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Autophagy in Dendritic Cells and B Cells Is Critical for the Inflammatory State of TLR7-Mediated Autoimmunity

Chi G. Weindel,* Lauren J. Richey, † Abhiruchi J. Mehta,‡ Mansi Shah,‡ and Brigitte T. Huber*†‡

Individuals suffering from autoimmune disorders possess a hyperactive cellular phenotype where tolerance to self-antigens is lost. Autophagy has been implicated in both the induction and prevention of autoimmunity, and modulators of this cellular recycling process hold high potential for the treatment of autoimmune diseases. In this study, we determine the effects of a loss of autophagy in dendritic cells (DCs), as well as both B cells and DCs, in a TLR7-mediated model of autoimmunity, similar to systemic lupus erythematosus, where both cell types are critical for disease. Although a loss of DC autophagy slowed disease, the combined loss of autophagy in both cell types resulted in a lethal sepsis-like environment, which included tissue inflammation and hyperproduction of inflammasome-associated cytokines. Ablation of B cell signaling reversed this phenotype, indicating that activation of these cells is an essential step in disease induction. Thus, autophagy plays a dichotomous role in this model of disease. The Journal of Immunology, 2017, 198: 1081–1092.

Inflammation is a tightly controlled biological reaction to cellular insults. An inflammatory response is required for the efficient clearance of bacterial and viral pathogens, as well as removal of cellular debris following tissue damage. Inflammation can also occur in the absence of infection. Detrimental effects of sterile inflammation are readily observed in autoimmune diseases in which inflammation is predisposed by genetic susceptibility and compounded by environmental triggers (1–4). Severe flares of inflammation mark the prototypical autoimmune disease, systemic lupus erythematosus (SLE). Inflammation in SLE is thought to be driven by antinuclear Abs (ANAs) as a result of a loss of self-tolerance (5).

Despite the identification of numerous SLE susceptibility genes via genome-wide association studies, the etiology of this disease remains poorly understood (6, 7). Notably, several single nucleotide polymorphisms associated with SLE have been mapped to autophagy-related genes (ATGs) (8, 9). Targeted macroautophagy, the canonical autophagy pathway herein referred to as autophagy, is a key regulator of cellular homeostasis as well as immune system function; that is, it controls recognition and clearance of extracellular pathogens (10–12), mediates cytokine production and release (13–15), and promotes the proliferation and survival of adaptive immune cells (16–19). Thus, it is reasonable to think that alterations in autophagy contribute to SLE predisposition and/or disease progression. Based on genetic linkage and cellular functions, modulators of autophagy have an excellent potential as therapeutics for autoimmune diseases where self-tolerance has been lost (reviewed in Refs. 20, 21). However, because autophagy is critical for multiple aspects of the immune system, its different functions may play opposing roles in autoimmunity. Promising results have been reported in lupus patients when treated with either inhibitors or inducers of autophagic processes (22).

To better understand the role of autophagy in SLE, we used a mouse model of autoimmunity, mediated by overexpression of the RNA-sensing innate immune receptor TLR7 (Tlr7.1 transgenic [tg]) (23, 24). We showed recently that B cell autophagy is required for the induction of autoimmunity (25). In particular, Tlr7.1 tg mice harboring B cells deficient in autophagy did not make ANAs, lacked a type I IFN signature, and did not develop glomerulonephritis (25), all hallmarks of SLE.

Although B cells are central in SLE, dendritic cells (DCs) also play an important role in this disease, because they are the main producers of type I IFNs and are essential APCs for T cell activation, both of which contribute to disease progression in humans and in mouse models (26–30). Autophagy is required for IFN-α production by plasmacytoid DCs (pDCs) during viral infection (11), as well as for Ag presentation by myeloid DCs (mDCs) (12, 31, 32). Therefore, disrupting autophagy in these cells has the potential to reduce autoimmune symptoms. Thus, we compared disease in Tlr7.1 tg mice with either a DC-specific ablation of autophagy or a combined loss of autophagy in DCs and B cells.

As predicted, an autophagy knockout (ko) in DCs slowed disease progression and reduced IFN-α production; however, Tlr7.1 tg mice lacking autophagy in both cell types developed a rapid and lethal inflammatory condition, reminiscent of sterile sepsis, suggesting that autophagy plays a dichotomous role in disease progression. The massive inflammatory response in the latter mice was not driven by ANAs, but instead was driven by autoantibodies against cytoplasmic material, such as cardiolipin (CL), a diphosphatidylglycerol lipid found in the mitochondrial membrane (33). These data demonstrate that B cells lacking autophagy maintain the capacity to respond to some self-antigens,

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Abbreviations used in this article: ANA, antinuclear Ab; APS, antiphospholipid syndrome; ATG, autophagy-related gene; Bko, Cd19-cro+/- Arg5’/5’; CL, cardiolipin; DBko, Cd11c-cro+/- Cd19-cro+/- Arg5’/5’; DC, dendritic cell; Dko, Cd11c-cro+/- Arg5’/5’; EMH, extramedullary hematopoeisis; ko, knockout; LAP, LC3-associated phagocytosis; LN, lymph node; mDC, myeloid DC; mtDNA, mitochondrial DNA; MZ, marginal zone; PAS, periodic acid–Schiff; pDC, plasmacytoid DC; SLE, systemic lupus erythematosus; tg, transgenic.

