Investigating the Role of Myeloid Cell Autophagy in Autoimmune Disease

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Abstract

Autophagy is a highly regulated cellular mechanism for the removal and/or recycling of damaged/excess organelles. Autophagy also has multiple roles to play in innate and adaptive immune responses. Systemic lupus erythematosus (SLE) is a heterogenous autoimmune disease characterised by loss of multiple tolerance checkpoints, pathogenic autoantibody production, and multisystem inflammation. Studies have focused on defects of autophagy in B and T cells leading to loss of tolerance in SLE. However, the importance of myeloid cell autophagy in the regulation of SLE has not been clearly elucidated yet.

Here, I investigated the impact of myeloid cell-specific autophagy deficiency on symptoms of autoimmunity in the *Lyn*$^{-/-}$ mouse model of lupus-like autoimmunity. Specifically, I investigated whether autophagy deficiency in myeloid cells regulates cytokine release, autoantibody production, B cell development, splenomegaly, and glomerulonephritis. I demonstrated that ablation of autophagy in myeloid cells resulted in the decreased production of specific autoantibodies and serum levels of the cytokines interleukin (IL)-6 and IL-10.

I further investigated the role of autophagy in the modulation of IL-6 and IL-10 release from myeloid cells *in vitro*. I stimulated human and mouse monocytes/macrophages with the mTOR inhibitor Torin-1 to induce autophagy in the presence of different TLR ligands and measured cytokines levels, including IL-6 and IL-10. Similarly, I investigated the impact of autophagy inhibition/deficiency on IL-6 and IL-10 release using the autophagy inhibitor 3-methyladenine (3-MA) or small interfering RNA (siRNA) against Beclin 1 (Atg6) and Atg7. The results indicate that autophagy and/or beclin-1 play a complex role in the release of IL-6 in response to TLR agonists. In contrast to IL-6, the TLR-dependent release of IL-10 by monocytes/macrophages was consistently inhibited in autophagy-defective cells and increased by treatment with Torin-1. The effects of autophagy on IL-10 were observable at the level of

mRNA, suggesting a mechanism upstream of protein production, either in the TLR signalling pathway or transcription process.

In an *in vivo* model of LPS-induced endotoxemia, I determined the effect of myeloid cell autophagy on the release of multiple cytokines and local myeloid cell populations, comparing responses in *Atg7*$^{fl/fl}$ LysMCre mice to wild type animals. I observed that the levels of IL-1α, IL-1β, IL-18, IL-23, IL-17A, IL-12, IL-6, and IFN-γ were significantly increased in the peritoneal cavity of autophagy-deficient mice compared to control mice, while in the serum levels of IL-1α, IL-1β, IL-18, and IFN-γ were significantly higher.

Taken together, my data highlight complex and context-dependent roles for autophagy in the regulation of IL-6 and IL-10 release. Moreover, the effects of autophagy inhibition on these and other cytokines differ in models of acute and chronic inflammation. My work extends our understanding of how autophagy contributes to the regulation of inflammation, particularly in myeloid cells, and offers new insights into how this may contribute to wider effects in autoimmune diseases, such as SLE. A complete understanding of how autophagy in different cell types regulates both protective and pathologic effects in disease is critical if autophagy-based therapeutic interventions are to be sought for human diseases.

Declaration

This thesis is an original work of my research and 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.

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Date: 26th May 2021

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List of Abbreviations

3-MA: 3-Methyladenine

AD: Alzheimer's disease

AIM2: Absent in melanoma 2

AMPK: AMP-activated protein kinase

ANOVA: Analysis of Variance

APC: Antigen Presenting Cell

APP: Amyloid precursor protein

Atg: Autophagy-specific genes

BAFF: B-cell–activating factor of the TNF family

Bcl2: B-cell lymphoma 2

Becn1: Beclin1

Bif-1: Bax-interacting factor-1

BMDC: bone marrow-derived dendritic cells

BMM: bone marrow-derived macrophages

CD: Crohn's disease

ChIP: Chromatin immunoprecipitation

CMA: Chaperone-mediated autophagy

DC: Dendritic cell

ds-DNA: Double-stranded DNA

ELISA: Enzyme-Linked Immunosorbent Assay

ELISA: Enzyme-linked immunosorbent assay

ENA: Extractable Nuclear Antigens

ER: Endoplasmic reticulum

FACS: Fluorescence-Activated Cell Sorting

Foxp3: Forkhead box p3

GC: Germinal Centre

GN: Glomerulonephritis

GWAS: Genome-wide association study

HBSS: Hank's balanced salt solution

HD: Huntington's disease

HF: Heart failure

HLA: Human leukocyte antigen

Htt: Huntingtin

IBD: Inflammatory Bowel Disease

iBMM: immortalised bone marrow-derived macrophages

IFN: Interferon

IgG: Immunoglobulin G

IL: Interleukin

IRFs: Interferon Regulatory Factors

IRGM: Immunity Related GTPase M

JAK2: Janus Kinase 2

JNK: c-Jun amino-terminal kinase

LAMP-2A: Lysosomal-associated membrane protein 2A

LAP: LC3-associated phagocytosis

LC3: Light Chain 3

LPS: Lipopolysaccharide

MAb: Monoclonal antibody

MAPK: Mitogen-Activated Protein Kinase

mDCs: myeloid dendritic cells

MHC: Major Histocompatibility Complex

MIF: Macrophage migration inhibitory factor

MPT: Mitochondrial permeability transition

mTOR: mechanistic target of rapamycin

MYD88: Myeloid Differentiation Factor 88

NET: Neutrophil extracellular trap

NFκB: Nuclear Factor kappa light chain enhancer of activated B cells

NLRP: NOD-like receptor proteins

NOD: Nucleotide-binding oligomerisation domain

NZB/W: New Zealand black x New Zealand white

Pam3CSK4: Pam3Cys-Ser-(Lys)4

PAMPs: Pathogen-associated molecular patterns

PAS: Periodic acid Schiff

PBMC: Peripheral Blood Mononuclear Cells

PD: Parkinson's disease

PI: Phosphatidylinositol

PI3-K: Phosphatidylinositol 3-kinase

PI3P: Phosphatidylinositol triphosphate

qPCR: Quantitative Polymerase Chain Reaction

RA: Rheumatoid Arthritis

RMQ: Resiquimod

ROS: Reactive oxygen species

RSV: Respiratory syncytial virus

SHP2: Src homology-2 domain-containing phosphatase 2

SIRT1: Silent information regulator 1

SLE: Systemic Lupus Erythematosus

SNPs: Single-nucleotide polymorphisms

SS: Sjogren's syndrome

SSc: Systemic sclerosis

STAT: Signal Transducer and Activator of Transcription

TAB2: TAK1-binding protein2

TAK1: Transforming growth factor beta-activated kinase 1

TGF-β: Transforming growth factor-β

TLR: Toll-Like Receptor

TNF: Tumour necrosis factor

Tor 1: Torin 1

Treg: T Regulatory cell

ULK1: UNC-51-like kinase 1

Vps34: Vesicular protein sorting 34

WT: Wild type

Evidence of Research output

Manuscripts in preparation

1. **Manuscript 1:** Effects of Myeloid cell autophagy in models of acute and chronic inflammation.
2. **Manuscript 2:** Characterisation of the role of D-dopachrome tautomerase (D-DT) in inflammation.
3. **Manuscript 3:** Cross-talk between Autophagy and Cytokines. (Review)

Presentations at Conferences

1. **2018, Poster presentation,** "Role of myeloid cell autophagy in a mouse model of systemic lupus erythematosus", MHTP Translational Research Symposium, Monash University, Monash Health, Melbourne, Victoria, Australia.
2. **2018, Poster presentation,** "Role of myeloid cell autophagy in a mouse model of systemic lupus erythematosus", Australasian Society for Immunology (ASI), Perth, Western Australia.
3. **2019, Poster presentation,** "Role of myeloid cell autophagy in a mouse model of systemic lupus erythematosus", British Society for Immunology Congress (BSI), Liverpool, United Kingdom.
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Chapter 1-Literature Review

1.1 Autophagy

Autophagy has emerged as an essential cellular homeostasis program that eliminates damaged/excess organelles, proteins, and cell membranes and recycles intracellular components, thereby playing a critical role in health and disease. The term "autophagy" was first introduced by Christian de Duve in 1963, is combined with the Greek word *auto* ("self") *phagein* ("to eat") (Wesselborg and Stork, 2015). Japanese scientist Yoshinori Ohsumi received the Nobel Prize in Physiology or Medicine in 2016 for establishing the mechanisms underlying autophagy, which further paved the way for understanding the mechanisms and the physiological association between autophagy and health and diseases (Van Noorden and Ledford, 2016, Levine and Klionsky, 2017). Autophagy is associated with a multitude of essential cellular and physiological processes, including cell development and differentiation, degradation of unnecessary or damaged organelles, starvation, breakdown of aberrant structures, innate and adaptive immune response, suppression of tumour, lifespan extension, cell death, and apoptosis (Mizushima et al., 2008, Levine and Kroemer, 2008, Tao and Drexler, 2020, Chen et al., 2016, Zhou et al., 2019a, Koenig et al., 2020). Autophagy occurs in all types of cells at a basal level but can also be triggered in response to a variety of stimuli/stresses, including pathogen infection, hypoxia, oxidative stress, endoplasmic reticulum stress, chemical stress, and adverse environmental conditions like starvation and growth factor deprivation (Ornatowski et al., 2020, Pecoraro et al., 2020). In most cases, it is a protective, pro-survival response and plays a crucial role in maintaining cell homeostasis and health by recycling essential amino acids and degrading potentially toxic cargo, including defective cellular components and aggregate-prone proteins (Levine and Kroemer, 2008).

Autophagy is a cellular degradation process that entails the engulfment of cytosolic constituents, their transport to lysosomes, and proteolytic digestion of the contents. Three distinct types of autophagy have been identified, termed macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Hayashi-Nishino et al., 2010, Mizushima and Murphy, 2020). Macroautophagy is the major autophagy process by which significant portions of the cytoplasm and cellular contents are sequestered into a double-membrane vacuole that degrades and recycles them for reuse (Feng et al., 2014, Klionsky and Codogno, 2013). In microautophagy, the lysosome takes up cytosolic constituents by the direct lysosomal membrane invagination, allowing for intracellular degradation (Mizushima, 2007, Paolini et al., 2018). Chaperone-mediated autophagy (CMA) does not require autophagosome formation but is similarly driven by physiological stresses, including starvation (Campbell et al., 2018). In this process, targeted proteins are translocated across the lysosomal membrane, forming a protein complex with a chaperone, including heat shock protein-70 (Hsc-70), and recognised by the lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A). Once bound to LAMP-2A, the protein unfolds and moves to the lumen via a LAMP-2A-enriched translocation complex, where it is degraded by the lysosome (Kaushik et al., 2008, Caballero et al., 2021).

Macroautophagy, referred to as “autophagy” from here, begins with an isolation membrane, commonly known as a phagophore, that is likely generated from the endoplasmic reticulum (ER) and/or the trans-Golgi network and endosomes (Glick et al., 2010, Wei et al., 2018). However, the site of the phagophore formation in mammalian cells is controversial. Some other possible membrane sources have been found, including plasma membrane (Cuervo, 2010), mitochondria (Hailey et al., 2010, Rambold and Lippincott-Schwartz, 2010), ER-mitochondria contact sites (Hamasaki et al., 2013, Morel, 2020), and recycling endosomes (Longatti and Tooze, 2012). The phagophore then extends to engulf intracellular cargo, such

as protein oligomers, organelles, and ribosomes, and sequesters them in a double membrane autophagosome (**Fig. 1.1**) (Backer, 2008, Wei et al., 2018). The loaded autophagosome develops by fusing with the lysosome, allowing lysosomal proteases to degrade the autophagosome contents. The maturation of newly formed autophagasome occurs through fusion with vesicles developed from the endolysosomal compartments (Zhao et al., 2021). Then the autophagosome contents are broken down by lysosomal proteases. Permeases in the lysosomal membrane and transporters allow amino acids produced inside the lysosome and other decay by-products to transfer into the cytosol, where they can be recycled and synthesised macromolecules (Mizushima, 2007, Glick et al., 2010). Thus, autophagy may be considered a cellular 'recycling factory' that increases energy efficiency in cells via ATP production and facilitates the regulation of damage by destroying non-functional proteins and organelles (Glick et al., 2010).

In the mammalian cell, autophagy is tightly controlled to prevent its inappropriate activation. The process of autophagy can be regulated by numerous autophagy-specific genes (Atg) and by multiple signalling pathways leading to and influenced by the mechanistic target of rapamycin (mTOR) (Botbol et al., 2016). The serine/threonine-protein kinase mTOR exists in two distinct cellular complexes, termed mTOR complex 1 (mTORC1) and mTORC2 (Saxton and Sabatini, 2017). mTORC1 has been found to be associated with several cellular processes, including ribosomal biogenesis, cellular stress such as DNA damage, and hypoxia (Laplante and Sabatini, 2012, Feng et al., 2018, de la Cruz López et al., 2019). In comparison to the mTORC1, little is known about the mTORC2 pathway. Several studies reported that the growth factors predominantly regulate the mTORC2 pathway via the PI3K signalling pathway (Zinzalla et al., 2011, Laplante and Sabatini, 2012, Fu and Hall, 2020). AMP-activated protein kinase (AMPK) promotes autophagy via the inactivation of mTORC1 and direct phosphorylation of the serine/threonine kinase ULK1 (Kim et al., 2011). ULK1 phosphorylates

Ambra1 during starvation, releasing Ambra1 and the PIK3C3 complex from microtubules and allowing the PIK3C3 complex to relocalise to the ER, which is the main organelle involved in autophagosome formation (Di Bartolomeo et al., 2010).

The role of PI3-kinases, notably Vps34 (vesicular protein sorting 34) and its interacting protein Atg6/Beclin-1, is relatively well understood in phagophore formation and autophagy (Backer, 2008). Vps34 produces the phosphatidylinositol triphosphate (PI3P) from phosphatidylinositol (PI), and it is required for phagophore elongation and the recruitment of additional Atg proteins to the phagophore (Xie and Klionsky, 2007, He et al., 2019). Autophagy-related proteins (ATG) are essential mediators of the autophagy pathway, and at least 35 ATG genes have been discovered in yeast (Han et al., 2009, Ye et al., 2018). Two ubiquitin-like conjugation systems, microtubule-associated protein light-chain (LC3)-PE (phosphatidylethanolamine) conjugation and Atg5-Atg12-Atg16L conjugation, have been shown to be involved in the autophagosome membrane elongation process (Zhou et al., 2011b, Fang et al., 2021). LC3 is cleaved by Atg4 cysteine protease to LC3-I, which is then conjugated to PE to generate LC3-II in the presence of Atg3, Atg7, and Atg16L in complex, which facilitates the phagophore expansion step in macroautophagy (**Fig. 1.1**). The mechanism of autophagy is complex, and its physiological functions in cells are context dependant. Thus, understanding the role of autophagy in various diseases is important for the discovery of novel drugs.

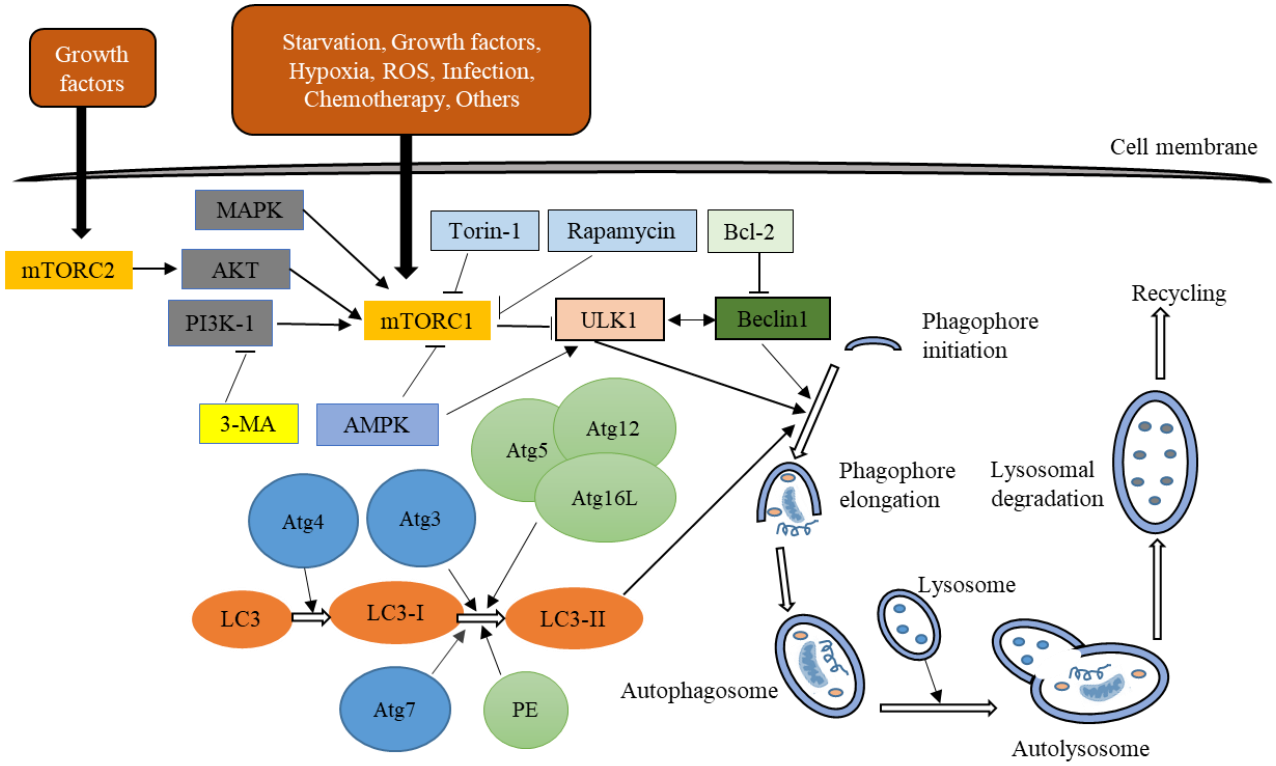


Figure 1.1 Diagrammatic representation of the macroautophagy process
Macroautophagy is modulated by a signalling cascade involving AMP-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR). Signals from a variety of sources, including growth factors, hypoxia, reactive oxygen species (ROS), energy levels, amino acids, pathogen-associated molecular patterns, and cytokines, are sensed and integrated by this mechanism. In response to nutrient starvation or other stimuli, mTOR act as a negative regulator of autophagy by regulating UNC-51-like kinase 1 (ULK1) via phosphorylating and destabilising the serine/threonine-specific protein kinase ULK1. Alternatively, under Nutrient deprivation, AMP-activated Protein Kinase (AMPK) inhibits mTOR activation, which in turn facilitates autophagosome formation. A number of essential molecular components are involved in the induction and completion of the complex autophagy pathway. Numerous autophagy-related proteins (ATG proteins) potentially regulate the process. In macroautophagic, cytoplasmic constituents and dysfunctional organelles are engulfed by the elongated phagophore, resulting in a double membrane autophagosome. The autophagosome can ingest large amounts of cytoplasm, or it can precisely target cargos. After fusing with lysosomes, autolysosomes are formed, which hydrolyse the sequestered components. Lysosomal enzymes break down the inner membrane of autophagosomes, and the cargoes and the essential biomolecules are recycled back into the cytoplasm.

1.2 LC3-Associated Phagocytosis (LAP)

Microtubule-associated protein light chain 3 (LC3), previously considered as a primary marker of autophagy, is also found to involve in LC3-associated phagocytosis (LAP) (Inomata et al., 2020, Asare et al., 2020). LAP is a unique autophagosome-independent process wherein

autophagy components are recruited to cargo-containing single membrane phagosomes, or "LAPosomes" for digestion and clearance (Asare et al., 2020). There is a functional and mechanistic difference between the LAP and autophagy process. It is important to note that LAP is activated upon phagocytosis of particles carrying ligands that engage cell-surface receptors, such as FcRs, TLR1/2, TLR2/6, TLR4, and T cell receptor (TCR)-interacting molecule (TRIM) (Heckmann et al., 2017, Huang et al., 2018). LAP does not require a pre-initiation complex, but it does require Beclin1, ATG5, and ATG7 (Martinez et al., 2015, Asare et al., 2020). Furthermore, LAP also requires Class III phosphoinositide 3-kinase-associated protein, Rubicon, which is not essential for autophagy (Martinez et al., 2015). The Class III PI(3)K complex in autophagy and LAP are similar but vary in some components. The main components of the PI(3)K complex are Beclin 1, VPS15, and VPS34, and these are commonly associated with both autophagy and LAP (Martinez et al., 2015). However, Rubicon is required for the Class III PI(3)K complex and the LAPosome interaction and subsequent production of Phosphatidylinositol 3-phosphate, PI (3)P (Martinez et al., 2015, Wong et al., 2018). Another notable difference is the rapidity in which LAP occurred. LC3 II is detectable on LAPosomes within 10 minutes of phagocytosis, while autophagosomes can take hours to form (Martinez et al., 2011).

1.3 Autophagy and Disease

Autophagy, particularly dysfunctional autophagy, has been found to be associated with diverse human diseases and regulate the function of cells of the immune system. Autophagy can play a protective or pathological role in various diseases, even at different phases of the same disease (Rubinsztein et al., 2012, Khandia et al., 2019, Bar-Yosef et al., 2019, Corti et al., 2020). Different natural products, kinase inhibitors (mTOR inhibitors), and antiangiogenic agents can trigger autophagy, and overactivation of autophagy might contribute to pathology in systemic lupus erythematosus (SLE), rheumatoid arthritis, and cancer (Liang et al., 1999, Harr et al.,

2010, Xu et al., 2015, Liu et al., 2016c, Liu et al., 2020, Chen et al., 2018, Su et al., 2016). Conversely, loss or disruption of autophagy, either as a result of specific polymorphisms, by siRNA silencing of autophagy genes or inhibition with pharmacological inhibitors, accounts for some of the effects of ageing, inflammatory bowel disease (IBD), diabetes, and neurodegenerative disorders, such as Alzheimer's, Parkinson's and Huntington's diseases (Jung et al., 2008, Shintani and Klionsky, 2004, Chen et al., 2010, Levine et al., 2011, Connor et al., 2012, Munoz et al., 2005, Guo et al., 2018, Fujikake et al., 2018).

Importantly, autophagy has been demonstrated to influence inflammation through the regulation of inflammatory molecules, including cytokines, signalling molecules, and inflammasome components (Mintern and Villadangos, 2012, Mintern and Harris, 2015, Harris et al., 2017, Sun et al., 2017, Dai et al., 2017, Vomero et al., 2020). Targeting particular regulatory molecules involved in the autophagy process may have a significant impact on controlling the disease. Numerous studies reported that Pharmacological inhibition or activation of autophagy by small molecule or pharmacological agents could significantly affect diseases including cancer, tuberculosis, sepsis, heart disease, diabetes, Huntington's disease, and autoimmune and inflammatory disease including colitis, rheumatoid arthritis (RA), and SLE (**Table 1.1**). Induction or inhibition of autophagy may have therapeutic effects by controlling cell survival or death. Thus, a better understanding of the autophagic machinery, the pharmacology, and mode of action of autophagy modulators could ultimately facilitate designing an autophagy-based therapeutic strategy for treating various human diseases.

Table 1.1: Effect of autophagy modulators in different human diseases

Associated Diseases	Agent/Small molecules	Modulation of Autophagy	Effects on disease	References
Cancer	Everolimus,	Activation	Inhibit mTORC1 signalling, Stimulate autophagy-mediated cyclin D1 degradation in breast cancer cells	(Chen et al., 2019a)
	Temsirolimus		Increase autophagy and inhibit PI3K/Akt/mTOR pathway in the lung cancer cells	(Jeong et al., 2012)
	Chloroquine, Hydroxy chloroquine	Inhibition	Induce high concentration of lysosomal enzymes into the cytosol of cancer cells and activate Bcl-2 family proteins, eventually leading to cell death; induce p53-dependent apoptosis or cell cycle arrest.	(Zhang et al., 2015b)
Heart disease	Sulfaphenazole, Rapamycin	Activation	Inhibits cardiomyocyte hypertrophy by promoting autophagy via MEK/ERK-dependent up-regulation of Beclin-1; inhibition of mTOR promoted the degradation of protein aggregates via autophagy	(Gu et al., 2016, Salabei and Conklin, 2013)
	Trichostatin A, Propofol, Urocortin	Inhibition	Activates PI3 kinase/Akt pathway and inhibits autophagy through inhibition of Beclin-1 and activation of mTOR during ischemia/reperfusion	(Noh et al., 2010, Valentim et al., 2006)
Infectious disease	AR-12 [2-amino-N-[4-[5-(2 phenanthrenyl)-3-(trifluoromethyl)-1H-	Activation	Up-regulation of Atg13 and PERK, which induces autophagy and facilitates the clearance of intracellular viruses and/or unfolded proteins	(Booth et al., 2016)

	pyrazol-1-yl] phenyl]-acetamide], 1α,25-dihydroxycholecalciferol, a form of vitamin D			
Diabetes	Liraglutide, Exenatide	Activation	Autophagy induction through upregulation of ATG5, AMPK and PI3K/Akt; Hepatoprotective effect by autophagy induction through enhanced the expression levels of Beclin-1 and LC3A/B-II/I	(Fan et al., 2018, Candeias et al., 2018)
	Metformin	Inhibition	Attenuation of hyperglycemia-induced endothelial impairment by autophagy inhibition	(Niu et al., 2019)
Neurodegenerative disease	Rilmenidine, Trehalose	Activation	Upregulation of mTOR-independent autophagy; upregulating autophagy-related genes including Lc3, Becn1, Sqstm1 and Atg5 at mRNA level	(Castillo et al., 2013, Frake et al., 2015)
Rheumatoid arthritis (RA)	Temsirolimus, Methotrexate	Activation	Stimulate autophagy through inducing autophagosome formation and enhancement of autophagic flux	(Xu et al., 2015)
	3MA, Chloroquine	Inhibition	Inhibits autophagy at an early stage of autophagosome formation, enhance immune-negative molecules such as interleukin 10 (IL-10), transforming growth factor β and IL-35, enhance Tregs production.	(Xiu et al., 2014, Dai et al., 2016)

Crohn's disease (CD)	5-ASA Mesalamine/ Sulfasalazine hydrogen sulphide (H_2S)	Activation	Reduces colon epithelial cell proliferation and induces protective autophagy by releasing hydrogen sulphide (H_2S) through AMPK and inhibiting mTOR, as well as lowering TNF-α expression.	(Wu et al., 2012b, Fiorucci et al., 2007)
Systemic lupus erythematosus (SLE)	Dexamethasone, Vitamin D, Rapamycin	Activation	Induce autophagy promotion by inhibiting mTOR phosphorylation; interferes with lysosomal acidification, inhibiting the degradation of autophagic vacuoles.	(Harr et al., 2010, Oaks et al., 2016, Zhang et al., 2020a)
	Chloroquine	Inhibition	Th17/Treg-mediated immunity balance is restored, reduce the level of inflammatory cytokinesa and improved SLE	(An et al., 2017)

1.3.1 Autophagy and Cancer

In recent years, the association between autophagy and cancer has gained extensive attention. Autophagy and numerous autophagy-related proteins have been found to play intricate and diverse roles in the immune system (Li et al., 2014, Webster, 2018, Morishita and Mizushima, 2019, Liu et al., 2020, Scherr et al., 2020). The multifaceted roles of autophagy in the immune system potentially lead to a context-dependent involvement for autophagy in cancer (Dower et al., 2018, Yazdani et al., 2019, Zhang et al., 2015b). In different stages of cancer progression, the function of autophagy likely differs; in the earliest stages of tumour growth and development, autophagy may have a protective effect against tumorigenesis; however, autophagy can enhance the growth and development of tumour by undermining the stress responses, thus increasing disease development after primary tumours or their metastases have been established (White, 2012, Janku et al., 2011, Thorburn et al., 2014, Galluzzi et al., 2015, Mulcahy Levy and Thorburn, 2020).

The association between autophagy and cancer was established based on two principal observations (Mah and Ryan, 2012, Lu and Harrison-Findik, 2013, Xu and Hu, 2019). First, several studies demonstrated that the monoallelic deletion of *BECN1* gene had been found in human ovarian, breast, and prostate cancer cells (Aita et al., 1999, Liang et al., 1999, Laddha et al., 2014, Wu et al., 2018a, Xu et al., 2017). Beclin 1, the yeast Atg6/vacuolar protein sorting (Vps)-30 mammalian orthologue, plays a key role in autophagy. It binds to the class III PtdIns 3-kinase, Vps34, to form the Beclin 1-Atg14-Vps34-Vps15 complex, which is essential for the localisation of downstream autophagic proteins at the site of the autophagosome formation and the induction of autophagy (Liang et al., 1999, He and Levine, 2010, Menon and Dhamija, 2018, Hill et al., 2019). Ectopic overexpression of BECN1 has also been found in MCF7 cells that have significantly low levels of endogenous Beclin 1, leading to the autophagy activation, inhibited proliferation as well as suppression of tumorigenesis (Liang et al., 1999, Mah and Ryan, 2012, Vega-Rubín-de-Celis, 2019). In gastric and colorectal cancer, mutations in several autophagy genes, such as Atg2B, Atg5, Atg9B, Atg12, and UVRAG, indicating that mutations in these genes may promote tumorigenesis by modifying the autophagy process (Kim et al., 2008, Kang et al., 2009, He et al., 2015).

Using transgenic mouse models lacking autophagy regulators, numerous studies demonstrated the mechanism of autophagic regulation in tumour growth and progression. Atg4C and Endophilin B1(BIF1) knockout mice and mice hemizygous for Becn1 have shown an increased incidence of tumour development (Mariño et al., 2007, Takahashi et al., 2007, Yue et al., 2003). It has also been reported that alteration of ATG4D expression results in fibroid-like cell phenotype by inhibition of autophagy, consequently, enhances the growth of human pelvic tumours (Andaloussi et al., 2017). However, increasing evidence suggests that autophagy plays a multifaceted and controversial role in tumorigenesis. Autophagy can facilitate the survival of tumour cells but may also lead to cell death (Degenhardt et al., 2006, Rosenfeldt and Ryan,

2011, Noguchi et al., 2020). In addition to this, cancer therapeutics can upregulate or suppress autophagy, and upregulation of autophagy may be either pro-survival or pro-death for tumour cells in cancer therapies (Levy and Thorburn, 2011, Wilkinson and Ryan, 2010, Liu et al., 2013a, Chen et al., 2019a, Jeong et al., 2012, Zhang et al., 2015b). High levels of autophagy have been found to play a vital role in pancreatic ductal adenocarcinoma (PDA) (Perera et al., 2015). Ras-driven pancreatic tumours promote the nuclear localization of the master regulatory MiT/TFE family of transcription factors, which activates transcription programs for autophagy and lysosomal biogenesis (Perera et al., 2015, White et al., 2015, Ferguson, 2015).

In various cancer cells, knocking down or ablation of key autophagy genes has been found to decrease cell survival and tumour progression, suggesting a potential role of autophagy in cancer (Yang et al., 2014, Wen et al., 2019, Lauzier et al., 2019). Using Cancer Genome Atlas (TCGA) datasets, Wen et al. found that in glioma patients, ATG4C mRNA expression was associated with the pathological glioma grades, suggesting individuals with relatively high ATG4C mRNA expression were susceptible to developing high-grade glioma (Wen et al., 2019). In an ectopic xenograft nude mice model, they found that depletion of ATG4C enhanced p21 and p53 expression with decreasing Cyclin E expression, suggesting ATG4C ablation inhibited the proliferation of glioma cells, possibly promoting G1/S Phase Cell Cycle Arrest (Wen et al., 2019). In a recent study, Poillet-Perez et al. demonstrated that in the hepatocytes, the autophagy process might promote the growth of tumour primarily through metabolic and immune mechanisms relying on mutational burden: by promoting regulatory T-cell activity and inhibiting interferon genes stimulator and interferon-γ, that facilitates high-TMB tumours growth or by maintaining circulating arginine in low TMB tumours (Poillet-Perez et al., 2020, Poillet-Perez et al., 2018). Therefore, the precise function of autophagy in cancer is likely context-dependent and understanding the mechanism by which manipulation of autophagy modulates the tumour immune response is essential for the development of cancer therapies.

1.3.2 Cardiovascular diseases

Autophagy has a dual function in many cardiovascular disorders, depending on the timing and severity of the activation and the cell type(s) involved. For example, decreased basal autophagy has been documented to encourage heart failure (HF) (Nakai et al., 2007, Nishino et al., 2000). Activation of autophagy has been shown in several studies to reduce the accumulation of aberrant proteins and other intracellular aggregates associated with cardiovascular disease (Pattison and Robbins, 2011, Bhuiyan et al., 2013, Khandia et al., 2019). However, increased autophagy may also be risky for the heart, leading to excessive intracellular component degradation and cell death of cardiomyocytes (Zhu et al., 2007a, Gustafsson and Gottlieb, 2009). Perturbations in autophagy have been related to heart disorders, including cardiomyopathies, cardiac hypertrophy, heart failure, ischemic heart disease, and damage due to ischemia-reperfusion (Pattison et al., 2011, Noh et al., 2010, Valentim et al., 2006). Autophagy is beneficial in cardiomyopathy caused by the aggregation of misfolded proteins. In proteotoxic cardiomyopathy, caused by aggregation of the alpha B-crystallin mutant, Atg7-dependent autophagy reduces the accumulation of amyloid oligomers in cardiomyocytes, indicating that stimulation of autophagy can boost cardiac function and reduce ventricular remodelling (Tannous et al., 2008). On the other hand, autophagy inhibition by partial genetic deletion of *Becn1* accelerates ventricular dysfunction in the alpha B-crystallin mutant mice (Verheye et al., 2007). Increased numbers of autophagosomes are evident in macrophages from atherosclerotic plaques, and autophagy may stabilise plaques by preventing apoptosis and necrosis and by maintaining clearance of apoptotic cells by efferocytosis (Liao et al., 2012, Nakai et al., 2007, Shao et al., 2016) (*see* **section 1.5.3**).

Autophagy is essential for the degradation of dysfunctional long-lived proteins and to clear defective organelles, thereby maintaining cellular homeostasis as well as the function and survival of cardiomyocyte (Shimomura et al., 2001, Chun and Kim, 2018, Aman et al., 2021).

Autophagy may also protect the heart from various stresses (Maejima et al., 2015). In the heart, the level of autophagy is altered not only in response to stress, such as ischemia/reperfusion, but also in response to stress caused by cardiovascular conditions, including cardiac hypertrophy and heart failure. Autophagy is rapidly activated in these conditions and performs a protective function by promoting cell survival. In the stressed heart, autophagy activation supplies energy substrates and facilitates cellular remodelling (Zhu et al., 2007a). The degree of autophagic development in a pressure overload model corresponds with the level of hypertrophic growth and the rate of transition to heart failure (Cao et al., 2011). The pathological remodelling response is exacerbated by cardiomyocyte-specific overexpression of *BECN1* (Cao et al., 2011, Cao and Hill, 2011). Conversely, HF is partially saved by *BECN1* Haploinsufficiency (Cao and Hill, 2011). These findings suggest that autophagy under conditions of extreme pressure overload can be maladaptive. Autophagy in cardiomyocytes seems to function largely as a pro-survival process under normal or mildly stressed conditions. However, when autophagy becomes overactive, it can have a deleterious effect on cells (Cao and Hill, 2011, Jia and Sowers, 2015).

1.3.3 Diabetes

Diabetes mellitus is a long-term metabolic condition characterised by hyperglycaemia and metabolic modifications caused by a combination of insulin resistance and/or β cell dysfunction (Kopan et al., 2018). Type 1 diabetes is primarily caused by an autoimmune attack of T cells on pancreatic β cells that contributes to their destruction and insulin deficiency, leading to hyperglycaemia (Katsarou et al., 2017). Type 2 diabetes, on the other hand, is an age-and lifestyle-related condition that affects various organs and tissues and is caused by a combination of insulin resistance and/or cell dysfunction (Nathan et al., 2009). Diabetes mellitus has been linked to an abnormal level of autophagy in β cells (Bhattacharya et al., 2018), and autophagy plays a role in the pathogenesis of both type 1 and type 2 diabetes (Fan

et al., 2018, Candeias et al., 2018). It has been suggested that in type 1 diabetes, the regulatory mechanism of autophagy in insulin-producing β-cells differs from those following the development of insulin resistance in type 2 diabetes (Bhattacharya et al., 2018, Marasco and Linnemann, 2018). In people with type 1 diabetes, a lack of insulin synthesis can cause excessively high blood glucose levels, whereas in type 2 diabetes, resistance to the cellular effects of insulin is impaired, and/or β-cells gradually lose the capacity to produce enough insulin (2013, Jung et al., 2008). Autophagy plays a vital role in diabetes and metabolic diseases because insulin and glucagon produce pancreatic cells are well-defined autophagy regulators (Yamamoto et al., 2018, Niu et al., 2019). It has been reported that in human autophagy signalling is inhibited by insulin, whereas glucagon stimulates autophagy (Ebato et al., 2008, Moruno et al., 2012, Møller et al., 2018). Basal autophagy is critical for the regulation of proper β-cell functioning (Jung et al., 2010, Lee et al., 2019). Moreover, mice lacking the Atg7 gene showed a significant decrease in β-cell mass and insulin level as well as hyperinsulinemia and hyperglycaemia, suggesting a crucial role for autophagy in islet function (Ebato et al., 2008, Zhou et al., 2009). Autophagy participates in the downregulation of insulin receptors and ER stress-mediated insulin resistance, and conversely, insulin resistance also suppresses autophagy (Liu et al., 2009, Bernales et al., 2007). Defective autophagy may contribute to mitochondrial malfunction and ER stress since it is anticipated to play a role in the maintenance of mitochondrial or ER function (Rodriguez-Enriquez et al., 2006, Kesidou et al., 2013, Biczo et al., 2018).

1.3.4 Neurodegenerative diseases

Brain cells, such as neurons, are post-mitotic and thus require tightly regulated protein quality control strategies. Autophagy maintains cellular homeostasis and is essential for neuronal cells. Impaired autophagy in neurons provokes improper homeostasis and neurodegenerative phenomena (Frake et al., 2015). Neuronal cell autophagy is required for anti-inflammatory

activity in glial cells, synaptic plasticity, oligodendrocyte development, and myelination processes. Autophagy is required for the elimination of aggregated proteins (aggresomes), and inhibiting basal autophagy causes neurodegeneration in the central nervous system (Chu, 2006, Kiriyama and Nochi, 2015). Autophagic flux is the balance between the formation of autophagosome and autophagic degradation, the disruption of which results in neuronal cell death (Kroemer and Levine, 2008, Lee, 2012, Cui et al., 2017). Excessive autophagy, on the other hand, might result in cytosolic component breakdown and likewise lead to neuronal cell death (Rubinsztein, 2006). Moreover, dysregulation of autophagic flux has been associated with neurodegeneration (Kiriyama and Nochi, 2015).

Increasing evidence suggests that alterations in autophagy-related genes and altered autophagic flux are associated with neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease (Rubinsztein et al., 2012, Mizushima, 2007, Janda et al., 2012, Kiriyama and Nochi, 2015, Webb et al., 2003, Sandhof et al., 2020). Knockdown of Becn1 in mice, enhanced accumulation intraneuronal amyloid beta, deposition of extracellular Abeta, and neurodegeneration as well as causing microglial alterations and severe neuronal ultrastructural abnormalities (Pickford et al., 2008). Also, Beclin1 overexpression in mice disrupted neuronal autophagy, consequently decreased the α-syn accumulation and alleviated the associated neuronal pathologic features (Pickford et al., 2008). Beclin 1 was shown to be considerably lower in the brains of Alzheimer's disease patients, and loss of Beclin 1 in amyloid precursor protein (APP) transgenic mice resulted in impaired autophagic activity in neurons, as well as faster β-amyloid accumulation and increased neurodegeneration (Lee and Gao, 2008, Bieri et al., 2018). Beclin 1 upregulation, on the other hand, reduced amyloid accumulation (Pickford et al., 2008, Jaeger et al., 2010).

Huntington's disease is a progressive, autosomal dominant, incurable neurodegenerative disorder associated with the expansion of the polyglutamine (polyQ) tract in huntingtin (Htt)

protein (Hoogeveen et al., 1993, Dabrowska et al., 2018). The polyQ tract is a part of a protein that consists of a sequence of multiple glutamine units, and various inheritable neurodegenerative diseases can arise if a polyglutamine tract in a certain gene becomes excessively long due to a mutation (Sarkar et al., 2007, Lieberman et al., 2019). Autophagy has been found to involve in controlling the turnover of Htt, which is the major component of the nuclear and cytosolic inclusions identified in the neurons affected in Huntington's disease (Jia et al., 2007, Croce and Yamamoto, 2019, Cortes and La Spada, 2014). Knockdown or knockout of the *Atg* genes, such as *Atg5* or *Atg7*, in *Caenorhabditis elegans*, enhances the aggregates formation and toxicity of the polyQ expansion proteins (Sarkar, 2013, Vilchez et al., 2014). Other studies using various models of mammalian cells, flies, and rodents have shown an increase in HD phenotypes after treatment with different autophagy inducers (Ravikumar et al., 2004, Southwell et al., 2017). The mTOR inhibitor rapamycin significantly reduced photoreceptor degeneration in a HD fly model (Sarkar et al., 2008). In addition, lithium, an mTOR independent autophagy inducer, substantially decreased neurodegeneration in the fly model of HD (Williams et al., 2008). Similar results were observed in zebrafish models of HD. The mTOR-dependent (rapamycin) and mTOR independent (verapamil, clonidine, calpastatin, and 2′5′-dideoxyadenosine) autophagy inducers have been shown to reduce mutant huntingtin aggregate count in the HD zebrafish retina (Sarkar et al., 2011, Rose et al., 2010). Autophagy enhancers have also been found to be beneficial in HD mice (Kovács et al., 2017, Bailus et al., 2021). Thus, Autophagy upregulation provides a possible treatment strategy for various neurodegenerative diseases.

1.3.5 Crohn's Disease

Crohn's disease (CD), also known as inflammatory bowel disease (IBD), is a chronic relapsing gastrointestinal disorder. CD is characterized by transmural inflammation, which affects all layers of the intestinal wall and causes fibrosis, strictures, and fistulae (Kobayashi et al., 2020).

Both environmental factors and genetic predisposition contribute to CD (Armitage et al., 2004, Alirezaei et al., 2009, Kobayashi et al., 2020). Impaired autophagy has also been associated with the disease, and there are multiple points where autophagy can affect immune responses contributing to the development and pathogenesis of CD (Iida et al., 2017, Lassen and Xavier, 2017, Shao et al., 2021, Wang et al., 2017). Susceptibility to CD has been linked to genetic variations in the genes coding for Atg16L1 and another key autophagy-related gene encoding immunity-related GTPase family M protein (IRGM) (Saitoh et al., 2008, Ajayi et al., 2019). These two autophagy-related genes are known to be critical for autophagy as well as antigen presentation (McCarroll et al., 2008, Fletcher et al., 2018). It has been demonstrated that Atg16L1 deficient mice are highly susceptible to dextran sulphate sodium-induced colitis (Saitoh et al., 2008). Moreover, macrophages from Atg16L1-deficient macrophages produce increased IL-1β in response to LPS and the TLR3 ligand poly (I: C) (Saitoh et al., 2008). In CD, Atg16L1 deficient Paneth cells similarly showed higher expression of inflammatory cytokines (Saitoh et al., 2008), and mice lacking Atg16L1 in CD11c+ DCs produced more proinflammatory cytokines such as IL-1β and TNF-α (Zhang et al., 2017a). Moreover, the production of adipocytokines, leptin, and adiponectin by Paneth cells has increased in Atg16L1 and Atg5 deficient mice (Cadwell et al., 2008).

In humans, two IRGM polymorphisms have been associated with CD, and *Irgm1* -deficient mice have are less capable of fighting intracellular bacteria and more vulnerable to infection (Brest et al., 2011). Optimal IRGM expression is required for effective xenophagy (autophagy directed against pathogens). Both over-expression and under-expression of IRGM can reduce the efficacy of xenophagy (Jostins et al., 2012). CD can be prevented by effectively limiting symbionts and pathogens in the gut environment (Cadwell et al., 2008), which is demonstrated by the finding of irregular gut microbiotic profiles in CD patients (Nell et al., 2010, Jones et al., 2013). Autophagy/xenophagy can control the gut microbial profile and restrict pathogenic

bacterial invasion (Haq et al., 2019). In addition, autophagy further facilitates commensal bacteria tolerance by controlling the consequence of the interplay between T-cell antigen-presenting cells and modulating the cytokine profile in the gut to avoid an extensive pro-inflammatory response (Bhonde et al., 2008, Jones et al., 2013).

1.4 Polymorphisms in autophagy-related genes

Autophagy gene polymorphisms have been shown to be correlated with the development of different chronic inflammatory diseases (**Table 1.2**). In particular, genetic polymorphisms of *ATG5* (rs510432, rs506027, and rs548234) and *ATG16L1* (rs10210302 and rs2241880) are associated with susceptibility to systemic lupus erythematosus, sepsis, RA and Crohn's disease (Gateva et al., 2009, Nuij et al., 2017, Shao et al., 2017, Lu et al., 2011, Lacher et al., 2009). A previous study has also reported that *ATG16L1* (rs2241880) and *ATG5* (rs2245214) polymorphisms are related to a higher risk of developing Paget's disease of bone (PDB) (Usategui-Martín et al., 2015). Further findings have confirmed SNPs in *ATG16L1* (rs2241880) and *IRGM*) are associated with increased susceptibility to Crohn's Disease (rs13361189, rs4958847, and rs10065172) (Parkes et al., 2007, Prescott et al., 2010, Moon et al., 2013, Sehgal et al., 2012, Lu et al., 2014). Polymorphisms in *ATG16L1* (rs2241880) and *IRGM* (rs13361189 and rs4958847) also have a weak association with ulcerative colitis (UC) susceptibility (Palomino-Morales et al., 2009). One *IRGM* isoform (*IRGMd*) has also been linked to mitochondrial dysfunction (Singh et al., 2010). It has been reported that IRGM controls mitochondrial, nuclear fission by translocating to the inner membrane of the mitochondria (Singh et al., 2010).

Another study has shown that the *ATG7 V471A* polymorphism plays an important role in Huntington disease (HD) pathogenesis and causes an approximately four years earlier onset of the disease, particularly in the Italian population (Metzger et al., 2010, Metzger et al., 2013). The rare p.471A allele might impair the activity of *ATG7* and ultimately disrupt the autophagic

process and breakdown of mutant huntingtin (mhtt). ATG7 deficiency in mice results in a loss of cerebellar and cortical neurons and the development of intracellular aggregates that are not degraded by proteasomes, suggesting that ATG7 malfunction might contribute to neurodegeneration (Komatsu et al., 2006).

Table 1.2: Polymorphisms in autophagy-related genes in different human diseases

Gene	SNP	Disease-associated	Function in Autophagic Regulation	Modulation of Autophagy protein	References
ATG16L1	rs2241880, rs10210302	IBD and Crohn`s disease	Associates with isolation membrane in complex with Atg 5-Atg 12; assists in autophagosomal elongation	Activation	(Lacher et al., 2009, Nuij et al., 2017)
ATG5	rs2245214, rs510432, rs506027, rs548234	SLE, sepsis, RA	Forms a complex with Atg12; assists in autophagosomal elongation	Activation	(Gateva et al., 2009, Shao et al., 2017, Lu et al., 2011)
IRGM	rs72553867, rs10065172 rs4958847, rs13361189	Crohn's disease, sepsis	Interacts with ULK1 and Beclin1 and control the formation of autophagy initiation complexes.	Inhibition	(Moon et al., 2013, Sehgal et al., 2012, Lu et al., 2014)
ATG7	V471A, rs1375206	Huntington disease, Parkinson's disease	Interacts with LC3 and Atg12 and controls autophagic vesicle expansion, affects the function of Atg7 and inhibits autophagy	Inhibition	(Metzger et al., 2013, Zhao et al., 2020)

1.5 Autophagy in immunity and inflammation

Several studies reported that autophagy acts as a regulator and effector in both innate and adaptive immunity (Gross et al., 2009, Clarke et al., 2018, Tao and Drexler, 2020, Metur and Klionsky, 2021). Importantly, autophagy plays a vital role in both activating and suppressing immunological and inflammatory responses. In particular, autophagy controls a number of immunological processes, such as recognition and destruction of the pathogen, antigen presentation, efferocytosis, lymphocyte development, and effector function, regulation of inflammatory cytokines and signalling molecules and (Merkley et al., 2018, Chan et al., 1997, Harris, 2011, Harris et al., 2017). Moreover, multiple immune and inflammatory signals regulate the activation and inhibition of autophagy (Harris, 2011, Ge et al., 2018, Wu et al., 2016).

1.5.1 Autophagy and pathogens

Autophagy has a protective role to play in the host immune response to a number of intracellular bacterial pathogens, such as *Salmonella enterica, Streptococcus pyogenes,* and *Mycobacterium tuberculosis* (**Fig. 1.2A**) (Gutierrez et al., 2004, Birmingham et al., 2006, Huang and Klionsky, 2007). In many of these cases, autophagosomes can engulf pathogen-containing phagosomes, leading to killing and lysosomal degradation (Ogawa et al., 2005). However, some pathogenic organisms such as *Shigella flexneri, Listeria monocytogenes, Brucella abortus, Staphylococcus aureus, Porphyromonas gingivalis, Coxiella burnetii,* and *Legionella pneumophila* are able to subvert autophagy in host cells to acquire nutrients for replication (**Fig. 1.2B**) (Webster, 2006, Dorn et al., 2002, Bélanger et al., 2006, Schnaith et al., 2007, Gutierrez et al., 2005, Amer and Swanson, 2005, Ogawa and Sasakawa, 2006, Huang and Klionsky, 2007). It is suggested that pathogen containing phagosomes can fuse with autophagosomes to create the protected vacuoles, and inhibition of autophagy is host-protective (Espert et al., 2007).

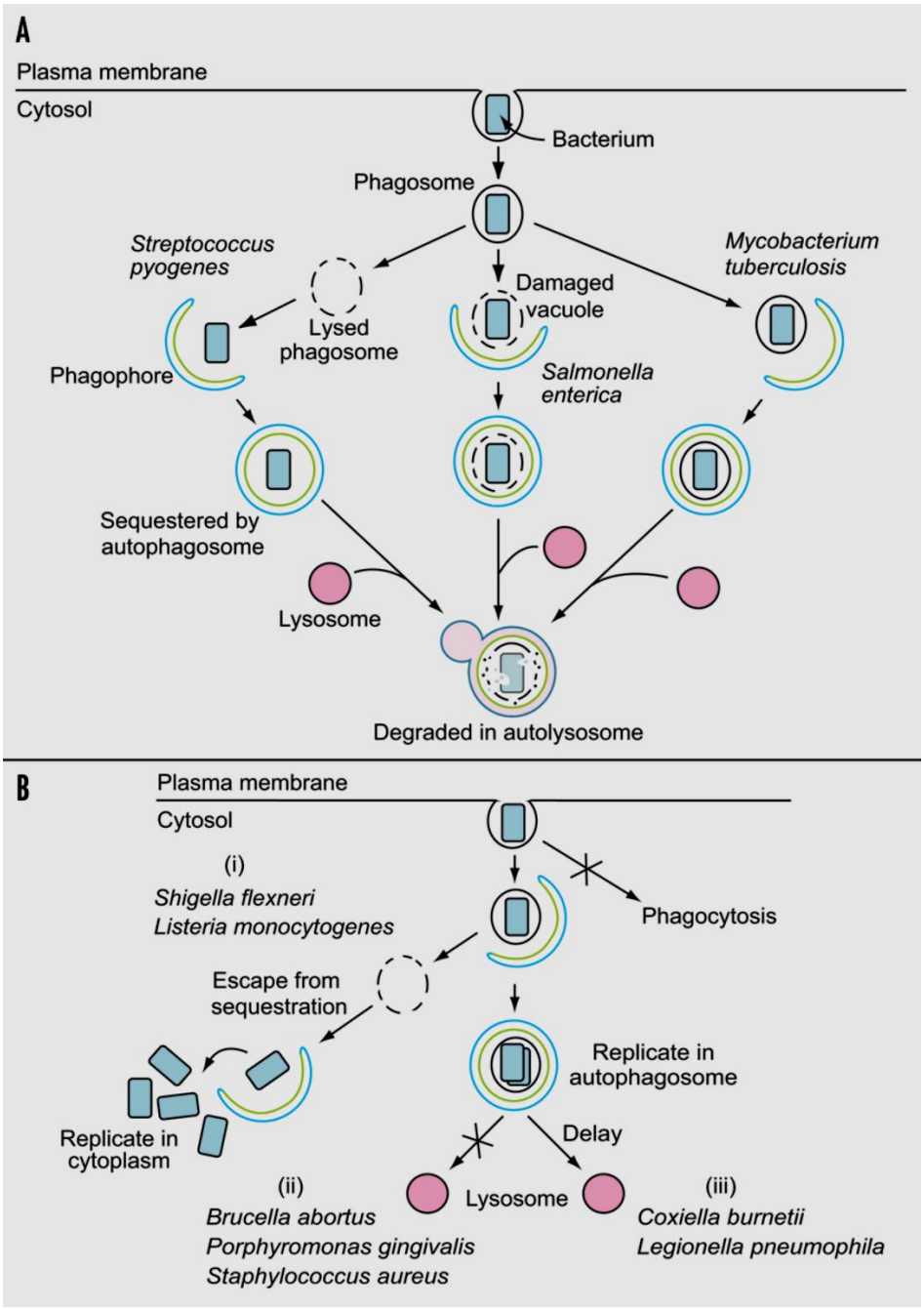


Figure 1.2 Interplay between autophagy and various intracellular pathogens in cell
(A) Autophagy is involved in the destruction of some intracellular bacteria. Upon autophagy activation, phagosomes containing pathogens are caught by the autophagic vacuole and then degraded by fusion with the lysosome. (B) Some bacteria exploit autophagy to release into the cytoplasm or use it for their replication (i) Bacteria such as *Shigella flexneri* and *Listeria monocytogenes* have the ability to lysis the phagosome and then make their way into the cytoplasm where they can reproduce. (ii) Other bacteria, including *Brucella abortus*, *Staphylococcus aureus* and *Porphyromonas gingivalis*, facilitate entry into and inhibit the autophagosome fusion with the lysosome and multiply in autophagosomes. (iii) Some other bacteria, for example, *Coxiella burnetii* and *Legionella pneumophila*, accelerate entrance into the autophagosome and inhibit fusion with the lysosome, giving them more time to complete their multiplication inside the autophagosome. Taken from (Huang and Klionsky, 2007).

Viruses infect and reproduce within their hosts by inserting their genetic material (DNA or RNA) into the cytosol. Upon virus infection, host cells can release type 1 interferons (IFNs), and IFN-γ has been found to upregulate autophagy (Liang et al., 1998, McNab et al., 2015, Singh et al., 2018). IFN-α can activate protein kinase C-related kinase (PKR) to inhibit the synthesis of protein and limit viral replication. Several studies support autophagy's antiviral activity in the fight against viral infection. For example, Beclin 1 overexpression in neurons prevents the replication of the Sindbis virus (SINV), which is the cause of fatal encephalitis (Huang and Klionsky, 2007, Liang et al., 1998). Furthermore, ATG5 deficiency causes delayed clearance of SINV, enhanced accumulation of p62 adaptor protein, and promotes neuronal cell death (Orvedahl et al., 2010).

1.5.2 Autophagy and antigen presentation

Autophagy plays a major role in antigen processing and presentation. Autophagosomes can present endogenous and exogenous antigens via the major histocompatibility complex (MHC) Class I and Class II pathways (Blum et al., 2013). MHC-I molecules present endogenous antigens to CD8+ T cells following degradation by the proteasome and translocation to the endoplasmic reticulum (ER). In contrast, MHC-II molecules present extracellular antigens from lysosome-derived organelles to CD4+ T cells (Wieczorek et al., 2017). However, certain exogenous antigens can be cross presented by MHC Class I molecules, and endogenous proteins can also be presented by MHC Class II molecules (Mintern et al., 2015, Embgenbroich and Burgdorf, 2018, Germic et al., 2019).

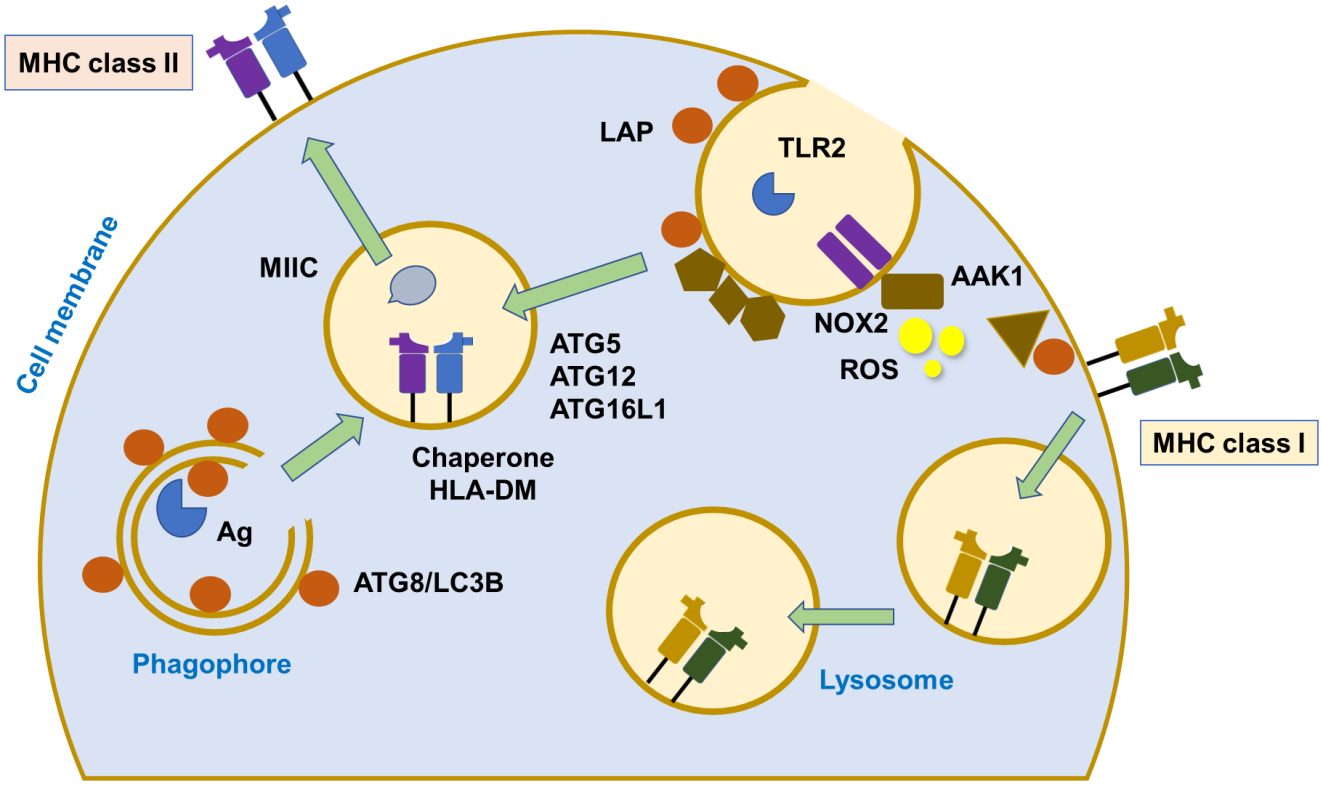


Figure 1.3 Mechanism of regulating MHC restricted antigen presentation by the macroautophagic machinery
Macroautophagy process entails the engulfment of cytoplasmic constituents and autophagosomes formation that can integrate with MHC class II-containing compartments. MHC class II molecules are assembled with peptides in MHC class II containing compartments (MIICs). They receive intracellular antigens (Ag) through macroautophagy and extracellular antigens through phagocytosis. During LC3-associated phagocytosis (LAP), LC3B are recruited on the surface of the phagosomes. ATG8/LC3B is bound to the cytoplasmic side of phagosomes by the ATG5-ATG12-ATG16L1 complex, which then transfers the endocytosed antigen to MIICs. The cargo of LC3/Atg8-associated phagosomes is efficiently processed for extended antigen presentation on MHC class II molecules that are loaded with lysosomal degradation products in MIICs with the help of the chaperone HLA-DM. The macroautophagy machinery, on the other hand, restricts MHC class I restricted antigen presentation, allowing MHC class I internalisation and lysosome mediated degradation. Then the adaptor-associated kinase 1 (AAK1) is transferred to MHC class I molecules for subsequent internalisation. Modified from (Münz, 2021).

Although a direct role for autophagy in the usual pathway for MHC class I presentation has not been found yet, studies have revealed possible functions for autophagy in modulating class I-mediated cross-presentation of exogenous antigens (Li et al., 2008, Shen and Rock, 2006, Mintern et al., 2015). Antigen-presenting cells (APC), such as dendritic cells (DC), internalise and break down antigens from the extracellular environment and presents the resultant peptides

to the cell surfaces in association with MHC class I molecules during the process of cross-presentation (Raghavan et al., 2008, Embgenbroich and Burgdorf, 2018). It has been reported that the immune surveillance of pathogen-infected cells and tumours, and the establishment of an adequate cytotoxic T cell response to these infections or tumorigenesis, are both accompanied by the class I-restricted cross-presentation system (Vyas et al., 2008, Crotzer and Blum, 2009, Gaudino and Kumar, 2019). Autophagy can be activated in cancer cells due to starvation or chemotherapy. Autophagosomes carrying tumour antigens may also be released into the extracellular environment, where they can be internalized by APC and Dc. However, it is still unclear where and how tumour antigens present in autophagosomes intersect with MHC class I molecules in DC. It has been found that these tumour peptides can be processed and presented by DC in the context of MHC class I for the activation of cytotoxic CD8-T-cells (Dengjel et al., 2005, Noubade et al., 2019).

Recent studies have demonstrated the involvement of autophagy in MHC class II-restricted antigen presentation (Valečka et al., 2018, Münz, 2021, Ligeon et al., 2021). It has been shown that autophagy can assist in the delivery of cytosolic constituents or pathogens to the lysosome and lead to antigen presentation by MHC class II molecules (**Fig. 1.3**) (Cooney et al., 2010, Münz, 2021). Professional APC, such as macrophages, DC and B cells, and also cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs), have been found to express MHC class II proteins on their surfaces (English et al., 2009, Uhl et al., 2009, Crotzer and Blum, 2009, Yamano et al., 2015, Breed et al., 2018). MHC class II molecules also regulate signal transduction contributing to B cell activation (Clement et al., 1986, Katikaneni and Jin, 2019). Several experimental results revealed that monoclonal antibodies (MAb) specific to MHC class II molecules inhibit activation of B-cells by antigens (Forsgren et al., 1984, O et al., 2014, Spanier et al., 2016). O et al. (2014) reported that MHC-II molecules contribute to antigen-specific antibody production and confer effective defence against

influenza virus infection in MHC-II KO mice (O et al., 2014). Cytoplasmic or nuclear antigens can be sequestered into autophagosomes that fuse with mature endosomes, allowing antigens to associate with MHC class II molecules (Zhou et al., 2005, Schmid et al., 2007). Lysosomal proteases can process the cytoplasmic or nuclear antigens before attaching to class II molecules and presenting them to CD4-T cells (Schmid et al., 2007, Elmore, 2007, Jurewicz and Stern, 2019).

1.5.3 Autophagy and efferocytosis

Apoptosis is an evolutionarily conserved cell death process, required to control and maintain tissue growth and homeostasis (Arandjelovic and Ravichandran, 2015, Kurosaka et al., 2003, Singh et al., 2019). Apoptosis is required to prevent the outflow of intracellular substances and regulate the immunological response against autoantigens generated (Youle and Strasser, 2008, Li et al., 2020a). Dysregulation of apoptosis or impaired removal of dying cells has been associated with the pathogenesis of cancer, neurodegenerative diseases, heart disease, Psoriasis, SLE, and other complex disorders (Condeelis and Pollard, 2006, Leuner et al., 2007, Choudhury et al., 2005, Hahn et al., 2007, Savill et al., 2002, Weatherhead et al., 2011, Arneth, 2019). The effect of apoptosis on immunity has been extensively investigated, and numerous studies indicate an association between apoptosis and autoimmunity (Ruiz-Argüelles et al., 2007, Clancy et al., 2006, Lleo et al., 2007, Schiller et al., 2008, Weatherhead et al., 2011, Mayer et al., 2020, Krawczyk et al., 2020). Recent findings have suggested that autoantigens are found inside apoptotic bodies and that apoptotic cells are crucial for antigen presentation, activation of innate immunity, and regulation of cytokine production (Mandron et al., 2008, Baudino et al., 2014, Lawlor et al., 2015, Kourtzelis et al., 2020, Bellone, 2000, Trahtemberg and Mevorach, 2017, Karbian et al., 2020).

The process of apoptotic cell clearance is termed efferocytosis, and different professional phagocytes (e.g., macrophages and dendritic cells) and non-professional phagocytes (e.g.,

epithelial cells and fibroblasts) play a key role in this process (Lee et al., 2016a, Yurdagul et al., 2017). Efferocytosis is another essential process for maintaining tissue homeostasis, which is mechanistically different from classic phagocytosis (Doran et al., 2020). This process comprises multiple steps, including apoptotic cell finding, binding, internalisation, and degradation (Ravichandran, 2010, Liu et al., 2014a, Doran et al., 2020, Boada-Romero et al., 2020). Previous studies reported that autophagy and efferocytosis are connected and that inhibition of autophagy results in defective efferocytosis (Guarente, 2011). Resveratrol, a Sirt1 activator, has been shown to enhance efferocytosis of oxidised low-density lipoprotein (ox-LDL)-induced apoptotic RAW264.7 cells via activation of autophagy (Liu et al., 2014a). It has been observed that efferocytosis of apoptotic murine RAW264.7 macrophages was significantly increased following incubation with the proper dose of Respiratory syncytial virus (RSV) compared to the autophagy inhibitor 3-MA and Sirt1 inhibitor Nicotinamide (NAM) (Liu et al., 2014a). Decreased Sirt1 protein can result in inflammation via autophagy dysregulation and high levels of the acetylated nuclear factor-κB (NF-κB) (Chen et al., 2001, Albani et al., 2009). In a cellular model (SK-N-BE cells) of oxidative stress, Sirt1 has been shown to regulate the autophagy process by promoting autophagosomes formation (Albani et al., 2009). Inhibition of Sirt1 in human macrophages leads to the deregulation of other nutrient-sensing pathways, including mTOR and AMPK, resulting in impaired autophagy, which in turn promotes NF-κB activation, accumulation of autophagy marker proteins (p62/Sqstm1) and inhibits light chain (LC) 3-II expression in THP-1 cells (Takeda-Watanabe et al., 2012, Mocholi et al., 2018).

