Immunology & Cell Biology 2022; **100**: 605–623
www.wileyonlinelibrary.com/journal/icb

ORIGINAL ARTICLE

OPEN

Investigating immunoregulatory effects of myeloid cell autophagy in acute and chronic inflammation

Md Abul Hasnat[1], Ianlan Cheang[1], Wendy Dankers[1], Jacinta PW Lee[1], Lynda M Truong[1], Mehnaz Pervin[1], Sarah A Jones[1], Eric F Morand[1], Joshua D Ooi[2] & James Harris[1]

1 Centre for Inflammatory Diseases, Department of Medicine, School of Clinical Sciences at Monash Health, Faculty of Medicine, Nursing and Health Sciences, Monash University, Clayton, VIC, Australia
2 Regulatory T Cell Therapies Group, Centre for Inflammatory Diseases, Department of Medicine, School of Clinical Sciences at Monash Health, Faculty of Medicine, Nursing and Health Sciences, Monash University, Clayton, VIC, Australia

Keywords
Atg7, autoimmunity, IL-10, IL-6, Lyn, systemic lupus erythematosus

Correspondence
James Harris, Cell Biology Assays, Manufacturing, CSIRO, Building 75, Monash University Clayton Campus, Clayton, VIC 3800, Australia.
E-mail: jim.harris@monash.edu

Present addresses
Wendy Dankers, Department of Rheumatology and Clinical Immunology, Amsterdam Institute for Infection & Immunity, Amsterdam University Medical Centers, Amsterdam, The Netherlands

Jacinta PW Lee, Department of Medicine, Addenbrookes Hospital, University of Cambridge, Cambridge, UK

James Harris, Cell Biology Assays, Manufacturing, Commonwealth Scientific and Industrial Organization (CSIRO), Clayton, VIC, Australia

Received 2 March 2022;
Revised 8 and 30 May 2022;
Accepted 30 May 2022

doi: 10.1111/imcb.12562

Immunology & Cell Biology 2022; **100**: 605–623

Abstract

Studies have highlighted a critical role for autophagy in the regulation of multiple cytokines. Autophagy inhibits the release of interleukin (IL)-1 family cytokines, including IL-1α, IL-1β and IL-18, by myeloid cells. This, in turn, impacts the release of other cytokines by myeloid cells, as well as other cells of the immune system, including IL-22, IL-23, IL-17 and interferon-γ. Here, we assessed the impact of genetic depletion of the autophagy gene *Atg7* in myeloid cells on acute and chronic inflammation. In a model of acute lipopolysaccharide-induced endotoxemia, loss of autophagy in myeloid cells resulted in increased release of proinflammatory cytokines, both locally and systemically. By contrast, loss of *Atg7* in myeloid cells in the $Lyn^{-/-}$ model of lupus-like autoimmunity resulted in reduced systemic release of IL-6 and IL-10, with no effects on other cytokines observed. In addition, $Lyn^{-/-}$ mice with autophagy-deficient myeloid cells showed reduced expression of autoantibodies relevant to systemic lupus erythematosus, including anti-histone and anti-Smith protein. *In vitro*, loss of autophagy, through pharmacological inhibition or small interfering RNA against *Becn1*, inhibited IL-10 release by human and mouse myeloid cells. This effect was evident at the level of *Il10* messenger RNA expression. Our data highlight potentially important differences in the role of myeloid cell autophagy in acute and chronic inflammation and demonstrate a direct role for autophagy in the production and release of IL-10 by macrophages.

INTRODUCTION

Myeloid cells, which include neutrophils, monocytes/macrophages and dendritic cells, play multiple critical roles in the promotion and regulation of inflammatory responses. Macroautophagy (hereafter referred to as autophagy) is a catabolic process for the lysosomal degradation of cytosolic components, including macromolecules and organelles. Autophagy involves the formation of a double-membraned autophagosome, which engulfs cytoplasmic targets and fuses with lysosomes for proteolytic degradation of the cargo.

Studies have shown that loss of autophagy in myeloid cells promotes the release of proinflammatory cytokines, including interleukin (IL)-1α, IL-1β, IL-18 and IL-23, *in vitro*.[1–3] Conversely, stimulation of autophagy limits the production of IL-1β and IL-23 *in vitro*.[1,2] In addition, loss of autophagy in human and mouse macrophages increases the release of the pleiotropic immunomodulatory molecule macrophage migration inhibitory factor (MIF) in response to toll-like receptor (TLR) ligation.[4,5] MIF has multiple proinflammatory roles within the immune system, including regulation of NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome activation, cytokine release, chemotaxis and autophagy.[6,7] Moreover, through regulation of IL-1 family members and IL-23, autophagy in myeloid cells can influence responses of other immune cells, particularly γδ T cells, promoting the release of IL-17, IL-22 and interferon-γ (IFNγ) *in vitro*.[2] Loss of autophagy has also been shown to promote the release of IL-6 by human and mouse macrophages in response to infection with *Borrelia burgdorferi*, the causative agent of Lyme disease.[8,9]

Loss of autophagy *in vivo*, through genetic depletion of the autophagy-related gene *Atg5* or the microtubule-associated proteins 1A/1B light-chain 3 gene *Map1lc3b*, also results in increased release of IL-1 family cytokines and IL-17A in response to bacterial and viral infections, as well as in the context of severe neutrophilic asthma.[10–12] Conversely, rapamycin, which induces autophagy, reduces serum levels of both IL-1β and IL-23 in a mouse model of lipopolysaccharide (LPS)-induced endotoxemia.[1,2] Similarly, in a mouse model of dextran sulfate sodium–induced colitis, genetic depletion of the autophagy gene *Atg16l1* in hematopoietic cells led to greatly increased serum levels of IL-1β and IL-18, coupled with greater loss of body weight and decreased survival.[3] Together, these data would suggest that myeloid cell autophagy has a protective, anti-inflammatory role to play in acute inflammatory diseases.

The role of autophagy in chronic autoimmune diseases, by contrast, is less clear and potentially more complicated. In humans, polymorphisms in the genes encoding the autophagy proteins ATG2a, ATG4a, ATG4d, ATG16L1, death-associated protein, immunity-related GTPase M (IRGM) and unc-51 like autophagy activating kinase 1 (ULK-1) have been associated with increased susceptibility to Crohn's disease.[13–18] Similarly, genetic association studies have suggested protective roles for *IRGM*, *ATG5* and *ATG7* against the autoimmune disease systemic lupus erythematosus (SLE).[19,20] SLE involves the formation of immune complexes containing autoantigens that are potentially exposed to B cells when apoptotic debris is not cleared effectively, a process that involves a noncanonical form of autophagy called LC3-associated phagocytosis (LAP). This process involves the recruitment of LC3 to phagosomes containing various cargo, including cell debris, immune complexes and some pathogen-associated molecules, and utilizes multiple molecular components of the autophagy pathway, including ATG5, ATG7 and Beclin-1 (BECN1).[21] In one study, mice lacking proteins required for LAP in myeloid cells spontaneously developed characteristics of autoimmunity, including increased serum levels of anti-double–stranded DNA (dsDNA) and anti-nuclear autoantibodies, immunoglobulin G and complement component C1q deposition in the kidneys, coupled with kidney damage, and expression of IFN signature genes.[22] Moreover, mice deficient in *Atg7* in myeloid cells had a reduced capacity to degrade apoptotic cells *in vivo* and produced higher levels of IL-1β, IL-6 and chemokine (C–C motif) ligand 4, but lower levels of IL-10, in response to dying cells.[22]

However, other studies have suggested that loss of autophagy/LAP may be protective in mouse models of SLE. Adoptive transfer of *Becn1*$^{-/-}$ macrophages significantly decreased anti-dsDNA antibody levels, reduced immune complex deposition and lessened proteinuria and glomerulonephritis (GN) in activated lymphocytes–derived DNA–induced murine lupus.[23] Moreover, increased levels of autophagy have been reported in T and B cells from mice with lupus-like disease, as well as in peripheral blood mononuclear cells of patients with SLE.[24–26] However, it is unclear whether this represents a pathological or protective role, and whether in humans it may also be influenced by medication. Here, we investigated the effects of *Atg7* deletion in myeloid cells in mouse models of acute (LPS-induced endotoxemia) and chronic [Lck/yes-related novel tyrosine kinase (*Lyn*$^{-/-}$]) model of autoimmunity] inflammation. Loss of *Atg7* in myeloid cells increased the release of multiple proinflammatory cytokines in the context of acute inflammation, including IL-1 family cytokines, IL-17A and IL-23. However, in the chronic model, loss of myeloid cell *Atg7* resulted in reduced serum levels of IL-6 and IL-10 and significantly lowered serum levels of anti-histone and anti-Smith protein (anti-SM) autoantibodies. *In vitro*, inhibition of autophagy in human and mouse macrophages similarly inhibited IL-10 release in response to TLR agonists. Our data highlight differences in the role of myeloid cell autophagy/ATG7 in acute and chronic inflammation and suggest a direct role in the regulation of IL-10 production and release by macrophages.

RESULTS

Loss of *Atg7* in myeloid cells increases proinflammatory cytokine responses in an acute model of inflammation *in vivo*

Previous studies have shown that loss of autophagy, both *in vitro* and *in vivo*, increases proinflammatory cytokine responses to inflammatory stimuli, including increased release of IL-1α, IL-1β, IL-18, IL-23 and IL-17.[1–3,10,12] To investigate the effects of *Atg7* depletion in myeloid cells on acute inflammation, we used a mouse model of LPS-induced endotoxemia. Four hours after intraperitoneal injection of LPS into wild-type (WT) or $Atg7^{fl/fl}$ LysMCre mice, the release of 11 different cytokines was measured by Luminex assay, both locally in the peritoneal cavity and systemically in the serum. In the peritoneal cavity, levels of IL-1α, IL-1β, IL-6 and IL-12p70 were all significantly higher in the LPS-treated $Atg7^{fl/fl}$ LysMCre mice than in the LPS-treated WT animals (Figure 1a). In addition, levels of IFNγ, IL-10, IL-17A and IL-23 were significantly increased in LPS-treated $Atg7^{fl/fl}$ LysMCre mice compared with saline-injected animals, whereas these cytokines were not significantly raised in WT mice injected with LPS, compared with saline controls (Figure 1a). Levels of tumor necrosis factor superfamily, member 13b [B-cell activating factor (BAFF)], IFNα and IL-18 were not significantly different between the two groups, nor between saline- and LPS-treated animals (Figure 1a). In the serum of LPS-treated $Atg7^{fl/fl}$ LysMCre mice, levels of IL-1α, IL-1β, IL-17A, IL-18 and IFNγ were all significantly higher than in LPS-treated WT animals, whereas IL-10 was increased in $Atg7^{fl/fl}$ LysMCre mice, but not in WT animals, in response to LPS injection (Figure 1b). Serum levels of BAFF, IFNα, IL-6, IL-12p70 and IL-23 were not significantly different between groups (Figure 1b).

