mg/ml by wet weight.
12. Prepare 1.5-ml aliquots in 5-ml tubes and store at −20°C until needed.

    Sheep or rabbits can be immunized in a number of different ways. The adjuvant and frequency of dosing can vary according to local practice and ethical requirements. It is important to regularly bleed and assess the renal function of the animal, as (uncommonly) they can themselves lose tolerance to their own GBM and develop autoimmune anti-GBM GN that can progress rapidly.

ABSORPTION AND PRECIPITATION OF A GLOBULIN FRACTION

SUPPORT PROTOCOL 2

Before it can be used, sheep serum containing antibodies against mouse basement membrane proteins must first be incubated with mouse RBC to absorb and remove any antibodies that may react with mouse RBC. If this is not done, these RBC-reacting antibodies may cause hemolysis of mouse RBC when injected into mice. RBC-adsorbed serum is then precipitated using saturated ammonium sulfate (SAS). Preparing an IgG fraction using protein G purification has been done, but this had no significant advantages in the authors' hands.

Before starting, ensure all reagents are made with autoclaved, distilled water, all tubes are sterile, larger containers scrubbed and/or autoclaved, and, if possible, all steps performed at 4°C. LPS contamination should be avoided, as it will alter the nephritogenicity of the antibodies (Karkar and Rees, 1997) and may result in batch-to-batch variability.

Materials

Sheep serum, sheep anti-mouse GBM (and if required, non-immune sheep serum)
Bottle of water (same shape and volume of serum)
Normal (i.e., untreated) mice (rats, if needed) of any strain
3.3% (w/v) tri-sodium citrate in distilled water
0.9% (w/v) NaCl
Borate buffer (see recipe)
Saturated ammonium sulfate (SAS, see recipe)
PBS, ice cold

37° and 60°C water baths
Thermometer
Timer
5-, 10-, and 50-ml tubes
26-G needles
1-ml syringes

Refrigerated centrifuge
Roller
Stirrer
5-liter containers
Spectrophotometer
UV cuvette
100-μm mesh

Heat inactivate sheep serum

1. If frozen, remove serum from freezer and leave overnight at 4°C.
2. Preheat a water bath to 60°C.
3. Place frozen sheep serum together with the water bottle for 10 to 20 sec in a cold water bath (4° to 10°C) or under cold tap water, then place in a 37°C water bath.
4. When thawing is complete, gently mix the serum. Transfer the thawed, mixed serum, together with the bottle of water containing a thermometer from the 37°C bath to the 60°C bath.
5. Gently agitate both bottles. When the thermometer indicates 56°C, set a timer and incubate for 30 min.

   Using a control bottle ensures that the serum has reached 56°C so that complement will be inactivated. It is important to decomplement the sheep serum first, as the presence of sheep complement in the serum may result in the lysis of mouse RBC.

Perform absorption of sheep globulin against RBC

6. Collect blood from normal (i.e., untreated) mice of any strain into anti-coagulant (3.3% tri-sodium citrate)-containing tubes.

   It is preferable to absorb against mouse RBC two times, but if not enough mouse blood can be obtained, it is possible to first bleed some rats and absorb with their RBC prior to absorbing with mouse RBC.

7. Centrifuge 15 to 30 min at 300 × *g*, 4°C.
8. Remove plasma and keep for other uses if needed.
9. Wash RBC in sterile 0.9% NaCl.
10. Centrifuge as in step 8 and discard the supernatant.
11. Wash again following steps 9 and 10; RBCs should now remain.
12. Add the heat-inactivated sheep serum to tube(s) containing RBC at a ratio of 1:9 (v/v) RBC/serum.

    It is important that RBCs are used relatively promptly to avoid RBC lysis and hemoglobin contamination of the supernatant.

13. Mix and leave on a roller overnight at a low setting at 4°C or 2 hr at room temperature.
14. Centrifuge 10 min at 300 × *g*, 4°C.
15. Collect the supernatant (containing the globulin fraction) and discard RBC.

    If not enough mouse/rat blood is available at one time for both adsorption steps, at this stage, supernatant can be frozen and stored for several months at −20°C prior to the second adsorption.

16. Absorb the serum a second time against fresh (new) RBC following steps 12 to 15.

Perform SAS precipitation of a globulin fraction

17. To *x* ml of serum, add an equal volume of borate buffer.
18. Stir 15 min at 4°C.
19. Add two times the volume (compared with that of the serum) of filtered SAS, drop by drop, while stirring at 4°C.
20. Stir 1 hr at 4°C.
21. Centrifuge 30 min at 1750 × *g*, 4°C.
22. Discard the supernatant.
23. Resuspend pellet in cold PBS (<*x* ml).
24. Repeat steps 17 through 23.
25. Dialyze in a 5-liter container for 48 hr against PBS at 4°C.
26. Determine the concentration of sheep IgG by measuring the absorbance at 280 nm using a spectrophotometer and calculate by using the following formula:

    concentration of sheep globulin (mg/ml) = A_{280}/1.414 × dilution factor
    where, 1.414 is the extinction coefficient of IgG.
27. Filter through a 100-μm mesh.
28. Dispense into 5- to 10-ml aliquots and store up to 2 to 3 years at −20°C.

DETERMINATION OF OPTIMAL DOSES OF SHEEP ANTI-MOUSE GBM GLOBULIN

SUPPORT PROTOCOL 3

Each batch of anti-GBM globulin must be first dose tested for its efficacy in inducing GN to determine the appropriate dose.

Materials

C57BL/6 mice (male, 6 to 12 weeks old)
Sheep anti-mouse GBM globulin (see Support Protocols 1 and 2)

Metabolic cages
26- and 30-G needles
1-ml syringes
Heat lamp or heat pad
Mouse retaining cone (or similar apparatus to facilitate i.v. injection)
5-ml tubes

1. Two to three days prior to starting experiments, place mice in metabolic cages for 24 hr, collecting all urine made over this 24-hr period in 5-ml tubes for determination of baseline proteinuria.
2. To determine the dose of sheep anti-mouse GBM globulin to use for non-accelerated model of renal disease, inject three to four (or more) different doses of sheep anti-GBM globulin i.v. into C57BL/6 mice (five to six mice per dose) using a 1-ml syringe and 26-G needle. To determine the globulin dose to inject for the accelerated anti-GBM disease model, immunize mice with NSG/FCA (as in Basic Protocol 2) 4 days before administering different doses of sheep anti-GBM globulin i.v. As mice have already been sensitized to NSG, the dose of anti-GBM globulin required for the accelerated model will be lower than that needed to induce comparable renal injury using the non-accelerated model.

Mice used for dose testing should be of the same sex and age (6 to 12 weeks old) as the mice to be used in experiments. The size, age, and gender of mice may affect the efficiency of anti-GBM antibodies in inducing GN and any potential adverse effects they may have. Mice should be checked daily throughout the entire course of GN for any signs of ill-health.

3. Collect urine from mice over 24 hr to assess urinary protein excretion.

   The presence and extent of proteinuria in the first 24 hr indicates the degree of heterologous injury.

4. To assess autologous injury (7 to 21 days after anti-GBM antibody injection or 10 days after anti-GBM antibody injection in the accelerated anti-GBM model), collect urine during the last 24 hr of experiments (for determination of proteinuria) and obtain kidneys for assessment of histological injury (by PAS or staining on formalin-fixed, paraffin-embedded tissues).

   Histological injury is assessed most commonly by determining the proportion of glomeruli affected by the presence of segmental or global necrosis, and the percentage of glomeruli affected by crescent formation (defined as two or more cell layers in Bowman's space). The severity of glomerular injury in C57BL/6 wild type mice can vary.

SUPPORT PROTOCOL 4

THREE-LAYER IMMUNOHISTOCHEMICAL STAINING TO ASSESS INTRARENAL LEUKOCYTE ACCUMULATION

Given the involvement of leukocytes in this model, assessment of their infiltration is often an important endpoint. Most monoclonal antibodies that target mouse leukocyte subset markers (including those specific for CD4+ cells) do not recognize antigens after paraffin embedding; therefore, they need to be used on frozen tissues.

Materials

PLP-fixed OCT-frozen kidneys (and spleen, as positive controls)
Blocking solution: 10% normal swine serum, 10% normal sheep serum in 5% BSA/PBS
Primary antibody and isotype controls diluted in 1% BSA/PBS (see Table 15.26.1)
PBS-Tween 20 solution containing 0.05% (v/v) Tween 20
Absolute methanol
30% hydrogen peroxide (H_2O_2) solution
Avidin-biotin blocking kit (Vector Laboratories)
Secondary antibody solution: 10% normal mouse serum diluted in PBS with rabbit anti-rat biotinylated antibody (DAKO) at 1:100
Tertiary antibody solution: Vectastain ELITE Standard ABC solution (Vector Laboratories)

Table 15.26.1 Antibodies and Isotype Controls Used for Renal Leukocyte Staining

Leukocyte	Antibody (clone)	Isotype control	Antibody concentration (μg/ml)
Macrophage	Rat anti-mouse CD68 (FA-11)[a]	Rat IgG2a	10
CD4+ T cell	Rat anti-mouse CD4 (GK1.5)	Rat IgG2b	20
Neutrophil	Rat anti-mouse Gr-1 (RB6-8C5)[b]	Rat IgG2b	2–5
CD8+ T cell	Rat anti-mouse CD8 (53-6.7)	Rat IgG2a	20

[a]Macrophages comprise a heterogeneous population and express a number of different markers. This marker is accepted in the literature as a reasonable marker of effector macrophages in glomeruli. Note that F4/80 does not reliably detect macrophages within glomeruli (Masaki et al., 2003).
[b]Anti-Gr-1 is also known as anti-Ly6G/C and also recognizes a subset of monocytes. An anti-Ly6G antibody (clone 1A8) can also be used to detect neutrophils more specifically, but this antibody detects neutrophils in only some strains of mice (including C57BL/6 mice).

3,3′ Diaminobenzidine tetrahydrochloride (DAB) tablets (Sigma-Aldrich)
Hematoxylin
Acid ethanol solution
Scott's tap water solution
Histosol solution
DPX mounting medium

Cryostat
Superfrost slides and cover slips
PAP pen
Humidity chamber
Coplin jars
Platform rocker
Aluminium foil
0.22-μm filter
3-ml transfer pipets
Oven or hair dryer

1. Prepare 6-μm thick sections of PLP-fixed kidneys/spleen using a cyrostat. As an isotype control is required for staining, prepare extra sections of at least one kidney/spleen.

   Sections of spleen act as a positive control.

   Cut sections can be stored up to 1 week at −80°C.

2. Immediately prior to staining, allow sections to dry on slides for 20 min at room temperature, and then mark around each section with a PAP pen.

3. Pre-incubate each kidney/spleen with 60 μl of blocking solution for 30 min in a humidity chamber (with water added to slide channel, to provide constant humid environment) at room temperature.

4. Tip solution off (do not wash).

5. Apply 60 μl of the primary antibody and appropriate isotype control solutions to each kidney/spleen and incubate in the humidity chamber overnight at 4°C or 1 hr at room temperature.

6. Wash by placing slides in a Coplin jar filled with 0.05% PBS-Tween 20 solution on rocker for 3 min; repeat three times.

   This and subsequent steps can be performed at room temperature.

7. Block endogenous peroxidase by placing slides in a Coplin jar containing absolute methanol and 1% hydrogen peroxide (diluted from 30% hydrogen peroxide) solution for 20 min at room temperature in a fume hood. Cover with aluminum foil to protect slides from light.

8. Wash for 3 min in PBS-Tween 20 solution on rocker; repeat three times.

9. Block using the avidin-biotin blocking kit as follows:

   a. Apply avidin solution for 15 min.
   b. Wash for 3 min in PBS-Tween 20 solution on rocker; repeat two times.
   c. Apply biotin solution for 15 min.
   d. Wash for 3 min in PBS-Tween 20 solution on rocker; repeat two times.

10. Apply 60 μl of secondary antibody solution to each kidney/spleen section and leave in the covered slide chamber for 1 hr at room temperature. While this staining step

is occurring, the ABC solution can be made and kept at room temperature for the next staining step as 40 min is required for the A and B components to complex together.

11. Wash for 3 min in PBS-Tween 20 solution on rocker; repeat three times.
12. Apply tertiary antibody solution (ABC solution) to each kidney/spleen section and leave in the covered slide chamber for 40 min at room temperature.

    For each 2 ml, the ABC solution should contain 1984 µl of 1% BSA/PBS, 18 µl of A solution, and 18 µl of B solution (it is important to note that this differs to the protocol supplied by the manufacturer).

13. Wash for 3 min in PBS-Tween 20 solution on rocker; repeat three times.
14. To develop the stain with DAB brown/hydrogen peroxide solution, dissolve one DAB brown tablet in 20 ml of PBS and filter through a 0.22-µm filter in a fume hood. Add 10 µl of 30% hydrogen peroxide and protect this solution from light exposure by covering vessel with aluminum foil.
15. Lay the slides flat in a staining chamber and apply the DAB brown/H_2O_2 solution with a 3-ml transfer pipet to the kidney sections.

    As the stain can develop rapidly, it is necessary to stop the stain and check the sections every 30 sec. This is to ensure that excessive background staining is not developing, limiting the recognition of the stained leukocytes. To halt the staining, quickly tip the solution off and place slides in a Coplin jar of distilled water. Slides can be checked with the light microscope (taking care to ensure the kidney sections do not dry out while using the microscope). Use the isotype control antibody to ensure that staining is specific for the leukocyte of interest.

    Developing the sections for each leukocyte stain will require a different period of time.

16. Once renal leukocyte staining is completed, counterstain briefly with hematoxylin for 10 to 20 sec, based on the desired intensity of the counter stain.
17. Rinse slides in a Coplin jar for 3 min in running tap water.
18. Dip in acid ethanol solution for 1 sec.
19. Dip in Scott's tap water solution for 30 sec.
20. Rinse in running tap water for 3 min.
21. Dry completely in an oven or with a hair-dryer.
22. Place in Histosol solution for 10 min in a fume hood; repeat two times, placing slides in fresh Histosol each time for 10 min.
23. Cover with coverslips using DPX mounting glue.
24. Allow to dry overnight at room temperature.

SUPPORT PROTOCOL 5

DAB BLACK STAINING WITH NUCLEAR FAST RED COUNTERSTAIN TO ASSESS INTRARENAL LEUKOCYTE ACCUMULATION

DAB black can be used instead of DAB brown to detect positive staining in cases where more sensitive detection may be required.

Additional Materials *(also see Support Protocol 4)*

DAB black solution (see recipe)
Nuclear Fast Red solution (see recipe)

1. Perform Support Protocol 4, steps 1 through 3.
2. Place slides into a Coplin jar containing DAB black solution in a fume hood and incubate for 30 min.
3. To develop the stain, add 333.2 μl (for each 100 ml of DAB black solution) of 3% H_2O_2 to the Coplin jar containing the slides and DAB black solution, protected from light in a fume hood. Remove slides every 30 sec to 2 min and place in a Coplin jar of tap water to stop developing. Check sections under the light microscope to assess the level of staining, ensuring kidney sections do not dry out.

   The slides can be replaced in the DAB black/H_2O_2 solution if further staining is required, checking every 2 min.

4. Wash in running water for 30 min.

   Washing in running water will intensify the black color of the stain.

5. When appropriately stained, counter stain by placing the slides in a Coplin jar containing the Nuclear Fast Red solution for 30 sec (or up to 2 min, based on the desired intensity of the counter stain). Wash briefly in tap water.
6. Dehydrate through graded alcohols, by dipping 1 sec through 30%, 50%, 70%, and 100% ethanol solutions.
7. Dry slides in an oven or with a hair dryer.
8. Place slides in Histosol solution in a fume hood for 10 min; repeat two times in fresh Histosol solution each time for 10 min.
9. Cover slides with coverslips using DPX mounting glue.
10. Allow to dry overnight at room temperature.

DETECTION OF MOUSE IgG OR C3 DEPOSITION IN GLOMERULI BY IMMUNOFLUORESCENCE

SUPPORT PROTOCOL 6

Mouse anti-sheep Ig antibodies and complement (mediators of glomerular injury in some mouse strains) are deposited in glomeruli of mice that develop anti-GBM Ig GN and can be detected by immunofluorescence. Determining the appropriate concentration of antibody should be conducted through a pilot experiment, using dilutions of 1:100, 1:250, and 1:500. The pilot protocol is described below. Ideal negative control specimens for this staining include kidney/spleen samples from a strain of an immunoglobulin-deficient mouse (e.g., a *Rag1–/–*, *Rag2–/–* or mu-chain–/– mouse) and a normal C57BL/6 mouse (to assess baseline).

Materials

Non-fixed OCT-frozen kidneys (and spleens) from mice with non-accelerated or accelerated anti-GBM GN (see Basic Protocols 1 and 2, respectively)
Acetone
15% normal sheep serum in 5% BSA/PBS or 15% normal goat serum in 5% BSA/PBS
Sheep anti-mouse Ig-FITC (Silenus) *or* goat anti-mouse C3-FITC (Cappel)
1% BSA/PBS
PBS
Fluorescent mounting medium (Dako)

Cryostat
Superfrost slides and coverslips

Coplin jars
PAP pen
Humidity chamber

1. Cut snap frozen kidney/spleen sections to a thickness of 4 μm using a cryostat and Superfrost slides.
2. Dry slides 30 min at room temperature. During this step, cool the acetone by filling a Coplin jar with acetone and placing it on ice in a fume hood.
3. When slides are completely dry, place them in a Coplin jar filled with acetone for 2 to 3 min in a fume hood.
4. Remove from acetone and dry slides 5 min at room temperature in a fume hood.
5. When slides are completely dry, draw around each kidney/spleen section with a PAP pen.
6. Block slides by pre-incubating 30 min in 60 μl (for each kidney/spleen section) of 15% normal sheep serum in 5% BSA/PBS solution if using a sheep antibody *or* 15% normal goat serum in 5% BSA/PBS solution if using a goat antibody.

   This step is not required if staining for sheep Ig deposition.
7. Pour off the blocking solution (do not wash).
8. Apply primary antibodies diluted in 1% BSA/PBS. Cover to protect from light in a humidity chamber and incubate for 1 hr at room temperature.

   For each antibody, use the pre-determined concentration that permits the best discrimination of IgG/C3/Fibrinogen deposition without excessive background staining.

   From this point forward slides should always be protected from light to prevent fading.
9. Wash in PBS for 5 min in a covered Coplin jar; repeat two times.
10. To cover the slides, add 1 drop of fluorescence mounting medium to the coverslip and carefully apply the coverslip to the slide, taking care not to move the coverslip over the sections.
11. Keep covered from light and store at 4°C until ready to use.

    When assessing the sections with the fluorescent microscope, never leave the section exposed to fluorescent light longer than necessary, to avoid bleaching of the fluorescence. Assess the sections in a dark room and keep slides protected from light when not being viewed on the microscope. Briefly assess all sections to determine that the appropriate controls have worked, and to determine the spectrum of scoring and how they are to be scored. Assess and score immunofluorescence promptly (ideally within 48 hr), as staining fades over time.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***APPENDIX 5****.*

Borate buffer

To prepare 1 liter, add:
6.184 g boric acid
9.532 g di-sodium-tetraborate
4.33 g NaCl
Bring up to 1 liter with distilled water
Adjust pH to 8.3 with 1 N NaOH or HCl
Store up to 2 months at 4°C

DAB Black solution

In a fume hood, add 2.5 ml of 1% cobalt solution and 2.0 ml of 1% ammonium nickel sulfate solution to 82 ml of 0.1 M phosphate buffer (see recipe).

Separately, dissolve 500 mg of 3,3 diaminobenzidine tetrahydrochloride hydrate (Sigma, cat. no. D5637) in 10 ml of distilled water, dispense into 1-ml microcentrifuge tubes, and freeze until required. Add 1.0 ml of this DAB Black solution to the previous solution above.

Bring up the volume to 100 ml with additional 0.1 M phosphate buffer (see recipe) and filter the solution through a 0.22-μm filter prepare fresh.

Unused solution should be discarded as chemical waste.

Nuclear Fast Red

Prepare 500 ml of 5% aluminum sulfate solution (i.e., 25 g aluminium sulfate dissolved in 500 ml distilled water). Add 0.5 g Nuclear Fast Red (Sigma) to this solution and mix well. Filter through a no. 1 Whatman paper or 5B Advantec paper. Store up to 6 months at room temperature.

Paraformaldehyde-periodate-lysine solution

Use appropriate personal protective equipment when weighing out and working with chemicals. Make up solution in a fume hood.

Solution A: add 10.69 g L-lysine to 300 ml of distilled water and mix well. Bring pH to 7.4 using fresh 0.1 M Na_2HPO_4 (1.42 g Na_2HPO_4 dissolved in 100 ml of distilled water). Dilute to 600 ml total volume with PBS.

Solution B: add 16 g paraformaldehyde to 200 ml of distilled water and dissolve by heating the beaker on a magnetic heat block to 60°C (do not exceed 60°C). Add 3 to 4 drops of 0.1 M NaOH to dissolve mixture completely. This solution must be completely dissolved before proceeding.

Add solution B to solution A and stir. Add 1.73 g Na periodate and stir. Adjust pH to 7.4 with 3 M NaOH. Store in 15-ml aliquots up to 3 years at −20°C. If the solution is thawed, discard the PLP as chemical waste. Do not refreeze and reuse.

Phosphate buffer, 0.1 M

Add 3.12 g sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$; Univar) and 11.28 of di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4 anhydrous; Univar) to a 1-liter bottle and fill up to 1 liter with deionized water. Adjust pH to 7.0 with 1 N HCl. Store up to 6 months at room temperature.

Saturated ammonium sulfate (SAS)

Heat distilled water to 60°C
Add dry ammonium sulfate until no more dissolves
Place at 4°C and allow to cool
Filter through a no. 1 Whatman paper and then through a 0.2-μm filter
Store up to several months at 4°C

COMMENTARY

Background Information

These models using heterologous globulin that target the glomerulus have been used for many years, since the original report by Masugi (1934). Studies using these models have contributed particularly to the understanding of how immune effectors target the glomerulus in severe and rapidly progressive forms of glomerulonephritis, and how immune responses induce glomerular injury. In addition

to mice, studies have used rats, especially the susceptible Wistar Kyoto strain (Huang et al., 1994; Aitman et al., 2006), and rabbits (Erlich et al., 1996) as hosts. To use these models effectively to define the immunopathology of severe proliferative nephritis, it is important to understand the nature of the models, what they mean, and their strengths and weaknesses. A significant problem exists with nomenclature. The three common terms used to describe these models (anti-glomerular basement membrane disease, nephrotoxic serum nephritis, and Masugi nephritis) all lead to confusion as to the mechanisms and mediators of injury.

The term "anti-glomerular basement membrane glomerulonephritis" has been used commonly for many years. Although the passively transferred heterologous antibodies target and bind to glomerular basement membranes, they recognize a number of targets separate from the autoimmune target of human anti-glomerular basement membrane disease, α3(IV)NC1. In the heterologous phase, some of the effector mechanisms are similar to those induced by anti-glomerular basement membrane antibodies in humans, although heterologous globulin tends to induce more neutrophil-mediated injury, while autologous antibodies tend to recruit macrophages (Boyce and Holdsworth, 1989). In the autologous phase, adaptive immunity to sheep globulin recognizes it planted on the GBM, i.e., the heterologous antibody acts as a foreign planted antigen. Autoimmunity to the GBM does not develop, but some of the effector mechanisms are similar to those seen in human anti-GBM GN. A further drawback in using the term "anti-GBM glomerulonephritis" is the existence of models of anti-GBM GN that are autoimmune and induced by breaking tolerance—confusingly these are often called experimental autoimmune glomerulonephritis or experimental autoimmune glomerulonephritis (EAG)—meaning that the two models, one autoimmune and one with no autoimmune component, are both known as "experimental anti-GBM GN."

Due perhaps to resistance to the term anti-GBM GN and the desire to avoid confusion, "nephrotoxic serum nephritis" is a term now commonly used. Although using this term does avoid confusing these models with autoimmune anti-GBM GN, it implies that the injury that occurs is due to "nephrotoxic serum" or a nephrotoxin as opposed to an immunological process. Using this term risks understating and undervaluing the clear, well-defined immunological principles that not only underpin these models, but also that have been instructive over many years. Some researchers use these models to study the nature of kidney injury generically. While this is a valid approach, the involvement of the immune system remains critical in the expression of the model. Any intervention in the autologous phase of this model, particularly if the intervention has the capacity to influence systemic immunity to sheep globulin, must include assessment of immunity to sheep globulin, the key variable determining injury in the autologous phase of disease.

The eponymous nature of the term "Masugi nephritis" encapsulates both its advantages and disadvantages. The advantage of using this term is that it avoids erroneously implying autoimmune or toxin-induced injury. However, its disadvantage is that the term is neutral and offers no insights into the nature of the model or the condition that it models.

Using the terms "anti-GBM glomerulonephritis" or "Masugi nephritis" and avoiding the term "nephrotoxic serum nephritis" are proposed. Any confusion between models of autoimmune anti-GBM GN and models induced by sheep globulin can be mitigated by using the terms "heterologous phase" or "autologous phase," and adding the descriptors "non-accelerated" or "accelerated." While cumbersome, this system more accurately describes the immunology underpinning kidney injury in these models and avoids any misapprehension that the kidney is being injured by an ill-defined "nephrotoxin." An alternative nomenclature is also possible, e.g., heterologous phase injury could be described as "passive in situ immune complex glomerulonephritis" and autologous phase injury as "planted antigen glomerulonephritis." However, the introduction of yet another series of descriptors may incite further confusion.

Heterologous phase injury effectively results from the passive transfer of heterologous globulin. While different host species and different species of donor antibodies engage effectors in a slightly different manner, generally, injury is neutrophil mediated and, in mice, there is only a limited role for complement (Tipping et al., 1994). Heterologous injury has in the past been made more complement dependent by enriching the sheep anti-mouse GBM for IgG subclasses that better fix mouse complement (Quigg et al., 1998).

In heterologous disease, innate components responding to the injected heterologous

anti-GBM globulin induce proteinuria within 24 hr (Kitching et al., 2002; Kuligowski et al., 2006; Devi et al., 2013). Maximal neutrophil accumulation in glomeruli occurs 2 to 4 hr after the administration of anti-GBM antibodies. There is a tendency for injury, including proteinuria, to subside after 24 to 48 hr, although administration of very high doses of anti-GBM antibodies can cause ongoing heterologous injury for 5 to 7 days. The value of this passive transfer phase of the model is that it is robust, immediate, predictable, and useful for studying mechanisms of leukocyte recruitment (Devi et al., 2013), neutrophil-mediated injury, and, in some systems, complement (Quigg et al., 1998).

Autologous phase injury in a "non-accelerated" model develops as adaptive immunity to sheep globulin after ~5 days. The systemic administration of sheep protein induces adaptive immunity, both cellular and humoral, to this foreign antigen, which then targets the sheep globulin bound in the mouse kidney. This autologous phase better reflects the rapid development of glomerulonephritis in humans, which most commonly results from adaptive immune responses targeting the glomerulus after the development of systemic immunity or autoimmunity to nephritogenic antigens. Autologous immunity can be established by sensitization to sheep globulin prior to injection of anti-GBM globulin that then acts as a target antigen within the glomerulus.

There are a variety of effector mechanisms that influence disease, with the relative contributions of humoral and cellular mediators dependent on several factors, including mouse strain and the immunization protocol. In the authors' hands, it has been found that severe, rapidly progressive autologous phase disease in C57BL/6 mice tends to mediate mainly by delayed-type hypersensitivity cell–mediated effectors, including Th1 cells and macrophages (Huang et al., 1997b; Kitching et al., 1999). Glomerular crescent formation occurs in the absence of autologous antibodies (Li et al., 1997) or CD8+ cells (Li et al., 2000). In BALB/c mice, autologous phase injury is mediated by autologous antibody, complement, and neutrophils (Huang et al., 1997a). Exploring these phenomena has been instructive in defining the pathogenesis of rapidly progressive forms of glomerulonephritis, e.g., by demonstrating the potential role of effector CD4+ cells and T cell cytokines (Holdsworth et al., 1999; Kitching et al., 2000a). Other users of a similar model induced by a large dose of heterologous globulin to naïve mice (using the non-accelerated model) found that early phase disease (days 4 to 7) is IL-17A-mediated and have used this model to study its effects on intrarenal chemokines and innate immune cells in its early stages (Paust et al., 2009; Hochheiser et al., 2011; Riedel et al., 2012). Tarzi et al. (2002) found in their version of accelerated autologous phase anti-GBM glomerulonephritis that FcγRs are important in autologous injury.

The contributions of autologous antibody and FcgR have also been demonstrated in a variant of the model, used mainly in rats but also in mice, known as "passive autologous phase" anti-GBM glomerulonephritis (Boyce and Holdsworth, 1989; Huang et al., 2000; Li et al., 2009). In this model, a low, subnephritogenic dose of heterologous anti-GBM globulin is injected, followed by passive transfer of polyclonal autologous antibody. For example, low-dose anti-GBM antibodies injected into rats can be followed by transfer of rat anti-sheep IgG antibodies, with the resulting injury being macrophage and FcR mediated.