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Materials and Methods

Mice

All mice used in this study were on a C57BL/6J background and were analyzed at either 3, 5, or 14 mo of age. C57BL/6J and Cd11c-/- Cre mice [C57BL/6-Tg[Il6cCre-2]494Gum/J] were purchased from The Jackson Laboratories (Bar Harbor, ME). Tlr7.1 tg mice [C57BL/6-Tg(Tlr7b)1Boll] were produced at the National Institutes of Health and generated by recombining the bacterial artificial chromosome clone RP23-1392P1 as previously described (23). Mice were bred at Tufts University School of Medicine; Cd11c-cre+/-, Atg5-/- (Dko) mice and Cd11c-cre+/-, Cd19-cre+/-, Atg5-/- (Dbko) mice were generated by intercrossing Cd11c-Cre and Cd19-cre+/-, Atg5-/- (Bko) mice [B6.129P2(Cd19tm1(cre)Cgn Atg5tm1Myok)] (17), respectively. Bko mice were obtained from H. Virgin (Washington University, St. Louis, MO), at the ninth generation of backcrossing to C57BL/6J, and were backcrossed at Tufts University for an additional generation. Tlr7.1 tg mice with one copy of Atg5-/- were crossed with Dko or Dbko mice to generate experimental cohorts. Tlr7.1 tg mice with one copy of Atg5-/- and one copy of Cd19-cre+/- were bred to Dbko mice to generate cohorts containing Tlr7.1 tg Dbko Cd19ko mice. All animals were housed, bred, and studied at Tufts University School of Medicine under approved Institutional Animal Care and Use Committee guidelines.

Flow cytometry

Single-cell suspensions of spleen and lymph node (LN) were made in 1× PBS (0.137 M NaCl, 2.7 mM KCl, 5.3 mM Na2HPO4, 1.8 mM KH2PO4), 2% FBS (Atlanta Biologicals, S11550) using 70-μm nylon cell strainers (BD Falcon, 352350). Peritoneal cells were isolated by lavage (1× PBS, 2% FBS). Cells were blocked for FCGR2B/CD32 (eBioscience, 14-0161-85) and stained with the following Abs from eBioscience: CD11c FITC (47-0112-82), and allophycocyanin–eFluor CD11b (47-0112-82); and BD Pharmingen GR1/Ly6G (45-5931-80), B220 allophycocyanin–eFluor 780 (11-5931-82), PDCA1 PerCP–eFluor 710 (46-3172-80), and Streptavidin-HRP (R&D Systems, 4800-30-06) was added at 1:1000 and read at 450 nm with SoftMax Pro using an EMax (Molecular Devices).

Histology

Tissue were fixed with 10% neutral buffered formalin (Sigma-Aldrich, HT501640) and embedded in paraffin; 5-μm sections were cut and stained with H&E. Two sections were stained with periodic acid–Schiff (PAS; Rowley Biochemical, SO-429). The pathologist performed a blinded evaluation of kidney sections for membranoproliferative glomerulonephritis using the following scoring system: 1, no areas to focal areas of minimal segmental PAS+ mesangial thickening; 2, focal to generalized mild segmental PAS+ mesangial thickening; 3, general multifocal or mild multifocal in both parenchymal and portal areas. Lung histology was scored as follows: 0, absent; 1, very slight multifocal or mild focal and suppurative; 2, mild multifocal and suppurative; 3, moderate multifocal or mild multifocal in both parenchymal and portal areas; 4, severe multifocal expansions of both megakaryocytes and myeloid cells in portal and parenchymal areas; Lung megakaryocyte and myeloid cells were analyzed using an LSM II with FACSDiva software (BD Biosciences). Secondary analysis was performed using FCS Express 4 research edition (De Novo Software).