To determine whether loss of *Atg7* affected myeloid cell development or numbers, we examined populations in the peritoneal cavities of the animals. No significant differences were observed between WT and $Atg7^{fl/fl}$ LysMCre mice in the frequency of $CD115^{+}CD11b^{+}Ly6C^{-}$ or $CD115^{+}CD11b^{+}Ly6C^{+}$ monocytes, $CD115^{-}CD11b^{+}CD11c^{-}MHC\ II^{-}$ or $CD115^{-}CD11b^{+}CD11c^{-}MHC\ II^{+}$ macrophages or $CD115^{-}CD11b^{-}CD11c^{-}$ or $CD115^{-}CD11b^{-}CD11c^{+}$ dendritic cells (Supplementary figures 1 and 2). These data suggest that the increase in cytokine production observed in the $Atg7^{fl/fl}$ LysMCre mice was not because of increased or altered populations of myeloid cells. Together, these data demonstrate that, in an acute model of inflammation, loss of *Atg7* in myeloid cells increases the release of multiple proinflammatory cytokines.

Loss of myeloid cell autophagy alters specific cytokine and autoantibody responses in a chronic model of inflammation

We next looked at whether loss of autophagy in myeloid cells similarly influences inflammatory responses in a chronic model of inflammation. Mice deficient in the *Lyn* gene spontaneously develop a well-characterized autoimmune disease as they age, similar to human SLE, with splenomegaly, autoantibody production and severe immune complex–mediated GN being the most common pathological features observed.[27,28] Lyn is an Src family, nonreceptor tyrosine kinase that is a key mediator of signal transduction pathways in B cells and myeloid cells.[29–31] Disease in $Lyn^{-/-}$ mice has been attributed to hyper-reactivity of *Lyn*-deficient B cells as a consequence of the unique role of Lyn in downregulating B-cell receptor activation.[30] In addition, proinflammatory cytokines, including IL-6, IL-12 and IL-17A, have been shown to be raised in the serum of $Lyn^{-/-}$ mice.[32,33] Here, we bred our $Atg7^{fl/fl}$LysMCre mice with $Lyn^{-/-}$ animals and measured pathological indicators of disease, including changes in body weight, splenomegaly, GN, autoantibody production, splenic B-cell populations and serum cytokines, in young (< 120 days) and aged mice (> 200 days).

In the young mice, no significant differences were seen in body weight or spleen weight between any of the groups (Figure 2a, b). As measures of GN, segmental necrosis and crescent formation were investigated, but the latter was only observed in a small number of mice with no significant differences between groups (data not shown). Segmental necrosis was significantly increased in young $Lyn^{-/-}$ mice, compared with WT (Figure 2c, d). While $Lyn^{-/-}Atg7^{fl/fl}$LysMCre (double knockout) mice were not significantly different from $Atg7^{fl/fl}$LysMCre animals, they did show increased segmental necrosis compared with WT controls and trended numerically lower than $Lyn^{-/-}$ mice (Figure 2c, d). In the double-knockout mice, a significant decrease in body weight was observed in the aged animals, but only compared with WT controls, not $Atg7^{fl/fl}$LysMCre mice (Figure 2e). Spleen weight, however, was significantly increased in both the aged $Lyn^{-/-}$ and double-knockout animals compared with their respective controls (Figure 2f). However, no significant difference in splenomegaly was observed between the $Lyn^{-/-}$ and double-knockout mice. In the aged mice, both $Lyn^{-/-}$ and double-knockout mice had significantly higher necrotizing GN than their respective controls, but with no difference observed between the two groups (Figure 2g, h). These data indicate that disease in the $Lyn^{-/-}$ and double-knockout mice was comparable, suggesting no net effect of *Atg7*

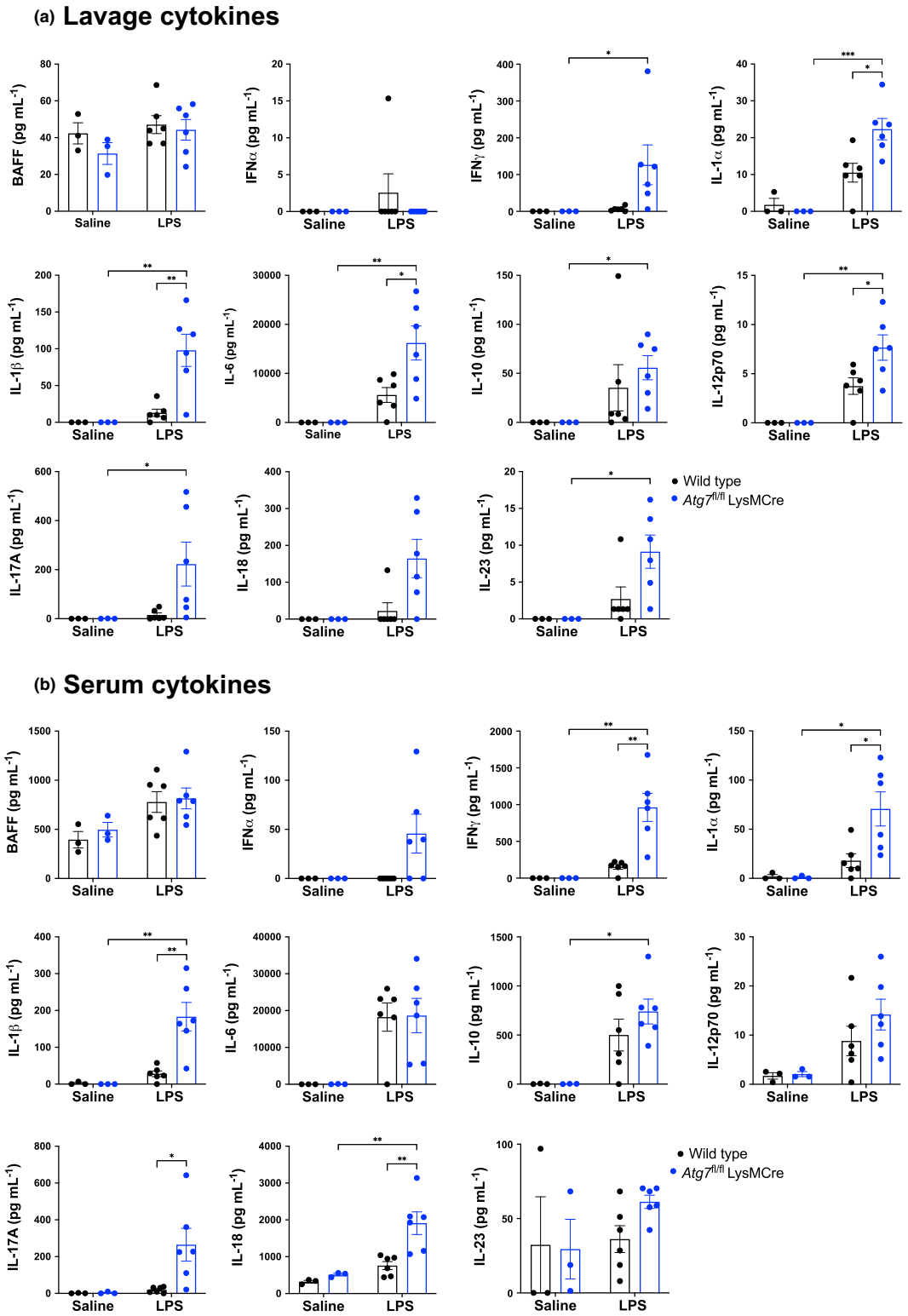

Figure 1. Effects of *Atg7* depletion in myeloid cells on local and systemic cytokine release in a mouse model of sepsis. Wild-type and *Atg7*$^{fl/fl}$ LysMCre mice were injected with saline control or lipopolysaccharide (LPS; 50 μg in 200 μL saline) and killed after 4 h. Cytokine release was measured in **(a)** peritoneal lavage fluid and **(b)** serum by multiplex assay. $N = 3$ or 6 animals per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$. BAFF, B-cell activating factor; IFN, interferon; IL, interleukin.

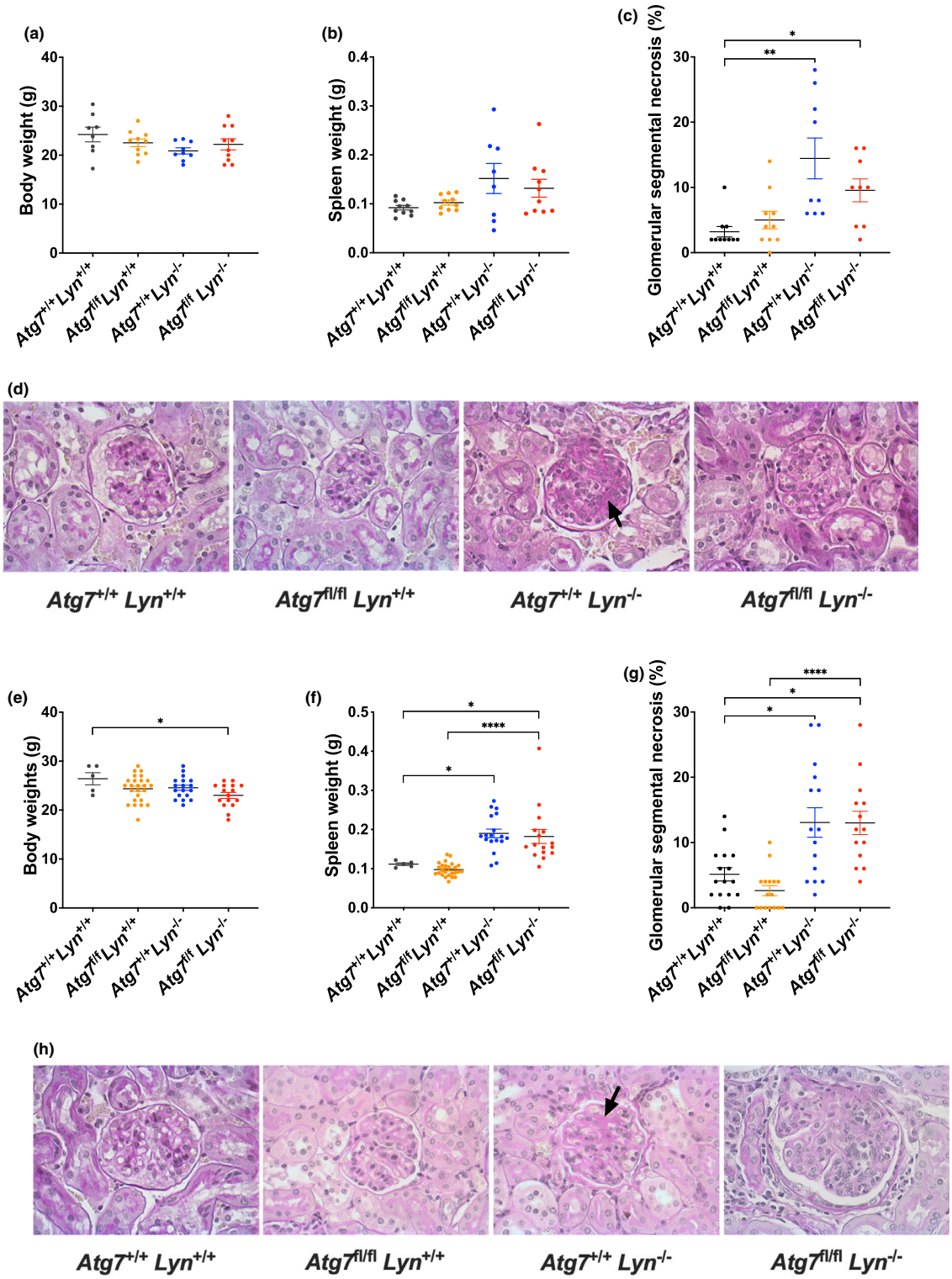

Figure 2. Effects of *Atg7* depletion in myeloid cells on measures of disease in a mouse model of lupus-like autoimmunity. Standard measures of autoimmune disease progression were taken from **(a–d)** young (< 120 days) and **(e–g)** aged (> 200 days) $Atg7^{+/+}Lyn^{+/+}$, $Atg7^{fl/fl}Lyn^{+/+}$, $Atg7^{+/+}Lyn^{-/-}$ and $Atg7^{fl/fl}Lyn^{-/-}$ mice, including **(a, e)** body weight, **(b, f)** spleen weight and **(c, d, g, h)** glomerular segmental necrosis. Images are representative of the respective populations. Arrows point to examples of glomerular segmental necrosis. $N = 5–25$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.001$.

deficiency in the myeloid cell compartment on these standard measures of disease progression.