Conventionally, studies of non-accelerated autologous phase anti-GBM glomerulonephritis have ended at 21 days and in the accelerated autologous model at 10 days (20 days after initial sensitization), when a mature adaptive immune response sheep globulin (the nephritogenic antigen) has developed. At this time point, significant glomerular injury with moderate tubulointerstitial injury is evident. Progressive disease has been studied at later time points (Summers et al., 2011), making this model of some value in examining mechanisms of renal fibrosis. If this model is to be used in this way, it is important not to omit assessment of systemic immunity, as it is this ongoing adaptive immune response that drives progressive disease. This is in contrast to some other models of progressive renal disease (e.g., experimental obstructive uropathy or renal mass reduction models).

Most studies use anti-GBM globulin raised by injecting antigen from the same species as the eventual recipient of the antibodies (i.e., mouse cortex injected into sheep induces sheep anti-mouse GBM antibodies that are then injected into mice). However, similar models have been induced in mice with antibodies that were raised from membrane fractions of rats (Ophascharoensuk et al., 1998).

Critical Parameters

There are several critical parameters in inducing these models.

Variation in the nephritogenicity of sheep anti-mouse GBM globulin

As sheep are outbred animals, their immune response to the injected mouse kidney cortex varies, and each polyclonal antiserum varies considerably. Therefore, it is important to test and characterize the serum from each sheep (as well as each batch from each sheep). It is preferable to immunize more than one sheep (e.g., three to four sheep), as antibodies derived from an individual sheep may not always be pathogenic on testing in mice.

It is useful to prepare moderately large batches of globulin at any one time from sheep serum (the limiting factor often being the availability of enough mouse and rat RBC for adsorption). Although the method of preparation is standardized (see Support Protocol 2), there may be differences in the dose needed to induce proteinuria. Therefore, a limited study should be performed prior to using each new batch of globulin. Lipopolysaccharide contamination should ideally be avoided. Although adding LPS to preparations enhances their nephritogenicity (Karkar and Rees, 1997), LPS may also enhance toxicity, and variable contamination may contribute to batch to batch variation.

Dose of anti-GBM globulin used

Generally, within a single sheep, the higher the dose of antibody injected the more injury that occurs. Injection of very large doses of antibody can result in substantial and prolonged heterologous injury that may confound assessment of early autologous injury, limiting the relevance of results to human disease. Using some antisera preparations, it has been possible to trigger accelerated autologous phase disease after injecting only a small dose of anti-GBM globulin (Huang et al., 1997a,b). The dose used can be one that does not induce measurable proteinuria (known as a subnephritogenic dose). In this setting (planting a foreign antigen in glomeruli of immunized mice), only minimal injury is induced by the antigen as an antibody, and almost all the injury by the host's autologous immune respond to sheep globulin as a planted foreign antigen.

Mouse strain, size, and sex

Different strains of mice exhibit different degrees of susceptibility and patterns of injury, in large part due to differences in the development of effector responses (Huang et al., 1997a,1997b; Kitching et al., 1999), but also due to intrarenal factors (Fu et al., 2007). Generally, smaller mice develop more severe disease for any given dose of anti-GBM antibodies. For this reason, using mice of a consistent age and sex is preferable. However, if is necessary to experiment on mice of variable sizes, antibodies can be injected on a milligram per gram basis (Kitching et al., 2000b). Studies that formally assess differences in responses between male and female mice in these models (after controlling for size differences) have not been published.

Accurate assessment of glomerular crescent formation

In immune renal disease, glomerular crescent formation, defined by extracapillary proliferation of epithelial cells and infiltration of leukocytes, portends a poor prognosis. In experimental models of murine glomerulonephritis, it is important to accurately assess glomerular crescent formation. In most species, the parietal epithelium (the layer of epithelial cells that line Bowman's capsule) is composed of squamous (flattened) epithelial cells, making proliferation within the urinary space relatively easy to assess. However, many normal (and diseased) mouse glomeruli exhibit cuboidal parietal epithelial cells, which needs to be taken into account when examining evidence of glomerular crescent formation in mice. Murine glomeruli are also comparatively small with narrow urinary spaces, and have a tendency to form capsular adhesions in disease. While it may be tempting to overinterpret signs of glomerular crescent formation, seeking clear evidence of two layers of cells (excluding the normal layer of podocytes) inside Bowman's capsule and within the urinary space is recommended before determining that a glomerulus has been affected by crescent formation (Kitching et al., 2000b). In humans, crescents fibrose over time. In these models, crescents occur relatively early, and therefore at time points commonly examined, most crescents are cellular. The presence of segmental areas of necrosis (hypocellular regions with the accumulation of eosinophilic or PAS positive material) is common in these models and in human rapidly progressive glomerulonephritis, and represents a valid histological outcome in these models (Kitching et al., 2005).

Troubleshooting

If glomerular disease is not severe enough

In non-accelerated injury, consider either increasing the dose of anti-GBM globulin or using an accelerated model of injury. In accelerated injury, there are a number of options, e.g., increasing the time between immunization and i.v. challenge, increasing the dose of sheep globulin, or altering the adjuvant to increase the concentration of *M. tuberculosis*, which may result in more severe disease, but also increases the risk of an anaphylactoid death at challenge.

If glomerular disease is too severe, or mice are dying

Consider the reverse of some of the suggestions outlined above. In addition, uncommonly, some antisera/preparations seem to have a high toxicity-to-efficacy ratio, for reasons that are unclear, but which may include LPS contamination. If, after reducing the dose of anti-GBM globulin, a point is reached where significant injury cannot be induced without inducing toxicity, consider preparing a different batch of antibody, carefully avoiding any contamination, or using antisera from a different sheep.

Anticipated Results

In autologous phase disease, mice should develop moderate to severe glomerular disease. A significant proportion of glomeruli should be exhibiting areas of segmental necrosis and/or glomerular crescents (two or more layers of cells in the urinary space). There is usually significant moderate tubulointerstitial injury, with protein casts, tubular atrophy, and interstitial infiltrates. Functionally, mice develop at least moderate proteinuria and in the autologous phase, some renal impairment. This can be measured by assessing urinary protein or albumin excretion and by measuring serum urea (blood urea nitrogen, BUN). Serum creatinine can be used but can be difficult to accurately assess in mice (Dunn et al., 2004).

Time Considerations

An autologous phase experiment with standard time points takes 21 days. Acquiring the relevant endpoints following the end of the experiment takes 2 to 6 weeks, depending on the nature and number of endpoints assessed. Experiments performed in heterologous injury, occurring over only 24 hr, can be completed more quickly, with assessment of more endpoints (histology, functional assessment, and assessment of neutrophil accumulation) being possible within 1 to 2 weeks. These time considerations assume that the researcher already has generated (or has in their possession) sheep anti-mouse GBM globulin and knows the correct dose to administer to mice.

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Chapter 4: FMS-like tyrosine kinase 3 ligand treatment does not ameliorate experimental rapidly progressive glomerulonephritis.

Declaration

Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design Performing the experiments Data analysis Writing and revising the manuscript	70

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Kim O'Sullivan	Performed the fluorescent microscopy, including the experimental design, technical work and data analysis	5
Peter Eggenhuizen	Assistance with performing experiments	
Stephen Holdsworth	Review of the manuscript	
A. Richard Kitching	Writing and revising the manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 18/3/15
Main Supervisor's Signature	[redacted]	**Date** 18/3/15

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

FMS-like tyrosine kinase 3 ligand treatment does not ameliorate experimental rapidly progressive glomerulonephritis

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Abstract

Fms-like tyrosine kinase 3-ligand (FL) is a growth factor that may expand dendritic cell and regulatory T cell populations. We hypothesised that FL-induced regulatory T cells would protect mice from experimental rapidly progressive glomerulonephritis**.** To determine if FL was able to enhance regulatory T cell populations, C57BL/6 mice received 10 days of daily intraperitoneal injections of either FL or phosphate buffered saline. To induce accelerated autologous-phase anti-mouse glomerular basement membrane glomerulonephritis, mice were sensitized to sheep globulin 4 days prior to the induction of glomerulonephritis with sheep anti-mouse glomerular basement membrane globulin, and experiments ended 10 days later. FL was administered before, throughout and during the sensitization phase of this glomerulonephritis model. Renal disease and systemic immunity to the nephritogenic antigen were assessed. FL increased regulatory T cell and plasmacytoid dendritic cell proportions within spleen and lymph nodes. FL administration prior to glomerulonephritis did not protect mice from renal injury. When FL was given throughout the model, FL treated mice had reduced survival, with more interstitial neutrophils and glomerular CD11c+ cells than controls. Systemic immune responses showed increased IL-17A production from splenocytes, with more CD11c+ cells, but reduced plasmacytoid dendritic cell proportions in spleen and lymph nodes, despite increased regulatory T cell proportions. Under homeostatic conditions, FL expanded regulatory T cell and plasmacytoid dendritic cell populations, but FL enhanced systemic inflammatory responses and conventional dendritic cell populations when given during experimental glomerulonephritis, suggesting selective attempts to suppress pathogenic immunity by dendritic cell manipulation may be harmful.

Introduction

Dendritic cells (DCs) are a heterogeneous population of professional antigen presenting cells, derived initially from common myeloid and common lymphoid progenitors in the bone marrow, which terminally differentiate in lymphoid and non-lymphoid tissues. They are broadly characterized into two groups: conventional or classical DCs (cDCs) and plasmacytoid DCs (pDCs) [1, 2]. In the mouse, cDCs include migratory and lymphoid-tissue resident DCs, which express CD11c and MHC II and are further characterized by the expression of other surface markers, including CD11b, CD103 and CD8. pDCs are found in lymph nodes (LN) or circulating within blood, and are $CD11c^{low\text{-}intermediate}$, have low MHC II expression and express other surface markers including B220 and murine pDC antigen 1 (PDCA-1) [3-5]. pDCs promote the maturation of cDCs, regulate antigen specific CD4+ T cell proliferation, effector T cell (Teff) production of IFN γ and CD4+foxp3+ Treg homeostasis in mucosal lymphoid tissue [6]. Fms-like tyrosine kinase 3 (FLT3) ligand (FL) is a growth factor that differentiates, matures and expands DCs following ligation of the FLT3 receptor expressed on the surface of hematopoietic precursors [7, 8]. It is expressed in steady state conditions to maintain cDC and pDCs, produced by hematopoietic and stromal cells [9]. Mice deficient in FL lack DCs [10].

Exogenous administration of FL to mice expands DCs within the spleen and lymphoid tissues, and increases tissue resident DC populations, including those in the thymus and kidney [11-13], as well as expanding regulatory T cell (Tregs) populations [14, 15]. Administration of FL to humans also expands immature myeloid DCs, pDCs and Tregs in peripheral blood, and is being explored as immunotherapy for certain malignancies [16-18].

Rapidly progressive glomerulonephritis (RPGN) is characterized by cellular crescents and fibrinoid necrosis of the glomerular tuft. Current therapies are largely non-specific immunosuppressive agents. There is increasing evidence from animal and human studies that Tregs are protective in RPGN [19-24]. Therefore, we hypothesized that exogenous FL may protect mice in experimental RPGN by suppressing nephritogenic immunity via expansion of pDCs and Tregs.

Materials and Methods

Experimental design

Male C57BL/6 mice, aged 6-10 weeks were purchased from Monash Animal Research Platform (Monash University) and were housed in specific pathogen free conditions (Monash Medical Centre Animal Facility, Clayton, Victoria, Australia). Studies were performed in accordance with the National Health and Medical Research Council's Australian code for the care and use of animals for scientific purposes and were approved by the Monash University Animal Ethics Committee B (Ethics Number MMCB12/42). Aged matched mice were randomly assigned to experimental groups, had free access to water and food throughout experiments and were reviewed daily by both the researchers and animal facility staff. Mice were humanely euthanized with carbon dioxide at the completion of experiments or if mice showed any signs of the following: lethargy, persistent recumbency, hunched posture, rough coat or loss of body condition.

FL–Ig (human/human, BioXcell, West Lebanon, NH, USA; as cited in [25, 26]) was delivered as 10 μg in 200 μL PBS daily intraperitoneal injections for 10 days, as previously published [15]. Control mice received the same volume of PBS at the same times. Initial assessment of the effect of FL vs PBS on naïve mice was made with 4 mice per group. The model of RPGN used was an accelerated autologous phase anti-glomerular basement membrane (GBM) model (n=6-9 each group; numbers were determined based on prior experience with this model [19]). Mice were sensitized s.c. with 0.5mg normal sheep globulin (in Freund's complete adjuvant [FCA]) to the right and left tailbase. Four days later, sheep anti-mouse GBM globulin was injected intravenously into the tail-vein and mice were humanely killed after a further 10 days. For delayed type hypersensitivity (DTH), 0.5mg sheep globulin or horse globulin (as a control) was injected into the right and left hind footpads, respectively, and footpad swelling was measured 24hrs later with a micrometer (n=4 each group). Statistical analysis was performed on Graphpad Prism 6 software. Data are presented as mean (±SEM) or median (range); assessment of 2 groups was performed with a student's t test or Mann Whitney test for parametric and non-parametric data, respectively. Differences in survival were assessed with a log-rank test. Significant values were defined as $P<0.05$.

Assessment of functional and histological injury

Mice were placed on metabolic cages to collect urine prior to the end of experiments. Proteinuria was assessed by Bradford's assay. Serum was collected from mice and urea levels were measured. To assess renal histology, 3 μ m-thick formalin-fixed, tissue processed, then paraffin embedded kidney sections were stained with Periodic Acid Schiff's reagent. Forty glomeruli were assessed for glomerular segmental necrosis and crescent formation (primary outcome). CD4+ T cells, macrophages, and neutrophils were detected by immunoperoxidase staining of 6 μ m-thick, periodate lysine paraformaldehyde-fixed, frozen kidney sections as previously described [27]. Primary antibodies used were: CD4+ T cells (anti-CD4, GK1.5), macrophages (anti-CD68, FA/11), neutrophils (anti-Gr-1, RB6-8C5) with isotype controls being IgG2b, IgG2a and IgG2b, respectively. For leukocyte infiltration, 20 glomeruli and 10 interstitial high-powered fields were assessed.

Assessment of immunity by flow cytometry

Lymph nodes (cervical, axillary, brachial, inguinal, mesenteric and para-aortic, pooled for each animal) and spleens were harvested and single cell suspensions were created. One million cells were stained for flow cytometric analyses using the following antibodies: PDCA-1 (eBioscience, San Diego, CA, USA; Bio927), CD11c (BD Biosciences, North Ryde, NSW, Australia; HL3), CD4 (BD Biosciences; GK1.5), foxp3 (eBioscience; FJK-16s), CD25 (BD Biosciences; PC61). For intracellular cytokine staining, cells were fixed with eBioscience Fixation/Permeabilization concentrate, diluent and staining buffer according to the manufacturer's protocol. Flow cytometry was performed on BD FACS Canto II and data analysed using FlowJo software (TreeStar, OR, USA).

Assessment of systemic immune responses to sheep globulin

Cultured cell supernatant was collected after stimulating 4 x 10^6 splenocytes with 100μg sheep globulin in 1mL RPMI-Complete (containing RPMI, 10% foetal calf serum, 50μM 2ME, 10mL penicillin-streptomycin and 5mL of L-glutamine) for 72 hours. IL-17A, IFN γ and IL-4 concentrations were measured by ELISA [28, 29] and other cytokines were measured with the Mouse Inflammation cytometric bead array (BD Biosciences). EliSpot assays for IFN γ (BD Biosciences) and IL-17A (eBioscience) were performed as previously described [30]. Spots were enumerated using an AID EliSpot platereader and software (v4.0, Autoimmun Diagnostika GmbH,

Strassberg, Germany). Mouse anti-sheep IgG antibody levels were measured on diluted serum samples by ELISA [31].

Assessment of CD11c+ DCs in the kidney

To assess renal DCs, immunofluorescent staining of frozen kidney sections was performed using an anti-CD11c antibody (BD Biosciences; HL3) and mounted with DAPI mounting media (Molecular Probes). Images were acquired on a Nikon C1 confocal laser scan head attached to a Nikon Ti-E inverted microscope (Nikon, Tokyo, Japan) using 488 and 561nm lasers. Renal DCs were assessed in three areas: within glomeruli, in the periglomerular region (within 3 cells of Bowman's capsule) or within a high-powered interstitial field. Twenty glomeruli and periglomerular regions and 10 high-powered fields were assessed for each animal, with CD11c+ immunofluorescence for each of these regions being analysed by Image J software (NIH, Bethesda, MD, USA).

Results

Effects of FL on Tregs and DCs

Daily administration of FL for 10 days (Fig. 1A) macroscopically increased spleen size and the size of all LN groups. When quantified, there were increased splenic cell numbers, with a trend towards increased cell numbers in pooled LN (Fig. 1B). CD11c+ cells were also increased in the spleen and LN after FL administration (Figs. 1C-D). Proportions of pDCs (CD11c+PDCA-1+/CD11c+ cells) were significantly elevated in the spleen, with a trend towards increase in the LN (Figs. 1C and E). The proportion of Tregs (CD4+foxp3+/CD4+ cells) in FL treated animals was significantly increased (Figs. 1F-G).

To determine if increased populations of pDCs and Tregs induced by FL protected mice from generating effector T cell responses, PBS or FL was administered to mice for 10 days, then mice were sensitized to sheep globulin. Mice were then culled 4 or 10 days after sensitization (Fig. 2A). At 4 and 10 days post-sensitization, the numbers of splenocytes and LN cells were not different between PBS and FL treated mice (Figs. 2B-C). Four days after sensitization to sheep globulin, the proportion of CD11c+ cells remained elevated in the LN, but not the spleen of FL treated mice (spleen PBS 20.9±2.5 vs FL 19.4±2.6%, P=0.7; LN PBS 6.6±0.3 vs FL 9.6±0.4%, P<0.005). FL treated mice had higher proportions of pDCs in spleen and LN (Fig. 2D), Ten days after sensitization to sheep globulin, FL treated mice had a reduced proportion of CD11c+ cells in the LN (spleen PBS 4.3±2.2 vs FL 2.4±0.1%, P=0.44; LN PBS 1.2±0.03 vs FL 0.9±0.02%, P<0.001), but there was no longer any detectable difference in pDC proportions (Fig. 2E). No significant differences in Treg proportions were identified four days after sheep globulin sensitization between mice that had been treated with PBS or FL (spleen PBS 7.2±0.8 vs FL 8.6±0.3%, P=0.16; LN PBS 8.6±0.1 vs FL 9.5±0.5%, P=0.11). Four days after sheep globulin sensitisation, FL mice developed increased dermal DTH to sheep globulin, suggesting elevated pDC populations did not suppress antigen-specific immunity, but rather enhanced effector T cell function (Fig. 2F). When mice were challenged with sheep globulin 10 days after sensitization, DTH was present to a similar degree in PBS and FL treated groups (Fig. 2G).

FL administered prior to the induction of nephritogenic immunity

When FL or PBS was administered for 10 days before the model of RPGN (with treatment ending the day before mice were immunized with sheep globulin), injury was similar in both groups of mice. No significant differences in renal functional injury (proteinuria or urea; Fig. 3A-B) or histological damage (glomerular crescents or segmental necrosis; Fig. 3C-E) were found. While the total number of splenocytes or LN cells was not different between groups, the proportion of splenic CD11c+ cells was reduced in FL treated mice (Fig. 3F-G), but unaltered in the LN between groups. There was no difference in the proportion of pDCs (Fig. 3H), nor was there a difference in the proportion of activated T cells (CD4+CD25+/CD4+ cells) in the spleen and LN between the two groups (spleen PBS 4.4±0.4 vs FL 5.5±0.4%, P=0.12; LN PBS 34.1±3.6 vs 35.9±2.8%, P=0.7). With respect to cellular immune responses, FL treated mice had reduced IL-17A levels in sheep globulin stimulated splenocyte supernatant compared to controls, but no differences were found in IFN γ, IL-4 or IL-10 concentrations (Table 1); TNF, IL-12p70 and IL-6 concentrations were below the threshold for detection in both groups. FL treated animals had a trend towards lower serum mouse anti-sheep immunoglobulin titres (Fig. 3I). Therefore, although FL did not protect mice when given prior to the induction of crescentic GN, some elements of cellular and humoral immune responses were attenuated by FL therapy, suggesting that FL might alter renal injury if delivered throughout the entire course of this disease model.

FL treatment beginning at sensitization, continuing throughout the disease model

When FL treatment commenced on the day mice were sensitized to sheep globulin and continued through the course of disease (Fig. 4A), more FL treated animals unexpectedly developed signs of renal failure, and met criteria for humane euthanasia, compared to PBS treated controls, occurring after sheep anti-mouse GBM globulin administration during nephritis (Fig. 4B); the experiment was terminated early at day 10 of the model. A repeat experiment was performed, with animals being euthanized at day 9 (five days after anti-GBM administration; Fig. 5A). Mice in both groups sustained significant functional renal injury (serum urea; PBS 121±16 vs FL 126±10 mmol/L, P=0.78), with comparable proteinuria and histological damage, marked by widespread glomerular segmental necrosis (Figs. 5B-E). FL treated mice had enhanced interstitial neutrophil recruitment compared to controls

(Table 2). Renal CD11c+ cells were increased within glomeruli of FL treated mice, with a trend to increase in periglomerular and interstitial regions (Figs. 5F-I).

Systemic inflammatory immune responses were heightened in the FL treated mice, with significantly increased IL-17A production, and a trend towards enhanced IFN γ production by splenocytes (Figs. 6A-B). IL-6 concentrations were not different between groups (median with range, PBS 0 [0-12] vs FL 0 [0-14], P=0.29). IL-12p70, TNF and IL-10 concentrations were undetectable in both groups. Within the spleen and LN, cell numbers were similar between groups, but FL treated mice had significantly increased proportions of CD11c+ cells and reduced proportions of pDCs (Figs. 6C-F). FL treated mice had elevated proportions of activated (CD4+CD25+) T cells and Tregs (CD4+CD25+foxp3+), with a similar proportion of effector T cells (CD4+CD25+foxp3-) within LN, but in the spleen FL treated mice had fewer activated T cells and Teff (Figs. 6G-J). Therefore, FL administration throughout experimental RPGN promoted maturation of DCs towards a conventional DC phenotype. It did not suppress nephritogenic immunity, but rather FL enhanced systemic immunity and T cell activation.

FL administered before and during the induction of nephritogenic immunity

This model of RPGN relies on planting sheep globulin, a foreign antigen, within the glomerulus of sensitized mice. It was possible that exogenous FL, administered at the time of anti-GBM globulin injection, enhanced the maturation and expansion of cDCs in sensitized mice rather than polarizing precursor DCs towards this phenotype. We sought to determine if FL could induce pDCs and enhance Treg populations in steady state conditions and suppress subsequent antigen-specific responses. Therefore, we administered FL before and during the sensitization period (i.e. when DCs were playing a key role in inducing immunity to sheep globulin) but discontinued it at the time nephritis was induced (Fig. 7A).

Compared to controls, FL treated mice showed similar functional injury, with a trend towards fewer glomerular crescents and less segmental necrosis (Figs. 7B-E). There was no difference in splenocyte number between groups, but LN cell numbers were reduced in FL treated mice (Fig. 7F). Splenic regulatory T cell populations of FL treated animals were enhanced, but fewer Tregs were present in the LN of FL treated mice (Fig. 7G). There were no significant differences in the proportions of CD11c+ cells and pDCs between groups (CD11c+ spleen PBS 1.3±0.3 vs FL

0.8±0.1%, P=0.11; CD11c+ LN PBS 1.9±0.1 vs FL 1.8±0.3, P=0.8 and pDCs spleen PBS 1.4±0.6 vs FL 1.3±0.7%, P=0.91; pDCs LN PBS 4.5 ±1.2 vs FL 5.3±1.2%, P=0.67). There was no difference in IFN γ, IL-17A, IL-4 or IL-10 concentrations in stimulated splenocyte supernatant (Table 3); IL-6, IL-12p70 and TNF concentrations were undetectable. Serum antigen specific IgG antibodies between groups were not different (Fig. 7H). Therefore, FL administration before and during the sensitization phase did not result in enhanced injury as seen when FL was given throughout the nephritis phase, but did not protect mice from effector T cell mediated renal injury.

Discussion

We have shown that FL administered under homeostatic conditions expands Treg and DC populations. DC expansion was not restricted to pDCs, but both cDC and pDC populations were increased. Despite inducing Tregs, FL given prior to autologous phase accelerated anti-GBM GN did not result in significant protection from renal injury, although we found evidence that some elements of systemic immunity were attenuated, with reduced IL-17A production from splenocytes. When FL was given throughout the nephritis model, FL treated mice exhibited excess mortality. This was likely, but not certain, to be due to renal disease. We repeated this model, euthanizing mice earlier to assess renal injury and systemic immune responses prior to mice becoming too unwell. We found similar, but severe, glomerular injury in both groups. However, glomerular CD11c+ DCs and neutrophil recruitment to the renal interstitium was increased in FL treated mice compared to controls, indicating greater pro-inflammatory local immune responses had developed in the FL group. Systemic immune responses were also increased in the FL group, with enhanced Th17 cellular immunity and increased cDC populations in secondary lymphoid organs. Therefore, FL administration throughout RPGN polarized naïve T cells towards an effector rather than a regulatory phenotype.

Despite the persistence of a higher proportion of pDCs after FL had been given for 10 days, mice subsequently sensitized to sheep globulin had enhanced dermal DTH 4 days later. Therefore, despite enhanced Treg populations and pDCs at the time of sensitization, the induction of immunity to sheep globulin enhanced effector T cell responses, indicating effective antigen presentation by the DC populations. When FL was given before and during sensitization, but not during nephritis, renal injury or systemic immune responses were not altered. These findings suggest that when FL was given in steady-state conditions, expanded DC populations and Treg induction were capable of modulating effector T cell responses and cytokine release.

There are variable reports of the effectiveness of FL therapy in experimental autoimmune disease. In the NOD mouse type I diabetes model, pancreatic beta cell destruction was prevented by administering FL prior to the development of pancreatic injury in young, but not in older mice, despite increasing DC and Treg populations in spleen and pancreatic LN [32, 33]. The onset of diabetes was accelerated by FL therapy given to NOD mice with detectable auto-reactive T cells

with increased pro-inflammatory cytokine expression [33]. FL attenuated inflammatory bowel disease in the TNFΔARE mouse model, by enhancing Tregs [34].

In models of T cell dependent inflammation or asthma/airways disease induced by foreign antigens, FL therapy has also provided mixed results. Experimental methylated bovine serum albumin induced arthritis was not significantly attenuated by FL treatment [35], whereas treatment with sumatinib (a tyrosine kinase receptor inhibitor directed against the FLT3 receptor) protected mice from joint damage [35, 36]. FL therapy did protect mice from allergic airways inflammation, with evidence for either a Th2 to Th1 shift in immunity [37, 38] or enhanced Treg function and recruitment [39, 40]. Similarly, experience with FL in models of transplantation provides conflicting results. Allogeneic T cell responses were reduced and mouse survival increased when bone marrow recipients were pre-treated with FL in a model of experimental graft vs host disease [15], and when FL treated renal DCs were transferred into mice receiving allogeneic cardiac transplants [13]. However, FL administration to donors prior to liver transplantation expanded myeloid and lymphoid DCs in the grafts, worsened graft survival, with evidence of heightened Th1 immune responses and less apoptosis of alloreactive T cells [41].

In vitro and *in vivo* studies suggest DC maturity directs their suppressive or inflammatory capacity. In steady state, DCs from mice receiving FL had an immature phenotype and were poor stimulators of T cell proliferation in co-culture *ex vivo*, but they could induce expansion of regulatory cells [13, 15, 42, 43], without requiring MHCII expression [15]. However, lipopolysaccharide (LPS)-matured FL-induced DCs potently induced naïve T cell proliferation without Treg expansion [13]. Bone marrow derived DCs cultured with FL plus IFNα or LPS had increased surface expression of activation markers and enhanced T cell proliferation compared to bone marrow DCs cultured with FL alone. Therefore, in an inflammatory milieu, FL treated DCs mature and take on a more conventional phenotype [42]. Similarly, compared to FL treated mice receiving peptide immunisation alone, FL treated mice receiving immunisation with peptide and adjuvant (FCA) had enhanced T cell activation and proliferation, and more DCs with a mature phenotype [44]. In the presence of toll like receptor 9 agonists or inactivated influenza virus, pDCs transformed into cells exhibiting a cDC phenotype, with increased IFNα production and the capacity to stimulate T cells in vitro [45]. Transfer of immature FL treated DCs into mice prior to experimental autoimmune encephalitis (EAE) induction (with myelin oligodendrocyte

glycoprotein/FCA) resulted in less severe disease than mice pre-treated with TNF and LPS matured FL DCs [46]. When FL therapy was initiated five days prior to EAE induction and continued for a further five days, FL treated mice developed increased disease severity compared to controls [47], whereas treatment of mice with established EAE with a FL inhibitor (CEP-701, which induced apoptosis of mature DCs) resulted in improvement in disease progression [48]. These studies suggest that although FL therapy promotes immature DCs and Tregs in steady state, these DCs can become polarised towards a conventional phenotype in the presence of pro-inflammatory cytokines and proteins. This may explain some of the conflicting reports about the immunomodulatory effects of FL, and why FL was not protective when given during our experimental crescentic GN model, which relies on an adaptive immune response to sheep globulin planted along the GBM in sensitized mice [46].

The current studies have shown that FL administered throughout a murine model of RPGN heightened inflammatory effector T cell immune responses, resulting in reduced animal survival. The pitfalls of FL therapy demonstrated in this work highlight the challenges inherent in manipulating cellular immunity to promote Tregs in immune renal disease.