1.5.4 Autophagy and T cell biology

Autophagy plays a vital role in maintaining T cell homeostasis, exerting both positive and negative regulation of signalling pathways (Andrade et al., 2006, Wu et al., 2018b) (**Table 1.3**). TCR engagement promotes autophagy in CD4+ and CD8+ T-cells, and TCR engagement,

along with costimulatory molecules and cytokine signalling, is essential for complete T-cell activation (Pua et al., 2007, Hubbard et al., 2010, Andrade et al., 2006). JNK, a mitogen-activated protein kinase (MAPK) that is activated downstream of the TCR, has been suggested as a potential contributor to autophagy induction in CD4+ T cells (Pua et al., 2007, Zhou et al., 2015). Interestingly, autophagy has been found to adjust its cargo selection upon T-cell activation by excluding mitochondria from degradation and favouring sequestration of soluble cytosolic content (Andrade et al., 2006, Jia et al., 2015) instead. Selective cargo degradation may be associated with regulating activation-induced responses in T cells (Mocholi et al., 2018, Paul et al., 2012).

It has also been shown that in effector T cells, autophagy-mediated turnover of BCL10 is needed to regulate TCR-induced activation of NF-κB (Dowling and Macian, 2018, Campanello et al., 2021). Autophagy has also been demonstrated to affect activated T cell activity and differentiation via regulating metabolic pathways (Whang et al., 2017). Studies have found an apparent association between autophagy and T-cell metabolism, where autophagic control of metabolic activity affects effector functions and cell differentiation (Andrade et al., 2006, Sena et al., 2013). For example, Inflammatory bowel disease is worsened in mice with ATG16L1-depleted T cells, with enhanced T helper (Th)2 differentiation and lower regulatory T cell (Treg) production, but no significant alterations in Th1 or Th17 populations (Kabat et al., 2016). However, in a mouse model of diet-induced steatosis, it has been found that ATG7-depleted T cells produced more IFN-γ and IL-17 in both CD4+ and CD8+ T cells, indicating differentiation bias between Th1 and Th17 phenotypes in CD4+ T cells (Amersfoort et al., 2018).

Table 1.3: Autophagy regulates T cells through different pathways

Pathways	Notes	References
T cell receptor (TCR) signalling pathway	TCR stimulation is essential for complete activation of T cells, and activated T cells induce AKT/mTOR activity. TCR signalling is important for the production of mitochondrial ROS, that contributes to T cell activation.	(Powell et al., 1999, Wong et al., 2012)
Mammalian target of rapamycin (mTOR) pathway	T cell proliferation is directly regulated by transcriptional regulation of cell cycle regulators, and it has been proposed as an important regulator of T cell activation-induced cell growth and proliferation. mTOR regulates Th1, Th2, Th17, and Treg differentiation. mTOR also has been shown to regulate CD8+ memory formation.	(Iritani et al., 2002, Wang et al., 2011, Delgoffe et al., 2009, Delgoffe et al., 2011, Lee et al., 2010, Yang et al., 2013, Zeng et al., 2013, Araki et al., 2009)
AMP-activated protein kinase (AMPK) pathway	In glucose-starvation, AMPK has been found to support CD8+ T cell effector activity.	(Geltink et al., 2018, O'Neill and Hardie, 2013)
Cytokine signalling pathway	The cytokines IL-7 and IL-15, along with IL-2 and IL-4, induce autophagy in T cells.	(Botbol et al., 2015)

1.5.5 Autophagy and inflammasomes: regulation of IL-1 family cytokines

Autophagy can influence immune responses by regulating cytokine secretion, particularly members of the IL-1 cytokine family. The IL-1 cytokine family includes IL-1α, IL-1β, IL-18, IL-33, IL-36, IL-37, and IL-38, which have numerous immune and physiological effects (Fiorentino et al., 2016, Xu et al., 2014, Gao et al., 2017b, Dinarello, 2018). Importantly, IL-1α, IL-1β, and IL-18 are effective pyrogens that exert a pathogenic role in inflammation if secreted in a chronic and/or uncontrolled manner (Dinarello, 2018, Harris, 2013), and loss of

autophagy causes excessive release of IL-1α, IL-1β and IL-18 in response to Toll-like receptor 3 (TLR3) and TLR4 ligands (Saitoh et al., 2008, Harris et al., 2011).

Production of biologically active IL-1β and IL-18 typically occurs in two stages. First, Toll-like receptors (TLRs) recognize external and intracellular pathogen-associated molecular patterns (PAMPs) that lead to the transcription of pro-IL-1 and pro-IL-18 cytokines through NF-kB signalling pathway. Secondly, activation of an inflammasome complex occurs, ultimately leading to caspase-1 activation, which then cleaves pro-IL-1β and pro-IL-18 into their mature forms for secretion (Harris et al., 2017, Rönnblom and Elkon, 2010). An inflammasome is a cytosolic protein complex that includes pattern-recognition receptors (PRRs) like NOD-like receptors (NLRs), including NLRP1, NLRP3, and NLRC4 or absent in melanoma 2 (AIM2), as well as the adaptor protein apoptosis-associated speck-like protein carrying caspase recruitment domain (ASC) and the protease caspase-1 (Schroder and Tschopp, 2010). Each NLR forms its own inflammasome, and the NLRP3 inflammasome has been characterised very well (Lamkanfi and Dixit, 2014).

The NLRP3 inflammasome can be activated by a number of stimuli or and multiple cellular and molecular events, including reactive oxygen species (ROS), cytokines mitochondrial DAMPs and ATP, crystalline structures (e.g., uric acid crystals), fibrillar proteins (e.g., β-amyloid fibrils), and environmental irritants (e.g., silica, alum), lysosomal damage (Davis et al., 2011, Lang et al., 2018, Yang et al., 2019, Kelley et al., 2019). NLRP1 and NLRC4 inflammasomes have been found to be activated by PAMPs such as muramyl dipeptide and flagellin, respectively (Broz and Dixit, 2016, Yi et al., 2019, Gram et al., 2021). The AIM2 inflammasome is activated by dsDNA, which can come from the host (nucleic and mitochondrial dsDNA) or from the pathogen (Guo et al., 2015, Morrone et al., 2015).

Several studies demonstrate that autophagy contributes to regulating inflammasomes and IL-1 family cytokines, limiting excessive inflammation (**Fig. 1.4**). Lack of the autophagy protein Atg16L1 contributes to increased activation of caspase-1 and a high level of IL-1β and IL-18 production in fetal liver macrophages after treatment with lipopolysaccharide (LPS, endotoxin; a TLR4 ligand) (Saitoh et al., 2008). Similarly, genetic (siRNA) or pharmacological (3MA) inhibition of autophagy significantly enhanced IL-1β and IL-18 release by macrophages in response to TLR3 and TLR4 ligands (Saitoh et al., 2008). This effect was influenced by potassium efflux, reactive oxygen species (ROS) and Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) (Saitoh et al., 2008). A second study confirmed these findings and demonstrated increased IL-1α secretion by autophagy-deficient macrophages (Harris et al., 2011). This study also suggested a partial role for NLRP3 in this effect (Harris et al., 2011). Other studies demonstrated a specific role for mitochondrial ROS and mitochondrial DNA in this response, suggesting that defective autophagy may lead to the accumulation of damaged mitochondria in cells treated with TLR ligands, leading to activation of the NLRP3 inflammasome (Nakahira et al., 2011, Shimada et al., 2012).

Another study demonstrated that autophagosomes degrade the inflammasome components, including NLRP3 and ASC, via the specific autophagic cargo receptor p62 (Shi et al., 2012) (**Fig. 1.4**). Similarly, Autophagosomes have been demonstrated to sequester IL-1β directly (Zhang et al., 2015a). Autophagy induction during or after LPS stimulation reduces the level of intracellular IL-1β in cells and suppresses its secretion in response to NLRP3 activators (Harris et al., 2011), suggesting autophagy plays a role in the turnover of intracellular pro-IL-1β. However, another research found that in dendritic cells, IL-1β (and IL-1α) are turned over by proteasomes rather than autophagosomes (Ainscough et al., 2014). Moreover, two other studies have demonstrated a role for autophagosomes in facilitating the unconventional secretion of IL-1β (Dupont et al., 2011, Zhang et al., 2015a) (**Fig. 1.4**). Thus, the precise role

of autophagy in the degradation and/or release of IL-1β appears to be complicated and possibly context-dependent.

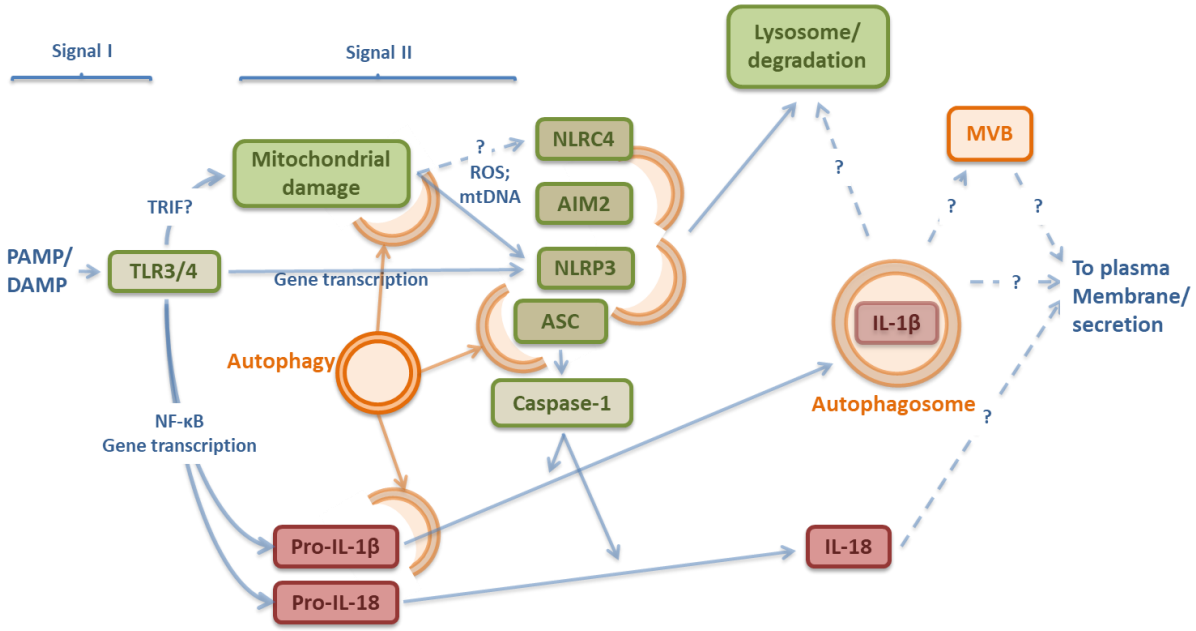


Figure 1.4 Inflammasome-dependent IL-1/IL-18 production is regulated via autophagy. Two signals are necessary for caspase-1 dependent inflammasome activation. The priming signal (signal I) requires the interaction of a receptor for pattern recognition, including a Toll-like receptor (TLR). As a consequence, NF-κB is activated, and the transcription of pro IL-1β, pro IL-18, and other components is upregulated. An exogenous Pathogen Associated Molecular Pattern (PAMPs) molecules or endogenous molecules induced by Damage Associated Molecular Patterns (DAMPs) provides the second signal (signal II), resulting in the assembly of inflammasome complex and caspase-1 activation. Taken from (Harris et al., 2017)

Aberrant activation of inflammasomes and the release of IL-1 family cytokines are involved in the development and pathology of multiple autoimmune and inflammatory diseases including type 2 diabetes, atherosclerosis, IBD, multiple sclerosis, lupus (particularly lupus nephritis), rheumatoid arthritis, Alzheimer's disease, Parkinson's disease and cryopyrinopathies (for example, chronic infantile neurologic cutaneous articular syndrome and Muckle-Wells syndrome) (Garlanda et al., 2013, Bronietzki et al., 2015, Ahmed et al., 2017, D'Espessailles et al., 2018, Zahid et al., 2019, Hong et al., 2019, Unterberger et al., 2021, Jäger et al., 2020).

Thus, regulation of IL-1 family cytokines by autophagy, both directly and through effects in inflammasome activation, highlights a potential therapeutic strategy for the treatment of various diseases.

1.5.6 Autophagy and other cytokines

The interaction between autophagy and cytokines may be a key component for orchestrating the innate and adaptive immune system (Deretic and Levine, 2018, Metur and Klionsky, 2021). Different cytokines have been shown to control autophagy. For example, the pro-inflammatory cytokine interferon (IFN)-γ activates autophagy to promote the killing of infectious pathogens such as *Mycobacteria* and *Chlamydia* (Gutierrez et al., 2004, Al-Zeer et al., 2013). Autophagy is also induced by IL-1 (α and β), tumour necrosis factor (TNF), IL-6 and IL-17, whereas autophagy is inhibited by IL-4, IL-13, IL-23, IL-33 and IL-10 (Harris and Keane, 2010, Shi and Kehrl, 2010, Harris et al., 2007, Harris et al., 2009, Park et al., 2011, Van Grol et al., 2010, Shen et al., 2017, Cha et al., 2014, Hu et al., 2021, Ramakrishnan et al., 2020). These findings strongly support the idea that autophagy is a key pathway in immune responses. Autophagy, on the other hand, may control the release of many cytokines, including IL-1 (α and β) (Harris et al., 2011, Harris, 2013, Claude-Taupin et al., 2018, Iula et al., 2018).

Autophagy and IFN-γ

IFN-γ is a fundamental pro-inflammatory cytokine produced mostly by natural killer cells and active CD4+ or CD8+ T cells. IFN-γ has been shown to play a crucial role in both innate and adaptive immunity (Brasseit et al., 2018, Mulder et al., 2017) by stimulating several immunomodulatory molecules and contributes to numerous inflammatory and autoimmune diseases (Tu et al., 2011). IFN-γ facilitates antigen presentation, cell proliferation, and viral and bacterial removal via increasing autophagy (Gutierrez et al., 2004, Al-Zeer et al., 2009, Chang et al., 2010, Assani et al., 2014). This autophagy activation then induces the release of

IFN-γ in a positive feedback loop (Chang et al., 2010). It was reported that both IFN-γ and amino acid starvation induce the ubiquitination of Beclin 1, which contributes to the induction of autophagy in macrophages (Shi and Kehrl, 2010, Chen et al., 2019b).

Autophagy and TNF

TNF is a pleiotropic cytokine that regulates pro-inflammatory responses, cell growth, differentiation, and death, acting as a key mediator in autoimmune and inflammatory diseases and infection control (Kumar et al., 2017, Gough and Myles, 2020). Growing data supports the hypothesis that TNF and autophagy interact with each other (Jia et al., 2006, Zheng et al., 2017, Winer et al., 2018, Orvedahl et al., 2019). TNF has been shown to induce necroptosis and autophagy in murine fibrosarcoma L929 cells by inhibiting the p38 MAPK-NF-B pathway (Ye et al., 2011). Conversely, autophagy may down-regulate TNF, which is dependent on the generation of ROS (Pan et al., 2015). A negative correlation is observed between NLRC5, a newly identified sub-class of the NLR family, and autophagy in endometriosis. Induction of autophagy in ovarian endometriosis patients' ectopic endometrial stromal cells (EESCs) leads to NLRC5-mediated suppression of IL-6 and TNF-α expressions, whereas autophagy inhibition limits NLRC5-mediated suppression of IL-6 and TNF-α expressions in EESCs, which in turn reduces inflammation in endometriosis (He et al., 2020).

Autophagy and IL-6

IL-6 is another pleiotropic cytokine produced by immune cells such as monocytes, lymphocytes, and macrophages. IL-6 plays an important function in inflammation and contributes to pathogenesis in multiple diseases. IL-6 has been shown to regulate the autophagic process by both inhibitory and stimulatory effects (Delk and Farach-Carson, 2012, Kang et al., 2012, Qin et al., 2015). IL-6-mediated STAT3 signalling has been linked to the regulation of autophagic cell death, which reduces arsenite-induced renal injury (Kimura et al., 2010). Another study reported that in U937 cells, IL-6 suppresses starvation-induced

autophagy and stimulates STAT3 to modulate the effect of Bcl-2 on Beclin 1 and VPS34 (Qin et al., 2015). STAT3 has been described as a Bcl-2 transcriptional activator that can positively control Bcl-2 expression (Alas and Bonavida, 2001, Real et al., 2002). Furthermore, Bcl-2 is an anti-autophagic effector protein that interacts with Beclin 1 to prevent autophagy induction (Qin et al., 2015).

Autophagy and IL-10

IL-10 is an immunomodulatory cytokine important to maintain a balance of immune homeostasis (Fiorentino et al., 2016). IL-10 suppresses both innate and adaptive immune responses as well as inhibits the production of numerous pro-inflammatory cytokines by monocyte and macrophages (Lin et al., 2017, Rojas et al., 2017, Mazer et al., 2019). As a major immune system regulator, autophagy may be anticipated to influence IL-10 development by macrophages and DCs, and IL-10 also has several regulatory effects on autophagy (Du et al., 2014a, Kishore et al., 2015, Lin et al., 2017). IL-10 prevents angiotensin II-induced pathological autophagy via modulating PI3K/Akt/mTORC1 signalling and enhancing the interaction of Bcl2 and Beclin1, which may reduce the anti-apoptotic activity. Pharmacological or molecular inhibitors of Akt and mTORC1 signalling, on the other hand, may reduce IL-10-inhibited Ang II-induced autophagy (Kishore et al., 2015). Furthermore, in murine macrophages, IL-10 signalling prevents autophagy and stimulates the class I PI3K pathway, that lead to p70S6K phosphorylation via activating Akt and mTORC1 (Park et al., 2011).

Autophagy and MIF

Macrophage migration inhibitory factor (MIF) is a pleiotropic pro-inflammatory cytokine that plays an important role in both innate and adaptive immune responses (Zernecke et al., 2008, Damle et al., 2017). MIF is widely distributed in a number of immune cells, such as macrophages, DCs, and T cells, also in most non-immune cells (Zernecke et al., 2008, Harris

et al., 2019). MIF is constitutively released into multiple cell types and is found in serum and healthy tissues, often at high concentrations (Calandra et al., 1994, Murakami et al., 2002, Dankers et al., 2020). Several recent studies implicated MIF in the regulation of autophagy, though the results are conflicting.

MIF has been found to activate autophagy in various cells including cardiomyocytes, endothelial cells, and a human hepatoma cell line (Chuang et al., 2012, Xu et al., 2016, Lai et al., 2015). In contrast, some studies revealed an inhibitory effect of MIF on autophagy in breast cancer cell lines and cardiomyocytes (Liu et al., 2014b, Wu et al., 2012a, Xu and Ren, 2015). Inhibition of autophagy increased MIF secretion in LPS-stimulated macrophages (Lee et al., 2016c). MIF is also associated with the pathogenesis of various autoimmune and inflammatory diseases, including sepsis, rheumatoid arthritis, and SLE (Morand, 2005). Thus, MIF is one of the key mediator of inflammation, and targeting MIF may offer therapeutic benefits in different diseases.

Autophagy and the IL-23-IL-17 axis

Autophagy influences the production of another inflammatory cytokine, IL-23, by regulating IL-1β secretion (Peral de Castro et al., 2012). In both macrophages and DCs, inhibition of autophagy results in an increase in IL-23 secretion, which is directly mediated by IL-1β, whereas activation of autophagy decreases secretion of IL-23 in response to LPS *in vivo* (Jones et al., 2013). It has been shown that IL-23, in combination with IL-1 (α or β), promotes the differentiation and expansion of Th17 cells from naive CD4 T cells and release of IL-17 by innate γδ T cells (Peral de Castro et al., 2012). Indeed, supernatants from LPS primed DCs stimulated with the autophagy inhibitor 3-MA, high in IL-1 and IL-23, increase the release of IL-17, as well as IL-22 and IFNγ, by γδ T cells (Peral de Castro et al., 2012). Numerous

autoimmune diseases, such as multiple sclerosis, psoriasis and asthma, are closely linked with the production of IL-23 and IL-17 cytokines (Jones et al., 2013).

Animals lacking the *Atg5* gene in myeloid cells show increased bacillary burden and more severe pulmonary inflammation as evidenced by neutrophil infiltration and an enhanced IL-17 response with a high level of IL-1α in response to *M. tuberculosis* infection than the autophagy-proficient mice (Castillo et al., 2012). Likewise, mice lacking the *Atg5* gene in CD11c+ cells lead to spontaneous airway hyper-reactivity and substantial neutrophilic inflammation in the lung, with a high level of IL-1 and IL-17A compared to the wild-type (WT) mice (Suzuki et al., 2016). Another study has reported that autophagy protein MAP1-LC3b (LC3b) deficient mice showed higher IL-17A-dependent lung pathology when infected with respiratory syncytial virus (RSV) (Reed et al., 2015). In a recent study, Mathur et al. demonstrated that autophagy induction in Cx3Cr1+ mononuclear cells inhibits IL-23/IL-22 axis-mediated inflammatory response in the TNBS-induced fibrosis mice (Mathur et al., 2019). These findings suggest that autophagy plays a pivotal role in regulating inflammation via modulation of the production and release of various inflammatory cytokines.

In this chapter, I have summarised the association between autophagy and the production of important cytokines (**Table 1.4 and Table 1.5**). Thus, modulation of the interactions of autophagy and cytokines might be a potential approach for controlling immune responses in clinical settings.

Table 1.4: Effect of different cytokines on autophagy

Cytokine	Main Sources	Modulation of Autophagy	Mechanism of Action	References
IFN-γ	Th1 cells, NK cells, NKT cells	Induces autophagy	Increases autophagosome formation and lysosomal trafficking decreases inflammatory cytokine production, Upregulate Erk2, JAK2 and MAPK signalling pathway	(Matsuzawa et al., 2012, Chen et al., 2017)
TNF-α	Macrophages, NK cells, CD4+ lymphocytes, adipocyte	Induces autophagy	Upregulates the expression of autophagy gene Atg7, Beclin 1 and LC3-II, inhibits p38/NF-κB signalling pathway.	(Lin et al., 2013b, Ye et al., 2011)
TGF-β	Macrophages, T cells, Fibroblasts	Induces autophagy	Activates TAK1 (TGF-β-activated kinase 1) and Akt, induces reactive oxygen species (ROS), Up-regulates autophagic protein marker LC3, beclin1, Atg5 and Atg7 expression	(Xu et al., 2012, Pan et al., 2015)
		Inhibits autophagy	Activate Akt and mTOR signalling pathway in fibroblasts	(Zhai et al., 2017)
MIF	Macrophages, dendritic cells and T cells	Induces autophagy	Activates RhoA-ROCK1 pathway, Upregulates Bcl2 expression, induces ROS production	(Xu et al., 2016) (Lai et al., 2015, Chuang et al., 2012)
IL-1	Macrophages, monocytes	Induces autophagy	increases the intracellular Ca2+ level, and autophagic marker LC3-II expression induces mitochondrial damage and increases ROS level	(Xu et al., 2014) (Gao et al., 2017b)
IL-2	Activated T cells	Induces autophagy	Increases serum levels of IFN-γ, IL-6, and IL-18 and translocates high mobility group box 1 (HMGB1) from the nucleus to the cytosol	(Kang et al., 2013)
IL-4	Th2-cells, NKT cells, B cells	Induces autophagy	Activates ATG14-BECN1-PIK3R4-PIK3C3 complex that is essential for the nucleation, elongation and formation of autophagosomes	(Xia et al., 2018)

IL-6	Macrophages, T-cells, adipocyte	Inhibits autophagy	Induces the phosphorylation of mTOR, inhibits starvation-induced phagosomal acidification and autophagy formation, downregulates beclin1, LC3-II, Atg7 and Atg12–Atg5 complex	(Roca et al., 2009) (Dutta et al., 2012)
		Induces autophagy	Increase LC3-II expression and autophagosome formation, upregulates Bcl2 expression	(Martinez-Outschoorn et al., 2011)
IL-10	Monocytes, Macrophages, T-cells, B-cells	Induces autophagy	Upregulates LC3-II autophagic marker in fibroblasts	(Santarelli et al., 2014)
		Inhibits autophagy	Activates the JAK/STAT3 and PI3K/Akt/mTORC1 pathways upregulates Bcl2 expression	(Kishore et al., 2015) (Park et al., 2011)
IL-12	Dendritic cells, macrophages, neutrophils	Induces autophagy	Inhibits AMPK and activates PI3K/Akt signalling pathway	(Lin et al., 2017)
IL-13	Th2 cells	Induces autophagy	Increases intracellular ROS generation and MUC5AC (mucin 5AC) secretion	(Dickinson et al., 2016)
IL-17	Th17 cell, NK cells, NKT cells	Induces autophagy	Enhances LC3-II expression and autophagosomes, up-regulates JAK2/STAT3 signalling pathway.	(Orosz et al., 2016) (Wu et al., 2017a)
		Inhibits autophagy	Enhances expression of Bcl2, reduces the expression or activity of autophagic protein BECN1, PIK3C3, PIK3R4, and ATG14, up-regulates TAK1-binding protein 2 (TAB2) and TAK1-binding protein 3 (TAB3)-inducing p38 mitogen-activated protein kinase (MAPK) pathways	(Liu et al., 2013c) (Zhou et al., 2016)
IL-23	Monocytes, dendritic cells	Inhibits autophagy	Activates AKT/mTOR/NF-κB signalling pathway	(Zheng et al., 2018)

IL-27	Monocytes, macrophages, dendritic cells, and microglial cells	Inhibits autophagy	Downregulated IFN-induced autophagosome generation and phagosome maturation activates JAK–PI3K–mTOR pathway and upregulation of the anti-apoptotic protein Mcl-1	(Sharma et al., 2014)
IL-33	Endothelial cells	Inhibits autophagy	Inhibits apoptosis, enhances expression of Bcl2 and inhibits autophagic marker LC3-II and Beclin1 decreases TNF-α and IL-1β secretion	(Gao et al., 2017a)

Table 1.5: Effect of autophagy on different cytokines

Interaction	Mechanism of action	Reference
Autophagy promotes IFN-γ production	Inhibits the expression of ROS and Src homology-2 domain-containing phosphatase 2 (SHP2), sustains Jak2-STAT1 activation and facilitates IFN-γ-induced cellular inflammation.	(Chang et al., 2010) (Liu et al., 2015)
Autophagy induces TNF-α production	Increase the secretion of TNF-α by regulating the p38 MAPK signalling pathway.	(Liu et al., 2016a)
Autophagy inhibits TNF-α release	Autophagy increased Silent information regulator 1 (SIRT1) expression via ROS dependent manner, which is required for nuclear translocation of a member of Forkhead box O (FoxO3A) and subsequent LC3II expression.	(Pun et al., 2015)
Autophagy reduces IL-17 induction	Autophagosome-modulated IL-1 release, together with the release of transforming growth factor (TGF)-β and IL-6, drives the differentiation of the Th17 cells, implying autophagic regulation of the Th1/Th17 balance.	(Said et al., 2014) (Reed et al., 2015)
Autophagy promotes IL-1β secretion	Activates caspase-1 followed by inflammasome formation	(Dupont et al., 2011)
Autophagy inhibits IL-1β secretion	Activates p62 degradation via lysosomal and proteasomal pathways, downregulates IL-23 secretion	(Lee et al., 2012)

		(Peral de Castro et al., 2012)
Autophagy enhances IL-10 production	Inhibits NF-κB activity and up-regulates anti-inflammatory cytokine expression, enhances the expression of the autophagosome marker LC3 through activation of AMPK, inhibits Bcl-2 and promotes hVps34/Beclin-1 complex formation, which increases anti-inflammatory cytokines such as TNF-α, IL-6 and IL-10, Promotes IL-10 production in macrophage via activation of the Cav1-Notch1/HES1 signalling pathway	(Du et al., 2014a) (Xu et al., 2019b)
Autophagy may reduce IL-10 production	Decreases apoptosis in mechanically injured spinal cord neurons through mTOR signalling or enhances autophagy-related protein Beclin1 and LC3B, decreases the inflammation factors IL-1β, TNF-α and IL-10	(Wang et al., 2015)
Autophagy inhibits TGF-β signalling	Autophagy deficiency results in an elevated level of TGF-β in obstructed kidneys; autophagy negatively affects TGF-β signalling through reduction of p62.	(Ding and Choi, 2014) (Araki et al., 2015)

1.6 Autophagy in autoimmune disease

Autoimmune diseases encompass a wide range of conditions in which the immune system generates autoantibodies that mistakenly attack the body's own cells, tissues, and organs. They are typically characterised by chronic inflammation, leading to the accrual of tissue and organ damage. The mechanisms underlying the development of autoimmunity are, in many cases, not well understood and most likely diverse. Autophagy has been known to be important in the removal of apoptotic cells, and it is essential not only for the removal of foreign or undesirable elements but also for effective energy recycling during metabolic stress. Autophagy also modulates immune reactions by influencing the immune cell activity, as discussed above, regulating antigen presentation, efferocytosis, cytokine responses, and the development and

homeostasis of lymphocytes. Not surprisingly, defects in autophagy have been related to multiple inflammatory and autoimmune conditions (**Table 1.6**).

Table 1.6: Role of autophagy in autoimmune disease

Associated diseases	Autophagy related genes and proteins	Effect of autophagy	References
Rheumatoid arthritis (RA)	ALFY, p62	Regulating the survival of RA synovial fibroblasts	(Kato et al., 2014)
	ATG7, optineurin	Regulating osteoclast differentiation and bone resorption	(Lin et al., 2013a) (Lee et al., 2020)
	LC3, p62/SQSTM1	Regulates survival of RA synovial fibroblasts	(Connor et al., 2012)
	LC3-II, p62	Regulating pro-inflammatory cytokine production	(Chen et al., 2018)
Psoriasis	ATG16L1, SQSTM1	Regulating keratinocytes proliferation	(Lee et al., 2011)
	PI3K/AKT/mTOR	Regulating IL-17 production	(Varshney and Saini, 2018)
Ankylosing spondylitis (AS)	IRGM, ULK1, LC3, Beclin 1, and ATG5	Autophagic protein is decreased in AS patients	(Xia et al., 2017, Zhang et al., 2017b)
Multiple sclerosis (MS)	ATG5, immune-related GTPase M (IRGM)1, ATG16L2	Expressed differentially	(Xu et al., 2010) (Igci et al., 2016, Liang and Le, 2015)
	Beclin-1, ATG7, mTOR	Knockdown or inhibition of autophagy shows a protective role	(Bhattacharya et al., 2014, Rioux et al., 2007)
Sjogren's syndrome	LAMP2A	Autophagy flux is significantly diminished, and the expression of the CMA marker LAMP2A is significantly reduced	(Li et al., 2018)
Inflammatory bowel disease (IBD)	ATG16L1, IRGM	SNPs, IL-17a, and IL-1beta production	(McCarroll et al., 2008, Saitoh et al., 2008)

	ATG16L1, nucleotide-binding oligomerization domain-containing protein 2	Regulating IL-1beta production	(Homer et al., 2010, Kemp et al., 2015)
SLE	Atg5	Regulates IFN and pro-inflammatory cytokine secretion	(Zhou et al., 2011b, Schrijvers et al., 2011)
	Atg7	Regulates plasma cell differentiation	(Pua et al., 2009, Feng et al., 2008)
	Mammalian target of rapamycin (mTOR), Becline-1, light-chain 3 and p62	Expressed differentially	(Wang et al., 2008) (Wu and Sun, 2011) (Wu et al., 2017b) (Zhao et al., 2017)
	AMBRA1 and UNC-51-like kinase 1 (ULK1)	UVB induced lower expression of AMBRA1 and ULK1	(He and Klionsky, 2009)

1.6.1 Autophagy and Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a chronic autoimmune disorder that can affect multiple organs, including lungs, heart, vascular system, eyes and skin (Bartok and Firestein, 2010). Chronic joint inflammation as well as damage of bone and cartilage are common characteristics of RA, which may contribute to chronic joint pain and eventually loss of function. RA synovial fibroblasts (RASF) residing in the joint is known to play a crucial role in the pathogenesis of RA (Turner and Filer, 2015). In RA patients, synovial fibroblasts show resistance to apoptotic stimuli and apoptosis is downregulated, whereas the autophagy process is upregulated (Dai and Hu, 2016). It has been reported that autophagy plays a role in the control of cell death pathway in rheumatoid arthritis synovial fibroblasts. In rheumatoid arthritis synovial fibroblasts during extreme endoplasmic reticulum (ER) stress, decreased expression of autophagy-linked FYVE protein (ALFY) and the development of p62-positive polyubiquitinated protein aggregates

accelerate the cell death (Kato et al., 2014). An autophagy adaptor/receptor protein, optineurin, has been found to be increased in Rheumatoid Arthritis Synovial Fibroblasts and plays an ameliorative role in RA (Lee et al., 2020).

In RA, joint damage is mediated predominately by proinflammatory cytokines, including IL-1α/β and TNF, that promotes synovial fibroblast production of the growth factors, proteinases, adhesion molecules and chemokines, which are characteristic of the arthritic joint (Bartok and Firestein, 2010, Neumann et al., 2010, Mateen et al., 2016, Alam et al., 2017, Lin et al., 2013a). TNF promotes the proliferation and differentiation of B-lymphocytes, T-lymphocytes and NK cells and enhances the release of pro-inflammatory cytokines, including IL-1, IL-6, and IL-8, that leads to inflammation and damage of bone and cartilage (Alam et al., 2017). TNF also trigger the expression of adhesion molecules on endothelial cells while limiting the number of Treg cells, resulting in increased angiogenesis and a more intense experience of pain (Mewar and Wilson, 2011). Therefore, TNF inhibitors are one of the authorized therapies for RA, which target and inhibit TNF, consequently modulate the inflammatory signalling associated with RA (Radner and Aletaha, 2015).

Autophagy in RA synovial fibroblasts is enhanced to counter Endoplasmic reticulum (ER) stress and sustain cell survival (Connor et al., 2012). It has also been found that Patients with active RA have higher levels of autophagy in their synovial tissues, which is associated with the severity of the disease (Zhu et al., 2017, Xiu et al., 2014, Wu et al., 2012b). Peripheral blood mononuclear cells from RA patients had higher LC3-II and p62 protein expression than healthy controls, indicating an association link between autophagy activation and RA-related inflammation (Chen et al., 2018). The role of autophagy in immune cells in the RA joint is still unclear but considering its involvement in regulating numerous cytokines in innate immune cells, it is certainly worthy of further investigation.

1.6.2 Autophagy and Psoriasis

Psoriasis is a recurrent autoimmune skin disease that is characterized by excessive hyperproliferation and aberrant differentiation of keratinocytes (Lee et al., 2011). In psoriasis immunopathogenesis, proinflammatory cytokines synergistically trigger keratinocytes to produce chemokines (CCL20, CXCL1, CXCL2, and CXCL8) antimicrobial peptides (S100A7, S100A8, S100A9, S100A12, DEFB4A/DEFB2 and CAMP/LL37), and other inflammatory stimuli that attract and stimulate pathogenic IL17A-producing T (Th17) cells and neutrophils, and further amplify the IL23-IL17 axis (Nestle et al., 2009, Wang et al., 2021). In psoriatic skin lesions, increased expression of p62/*SQSTM1* protein has been observed (Lee et al., 2011). Furthermore, a high level of autophagy-related protein ATG16L1 was found in DCs from psoriatic arthritis patients (Wenink et al., 2011). It has also been suggested that autophagy inhibition via activation of PI3K/AKT/mTOR signalling could be a therapeutic strategy for IL-17A-mediated psoriasis (Varshney and Saini, 2018). AP1S3 is a protein involved in autophagosome formation, and its deficiency disrupts autophagy in keratinocyte, leading to abnormal accumulation of p62, consequently up-regulate IL-1 signalling and increase the expression of IL-36α, which is an important mediator of skin inflammation (Mahil et al., 2016). In addition, expression of IL-36 and IL-1 signalling were upregulated in keratinocytes from patients' with AP1S3 mutations (Mahil et al., 2016), suggesting a critical role of autophagy in psoriasis.

1.6.3 Autophagy and ankylosing spondylitis

Ankylosing spondylitis (AS) is a hereitable systemic inflammatory disorder characterized by persistent pain and stiffness; in severe cases, the spinal vertebrae can fuse together (Yu et al., 2015b, Mauro et al., 2021). Patient mobility decreases as the condition progresses, and many people eventually suffer from body disability (Ghasemi-Rad et al., 2015). Inflammation typically occurs in the sacroiliac joints and leads to irregular erosion and sclerosis of

subchondral bones, after which tissues are eventually replaced by fibrocartilage and ossified with matrix protein deposition. In the advanced stages of the disease, fibrous replacement and osteoproliferation lead to ankylosis (fusion of the joints) of the spine (Zhu et al., 2019).

Along with an important role for HLA-B27 in AS pathogenesis, various inflammatory mediators, such as TNF, IL-6, and IL-23/IL-17, play a dominant role in these inflammatory and proliferative AS cascades (Zinovieva et al., 2009, Wendling and Claudepierre, 2013). Previous studies have shown that there are multiple genes associated with both AS and CD (Simmons et al., 2000, Zucchelli et al., 2011, Cai et al., 2016, Burton et al., 2007), and a genome-wide association study (GWAS) has identified a collection of CD susceptive genes in a large cohort of AS patients (Barrett et al., 2008, Smale et al., 2001). Some AS patients show symptoms such as chronic diarrhoea and abdominal pain, rectal bleeding, iron deficiency, fever and weight loss, that are close to those seen in CD patients, and *vice versa* (Brophy et al., 2001, Xia et al., 2017, Sanz Sanz et al., 2018). Since both AS and CD have a high heritability, it's likely that these two diseases have significant genetic overlap and have common pathogenic pathways. *IRGM* gene has been shown to be correlated with AS vulnerability in a Chinese female population (Xia et al., 2017). Another study reported that ULK1 polymorphisms (rs9652059 SNP and three haplotypes) are also associated with susceptibility to AS in a Chinese Han population (Zhang et al., 2017b). Recently, decreased *MAP1LC3B*, *BECN1*, and *ATG5* have been reported in AS patients, along with significant negative associations of their expression levels with severity of spinal damage in AS patients were reported (Park et al., 2017). These findings suggest that autophagy may act as a defensive or homeostatic mechanism against AS and that compromised autophagy may be a critical mechanism in the development of spinal damage in AS.

1.6.4 Autophagy and systemic sclerosis (scleroderma)

Systemic sclerosis (SSc) or scleroderma is a complex autoimmune condition characterised by high levels of collagen deposition and tissue fibrosis, which may be represented as an excessive wound healing process induced by the presence and persistence of active fibroblasts (Frech et al., 2014, Sobolewski et al., 2019). Autophagy dysfunction has been shown to be associated with SSc pathogenesis. Previous research has found that skin samples from systemic sclerosis (SSc) patients exhibit higher levels of LC3 immunoreactivity than healthy controls, while another study has shown that autophagic flux is decreased in SSc fibroblasts (Dumit et al., 2014, Zhang et al., 2020b). Therefore, the involvement of autophagy in SSc remains controversial. A recent study reported that peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) is overexpressed in SSc and regulates fibroblast activation and autophagy (Zhang et al., 2020b). PGC-1α knockout in fibroblasts inhibits autophagy activation in fibrotic conditions and ameliorates experimental fibrosis (Zhang et al., 2020b). A high level of the autophagy-related gene (ATG)5 and immune-related GTPase M (IRGM) 1, as well as a low level of ATG16L2, was observed in T-cells in the experimental autoimmune encephalomyelitis and in tissues from active relapsing-remitting MS brains (Alirezaei et al., 2009, Xu et al., 2010, Liang and Le, 2015). However, more investigation is necessary to fully comprehend the role of autophagy in this debilitating disease.

1.6.5 Autophagy and Sjogren's syndrome

Sjogren's syndrome (SS) is a heterogeneous autoimmune disorder characterized by lymphocyte infiltration and inflammation of the lacrimal and salivary glands, resulting in a lack of adequate tear and saliva production, causing severe dry eyes and mouth symptoms (Mitsias et al., 2006, Li et al., 2018). Patients are categorised as having a primary or secondary SS due to the involvement of other concurrent autoimmune disorders. Primary SS is not linked to any other autoimmune disease (Ramos-Casals et al., 2012). Secondary SS, on the other hand, can arise

in the context of another autoimmune disorder, most commonly rheumatoid arthritis (Ramos-Casals et al., 2012, Li et al., 2018). There is limited evidence of a role for autophagy in SS. Previous research has found autophagy dysregulation in the salivary glands of SS mice models, as well as a protective function for autophagy in salivary gland epithelial cells following endoplasmic reticulum stress (Voynova et al., 2020, Katsiougiannis et al., 2019). T cells are a key component of the lymphocytic infiltration at an initial stage of the disease, which involves the lack of immunity to self-antigens and the release of several pro-inflammatory cytokines correlated with local inflammation (Mingueneau et al., 2016). The level of autophagy in peripheral blood T lymphocytes has been shown to be positively correlated with SS disease activity indexes (Alessandri et al., 2017). A low level of LAMP2A, a chaperone-mediated autophagy (CMA) marker, was observed in the salivary glands from MRL/lpr mice than the control mice, indicating a link between autophagy processes and SS condition in mice (Li et al., 2018). Further work is needed to ascertain the role of autophagy in SS and whether it might be context dependent.

1.6.6 Autophagy and systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a systemic multiorgan autoimmune disease of unknown aetiology characterized by T and B cell dysfunction and generation of antinuclear antibodies (**Fig. 1.5**) (Lisnevskaia et al., 2014, Nagy et al., 2005). SLE is an acute and chronic disease that affects multiple organs and tissues, such as skin, heart, kidneys, joints, and/or nervous system and mostly affects young adult women (female: male ratio 9:1, most commonly develops between the ages of 15-44) (Connelly et al., 2013, Kaul et al., 2016). In Australia, SLE affects 1 in 1,000 people and is more prevalent and more severe in indigenous Australians and individuals of Asian ethnicity (Vincent et al., 2012, Golder et al., 2013). A complex interplay of genetic, environmental., such as infectious agents, UV light, hormonal factors and drugs, appear to contribute to disease development (Harley et al., 2009).

Although numerous studies have shown that autophagy-related defects may be implicated in SLE pathogenesis, the autophagy process has yet to be thoroughly characterized in SLE. Lack of Atg5-dependent processes, including negative thymic selection, control of type 1 IFN and production of proinflammatory cytokines, antigen presentation and efferocytosis, may lead to autoimmunity and inflammation in SLE (Zhou et al., 2011b, Schrijvers et al., 2011, Arnold et al., 2016). However, whether this is attributed to defects in autophagy or other function of ATG5 is not clear yet. The inadequate clearance of apoptotic cells may contribute to the development and accumulation of nuclear autoantigens in various tissues of SLE patients (Munoz et al., 2005). Phagocytosis and/or efferocytosis by macrophages and neutrophils is decreased in patients with SLE, and this impaired clearance of cellular debris may lead to the buildup of apoptotic material (Munoz et al., 2005). As already discussed, autophagy has an crucial role to play in efferocytosis (Martinet and De Meyer, 2009, Arsov et al., 2011, Boada-Romero et al., 2020), suggesting that autophagy, particularly in professional phagocytes such as macrophages, may be critical in limiting the development of autoantibodies against nuclear antigens.

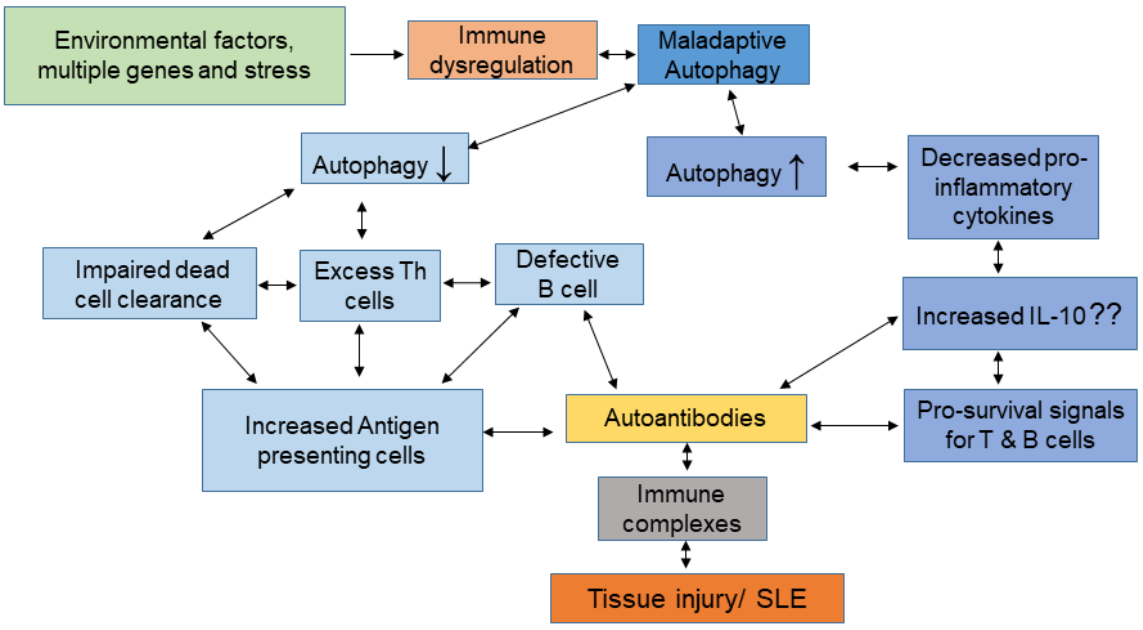


Figure 1.5 Schematic diagram of autophagy in SLE
Environmental factors, multiple genes, stress develop immune dysregulation, which ultimately causes maladaptive autophagy. Insufficient autophagy results in impaired dead cell clearance and an excess T-cell helper, which produces increased antigen-presenting cells, defective B cells, and inflammatory cytokines. Conversely, increased autophagy promotes T and B cell survival. All of these are involved in the pathogenic autoantibodies production and immune complexes deposition and thus play a critical role in developing SLE in human.

Autophagy has been found to be necessary for T cell activation and their survival after stimulation and differentiation (Stephenson et al., 2009). Animal studies regarding T cell-specific ablation of *Atg5* or *Atg7* suggest that mitochondrial quality control and recycling are crucial for T cell survival (Pua et al., 2009, Feng et al., 2008). Moreover, excessive autophagy is deleterious to the survival of T cells, is tightly regulated by IRGM in mice (Feng et al., 2008). Nucleotide polymorphisms in autophagy-related genes have been associated with susceptibility to SLE in genome-wide association (GWAS) studies. At least five single-nucleotide polymorphisms (SNPs) close to and in the *ATG5* locus have been linked to the onset and/or progression of SLE (Zhou et al., 2011b). *ATG7 (rs11706903)* and *IRGM (rs10065172)* polymorphisms have also been discovered as SLE susceptibility genes (Ramos et al., 2011). Positive gene-gene interactions have been observed among some autophagy-related genes, including *ATG5, ATG7* and *IRGM*, indicating autophagy is involved in SLE pathogenesis (Lo and Tsokos, 2012, Zhou and Zhang, 2012). Thus, these studies identify autophagy as a potential therapeutic target in SLE, with the possibility to uncover a crucial disease mechanism.

Autophagy has been shown to be regulated by a variety of medications utilized or in preclinical studies for the treatment of SLE. The American Food and Drug Administration authorized glucocorticoids for SLE treatment in 1950, and they have had considerable success in the treatment of SLE (Jones and Morand, 2016, Mejía-Vilet and Ayoub, 2021). Glucocorticoids have been shown to promote autophagy via suppressing the inositol triphosphate dependent calcium signals (Harr et al., 2010).

As early as the 1980s, triptolide extracted from *Tripterygium wilfordii* Hook F (a vine used in Chinese herbal medicine) was used for the treatment of autoimmune diseases such as psoriasis, RA and SLE (Zhao et al., 2016). Triptolide triggers autophagy via activating ULK1 and Beclin1 while inhibiting mTOR (Zhao et al., 2016). Vitamin D supplementation has been recommended for individuals with SLE because higher vitamin D levels can reduce inflammatory and haemostatic indicators, potentially leading to clinical improvement (Abou-Raya et al., 2013). Vitamin D3, the active form of vitamin D, has been reported to induce autophagy (Wu and Sun, 2011) by acting at different stages of the autophagy process, including activation, elongation and degradation via various signalling pathways including, Beclin1, Bcl-2, PI3KC3, mTOR, CDK cathelicidin and calcium metabolism (Wang et al., 2008, Wu and Sun, 2011, Yu et al., 2015a). Deficiency of vitamin D has also been shown to regulate the level of autophagy related genes in PBMCs and T-cell subsets, consequently decrease the autophagy process in patients with active SLE (Zhao et al., 2017). Furthermore, the expression of Becline-1, LC3 and p62 was positively associated with SLE Disease Activity Index (SLEDAI) and autoantibody production in PBMCs from SLE patients (Wu et al., 2017b).

It was discovered that the most popular medication for SLE, hydroxychloroquine, inhibits autophagy, especially the LAP-mediated autophagy process (Boya et al., 2005, Yin et al., 2018, Cook et al., 2014). In addition, low-dose Cyclosporine A has also been used to treat SLE patients (Sheikholeslami et al., 2018, Cruz-Pérez et al., 2018). It can prevent autophagosome proliferation by inhibiting the mitochondrial permeability transition (MPT), that can lead to mitochondrial depolarisation and eventual sequestration of mitochondria into autophagosomes as a result of autophagic stimulation in rat hepatocytes (Elmore et al., 2001). Furthermore, in clinical studies, a nuclear ribonucleoprotein, P140, was found to dramatically reduce the level of IgG anti-dsDNA antibody and SLE disease activity (Muller et al., 2008, Zimmer et al., 2013). Another research has shown that by interacting with the HSC70 chaperone in MRL/lpr

B cells, P140 can enhance the accumulation of autophagic markers such as p62/SQSTM1 and LC3II, which is consistent with the down-regulated lysosomal degradation during autophagic flux (Page et al., 2011). According to the previous research, various SLE medications may affect the autophagy process differently, and the role of autophagy in lupus can be beneficial or detrimental. Therefore, the context-dependent role of autophagy in SLE and the balance between innate immunity and adaptive immunity should be taken into account when evaluating autophagy as a therapeutic stratagy.

In SLE, cytokine secretion is dysregulated, which leads to immune dysfunction and causes tissue inflammation and organ damage. It has been found that inflammatory cytokines, such as type I and type II IFN, TNF, IL-1, IL-6, IL-18, and MIF, as well as immunomodulatory cytokines including IL-10 and TGF-β, plays a crucial role in SLE (Park et al., 1998, Merkley et al., 2018, Rönnblom and Elkon, 2010, Wu et al., 2016). SLE patients have higher levels of IL-10 in serum than healthy controls, and these have been linked to disease development (Godsell et al., 2016, Abd Elazeem et al., 2018). IL-10 stimulates B cell proliferation and Ig class switching, contributing to a high level of antibody production in SLE (Rousset et al., 1992). The level of IL-6 is also increased in the serum of patients with SLE, and this cytokine has a significant impact on both B cells and T cells (Kitani et al., 1992). SLE B cells are hypersensitive to IL-6, and anti-IL-6 mAb can inhibit the generation of anti-dsDNA autoantibodies (Liang et al., 2006). In addition to this, high levels of TNF and soluble TNF receptors are associated with disease development in SLE patients (Studnicka-Benke et al., 1996, Weckerle et al., 2012, Idborg et al., 2018). The mRNA level of TNF adapter proteins is reduced considerably in SLE PBMCs and negatively correlated with the SLE disease activity index (SLEDAI), likely as a secondary defect in apoptosis (Zhu et al., 2007b). IL-1 and IL-1 also primarily serve as regulators of the host inflammatory response to infections (Hadadi et al., 2016, Dinarello, 2018, Kaneko et al., 2019). High serum IL-1 levels are likely to correspond

with SLE disease activity as IL-1 can co-stimulate B and T lymphocyte responses (Brugos et al., 2010, Sturfelt et al., 1997).

MIF also plays a critical role in regulating B and T cell activity (Bacher et al., 1996, Lang et al., 2018, Alibashe-Ahmed et al., 2019). Several studies demonstrated that MIF is associated with the pathogenesis of SLE (Foote et al., 2004, Lang et al., 2015, Tu et al., 2019). SLE disease activity has been linked to the high level of MIF in serum (Foote et al., 2004, Gamez-Nava et al., 2020). The interaction between autophagy and cytokines may be critical for orchestrating innate and adaptive immune systems (Singh et al., 2010, Paunovic et al., 2018, Yin et al., 2018, Biasizzo and Kopitar-Jerala, 2020). Thus, autophagy is considered to have multiple roles in the occurrence, progression, and severity of SLE. These roles may be cell and context-specific and may potentially be antagonistic to one another, in some cases promoting pathology, in others more protective. For this reason, understanding the different roles for autophagy in SLE may be critical for developing new, specific therapeutic avenues.

1.7 Rationale for the current study

Autophagy is present at a basal level in all cell types to eliminate undesirable cytoplasmic content and maintain intracellular homeostasis (He and Klionsky, 2009, Perrotta et al., 2020). If autophagy is limited, redundant cytoplasmic material cannot be eliminated and may act as autoantigens to trigger B cells to develop autoantibodies, leading to developing autoimmune diseases. However, autophagy eventually destroys the cells when hyper-activated, potentially leading to extracellular autoantigen accumulation (Wang and Law, 2015). As a result, both up-regulation and down-regulation of autophagy may play a role in autoimmune disease pathogenesis. Autophagy is involved in almost every stage of both innate and adaptive immune processes (Levine et al., 2011). In order to improve successful and more precise therapeutic techniques, it is crucial to understand the dynamic interaction between autophagy and autoimmune diseases.

Autophagy can have various functions in autoimmune diseases based on the cells involved, and the final effects of pharmacological modulation may depend on the balance of multiple different effects. SLE is a systemic autoimmune disease that is complex and highly polymorphic. Many studies on SLE pathogenesis focus on defects of B and T cell-dependent tolerance as an underlying cause of this disease. However, the involvement of myeloid cells in the regulation of SLE is not clearly understood. Myeloid cells are the most commonly found nucleated hematopoietic cells in the body, comprising various cells including macrophages, monocytes, dendritic cells (DCs), neutrophils, and mast cells, all of which play diverse roles. During the invasion of the pathogen, myeloid cells, such as monocytes, are promptly transported into local tissues by various chemokine receptors, where they are activated for differentiation into macrophages, proficient in phagocytosis and able to direct subsequent immunity by producing cytokines and chemokines (Stegelmeier et al., 2019, Bashant et al., 2020).

As I have outlined in this chapter, many studies have shown that inhibiting autophagy in myeloid cells can contribute to increased inflammation, while induction of autophagy is generally anti-inflammatory. Moreover, autophagy is important for efferocytosis, a critical process for the clearance of apoptotic cells that might otherwise lead to the development of autoantibodies. The pathogenesis of SLE is clearly defined by autoantibody production and hyperactivated B cells and T cells as well as by pathogenic interactions among myeloid cells, B cells and T cells (Scapini et al., 2010, Jones et al., 2016). In this study, I have interrogated the role of myeloid cell autophagy in a mouse model of SLE-like autoimmunity. The aim of this work was to determine the net effect of myeloid cell autophagy on the development and severity of autoimmunity in SLE. In addition, I have studied further the role of autophagy in regulating the key immunoregulatory cytokines IL-6 and IL-10, both important in SLE pathogenesis.

Chapter 2: Myeloid cell autophagy regulates autoimmunity in a mouse model of lupus.

Abstract

Systemic lupus erythematosus (SLE) is a heterogenous autoimmune disorder characterized by a loss of tolerance against self-antigens, high levels of autoantibodies production, and multi-system manifestations. Dysregulation of autophagy has been associated with multiple autoimmune diseases, including SLE. However, the role of myeloid cell-specific autophagy in SLE pathogenesis and disease progression has not been addressed. This study sought to investigate whether altered autophagy in myeloid cells is associated with the pathogenesis of SLE.

Here, I investigated the development of the lupus phenotype in *Lyn*-deficient (*Lyn-/-*) mice in which autophagy was genetically ablated by myeloid cell-specific deletion of autophagy-related gene 7 (*Atg7*). I studied the potential effect of myeloid-specific autophagy on the SLE-associated inflammatory responses and disease severity by evaluating serum autoantibodies and cytokine levels, immune complex deposition, and renal pathological features. I found that the loss of autophagy in myeloid cells in lupus mice contributed significantly to specific readouts of SLE pathogenesis, including regulation of anti-SM, anti-dsDNA, and anti-histone autoantibodies and inhibition of IL-6 and IL-10. Thus, the findings from this study reveal that myeloid cell autophagy has novel effects on specific processes in SLE pathogenesis.

2.1 Introduction

Autophagy is a highly conserved lysosome-mediated catabolic process that helps cells to degrade unnecessary cytoplasmic constituents and recycle nutrients in a regulated manner. Apart from its physiological role in controlling cell homeostasis and removing dead cells, autophagy is a part of the cellular response to different types of stress, and its diverse

associations with immune responses and inflammation have attracted considerable interest (Levine et al., 2011, Levine and Kroemer, 2008, Bhattacharya and Eissa, 2013, Choi et al., 2013). Autophagy is implicated in both innate and adaptive immune responses, such as the formation of neutrophil extracellular traps (NETs), recognition of pathogen, antigen processing and presentation, lymphocyte and monocyte development and function, inflammasome activation, cytokine secretion, and regulation of inflammation, thus plays a vital in immune homeostasis (Levine et al., 2011, Münz, 2010, Saitoh et al., 2008, Liu et al., 2016c). Autophagy occurs constitutively under normal conditions but can be further stimulated by starvation, growth factors, environmental stresses, and cytokines (Lim et al., 2021). A basal level of autophagy is always needed to ensure a healthy and balanced cytosolic environment (Jones et al., 2013). However, perturbations in autophagy have been implicated in several autoimmune diseases. Analysis of mice with systemic or tissue-specific deletion of autophagy-related genes (*Atg*), combined with studies of genetic polymorphisms in humans, has demonstrated an association between dysregulated autophagy and various pathologies, such as autoimmune diseases, neurodegenerative diseases, infectious diseases, metabolic disorders, and cancer (Levine et al., 2011, Choi et al., 2013, Rubinsztein et al., 2011, White, 2012, Murrow and Debnath, 2013, Nixon, 2013, Zhou et al., 2011b, Pierdominici et al., 2012, Marchiando et al., 2013, Lee et al., 2016b, Yamamoto et al., 2018, Sato et al., 2018, Aqbi et al., 2018).

SLE is one of the most complex and multifaceted autoimmune disorders characterised by the presence of autoantibodies against nuclear antigens and the formation and deposition of immune complexes, resulting in chronic inflammation and multi-organ injury (Gualtierotti et al., 2010, Qi et al., 2019). SLE is highly heterogenous and can affect various organs and tissues, including the skin, joints, kidneys, and the central nervous system (Kaul et al., 2016). Although it is established that both genetic predisposition and environmental factors may lead to the disease onset and progression, the aetiology and pathogenesis of SLE are not fully understood.

For example, environmental factors such as ultraviolet light exposure and viral infection, as well as administration of certain drugs like procainamide and hydralazine, can contribute to the development and progression of SLE, together with genetic factors that influence disease susceptibility (Bentham et al., 2015, Solhjoo et al., 2021). The genes of the major histocompatibility complex (MHC) have received the most attention in terms of their contribution to human SLE (Mok and Lau, 2003). Human leukocyte antigen (HLA) class II gene polymorphisms have been linked to SLE susceptibility in population studies (Matzaraki et al., 2017). HLA DR2 and DR3 are often linked to SLE in patients of various ethnicities, with a relative risk of two to five times higher than the general population (Pisetsky, 2008, Tsao, 2002).

A number of studies have suggested a critical role of autophagy in the origin, progression, and severity of SLE (Zhou et al., 2011b, Gateva et al., 2009, Clarke et al., 2015, Arnold et al., 2016, Zhou et al., 2019b). Autophagy plays a critical role in the turnover of dysfunctional or damaged proteins and organelles and is involved in cytokine production and autoantigen presentation (Yin et al., 2018, Abdel Fattah et al., 2015, Liu et al., 2020). T and B cells from lupus animal models, as well as PBMCs from SLE patients, have shown a higher level of autophagy (Clarke et al., 2015, Alessandri et al., 2012, Miller et al., 2008, Gros et al., 2012, Watanabe et al., 2008), although it is unclear whether this is a protective or pathogenic response. However, inhibition of autophagy has been shown to partially suppress plasma cell differentiation (Clarke et al., 2015), which further suppresses high-affinity autoantibody production (Liu and Davidson, 2012).

Studies have shown that autophagy downregulates inflammatory responses in macrophages and dendritic cells, particularly the release of pro-inflammatory cytokines (Abdel Fattah et al., 2015, Stranks et al., 2015, Cadwell, 2016, Harris et al., 2017). However, the importance of autophagy in myeloid cells for pathogenesis in systemic lupus erythematosus (SLE) has not

been elucidated. Myeloid cells such as neutrophils, macrophages, and dendritic cells are the most abundant nucleated hematopoietic cells in the body. Myeloid cells are immediately recruited into local tissues following pathogen invasion by different chemokine receptors (Kawamoto and Minato, 2004). Myeloid cells are activated there for phagocytosis and release of inflammatory cytokines, thus playing a major role in innate immunity and subsequent adaptive immune responses (Hirayama et al., 2017, Ifergan and Miller, 2020). It has been demonstrated that excess BAFF production by hyperactive myeloid cells contributes to inflammation and autoimmunity in part by inducing the release of IFN-γ by T cells (Scapini et al., 2010). In this study, I investigated the role of myeloid cell autophagy in a mouse model of lupus-like autoimmunity by crossing autophagy-deficient *Atg7*$^{fl/fl}$ LysMCre mice with Lyn-deficient (*Lyn*$^{-/-}$) mice. In the LysMCre mice, the Cre recombinase is under the control of the myeloid-specific *Lyz2*/*LysM* (lysozyme 2) promotor, so when crossed with *Atg7*$^{fl/fl}$ mice, *Atg7* is conditionally deleted in myeloid cells.

Lyn (Lck/yes-related novel tyrosine kinase) is one of several Src family tyrosine kinases that play a crucial function in a number of signalling cascades in B cells, DCs, and myeloid cells (Xu et al., 2005, Scapini et al., 2009, Ma et al., 2019b). Mice lacking Lyn acquire an autoimmune condition similar to lupus, including developing splenomegaly, production of anti-nuclear antibodies (ANA), and immune complex-deposition in kidneys results in severe glomerulonephritis (GN) (Hibbs et al., 1995, Lamagna et al., 2014, Nataraja et al., 2021). Lyn has been shown to phosphorylate inhibitory receptors in B cells with immunoreceptor tyrosine-based inhibitory motifs (ITIM), consequently activating signalling pathways such as cytokine production, proliferation, and migration (Xu et al., 2005, Mkaddem et al., 2017). Thus, Lyn deficiency in mice is associated with Lyn-deficient B cells hyper-reactivity as a consequence of the Lyn specific downregulation of B-cell receptor activation via the phosphorylation of inhibitory molecules and receptors (Scapini et al., 2009, Hua et al., 2014). Several Pro-

inflammatory cytokines, including IL-6, IL-10, and BAFF, also have also been found to play a critical role in this phenotype, which is comparable to SLE in human (Tsantikos et al., 2010, Nataraja et al., 2021, Su et al., 2012).

Several studies have revealed that Lyn is engaged in autophagy, interacts with Atg7, or is involved in different autophagy-like processes in pathogen-infected conditions (Li et al., 2016, Lupo et al., 2016, Li et al., 2020b). Recruitment of LC3 to *Pseudomonas aeruginosa* (Pa) pathogen-containing phagosomes in alveolar macrophages was inhibited by genetic depletion of *Lyn*. Moreover, this infection-induced autophagy was reduced by a Lyn inhibitor, and this role of Lyn is dependent on its kinase activities (Li et al., 2016). However, *in vitro* (in MH-S cells transfected with Lyn siRNA) and *in vivo* (in *Lyn*-/- mice) studies showed that inhibition of Lyn did not change the autophagic marker LC3 (either conversion of LC3 I to LC3 II or $LC3^{+}$ puncta formation) in uninfected controls or in response to rapamycin (Li et al., 2016), suggesting that the effects of Lyn on autophagy are specific to infection and do not affect basal autophagy. Interestingly, this study also demonstrated that the recruitment of LC3 to phagosomes was not the same as L3-associated phagocytosis (LAP), but rather a specific form of infection-induced autophagy (Li et al., 2016).

An association between increased levels of active Lyn and higher levels of the autophagy-related proteins Ulk1 and Atg7 were observed in neuroacanthocytoses, which results in delayed clearance of mitochondria and lysosomes, as well as dysregulation of red blood cell homoeostasis. However, no association was found between Lyn and Ulk1 or Atg7 in healthy red cells (Lupo et al., 2016), again suggesting that the effects of Lyn on autophagy are disease-specific. Lyn also interacts with the autophagy process to mediate the evacuation of virus particles from infected cells. A recent study reported that viral progenies use secretory organelles generated from autophagosomes to escape host cells in a Lyn-dependent manner (Li et al., 2020b). Together, these studies suggest that Lyn may affect the autophagic process, but

apparently only in the context of specific disease-related stimuli. Whether loss of Lyn alters autophagy during the development of autoimmune disease is not yet clear.