Next, we looked at serum autoantibody levels in young and aged mice. Previous studies have shown that $Lyn^{-/-}$ mice produce higher levels of anti-nuclear antibodies (ANAs) and autoantibodies to extractable nuclear antigens, including dsDNA (anti-dsDNA), Smith protein (anti-SM) and anti-histone, comparable to patients with SLE.[27,32] Using a panel of human extractable nuclear antigens modified for mouse antibodies, we found that in young mice only anti-dsDNA autoantibodies were significantly increased in the $Lyn^{-/-}$ mice, compared with WT controls, but were not significantly different from those in the double-knockout animals (Supplementary figure 3). Compared with $Atg7^{fl/fl}$LysMCre mice, double-knockout animals had significantly higher levels of anti-CENP (centromere protein), anti-SM, anti-PMSci, anti-histone, anti U1-RNP, anti-dsDNA, anti-TRIM21, anti-SMRNP, anti-ribosomes and anti-Jo-1 (Supplementary figure 3). Compared with WT animals, double-knockout mice had higher levels of anti-histone and anti-dsDN (Supplementary figure 3).

In the aged mice, levels of anti-dsDNA, anti-histone, anti-Jo-1, anti-PmScl, anti-ribosomes, anti-Scl70, anti-SM, anti-SMRNP, anti-SSB, anti-TRIM21 and anti-U1-RNP were all significantly raised in $Lyn^{-/-}$ mice, compared with WT controls (Figure 3). Moreover, levels of anti-histone and anti-SM were significantly lower in double-knockout animals than in the $Lyn^{-/-}$ mice, while levels of anti-dsDNA, anti-Jo-1, anti-PmScl, anti-ribosomes, anti-Scl70, anti-SMRNP, anti-TRIM21 and anti-u1-RNP all similarly trended lower in double knockouts, albeit nonsignificantly (Figure 3). In a previous study, loss of genes encoding proteins involved in autophagy/LAP, including *Atg7*, resulted in a spontaneous increase in production of anti-dsDNA and anti-nuclear antibodies in aged animals. Here, when comparing WT mice with $Atg7^{fl/fl}$LysMCre animals, we observed a significant increase in levels of anti-SSB, but significantly lower levels of anti-SSA, anti-histone, anti-PCNA, anti-dsDNA, anti-TRIM21 and anti-ribosomes (Figure 3). These data suggest a largely protective effect of *Atg7* deficiency in myeloid cells against autoantibody production.

To determine whether loss of *Atg7* in myeloid cells might be influencing autoantibody production through an indirect effect on B-cell populations, we investigated these in the spleens of our animals using flow cytometry (see Supplementary figure 4 for the gating strategy). In young mice, the number of plasma cells was significantly increased in the $Lyn^{-/-}$ mice, compared with WT animals (Figure 4a). Moreover, plasma cell numbers in the double-knockout mice were significantly lower than in the $Lyn^{-/-}$ animals (Figure 4a). In the aged mice, however, while plasma cell numbers were similarly increased in the $Lyn^{-/-}$ and double-knockout mice, no significant difference was observed in plasma cell numbers between these genotypes (Figure 4b), suggesting that the effect in young mice is transient. Whether this early effect on plasma cell numbers could account for the difference in autoantibody production is unclear, but it is notable that autoantibody levels in the young mice do not appear to follow the plasma cell numbers (Supplementary figure 3). Compared with WT mice, numbers of B cells and nongerminal center B cells were significantly lower in spleens of $Lyn^{-/-}$ and double-knockout animals, but no differences were seen between the two genotypes in these, or any other, B-cell populations (Figure 4). Interestingly, plasma cell numbers were highest in the aged $Atg7^{fl/fl}$LysMCre mice, although not statistically significantly higher than in the double-knockout animals.

Finally, we looked at serum cytokines in both young and aged mice. In the young mice, serum levels of BAFF, IL-6, IL-10 and IL-17A were significantly higher in $Lyn^{-/-}$ and double-knockout animals, compared with their respective controls (WT and $Atg7^{fl/fl}$LysMCre, respectively; Figure 5a). However, no differences were observed between $Lyn^{-/-}$ and double-knockout mice (Figure 5a). In the aged mice, levels of BAFF were significantly raised in both $Lyn^{-/-}$ and double-knockout mice, but with no difference observed between the two (Figure 5b). However, serum levels of both IL-6 and IL-10 were significantly raised only in the $Lyn^{-/-}$ mice and were significantly lower in the double-knockout animals (Figure 5b), suggesting that loss of *Atg7* in myeloid cells has an inhibitory effect on IL-6 and IL-10 release in this model. Levels of IFNγ were also significantly raised in the $Lyn^{-/-}$ mice, but not in the double-knockout animals, although the difference in levels between these two genotypes was not significant (Figure 5b). Levels of IL-17A, by contrast, were only significantly raised in the double-knockout group, although again were not significantly different between $Lyn^{-/-}$ and double-knockout mice (Figure 5b). These data may suggest that effects on IL-6 and/or IL-10 might, in turn, contribute to the differences observed in autoantibody production between these genotypes (Figure 3).

Interestingly, levels of BAFF, IL-18 and IFNγ were all significantly raised in aged $Atg7^{fl/fl}$LysMCre mice, compared with WT controls (Figure 5b). These data would suggest that in the absence of genetically driven autoimmune disease, loss of *Atg7* in myeloid cells is more generally proinflammatory and may explain previous observations of spontaneous autoimmunity in these mice.[22] However, we did not see any obvious indication of autoimmune disease in our animals.

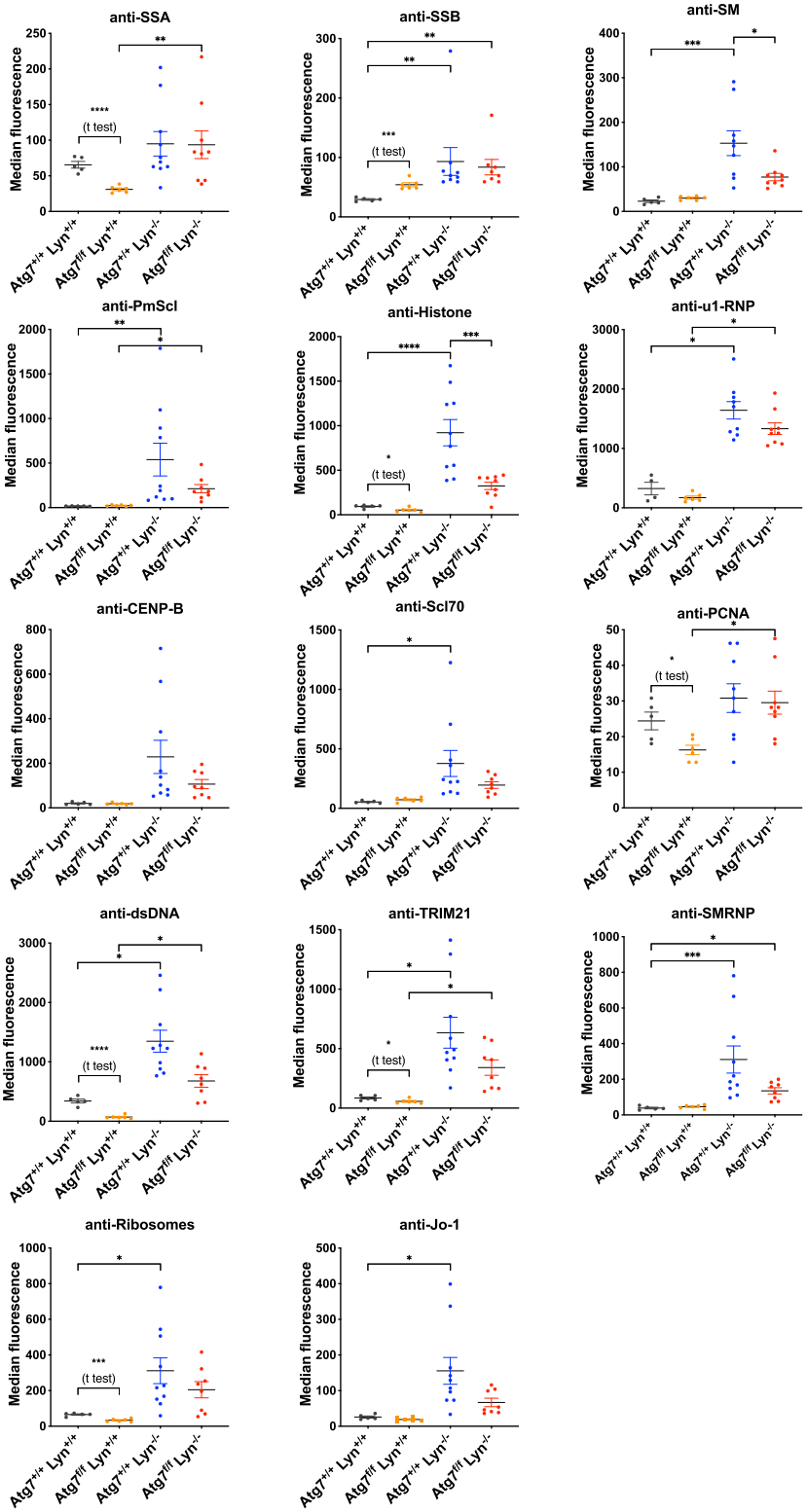

Figure 3. Effects of *Atg7* depletion in myeloid cells on autoantibody production in a mouse model of lupus-like autoimmunity. Serum autoantibodies were measured using a modified extractable nuclear antigen test in aged (> 200 days) $Atg7^{+/+}$ $Lyn^{+/+}$, $Atg7^{fl/fl}$ $Lyn^{+/+}$, $Atg7^{+/+}$ $Lyn^{-/-}$ and $Atg7^{fl/fl}$ $Lyn^{-/-}$ mice. N = 4–10 mice per group. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001.

