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*J Immunol* 2015; 194:5407-5416; Prepublished online 17 April 2015;
doi: 10.4049/jimmunol.1402277

http://www.jimmunol.org/content/194/11/5407

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2015/04/17/jimmunol.1402277.DCSupplemental

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Critical Role for IL-18 in Spontaneous Lung Inflammation Caused by Autophagy Deficiency

Elmoataz Abdel Fattah,* Abhisek Bhattacharya,* Alan Herron,† Zeenat Safdar,* and N. Tony Eissa*†

Autophagy has been recognized as an important regulator in many biological processes (1). As such, there is increasing interest in understanding the roles of autophagy in human diseases. Recently, mutations in autophagy genes were linked to several disorders (2). However, the role of autophagy in organ physiology and pathology needs to be elucidated as a prelude to better understanding of its role in human diseases. In the lung, autophagy is most likely critical for several important biological pathways implicated in lung diseases. Collectively, inflammatory lung injury syndromes account for the majority of morbidity and mortality associated with lung diseases and critical illness. One significant gap in our knowledge is the role that each cell type plays in orchestrating the injury. This issue is complicated by the extensive interactions between different cells in the lungs and the alveolar space. The use of cell-specific genetically engineered mouse models could greatly enhance our understanding of the roles of various cell types in lung injury pathogenesis. Macrophages are important cells in host defense. Resident macrophages in different tissues, including lung alveolar macrophages, ensure continuous immune surveillance and play a critical role in activating the cascade of immune responses. Autophagy deficiency in macrophages has been shown to result in increased inflammasome activity (2). Furthermore, activation of autophagy limits IL-1β production by targeting ubiquitinated inflammasomes for destruction (3). However, the consequences of autophagy deficiency in vivo are much less understood. In this study, we describe the development of spontaneous sterile lung inflammation in mice lacking Arg7 or Atg5 in myeloid cells. This inflammation was largely driven by IL-18 subsequent to constitutive inflammasome activation. This study reveals essential role of autophagy as a negative regulator of lung inflammation and identifies IL-18 as a critical mediator in lung injury due to autophagy deficiency.

Materials and Methods

Reagents and Abs

LPS (Escherichia coli O111:B4), DNase, collagenase, bleomycin sulfate, and mouse rM-CSF were from Sigma-Aldrich. Percoll was from GE Health Care. IL-1R antagonist (Anakinra) was from Biovitrum. IL-17 neutralizing Ab (MAB421), rat IgG2a IL-17 isotype control (MAB006), and rat IgG1 IL-18 isotype control (MAB005) were from R&D Systems. IL-18 neutralizing Ab (D048-3) was from MBL International. L chain 3B Ab was previously described (4). ATG7 Ab was from Rockland Immunochemicals (600-401-487). SQSTM1 (P62) Ab was from American Research Products (03-GP62-C-1).

Mice

We purchased LysM-cre mice from The Jackson Laboratory. Atg7Cre-tdTom was obtained from M. Komatsu. To specifically delete Atg7 and Atg5 from myeloid cells, we crossed Atg7Cre-tdTom and Atg5Cre-tdTom mice to LysM-cre, both on a C57BL/6 background (5). Age- and sex-matched Atg7Cre-tdTom/LysM-cre or wild-type (Atg7cre) mice were examined in each experiment. Mice were housed within a specific pathogen-free vivarium. Atg7Cre-tdTom/LysM-cre mice were rederived into a higher barrier 4 vivarium. Institutional Animal Care and Use Committee approved the research protocol.

LPS and bleomycin challenges

Intraperitoneal administration of LPS was done using a dose of 10 mg/kg for males and 20 mg/kg for females (6). Intranasal challenge of bleomycin was done as previously described (7). Briefly, bleomycin was dissolved in PBS at a concentration of 2.5 UKg/ml. Mice were anesthetized with isoflurane, and bleomycin in 50 μl PBS or PBS alone was instilled to the airway by inhalation through the nasal openings.