Cytokine ELISA

Cytokine levels of serum IL-18 were determined as follows: plates were coated with 50 μl of rat anti-mouse IL-18 mAb (MBL International, DO47-3) at 1:1000 in 1 × PBS and incubated overnight at 4°C. Plates were blocked for 1 h in 1× PBS, 1% BSA. Standards were diluted 1:1 starting at 4 ng/ml (MBL International, DO48-3) and samples, diluted 1:4 in blocking buffer, were added to the plate and incubated overnight at 4°C. Detection Ab, 50 μl of polyclonal rat anti-mouse IL-18 (MBL International, DO48-6), was used at a 1:2000 dilution in blocking buffer and incubated 1 h at room temperature. Streptavidin-HRP (R&D Systems, 4800-30-06) was added at 1:1000 and incubated for 20 min at room temperature. For detection, 50 μl of tetramethylbenzidine substrate (BD Biosciences, 555214) was added, and reactions were stopped with 50 μl of 2N H2SO4. For all cytokine ELISAs, plates were read at 450 nm with SoftMax Pro using an EMax (Molecular Devices).
line to mice containing a Cd11c-Cre–mediated DC-specific conditional deletion of Atg5 (Dko) (see Materials and Methods for a full list of the genotypes and abbreviations used). As shown in Fig. 1A, a loss of DC autophagy significantly improved the survival of Tlr7.1 tg mice, increasing the median survival from 28 to 40 wk. However, as these mice aged beyond 40 wk, their health rapidly declined, leading to a precipitous death. Because DCs are major producers of IFN-α, we hypothesized that the increased survival of these mice was caused by lower production of this cytokine and a reduced IFN signature. To test this, we screened

![FIGURE 1. Tlr7.1 tg mice lacking DC autophagy have increased lifespan and reduced IFN-α. (A) Survival of Tlr7.1 tg mice (red line) compared with Tlr7.1 tg Dko mice (blue line). Mice were observed during 58 wk. Controls (n = 13), Tlr7.1 tg (n = 14), and Tlr7.1 tg Dko (n = 9) mice are shown. *p < 0.05 by a Mantel–Cox log-rank test. (B–G) Sera from age-matched nonmoribund mice between 16 and 20 wk of age were tested for cytokines using multiplex immunoassays: (B) IFN-α; (C) IL-18 and IL-1β, (D) IL-12p70, IL-6, and IL-10, (E) G-CSF, (F) IL-2 and IFN-γ, (G) IL-4 and IL-5. Each point is representative of an individual animal. (H) Representative H&E sections of liver for controls (left panels), Tlr7.1 tg (right center panel), and Tlr7.1 tg Dko mice (far right panel). Original magnification ×10. (I) HEp-2 slides used to detect ANAs (IgG-FITC) in 16- to 20-wk-old mice. Original magnification ×20. Results are based on at least six mice per genotype. *p < 0.05 **p < 0.01, ***p < 0.005 by a Kruskal–Wallis test with a Dunn posttest.]

### FIGURE 1

(A) Survival of Tlr7.1 tg mice (red line) compared with Tlr7.1 tg Dko mice (blue line). Mice were observed during 58 wk. Controls (n = 13), Tlr7.1 tg (n = 14), and Tlr7.1 tg Dko (n = 9) mice are shown. *p < 0.05 by a Mantel–Cox log-rank test. (B–G) Sera from age-matched nonmoribund mice between 16 and 20 wk of age were tested for cytokines using multiplex immunoassays: (B) IFN-α; (C) IL-18 and IL-1β, (D) IL-12p70, IL-6, and IL-10, (E) G-CSF, (F) IL-2 and IFN-γ, (G) IL-4 and IL-5. Each point is representative of an individual animal. (H) Representative H&E sections of liver for controls (left panels), Tlr7.1 tg (right center panel), and Tlr7.1 tg Dko mice (far right panel). Original magnification ×10. (I) HEp-2 slides used to detect ANAs (IgG-FITC) in 16- to 20-wk-old mice. Original magnification ×20. Results are based on at least six mice per genotype. *p < 0.05 **p < 0.01, ***p < 0.005 by a Kruskal–Wallis test with a Dunn posttest.
sérum on an array of SLE-associated inflammatory cytokines. Whereas the Tlr7.1 tg mice presented with a significant increase in IFN-α, this was not seen in Tlr7.1 tg Dko mice, indicating a defect in type 1 IFN production (Fig. 1B). Alternatively, IL-18 and, to a lesser extent, IL-1β levels were elevated in the latter mice (Fig. 1C). These two cytokines require cleavage by caspase-1 for secretion and, therefore, are dependent on inflammasome activation (35, 36). Aside from these clear differences, other SLE-associated cytokines were similarly elevated in Tlr7.1 tg and Tlr7.1 tg Dko mice, including IL-12p70, IL-6, and IL-10 (Fig. 1D); growth factors (Fig. 1E) and Th1 and Th2 cytokines were also unchanged (Fig. 1F, 1G). Considering the increased percentage of marginal zone (MZ) B cells was depleted in both groups of Tlr7.1 tg mice, indicating B cell activation (Supplemental Fig. 1D). Consistent with this, the percentages of CD80+ B cells were significantly increased in both Tlr7.1 tg and Tlr7.1 Dko mice (Supplemental Fig. 1E).

Thus, we conclude that B cells are the primary inducers of disease in these mice and are capable of driving disease progression, whereas lower production of IFN-α in Tlr7.1 tg Dko mice slows disease.