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Figures

Figure 1

The effects of 10 days of intraperitoneal FL on DC and Treg populations.

(A) Experimental design, where FL or PBS was administered intraperitoneally to mice for daily for 10 days. (B) Total spleen and pooled LN cell number. (C) Representative FACS plots showing proportions of pDCs (CD11c+PDCA-1+ cells) in the spleen, gating on all CD11c+ leukocytes. (D) Proportions of CD11c+ cells in the spleen and LN. (E) Proportions of pDCs in the spleen and LN. (F) Representative FACS plots showing Tregs in the spleen, gating on lymphocytes, staining for CD4+ and foxp3+ cells. (G) Proportion of Tregs in the spleen and LN. Black bars represent PBS treated mice. White bars represent FL treated mice. n=4 per group. *P<0.05, **P<0.01, ***P<0.001.

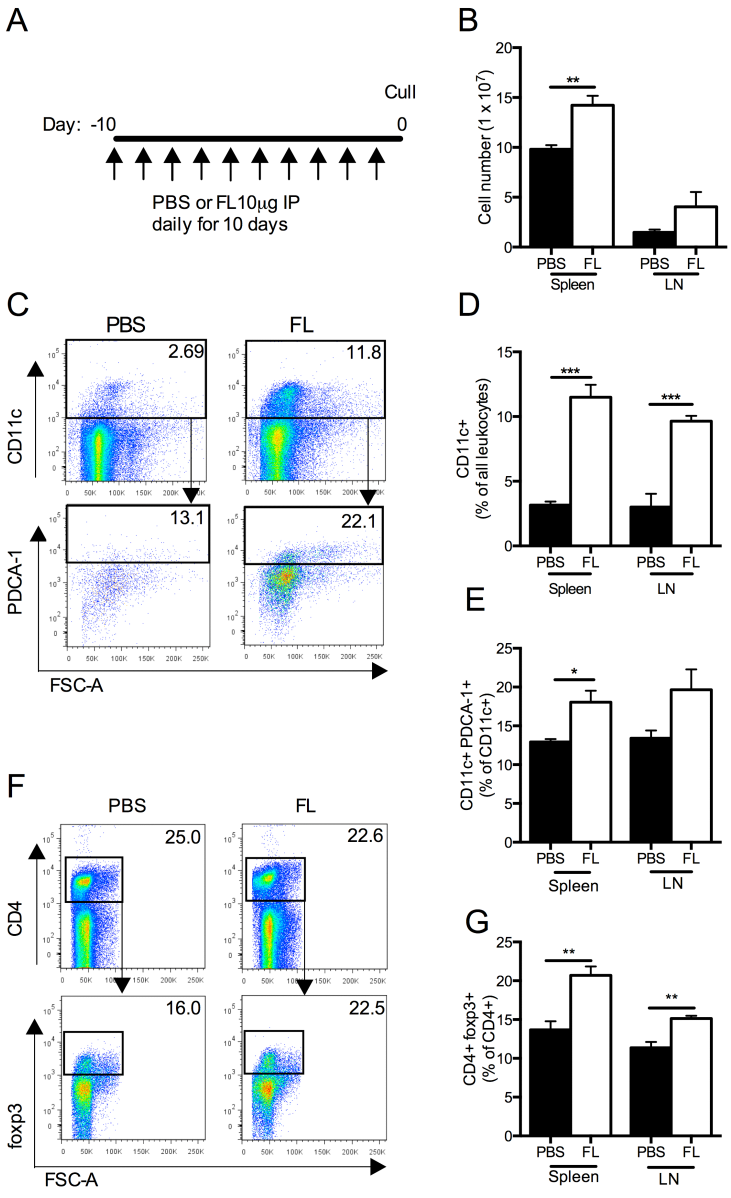


Figure 2

FL therapy expands pDCs but increases delayed type hypersensitivity four days after sensitization to sheep globulin.

(A) Experimental design. (B, C) Total spleen and LN cell number 4 and 10 days after sensitization, respectively. (D, E) Proportion of pDCs in the spleen and LN 4 and 10 days post-sensitization to sheep globulin respectively. (F, G) Footpad swelling in mice 4 and 10 days post-sensitization to sheep globulin, respectively. Black bars represent PBS treated mice. White bars represent FL treated mice. n=4 per group. *P<0.05, **P<0.01.

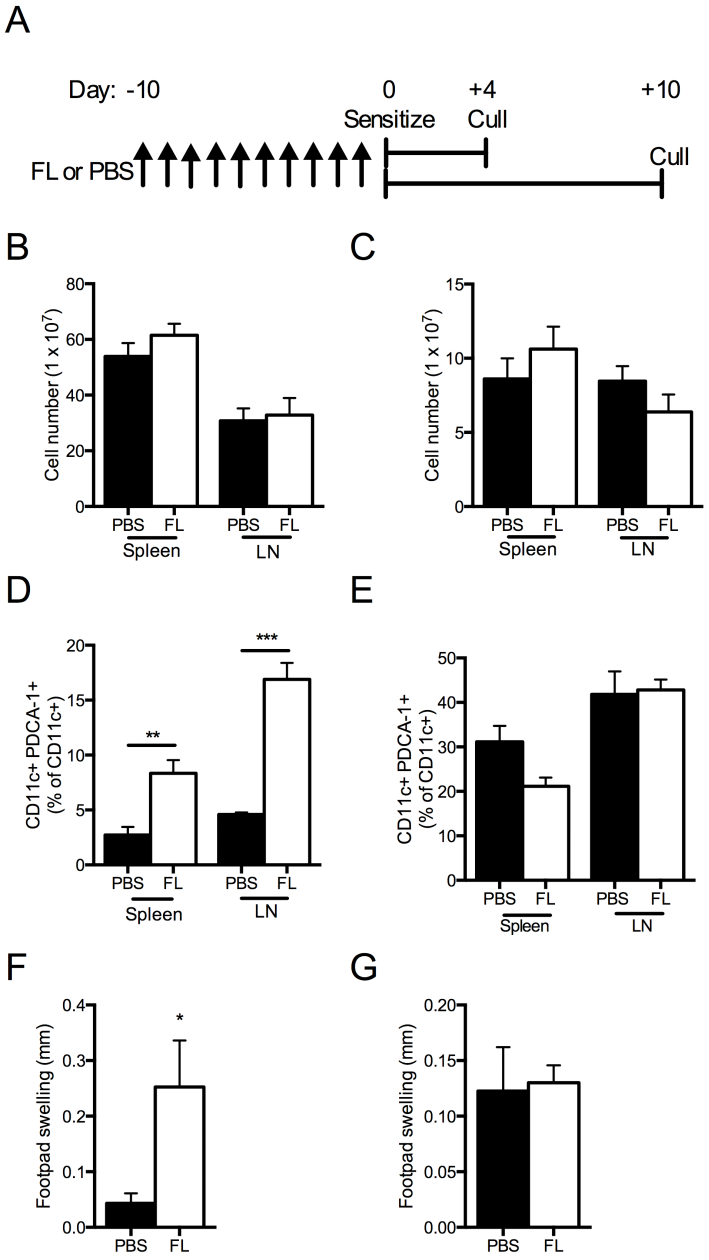


Figure 3

FL administered before accelerated autologous phase anti-GBM disease did not protect against nephritis, but altered systemic immunity.

(A) Proteinuria (dotted line represents measured level in non-nephritic WT mice, n=4). (B) Serum urea (dotted line represents measured level in non-nephritic WT mice, n=4). (C) Proportions of glomeruli with crescents and (D) glomerular segmental necrosis. (E) Representative images of glomeruli from PBS and FL treated mice taken at high power (400x, PAS stain). (F) Total spleen and LN cell number. (G) Proportion of CD11c+ cells from leukocytes in spleen and LN. (H) Proportion of pDCs in spleen and LN. (I) Serum mouse anti-sheep IgG antibody levels. Black bars represent PBS treated mice. White bars represent FL treated mice. OD, optical density. n=6 per group. *P<0.05.

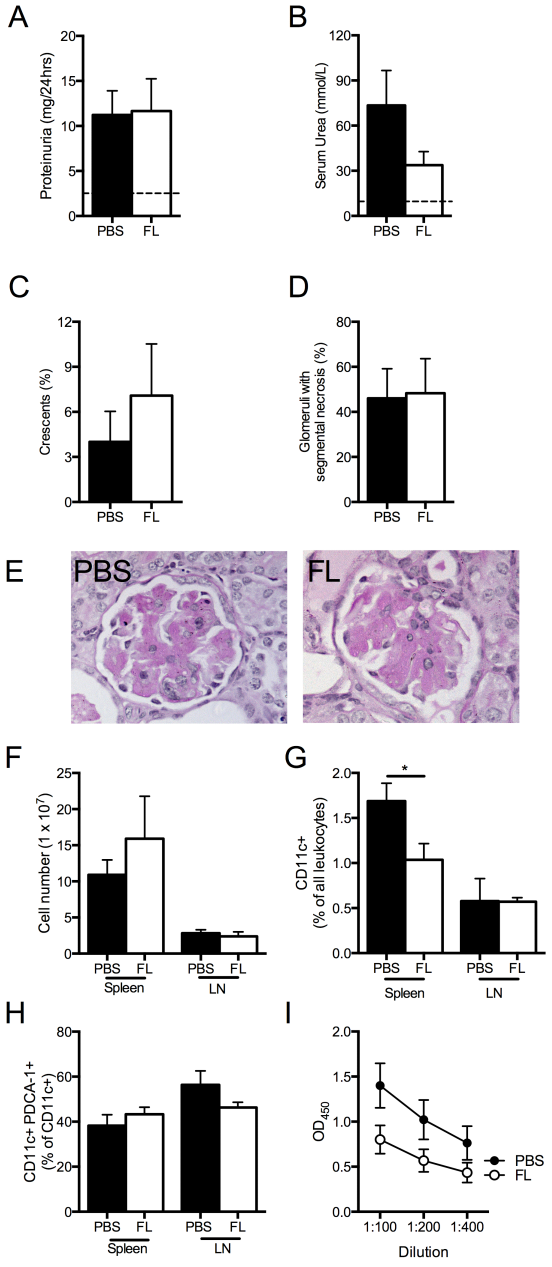


Figure 4

FL administered throughout accelerated autologous phase anti-GBM disease reduced mouse survival.

(A) Experimental design. (B) Survival following the induction of nephritis. Black bars represent PBS treated mice. White bars represent FL treated mice. n=9 per group. *P<0.05.

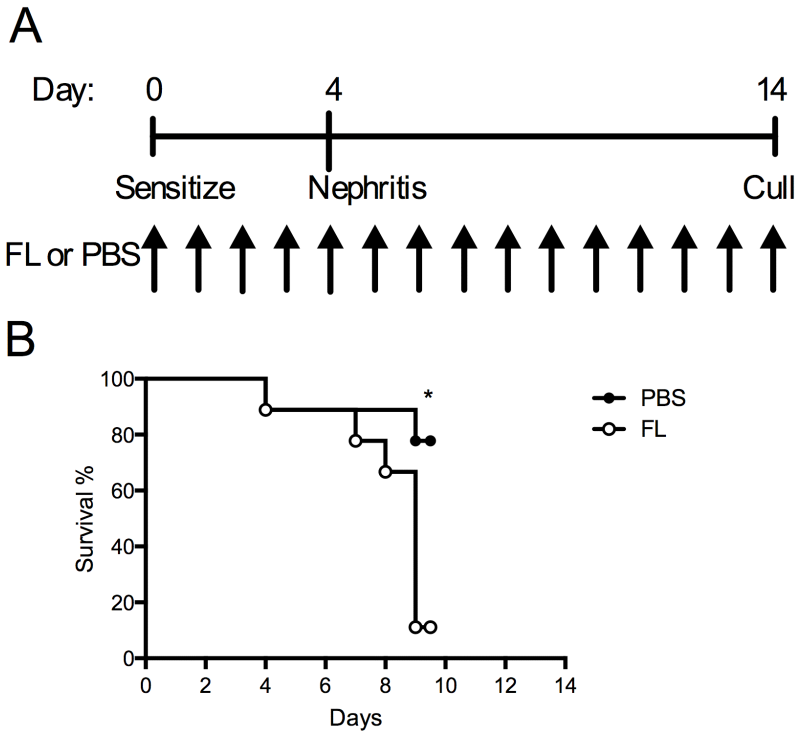


Figure 5

FL therapy throughout accelerated anti-GBM disease did not protect mice from nephritis.

(A) Experimental design. (B) Proteinuria. (C) Percentage of glomerular crescents and (D) glomeruli with segmental necrosis. (E) Representative images of glomeruli from PBS and FL treated mice taken at high power (400x, PAS stain). (F-H) gcs, periglomerular and interstitial hpf CD11c+ immunofluorescence assessed by fluorescent microscopy of renal sections. (I) Representative images of CD11c+ immunofluorescent staining within the interstitial, glomerular and periglomerular regions. Black bars represent PBS treated mice. White bars represent FL treated mice. AU, arbitrary units; gcs, glomerular cross section; hpf, high-powered field. n=7 per group. *P<0.05.

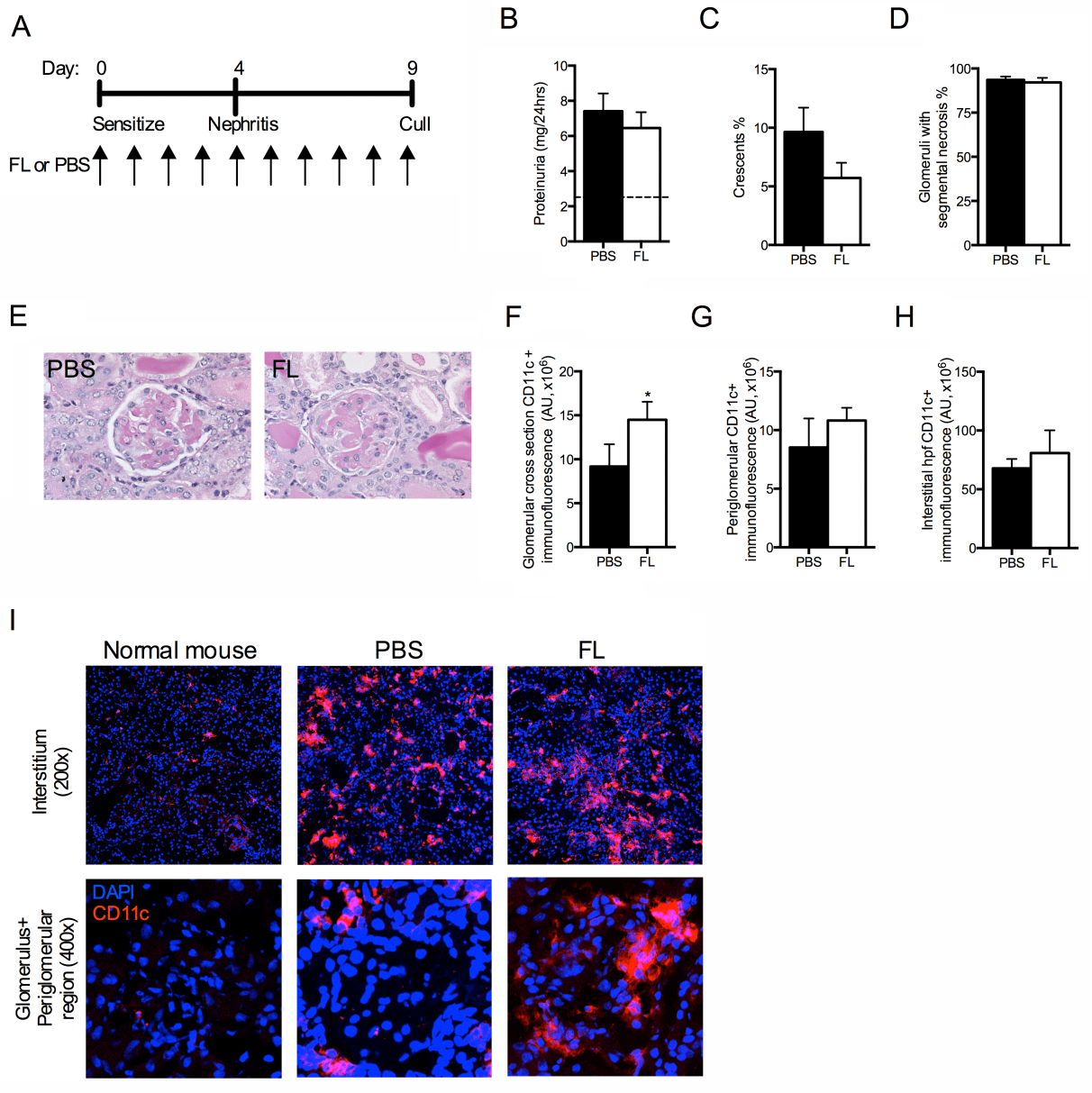


Figure 6

Cellular pro-inflammatory immune responses were heightened when FL was given during accelerated anti-GBM disease.

(A) IL-17A+ spots and (B) IFN γ + spots per 1 million stimulated splenocytes, measured by EliSpot. (C) Total spleen and LN cell number. (D, E) Proportion of CD11c+ cells and pDCs in the spleen and LN. (F) Representative FACS plots of splenocytes stained for CD11c and PDCA-1. (G) Proportion of activated T cells (CD4+CD25+/CD4+) in spleen and LN. (H) Representative FACS plots of pooled LN cells stained for CD4, CD25 and foxp3. (I, J) Proportions of Tregs (CD4+CD25+foxp3+/CD4+) and Teff (CD4+CD25+foxp3-/CD4+) in spleen and LN. Black bars represent PBS treated mice. White bars represent FL treated mice. n=7 per group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

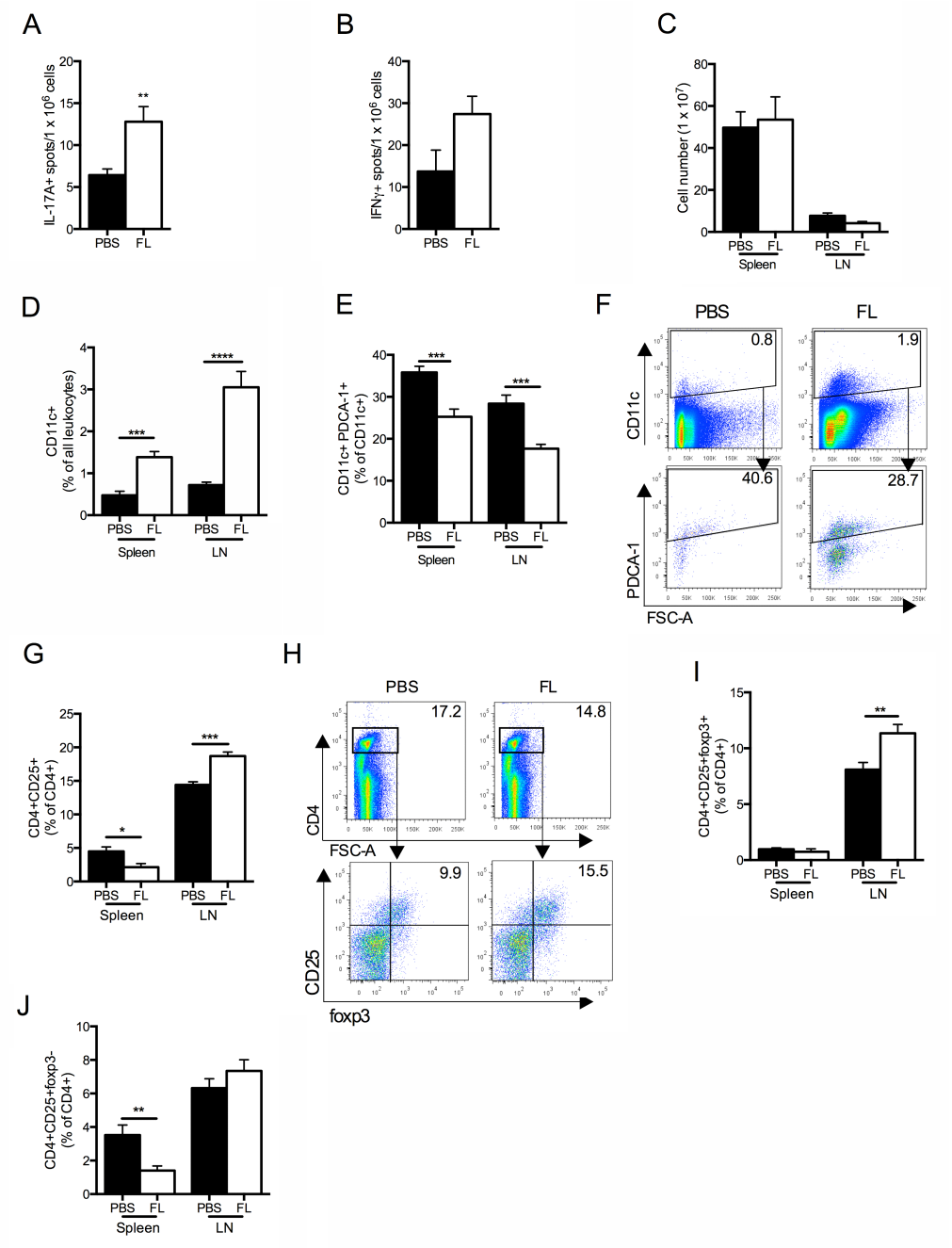
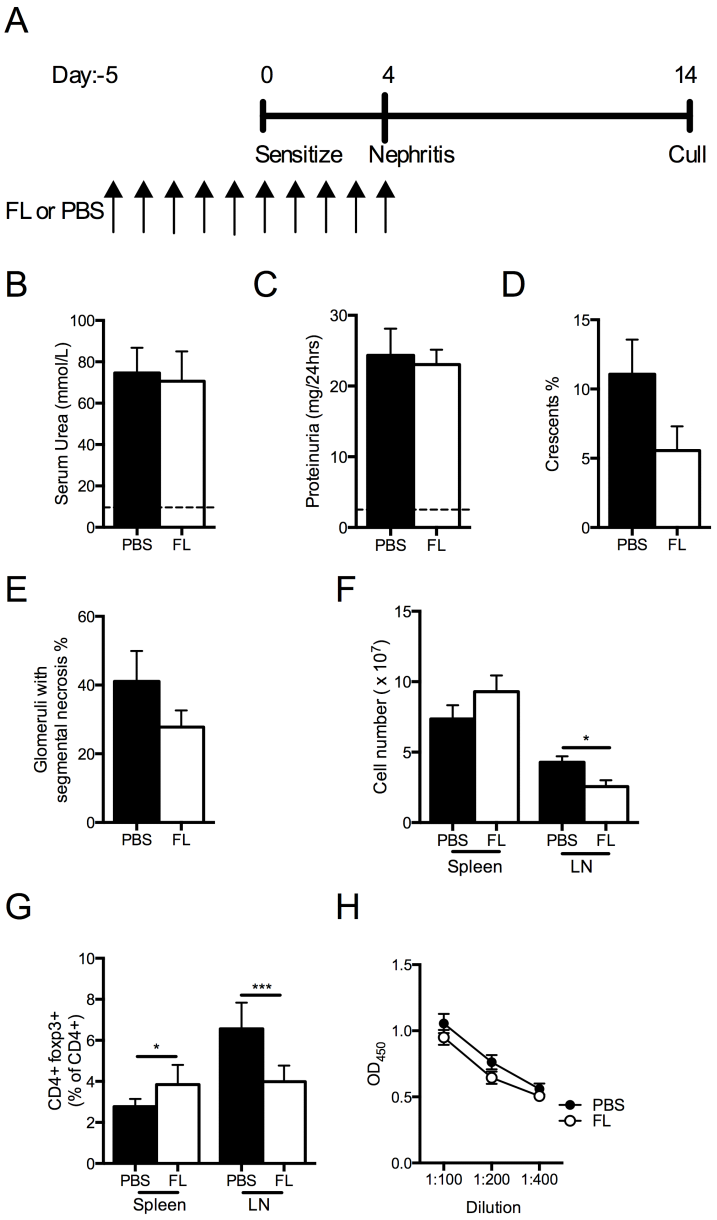


Figure 7

Effects of FL given during sensitization, but not during the nephritic phase of injury.

(A) Experimental design. (B) Serum urea (dotted line represents measured level in non-nephritic WT mice, n=4). (C) Proteinuria (dotted line represents measured level in non-nephritic WT mice, n=4). (D, E) Percentage of glomerular crescents and segmental necrosis. (F) Total spleen and LN cell number. (G) Proportion of Tregs in the spleen and LN. (H) Serum mouse anti-sheep IgG antibody concentrations. Black bars represent PBS treated mice. White bars represent FL treated mice. OD, optical density. n=7 for PBS group and 9 for FL group. *P<0.05, ***P<0.001.



Tables

Table 1

Cytokine concentrations in stimulated splenocyte supernatant for mice receiving PBS or FL for 10 days prior to commencement of the anti-GBM disease model.

	Concentration (pg/mL)		
Cytokine	**PBS** (n=5)	**FL** (n=7)	P
IL-17A	768 ± 130	290 ± 32	0.0019
IFN γ	232 ± 53	332 ± 211	0.63
IL-4	1381 ± 522	710 ± 231	0.22
IL-10	0 (0-12)	0 (0-293)	0.92

Data presented as mean±SEM, except for IL-10, presented as median (range) as data is non-parametric. TNF, IL-12p70 and IL-6 were not detectable.

Table 2

Intrarenal leukocytes in mice treated with PBS or FL throughout anti-GBM disease until day 9.

	Number of cells	
	PBS (n=7)	**FL** (n=7)
Glomeruli (c/gcs)		
Neutrophils	3.0 ± 0.3	2.4 ± 0.3
CD4+ T cells	0.2 ± 0.03	0.2 ± 0.03
CD68+ Macrophages	1.5 ± 0.2	1.7 ± 0.1
Interstitium (c/hpf)		
Neutrophils	11.4 ± 1.2	15.9 ± 1.3*
CD4+ T cells	9.8 ± 1.8	8.0 ± 1.2
CD68+ Macrophages	32.9 ± 2.5	34.7 ± 2.6

c/gcs, cells per glomerular cross section; c/hpf, cells per high power field. *P=0.02.

Table 3

Cytokine concentrations in stimulated splenocyte supernatant for mice receiving PBS or FL before and during sensitization to the nephritogenic antigen in the anti-GBM disease model.

	Concentration (pg/mL)		
Cytokine	**PBS** (n=7)	**FL** (n=9)	P
IL-17A	173 ± 49	200 ± 77	0.78
IFN γ	332 ± 45	447 ± 79	0.3
IL-4	1100 ± 213	711 ± 227	0.24
IL-10	22 (0-218)	0 (0-74)	0.57

Data presented as mean±SEM, except for IL-10, presented as median (range) as data is non-parametric. IL-6, IL-12p70 and TNF were not detected.

Chapter 5: Induced regulatory T cells are phenotypically unstable and do not protect mice from rapidly progressive glomerulonephritis.

Declaration

Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design Performing the experiments Data analysis Writing and revising the manuscript	80

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Maliha Alikhan	Performed the renal digestion for flow cytometry, including the experimental design, technical work and data analysis	
Stephen Holdsworth	Review of the manuscript	
A. Richard Kitching	Writing and revising the manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 18/3/15
Main Supervisor's Signature	[redacted]	**Date** 18/3/15

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Induced regulatory T cells are phenotypically unstable and do not protect mice from rapidly progressive glomerulonephritis

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Running Headline: iTregs are unstable and do not limit nephritis

Abstract

Regulatory T cells (Tregs) are a suppressive CD4+ T cell subset that holds therapeutic potential for inflammatory renal diseases, such as rapidly progressive glomerulonephritis. Polyclonal Foxp3+ induced Tregs (iTregs) were generated *in vitro* using IL-2, TGF-β and all-trans retinoic acid. The iTregs exhibited typical surface receptor expression and *in vitro* suppressive ability similar to natural Tregs. However, these iTregs did not regulate antigen specific delayed type hypersensitivity and systemic inflammatory immune responses, displaying an unstable phenotype by losing Foxp3 expression. In experimental rapidly progressive glomerulonephritis, transferred iTregs did not prevent renal injury or modulate systemic Th1 immune responses. Despite adding a monoclonal antibody against IL-12p40 to iTreg cultures, which enhanced their suppressive phenotype *in vitro* and regulated dermal delayed type hypersensitivity *in vivo*, iTregs induced in the presence of anti-IL-12p40 also did not protect against renal injury upon transfer, losing Foxp3 expression, especially in the transferred cells recruited to the kidney. Modifying iTreg culture conditions, by adding rapamycin or using RORγt-/- CD4+ T cells, did not enhance their stability or induce a suppressive phenotype *in vivo*. Therefore, iTregs induced via several protocols suppress T cell proliferation *in vitro*, but do not regulate experimental rapidly progressive glomerulonephritis and are unstable in this inflammatory milieu *in vivo*.

Introduction

Most forms of rapidly progressive glomerulonephritis (RPGN) are mediated by abnormal adaptive immune responses, where CD4+ T cell subsets not only promote pathological antibody deposition, but also act as cellular effectors in the kidney. However, regulatory T cells (Tregs), a suppressive T cell subset, could limit injury in these conditions. Tregs are reduced in number or function in human diseases causing RPGN, such as systemic lupus erythematosus (1) and ANCA-associated vasculitis (2-5) and play a role in restoring tolerance in anti-glomerular basement membrane (GBM) disease (6, 7). Tregs express the transcription factor Foxp3 (8), and are generated in the thymus (known as natural Tregs, nTregs). These nTregs mediate central and peripheral tolerance, preventing autoimmune effector T cell (Teff) responses and regulating inflammation. Tregs can also be generated in the periphery from naïve CD4+ T cells, upon ligation of their T cell receptor, appropriate co-stimulation and cytokine stimulation, and are known as adaptive or induced Tregs (iTregs). TGF-β is required for the development of iTregs, upregulating Foxp3 expression when CD4+ T cells are stimulated with IL-2 (9).