Histology and immunohistochemistry

Mice were sacrificed, and organs were harvested, fixed, and embedded in paraffin blocks. Paraffin-embedded organs were deparaffinized, rehydrated,
sectioned, and stained for H&E. Sections from fixed inflated lung were stained for periodic acid–Schiff (PAS) for detection of mucin glycoprotein, or Masson’s Trichrome for collagen. For immunohistochemistry of lung sections, rat monoclonal C4A5/B220 (B cell marker) from BD Pharmingen, rat monoclonal anti F4/80 (macrophages marker) from Abcam, or MUC5AC clone 4SM1 from Thermo Scientific were used as primary Abs.

Quantification of immunohistological findings

The number of B220−, PAS−, and muc5ac-positive cells were counted on nonoverlapping high power fields (original magnification ×400) of lung parenchyma (for B220) or airway epithelium (for muc5ac and PAS) beginning at the periphery of the section. Two separate stained sections for each Ab were counted per mouse, and the mean number of positive cells was reported. For peribronchial trichrome staining, area was outlined and quantified using a light microscope and ImageJ software from National Institutes of Health. Results are expressed as the area of trichrome staining per micrometer length of basement membrane of bronchioles. To estimate the level of lung fibrosis following bleomycin challenge, the Ashcroft score was determined, as previously reported (8). The amount of soluble collagen in bronchoalveolar lavage (BAL) and lung tissue homogenates was quantified using the Sircol assay from Biocolor (Carrick, U.K.). Lung inflammation score was quantified, as previously described (9).

Evaluation of cellular composition of BAL

Mice were sacrificed by CO2 asphyxiation, and BAL was done by instilling 0.8 ml PBS in trachea and then withdrawing it. Differential cell count of BAL was done on cytopsin by evaluating 200 cells based on characteristic morphology.

Bone marrow–derived macrophages

Bone marrow cells from 6- to 12-wk-old mice were cultured in DMEM containing 32 ng/ml mouse M-CSF for 7 d to differentiate into macrophages.

Flow cytometry

Lung leukocytes were isolated, as previously described (10). Lung leukocytes as well as single-cell suspensions from spleen, thymus, and bone marrow were stained by Abs against CD16/CD32, CD45-APC, Lys6G-PE, Lys6G/Ly6c-PE, CD45/B220- FITC, CD3e-PE-Cy7, CD4-, PE-Cy7, or CD8a-FITC, all from BD Pharmingen, or F4/80-Pe-Cy7 from eBioscience. Dead cells were excluded by Sytox blue from Invitrogen. Data were collected with FACS LSR Fortessa from BD Biosciences and analyzed using FlowJo software from Tree Star. Total number of leukocytes was obtained by multiplying total number of cells by the frequency of CD45+ cells obtained from flow cytometry analysis. Total cell numbers from single-cell suspensions of lungs, spleens, thymus, bone marrow, and BAL were counted using hemocytometer. Bone marrow proliferation was assessed using Click-iT EDU Flow Cytometry Assay Kit from Molecular Probes. Mice were injected i.p. for 3 h with 200 µg 5-ethyl-2′-deoxyuridine (EDU) and sacrificed, and bone marrow cells were analyzed for proliferative activity.

ELISA

IL-1β, IL-17, TGF-β, IL-6, and IL-33 were quantified using Quantikine ELISA kits from R&D Systems. Mouse IL-18 ELISA kit from MBL International was used to measure serum and BAL IL-18. Serum IgE was measured using BD optEIA ELISA kit from BD Biosciences. Levels of 32 cytokines and chemokines (eotaxin, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 [p40], IL-12 [p70], IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC-like, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, VEGF) were simultaneously measured in mouse serum using a Milliplex Mouse Cytokine/Chemokine 32plex assay from Millipore on a luminex-based multi-analyte plate from BioPlex and Bio-Rad.

ELISPOT

Single-cell suspensions from the lungs and spleens were obtained after flushing the systemic and pulmonary circulation with PBS; tissues were cut into small portions and then dispersed through 40-µm nylon filters. RBCs were lysed in ammonium-chloride-potassium lysing buffer, and remaining cells were washed twice and resuspended to 10⁶ cells/ml (11). The numbers of IL-1β–producing cells were quantified by aliquoting 10⁶ cells/100 µl to 96-well microtiter plates precoated with monoclonal anti-mouse IL-1β from Millipore. The numbers of IL-17–, IL-4–, and IFN-γ–producing cells were quantified by ELISPOT Mouse Development Module. IL-1β Abs, ELISPOT kits, and color development reagents were from R&D Systems.