**Loss of DC and B cell autophagy results in severe lethality, splenomegaly, and lymphadenopathy**

Because survival was increased in Tlr7.1 tg mice with a DC loss of autophagy (Dko), likely due to a reduction in IFN-α levels, whereas B cell activation remained unchanged, we reasoned that combining a loss of DC and B cell autophagy might ablate disease completely in Tlr7.1 tg mice. We therefore bred the Tlr7.1 tg line to mice containing a combined DC and B cell conditional deletion of Atg5 (DBko). Surprisingly, we observed a drastically different phenotype than predicted; most significantly, these mice succumbed to rapid death with a median survival of 20 wk (Fig. 2A, solid line). A considerable increase in the weight of spleens and lymph nodes (Fig. 2B, C) was observed, with a corresponding increase in white pulp (WP) and red pulp (RP) (Fig. 2D, E).

**Loss of DC and B cell autophagy results in severe DKO.** (A) Survival of Tlr7.1 tg mice (red line) compared with Tlr7.1 tg DBko mice (blue line). Mice were observed during 58 wk. Controls (n = 13), Tlr7.1 tg (n = 13), and Tlr7.1 tg DBko (n = 11) mice are shown. *p < 0.05 by Mantel–Cox log-rank test. Spleens and LNs were weighed and normalized as a percentage of body weight at (B) 12–14 wk and at (C) 57 wk for controls. Each point is representative of an individual animal. Results are based on at least three independent experiments. (D and E) H&E sections of spleen (D) white pulp (WP) and red pulp (RP) for controls (left panel), Tlr7.1 tg (center panel), and Tlr7.1 tg DBko mice (right panel). Original magnification ×4. Results are based on at least six mice per genotype. (E) LNs showing nuclei (hematoxylin, blue) and cytoplasm (eosin, pink) for Tlr7.1 (upper panel) and Tlr7.1 tg DBko (lower panel) mice. Original magnification, ×40. (F) Flow cytometry of nucleated cells for spleen (left plot), LNs (middle plot), and peritoneum (right plot). Results are based on at least three independent experiments with mice between 12 and 14 wk of age. *p < 0.05, **p < 0.01, ***p < 0.005 by a Kruskal–Wallis test with a Dunn posttest.
(Fig. 2B, left panel) and LNs (Fig. 2B, right panel) was apparent in these mice. Aged control mice with a double autophagy ko (DBko) also developed significant increases in the weight of secondary lymphoid organs over time (Fig. 2C). The phenotype of these mice was strongly enhanced by the Tlr7.1 tg, as the spleens of Tlr7.1 tg DBko mice at 12 wk of age accounted for as much as 12% of total body weight, compared with only 3% in Tlr7.1 tg mice. Additionally, the spleens of Tlr7.1 tg DBko mice showed a complete loss of architecture, enhanced myelopoiesis and erythropoiesis, and an expansion of the white pulp as well as the red pulp (Fig. 2D). The LNs of these mice had a similar expansion of cell populations than did those of their Tlr7.1 tg littermates, whereas their peritoneal cavity cell numbers were normal (Fig. 2E). This latter finding is likely due to the fact that a B cell loss of autophagy inhibits development of B1 cells, which are mostly found in the peritoneum (17).

The early mortality of the Tlr7.1 tg DBko mice, combined with the increased size, weight, and cellularity of their secondary lymphoid organs, suggested excessive levels of tissue inflammation in these mice. Consistent with this, the dramatic cellular expansion in the spleens and LNs consisted of myeloid cell populations; notably, increased numbers of mDCs, neutrophils, monocytes, and macrophages were observed (Fig. 3A, 3B; see Supplemental Fig. 2 for myeloid cell gating strategy), whereas Tlr7.1 tg mice only showed a mild increase in splenic mDCs at this early time point (Fig. 3A, left plot). Additionally, an increase

![FIGURE 3. Loss of B cell and DC autophagy results in a systemic expansion of myeloid lineage cells in Tlr7.1 tg mice. (A and B) Myeloid and granuloid cell populations in the spleen (A) and LNs (B), as well as activated DCs (C and D) and inflammatory monocytes (E and F), were analyzed by flow cytometry. mDCs (CD11c+CD11b+B220-), monocytes (CD11b+GR1low/CD11c- [forward scatter/side scatter]), macrophages (CD11b+GR1low/CD11c- [forward scatter/side scatter]), neutrophils (CD11b+GR1+CD11c-), activated DCs (CD11c-B220-CD80+), inflammatory monocytes (CD11b+GR1low/CD11c-Ly6Chigh). Each point is representative of an individual animal. *p < 0.05, **p < 0.01, ***p < 0.005 by a Kruskal–Wallis test with a Dunn posttest.](http://www.jimmunol.org/)
inflammatory markers was seen on splenic and LN cells of Tlr7.1 tg DBko mice, including CD80$^{\text{high}}$ DCs (Fig. 3C, 3D) and Ly6C$^{\text{high}}$ monocytes (Fig. 3E, 3F).