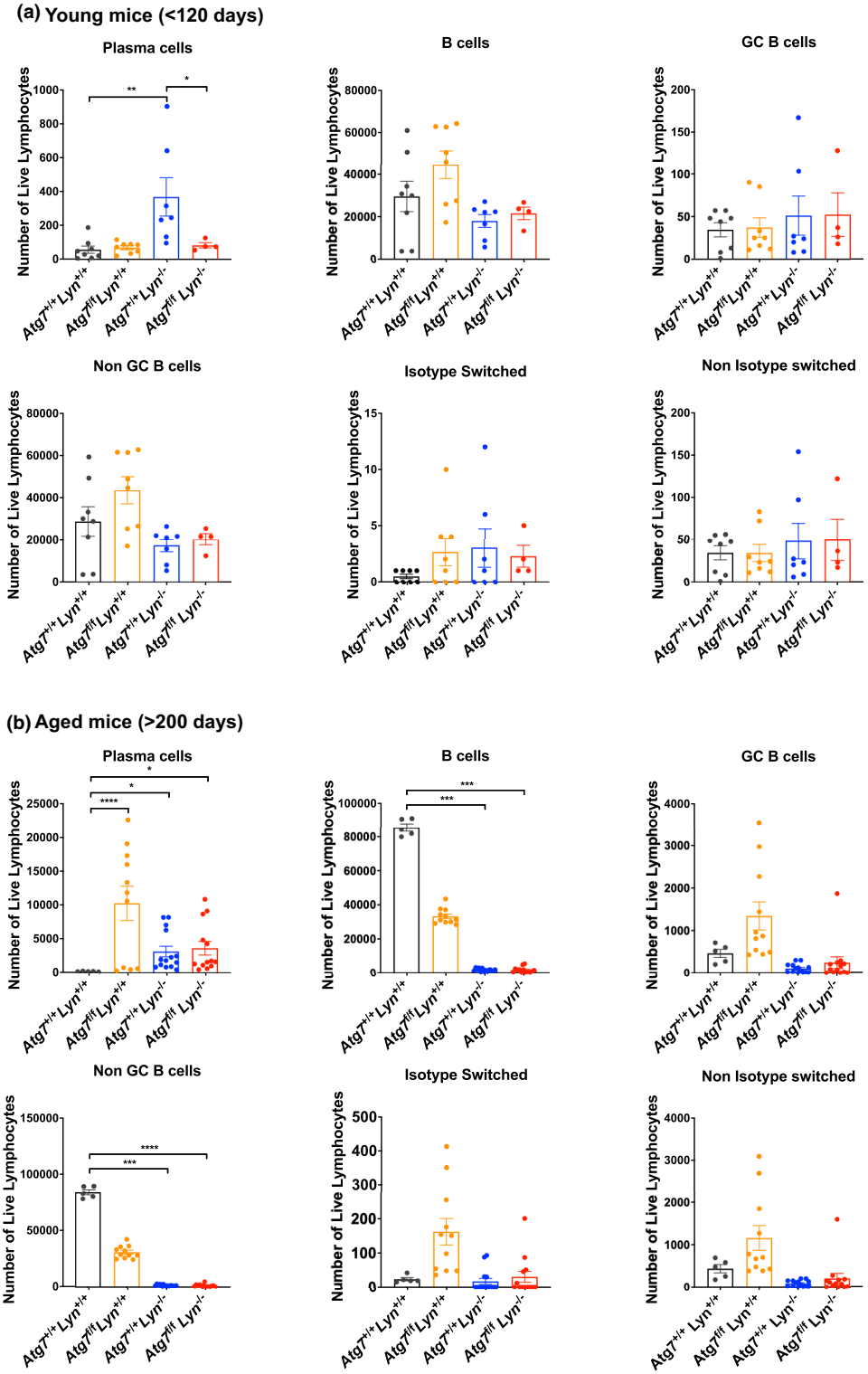

Figure 4. Effects of *Atg7* depletion in myeloid cells on splenic B-cell populations in a mouse model of lupus-like autoimmunity. Flow cytometry was used to measure specific B-cell populations in the spleens of **(a)** young (< 120 days) and **(b)** aged (> 200 days) $Atg7^{+/+}$ $Lyn^{+/+}$, $Atg7^{fl/fl}$ $Lyn^{+/+}$, $Atg7^{+/+}$ $Lyn^{-/-}$ and $Atg7^{fl/fl}$ $Lyn^{-/-}$ mice. Data are expressed as total number of plasma cells, B cells, germinal center (GC) B cells, non-GC B cells, isotype-switched B cells and nonisotype-switched B cells. N = 4–14 mice per group. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001.

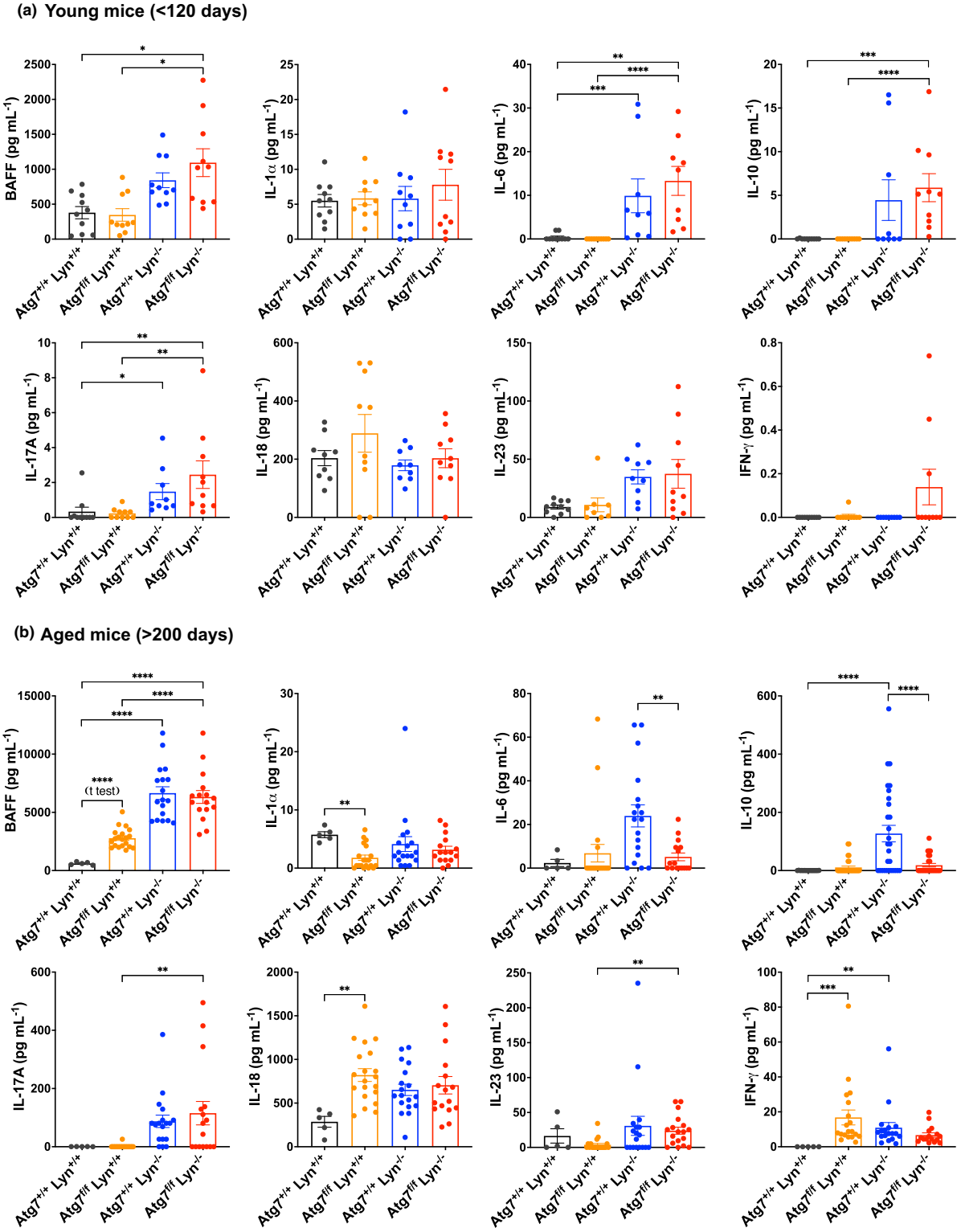

Figure 5. Effects of *Atg7* depletion in myeloid cells on serum cytokines in a mouse model of lupus-like autoimmunity. Multiplex analysis was used to measure serum cytokines from **(a)** young (< 120 days) and **(b)** aged (> 200 days) $Atg7^{+/+}Lyn^{+/+}$, $Atg7^{fl/fl}Lyn^{+/+}$, $Atg7^{+/+}Lyn^{-/-}$ and $Atg7^{fl/fl}Lyn^{-/-}$ mice. $N = 5–32$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$. BAFF, B-cell activating factor; IFN, interferon; IL, interleukin.

As T regulatory cells are known to suppress immune responses through secretion of IL-10, we checked whether splenic populations were different between the *Lyn*$^{-/-}$ and double-knockout animals. While both genotypes showed a significantly increased frequency of FoxP3^{+}CD25^{+} T regs, the frequency was not different between the *Lyn*$^{-/-}$ and double-knockout animals (Supplementary figure 5). Thus, we hypothesize that the differences in IL-10 and IL-6 seen between the *Lyn*$^{-/-}$ and double-knockout mice are due to differences in secretion by myeloid cells.

Loss of autophagy in myeloid cells inhibits IL-6 and IL-10 release *in vitro*

The reduced serum IL-6 and IL-10 in the double-knockout mice, compared with *Lyn*$^{-/-}$ animals, led us to investigate whether loss of autophagy similarly inhibits IL-6 and IL-10 release by myeloid cells *in vitro*. Previous studies have shown that loss of autophagy increases IL-6 release by human and mouse macrophages infected with *Borrelia burgdorferi*.[8,9] However, we have previously reported that the autophagy inhibitor 3-methyladenine (3-MA) either inhibits or has no effect on IL-6 release by LPS-treated murine dendritic cells, while small interfering RNA (siRNA) knockdown of *Atg7* or *Becn1* had no effect on LPS-induced IL-6 release by murine immortalized bone marrow–derived macrophages (iBMMs).[1,2] Similarly, TLR-dependent IL-6 release in the context of autophagy deficiency in murine RAW 264.7 macrophages was inconsistent here. In response to the TLR4 ligand LPS, 3-MA had no effect on IL-6 release after 6 or 16 h (Figure 6a, b). However, in response to the TLR7/8 ligand R-848 (Resiquimod), 3-MA inhibited IL-6 release after 6 h (Figure 6c), but not after 16 h (Figure 6d). The lack of inhibition after 16 h may be due to the fact 3-MA was only added at the start of the experiment, rather than continuously throughout. Similarly, treatment with *Becn1* siRNA had no effect on LPS-induced IL-6 release by RAW 264.7 cells but did inhibit IL-6 release in response to R-848 treatment after 16 h (Figure 6e–g). These data suggest a complex relationship between autophagy, TLRs and IL-6. Nonetheless, the data do support a protective effect of autophagy inhibition on IL-6 release in the context of TLR7 activation and could explain the effects seen in the *Lyn*$^{-/-}$*Atg7*$^{fl/fl}$ LysMCre mice (Figure 5), especially as *Lyn*$^{-/-}$ cells have previously been shown to have hyperactivated MyD88 signaling and increased cytokine responses to the TLR7 agonist R837.[34,35]

By contrast, the effect of autophagy inhibition and induction on IL-10 release by myeloid cells was more consistent. Treatment with 3-MA for 16 h abrogated both LPS- and R-848-dependent IL-10 release in murine RAW 264.7 cells in a dose-dependent manner (Figure 6h, i). In these experiments, significant IL-10 release was not observed after 6 h (data not shown). To confirm these findings further, we used siRNA against *Becn1* in RAW 264.7 cells treated with LPS or R-848. Knockdown of *Becn1* resulted in significantly reduced IL-10 release in response to both TLR ligands (Figure 6j, k).