IL-1R blockade and Ab neutralization of IL-17 or IL-18

In survival studies, following LPS administration, 25 mg/kg Anakinra was injected s.c. every 4 h for 12 h (12). Abs against IL-18 (15 µg) or isotype control were injected i.p. every 4 h for 12 h. To determine the effect of IL-1β on spleen weight, 100 mg/kg Anakinra was s.c. injected 30 min before and 3 h after LPS. For neutralizing the lung inflammation, Anakinra was injected s.c. daily for 8 d at a dose of 200 mg/kg. Control mice, not receiving Anakinra, were injected s.c. with PBS. Neutralizing IL-17 Ab (100 µg) or isotype control was injected i.p. three times every other day. IL-18 Ab (25–50 µg) or isotype control was injected i.p. two or three times every other day. Mice were sacrificed 24 h following the last injection.

Statistical analysis

Paired results were compared using Student t test. A two-way ANOVA followed by Bonferroni correction was used for multiple comparisons. Survival was evaluated with the log-rank test. All analyses were carried out with GraphPad Prism Version 5.00 Software.

Results

Autophagy deficiency in myeloid cells results in spontaneous lung inflammation

Mice with deficiency of key autophagy gene Atg5 or Atg7 die in the first day after birth (5). To generate myeloid-specific Atg7 knockout mice, we crossed mice bearing an Atg7tm2 allele with LysM-Cre transgenic mice, which express Cre recombinase specifically in cells of myeloid origin, including macrophages and neutrophils (4). Mice were raised in a specific pathogen-free barrier facility. The resulting mice, Atg7tm2Cre+ (henceforth termed Atg7Dmice), were viable with normal growth pattern similar to age-matched wild-type controls (Atg7Dmice). Consistent with conditional Atg7 knockout in macrophages, bone marrow–derived macrophages from Atg7Dmice exhibited marked reduction of ATG7 (Fig. 1A). Furthermore, accumulation of SQSTM1 (also known as p62), an autophagy substrate, and the diminished lipidation of the microtubule-associated protein 1 chain 3B from type I to II confirmed autophagy deficiency in these cells (13). Examination of spleen, liver, gastrointestinal tract, and brain from Atg7Dmice and Atg7tm2mice revealed no gross abnormalities. Surprisingly, Atg7tm2mice developed spontaneous lung inflammation characterized by increased infiltration of inflammatory cells in lung tissues, submucosal thickening, and increased collagen deposition. Lung inflammation was observed in 2-mo-old mice and persisted in mice evaluated up to 10 mo of age (Fig. 1B–D). Flow cytometry analysis of single-cell suspension from lung leukocytes showed that Atg7tm2mice had a higher number of total leukocytes, as indicated by the leukocyte common Ag/CD45 Ab staining (10), including higher number of neutrophils, as indicated by the Ly6G Ab staining and higher percentages of neutrophils and lymphocytes (Fig. 1E, 1F). Immune staining of lung tissue revealed that Atg7tm2mice accumulated macrophages and B220+ cells, and concomitant serum analysis indicated elevated IgE levels (Supplemental Fig. 1A–C).

Analysis of bronchoalveolar lavage (BAL) revealed that Atg7tm2mice exhibited accumulation of leukocytes, compared with wild-type Atg7Dmice (Fig. 1G). Differential cell count of BAL showed that Atg7tm2mice had higher percentages of neutrophils and lymphocytes (Fig. 1H). Furthermore, Atg7tm2mice had a higher number of leukocytes in bone marrow, including macrophages and B lymphocytes (Supplemental Fig. 1D–F). To identify a possible cause for increased number of cells in bone marrow from Atg7tm2mice, we investigated the proliferation of total leukocyte population in bone marrow after i.p. injection of EDU. Flow cytometry analysis of these cells showed that leukocytes of Atg7tm2mice incorporated more EDU, indicating increased proliferative activity (Supplemental Fig. 1G). The above findings indicated that...
autophagy in myeloid cells was essential for the maintenance of normal lung homeostasis.