**Loss of DC and B cell autophagy promotes inflammasome activation and organ damage**

The inflammatory condition of Tlr7.1 tg mice is well described and consists of a myeloid cell expansion, liver and kidney inflammation, as well as elevated cytokines, including IFN-α, TNF-α, and IL-6, seen between 12 and 24 wk (23). To determine whether the lethality and severe secondary lymphoid expansion in Tlr7.1 tg DBko mice was due to an accelerated disease, occurring earlier and beyond that observed in Tlr7.1 tg littermates, we determined the levels of SLE-associated cytokines in the sera of Tlr7.1 tg and Tlr7.1 tg DBko mice between 12 and 14 wk of age and assessed organ inflammation. As shown in Fig. 4, we detected a dramatically different cytokine profile in the latter mice; namely, IL-18 (Fig. 4A, left panel) and IL-1β (Fig. 4A, right panel) were highly elevated far above the levels seen in Tlr7.1 tg Dko mice (Fig. 1C). These data indicate that loss of autophagy in B cells is enhancing the cytokine phenotype observed in Tlr7.1 tg Dko mice. Increased serum IL-18 was also observed in Dko and DBko control mice, suggesting increased inflammasome activation in the absence of autophagy. Consistent with a highly inflammatory condition, TNF-α was also significantly elevated in Tlr7.1 tg DBko mice (Fig. 4B). Alternatively, IFN-α was not significantly elevated (Fig. 4C). This was surprising, because Tlr7.1 tg DBko mice showed a severe myeloid expansion at this time point. Thus, the lack of IFN-α indicates that the expansion of myeloid cells was not being driven by type 1 IFNs. At this age, Tlr7.1 tg mice with working autophagy had normal levels of IFN-α, consistent with their early stage in disease progression. Tlr7.1 tg DBko mice also had increased levels of IL-12p70, IL-6, and IL-10 (Fig. 4D), as well as multiple T cell cytokines (Fig. 4E, 4F). Taken together, these data suggest that Tlr7.1 tg DBko mice develop a severe inflammatory condition, reflective of increased inflammasome activity, as well as T cell activation. Importantly, their inflammatory phenotype occurs at an earlier time point and does not include IFN-α, the signature cytokine of SLE; thus, inflammation is driven by a different mechanism than in Tlr7.1 tg mice with working autophagy. We conclude that Tlr7.1 tg DBko mice develop a lethal inflammatory condition that is distinct from the inflammation present in Tlr7.1 tg mice.

The distinct cytokine profile of Tlr7.1 tg DBko mice mirrored a condition resembling sterile sepsis, which is typically accompanied by systemic organ damage. Therefore, we surveyed multiple organs for signs of inflammation. As expected, the livers of both Tlr7.1 tg and Tlr7.1 tg DBko mice showed cellular infiltrates and inflammation (Fig. 5A, Supplemental Table I). Notably, however, the latter developed a more widespread condition, with lymphohistiocytic and suppurative inflammation in both the portal and parenchymal areas (Fig. 5A, far right panel). In addition to increased inflammation, the livers of Tlr7.1 tg DBko mice showed increased EMH, consisting of myeloid cells and megakaryocytes (Fig. 5B, Supplemental Table I).

**FIGURE 4.** Inflammasome-associated cytokines are elevated upon loss of DC and B cell autophagy. Sera from age-matched nonmoribund mice between 12 and 14 wk of age were tested for cytokines using a multiplex immunoassay. (A) IL-18 and IL-1β, (B) TNF-α, (C) IFN-α, (D) IL-12p70, IL-6, and IL-10, (E) IL-2 and IFN-γ, and (F) IL-4 and IL-5. Each point is representative of an individual animal. *p < 0.05, **p < 0.01, ***p < 0.005 by a Kruskal–Wallis test with a Dunn posttest.
Unlike the Tlr7.1 tg mice that do not develop severe lung inflammation (23), 90% of the Tlr7.1 tg DBko mice showed lung infiltrates of lymphocytes or macrophages, as well as megakaryocytes, ranging from mild to severe. Additionally, a thickening of the interstitium was observed, indicative of an inflammatory state (Fig. 5C, right panel, SD, summary, Supplemental Table I). Interestingly, 67% of aged DBko control mice also possessed a slight increase in lung inflammation due to macrophage and neutrophil infiltrates (Fig. 5C, middle left panel).

Tlr7.1 tg mice develop glomerulonephritis, a characteristic of SLE (23, 37). Thus, the inflammatory state of Tlr7.1 tg DBko kidneys was determined. Early in disease progression the glomeruli of these mice scored more severely on a pathology index than did those of Tlr7.1 tg mice (Table I). However, their kidneys did not worsen with age; rather, they maintained a lower pathology score, whereas Tlr7.1 tg DBko mice (Fig. 7A; see Table I), unlike those of their Tlr7.1 tg littermates (Fig. 6A, middle left panel, Table I), did not develop more severe kidney pathology as disease progressed (Table I). Overall, our data demonstrate that Tlr7.1 tg DBko mice have multiple and more widespread organ damage than do Tlr7.1 tg mice, consistent with a sterile sepsis-like inflammatory condition.