The findings from this study indicate that, in *Lyn*$^{-/-}$ mice, loss of autophagy in myeloid cells had specific effects, including lowering levels of anti-SM, anti-dsDNA, and anti-histone autoantibodies, as well as lowering levels of IL-6 and IL-10. However, these effects were lost with increased age, and no overall effects on pathology, including splenomegaly and kidney disease, were observed. These results suggest that autophagy may have specific roles to play in myeloid cell responses, which in turn impact the responses of other cells, particularly autoantibody-producing plasma cells, but that the effects of myeloid cell autophagy on disease onset and progression are unclear and likely multifactorial.

2.2 Materials and methods

Animals

Lyn$^{-/-}$ mice (Hibbs et al., 1995, Tsantikos et al., 2010) were originally obtained from Margaret Hibbs (Monash University) and have been maintained in our lab for the past 5 years. Crossing of *Lyn*$^{-/-}$ mice with *Atg7*$^{fl/fl}$ LysMcre mice was initially overseen by Jacinta Lee in our lab (Rheumatology Research Group), and colonies were maintained by Jacinta Lee and Wendy Dankers (Rheumatology Research Group) over the course of this work. *Lyn* genotyping was performed by Transnetyx, Inc (Cordova, TN, USA) using real-time PCR. Lyn-deficient (*Lyn*$^{-/-}$) C57BL/6 mice were crossed with *Atg7*$^{fl/fl}$ LysMCre heterozygote mice to create double-knockouts. Cre- mediated deletion of *Atg7* in myeloid cells (LysM-expressing) was confirmed by PCR in my lab. Genotyping of animals was conducted by myself, Jacinta Lee and Wendy Dankers (Rheumatology Research Group). For "wild type" (WT) controls, *Atg7*$^{+/+}$ LysMcre$^{+/-}$ mice were used, and for all analyses, WT, *Atg7*$^{fl/fl}$ LysMCre (*Atg7*$^{fl/fl}$ *Lyn*$^{+/+}$), *Lyn*$^{-/-}$ (*Atg7*$^{+/+}$ *Lyn*$^{-/-}$) and *Atg7*$^{fl/fl}$ LysMCre x *Lyn*$^{-/-}$ (*Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$) mice were compared. All mice were

housed in a specific pathogen-free barrier facility. All animals were housed in identical conditions in adjacent cages within the Monash Medical Centre animal facility (run by the Monash Animal Research Platform). Mice were studied at less than 100 days ("young mice") or after 200 days ("old mice"). Old mice were further divided into two groups; group1: age less than or equal 250 days and group 2: age more than 250 days. Both male and female mice were used throughout the study (**Table 2.1**). All studies and procedures were approved by Monash University Ethics Committee (MMC-B).

Table 2.1: Demographic parameters of experimental mice

Phenotype			**Wild type**	**Atg7 Knockout**	**Lyn Knockout**	**Double knockout**
Genotype			*Atg7*$^{+/+}$*Lyn*$^{+/+}$	*Atg7*$^{fl/fl}$ *Lyn*$^{+/+}$	*Atg7*$^{+/+}$ *Lyn*$^{-/-}$	*Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$
N			40	70	48	44
Sex	Female		25	35	25	27
	Male		15	35	23	17
Age	Young mice	< 100 days	10	10	10	10
	Old mice	≤ 250 days	5	25	19	16
		> 250 days	25	35	19	18

Histology of Kidneys

Mice were sacrificed by CO_2 asphyxiation. Kidneys were harvested and cut in half laterally, fixed in 4% formalin, and embedded in paraffin. Then the formalin-fixed sections were stained with Periodic acid Schiff (PAS) staining for histopathological analysis. The longitudinal section allows histological examination of both renal poles and helps evaluate any localised lesions. Glomerular damage was scored in a blinded fashion by Dr. Josh Ooi (Monash University) for glomerular necrosis and crescent formation. Glomerular necrosis was defined

as an accumulation of periodic acid-Schiff-positive material in ≥50% of the glomerulus, while two or more layers of cells in Bowman's space were considered as a glomerular crescent. A minimum of 50 consecutive glomeruli per mouse was examined, and results were expressed as the percentage of necrosis per glomerular cross-section. However, no glomerular crescents were observed in my experimental mice.

Extractable Nuclear Antigens (ENA) test

Autoantibodies against the extractable nuclear antigen (ENA) in sera were measured by the FIDIS Connective Profile MX 117 kit (Theradiag) as per the manufacturer's protocol, with the variation of the use of an anti-mouse secondary antibody (Waibel et al., 2015). This test uses multiplex technology with addressable laser bead immunoassay, which enables the simultaneous detection of numerous antibody specificities in a single step. PE-conjugated polyclonal anti-mouse IgG F(ab')2 secondary antibody (eBioscience, cat. no. 12-4010) was used to detect autoantibodies. Fluorescence was quantified using a BD FACSCanto II (BD Bioscience). Champa Nataraja and Wendy Dankers (Rheumatology research Group) assisted with the analysis of ENA data.

Flow cytometry for identification of B cell subsets

Live single-cell suspensions were prepared from spleens and stained with propidium iodide (PI, 1 in 100 dilutions), CD45RA/B220 (Phycoerythrin, PE-Cy7, 1 in 400 dilutions), CD138 (PE, 1 in 400 dilutions), GL7 (Pacific Blue, PB, 1 in 200 dilutions), PNA (Fluorescein Isothiocyanate, FITC, 1 in 400 dilutions), and IgG1 (Allophycocyanin, APC, 1 in 400 dilutions). FACS buffer (HBSS with 2% FCS) was used for the experiment. All antibodies were from BioLegend. Cells were analysed on a BD fluorescence-activated cell sorting (FACS) Canto II (BD Bioscience) after exclusion of dead cells with PI. Data were analysed using FlowJo version 10.6.2 cell cycle analysis software (Tree Star, Ashland, OR). Cell populations

were defined as follows: plasma cells: $B220^{low}CD138^{hi}$; conventional B cells: $B220^{hi}$ $CD138^{low}$; GC B cells: $B220^{hi}GL7^{hi}PNA^{hi}$; non-GC B cells: $B220^{hi}GL7^{low}PNA^{low}$; isotype switched: $B220^{hi}GL7^{hi}PNA^{hi}IgG1^{hi}$; non-isotype switched: $B220^{hi}GL7^{hi}PNA^{hi}IgG1^{low}$.

T regulatory cells (Tregs) analysis

Live single-cell suspensions were prepared from spleens, and a combination of CD4-PB, CD25-APC, and Anti-FoxP3-PE markers are used to identify the frequency of Tregs (Hori et al., 2003). All antibodies were from BD Biosciences (San Diego, CA). Briefly, mouse splenocytes were first stained with CD4-PB (1 in 400 dilutions) and CD25-APC (1 in 100 dilutions) and incubated for 20 minutes at room temperature (RT) in the dark. Cells were then fixed with fixation buffer (BD Pharmingen™ Mouse Foxp3 Fixation Buffer) and incubated for 30 minutes at 4°C in the dark. To permeabilize the cells, permeabilization buffer (BD Pharmingen™ Mouse Foxp3 Permeabilization buffer) was used for 30 minutes at 37°C in the dark. Cells were then stained with Anti-Forkhead box protein P3- Phycoerythrin monoclonal antibody (Anti-FoxP3-PE mAB) and incubated for 20 minutes at RT in the dark. The Treg population was identified as $CD4^+$ $CD25^+$ $FoxP3^+$. Cells were analysed by FACSCanto II flow cytometry (BD Biosciences) and analysed with FlowJo version 10.6.2 cell cycle analysis software (Tree Star, Ashland, OR).

Serum cytokines analysis by Luminex assays

Blood was collected by cardiac puncture and stored overnight at 4°C, followed by centrifugation at 1500 g for 15 minutes. Serum was collected and 11 different cytokines measured using a procartaplex multiplex assay (Mouse 11-plex ProcartaPlex™ Kit, Invitrogen); BAFF, IFN-α, IFN-γ, IL-1α, IL-1β, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-6 as per the manufacturer's protocol. Serum samples were used at a 1:1 dilution in 1x universal Assay Buffer (provided with the kit). Serum samples were added to a colour-coded

beads mixture that was pre-coated with analyte-specific capture antibodies. An antibody-antigen sandwich was created by adding biotinylated detection antibodies specific to the analytes of interest. A hand-held magnetic plate washer was used throughout the assay. Finally, readings were taken using a Bio-plex MAGPIX^{TM} multiplex reader (Bio-Rad).

Statistical analyses

GraphPad Prism software (version 8.0.2) was used to conduct the statistical analysis. Results are expressed as the mean ± SEM. For all *in vivo* experiments, at least 4-8 mice were used (biological replicates). In most cases, these were analysed in a single group although in some experiments the different age groups were analysed separately and for ENA analysis, multiple analyses were conducted and data pooled. Data were tested for normality by the Shapiro-Wilk test, and statistical differences were determined using one-way ANOVA followed by Tukey's posthoc test. In addition, I used a *t* test to examine two groups individually if the difference was not significant by ANOVA and the groups were independent of the others in the experiment. Statistically significant differences between data groups are indicated by an asterisk(s), whereas nonsignificant differences are either unmarked or highlighted by "*ns*".

2.3 Results

2.3.1 Effects of autophagy deficiency in myeloid cells on the release of cytokines in *Lyn*$^{-/-}$ mice

Previous reports have demonstrated altered cytokine production in Lyn kinase-deficient mice. In particular, *Lyn*$^{-/-}$ mice showed expansion of IL-6 and IL-10 producing B cells which contribute to disease progression (Scapini et al., 2011, Tsantikos et al., 2010). It was also reported that *Lyn* deficiency leads to exaggerated pro-inflammatory cytokine production such as BAFF, IFN-α, IL-17, and IL-12p40 (Scapini et al., 2010, Lamagna et al., 2014). Here, I investigated whether autophagy deficiency in myeloid cells altered the production of pro-inflammatory cytokines in disease and non-disease conditions. Using Luminex technology, 11 different serum cytokines (BAFF, IFN-α, IFN-γ, IL-1α, IL-1β, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-6) were measured in the serum of mice at different ages. In young mice (<100 days), serum cytokines were not altered in autophagy-deficient mice (*Atg7*$^{fl/fl}$ *Lyn*$^{+/+}$) compared to WT mice (*Atg7*$^{+/+}$ *Lyn*$^{+/+}$) (**Fig. 2.1**). Levels of IL-17A were significantly increased in *Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$ mice compared to WT and *Atg7*$^{fl/fl}$ *Lyn*$^{+/+}$ mice, although levels were also raised in *Atg7*$^{+/+}$ *Lyn*$^{-/-}$ mice, and no difference was observed between the *Atg7* deficient and *Lyn*$^{-/-}$ mice (**Fig. 2.1B**). Moreover, the levels of IL-17A in the serum were low overall (<10 pg/ml). Serum levels of Baff and IL-6 were significantly higher in *Atg7*$^{+/+}$ *Lyn*$^{-/-}$ and *Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$ mice, although I did not observe any significant difference between the two groups, suggesting their release is disease, not autophagy, related (**Fig. 2.1A and G**). Levels of IL-23 were also raised in both *Atg7*$^{+/+}$ *Lyn*$^{-/-}$ and *Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$ mice (**Fig. 2.1E**). Overall, this data indicates that autophagy deficiency in myeloid cells did not have any significant effect in the early stage of the disease.

In mice aged 101-250 days, serum levels of BAFF, IL-17A, IL-18, IL-23, IL-6, and IL-10 were significantly raised in *Atg7*$^{+/+}$ *Lyn*$^{-/-}$ mice (**Fig. 2.2**). Of these, IL-6 and IL-10 were significantly lower in *Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$ mice (**Fig. 2.2G and H**), suggesting that loss of myeloid cell autophagy

significantly impairs production and/or release of these cytokines in the later stages of the disease. However, this effect was no longer apparent in the mice aged >250 days (**Fig. 2.3**), suggesting that at the very late stages of the disease, the effects of myeloid autophagy impairment are superseded by other pathological processes. It is notable that total cytokine levels in these highly aged mice were considerably lower than in the "middle-aged" group, suggesting that overall immune responses may become exhausted with age. Levels of MIF in older mice (>100 days) were also measured separately by ELISA, but no differences were observed between any of the genotypes (Supplementary Figure S3, Appendix II).

Interestingly, in the mice aged 101-250 days, levels of IL-18 were significantly raised in the autophagy-deficient mice (*Atg7*$^{fl/fl}$ *Lyn*$^{+/+}$) compared to WT mice (**Fig. 2.2C**), suggesting a role for myeloid cell autophagy in its release, independent of autoimmunity. This is interesting in the context of previous studies that have demonstrated a role for autophagy in the release of IL-1 family cytokines, particularly IL-1β and IL-18 (Saitoh et al., 2008, Harris et al., 2011).

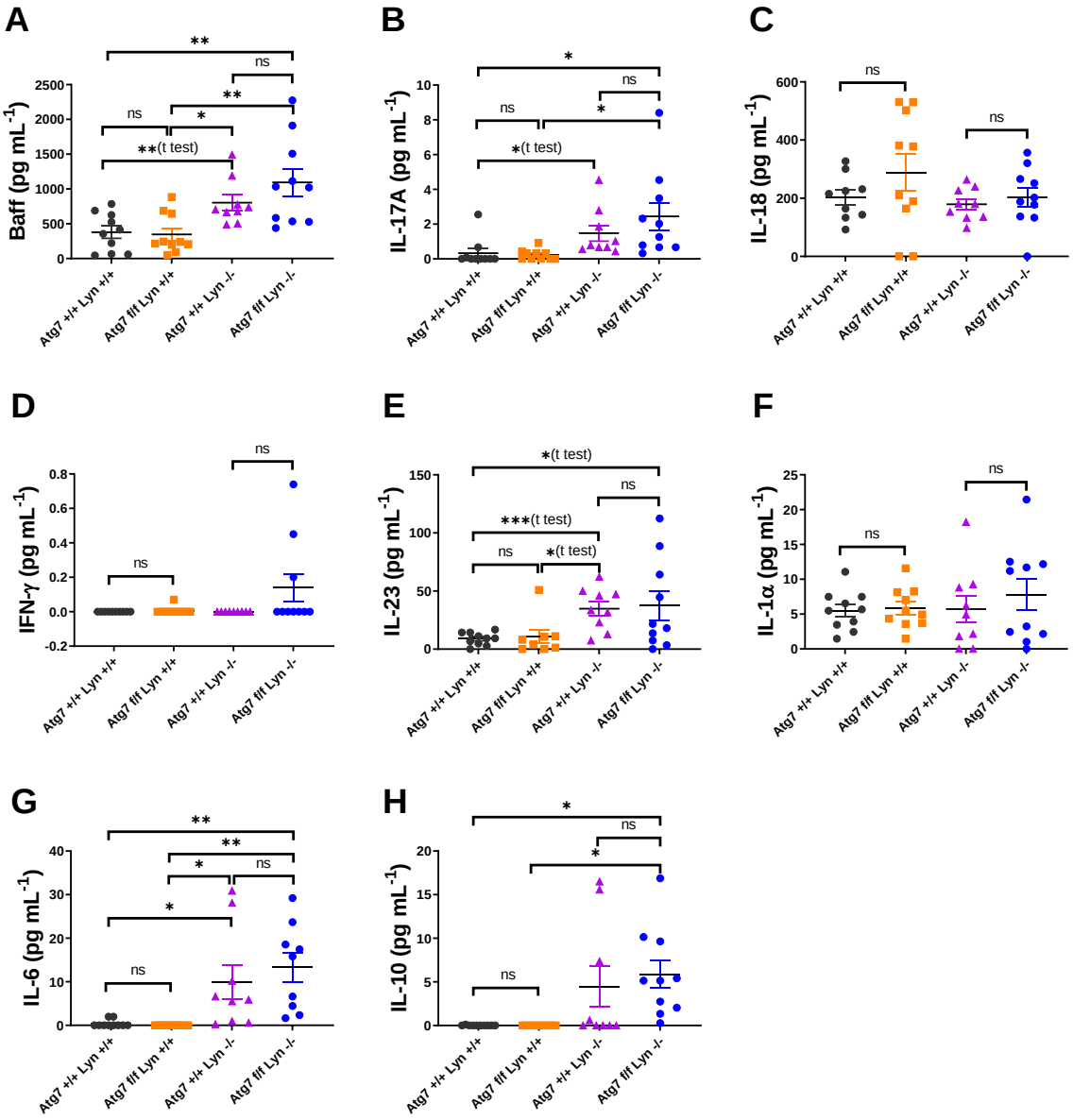


Figure 2.1 Myeloid cell autophagy regulates serum cytokines in experimental young mice (age < 100 days)

The level of secreted cytokines in serum was measured by Luminex assay. N=8-10 per group. The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test and t-test; *p < 0.05; **p < 0.01; ns: not significant.

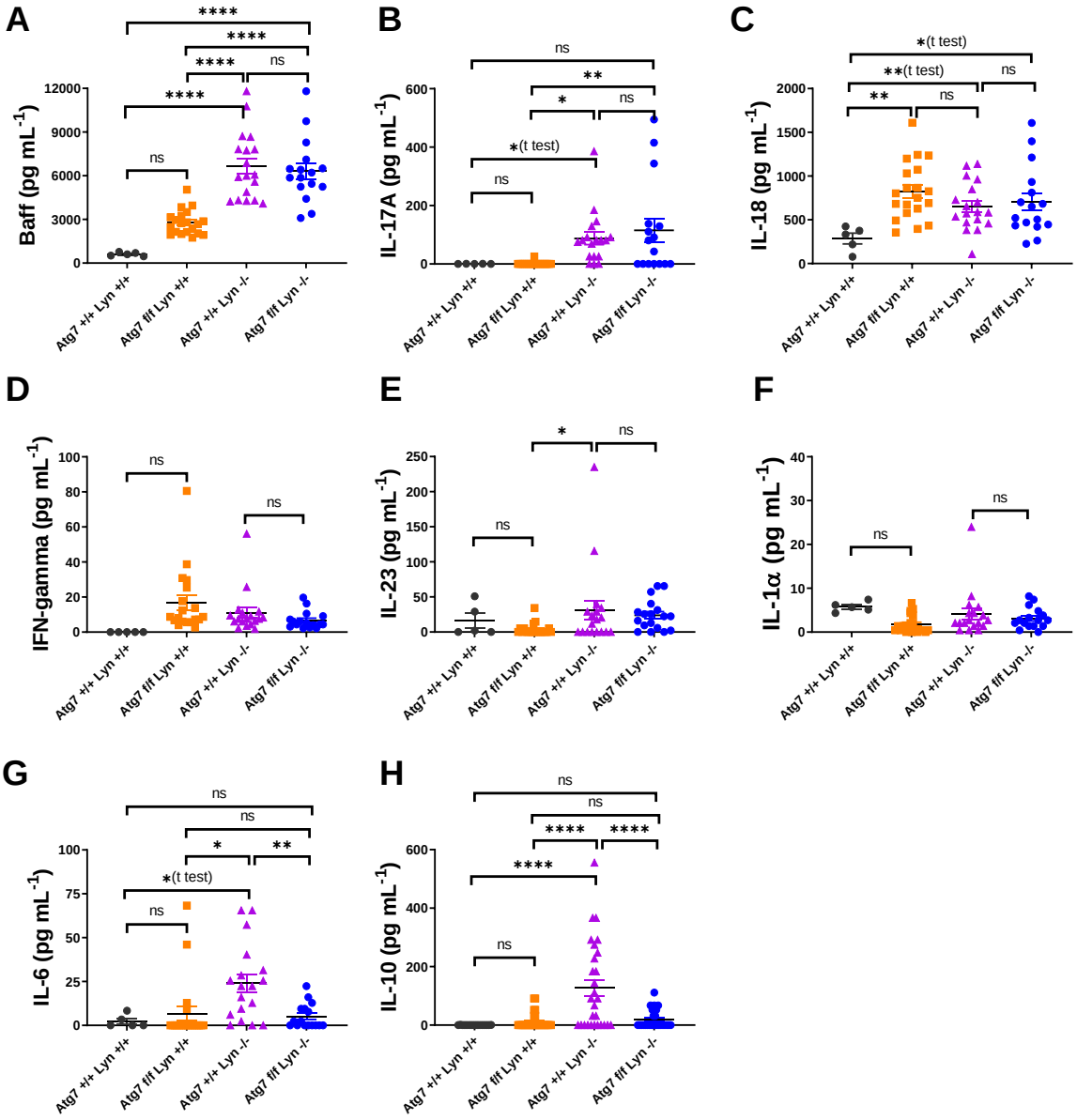


Figure 2.2 Myeloid cell autophagy regulates serum cytokines in experimental old mice (group 1, age ≤ 250 days)

The level of secreted cytokines in serum was measured by Luminex assay. N= 5-20 per group. The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test and t-test; *p < 0.05; **p < 0.01; ****p< 0.0001 ns: not significant.

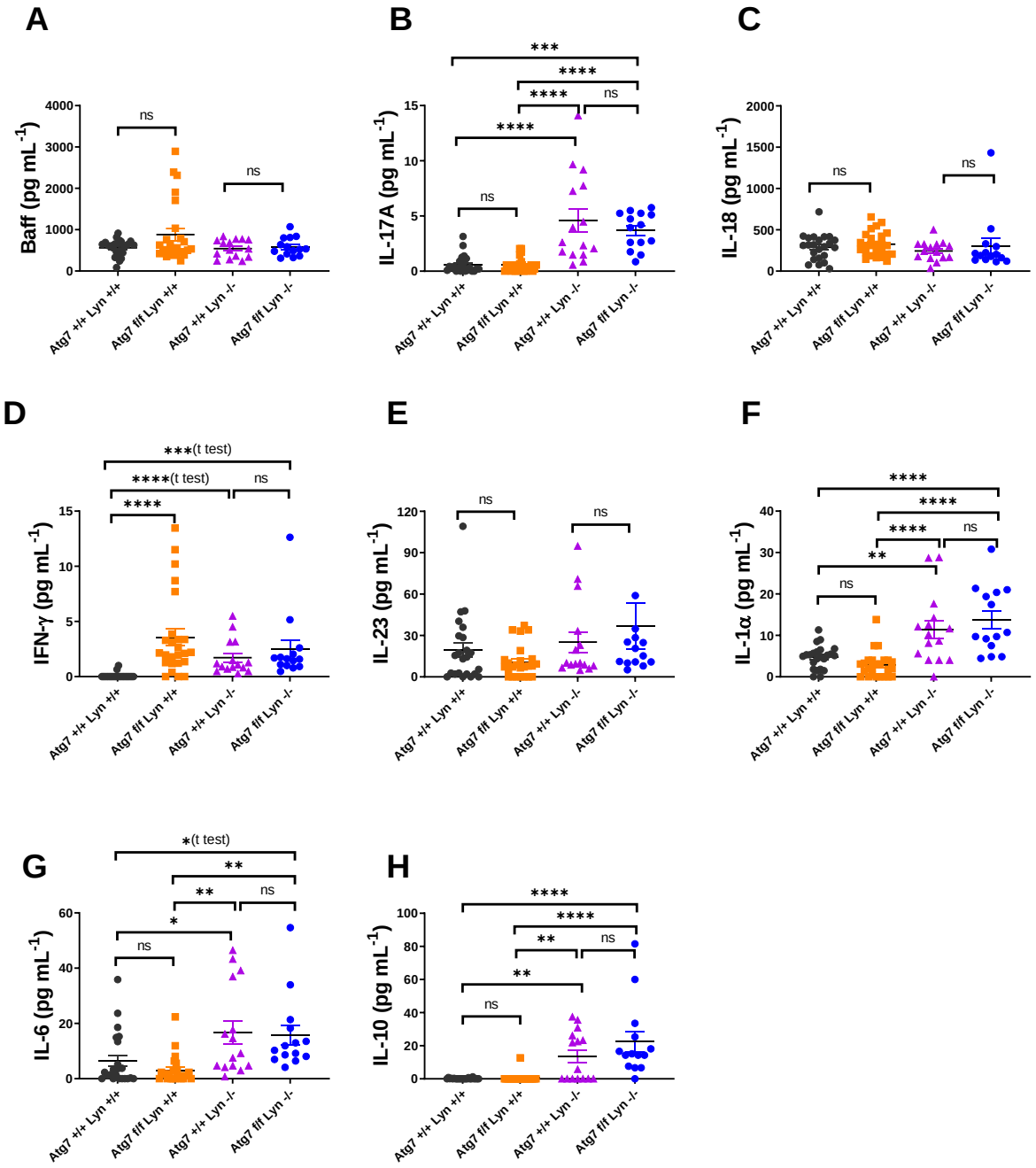


Figure 2.3 Myeloid cell autophagy regulates serum cytokines in experimental old mice (group 2, age > 250 days)

The level of secreted cytokines in serum was measured by Luminex assay. N= 14-25 per group. The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test and t-test; *p < 0.05; **p < 0.01; ****p < 0.0001 ns: not significant.

2.3.2 Effects of autophagy deficiency in myeloid cells on autoantibody production

Autoantigens play a key role in SLE disease development, and more than 160 autoantigens have been described in the sera of SLE patients, though only a few of these are routinely tested (Sherer et al., 2004). Primary concomitants in the SLE pathogenesis include higher autoantigenic exposure and reduced clearance of autoantigens (Pravda, 2019). Anti-double-stranded DNA (anti-dsDNA), Anti-Smith (anti-SM), anti-histone, anti-JO-1, and anti-Sjögren's syndrome-related antigen A and B (SSA/Ro, and SSB/La, respectively) are commonly used as diagnostic markers in SLE (Hoffman et al., 2004, Tang et al., 2010). To measure the autoantigens in the experimental mice, a modified ENA panel (CENP-B, dsDNA, Histone, Jo-1, PCNA, pmScl, Ribosomes, Scl-70, SM, SMRNP, SSA, SSB, TRIM21 and U1RNP) test was performed, and mice separated by age as in 2.3.1.

Levels of anti-dsDNA, anti-histone, anti-SM, and anti-TRIM21 were significantly raised in both Atg$7^{+/+}$ *Lyn*$^{-/-}$ and *Atg*$7^{fl/fl}$ *Lyn*$^{-/-}$ mice (**Fig. 2.4**). However, no significant difference between the two groups was observed (**Fig. 2.4**). In mice aged 101-250 days, serum levels of anti-dsDNA, anti-Histone, anti-Jo-1, anti-PCNA, anti-pmScl, anti-Ribosomes, anti-Scl-70, anti-SM, anti-SMRNP, anti-TRIM21 and anti-U1RNP were significantly increased in *Atg7*$^{+/+}$ *Lyn*$^{-/-}$ mice, compared to WT and *Atg7*$^{fl/fl}$ *Lyn*$^{+/+}$ mice (**Fig. 2.5**). Of these, anti-SM, anti-dsDNA and anti-histone were significantly decreased in *Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$ mice (**Fig. 2.5**), suggesting that myeloid cell autophagy has a significant impact on the production of specific autoantibodies in this model of lupus. However, similar to the effects observed on cytokine release, this effect was lost in older mice (>250 days) (**Fig. 2.6**), suggesting that eventually, the disease overcomes any protection offered by the autophagy deficiency.

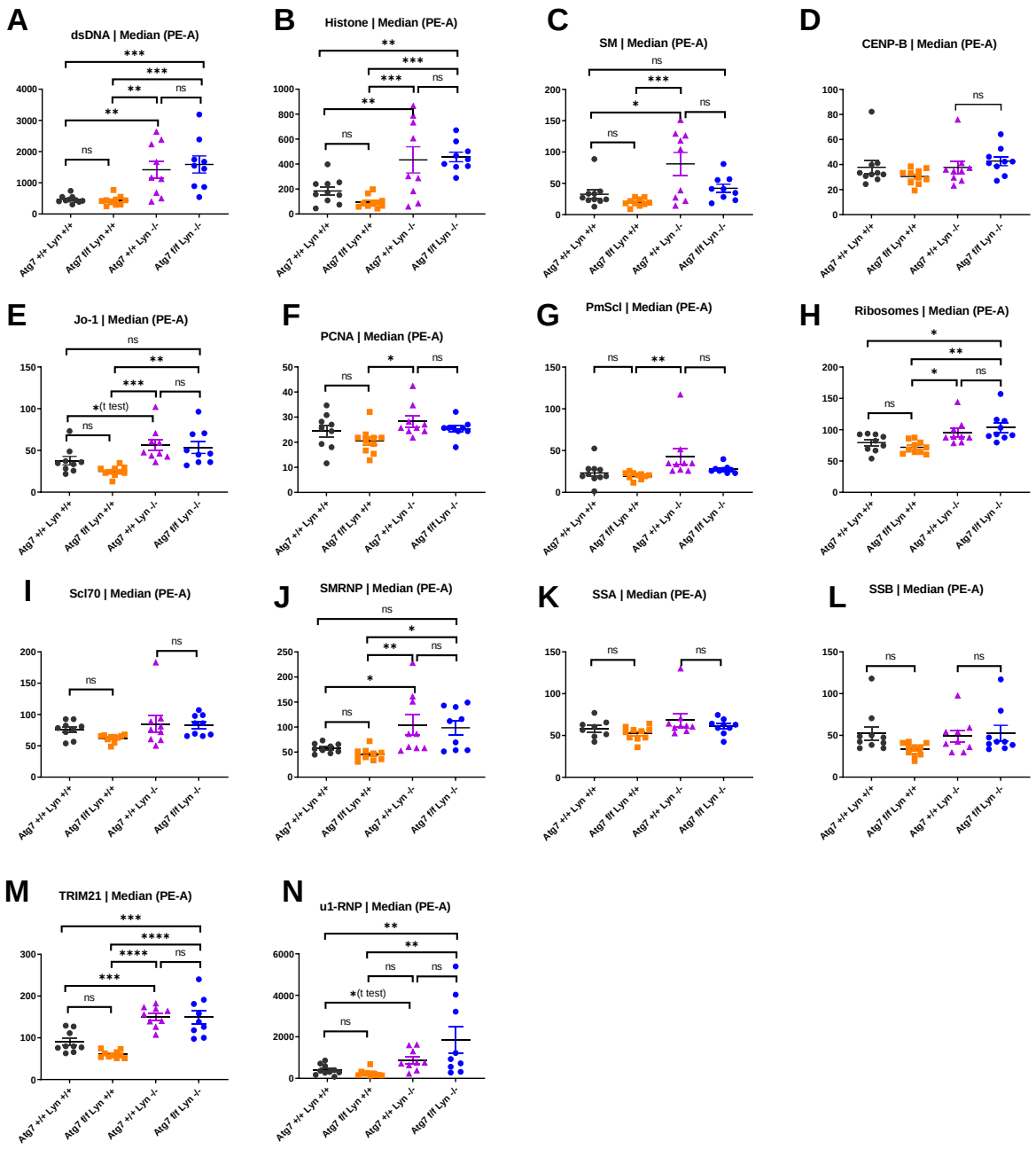


Figure 2.4 Autoantibody profile in experimental young mice (age < 100 days)
14 different autoantibodies against different autoantigens (CENP-B, dsDNA, Histone, Jo-1, PCNA, pmScl, Ribosomes, Scl-70, SM, SMRNP, SSA, SSB, TRIM21 and U1RNP) were analyzed by Extractable Nuclear Antigens (ENA) test. N=8-10 per group. The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test and t-test. *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.0001.

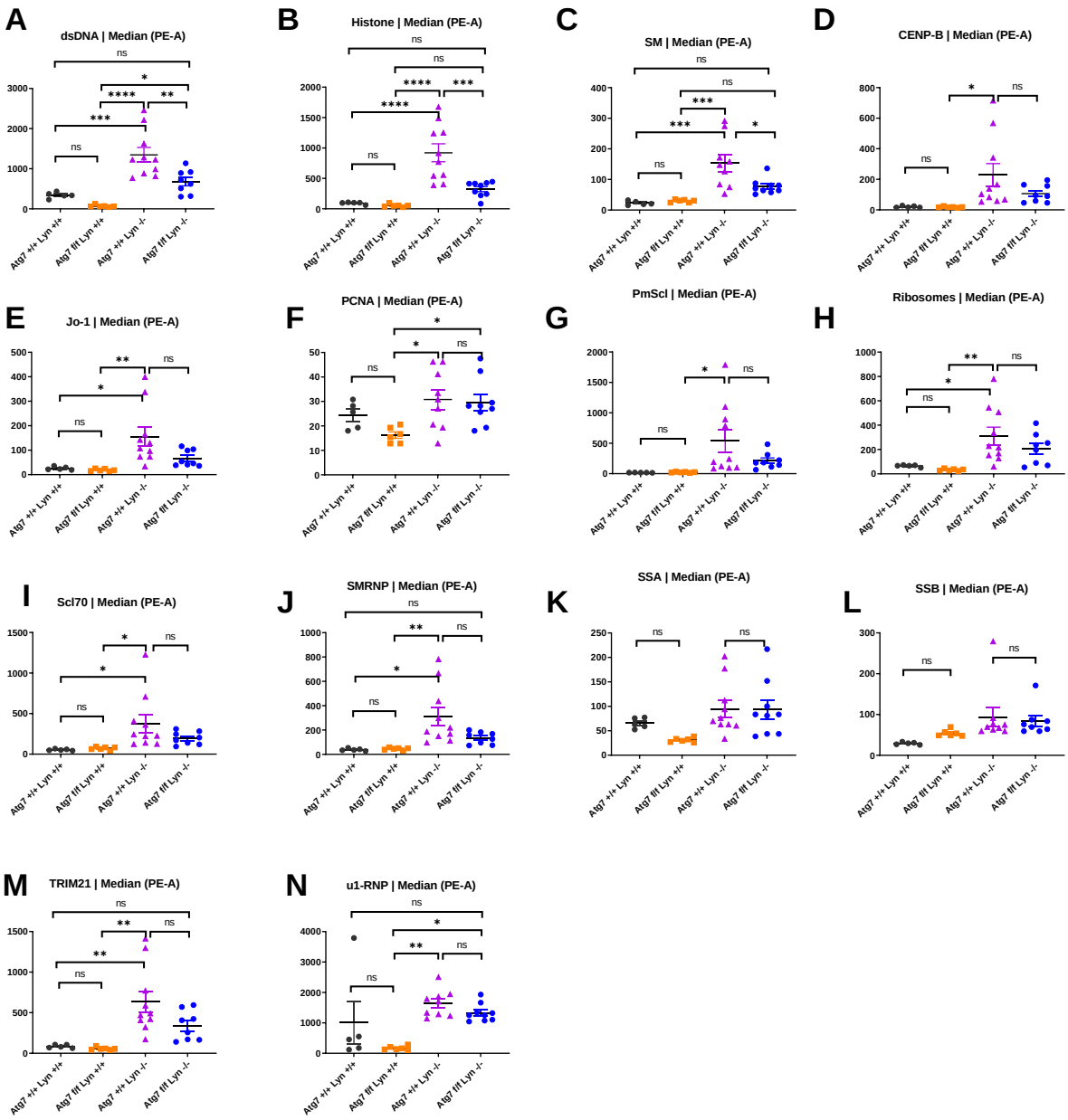


Figure 2.5 Autoantibody profile in experimental old mice (group 1, age ≤ 250 days)
14 different autoantibodies against different autoantigens (CENP-B, dsDNA, Histone, Jo-1, PCNA, pmScl, Ribosomes, Scl-70, SM, SMRNP, SSA, SSB, TRIM21 and U1RNP) were analyzed by Extractable Nuclear Antigens (ENA) test. N=8-10 per group. The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test. *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.0001.

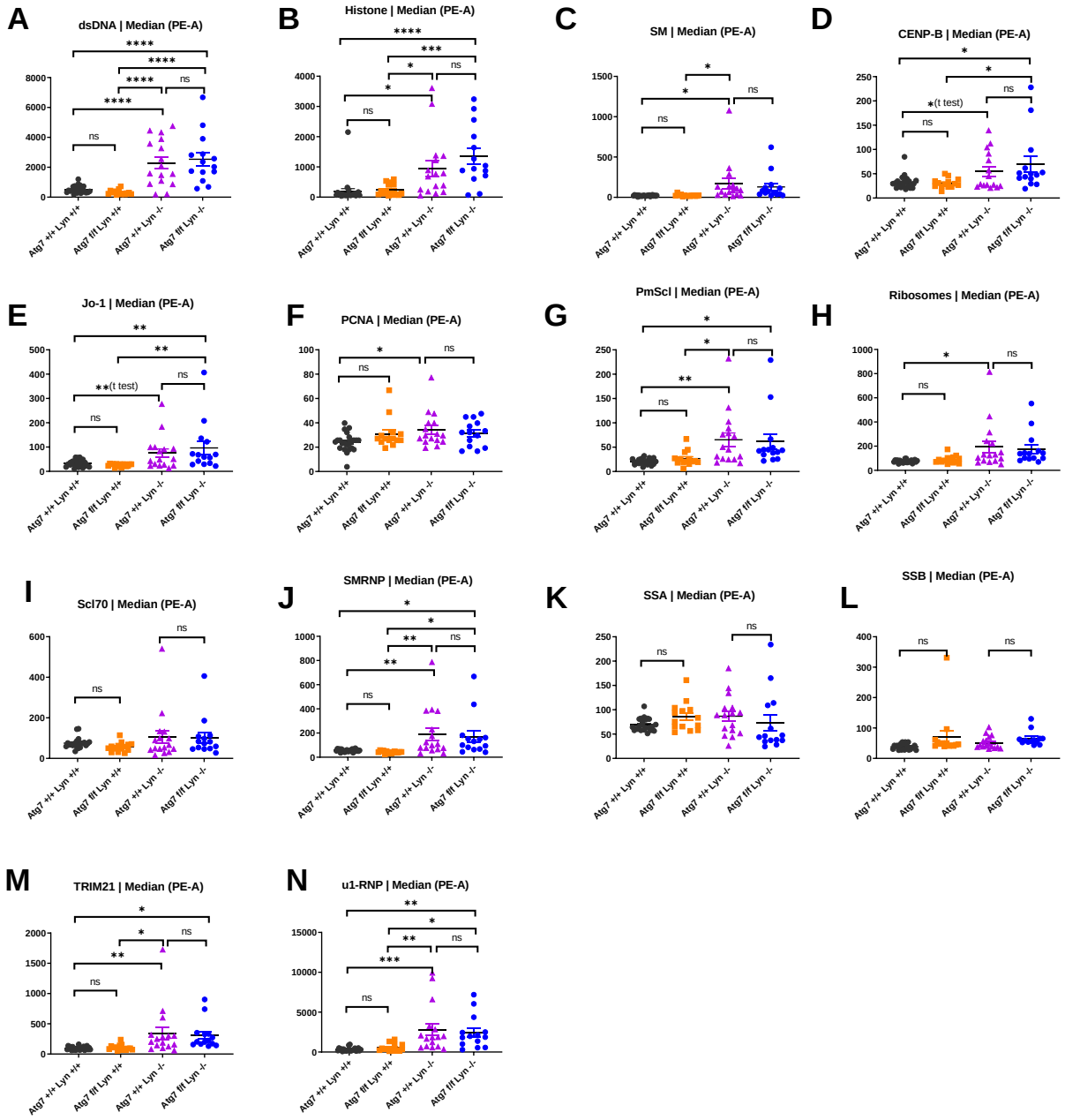


Figure 2.6 Autoantibody profile in experimental old mice (group 2, age > 250 days)
14 different autoantibodies against different autoantigens (CENP-B, dsDNA, Histone, Jo-1, PCNA, pmScl, Ribosomes, Scl-70, SM, SMRNP, SSA, SSB, TRIM21 and U1RNP) were analyzed by Extractable Nuclear Antigens (ENA) test. N=8-10 per group. The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test and t-test. *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.0001.

2.3.3 Effects of autophagy deficiency in myeloid cells on B cell populations

B cells play a major role in the development of autoimmune disease in *Lyn*-deficient mice (Nishizumi et al., 1995, Hibbs et al., 1995, Chan et al., 1997). Based on the effect of autophagy deficiency on the production of autoantibodies seen here, splenic B cell populations were analysed by flow cytometry, specifically evaluating various cell surface receptors that reflect the development and activation status of B cell subsets (**Figs. 2.7**).

Plasma cell accumulation is a characteristic feature in *Lyn* deficient mice (Chan et al., 1997), and in young mice, an increase in this population was observed in $Atg7^{+/+}$ $Lyn^{-/-}$ (**Fig. 2.8A**). Interestingly, this population was not increased in the $Atg7^{fl/fl}$ $Lyn^{-/-}$ mice (**Fig. 2.8A**), indicating a role for autophagy in regulating specific mechanisms in myeloid cells that subsequently mediate plasma cell development. However, in aged mice (both 101-250 days and >250 days), no difference was seen in plasma cell numbers between $Atg7^{+/+}$ $Lyn^{-/-}$ and $Atg7^{fl/fl}$ $Lyn^{-/-}$ mice, although plasma cell numbers were increased in the "middle-aged" $Atg7^{fl/fl}$ $Lyn^{+/+}$ mice compared to all other groups (**Fig. 2.9A**). Similarly, in this age group, the $Atg7^{fl/fl}$ $Lyn^{+/+}$ mice had higher numbers of germinal centre (GC) B cells, and isotype switched B cells (**Fig. 2.9C and E**), again suggesting that myeloid cell autophagy impacts B cell development, although in this case independently of disease.

B cell numbers were significantly lower in all aged knockout mice (**Fig. 2.9B and 2.10B**), while GC B cells, non-GC B cells, isotype-switched cells, and non-isotype switched cells were all decreased in both *Lyn* deficient and double knockout aged mice (**Fig. 2.9 and 2.10**). This is in line with previous studies that have demonstrated that Lyn deficiency directly affects B cell development, with a 30-50% reduction in B cell maturation (Liu et al., 2010, Sakaguchi, 2004, Ehrenstein et al., 2004). However, Double knockout mice were not significantly different from the Lyn deficient mice for any of these, suggesting that the loss of myeloid cell autophagy does not impact these B cell populations in the setting of *Lyn* deficiency.

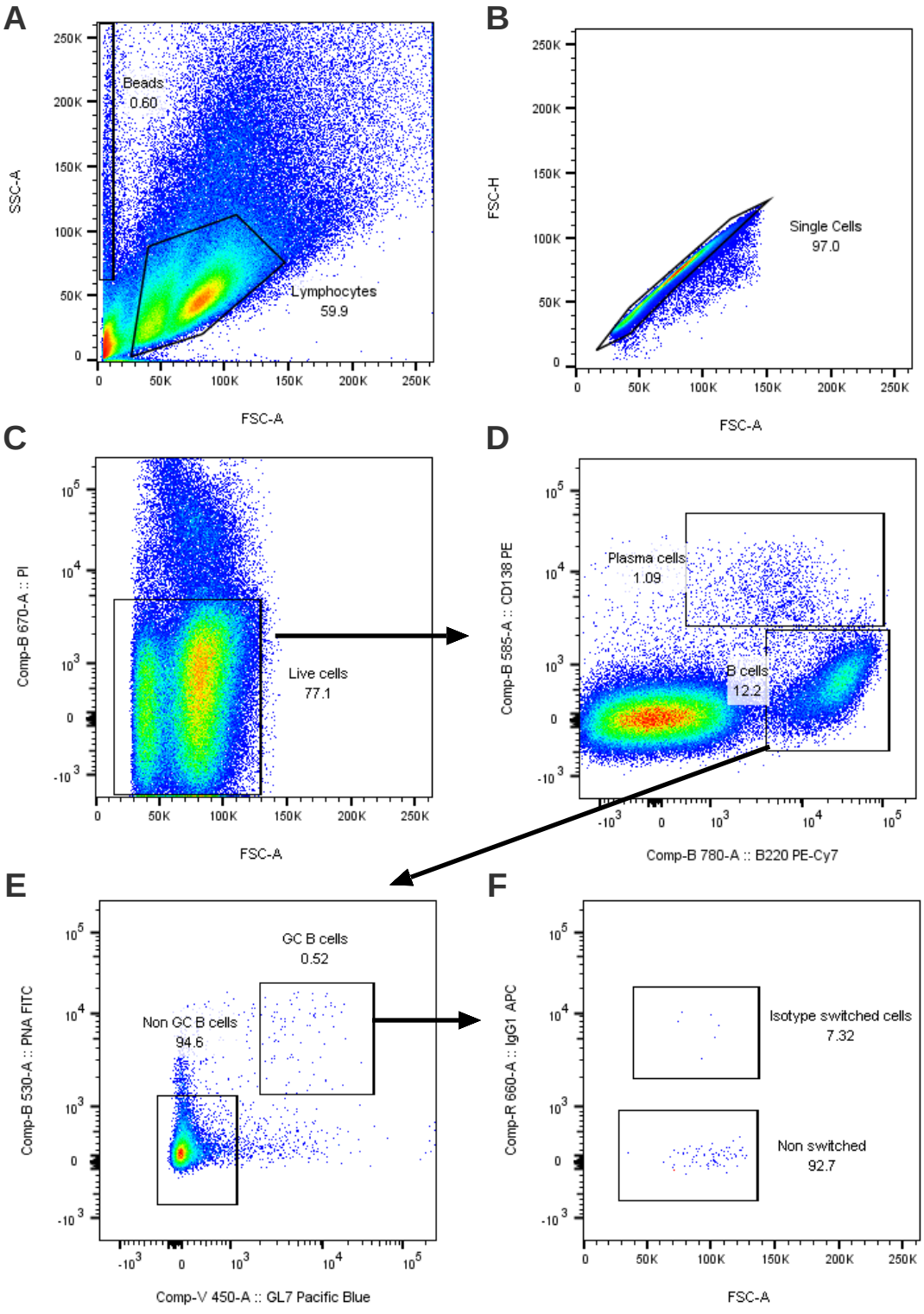


Figure 2.7 Gating strategy for splenic B cell compartments in experimental mice
B cells population were analysed by flow cytometry. The following cell populations were identified: plasma cells ($B220^{low}CD138^{hi}$), conventional B cells ($B220^{hi}CD138^{low}$), GC B cells ($B220^{hi}GL7^{hi}PNA^{hi}$), non-GC B cells ($B220^{hi}GL7^{low}PNA^{low}$), isotype switched ($B220^{hi}GL7^{hi}PNA^{hi}IgG1^{hi}$), non-isotype switched ($B220^{hi}GL7^{hi}PNA^{hi}IgG1^{low}$).

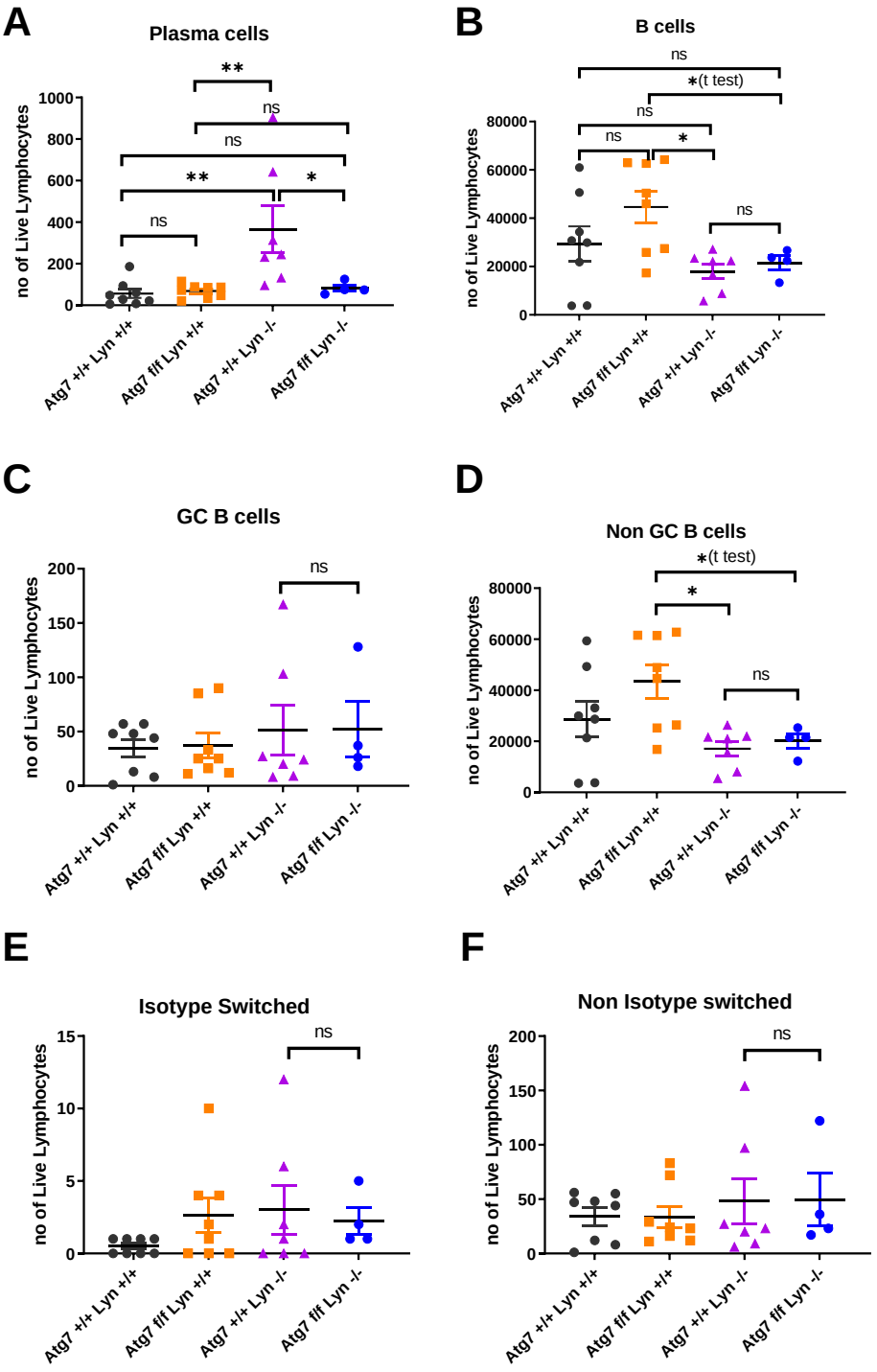


Figure 2.8 Effect of myeloid cell autophagy on splenic B cell compartments in experimental young mice (age < 100 days)

Splenic B cells were analyzed by flow cytometry. N=4-8 per group. The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test and t-test. *P < 0.05; **P < 0.01.

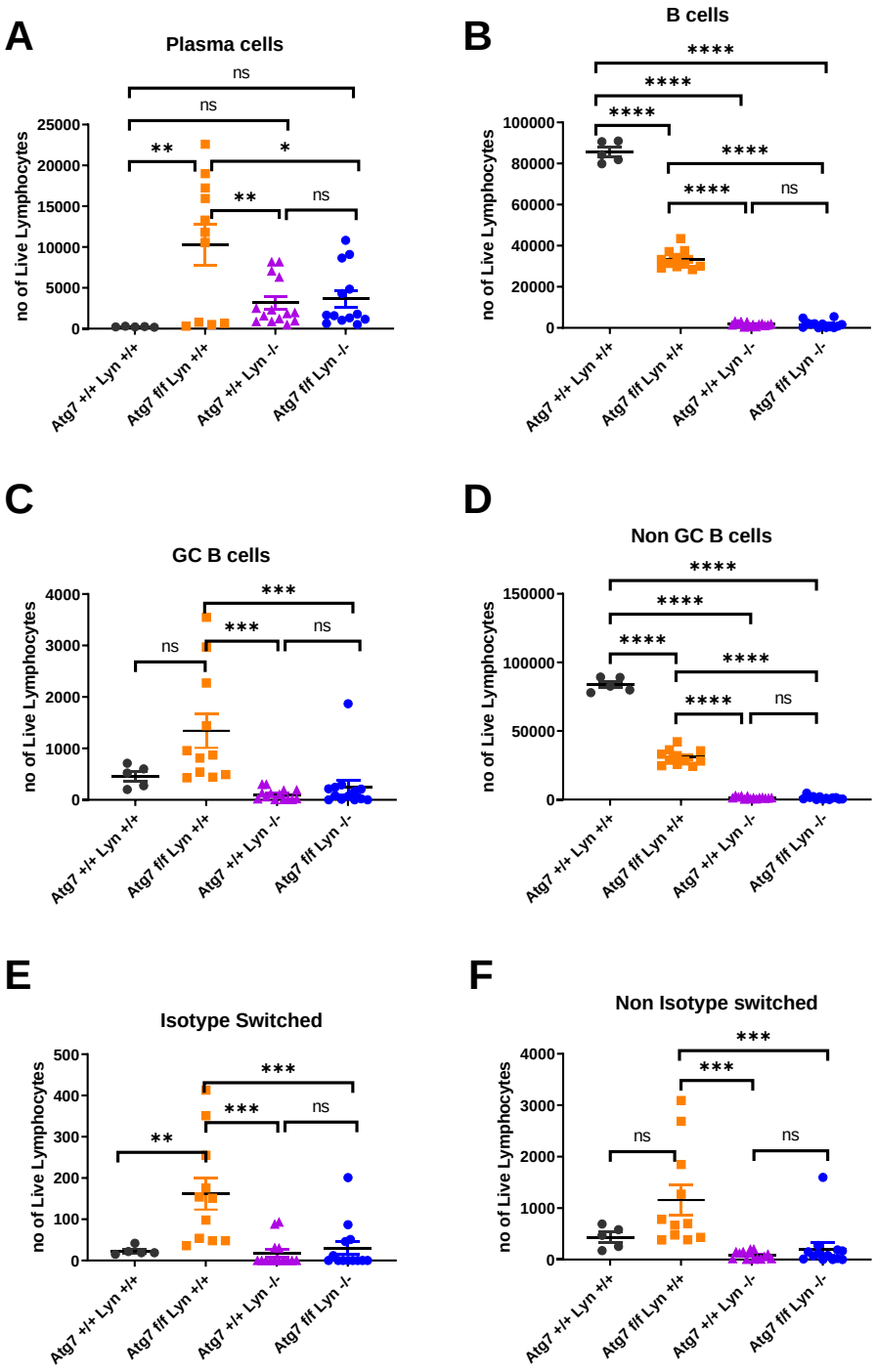


Figure 2.9 Effect of myeloid cell autophagy on splenic B cell compartments in experimental old mice (group 1, age ≤ 250 days)

Splenic B cells were analyzed by flow cytometry. N= 5-14 per group. The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test. **P < 0.01; ***P < 0.001 **** P < 0.0001.

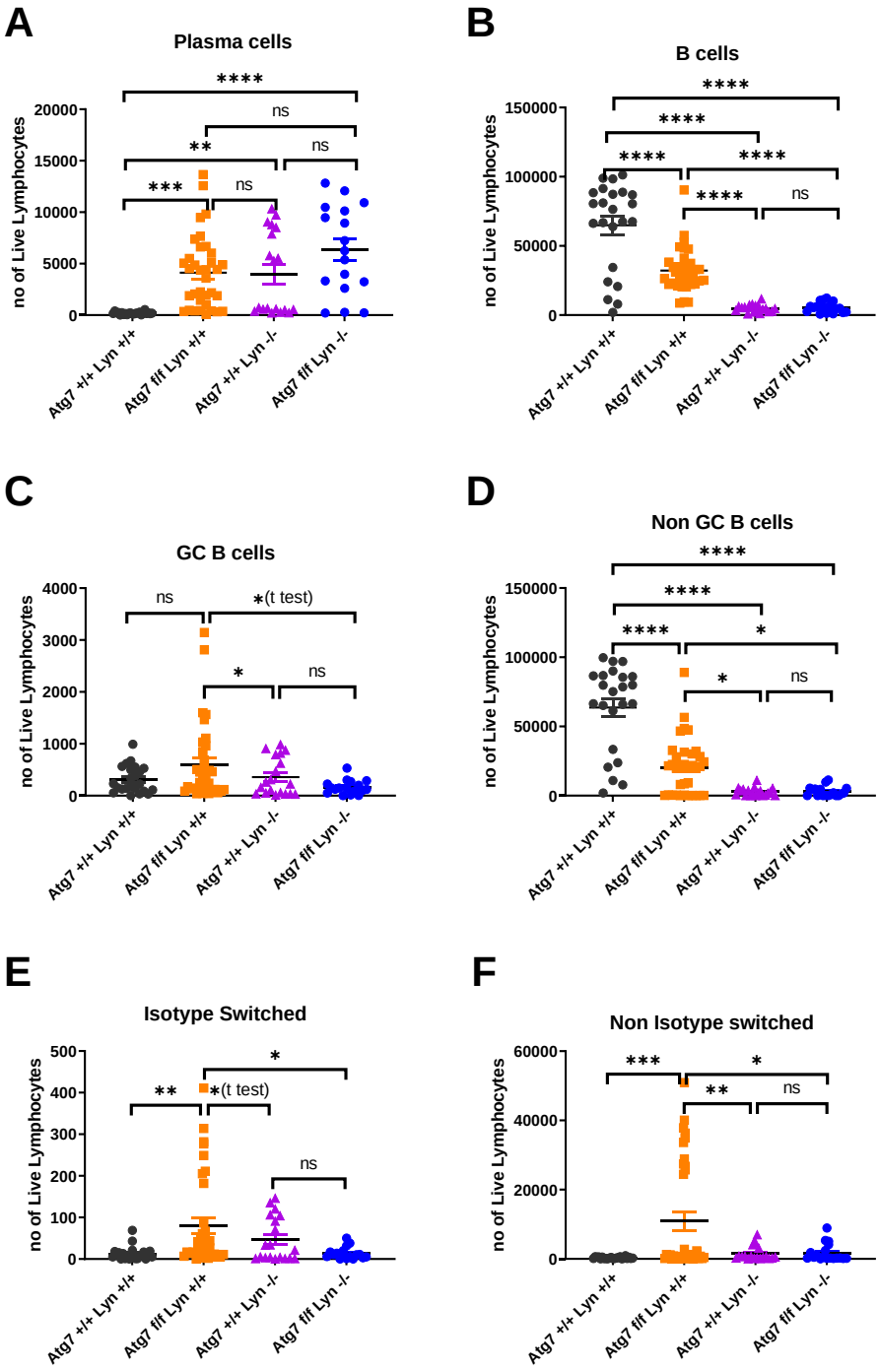


Figure 2.10 Effect of myeloid cell autophagy on splenic B cell compartments in experimental old mice (group 2, age > 250 days)
Splenic B cells were analyzed by flow cytometry. N= 17-35 per group. The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test and t-test. *P < 0.05; **P < 0.01; ***P < 0.001 **** P < 0.0001.

2.3.4 Regulatory T cells are increased in Lyn deficient mice

T cells co-expressing CD4, CD25, and the Forkhead box p3 (Foxp3) transcription factor are known as regulatory T cells (Treg) (Liu et al., 2010). Treg cells have the ability to inhibit effector cells and decrease a wide variety of immune responses, including those linked to autoimmune diseases, including type 1 diabetes, multiple sclerosis, SLE, RA, and others (Sakaguchi, 2004, Ehrenstein et al., 2004, Balandina et al., 2005). Tregs are essential in the maintenance of immune homeostasis and self-tolerance (Campbell and Koch, 2011, Wan and Flavell, 2007). Tregs can inhibit T cell proliferation and cytokine production (particularly IL-2 production) and play critical roles in preventing autoimmune diseases (Horwitz, 2008, Scalapino et al., 2006). In animals, Treg function seems to decrease with advancing age (Zhao et al., 2007). It has previously been reported that $CD25^{high}$ Tregs from aged animals were less effective at inhibiting the pro-inflammatory activity of $IL\text{-}17^{+}$ than cells from young mice (Sun et al., 2012).

Here, I measured Tregs in young mice to determine whether they are involved in the early stage of disease in *Lyn*$^{-/-}$ animals (**Fig. 2.11)**. Using flow cytometry to identify CD4+CD25+FOXP3+ cells, Treg cells were significantly increased in *Lyn* deficient and double knockout mice compared to WT and *Atg7*$^{fl/fl}$ *Lyn*$^{+/+}$ mice (**Fig. 2.12**). There was no significant difference between the *Atg7*$^{+/+}$ *Lyn*$^{-/-}$ and *Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$ mice, suggesting that myeloid cell autophagy does not contribute to the regulation of Tregs in *Lyn* deficient mice.

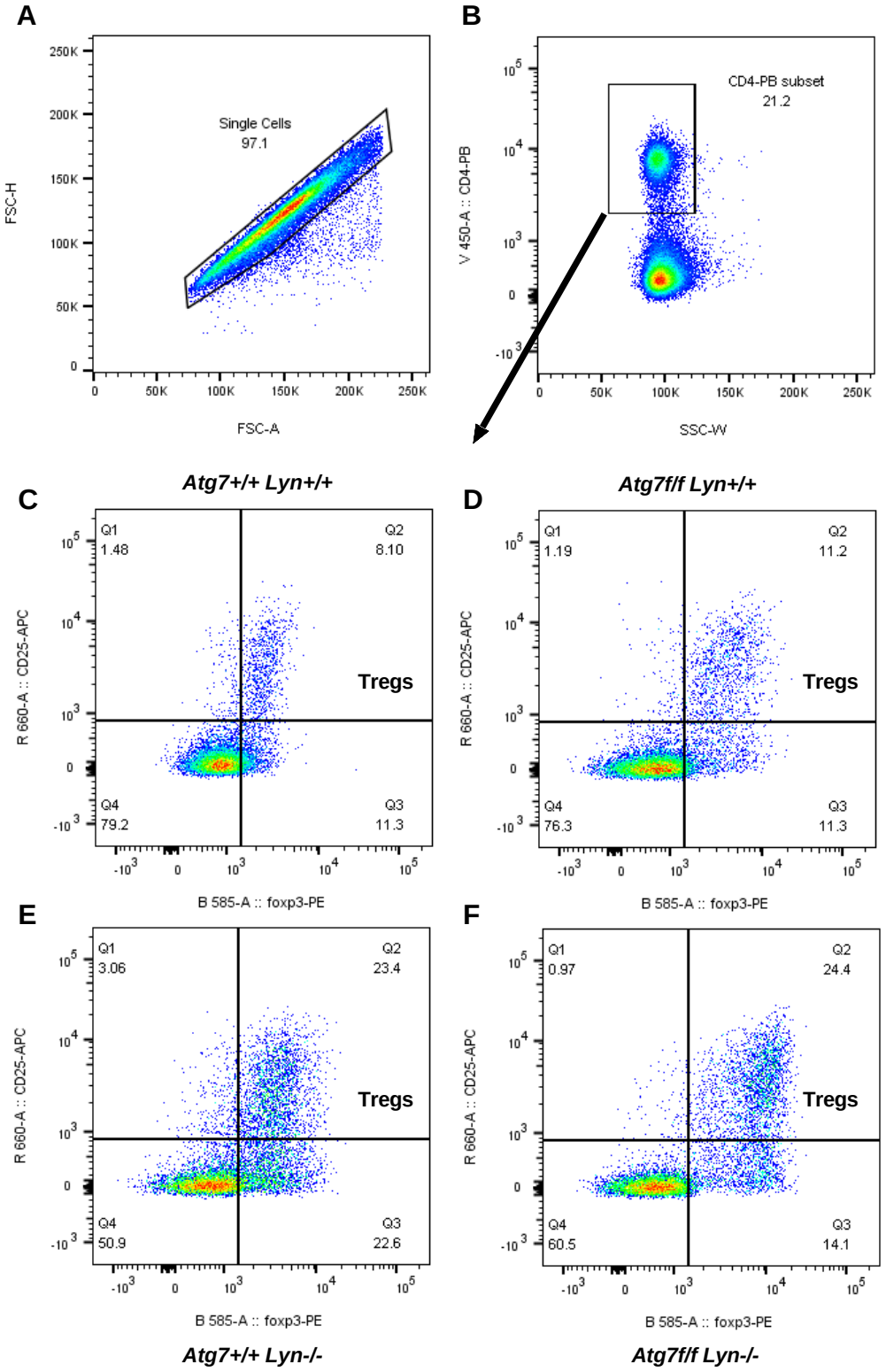


Figure 2.11 Gating strategy for CD4+ T regulatory cells (Tregs) in the spleen
CD4+ T cells population were analysed by flow cytometry, and $CD4^{+}CD25^{+}Foxp3^{+}$ were identified as Tregs. Tregs from different groups of mice were shown (C-F)

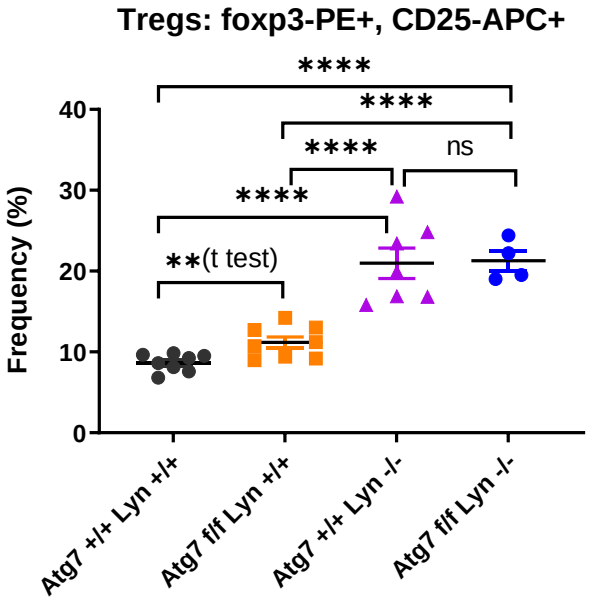


Figure 2.12 Frequency of regulatory T (Treg) cells in the spleen
Splenic T regulatory cells (Treg) were analyzed by flow cytometry. The percentages of Tregs within the CD4+ T cell populations were presented in the graph. N=4-8 per group; The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test and t-test. ****p < 0.0001; ns: not significant.