Mice were housed in pathogen-free environment, and repeated screenings failed to show any evidence of infection. To further rule out the possibility that an occult infectious agent contributed to the observed phenotype, we rederived \( \text{Atg7}^{D} \) and control \( \text{Atg7}^{f/f} \) mice into a higher barrier 4 facility. Histological analysis of lung tissues of rederived mice confirmed sustained lung inflammation in \( \text{Atg7}^{D} \) mice, compared with control mice (Supplemental Fig. 2A–C). Furthermore, BAL of rederived \( \text{Atg7}^{D} \) mice had increased total leucocyte count and differential cell counts of neutrophils and lymphocytes. These results confirm the sterile phenotype of lung inflammation observed in \( \text{Atg7}^{D} \) mice.

Mucus metaplasia in the lungs of \( \text{Atg7}^{D} \) mice

We next investigated evidence of tissue injury and remodeling as a result of lung inflammation in \( \text{Atg7}^{D} \) mice. Goblet cell metaplasia is a common pathological feature that occurs in several inflammatory lung diseases. \( \text{Atg7}^{D} \) mice exhibited increased mucus production and accumulation of mucin in the airway epithelia (Fig. 2). These findings were revealed by PAS staining and confirmed by immunohistochemical analysis using mucin-specific Muc5ac Ab. Quantification of PAS- and Muc5ac-positive cells in the airway epithelium revealed higher numbers of positive cells in \( \text{Atg7}^{D} \) mice compared with wild-type \( \text{Atg7}^{f/f} \) mice. These results indicated that lung inflammation in \( \text{Atg7}^{D} \) mice was associated with goblet cell metaplasia.

Lung inflammation and mucus metaplasia in \( \text{Atg5}^{D} \) mice

To rule out the possibility that the phenotype observed in \( \text{Atg7}^{D} \) mice was caused by loss of nonautophagic functions of \( \text{Atg7} \), we generated myeloid-specific \( \text{Atg5}^{D} \) mice by breeding mice bearing an \( \text{Atg5}^{flox} \) allele with LysM-Cre transgenic mice. Similar to data obtained from \( \text{Atg7}^{D} \), lung tissues of \( \text{Atg5}^{D} \) mice revealed increase in the infiltration of leukocytes (Supplemental Fig. 2D–I). \( \text{Atg5}^{D} \) mice also exhibited mucus cell metaplasia in the airway epithelia and had a higher number of total leukocytes, particularly neutrophils, in lung tissues and BAL. These results confirmed the data obtained from \( \text{Atg7}^{D} \) mice and further suggested that autophagy deficiency, and not autophagy-independent gene-specific function, was responsible for the lung phenotype observed.

Constitutive inflammasome activation in \( \text{Atg7}^{D} \) mice

Tissue damage and disruption of cellular homeostasis are the hallmarks of inflammation. Inflammasomes are protein complexes that play a critical role in the recognition of danger-associated molecular patterns from damaged tissue or dying cells (14). Secretion of the active form of the potent proinflammatory cytokines

![FIGURE 1. Atg7D mice presents with spontaneous lung inflammation. Bone marrow-derived macrophages (BMDM) from wild-type (Atg7f/f) or Atg7D mice were analyzed by immunoblotting (A). Lung tissues from (B) 2-, (C) 6-, or (D) 10-mo-old mice were analyzed by H&E staining. Dotted line in (A) indicates where parts of the image were joined. Flow cytometry analysis of single-cell suspensions of the lungs was done using the pan leukocytic marker anti-CD45 (E) or anti-Ly6G (F). Quantification graphs are shown. BAL from 2- to 4-mo-old Atg7D or control Atg7f/f mice was analyzed for total cell count (G) or differential cell count (H). Scale bar, 50 μm. Data are mean ± SEM, n = 3–8 mice/genotype. *p < 0.05, **p < 0.01. Lymph, lymphocyte; MΦ, macrophage; PMN, polymorphonuclear neutrophil.](http://www.jimmunol.org/DownloadedFrom/)
FIGURE 2. Mucus hypersecretion and goblet cell metaplasia in Atg7f/f mice. PAS staining was performed on lungs from (A) 2-, (B) 6-, or (C) 10-mo-old control Atg7f/f or Atg7Δ mice. Quantitative analyses of results are shown (D). Lung tissues from 6-mo-old mice were immunostained for muc5ac, and the quantitation of positive cells is shown (E). Scale bar, 50 μm. Data are shown as mean ± SEM, n = 4–8 mice/genotype. ∗p < 0.05, **p < 0.01.