All-trans retinoic acid (ATRA), a vitamin A derivative produced by dendritic cells (DCs), is an important cofactor with TGF-β for the generation of Foxp3+ Tregs from naïve CD4+ T cells, upon antigen presentation by these DCs or during *in vitro* culture (10, 11). It enhances Foxp3 and antagonizes RORγt expression and IL-17A production in cultured CD4+T cells (even in Th17 inducing conditions) (11-13). Treatment of human nTregs with ATRA increases the expression of surface receptors with suppressive effects (such as CD103, CTLA-4, GITR, PD-L1 and membrane bound TGF-β), and stabilizes the phenotype of these nTregs, making them resistant to Th1 and Th17 conversion (14). As nTregs make up only a small population of all circulating CD4+ T cells, generating iTregs *in vitro* allows the therapeutic potential of Tregs to be more easily investigated.

In experimental RPGN, endogenous Tregs limit immunity and tissue injury (15, 16), and nTreg transfer also constrains disease (17). Therefore, restoring tolerance and limiting renal injury in RPGN by exploiting the immunomodulatory properties of Tregs is an attractive potential therapeutic strategy. We generated polyclonal iTregs from

naïve CD4+ T cells using ATRA, TGF-β and IL-2, and aimed to stabilize the Treg phenotype by supplementing media with anti-IFNγ, anti-IL-4 and anti-IL-12p40 monoclonal antibodies (mAb). These cells did not maintain their regulatory phenotype or induce immunomodulation *in vivo*. Further modifications to these standard protocols were similarly non-immunosuppressive *in vivo*.

Results

iTreg phenotype following in vitro culture

Culture of naïve CD4+ T cells in Treg polarizing conditions (ATRA, TGF-β, IL-2, with anti-CD3, anti-CD28, anti-IFNγ and anti-IL-4 mAb) induced Foxp3 expression in >90% of CD4+ T cells (hereto referred to as "iTregs"; Figure 1a). After 5 days of culture, iTregs produced IL-10, IFNγ and TNF, with minimal IL-6 and IL-17A production (Figure 1b). To diminish pro-inflammatory cytokine production by these iTregs, the media and cytokine mix was supplemented with a neutralizing anti-IL-12p40 mAb, to limit IL-12 and IL-23 signalling, required for IFNγ and IL-17A production by the cultured cells (referred to as "iTregs+anti-IL-12p40"). The iTregs+anti-IL-12p40 had comparable Foxp3 expression to iTregs, and produced less IFNγ, TNF and IL-10 (Figure 1a and b).

Flow cytometry confirmed that both iTregs and iTregs+anti-IL-12p40 expressed surface markers consistent with Tregs (Figure 1c). Expression of the nuclear transcription factor Helios, indicating the presence of nTregs (18, 19), was low, confirming that the cells were iTregs (Figure 1c). In an *in vitro* co-culture system, both types of iTregs suppressed Teff proliferation more effectively than nTregs (from naïve Foxp3-GFP mice), with anti-IL-12p40 mAb induction further improving the suppressive capacity of iTregs (Figure 1d and e).

The expression of chemokine receptor, surface marker and transcription factor mRNA was assessed (Figure 2a-m). Compared to expression by nTregs, iTregs and iTregs+anti-IL-12p40 had reduced CCR5 and CCR6 (Figure 2b and c), but increased CCR8 expression (Figure 2e). Adding anti-IL-12p40 mAb to iTreg cultures also enhanced CD103 (*Itgae*) expression (Figure 2l). Both types of iTregs had similar CTLA-4 expression to nTregs (Figure 2j). While there was no difference in Tbet mRNA expression, the iTregs and iTregs+anti-IL-12p40 showed less GATA3 expression than nTregs (Figure 2k-l). *Rorc* mRNA expression (encoding RORγt) was increased 28 and 30-fold in iTregs and iTregs+anti-IL12p40, respectively.

Effect of iTregs and iTregs+anti-IL-12p40 in modulating immune responses to sheep globulin (SG) in vivo

To determine the optimal dose of cells to transfer *in vivo*, different doses of iTregs or iTregs+anti-IL-12p40 were transferred into CD45.1 (Ly5.1) congenic mice that were then sensitized to SG, the nephritogenic antigen used in the subsequent models of nephritis. Dermal delayed type hypersensitivity (DTH) responses were assessed 10 days later. At transfer, >90% of the cultured iTregs were Foxp3+ (data not shown). DTH responses were not reduced in mice treated with iTregs (between 1-10x10^6 cells/mouse), but transfer of 10x10^6 iTregs+anti-IL-12p40 into mice suppressed dermal DTH (Figure 3a and b).

Systemic Th1 responses (IFNγ) were increased in mice treated with 10x10^6 iTregs, but reduced in mice given 10x10^6 iTregs+anti-IL-12p40 (Figure 3c and d). Although similar systemic Th17 responses (IL-17A) were seen between controls and mice treated with iTregs, IL-17A was reduced in mice receiving 10x10^6 iTregs+anti-IL-12p40 compared to lower doses (Figure 3e and f). A population of transferred cells (CD45.2+) could be identified in spleens of recipient Ly5.1 mice, particularly in the groups receiving the larger doses of iTregs or iTregs+anti-IL-12p40. Compared to >90% Foxp3 expression by iTregs at transfer, Foxp3 expression was reduced (to ~49-56%) in the recovered iTregs, suggesting the regulatory phenotype of these cells is unstable *in vivo*. Transferred iTregs+anti-IL-12p40 were more stable, with ~83-93% being Foxp3+ (Figure 3g-j). Humoral immunity, measured by serum anti-SG IgG titers, was not altered in mice receiving iTregs or iTregs+anti-IL-12p40 (data not shown). Therefore, iTregs cultured without anti-IL-12p40 were unstable and promoted Th1 responses *in vivo* whereas mice receiving 10x10^6 iTregs+anti-IL-12p40 had reduced cellular immunity and these iTregs better maintained their regulatory phenotype. In subsequent adoptive transfer studies in experimental RPGN, 10x10^6 cells/mouse was the dose of cells used.

Transfer of iTregs in experimental rapidly progressive GN

To determine if iTreg transfer would protect mice from glomerulonephritis, a model of RPGN ("accelerated autologous phase anti-GBM GN") was used. Mice were sensitized to SG and injected with 10mg of anti-GBM globulin 4 days later (20). 10x10^6 iTregs, iTregs+anti-IL-12p40 or media (control) was transferred into mice on the days of sensitization and anti-GBM globulin administration. The experiment was

terminated 8 days after anti-GBM globulin as mice developed severe GN, with a tendency for reduced survival in media and iTreg treated groups (Figure 4a; *P*=0.08). In mice euthanized 8 days after anti-GBM globulin, severe GN with widespread segmental necrosis was observed (Figure 4b).

A lower dose (5mg) of anti-GBM globulin was administered to sensitized mice, for milder injury, to allow better assessment of the iTregs' immunomodulatory capacity. $10x10^6$ iTregs, iTregs+anti-IL-12p40 or media was transferred into mice on the day of sensitization. There were no differences in functional or histological renal injury between groups (Figure 5a-e). While CD4+ T cell and neutrophil recruitment to glomeruli was unchanged, glomerular macrophage infiltration was increased in mice treated with iTregs+anti-IL-12p40 (Figure 5f-h). Interstitial CD4+ T cell, macrophage and neutrophil recruitment was similar between groups (Figure 5i-k). Systemic immune responses were increased in mice receiving iTregs+anti-IL-12p40, with increased IFNγ production, and a trend towards greater IL-17A production (Figure 6a-b). Serum antigen-specific antibodies were comparable between all groups (data not shown). Assessment of the transferred (CD45.2+) cells in the spleen showed that only 62% and 64% of the iTregs and iTregs+anti-IL-12p40, respectively, remained Foxp3+ (Figure 6c), indicating an unstable phenotype. A small population of CD45.2+ cells were identified among renal CD4+ cells in both groups receiving cells, but the proportion of CD45.2+ cells that maintained Foxp3 expression was low (Figure 6d and e). Therefore, iTregs, induced with or without anti-IL-12p40, do not protect against the development of RPGN, but enhance systemic Th1 immunity and on migration to the kidney lose Foxp3 expression and regulatory phenotype.

A suppressive iTreg phenotype could not be promoted through the inhibition of RORγt expression or by rapamycin treatment

Recent reports have suggested Tregs are a heterogeneous population, with some Tregs acquiring a Th1 and Th17 phenotype (21-24). To explore whether deleting RORγt stabilized their phenotype, iTregs were generated from naïve CD4+RORγt-/- T cells. After 5 days of culture, RORγt-/- iTregs had comparable Foxp3 expression to iTregs (data not shown), and produced significantly less IL-17A and TNF, comparable IFNγ and increased IL-10 (Supplementary Figure 1a). However, transfer of RORγt-/- iTregs into mice in whom GN was induced did not limit renal injury (Supplementary Figure 1b-d).

Rapamycin, an inhibitor of the mammalian target of rapamycin, can promote iTregs in *in vivo* animal models of transplantation and enhance Treg stability in *in vitro* mouse and human iTregs (25-30). iTregs were generated using TGF-β, IL-2, rapamycin and ATRA (referred to as "Rapa iTregs") (25, 26, 31). ATRA was required for the generation of high Foxp3 expression in CD4+ T cells (no ATRA 39%; with ATRA 92%). Results from experiments using Rapa iTregs are presented in Supplementary Figure 2. Despite comparable CD25, GITR, ICOS and Helios expression to iTregs, Rapa iTregs produced more IL-4, IFNγ and IL-10 (Supplementary Figure 2a-b). Rapa iTregs were less effective in suppressing Teff proliferation than nTregs and exhibited reduced CXCR3, CXCR5 and CTLA-4 mRNA expression (Supplementary Figure 2c-m). Rapa iTregs transferred into SG-sensitized mice did not suppress dermal DTH and were associated with higher systemic Th1 immunity, with 80% retaining Foxp3 expression upon recovery from the spleen (Supplementary Figure 2n-q). Therefore, the addition of rapamycin does not enhance a regulatory phenotype in iTregs.

iTreg and iTreg+anti-IL-12p40 phenotype changes upon transfer into sensitized mice

We hypothesized that more iTregs and iTregs+anti-IL-12p40 express IFNγ and IL-17A after transfer into mice with antigen-driven inflammation. $10x10^6$ iTregs, iTregs+anti-IL-12p40 or media were transferred into Ly5.1 congenic mice sensitized to SG and mice culled 6 days later, so the proportion of transferred cells (CD45.2+) expressing IFNγ and IL-17A could be determined. Both iTregs and iTregs+anti-IL-12p40 recovered from the spleens of sensitized mice showed loss of Foxp3 expression (72.2±2.4% and 69.3±2.4% of recovered CD45.2+CD4+ cells, respectively, were Foxp3+). iTregs and iTregs+anti-IL12p40 demonstrated a significant increase in IFNγ expression (Figure 7a-b) compared to before transfer. While very few iTregs or iTregs+anti-IL-12p40 produced IL-17A during *in vitro* culture, there was a trend towards increased IL-17A expression by these cells after transfer into sensitized mice (Figure 7c). These data confirm that the iTreg and iTregs+anti-IL12p40 phenotype is unstable, and that more of these cells acquire the capacity to produce Teff cytokines after transfer into mice that are sensitized to the nephritogenic antigen.

Discussion

Tregs may be a novel form of cellular therapy for immune and inflammatory diseases and in transplantation. Tregs have been defined as key regulators of the immune system and the concept that these cells could suppress pathological inflammation is an attractive one. However, endogenous Tregs exist only as a small proportion of the total CD4+ T cell population. Thus reliable methods of inducing Tregs from naïve CD4+ T cells or expanding nTregs *ex vivo* are desirable, to explore the use of Tregs in re-establishing tolerance in disease. We used modifications of standard culture protocols to generate polyclonal iTregs *ex vivo*, and tested the hypothesis that these Foxp3+ iTregs could suppress disease and prevent nephritogenic immunity in experimental RPGN. These studies demonstrate that available methods of *ex vivo* induction and expansion of iTregs generate cells that can regulate immunity *in vitro*, but are phenotypically unstable in nephritis *in vivo,* indicating they are not a viable therapeutic option in severe immune renal disease.

We generated polyclonal iTregs with >90% Foxp3 expression from naïve CD4+ T cells using ATRA and TGF-β, similar to standard protocols. These iTregs displayed a regulatory phenotype *in vitro*, including suppressive surface markers and inhibition of Teff proliferation, consistent with published findings (12, 25, 26, 32, 33). However, as iTregs produced IFNγ and TNF, a neutralizing IL-12p40 mAb was added to cultures, which further enhanced their suppressive ability *in vitro*. We assessed the regulatory capacity of iTregs and iTregs+anti-IL-12p40 *in vivo* using dermal DTH, where mice were sensitized and re-challenged to SG. While iTregs did not suppress DTH, iTregs+anti-IL-12p40 reduced DTH and pro-inflammatory cytokine production. Despite limiting DTH, *in vivo* transfer of iTregs+anti-IL-12p40 did not protect mice from RPGN. Both iTregs and iTregs+anti-IL-12p40 demonstrated an unstable phenotype, with loss of Foxp3 expression when transferred cells were recovered from the spleen and kidney. Modifying the iTregs by using rapamycin in culture or RORγt-/- CD4+ T cells did not suppress inflammatory immune responses or RPGN.

We have shown that iTregs were phenotypically unstable upon transfer, losing Foxp3 expression and producing pro-inflammatory cytokines. Other studies have demonstrated that iTregs could change phenotype in models of alloimunity. Beres et

al. found that transfer of iTregs with mismatched bone marrow did not protect from experimental graft versus host disease (GVHD); the majority of recovered iTregs were Foxp3- after 10 days, with a significant proportion producing IFNγ (34). Although ATRA enhanced the proportion of iTregs expressing Foxp3, it did not render the cells more phenotypically stable (34). Similarly, in murine xeno-GVHD, ATRA expanded Tregs from human CD4+CD25+ cells produced IFNγ and IL-17A after transfer (26). These data, together with our results showing both loss of Foxp3 and the induction of IFNγ and IL-17A in cells after transfer, show that the stability of iTregs in an inflammatory microenvironment is a significant issue.

Adoptive transfer studies of nTregs in experimental RPGN demonstrated the recipients had reduced systemic IFNγ and TNF production and fewer renal leukocytes, but the transferred nTregs were found in secondary lymphoid organs, not within nephritic kidneys (17), suggesting that modulation of systemic immunity is an important mechanism of action by Tregs. However, other studies have demonstrated that endogenous Tregs home to the kidney in experimental RPGN (15), with Treg depletion (at the time of or after renal injury is induced) altering both systemic immunity and renal Treg infiltrates (15, 16), suggesting Tregs can migrate to the kidney to suppress local inflammatory responses. Interactions between tissue chemokines and chemokine receptors on Tregs are important for Treg migration to sites of inflammation, for example, CCR4, CCR8 and CXCR3 aids Treg migration to the dermis (35-38). Less is known about Treg localization to the kidney, but CCR6 is likely to be important (39, 40). The iTregs (generated with or without IL-12p40) exhibited reduced CCR6 and CCR5 and enhanced CCR8 and CD103 expression. These features may have limited the recruitment of the (unstable) iTregs to the kidney and, together with the reduced proportions of transferred cells remaining Foxp3+ within the kidney, contributed to lack of regulatory effect.

A further reason why iTregs did not suppress nephritogenic immunity may relate to their polyclonality, as polyclonal Tregs may have less efficacy in regulating antigen-specific immune responses than antigen-specific Tregs. Studies reporting successful modulation of autoimmunity with *ex vivo* generated iTregs utilize CD4+ T cells from transgenic mice with a TCR specific for autoantigens, including models of autoimmune gastritis and diabetes (41-44). Nonetheless, there are some reports of polyclonal *ex vivo* iTregs regulating experimental inflammatory diseases, including in

experimental autoimmune encephalomyelitis (45), colitis induced by T cell transfer into SCID mice (46), experimental dust mite-induced asthma (9), lupus-like chronic GVHD (47), and a murine corneal allograft model (48).

While systemic administration of rapamycin promotes iTregs *in vivo*, with increased experimental skin graft survival (29) and improved outcomes in a experimental GVHD (31), studies using rapamycin to generate iTregs *in vitro* demonstrate mixed results in generating efficacious iTregs (26, 27). In our hands, Rapa iTregs did not have a suppressive phenotype *in vitro,* and did not suppress Th1 immune responses in dermal DTH. Epigenetic modifications of the *Foxp3* gene locus may be important in determining the stability of Tregs (49). Demethylation of CpG islands in the Treg specific demethylated region of the *Foxp3* locus can be enhanced by ATRA (14), and further improved by concurrent rapamycin treatment (50), suggesting that further exploration of the use of rapamycin in stabilizing iTreg phenotype may be warranted.

Despite high Foxp3 expression in our cultured iTregs and iTregs+anti-IL12p40, RORγt expression was unregulated. RORγt and Foxp3 can be co-expressed in naïve CD4+ T cells, and TGF-β-induced Foxp3 inhibits RORγt activity (51). Therefore, it is possible that during *in vitro* culture conditions, Foxp3 expression was sufficient to restrict RORγt-mediated IL-17A transcription, but the *in vivo* inflammatory milieu encountered by iTregs may have contributed to their loss of Foxp3 expression, removing antagonism of RORγt, promoting conversion to a Teff phenotype. In view of the substantial RORγt expression of our iTregs, we examined a functional role for RORγt in the effects of iTregs. While RORγt-/- iTregs had reduced IL-17A and TNF production compared to iTregs, in experimental RPGN they did not protect mice from injury, showing that excessive RORγt expression in itself was not the primary reason for the lack of effect of iTregs.

In conclusion, while iTregs can be generated *in vitro* with TGF-β and ATRA, yielding a high proportion of Foxp3+ cells and effectively suppressing Teff proliferation *in vitro*, they were unstable in phenotype when used in experimental RPGN, losing Foxp3 expression and promoting Th1 responses. These iTregs had some phenotypic differences from nTregs in chemokine receptor expression, which may restrict their

trafficking to the kidney. Thus, transfer of *in vitro* generated polyclonal iTregs is not yet a viable therapeutic strategy in GN. If iTregs are to be a potential therapy for human GN, further work is required to stabilize their phenotype to ensure they will have suppressive, and not detrimental pro-inflammatory actions. The current studies highlight the need for the development of strategies to stabilize iTreg phenotype, ensuring they maintain a suppressive phenotype *in vivo*.

Methods

Animals were housed in specific pathogen free facilities at Monash Medical Centre Animal Facility (Melbourne, Australia). Foxp3-GFP and RORγt-/- mice (15, 52) were bred-in house. Ly5.1 congenic mice were from the Walter and Eliza Hall Institute (Melbourne, Australia). Experiments were performed according to the National Health and Medical Research Council (NHMRC) code for the care and use of animals for scientific purposes and were approved by Monash University Animal Ethics Committee B (MMCB13/35). Male mice between 6-10 weeks were used. Mice were euthanized at the completion of experiments or if showing signs of lethargy, persistent recumbency, hunched posture, rough coat or loss of body condition. Data are presented as mean (±SEM), using student's t-test (2 groups) or ANOVA (3 or more groups). Differences in survival were assessed with a log-rank test. Significance was defined as $P<0.05$.

In vitro induction of Tregs and isolation of natural Tregs

From single cell suspensions of spleens and lymph nodes of naïve Foxp3-GFP or RORγt-/- mice, CD4+ T cells were purified using L3T4 microbeads (Miltenyi Biotec GmbH, Germany). iTregs were induced using a method adapted from published protocols (53, 54). 24-well plates were coated with 1mL anti-CD3 (BioXcell, West Lebanon, NH, 17A2; 10μg/mL; overnight 4^{o}C) then washed twice with PBS before use. CD4+ T cells ($0.5x10^{6}$/well) were cultured in 1mL of RPMI-complete (RPMI with 10% fetal calf serum, 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine, 50μM 2ME) with anti-CD28 (BioXcell, 37.51; 2μg/mL), ATRA (Sigma-Aldrich, Sydney, Australia; 1pmol/L), recombinant human (rh) TGF-β1 (Biolegend, San Diego, CA; 20ng/mL), IL-2 (eBioscience, San Diego, CA; 7.29ng/mL), anti-IFNγ (BioXcell, R4-6A2; 10μg/mL) and anti-IL-4 (11B11, in-house; 500ng/mL). A neutralizing anti-IL-12p40 mAb (C17.8; in-house; 20μg/mL (55)) was added to some cultures (iTregs+anti-IL-12p40). For Rapa iTregs, CD4+ T cells from naïve Foxp3-GFP were cultured in anti-CD3 coated plates with RPMI-complete, anti-CD28, rhTGF-β1, IL-2 and rapamycin (Life Technologies, Frederick, MD; 10ng/mL) with ATRA (1pmol/L) (25, 26, 31).

Cells were incubated at 37°C with 5% CO_2 for 3 days. Supernatants were aspirated (stored at -80°C), and replaced with 1mL of RPMI-complete with IL-2. Cells were harvested on day 5. To obtain nTregs, isolated CD4+ cells from spleens and lymph nodes of naïve Foxp3-GFP mice, were sorted on GFP using a Mo-Flo XDP cell sorter, with >97% cells being CD4+Foxp3+.

Treg suppressive assay, cytokine production and mRNA expression

T effectors (Teff) were naïve CD4+ T cells from the spleens of Ly5.1 mice, and were labeled with Cell Trace Violet (CTV) cell proliferation kit (Life Technologies; 10μM). Co-cultures of Teff ($1x10^5$) with serial dilutions of nTregs, *in vitro* generated iTregs, iTregs+anti-IL-12p40 or Rapa iTregs were stimulated with plate-bound anti-CD3 (10μg/mL), soluble anti-CD28 (0.4μg/mL) and RPMI-complete (72hr, 37^0C, 5% CO_2), to compare suppression of Teff proliferation by FACS (56). For cytokine production, supernatants from cultured iTregs were assayed using a Mouse Th1/Th2/Th17 cytometric bead array (BD Biosciences, North Ryde, NSW, Australia). mRNA was extracted from $1x10^6$ cells (Qiagen RNeasy Mini kits, Victoria, Australia). cDNA was generated using the Applied Biosystems (Foster City, CA) high capacity cDNA reverse transcription kit. RT-PCR was performed using Taqman or Power SYBR Green mastermix and probes (sequences available upon request; Life Technologies, Victoria, Australia) in a Rotor-Gene RG3000 RT thermal cycler (Corbett Life Science, Qiagen).

Assessment of immune responses to SG immunization

Ly5.1 congenic mice were sensitized to sheep globulin (SG; 0.5mg in Freund's complete adjuvant [FCA]) to the tailbase subcutaneously and iTregs or media (control) were injected into the tail vein on the same day. After 9 days, 0.5mg SG or horse globulin (control) were injected into the right and left footpads, respectively. On day 10, mice were euthanized and footpad swelling was measured, assessing dermal DTH. EliSpot assays for IFNγ (BD Biosciences) and IL-17A (eBioscience) were performed using $1x10^6$ splenocytes/well (in duplicate), stimulated with SG (57). Spots were enumerated by EliSpot platereader (Autoimmun Diagnostika GmbH, Strassberg, Germany). Serum mouse anti-sheep IgG antibody levels were measured by ELISA (58).

Experimental accelerated anti-GBM disease model and adoptive transfer studies

"Accelerated autologous phase anti-GBM GN" as a model of RPGN, was induced in mice sensitized subcutaneously with 0.5mg of SG/FCA to the tailbase at day -4, then sheep anti-mouse GBM globulin (as a nephritogenic antigen) was injected intravenously on day 0 (5mg or 10mg for mild or severe RPGN, respectively). Experiments ended on day 10. Adoptive transfer of iTregs was performed in a model of severe RPGN; mice received $10x10^6$ cells iTregs, iTregs+anti-IL-12p40 or an equivalent volume of media on day -4 and day 0 (for experiments with RORγt-/- iTregs, cell doses were $10x10^6$ and $1.25x10^6$/mouse on day -4 and 0, respectively). In the model of milder RPGN, $10x10^6$ iTregs, iTregs+anti-IL-12p40 or media were transferred on day -4 only.

Assessment of renal injury and renal leukocytes

Urine was collected from mice to assess proteinuria, by Bradford's assay, and urinary creatinine. Paraffin-embedded Periodic acid Schiff 3μm sections were assessed for glomerular segmental necrosis and crescent formation (≥40 glomeruli/mouse) and interstitial injury (10 high powered fields [hpf] at 200x (15)). Renal leukocyte accumulation was assessed in periodate lysine paraformaldehyde fixed 6μm-thick sections (≥20 glomeruli and 10 interstitial hpf/mouse) (59). Scoring of interstitial macrophage accumulation was graded based on the percentage of the interstitial hpf containing macrophages (0, no macrophages, 1:0-25%, 2:25-50%, 3:50-75% or 4:75-100%). Primary mAb were: CD4+ T cells (anti-CD4, GK1.5), macrophages (anti-CD68, FA/11), neutrophils (anti-Gr-1, RB6-8C5) with isotype control mAbs. Kidneys were digested with 1mL Hank's Buffered Saline Solution, 4mg/mL collagenase D and 100ug/mL DNase I (30 min at 37^{o}C), red blood cells lysed and CD45+ cells isolated, using CD45 microbeads (Miltenyi) and cell depletion columns, as previous described (60).

Flow Cytometry

Cells were stained with mAbs from eBioscience: CD45.1 (A20), CD45.2 (104), CD4 (RM4-5), ICOS (7E.17G9), GITR (DTA-1), IL-17A (17B7); BD Biosciences: CD25 (PC61), CTLA-4 (CD152, UC10-4F10-11), CD44 (IM7), CD69 (H1.2F3), IFNγ (XMG1.2); Biolegend: Helios (22F6), CD45 (30-F11) and propidium iodide. Intracellular cytokine staining was performed using eBioscience Foxp3

fixation/permeabilization kit for IL-17A and BD Biosciences Cytofix/Cytoperm for IFNγ. Cells were stimulated with 50ng/ml PMA and 1μg/ml ionomycin (1 hr), then 10μg/ml brefeldin A (4 hr) before staining and fixation/permeabilization. FACS was performed on a FACSCanto II instrument (BD Biosciences). Data was assessed using FlowJo software (Treestar, Oregan) with single color and fluorescence minus one control samples.

Disclosure

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Titles and Legends

Figure 1

In vitro cultured iTregs and iTregs treated with anti-IL-12p40 monoclonal antibody have a regulatory T cell phenotype.

(**a**) Representative FACS plot showing CD4+Foxp3+ expression on day 5 of *in vitro* culture for iTregs and iTregs+anti-IL-12p40. (**b**) Assessment of supernatant at day 5 from cultured iTregs and iTregs+anti-IL-12p40 for expression of IL-4, IL-6, IL-17A, TNF, IFNγ and IL-10 (cells harvested from different wells, *n*=6 per group). (**c**) Representative histograms showing the expression of the surface markers CD25, GITR, ICOS and the nuclear transcription factor Helios, by iTregs and iTregs+anti-IL-12p40, gated on CD4+Foxp3+ cell populations, following 5 days of culture. Black line represents fluorescence minus one control, red line represents iTregs, blue line represents iTregs+anti-IL-12p40. (**d**) Representative histograms from the Treg suppressive assay, demonstrating Teff (labeled with CTV) proliferation following 72 hrs of *in vitro* co-culture with Tregs. Teff:Treg ratio is displayed above each FACS plot. Co-cultures were plated in triplicate and Teff proliferation was analyzed by FACS. Black, red and blue lines indicate nTregs (sorted from naïve Foxp3-GFP+ mice), cultured iTregs and iTregs+anti-IL-12p40, respectively. (**e**) Percentage of suppression of Teff proliferation at varying Teff:Treg ratios. Results represent the mean of co-cultures performed in triplicate, on 2 separate occasions. * $P<0.05$, ** $P<0.01$, **** $P<0.0001$. CTV, Cell Trace Violet; NS, not significant; iTreg, induced Treg; nTreg, natural Treg; Teff, effector T cell; Treg, regulatory T cell.