To determine whether this effect of autophagy on IL-10 release was upstream or downstream of IL-10 transcription/translation, we looked at *Il10* messenger RNA (mRNA) levels in response to pharmacological and genetic regulation of autophagy. As with protein release, levels of *Il10* mRNA in response to LPS were significantly reduced by treatment with 3-MA (Figure 6l). Likewise, siRNA knockdown of *Becn1* significantly inhibited *Il10* mRNA expression in response to both LPS and R-848 (Figure 6m), suggesting that regulation of IL-10 by autophagy occurs upstream of transcription/translation. Previous studies have shown that IL-1β is sequestered in autophagosomes[1] and may utilize autophagy as part of an unconventional secretory pathway.[36,37] To determine whether the same might be true for IL-10, we looked at intracellular localization of IL-10 in iBMMs stably expressing GFP-LC3 treated with LPS over time. As has previously been shown,[1,38] LPS induced autophagosome formation, increasing over time up to 16 h (Supplementary figure 6). However, IL-10 was not observed to colocalize with LC3 at any time point (Figure 7). These data demonstrate that autophagy regulates myeloid cell IL-10 production at the level of mRNA transcription, potentially explaining the inhibitory effects of *Atg7* knockout on serum IL-10 levels in *Lyn*$^{-/-}$ mice (Figure 5).

DISCUSSION

Many studies have demonstrated that loss or inhibition of autophagy and/or LAP in myeloid cells has significant proinflammatory effects, both *in vitro* and *in vivo*, particularly with respect to production and release of cytokines.[1–3,5,9,22,39,40] Here, we have demonstrated that in a model of acute inflammation, loss of *Atg7* in myeloid cells results in a significant increase in the release of proinflammatory cytokines, both locally and systemically, including IL-1α, IL-1β, IL-6, IL-12, IL-17A and IL-18 (the latter two only significant systemically). This supports previous *in vitro* studies that have demonstrated increased release of IL-1α, IL-1β, IL-18 and IL-23 by autophagy-/LAP-deficient myeloid cells. Moreover, previous studies have shown that the autophagy inducer rapamycin, which inhibits mTOR, inhibits both IL-1β and IL-23 release in a mouse model

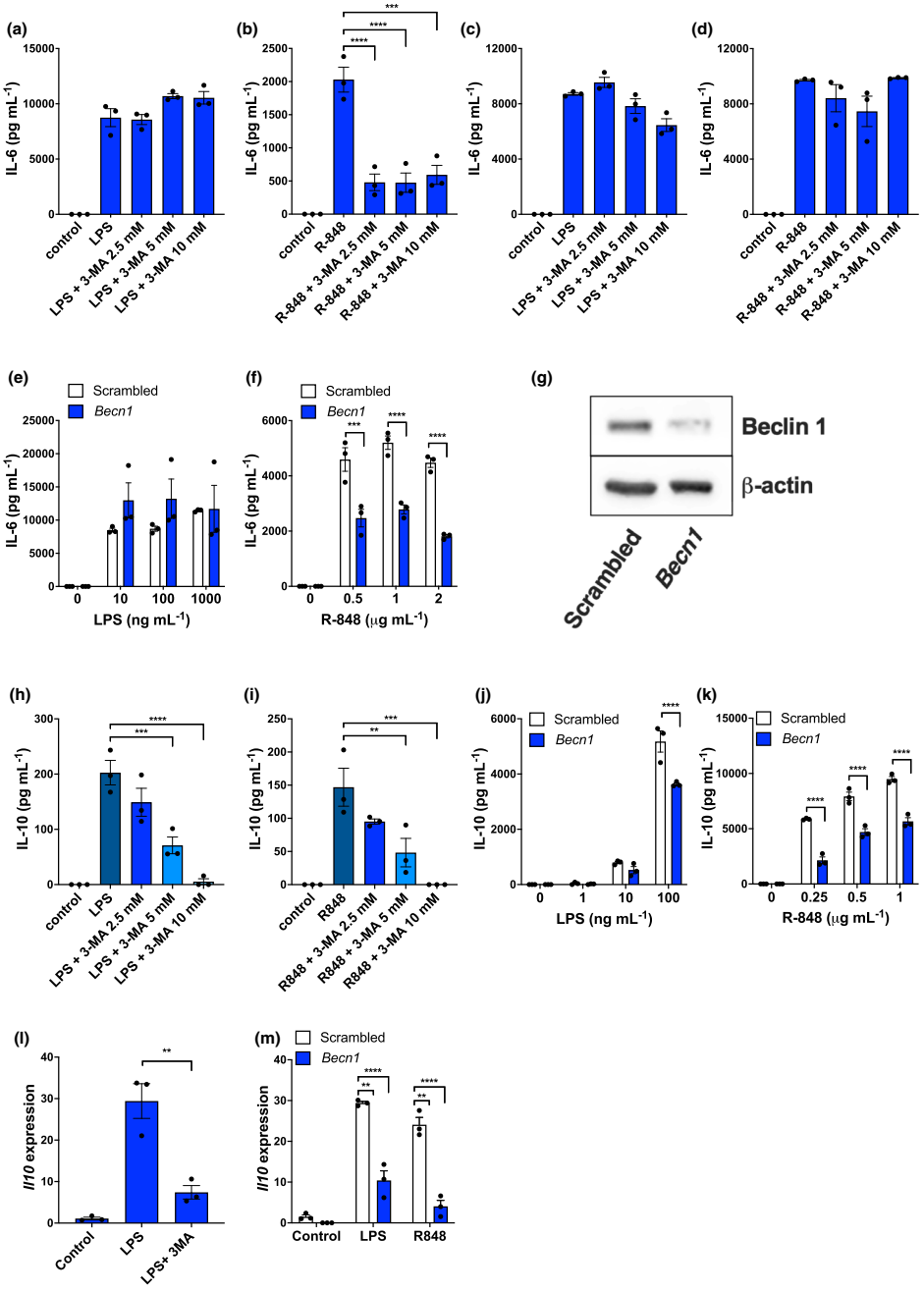

Figure 6. Inhibition of autophagy inhibits interleukin (IL)-6 and IL-10 release by macrophages *in vitro*. Mouse RAW 264.7 macrophages were treated with different concentrations of 3-methyladenine (3-MA) in combination with **(a, c)** 100 ng mL^{-1} lipopolysaccharide (LPS) or **(b, d)** 1 μg mL^{-1} R-848 for **(a, b)** 6 h or **(c, d)** 16 h and IL-6 release was measured by ELISA. **(e, f)** RAW 264.7 cells were transfected with nontargeting (scrambled) or small interfering RNA (siRNA) targeting *Becn1* and treated with different concentrations of **(e)** LPS or **(f)** R-848 for 16 h and IL-6 release was measured by ELISA. **(g)** Western blot confirmation of Beclin-1 protein knockdown in RAW 264.7 cells. The image is representative of three independent experiments. The full Western blot is shown in Supplementary figure 7. **(h, i)** RAW 264.7 macrophages were treated with different concentrations of 3-MA in combination with **(h)** 100 ng mL^{-1} LPS or **(i)** 1 μg mL^{-1} R-848 for 16 h and IL-10 release was measured by ELISA. **(j, k)** RAW 264.7 cells were transfected with nontargeting (scrambled) or *Becn1* siRNA and treated with different concentrations of **(e)** LPS or **(f)** R-848 and IL-10 release was measured by ELISA. **(l)** RAW 264/7 cells were treated with LPS (100 ng mL^{-1}) with or without 3-MA (10 mM) for 4 h and *Il10* messenger RNA (mRNA) expression was measured by quantitative PCR (qPCR). **(m)** RAW 264.7 cells were transfected with scrambled or *Becn1* siRNA and treated with LPS or R-848 for 4 h. Expression of *Il10* mRNA was measured by qPCR. $N = 3$ independent experiments. $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$.

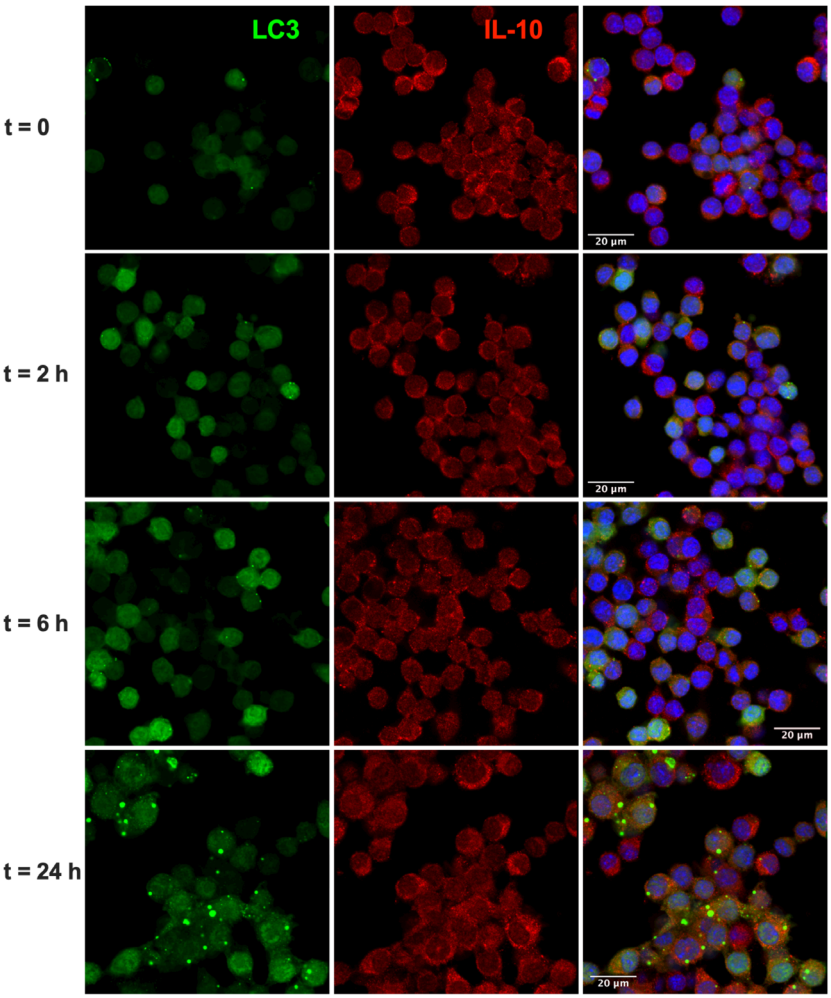

Figure 7. Interleukin (IL)-10 is not sequestered into autophagosomes. Confocal analysis of immortalized bone marrow–derived macrophages stably expressing GFP-LC3 (green). Following treatment with lipopolysaccharide (100 ng mL^{-1}) for 2–16 h, cells were fixed and stained with antibody against IL-10 (red). Images are representative of three independent experiments.

of LPS-induced endotoxemia,[1,2] highlighting an anti-inflammatory role for autophagy/LAP. Similarly, *in vivo* studies of bacterial and viral infection, as well as severe neutrophilic asthma, have demonstrated increased release of IL-1 family cytokines and IL-17 in mice with compromised myeloid cell autophagy.[10–12] Here, we also show that loss of *Atg7* in myeloid cells increases local, but not systemic, release of IL-6 and IL-12. Further studies investigating the difference in local and systemic responses would be of interest, especially given the relatively high variation between animals in this study. Together, these data suggest a clear role for myeloid cell autophagy/LAP in dampening acute inflammation. Additional studies are needed to determine whether the effects observed here are through macroautophagy or LAP, or a combination of the two. Repeating these experiments using mice with genetic deletion of LAP-specific genes, such as *Uvrag* or *Rubcn* (Rubicon), and/or with genes required for canonical autophagy, but dispensable for LAP, such as *Ulk1* or *fip200*,[21] would allow for a clearer picture of the relative contribution. Interestingly, a recent study has demonstrated that ATG7 is not required for LC3–PE conjugation in thioglycolate-elicited mouse peritoneal macrophages, but is required for this process in naïve peritoneal macrophages and BMMs.[41] While this study did not address whether LAP and/or canonical autophagy was involved, it does open the possibility that effects of *Atg7* deletion on inflammatory responses may differ depending on metabolic and/or activation status of the cells.