IL-1β and IL-18 occurs as a result of inflammasome complex assembly and activation. Deficiency of autophagy has been linked to hyperactivity of the inflammasome (2). We reasoned that lung inflammation in Atg7Δ mice might be caused by a constitutive inflammasome activity. To test this hypothesis, we examined single-cell suspensions from the lungs of Atg7Δ mice by ELISPOT. The number of cells producing IL-1β was markedly elevated in lungs from Atg7Δ mice (Fig. 3A). Inflammasome activation has been shown to potentiate Th17 cell–dominant immune responses (15). Furthermore, pulmonary inflammation induced by direct intranasal IL-1β challenge in mice is mediated by IL-17 (16). To identify the nature of the Th immune response mediating the spontaneous inflammation in the lung, we used ELISPOT to determine the number of cells that produce IFN-γ (for Th1), IL-4 (for Th2), or IL-17 (for Th17). Atg7Δ mice yielded higher number of IL-17 cells in lungs (Fig. 3B). The numbers of IFN-γ– or IL-4–producing cells, in lungs and spleens, were similar between Atg7f/f and Atg7Δ mice (data not shown). IL-18, another inflammasome product, has been shown to be important in lung inflammation associated with Influenza A virus infection (17). In our study, BAL fluid showed that Atg7Δ mice had higher levels of IL-1β and IL-18, compared with barely detectable levels in Atg7f/f mice (Fig. 3C). Importantly, in Atg7Δ mice, IL-18 concentration was ∼7-fold higher than IL-1β, suggesting an important role for IL-18 in lung inflammation observed in these mice. IL-1β and IL-18 were also higher in serum of Atg7Δ mice, compared with control mice (Fig. 3D).

Increased IL-1β, IL-18, and IL-17 in the lungs of Atg7Δ mice suggested that pulmonary neutrophilia was driven by the increase in these cytokines (18, 19). A recent study has shown that IL-17 plays a role in bleomycin-induced lung inflammation and fibrosis in mice, and that induction of collagen by IL-17 was TGF-β dependent (20). To investigate possible biological consequences of inflammasome activation and subsequent increase in IL-17 in the lungs of Atg7Δ mice, we examined latent TGF-β levels. BAL from Atg7Δ mice contained higher amounts of TGF-β than in control Atg7f/f mice (Fig. 3C). It has been previously shown that TGF-β and IL-6 can promote the differentiation of naive CD4 cells to IL-17–producing T cells (21). Analysis of BAL from Atg7Δ mice indicated substantial increase in IL-6 compared with BAL from Atg7f/f mice (Fig. 3C).

Lung inflammation in Atg7Δ mice is primarily mediated by IL-18

Our data above revealed upregulation of inflammasome-associated cytokines IL-1β, IL-17, and IL-18 in Atg7Δ mice. Unlike IL-1β, pro–IL-18 is presorted inside macrophages, and it is readily secreted upon inflammasome activation, without the need for a priming step (14, 22, 23). Thus, we speculated that IL-18 might have an important role in lung inflammation in Atg7Δ mice. We used neutralization strategies to determine the relative contribution of each cytokine to the causation of lung inflammation in these mice. Treatment of Atg7Δ mice with the IL-1R antagonist (Anakinra) or the use of neutralizing Abs against IL-17 had little effect on lung inflammation in Atg7Δ mice. In contrast, the use of Abs against IL-18 markedly prevented lung inflammation in Atg7Δ mice compared with mice injected with isotype control Abs (Fig. 4A, 4B). Furthermore, only IL-18 Abs treatment led to substantial reduction in the total number of leukocytes in BAL of Atg7Δ mice (Fig. 4C, 4D). Differential cell count showed that IL-18 treatment almost completely inhibited the recruitment of lymphocytes and neutrophils in the BAL of Atg7Δ mice (Fig. 4E). Despite having no effect on the tissue inflammation score or total number of leukocytes in BAL, IL-17 Ab treatment was effective in reducing the number of neutrophils in BAL. These results suggest that lung inflammation, in Atg7Δ mice, was primarily driven by IL-18.