2.3.5 Autophagy deficiency does not affect spleen and body weight in Lyn-deficient mice

Splenomegaly has previously been shown to occur in *Lyn*-deficient mice due to the accumulation of plasma cells and Mac1^{+} lymphoblasts as early as 12 weeks of age (Nishizumi et al., 1995). Here, body and spleen weight were unchanged among all the groups of young mice aged less than 100 days (**Fig. 2.13A and B).** In mice aged 101-250 days and >250 days, spleen weight was significantly increased in both *Atg7*$^{+/+}$ *Lyn*$^{-/-}$ and *Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$ mice compared to controls (**Fig. 2.13D and F**), but no differences were seen between these two groups. In mice aged 101-250 days and >250 days, body weight was significantly lower in the double knockout group compared to WT controls (**Fig. 2.13C and E).** In the oldest group of mice aged >250 days, bodyweight was significantly lower in the *Atg7*$^{+/+}$ *Lyn*$^{-/-}$mice compared to WT controls (**Fig. 2.13E),** but no significant differences were seen between WT controls and *Atg7*$^{+/+}$ *Lyn*$^{-/-}$mice aged 101-250 days (**Fig. 2.13C)**. In all, these data suggest that while

both spleen and body weight are affected by Lyn deficiency, loss of myeloid cell autophagy does not contribute to this.

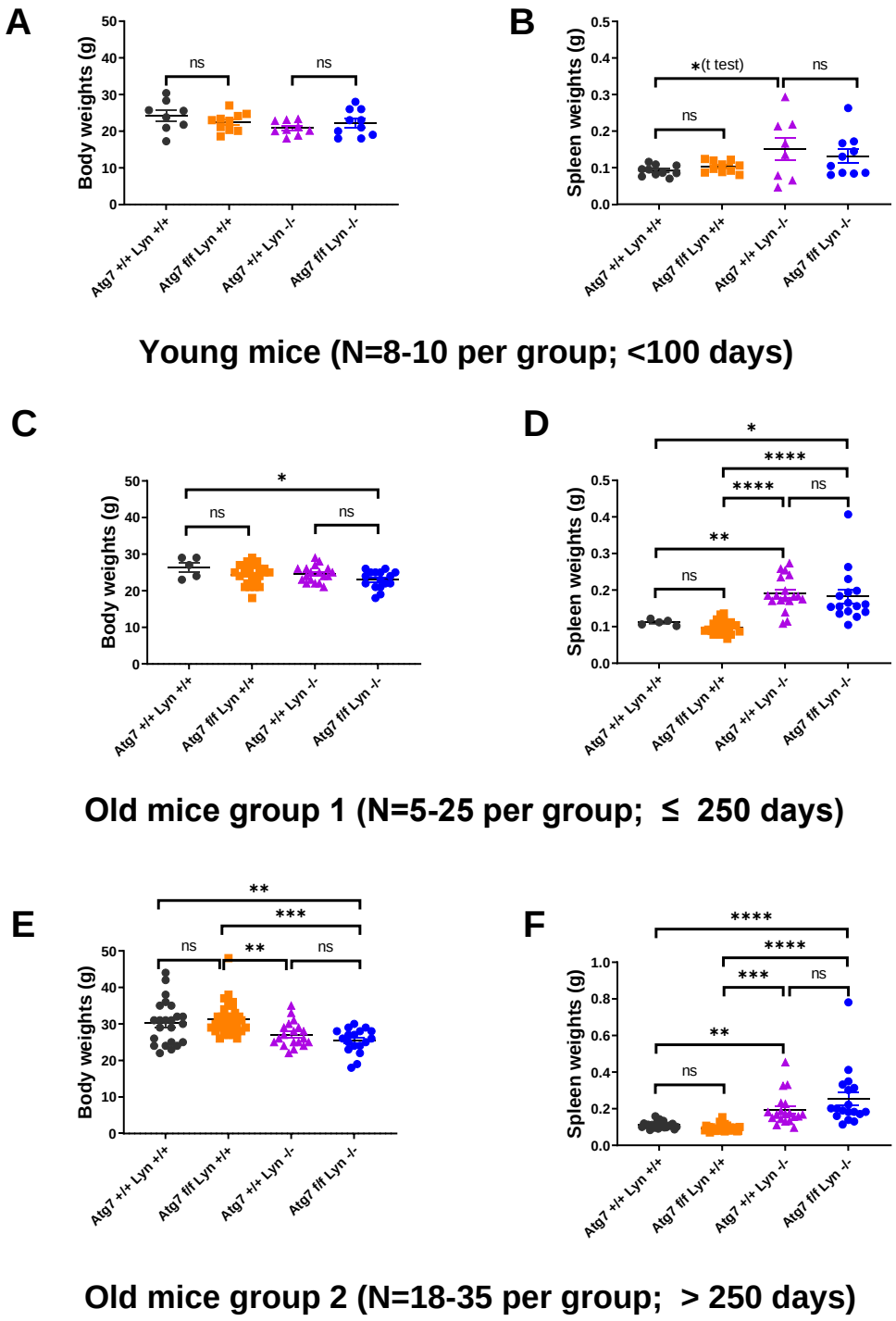
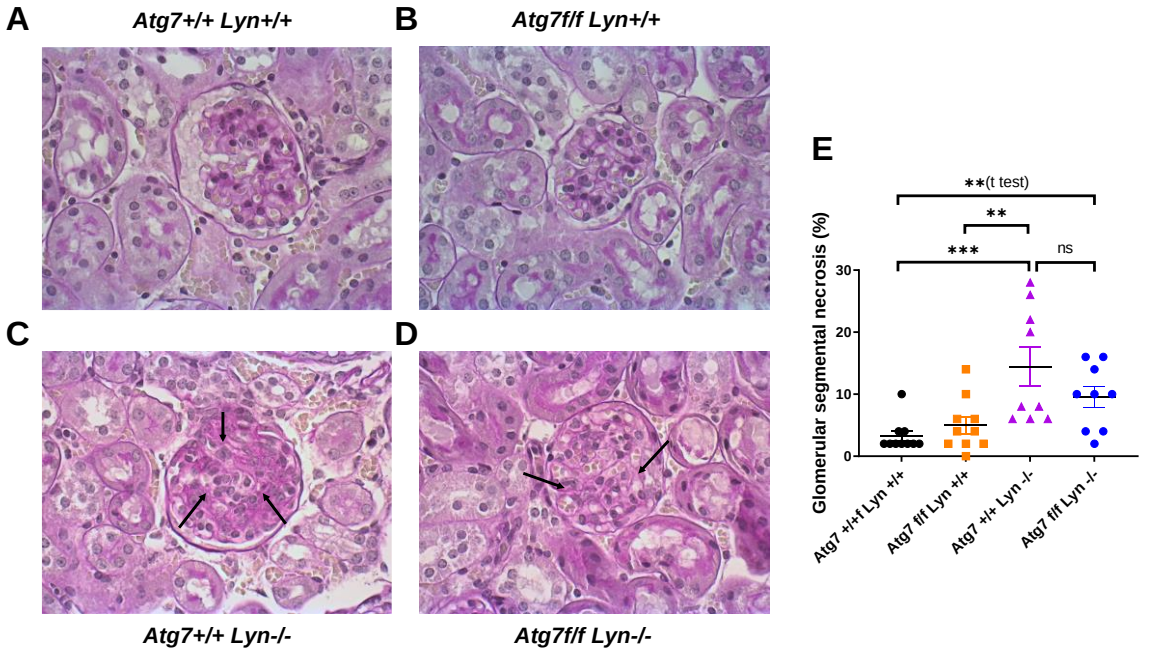


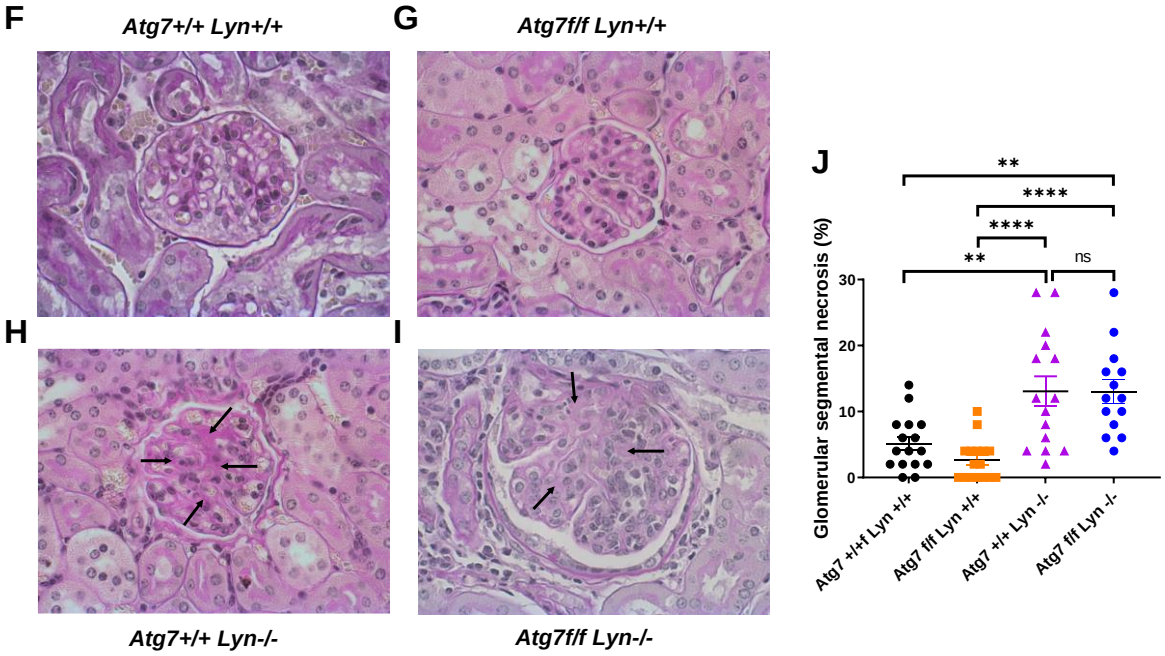
Figure 2.13 Effect of myeloid cell autophagy on body and spleen weights of mice
Body and spleen weights (g) of different experimental groups of mice at different ages (days). The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test and t-test; *P < 0.05; **P < 0.01; **** P < 0.0001: ns: not significant.

2.3.6 Histopathology in Lyn deficient mice

Glomerulonephritis (GN) is a characteristic feature in *Lyn* deficient mice, developing as a result of immune complex deposition as early as 6 to 8 weeks of age (Nishizumi et al., 1995, Hibbs et al., 1995). To determine whether myeloid-specific autophagy deficiency affect glomerulonephritis in the Lyn-deficient mice, I compared the development of glomerulonephritis in Lyn-deficient mice (Atg7+/+ Lyn-/- mice) with that in myeloid-specific autophagy and Lyn deficient double knockout mice (Atg7fl/fl Lyn-/-) by histopathological analysis of Kidney. In young mice, glomerular segmental necrosis was increased in $Atg7^{+/+}$ $Lyn^{-/-}$ mice compared to WT and $Atg7^{fl/fl}$ $Lyn^{+/+}$ mice (**Fig. 2.14A-E**). Interestingly, in these young mice, segmental necrosis was not similarly increased in double knockout mice, suggesting a potential protective effect of *Atg7* deficiency in myeloid cells in the early stages of disease development (**Fig. 2.14A-E**). However, this effect was lost in aged mice with segmental necrosis significantly increased in both *Lyn* deficient and double knockout mice group (**Fig. 2.14F-J**), suggesting that any protective effect of *Atg7* deletion is transient and over-ridden as the disease progresses. In non-disease conditions, loss of myeloid cell autophagy did not affect GN development at any age.



Young mice (N= 9-10 per group; < 100 days)



Old mice (N= 14-16 per group; > 250 days)

Figure 2.14 Effect of Autophagy deficiency in lupus mice

Kidney sections from the different genotypes of young (A) and aged mice (B) were stained with PAS to assess pathological changes associated with glomerulonephritis (original magnification ×40). Sections from individual mice (N= 8-10 per group for young mice and N=14-16 per group for aged mice) were scored in a blinded fashion for cellular proliferation, segmental necrosis (glomeruli affected per 100 glomeruli), and crescent formation. The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test and t-test; **P < 0.01; ***P < 0.001 **** P < 0.0001.

2.4 Discussion

The *Lyn* deficient mice model is a single gene defect model that causes hyperactive intracellular signalling responses and represents a widely used genetic model for autoimmune and inflammatory diseases like SLE (Xu et al., 2005, Scapini et al., 2009). Lyn deficient mice develop autoimmune symptoms over time that are comparable to human SLE (Hibbs et al., 1995). In lupus patients, the expression of *LYN* expression is decreased than the healthy controls, and functional abnormalities in Lyn ubiquitination have been attributed to an increased risk of SLE (Liossis et al., 2001, Flores-Borja et al., 2005). *Lyn* kinase is mainly expressed in B cells, and myeloid cells and changes in *Lyn* expression or activity contribute to altered B cell function, hyperresponsiveness to BCR stimulation and SLE-like autoimmune disease (Xu et al., 2005, Hibbs et al., 1995, Nishizumi et al., 1995). In the present study, we bred myeloid-specific autophagy deficiency into the Lyn knockout mouse model of lupus to gain insight into the mechanisms by which myeloid-specific autophagy deficiency regulates the development of autoimmunity.

In this study, an increased level of serum cytokines, high level of autoantibodies, splenomegaly as well as severe glomerulonephritis were observed in *Lyn* deficient lupus mice compared to control mice. Furthermore, B cells population was decreased, and plasma cells were increased in *Lyn* deficient animals compared to wild type. Myeloid-specific autophagy deficiency in *Lyn*-/- mice contributed significantly to the inhibition of pro-inflammatory cytokines IL-6 and IL-10 production. Consequently, lack of myeloid-specific autophagy could robustly decrease the anti-dsDNA antibodies level, which may be attributed to the significantly reduced IL-6 and IL-10 production. Our recent study demonstrated that Lyn deficient lupus mice also develop splenomegaly, glomerulonephritis, produce high anti-dsDNA antibody and elevated cytokine expression, and all these disease measures of SLE are aggravated by GILZ deficiency (Nataraja et al., 2021).

Both autophagy and myeloid cells play an important role in autoimmune disease progression though the role of autophagy in myeloid cells in autoimmune disease is not well understood. A few studies have been conducted on the association of myeloid cell autophagy with various diseases, including diabetes and colitis, rhinosinusitis, Ischemic Brain Injury, and sepsis (Lee et al., 2016b, Choi et al., 2018, Kotoda et al., 2018, Orvedahl et al., 2019). Choi et al reported that Impaired autophagy provokes eosinophilic and type 2 inflammation in a mouse model of eosinophilic chronic rhinosinusitis (ECRS) mouse model, and this pathologic change largely relies on altered macrophage function (Choi et al., 2018). They found that autophagy deficiency in macrophages increased the level of IL-1β and macrophage ablation or blockade of IL-1 receptor alleviated the eosinophilic inflammation and sinonasal anatomic abnormalities related to autophagy deficiency.

Loss of autophagy also affects the clearance of dying cells, which has been proposed to underlie the pathogenesis of SLE (Moulton et al., 2017). Activation of autophagy is crucial for the survival and differentiation of monocytes into macrophages, and inhibition of apoptosis by autophagy is necessary for this differentiation (Zhang et al., 2012). I did not look at myeloid cell populations in *Atg7*$^{fl/fl}$ mice in this chapter. However, In LPS induced endotoxemia, I observed increased pro-inflammatory cytokines in *Atg7*$^{fl/fl}$ LysMcre mice but no difference in the number of monocytes or macrophages (Chapter 3; Fig. 3.19). This would suggest that loss of Atg7 and/or autophagy in myeloid cells does not have a significant effect on myeloid cell populations *in vivo*. Previous studies have suggested that autophagy is required for the differentiation of human monocytes into macrophages *in vitro* (Zhang et al., 2012, Jacquel et al., 2012, Bhattacharya et al., 2021). Whether this suggests differences in the role of autophagy in monocyte differentiation *in vivo* versus *in vitro*, or in mouse versus human is not clear. However, studies from our lab have previously suggested that *Atg7*$^{fl/fl}$ bone marrow cells can

differentiate into macrophages comparable to WT controls (Juliana Vago and James Harris, unpublished observation).

Thus, autophagy may have critical roles in SLE by affecting monocytes and/or macrophages (Zhang et al., 2012, Li et al., 2014). Indeed, autophagy in macrophages may be perturbed in the context of autoimmunity. Genes that encode Beclin1, Atg5, and Atg12 were substantially up-regulated in macrophages of ALD-DNA induced lupus mice and patients with SLE (Li et al., 2014, Zhang et al., 2012). While levels of mRNA do not always equate to levels (or function) of their corresponding proteins, these studies may suggest that autophagy is up-regulated in SLE. If so, whether this is a protective or pathological response is not clear. However, my own data suggest that loss of an autophagy gene, *Atg7*, in myeloid cells, may have specific protective effects, at least in terms of autoantibody production and release of IL-6 and IL-10. Together, this may suggest a pathological role for autophagy in certain aspects of SLE disease progression. However, whether autophagy also has some protective effects is not fully clear, especially as I was ultimately unable to detect any significant differences in the overall disease progression. Moreover, the effects of autophagy may be very different in different cell types. It is also worth noting that both genetic ablation of autophagy and hyperactive autophagy are extreme situations that likely have both beneficial and harmful effects. In the course of SLE disease, the role of autophagy is probably much more nuanced and context dependent.

In *Lyn* deficient mice, several potential mechanisms are involved in the initiation of the inflammation/autoimmunity. Deletion of *Lyn* has effects on autoantibody production and B cells development and differentiation. (Ma et al., 2019a, Lee et al., 2016b). Deletion of *Lyn* causes myeloid expansion and the development of autoimmune characteristics similar to, but not as severe as, the disease in total $lyn^{-/-}$ mice, suggesting an important role for these cells in pathogenesis (Scapini et al., 2010). Here, I have found that loss of *Atg7* in myeloid cells

significantly reduced the production of specific autoantibodies in *Lyn* deficient mice aged 101-250 days, namely anti-dsDNA, anti-histone, and anti-SM. These autoantibodies are commonly found in patients with SLE (Cozzani et al., 2014). Interestingly, in another study, adoptive transfer of *Becn1*$^{-/-}$ macrophages has been shown to significantly reduce the level of anti-dsDNA and renal immune complex deposition, decrease proteinuria level and alleviate glomerulonephritis in ALD-DNA induced murine lupus (Li et al., 2014). Thus, my data are consistent with and further expand this finding.

In this study, I found that in mice aged (101-250 days), serum levels of BAFF, IL-1α, IL-17A, IL-18, IL-6, and IL-10 were significantly raised in *Lyn-/-* mice. Among these, BAFF, IL-1α, and IL-23 all trended non-significantly numerically reduced in double knockout mice (101-250 days) mice than in *Lyn-/-* mice, while IL-6 and IL-10 were significantly reduced. In accordance with this, we recently reported that *Lyn-/-* mice express a higher level of multiple cytokines, and *GILZ* deficiency in young mice exacerbates the expression of IL-18, IL-12, and IL-17A (Nataraja et al., 2021). It has also been demonstrated that the *IL-23A* mRNA levels were significantly increased in SLE patients compared to the healthy controls (Du et al., 2014b, Miteva et al., 2020).

Interestingly, Li and colleagues (2014) suggested that the protective effect of transferring *Becn1*$^{-/-}$ macrophages on anti-dsDNA autoantibody production might be attributed to a reduction in IL-6 and TNF-α production (Li et al., 2014). In this study, I found that loss of Atg7 in myeloid cells resulted in decreased IL-6 production in mice aged 101-250 days. IL-6 is increased in the serum of patients with SLE, and it has a significant impact on both B cells and T-cells (Kishimoto, 2006). IL-6 can promote the differentiation of B cells into plasma cells, and IL-10 acts as an effective B cell stimulator that stimulates the activation, proliferation, and differentiation of B cells (Suematsu et al., 1989, Iyer and Cheng, 2012). IL-6 regulates the accumulation of myeloid cells in inflamed organs, and the removal of over-activated myeloid

cells can improve the outcome in different autoimmune diseases (Kishimoto, 2006). It has been found that therapies targeting IL-6 are highly effective in treating other autoimmune disorders, including rheumatoid arthritis. (Nishimoto et al., 2003, Nishimoto et al., 2004, Woo et al., 2005). Thus, the results from my study further suggest a role for IL-6 in lupus pathology and confirm a role for autophagy in regulating IL-6. These data do not tell us which cells are secreting the IL-6, whether it directly affects the myeloid cells, or whether IL-6 release from other cells is affected. This is, however, further investigated and demonstrated in Chapter 3.

I also found that serum levels of IL-10 were significantly lower in double knockout mice compared to the *Atg7*$^{+/+}$ *Lyn*$^{-/-}$ group. This represents, to my knowledge, a previously unrecognised role for autophagy in cytokine regulation. The role of IL-10 in SLE is potentially complex. High levels of serum IL-10 are observed in SLE patients (Park et al., 1998), and it has been shown to stimulate B cell proliferation and Ig class switching, leading to increased antibody production (Rousset et al., 1992). Conversely, IL-10 has immunosuppressive effects in *Lyn*$^{-/-}$ mice. IL-10 is essential for inhibiting IFN-γ-and IL-17-producing T cells that promote inflammation and autoimmune diseases (Damsker et al., 2010). IL-10 deficiency results in a drastic increase in the release of pro-inflammatory mediators, such as IL-6 and IL-1, which can contribute to reduced control of Foxp3 expression in CD4+ Tregs in *Lyn-/-* mice (Scapini et al., 2011). This latter phenomenon may result in the loss of Treg activity, and one essential immunomodulatory function of IL-10 probably is to maintain the capacity of the expanded CD4+ Tregs to inhibit the development of disease in *lyn*$^{-/-}$ mice (Wan and Flavell, 2007, Tsantikos et al., 2009). IL-10 also specifically restricts the spread of hyperactivated myeloid cells that develop high levels of pro-inflammatory cytokines, which facilitate pathogenic cross-talk between myeloid cells and T cells (Scapini et al., 2010). This could suggest that the observed lower level of IL-10 in this mouse model might have had an autocrine effect on myeloid cells, allowing them to become more hyperactive and release more pro-inflammatory

cytokines. As higher levels of pro-inflammatory cytokines were not seen in this model (and the level of IL-6 was in fact reduced) it would appear that this is not the case here. However, future studies might look at myeloid cell populations within sites of inflammation/damage, such as the kidneys, where localised effects may be more pronounced than those seen systemically. For example, lower IL-10 at sites of inflammation may promote increased pro-inflammatory and chemotactic responses, leading to increased immune cell infiltration and greater tissue damage. In this study, however, no difference was observed in kidney disease, again suggesting that the lower IL-10 levels do not observably drive increased myeloid cell activity.

Here I found that loss of myeloid cell autophagy downregulates both IL-6 and IL-10 release. This may account for the effects on autoantibody production. Anti-mouse IL-6 receptor (IL-6R) antibody, MR16-1, has been shown to suppress the production of all IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) of anti-DNA antibody production and ameliorated the development of autoimmune disease in NZB/W F1 mice (Mihara et al., 1998). It has also been demonstrated that abnormally increased IL-10 levels appear to contribute to spontaneous B-cell hyperactivity; as a consequence, it can directly result in autoantibody production by plasma cells (Peng et al., 2013). While both cytokines potentially increase B cell differentiation/activity, the only difference observed in splenic B cell populations between *Atg7*$^{+/+}$ *Lyn*$^{-/-}$ and *Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$ mice were in plasma cell numbers in young mice; an age at which differences in these cytokines were not seen. This would suggest that effects on B cell numbers are not key to the effects seen here.

Thus, how myeloid cell autophagy influences autoantibody production in this model is not yet clear, although I may suggest possible mechanisms. Firstly, changes in levels of IL-6 and/or IL-10 may directly influence autoantibody production by plasma cells, affecting cell activity rather than differentiation/proliferation. Secondly, effects on efferocytosis may have a role to play. A previous study has demonstrated that ablation of autophagy in myeloid cells can induce

spontaneous autoimmunity, including autoantibody production, due at least in part to defects in efferocytosis by myeloid cells (Clarke et al., 2015). Efferocytosis is important for the clearance of dead cells, which could otherwise accumulate, rupture and lead to exposure to multiple autoantigens. However, while this might explain how autophagy-deficient animals might develop spontaneous autoimmunity, it does not explain why the loss of autophagy is apparently protective against the development of autoantibodies seen in our experiments, as we would expect decreased efferocytosis to result in increased autoimmunity. It is possible that in the *Lyn*$^{-/-}$ animals, cell death already supersedes efferocytosis capacity. This may instead point to a third possibility: effects of myeloid cell autophagy on autoantigen processing and/or presentation.

Antigen presentation is crucial to the initiation of an immune response that ensures adequate pathogen clearance from the infected host (Patterson and Mintern, 2012). Major histocompatibility complex (MHC)-restricted antigen presentation is vital for the specificity of immunity and demonstrates an essential interaction between innate and adaptive immune pathways (Merkley et al., 2018). Studies have shown that pharmacological and genetic inhibition of autophagy decreased efficient MHCII presentation of endogenous (Paludan et al., 2005) and MHC I cross-presentation of exogenous antigen (Li et al., 2008). MHC class II molecules also play a role in signal transduction, which contributes to B cell activation (Clement et al., 1986) and several findings indicate that monoclonal antibodies (MAb) specific to MHC class II molecules inhibit antigen-induced B-cell activation (Forsgren et al., 1984, O et al., 2014). The data here showed that there is a clear effect of deficient myeloid cell autophagy/loss of *Atg7* on autoantibody production. Thus, this could be due to a decrease in MHC class II restricted presentation of autoantigens and/or class II dependent B cell activation, both of which could lead to increased autoantibody production. However, further studies are

needed to understand the involvement of autophagy pathways in myeloid cell antigen presentation and autoantibody production in this model.

In addition, we hypothesized that autophagy deficiency in *Lyn* deficient lupus mice could impact downstream autoimmune pathologies, such as splenomegaly and nephritis. Splenomegaly is a characteristic feature of *Lyn* deficient mice (Jones et al., 2016, Pore et al., 2018, Nataraja et al., 2021), which have been infrequently observed in SLE patients (Zhang et al., 2019), develops as a manifestation of active SLE (Colmegna et al., 2005). Sustained autoantigen and immune complex deposition in the spleen can increase the progression of SLE (Zhang et al., 2019). It has been demonstrated that splenomegaly is caused by splenic vessel inflammation, lymphoid hyperplasia (Dixon et al., 1978), extramedullary haematopoiesis due to increased production of myeloid growth factors (Gemery et al., 2016, Gottschalk et al., 2015) and in *Lyn* deficient mice, caused by an accumulation of plasma cells, and Mac-1+ lymphoblasts (Nishizumi et al., 1995). I found that *Lyn* deficient lupus mice spontaneously developed splenomegaly, and the architectural damage of the spleen in Lyn deficient mice may be attributed to the production and deposition of pathogenic IgG, a major pathological component for spleen inflammation and immunological abnormalities. It has also been reported that Lyn- deficient mice produce IL-6 that trigger hyperactivation of B and T cells and splenomegaly (Tsantikos et al., 2010). In this study, I found that Lyn deficient mouse produce high level of IL-6 and severe splenomegaly, while increases in IL-6 in Lyn-deficient mice were decreased in autophagy deficient double knockout mice, however loss of myeloid cell autophagy does not significantly alleviate splenomegaly.

Lupus nephritis is one of the most severe organ manifestations of human SLE. The kidneys of Lyn-deficient mice contain infiltrating inflammatory leukocytes resulting in glomerulonephritis as well as deposition of IgG autoantibodies and fixation of complement component C3 (Yu et al., 2001). We have previously reported that *Lyn* deficient mice

developed splenomegaly severe glomerulonephritis, while Glucocorticoid-induced leucine zipper (GILZ) deficiency in *Lyn* deficient mice increased early damage to the spleen and exacerbated Glomerulonephritis (Nataraja et al., 2021). In the current study, I also observed that the kidneys of Lyn-deficient lupus mice showed enlarged glomeruli filled with leukocytes. Autophagy deficient double knockout mice ameliorate autoantibody production but do not significantly reduce associated severe kidney pathology. Overall, this study suggests myeloid-specific *Atg7* deficiency did not significantly alleviate splenomegaly and remit the glomerulonephritis. It is possible that the severe lesion on the kidney caused by *Lyn* deficiency was so intense that autophagy deficiency did not significantly impact. However, In the current study, I only assessed the effect of myeloid-specific autophagy on effect in 2 age groups of mice, such as young mice (< 100 days old) and old mice (> 250 days old). The effect of autophagy deficiency on kidney damage might be associated with age in this context. Lyn-deficient mice have been reported to develop glomerular immune complex deposition at the age of 6–8 weeks; subsequently, progressive kidney damage deteriorates with ageing (Hibbs et al., 1995, Nishizumi et al., 1995). Our lab has previously demonstrated that GILZ- deficient older mice also acquire moderate immune complex-mediated glomerulonephritis (Jones et al., 2016). This indicates that the age of experimental mice is an important factor regarding SLE disease progression. Also, in this study, I only evaluated anti-nuclear antibody (ANA) production, cytokine release, splenomegaly, and kidney pathology. It is possible that other disease outcomes, such as proteinuria levels and immune complex deposition, may be affected. A previous study reported that adoptive transfer of Beclin1 knockdown macrophages in lupus mice could significantly reduce anti-dsDNA antibody levels, inhibit the immune complex deposition and reduce proteinuria (Li et al., 2017). In addition to this, autophagy has both positive and negative effects on the pathogenesis of SLE; thus, the net effect was therefore not significant. The results further suggest that as the mice age, the *Lyn* deficiency may override

any protective effects of autophagy. Analysis of younger mice (less than 100 days) was also performed to assess effects during the establishment of SLE. However, these mice did not display significant differences in autoantibodies or serum cytokines between *Lyn* deficient and double deficient groups. This would suggest that the effects of autophagy deficiency in myeloid cells are not due to differences in the establishment of disease in this model.

In the non-lupus mice, it is notable that only IL-18 was expressed at different (higher) levels in the *Atg7*$^{fl/fl}$ mice. Previous studies have shown that loss of autophagy can increase the release of IL-1 family cytokines, including IL-1α, IL-1β, and IL-18, in response to specific stimuli (Harris, 2013, Xu et al., 2019a). We did not see a similar increase in systemic IL-1α here, and IL-1β was not detected. Thus, whether the mechanism here is similar to that previously shown is unclear. It would be interesting to look at the release of IL-1α, IL-1β, and IL-18, particularly in the kidneys, where the disease is more localised. Nonetheless, IL-18 is of interest in the study of lupus disease progression, as it has been implicated in pathogenesis both in mouse models and human disease (Hoshino et al., 2001, Mende et al., 2018). Furthermore, a positive correlation between plasma cell proliferation and IL-18 was observed in a previous report (Tsirakis et al., 2013), which may help to understand why the loss of myeloid cell autophagy was associated with changes in B cell and plasma cell populations. However, cell numbers were not different between the *Lyn*$^{-/-}$ and double knockout animals. This would suggest that any effect of IL-18 on B cell/plasma cells is independent of disease and that two separate mechanisms may be at play to drive IL-18 in diseased and non-diseased animals. Importantly, this observation may at least further explain why autophagy-deficient mice have been shown to develop lupus-like autoimmunity spontaneously (Xu et al., 2019a). Given the potential importance of IL-18 in lupus, further study of this phenomenon is warranted.

It has been revealed in several studies that Lyn itself may play a critical role in the autophagy process in the context of disease and/or infection (Lupo et al., 2016, Li et al., 2016, Li et al.,

2020b). Inhibition of Lyn inhibits autophagy in *Pseudomonas aeruginosa* (Pa) infected macrophages and delays bacterial clearance (Li et al., 2016). Whereas, in Chorea-acanthocytosis, a neurodegenerative disease, reduced clearance of lysosomes and mitochondria in erythroid cells was associated with the accumulation of active Lyn and the autophagy proteins Ulk1 and Atg7 (Lupo et al., 2016). Moreover, Lyn plays a key role in the secretion of viral progenies via autophagosome-derived organelles and a significant reduction in the abundance of extracellular virus particles was observed in Lyn-deficient Dengue- and Zika-infected HeLa, Vero, HepG2 and Huh7 cells (Li et al., 2020b). Importantly, however, none of these studies revealed – or suggested - a role for Lyn in basal autophagy, but rather suggested it directs autophagy in very specific circumstances. Whether or not such a connection exists in the context of autoimmunity in $Lyn^{-/-}$ mice is not clear but warrants further study. Any such role for Lyn in the autophagic process would potentially impact - or mask - the effects of *Atg7* deficiency in myeloid cells observed here. Further studies using other models of lupus-like autoimmunity would help to clarify this potential issue.

Taken together, my findings suggest that myeloid cell-specific autophagy plays complex and apparently specific roles in the progression of SLE in *Lyn* deficient mice. The clinical manifestations of SLE in patients are complex and can affect haematological, dermatological, renal, cardiac, vascular, pulmonary, ocular, gastrointestinal, musculoskeletal, and neuropsychiatric systems (Cojocaru et al., 2012). The *Lyn* deficient mice recapitulate only some of these symptoms and so, like most other animal models, are limited. Thus, future research should consider the potential effects of myeloid-specific autophagy in human SLE patients. In addition, future experiments examining the role of other autophagy genes in SLE models could help understand the pathways they modulate and the devastating impact that can result when they are interrupted and identify new therapeutic targets. In conclusion, our results indicate a novel role of myeloid-specific autophagy in modulating the development of SLE-

like disease in Lyn-deficient mice and suggest that myeloid-specific autophagy may be a new target for the development of therapeutic strategies for SLE.

Chapter 3-Investigating the role of autophagy in the regulation of cytokines relevant to SLE

Abstract

Autophagy is a degradative pathway that is critical for maintaining cellular homeostasis, and it has been reported as an intrinsic cellular defence mechanism in both innate and adaptive immune responses. The aim of this study was to understand the role of autophagy in regulating cytokines, particularly those involved in SLE pathogenesis. Here, I investigated the effects of autophagy induction and inhibition on the release of IL-6 and IL-10 from murine macrophages *in vitro*. I stimulated cells with the autophagy inducer Torin-1 (a mTOR inhibitor) in the presence of the TLR ligands LPS (TLR4), PAM3CSK4 (TLR2/TLR1), and resiquimod (R848) (TLR7/8) and cytokine release were measured by ELISA. Likewise, I used the autophagy inhibitor 3-methyladenine (3-MA) or small interfering RNA (siRNA) against *Becn1* (*Atg6*) and *Atg7* to examine the effects of autophagy impairment on IL-6 and IL-10 release. I found that macrophages readily release IL-10 in response to different TLR ligands. This was significantly inhibited when autophagy was inhibited and increased when autophagy was induced, indicating autophagy positively regulates IL-10 expression/release in macrophages. This process occurred at the mRNA level. However, autophagy did not directly modulate the production and release of IL-6. Furthermore, using WT and mice lacking *Atg7* in myeloid cells ($Atg7^{fl/fl}$ LysMCre), I determined the effect of myeloid cell autophagy on cytokine production in response to acute inflammation *in vivo*. Mice were intraperitoneally injected with LPS, and the level of multiple cytokines was measured. *Atg7* deficient mice had significantly higher levels of IL-1α, IL-1β, IL-18, IL-23, IL-17A, IL-12p70, and IL-6, suggesting autophagy is a key regulator of cytokine production in acute inflammation. Thus, our study provides important insight into the autophagic regulation of cytokines, which may help in the future development of autophagy-targeting therapies.

3.1 Introduction

Autophagy is now recognised as a key mechanism for the regulation of many important innate immune cell responses, including the clearance of intracellular bacteria, efferocytosis, and antigen presentation (Deretic, 2010, Münz, 2010, Harris, 2011). Several studies have also shown that autophagy regulates the production and/or release of multiple cytokines from myeloid cells (Harris, 2011, Harris et al., 2018, Ge et al., 2018). Indeed, the interaction between autophagy and cytokines may represent a key mechanism for regulating the function of the innate and adaptive immune systems (Klionsky, 2007). Cytokines have critical roles in modulating both the innate and adaptive immune responses, and a fine balance of cytokines is needed retaining immune homeostasis. TLRs (Toll-like Receptors) are pattern recognition receptors that serve as a connection between innate and adaptive immunity (Li and Wu, 2021). They can detect both external pathogen-associated molecular patterns (PAMPs) and internal damage-associated molecular patterns (DAMPs) (Zhang and Liang, 2016, Yu and Feng, 2018). To date, 10 subtypes of TLR (TLR1–TLR10) in human and 12 subtypes (TLR1–TLR9, TLR11–TLR13) in mouse have been identified (Wang et al., 2016). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are cell-surface TLRs, whereas TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 are intracellular TLRs that are found in the endosome (**Fig. 3.1)** (Kawai and Akira, 2010, Celhar et al., 2012). TLRs trigger the activation of the transcription factors NF-kB and IRFs (Interferon Regulatory Factors), which control the outcome of innate immune responses by attracting particular adaptor molecules (Kawasaki and Kawai, 2014).

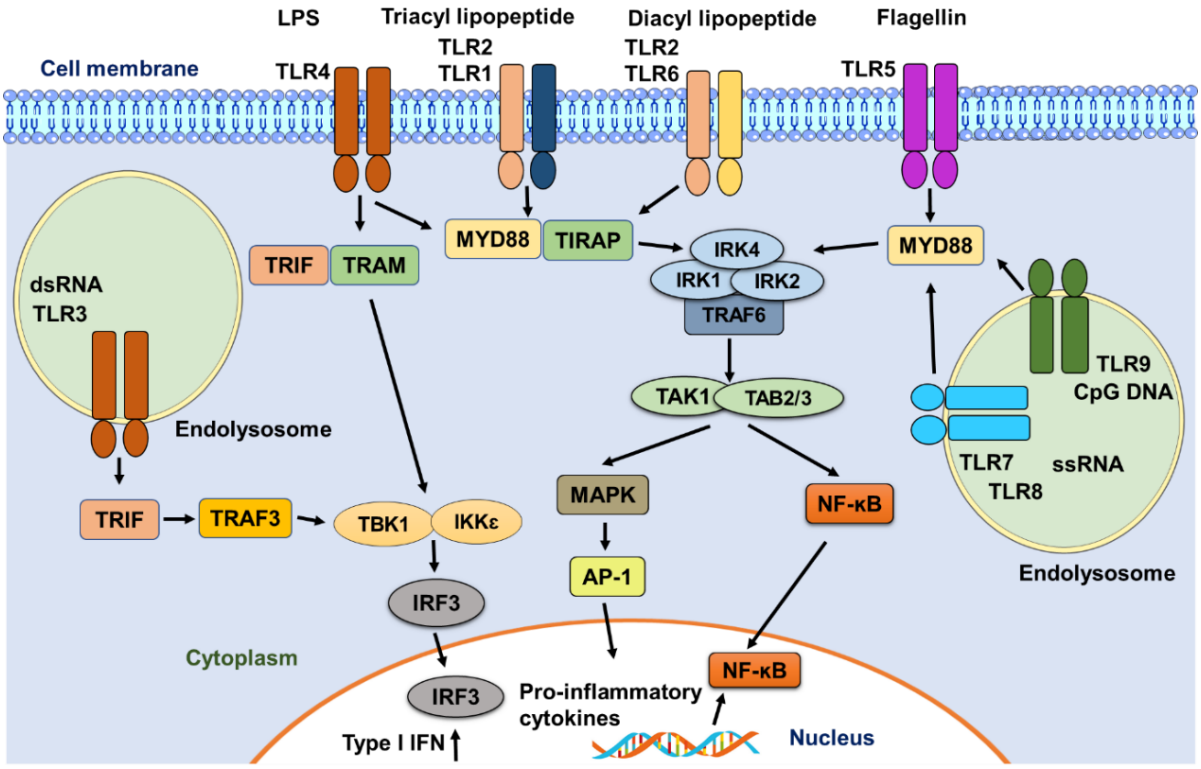


Figure 3.1: Mechanisms of TLR-mediated immune responses in cell
TLRs 1, 2, 4, 5, and 6 are expressed on the surface of the cell and recognise molecular components located on the pathogen's surfaces. TLRs 3, 7, 8, and 9, in contrast, are expressed intracellularly on the membranes of endosomal compartments, where they recognise nucleic acids. TLR1–TLR2 detects triacyl lipopeptides, while TLR2–TLR6 recognise diacyl lipopeptides. TLR4 recognises and responds to bacterial LPS, whereas TLR5 recognises bacterial flagellin. TLR3 mediates recognition of dsRNA. TLR7/8 primarily recognise ssRNA. TLR9 directs responses to bacterial and viral CpG DNA. All TLRs signal through myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathways. MyD88 is required for TLR 1, 2, 5, 7, and 9 mediated responses, whereas TLR3 signals via the TRIF-dependent pathway. TLR4 can signal through both MyD88 and TRIF dependent pathways. TNF receptor-associated factor-6 (TRAF6), and members of the interleukin-1 receptor-associated kinase (IRAK) family are recruited by the adapter MyD88, which leads to IκB phosphorylation. After the proteasomal degradation of IκBs, NF-κB translocates into the nucleus. TAK1 can also stimulate the MAP kinase pathway, which activates AP-1 phosphorylation. NF-κB and AP-1 regulate inflammatory responses by producing inflammatory cytokines. In addition, TLR4 also recruits TRAM and TRIF, which then interacts with serine/threonine-protein kinase 1 (TBK1). IKK-ε mediates the phosphorylation and nuclear translocation of interferon regulatory factor 3 (IRF3), resulting in the induction of type-1 interferon (IFN). TLRs protect against a wide range of infections, but their improper or uncontrolled activation can result in chronic inflammatory and autoimmune diseases. Modified from (van Duin et al., 2006).

Autophagy inhibition, either pharmacologically or genetically, has been found to enhance the release of the IL-1 family cytokines IL-1α, IL-1β, and IL-18 in response to TLR3 and TLR4

ligands (Harris et al., 2011, Iula et al., 2018, Saitoh et al., 2008). Moreover, IL-1β, released by autophagy-deficient cells in response to TLR agonists, promotes the autocrine release of IL-23 by macrophages and dendritic cells (Peral de Castro et al., 2012). Conversely, induction of autophagy with rapamycin, both *in vivo* and *in vitro*, has the opposite effect; inhibiting the release of both IL-1β and IL-23 (Peral de Castro et al., 2012, Harris et al., 2011). As IL-23, in combination with IL-1α or IL-1β, has been shown to polarise $CD4^+$ T cells to a Th17 phenotype and induce the release of IL-17 from both Th17 cells and innate γδ T cells (Cho et al., 2006, Sutton et al., 2006, Sutton et al., 2009), it might be expected that an increase in the release of IL-1 and IL-23 by autophagy impaired cells should promote IL-17 release by T cells. This has been shown to be the case, both *in vitro* and *in vivo*. Supernatants from mouse dendritic cells treated with 3-MA, rich in IL-1α, IL-1β, and IL-23, have been shown to strongly induce the release of IL-17A, as well IFNγ and IL-22, by T cells, particularly γδ T cells (Peral de Castro et al., 2012). Moreover, splenocytes from mice deficient in *Atg5* in myeloid cells ($Atg5^{fl/fl}$ LysMcre) and infected with *Mycobacterium tuberculosis* produce more IL-17A than WT controls following re-stimulation with a synthetic mycobacterial antigen *ex vivo* (Castillo et al., 2012). Similarly, following infection with a respiratory syncytial virus (RSV), mice lacking the autophagy protein LC3B developed IL-17A-induced lung damage, and RSV infected $Maplc3b^{-/-}$ $CD11b^+$ DCs trigger the release of IL-17A from $CD4^+$ T cells in an IL-1-dependent mechanism (Reed et al., 2015). It has also been found that Mice with Atg5 deficiency in CD11c+ cells showed rapid airway hyperreactivity and extensive neutrophilic lung inflammation, with high levels of IL-1, IL-1, IL-23, and IL-17A in the lungs (Suzuki et al., 2016, McGeachy et al., 2009, Chen et al., 2014).

Studies have implicated multiple cytokines in the pathology of SLE, including IFN-γ, IL-1α, IL-1β, IL-10, IL-17, IL-18, IL-23, and MIF, all of which have been shown to be regulated by autophagy (Merkley et al., 2018, Rönnblom and Elkon, 2010, Harris et al., 2017). The findings

from my project, presented in Chapter 2, also suggest a role for myeloid cell autophagy in the regulation of IL-6 and IL-10 release in a mouse model of lupus-like autoimmunity. A number of studies have highlighted pathological roles for IL-6 and IL-10 in SLE (Llorente et al., 1993, Linker-Israeli et al., 1991, Llorente et al., 1994, Sugiyama et al., 1995).

IL-6 was first considered as a B-cell stimulatory factor, but it also regulates numerous cellular and biological functions, such as B-cell differentiation, maturation, and immunoglobulin secretion, synthesis of acute-phase protein, renal mesangial cell proliferation, activation of bone marrow progenitor, and differentiation of cytotoxic T-cells (Tanaka et al., 2014, Okada et al., 1988). In human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 stimulated peripheral blood monocytes, IL-6 was found to regulate the monocytes differentiation from DCs to macrophages (Chomarat et al., 2000, Mitani et al., 2000). IL-6 can function on its own or with other cytokines, such as by regulating the expression of IL-21, to enable the process of differentiation of B cells into Ig-producing cells, as well as the proliferation and differentiation of T cells (Tsokos et al., 1986, Kishimoto and Hirano, 1988). Studies also demonstrate IL-6 as a critical factor in the generation of Th17 cells (Ivanov et al., 2006, Yang et al., 2008). In the presence of TGF-β, IL-6 enhances Th17 differentiation by suppressing activation of STAT1 and STAT5, while IL-6 also inhibits the transcription factor forkhead box P3 (FOXP3), resulting in a reduction in regulatory T cells in human PBMC (Mangan et al., 2006, Gao et al., 2012).

Within the immune system, IL-6 is primarily produced by monocytes and macrophages, but T and B cells can also produce this cytokine (Baumann et al., 1984, Freeman et al., 1989). IL-6 plays a crucial role in the host immune response to pathogens, and a role for IL-6 in viral infections has been reported in experimental models of influenza and pneumonia, using IL-6-deficient mice (Lauder et al., 2013, Yang et al., 2017). It has been demonstrated that IL-6 plays a critical role in the survival of these pathogen-infected mice by enhancing optimal control of

the T-cell response, inflammatory response, tissue remodelling that promotes lung recovery, macrophage migration, and phagocytic behavior, and prevention of viral-induced apoptosis in lung epithelial cells (Yang et al., 2017, van der Poll et al., 1997, Lauder et al., 2013). Indeed, mice lacking IL-6 are more vulnerable to infection with a variety of pathogens, such as *Mycobacterium tuberculosis*, *Listeria monocytogenes*, vaccinia virus, and vesicular stomatitis virus, where compromised pathogen clearance was related to the production of suboptimal T-cell responses in the knockout animals in each case (Kopf et al., 1994, Ladel et al., 1997, van der Poll et al., 1997). Overall, these data imply that the role of IL-6 in response to infection with many pathogens is multifactorial and protective.

Increased IL-6 levels have also been found in numerous autoimmune diseases, such as SLE, RA, systemic-onset juvenile idiopathic arthritis (JIA), osteoporosis, and psoriasis (Metsärinne et al., 1992, Linker-Israeli et al., 1991). Elevated levels of IL-6 in serum, urine, and renal glomeruli in active SLE patients and in murine SLE models indicate that IL-6 plays a role in disease pathogenesis (Spronk et al., 1992, Kitani et al., 1992). Mrl/lpr mice, an animal model used to study SLE, have elevated age-related IL-6 and soluble IL-6R serum levels (Suzuki et al., 1993, Tang et al., 1991). IL-6-deficient Mrl/lpr mice show delayed initiation of nephritis and greater long-term survival, again suggesting a pathogenic role for IL-6 in this model (Cash et al., 2010). Similarly, IL-6 receptor blockade suppressed the generation of IgG antibodies in lupus-prone NZB/W F1 mice and the subsequent development of autoimmune disease, while exogenous IL-6 administration accelerated glomerulonephritis in these animals (Mihara et al., 1998, Finck et al., 1994). A study has also shown that blocking IL-6 targets not only autoreactive B cells in NZB/W F1 mice but also inhibits autoreactive T cells (Liang et al., 2006). Individuals with active lupus nephritis show a high level of IL-6 in the urine, and IL-6 expression is increased in the kidneys of lupus nephritis patients.

IL-10 is a multifunctional cytokine that inhibits/regulates monocytes, macrophages, T cells, DCs, and NK cells (Lourenço and La Cava, 2009). It inhibits class II MHC and the co-stimulatory molecule B7-1/B7-2 in monocytes, macrophages, and dendritic cells, with corresponding inhibition of pro-inflammatory cytokine and chemokine secretion (Moore et al., 2001, de Waal Malefyt et al., 1991, Caux et al., 1994, Péguet-Navarro et al., 1994). A previous report showed that IL-10 suppresses IFNγ-dependent MHC-II transcription, compounding the direct inhibitory effect of IL-10 on MHC-II expression (Chan et al., 2010). Although IL-10 is categorized as a Th2 cytokine, it has been shown to inhibit a variety of inflammatory responses and is a key player in regulating immune homeostasis (O'Garra and Murphy, 2009). Moreover, IL-10 is also an important cytokine that promotes B-cell survival and autoantibody production (Burt et al., 1998, Rönnblom and Elkon, 2010).

The role of IL-10 in SLE is complex and diverse. IL-10 is involved in the activation of B-cell but can also significantly suppress the antigen-presenting cells and $CD4^+$ T cells (Mosser and Zhang, 2008). IL-10 stimulates proliferation of B cell and Ig class switching in SLE, resulting in increased autoantibody development (Rousset et al., 1992). Plasma levels of IL-10 are increased in SLE patients with active disease and correlate with measures of disease activity (Park et al., 1998, Godsell et al., 2016). Moreover, Ishida and co-workers found that Anti-IL-10 mAb treatment in NZB/W F1 mice prevented the development of autoimmune symptoms and improved survival (Ishida et al., 1994). This increased survival was accompanied by a decrease in anti-dsDNA autoantibodies, decreased proteinuria, and decreased incidence and severity of glomerular lesions (Ishida, 1994). Furthermore, it has been reported that continuous administration of IL-10 to NZB/W F1 mice from 4 weeks of age accelerates the development of autoimmunity (Ishida, 1994).

As a crucial regulator of the immune system, autophagy might be expected to impact the release of IL-6 and IL-10 by macrophages and DCs (Wu et al., 2016). A previous study has shown that

pharmacological inhibition of autophagy in human PBMC infected with *Borrelia burgdorferi* (the causative agent of Lyme disease) *ex vivo* increases both IL-1β and IL-6 release (Buffen et al., 2013). However, other studies have suggested that autophagy does not directly regulate IL-6 release by myeloid cells in response to TLR ligands (Harris et al., 2011, Peral de Castro et al., 2012). Moreover, while IL-10 has been shown to have (mostly inhibitory) effects on autophagy (Wu et al., 2016, Park et al., 2011), a role for autophagy in IL-10 regulation has not previously been demonstrated.

In chapter 2, I reported that the level of IL-10 is increased in the serum of *Lyn*$^{-/-}$ mice but significantly downregulated in *Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$ mice. Here, I examined whether this downregulation of pro-inflammatory cytokines is directly mediated by autophagy in myeloid cells. I assessed the effect of autophagy on the production of myeloid-cell-derived IL-6 and IL-10 induced by different TLR ligands using different cell lines. I demonstrated that loss of autophagy inhibits the expression of *Il10* mRNA and release of IL-10 protein, while induction of autophagy had the opposite effect. Inhibition/induction of autophagy had inconsistent effects on IL-6 release, potentially suggesting context-dependent effects of autophagy on IL-6 release. I further evaluated the role of autophagy in an *in vivo* model of intraperitoneal (i.p) challenge induced by LPS in *Atg7*$^{fl/fl}$ LysMCre mice. Analysing the peritoneal lavage fluid from the mice, I reveal that loss of myeloid cell autophagy results in increased expression of IL-1α, IL-1β, IL-18, IL-23, IL-17A, IL-12p70, and IL-6. Together, these findings suggest autophagy plays a key role in the regulation of pro-inflammatory cytokines and immune response.

3.2 Materials and methods

3.2.1 Cells and culture conditions

Mouse RAW 264.7 macrophages, THP-1 cells (a human monocyte-like cell line), immortalised bone marrow-derived macrophages (iBMM), and primary bone marrow-derived macrophages

(BMM) and dendritic cells (BMDC) from wild type and *Atg7*$^{fl/fl}$LysMCre mice were used. Cells were grown and cultured in complete RPMI medium (Gibco, 11875–093) enriched with 10% fetal bovine serum (JRH Biosciences, 12103), penicillin (50 U/ml), streptomycin (50 mg/ml), and L-glutamine(2 nM) (Gibco, 10378–106). For primary cells, bone marrow was extracted from femurs and tibias of mice, and BMM differentiated in complete RPMI supplemented with 15% culture supernatant from MCSF-transduced L929 cells (L-cell conditioned medium) for seven days.

3.2.2 Cell treatments

Cells were plated at 5 x 10^5 cells/well in 200 µL medium and stimulated *in vitro* with the TLR4 ligand Lipopolysaccharide (LPS, 100 ng/mL) (Sigma-Aldrich), the TLR7/8 ligand resiquimod (R848, 1 µg/mL) (Sigma-Aldrich), the TLR3 ligand Poly (I:C) (20 µg/mL) (Sigma-Aldrich), and the TLR 1/2 ligand PAM_3Cys-Ser-$(Lys)_4$ (PAM3CSK4, 200 ng/mL) (Sigma-Aldrich) unless otherwise indicated for 6 or 24 h with or without autophagy inhibitor 3 -methyladenine (3-MA) (2.5 - 10 mM) or the autophagy inducer Torin-1 (Tor 1) (0.25 - 1µM). After stimulation, cell culture supernatants were harvested for cytokine analysis. To assess the role of autophagy on cytokine release in infection and damage conditions, I used different types of surface-expressed (TLR 1/2 and 4) and intracellular (TLR 3 and 7/8) TLR agonists in this work (**Fig 3.1**). TLR1/2, TLR3, and TLR4 agonists were used to recapitulate inflammation induced by bacterial products, while the TLR7/8 agonist R848 was chosen to mimic autoantigen-induced inflammation.

3.2.3 siRNA transfection

RAW264.7 cells were transfected with siRNA using an Amaxa Nucleofector™ Device (Lonza, Victoria, Australia) as previously stated (Harris et al., 2007). In brief, cells were counted and

resuspended in 100 µl electroporation buffer (BTX Harvard Apparatus, 45–0803). Then 400 nM *Becn1*, *Atg7* or non-targeting (scrambled) ON-TARGETplus siRNA (SMARTpool, GE Healthcare Dharmacon) was added into the particular cell suspension. The cell suspension was transferred to a transfection cuvette, placed on the Amaxa Nucleofector™ device, and electroporated (Lonza, Victoria, Australia). Transfected cells were transferred to a cell culture flask at a density of 5 x 10^5 cells/ml. After 24 hours, the cell culture media was changed, and the cells were incubated for another 24 hours before the stimulation. Knockdown of Beclin1 and Atg7 protein was validated by western blot for each experiment.

3.2.4 Western blot analysis

Western Blot analysis were performed according to a protocol described by Lee et al (Lee et al., 2016c). Cells were stimulated, washed twice with phosphate-buffered saline (PBS), and then lysed with either RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM Tris pH 8) or 2 percent Igepal CA-130 (Sigma, I8896), in Tris buffer. Cell lysates were missed with Laemmli buffer (under reducing conditions), heated at 95°C for 5min. Lysates were separated on 4–12% NuPAGE bis-tris gels (Novex, NP0322PK2) and transferred to PVDF membranes. The membranes were then blocked with 5% skimmed milk in PBS with 0.1 % Tween™ 20 (PBS/T) for 1 h and incubated with primary antibodies overnight at 4℃ (diluted 1:1,000 in 2.5% BSA). The next day, the membranes were incubated with fluorescent secondary antibodies (1:10,000 in 2.5% BSA) for 60 min at room temperature. After washing with PBS-T, the blots were soaked with enhanced chemiluminescence (ECL) Western blotting detection solution (GE Healthcare, Buckinghamshire, UK) for 1 min, and images were taken using ImageQuant LAS4000 mini (GE Healthcare).

3.2.5 Quantitative PCR

Following treatments, total RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen, #74134), according to the manufacturer's protocol. The MultiScribe reverse transcriptase (Applied Biosystems) was used to prepare complementary DNA (cDNA) from 0.5 g of total RNA using the M-MLV reverse transcription protocol. Quantitative real-time PCR analyses were conducted on duplicate samples using the SYBR Green I Master kit and a LightCycler® 480 Detection system, according to the manufacturer's protocol (Roche Applied Science). The PCR settings were 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. The 2^ddCT formula was used to calculate the relative mRNA level of a particular gene expression.

3.2.6 *In vivo* intraperitoneal (i.p) LPS challenge

Female C57BL/6 mice (20–30 weeks old) were injected intraperitoneally (i.p) with LPS (50 µg/200µL). LPS was prepared in sterile saline before use. Control mice received saline (200µL). Mice were monitored every hour for signs of severe distress and euthanised by CO_2 asphyxiation 4 h after injection. Blood was collected by cardiac puncture and stored overnight at 4℃, followed by centrifugation at 1500 g for 15 minutes. Sera were collected and stored at -80℃ for further analysis. Peritoneal cavities were lavaged with 3 ml ice-cold sterile PBS, and the lavage fluids were collected and stored at -20℃. Mice were housed in a specific pathogen-free (SPF) conditions at the Monash Medical Centre (MMC) Animal Facility. All studies and procedures were conducted in accordance with protocols approved by the animal care and use committee of Monash University (MMCB).

3.2.7 Cytokine measurements

Secreted cytokines from *in vitro* assays were measured by ELISA of culture supernatants according to the manufacturer's protocols [Biolegend for mouse IL-6 (431302), IL-10 (431411), TNF (430902), and IL-1β (432601)]. Levels of MIF release were also measured by ELISA (R&D systems mouse MIF DuoSet, DY1978) in some experiments, and data from these is shown in Appendix II (Supplementary Figures S1 and S2). Colourimetric readings were taken on a Tecan Infinite M1000 Pro multifunction plate reader. For the measurement of cytokines from *in vivo* experiments, a multiplex assay was used. Concentrations of BAFF, IFNα, IFNγ, IL-10, IL-1α, IL-1β, IL-18, IL-12p70, IL-6, IL-23A, and IL-17A, were measured in serum and lavage as described in the manufacturer's protocol (Mouse 11-plex ProcartaPlex™ Kit, Invitrogen). Serum and lavage samples were used undiluted in a universal Assay Buffer. The samples were mixed with color-coded beads that had been pre-coated with analyte-specific capture antibodies. An antibody-antigen sandwich was created by adding biotinylated detection antibodies specific to the analytes of interest. A hand-held magnetic plate washer was used throughout the assay. Readings were taken using a Bio-plex MAGPIX^{TM} multiplex reader (Bio-Rad).

3.2.8 Analysis of peritoneal myeloid cell subsets by flow cytometry

Single-cell suspensions were prepared from peritoneal lavage and stained with CD11b-APC/Cy7, MHC-II-PB, B220-PE/Cy7, F4/80-FITC, Ly6c-PE, CD11c-PerCP/cy5.5, and CD115-APC. Cells were analysed on a BD fluorescence-activated cell sorting (FACS) Canto II (BD Bioscience). Data were analysed using FlowJo version 10.6.2 cell cycle analysis software (Tree Star, Ashland, OR).

3.2.9 Statistics

Statistical differences were determined using one-way ANOVA followed by Tukey's posthoc test and unpaired t-test. Statistically significant differences between data groups are indicated by an asterisk(s), whereas nonsignificant differences are either unmarked or highlighted by "*ns*". Statistical analyses were performed using GraphPad Prism software (version 8.0.2, GraphPad Software, La Jolla, CA). Error bars represent SEM, which is based on biological replicates. Data were tested for normality by the Shapiro-Wilk test, where appropriate.

3.3 Results

3.3.1 Autophagy does not regulate IL-6 release from murine macrophages

In Chapter 2, I demonstrated that myeloid cell-specific autophagy deficiency has a significant inhibitory effect on levels of IL-6 and IL-10 in serum from *Lyn* deficient mice. However, it is not clear from this result whether loss of autophagy directly attenuates pro-inflammatory cytokine release from myeloid cells. I, therefore, tested the effect of autophagy inhibition and induction on the release of IL-6 and IL-10 by macrophages *in vitro*. In RAW cells, after 6 h, 3-MA had apparently contrasting effects on LPS-induced IL-6 release, with inhibition at 2.5 mM and stimulation at 5 and 10 mM though the effect was very small (**Fig. 3.2A**). Torin 1 clearly inhibits LPS-induced IL-6 release at 6 h (**Fig. 3.2B**), which is similar to previous work from my lab (Lee et al., 2016b). In response to reisquimod, both 3-MA and Torin -1 are clearly inhibitory (**Fig. 3.2C, D**). All of these effects are lost after 24 h (**Fig. 3.2E-H**). At 6 h iBMMs show similar effects in terms of 3-MA along with LPS (**Fig. 3.3A)**, but potentially opposite effects with Torin 1 (**Fig. 3.3B)**. Both 3-MA and Torin-1 significantly increased IL-6 in response to R848 (**Fig. 3.3C, D)**, and it might not be an autophagic effect. TNF is altered in various ways (**Fig. 3.4 and 3.5)**. Whether this is autophagy-specific is not clear. I also tested the effect in primary BMM from WT and *Atg7* KO mice. *Atg7* KO did not decrease IL-6 in

response to 24 h LPS and R848 treatment (**Fig. 3.6**), which confirms again that autophagy does not regulate IL-6 production in macrophage. The LPS result is more in line with previously published work (Peral de Castro et al., 2012, Harris et al., 2011). However, it could be worthwhile to observe the effect after 6 hours of a treatment since the effect after 24 hours is complicated by a number of secondary effects.

To further interrogate these effects, I used a siRNA transfection approach to knockdown the autophagy genes *Atg7* and *Becn1* in RAW264.7 macrophages and stimulated the cells with LPS or R848 for 24 h **(Fig. 3.7)**. Target knockdown was confirmed by western blot analysis (**Fig. 3.7A**). Similar to the observations in *Atg7*$^{fl/fl}$ primary BMM, siRNA knockdown of *Atg7* or *Becn1* had no effect on IL-6 release by RAW264.7 cells in response to LPS **(Fig. 3.7B)**. However, IL-6 release was significantly abrogated in cells transfected with siRNA against *Becn1*, but not *Atg7*, in response to R848 **(Fig. 3.7C)**. It is not clear why the loss of *Atg7* did not likewise inhibit IL-6 release, but this might suggest either a role for autophagy in TLR7/8 responses or an autophagy-independent role for beclin 1. However, future work would look more closely at the timing/dynamics of these effects. Together, these findings indicate that autophagy has a potentially complex and context-dependent role to play in the release of IL-6 from macrophages in response to different inflammatory stimuli, potentially accounting for the effects seen on IL-6 in *Lyn*$^{-/-}$ mice (Chapter 2).

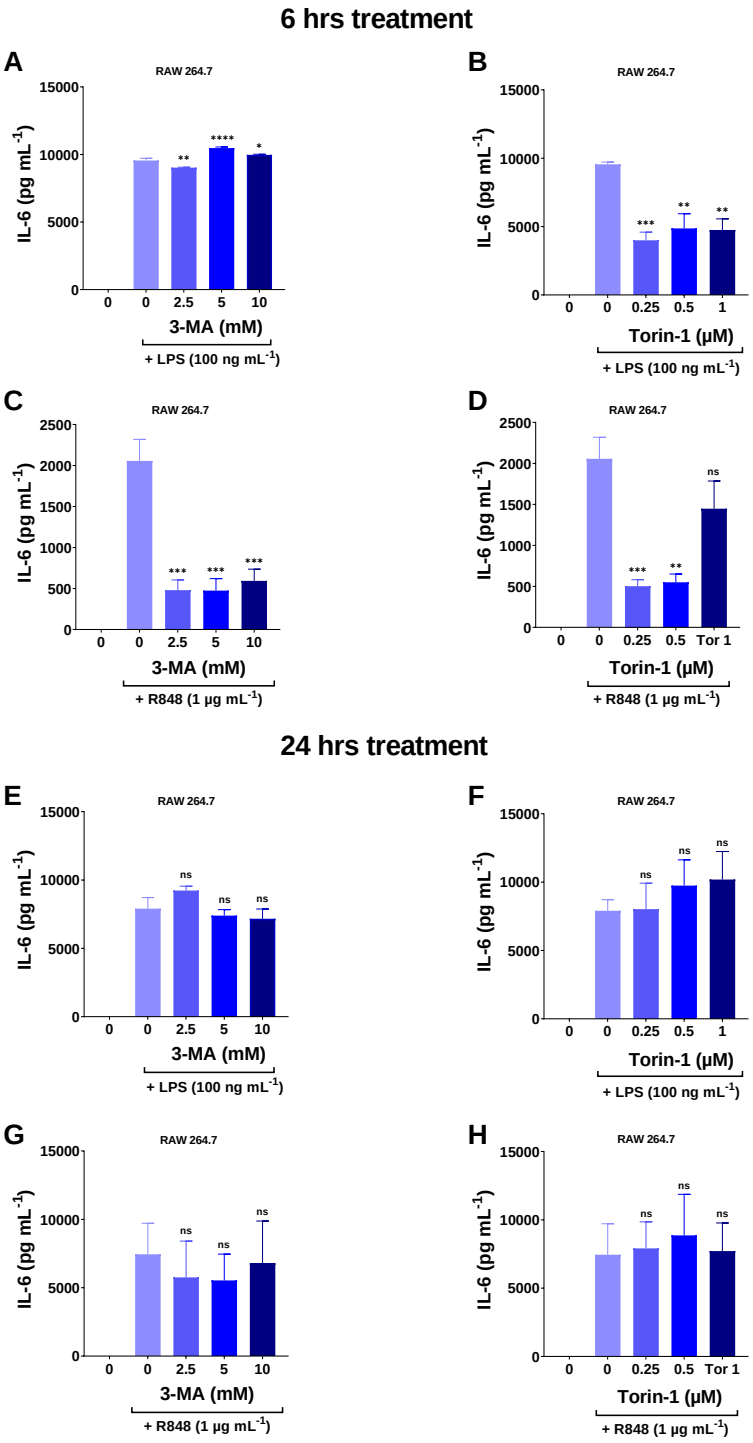


Figure 3.2 Autophagy regulating drugs do not alter IL-6 release in response to TLR ligands in RAW264.7 macrophages

Effect of the autophagy inhibitor 3 -methyladenine (3-MA) (2.5 - 10 mM) and the autophagy inducer Torin-1 (Tor 1) (0.25 - 1μM) in combination with LPS (100 ng/mL) or Resiquimod (R848, 1 μg/mL) for 6 hrs and 24 hrs. IL-6 secretion was measured by ELISA. Bars represent means ± SEM of 3 independent experiments. The significance of differences was determined using one-way ANOVA with Dunnett's multiple comparison test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to cells treated with LPS and R848.