The role of autophagy and LAP in the setting of chronic autoinflammatory disease, by contrast, is less clear and likely complex and multifaceted. One study found that mice with myeloid-specific genetic depletion of genes involved in LAP, including *Atg7*, spontaneously developed symptoms of autoimmunity, including increased autoantibody production, kidney damage with immune complex and C1q deposition in the glomeruli, and raised serum levels of multiple proinflammatory cytokines.[22] Here, we found evidence of increased anti-SSB autoantibodies, but conversely decreased anti-SSA, anti-histone, anti-PCNA, anti-dsDNA, anti-TRIM21 and anti-ribosomes autoantibodies. We did not observe differences in kidney disease but did observe raised levels of BAFF, IL-18 and IFNγ in the serum of aged $Atg7^{fl/fl}$LysMCre mice, compared with WT controls. It is unclear why we failed to recapitulate the previous observations, although we can hypothesize that the housing conditions of the mice may have differed in a way that influenced the results, possibly through exposure to different pathogens. Besides, in the previous study, smaller groups of animals ($n = 3$, *versus* 10–30 in the present study) were studied and total autoantibody levels measured, whereas here we have looked at specific autoantibodies. In the context of $Lyn^{-/-}$-driven autoimmunity, loss of *Atg7* in myeloid cells did not, as might have been expected, worsen the disease, but instead appeared potentially protective, lowering serum levels of autoantibodies, along with IL-6 and IL-10. Interestingly, this agrees with a previous study demonstrating that adoptive transfer of $Becn1^{-/-}$ macrophages significantly decreased anti-dsDNA antibody levels, reduced immune complex deposition and alleviated proteinuria and GN in activated lymphocytes–derived DNA–induced murine lupus. Moreover, this coincided with a decrease in levels of IL-6 and tumor necrosis factor.[23] However, in our $Lyn^{-/-}$ model, measures of disease severity, including loss of body weight, splenomegaly and GN were not significantly different in the $Lyn^{-/-}Atg7^{-/-}$ mice. This may suggest that, at least in this specific model, the protective effects of autophagy on specific autoantibody production are not sufficient to overcome disease progression. This may highlight important differences between the different models of lupus-like autoimmunity.

How loss of *Atg7* in myeloid cells reduces autoantibody production remains to be elucidated, but several possibilities exist. One is that altered myeloid cell activity might influence plasma cell populations and/or activity. Interestingly, our data do show that loss of *Atg7* in myeloid cells in *Lyn*-sufficient mice results in increased numbers of plasma cells. Such an effect could at least partially explain why autoantibody production was previously reported to be higher in mice in which genes required for LAP were deficient in myeloid cells.[22] However, as already noted, we only observed increased anti-SSB production in our $Atg7^{fl/fl}$ mice, with levels of multiple other autoantibodies decreased. This increase in plasma cell populations may be driven by the increased cytokines observed in the same mice, particularly BAFF, which promotes B-cell survival.[42] Whether IL-18 and/or IFNγ, both similarly raised in $Atg7^{fl/fl}$ mice, influence B-cell survival and/or activity remains unclear, although both have been found to be raised in the plasma of patients with SLE.[43,44] Moreover, serum IL-18 correlates with renal disease in SLE[44] and has been shown to correlate with plasma cell proliferation,[45] while IFNγ has been shown to increase the release of the soluble B-lymphocyte stimulator (BLyS) by human monocytes, which promotes B-cell activation and maturation.[46] In the context of *Lyn* deficiency, loss of *Atg7* resulted in decreased plasma cell numbers in the young mice, compared with $Lyn^{-/-}Atg7^{+/+}$ animals. However, this was not seen in the aged mice, in which autoantibody levels were decreased in the double-knockout animals, suggesting that changes in plasma cell development are unlikely to be responsible for the effects seen on autoantibody levels.

A more likely explanation for the effects seen on autoantibody levels, based on our data, is that changes in cytokine release alter plasma cell activity and autoantibody production. Serum levels of IL-6 and IL-10 were significantly lower in double-knockout mice than in the $Lyn^{-/-}$ animals, in which they were significantly raised compared with WT controls. Both IL-6 and IL-10 play crucial roles 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.[47,48] Importantly, IL-6 has been proposed to drive autoantibody production in $Lyn^{-/-}$ mice.[33] Moreover, a previous study demonstrated that although the number of B lymphocytes from patients with SLE was unaffected by treatment with an anti-IL-10 monoclonal antibody *ex vivo*, the production of antibodies by these cells was inhibited.[49] Similarly, in a severe combined immunodeficient mouse model, anti-IL-10 monoclonal antibody significantly decreased autoantibody production.[49] 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.

Other possible explanations for the decrease in autoantibody seen in double-knockout mice include effects of myeloid cell *Atg7* deletion on the uptake of apoptotic cells (efferocytosis) and/or antigen processing and presentation. Efferocytosis is important for the

clearance of dead and dying cells, which could otherwise accumulate, rupture and expose multiple autoantigens.[50] Martinez *et al.*[22] demonstrated that loss of genes required for LAP in myeloid cells, including *Atg7*, results in decreased efferocytosis and degradation of apoptotic cells. However, this would not explain why the loss of *Atg7* is protective against the development of autoantibodies seen in this study, 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 as a result of loss of autophagy are inconsequential. This may instead point to the effects of myeloid cell autophagy on autoantigen processing and/or presentation. Studies have shown that pharmacological and genetic inhibition of autophagy decreases efficient major histocompatibility complex class II (MHC II) presentation of endogenous antigens.[51,52] Autophagosomes have been shown to fuse directly with MHC II loading compartments,[53,54] while fusing viral and tumor antigens to LC3-II, which is found on autophagosomal membranes, improves presentation to $CD4^+$ T cells.[54–56] Autophagy has also been implicated in MHC I cross-presentation of exogenous antigens.[57,58] MHC I presentation is based on proteasomal antigenic peptide processing and peptide transport from endosomal compartments to the cytosol, in contrast to autophagy, which transports cytosolic components to endocytic compartments. However, autophagy substrates may be redirected to proteasomes for processing and MHC I–restricted presentation when autophagy is disrupted.[59] In a recent study, it was reported that there was a reduction in autoreactive $CD4^+$ T cells in the absence *Atg5* in dendritic cells, which prolonged the initiation of disease and decreased clinical severity relative to mice with *Atg5*-sufficient dendritic cells.[60] Thus, defective autoantigen processing and/or presentation in autophagy-deficient myeloid cells, coupled with changes in cytokine release, could explain why autoantibody production is reduced in $Atg7^{-/-}Lyn^{-/-}$ mice. However, further work is needed to investigate whether this is a mechanism at play here.

We hypothesized that the effects of *Atg7* deletion in myeloid cells of $Lyn^{-/-}$ mouse on serum levels of IL-6 and IL-10 were due to direct effects on those cells. Thus, we tested the effects of autophagy inhibition on macrophages *in vitro*. Using 3-MA as a pharmacological inhibitor of autophagy and knockdown of *Becn1* with siRNA, we found that inhibition of autophagy/LAP in mouse macrophages lowered IL-6 release in response to the TLR7 agonist R-848, but not the TLR4 agonist LPS. The reasons for this difference are not clear, although while TLR7 signals through an MyD88-dependent pathway, LPS triggers both MyD88 and TRIF pathways, raising the possibility that the two pathways respond differently to autophagy/LAP inhibition. However, previous studies have shown that effects of autophagy inhibition on IL-1 family cytokine release are dependent on TRIF signaling,[1,3] so this explanation seems unlikely. The effects of autophagy/LAP inhibition on IL-10 release were, by contrast, more consistent. Both 3-MA and siRNA against *Becn1* inhibited the release of IL-10 in response to both LPS and R-848 and this effect was observed at the level of *Il10* mRNA. Thus, the inhibition of autophagy/LAP in myeloid cells directly regulates IL-10 production in these cells *in vitro*, and this may explain the decrease in serum IL-10 observed in $Atg7^{fl/fl}Lyn^{-/-}$ mice. However, it is interesting to note that in the acute model of LPS-induced endotoxemia, levels of IL-10 were not lower in the $Atg7^{fl/fl}$LysMCre mice. As this model was characterized by increased proinflammatory cytokine release in the *Atg7*-deficient animals, this result may reflect the more inflammatory milieu, with proinflammatory cytokines affecting the production and release of IL-10, as well as possible effects on nonmyeloid cells. Nonetheless, our data suggest that direct regulation of IL-10 release by autophagy/LAP in myeloid cells may contribute to autoimmunity in some circumstances. Moreover, the data further highlight the largely proinflammatory consequences of autophagy/LAP inhibition in myeloid cells demonstrated by other studies.[1–3,9]

To conclude, we have demonstrated differential roles for myeloid cell *Atg7* in acute and chronic inflammation. Loss of *Atg7* in myeloid cells in a mouse model of acute LPS-induced inflammation resulted in increased release of multiple proinflammatory cytokines, whereas in a model of chronic autoimmunity specific autoantibody production and serum levels of IL-6 and IL-10 were reduced in mice deficient in myeloid cell *Atg7*. Inhibition of autophagy/LAP in mouse and human macrophages *in vitro* similarly resulted in reduced production and release of IL-10. Our data highlight a potentially protective role for myeloid cell autophagy/LAP in acute inflammation; a role that could potentially be targeted therapeutically in diseases such as sepsis. However, our data also highlight a more nuanced role for autophagy/LAP in chronic inflammatory and autoimmune diseases that warrants further investigation.

METHODS

Animals

$Atg7^{fl/fl}$LysMCre mice were bred under specific pathogen-free conditions at the Monash Animal Research Platform facility located at the Monash Medical Centre, Block E, from $Atg7^{loxp/loxp}$ mice, originally obtained from Maasaki Komatsu,

Tokyo Metropolitan Institute of Medical Science,[61] and LysMCre mice from our own facility. These mice were crossed with $Lyn^{-/-}$ mice, obtained from Margaret Hibbs, Monash University Central Clinical School.[27] For the $Lyn^{-/-}$ studies, mice were studied at < 120 days (young mice) and > 200 days (aged mice), allowing for clear dichotomy of disease progression, as previously published.[32,33] All animals were on a C57Bl/6 background and were housed under identical conditions in adjacent cages within our facility. All procedures were approved by the Monash Medical Centre Animal Ethics Committee.

Cell culture

Mouse RAW 264.7 cells and iBMMs were cultured in RPMI-1640 medium (catalog number 21870–076; Gibco/Thermo Fisher, VIC, Australia), supplemented with fetal calf serum (catalog number 12103; JRH Biosciences, VIC, Australia), 2 nM L-glutamine, 50 U mL^{-1} penicillin and 50 μg mL^{-1} streptomycin (catalog number 10378–106; Gibco/Thermo Fisher; complete medium). iBMMs stably expressing GFP-LC3[1] were cultured in complete medium with puromycin dihydrochloride (5 μg mL^{-1}; P9620; Sigma/Merck, VIC, Australia).