Loss of autophagy promotes B cell recognition of mitochondrial Ags in Tlr7.1 tg mice

The elevated serum IL-18 and IL-1β present in the Tlr7.1 tg DBko mice (Fig. 4A) and Tlr7.1 tg Dko mice (Fig. 1C) indicates increased inflammasome activity. Interestingly, IL-18 levels in Tlr7.1 tg DBko mice were nearly twice those of Tlr7.1 tg Dko mice. These data combined with the increased inflammation in Tlr7.1 tg DBko mice compared with Tlr7.1 tg Dko mice indicated that a B cell loss of autophagy enhanced the inflammasome activation and septic condition. Because B cells lacking autophagy were unable to produce ANAa in Tlr7.1 tg mice (25), we sought to confirm this phenotype in Tlr7.1 tg DBko mice. As expected, sera from DBko and Tlr7.1 tg DBko mice lacked nuclear and nucleolar staining on HEp-2 slides (Fig. 6A, middle left and far right panels, Table I), unlike those of their Tlr7.1 tg littermates (Fig. 6A, middle right panel, Table I). However, bright staining of either cytoplasmic components or the nuclear/cellular membrane was present in the sera of the latter (Fig. 6A, far right panels, Table I). Because the speckled cytoplasmic pattern resembled mitochondrial staining, we tested for Abs against CL, a phospholipid that is a major component of the mitochondrial inner membrane, but is exposed to the cytosol upon mitochondrial damage (38). As shown in Fig. 6B, anti-CL IgG was increased in Tlr7.1 tg DBko mice. Whereas both Tlr7.1 tg and Tlr7.1 tg DBko mice had similarly elevated total serum IgM levels (Fig. 6C, left graph), the total IgG level of Tlr7.1 tg DBko mice was significantly increased (Fig. 6C, right graph), and IgG1 accounted for most IgG in these mice (Fig. 6D). Alternatively, IgG2a and IgG2b were the predominant subtypes in Tlr7.1 tg mice (Fig. 6E), as previously reported (24, 37). The levels of IgG2c and IgG3 were similar for all mice (Fig. 6E). These data indicate that an autophagy ko in B cells prevents production of ANAs, but is permissive for production of other autoantibodies. Thus, we conclude that nuclear and cytoplasmic Ags are processed by different cellular machinery.

IL-18 hyperproduction and lethality in Tlr7.1 tg DBko mice is dependent on BCR function

In addition to the presence of anti-CL IgG and high IgG1, B cells were increased in the LNs of Tlr7.1 tg DBko mice (Fig. 7A; see

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**Table I. Kidney pathology and ANA patterns for nonmoribund and moribund mice**

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Supplemental Fig. 3 for lymphocyte gating strategy), and these cells had higher levels of the activation marker CD80 (Fig. 7B, Supplemental Fig. 2A). B cell numbers and activation were similarly increased in the spleens (Fig. 7C, 7D). B cell subsets were also altered in these mice, with reduced numbers of follicular and MZ B cells (Fig. 7E, Supplemental Fig. 2B). Splenic plasmablasts were increased, but plasma cell numbers remained unchanged (Fig. 7F, Supplemental Fig. 2C). Although total T cell numbers were unchanged in Tlr7.1 tg DBko mice, increased T cell activation (CD69) was seen (Fig. 7G, Supplemental Fig. 2D). The increase in B cell activation was surprising, given the defects reported to be associated with a loss of B cell autophagy (19, 39).

To test whether the inflammatory condition of Tlr7.1 tg DBko mice was due to the hyperactivation of their B cells, we created triple ko mice, combining a Cd19 ko with the B cell and DC loss of autophagy on the Tlr7.1 tg background, and analyzed the survival of these mice. CD19 is critical for BCR function, because its loss decreases the B cell response to Ag. As shown in Fig. 7H, loss of CD19 rescued Tlr7.1 tg DBko mice from premature death. Interestingly, the survival curve of these mice was similar to that of Tlr7.1 tg mice with a B cell loss of autophagy, where disease is prevented (25). Furthermore, aged Tlr7.1 tg DBko Cd19ko mice showed a reduction in serum IL-18, similar to the levels in DBko controls (Fig. 7I). These data suggest that the lethal inflammatory disease in Tlr7.1 tg DBko mice is initiated by B cells responding to intra- or extracellular autoantigenic stimuli and is augmented by the Tlr7.1 tg and a loss of DC autophagy.

### Discussion

Using a TLR7-mediated model of autoimmunity (Tlr7.1 tg) (23), we have previously shown that loss of B cell autophagy prevents disease, indicating that autophagy is required for disease development (25). In this study, we demonstrate that autophagy deficiency in DCs slowed disease progression but resulted in secondary effects. However, when autophagy was absent in both B cells and DCs, a severe and lethal inflammatory state was induced. These results are especially interesting, given that autophagy-associated genes have confounding roles in SLE development in humans, where polymorphisms have positive disease association only in specific populations or are dependent on other functional factors, such as IL-10 (9, 40, 41).