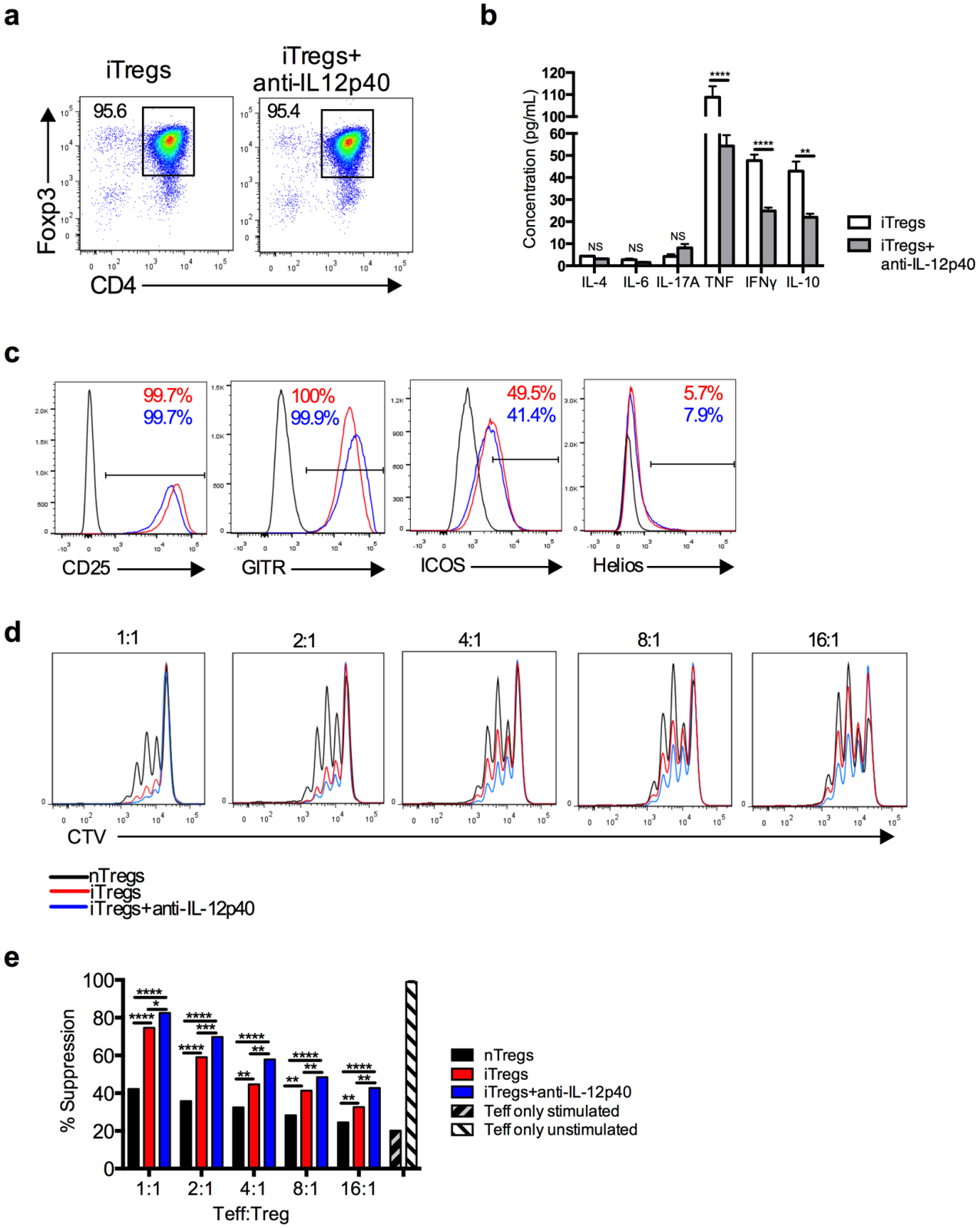

a
iTregs
iTregs+
anti-IL12p40
95.6
95.4
Foxp3
CD4
b
Concentration (pg/mL)
NS
NS
NS
****
****
**
IL-4
IL-6
IL-17A
TNF
IFNγ
IL-10
iTregs
iTregs+
anti-IL-12p40
c
99.7%
99.7%
100%
99.9%
49.5%
41.4%
5.7%
7.9%
CD25
GITR
ICOS
Helios
d
1:1
2:1
4:1
8:1
16:1
CTV
nTregs
iTregs
iTregs+anti-IL-12p40
e
% Suppression
Teff:Treg
1:1
2:1
4:1
8:1
16:1
nTregs
iTregs
iTregs+anti-IL-12p40
Teff only stimulated
Teff only unstimulated

Figure 2

RT PCR expression of chemokine receptors, regulatory molecules and T cell nuclear transcription factors highlights some differences between nTregs (from sorted GFP+ cells from naïve Foxp3-GFP mice), iTregs and iTregs+anti-IL-12p40.

mRNA was extracted from $1x10^6$ cells collected on different occasions (sorted nTregs, *n*=4; cultured iTregs and iTregs+anti-IL12p40, *n*=6). Gene of interest has been compared to 18S expression and presented as relative expression to nTregs (control group). (**a**) CCR4, (**b**) CCR5, (**c**) CCR6, (**d**) CCR7, (**e**) CCR8, (**f**) CCR9, (**g**) CXCR3, (**h**) CXCR5, (**i**) CD103 (*Itgae*), (**j**) CTLA-4, (**k**) Tbet, (**l**) GATA3 and (**m**) RORγt (*Rorc)* between groups. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. iTreg, induced Treg; nTreg, natural Treg; Treg, regulatory T cell.

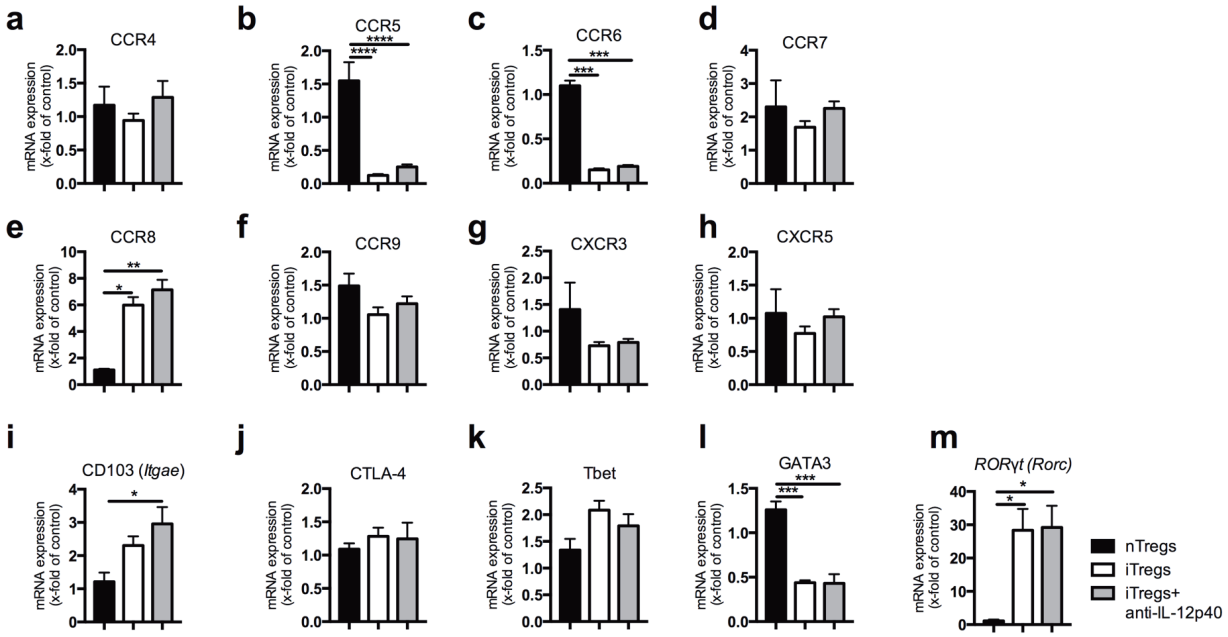


Figure 3

At a dose of 10x10^6 cells/mouse, iTregs enhanced, but iTregs+anti-IL-12p40 attenuated, Th1 immune responses in mice sensitized to sheep globulin (SG) for 10 days.

Dermal DTH, assessed 10 days after sensitization to SG (measured by footpad swelling 24 hrs after re-challenge with SG into footpad) for different doses of (**a**) iTreg or (**b**) iTreg+anti-IL-12p40. EliSpot measurement of IFNγ+ spots/1x10^6 stimulated splenocytes in mice treated with either (**c**) iTregs or (**d**) iTregs+anti-IL-12p40. EliSpot measurement of IL-17A+ spots/1x10^6 stimulated splenocytes in mice treated with either (**e**) iTregs or (**f**) iTregs+anti-IL-12p40. Proportion of transferred cells (CD45.2+) retaining CD4 and Foxp3 expression, recovered from the spleen of recipient (Ly5.1 congenic) mice treated with either 10 x10^6 (**g**) iTregs or (**h**) iTregs+anti-IL-12p40 (3x10^6 splenocytes were stained for flow cytometry, gating on live single cells, with ≥1 million events collected per mouse). Representative FACS plots of splenocytes from mice receiving 10x10^6 (**i**) iTregs or (**j**) iTregs+anti-IL-12p40, respectively. **P*<0.05, ***P*<0.01, ****P*<0.001. DTH, delayed type hypersensitivity; iTreg, induced Treg; ND, not detected; Treg, regulatory T cell.

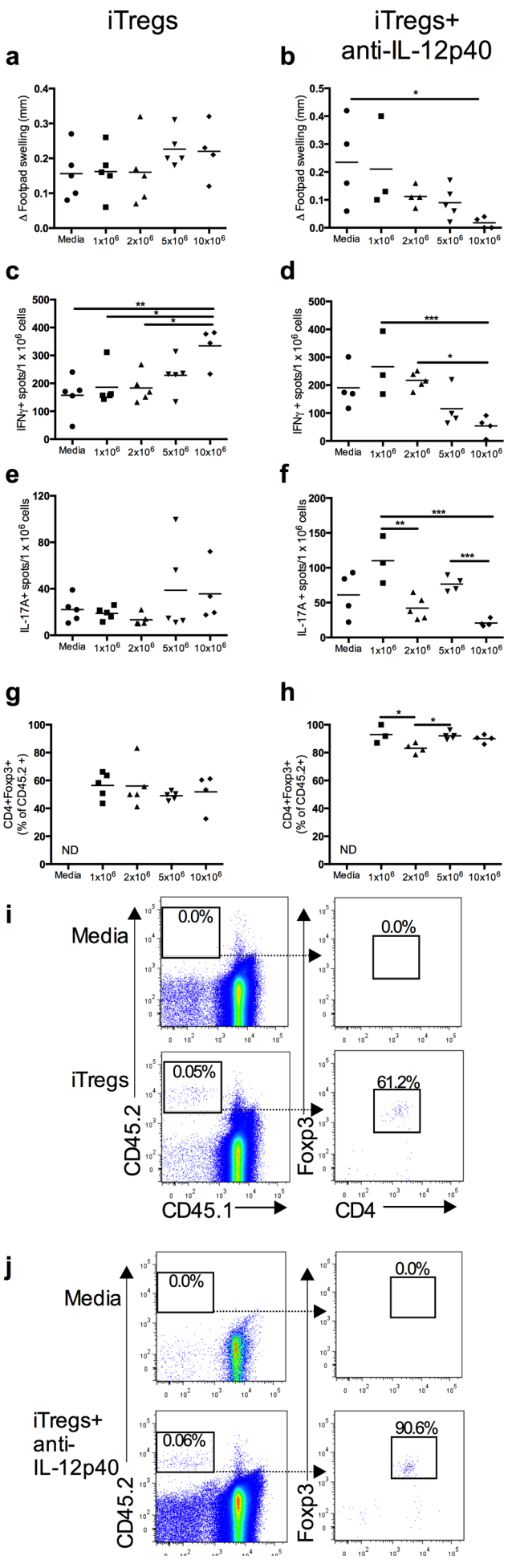

iTregs
iTregs+
anti-IL-12p40
a
b
c
d
e
f
g
h
i
j
Δ Footpad swelling (mm)
IFNγ+ spots/1 x 10^6 cells
IL-17A+ spots/1 x 10^6 cells
CD4+Foxp3+
(% of CD45.2+)
ND
Media
1x10^6
2x10^6
5x10^6
10x10^6
0.0%
0.05%
61.2%
0.06%
90.6%
CD45.2
CD45.1
Foxp3
CD4
iTregs
iTregs+
anti-
IL-12p40

Figure 4

iTregs and iTregs+anti-IL-12p40 did not protect mice from severe RPGN.

(**a**) Survival between mice receiving media (n=5), $10x10^6$ iTregs (n=9) or iTregs+anti-IL-12p40 (n=9) in a model of severe RPGN (using the accelerated anti-GBM disease model, with mice sensitized subcutaneously to SG/FCA receiving 10mg of sheep anti-GBM globulin intravenously), terminated early at day 8 following sheep anti-mouse GBM antibody administration (P=0.08). (**b**) Representative PAS stained glomeruli from media, iTreg and iTreg+anti-IL-12p40 treated mice, showing severe glomerular segmental necrosis (400x; scale bar=50μm). iTreg, induced Treg; PAS, Periodic Acid Schiff; Treg, regulatory T cell.

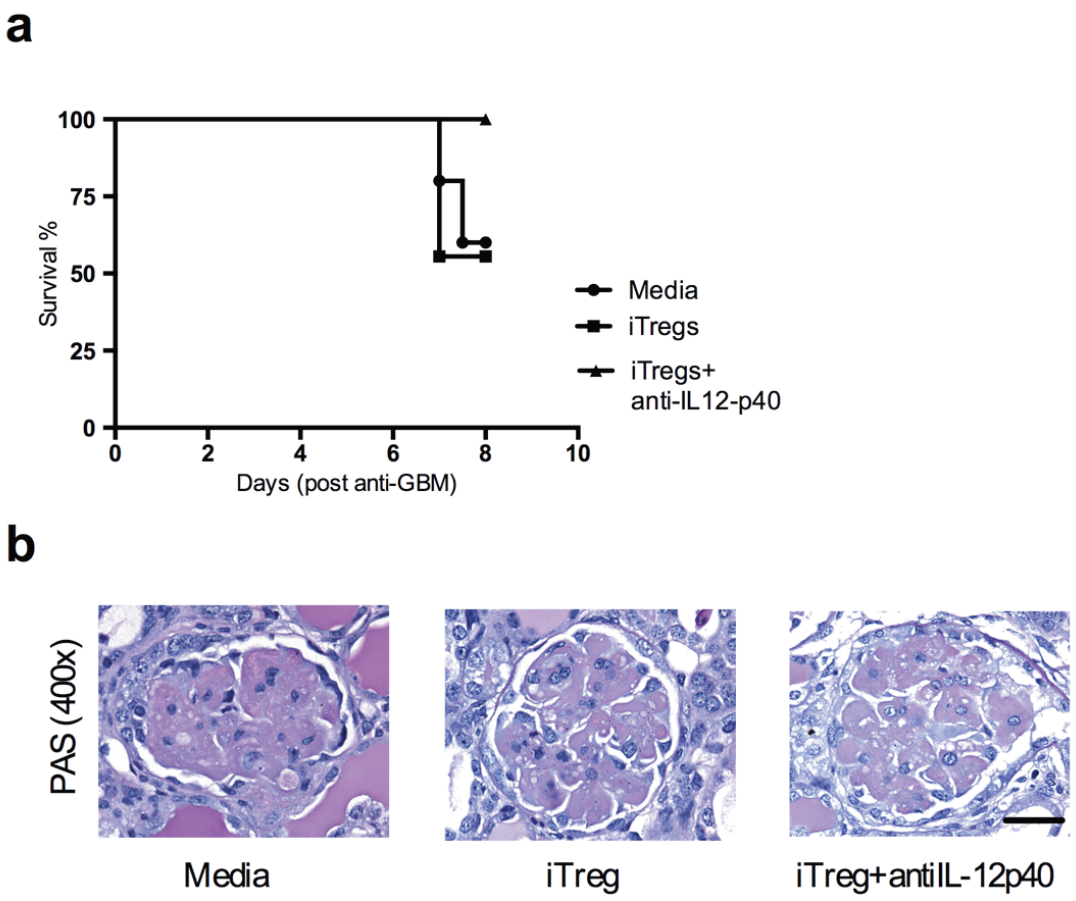


Figure 5

iTregs and iTregs+anti-IL-12p40 treated mice were not protected from anti-GBM disease in a model of milder RPGN.

Ly5.1 congenic mice were sensitized subcutaneously to SG/FCA and given media (*n*=7), 10x10^6 iTregs (*n*=8) or 10x10^6 iTregs+anti-IL-12p40 (*n*=9). Intravenous sheep anti-mouse GBM globulin (5mg) was administered 4 days later, before mice were euthanized after a further 10 days. Renal injury was assessed by (**a**) serum urea (dotted line represents non-nephritic WT mice; *n*=4), (**b**) urine protein/creatinine ratio (dotted line represents non-nephritic WT mice; *n*=4), (**c**) percentage of glomeruli with segmental necrosis and (**d**) interstitial injury score. (**e**) Representative images of PAS stained glomeruli (400x; scale bar=50μm). Immunohistochemical staining on periodate lysine paraformaldehyde-fixed frozen kidney sections was performed to quantify (**f**) CD4+ cells, (**g**) macrophages and (**h**) neutrophils within glomeruli and (**i**) CD4+ cells, (**j**) macrophages and (**k**) neutrophils within the cortical interstitium. **P*<0.05. c/gcs, cells per glomerular cross section; c/hpf, cells per high powered field; iTreg, induced Treg; PAS, Periodic Acid Schiff; Treg, regulatory T cell.

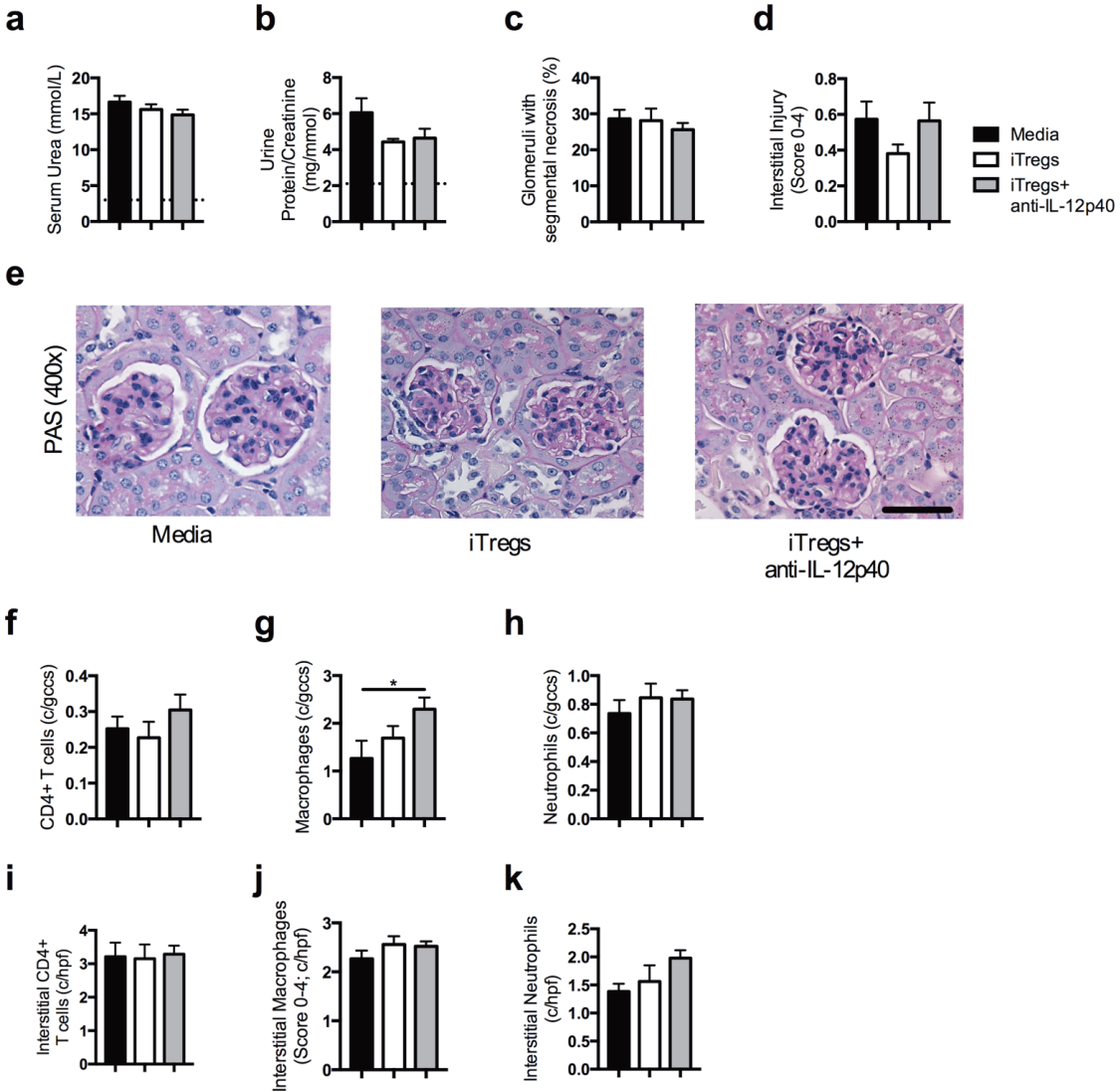


Figure 6

iTregs+anti-IL-12p40 treated mice, with milder RPGN, had enhanced Th1 cellular immune responses, with iTregs and iTregs+anti-IL-12p40 demonstrating an unstable phenotype and restricted renal recruitment.

EliSpot measurement of (**a**) IFNγ+ and (**b**) IL-17A+ spots/1x10^6 stimulated splenocytes, respectively, in mice treated with either media (*n*=7), 10x10^6 iTregs (*n*=8) or 10x10^6 iTregs+anti-IL-12p40 (*n*=9). (**c**) Proportion of splenocytes expressing CD4 and Foxp3 on CD45.2+ cells from recipient (Ly5.1 congenic) mice, highlighting a change in phenotype of the transferred iTregs and iTregs+anti-IL-12p40 (3x10^6 splenocytes were stained for flow cytometry, gating on live single cells, with ≥1 million events collected per mouse). (**d**) Proportion of renal CD45.2+ cells expressing Foxp3 in mice receiving iTregs or iTregs+ani-IL-12p40, assessed by flow cytometry. (**e**) Concatenated FACS plots of renal leukocytes, demonstrating the presence of a small population of transferred iTregs and iTregs+anti-IL-12p40, with diminished Foxp3 expression, within the kidney. **P*<0.05. ND, not detected; iTreg, induced Treg; Treg, regulatory T cell.

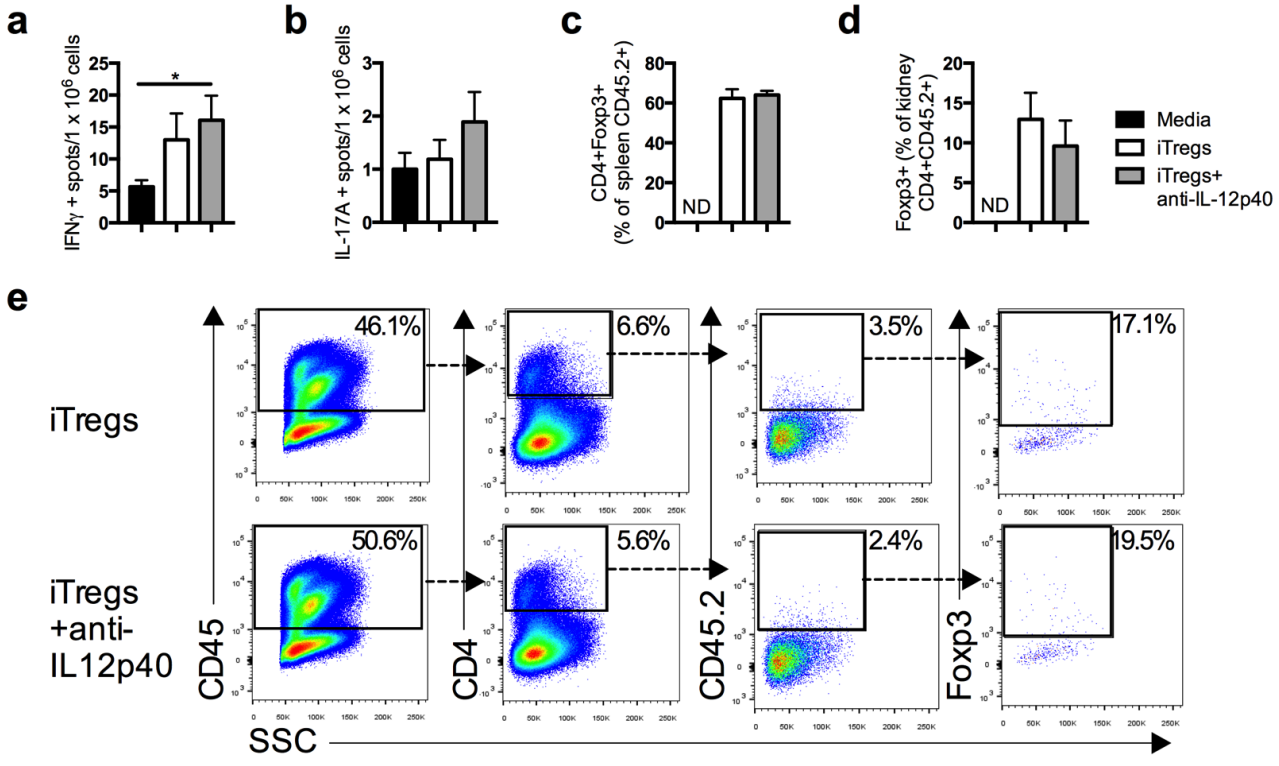
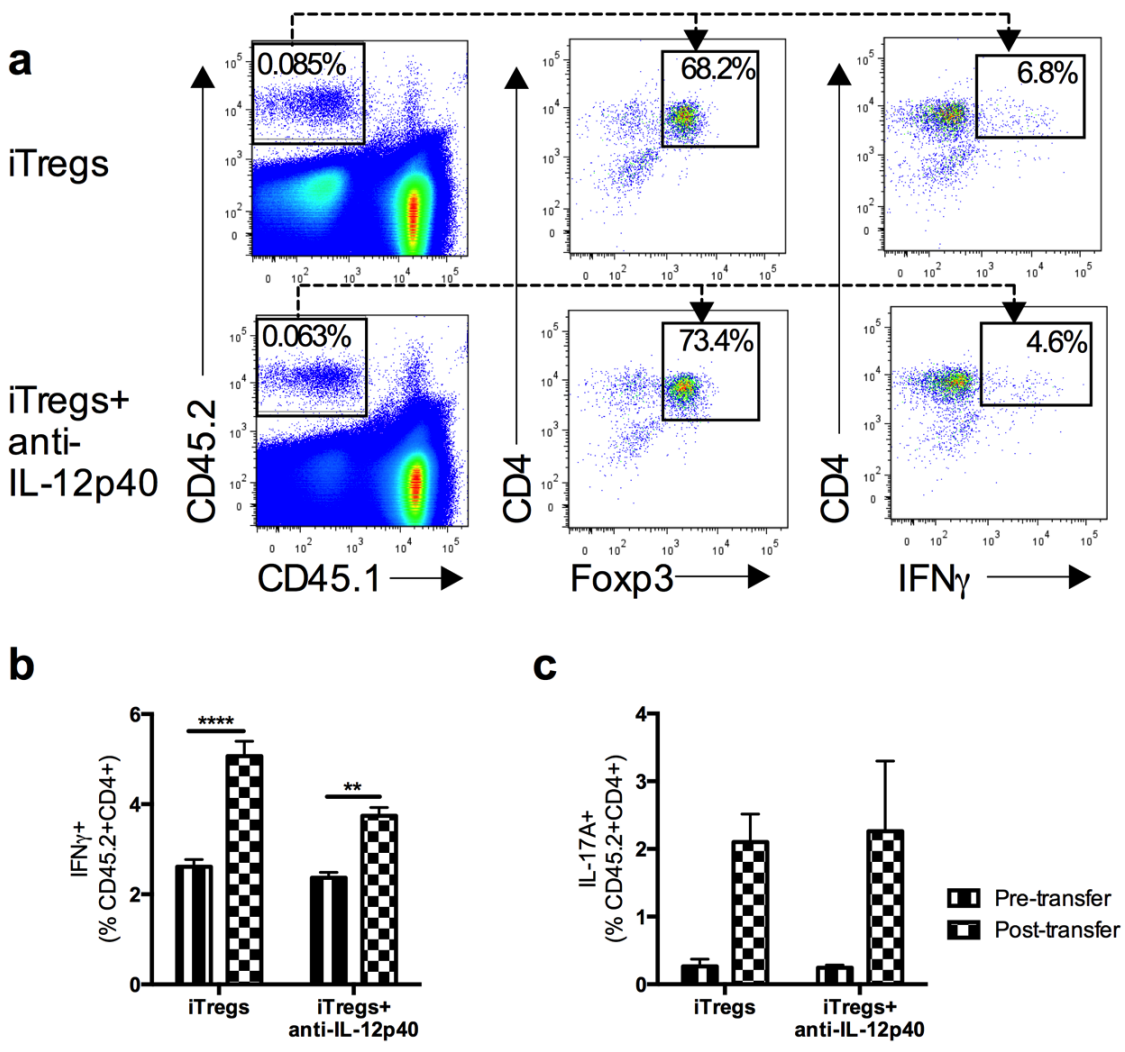


Figure 7

iTregs and iTregs+anti-IL-12p40 change phenotype 6 days after transfer into mice sensitized to sheep globulin, with increased IFNγ expression.