Reagents

3-MA (catalog number M9281; 3-MA, Sigma/Merck) was freshly prepared for each experiment in phosphate-buffered saline (PBS; 8 g L^{-1} NaCl, 0.2 g L^{-1} KCL, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4) at 50°C. LPS (catalog number L2630) and R-848 (catalog number SML0196; Resiquimod) were from Sigma/Merck.

LPS-induced endotoxemia

Female mice (20–30 weeks old) were injected intraperitoneally with LPS (50 μg in 200 μL sterile saline). Control mice received sterile saline only (200 μL). Mice were killed by CO_2 asphyxiation 4 h after injection, an optimum time point chosen based on previous studies.[1,2,7] Blood was collected by cardiac puncture for analysis of serum cytokines. Peritoneal cavities were flushed with 3 mL ice-cold sterile PBS, and the lavage fluids centrifuged to separate cells (for flow cytometry) and supernatants (for cytokine analyses). Supernatants were stored at −20°C.

Spleen weights and histology of kidneys

Spleens were harvested and weights measured in milligrams. Kidneys were harvested and cut in half laterally, fixed in 4% formalin, embedded in paraffin and stained with periodic acid–Schiff staining for histopathological analysis. Glomerular damage was scored in a blinded fashion for glomerular necrosis, defined as an accumulation of periodic acid–Schiff–positive material in 50% or more of the glomerulus, while glomerular crescents were defined as two or more layers of cells in Bowman's space. A minimum of 50 consecutive glomeruli per mouse was examined, and results were expressed as the percentage of necrosis per glomerular cross section.

Measurement of autoantibodies

Autoantibodies against extractable nuclear antigen in sera (diluted 1/200 in PBS) were measured using the FIDIS Connective Profile kit (catalog number MX 117; Theradiag, Croissy-Beaubourg, France) according to the manufacturer's protocol, with the variation of the use of an anti-mouse secondary antibody.[62] This test uses multiplex technology with addressable laser bead immunoassay, which offers simultaneous detection of multiple antibody specificities, allowing a single-step screening and confirmation. To detect autoantibodies, phycoerythrin (PE)-conjugated polyclonal anti-mouse IgG F(ab′)2 secondary antibody (catalog number 12–40410; eBioscience/Thermo Fisher) was used, and fluorescence was measured using a BD FACSCanto II (BD Bioscience, VIC, Australia).

Flow cytometry

For analysis of B-cell populations and T regulatory cells, live single-cell suspensions were prepared from spleens and stained with propidium iodide (PI; catalog number P4864; Sigma/Merck), CD45R/B220-PE-Cy7 (1/400; catalog number RA3-6B2; BioLegend, San Diego, CA, USA), CD138-PE (1/400; catalog number 281–2; BioLegend), GL7-Pacific Blue (1/200; BioLegend), peanut agglutinin (PNA)–fluorescein isothiocyanate (1/400; catalog number L7381; Sigma/Merck), IgG1-APC (1/400; catalog number RMG1-1; BioLegend), CD4-Pacific Blue (1/400; catalog number RM4-5; BD Biosciences), CD25-APC (1/100; catalog number PC61; BD Biosciences) and FoxP3-PE (catalog number MF23; BD Biosciences). Antibodies were diluted in FACS buffer (Hanks' Balanced Salt Solution with 2% fetal calf serum). For staining with anti-FoxP3, cells were fixed and permeabilized with the Mouse FoxP3 buffer set (BD Biosciences). B-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, $B220^{hi}GL7^{hi}PNA^{hi}IgG1^{hi}$; nonisotype switched, $B220^{hi}GL7^{hi}PNA^{hi}IgG1^{low}$. T regulatory cells were identified as $CD4^{+}CD25^{+}FoxP3^{+}$. Cells were analyzed on a BD FACSCanto II (BD Biosciences) after exclusion of PI^{+} dead cells. Data were analyzed using FlowJo version 10.6.2 cell cycle analysis software (BD Biosciences).

Measurement of peritoneal lavage and serum cytokines

Blood was collected by cardiac puncture and stored overnight at 4°C, followed by centrifugation at 1500*g* for 15 min. Serum was collected and 11 different cytokines were measured using a ProcartaPlex multiplex assay (Mouse custom 11-plex ProcartaPlex Kit; Invitrogen/Thermo Fisher), as per the manufacturer's protocol: BAFF, IFNα, IFNγ, IL-1α, IL-1β, IL-10, IL-12p70, IL-17A, IL-18, IL-23 and IL-6. A handheld magnetic plate washer was used throughout the assay.

Readings were taken using a Bio-plex MAGPIX multiplex reader (Bio-Rad, NSW, Australia).

Measurement of cytokines in cell supernatants

Cytokines were measured in cell culture supernatants by ELISA (Biolegend; mouse IL-6: catalog number 431301, mouse IL-10: catalog number 431411 and human IL-10: catalog number 430601), according to the manufacturer's protocols.

siRNA transfection

RAW 264.7 cells were transfected with siRNA as previously described.[63] In brief, cells were resuspended in 100 mL BTXpress electroporation buffer (catalog number 45–0803; Harvard Apparatus, Holliston, MA, USA) with 400 nM *Becn1* or nontargeting (scrambled) ON-TARGETplus siRNA (SMARTpool; Dharmacon, Lafayette, CO, USA) and electroporated using an Amaxa Nucleofector device (Lonza, Basel, Switzerland). Cells were cultured at 5×10^5 cells mL^{-1}, medium replaced after 24 h and incubated for a further 24 h prior to final stimulation.

Western blot analysis

Following stimulations, cells were washed two times with PBS and lysed with either 2% IGEPAL CA-130 (catalog number I8896; Sigma) in Tris buffer, as previously described,[1] or radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris pH 8.0). Lysates were boiled with Laemmli buffer (under reducing conditions), loaded and separated on 4–12% NuPAGE bis-tris gels (catalog number NP0322PK2; Novex/Thermo Fisher) and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h with 5% skimmed milk in PBS with 0.1% Tween 20 and stained with primary antibodies overnight at 4°C (1:1000 dilution in 2.5% bovine serum albumin). Primary antibodies were rabbit anti-Beclin-1 (catalog number sc-11 427; Santa Cruz) and mouse anti-β-actin (clone AC-15; Sigma). The membranes were then probed with horseradish peroxidase–conjugated secondary antibodies (diluted 1: 10 000 in 2.5% bovine serum albumin) for 60 min at room temperature. After washing with PBS with 0.1% Tween 20, the blots were developed with enhanced chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and scanned in a Kodak IS4000R imager (Fisher Scientific).

Quantitative PCR

Following treatments, total RNA was extracted from cells using the RNeasy Plus Mini Kit (catalog number 74 134; Qiagen, Hilden, Germany), according to the manufacturer's protocol. Complementary DNA was prepared from 0.5 μg of total RNA according to the M-MLV reverse transcription protocol using MultiScribe reverse transcriptase (catalog number 4 311 235; Thermo Fisher). Quantitative real-time PCR analyses were performed on duplicate samples using LightCycler 480 SYBR Green I Master kit (Roche, Basel, Switzerland). The PCR conditions were 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Values are expressed as relative mRNA level of specific gene expression as obtained using the 2^{ddCT} formula.

Confocal microscopy

iBMMs stably expressing GFP-LC3 were cultured on 18-mm diameter coverslips (number 1.5, 0.17 mm thickness) in 12-well tissue culture plates. Cells were prepared and fixed as previously described.[64] In brief, after treatment with LPS (100 ng mL^{-1}), cells were fixed with paraformaldehyde (2% in PBS) for 30 min at room temperature. They were then permeabilized with 0.1% Triton X-100 in PBS and blocked with 1% bovine serum albumin and 0.5% goat serum in PBS for 1 h at room temperature. Cells were stained with rabbit polyclonal antibody against IL-10 (catalog number ab175471; Abcam, Cambridge, UK) for 1 h at room temperature and then secondary stained with Alexa-Fluor 568–conjugated anti-rabbit monoclonal antibody (Abcam; catalog number ab9969) for 1 h at room temperature. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; 5 μg mL^{-1}; catalog number D9542; Sigma) for 10 min and then cells mounted on slides with fluorescence mounting medium (catalog number S3023; Dako). Cells were observed on an Olympus OM-10 confocal microscope and images captured for quantitation of $LC3^+$ autophagosomes. At least 100 cells (mean 214.9, range 140–314) were counted per sample and the experiment was repeated three times. Processing and analysis of staining and localization of IL-10/LC3 were achieved using FIJI and Adobe Photoshop software.

Statistical analyses

All data were tested for normal distribution using a combination of Anderson–Darling, D'Agostino and Pearson, Shapiro–Wilk and Kolmogorov–Smirnov tests. Normally distributed data were analyzed for significant differences between groups using a one-way ANOVA with Šídák's multiple comparison test. Data that were not normally distributed were tested for differences using a Kruskal–Wallis test with Dunn's multiple comparison test. For direct comparison between two groups, with normally distributed data, *t*-tests were conducted.

ACKNOWLEDGMENTS

The authors acknowledge the hard work and invaluable assistance of the staff at the Monash Medical Centre Animal Facility (MMCAF). This work was supported by a Project Grant (1068040) from the Australian National Health and Medical Research Foundation (NHMRC) and by donations from the Lions Rheumatism and Arthritis Medical Research

Foundation Australia. Open access publishing facilitated by Monash University, as part of the Wiley - Monash University agreement via the Council of Australian University Librarians.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Md Abul Hasnat: Data curation; formal analysis; investigation; methodology; writing – review and editing. **IanIan Cheang:** Formal analysis; investigation; writing – review and editing. **Wendy Dankers:** Data curation; formal analysis; methodology; writing – review and editing. **Jacinta Lee:** Data curation; investigation; methodology; writing – review and editing. **Lynda Truong:** Formal analysis; investigation; writing – review and editing. **Mehnaz Pervin:** Investigation; writing – review and editing. **Sarah Jones:** Methodology; writing – review and editing. **Eric Morand:** Funding acquisition; writing – review and editing. **Joshua D Ooi:** Formal analysis; investigation; methodology; supervision; writing – review and editing. **James Harris:** Data curation; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; writing – original draft; writing – review and editing.