Overall, our observations suggest that autophagy may have opposing roles in the development versus progression of disease.

The importance of DC autophagy in disease progression is evident by the increased mean survival and reduced IFN-α present in the sera of Tlr7.1 tg Dko mice, slowing down TLR7-mediated autoimmune development. However, over time disease was induced in these mice, and ANA production, liver inflammation, and glomerulonephritis were present at similar levels in aged mice from both groups. Because elevated type 1 IFN expression is restricted to pDCs, even in the presence of TLR7 overexpression (30), the phenotype of Tlr7.1 tg Dko mice suggests that a loss of DC autophagy is inhibiting the function of this cell type. In support of this, the improved survival of Tlr7.1 tg Dko mice is similar to the increased survival seen in Tlr7 tg mice that are unable to develop pDCs due to Tcf4 haplodeficiency (42). These latter mice showed reduced glomerulonephritis, although ANA production persisted. It has been shown that type 1 IFN upregulates TLR7 activity through a positive feedback loop in B cells (43). The presence of ANA and B cell activation in Tlr7.1 tg Dko mice, as well as Tlr7 tg Tcf4 haplodeficient mice, indicates that B cells in Tlr7 tg mice do not require IFN-α produced by pDCs for autoantibody production.

The clearance of apoptotic and necrotic cells, as well as microbes, by macrophages is dependent on LC3-associated phagocytosis (LAP), a pathway that relies on several components of the canonical autophagy machinery, including ATG5,
ATG7, and Beclin (44). It has been shown that defects in this pathway result in increased production of proinflammatory cytokines (44, 45). Additionally, mice with a LysM-cre–mediated deletion of LAP components in macrophages, monocytes, neutrophils, and mDCs develop an SLE-like disease over time, which includes autoantibody production and glomerulonephritis. Disease progression in these mice was caused by the defective degradation of phagocytized dead cells (45). In contrast, all mice in our study had functional ATG5 in macrophages, monocytes, and neutrophils. Furthermore, control Dko and DBko mice never developed autoantibodies (C.G. Weindel, A.J. Mehta, and B.T. Huber, unpublished data). These findings indicate that mDCs, pDCs, and B cells are dispensable for the clearance of apoptotic debris through LAP. In addition to the clearance of apoptotic debris, LAP is also critical for TLR9-dependent IFN-α production following the uptake of DNA immune complexes in pDCs (15). LAP-dependent clearance of RNA-containing complexes has not been described; nevertheless, the IFN-α defect in Tlr7.1 tg Dko mice indicates that TLR7-dependent IFN-α production by pDCs may also rely on LAP.

FIGURE 7. The sepsis-like state of Tlr7.1 tg DBko mice is dependent on BCR activation. (A–D) Total LN B cell numbers (A) and activation markers (B220+ CD3+ CD80+) (B) and total splenic B cells (C) and activation markers (D) are shown (total B cells, B220+CD3+; activated B cells, B220+CD3+ CD80+). (E–G) Splenic lymphocyte subsets: (E) follicular (FO) B cells and MZ B cells, (F) plasmablasts and plasma cells, and (G) T cell activation are shown (FO B cells, B220+CD3+CD21+CD23+; MZ B cells, B220+CD3+CD21low/−CD23+; plasmablasts, B220lo/cmCD138+; plasma cells, B220+CD138hi; and activated T cells, B220+CD3+CD69+). Each point is representative of an individual animal. Results are based on at least three independent experiments with mice between 12 and 14 wk of age. *p < 0.05, **p < 0.01, ***p < 0.005 by a Kruskal–Wallis test with a Dunn posttest. (H) Survival of Tlr7.1 tg DBko mice (blue line) compared with Tlr7.1 tg DBko C19ko mice (dotted blue line). Curves of mice were measured during 58 wk. Controls (n = 14), Tlr7.1 tg (n = 9), Tlr7.1 tg DBko (n = 8), and Tlr7.1 tg DBko C19ko (n = 5) mice are shown. *p < 0.05 by a Mantel–Cox log-rank test. (I) ELISA for serum IL-18 in mice aged 58 wk. *p < 0.05 by a Kruskal–Wallis test with a Dunn posttest.
Given the importance of autophagy for B cell activation and plasmablast development, it was surprising that the Tlr7.1 tg DBko mice developed a severe and lethal inflammatory condition. Their pervasive inflammation in the lung, liver, and kidney, as well as the increase in inflammatory cytokines and expansion of inflammatory myeloid cells, was reminiscent of a sterile systemic inflammatory response, sepsis in the absence of acute infection (reviewed in Ref. 46). Consistent with this, the two cytokines associated with activation of the inflammasome, IL-1β and IL-18, were present in the sera of these mice. Interestingly, IL-18 levels were more prominently elevated than IL-1β, which could be due to differences in turnover or assay sensitivity. It is also possible that IL-1β is produced secondarily to IL-18. Notably, IL-1β was undetectable in aged Dko and DBko controls (C.G. Weindel and B.T. Huber, unpublished data), whereas levels of IL-18 were significantly elevated. The fact that IL-18 and IL-1β levels were not increased in Tlr7.1 tg Bko mice (25) indicates that autophagy requirements for maintaining homeostasis and survival are cell type specific.