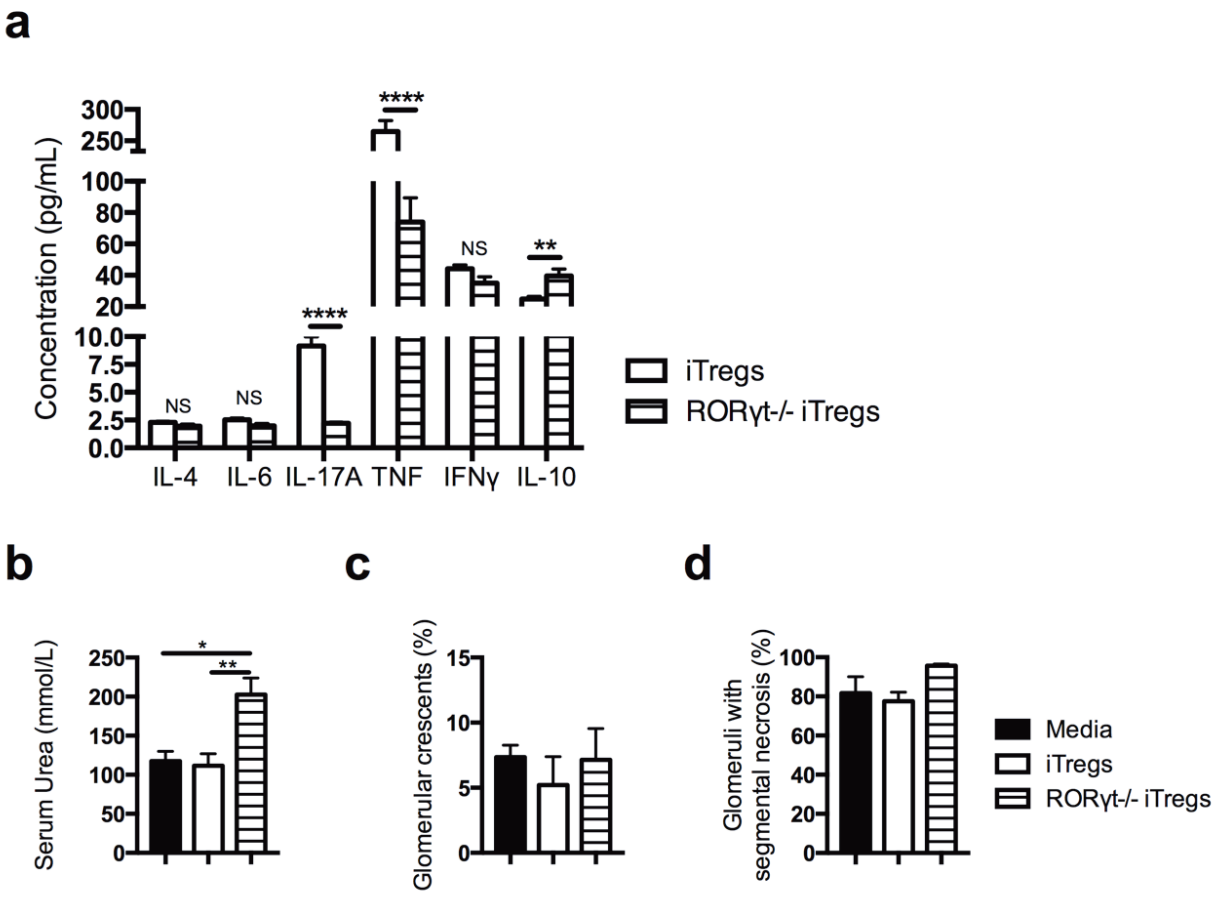
Ly5.1 congenic mice were sensitized to SG/FCA subcutaneously and received 10x10^6 iTregs (*n*=7) or iTregs+anti-IL-12p40 (*n*=9) intravenously, before euthanasia 6 days later. Immediately pre-transfer iTregs and iTregs+anti-IL-12p40 were assessed by FACS (*n*=3, taken from different culture wells). (**a**) Concatenated FACS plots of splenocytes from recipient mice, assessing the proportion of transferred iTregs and iTregs+anti-IL-12p40 (CD45.2+) expressing CD4, Foxp3 and IFNγ. (**b**) Comparison of the proportion of iTregs and iTregs+anti-IL-12p40, recovered from the spleens of recipient mice, expressing IFNγ pre- and post-transfer. iTregs and iTregs+anti-IL-12p40 were identified by flow cytometry as CD45.2+CD4+ cells. (**c**) Comparison of the proportion of iTregs and iTregs+anti-IL-12p40, recovered from the spleens of recipient mice, expressing IL-17A pre- and post-transfer. ***P*<0.01, *****P*<0.0001. iTreg, induced Treg; Treg, regulatory T cell.



Supplementary Figure 1

Assessment of the phenotype of iTregs derived from RORγt-/- mice and the ability of these RORγt-/- iTregs to suppress renal injury and systemic immune responses in the model of severe RPGN.

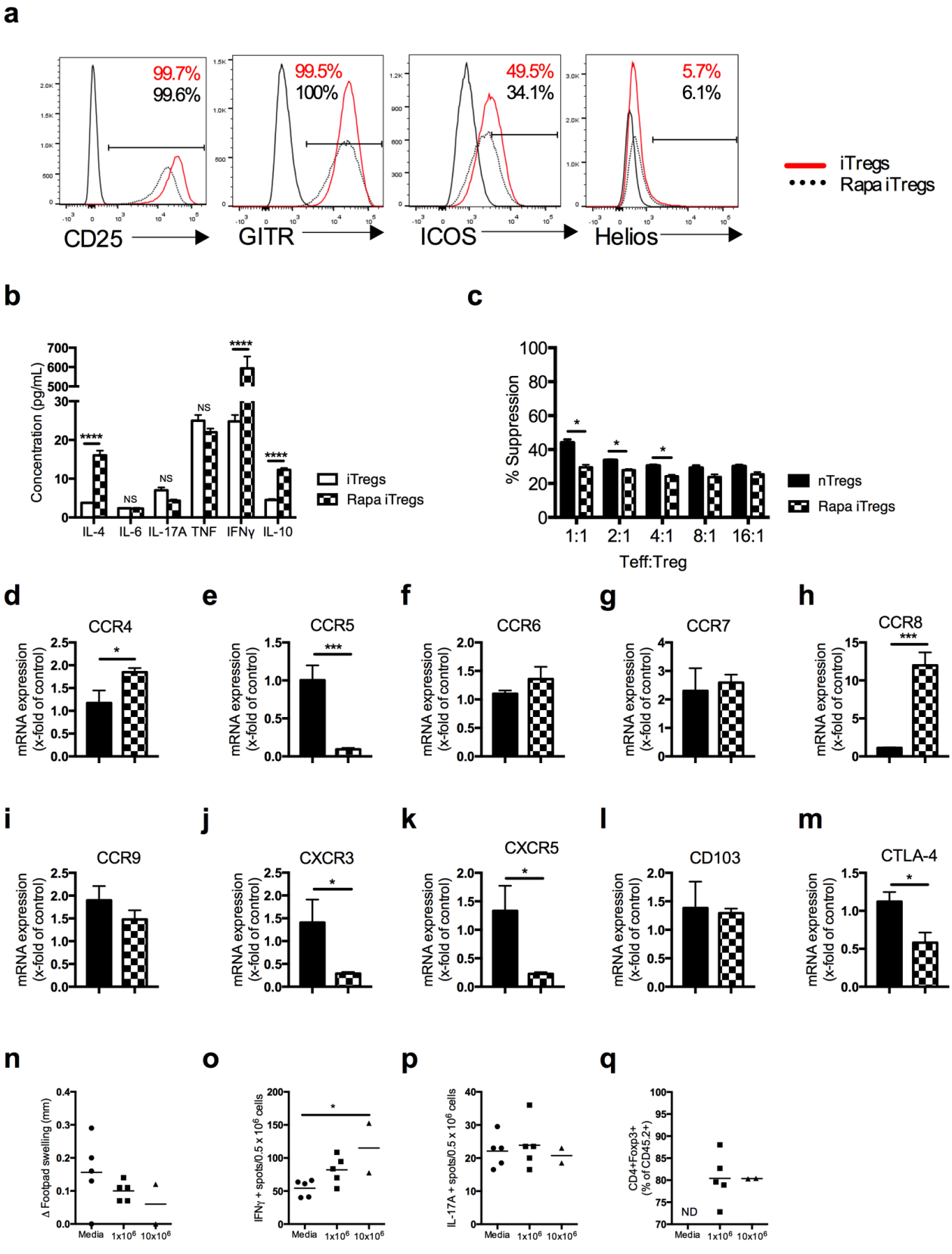
(**a**) Assessment of supernatant at day 5 from cultured iTregs and RORγt-/- iTregs for the expression of IL-4, IL-6, IL-17, TNF, IFNγ and IL-10 (cells harvested from different wells, *n*=6 per group). Ly5.1 congenic mice were sensitized subcutaneously to SG/FCA and given media (*n*=4), 10x10^6 iTregs (*n*=6) or 10x10^6 iTregs+anti-IL-12p40 (*n*=5). Intravenous sheep anti-mouse GBM globulin (10mg) was administered 4 days later, and media or 1.25x10^6 iTregs or iTregs+anti-IL-12p40 were transferred on the same day, before mice were euthanized after a further 10 days. Renal injury was assessed by (**b**) serum urea, (**c**) percentage of glomeruli with crescents and (**d**) percentage of glomeruli with segmental necrosis. *P<0.05, **P<0.01, ****P<0.0001. iTreg, induced Treg; Treg, regulatory T cell.



Supplementary Figure 2

Assessment of the phenotype of iTregs generated *in vitro* with rapamycin and ATRA.

iTregs were generated using anti-CD3, anti-CD28, TGF-β, IL-2, Rapa (10ng/mL) and ATRA. (**a**) Representative histograms showing the expression of the surface markers CD25, GITR, ICOS and the nuclear transcription factor Helios, gated on CD4+Foxp3+ cell populations, following 5 days of culture for iTregs and Rapa iTregs. Black line represents fluorescence minus one control, red line represents iTregs, black dotted line represents Rapa iTregs. (**b**) Assessment of supernatant at day 5 from cultured iTregs and Rapa iTregs for the expression of IL-4, IL-6, IL-17, TNF, IFNγ and IL-10 (cells harvested from different wells, *n*=6 per group). (**c**) Percentage of suppression of proliferation of CTV labeled Teff in co-cultures (72hrs) with either nTregs (from sorted GFP+ cells from naïve Foxp3-GFP mice) or Rapa iTregs, at varying Teff:Treg ratios. Results represent the mean of co-cultures performed in triplicate. mRNA was extracted from $1x10^6$ cells collected on different occasions (sorted nTregs, *n*=4; cultured Rapa iTregs, *n*=6). Gene of interest has been compared to 18S expression and presented as relative expression to nTregs (control group) (**d**) CCR4, (**e**) CCR5, (**f**) CCR6, (**g**) CCR7, (**h**) CCR8, (**i**) CCR9, (**j**) CXCR3, (**k**) CXCR5, (**l**) CD103 (*Itgae*) and (**m**) CTLA-4. (**n**) Dermal DTH assessed 10 days after sensitization to SG (measured by footpad swelling 24 hrs after re-challenge with SG into footpad) between mice treated with media, $1x10^6$ or $10x10^6$ Rapa iTregs, respectively. EliSpot measurement of (**o**) IFNγ+ spots and (**p**) IL-17A/$0.5x10^6$ stimulated splenocytes in mice treated with media, $1x10^6$ or $10x10^6$ Rapa iTregs. (**q**) Proportion of transferred cells (CD45.2+), assessed by flow cytometry, retaining CD4 and Foxp3 expression, recovered from the spleen of recipient (Ly5.1 congenic) mice ($3x10^6$ splenocytes were stained for flow cytometry, gating on live single cells, with ≥1 million events collected per mouse). **P*<0.05, ****P*<0.001, *****P*<0.0001. ATRA, all-trans retinoic acid; CTV, cell trace violet; iTreg, induced Treg; ND, not detected; Rapa, rapamycin; Teff, effector T cell; Treg, regulatory T cell.


a
99.7%
99.6%
99.5%
100%
49.5%
34.1%
5.7%
6.1%
CD25
GITR
ICOS
Helios
iTregs
Rapa iTregs
b
Concentration (pg/mL)
IL-4
IL-6
IL-17A
TNF
IFNγ
IL-10
NS
****
iTregs
Rapa iTregs
c
% Suppression
Teff:Treg
1:1
2:1
4:1
8:1
16:1
nTregs
Rapa iTregs
d
CCR4
e
CCR5
f
CCR6
g
CCR7
h
CCR8
mRNA expression (x-fold of control)
i
CCR9
j
CXCR3
k
CXCR5
l
CD103
m
CTLA-4
n
Δ Footpad swelling (mm)
o
IFNγ + spots/0.5 x 10^6 cells
p
IL-17A + spots/0.5 x 10^6 cells
q
CD4+Foxp3+ (% of CD45.2+)
ND
Media
1x10^6
10x10^6

Chapter 6: IL-17RA on leukocytes and tissues cells mediates experimental rapidly progressive glomerulonephritis.

Declaration

Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 6

Declaration by candidate

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design Performing the experiments Data analysis Writing and revising the manuscript	70

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Kim O'Sullivan	Performed the fluorescent microscopy, including the experimental design, technical work and data analysis	10
Peter Eggenhuizen **Stephen Holdsworth**	Assistance with performing experiments Review of the manuscript	
A. Richard Kitching	Writing and revising the manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 18/3/15
Main Supervisor's Signature	[redacted]	**Date** 18/3/15

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

IL-17RA on leukocytes and tissues cells mediates experimental rapidly progressive glomerulonephritis

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Running Headline: Leukocyte and tissue cell IL-17RA mediate GN

Abstract

Interleukin (IL)-17A and IL-17F are proinflammatory cytokines, signalling through a receptor complex of IL-17RA and IL-17RC subunits. We sought to define the role of IL-17RA expression by leukocytes and stromal cells, using bone marrow chimeras with either bone marrow (BM) or tissue cell (TC) deficiency of IL-17RA, in a model of glomerulonephritis induced by sheep anti-mouse glomerular basement membrane globulin. IL-17RA-/- mice had reduced glomerular crescent formation, neutrophils and macrophages compared to wildtype mice. BM-TC+ mice had less glomerular segmental necrosis and neutrophils, suggesting leukocyte IL-17RA expression is important for neutrophil chemotaxis in GN. IL-17RA expression was required on both BM and TC for maximal systemic IFNγ expression. The focus on IL-17 in studies in glomerulonephritis has been on cellular immunity, but in the absence of IL-17RA, antigen-specific humoral immune responses were impaired. Glomerular IgG and C3 deposition was reduced in BM+TC- and BM-TC+ mice, respectively, compared to BM+TC+ mice. Humoral immunity was impaired in BM and TC deficient chimeras. BM+TC- mice had fewer B cells expressing CXCR5. IL-17RA-/- mice had abnormal germinal center development after immunization, with less B and T follicular helper cell CXCR5 expression, explaining the impaired humoral immunity. IL-17RA expression on both leukocytes and stromal cells is required for the evolution of adaptive immunity, particularly humoral immunity, in experimental glomerulonephritis.

Introduction

Interleukin (IL)-17 cytokines are a family of structurally related cytokines, which signal through a unique receptor family. The two best characterized IL-17 cytokines, IL-17A and IL-17F, have ~50% sequence homology and similar proinflammatory effects (with IL-17A being more potent), signalling as IL-17A or IL-17F homodimers or as IL-17A/F heterodimers through a receptor complex composed of IL-17RA and IL-17RC subunits (1-6). IL-17RA is ubiquitously expressed (7-9). IL-17RC is expressed on vascular and lymphatic endothelial cells, fibroblasts, synoviocytes, glandular and epithelial cells (including human renal proximal tubular cells) and mouse mesangial cells, with minimal expression on lymphocytes (10-12). In response to IL-17A, epithelial and endothelial cells produce inflammatory cytokines and growth factors, including IL-1β and IL-6 (13, 14), and have increased expression of neutrophil attracting chemokines (15-17). IL-17RA complexes with other IL-17 receptor subunits (IL-17RB, RD and RE) to permit signalling of other IL-17 cytokines, such as IL-17E (also known as IL-25) and IL-17C (18-20). Therefore, absence or blockade of IL-17RA abrogates all IL-17 cytokine signalling.

CD4+ T helper (Th) 17 cells are generated from naïve or memory T cells in the presence of TGFβ and IL-6, enabling the nuclear translocation of signal transducer activator of transcription (STAT) protein 3, thereby upregulating expression of RORγt (the lineage-specific transcription factor for Th17 cells). Th17 cells produce IL-17A/F and IL-21 (14, 21), and express the IL-23 receptor, with IL-23 stabilizing and expanding the Th17 subset (22-25). IL-17A/F are also produced by innate immune cells, including γδT cells, lymphoid tissue inducer cells, invariant NKT cells, mast cells and neutrophils (26-32), and some stromal cells (33, 34). IL-17A/F protects the host from infections, particularly those at mucosal sites, including fungal and Gram negative extracellular pathogens ((35), reviewed in (36, 37)). However Th17 cytokines and IL-23 have been implicated in the pathogenesis of several autoimmune conditions, including psoriasis, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease (33, 38-45).

Human observations suggest that IL-17A and Th17 cells contribute to the pathogenesis of rapidly progressive glomerulonephritis (GN). Patients with ANCA-associated vasculitis (AAV) have an expanded population of Th17 cells in peripheral blood (46) and raised IL-17A and IL-23 concentrations in sera (47), while IL-17+ cells are present in renal biopsies of patients with AAV (31). Patients with lupus nephritis (LN) also have a higher frequency of circulating Th17 cells (correlating with severe renal involvement), higher serum levels of IL-17A, IL-6 and IL-23 than healthy controls (48) and increased intrarenal expression of Th17 cytokines (48). Experimentally, in murine models of anti-myeloperoxidase GN and lupus, Th17 cells and IL-17A and IL-23 contribute to renal injury (49-55) and in experimental autoimmunity to the non-collagenous domain of α3 type IV collagen, the Goodpasture antigen, mice deficient in IL-23, but not IL-12, were protected from GN and developed lower autoantibody titers (56). In autologous phase “anti-GBM” disease, RORγt-/- and IL-23p19-/- mice are protected from renal injury (57, 58), although it is likely that Th17 and Th1 responses are temporally coordinated, suggesting an interplay between these adaptive immune responses (59-61).

Given the differential expression of IL-17RA and IL-17RC, we examined the role that IL-17RA plays in a murine autologous phase “anti-GBM” GN, to establish the contribution of leukocyte and stromal cell expression of IL-17RA in mediating glomerular inflammation and the role of IL-17A/F signalling in the evolution of adaptive immunity, both cellular and humoral, in this model.

Results

IL-17RA-/- mice are protected from GN at day 21

At day 21 in non-accelerated autologous phase “anti-GBM” GN, IL-17RA-/- mice had fewer glomerular crescents, but a similar degree of focal necrosis and no difference in interstitial injury, compared to wildtype (WT) mice (Figure 1a-d). Serum urea levels were similar between the groups, but IL-17RA-/- mice had more proteinuria (Figure 1e and f). There were fewer neutrophils and macrophages within glomeruli of IL-17RA-/- mice (Table 1). Serum antibodies against the nephritogenic antigen, sheep globulin (SG), were significantly reduced in IL-17RA-/- mice (Figure 2a). Semi-quantitative immunofluorescent staining for mouse IgG and C3 deposition within glomeruli was performed, showing IgG deposition in glomeruli was not significantly reduced in IL-17RA-/- mice, and C3 deposition was unchanged (Figures 2b-c).

IL-17RA expression on leukocytes and tissue cells is required for renal inflammation at day 21

Bone marrow (BM) chimeric mice (from WT, Ly5.1 congenic and IL-17RA-/- mice) were generated to delineate the role of IL-17RA expression on renal tissue cells (TC) in mediating inflammatory responses: bone marrow (BM)+ tissue cell (TC)+ (a control for irradiation), BM-TC+ (lacking IL-17RA on bone marrow derived cells) and BM+TC- (IL-17RA deficient on tissue cells). Donor chimerism was 96% (data not shown), consistent with previous studies (62, 63). Non-accelerated anti-GBM disease was induced and experiments ended at day 21. Serum urea levels were similar (BM+TC+ 29.8±5.2, BM-TC+ 21.7±1.5, BM+TC- 21.8±3.0mmol/L; P=0.22). BM-TC+ mice were relatively protected from glomerular segmental necrosis, but crescent formation and interstitial injury not significantly different between groups (Figures 3a-d). There were fewer neutrophils within glomeruli of the BM-TC+ group than the BM+TC- group (Table 2), but glomerular CD4+ T cell and macrophage numbers were similar. There was no significant difference in the mRNA expression of key intrarenal cytokines between the groups (Table 3). There were trends to reduced expression of mRNA for several chemokines, reaching significance in the case of CCL3 in mice lacking IL-17RA expression on TC cells (Table 3).

Expression of IL-17RA on both BM and TC was required for optimal antigen-stimulated IFNγ production (Figure 4a and b). Proportions of CD4+ T cells (in spleens) were similar in all groups (Figure 4c and d) and proportions of CD25+, CD69+ and Foxp3+ cells (Figure 4e-g) were similar compared with BM+TC+ mice (but IL-17RA BM-TC+ mice had fewer CD25+, CD69+ and Foxp3+ cells than BM+TC- mice). Assessing humoral responses, serum mouse anti-sheep IgG antibodies were reduced in both BM-TC+ and BM+TC- mice (Figure 5a). IgG1 antibody titers were similar (Figure 5b) but anti-sheep IgG2b antibodies were significantly lower in BM+TC- mice (Figure 5c, IgG3 titers were low in all groups, data not shown). In the kidney, compared to the BM+TC+ group, there was less IgG deposition in glomeruli in the BM+TC- group, with a trend to reduced deposition in the BM-TC+ group (Figure 5d). Complement (C3) deposition was significantly reduced in the BM-TC+ group, with a trend to reduced deposition in the BM+TC- group (Figure 5e and f).

Germinal centers have abnormal architecture in IL-17RA-/- mice

To define the mechanisms that underpin the reduced humoral immunity in IL-17RA deficiency, WT and IL-17RA-/- mice were sensitized to SG, and mice were culled 10 days later. As anticipated, IL-17RA-/- mice developed lower titers of antigen-specific antibodies (Figure 6a). Germinal center (GC) development was compared between WT and IL-17RA-/- mice, using four color immunofluorescent staining of spleen sections. IL-17RA-/- mice had significantly fewer centroblasts (peanut agglutinin [PNA] positive area) per um^2 of spleen compared to WT mice and distinct GCs, with centroblasts adjacent to follicular dendritic cells (in green), were not apparent in IL-17RA-/- mice (Figure 6b and c). Both follicular B cells and T follicular helper cells (TFH) express CXCR5, permitting them to migrate towards CXCL13 into GC follicles so that B-T interactions can occur, resulting in mature humoral responses (64, 65). To identify whether GC formation was perturbed as a consequence of altered CXCR5 expression on lymphocytes, splenocytes from immunized WT and IL-17RA-/- mice were assessed. Expression of CXCR5 was reduced in IL-17RA-/- mice on both CD19+ B cells and CD4+ T cells, (Figure 6d-g). Therefore, IL-17RA-/- mice have impaired humoral immunity due to abnormal GC formation, with impaired B cell expression of CXCR5 and fewer TFH cells.

IL-17RA plays a role in coordinating the evolving cellular and humoral immune responses in GN at day 7 as renal injury is being established

To assess the role that IL-17RA may play in establishing a mature immune response, further groups of BM chimeric mice were studied seven days after anti-GBM antibody administration. Renal disease was mild at this early stage of the autologous phase, with no differences in histological or functional injury seen between mice, although BM+TC- mice had fewer interstitial macrophages and more interstitial neutrophils than BM+TC+ mice (Table 4). The three groups had comparable IFNγ and IL-17A production by stimulated splenocytes and low titers of mouse anti-sheep globulin antibodies (Supplementary Figure 1), findings consistent with immature autologous adaptive immune responses at this early timepoint.

To assess the role that IL-17RA may play in establishing humoral and cellular immunity, we assessed splenic CD4+ T cells for expression of the early activation marker CD69. TC expression of IL-17RA was required for optimal expression of CD69 on CD4+ T cells (Figure 7a and b). At this early stage, proportions of TFH (CD4+CXCR5+) cells were low, and similar between groups (Figure 7c). While there was no measurable difference in the intensity of staining of centroblasts within the spleen between the chimeric groups (Figure 7d), BM+TC- mice had fewer CD19+ cells expressing CXCR5 (Figure 7e), with significantly lower CXCR5 MFI (Figure 7f and g).

Discussion

IL-17RA-/- mice are protected from renal injury in non-accelerated autologous phase anti-GBM disease. This commonly used model of nephritis is induced by a single injection of a heterologous globulin, usually of sheep origin, directed against renal basement membranes. After the initial phase of “heterologous injury”, occurring within hours and due to the action of the sheep anti-mouse antibody binding to the GBM as an antibody, a second phase of injury, “autologous injury”, develops from around 6 days of nephritis, and subsequent injury is mediated by both cellular and humoral effectors targeting sheep globulin present in the kidney (and systemically) as a foreign antigen. In this phase of disease, by day 21, IL-17RA promotes renal injury, T cell and humoral responses. While IL-17RA expression on leukocytes is required for the full expression of glomerular injury, IL-17RA expression on both leukocytes and radioresistant tissue cells promotes cellular immune responses and IL-17RA expression on tissue cells is important in the co-ordination of cellular and humoral adaptive immune responses.

Renal tubular cells express IL-17RA and IL-17RC, producing proinflammatory cytokines and chemokines in response to IL-17A/F (11, 66), and most studies of IL-17A/F and Th17 cells in experimental glomerulonephritis have focused on its effects on cellular immunity (57-61, 67). However in IL-17RA-/- mice humoral immunity to the nephritogenic antigen was impaired, with a trend to reduced IgG deposition within glomeruli. Both BM+TC- and BM-TC+ chimeric mice had reduced serum antigen-specific IgG antibodies at day 21, with glomerular IgG deposition of being reduced in BM+TC- mice, with a trend to reduction in BM-TC+ mice. Although severe glomerular injury in this model is usually associated with dependence on cellular effectors, humoral responses also contribute to injury. Glomerular IgG and C3 in deposition is well documented, indicating a pathogenic humoral response to the nephritogenic antigen (68, 69). In further experiments, we showed that SG-immunized IL-17RA-/- mice had abnormal GC formation in IL-17RA-/- mice and fewer B cell centroblasts. By flow cytometry, fewer splenic B cells expressed CXCR5 and there was a deficiency in splenic TFH cells (CD4+CXCR5+ cells) in immunized IL-17RA-/- mice compared to WT. CXCR5 expression is required for lymphocyte migration to the B-T border of GCs to permit B cell somatic hypermutation and Ig class switching. At day 7 of GN, prior to differences in antigen-specific IgG titers being detectable in sera, IL-

17RA deficiency on TC resulted in impaired splenic CD19+ B cell expression of CXCR5. Recent studies have suggested a role for IL-17A and Th17 cells in humoral immunity (70-75). IL-17A promotes B cell survival (70). *In vitro* polarized Th17 cells stimulated B cell proliferation *in vitro*, promoting antibody IgG class switching (71), but it has been unclear whether IL-17A enhances B cell class switching and which antibody subclasses are generated (71-74). Th17 cells, transferred into IL-17RA-/- or T cell deficient mice, could induce antibody class switching, thereby acting as TFH cells, but recipients had reduced IgG2b levels, with smaller GCs (71). Lupus prone BXD2 mice display high IL-17A levels, have well-developed GCs and Th17 cells located adjacent to PNA+ GC regions, and IL-17 neutralization attenuated these changes (75). We have shown that stromal cell expression of IL-17RA is required for IgG2b antibody generation (a subclass that engages FcγR and fixes complement) and for optimal B cell expression of CXCR5. SG-immunized IL-17RA-/- mice were relatively TFH deficient, though others have not found this (76), potentially due to difference in defining TFH or the antigen used.

IL-17RA-/- mice were protected from glomerular injury at day 21, with fewer neutrophils and macrophages recruited to glomeruli. BM-TC+ mice were also protected from GN, with fewer glomerular neutrophils. IL-17RA, RORγt and innate cell IL-17A expression have recently been found to be required for neutrophil and macrophage recruitment in models of rapidly progressive GN (57, 66, 67). We have shown that at day 21 of this model, BM and TC expression of IL-17RA was required for optimal systemic IFNγ production, highlighting the collective role of IL-17 cytokines in adaptive immunity and Th1 effector responses. Recent studies using non-accelerated and accelerated models of anti-GBM GN have suggested IL-17A signalling occurs upstream of IFNγ, and may be regulated by IFNγ. Th17 cell numbers peak at days 7-10 in nephritic kidneys, whereas Th1 cells increase from day 7 onwards, with IL-17A being important in early GN and IFNγ being required to perpetuate injury in established GN (59). IL-17A-/- mice were protected from renal injury at day 6, but had more severe GN at day 21, with increased Th1 responses (60). RORγt-/- mice were also protected from GN, and RAG-1-/- mice repopulated with CD4+ T cells from RORγt-/- mice, showed attenuated glomerular injury, with impaired splenic Th1 responses compared to recipients of wild type CD4+ T cells (57), supporting a role for Th17 cells in promoting, rather than suppressing Th1 immunity. Anti-IL-17A given to WT mice throughout GN attenuated renal injury (66).

These findings are consistent with the evolution of responses to a foreign antigen, with early Th17 responses being followed by later Th1 dominant responses. Our findings in this model, while not dissimilar from some of these results, use mice deficient in all elements of IL-17 family member signalling.