REFERENCES

1. Harris J, Hartman M, Roche C, *et al*. Autophagy controls IL-1β secretion by targeting pro-IL-1β for degradation. *J Biol Chem* 2011; **286**: 9587–9597.
2. Peral de Castro C, Jones SA, Ní Cheallaigh C, *et al*. Autophagy regulates IL-23 secretion and innate T cell responses through effects on IL-1 secretion. *J Immunol* 2012; **189**: 4144–4153.
3. Saitoh T, Fujita N, Jang MH, *et al*. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1β production. *Nature* 2008; **456**: 264–268.
4. Dankers W, Hasnat MA, Swann V, *et al*. Necrotic cell death increases the release of macrophage migration inhibitory factor by monocytes/macrophages. *Immunol Cell Biol* 2020; **98**: 782–790.
5. Lee JP, Foote A, Fan H, *et al*. Loss of autophagy enhances MIF/macrophage migration inhibitory factor release by macrophages. *Autophagy* 2016; **12**: 907–916.
6. Harris J, VanPatten S, Deen NS, Al-Abed Y, Morand EF. Rediscovering MIF: New tricks for an old cytokine. *Trends Immunol* 2019; **40**: 447–462.
7. Lang T, Lee JPW, Elgass K, *et al*. Macrophage migration inhibitory factor is required for NLRP3 inflammasome activation. *Nat Commun* 2018; **9**: 2223.
8. Buffen K, Oosting M, Li Y, Kanneganti TD, Netea MG, Joosten LA. Autophagy suppresses host adaptive immune responses toward *Borrelia burgdorferi*. *J Leukoc Biol* 2016; **100**: 589–598.
9. Buffen K, Oosting M, Mennens S, *et al*. Autophagy modulates *Borrelia burgdorferi*-induced production of interleukin-1β (IL-1β). *J Biol Chem* 2013; **288**: 8658–8666.
10. Castillo EF, Dekonenko A, Arko-Mensah J, *et al*. Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. *Proc Natl Acad Sci USA* 2012; **109**: E3168–E3176.
11. Reed M, Morris SH, Owczarczyk AB, Lukacs NW. Deficiency of autophagy protein Map1-LC3b mediates IL-17-dependent lung pathology during respiratory viral infection via ER stress-associated IL-1. *Mucosal Immunol* 2015; **8**: 1118–3110.
12. Suzuki Y, Maazi H, Sankaranarayanan I, *et al*. Lack of autophagy induces steroid-resistant airway inflammation. *J Allergy Clin Immunol* 2016; **137**: 1382–1389. e9.
13. Anderson CA, Boucher G, Lees CW, *et al*. Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. *Nat Genet* 2011; **43**: 246–252.
14. Brinar M, Vermeire S, Cleynen I, *et al*. Genetic variants in autophagy-related genes and granuloma formation in a cohort of surgically treated Crohn's disease patients. *J Crohns Colitis* 2012; **6**: 43–50.
15. Hampe J, Franke A, Rosenstiel P, *et al*. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet* 2007; **39**: 207–211.
16. Henckaerts L, Cleynen I, Brinar M, *et al*. Genetic variation in the autophagy gene ULK1 and risk of Crohn's disease. *Inflamm Bowel Dis* 2011; **17**: 1392–1397.
17. Rioux JD, Xavier RJ, Taylor KD, *et al*. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet* 2007; **39**: 596–604.
18. Wellcome Trust Case Control Consortium, Craddock N, Hurles ME, *et al*. Genome-wide association study of CNVs in 16,000 cases of eight common diseases and 3,000 shared controls. *Nature* 2010; **464**: 713–720.
19. Ramos PS, Criswell LA, Moser KL, *et al*. A comprehensive analysis of shared loci between systemic lupus erythematosus (SLE) and sixteen autoimmune diseases reveals limited genetic overlap. *PLoS Genet* 2011; **7**: e1002406.
20. Zhou XJ, Lu XL, Lv JC, *et al*. Genetic association of PRDM1-ATG5 intergenic region and autophagy with systemic lupus erythematosus in a Chinese population. *Ann Rheum Dis* 2011; **70**: 1330–1337.
21. Heckmann BL, Green DR. LC3-associated phagocytosis at a glance. *J Cell Sci* 2019; **132**: jcs222984.
22. Martinez J, Cunha LD, Park S, *et al*. Noncanonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. *Nature* 2016; **533**: 115–119.
23. Li B, Yue Y, Dong C, Shi Y, Xiong S. Blockade of macrophage autophagy ameliorates activated lymphocytes-derived DNA induced murine lupus possibly via inhibition of proinflammatory cytokine production. *Clin Exp Rheumatol* 2014; **32**: 705–714.
24. Alessandri C, Barbati C, Vacirca D, *et al*. T lymphocytes from patients with systemic lupus erythematosus are resistant to induction of autophagy. *FASEB J* 2012; **26**: 4722–4732.
25. Clarke AJ, Ellinghaus U, Cortini A, *et al*. Autophagy is activated in systemic lupus erythematosus and required for plasmablast development. *Ann Rheum Dis* 2015; **74**: 912–920.

26. Gros F, Arnold J, Page N, *et al*. Macroautophagy is deregulated in murine and human lupus T lymphocytes. *Autophagy* 2012; **8**: 1113–1123.
27. Hibbs ML, Tarlinton DM, Armes J, *et al*. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell* 1995; **83**: 301–311.
28. Lamagna C, Hu Y, DeFranco AL, Lowell CA. B cell-specific loss of Lyn kinase leads to autoimmunity. *J Immunol* 2014; **192**: 919–928.
29. Xu Y, Harder KW, Huntington ND, Hibbs ML, Tarlinton DM. Lyn tyrosine kinase: accentuating the positive and the negative. *Immunity* 2005; **22**: 9–18.
30. Scapini P, Pereira S, Zhang H, Lowell CA. Multiple roles of Lyn kinase in myeloid cell signaling and function. *Immunol Rev* 2009; **228**: 23–40.
31. Bolen JB, Rowley RB, Spana C, Tsygankov AY. The Src family of tyrosine protein kinases in hemopoietic signal transduction. *FASEB J* 1992; **6**: 3403–3409.
32. Nataraja C, Dankers W, Flynn J, *et al*. GILZ regulates the expression of pro-inflammatory cytokines and protects against end-organ damage in a model of lupus. *Front Immunol* 2021; **12**: 652800.
33. Tsantikos E, Oracki SA, Quilici C, Anderson GP, Tarlinton DM, Hibbs ML. Autoimmune disease in Lyn-deficient mice is dependent on an inflammatory environment established by IL-6. *J Immunol* 2010; **184**: 1348–1360.
34. Ban T, Sato GR, Nishiyama A, *et al*. Lyn kinase suppresses the transcriptional activity of IRF5 in the TLR-MyD88 pathway to restrain the development of autoimmunity. *Immunity* 2016; **45**: 319–332.
35. Lamagna C, Scapini P, van Ziffle JA, DeFranco AL, Lowell CA. Hyperactivated MyD88 signaling in dendritic cells, through specific deletion of Lyn kinase, causes severe autoimmunity and inflammation. *Proc Natl Acad Sci USA* 2013; **110**: E3311–E3320.
36. Dupont N, Jiang S, Pilli M, Ornatowski W, Bhattacharya D, Deretic V. Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1β. *EMBO J* 2011; **30**: 4701–4711.
37. Zhang M, Kenny SJ, Ge L, Xu K, Schekman R. Translocation of interleukin-1β into a vesicle intermediate in autophagy-mediated secretion. *Elife* 2015; **4**: e11205.
38. Delgado MA, Elmaoued RA, Davis AS, Kyei G, Deretic V. Toll-like receptors control autophagy. *EMBO J* 2008; **27**: 1110–1121.
39. Harris J. Autophagy and cytokines. *Cytokine* 2011; **56**: 140–144.
40. Harris J, Lang T, Thomas JPW, Sukkar MB, Nabar NR, Kehrl JH. Autophagy and inflammasomes. *Mol Immunol* 2017; **86**: 10–15.
41. Vujic N, Bradic I, Goeritzer M, *et al*. ATG7 is dispensable for LC3-PE conjugation in thioglycolate-elicited mouse peritoneal macrophages. *Autophagy* 2021; **17**: 3402–3407.
42. Schweighoffer E, Tybulewicz VL. BAFF signaling in health and disease. *Curr Opin Immunol* 2021; **71**: 124–131.
43. Kokic V, Martinovic Kaliterna D, Radic M, Perkovic D, Cvek M, Capkun V. Relationship between vitamin D, IFN-γ, and E2 levels in systemic lupus erythematosus. *Lupus* 2016; **25**: 282–288.
44. Mende R, Vincent FB, Kandane-Rathnayake R, *et al*. Analysis of serum interleukin (IL)-1β and IL-18 in systemic lupus erythematosus. *Front Immunol* 2018; **9**: 1250.
45. Tsirakis G, Pappa CA, Kaparou M, *et al*. The relationship between soluble receptor of interleukin-6 with angiogenic cytokines and proliferation markers in multiple myeloma. *Tumour Biol* 2013; **34**: 859–864.
46. Harigai M, Kawamoto M, Hara M, Kubota T, Kamatani N, Miyasaka N. Excessive production of IFN-γ in patients with systemic lupus erythematosus and its contribution to induction of B lymphocyte stimulator/B cell-activating factor/TNF ligand superfamily-13B. *J Immunol* 2008; **181**: 2211–2219.
47. Suematsu S, Matsuda T, Aozasa K, *et al*. IgG1 plasmacytosis in interleukin 6 transgenic mice. *Proc Natl Acad Sci USA* 1989; **86**: 7547–7551.
48. Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. *Crit Rev Immunol* 2012; **32**: 23–63.
49. Llorente L, Zou W, Levy Y, *et al*. Role of interleukin 10 in the B lymphocyte hyperactivity and autoantibody production of human systemic lupus erythematosus. *J Exp Med* 1995; **181**: 839–844.
50. Mahajan A, Herrmann M, Munoz LE. Clearance deficiency and cell death pathways: a model for the pathogenesis of SLE. *Front Immunol* 2016; **7**: 35.
51. Paludan C, Schmid D, Landthaler M, *et al*. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science* 2005; **307**: 593–596.
52. Münz C. Autophagy proteins in antigen processing for presentation on MHC molecules. *Immunol Rev* 2016; **272**: 17–27.
53. Kasai M, Tanida I, Ueno T, *et al*. Autophagic compartments gain access to the MHC class II compartments in thymic epithelium. *J Immunol* 2009; **183**: 7278–7285.
54. Schmid D, Pypaert M, Münz C. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. *Immunity* 2007; **26**: 79–92.
55. Fonteneau JF, Brilot F, Münz C, Gannagé M. The tumor antigen NY-ESO-1 mediates direct recognition of melanoma cells by $CD4^+$ T cells after intercellular antigen transfer. *J Immunol* 2016; **196**: 64–71.
56. Jin Y, Sun C, Feng L, *et al*. Regulation of SIV antigen-specific $CD4^+$ T cellular immunity via autophagosome-mediated MHC II molecule-targeting antigen presentation in mice. *PLoS One* 2014; **9**: e93143.
57. Li Y, Wang LX, Yang G, Hao F, Urba WJ, Hu HM. Efficient cross-presentation depends on autophagy in tumor cells. *Cancer Res* 2008; **68**: 6889–6895.
58. Mintern JD, Macri C, Chin WJ, *et al*. Differential use of autophagy by primary dendritic cells specialized in cross-presentation. *Autophagy* 2015; **11**: 906–917.

59. Wenger T, Terawaki S, Camosseto V, *et al*. Autophagy inhibition promotes defective neosynthesized proteins storage in ALIS, and induces redirection toward proteasome processing and MHCI-restricted presentation. *Autophagy* 2012; **8**: 350–363.
60. Keller CW, Sina C, Kotur MB, *et al*. ATG-dependent phagocytosis in dendritic cells drives myelin-specific $CD4^+$ T cell pathogenicity during CNS inflammation. *Proc Natl Acad Sci USA* 2017; **114**: E11228–E11237.
61. Komatsu M, Waguri S, Ueno T, *et al*. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol* 2005; **169**: 425–434.
62. Waibel M, Christiansen AJ, Hibbs ML, *et al*. Manipulation of B-cell responses with histone deacetylase inhibitors. *Nat Commun* 2015; **6**: 6838.
63. Harris J, De Haro SA, Master SS, *et al*. T helper 2 cytokines inhibit autophagic control of intracellular *mycobacterium tuberculosis*. *Immunity* 2007; **27**: 505–517.
64. Harris J. Staining MIF in cells for confocal microscopy. *Methods Mol Biol* 2020; **2080**: 85–91.

SUPPORTING INFORMATION

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