The lung pathology observed in Atg7Δ mice suggested that autophagy in myeloid cells has an important protective role against lung inflammation. To elucidate such role, we investigated the role of autophagy in two clinically relevant models of lung inflammation, namely acute lung injury secondary to sepsis and interstitial lung fibrosis secondary to bleomycin exposure.

Enhanced susceptibility of Atg7Δ mice to endotoxemia

Sepsis syndrome can lead to septic shock that is associated with high rate of mortality (12). Sepsis is associated with increased IL-1β and IL-18 production and other systemic inflammatory responses, including lung injury. Endotoxins such as LPS play an important role in the above responses. We conducted studies to test the susceptibility of Atg7Δ mice to endotoxins. We injected mice with i.p. LPS and evaluated cytokine production, survival, and degree of lung inflammation. Levels of IL-1β, IL-18, and IL-17 were substantially elevated...
in Atg7D mice, compared with control mice, as early as 4–6 h following LPS injection (Fig. 5A–C). We next evaluated survival of Atg7f/f and Atg7D mice in response to LPS, injected i.p. at a dose of 10 mg/kg for males and 20 mg/kg for females (6). Survival rate was significantly reduced for both male and female Atg7D mice, compared with control mice (Fig. 5D, 5E). In addition, Atg7D mice died much earlier than Atg7f/f mice.

We then wanted to determine the effect of IL-18 neutralization on mice survival in LPS-induced sepsis. We coadministered mice with LPS and IL-18 Abs or with LPS and isotype control (19). IL-18 neutralization, following LPS injection, improved survival of Atg7f/f mice, but had no significant effect on survival of Atg7D mice (Fig. 5F). These data suggest that IL-18 is important in endotoxin-induced lethality. However, in autophagy-deficient mice, the increase of other inflammatory mediators, including IL-1β, IL-18, TGF-β, and IL-6, plays a more important role in endotoxin-induced lethality.

To determine the relative contribution of the increased IL-1β on the enhanced endotoxin-induced lethality in Atg7D mice, we cotreated mice with LPS and Anakinra (12). IL-1R blockade attenuated LPS lethality by reducing the frequency of death and delaying its onset in Atg7D mice (Fig. 5G). These findings are consistent with an important role for IL-1β in endotoxin-induced death in Atg7D mice. The incomplete prevention of LPS lethality probably reflects the upregulation of other proinflammatory cytokines downstream of inflammasome activation in Atg7D mice. These data suggested that, whereas IL-1β was essentially dispensable for lung inflammation, it played a more important role in endotoxin-induced lethality.

LPS induces higher levels of serum cytokines in Atg7D mice
Four hours following LPS injection, the levels of 32 cytokines and chemokines were measured in the serum from Atg7f/f mice or Atg7D mice. Compared with control mice, Atg7D mice had increased serum level of IFN-γ, IL-3, IL-13, LIF, RANTES, and TNF-α in response to LPS (Supplemental Fig. 3A, and data not shown). Atg7D mice exhibit enhanced lung inflammation after LPS challenge
Acute lung injury is a major cause of morbidity and mortality in patients with sepsis. LPS challenge in mice is often used as a model to study acute lung injury of sepsis. Intraperitoneal injection of LPS leads to increase in the recruitment of macrophages to the BAL and
neutrophils to the lung (24). We, therefore, evaluated lung inflammation in Atg7\textsuperscript{-/} and Atg7\textsuperscript{+/} mice after i.p. LPS injection. Histological analysis of lung tissues showed that Atg7\textsuperscript{-/} mice displayed a marked increase in the infiltration of inflammatory leukocytes to lungs, compared with Atg7\textsuperscript{+/} mice (Fig. 6A). Furthermore, there was increased goblet cell metaplasia after LPS injection in Atg7\textsuperscript{-/} mice. PAS staining for the glycoprotein content of mucin revealed that Atg7\textsuperscript{-/} mice contained much higher PAS-positive epithelial cells in the airways (Fig. 6B). Collagen deposition was increased in lungs of Atg7\textsuperscript{-/} mice, as early as 6 h following LPS injection and at death, suggesting the development of profibrotic lesions (Fig. 6C, 6D). BAL of Atg7\textsuperscript{-/} mice had higher