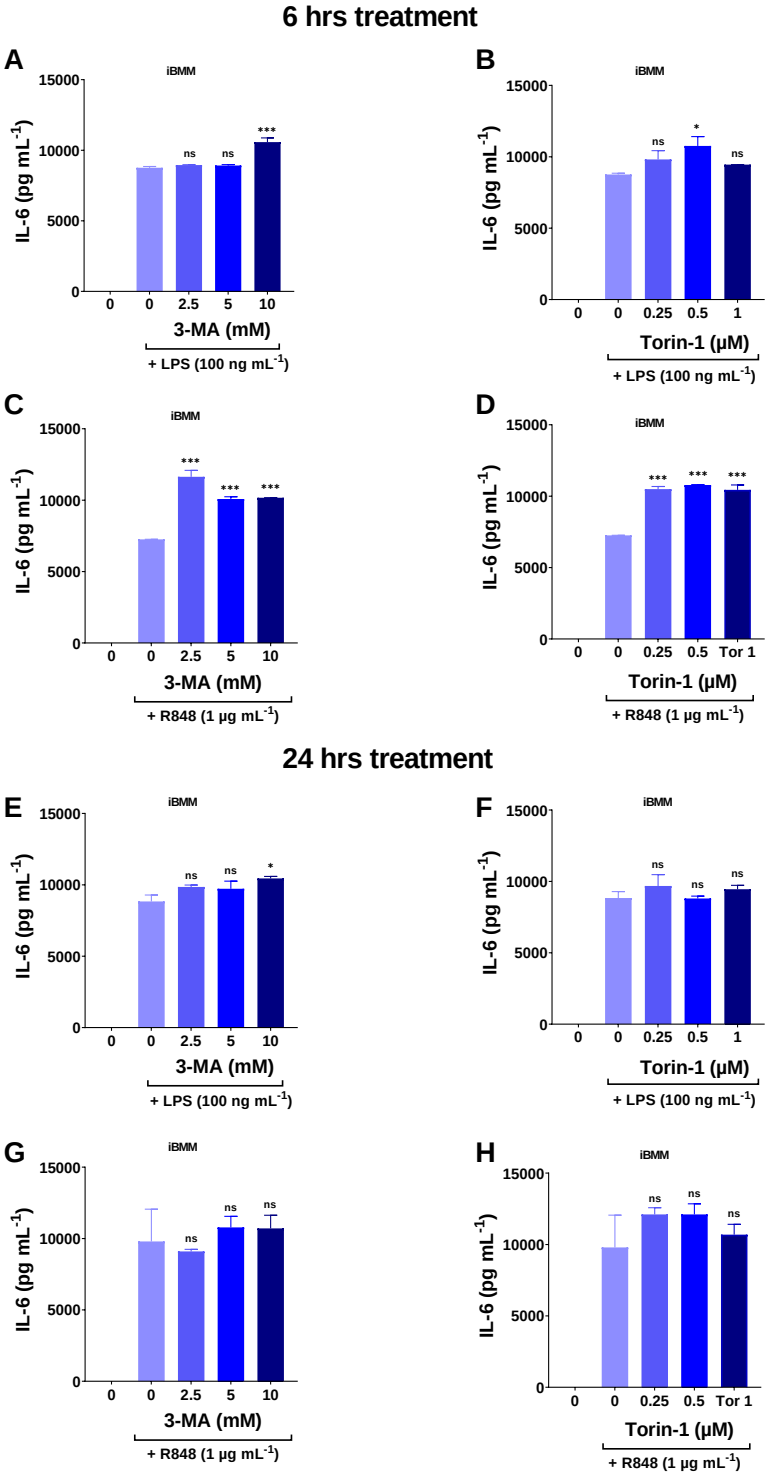


Figure 3.3 Autophagy regulating drugs do not alter IL-6 release in response to TLR ligands in iBMM cells

Effect of the autophagy inhibitor 3 -methyladenine (3-MA) (2.5 - 10 mM) and the autophagy inducer Torin-1 (Tor 1) (0.25 - 1μM) in combination with LPS (100 ng/mL) or Resiquimod (R848, 1 μg/mL) for 6 hrs and 24 hrs. Secretion of IL-6 was measured by ELISA. Bars shows means ± SEM of 3 separate experiments. The significance of differences was determined using one-way ANOVA with Dunnett's multiple comparison test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to cells treated with LPS and R848.

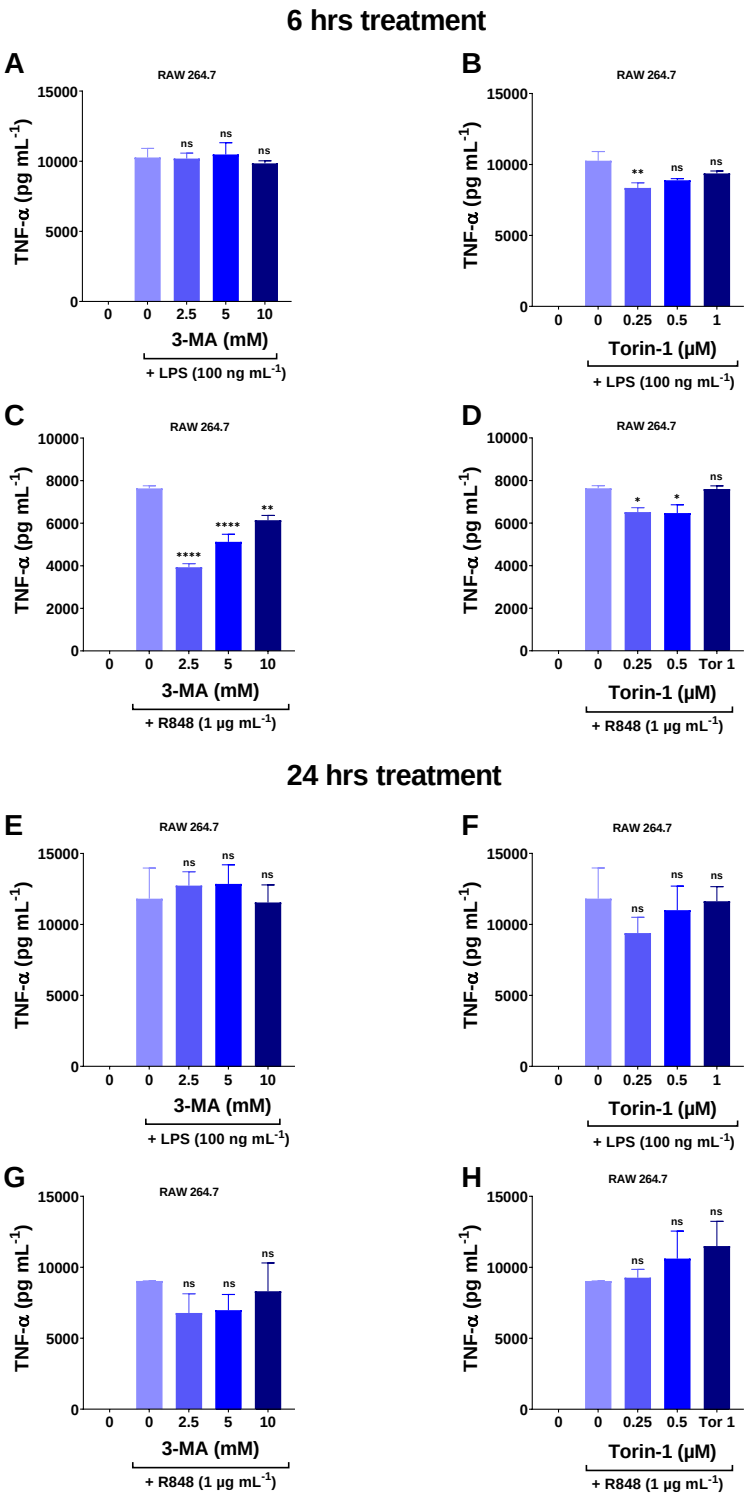


Figure 3.4 Autophagy regulating drugs do not alter TNF-α release in response to TLR ligands in RAW264.7 macrophages

Effect of the autophagy inhibitor 3 -methyladenine (3-MA) (2.5 - 10 mM) and the autophagy inducer Torin-1 (Tor 1) (0.25 - 1μM) in combination with LPS (100 ng/mL) or Resiquimod (R848, 1 μg/mL) for 6 hrs and 24 hrs. TNF-α secretion was quantified by ELISA. Values are means ± SEM of 3 separate experiments. The significance of differences was determined using

one-way ANOVA with Dunnett's multiple comparison test; * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 compared to cells treated with LPS and R848.

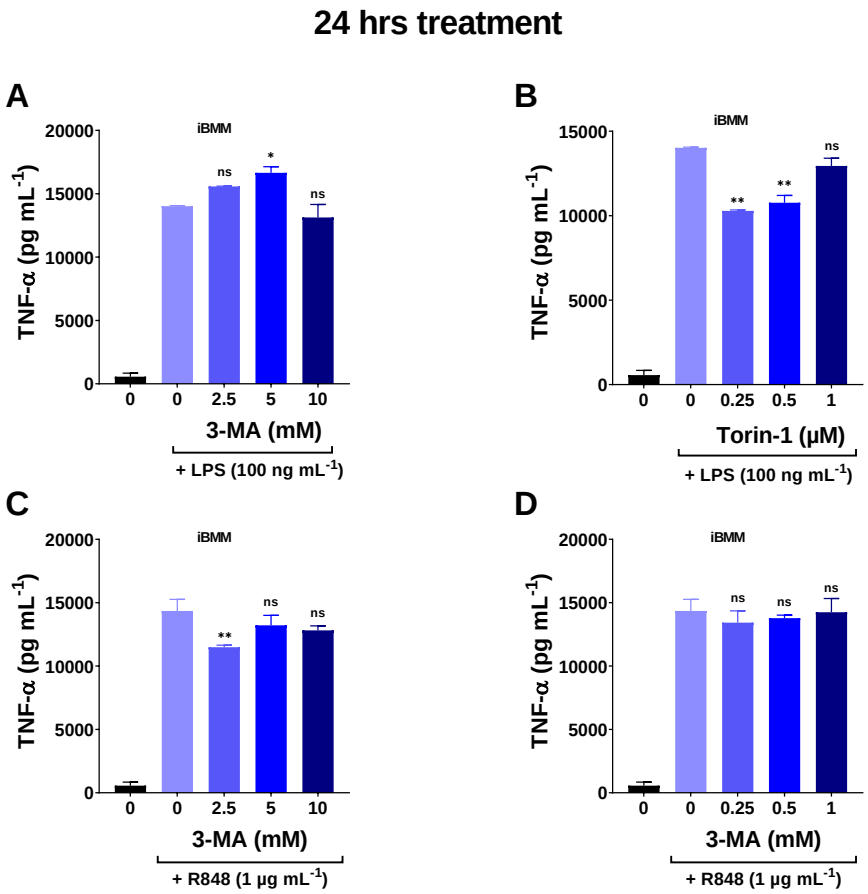


Figure 3.5 Autophagy regulating drugs do not alter TNF-α release in response to TLR ligands in iBMM cells

Effect of the autophagy inhibitor 3 -methyladenine (3-MA) (2.5 - 10 mM) and the autophagy inducer Torin-1 (Tor 1) (0.25 - 1μM) in combination with LPS (100 ng/mL) or Resiquimod (R848, 1 μg/mL) for 24 hrs. TNF did not release after 6 hrs treatment. Secretion of TNF-α was measured by ELISA. Bars represent means ± SEM of 3 separate experiments. The significance of differences was determined using one-way ANOVA with Dunnett's multiple comparison test; * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 compared to cells treated with LPS and R848.

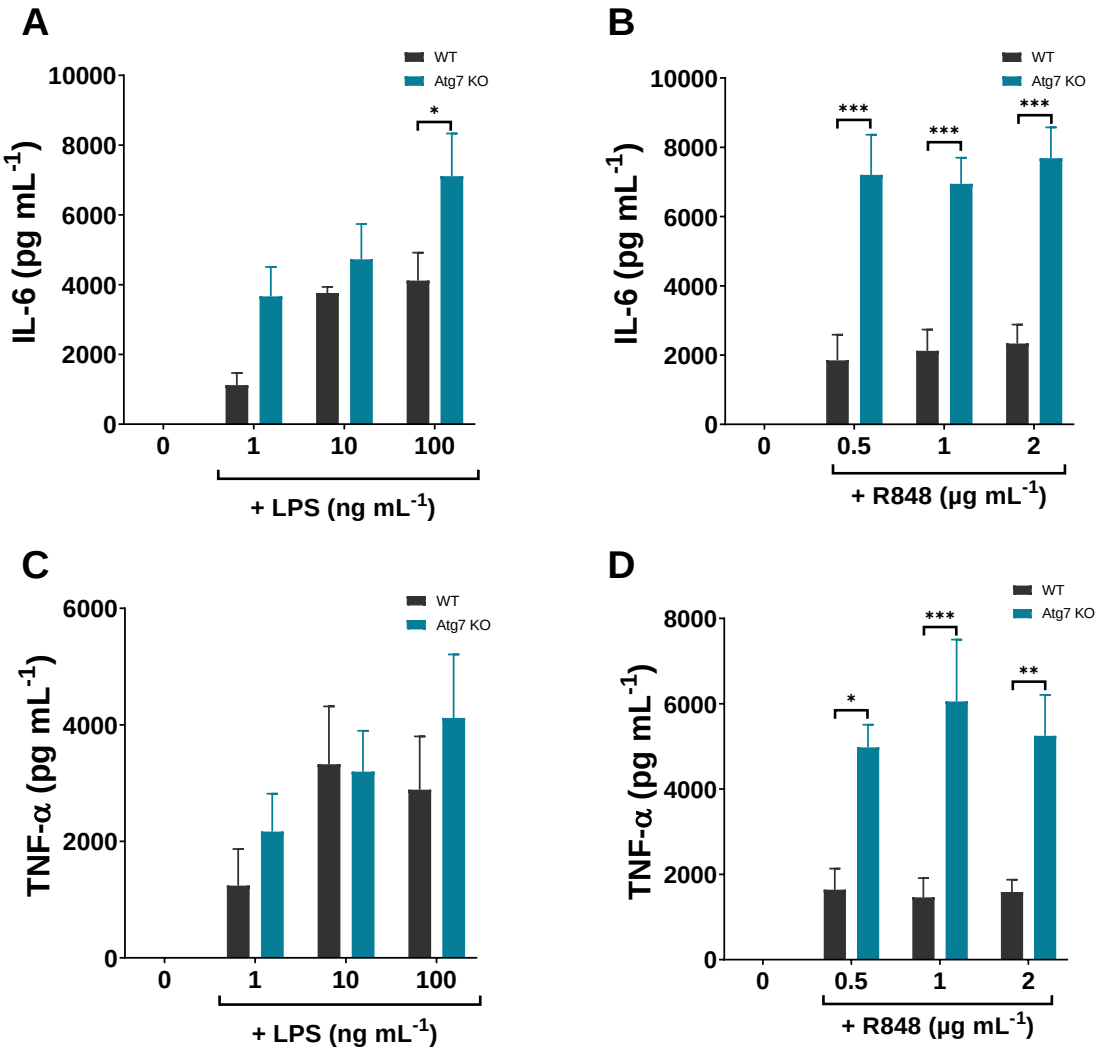


Figure 3.6 Autophagy regulating drugs do not alter IL-6 release in response to TLR ligands in Primary BMM cells

Primary BMM cells from WT and *Atg7*$^{fl/fl}$ LysMCre mice were primed with LPS (1 - 100 ng/mL) and R848 (0.5 - 2 µg/mL) for 24 hr. IL-6 and TNF secretion were measured by ELISA. Bars represent means ± SEM of 3 independent experiments. The significance of differences was determined using two-way ANOVA with Sidak's multiple comparison test. *P < 0.05, ** P < 0.01, *** P < 0.001 significant difference between WT and Atg7 KO groups.

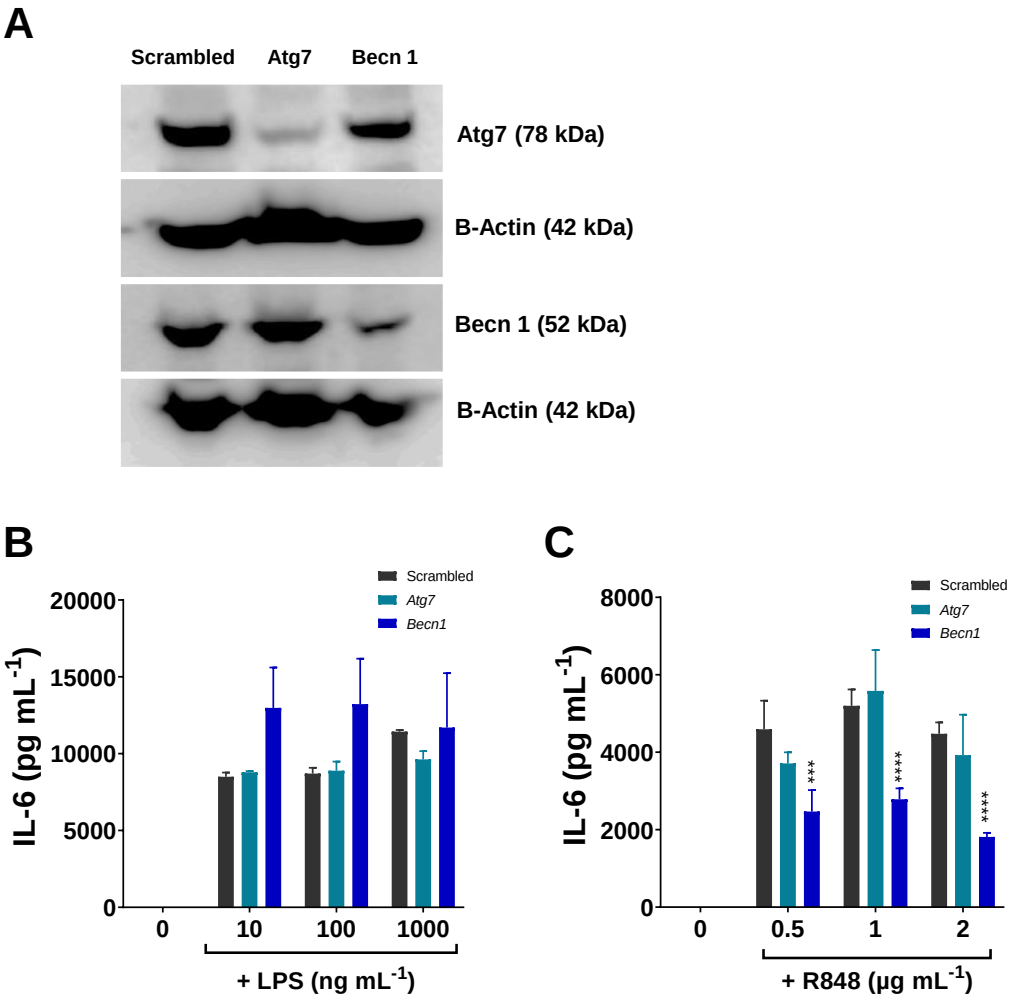


Figure 3.7 IL-6 in RAW264.7 macrophages transfected with siRNA against *Atg7* and *Becn1*

(A) Western blot analysis of Atg7 and Becn1 protein in the lysates from RAW264.7 cells transfected with siRNA against Atg7, Becn1, or non-targeting (scrambled, scr) siRNA for 24 h. IL-6 in RAW264.7 macrophages transfected with siRNA against *Atg7* and *Becn1*. Cells were stimulated with **(B)** LPS (10-1000 ng/ml), **(C)** R848 (0.5-2 µg/ml) for 24 h. Bars represent the mean of three separate experiments ± SEM. The significance of differences was determined using two-way ANOVA with Tukey's multiple comparison test; *** $P < 0.001$, **** $P < 0.0001$ compared to scrambled siRNA transfected cells.

3.3.2 Autophagy regulates IL-10 release by macrophages

I next investigated whether autophagy regulates the release of IL-10 by macrophages. Treatment of RAW264.7 macrophages or human THP-1 monocytic cells with 3-MA significantly inhibited IL-10 secretion in response to LPS and R848, as well as the TLR3 ligand poly(I:C) in both cell types (**Fig. 3.8A, C, E and 3.9A, C, E**). Conversely, in RAW26.7 cells,

the autophagy inducer Torin-1 significantly increased LPS and R848-dependent IL-10 secretion in a dose-dependent manner (**Fig. 3.8B, D**). However, lower doses of Torin 1 actually inhibited IL-10 release in response to poly(I:C) (**Fig. 3.8F**). These results suggest that autophagy may have a direct effect on IL-10 release from mouse and human myeloid cells. Interestingly, Torin-1 had either no effect on or inhibited IL-10 release by THP-1 cells in response to all stimuli (**Fig. 3.9B, D, F**). To evaluate the result in primary cells, I treated BMM cells from WT and *Atg7*$^{fl/fl}$ mice with LPS or R848 for 24 hr (**Fig. 3.10A, B**). IL-10 secretion was significantly decreased in *Atg7*$^{fl/fl}$ cells in response to LPS (**Fig. 3.10A**) and non-significantly decreased in *Atg7*$^{fl/fl}$ cells in response to R848 (**Fig. 3.10B**), further confirming the effects of pharmacological inhibition of autophagy.

To further confirm these results, I used siRNA against *Atg7* and *Becn1* to investigate the effects of autophagy deficiency on IL-10 release. Target knockdown was confirmed by western blot analysis (**Fig. 3.11A**). Knockdown of both *Atg7* and *Becn1* significantly inhibited IL-10 production in response to LPS, R848, the TLR2/1 agonist PAM3CSK4, and poly(I:C) (**Fig. 3.11B-E**). As a control, TNF secretion was also measured. Knockdown of *Atg7* or *Becn1* had some effects on TNF release in response to LPS and R848 (**Fig. 3.12**). At higher doses of LPS, knockdown of *Becn1* significantly increased TNF secretion (**Fig. 3.12A**). However, in response to R848, knockdown of *Becn1* significantly decreased TNF secretion (**Fig. 3.12B**). No effect on TNF was observed in responses to poly(I:C), although it is notable that levels of protein were significantly lower than in response to the LPS and R848. Together, these results suggest that IL-10 release from RAW264.7 macrophages is regulated by autophagy, with inhibition of autophagy abrogating its release and induction of autophagy inducing greater secretion.

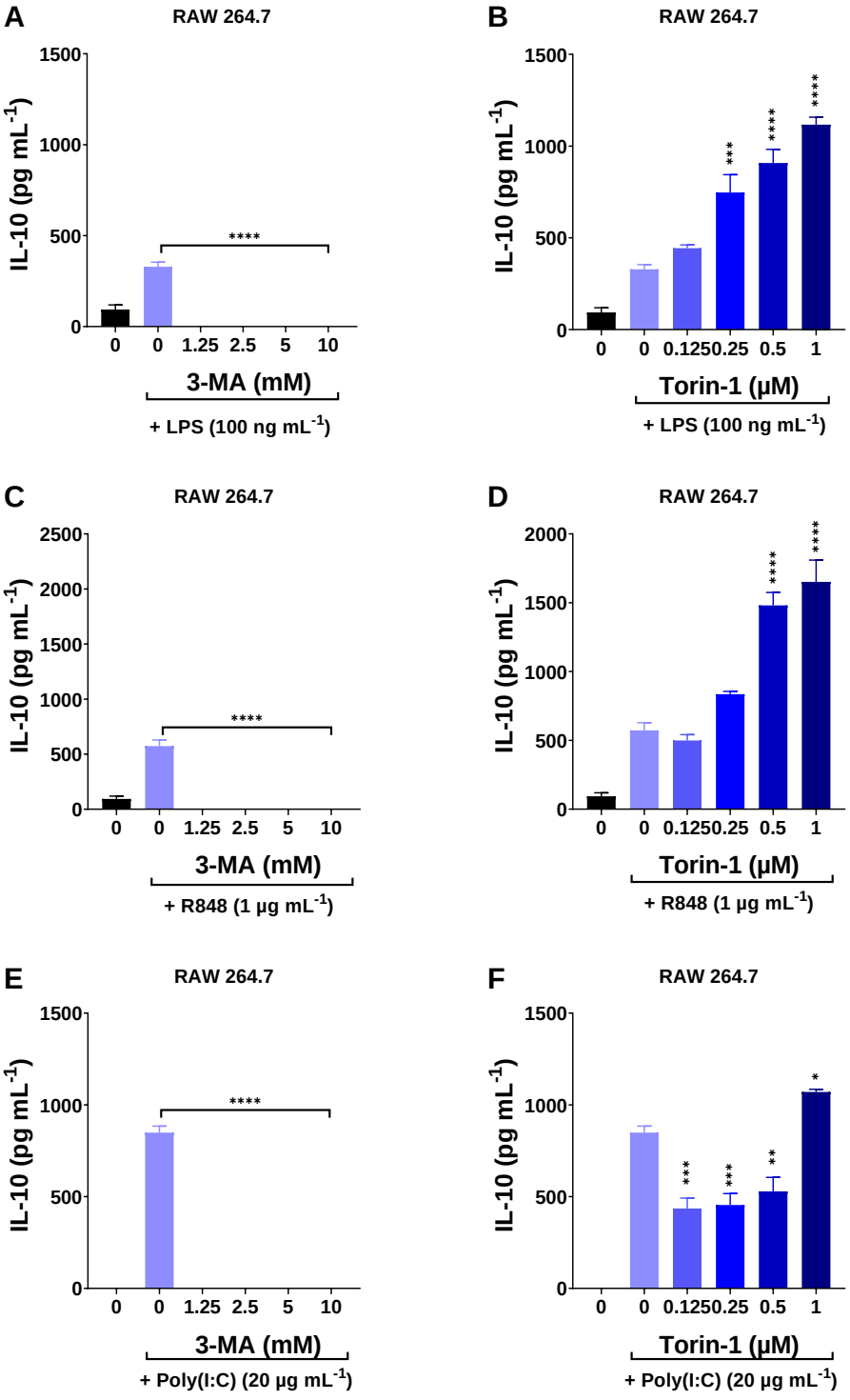


Figure 3.8 Autophagy regulating drugs alter IL-10 released in response to different TLR ligands in RAW264.7 macrophages

Effect of 3-MA (1.25 - 10 mM) and Torin-1 (0.125 - 1μM) on IL-10 release after 24 h treatment. TLR4, LPS (100 ng/mL); TLR7/8, R848 (1 μg/ml); and TLR3, Poly(I:C) (20 μg/mL). IL-10 secretion was measured by ELISA. Bars represent means ± SEM of 3 separate experiments. The significance of differences was determined using one-way ANOVA with Dunnett's multiple comparison test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to cells treated with TLR ligands.

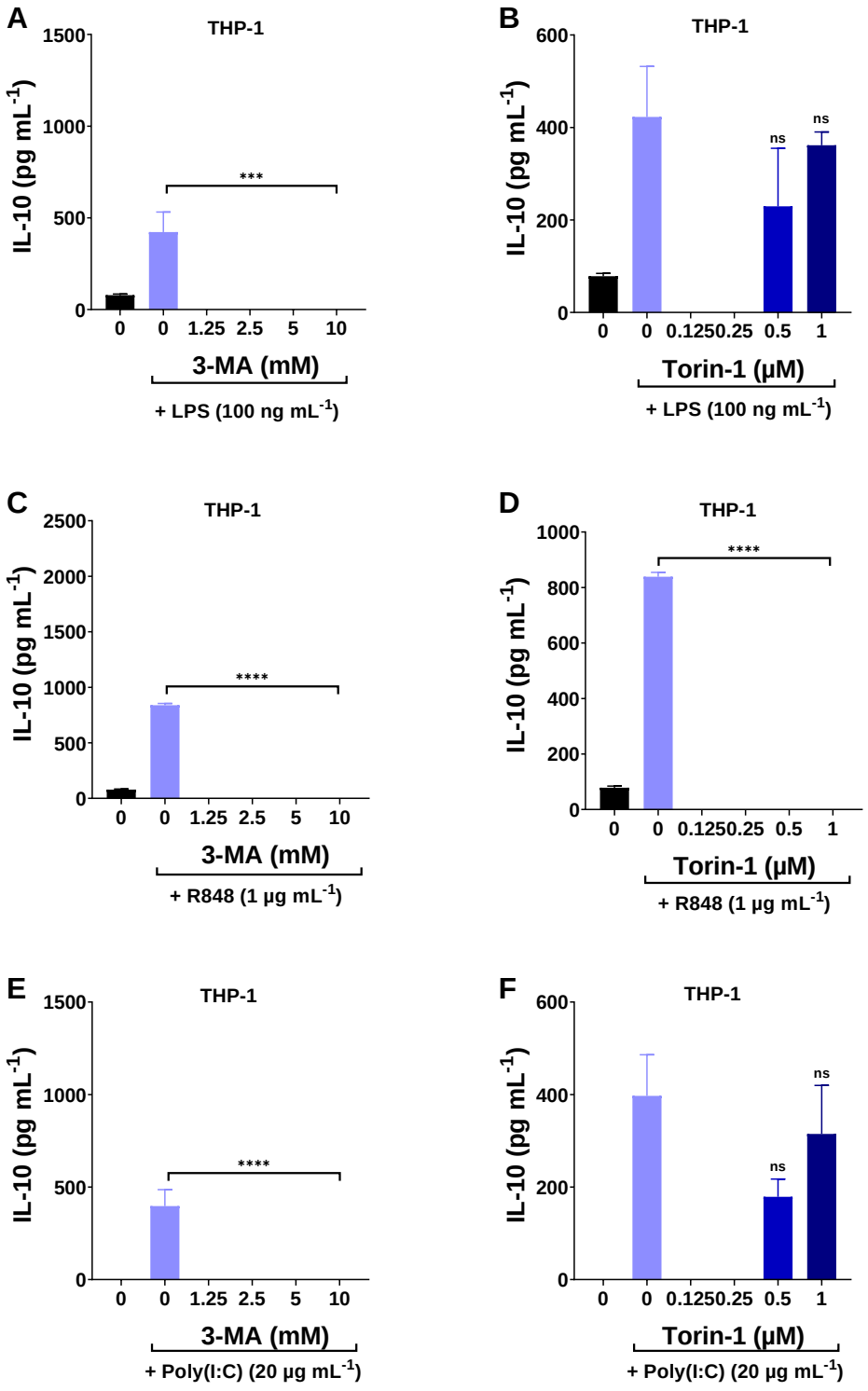


Figure 3.9 Autophagy regulating drugs alter IL-10 released in response to different TLR ligands in THP-1 cells

Effect of 3-MA (1.25 - 10 mM) and Torin-1 (0.125 - 1μM) on IL-10 release after 24 h treatment. TLR4, LPS (100 ng/ml); TLR7/8, R848 (1 μg/ml); and TLR3, Poly(I:C) (20 μg/ml). IL-10 secretion was measured by ELISA. Bars represent means ± SEM of 3 separate experiments. The significance of differences was determined using one-way ANOVA with

Dunnett's multiple comparison test; *** P < 0.001, **** P < 0.0001 compared to cells treated with TLR ligands.

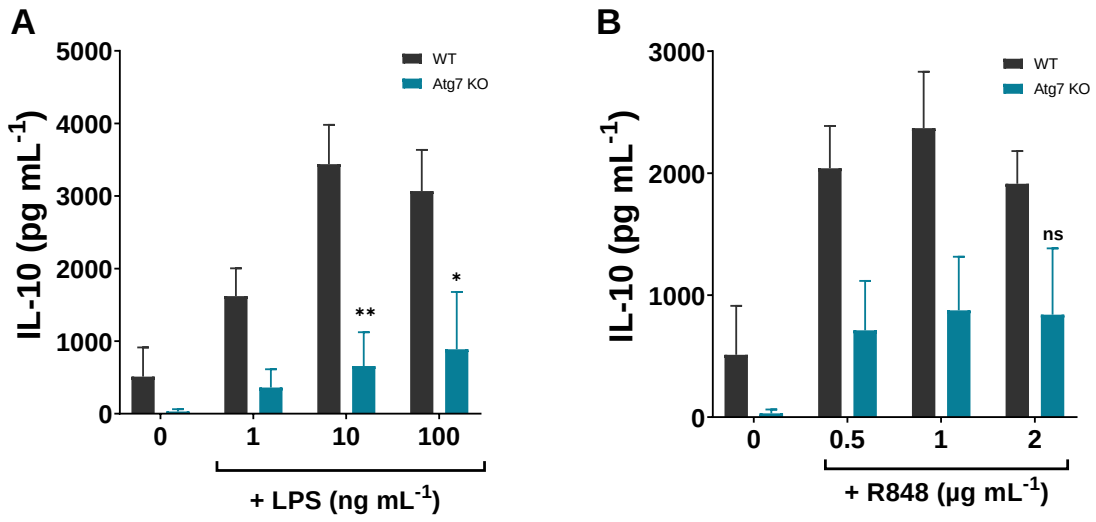


Figure 3.10 IL-10 released in response to different TLR ligands in wildtype and Atg7 ko primary BMM cells

Primary BMM cells from WT and *Atg7*$^{fl/fl}$ LysMcre mice were treated with LPS or R848 for 24 hr. secretion of IL-10 was measured by ELISA. Data are represented as the mean ± SEM of 3 separate experiments. The significance of differences was determined using two-way ANOVA with Sidak's multiple comparison test; * P < 0.05, ** P < 0.01 significant difference between WT and Atg7 KO groups.

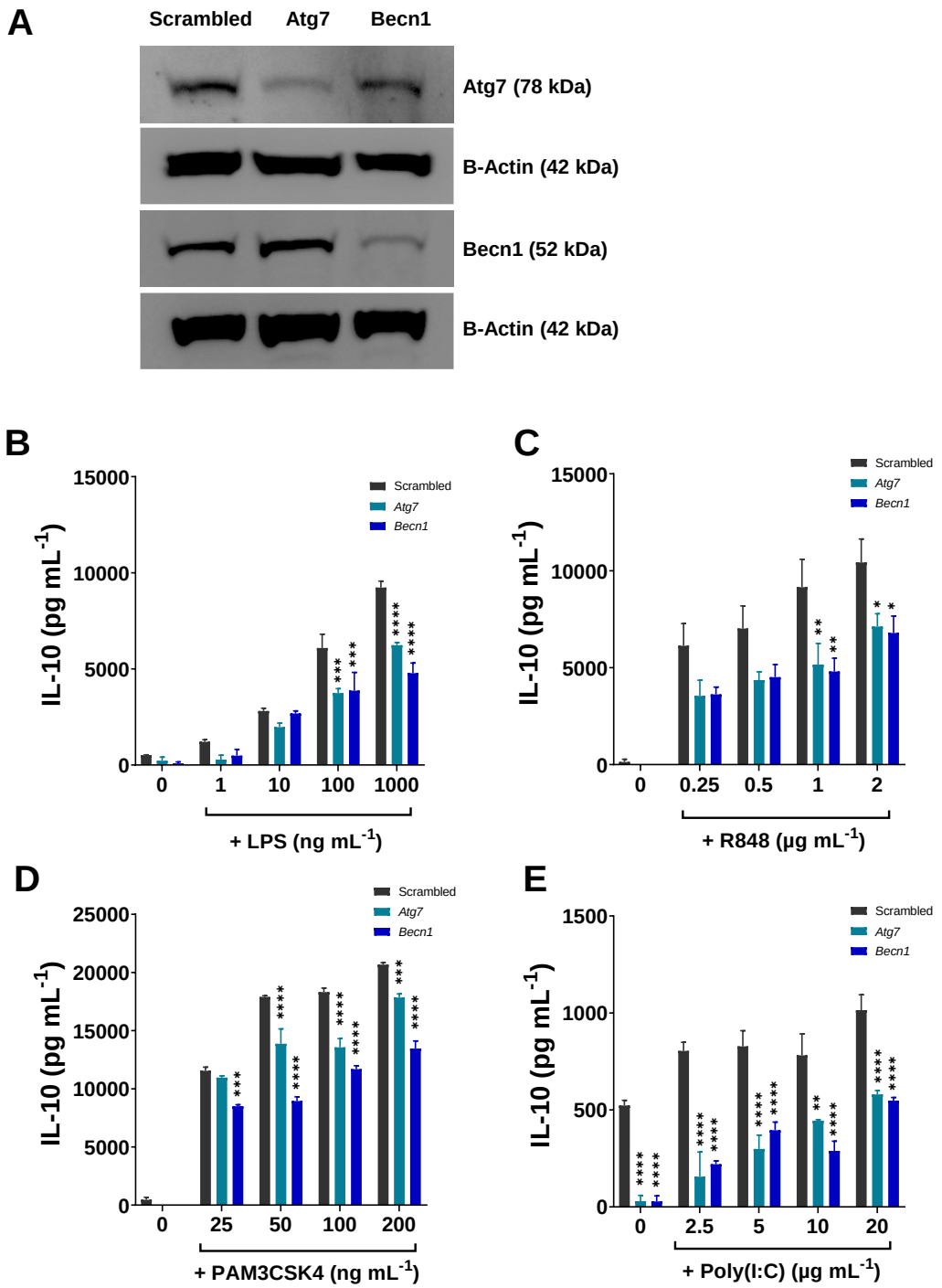


Figure 3.11 Knockdown of autophagy genes inhibits IL-10 production in response to different TLR ligands

(**A**) Western blot analysis of Atg7 and Becn1 protein in lysates from RAW264.7 cells transfected with siRNA against *Atg7*, *Becn1*, or nontargeting (scrambled, scr) siRNA for 24 h. IL-10 in RAW264.7 macrophages transfected with siRNA against *Atg7* and *Becn1*. Cells were stimulated with **(B)** LPS (1-1000 ng/ml), **(C)** Resiquimod (0.5-2 µg/ml), **(D)** PAM3CSK4 (25-200 ng/ml), and **(E)** PolyIC (2.5- 20 µg/ml) for 24 h. Data are represented as the mean± SEM of 3 separate experiments. The significance of differences was determined using two-way ANOVA with Tukey's multiple comparison test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to scrambled siRNA transfected cells.

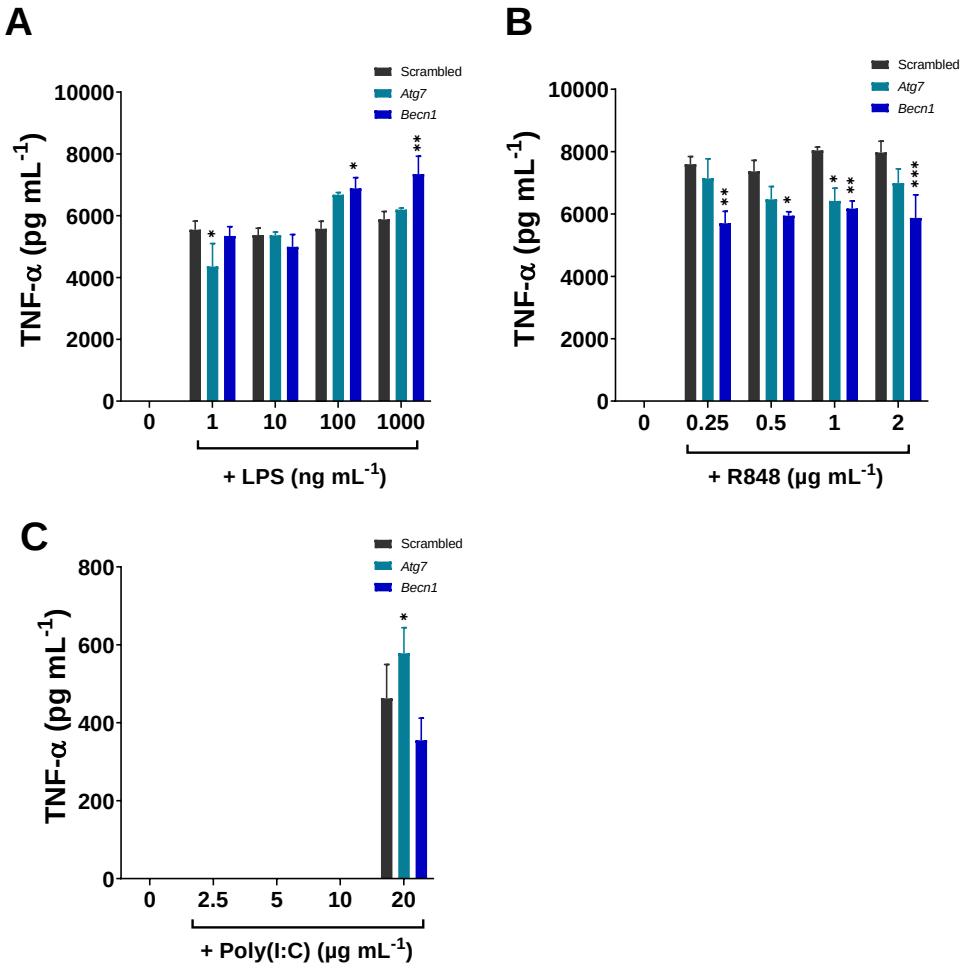


Figure 3.12 Knockdown of autophagy genes does not inhibit TNF production in response to different TLR ligands

TNF in RAW264.7 macrophages transfected with siRNA against *Atg7* and *Becn1*. Cells were treated with **(A)** LPS (1-1000 ng/ml), **(B)** resiquimod (0.5-2 µg/ml), and **(C)** PolyIC (2.5- 20 µg/ml) for 24 h. The error bars represent means ± SEM of 3 separate experiments. The significance of differences was determined using two-way ANOVA with Tukey's multiple comparison test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to scrambled siRNA transfected cells.

3.3.3 Autophagy regulates IL-10 mRNA transcription in RAW264.7 cells

To determine whether the effect of autophagy on IL-10 is mediated at the level of mRNA, I measured *Il10* mRNA levels in RAW264.7 cells treated with LPS and 3-MA or Torin-1. The data show that, similar to protein release from the cells, treatment with 3 MA significantly inhibits *Il10* mRNA expression, whereas Torin-1 significantly increased *Il10* mRNA (**Fig.**

3.13A). Levels of *Atg7* and *Becn1* mRNA were also significantly altered by 3MA treatments (**Fig. 3.13B and 3.13C)**. To confirm the specific role of autophagy in this observation, RAW264.7 cells were transfected with siRNA against *Atg7* or *Becn1*, or scrambled control, treated the cells with LPS or R848 and again looked at *Il10* mRNA. Knockdown of proteins was again confirmed by Western blot (**Fig. 3.14A**). In agreement with the 3-MA data, knockdown of *Becn1* resulted in lower *Il10* mRNA expression in response to LPS stimulation, while knockdown of both *Atg7* and *Becn1* inhibited expression of *Il10* mRNA in response to R848 (**Fig. 3.14B**). I also tested *Atg7* and *Becn1* mRNA levels (**Fig. 3.14C, D)**. Knockdown of *Becn1* significantly increased *Atg7* mRNA levels in response to both LPS and R848, while knockdown of *Atg7* non-significantly inhibited *Atg7* mRNA in response to LPS (**Fig. 3.14C**). Knockdown of *Atg7* and *Becn1* non-significantly inhibited *Becn1* mRNA expression in response to both LPS and R848 (**Fig. 3.14D**). Together, this data suggests that the effects of autophagy on IL-10 secretion are mediated either at the transcriptional level, either through a direct effect on transcription or upstream of it.

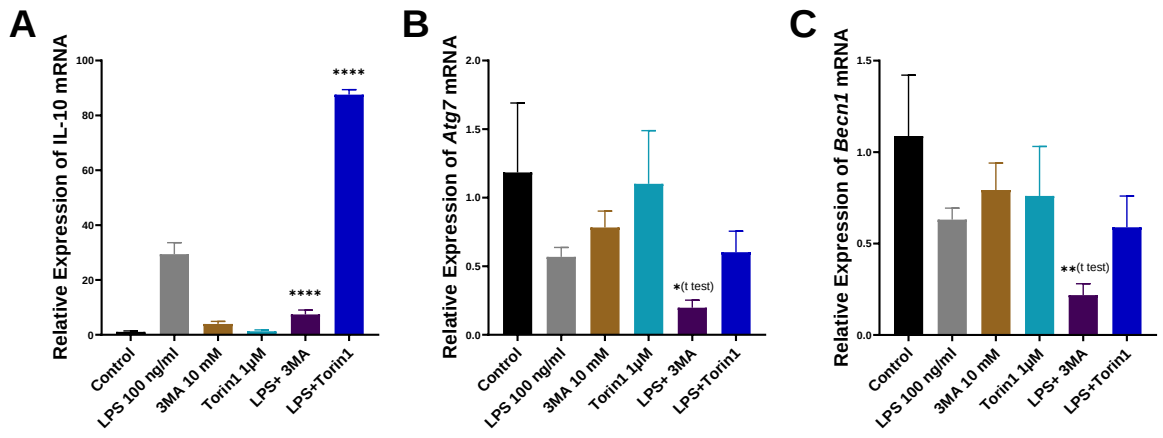


Figure 3.13 IL-10 mRNA transcription is regulated by autophagy regulating drugs
Effect of autophagy-regulating drugs in combination with LPS (100 ng/ml) on **(A)** IL-10 mRNA, **(B)** *Atg7* mRNA, and **(C)** *Becn1* mRNA. IL-10 mRNA was measured by qPCR after 4h. All data are represented as mean ± SEM of 3 independent experiments. The significance of differences was determined using one-way ANOVA with Dunnett's multiple comparison test and unpaired t-test; *p < 0.05; **p < 0.01; ****p < 0.0001 compared with LPS treated group.

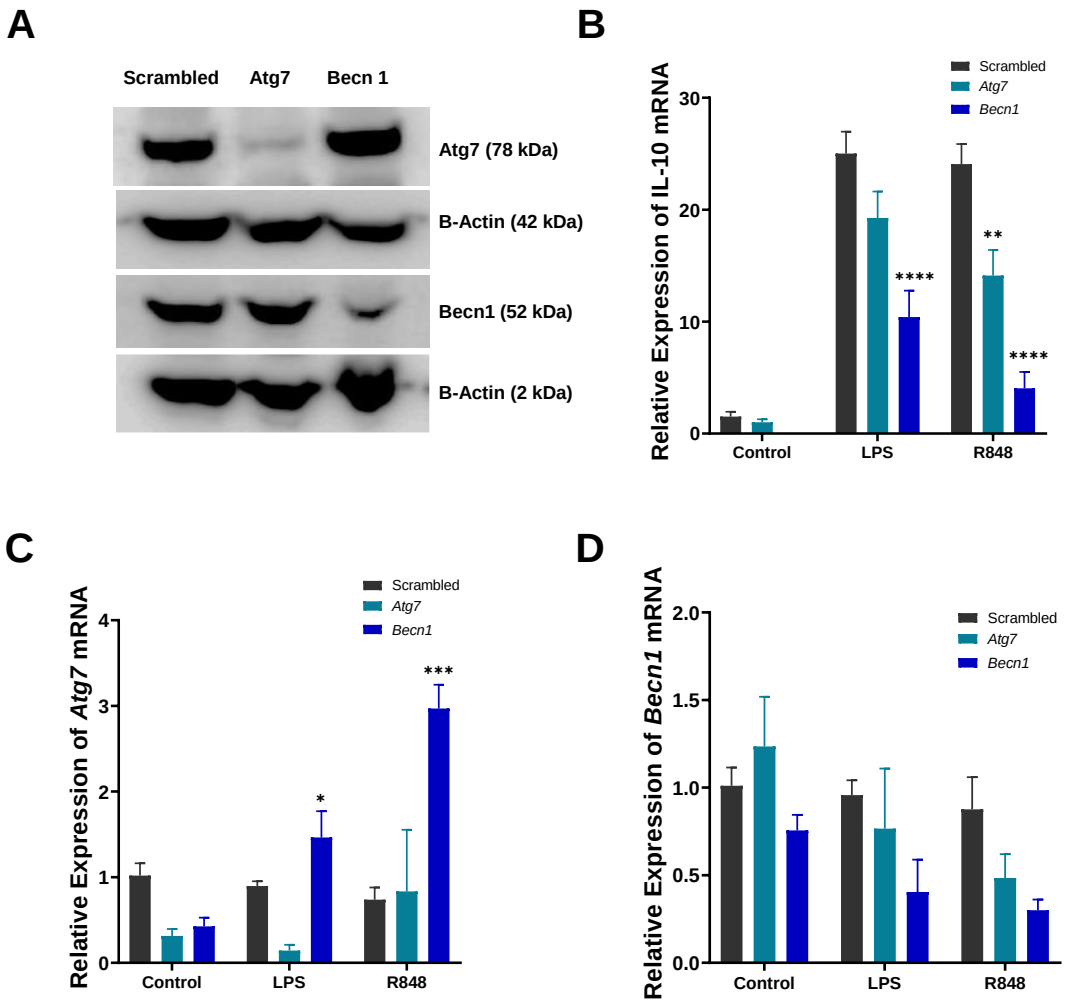


Figure 3.14 The effects of Knockdown of autophagy genes on transcription and processing of IL-10

(A) Western blot analysis of ATG7 and Becn1 protein in lysates from RAW264.7 cells transfected with siRNA against *Atg7*, *Becn1*, or non-targeting (scrambled, scr) siRNA for 4 h. **(B)** IL-10, **(C)** *Atg7* and **(D)** *Becn1* mRNA expression in *ATG7* and *Becn1* siRNA transfected RAW cells stimulated with LPS (100 ng/mL) or R848 (1 □g/mL). The mRNA level was measured by qPCR. Results are expressed as mean ± SEM of 3 separate experiments. The significance of differences was determined using two-way ANOVA with Tukey's multiple comparison test; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, compared to scrambled siRNA transfected cells.

3.3.4 Effect of autophagy on IL-10 release in iBMM

To further confirm the observations of autophagic regulation of IL-10 in macrophages, I evaluated the response in murine iBMM. As with RAW264.7 cells, 3-MA inhibited the release of IL-10 in response to R848 and PAM3CSK4 (**Fig. 3.15**). However, 3-MA did not affect IL-

10 secretion in LPS stimulated iBMM (**Fig. 3.15A**). I hypothesised that this might reflect differences between iBMM and RAW264.7 cells with respect to inflammasome activation and IL-1β release. A previous study has demonstrated that RAW264.7 macrophages do not express the critical inflammasome protein ASC (Pelegrin et al., 2008). Other studies have demonstrated that inhibition of autophagy in iBMM increases IL-1β release in response in a TRIF-dependent manner, meaning that this only occurs in response to TLR3 and TLR4 agonists (Harris et al., 2011, Saitoh et al., 2008). I found that RAW264.7 cells do not release IL-1β in response to LPS and 3-MA (data not shown), whereas iBMM do (**Fig. 3.15B**), thus representing a potentially crucial difference between these two macrophage lines. Importantly, several studies have demonstrated that IL-10 can inhibit the expression, processing, and signalling of IL-1β (Kwong et al., 1998, Jenkins et al., 1994, Ipseiz et al., 2020, Sun et al., 2019), suggesting it may be a key regulator of IL-1-dependent inflammation. Conversely, the effects of IL-1β on IL-10 release are not well understood. It is possible that increased IL-1β release in response to LPS and 3-MA in the iBMM is accompanied by an increase in IL-10 as a negative feedback mechanism, thus negating the inhibitory effects of 3-MA on IL-10.

To test this further, I stimulated iBMM cells with agonists for TLR2 (PAM3CSK4) and TLR7/8 (R848) in the presence of 3-MA and measured the level of both IL-10 and IL-1β. IL-10 released in response to both R848 and PAM3CSK4 was significantly inhibited by 3-MA (**Fig. 3.15C, E**). In contrast, levels of IL-1β in response to resiquimod and PAM3CSK4 + 3-MA, while raised, were not significantly increased and were notably lower than those seen in response to LPS + 3-MA (**Fig. 3.15D, F**). Thus, these data may suggest that IL-10 release by iBMM is increased in response to increased IL-1β and this may explain why IL-10 release in response to LPS is not inhibited by 3-MA.

A previous study has suggested that IL-1β released in response to autophagy inhibition is partially reliant on the activation of the NLRP3 inflammasome (Harris et al., 2011). To test this here, I stimulated iBMM cells with LPS, R848 or PAM3CSK+ 3-MA in the presence of the potent NLRP3 inhibitor MCC950 (Coll et al., 2015). I found that the levels of IL-10 were unaffected by NLRP3 inhibition (**Fig. 3.15A, C, E**). Surprisingly, IL-1β release was also unaffected by treatment with MCC950 (**Fig. 3.15B, D, F**), suggesting that its release in these experiments was independent of NLRP3. As a positive control, MCC950 was also tested in assays in which NLRP3 was specifically activated with the bacterial toxin nigericin, following priming with LPS for 4 h. In this experiment, MCC950 abrogated NLRP3-dependent IL-1β release (**Fig. 3.16)**, indicating that the MCC950 did work. In addition, this same batch of inhibitor was used to demonstrate NLRP3 specificity in a recent publication from our lab (Dankers et al., 2020). Taken together, these data suggest the possibility that the release of IL-10 is differentially regulated by autophagy and IL-1 signalling in LPS-stimulated iBMM, with the net effect of no significant change in levels.

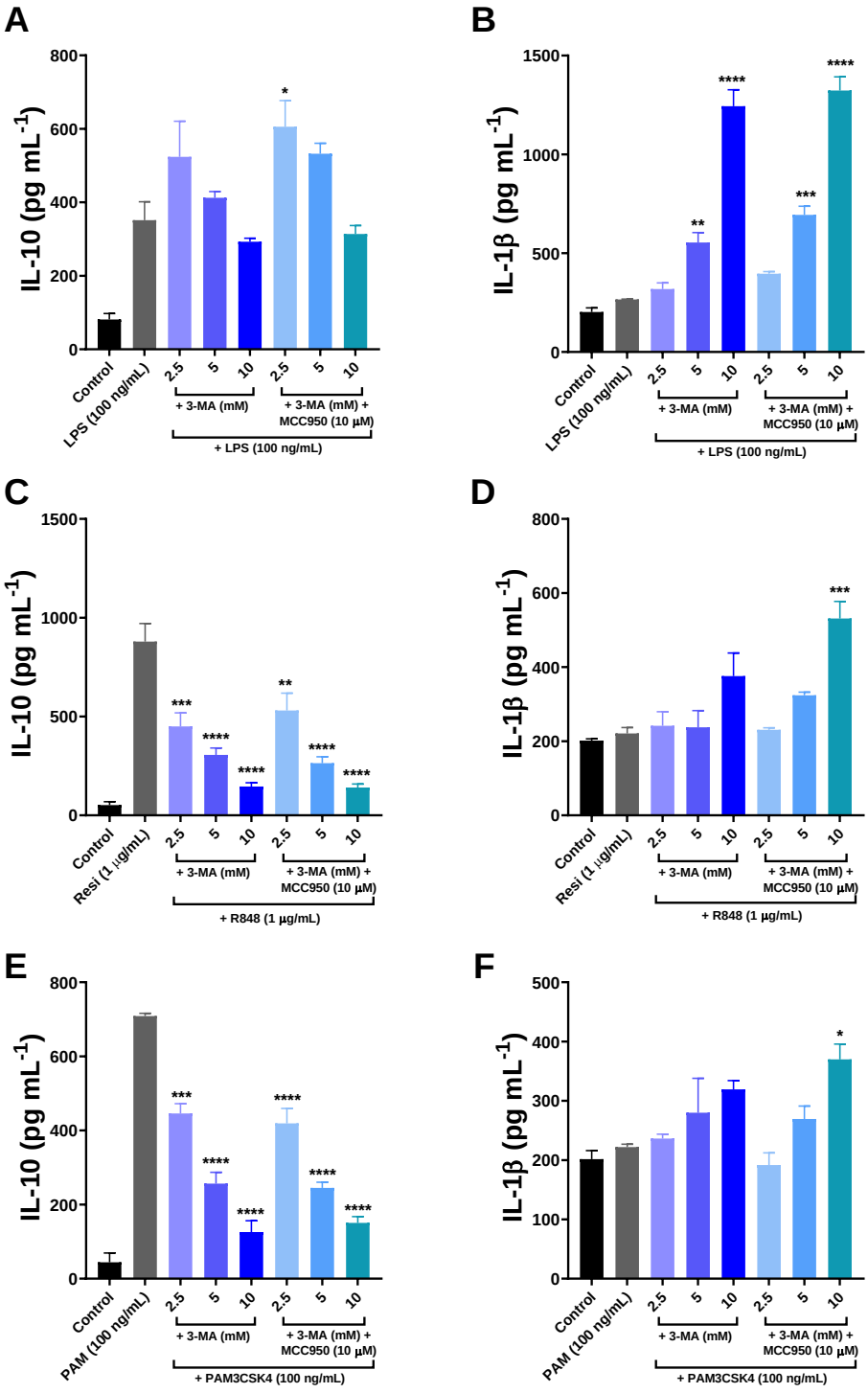


Figure 3.15 IL-10 and IL-1β release by iBMM in response to autophagy inhibition and regulation of NLRP3 inflammasome

Effect of the autophagy inhibitor 3 -methyladenine (3-MA) (2.5 - 10 mM), with or without the NLRP3 inflammasome inhibitor MCC950 on IL-10 and IL-1β release from iBMM cells stimulated for 24 hr with **(A, B)** LPS (100 ng/ml), **(C, D)** Resiquimod (R848, 1 µg/ml), and **(E, F)** PAM3CSK4 (100 ng/ml). IL-10 and IL-1β secretion were measured by ELISA. Data are represented as mean ± SEM of 3 independent experiments. The significance of differences was

determined using one-way ANOVA with Tukey's multiple comparison test; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, compared to cells treated with different TLR ligands.

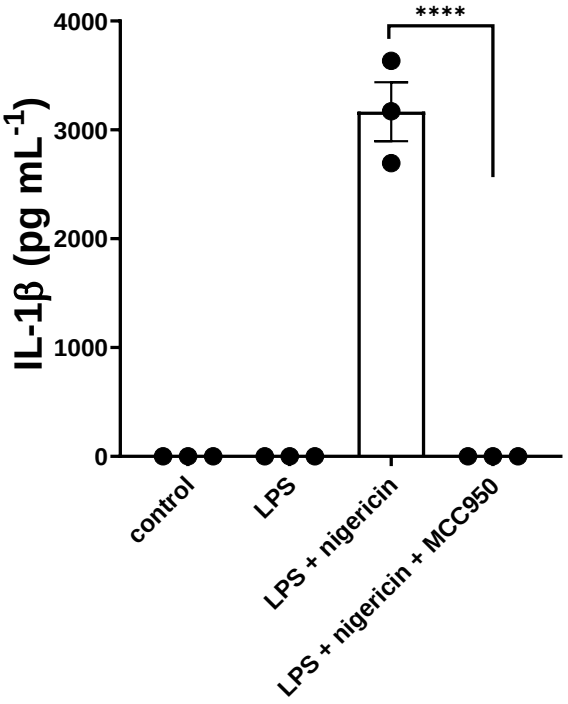


Figure 3.16 NLRP3 activated with the bacterial toxin nigericin
iBMM were primed with LPS (100 ng/ml) for 4 h, then MCC950 (10 uM) for 30 min, followed by nigericin (5 µM) for 45 min. Release of IL-1β was measured by ELISA. Data represents mean ± SEM of 3 separate experiments. The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test; **** $P < 0.0001$.

3.3.5 Myeloid cell autophagy regulates cytokine production in response to intraperitoneal (i.p) LPS challenge *in vivo*

To determine whether myeloid cell autophagy regulates cytokine responses to an acute inflammatory challenge *in vivo*, WT and *Atg7*$^{fl/fl}$ LysMCre mice were injected intraperitoneally (i.p) with R848, and cytokines (IL-6, IL-10) release both in the peritoneal cavity and serum was measured using ELISA. However, I did not see any significant change in levels of IL-6 and IL-10 after 2, 4, and 6 hr treatment in experimental mice (data not shown). Therefore, A second experiment in which LPS was injected i.p. Cytokine release both in the peritoneal cavity and serum, was measured using a multiplex assay. Previous studies have shown that in *in vivo*

LPS challenge experiments, induction of autophagy by the mTOR inhibitor rapamycin specifically abrogated effects of LPS on serum IL-1β and IL-23 release (Harris et al., 2011, Peral de Castro et al., 2012), suggesting that autophagy downregulates pro-inflammatory responses. In agreement with this, levels of IL-1β, along with the other IL-1 family cytokines IL-1α and IL-18, and IL-23 were significantly higher in the peritoneal lavage fluid of *Atg7*$^{fl/fl}$ LysMCre mice in response to LPS, compared to WT animals (**Fig. 3.17A-D**). In addition, levels of IL-17A, IFNγ and IL-12p70 were all higher in the peritoneal lavage fluid of *Atg7*$^{fl/fl}$ LysMCre mice (**Fig. 3.17E-G**). Interestingly, levels of IL-6 were also increased (**Fig. 3.17H**), while levels of IL-10 were not significantly different between *Atg7*$^{fl/fl}$ LysMCre and WT mice (**Fig. 3.17I**).

Cytokine responses in serum from the LPS injected WT, and *Atg7*$^{fl/fl}$ LysMCre mice were also measured. Levels of serum IL-1β, along with IL-1α and IL-18, were significantly increased in *Atg7*$^{fl/fl}$ LysMCre mice in response to LPS (**Fig. 3.18A-C**). However, the level of IL-23 was unchanged in *Atg7*$^{fl/fl}$ LysMCre mice compared to WT mice (**Fig. 3.18D**). Levels of IL-17A, IFNγ were also higher in *Atg7*$^{fl/fl}$ LysMCre mice (**Fig. 3.18E-F**), but levels of IL-12p70, IL-6, and IL-10 were not significantly altered in autophagy knockout mice compared with wild type (**Fig. 3.18G-I**).

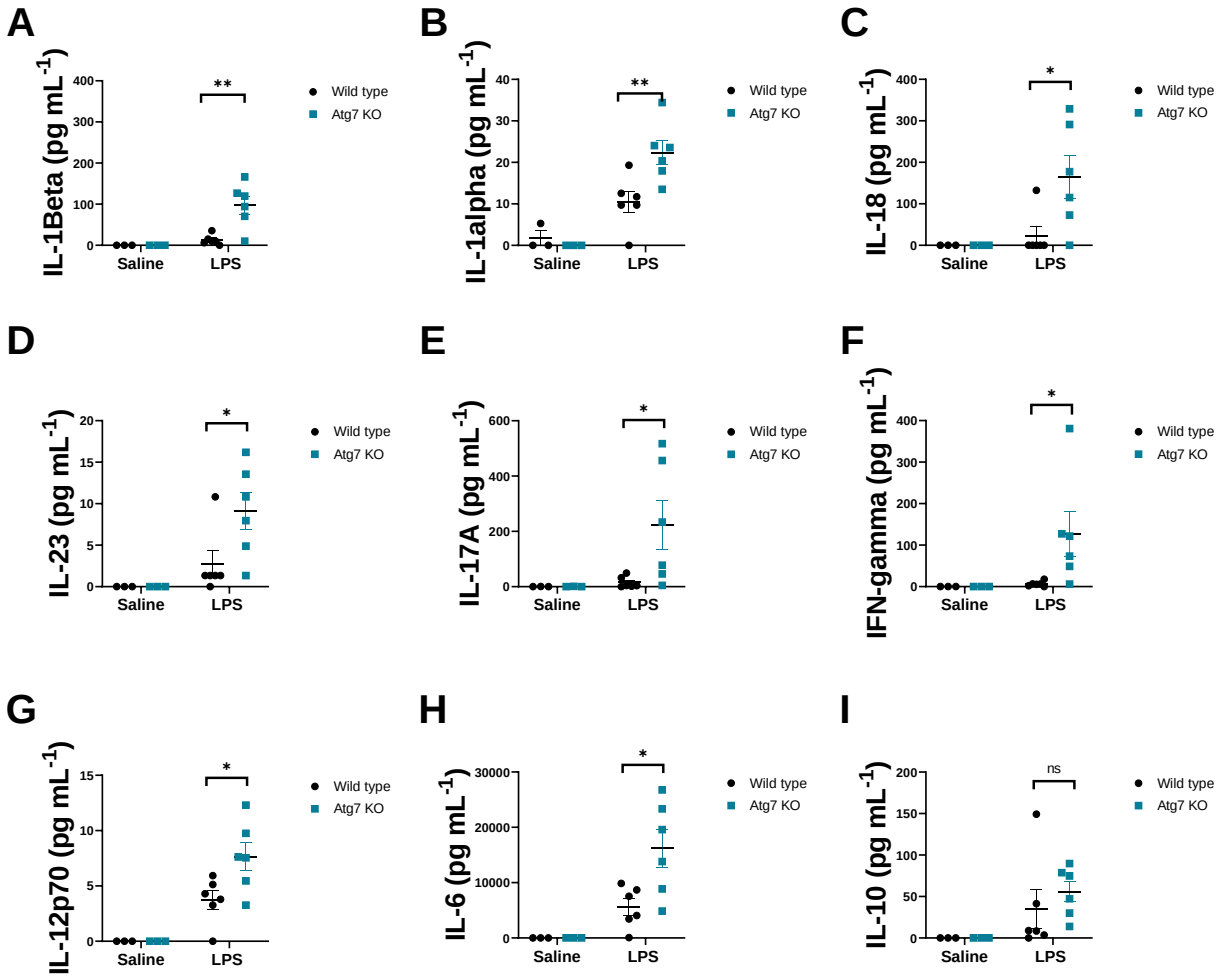


Figure 3.17 Atg7 Knockout mice show increased intracellular cytokines level in peritoneal lavage fluid

Wildtype (WT) and *Atg7*$^{fl/fl}$ LysMCre mice were injected intraperitoneally (i.p) with saline (200 µL) and LPS (50 µg/mouse) (n=3-6 per group) for 4 h. Peritoneal lavage from each mouse was collected with 3mL PBS. The level of secreted cytokines was measured by Luminex assay in peritoneal lavage fluid. All data are mean ± SEM. The significance of differences was determined using two-way ANOVA with Sidak's multiple comparison test; * $P < 0.05$, ** $P < 0.01$, significant difference between WT and Atg7 KO groups.