In addition to myeloid cell activation in Tlr7.1 tg DBko mice, we also observed increased B and T cell activation and elevated IgG. The high level serum levels of IgG1 in Tlr7.1 tg DBko mice, compared with IgG2a and IgG2b in Tlr7.1 tg mice, suggest that autophagy-deficient B cells respond differently to Ag than do B cells with working autophagy. Furthermore, the increase in B cell activation and total B cell numbers in Tlr7.1 tg DBko mice indicates that these cells were surviving and undergoing class switch recombination in the absence of functioning autophagy (18). Whereas Tlr7.1 tg DBko mice lacked ANAs, they produced autoantibodies against cytoplasmic or membranous determinants, demonstrating that Abs were being made against organelles. This was substantiated by the finding that these mice made anti-CL Abs, indicative of damaged mitochondria.

It has been shown in some cell types that mitophagy occurs through ATG5/ATG7-independent autophagy (47, 48), and the vesicular maintenance of mitochondria is complementary to the autophagy machinery (49). Therefore, the loss of ATG5 may allow clearance of damaged mitochondria under basal conditions. Consistent with this, control mice lacking DC and B cell autophagy did not make elevated anti-CL Abs, suggesting that the steady-state maintenance of mitochondria was not perturbed. The fact that TLR7 overexpression was required for the severe inflammatory state and anti-CL Ab production indicates that excessive cellular activation and accumulated damage might exhaust the noncanonical autophagy machinery. Alternatively, cellular stressors of mitochondria could be initiated prior to apoptosis. In support of this, CL is depleted from damaged mitochondria in neurons prior to mitochondrial expulsion from cells (50); furthermore, in these cells, CL binds directly to LC3, resulting in clearance by ATG7-dependent mitophagy (33). The presence of anti-CL Abs and damaged mitochondria in Tlr7.1 tg DBko mice is of particular interest, given that circulating mitochondrial DNA (mtDNA) is found in the plasma of trauma patients (51). Acting as a damage-associated molecular pattern, mtDNA causes inflammation and is a predictor of outcome in sepsis patients (52, 53). The role of damaged mitochondria in sepsis and acute organ damage has only recently been appreciated and is poorly understood. The phenotype of Tlr7.1 tg DBko mice suggests that autophagy plays a role in the cellular response to tissue damage, thus providing a potential target for direct intervention.

Either B cells or DCs from Tlr7.1 tg DBko mice could be a source of the damaged mitochondria, resulting in CL exposure to the cytoplasm. A loss of autophagy in B cells has been shown to result in endoplasmic reticulum stress, the unfolded protein response, and increased cell death following activation (19). Endoplasmic reticulum stress can also lead to reactive oxygen species and mitochondrial damage (54); mitochondrial damage and release of mtDNA, in turn, are known inflammasome activators (55, 56). Although this would suggest that autophagy-deficient B cells respond to their own damaged mitochondria, thereby augmenting the inflammatory response seen in Tlr7.1 tg Dko mice, our findings that Tlr7.1 tg Bko mice had normal levels of serum IgG, IL-18, and IL-1β is not consistent with this hypothesis (25). Additionally, the slight but significant increase of IL-18 in Dko and DBko controls suggests that the IL-18 production present is a direct result of a DC-specific loss of ATG5. Therefore, Tlr7.1 tg DBko B cells could be responding to extracellular Ag, such as the cytoplasmic components of dying DCs. It has been shown that B cells do not require autophagy for activation through the BCR in C57BL/6 lpr/lpr mice (57). The notable reduction of disease in Tlr7.1 tg DBko C1d9ko mice, deficient in BCIR function, supports this latter hypothesis. Alternatively, high levels of IL-18 have been shown to result in increased Ab production in B cells, which is mitigated by a loss of CD19 (58). The decrease in IL-18 levels in Tlr7.1 tg DBko C1d9ko mice, similar to that of DBko and Dko controls, further illustrates the critical role of both B cell function and IL-18 in augmenting disease.

Abs against phospholipids such as CL are associated with multiple immunological disorders, including antiphospholipid syndrome (APS), rheumatoid arthritis, and scleroderma. Patients suffering from APS share characteristics of SLE patients, that is, hematological malignancies and pregnancy complications (59). Anti-CL Abs have also been found in SLE patients in conjunct with ANAs (60, 61). How these autoantibodies develop and contribute to disease pathogenesis remains an unanswered question. B cell depletion therapy has been shown to significantly reduce the number of anti-CL Abs in SLE patients (62) and could, therefore, provide a therapeutic alternative for patients with APS. Our study sheds light on potential cellular mechanisms contributing to the production of lipid autoantibodies in these diseases.

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Disclosures

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References