**FIGURE 4.** IL-18 is required for lung inflammation in Atg7\textsuperscript{-/} mice. (A and C) Representative images from Atg7\textsuperscript{-/} mice treated with saline or Anakinra (upper panels), isotype control or IL-17 Ab treatment (middle panels), or isotype control or IL-18 Ab (lower panels). (A) Lung tissues were analyzed by H&E staining. (B) Quantification of the inflammatory score. (C) BAL was analyzed by HEMA-3 staining. Total cell count (D) and differential count (E) were determined. Scale bar, 50 \( \mu \)m. Data are shown as mean ± SEM, \( n = 3–13 \) mice/treatment. *\( p < 0.05 \), **\( p < 0.01 \).

**FIGURE 5.** Increased cytokine production and reduced survival in Atg7\textsuperscript{-/} mice in response to LPS. (A–C) Following i.p. LPS injection in mice, serum levels of IL-1β (A), IL-18 (B), and IL-17 (C) were evaluated. (D–F) Kaplan–Meier survival curves of mice were recorded following injection of LPS (D and E) or LPS plus anti-IL-18 Ab or isotype control (F) and LPS plus IL-1R antagonist Anakinra (G). Data are mean ± SEM, \( n = 3–6 \) mice/genotype for each time point. *\( p < 0.05 \), **\( p < 0.01 \).
total cell count following LPS injection, compared with Atg7<sup>f/f</sup> mice (Supplemental Fig. 3B). Differential cell count of BAL showed that Atg7<sup>△</sup> mice exhibited a significantly higher percentage of lymphocytes and neutrophils following LPS injection (Supplemental Fig. 3C). In contrast, Atg7<sup>f/f</sup> mice did not show any relevant changes in the percentages of lymphocytes and neutrophils, as most of the cells recovered in BAL from these mice (Atg7<sup>△</sup>) before and after LPS injection were macrophages. The findings in Atg7<sup>△</sup> control mice are consistent with a recent study that found no changes in the number of neutrophils or in the concentration of IL-1β in the BAL, following i.p. injection of LPS (25). Similarly, in our study, levels of IL-1β, IL-18, and IL-17 in Atg7<sup>△</sup> control mice were largely unchanged 6 h post-LPS (Fig. 6D–F). In contrast, there was marked increase in IL-1β, IL-18, and IL-17 in BAL of Atg7<sup>△</sup> mice. These data suggested that lack of Atg7 in myeloid cells led to failure of lung homeostatic protective mechanisms in Atg7<sup>△</sup> mice.

In addition to increased cytokines and lung inflammation, our studies revealed that Atg7<sup>△</sup> mice rapidly developed marked increase in the size of the spleen within 4 h of LPS injection. In contrast, there was no significant change in spleen size in similarly treated Atg7<sup>f/f</sup> mice (Supplemental Fig. 3D). A previous study found that mice with conditional disruption of the IKK-β in myeloid cells, which led to increased inflammasome activity, developed splenomegaly and neutrophilia, and that these inflammatory changes were dependent on the presence of IL-1R (18). In our study, concomitant Anakinra treatment partially neutralized the increase in the size of the spleen seen after LPS injection in Atg7<sup>△</sup> mice, whereas it had no significant effect on spleens of Atg7<sup>f/f</sup> mice (Supplemental Fig. 3D). These data indicated that autophagy deficiency in myeloid cells predisposed mice to a severe form of lung inflammation and splenomegaly in response to LPS. We then investigated whether Atg7<sup>△</sup> mice would also exhibit greater predisposition to pulmonary fibrosis.