TC expression of IL-17RA was important in activation of early antigen-specific immune responses. In secondary lymphoid organs, lymph node stromal cells (LNSC) are increasingly being recognized as participants in the development of adaptive cellular immunity; they can present antigen to CD4+ T cells via MHCII, and may negatively regulate CD4+ T cell proliferation and survival (77, 78). LNSC can endogenously express MHCII or may acquire it via hematopoietic dendritic cells (DCs) (78). Our results suggest IL-17RA expression on stromal cells enhance early CD4+ T cell activation in response to exposure to a foreign antigen. IL-17A enhances DC expression of CD80, CD86 and MHC II, with increased T cell proliferation and polarization to a Th1 phenotype *in vitro* and *in vivo* (79-81). Therefore, IL-17RA expression on radioresistant and hematopoietic cells is important in the full development of Th1 immune responses in this model of nephritis.

At day 21, BM-TC+ mice were protected from glomerular segmental necrosis, with fewer glomerular neutrophils, suggesting IL-17RA signalling on leukocytes is important for neutrophil chemotaxis to the kidney. There are a number of potential mechanisms by which neutrophil recruitment may be diminished in nephritis, including in association with fewer circulating blood neutrophils (82), less autologous antibody deposited in glomeruli (68), impairment in complement activation (83) or altered chemokine expression (63, 84). Although histological indices of GN were reduced, proteinuria was increased in nephritic IL-17RA-/- mice and in BM+TC- chimeric mice. The mechanism behind this finding is uncertain, although the action of IL-17A on podocytes is unclear, with one study suggesting IL-17A enhances podocyte apoptosis (85).

In conclusion, IL-17 cytokines signalling through IL-17RA promote local renal injury and adaptive immunity, especially humoral immunity in experimental GN. Both BM and TC cell IL-17RA play a role in both these areas. IL-17RA deficiency impairs TFH

and B cells in inducing nephritogenic autoantibodies. In addition to their roles in cellular immunity, IL-17 cytokines and IL-17 receptors may play significant roles in forms of nephritis mediated by humoral immunity.

Methods

Induction of non-accelerated anti-GBM disease and experimental design

C57BL/6J (WT) and Ly5.1 congenic mice were bred in specific-pathogen free conditions at Monash Animal Research Platform and the Walter and Eliza Hall Institute (purchased through Monash Animal Services). IL-17RA-/- mice on an C57BL/6 background (from Dr J Tocker, Amgen, Seattle, WA) were bred at Monash Medical Centre Animal Facility. Experiments were conducted on age-matched 6-10 week old male mice, approved by Monash University Animal Ethics Committee B (MMC B10/20) and performed in accordance with the National Health and Medical Research Council's Australian code for the care and use of animals for scientific purposes.

To induce 'non-accelerated GN' in WT and IL-17RA-/- mice, 13mg sheep anti-mouse GBM globulin was injected intravenously intravenously (*n*=6-8 per group) (86). Mice were euthanized 21 days later by CO_2 inhalation. Bone marrow chimeric mice were generated from WT, Ly5.1 congenic and IL-17RA-/- mice; 5-6 week old mice received whole body irradiation, followed by injection of BM cells ($5x10^6$/mouse), and reconstitution for 8 weeks (87). Mice received 14mg of sheep anti-mouse GBM antibody (*n*=8-12 per group) and were culled 7 or 21 days later. For assessment of humoral responses in WT and IL-17RA-/- mice, mice were sensitized to 0.5mg normal sheep globulin (SG) in Freund's complete adjuvant, subcutaneously at the base of tail (*n*=7 per group). Mice were culled 10 days later.

Analyses were performed on Graphpad Prism Software (version 6.0e). Differences between 2 groups were tested using a t test (for parametric data) and differences between 3 groups were performed by ANOVA, with Tukey test for multiple comparisons. Data is expressed as mean±SEM. *P* values were significant if $P<0.05$.

Assessment of renal Injury

Paraffin-embedded Periodic acid Schiff 3μm-sections of kidney were assessed for glomerular segmental necrosis, crescent formation (≥40 glomeruli/mouse) and interstitial injury (assessed on 10 medium powered fields (200x), as described (86,

88, 89). Leukocyte infiltration was assessed in periodate lysine paraformaldehyde fixed 6μm-thick sections of kidney (≥20 glomeruli and 10 interstitial high powered fields [400x]/mouse) as previously described (86, 90). The following primary antibodies were used: CD4+ T cells (anti-CD4, GK1.5), macrophages (anti-CD68, FA/11), neutrophils (anti-Gr-1, RB6-8C5) with isotype control mAbs (86). Urine was collected using metabolic cages, for measurement of proteinuria (by Bradford's assay) and creatinine. Urea was measured from mouse serum.

Systemic cellular and humoral immune responses

Single cell suspensions of spleens were made and red blood cells lysed. Splenocytes ($4x10^6$ cells/mL) were stimulated with 100μg/mL of SG in RPMI-complete (10% fetal calf serum, 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine, 50μM 2ME), incubated for 72h at 37°C with 5% CO_2, and supernatant was stored at -80°C. IFNγ, IL-17A and IL-5 levels were assessed by ELISA as described (90, 91). EliSpot assays for IFNγ (BD Biosciences, North Ryde, NSW, Australia) and IL-17A (eBioscience, San Diego, CA) were performed according to the manufacturer's instructions; spots were enumerated by EliSpot platereader (Autoimmun Diagnostika GmbH, Strassberg, Germany). Assessment of mouse anti-sheep IgG and IgG subclass levels were made on sera diluted in PBS by ELISA and measured at $OD_{450,}$ with the following antibodies; sheep anti-IgG-HRP (GE Healthcare UK), goat anti-mouse IgG1-HRP, IgG2b-HRP and IgG3-HRP (all Southern Biotechnology Associates, Birmingham, AL).

Flow cytometry

Splenocytes were stained for flow cytometric analyses with the following antibodies (BD Pharmingen): CD4 (GK1.5), CD44 (IM7), CD69 (H1.2F3), CD19 (1D3), CD25 (PC61) and CXCR5 (2G8). Propridium iodide used to assess live cell populations. Cells were fixed, permeabilized and stained for Foxp3 using eBioscience Foxp3 staining buffer and anti-Foxp3 (FJK-16s). Data analyses were performed on FlowJo software (TreeStar, OR).

Real-time polymerase chain reaction (RT-PCR)

Kidneys were homogenized and mRNA was extracted using standard laboratory techniques. cDNA was generated using the Applied Biosystems (Foster City, CA) high capacity cDNA reverse transcription kit using a Veriti Thermal Cycler. RT-PCR was performed using Taqman or Power SYBR Green mastermix and probes (sequences available upon request; Life Technologies, Victoria, Australia) in a Rotor-Gene RG3000 RT thermal cycler (Corbett Life Science, Qiagen). Samples were run in duplicate, standardized to 18S expression and referenced to the BM+TC+ group.

Assessment of splenic germinal centers (GC) and glomerular IgG and C3 deposition

GC development was assessed by multicolor fluorescence microscopy of stained snap frozen sections of mouse spleen, based on the method of Hsu et al. (75), using the following antibodies: CD21/35 (BD Biosciences, 76G), CD4 (American type culture collection, VA, GK1.5) conjugated to Alexa Fluor 594 (Molecular Probes, Eugene, Oregon), IgM (eBioscience, II/41), and biotinylated peanut agglutinin (PNA; Vector Laboratories, Burlingame, CA) conjugated to V450-streptavidin (BD Biosciences), identifying follicular dendritic cells, CD4+ T cells, IgM expressing B cells and B cell centroblasts, respectively. Sections were assessed at 100X and scoring of GCs was based on PNA+ cells/μm^2.

Glomerular mouse anti-sheep IgG and C3 deposition was assessed using sheep anti-mouse Ig-FITC (Chemicon, Victoria, Australia, AP 326F) and goat anti-mouse C3–FITC (Cappell West Chester, PA) on frozen kidney sections. Twenty glomeruli were assessed for each animal, at 200X. Images were acquired on a Nikon C1 confocal laser scan head attached to a Nikon Ti-E inverted microscope (Tokyo, Japan) using 488 and 561nm lasers. Quantification of immunofluorescent staining was assessed with Image J software (NIH, Bethesda, MD).

Disclosure

The authors declare no conflict of interest.

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Titles and Legends

Figure 1

IL-17RA-/- mice were protected from experimental GN at day 21.

Non-accelerated anti-glomerular basement membrane disease was induced in WT (*n*=8) and IL-17RA-/- (*n*=6), and mice were euthanized at day 21. (**a**) Representative periodic-acid Schiff (PAS) stained glomerular cross sections at high power (400x) from WT and IL-17RA-/- mice. Histological injury, showing (**b**) glomerular crescents, (**c**) glomeruli with segmental necrosis and (**d**) interstitial injury. (**e**) Serum urea levels were similar between groups but (**f**) urine protein:creatinine ratios were higher in IL-17RA-/- mice. **P*<0.05, ****P*<0.001.

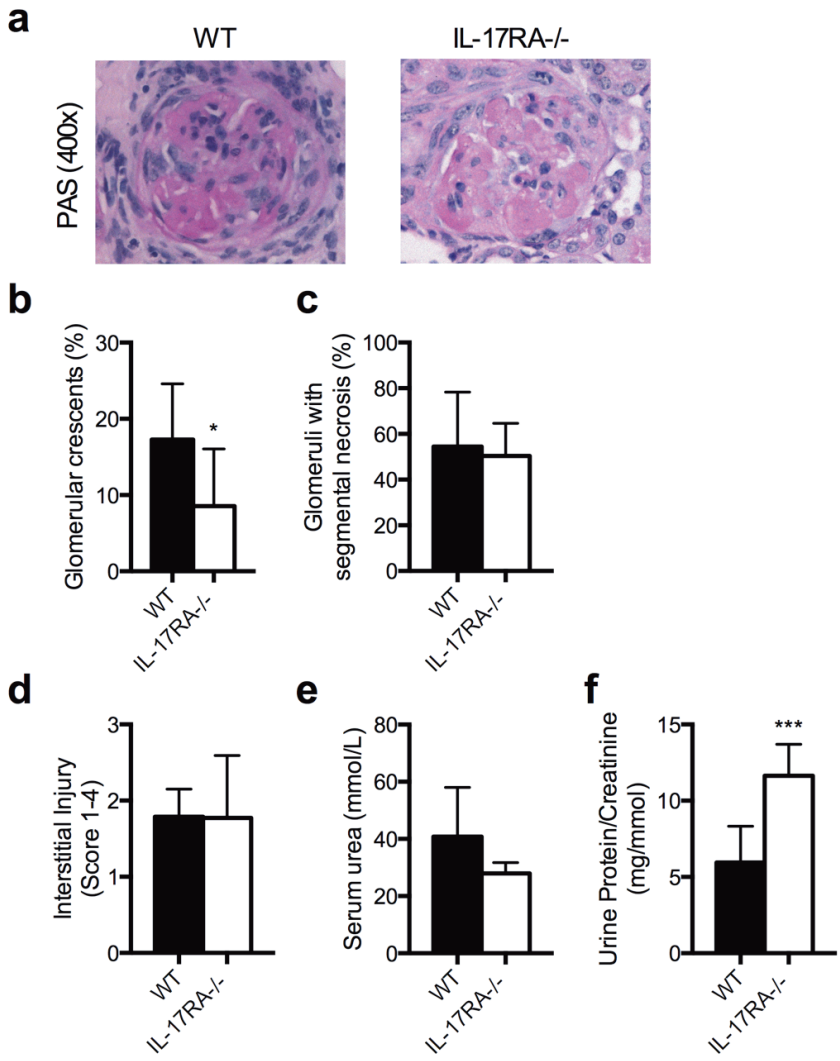


Figure 2

IL-17RA-/- mice had impaired humoral immune responses.

Non-accelerated anti-glomerular basement membrane disease was induced in WT (*n*=8) and IL-17RA-/- (*n*=6), and mice were euthanized at day 21. (a) Serum mouse anti-sheep IgG titers. Semiquantitative immunofluorescent staining of (**b**) mouse IgG and (**c**) C3 deposition within glomeruli. **P*<0.05. A.U, arbitrary units.

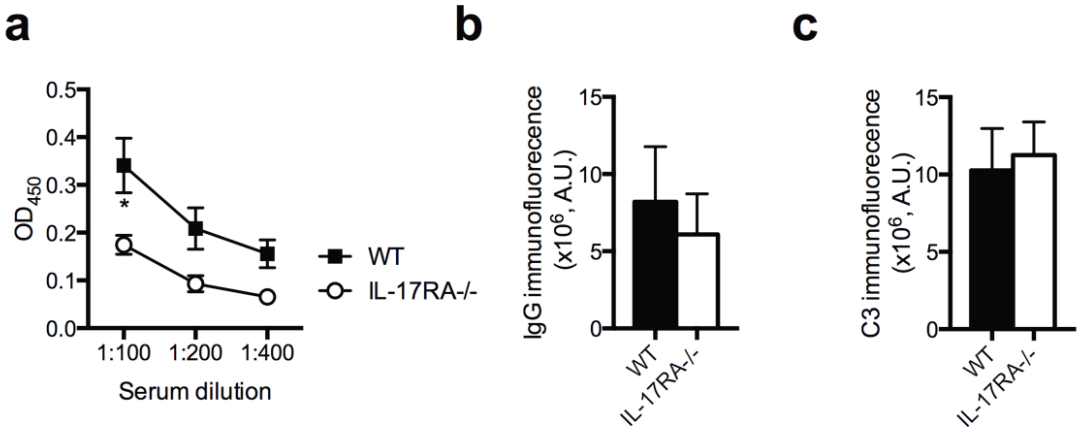


Figure 3

Assessment of renal injury in mice deficient in IL-17RA expression on either bone marrow (BM) or tissue cells (TC) at day 21 following experimental GN.

BM chimeric mice were made, being BM+TC+ (*n*=10), BM-TC+ (*n*=9) or BM+TC- (*n*=8). Non-accelerated anti-glomerular basement membrane disease was induced and mice were euthanized 21 days later. (**a**) Glomerular segmental necrosis, (**b**) crescents and (**c**) interstitial injury. (**d**) Representative images of Periodic acid Schiff (PAS) stained kidneys taken at low power (200X) from BM+TC+, BM-TC+ and BM+TC- mice. ***P*<0.01.

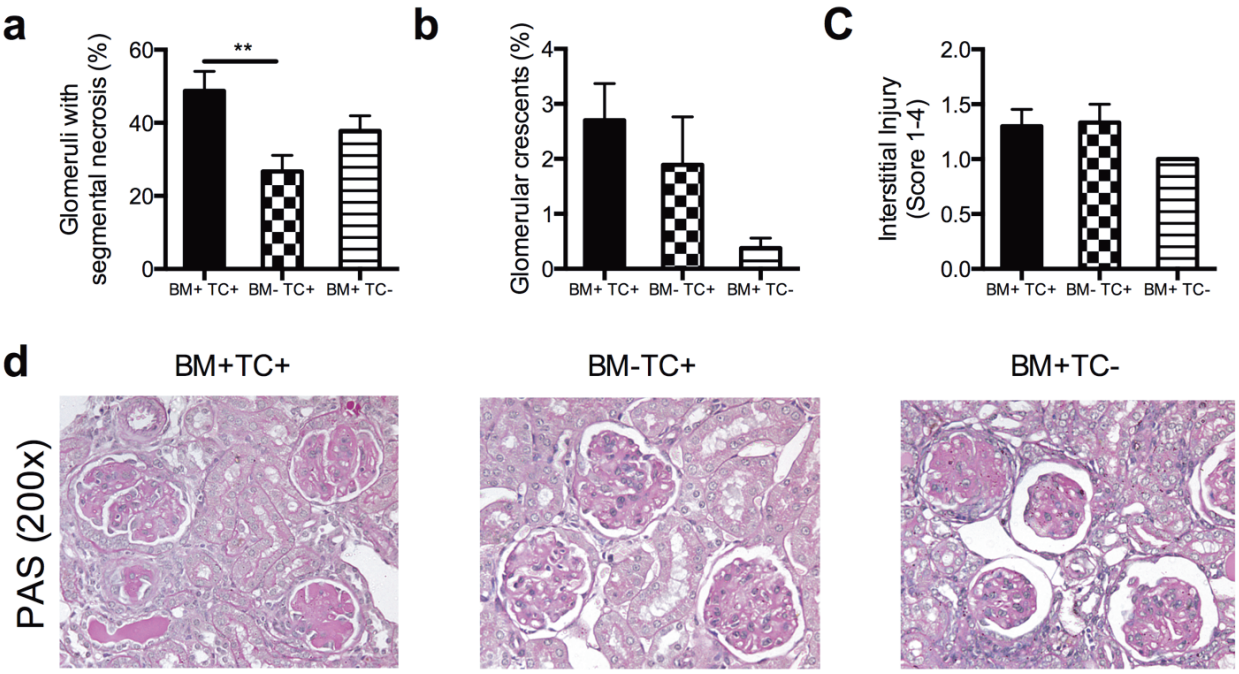


Figure 4

Assessment of cellular immune responses in bone marrow (BM) chimeric mice at day 21 of experimental GN.

EliSpot assessment of (**a**) IL-17A+ and (**b**) IFNγ+ spots/2x10^6 sheep globulin stimulated splenocytes. (**c**) Proportion of CD4+ cells in spleen assessed by flow cytometry. (**d**) Representative FACS plots of splenocytes showing the proportion of CD4+ and CD25+ cells. Proportion of CD4+ cells in spleen expressing (**e**) CD25, (**f**) CD69 or (**g**) Foxp3. *P<0.05, **P<0.01.

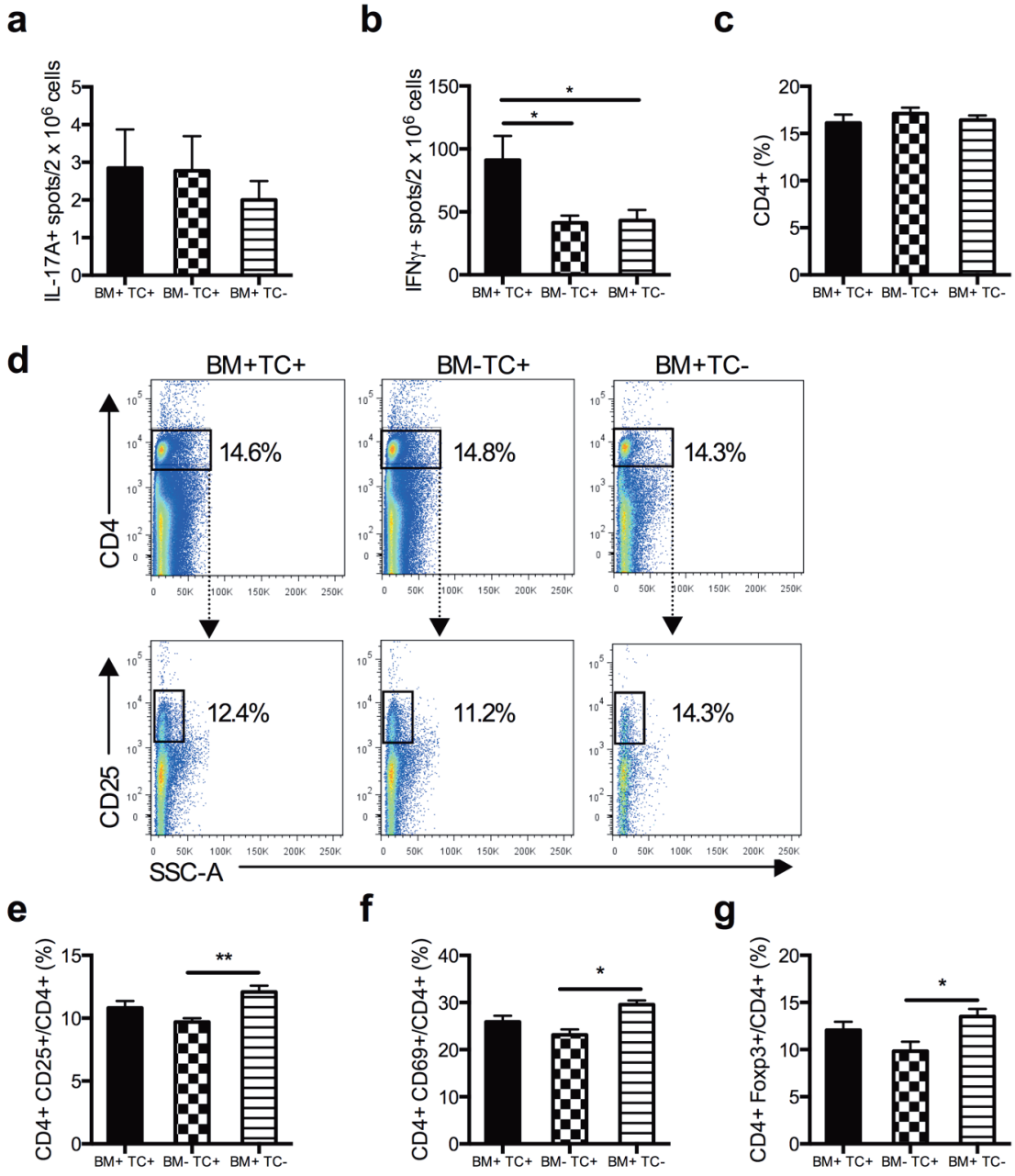


Figure 5

Assessment of humoral immune responses in bone marrow (BM) chimeric mice at day 21 of experimental GN.

Serum mouse anti-sheep (**a**) IgG, (**b**) IgG1 and (**c**) IgG2b antibodies. Semiquantitative immunofluorescent staining of (**d**) mouse IgG and (**e**) C3 deposition within glomeruli. (**f**) Representative images (taken at 1000x) showing mouse IgG and C3 staining of glomeruli in chimeric mice (scale bar = 50μm). **P*<0.05, ***P*<0.01. *****P*<0.0001 compared to BM+TC+. A.U, arbitrary units.

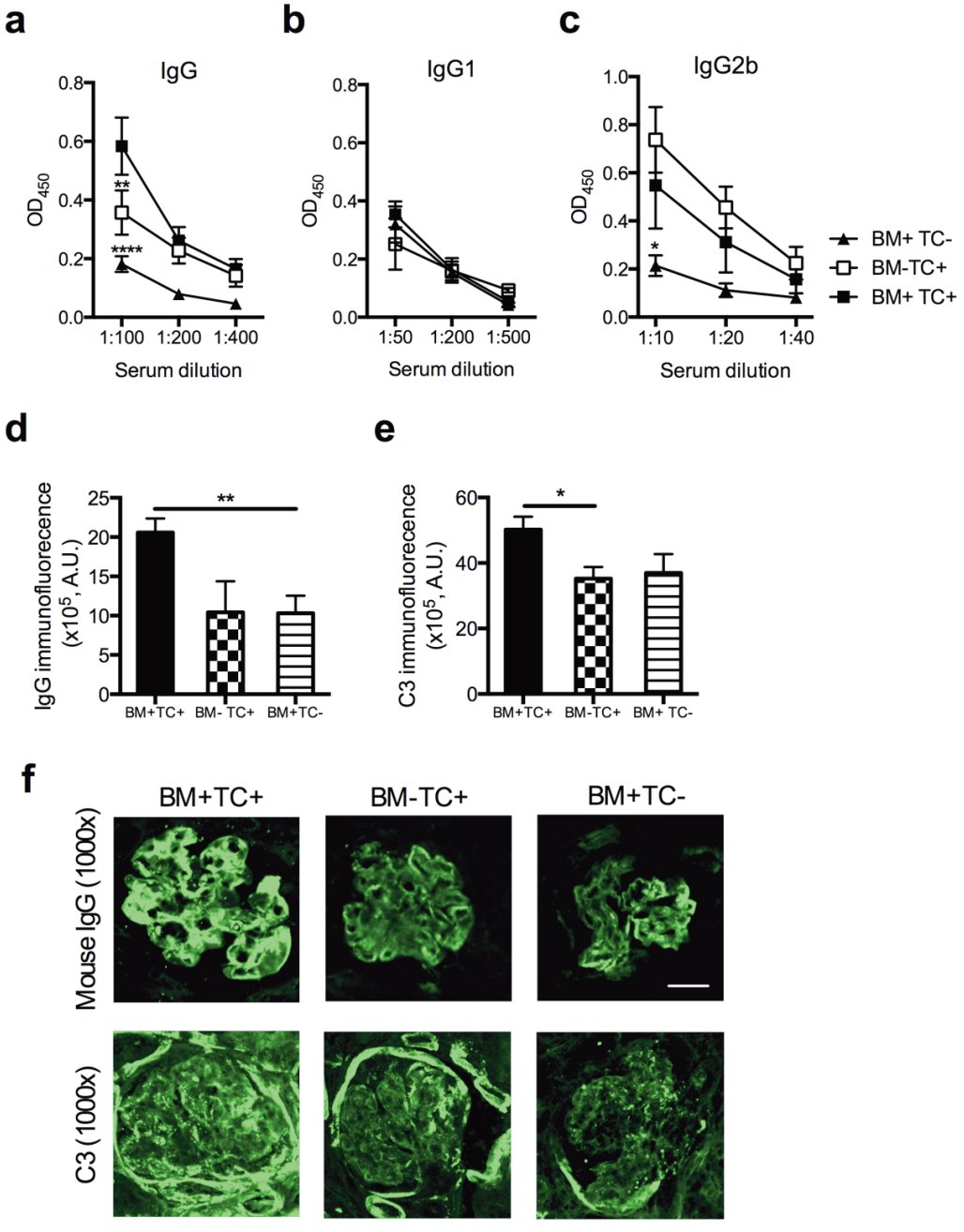


Figure 6

Germinal center architecture is abnormal inIL-17RA-/- mice immunized to sheep globulin.

WT (*n*=6) and IL-17RA-/- (*n*=7) mice were immunized to normal sheep globulin (0.5mg) in Freund's complete adjuvant, subcutaneously, and euthanized 10 days later. (**a**) Serum mouse anti-sheep IgG antibodies. (**b**) Quantification of the area of spleen stained for peanut agglutinin (PNA; representing B cell centroblasts) in immunized WT and IL-17RA-/- mice, by image J analysis of immunofluorescent staining of frozen spleen sections. (**c)** Representative fluorescent microscopy images of stained frozen spleen sections (100x) from immunized WT and IL-17RA-/- (scale bar = 100μm). Assessment by flow cytometry of splenocytes for (**d**) the proportion of CD19+ B cells expressing CXCR5 and (**e**) mean fluorescence intensity (MFI) of CXCR5 expression. Identification by flow cytometry of (**f**) splenic T follicular helper cells, by assessment of CXCR5 expression on CD4+ cells and (**g**) mean fluorescence intensity (MFI) of CXCR5 expression. **P*<0.05, ***P*<0.01, *****P*<0.0001.