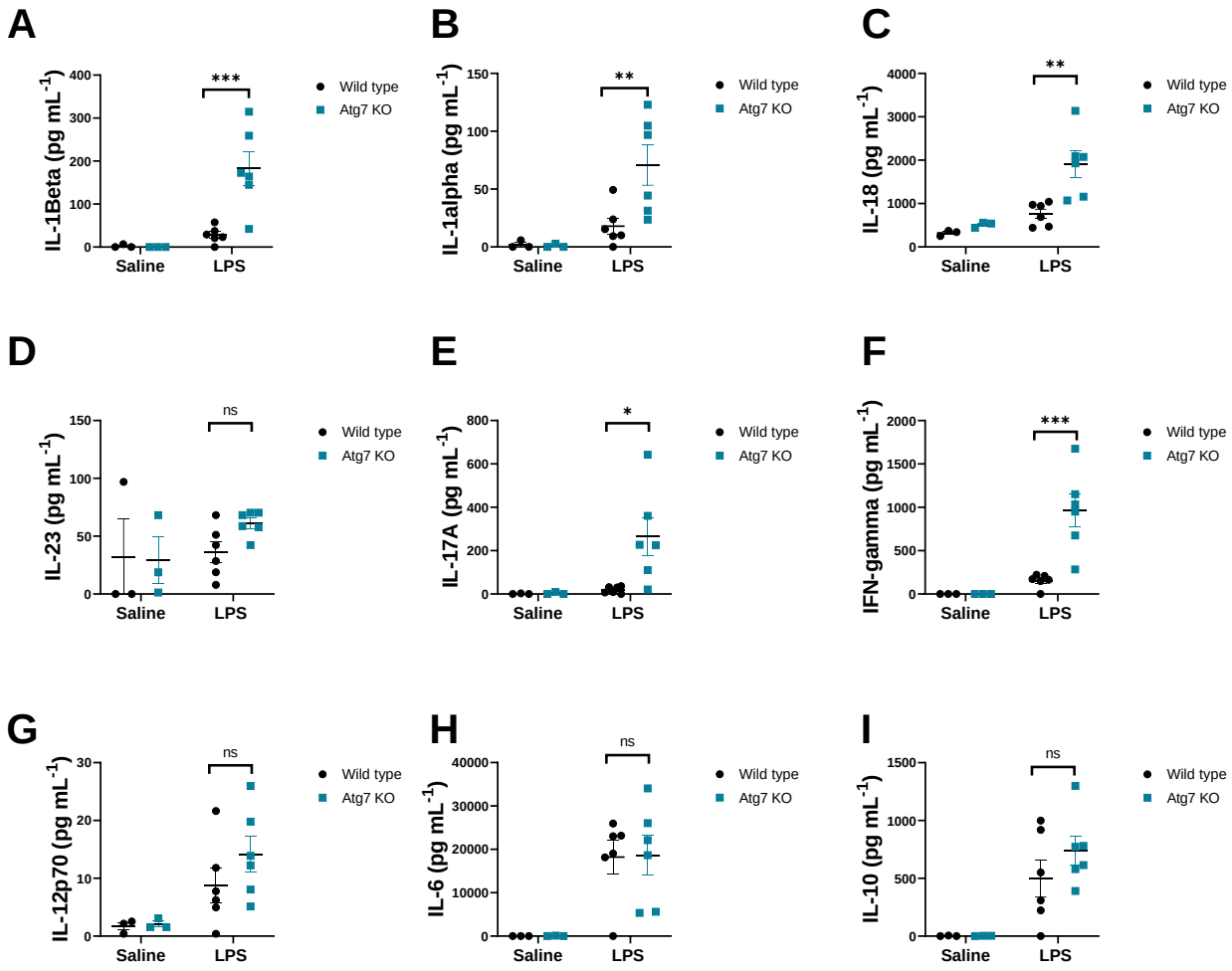


Figure 3.18 Atg7 Knockout mice show increased intracellular inflammatory cytokine level in serum

Wildtype (WT) and *Atg7*$^{fl/fl}$LysMCre mice were treated intraperitoneally (i.p) with saline (200 µL) and LPS (50 µg/mouse) (n=3-6 per group) for 4 h. Blood serum from each mouse was collected and analysed. The level of secreted cytokines in serum was measured by Luminex assay. All data are mean ± SEM. The significance of differences was determined using two-way ANOVA with Sidak's multiple comparison test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significant difference between WT and Atg7 KO groups.

3.3.6 LPS challenge *in vivo* regulates peritoneal monocyte subsets.

Peritoneal macrophages/monocytes play a crucial role in controlling infectious and inflammatory diseases by a variety of effector and regulatory functions (Dahdah et al., 2014, Okabe and Medzhitov, 2014). Significant interest has now been focused on uncovering the unique subpopulation of monocytes that give rise to tissue-resident macrophages in steady-state or in various clinical contexts. Here, I studied the distribution of peritoneal macrophages/monocytes according to the expression pattern of different membrane markers; $CD115^+$ $CD11b^+$ monocytes (further subdivided into Ly6C positive and negative monocytes), $CD115^-$ $CD11b^-$ dendritic cells (further subdivided into CD11c positive and negative) and $CD115^-$ $CD11b^+$ $CD11c^-$ macrophages (further subdivided into MHC II positive and negative) (**Fig. 3.19**). Ly6c positive monocytes are rapidly recruited to sites of inflammation, and Ly6c negative monocytes patrol blood vessels and in the tissue comprise resident microglia (Cedric Auffray et al., 2009). No significant differences in any myeloid cell subsets were observed between LPS challenged WT and myeloid cell autophagy-deficient (*Atg7*$^{fl/fl}$ LysMCre) mice (**Fig. 3.20**). These data indicate that loss of Atg7 does not alter the response to LPS in terms of myeloid cell populations.

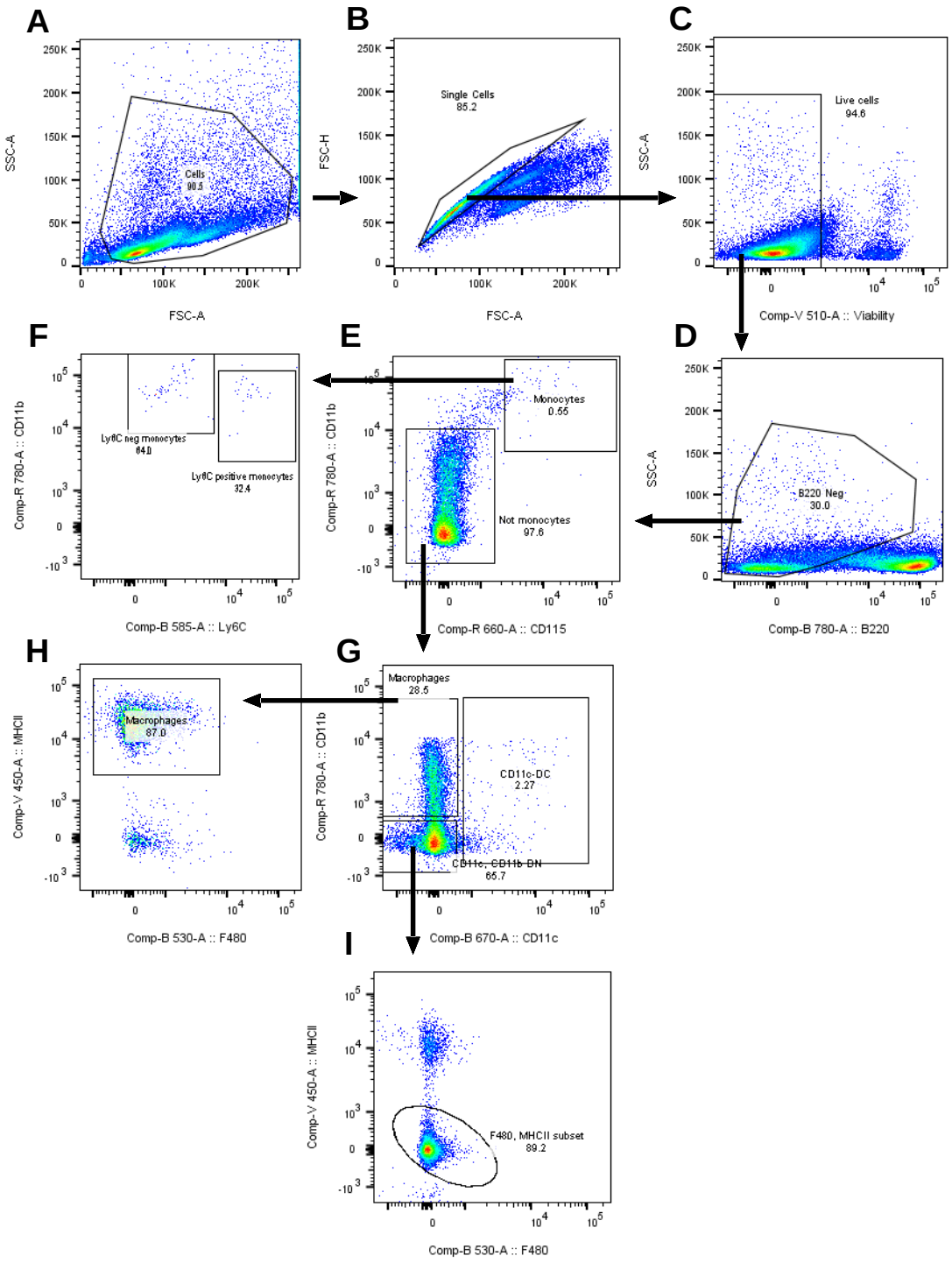


Figure 3.19 Gating strategy for myeloid cells subsets in peritoneal lavage
(A-I) To identify specific cell populations by flow cytometry, a sequential gating strategy was used. The following cell populations were identified: monocytes ($CD115^+$ $CD11b^+$), Ly6C positive monocytes ($CD115^+$ $CD11b^+$ $Ly6c^+$), Ly6C negative monocytes ($CD115^+$ $CD11b^+$ $Ly6c^-$), CD11c positive dendritic cells ($CD115^-CD11b^-CD11c^+$), CD11c negative dendritic cells ($CD115^-CD11b^-CD11c^-$), macrophages ($CD115^-CD11b^+CD11c^-$) and MHC II positive macrophages ($CD115^-CD11b^+CD11c^-$ $MHCII^+$)**.**

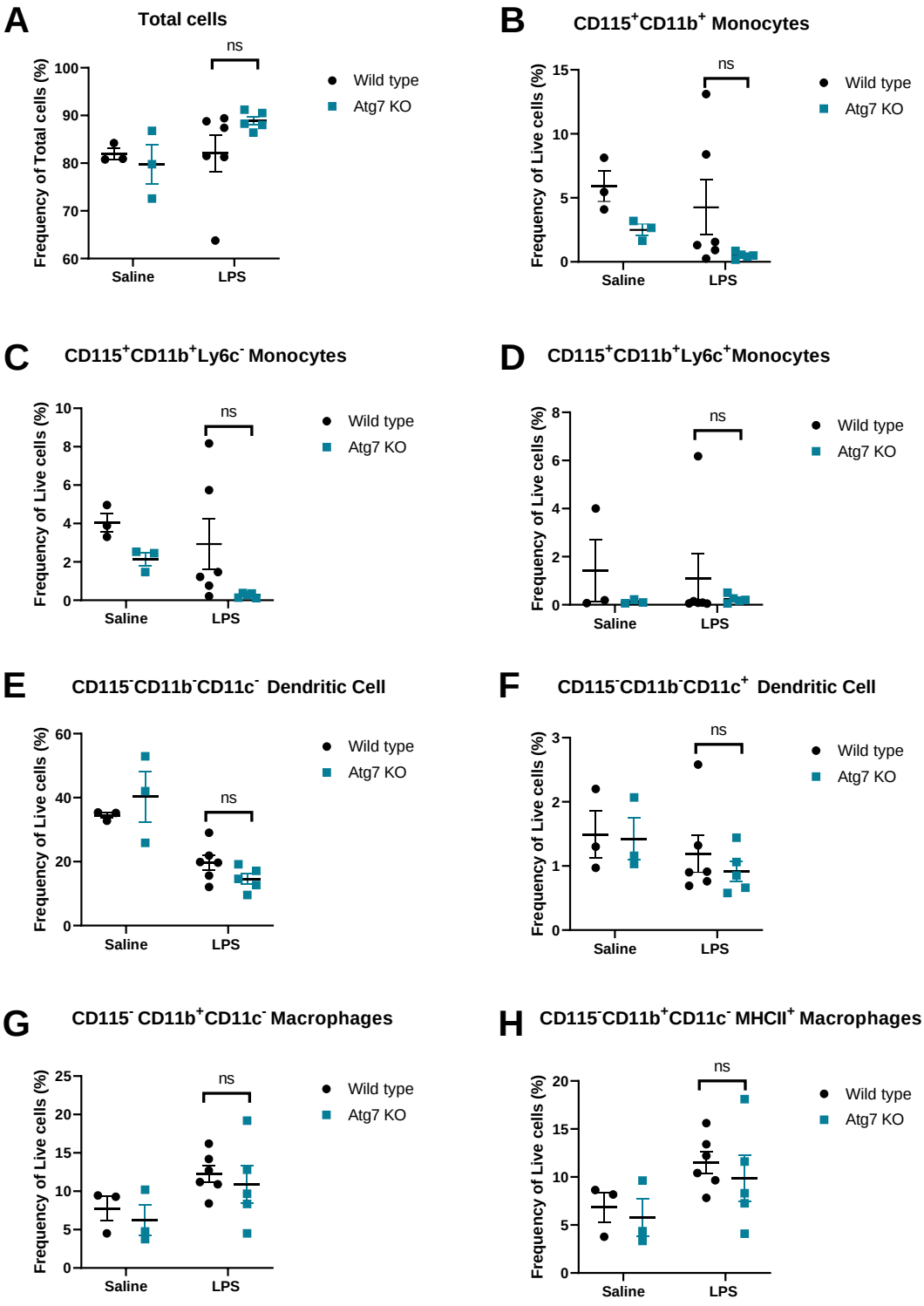


Figure 3.20 LPS did not affect myeloid cells subsets in peritoneal lavage
Wildtype (WT) and *Atg7*$^{fl/fl}$ LysMCre mice (n=3-6 per group) were treated intraperitoneally (i.p) with saline (200 µL) or LPS (50 µg/mouse in 20 µL saline). (A) Total cells (B) Monocytes (C) Ly6c$^-$ monocytes (D) Ly6c$^+$ monocytes (E) CD11c$^-$ DC (F) CD11c$^+$ DC (G) Macrophages and (H) MHCII$^+$ macrophages were analysed. All data are mean ± SEM. The significance of differences between groups was determined using two-way ANOVA with Sidak's multiple comparison test; * $P < 0.05$, ** $P < 0.01$.

3.4 Discussion

Autophagy regulates both innate and adaptive immune responses (Schmid and Münz, 2007), and the role of autophagy in immune responses has been studied intensively in different pathological conditions, including cancer, heart disease, infectious disease, neurodegenerative disorders, RA, Crohn's disease and SLE (Liang et al., 1999, Gu et al., 2016, Booth et al., 2016, Castillo et al., 2013, Xu et al., 2015, Fiorucci et al., 2007, Dutta et al., 2012). Autophagy has an impact on the development, homeostasis, and survival of inflammatory cells such as macrophages, lymphocytes, and neutrophils, all of which are important in developing and controlling inflammation. Autophagy has been shown to have an influence on the maturation of pathogen-containing phagosomes and MHC class II presentation of viral antigens (Gutierrez et al., 2004, Orvedahl et al., 2007).

Multiple cytokines have been found to regulate autophagy in macrophages and DC (Harris, 2011). For example, IL-1α, IL-1β, IFN-γ, and TNF-α has been shown to enhance autophagosome formation in macrophages (Shi et al 2008; Harris & Keane 2010, Gutierrez et al 2004), while IL-4, IL-13, and IL-10 suppress autophagy (Harris et al., 2007, Van Grol et al., 2010, C et al., 2011, Park et al., 2011, Harris et al., 2009). Additionally, inflammatory factors have been shown to cause autophagy, including LPS and other TLR agonists (Paludan et al., 2005, Delgado et al., 2008, Xu et al., 2007, Harris, 2011). Conversely, inhibition of autophagy facilitates IL-1β production release due to the intracellular aggregation of endogenous inflammasome-activating factors, including mitochondrial DNA and ROS (Harris et al., 2011, Zhou et al., 2011a, Kleinnijenhuis et al., 2011, Saitoh et al., 2008).

Macrophages are essential for the host defence system. Macrophages deficient of Atg16L1 or Atg7, key components of the autophagy process, produce more interleukin (IL)-1β and IL-18 in response to inflammatory stimulation by TRIF-dependent toll-like receptor (TLR) signalling pathways (Saitoh et al., 2008, Harris et al., 2011). TLRs play an instructive role in innate and

adaptive immunity by recognising specific molecular patterns from pathogens. Moreover, TLR signalling can also accelerate phagosome maturation and fusion of phagosomes and lysosomes in macrophages/monocytes, which is dependent on autophagic machinery, such as ATG5 and ATG7, resulting in rapid acidification and facilitate destruction of the ingested organism (Sanjuan et al., 2007). TLR signalling primarily triggers regulatory factors for interferon and activation of nuclear factor-κB (NF-κB) and AP1. This induces type I interferon and inflammatory cytokines to be released (Akira and Takeda, 2004, Kawai and Akira, 2006). In this study, I found that the regulation of autophagy with drugs, genetic depletion or siRNA could regulate IL-6 and IL-10 production in response to different TLR ligands such as LPS, Resiquimod, and PAM3CSK4 in various macrophages. In particular, I have confirmed that autophagy can directly and consistently influence the secretion of IL-10.

Increasing evidence indicates that IL-6 and IL-10 contribute to the pathogenesis of SLE by enhancing autoantibody production and immune complex deposition (Tanaka et al., 2014, Facciotti et al., 2020). In chapter 2, using a mouse model of lupus, I discovered that autophagy has a role in regulating the level of inflammatory cytokines and SLE-like autoimmunity. However, the mechanism behind it remains unclear. Previous studies have reported these cytokines have diverse or even opposite effects on different cell types in a context-dependent manner. Therefore, to evaluate the effect of autophagy in the regulation of the cytokines, I used various types of macrophages and observed a pattern of autophagic regulation of cytokines.

It has been reported that when TLRs are activated, several cell types in the body, such as neutrophils and monocytes or macrophages, can synthesise IL-6 (Chalaris et al., 2007). IL-6 has been shown to regulate the autophagy process by both inhibitory and stimulating effects (Delk and Farach-Carson, 2012, Kimura et al., 2010). However, the molecular mechanisms of the regulation of IL-6 by autophagy remain unclear. In this study, I investigated whether autophagy directly regulates IL-6 production in different types of myeloid cells stimulated with

TLR4 agonist LPS or the TLR7/8 agonist resiquimod (R848), in the presence of the type III PI3K inhibitor (an autophagy inhibitor) 3-methyladenine (3-MA) and induction of autophagy, with the mTOR inhibitor (an autophagy inducer) Torin-1. Other PI3K inhibitors, such as wortmannin and LY294002, have been proposed to suppress autophagy by inhibiting the class III PI3K (Petiot et al., 2000). However, these inhibitors primarily target class I PI3K, which is essential in the AKT signalling pathway and may, in some cases, increase autophagy over prolonged use (Wu et al., 2010). Similarly, 3-MA has been reported to inhibit class I PI3K (Wu et al., 2010, Chicote et al., 2020), so it may also be regulating IL-10 release through effects on Akt signalling rather than (or as well as) via type III PI3K and may actually induce autophagy over longer time periods (Wu et al., 2010). To address this, more specific targeted inhibition of both the class IPI3K/AKT pathway and the class III Pi3K pathway would allow us to better distinguish between autophagy-dependent and PI3K non-autophagy dependent roles. More specific type I PI3K inhibitors are commercially available, such as idelalisib (CAL101), alpelisib (BYL719), copanlisib (BAY 80–6946), and duvelisib (IPI-145) (Fang et al., 2020). Further studies could test these inhibitors for effects on IL-10 release. Likewise, siRNA knockdown of AKT (Harris et al., 2007) and/or other targets in the AKT pathway would similarly determine whether this effect is dependent on autophagy or class I PI3K/Akt signalling. Similarly, reportedly more specific class III Pi3K inhibitors have been identified, including Compound 31 ((2*S*)-8-[(3*R*)-3-Methylmorpholin-4-yl]-1-(3-methyl-2-oxobutyl)-2-(trifluoromethyl)-3,4-dihydro-2*H*-pyrimido[1,2-*a*]pyrimidin-6-one) (Pasquier et al., 2015), SAR405 (Ronan et al., 2014)and VPS34-INI (Bago et al., 2014). The effects I observed here with 3-MA were also seen in cells treated with siRNA against both *Atg7* and *Becn1*, which would strongly suggest a role for autophagy in the release of IL-10. Nonetheless, future studies to determine the relative contributions of class I and class III PI3K would be of considerable interest.

In RAW264.7 macrophages, 3-MA had contrasting effects, but Torin 1 inhibits LPS-induced IL-6 release after 6 hours, which is consistent with previous research from my lab (Lee et al., 2016c). However, after 24 hours, all these effects are lost. In iBMM cells, both 3-MA and Torin-1 significantly increased IL-6 in response to R848, suggesting that it is not an autophagic reaction. Similar results are found in primary cells, and Atg7 KO did not decrease IL-6 in response to 24 h LPS and R848 treatment. In addition, siRNA knockdown of *Atg7* or *Becn1* had no effect on IL-6 release in response to LPS by RAW264.7 cells. However, in response to R848, IL-6 release was significantly abrogated in cells transfected with siRNA against *Becn1*. This could probably be an autophagy-independent regulation by beclin1 in response to TLR7/8. Since Becn1 are involved in autophagy and endocytosis (Ruck et al., 2011) and the Toll-like receptor 7/8-ligand resiquimod (R848) is an endosomal receptor (Petes et al., 2017), we cannot exclude the possibility that an endocytic function of Becn1 is a contributing factor in the regulation of IL-6. However, it is still unclear how Becn1 regulates the level of IL-6 via the endocytic pathway in response to TLR7/8 stimulation and how its various modes of regulation are altered/controlled in various tissues in respect to different physiological contexts. Thus, further work is required to uncover the mechanism of Becn1-mediated endocytic regulation of IL-6 in different cellular contexts. Also, it would be interesting to investigate how the Becn1 mediated endocytic pathway regulates IL-6 using Becn1-deficient mice. More research is required to better understand the function of autophagy and the specific component of the autophagy process, such as Atg genes in regulating IL-6 release by macrophages.

IL-10 plays an essential role in maintaining immune homeostasis, and it has both positive and negative effects on autophagy (Park et al., 2011, Martinez-Outschoorn et al., 2011). However, little is known about how autophagy regulates IL-10. The results from this study demonstrated that autophagy could regulate IL-10 production and secretion by macrophages; loss of autophagy inhibits IL-10 release while induction of autophagy with the mTOR inhibitor Torin-

1 promotes IL-10 release in response to TLR activation. Moreover, inhibition of IL-10 releases following autophagy inhibition was repeated in primary murine BMM, iBMM, and human THP-1 monocytic cells. Previous studies have shown that IL-10 regulates immune responses by inhibiting the antigen-presenting capacity of monocytes/macrophages through downregulation of class II MHC-restricted antigens to T cells (de Waal Malefyt et al., 1991, Mittal and Roche, 2015). Whether this is a mechanism at play in the *Lyn*$^{-/-}$ model presented here is unclear but warrants further investigation. However, in contrast to autophagic regulation of IL-10 in RAW264.7 and THP-1 cells, the autophagy inhibitor 3-MA did not inhibit the secretion of IL-10 from LPS-primed iBMM. There is a possibility that the dose of LPS (100 ng/ml) used in iBMM might be too high and mask the effect of 3-MA. The same dose was used in both RAW264.7 and THP-1 cells and did not mask the effects of 3-MA on IL-10 release, but future studies looking at different doses of LPS in iBMM are warranted as these cells may be more sensitive to LPS stimulation. One possible explanation for this lies in the difference between iBMM and RAW264.7 cells in terms of inflammasome activation and release of IL-1β. It has been demonstrated that RAW264.7 macrophages do not release mature IL-1β in response to inflammasome activation since they do not express ASC and thus have no caspase-1 inflammasome activity (Pelegrin et al., 2008). In accordance with this study, I found that these cells do not release IL-1β in response to LPS and 3-MA. Furthermore, previous research has shown that autophagic regulation of IL-1β release is dependent on the signalling molecule TRIF, which is involved in TLR3 and TLR4-dependent pathways (Harris et al., 2011, Saitoh et al., 2008).

Here, I showed that 3-MA did inhibit IL-10 in response to ligation of TLRs 2 and TLR7/8 that signal through MyD88, rather than TRIF. In response to both R848 and PAM3CSK4, the release of IL-10 was significantly inhibited by 3-MA, but in LPS primed iBMM, autophagy inhibitor 3-MA did not affect IL-10 release. Thus, it may be that IL-10 release is positively

regulated by both autophagy and IL-1β, such that inhibition of autophagy inhibits IL-10 while increased IL-1β release increases it. This would suggest that the two regulatory mechanisms involved (on IL-10) are independent of each other, counteracting the effects of each other. Interestingly, however, while serum and peritoneal levels of IL-10 were unaffected in *Atg7*$^{fl/fl}$ LysMCre mice in an *in vivo* model of endotoxemia, *Atg7*$^{fl/fl}$ BMM did release less IL-10 than WT cells *in vitro*.

A previous study suggested that in response to autophagy inhibition, the release of IL-1β is at least partially dependent on NLRP3 inflammasome activation (Harris et al., 2011). To examine NLPR3 inflammasome dependent release of IL-1β, I stimulated iBMM with LPS, R848 or PAM3CSK4 + 3-MA in the presence of the NLRP3 inhibitor MCC950. MCC950 is a potent inhibitor of NLRP3 inflammasome activation, directly targeting the NLRP3 ATP-hydrolysis motif in the NACHT domain (Coll et al., 2015, Coll et al., 2019). IL-10 released in response to all TLR agonists was unaffected by NLRP3 inhibition. Moreover, and surprisingly, IL-1β release was also unaffected upon stimulation of iBMM with MCC950, even in response to LPS + 3-MA. This would suggest that the release of IL-10 in these experiments was independent of NLRP3 inflammasome, but so was IL-1β release. Future studies with Knockout cells or mice (e.g. NLRP3 KO, caspase-1 KO, ASC KO) would be needed to confirm this finding. However, the release of IL-1β may have been dependent on TRIF signalling, as it was only significantly increased in response to 3-MA with the TLR ligand LPS and not with the TLR2 and TLR7/8 ligands PAM3CSK and R848, respectively, which signal through MyD88. It is notable that, at 10 mM 3-MA, IL-1β levels did increase in PAM3CSK and R848 treated cells, albeit not significantly, and at lower levels to those seen with LPS. This could suggest that the process is not solely TRIF-specific, or alternatively, could be due to contamination of the reagents with LPS.

How IL-1β is released in response to LPS + 3-MA remains to be elucidated. One possibility is that another in inflammasome is activated, such as the AIM2 inflammasome, as previous studies have shown that autophagosomes sequester AIM2 and ASC for lysosomal degradation (Liu et al., 2016b, Shi et al., 2012). Another possibility is that treatment of cells with LPS + 3-MA induces cell death and rupture, leading to passive release of IL-1β. While previous studies have suggested that this is not a major route of IL-1β in this setting (Lee et al., 2016c, Harris et al., 2011), our lab's recent work did demonstrate significant cell death in iBMM treated with LPS + 3-MA, leading to increased IL-1β and MIF (Dankers et al., 2020). Interestingly, MIF has been shown to be required for NLRP3 activation (Lang et al., 2018). In this context, it would be interesting to see if IL-10 in these experiments is affected by MIF inhibitors/MIF Knockout.

Interestingly, a study published this year reported that treatment of peripheral blood mononuclear cell (PBMC)-derived M2 like macrophages with rapamycin inhibited *IL10* mRNA expression, as well as intracellular protein levels. Moreover, this inhibition could be reversed with 3-MA, suggesting the opposite effect to that seen here (Maneechotesuwan et al., 2021). The difference could be due to the different experimental settings, particularly the conditions used to generate the M2 macrophages (treatment with IL-/13). It should also be noted that IL-10 release from cells was not measured in the study. Nonetheless, as with my data in iBMM, this study highlights the potential complexity of autophagic regulation of IL-10, which may be highly context-dependent.

Autophagy may regulate IL-1β through different intracellular mechanisms, including targeting inflammasome components and IL-1β itself (Shi et al., 2012, Harris et al., 2011), effects directed at the protein level. In the present study, I have shown pharmacological inhibition, and induction of autophagy regulates *Il10* mRNA, suggesting possible effects on transcription.

In addition, I have confirmed this in cells following siRNA targeting of *Atg7* and *Becn1* in RAW264.7 macrophages. These results suggest that autophagy regulates IL-10 production upstream of protein production. However, the exact mechanism by which autophagy controls *Il10* mRNA requires further study. It is possible that autophagy regulates a signalling molecule in the TLR pathways upstream of cytokine gene transcription or alternatively regulates a specific transcription factor.

I have tested the effect of autophagy regulating drugs on cytokine release in different TLR stimulated mouse and human macrophages. The autophagy inhibitor 3-MA inhibited IL-10 in both mouse and human macrophages. However, the autophagy inducer Torin 1 showed distinct effects in mouse and human macrophages, increasing IL-10 release in mouse cells, but inhibiting it in human THP-1 in response to R848. Notably, R848 is a TLR7/8 inhibitor, however, mouse TLR8 is non-functional due to the lack of 5 amino acids essential for RNA recognition (Heil et al., 2004, Ostendorf et al., 2020). Whether the presence of functional TLR8 changes the IL-10 response could be further investigated using mouse macrophages that express human TLR8 and/or by knocking down TLR8 in human cells.

A number of transcription family members have been identified as important regulators of IL-10, including specificity protein (Sp), signal transducers and activators of transcription (STAT), interferon regulatory factors (IRF), activator protein (AP), CCATT enhancer/binding protein (C/EBP), cAMP response element-binding protein (CREB), c-musculoaponeurotic fibrosarcoma factor (c-MAF), and nuclear factor κ-B (NF-κB) (Iyer and Cheng, 2012). In both myeloid and lymphoid cell types, STAT3 transcription factors play an essential role in the induction of IL-10 (Iyer and Cheng, 2012, Cuesta et al., 2003, Xu et al., 2009). Similarly, in bone marrow-derived macrophages, IL-27 directly up-regulates *Il10* transcription by activating and recruiting both STAT1 and STAT3 to the IL-10 promoter (Tone et al., 2000, Staples et al., 2007). It has also been demonstrated that IL-10 stimulation in macrophages or stimulation of

DCs by *Mycobacterium tuberculosis* or with lipopeptides and the LcrV antigen of *Yersinia pestis* can primarily activate the TLR2-MYD88 signalling pathway, leading to activation of MAPK and IL-10 transcription (Iyer and Cheng, 2012). Thus, future research is needed to discover where autophagy intersects with these pathways.

A recent study has demonstrated that ablating *Atg7* through Cx3cr1Cre in bone marrow-derived macrophages (BMDMs) increased IL-23 expression, contributing to upregulation of IL-22 expression and enhanced intestinal fibrosis induced by 2,4,6-trinitrobenzene sulfonic acid administration (Mathur et al., 2019), suggesting that impairments in autophagy in BMDMs may cause intestinal inflammation when pro-inflammatory cues are added. In my study, I observed increased pro-inflammatory cytokine release in response to i.p. injection of LPS; in particular, peritoneal levels of IL-1α, IL-1β, IL-18, IL-23, IL-17A, IL-12p70, IFN□ and IL-6 were significantly higher in *Atg7*$^{fl/fl}$ LysMCre mice compared to WT animals. In addition, systemic levels of IL-1α, IL-1β, IL-18, IL-17A and IFNγ were significantly increased in *Atg7*$^{fl/fl}$ LysMCre mice in response to LPS compared to WT mice. My results are in line with previous findings that mice lacking the autophagy protein Atg7 in myeloid cells secrete higher levels of different pro-inflammatory cytokines (Castillo et al., 2012). Ablating Atg7 from myeloid cells affected IL-6 locally, but IL-10 was not significantly affected. IL-10 secretion might be regulated by other IL-1 member cytokines (e.g., IL-1α, IL-1β and IL-18). Future studies looking at larger proteomics (large scale multiplex assays) would be useful to build a picture of the different cytokines affected by autophagy (or loss of autophagy)

In this study, I have shown that disruption of autophagy has an important impact on cytokine modulation, impacting both IL-6 and IL-10 secretion. It would be interesting to further assess the factors and the signalling pathways that are involved in the autophagy-mediated regulation of these and, potentially, other cytokines yet to be studied. Taken together, my work establishes that autophagy plays a critical role in modulating cytokines in inflammatory settings, adding

to growing evidence that autophagy has context-dependent roles in the production and release of multiple cytokines. Understanding the molecular mechanisms by which autophagy modulates the production, release and/or expression of these regulators of inflammation will advance our understanding of inflammatory immune responses and may lead to novel therapeutic approaches for autoimmune and inflammatory diseases, such as SLE.

Chapter 4: General Discussion

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disorder characterized by the involvement of autoantibodies directed against nuclear antigens, multisystem inflammation and severe clinical manifestations. SLE is predominantly diagnosed in people aged 15–44 years, with women accounting for about 90% of cases (Marder et al., 2015). As reported by the Australian Society of Clinical Immunology and Allergy (ASCIA), SLE affects more than 20,000 people in Australia and New Zealand. According to the Lupus Foundation of America, 1.5 million people in the United States (US), and at least five million people globally, have lupus (Schudrich et al., 2012), and the estimated incidence rate is 16,000 new cases per year. SLE imposes a considerable economic burden. It has been found that in USA, the average annual direct health care expenses of an individual with lupus were US$33,223 (Carter et al., 2016). The mean total costs for people with lupus (including direct and indirect expenditures) can reach $50,000 per year (Meacock et al., 2013, Barber and Clarke, 2017, Carter et al., 2016). Moreover, these numbers could be higher among individuals with lupus nephritis and more severe or active lupus (Carter et al., 2016). In a recent study, it has been reported that lupus low disease activity state (LLDAS) would be associated with reduced health care cost and a mean annual direct medical hospital cost is US$7,413 per patient per year in Australia (Yeo et al., 2020).

SLE has a wide range of clinical manifestations, including symptoms such as fever and malaise, and dermatological, respiratory, haematological, musculoskeletal, renal, cardiovascular, and neurological symptoms (Sciascia et al., 2018, Kamal and Khamashta, 2014, Rahman and Isenberg, 2008). Relapses and flares with alternating periods of remission are distinctive characteristics of SLE. As a result, the management of SLE is challenging. For many years, nonsteroidal anti-inflammatory medications, glucocorticoids, hydroxychloroquine, and immunosuppressive agents have been the mainstays in the treatment and management of SLE

(Chan et al., 2013). While many of these conventional medicines can be beneficial at controlling inflammation, they are predictably and consistently associated with multiple adverse effects, including diabetes, obesity, glaucoma and cardiovascular disease (Ruiz-Irastorza et al., 2012). Unfortunately, this makes treatment of SLE, a chronic inflammatory autoimmune disease, all the more challenging. Therefore, there is an urgent need for new therapeutic approaches to treat SLE.

The pathophysiology of SLE is highly complex. SLE is distinguished by the presence of numerous cellular and molecular dysregulation in the immune system, such as leukocyte activation and cytokine imbalance. There is evidence of a breakdown in B cell and T cell tolerance in SLE, and many aspects of B cell biology have been linked to its pathogenesis (Nashi et al., 2010). A distinctive feature of SLE is a disruption in B cell tolerance that results in the generation of autoantibodies that target self-antigens including extractable nuclear antigens (ENA) and DNA. Although the role of B cells in the initiation and perpetuation of SLE is complex, it is likely that they assist in the priming of autoreactive T cells, serve as antigen-presenting cells (APCs), and are primary source of inflammatory cytokines that contribute to immune dysregulation in SLE.

Several drugs that have been tested in SLE clinical trial, target B cell pathways (Chan et al 2013) (Chan et al., 2013). These therapeutic agents follow different approaches, ranging from targeting B cell-specific surface molecules (for example, CD20 or CD22) to constrain B cell survival by influencing several cytokines and signalling proteins, such as IL-6, IL-17, IL-21 and BAFF, as well as interfering with B cell antigen presentation via targeting co-stimulatory molecules (Murphy and Isenberg, 2019). However, these biological therapies have shown only minimal success in clinical trials. Thus, a clear unmet need exists for new therapeutics that can target important molecular mediators in the pathogenesis of SLE.

Autophagy is a fundamental essential lysosomal degradation system that preserves the cytoplasmic homeostasis by removing and recycling the protein aggregates and damaged organelles (Yim and Mizushima, 2020). Autophagy plays a vital role in activating and regulating several processes in both innate and adaptive immunity. Autophagy-related proteins (ATG) directly contribute to pathogen clearance through selective autophagy of microorganisms, coordinated responses with pattern recognition receptors, inflammasome formation, antigen presentation, and LC3-associated phagocytosis (Germic et al., 2019). In adaptive immunity, autophagy modulates antigen processing and presentation and regulates lymphocyte development (Arbogast and Gros, 2018). Accumulating evidence supports the hypothesis that dysfunctional autophagy may contribute to the pathogenesis of SLE (Jones et al., 2013, Clarke et al., 2015, Martinez et al., 2016, Allison, 2016, Lee et al., 2016c).

It has been reported that autophagy is activated in myeloid cells, particularly macrophages, of mice with lupus-like autoimmunity (Li et al., 2014). In the present set of study, I investigated the role of myeloid cell autophagy in the development of murine lupus by crossing autophagy-deficient *Atg7*$^{fl/fl}$LysMCre mice with *Lyn*$^{-/-}$ mice. In Chapter 2, I investigated the net effect of myeloid cell autophagy in the development and severity of autoimmunity in these *Lyn*$^{-/-}$ mice. In particular, I studied whether autophagy deficiency in myeloid cells regulates cytokine release, autoantibody production, B cell development and kidney pathology. In chapter 3, I further investigated the role of autophagy in regulating the key immunoregulatory cytokines IL-6 and IL-10, both of which have known roles to play in SLE pathogenesis. The major findings of my thesis are-

- Loss of myeloid cell autophagy decreases specific autoantibody production in *Lyn*$^{-/-}$ mice.
- Ablating autophagy in myeloid cells significantly reduces IL-6 and IL-10 in *Lyn*$^{-/-}$ mice.
- Autophagy regulates both IL-6 and IL-10 production in macrophages. Specifically;

- Autophagy and/or beclin-1 have a complex role to play in IL-6 release in response to TLR agonists, and;
- Loss of autophagy decreases the production of IL-10, whereas activation of autophagy increases IL-10 release in macrophages.

4.1 Myeloid cell autophagy regulates autoimmunity in a mouse model of lupus.

Lyn is a non-receptor tyrosine kinase of the Src family present predominantly in myeloid cells and B lymphocytes. Lyn is thought to be involved in the initiation of ITAM-receptor-mediated signalling (Mkaddem et al., 2017) and contributes to the development of autoimmune disease in Lyn deficient lupus mice (Brodie et al., 2018). In a genome-wide association study, two SNPs of Lyn (rs7829816 and rs2667978) demonstrated significant association in some SLE cohorts (Harley et al., 2008). The *Lyn* deficient mouse model is a single gene defect model, mice lacking *Lyn* progressively develop autoimmunity symptoms that closely resemble human SLE (Hibbs et al., 1995). In particular, *Lyn* deficiency in mice results in progressive autoimmunity characterized by autoantibody production, lymphocyte activation, splenomegaly, systemic inflammation, IgG immune complex deposition, and glomerulonephritis (Hibbs et al., 1995). *Lyn* deficient B cells are hyperreactive to antigen receptor activation, leading in abnormal B-cell selection and/or tolerance and self-reactive antibodies production (Xu et al., 2005, Chan et al., 1997). Notably, *Lyn* deficient B cells produce high levels of IL-6, which in turn drives autoantibody production (Tsantikos et al., 2010). *Lyn* depleted myeloid cells are also hyperresponsive to different stimuli, and several studies revealed its association with the pathogenesis of SLE (Scapini et al., 2009, Scapini et al., 2010, Charles et al., 2010). For example, overproduction of BAFF by *Lyn* deficient myeloid cells is critical in this model to sustain B-cell and activation of T-cell (Scapini et al., 2010). Importantly, in this model the SLE-like disease is characterised by autoantibody production as

well as pathogenic interactions between myeloid cells and T cells that result in inflammation, progression of the disease, and development of glomerulonephritis (Scapini et al., 2010). In agreement with these previous studies on *Lyn* deficecy (*Lyn*$^{-/-}$), I found these animals developed SLE-like symptoms including splenomegaly, systemic cytokine release, production of antinuclear antibodies (ANA), and glomerulonephritis. Moreover, I have shown that loss of the autophagy protein Atg7 in myeloid cells influences specific aspects of disease development.

Autophagy is essential for metabolic response to the nutrient deficit as well as the breakdown of damaged or malfunctioning proteins and organelles. Importantly, autophagy also regulates inflammatory signalling pathways in myeloid cells as well as the production and release of multiple cytokines (Harris, 2011, Mills et al., 2013). While autophagy in T and B cells has been studied in human SLE (Yang et al., 2015, Clarke et al., 2015, Liu et al., 2016c, Yin et al., 2018, Qi et al., 2019, Noguchi et al., 2020), the role of myeloid cell autophagy in the regulation of SLE has not been clearly elucidated. Thus, to investigate this further, I crossed *Lyn*$^{-/-}$ C57BL/6 mice with *Atg7*$^{fl/fl}$ x LysMCre$^{+/-}$ mice to create double-knockouts. This allows me to investigate the effect of inhibiting myeloid cell autophagy on the development of autoimmunity in this model. I hypothesised that loss of autophagy in myeloid cells might increase inflammatory responses, particularly with regard to the release of cytokines, such as MIF and IL-1 family members, as has been shown previously in other inflammatory contexts both *in vitro* and *in vivo* (Lee et al., 2016c, Harris, 2013).

Pathogenic autoantibodies are a hallmark of SLE and have been shown to play a necessary role in many of the manifestations of the disease (Nashi et al., 2010). In SLE, autoreactive B cells survive and proliferate to form plasma cells producing high-affinity autoantibodies (Liu and Davidson, 2012). In chapter 2, I demonstrated that loss of myeloid cell autophagy resulted in significantly reduced production of anti-SM, anti-dsDNA and anti-histone autoantibodies in lupus mice aged between 120 to 250 days. Other autoantibodies such as anti-CENP-B, anti-Jo-

1, anti-pm-Scl, anti-Ribosomes, anti-Scl-70, anti-SMRNP, anti-TRIM21 and anti-U1RNP also non-significantly reduced in this group of mice. Interestingly, this effect was diminished as these mice got older (>250 days), suggesting that the effects of autophagy ablation are either superseded by the disease progression in this model or autoantibody production becomes more generally exhausted with age.

In a previous study, *Atg7*$^{fl/fl}$ LysMCre mice were found to spontaneously produce higher levels of anti-dsDNA autoantibodies after 52 weeks (Martinez et al., 2016). However, we did not observe a significant difference in autoantibodies between the WT and *Atg7*$^{-/-}$ *Lyn*$^{+/+}$ ("non-lupus") animals. In the previous study, only three mice per group were studied, while I have included many more (n≥30 per group for aged mice and n=10 per group for young mice) (Martinez et al., 2016), and it might be a reason for the difference observed. Alternatively, differences in animal age, sex and housing between the sites involved may play a role. However, I did observe altered B cell populations in myeloid cell-specific *Atg7*$^{-/-}$, suggesting the potential for aberrant autoantibody production under the right circumstances. Further work examining this effect, including in other tissues such as bone marrow, would be of interest. The mechanism by which myeloid cell autophagy impacts autoantibody production in this model is not clear yet, but I can propose a number of possible roles:

4.1.1 Myeloid cell autophagy impacts B cell development/differentiation

In chapter 2, I have shown that myeloid-specific *Atg7*-deficient mice show differences in B cell populations compared to WT controls. In particular, numbers of plasma cells, germinal centre (GC) B cells, isotype-switched B cells and non-isotype switched B cells were all increased in myeloid-specific *Atg7*$^{-/-}$ mice. Conversely, numbers of splenic B cells and non-GC B cells were lower in the *Atg7*$^{fl/fl}$ mice. These findings indicate that autophagy deficiency in myeloid cells can significantly alter B cell populations in mice. To my knowledge, this is the first study to

demonstrate the effect of myeloid cell autophagy on B cell development/differentiation in a disease-free setting. This is potentially important, as a previous study has shown that that ablation of autophagy in myeloid cells can induce spontaneous autoimmunity, including autoantibody production, due to defects in efferocytosis by myeloid cells (Clarke et al., 2015) (discussed below). My data may suggest that this effect is not solely due to defects in efferocytosis but might also involve differences in B cell differentiation.

A previous study has shown that autophagy plays an essential role in plasma cell homeostasis, and enhanced autophagy has been observed in T and B cells from lupus mice and in PBMCs from SLE patients (Nishizumi et al., 1995). However, In the *Lyn*$^{-/-}$ SLE model, I did not see any significant difference in B cell subsets between aged *Lyn*$^{-/-}$ mice and double knockout (*Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$) mice (>30 weeks), suggesting that loss of myeloid cell autophagy does not impact significantly on B cell populations during disease. Numbers of plasma cells were decreased in younger (12-15 weeks) double knockout mice, compared to *Lyn*$^{-/-}$, which may indicate a role for myeloid cell autophagy on B cell-mediated events early on in disease development, but the effect did not correlate with autoantibody production and was no longer apparent in the older mice when autoantibody levels were different. Overall, this would suggest that changes in B cell development are unlikely to be responsible for the effect I observed on autoantibody levels.

4.1.2 Plasma cell autoantibody production directly influenced by changes in cytokine levels

Several studies have demonstrated that Aged *Lyn*$^{-/-}$ mice display increased production of inflammatory cytokines, including BAFF, IFN-γ, IL-6 and IL-10 (Tsantikos et al., 2010, Scapini et al., 2010, Scapini et al., 2011). My data is largely consistent with these studies, with serum levels of BAFF, IL-6, and IL-10 significantly increased in Lyn deficient mice compared to WT animals. In addition, I observed increased levels of IL-18 and IL-17A but did not see an

increase in IFN-γ. Interestingly, both IL-6 and IL-10 were significantly lower in double knockout *Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$ mice than Lyn-deficient animals, suggesting that loss of myeloid cell autophagy significantly impairs production and/or release of these cytokines. Importantly, IL-6 and IL-10 both play a crucial role in autoantibody production and B cell activity. IL-6 can promote the differentiation of B cells into plasma cells, and IL-10 effectively stimulates B cell activation, proliferation and differentiation (Suematsu et al., 1989, Iyer and Cheng, 2012). As already noted in chapter 2, I did not see significant differences in plasma cell numbers in *Atg7*$^{fl/fl}$ *Lyn*$^{-/-}$ mice compared to the *Lyn*$^{-/-}$ animals. However, IL-6 has been proposed to drive autoantibody production in *Lyn*$^{-/-}$ mice (Tsantikos et al., 2010). Moreover, a previous study demonstrated that, although the number of B lymphocytes from SLE patients was unaffected by treatment with anti-IL-10 mAb *ex vivo*, the production of antibodies by these cells was inhibited (Llorente et al., 1995). Similarly, in a SCID mouse model (lupus mice), anti-IL-10 mAb significantly decreased autoantibody production (Llorente et al., 1995). Taken together, the reduction of serum IL-6 and/or IL-10 in *Lyn*$^{-/-}$ *Atg7*$^{-/-}$ mice compared with *Lyn*$^{-/-}$ mice may explain the reduced autoantibody production observed in the same mice.

In the non-lupus mice, it is notable that serum IL-18 was increased in the *Atg7*$^{fl/fl}$ mice. Previous studies have shown that loss of autophagy can increase the release of IL-1 family cytokines, including IL-1α, IL-1β and IL-18, in response to specific stimuli (Xu et al., 2019a, Harris, 2013). I did not see a similar increase in systemic IL-1α here, and IL-1β was not detected. In this context, it would be interesting to investigate the local release of IL-1α, IL-1β and IL-18, particularly in the kidneys, where the disease is particularly conspicuous. Regardless, IL-18 is of interest in the study of lupus disease progression, as it has been implicated in pathogenesis both in mouse models and human disease (Hoshino et al., 2001, Mende et al., 2018). Furthermore, a positive correlation between plasma cell proliferation and IL-18 was observed in a previous report (Tsirakis et al., 2013), which may help understand why the loss of myeloid

cell autophagy was associated with B cell and plasma cell population changes. However, cell numbers were not different between the *Lyn*$^{-/-}$ and double knockout animals. Future research should consider the potential effects of IL-1 or IL-18 depletion on immune response using specific antagonists/neutralising antibodies or specific knockout mice.

4.1.3. Myeloid cell autophagy affects efferocytosis and antigen presentation

In lupus patients, substantial amounts of apoptotic cells accumulate in different tissues, including germinal centres (Shao and Cohen, 2011). Efferocytosis is important for the clearance of dead cells, which could otherwise accumulate, rupture and lead to exposure to multiple autoantigens. Two nuclear antigens, including double-stranded DNA (dsDNA) and the Sm antigens of the U-1 small nuclear ribonucleoprotein complex, are considered pathognomonic of SLE (Riemekasten and Hahn, 2005). Autoantigens are produced mostly from secondary necrotic cells due to improper apoptotic cell clearance or inadequate breakdown of DNA-containing neutrophil extracellular traps (NETs) (Mahajan et al., 2016). Follicular dendritic cells present these modified autoantigens to autoreactive B cells in the germinal centres of secondary lymphoid organs (Mahajan et al., 2016). These are the driving forces for breaking self-tolerance and generating autoantibodies, which are common features of SLE. Clarke et al (2015) demonstrated that ablation of autophagy in myeloid cells could induce spontaneous autoimmunity, including autoantibody production, as a result of defects in efferocytosis (Clarke et al., 2015). However, this would not explain why the loss of autophagy is protective against the development of autoantibodies seen in this studiy, as we would expect decreased autophagy to correlate with decreased efferocytosis and thus increased autoantigen exposure. It may also be possible that in the *Lyn*$^{-/-}$ mice, cell death already outweighs efferocytosis capacity, so further impacts due to loss of autophagy are inconsequential. This may instead point to the effects of myeloid cell autophagy on autoantigen processing and/or presentation or cytokine production.

Antigen presentation is crucial for the initiation of an immune response that ensures adequate pathogen removal from the infected host (Patterson and Mintern, 2012). Major histocompatibility complex (MHC)-restricted antigen presentation is essential to the specificity of immunity and marks an important interaction between innate and adaptive immune pathways (Merkley et al., 2018). Studies have shown that pharmacological and genetic inhibition of autophagy decreases efficient MHCII presentation of endogenous antigens (Paludan et al., 2005, Münz, 2016). It has been found that autophagosomes fused directly with MHC II loading compartments (Kasai et al., 2009, Schmid et al., 2007). Furthermore, fusing viral and tumour antigens to the ATG8 (autophagy-related gene 8) family protein LC3-II, which is found on autophagosomal membranes, improves presentation to CD4+ T cells (Schmid et al., 2007, Fonteneau et al., 2016, Jin et al., 2014). Autophagy has also been implicated in MHC I cross-presentation of exogenous antigens (Li et al., 2008, Mintern et al., 2015). However, the role of autophagy in MHC I presentation is still controversial. MHC I presentation is based on proteasomal antigenic peptide processing and peptide transport from endosomal compartments to the cytosol, while autophagy transports cytosolic components to endocytic compartments. Autophagy substrates may be redirected to proteasomes for processing and MHC I-restricted presentation when autophagy is disrupted (Wenger et al., 2012). In a recent study, it was reported that there was a reduction in autoreactive CD4+ T cells in the absence of *ATG5*, which prolonged the initiation of disease and decreased clinical severity relative to mice expressing *ATG5* in DC (Keller et al., 2017). Thus, defective autoantigen processing and/or presentation in autophagy-deficient myeloid could explain why autoantibody production is reduced in $Atg7^{-/-}$ $Lyn^{-/-}$ mice. Further work is needed to investigate whether this is a mechanism at play here. If so, a key and particularly interesting question is why it applies only to specific autoantigens/autoantibodies. Autophagy deficient mice

inoculated with specific infectious agents such as Polyoma virus, or Pneumococcal cell wall polysaccharide could be used to address this question.

In sum, my results suggest that autophagy deficiency in myeloid cells may exert a protective role against the development of specific autoantibodies in SLE. This might be linked to a reduction of MHC class II-restricted autoantigen presentation in autophagy-deficient myeloid cells (Paludan et al., 2005) and/or class II dependent B cell activation. This provides new insight into the complex role of autophagy in the development of autoimmunity and inflammatory processes. A better understanding of the mechanisms at play may ultimately lead to new therapeutic strategies.

4.2 Autophagic regulation of cytokines relevant to SLE

Numerous studies have shown the association of multiple cytokines in the pathogenesis of SLE, including TNFα, IFN-γ, IL-1α, IL-1β, IL-10, IL-17, IL-18, IL-23, MIF, all of which have been demonstrated to be regulated by autophagy (Merkley et al., 2018, Rönnblom and Elkon, 2010, Harris et al., 2011, Harris et al., 2017). Here, I investigated the production of various cytokines in response to inflammatory stimuli in normal and autophagy-deficient myeloid cells, particularly monocytes/macrophages. A better understanding of the regulation of these cytokines could provide a valuable perspective to the pathogenic mechanisms underlying human SLE, paving the way toward more effective therapeutic strategies.

In chapter 2, I showed that loss of autophagy in myeloid cells in a mouse model of lupus-like autoimmunity resulted in lower serum cytokines IL-6 and IL-10. This led to a more in-depth study of the role of autophagy in regulating IL-6 and IL-10 release by monocytes/macrophages in response to different TLR agonists *in vitro* in Chapter 3. In addition, I measured cytokine release in an *in vivo* mouse model of LPS-induced endotoxemia.

I hypothesise that myeloid cells may not be the major source of the IL-6 seen in the *Lyn*$^{-/-}$ mice as IL-6 release by macrophages was not directly affected by loss or induction of autophagy *in vitro.* Instead, as has already been reported, it may be predominantly B-cell derived (Chien and Chiang, 2017). The reduction observed in the double knockouts may be due to regulation of this response, possibly through regulation of other cytokines (such as IL-10). Or it may be that the myeloid cells behave different *in vivo* to *in vitro* due to all the other things being affected. IL-6 is a pleiotropic cytokine that has been associated with antigen-specific immune responses and inflammatory reactions (Cui et al., 2015, Kishimoto, 2006). This cytokine promotes the maturation of B lymphocytes and enhances Ig secretion (Yap and Lai, 2010). IL-6 has been shown to be associated with SLE disease activity (Linker-Israeli et al., 1991, Liu et al., 2013b), suggesting its potential as a biomarker and, in some cases, therapeutic target. In chapter 2, I demonstrated that serum IL-6 was significantly increased in *Lyn* deficient mice compared to WT mice, but not in autophagy-deficient Lupus mice (*Lyn*$^{-/-}$ *Atg7*$^{-/-}$), indicating, deficiency of myeloid cell autophagy impairs production and/or release of IL-6 from cells. In chapter 3, I further examined whether autophagy directly regulates the release of IL-6 in mouse macrophages in response to the TLR4 agonist LPS or the TLR7/8 agonist R848. I found that, in RAW264.7 macrophages, 3-MA had contrasting effects, but Torin 1 inhibits LPS-induced IL-6 release after 6 hours. However, after 24 hours, all these effects are lost as TLR stimulation has primary and secondary effects depending on treatment time. Both 3-MA and Torin-1 increased IL-6 in iBMM cells in response to R848, indicating that it is not an autophagic response. In primary cells, similar findings were obtained, and Atg7 KO did not reduce IL-6 in response to 24 h LPS and R848 treatment.

Furthermore, I found that IL-6 release was substantially reduced in cells transfected with siRNA against Becn1 in response to TLR7/8 agonist R848. This is most likely an autophagy-independent response to R848 by beclin1. Beclin1 has been shown to a key regulator of

autophagy and endocytosis (Noguchi et al., 2019, Ruck et al., 2011). TLR7/8 are endosomal innate immune sensors and, therefore, associated with endosomal trafficking (Petes et al., 2017). Thus, the results indicate that Becn1 may control IL-6 release via the endocytic pathway. However, how Becn1 controls the level of IL-6 through the endocytic pathway in response to TLR7/8 stimulation is still unknown, and further research is needed to confirm these novel findings by using IL-6 deficient mice. The results suggest that autophagy can play a complex and context-dependent role in the release of IL-6 from macrophages in response to various inflammatory stimuli, which could explain the IL-6 effects seen in Lyn-/- mice.

IL-10 has been found to play a crucial role in the growth, survival, differentiation, and function of B cells (Peng et al., 2013). Abnormal increase of IL-10 synthesis appears to contribute to the spontaneous hyperactivity of the B cell compartment, which may directly result in autoantibody generation by committed plasma cells, formation of immune complexes and eventually tissue and organ damage, key features of SLE pathogenesis (Peng et al., 2013, Facciotti et al., 2020). As discussed in chapter 2, the IL-10 level in the serum of *Lyn* deficient mice was increased compared to WT mice but decreased in the serum of autophagy-deficient Lupus mice (*Lyn*$^{-/-}$ *Atg7*$^{-/-}$). Based on the findings in chapter 2, I examined whether loss of autophagy in myeloid cells altered IL-10 responses *in vitro* in human monocytes and mouse macrophages. I found that the autophagy inhibitor 3-MA significantly inhibited IL-10 secretion by RAW264.7 macrophages. On the other hand, autophagy induction by the mTOR inhibitor Torin-1 increased IL-10 production in the same cells. Similarly, transfection of macrophages with siRNA against the autophagy genes *Becn1* (encodes Beclin 1) or *Atg7* resulted in significantly lower IL-10 secretion in response to different TLR agonists. I repeated the experiments and confirmed similar findings in THP-1 and BMM cells. Thus, the results suggest that autophagy promotes IL-10 release by macrophages.

Consequently, to investigate whether autophagy regulates the production of IL-10 at the transcriptional level, I measured the level of *Il10* mRNA in LPS stimulated RAW264.7 cells in response to 3-MA or Torin-1. The results demonstrated in chapter 3 suggest that autophagy significantly regulates the production of IL-10 at the transcriptional level, either through a direct effect on transcription or upstream of it, somewhere in the TLR signalling pathway. Understanding this transcriptional regulation of IL-10 by autophagy may help in the development of new strategies to control IL-10-mediated pathologies. Several studies have demonstrated that changes in autophagy activity could be associated with alteration in *Il10* transcription, which is primarily regulated by transcription factors Sp1 and Sp3; induction of autophagy may downregulate Sp1 protein expression (Tone et al., 2000, Brightbill et al., 2000, Kim et al., 2017, Maneechotesuwan et al., 2021). However, our results are in contrast with a recent study in which siRNA-mediated knockdown of LC3 in macrophages showed a maximal induction of *IL-10* transcription (Maneechotesuwan et al., 2021), as well as inhibition of autophagy, promotes IL-10 production, which reduces asthmatic inflammation. The difference may be caused by the different experimental settings, particularly the conditions under which M2 macrophages are produced. Additionally, this may be attributed to the possible complex and context-dependence regulation of IL-10 by autophagy. Thus, the potential mechanisms underlying the autophagic regulation of *Il10* transcription is not clearly understood and deserves further investigation.

The results found in chapter 3 may explain the reduced levels of IL-10 seen in the double knockout mice, suggesting that a significant proportion of the IL-10 released in this model may be myeloid-derived. This highlights a clear mechanism by which myeloid cell autophagy can impact disease progression and directly affect responses of other cells – for example, B cells – in this model. Another potential source of IL-10 in the disease could be Treg cells (Chaudhry et al., 2011). However, the proportions of Tregs (analysed by flow cytometry) were unchanged

in this model, suggesting that Tregs were not directly affecting the relative amounts of IL-10 secretion in these mice.

Thus, our findings from chapter 2 and 3 demonstrated that autophagy regulates the production and release of both IL-6 and IL-10, suggesting that disruption of autophagy has an important impact on inflammatory cytokine modulation. These findings reveal an important function for myeloid cell autophagy in regulating signalling pathways and inflammatory responses and shed light on the development of autophagy-targeted therapeutics for SLE. In addition to this, I further investigated autophagic regulation of inflammatory cytokines in response to an acute inflammatory challenge *in vivo* using WT and *Atg7*$^{fl/fl}$ LysMCre mice. In peritoneal lavage, the level of IL-1 family cytokines IL-1β and IL-18, and IL-23 as well as IL-17A, IFN-γ and IL-12p70 level were significantly higher in the peritoneal lavage fluid of *Atg7*$^{fl/fl}$ LysMCre mice in response to LPS, compared to WT animals, suggesting that autophagy depletion may regulate the production, processing and/or secretion of these cytokines.

In chapter 2, using the *Lyn* deficient mouse model of lupus, I have shown that *Lyn* deficient lupus mice produce a high level of cytokine IL-6, whereas deficiency of myeloid cell autophagy impairs production and release of IL-6. In contrast, in my LPS-induced endotoxemia model, I found that levels of IL-6 significantly increased in the peritoneal lavage fluid and in serum of autophagy knockout (*Atg7*$^{fl/fl}$ LysMCre) mice compared to the WT mice. This different role for myeloid cell autophagy in IL-6 release could be due to differences in the initiating inflammatory stimuli between the two models and/or the acute *vs* chronic nature of endotoxemia, compared to *Lyn*$^{-/-}$ autoimmunity. It has been demonstrated that IL-6 expression could be regulated by the activity of other pro-inflammatory cytokines, such as tumour necrosis factor α and interleukin-1β (Yang et al., 2004). In my endotoxemia model, IL-1β release was significantly increased in the *Atg7*$^{fl/fl}$ LysMCre mice; this may, in turn, drive the increased

release of IL-6 and other cytokines, including IL-23, as previously demonstrated (Peral de Castro et al., 2012).

Interestingly, in peritoneal lavage and serum, levels of IL-10 were not significantly different between *Atg7*$^{fl/fl}$ LysMCre and WT mice, whereas IL-10 level was significantly decreased in *Atg7*$^{-/-}$ *Lyn*$^{fl/fl}$ mice, compared to *Atg*$^{+/+}$ *Lyn*-/- animals. Again, this may reflect differences in IL-10 release and/or the role of myeloid cell autophagy in acute *vs* chronic inflammation and/or in response to different inflammatory stimuli. Other IL-1 family cytokines (e.g. IL-1α, IL-1β, and IL-18) can regulate IL-10 secretion.

IL-10 is an essential immune-regulatory cytokine with both pro-and anti-inflammatory activity (Hedrich et al., 2014). The role of IL-10 in the pathogenesis of SLE remains likely complex multi-factorial (Yap and Lai, 2010). A large body of evidence has shown that IL-10 inhibits macrophages and T cell activation and reduces pro-inflammatory cytokines production in immune cells (Joss et al., 2000, McBride et al., 2002, Moore et al., 2001, Schuetze et al., 2005, Wang et al., 2005). However, it has also been demonstrated that IL-10 contributes to B cell survival, proliferation, differentiation and antibody production through its B cell-stimulating abilities (Ouyang et al., 2011) and decreased auto-reactive B-cell apoptosis by enhancing Bcl-2 expression, leading to excessive autoantibody production in SLE (Levy and Brouet, 1994, Llorente et al., 1995, Godsell et al., 2016). Thus, depending on timing and context, IL-10 may be both protective and harmful in SLE. Here, I have similarly demonstrated a high level of IL-10 in the serum of *Lyn* deficient mice, while a lack of autophagy in myeloid cells appears to suppress IL-10 production. In parallel, I observed decreased autoantibody production and IL-6 release. However, measures of disease, including kidney damage, splenomegaly and body weight were not significantly different between *Lyn*$^{-/-}$ and *Lyn*$^{-/-}$ *Atg7*$^{fl/fl}$, suggesting no net effect of myeloid cell autophagy on disease in these mice Moreover, this would suggest that autoantibody levels (at least those influenced by myeloid cell autophagy) also do not

significantly alter disease outcomes in this model. Future studies looking at other models of lupus are required to determine whether these findings apply more generally or are specific to the *Lyn*$^{-/-}$ model.

In the Lyn model, loss of autophagy was not obviously pro-inflammatory, as we might have expected. However, it is worth noting that it was also not anti-inflammatory – IL-10 is generally anti-inflammatory, while IL-6 has both pro- and anti-inflammatory actions. So basically, no major effect on the inflammatory state. However, in the LPS model, loss of autophagy was very obviously pro-inflammatory (increased IL-1, IL-23, IL-12, IL-17A, IFNγ and IL-12p70). This confirms previously published data. So, in the context of TRIF signalling (e.g. TLR3/TLR4 agonists), autophagy is anti-inflammatory, but for other stimuli, it may have different or no effects. The Lyn model is not a TLR3/4 dependent one, so this would explain why the loss of autophagy was not pro-inflammatory. However, our mice are housed in SPF conditions, so they have minimal exposure to bacterial/viral infections (which would trigger TLR3/4-dependent signalling activation). In the real world, patients with lupus are exposed to infections, and in some cases, these may be linked to worse disease. In fact, many have postulated that chronic infections might trigger SLE (Riemekasten and Hahn, 2005, Doria et al., 2008, Kaul et al., 2016, Qiu et al., 2019). Viral or bacterial infections can activate the innate immune system that promotes the activation and expansion of autoreactive B and T cells (Riemekasten and Hahn, 2005). Infectious agents, such as EBNA-1 and EBNA-2 of EBV (Sundar et al., 2004), Pneumococcal cell wall polysaccharide (Sharma et al., 2001) and HIV (González et al., 1996) can generate autoantibodies against lupus autoantigens. It has been demonstrated that purified infectious BK virus inoculation in rabbits resulted in a break of immune tolerance, leading to the production of autoantibody against highly conserved antigens (Flaegstad et al., 1988). So, it may be that our model is missing a vital component – infections. Further studies could look at mouse models with the introduction of specific pathogens and see

whether loss of autophagy in this context does lead to increased inflammation and, ultimately, worse disease.

Previous studies have shown that both IL-6 and IL-10 can promote the differentiation of B cells into plasma cells (Suematsu et al., 1989, Iyer and Cheng, 2012). In this study, I have confirmed that autophagy has a consistent and direct effect on IL-10 secretion. Coupled with the lack of an effect on IL-10 producing Treg populations, this may suggest that the decrease in IL-10 observed in the *Atg7*$^{fl/fl}$ LysMcre x *Lyn*$^{-/-}$ mice is primarily due to decreased secretion by myeloid cells, although I cannot at this stage rule out the possibility that other cells may also be involved in this response. The role of autophagy in regulating IL-6 secretion by macrophages in vitro, on the other hand, is less clear and requires further investigation. Whether the observed decrease in serum IL-6 observed in the *Atg7*$^{fl/fl}$ LysMcre x *Lyn*$^{-/-}$ mice is due to reduced secretion by myeloid cells or is instead driven by other cells (perhaps in response to decreased IL-1 release), remains unclear. Nonetheless, both cytokines are clearly downregulated in *Atg7*$^{fl/fl}$ LysMcre x *Lyn*$^{-/-}$ mice and this may, in turn, contribute to the decreased autoantibody production observed.

Thus, the data are telling us that the role of autophagy in specific mechanisms of lupus pathology are perhaps more nuanced (effects on plasma cells, IL-6, IL-10 and specific autoantibodies) than in an acute inflammation model. However, more work is needed to understand the many possible roles of autophagy in SLE. Nonetheless, my work has highlighted biologically significant roles in autoantibody production and cytokine release that are important for understanding myeloid cell autophagy health and disease.

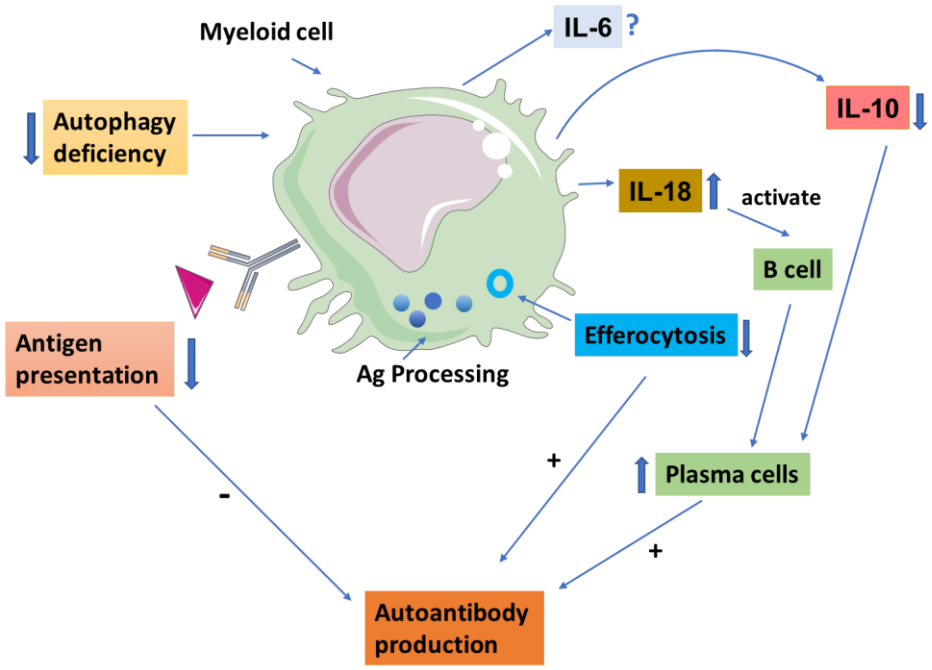


Figure 4.1 Proposed roles of myeloid cell autophagy in mouse models of SLE-like autoimmunity
Mouse model of SLE is characterized by hyperactivated B cells and pathogenic interactions between myeloid cells and T cells that facilitate inflammation, disease development, and nephritis. The inflammatory loop is partly regulated by B-cell activating factor (BAFF) and cytokine production by myeloid cells. IL-10 enhances B cell survival, proliferation, differentiation and antibody production while decreasing apoptosis of auto-reactive B-cell via enhancing Bcl-2 expression, leading to increased autoantibody production in SLE. Ablating autophagy in myeloid cells decreases IL-10 production and antigen presentation but stimulates IL-18 secretion. As a consequence, changes in cytokines level may directly influence autoantibody production by plasmablast differentiation.

4.3 Significance

Despite advances in overall disease management, there is a substantial unmet medical need for new therapies in SLE. My work provides new mechanistic insights into the role of myeloid cell autophagy in the pathogenesis of SLE. I have shown that myeloid cell autophagy blockade could have specific effects on pathogenesis, evidenced by decreased anti-dsDNA, anti-histone, and anti-SM autoantibody production. This is coupled with reduced production of IL-6 and IL-10. While these effects did not ultimately correlate with reduced disease, they highlight important ways in which myeloid cell autophagy might contribute to pathology in human SLE. I have also demonstrated an important role for autophagy in plasmablast differentiation at the

early stage of the disease. In SLE, the plasmablast population is often markedly expanded and is correlated with disease activity (Odendahl et al., 2000). I observed a reduced number of plasma cells in young mice, and it was previously reported that pharmacological inhibition of autophagy restricts human plasmablast differentiation *in vitro* (Clarke et al., 2015). These effects are all worthy of further investigation, both in different models of SLE and in SLE patients, to fully understand their potential significance. Importantly, my work highlights a previously unrecognized role for autophagy in the release of IL-10 from monocytes and macrophages. Further studies are needed to elucidate the precise mechanism(s) involved.

Dysregulation of cytokine is prevalent in SLE, and their protein and gene expression profiles could be used as markers of disease activity and severity of SLE. Biological agents targeting these cytokines could be promising therapies for SLE. My study suggests that autophagy regulates IL-10 cytokine expression, which helps to understand the specific molecular events that regulate the production of this important cytokine. Moreover, my work has expanded on previous studies, demonstrating the (other cytokines).

4.4 Limitations

Lyn kinase-deficient mice are a well-studied model of SLE. Deletion of *Lyn* results in hyperactive B cells and myeloid cells. *Lyn*-deficient mice develop progressive autoimmunity, characterized by dramatic proliferation of myeloid cells as well as B- and T-cell activation, leading to kidney inflammation (Scapini et al., 2010). There are obvious limitations in evaluating therapeutic targets in mouse models of SLE. In *Lyn*-deficient mice, I found that *Lyn* mice developed progressive autoimmunity, characterized by splenomegaly, presence of autoantibody in serum, high level of inflammatory cytokine and glomerulonephritis. In autophagy-deficient double knockout mice, level of autoantibody and cytokine was decreased, suggesting loss of autophagy negatively regulates symptoms of autoimmunity in lupus mice.

However, in some case, the protective role of autophagy in double knockout mice was age-specific; for example, in young mice (age less than or equal to 100 days) and older mice (age more than 250 days), there was no obvious ameliorative effect of autophagy on the level of autoantibody.

In both murine and human lupus, the kidney is a primary site of tissue damage. Deposition of immune complexes in glomeruli may contribute to the development of lupus nephritis (Onishi et al., 2015). In my study, I observed that *Lyn* deficient mice developed severe glomerulonephritis, whereas autophagy deficiency did not have any clear protective impact on the severity of nephritis. Therefore, I assume that autophagy might have a protective effect on glomerulonephritis. Still, at the advanced stage of the disease, due to the severe segmental necrosis in double knockout mice, the impact of autophagy was superseded. Although different mouse models have provided important insights into the pathogenesis of SLE, these models do not completely reflect the full spectrum and complexity of human SLE (Gunawan et al., 2017). There are also obvious differences between the human and mouse immune system, including lymphocyte frequencies (Mestas and Hughes, 2004). Besides, laboratory mice are housed in relatively germ-free conditions, whilst humans are directly exposed to pathogens that activate the immune system in multiple ways, including by TLR engagement and IFN signalling (Shamriz et al., 2016). Thus, to confirm our promising findings that myeloid cell-specific autophagy is a potential therapeutic target, more research is required in SLE patients.

4.5 Future directions

Although I have made some exciting discoveries, several outstanding questions and future challenges remain. More research in this area should include:

- Investigation of the role of myeloid cell-specific autophagy in other autoimmune and inflammatory diseases.

- Assessment of the role of myeloid cell-specific autophagy in human SLE; through investigation of cells *ex vivo*, as well as larger-scale proteomics and transcriptomics studies of inflammatory and autophagosomal markers.
- More detailed investigation of the autophagic regulation of IL-18 in innate and adaptive immune cells and in other animal models of autoimmune disease.
- Investigation of autophagic regulation of other cytokines, chemokines, growth factors, and serum biomarkers using large-scale proteomics.
- Investigation of the transcription factors and signalling cascades that can specifically induce IL-10 in various immune cells and how they are associated with autophagy.
- Identifying compounds that could target autophagy to regulate inflammatory activity with potential for therapeutic development.
- Investigating ways to target autophagy-regulating compounds to specific immune cells, such as monocytes and macrophages.

4.6 Conclusions

In summary, the findings from my study suggest that autophagy deficiency in myeloid cells plays a crucial role in regulating SLE like disease in mice. *Lyn* deficient myeloid cells contribute to the development of lupus disease through the release of multiple cytokines, and ablating autophagy in these cells significantly inhibited IL-6 and IL-10 release *in vivo* and *in vitro*. In addition, loss of autophagy of myeloid cells had knock-on effects on other cells, including plasma cells and led to reduced levels of specific autoantibodies. This work highlights the complex and nuanced role of myeloid cell autophagy in the development of autoimmune phenotypes. In the setting of acute LPS-induced inflammation, loss of autophagy in myeloid cells had a clear pro-inflammatory effect, increasing the release of IL-1 family cytokines, IL-23, IL-12, IL- and IL-17A. This again demonstrates the complexity of autophagy

in the regulation of inflammatory responses in myeloid cells. My findings offer new insight into molecular targets in the search for pathophysiological processes associated with SLE and inflammation, more generally, of potential significance for the future development of immune intervention strategies based on autophagy manipulation.

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Appendix

Appendix I: Publications accepted during candidature

1. Harris, J., Deen, N., Zamani, S. & Hasnat, Md. A. (2018). Mitophagy and the release of inflammatory cytokines. *Mitochondrion* **41**, 2-8. *Citations:* 34

This review article in *Mitochondrion* was written with my supervisor, Dr James Harris and lab colleagues, Nadia Deen and Shahrzad Zamani. I contributed to sections on Mitochondrial damage, autophagy and IL-1 family cytokines, based on aspects of my own literature review.

2. Dankers, W., **Hasnat, Md. A**., Swann, V., Alharbi, A., Lee, J. P. W., Cristofaro, M. A., Gantier, M. P., Jones, S. A., Morand, E. F., Flynn, J. A. & Harris, J. (2020). Necrotic cell death increases the release of macrophage migration inhibitory factor (MIF) by monocytes/macrophages. *Immunology and Cell Biology*. **98**, 782-790. *Citations:* 3

I contributed to a research article published in *Immunology & Cell Biology* from the Rheumatology Research Group. Here, I helped to investigate MIF release by myeloid cells in response to inflammatory stimuli.

3. Nataraja, C., Dankers, W., Flynn, J., Lee, J. P. W., Zhu, W., Vincent, F. B., Gearing, L. J., Ooi, J., Pervin, M., Cristofaro, M. A., Sherlock, R., **Hasnat, Md. A**., Harris, J., Morand, E. F. & Jones, S. A. (2021). GILZ regulates the expression of pro-inflammatory cytokines and protects against end-organ damage in a model of lupus. *Frontiers in Immunology* **12**, 652800.

I contributed to a research article published in *Frontiers in Immunology* from the Rheumatology Research Group. Here, I helped to investigate extractable nuclear antigens (ENA) and serum cytokine expression by Luminex assay in a mouse model of Lupus (Lyn-/-) crossed with *GILZ*$^{-/-}$ mice.

Appendix Ii:

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Review

Mitophagy and the release of inflammatory cytokines

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ABSTRACT

Mitophagy is a selective form of autophagy in which damaged or dysfunctional mitochondria are specifically targeted by autophagosomes for lysosomal degradation. Studies have demonstrated that loss of autophagy/mitophagy can lead to a build-up of cytosolic reactive oxygen species and mitochondrial DNA, which can, in turn, activate immune signalling pathways that ultimately lead to the releases of inflammatory cytokines, including IL-1α, IL-1β, IL-18, type I IFN and macrophage migration inhibitory factor (MIF). Moreover, release of these cytokines can subsequently promote the release of others, including IL-23 and IL-17. Thus, as well as being essential for normal cell homeostasis and mitochondrial health, mitophagy may represent an important regulatory mechanism controlling inflammatory responses in immune cells. This review discusses our current understanding of the mechanisms through which mitophagy regulates inflammatory cytokine release.

1. Introduction

Mitochondria are essential organelles with multiple roles in maintaining cellular homeostasis, not only through energy production, but also by controlling signalling cascades that regulate cell death, differentiation and aging (Lazarou 2015). Mitochondrial DNA encodes 13 polypeptides that assemble with nuclear gene products to form the respiratory chain, which drives ATP production via oxidative phosphorylation (Rich and Marechal 2010). By-products of this process are reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH) and superoxide (O_2^-) species. Normally, these mitochondria-generated ROS are controlled by an efficient system of endogenous antioxidant scavengers. However, mitochondrial dysfunction, resulting in the loss of membrane potential ($\Delta\Psi m$), can lead to the release of ROS, where they can cause oxidative damage to proteins, lipids and DNA. Moreover, cytosolic ROS may induce further release of ROS from mitochondria by triggering the mitochondrial permeability transition, an incompletely understood process that involves the opening of a channel in the inner mitochondrial membrane. This process is termed ROS-induced ROS release (RIRR) (Zorov et al. 2014). Thus, release of ROS from one mitochondrion could trigger ROS release from its neighbours, acting in a positive-feedback loop. While RIRR may have important physiological roles, it is also associated with disease processes, particularly post-ischaemic pathologies, such as arrhythmias (Zorov et al. 2006). Importantly, the excessive build-up of cytosolic ROS can lead to activation of inflammatory signalling pathways, resulting in the pathological release of pro-inflammatory molecules from immune cells (Harris et al. 2017). Thus, regulation of mitochondrial damage is critical for the maintenance of effective and appropriate immune responses.

1.1. Mitophagy: A specialised form of autophagy

Autophagy, specifically macroautophagy, is a highly conserved mechanism for the sequestration and lysosomal degradation of cytosolic constituents, including organelles (Mizushima et al. 2011). The process of (macro)autophagy is regulated by the products of numerous autophagy-related genes (Atg). It is characterized by the formation of a double-membrane autophagosome that targets and engulfs intracellular cargo, then fuses with lysosomes. Although present basally, autophagy is upregulated in response to many different stimuli, including amino acid starvation, growth factors and cytokines, environmental stresses and the presence of damaged organelles, including mitochondria (Klionsky et al. 2016). Typically, autophagy promotes cell survival in times of stresses, such as nutrient deprivation, but more recently it has also been shown to regulate inflammatory responses in immune cells (Harris 2011; Harris et al. 2017).

Mitophagy is a selective form of autophagy that specifically targets mitochondria for degradation (Lazarou 2015). In this process, a series of specific receptors/adaptors mediate signalling pathways that promote the recruitment of autophagic machinery to dysfunctional mitochondria, which are then engulfed by the double-membrane autophagosome (Nguyen et al. 2016). Upon subsequent fusion of the autophagosome with a lysosome, the damaged mitochondria are

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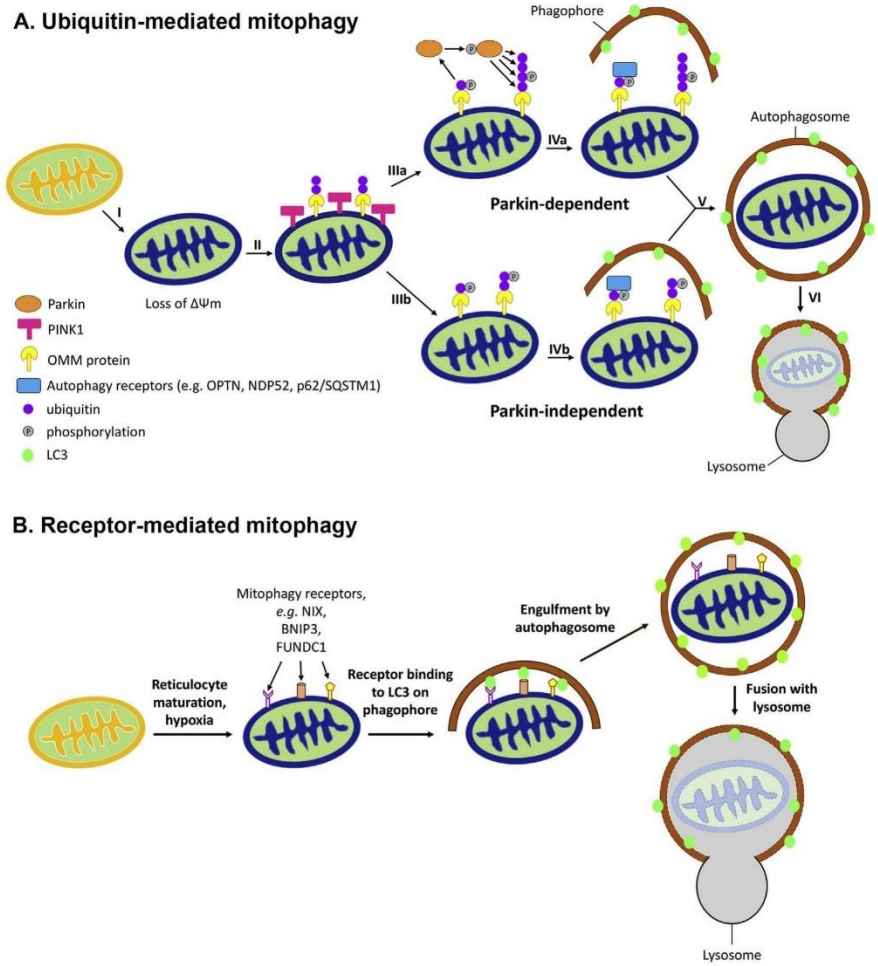

Fig. 1. Mechanisms of mitophagy. A. Ub-mediated mitophagy. Upon loss of mitochondrial membrane potential (I), PINK1 is stabilized on the outer mitochondrial membrane (OMM) (II), where it recruits and phosphorylates Parkin; PINK1 also phosphorylates Ub located on the OMM (IIIa). The phosphorylated Ub then recruits more Parkin to the OMM (dashed arrow), thereby providing PINK1 with more Parkin for phosphorylation (IIIa). Once activated by PINK1, Parkin accumulates Ub on OMM proteins (IIIa). The resulting Ub chains translocate the autophagy receptors (e.g. OPTN, NDP52, p62/SQSTM1) in close proximity to the damaged mitochondrion, where these receptors facilitate the biogenesis of a phagophore via the recruitment of the autophagy proteins (IVa). Autophagy receptors are also recruited to the damaged mitochondrion via binding to the PINK1-phosphorylated Ub located on the OMM (IIIb) and facilitate the downstream events of phagophore formation (IVb) independent of Parkin. The phagophore expands and encloses the damaged mitochondrion within an autophagosome (V), decorated with LC3, which can fuse with lysosomes for degradation of the cargo (VI). **B. Receptor-mediated mitophagy.** A number of receptors, including NIX, BNIP3 and FUNDC1 are expressed on the OMM in response to different stimuli and facilitate mitophagy via binding to LC3 on the phagophore. As before, the phagophore expands and encloses the damaged mitochondrion within an autophagosome, which can fuse with lysosomes for degradation of the cargo.

degraded in the resulting autolysosome. In mammals, two distinct but interconnected signalling cascades of mitophagy have been identified to date: ubiquitin (Ub)-dependent (Fig. 1A) and Ub-independent, or receptor-mediated (Fig. 1B) (Georgakopoulos et al. 2017; Lazarou 2015).

The most well-defined Ub-mediated pathway of mitophagy is driven by PINK1 (PTEN induced putative kinase 1) and Parkin: two proteins commonly mutated in an autosomal recessive form of Parkinson's disease characterized by mitochondrial dysfunction (Bonifati 2012; Kitada et al. 1998; Lazarou 2015; Valente et al. 2004). The molecular mechanisms of PINK1/Parkin-mediated mitophagy are complex and yet to be fully elucidated. Briefly, in response to the loss of ΔΨm, the serine/threonine kinase PINK1 accumulates on the outer mitochondrial membrane (OMM) and drives the recruitment and activation of the E3 Ub ligase Parkin to the OMM via phosphorylation at the highly conserved residue serine 65 (Fig. 1A) (Kondapalli et al. 2012; Narendra et al. 2010). PINK1 also phosphorylates Ub, the Parkin substrate, basally linked to the proteins present on the OMM (Kazlauskaite et al. 2014). This, in turn, facilitates the translocation of more Parkin from the cytosol to the OMM, since Parkin has a high affinity for these phosphorylated Ub (Kazlauskaite et al. 2014). Once completely activated by PINK1, Parkin builds up Ub chains onto the OMM proteins, which are again phosphorylated by PINK1 and recruit more Parkin. Therefore, PINK1 and Parkin function co-ordinately to accumulate Ub chains on the OMM, which is required to recruit autophagy receptors to mitochondria (Nguyen et al. 2016).

So far, five different autophagy receptors have been found to localize to the damaged mitochondria; p62/SQSTM1 (sequestosome 1), neighbour of BRCA1 gene 1 (NBR1), optineurin (OPTN), nuclear dot

protein 52 (NDP52) and Tax1-binding protein 1 (TAX1BP1) (Chan et al. 2011; Lazarou 2015; Sarraf et al. 2013). These receptors contain both Ub-binding domains (UBDs) and LC3-interacting regions (LIR), and are thereby able to simultaneously bind to ubiquitin chains on mitochondria and microtubule-associated proteins 1A/1B light chain 3 (LC3)/GABAA receptor-associated protein (GABARAP) present on autophagosomes, respectively (Wild et al. 2014). Of these receptors, OPTN and NDP52 appear to act as the main receptors/molecular adaptors of PINK1/Parkin-triggered mitophagy, linking between ubiquitinated mitochondria and autophagosomes (Nguyen et al. 2016). Interestingly, NBR1 and p62/SQSTM1 may not directly recruit autophagy machinery to damaged mitochondria during PINK1/Parkin-triggered mitophagy, but instead promote clustering of damaged mitochondria for their subsequent disposal into autophagosomes (Narendra et al. 2010).

Accumulating evidence suggest that OPTN and NDP52 can be recruited to mitochondria independent of Parkin (Lazarou 2015; Stolz et al. 2014; Wong and Holzbaur 2014). This observation is supported by the fact that OPTN and NDP52 preferentially bind to PINK1-phosphorylated OMM ubiquitin and that phosphorylation of basal OMM ubiquitin is sufficient for receptor recruitment at a low level in the absence of Parkin (Nguyen et al. 2016). By modulating the expression of Parkin, Lazarou et al. were also able to demonstrate that OPTN and NDP52 recruited to mitochondria by "conditionally targeted PINK1" in the cells lacking Parkin could promote mitophagy, while the addition of Parkin expression amplifies this (Lazarou et al. 2015) (Fig. 1A).

Specific receptors such as NIX (NIP3-like protein X), BNIP3 (BCL2/adenovirus E1B 19 kDa protein interacting protein 3) and FUNDC1 (FUN14 domain-containing 1) localized on OMM can also trigger mitophagy, independent of Ub (Georgakopoulos et al. 2017). These receptors recruit autophagy machinery to mitochondria through their LIR motifs (Fig. 1B). NIX (NIP3-like protein X) has been shown to promote mitophagy during the maturation of reticulocytes, whereas both BNIP3 and FUNDC1 trigger mitophagy during hypoxia. The lipid cardiolipin, when exposed to the OMM, can also promote mitophagy via LC3 binding (Chu et al. 2013; Liu et al. 2012; Schweers et al. 2007; Zhang et al. 2008).

1.2. Mitochondrial damage, autophagy and IL-1 family cytokines

1.2.1. IL-1 family cytokines and inflammasomes

The IL-1 family of cytokines includes, amongst others, IL-1α, IL-1β and IL-18. IL-1α and IL-1β bind the same receptor (IL-1R1) and share similar biological activities (Garlanda et al. 2013). The generation of biologically active IL-1β and IL-18 requires processing of inactive precursors (pro-IL-1β and pro-IL-18), which are produced in response to NF-κB activation. This processing is typically dependent on caspase-1 and commonly involves the activation of an inflammasome. Inflammasomes are multi-protein complexes that activate caspase-1 either directly (canonical inflammasome) or indirectly, via caspase-4/5 (caspase-11 in mice; non-canonical inflammasome) in response to multiple inflammatory or toxic stimuli (Guo et al. 2015; Vigano et al. 2015). These complexes contain a pattern recognition receptor, including members of the nucleotide-binding domain, leucine-rich repeat containing proteins (NOD-like receptors, NLRs), such as NLRP1, NLRP3 and NLRC4, or absent in melanoma 2 (AIM2)-like receptors (ALRs). In most cases, the NLR or ALR binds apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) leading to recruitment and activation of caspase-1. This results in cleavage of pro-IL-1β or pro-IL-18 into the mature, bioactive cytokines.

IL-1 family cytokines are key regulators of innate and adaptive immune responses, but IL-1α, IL-1β and IL-18 also play a pathological role in inflammation if released in an uncontrolled manner (Afonina et al. 2015; Garlanda et al. 2013). In particular, NLRP3-dependent IL-1β release, driven by monosodium urate (MSU) crystals in the joints, is responsible for pathology in gout (Gonzalez 2012). Moreover, gain-of-function mutations in the *NLRP3* (*CIAS1*) gene are associated with cryopyrin-associated periodic syndromes (CAPS), including familial cold autoinflammatory syndrome (FCAS), neonatal-onset multisystem inflammatory disease (NOMID) and Muckle-Wells syndrom (MWS) (Dode et al. 2002; Hoffman et al. 2001). NLRP3 activation has also been linked to other diseases, including type II diabetes, obesity-induced insulin resistance and some cancers (Menu and Vince 2011).

1.2.2. Autophagy, mitophagy and inflammasome activation

Numerous studies have demonstrated that loss or impairment of autophagy in macrophages and dendritic cells can lead to hyper-secretion of IL-1α, IL-1β and IL-18 in response to TLR3 or TLR4 ligands alone (Harris et al. 2011; Nakahira et al. 2011; Saitoh et al. 2008; Zhou et al. 2011). Importantly, this effect has been shown to be dependent on mitochondrial damage and the release of ROS. Macrophages deficient in autophagy/mitophagy accumulate dysfunctional/abnormal mitochondria that release ROS into the cytosol (Lee et al. 2016; Nakahira et al. 2011; Zhong et al. 2016; Zhou et al. 2011). ROS derived from NADPH oxidase, on the other hand, do not appear to be affected by autophagy inhibition in macrophages and dendritic cells (Harris et al. 2011; Lee et al. 2016). Treatment with LPS further increases mitochondrial dysfunction and the release of both ROS and mitochondrial DNA (mtDNA), leading to the presence of oxidized mtDNA, in the cytosol, which has been shown to directly bind NLRP3 to activate the inflammasome (Nakahira et al. 2011; Shimada et al. 2012; Zhou et al. 2011). Interestingly, the increased release of IL-1β and IL-18 appears to be dependent on Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), through which TLR3 and TLR4 signal (Harris et al. 2011; Saitoh et al. 2008). The reason for this is not clear, but whether the increased mitochondrial damage observed in LPS-treated cells is somehow dependent on TRIF signalling warrants further investigation.

While IL-1β release from dendritic cells treated with the autophagy inhibitor 3-MA is significantly lower in *Nlrp3*$^{-/-}$ cells, it is not completely abrogated, suggesting that some release is independent of NLRP3 (Harris et al. 2011). One possibility is that other inflammasomes are also activated in autophagy-deficient cells (discussed below). However, another possible explanation for this is that loss of autophagy increases cell death in response to TLR3/TLR4 ligation. Most secreted proteins with an N-terminal signal peptide traffic through the classical secretion pathway, via the endoplasmic reticulum and golgi apparatus. However, a number of secreted proteins, including IL-1α, IL-1β and IL-18, lack a classical signal peptide and are released by mostly undefined mechanisms, collectively termed unconventional secretion. This may be through an active secretory pathway or in some cases, at least in part, as a result of lytic cell death (Liu et al. 2014; Shirasaki et al. 2014). Indeed, inflammasome activation in macrophages and dendritic cells typically results in caspase-1-dependent cell death, termed pyroptosis (Bergsbaken et al. 2009). In the case of autophagy-deficient macrophages, pyroptosis may be increased as a result of increased NLRP3 inflammasome activation. Other forms of cell death can also be increased as a direct result of mitochondrial dysfunction, including apoptosis and necrosis (Parsons and Green 2010), potentially leading to further IL-1 release. However, it should be noted that other forms of cell death that may be less dependent on mitochondrial involvement, including necroptosis, have also been demonstrated to activate the NLRP3 inflammasome (Conos et al. 2017).

It should be noted that, to date, few studies have specifically implicated mitophagy, as distinct from autophagy more generally, in the regulation of inflammasome activation. Interestingly, Allam et al. demonstrated that macrophages from *Parkin*$^{-/-}$ mice do not produce more IL-1β in response to NLRP3-stimulating treatments, as might be expected if mitophagy were important in regulating inflammasome activation (Allam et al. 2014). Moreover, cells treated with LPS only did not produce IL-1β, in contrast to the many studies that have demonstrated increased IL-1β release in autophagy-deficient cells under these conditions. Importantly, it should be noted that these experiments were conducted over a much shorter time period (3 h LPS treatment versus

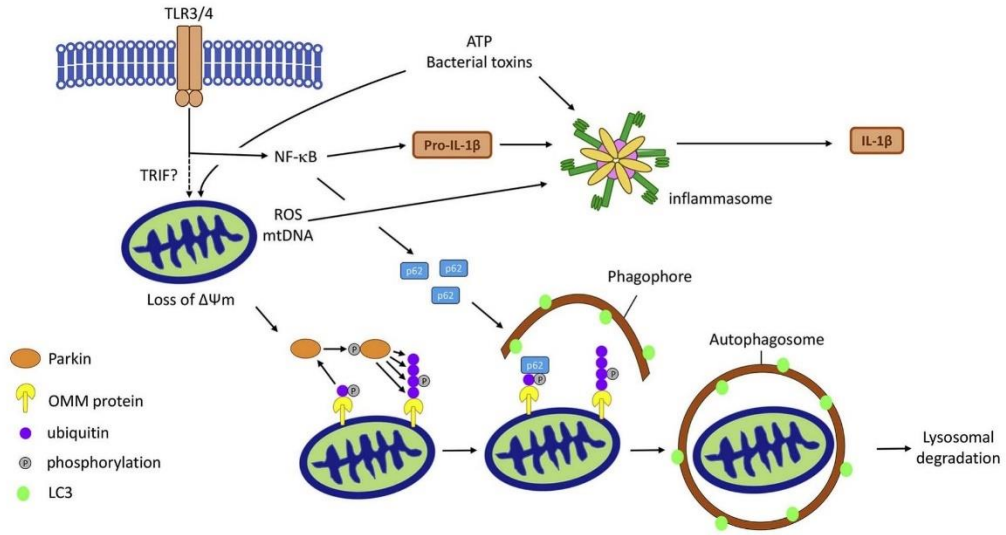

Fig. 2. Mitophagy and the inflammasome. Ligation of TLR3 or TLR4 results in activation of NF-κB, which in turn induces transcription and translation of pro-IL-1β, as well as the autophagy receptor p62/SQSTM1. Moreover, TLR3/4 signalling may result in increased mitochondrial damage via an unknown TRIF-dependent mechanism. Similarly, some NLRP3 activators, including ATP and bacterial toxins, induce mitochondrial dysfunction and loss of membrane potential (ΔΨm), resulting in Parkin-dependent ubiquitination of outer mitochondrial membrane (OMM) proteins. These ubiquitinated proteins are then recognised by p62/SQSTM1, which in turn recruits $LC3^{+}$ phagophores, which elongate and engulf the mitochondrion within an autophagosome. However, in the absence of functional autophagic machinery, damaged mitochondria can release reactive oxygen species (ROS) and DNA (mtDNA) that can further activate inflammasomes. This leads to activation of caspase-1, which can process pro-IL-1β into the mature bioactive cytokine.

overnight stimulations in other studies), which may suggest that timing and context are crucial to deciphering the role of autophagy/mitophagy in NLRP3 activation and the release of IL-1 family cytokines. In particular, the effect of Parkin deficiency over time would be worth investigating, as defective Parkin-mediated mitophagy may become more inflammatory over longer times, as is the case with autophagy deficiency (Harris et al. 2011; Saitoh et al. 2008).

However, a recent study has contradicted these results, demonstrating that macrophages from *Parkin*$^{-/-}$ mice do secrete more IL-1β in response to a similar selection of NLRP3 activators, although the precise timing of these experiments is not clear (Zhong et al. 2016). This study also demonstrated that NLRP3 activators induced mitochondrial damage, leading to their Parkin-dependent ubiquitylation and p62/SQSTM1-dependent mitophagy (Fig. 2). Moreover, p62/SQSTM1 mRNA and protein expression was significantly increased by LPS-induced NF-κB activation, which corresponds with other studies demonstrating that LPS and other TLR ligands also induce autophagosome formation (Delgado et al. 2008; Harris et al. 2011; Xu et al. 2007). This would suggest that autophagy/mitophagy might act as a negative feedback mechanism for the control of inflammasome activation. Further studies are clearly needed to clarify the interactions between Parkin-dependent mitophagy, autophagy and NLRP3 inflammasome activation. In this context, it is important to note that autophagy has also been shown to target inflammasome components for degradation (Shi et al. 2012), and this may represent an alternative, complimentary, pathway by which autophagy regulates inflammasome activation.

Control of dysfunctional mitochondria and accumulation of ROS/DNA may also link mitophagy to the activation of other inflammasomes. The bacterium *Pseudomonas aeruginosa* has been shown to induce mitochondrial damage and the release of mitochondrial DNA and ROS into the cytosol, which activates the NLRC4 inflammasome (Jabir et al. 2015). Another study demonstrated that high mobility group box 1 (HMGB1), when bound to DNA (HMGB1-DNA), activates the AIM2 inflammasome. However, HMGB1-DNA also activates autophagy via the receptor for advanced glycosylation endpoints (RAGE), which inhibits further AIM2 activation. This suggests that autophagy may function as a negative feedback mechanism to prevent excessive AIM2 activation (Liu et al. 2016).

1.2.3. Autophagy and unconventional secretion

While loss of autophagy increases the release of IL-1 family cytokines in response to TLR3 or TLR4 ligation, another study has demonstrated that induction of autophagy can also increase IL-1β release by macrophages following inflammasome activation, suggesting a role for autophagy in the unconventional secretion of the cytokine (Dupont et al. 2011). This process required the Golgi reassembly stacking proteins GRASP55 and Rab8. Moreover, autophagy similarly increased the unconventional secretion of HMGB1 and IL-18 (Dupont et al. 2011), although it is not yet clear whether either of these proteins are sequestered in autophagosomes.

A role for autophagosomes in the secretion of IL-1β was further confirmed by Zhang and colleagues, who also identified GRASP65 as an important factor in this process (Zhang et al. 2015). Moreover, hepatocyte growth factor receptor substrate (Hrs) and TSG101, both involved in multi-vesicular body (MVB) formation, were required, suggesting a role for MVBs. In this study, IL-1β could be seen on the early phagophore and then translocated across the phagophore membrane as the mature cytokine. IL-1β was then found in the space between the inner and outer membranes of the autophagosome. This translocation of IL-1β into the intermediate space was dependent on the chaperone protein Hsp90 and two KFERQ sequence motifs on IL-1β, suggesting a process similar to that of chaperone-mediated autophagy in which molecules are targeted directly to lysosomes (Tasset and Cuervo 2016; Zhang et al. 2015). It is not clear how IL-1β is delivered to the cell surface, although it may involve interaction between autophagy and a MVB or lysosome.

1.2.4. Autophagy and IL-1α

Loss of autophagy has also been shown to increase the secretion of IL-1α by macrophages and dendritic cells, although this is independent

of inflammasome activation (Castillo et al. 2012; Harris et al. 2011). Instead, increased ROS, possibly mitochondrial, appears to drive calpain-dependent secretion of IL-1α. However, this is independent of p62/SQSTM1 and dependent on Atg5 (Castillo et al. 2012), suggesting a different mechanism to that seen for NLRP3-dependent IL-1β release and possibly independent of Parkin-dependent mitophagy.

1.3. The IL-1 - IL-23 –IL-17 axis

Interleukin 1α and IL-1β both bind the type I IL-1 receptor (IL-1R1), which is ubiquitously expressed, and elicit multiple signalling cascades via MyD88 and IRAK4, ultimately leading to activation of the NF-κB, c-terminal Jun N-terminal kinase (JNK) and p38 MAPK pathways (Weber et al. 2010). Thus, IL-1 signalling results in the NF-κB-dependent induction of multiple pro-inflammatory genes, including IL-6 and IL-8 (Hoffmann et al. 2005; Krause et al. 1998). Perhaps, then, it is not surprising that autophagy has also been shown to regulate IL-6, IL-8 and many other cytokines, although other IL-1-independent pathways may also be involved (reviewed in Harris 2011). Interleukin-1β has also been shown to induce the secretion of IL-23 by human peripheral blood mononuclear cells and purified monocytes (Harris et al. 2008). Similarly, the increase in IL-1β release by autophagy-deficient macrophages and dendritic cells has been shown to induce the autocrine secretion of IL-23 (Peral de Castro et al. 2012). Conversely, LPS-stimulated secretion of IL-23 in vivo was inhibited in mice co-treated with the autophagy-inducing drug rapamycin (Peral de Castro et al. 2012).

Importantly, IL-1 (α and/or β), combined with IL-23, can elicit the secretion of IL-17 by innate and memory T cells (Cho et al. 2006; Sutton et al. 2006; Sutton et al. 2009), representing important cross-talk between innate and adaptive immune responses. Correspondingly, supernatants from autophagy-deficient dendritic cells treated with LPS (high in IL-1α, IL-1β and IL-23) drive IL-17A and IL-17F secretion, as well as IFN-γ and IL-22, by innate γδ T cells in vitro (Peral de Castro et al. 2012). Similarly, $LC3B^{-/-}$ $CD11b^{+}$ DCs infected with respiratory syncytial virus elicit IL-17 secretion from $CD4^{+}$ T cells in an IL-1-depednent manner and the infected mice display increased numbers of both innate (ILC and γδ T cells) and adaptive (CD4 + T cells) IL-17A-producing cells (Reed et al. 2015). Moreover, mice with Atg5 deficient myeloid cells infected with *Mycobacterium tuberculosis* were found to have a higher bacillary load, increased inflammation and produce more IL-1α and IL-17 than their autophagy-proficient littermates (Castillo et al. 2012). In another study, mice with Atg5 deficient $CD11c^{+}$ cells developed spontaneous airway hyper-reactivity and severe neutrophilic lung inflammation, with increased IL-1 and IL-17A levels in the lungs (Suzuki et al. 2016). Thus, these studies demonstrate that defective autophagy/mitophagy can have consequences beyond any specific effects on inflammasome activation.

1.4. Type 1 IFN

A number of studies have linked autophagy to type I IFN secretion, but with seemingly conflicting results. One study has demonstrated that plasmcytoid dendritic cells deficient in autophagy have impaired IFN-α responses following exposure to vesicular stomatitis virus (VSV), herpes simplex virus (HSV) or CpG (Lee et al. 2007). In contrast, $Atg5^{-/-}$ embryonic fibroblasts produce more IFN-α and IFN-β in response to VSV and dsDNA and are resistant to infection with VSV (Jounai et al. 2007; Tal et al. 2009). This response is dependent on mitochondrial dysfuntion and ROS, which enhances signalling via RIG-I-like receptors. Similarly, siRNA-mediated knockdown of beclin-1 (Atg6) or Atg7 in immortalized human hepatocytes increases expression of IFN-α and IFN-β in response to hepatitis C virus (HCV) (Shrivastava et al. 2011). More recently, studies have demonstrated that mtDNA released following Bak/Bax-dependent apoptosis induce type I IFN through the cGAS/STING/IRF3-dependent DNA sensing pathway (West et al. 2015; White et al. 2014). Moreover, HSV induces mtDNA stress, upregulating type I IFN expression, suggesting that release of mtDNA is an important cell intrinsic mediator of anti-viral responses (West et al. 2015). In turn, these findings would suggest that autophagy/mitophagy might act as an important regulator of Type I IFNs and anti-viral signalling.

1.5. Macrophage migration inhibitor factor (MIF)

Discovered in the 1960′s, MIF was one of the earliest cytokines identified (Bloom and Bennett 1966; David 1966). Since this time there has been significant scientific interest in MIF, which functions not only as a pro-inflammatory protein, but also as a stress and growth factor, and is released by cells of the anterior pituitary gland in a manner similar to a hormone (Lang et al. 2015). MIF is expressed in multiple immune cell types, including macrophages, dendritic cells, T and B cells, neutrophils, eosinophils and mast cells. The cytokine has an important role to play in the innate immune response to different bacteria, including *Salmonella* and *Mycobacterium* species, and can upregulate TLR4 and pro-inflammatory cytokines, including TNF-α, IL-1β and IFN-γ (Calandra and Roger 2003; Das et al. 2013; Koebernick et al. 2002). MIF has a pathogenic role to play in endotoxaemia (Calandra and Roger 2003) and MIF inhibition or deficiency is protective in animal models of rheumatoid arthritis, systemic lupus erythematosus, Ross River disease, osteoporosis and cardiac ischaemia-reperfusion injury (Gao et al. 2011; Gu et al. 2015; Herrero et al. 2011; Hoi et al. 2006; Leech et al. 1999; Santos et al. 2011; White et al. 2013).

We have recently demonstrated that, similar to IL-1 family cytokines, loss of autophagy in human and mouse macrophages leads to increased secretion of MIF in response to LPS (Lee et al. 2016). This was dependent on mitochondrial ROS, which accumulated in the cytosol of autophagy-deficient macrophages treated with LPS. MIF secretion was also increased in response to amino acid starvation, an observation similarly noted in another study (Chuang et al. 2012). Given that amino starvation is a strong inducer of autophagy, these results may seem contradictory. However, we found that MIF release was unaffected by other autophagy inducers (the mTOR inhibitors rapamycin, Torin 1 and AZD8055). We hypothesised that amino acid starvation led to rapid exhaustion of autophagosomes, due to degradation of autophagosomal components, coupled with a lack of de novo protein synthesis. In addition, starvation led to mitochondrial damage, ultimately allowing the accumulation of mitochondrial ROS in the cytosol (Lee et al. 2016). The mechanism behind ROS-induced MIF release remains elusive, although a previous study has demonstrated that H_2O_2 increases transcription of *Mif* mRNA (Harrison and Sumners 2009).

2. Conclusions

While many studies demonstrate clear links between mitochondrial dysfunction, autophagy and inflammatory processes and pathologies, the full extent to which selective mitophagy is involved remains incompletely understood. Future studies are required to fully elucidate this potentially crucial immune homeostatic mechanism. This is important not only for our understanding of the mechanisms that control inflammation, but also, by extension, for the development of new therapeutic and diagnostic strategies. Polymorphisms in autophagy-related genes have been shown to increase susceptibility to some inflammatory and autoimmune diseases (Jones et al. 2013), but similar studies on mitophagy-specific polymorphisms are currently lacking. In some cases, loss of selective mitophagy may be compensated for by an increase in non-selective autophagy, as has been demonstrated in a zebrafish model of dopaminergic neuron loss (Zhang et al. 2017). However, the short- and long-term effects of mitophagy impairment on inflammation warrant further attention.

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Appendix Iii:

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SHORT COMMUNICATION

Necrotic cell death increases the release of macrophage migration inhibitory factor by monocytes/macrophages

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Abstract

Macrophage migration inhibitory factor (MIF) is a pleiotropic inflammatory molecule with both cytokine and noncytokine activity. MIF is constitutively released from multiple cell types via an unconventional secretory pathway that is not well defined. Here, we looked at MIF release from human and mouse monocytes/macrophages in response to different stimuli. While MIF release was not significantly altered in response to lipopolysaccharide or heat-killed *Escherichia coli*, cytotoxic stimuli strongly promoted release of MIF. MIF release was highly upregulated in cells undergoing necrosis, necroptosis and NLRP3 inflammasome-dependent pyroptosis. Our data suggest that cell death represents a major route for MIF release from myeloid cells. The functional significance of these findings and their potential importance in the context of autoimmune and inflammatory diseases warrant further investigation.

INTRODUCTION

The activity of macrophage migration inhibitory factor (MIF) was first discovered in the 1960s by two separate groups.[1,2] However, MIF was not definitively cloned until the early 1990s[3,4]; since then, multiple biological roles have been assigned to it. MIF is a pleiotropic inflammatory molecule with both cytokine-like and cytokine-independent roles. In the context of immunity, MIF can inhibit macrophage migration *in vitro* but, contrary to this, also has chemotactic activity in monocytes, neutrophils, T cells and B cells.[5,6] MIF has also been shown to regulate the expression and release of multiple other cytokines, including tumor necrosis factor (TNF), interferon-γ, interleukin (IL)-2, IL-6, IL-8 and the IL-1 family cytokines IL-1α, IL-1β and IL-18.[7] Importantly, loss or inhibition of MIF is protective in animal models of a number of diseases, including systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and sepsis.[7] However, MIF is also proposed to have protective roles, including in wound healing and chronic lung disease.[8,9]

Studies have demonstrated the release of MIF from different cell types in response to a variety of stimuli, including microbial constituents, microorganisms and glucocorticoids.[3,4,10–12] It is not fully clear how this release is regulated, although the Golgi-related protein p115 may play a role in its unconventional secretion.[13] Interestingly, initial release of MIF is independent of transcription, but after 12 h MIF messenger RNA levels increase upon lipopolysaccharide (LPS) stimulation.[13,14] Unlike other classical proinflammatory cytokines, MIF is constitutively released by many cell types, including monocytes and macrophages and is present in serum and healthy tissues, often at high concentrations.[7] Moreover, in human neutrophils, proinflammatory stimuli do not appear to induce the release of MIF, which exists preformed in the cytosol. Instead, MIF, along with another cytokine, IL-16, is released only when the neutrophils become necrotic, secondary to apoptosis.[15] It remains to be determined whether the release of MIF from dead cells is a significant process across other cell types as well. Thus, here, we investigated the release of MIF by monocytes and macrophages *in vitro*, in response to both inflammatory stimuli and cell death. While we did not see a consistent effect of the TLR4 agonist LPS or heat-killed *Escherichia coli* on MIF release by human and mouse monocytes/macrophages, we observed significant MIF release in response to treatments that induce necrotic forms of cell death, including necrosis, necroptosis and pyroptosis. These data suggest that cell death represents a major mechanism of MIF release by innate immune cells.

RESULTS AND DISCUSSION

MIF release by myeloid cells in response to inflammatory stimuli

Studies have suggested that MIF is released by macrophage cell lines in response to treatment with LPS.[13,14] However, in our own studies, we have observed inconsistent responses to LPS treatment in primary mouse macrophages, mouse RAW264.7 macrophages and human THP-1 cells, with apparent stimulation of MIF release in some experiments, but no effect in others.[16] Thus, to test this further, we measured cytokine release by murine immortalized bone marrow macrophages (iBMMs) and undifferentiated human THP-1 monocyte-like cells treated with different concentrations of LPS and heat-killed *E. coli* for 6 h. In both cell types, TNF release was significantly stimulated by treatment with both LPS and heat-killed *E. coli* (Figure 1a–d). However, no consistent significant effect of LPS on MIF release was observed in either cells type at doses ranging from 1 to 1000 ng mL^{-1}, although considerable variation was observed between experiments (Figure 1a, b) . Likewise, no significant effect was seen at 0.1 ng mL^{-1} in iBMMs (Supplementary figure 1a) or 10 μg mL^{-1} in THP-1 cells (Supplementary figure 1b). Similarly, treatment with heat-killed *E. coli* had no significant effect on MIF release at any dose but did induce TNF (Figure 1c, d) and IL-6 (Supplementary figure 1c).

These data, coupled with our previous findings,[16] indicate that these inflammatory stimuli, contrary to the common perception in the literature, are not reliable inducers of MIF release by macrophages *in vitro*. Why our data differ from previous publications and why MIF release in the setting of LPS treatment is so variable are not clear. One hypothesis worth further investigation is that LPS treatment has dual effects on the pool of extracellular MIF, potentially stimulating release while simultaneously increasing its uptake via micropinocytosis and/or macropinocytosis. Studies have shown that LPS stimulates macropinocytosis in macrophages,[17] but whether MIF is taken up in macropinosomes remains to be determined. Based on these findings, we suggest that more detailed studies testing the effects of different stimuli, including other TLR agonists, on MIF release and uptake by mouse and human myeloid cells are warranted.

Notable from our data, as well as from previous studies,[13,16,18] is a relatively high level of constitutive MIF release by unstimulated cells. This is similarly true in healthy human plasma, with concentrations ranging from 0.1 to 30 ng mL^{-1}.[19,20] These observations might suggest a role for MIF akin to a growth factor, required for normal functioning of cells. To investigate the dynamics of this, we looked at MIF release by iBMMs and THP-1 following removal and replacement of the culture medium. In both cell types, extracellular MIF increased over 6 h, with significant release seen as early as 30 min after media change (Supplementary figure 2). Importantly, addition of LPS did not alter the dynamics of this release, nor the amount released by either cell type (Supplementary figure 2).

MIF is released by necrotic macrophages

As MIF was not released by cells in response to inflammatory stimuli and in light of the previous work showing that MIF is released by necrotic neutrophils,[15] we next looked at whether necrosis of monocytes/macrophages might elicit similar MIF release, using a number of different cytotoxic treatments. First, we treated iBMMs with hydrogen peroxide (H_2O_2), which can induce both apoptotic and nonapoptotic cell death, depending on concentration and cell type used.[21] Here, treatment of iBMMs with 1 mM H_2O_2 induced both cell death and MIF release (Figure 2a) Moreover, cell death

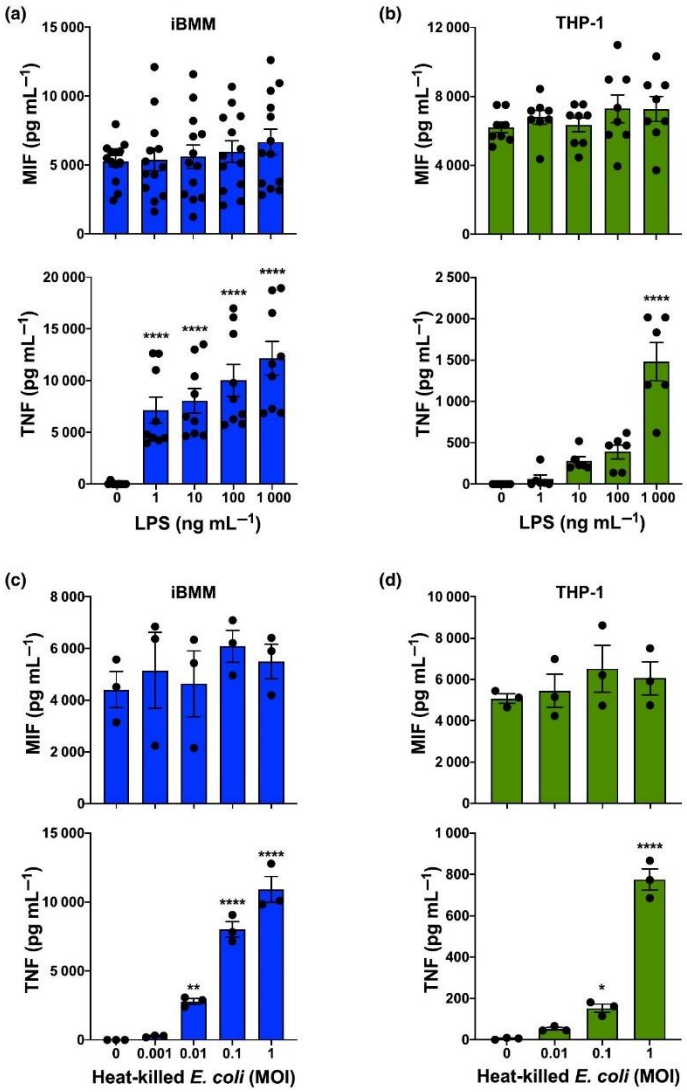

Figure 1. Bacterial products do not reliably induce macrophage migration inhibitory factor (MIF) release by myeloid cell lines. **(a)** Murine immortalized bone marrow-derived macrophages (iBMMs) and **(b)** human THP-1 monocytic cells were treated with lipopolysaccharide (LPS) at the stated concentrations for 6 h and culture supernatants tested for MIF (top) and tumor necrosis factor (TNF; bottom) release by ELISA. **(c)** iBMMs and **(d)** THP-1 cells were stimulated with heat-killed *Escherichia coli* (*E. coli*) at the stated multiplicity of infection for 6 h and culture supernatants tested for MIF (top) and TNF (bottom) release by ELISA. Data are means ± s.e.m. of 3–13 independent experiments. Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test; $*P < 0.05$, $**P < 0.01$, $****P < 0.001$, compared to unstimulated (0) cells. MOI, multiplicity of infection.

and MIF release significantly correlated with each other (Figure 2a, right panel). Similarly, when iBMMs were treated with 10% ethanol for 6 h, both cell death and MIF release were significantly increased, whereas lower concentrations of ethanol, which did not induce cell death, did not increase MIF release (Figure 2b). No TNF

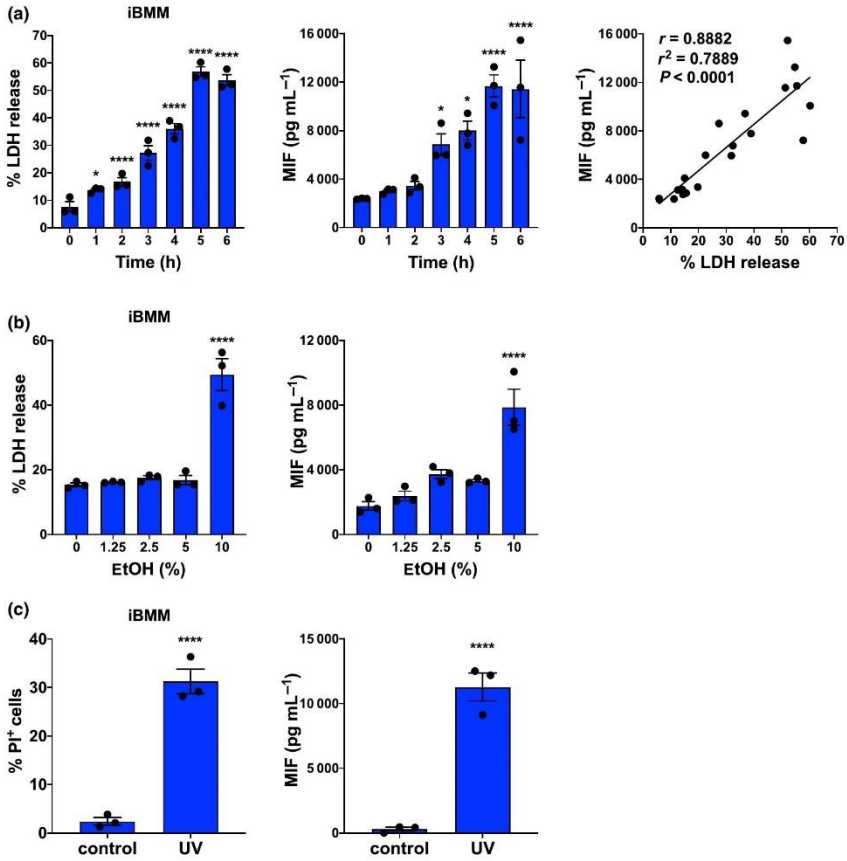

Figure 2. Macrophage migration inhibitory factor (MIF) is released by necrotic cells. **(a)** Murine immortalized bone marrow-derived macrophages (iBMMs) were treated with H_2O_2 (1 mM) for 0–6 h and cell death (left graph) and MIF release (middle graph) measured by lactate dehydrogenase (LDH) release and ELISA, respectively. Correlation (Pearson's *r*) and linear regression for these data were analyzed (right graph). **(b)** Cell death was induced in iBMMs by treatment with 1.25–10% ethanol (EtOH) for 6 h. **(c)** Necrosis was induced in iBMMs by irradiation with ultraviolet (UV) light (254 nm, 150 mJ cm^{-2}). Cell death was measured by LDH release **(a, b)** or by incorporation of propidium iodide (PI) **(c)** and testing MIF release tested by ELISA. Data are means ± s.e.m. of three independent experiments. Data were analyzed by **(c)** an unpaired Student's *t*-test or **(a, b)** one-way ANOVA with Dunnett's multiple comparisons test; $^*P < 0.05$, $^{****}P < 0.001$.

release was detected in the same culture supernatants, except a small amount (57.47 ± 16.82 pg mL^{-1}) with 5% ethanol (data not shown). Similar effects were observed when we exposed iBMMs to a lethal dose of ultraviolet (UV) irradiation; both cell death and MIF release were significantly increased after 4 h (Figure 2c).

MIF is released by cells undergoing necroptosis

Next, we treated iBMMs with LPS and the pan-caspase inhibitor Z-VAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O- methyl]- fluoromethylketone; Z-VAD), a combination known to induce receptor-interacting kinase-3-dependent programmed necrosis, also known as necroptosis.[22] Once again, both cell death and MIF release were concurrently increased in cells treated with LPS and Z-VAD (Figure 3a). Neither LPS nor Z-VAD alone induced cell death or MIF release (Figure 3a). We also induced necroptosis in THP-1 cells by treating them with a combination of TNF, the SMAC mimetic BV-6 and Z-VAD.[23] Again, MIF was released in response to cell death and this was abrogated with the inhibitor of necroptosis necrostatin-1[24] (Figure 3b). These data confirm that, as in neutrophils, MIF is released by monocytes/macrophages when they die.

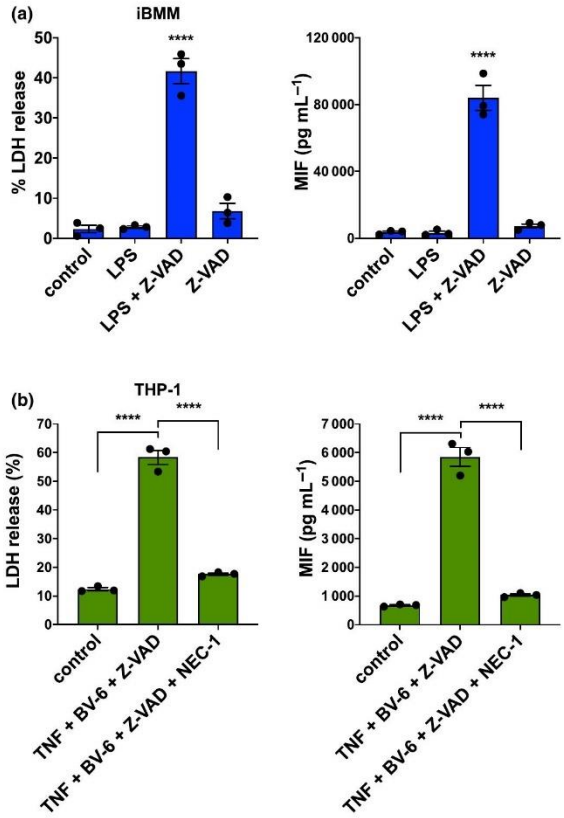

Figure 3. Macrophage migration inhibitory factor (MIF) is released by cells undergoing necroptosis. **(a)** Necroptosis was induced in murine immortalized bone marrow-derived macrophages (iBMMs) with lipopolysaccharide (LPS; 100 ng mL^{-1}) and the pan-caspase inhibitor Z-VAD-fmk (Z-VAD,50 μM) for 16 h. Cell death was measured by lactate dehydrogenase (LDH) release and MIF release by ELISA of cell supernatants. **(b)** Necroptosis was induced in human THP-1 cells by treatment with a combination of recombinant human tumor necrosis factor (TNF; 30 ng mL^{-1}), an SMAC mimetic (BV-6; 1 mM) and Z-VAD-fmk (25 μM) for 16 h. Necroptosis was inhibited with the RIP1 inhibitor necrostatin-1 (30 mM). Cell death was measured by LDH release and culture supernatants were tested for MIF release by ELISA. Data are means ± s.e.m. of three independent experiments. Date were analyzed by one-way ANOVA with **(a)** Dunnett's multiple comparisons test or **(b)** Tukey's multiple comparisons test; ****$P < 0.001$.

Moreover, this is the first study to show MIF release by cells undergoing necroptosis.

MIF release in response to inflammasome activation

Another form of necrotic cell death, pyroptosis, occurs in response to inflammasome activation.[25] Here, we looked at pyroptosis and MIF release in response to activation of the NLRP3 inflammasome. Treatment of LPS-primed iBMMs with the microbial toxin nigericin induced pyroptotic cell death, measured by lactate dehydrogenase (LDH) release, in a dose-dependent manner, with a concomitant increase in both MIF and IL-1β release (Figure 4a). These effects were abrogated by treatment with the NLRP3-specific inhibitor MCC950[26] (Figure 4a). Likewise, nigericin treatment of LPS-primed THP-1 cells increased cell death, along with MIF and IL-1β release, and this was again abrogated in the presence of MCC950 (Figure 4b). As pyroptosis is activated by caspase 1 or caspase 11 in mice (caspase 4/5 in humans),[25] we also treated LPS-primed iBMMs with nigericin (with or without Z-VAD). Interestingly, while IL-1β release was almost completely abrogated by Z-VAD, the effect on both cell death and MIF release was less pronounced (Figure 4c). This may suggest that another form of caspase-independent cell death was activated, promoting

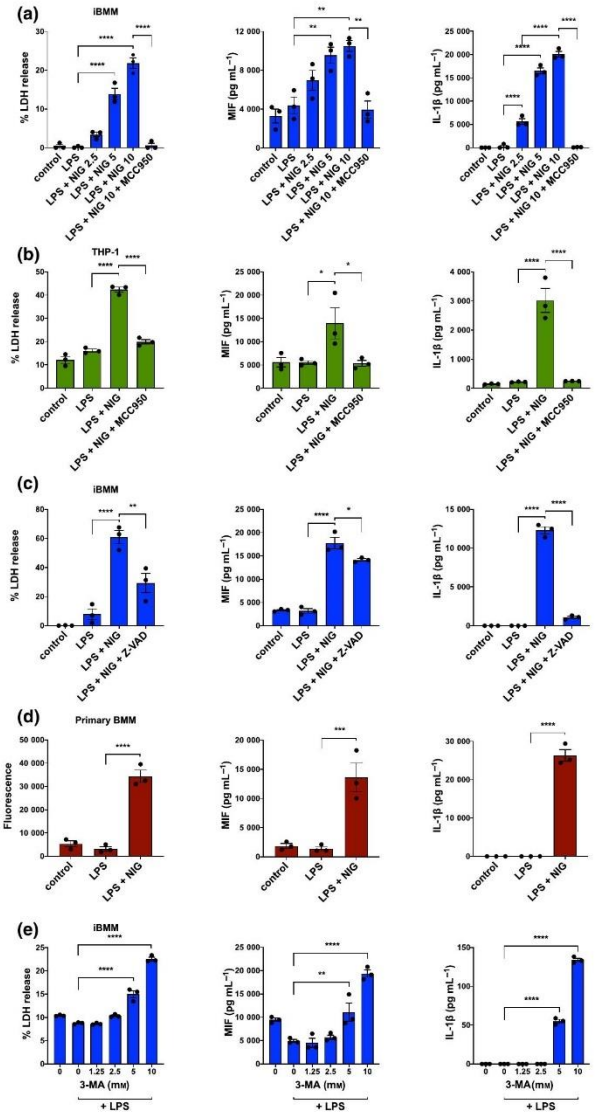

Figure 4. Macrophage migration inhibitory factor (MIF) is released by myeloid cells in response to NLRP3-dependent pyroptosis. **(a)** Murine immortalized bone marrow-derived macrophages (iBMMs) were primed with lipopolysaccharide (LPS; 100 ng mL^{-1}) for 4 h, then treated with nigericin (2.5, 5 or 10 mM) or nigericin (10 mM) with the NLRP3 inhibitor MCC950 (10 mM) for 1 h. Cell death was measured by lactate dehydrogenase (LDH) release (left graph); MIF release (middle graph) and interleukin-1β (IL-1β) release (right graph) in culture supernatants were measured by ELISA. **(b)** Human THP-1 cells were primed with LPS (100 ng mL^{-1}) for 4 h and then stimulated with nigericin (10 mM) with or without MCC950 (10 mM) for 1 h and cell death measured by LDH release; MIF and IL-1β release were measured by ELISA. **(c)** iBMMs were primed with LPS (100 ng mL^{-1}) for 4 h and then stimulated with nigericin (10 mM) with or without Z-VAD-fmk for 1 h and cell death measured by LDH release; MIF and IL-1β release were measured by ELISA. **(d)** Primary murine bone marrow-derived macrophages were primed with LPS (100 ng mL^{-1}) for 4 h and then stimulated with nigericin (10 mM) and cell death measured by incorporation of SYTOX green; MIF and IL-1β release were measured by ELISA. **(e)** iBMMs were treated with a combination of LPS (100 ng mL^{-1}) and 3-methyladenine (3-MA; 1.25–10 mM) for 16 h and cell death measured by LDH release; MIF and IL-1β release were measured by ELISA. Data are means ± s.e.m. of three independent experiments, or three mice **(d)**. Data were analyzed by one-way ANOVA with Tukey's multiple comparisons test; *$P < 0.05$, ** $P < 0.01$, ***$P < 0.005$, ****$P < 0.001$.