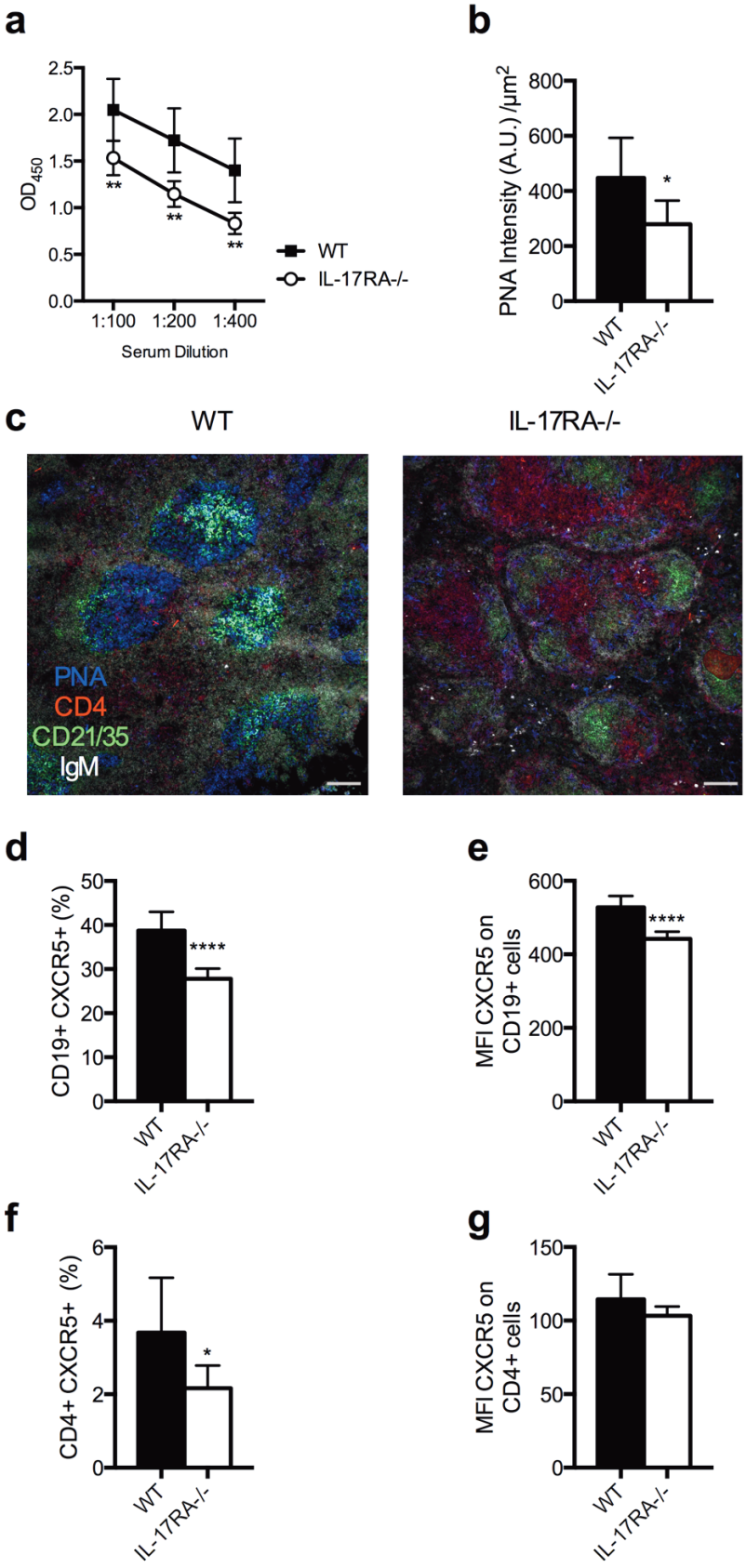
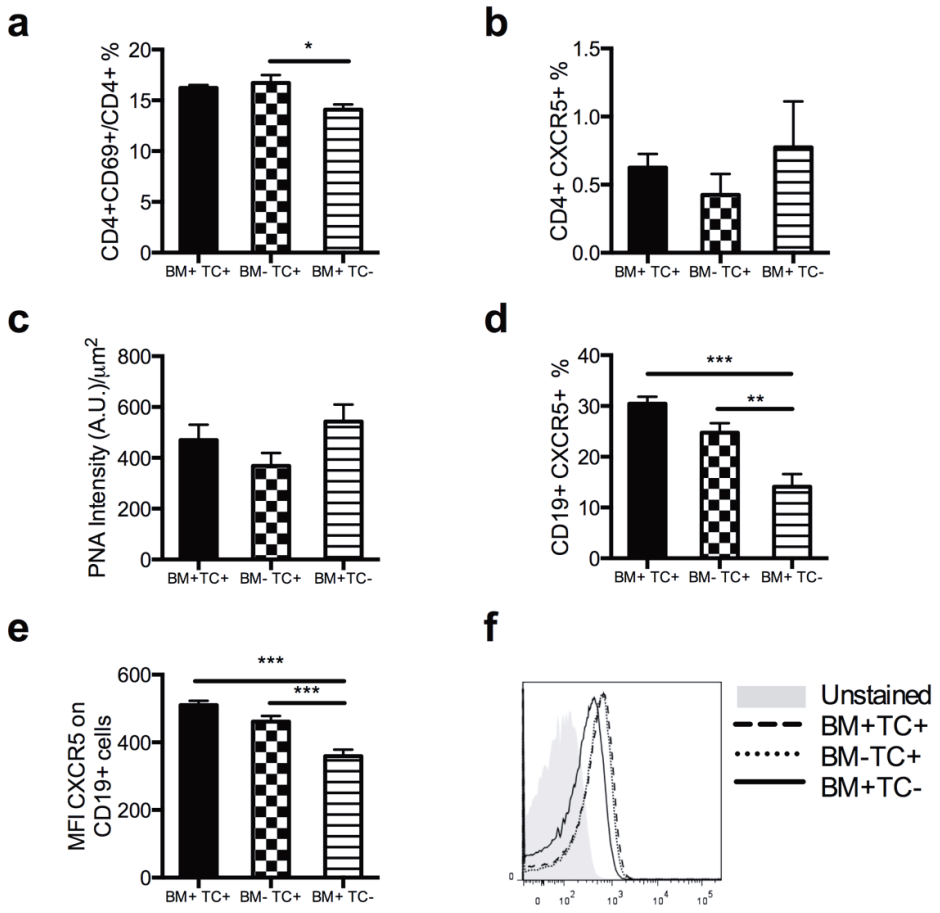

a
b
c
WT
IL-17RA-/-
PNA
CD4
CD21/35
IgM
d
e
f
g
OD450
Serum Dilution
1:100
1:200
1:400
WT
IL-17RA-/-
PNA Intensity (A.U.) /µm2
CD19+ CXCR5+ (%)
MFI CXCR5 on CD19+ cells
CD4+ CXCR5+ (%)
MFI CXCR5 on CD4+ cells

Figure 7

IL-17RA expression on bone marrow (BM) and tissue cells (TC) is required for T cell activation and B cell expression of CXCR5 at day 7 of experimental GN.

BM chimeric mice were made, being BM+TC+ (*n*=10), BM-TC+(*n*=12) or BM+TC- (*n*=10). Mice received sheep anti-mouse glomerular basement membrane globulin and were euthanized 7 days later. Proportion of splenic CD4+cells expressing (**a**) CD69 and (**b**) CXCR5 (TFH cells), assessed by flow cytometry. (**c**) Quantification of the area of spleen stained for peanut agglutinin (PNA; representing B cell centroblasts) by image J analysis of immunofluorescent staining of frozen spleen sections. (**d**) CXCR5+CD19+ B cells and (**e**) mean fluorescence intensity (MFI) of CXCR5 on B cells from the spleen. (**f**) Representative histograms showing CXCR5 expression on CD19+ B cells for BM+TC+, BM-TC+ and BM-TC+ groups. **P*<0.05, ***P*<0.01, ****P*<0.001.



Supplementary Figure 1

IL-17RA on bone marrow (BM) and tissue cells (TC) is involved in the co-ordination of the evolving immune response at day 7 of experimental GN.

BM chimeric mice were made, being BM+TC+ (*n*=10), BM-TC+ (*n*=12) or BM+TC- (*n*=10). Mice received sheep anti-mouse glomerular basement membrane globulin and were euthanized 7 days later. Assessment of renal injury was made on Periodic acid Schiff (PAS) stained kidney sections for (**a**) glomerular segmental necrosis. (**b**) Serum Urea. EliSpot assessment of (**c**) IL-17A+ and (**d**) IFNγ+ spots/2x10^6 sheep globulin stimulated splenocytes. (**e**) Serum mouse anti-sheep IgG antibodies.

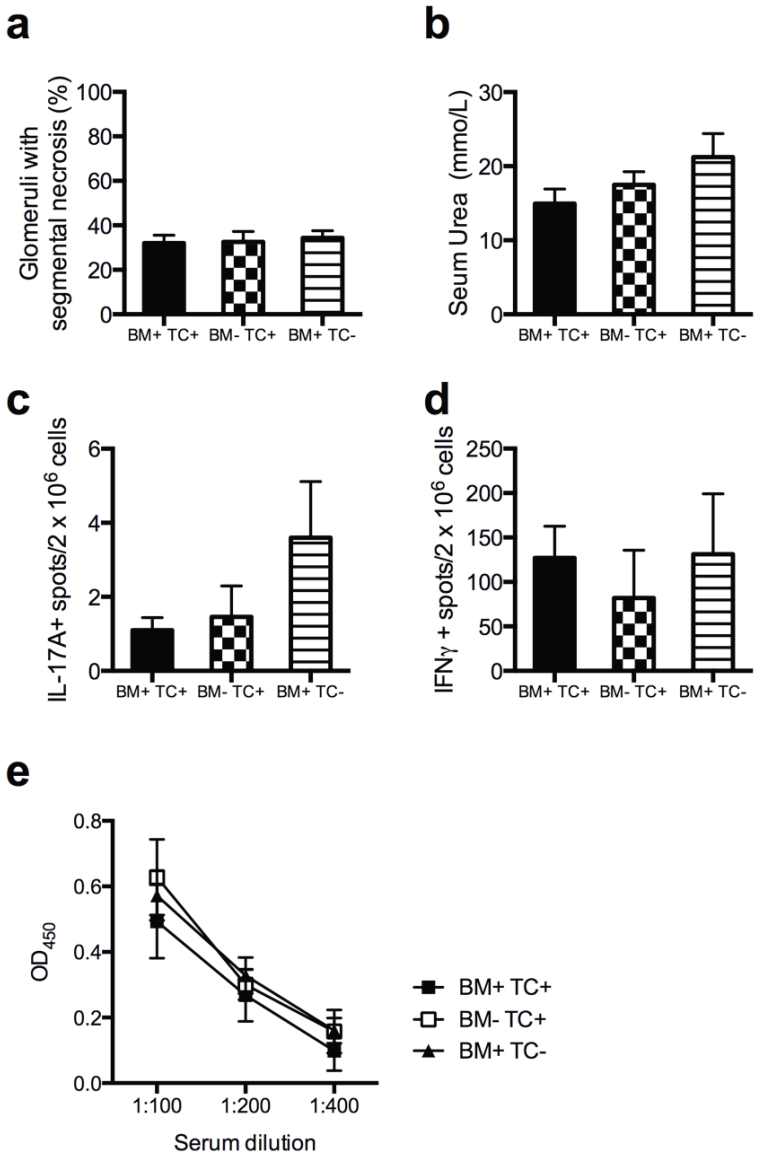


Table 1

Renal leukocyte infiltration at day 21 of experimental GN in WT and IL-17RA-/- mice.

Leukocyte	**WT** (*n*=8)	**IL-17RA-/-** (*n*=6)	***P* value**
Glomeruli	**Cells per gcs (mean±SEM)**		
Neutrophil	1.5 ±0.2	0.6±0.06	0.01
CD4+ T cell	0.25±0.04	0.15±0.04	0.1
Macrophage	2.4±0.3	0.9±0.3	0.009
Interstitium	**Cells per hpf (mean±SEM)**		
Neutrophil	1.8 ±0.3	1.3 ±0.2	0.21
CD4+ T cell	19.8 ±2.2	14.9 ±2.0	0.14
Macrophage	31.8 ±2.0	27.3 ±1.5	0.11

Abbreviations: gcs, glomerular cross section; hpf, high powered field (400x).

Table 2

Renal leukocyte infiltration at day 21 of experimental GN in WT and IL-17RA-/- bone marrow chimeric mice.

Leukocyte	**BM+ TC+** (*n*=10)	**BM-TC+** (*n*=9)	**BM+TC-** (*n*=8)	***P* value**
Glomeruli	**Cells per gcs (mean±SEM)**			
Neutrophil	0.9±0.2	0.6±0.1	1.1±0.1	0.01*
CD4+ T cell	0.3±0.1	0.4±0.1	0.3±0.1	NS
Macrophage	1.5±0.1	1.4±0.1	1.7±0.2	NS
Interstitium	**Cells per hpf (mean±SEM)**			
Neutrophil	0.7±0.1	0.7±0.3	0.6±0.1	NS
CD4+ T cell	14.6±1.5	11.7±2.6	16.7±2.6	NS
Macrophage	17.4±1.2	15.3±1.4	19.6±1.8	NS

(a) BM-TC+ vs BM+TC-. Abbreviations: BM, bone marrow; gcs, glomerular cross section; hpf, high powered field (400x); TC, tissue cell.

Table 3

Intrarenal cytokine and chemokine mRNA expression at day 21 of experimental GN in IL-17RA-/- bone marrow chimeric mice.

	mRNA expression (relative to 18S, indexed to BM+TC+)			***P* value**
	BM+ TC+ (*n*=10)	**BM-TC+ (*n*=9)**	**BM+TC- (*n*=8)**	
Cytokines				
IL-1β	2.2±0.9	1.4±0.5	1.4±0.4	0.41
IL-6	1.8±0.5	1.1±0.6	1.0±0.6	0.55
TGFβ1	0.6±0.2	0.4±0.2	1.6±0.6	0.1
CSF2	0.7±0.3	2.6±1.3	0.6±0.2	0.2
IFNγ	2.2±0.7	0.8±0.4	0.8±0.4	0.13
Chemokines				
KC/CXCL1	1.5±0.7	1.5±0.8	0.9±0.5	0.78
MIP-2/CXCL2	1.6±0.5	0.8±0.4	0.7±0.4	0.28
MCP-1/CCL2	1.4±0.5	0.9±0.3	0.7±0.3	0.38
MIP-1a/CCL3	1.5±0.5	0.3±0.1	0.3±0.1	0.04*
LARC/CCL20	1.1±0.3	0.8±0.4	0.8±0.3	0.78
RANTES/CCL5	0.8±0.2	0.9±0.3	0.7±0.2	0.86

*$P<0.05$. Abbreviations: BM, bone marrow; TC, tissue cell.

Table 4

Renal leukocyte infiltration at day 7 of experimental GN in IL-17RA-/- bone marrow chimeric mice.

Leukocyte	**BM+ TC+ (*n*=10)**	**BM-TC+** (*n*=12)	**BM+TC-** (*n*=10)	***P* value**
Glomeruli	**Cells per gcs (mean±SEM)**			
Neutrophil	0.6±0.05	0.7±0.1	0.8±0.1	0.29
CD4+ T cell	0.3±0.04	0.2±0.03	0.2±0.04	0.052
Macrophage	0.9±0.07	1.0±0.1	0.8±0.05	0.4
Interstitium	**Cells per hpf (mean±SEM)**			
Neutrophil	0.9±0.07	1.5±0.3	1.9±0.2	0.014[a]
CD4+ T cell	4.5±0.6	4.7±1.0	2.9±0.3	0.19
Macrophage	18.6±1.1	17.0±1.0	13.0±0.9	0.002[b,c]

(a) BM+TC+ vs BM+TC- $P<0.05$. (b) BM+TC+ vs BM+TC- $P<0.01$. (c) BM-TC+ vs BM+TC-. $P<0.05$. Abbreviations: BM, bone marrow; gcs, glomerular cross section; hpf, high powered field (400x); TC, tissue cell.

Chapter 7: Discussion, conclusions and future directions

Discussion, conclusions and future directions

Discussion

In human autoimmune diseases, tolerance to 'self' is lost, resulting in organ injury and inflammation, including glomerulonephritis, as a consequence of abnormal cellular and humoral immune responses. Understanding the events and mechanisms that lead to abnormal adaptive immune responses in autoimmune diseases is required, in order to prevent the development of these conditions or develop targeted therapies to suppress inflammation, restore tolerance and prevent disease relapse. Diseases that cause rapidly progressive glomerulonephritis (RPGN), such as anti-GBM disease, systemic lupus erythematosus (SLE) and ANCA-Associated vasculitis (AAV) are examples of autoimmune diseases where cellular and humoral immune responses are dysregulated. These diseases can result in permanent kidney failure; glomerulonephritis is the second most common cause of end-stage renal failure in Australia, recognised as the primary disease in 19% patients commencing dialysis in 2014, with at least 11% being defined as RPGN (2015). At present current therapies are non-selective immunosuppressives, which carry a number of toxicities and side effects for patients. In the case of SLE and AAV, these diseases are also relapsing in nature, and prevention of this requires long-term immunosuppression. Efficacious therapies that promote immunoregulatory responses and limit inflammatory ones, with minimal side effects are therefore desirable, hence the need for therapies that target cellular effectors and their cytokines in the treatment of these renal autoimmune diseases.

Recent studies exploring regulatory T cell (Treg) depletion or adoptive transfer of natural Tregs have found that Tregs exert a protective role in experimental RPGN (Ooi et al. 2011, Paust et al. 2011). However, exploiting Tregs as a novel cellular therapy poses challenges, particularly as Tregs comprise only a small population of total CD4+ T cells (~10% of the total CD4+ T cell population in a naïve mouse (Thornton and Shevach 1998)). Human studies have suggested that the ratio of circulating effector T cells (Teff) to Tregs is deranged in SLE, anti-GBM disease and AAV (Salama et al. 2001, Barath et al. 2007, Yang et al. 2009, Chavele et al. 2010, Morgan et al. 2010, Rimbert et al. 2011, Xing et al. 2012), skewed towards Teffs, and

there is evolving human and experimental evidence that Th17 cells are pathogenic in these diseases (Abdulahad et al. 2008, Crispin et al. 2008, Ooi et al. 2009, Paust et al. 2009, Shah et al. 2010, Steinmetz et al. 2010, Odobasic et al. 2011, Paust et al. 2012). Therefore, exploiting the immunoregulatory ability of Tregs and understanding the role of the pro-inflammatory cytokines IL-17A and IL-17F in RPGN, to establish the potential protective effects of systemic blockade of these cytokines, may provide two methods to 'reset' the ratio of Teff:Tregs in these diseases.

I hypothesised that:

1. Expanding endogenous Tregs through the administration of Fms-like tyrosine kinase (FLT) 3-ligand (FL), a growth factor that acts on the FLT3 receptor on haematopoietic progenitors promoting plasmacytoid dendritic cells (pDCs) and Tregs, would protect mice from experimental RPGN (Chapter 4).

2. Inducing Tregs from naïve CD4+ T cells, through the use of all-trans retinoic acid (ATRA) and TGFβ supplementation, would permit the adoptive transfer of induced Tregs (iTregs), protecting mice from experimental RPGN (Chapter 5).

3. IL-17 receptor A (IL-17RA) promotes renal injury and systemic inflammatory responses. I also aimed to characterise the specific effects of deficiency of IL-17RA expression on leukocytes or stromal cells (Chapter 6).

To test these hypotheses, I have utilised two related murine models of experimental RPGN ('non-accelerated' and 'accelerated' anti-GBM disease, detailed in Chapter 3). In these models, administration of sheep anti-mouse GBM Ab (either to naïve mice in the case of the non-accelerated model or to mice sensitised to sheep globulin in accelerated anti-GBM disease) induces renal injury similar to that seen in human anti-GBM disease, characterised by glomerular segmental necrosis and crescent formation, with linear murine IgG deposition along the GBM. The major caveat to this model is that immune responses are induced by exposure to a foreign antigen

(sheep globulin; SG); therefore, changes identified in immune responses may differ to that which occurs in human disease. Nevertheless, these robust, well-described models permit innate, T and B cell immune responses to be characterised.

Summary and Conclusions

Recent studies suggest that FL, through its effects on pDCs, enhances Treg populations in mice and humans (Darrasse-Jeze et al. 2009, Swee et al. 2009, Klein et al. 2013). In Chapter 4, I administered FL to naïve C57BL/6 mice for 10 consecutive days and confirmed that it expanded pDCs and increased Treg populations. I explored the effects of administering FL prior to sensitising mice to a foreign antigen (SG). When mice were rechallenged with SG to assess dermal delayed type hypersensitivity (DTH) 4 days later, no differences in Treg populations were identified, despite an increase in pDC proportion in spleen and lymph nodes in FL treated mice. However, dermal DTH was enhanced in FL treated animals, suggesting FL administration had promoted Teff responses, rather than regulating them. When FL was administered for 10 days prior to experimental RPGN, it did not protect mice from GN, but FL treated mice had reduced IL-17A production from stimulated splenocytes, suggesting an immunomodulatory effect. When FL was given throughout the experimental RPGN model, FL treated mice had worse survival than controls, and assessment of the underlying immune responses found comparable and severe renal injury in both the FL and PBS treated mice, but FL therapy increased renal CD11c+ cells and neutrophils within glomeruli. IL-17A production by splenocytes was enhanced with FL therapy, and the proportion of CD11c+ cells in the spleen and lymph nodes were increased. Amongst the CD11c+ DCs, the proportion of pDCs was reduced. FL treated mice had greater Tregs and activated CD4+ T cells in the lymph nodes compared to PBS treated mice. Administration of FL prior to and during the sensitisation phase of the model (but not following anti-GBM administration) did not protect mice from GN.

In summary, this work has shown that FL enhances pDC and Treg populations in steady state conditions. However, when given in experimental RPGN (dependent on immune responses to a foreign antigen), DCs develop a conventional phenotype within secondary lymphoid organs, Teff responses are enhanced, being

predominantly Th17 mediated, and local immune responses are also increased, with increased CD11c+ DCs and neutrophils being recruited to glomeruli. This highlights a potential pitfall in trying to manipulate Tregs via DCs.

In aiming to utilise Tregs as a cellular therapy, without manipulating other cellular effectors, I generated iTregs *in vitro* and explored their immunomodulatory potential in experimental RPGN in Chapter 5. Human studies are underway exploring Tregs as a cellular therapy in renal transplantation and chronic graft vs host disease (www.clinicaltrials.gov; NCT02091232, NCT01903473), and therefore may be therapeutic in renal autoimmune diseases. I induced Tregs *in vitro* from cultures of naïve CD4+ T cells from Foxp3-GFP mice in the presence of stimulation with anti-CD3, anti-CD28, IL-2, anti-IFNγ and anti-IL-4 antibodies, ATRA and TGFβ. After five days of culture, >90% of CD4+ T cells expressed Foxp3 and they had a regulatory phenotype, with expression of Treg surface markers and suppression of Teff proliferation in *in vitro* co-cultures. As these cells were still able to produce IFNγ and IL-17A, I aimed to minimise this through the administration of a monoclonal antibody to IL-12p40 (a subunit of both IFNγ and IL-23) during the culture process. Anti-IL-12p40 treated iTregs had greater ability to suppress Teff proliferation *in vitro* than iTregs induced without anti-IL-12p40. However, some important differences between natural Tregs and both types of iTregs were noted, particularly with respect to chemokine receptor expression.

I assessed the effectiveness of these iTregs to modulate immune responses in two models; a dermal DTH model, where mice were sensitised to SG, and the 'accelerated anti-GBM disease' model. Only iTregs cultured in the presence of anti-IL-12p40 were able to suppress dermal DTH and modulate IFNγ and IL-17A production by stimulated splenocytes, displaying a more 'stable' phenotype, with a greater proportion of the transferred cells remaining Foxp3+ when retrieved from the spleen. In experimental RPGN, neither iTregs nor iTregs+anti-IL-12p40 could protect mice from GN, and iTregs+anti-IL-12p40 enhanced proinflammatory Th1 responses in the spleen. A small population of the iTregs and iTregs+anti-IL-12p40 were identified within the kidneys but both had lost Foxp3 expression, indicating both types of iTregs had an unstable phenotype. To promote iTreg stability, I further modified the iTreg cultures. Firstly, as studies have shown Tregs can lose Foxp3 expression

and become Th1 or Th17 cells (Komatsu, Mariotti-Ferrandiz et al. 2009, Lee et al. 2009, Beres et al. 2011, Komatsu et al. 2014), I induced Tregs from naïve RORγt-/- CD4+ T cells (lacking the Th17 lineage defining transcription factor). Secondly, I added rapamycin to iTreg cultures, as rapamcyin may promote a stable regulatory phenotype in natural Tregs or CD4+ T cells (Gao et al. 2007, Golovina et al. 2011, Scotta et al. 2013). Neither of these modified iTregs were able to modulate proinflammatory responses in the DTH or experimental RPGN models. In summary, iTregs may be induced from naïve CD4+ T cells *in vivo* with a suppressive phenotype, but upon transfer into an inflammatory environment, they had unstable Foxp3 expression, failed to suppress Teff responses or protect mice from experimental RPGN, homing poorly to the kidney, possibly through reduced CCR6 expression.

Administration of FL promoted Th17 responses in the accelerated anti-GBM model, and a number of studies have identified Th17 cells as important cellular effectors in both the non-accelerated and accelerated anti-GBM models (Ooi, Phoon et al. 2009, Paust, Turner et al. 2009, Steinmetz, Summers et al. 2010, Odobasic, Gan et al. 2011, Ramani et al. 2014). In Chapter 6, I have explored the role of IL-17RA, a subunit of the receptor complex for a number of IL-17 family cytokines, in experimental RPGN, utilising the non-accelerated anti-GBM model, as this permits the assessment of evolving adaptive immunity following anti-GBM antibody administration. As commercially available humanised antibodies exist against IL-17A, IL-17RA and IL-12p40, have been shown to be safe for use in humans and effective in the treatment of psoriasis (Papp et al. 2008, Gottlieb et al. 2011, Strober et al. 2011, Tsai et al. 2011, Leonardi et al. 2012, Papp et al. 2012), targeting cytokines from the IL-23/IL-17A axis may offer another strategy for treating human RPGN.

IL-17RA-/- mice were protected from RPGN. Using bone marrow chimeric mice, IL-17RA expression on leukocytes and renal tissues cells were shown to be important in adaptive immune responses. In particular, leukocyte expression was important in glomerular segmental necrosis and neutrophil recruitment to glomeruli. Both were important in generating maximal Th1 effector responses systemically. Compared to controls, sensitised IL-17RA-/- mice had reduced antigen-specific humoral immune responses, abnormal germinal centre formation within the spleen, fewer T follicular helper (TFH) cells and fewer B cells expressing CXCR5, required to enable B cell

migration toward T cells, to allow antibody class switching. While both leukocyte and stromal cell expression of IL-17RA was important for the development of humoral immunity in experimental RPGN, stromal cell expression of IL-17RA was particularly important for IgG2b class switching and for the early expression of CXCR5 on B cells.

Future Directions

For Tregs to be an effective cellular therapy, they must have a stable regulatory phenotype, even in an inflammatory microenvironment. Therefore, future investigations should include optimising Treg phenotypic stability. Although I have shown that Treg induction from murine naïve CD4+ T cells did not generate such a population, other groups have explored expanding human Tregs *ex vivo*, which may permit culture of 'stable' Tregs. As Foxp3 is an unreliable marker of Tregs in humans (Gavin, Torgerson et al. 2006, Miyara et al. 2011), clarifying which surface markers denote 'stable', as well as a suppressive, Tregs is desirable. Along with intracellular Foxp3 expression by CD4+ T cells, potential human Treg surface markers include CD25+ (particularly being $CD25^{high}$), $CD127^{low/-}$ and CD45RA+ and expansion of these Tregs, and transfer into murine disease models have been able to modulate immune responses (Liu et al. 2006, Di Caro et al. 2011, Golovina, Mikheeva et al. 2011, Miyara and Sakaguchi 2011, Scotta, Esposito et al. 2013, Canavan et al. 2015). Studies are yet to indicate whether *in vitro* expanded Tregs (eTregs) traffic to inflamed organs in human disease, whether their regulatory effect is exerted through immunomodulation in secondary lymphoid organs or locally, or whether survival of eTregs could be monitored within peripheral blood, as a means of predicting disease relapse and establishing protocols for repeat dosing.

Another promising means of determining a stable Treg population involves identifying epigenetic modifications of the Foxp3 locus in Tregs. Demethylation of the Treg-specific demethylated region (TSDR) has been shown to occur in natural Tregs with a stable suppressive phenotype, whereas methylation at this site is evident in naïve CD4+ T cells or Tregs induced with TGFβ, regardless of Foxp3 expression (Floess et al. 2007, Lal et al. 2009, Lal et al. 2009). eTregs generated from CD4+CD25+CD127- T cells cultured with IL-2, with or without rapamycin, had hypomethylation of the TSDR, but eTregs cultured without rapamycin lost their

regulatory epigenetic signature with time *(in vitro)* (Rossetti et al. 2015), suggesting rapamycin may maintain demethylation and eTreg stability. Other important questions which remain to be clarified regarding demethylation of the TSDR include understanding the events that promote demethylation, whether demethylation of the TSDR can be correlated with specific surface markers (thereby making the selection of stable Tregs by flow cytometry easier) and whether strategies to prevent methylation of the TSDR could be utilised during *in vitro* culture of eTregs or administered systemically with transferred cells in experimental models.

I have shown that IL-17RA contributes to renal and systemic inflammatory responses, both cellular and humoral, in experimental RPGN. However, IL-17RA is a receptor subunit that is used by other IL-17 family cytokines for signalling. As knowledge of the function of these cytokines is still somewhat limited, some effects of IL-17RA deficiency or IL-17RA blockade may yet be further defined. I found that IL-17RA-/- mice had worse proteinuria, despite having milder GN, therefore, exploring the role of IL-17RA on podocyte integrity and function or establishing if other IL-17 family cytokines may have a protective effect on renal tubular epithelial cells is warranted.

As I found stromal cells were responsible for early B cell CXCR5 expression and aided antibody class switching to IgG2b, studying the mechanisms for this effect would be interesting. As lymph node stromal cells (LNSC) are radioresistant, further investigations to isolate these cells, confirm IL-17RA expression upon their surface and to assess the interaction between LNSC and B cells (ie, in terms of whether direct cell contact between these cell types or cytokines produced by LNSCs promote CXCR5 expression on B cells, and whether these effects are altered in the presence of inflammatory cytokines, such as IL-17A or TNF) could be pursued. Studying LNSC from immunised WT and IL-17RA-/- mice would also be instructive, to identify if cytokines that influence B cell development/maturation (for example IL-7 or B cell activating factor) are made by these stromal cells in an IL-17A or IL-17F dependent manner. Humoral immune responses mediated by IL-17RA may be an important line of investigation in forms of GN where deranged humoral immune responses are more clearly involved in renal disease, such as membranous GN or SLE.

Further characterisation of the TFH cells in immunised IL-17RA-/- mice is required; defining ICOS+ and PD-1+ expression may clarify if there are specific TFH subsets that are dependent on IL-17A or IL-17F signalling for optimal function or for maintenance of TFH cell number. TFH cells may be relevant in models of autoimmune disease like SLE or AAV, where diseases are relapsing in nature, and where auto-antibodies are defined and pathogenic. As TFH provide help to B cells, targeting this type of Th cell may provide a novel avenue to control autoimmunity. Blockade of cytokines produced by TFH, ie IL-21, could provide a novel therapeutic target in these diseases. At present, it is unclear if humoral immunity is disturbed in patients receiving anti-IL-17A or anti-IL-17RA mAb therapy, as studies utilising these agents have not reported specific immunodeficiencies. However, common side effects from these agents include nasopharygitis, and there are reports of disseminated Herpes zoster, therefore this area warrants further investigation.

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