MIF, but not IL-1β, release, as IL-1β processing is dependent on caspase 1 and/or caspase 8.[25,27] Similarly, MIF release was significantly increased in primary murine bone marrow-derived macrophages treated with LPS in combination with nigericin, along with IL-1β release and cell death (Figure 4d). Thus, these data demonstrate, for the first time, that MIF release is promoted by cell death in response to NLRP3 inflammasome activation. These data are interesting in the context of recent studies that have demonstrated that MIF is itself required for NLRP3 inflammasome-dependent IL-1β and IL-18 release by human and mouse monocytes/macrophages.[18,28] Moreover, inhibition or loss of MIF abrogates caspase-1-dependent pyroptosis.[18] Whether MIF released by necrotic/pyroptotic cells can further amplify NLRP3 activation in other cells is worthy of investigation.

Previous studies have demonstrated that loss of autophagy in macrophages results in increased release of both MIF and IL-1β in response to activation with TLR agonists.[29,30] In the case of IL-1b, this is at least partially NLRP3 dependent and is a result of a build-up of mitochondrial products that would otherwise be cleared by autophagosomes, including reactive oxygen species and mitochondrial DNA.[29,30] Therefore, we investigated whether MIF release in response to inhibition of autophagy with the PI3K inhibitor 3-methyladenine (3-MA) coincides with cell death. As shown in Figure 4e, cell death and the release of both MIF and IL-1β were increased in a dose-dependent manner in response to treatment with 3-MA. This again suggests that MIF release in response to inhibition of autophagy is a consequence of increased cell death, possibly through pyroptosis.

Taken together, our data suggest that while the release of MIF by monocytes/macrophages in response to inflammatory stimuli may be variable, its release in response to necrotic cell death is both consistent and substantial. Based on these observations, we caution researchers to ensure cell death is taken into consideration when assessing effects of different stimuli/treatments on MIF release. Our data also highlight the relatively high levels of constitutive MIF release by cells *in vitro*. We highlight this observation as an important consideration when designing and analyzing experiments to look at MIF release in response to different stimuli/treatments; investigators should consider whether media removal is necessary prior to treatment and, if so, ensure appropriate controls are used and attention to subsequent release dynamics is taken into account.

The data presented here complement a study by Roth and colleagues demonstrating that MIF is released by necrotic human neutrophils.[15] It is interesting to note that this same study also reported no effect of cytokines or microbial constituents on MIF release by neutrophils, suggesting that necrosis may be the major route of MIF release by neutrophils. Whether this is also true for monocytes/macrophages requires further study, but our data clearly demonstrate that necrotic cell death is a potent inducer of MIF release by these cells. Moreover, MIF was only released by neutrophils following secondary necrosis, not by cells undergoing apoptosis.[15] In the experiments reported here, we observed very little early apoptosis, so have been unable to determine whether MIF is released by macrophages undergoing apoptosis, or only following necrosis, although MIF release clearly increases with increased necrosis. To what extent cell death, either through necrosis or necroptosis, might be responsible for the increased levels of MIF seen in many different diseases[7] should, in our opinion, be investigated. Perhaps most importantly, our findings indicate that the contribution of MIF released by necrotic/necroptotic cells to pathologies associated with increased cell death and/or defective clearance of apoptotic cells should be elucidated.

METHODS

Reagents

LPS from *E. coli* 0111:B4, 3-methyladenine and propidium iodide were from Sigma (St. Louis, MO, USA); nigericin (sodium salt) from Cayman Chemical (Ann Arbor, MI, USA); MCC950 from InvivoGen (San Diego, CA, USA); recombinant human TNF from ImmunoTools (Friesoythe, Germany); necrostatin-1 from AdipoGen (San Diego, CA, USA); BV-6 from Selleck Chemicals (Houston, TX, USA); Z-VAD-fmk from AdooQ Bioscience (Irvine, CA, USA) and hydrogen peroxide (H_2O_2, 30%) from Univar Solutions (Downers Grove, IL, USA).

Cells

Murine iBMMs and human THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS, USA), 2 mM L-glutamine, 50 U mL^{-1} penicillin and 50 mg mL^{-1} streptomycin (Sigma-Aldrich) (complete medium). For all experiments, cells were transferred to 96-well tissue culture plates at a density of 2.5–5 × 10^5 cell mL^{-1} and incubated at 37°C with 5% CO_2 overnight prior to treatments. Unless otherwise stated, culture medium was not removed prior to treatment, to ensure constitutive MIF levels were maintained. Primary murine bone marrow-derived macrophages were cultured as previously described.[18] In brief, bone marrow cells were collected from femurs of C57BL/6 mice (used with the approval of the appropriate Monash University Ethics Committee) and differentiated in complete medium further supplemented with 15% L929 conditioned medium for 7 days. Cells were plated in 96-well tissue culture plates overnight prior to treatment.

ELISA

Cytokines in culture supernatants were measured by ELISA according to manufacturers' protocols. For human and mouse MIF, kits DY289 and DY1978, respectively, from R&D systems (Minneapolis, MN, USA) were used. For human and mouse IL-1β, kits 437004 and 432601, respectively, from BioLegend (San Diego, CA, USA) were used. For human and mouse TNF, kits 430201 and 430901, respectively, from BioLegend were used. For mouse IL-6, kit 431301 from BioLegend was used.

Induction of necrosis

Necrosis was induced in cells by treatment with 1 mM hydrogen peroxide (H_2O_2, from a 30% stock) for 1–6 h or treatment with absolute ethanol at 1.25%, 2.5%, 5% and 5% (v/v) for 6 h. In addition, necrosis was induced by irradiation with UV light. In brief, cell culture medium was removed and cells were carefully washed once with phosphate-buffered saline prior to UV exposure. Cells were irradiated in the absence of medium/phosphate-buffered saline with a UV dose of 150 mJ cm^{-2} for <150 s using a UVC cross-linker (Spectrolinker XL-1000; UV wavelength of 254 nm). Immediately after irradiation complete culture medium was added back to the cells (UV-treated and nontreated cells) and plates were incubated at 37°C with 5% CO_2 for 4 h. A matched plate was mock treated (without UV) for the same duration and used as a control.

Induction and inhibition of necroptosis

In iBMMs, necroptosis was induced by treatment with a combination of LPS (100 ng mL^{-1}) and Z-VAD-fmk (20 μM) for 16 h. In THP-1 cells, necroptosis was induced with a combination of recombinant human TNF (30 ng mL^{-1}), BV-6 (1 mM) and Z-VAD-fmk (25 μM), incubated for 16 h. To inhibit necroptosis in THP-1 cells, the RIP1 kinase inhibitor necrostatin (30 mM) was added to cells 30 min prior to treatment with TNF, BV-6 and Z-VAD-fmk.

Activation of the NLRP3 inflammasome and pyroptosis

To induce NLRP3 inflammasome activation, cells were first primed with LPS (100 ng mL^{-1}) for 4 h, and then treated with nigericin (2.5–10 mM) for 1 h. When used, the NLRP3-specific inhibitor MCC950 (10 μM) or the pan-caspase inhibitor Z-VAD-fmk (50 μM) was added 30 min prior to administration of nigericin. Inflammasome activation in autophagy-impaired iBMMs was recapitulated by treating cells with a combination of LPS (100 ng mL^{-1}) and the type III PI3K inhibitor 3-methyladenine (1.25–10 mM) for 16 h, as previously described.[16]

Measurement of cell death

Cell death was measured in three different ways. Release of lactate dehydrogenase from cells into the culture supernatant was measured using a Cytotoxicity Detection Kit^{PLUS} (Sigma-Aldrich) according to the manufacturer's protocol. Alternatively, incorporation of propidium iodide was measured by flow cytometry; propidium iodide was added to cells for 5 min at the end of experiments and then measured on a BD FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed using FlowJo version 10 (BD Life Sciences, Franklin Lakes, NJ, USA). Finally, incorporation of SYTOX Green nucleic acid stain (5 mM; Thermo Fisher, Carlsbad, CA, USA) was measured on a Tecan INFINITE M1000 multifunctional plate reader (excitation 504 nm, emission recorded at 523 nm).

Statistical analysis

Data are presented as means ± standard error of the mean (s.e.m.), based on a minimum of three repeats. Data were tested for normal distribution and differences between two groups were evaluated using an unpaired Student's *t*-test; differences between multiple groups were determined using one-way ANOVA with either Dunnett's multiple comparison test (when comparing all groups with a control) or Tukey's multiple comparison test (when comparing all groups against each other). Analyses were performed using GraphPad Prism version 8.3.1 (GraphPad Software, San Diego, CA, USA).

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTION

Wendy Dankers: Data curation; Formal analysis; Investigation; Methodology; Writing-original draft; Writing-review & editing. **Md Abul Hasnat:** Data curation; Investigation. **Vanesa Swann:** Investigation. **Arwaf Alharbi:** Investigation; Writing-review & editing. **Jacinta PW Lee:** Formal analysis; Investigation; Methodology. **Megan A Cristofaro:** Investigation; Writing-review & editing. **Michael P Gantier:** Conceptualization; Methodology; Resources; Writing-review & editing. **Sarah A Jones:** Conceptualization; Methodology; Writing-review & editing. **Eric F Morand:** Conceptualization; Funding acquisition; Project administration; Supervision; Writing-review & editing. **Jacqueline K Flynn:** Conceptualization; Formal analysis; Investigation; Methodology; Supervision; Writing-review & editing. **James Harris:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Writing-original draft; Writing-review & editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix Iiii:

frontiers in Immunology

ORIGINAL RESEARCH
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GILZ Regulates the Expression of Pro-Inflammatory Cytokines and Protects Against End-Organ Damage in a Model of Lupus

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Glucocorticoid-induced leucine zipper (GILZ) mimics many of the anti-inflammatory effects of glucocorticoids, suggesting it as a point of therapeutic intervention that could bypass GC adverse effects. We previously reported that GILZ down-regulation is a feature of human SLE, and loss of GILZ permits the development of autoantibodies and lupus-like autoimmunity in mice. To further query the contribution of GILZ to protection against autoimmune inflammation, we studied the development of the lupus phenotype in Lyn-deficient ($Lyn^{-/-}$) mice in which GILZ expression was genetically ablated. In $Lyn^{-/-}$ mice, splenomegaly, glomerulonephritis, anti-dsDNA antibody titres and cytokine expression were exacerbated by GILZ deficiency, while other autoantibody titres and glomerular immune complex deposition were unaffected. Likewise, in patients with SLE, *GILZ* was inversely correlated with *IL23A*, and in SLE patients not taking glucocorticoids, *GILZ* was also inversely correlated with *BAFF* and *IL18*. This suggests that at the onset of autoimmunity, GILZ protects against tissue injury by modulating pro-inflammatory pathways, downstream of antibodies, to regulate the cycle of inflammation in SLE.

Keywords: GILZ, glucocorticoid, systemic lupus erythematosus, glomerulonephritis, IL-23

INTRODUCTION

Glucocorticoid-induced leucine zipper (GILZ) exhibits a range of anti-inflammatory effects that invites significant interest as a potential target in developing an alternative therapeutic to glucocorticoids. Glucocorticoids continue to be used in the treatment of autoimmune diseases, particularly systemic lupus erythematosus (SLE), despite their predictable and severe adverse effects, because no safe and effective alternative has emerged. Glucocorticoids potently up-regulate GILZ, and thus understanding the potential for GILZ to protect against inflammation in SLE and other inflammatory conditions is imperative.

GILZ exerts anti-inflammatory actions by interacting with transcription factors to modulate important inflammatory signaling pathways, such as the NF-κB pathway, similar to the effects of glucocorticoids (1). As a result of its anti-inflammatory activity, GILZ is protective against damage in neuroinflammation (2), vascular, intestinal and liver inflammation (3–5), allergy (6), heart disease (7, 8), acute kidney injury (9), arthritis (10), psoriasis (11) and SLE. In SLE patients, active disease is associated with impaired GILZ expression (12). We previously showed that loss of GILZ exacerbates inflammation, exemplified by the development of a lupus-like phenotype in GILZ-deficient mice, characterized by antinuclear antibodies (ANA) and nephritis (12). Importantly, we have previously shown that GILZ is a non-redundant regulator of B cell activity, with GILZ deficiency resulting in heightened B cell activation and proliferation which was reversed by treatment with a cell-permeable GILZ fusion protein (12). These findings have important clinical correlates, for example, GILZ induction by glucocorticoids in most B cell subsets was negatively correlated with SLE disease activity (12). Additionally, others have independently confirmed that GILZ mRNA expression is negatively correlated with the SLE disease activity index (SLEDAI) (13, 14).

To gain further insights into the means by which GILZ protects against development of autoimmunity, we bred GILZ deficiency onto the Lyn knockout mouse model of lupus. Lyn is a member of the SRC family of protein tyrosine kinases that is a key negative regulator of signal transduction pathways in B cells, myeloid cells and dendritic cells (DC) (15, 16). Lyn phosphorylates inhibitory receptors in B cells that contain immunoreceptor tyrosine-based inhibitory motifs (ITIM) which initiate signaling events such as cytokine production, proliferation and migration (17). Thus, Lyn acts as a negative regulator of B cell activation, and consequently Lyn-deficient B cells are hyper-responsive to BCR ligand/signaling, with a lowered threshold to stimulation (18). Notably, Lyn-deficient mice spontaneously develop a well-characterized lupus-like autoimmune disease as they age that includes the loss of normal GILZ expression (12) and splenomegaly, autoantibody production, and severe immune complex-mediated glomerulonephritis (GN) (18, 19). Furthermore, pro-inflammatory cytokines such as BAFF, IL-6 and IFNγ play an important role in this phenotype, similar to human SLE (16, 20).

By examining the effect of GILZ deficiency on the $Lyn^{-/-}$ model of lupus, we identified that, while loss of GILZ did not further exacerbate autoantibody expression, it did increase early damage to spleen and kidneys. This was accompanied by the appearance of pro-inflammatory cytokines at an earlier age in $Lyn^{-/-}$ mice lacking GILZ than in those with sufficient GILZ. Thus, as appears to occur in human SLE, GILZ functions to limit the development of the inflammatory environment that contributes to end-organ damage.

MATERIALS AND METHODS

Animals

The generation of GILZ-deficient mice has been previously described (21, 22), as has the generation of Lyn-deficient C57Bl/6 mice (18, 20). All GILZ-deficient mice are male, since GILZ is X-linked and renders male mice sterile (22) and female GILZ-deficient mice cannot be bred. Mice were housed in specific pathogen-free conditions. We generated GILZ-deficient mice on a Lyn-deficient background ($GILZ/Lyn^{-/-}$) and compared them to wildtype (WT), GILZ knockout ($GILZ^{-/-}$), and Lyn knockout ($Lyn^{-/-}$) mice. WT and $GILZ^{-/-}$ mice were co-housed as littermate controls, and $Lyn^{-/-}$ and $GILZ/Lyn^{-/-}$ animals were co-housed as littermate controls. All animals were housed in identical conditions in adjacent cages within our facility. Animals in experimental groups were carefully age and sex matched, but with ages pooled into <150 days or >200 days old so as to adequately power our statistical analyses. All procedures were approved by the Monash Medical Centre Animal Ethics Committee.

Spleen Weights

Mouse spleens were harvested and spleen weights were measured in milligrams (mg). Curves were fit to a one-phase decay model with robust regression using GraphPad Prism Software 7.0b. The age spans of mice chosen to display in **Figure 1B** were 70-84 days (10-12 weeks) and 245-315 days (35-45 weeks) so as to ensure adequate sample size and no significant differences in age that may alter the interpretation of the results. The statistics for the age groups are shown in **Table 1**.

A Kruskal Wallis test followed by Dunn's correction for multiple comparisons found no significant differences between any age groups in the 70-84 day old cohort. In the 245-315 day cohort, there was a significant difference, ($GILZ^{-/-}$ mice significantly were older than $GILZ/Lyn^{-/-}$ mice, P=0.03), and no other significant differences were detected between ages of mice across genotypes.

Cytokine Measurements

Serum was isolated from whole blood obtained by cardiac puncture following CO_2 asphyxiation. Concentrations of BAFF, IFNγ, IL-10, IL-1α, IL-1β, IL-18, IL-12p70, IL-6, IL-23A and IL-17A, were measured in serum by Luminex as described in the manufacturer's protocol (Customized mouse 11-plex ProcartaPlex Kit, Invitrogen).

Autoantibody Measurement

Serum samples were used at 1:200 dilution for the identification of antibodies to extractable nuclear antigens (ENA) by flow cytometry. The FIDIS Connective Profile MX 117 kit (Theradiag) was used as per the manufacturer's instructions with the variation of the use of a PE-conjugated anti-mouse IgG F(ab')2 secondary antibody (eBioscience) for flow cytometry (23).

Histology and Immunofluorescence of Kidneys

Kidneys from 8 to 45-week-old male mice were frozen in Optimal Cutting Temperature (OCT) compound (Tissue Tek). Frozen sections were stained with PE-conjugated polyclonal anti-mouse IgG F(ab')2 secondary antibody (eBioscience) and FITC-conjugated goat IgG fraction to detect mouse complement component C3 (MP Biomedicals). Formalin fixed sections were stained with Haematoxylin and Eosin or periodic acid-Schiff

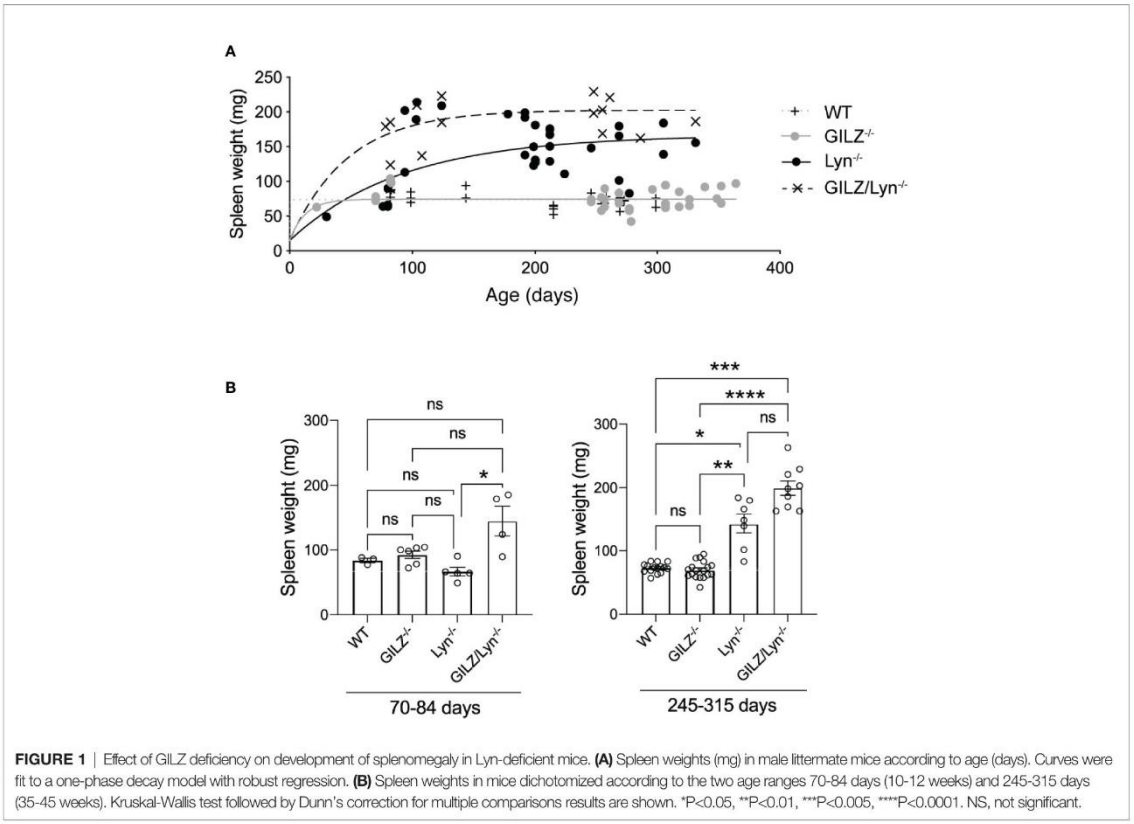

FIGURE 1 | Effect of GILZ deficiency on development of splenomegaly in Lyn-deficient mice. **(A)** Spleen weights (mg) in male littermate mice according to age (days). Curves were fit to a one-phase decay model with robust regression. **(B)** Spleen weights in mice dichotomized according to the two age ranges 70-84 days (10-12 weeks) and 245-315 days (35-45 weeks). Kruskal-Wallis test followed by Dunn's correction for multiple comparisons results are shown. *P<0.05, **P<0.01, ***P<0.005, ****P<0.0001. NS, not significant.

TABLE 1 | Number, mean and standard deviation of ages of mice included in the analysis shown in **Figure 1B**.

	70-84 days			245-315 days		
	n	*mean*	*SD*	*n*	*mean*	*SD*
WT	3	82.13	0.404	15	269.7	14.26
GILZ$^{-/-}$	6	78.05	6.24	16	275.1	19.65
Lyn$^{-/-}$	4	78.85	1.90	7	277.1	21.29
GILZ/Lyn$^{-/-}$	4	80.93	1.95	9	239.6	39.15

(PAS) stain. Glomerular segmental necrosis was defined as acellular areas of PAS positive staining (24).

Glomerular damage was scored in a blinded manner for segmental necrosis (shown as percentage of glomeruli affected) and crescent formation as described (25). IgG and C3 immunofluorescence staining were given an intensity score between 0–3, and an average of 20 glomeruli were scored for each mouse.

Measurement of Gene Expression

To assess GILZ expression in healthy subjects, we mined the publicly available datasets GSE123698 and GSE69832 for the probes corresponding to GILZ (gene name *TSC22D3*), as shown in **Supplemental Figure 2** (26, 27). To measure gene expression in patients with SLE, we mined the dataset GSE88884, which contains gene expression data from PBMC from n = 60 healthy subjects and n = 1,760 SLE patients enrolled in the ILLUMINATE-1 and ILLUMINATE-2 tabalumab phase III clinical trials (28, 29), taken at baseline. We extracted the data for the probes TC0X001262.hg.1:1, TC0X001262.hg.1:2 and TC0X001262.hg.1:3 that identified GILZ (gene name *TSC22D3*). Relative expression values for GILZ were determined by averaging the three probe set values for each subject, without batch correction, as with previous analyses. The strength of associations between GILZ and cytokine expression were determined based on Spearman's rank order correlation. Clinical data were kindly provided by Dr Robert Hoffman of Eli Lilly.

Statistical Analysis

All analyses and data visualization were performed using GraphPad Prism Software 7.0b and R software (version 4.0.2). Continuous and categorical data are presented as mean (standard deviation (SD)) or median (interquartile range) (IQR) and frequencies (percentage), respectively, according to data distribution. Choice of parametric/non-parametric test was guided by the assessment of continuous data distribution, as

examined by Shapiro-Wilk and Kolmogorov-Smirnov tests. Correlation between variables was analyzed using Spearman's rank correlation test. Difference in continuous variable between 2 or more than 2 groups were examined using Student's unpaired two-tailed *t*-test with Welch's correction, or Kruskal-Wallis test followed by Dunn's multiple comparison test, respectively, where appropriate. A P value of < 0.05 was deemed statistically significant.

RESULTS

GILZ Deficiency Increased Spleen Weight in Lyn-Deficient Mice

Splenomegaly occurs in some patients with active SLE and has also been described in both Lyn$^{-/-}$ and GILZ$^{-/-}$ mice (30). In Lyn$^{-/-}$ mice, splenomegaly is driven by the accumulation of plasma cells and Mac-1^{+} lymphoblasts, from as early as 12 weeks of age (31). GILZ-deficient mice develop splenomegaly as they age and isotype-switched plasma cells in spleen are increased (12). We therefore examined whether Lyn and GILZ synergized to regulate the processes that cause splenomegaly. We observed that Lyn-deficient mice developed splenomegaly at an early age, more so when GILZ was deficient, and spleen size progressively increased with aging (**Figures 1A, B**). While a Kruskal-Wallis test followed by Dunn's correction for multiple comparisons did not detect a statistically significant difference between Lyn$^{-/-}$ and GILZ/Lyn$^{-/-}$ mice of 35-45 weeks of age when all genotypes were included in the analysis (**Figure 1B**), a Student's t-test between these two genotypes showed a significant difference (P=0.0077). We did not observe splenomegaly in GILZ-deficient mice (**Figures 1A, B**), in contrast to our previous report (12), which is likely due to a change in housing conditions between these studies, as microbiota and cohousing may affect the phenotypes (32). A detailed analysis of the microorganisms detected within sentinel animals in the same room in which our colonies were housed in shown in **Supplemental Figure 1**, although we did not test whether the changes impacted on the development of an autoimmune phenotype in the GILZ$^{-/-}$ strain. However, splenomegaly observed in Lyn-deficient mice was hastened in the absence of GILZ, and was worsened over time (**Figures 1A, B**). This detailed kinetic study of the degree of splenomegaly confirms that GILZ deficiency is permissive of the development of autoimmunity triggered by underlying genetic factors.

GILZ has recently been reported to decrease in murine macrophages as a function of the mild inflammatory state that develops with age (33). We previously showed that Lyn$^{-/-}$ mice expressed lower GILZ, like patients with SLE, and this deficiency worsened with age (12). In the Lyn$^{-/-}$ model, the development of autoimmunity is exacerbated over time and severity increases with age (34). However, in wildtype mice (12), and in peripheral blood mononuclear cells from healthy subjects (26, 27) and patients with SLE (35), we detected no meaningful change in GILZ expression that occurred over the lifespan (**Supplemental Figure 2**). Thus, the exacerbation of the Lyn$^{-/-}$ phenotype with age, that occurred as a result of GILZ deficiency, was likely due to factors other than an age-related effect.

GILZ Deficiency Worsened Glomerulonephritis in Lyn-Deficient Mice

Morbidity in the Lyn$^{-/-}$ model is associated with the progressive development of renal injury. To determine GILZ whether deficiency worsens glomerulonephritis in the Lyn$^{-/-}$ model, we compared the development of glomerulonephritis in Lyn$^{-/-}$ mice with that in mice lacking both Lyn and GILZ (**Figure 2**). We observed that aged GILZ/Lyn$^{-/-}$ mice had more severe glomerulonephritis compared to Lyn-deficient mice, featuring significantly increased segmental necrosis and glomerular crescents on PAS staining of kidney sections (**Figure 2A** with representative images shown in **Figure 2B**), and reduced kidney size (**Figure 2C**). Therefore, kidney damage in Lyn$^{-/-}$ mice was exacerbated by GILZ deficiency.

GILZ Deficiency Effects on Serum Autoantibodies in Lyn-Deficient Mice

We next explored potential explanations for the heightened autoimmune phenotype observed in Lyn-deficient mice when GILZ was also deficient. The hyperactive B cells in Lyn$^{-/-}$ mice (20) and loss of B cell quiescence and tolerance we previously reported in GILZ$^{-/-}$ mice (12) each result in the development of lupus-like autoimmunity. As a result, both strains produce ANA, and antibodies to ENA (dsDNA, histone, Smith (SM) and U1RNP) (12, 18). We sought to determine whether GILZ deficiency worsened the lupus phenotype of Lyn$^{-/-}$ mice *via* exacerbation of autoantibody-mediated autoimmunity. While both Lyn$^{-/-}$ and GILZ/Lyn$^{-/-}$ mice expressed autoantibodies at a young age (**Figure 3A**), anti-dsDNA antibodies were present at significantly higher concentrations in the aged GILZ/Lyn$^{-/-}$ mice compared to Lyn$^{-/-}$ mice (>200 days) (**Figure 3B**); no other autoantibodies were significantly affected in aged GILZ/Lyn$^{-/-}$ mice. Thus, while GILZ deficiency did not alter overall serum autoantibody production in Lyn$^{-/-}$ mice in this study, it did significantly impact on an autoantibody specifically associated with nephritis in human SLE.

Loss of GILZ Did Not Alter Immune Complex Deposition in Kidneys of Lyn-Deficient Mice

Previous studies report that Lyn-deficient mice develop glomerular immune complex deposition as early as 6–8 weeks of age and cumulative damage to kidneys subsequently worsens with ageing (18, 31). We previously reported that GILZ$^{-/-}$ mice also develop mild immune complex-mediated glomerulonephritis as they age (12). In keeping with the generally equivalent levels of autoantibodies in Lyn$^{-/-}$ and GILZ/Lyn$^{-/-}$ mice, but in contrast to the higher levels of nephritis-associated anti-dsDNA antibodies, we detected negligible differences in glomerular immune complex deposition between mice of these two strains. While young mice (< 150 days old) of both Lyn$^{-/-}$ and GILZ/Lyn$^{-/-}$ strains showed deposition of C3 and IgG in the glomeruli, there was no significant difference in C3 deposition between the two genotypes (**Figure 4A** and quantified in **Figure 4B**). In aged mice, immunofluorescence staining of kidneys for C3 and IgG

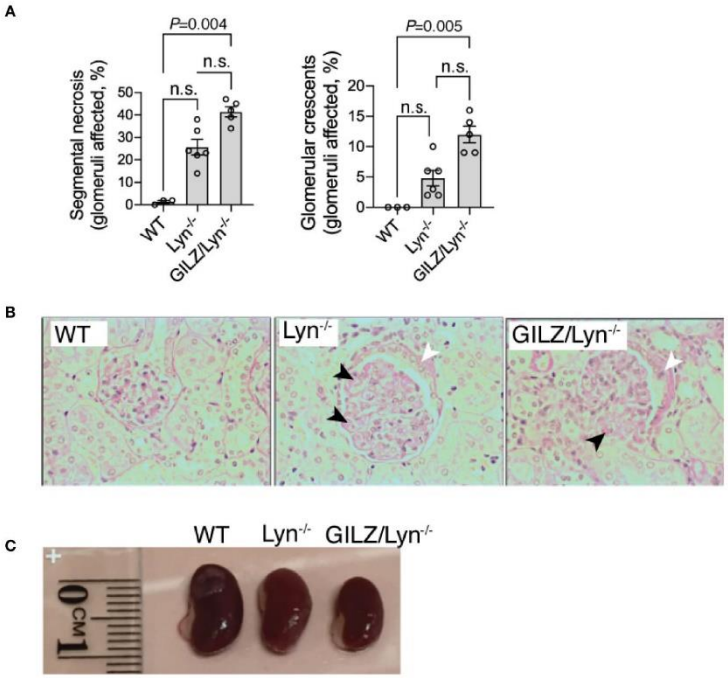

FIGURE 2 | Effect of GILZ deficiency on glomerulonephritis in Lyn-deficient mice. **(A)** Renal injury was assessed by measurement of segmental necrosis (glomeruli affected in %) and glomerular crescents (glomeruli affected in %) in mice older than 200 days of age. **(B)** Representative images of glomerular lesions stained with periodic acid-Schiff (PAS) stain are shown, with segmental necrosis indicated with black arrowheads and glomerular crescents with white arrowheads. **(C)** Indicative differences in kidney size between mouse strains. Statistical significance was determined using Kruskal-Wallis test with Dunn's correction for multiple comparisons (n=5-8). All data shown are mean ± SEM. NS, not significant.

showed immune complex deposition in both Lyn$^{-/-}$ and GILZ/Lyn$^{-/-}$ mice, with no significant difference in the extent of C3 deposition observed (**Figure 4C**). Rather, there was a trend toward reduced IgG staining in glomeruli of aged GILZ/Lyn$^{-/-}$ mice (**Figure 4C**). These observations suggest that GILZ deficiency worsened autoimmune disease outcomes in the Lyn$^{-/-}$ model *via* processes other than through effects on the degree of autoantibody immune complex deposition in kidneys.

GILZ Deficiency Affects Serum Cytokine Levels in Lyn-Deficient Mice

To further investigate the basis of the exacerbation of organ damage in GILZ/Lyn$^{-/-}$ mice, we tested whether GILZ deficiency altered cytokine production in Lyn$^{-/-}$ mice. Multiple cytokines were elevated in serum of young GILZ/Lyn$^{-/-}$ mice, with substantial variance between individual mice as observed in human SLE. BAFF, IFNγ, IL-10, IL-1α, IL-1β, IL-6 and IL-23A all trended non-significantly numerically higher in young GILZ/Lyn$^{-/-}$ mice than in Lyn$^{-/-}$ littermate controls, and elevations in IL-18, IL-12 and IL-17A were significant (**Figure 5A**). In aged mice, in which the autoimmune phenotype was well established, these early significant differences were all attenuated (**Figure 5B**).

GILZ Correlates With Cytokine Expression in Patients With SLE

To assess whether low GILZ expression is permissive of cytokine expression in human SLE, we mined the dataset GSE88884, which contains microarray data from PBMC from n =1,760 SLE patients. It is important to note that this study was limited to mRNA levels in PBMC rather than absolute amounts of circulating cytokines. Relationships with *GILZ* expression were strongest with *IL23A*, where a negative correlation was present in SLE patients (**Figure 6A**).

GILZ is a glucocorticoid-induced gene, and we assessed whether GILZ expression and its associations with cytokines were altered by glucocorticoid exposure in SLE patients. Healthy subjects (n = 60) and SLE patients not taking glucocorticoids (n = 460) had normally distributed and similar levels of expression of GILZ, with median, mean (± standard deviation) of 7.769, 7.727 (± 0.113) and 7.750, 7.749 (± 0.156) respectively. GILZ expression in PBMC from SLE patients taking

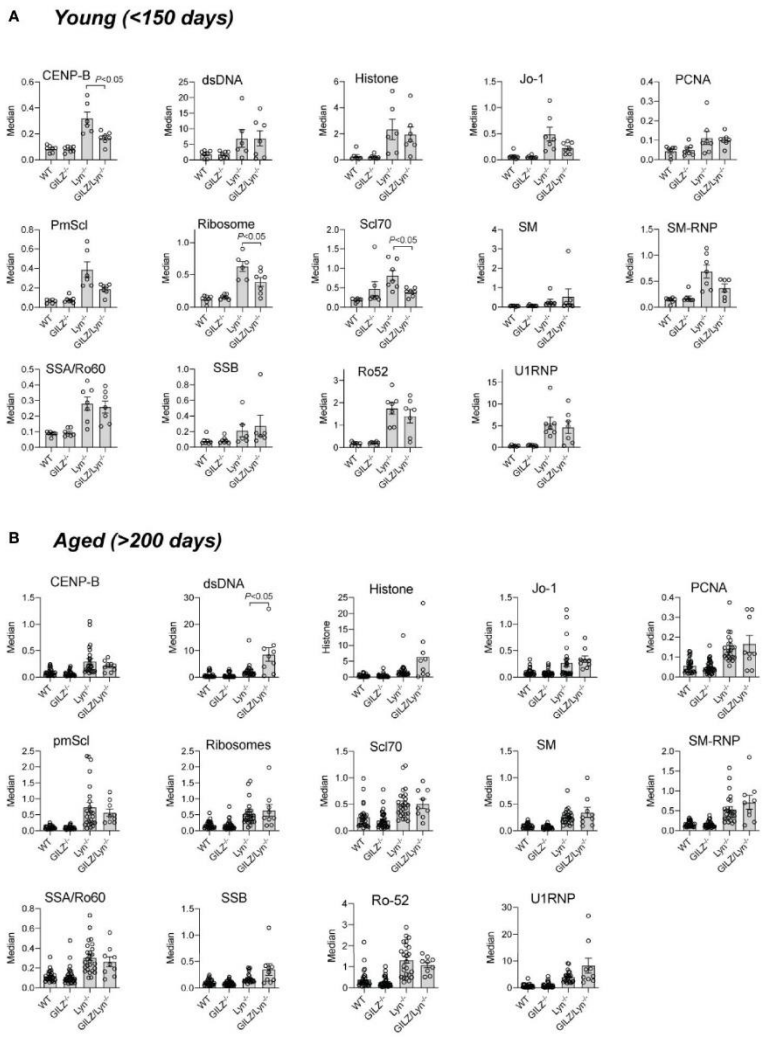

FIGURE 3 | Autoantibodies reactive to specified extractable nuclear antigens (ENA) in mouse sera. Detection of IgG F(ab)2 autoantibodies against extractable nuclear antigens (ENA) detected by flow cytometry of serum from **(A)** young mice (<150 days) or **(B)** older (>200 days). Bars show median fluorescence intensity ± SEM normalized to positive control ENA beads. *P*-values were derived from Student's *t*-test comparing $Lyn^{-/-}$ with GILZ/$Lyn^{-/-}$ mice. All data are presented as mean ± SEM (n = 6-24).

glucocorticoids (n = 1293) was median and mean of 7.771 and 7.795 (± 0.211), and was significantly higher than in patients not taking glucocorticoids (*P*=0.002; **Figure 6B**). Since increased *GILZ* expression by glucocorticoids treatment could potentially obscure associations, we analyzed the subset of 460 patients not taking glucocorticoids. The level of *IL23A* was moderately negatively correlated to the expression of *GILZ* in both subsets of patients taking and not taking glucocorticoids (**Figures 6C, D**). Other cytokines including *BAFF* and *IL18* were negatively correlated with GILZ expression in non-glucocorticoid-using patients, although the relationship between *GILZ* and *BAFF* did not reach significance (with *P* = 0.06) (**Figure 6C**).

DISCUSSION

The treatment of autoimmune diseases like SLE with GC results in beneficial suppression of autoimmune inflammation, but also exposes patients to major adverse effects. The discovery of targets for the potential generation of safer GC mimics is an

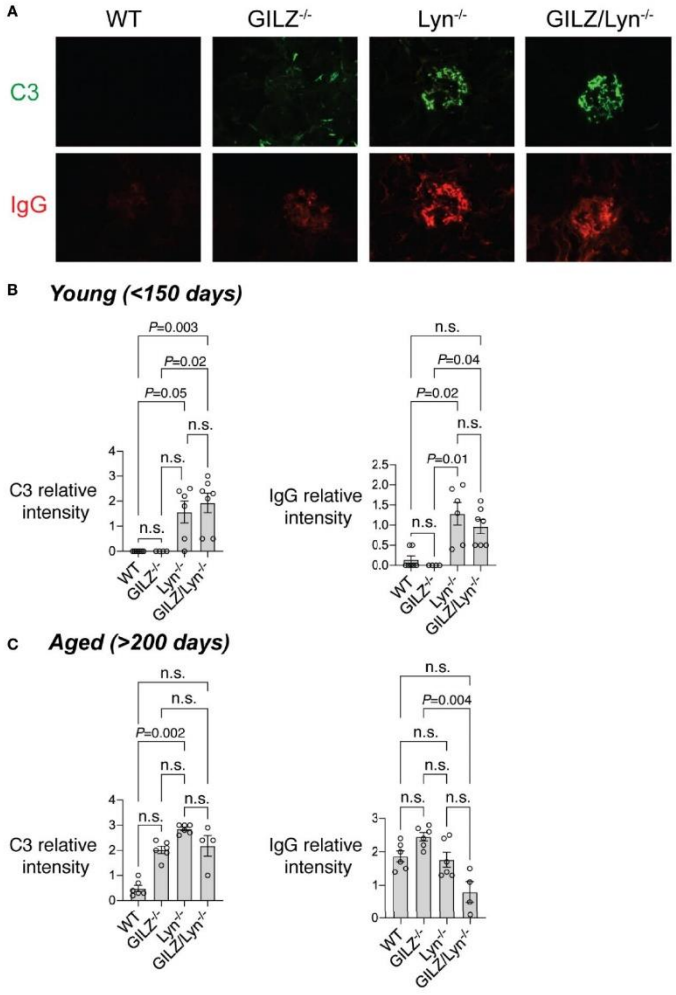

FIGURE 4 | Immunofluorescence staining of immune complex deposition in glomeruli. **(A)** Immune complex (IC) deposition in glomeruli measured by immunofluorescence (IF) staining of complement component C3 (green) and immunoglobulin G (IgG; red). Representative images are shown from mice around 100 days of age. **(B)** IC deposition in mice young than 150 days or **(C)** older than 200 days was scored by a blinded expert using a scale of 0–3. (n=4–7). Statistical significance was determined using Kruskal-Wallis test with Dunn's correction for multiple comparisons. All data are mean ± SEM. NS, not significant.

urgent unmet need. GILZ has been identified as a potential target for such an approach. Here, we investigated the effects of deficiency of GILZ on morbidity and mortality in Lyn$^{-/-}$ mice, to glean insights into the pathogenic pathways that are regulated by GILZ in SLE.

GILZ is highly expressed in both mouse and human lymphoid and myeloid subsets, and is particularly abundant in macrophages (36–38), and low in plasmacytoid DCs, the key source of type I IFN in SLE (39). GILZ restrains DC maturation and activation, inducing DC-mediated promotion of antigen-specific Treg development (40–42). GILZ is highly expressed in macrophages, with clear anti-inflammatory effects (38, 43, 44). Moreover, down-regulation of GILZ occurs in acute inflammatory models, such as the caecal ligation and puncture (CLP) model of sepsis, mirroring the situation in patients with sepsis, although restoration of GILZ was protective against bacteremia and systemic inflammation (45–47).

Both Lyn-deficient and GILZ-deficient mice spontaneously develop lupus-like autoimmunity, and SLE patients have lower Lyn and GILZ expression (12, 48, 49). One study showed that by 25 weeks of age, 42% of Lyn$^{-/-}$ mice were either developing

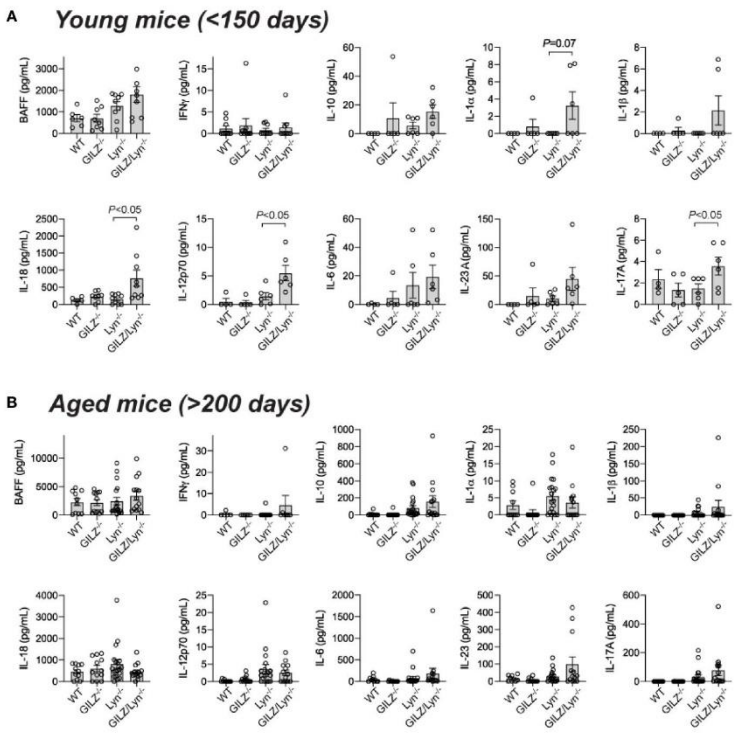

FIGURE 5 | Effect of GILZ deficiency on serum cytokine expression in Lyn-deficient mice. Measurement of serum cytokines (pg/mL) including BAFF, IFNγ, IL-10, IL-1α, IL-1β, IL-18, IL-12p70, IL-6, IL-23A and IL-17A by Luminex in **(A)** young (< 200 days), and **(B)** aged mice (> 200 days). *P*-values were derived from Student's *t*-test comparing $Lyn^{-/-}$ with $GILZ/Lyn^{-/-}$ mice. All data are presented as mean ± SEM (n = 4-20).

autoimmune disease or had succumbed (18). In SLE patients, severe autoimmune disease with multi-organ damage results in marked loss of life expectancy when compared to the general population (50).

Here we report that loss of GILZ resulted in earlier onset and worsened splenomegaly in Lyn-deficient mice, suggesting a non-redundant role for GILZ in modulating the severity of autoimmune disease in this model. Investigations of GILZ deficiency are restricted to male mice, since the GILZ gene is on the X chromosome and its deficiency renders male mice sterile, thus knockout females cannot be bred, although male mice in the strains used here develop lupus-like autoimmunity in a relatively consistent manner over time. Since whole body knockout mice were employed, our data do not allow us to conclude which cell types are responsible for the phenotype we observed. However, several organ systems were affected by GILZ deficiency in $Lyn^{-/-}$ mice.

Splenomegaly is an uncommon clinical manifestation of SLE that occurs particularly during active disease (30), the exact mechanism of which is not clearly understood, although it occurs almost universally in $Lyn^{-/-}$ mice. Splenomegaly is known to be an active process that occurs due to splenic vessel inflammation, lymphoid hyperplasia (51), extramedullary hematopoiesis due to increased production of myeloid growth factors (52, 53) and in Lyn-deficient mice, due to accumulation of plasma cells, and Mac-1^{+} lymphoblasts, as early as 12 weeks (31). It has been demonstrated that splenomegaly is an IL-6-dependent phenotype in Lyn-deficient mice (20), however increases in IL-6 in $Lyn^{-/-}$ mice were not further exacerbated in the absence of GILZ and mRNA expression of GILZ and IL-6 were significantly positively correlated in human SLE.

Lupus nephritis is a clinical phenotype that arises in 40–50% of SLE patients, and is associated with marked increases in morbidity and mortality. It is strongly associated with the presence of serum anti-dsDNA antibodies, and a recent phase III clinical trial has shown that outcomes are improved when neutralization of BAFF is added to glucocorticoids (54). Here, we confirmed the presence of immune complex-mediated nephritis in $Lyn^{-/-}$ mice, and observed an exacerbation of renal damage in the absence of GILZ. In the present study, $GILZ^{-/-}$ mice showed little evidence of splenomegaly, ENA, IgG or C3 in glomeruli of younger mice, or inflammatory cytokine expression, and for these reasons we did not assess the kidneys of $GILZ^{-/-}$ for glomerular injury. However, GILZ deficiency worsened the severity of the $Lyn^{-/-}$ phenotype, including glomerulonephritis.

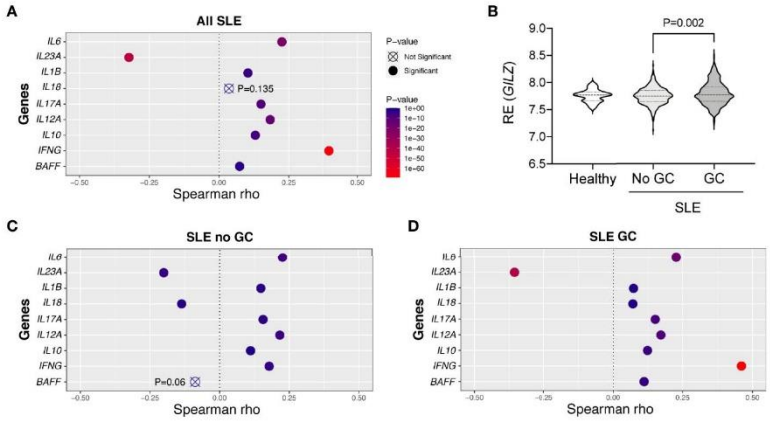

FIGURE 6 | Correlations between GILZ and cytokine mRNA expression in patients with SLE. (Panels **A**, **C**, **D**) Publicly available microarray data was obtained from PBMCs of healthy controls and patients with SLE enrolled in the tabalumab trial at baseline (GSE88884). Relative mRNA expression levels of *IFNG*, *IL12A*, *IL6*, *IL1B*, *IL10*, *IL17A*, *IL23A*, *BAFF* and *IL18* were mined from the dataset and their correlations with GILZ were determined. Panels depict the degree to which GILZ expression is correlated with individual cytokines based on Spearman rho values, including all SLE patients (n=1,760; panel **A**), or limited to patients not taking glucocorticoids (GC; n=460, panel **C**), or patients taking GC (n=1293 included, panel **D**). **(B)** Distributions of the relative expression (RE) of GILZ isoform 1 in the healthy subjects (n = 60) and SLE patients divided into those not taking (n = 460), and those taking (n = 1293), glucocorticoids. The *P*-value was derived by Kruskal-Wallis test followed by Dunn's correction for multiple comparisons.

This suggests that GILZ induction may represent a mechanism through which GC act in the treatment of lupus nephritis in humans, although this remains to be proven.

In studying the processes that contribute to the increased severity of lupus-like disease upon loss of both GILZ and Lyn, we found that while autoantibody production overall was not significantly altered by the loss of GILZ, anti-dsDNA antibodies that are strongly associated with nephritis in human SLE were significantly increased in older double-deficient mouse strain compared to $Lyn^{-/-}$. Despite this, glomerular immune complex deposition was not detectably increased in the absence of GILZ, suggesting that the effect of dysregulated BCR signaling in the absence of Lyn is a strong enough lesion in B cells that the absence of GILZ does not further exacerbate it and that effects on immune complex deposition do not explain worsened nephritis in the absence of GILZ.

Although SLE is an autoimmune disease characterized by hyperactive B cells, other cells including T cells and DC, and various cytokines, also play critical roles in SLE pathogenesis. SLE patients express increased levels of cytokines as products of innate and adaptive immune responses, and increased cytokine secretion is likely to drive tissue injury and end-organ damage in SLE. Interestingly, we found that GILZ deficiency permitted the early expression of an array of pro-inflammatory cytokines in young Lyn-deficient mice. The regulation of IL-17A and the possible regulation of the Th17-promoting cytokine IL-23A by GILZ in our study resonates with several previous reports including our own. We previously showed that GILZ inhibits IL-17A production and GILZ-deficient CD4 T cells produced increased IL-17 (21). Similarly, in acute kidney injury, GILZ was markedly suppressed by inflammation, and treatment with exogenous GILZ resulted in decreased IL-17, increased regulatory T cells (Treg) and IL-10, and prevention of cell death, demonstrating the renoprotective role of T cell GILZ (9). Indeed, a negative correlation was reported between GILZ mRNA and IL-17A levels in SLE patients (13, 14). Thus, our findings support the conclusion of these reports that GILZ is a regulator of the IL-17 axis, but suggest this effect has ramifications for organ damage in SLE.

While our study of gene expression levels in PBMC from SLE patients did not completely replicate our findings in the GILZ-deficient and GILZ-Lyn double-deficient mice, GILZ was robustly negatively correlated with expression of *IL23A*, an important Th17 driver, and with *BAFF* and *IL18* when studied in the subset of glucocorticoid-free patients. The role of BAFF has been comprehensively studied in SLE and neutralization of BAFF is approved for treatment of pediatric and adult SLE. Inhibition of BAFF is therapeutic in human lupus nephritis (54), and BAFF blockade in a phase III clinical trial of over 400 patients with SLE allowed glucocorticoid dose reduction (55). Thus, BAFF inhibition may occur downstream of glucocorticoid treatment, potentially mediated by GILZ.

The involvement of inflammasome activation, whence active IL-18 is derived, is comparatively understudied in SLE, although reports of associations of IL-18 in SLE are emerging. In two separate Asian populations, IL-18 predicted active renal SLE (56, 57), and an *IL18* gene polymorphism was identified to confer risk to renal involvement in SLE (57). A cross-sectional study of 28 SLE patients reported that IL-18 positively correlated with SLE disease activity, flares and anti-dsDNA expression (58). This same study identified that *IL23A* mRNA was strongly over-expressed in blood from patients with SLE (58). We recently

showed, in the largest study to date, that serum IL-18 was elevated in SLE compared to healthy subjects, and was significantly associated with the presence of nephritis (59). Interestingly, glucocorticoid treatment returned elevated IL-18 to control levels in a group of 30 previously untreated SLE patients (60); our data suggest that GC-induced GILZ may be at play in this response.

In conclusion, our findings demonstrate that any additional effect of GILZ on regulation of spontaneous B cell activation, which we previously described, is modest in the context of autoimmune disease development in the $Lyn^{-/-}$ mouse model. In contrast, GILZ appears to modulate cytokine-dependent inflammation in SLE, resulting in exacerbation of renal injury. This supports the hypothesis that GILZ supplementation could be beneficial in SLE, a concept that merits further investigation, such as through the use of GILZ transgenic animal models.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Monash Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

CN, WD, and JF planned and conducted experiments, analyzed data, and helped to prepare the manuscript. JL, WZ, FV, LG, JO, MP, MC, RS, and MH conducted experiments and/or analyzed data. JH, EM, and SJ planned experiments, analyzed data and prepared the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.652800/full#supplementary-material

Supplementary Figure 1 | Health summary from sentinel animals housed in the same room as the strains used in this study over the period 2013-2020. Only organisms that were detected are shown in this figure. The degree of coloring corresponds to the proportion of sampled animals that tested positive.

Supplementary Figure 2 | GILZ expression in healthy subjects in publicly available datasets **(A)** GSE123698 and **(B)** GSE69832 (26, 27). **(C)** GILZ expression in (n = 1,760) patients with SLE, determined by extracting the data for the probes TC0X001262.hg.1:1, TC0X001262.hg.1:2 and TC0X001262.hg.1:3, which identified GILZ, from the public dataset GSE88884 and averaging the three probe set values for each subject (28, 29).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix II: Investigate the relationship between MIF and autophagy in macrophages.

MIF is an essential effector molecule of the innate immune system. Unlike other cytokines, MIF is constitutively expressed by immune and endocrine cells and also by the epithelial lining of tissues, suggesting a role for MIF as a regulator of host responses to infection and stress. A number of recent studies, though with seemingly conflicting results, have highlighted the role of MIF in the regulation of autophagy. Since MIF is expressed constitutively in cells, autophagy may be a key regulator of its turnover and secretion. I looked at the impact of the pharmacological modulators of autophagy and siRNA against Atg7 and Beclin1 (Atg6) on the secretion of MIF by macrophages in response to LPS to determine whether autophagy functions as a regulator of MIF. I found that inhibition of autophagy with 3MA did not have any significant impact on MIF release by RAW264.7 cells in response to LPS stimulation. However, siRNA against Atg7 and beclin1 significantly decreased MIF release in macrophages (**Figure S1**). This contrasts with findings previously published by this lab (Lee et al., 2016c), where release of MIF by THP-1, RAW264.7 and iBMM cells was increased in response to autophagy inhibition. The reason for this difference is not clear, although our more recent study on MIF release by dying cells did also show increased MIF release by iBMM in response to autophagy inhibition with 3-MA (Dankers et al., 2020). Thus, one possibility is that in my experiments LPS + 3-MA did not induce cell death as it does in iBMM and THP-1.

I also investigated MIF release in response to different TLR ligands and found that MIF release was significantly increased upon treatment with 100 and 1000 ng/ml LPS, but unaffected by treatment with R848 or PAM3Cysk4 (**Figure S2**). However, this result with LPS was not consistent (see Figure S1 here and our recent publication) (Dankers et al., 2020). I also measured serum MIF in my experimental mice (old mice) and found a high level of MIF in different experimental groups, but no significant difference among the groups (**Figure S3**). Based on these data (particularly the inconsistencies), along with the fact autophagy and MIF had already been the subject of several previous publications I did not investigate MIF further, and instead concentrated on IL-6 and IL-10.

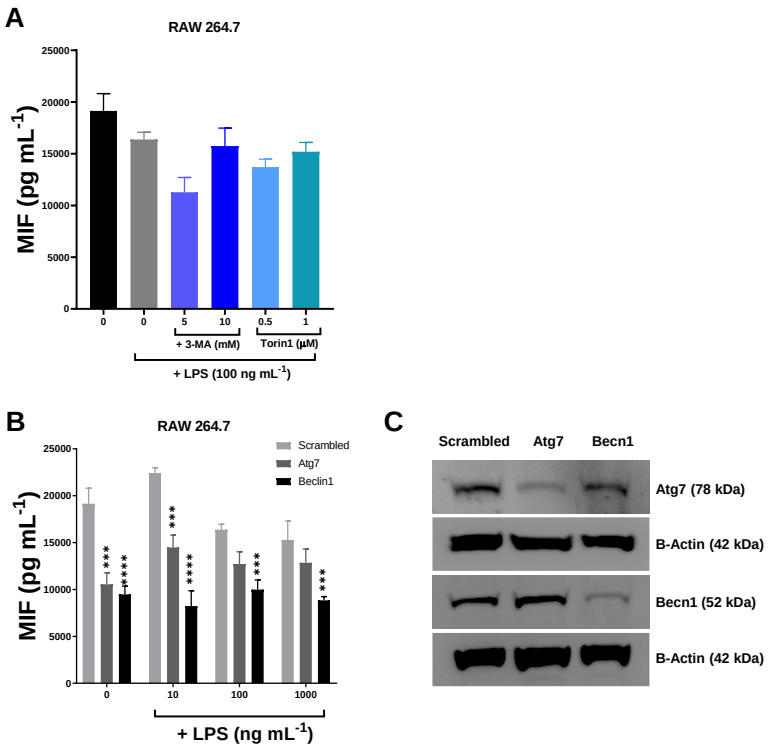

Figure S1: Effect of TLR4 ligand LPS and autophagy on MIF production in macrophage. (A) Effect of the autophagy inhibitor 3 -methyladenine (3-MA) (5 - 10 mM) and the autophagy inducer Torin-1 (Tor 1) (0.5 - 1μM) following treatment with LPS (100 ng/mL). (B) MIF in RAW264.7 macrophages transfected with siRNA against *Atg7* and *Becn1*. (C) Western blot analysis of Atg7 and Becn1 protein in lysates from RAW264.7 cells transfected with siRNA against *Atg7*, *Becn1*, or non-targeting (scrambled, scr) siRNA for 24 h. The significance of differences was determined using two-way ANOVA with Tukey's multiple comparison test; *** $P < 0.001$, **** $P < 0.0001$ compared to scrambled siRNA transfected cells.

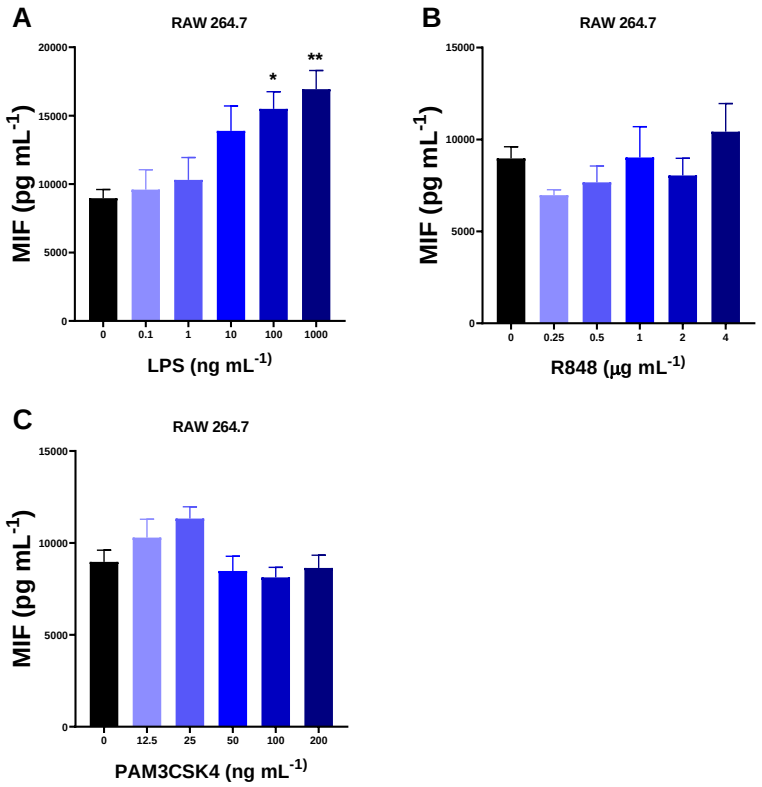

Figure S2: Effect of different TLR ligands on MIF release in macrophage.
(A) TLR4, LPS (0.1 - 1000 ng/mL); (B) TLR7/8, R848 (0.25 - 4 µg/mL); and TLR1/2, PAM3CSK4 (12.5 – 200 ng/mL). MIF was measured by ELISA. Bars represent means ± SEM of 3 separate experiments. * P < 0.05, ** P < 0.01 compared to cells treated with TLRs.

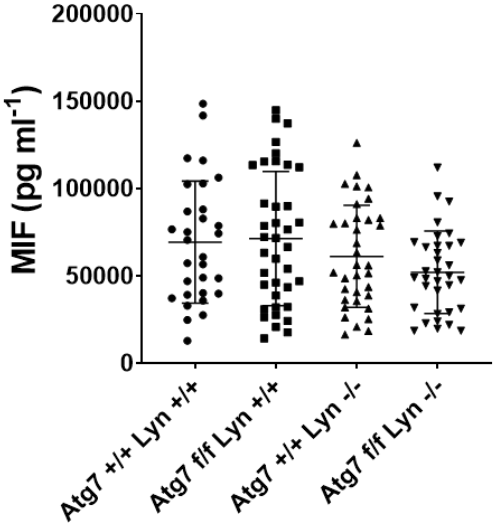

Figure S3: Level of MIF in serum of experimental mice (Old mice age > 100 days).
High level of MIF is observed in different experimental groups. N= 30-50 per group and mice were aged more than 30 weeks. The significance of differences was determined using one-way ANOVA with Tukey's multiple comparison test.

Appendix III: Participated in MIF/DDT experiment

My research group has long investigated the roles of MIF in inflammation and autoimmunity. A second member of the MIF family, D-dopachrome tautomerase (D-DT), has also been identified. While D-DT has been shown to have similar effects to MIF in vitro and in vivo, it's role in disease is less clear and may be somewhat different to that of MIF. Our group has generated *Ddt*$^{-/-}$ mice to test this further and fully characterise the role of D-DT in inflammation. One role of interest is in regulation of the NLRP3 inflammasome and the release of IL-1 family cytokines, particularly IL-1 and IL-18. Our group has recently demonstrated that MIF is required for NLRP3 inflammasome activation, so we are now investigating whether D-DT similarly regulates NLRP3-depednent IL-1 release. As part of this work, I helped with a mouse model of LPS-induced endotoxemia. I monitored the mice and helped to collect serum, spleen and peritoneal lavage samples, which I then tested for peritoneal lavage and serum cytokine levels by Luminex assay. Early data from this work suggests that loss of D-DT, similar to MIF, specifically results in decreased release of IL-1β in both the peritoneal cavity and serum (**Figure S4**), while other cytokines (IL-1, IL-10, IL-23, IL-6, TNF, IFN, IFNβ and IP-10) were unaffected (not shown).

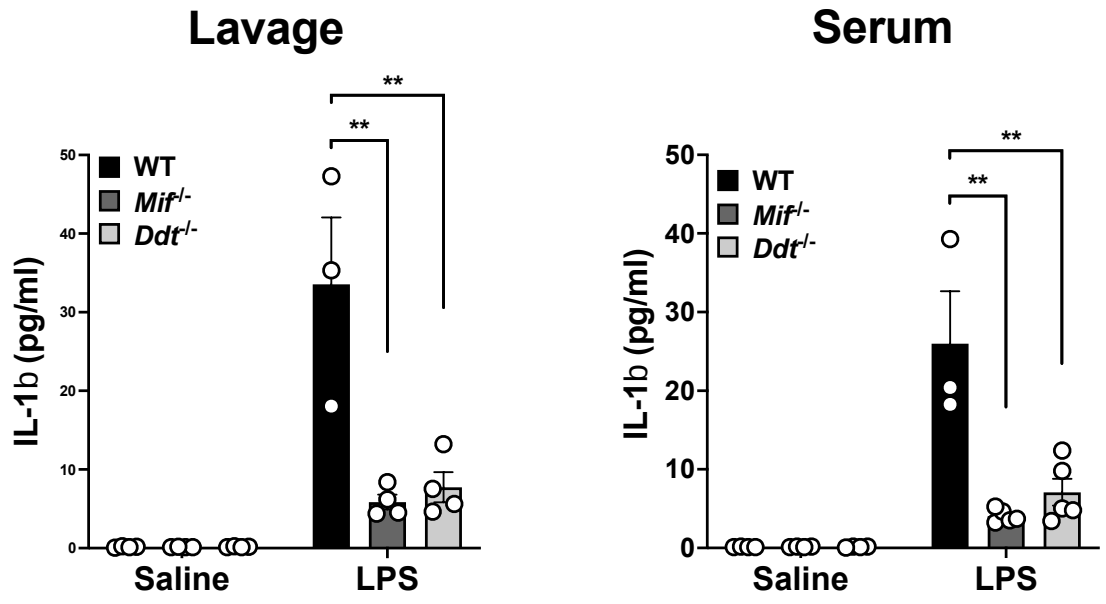

Figure S4. Level of D-DT in peritoneal cavity and serum of mice injected with LPS. Wildtype (WT), Mif-/- and Ddt-/- mice were treated intraperitoneally (i.p) with saline (200 μL) or LPS (50 μg/mouse) (n=3-6 per group) for 4 h. Peritoneal lavage and blood serum from each mouse was collected and analysed. The level of secreted cytokines in serum was measured by Luminex assay. All data are mean ± SEM. The significance of differences was determined using two-way ANOVA with Tukey's multiple comparison test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significant difference between WT and Atg7 KO groups.