**Increased collagen production in Atg7<sup>△</sup> mice**

One of the most deleterious outcomes of lung inflammation is pulmonary fibrosis, which leads to loss of the normal architecture of the lung and deterioration of respiratory function. Pulmonary fibrosis is characterized by fibroblast proliferation and deposition of extracellular matrix proteins such as collagen (26). Idiopathic pulmonary fibrosis is a fatal disease of unknown etiology characterized by deterioration of the respiratory function and progressive lung scarring and fibrosis (26). Although murine models are used frequently to study human lung fibrosis, mice are more resistant to the development of permanent fibrotic lesions (27). We hypothesized that lung inflammation will predispose Atg7<sup>△</sup> mice to lung remodeling in the form of increased deposition of extracellular matrix proteins. At the age of 2 mo, Atg7<sup>△</sup> mice exhibited a 2-fold increase in the content of collagen in lung tissues (Fig. 7D). Soluble collagen in the BAL was also increased by 10 mo of age in Atg7<sup>△</sup> mice (Supplemental Fig. 3E). These results indicated that lung inflammation in Atg7<sup>△</sup> mice was accompanied by lung remodeling and deposition of extracellular matrix.

**Atg7<sup>△</sup> mice exhibit enhanced susceptibility to bleomycin-induced lung fibrosis**

Mice challenged intranasally with bleomycin exhibit inflammation, accumulation of collagen, and fibrosis in the lungs, and this model has been used to study the pathogenesis of pulmonary fibrosis (7, 16, 20). Atg7<sup>f/f</sup> and Atg7<sup>△</sup> mice were subjected to intranasal bleomycin or PBS, and lungs were analyzed at 7 and 14 d post-challenge. Both Atg7<sup>f/f</sup> and Atg7<sup>△</sup> mice showed a moderate loss of body weight, but the percentage of weight loss was more in Atg7<sup>△</sup> mice (Fig. 7A). Mice instilled with PBS did not show any weight loss. Histological analysis of lung tissues, at day 14 after bleomycin challenge, showed that Atg7<sup>△</sup> mice presented with severe histopathological features of pulmonary fibrosis, compared with control Atg7<sup>f/f</sup> mice (Fig. 7B). Consistent with these findings, Atg7<sup>△</sup> mice showed pronounced trichrome staining compared with
Atg\textsuperscript{7\textmu} mice following bleomycin treatment (Supplemental Fig. 4A). The severity of lung fibrosis was scored using the Ashcroft scaling system (8). Furthermore, quantification of soluble collagen levels in lung homogenates and BAL was performed. Atg\textsuperscript{7\textmu} mice developed increased lung fibrosis and higher collagen levels (Fig. 7C–E). Moreover, levels of latent TGF-\textbeta and IL-6, considered mediators of fibrosis, were elevated in BAL of Atg\textsuperscript{7\textmu} mice compared with Atg\textsuperscript{ff} (Fig. 7F, 7G). Both Atg\textsuperscript{ff} and Atg\textsuperscript{7\textmu} mice showed a marked increase in the number of total leukocytes in BAL in response to bleomycin. However, total leukocyte count of BAL in Atg\textsuperscript{7\textmu} mice was higher (Fig. 7H). The percentages of lymphocytes and neutrophils were higher in Atg\textsuperscript{7\textmu} mice after bleomycin treatment (Supplemental Fig. 4B–D). Furthermore, Atg\textsuperscript{7\textmu}, but not wild-type, mice displayed a progressive splenomegaly following bleomycin challenge (Supplemental Fig. 4E). Collectively, these data suggested an important protective role for autophagy in lung inflammation and fibrosis in response to bleomycin challenge.

Discussion

There is a growing interest in translating autophagy studies into better understanding of human diseases (28). However, the in vivo roles of autophagy in relation to organ pathology are not well understood. Studies addressing such roles are likely to provide breakthroughs into consequences of autophagy aberration on human diseases. In this regard, our studies reveal several novel findings. They showed that autophagy in myeloid cells was required for normal lung homeostasis. In the absence of autophagy, there was constitutive inflammasome activation leading to a spontaneous sterile lung inflammation. This effect was primarily mediated by IL-18 production. An important finding of this study is elucidation of the differential roles of IL-1\textbeta and IL-18 in lung injury and sepsis. IL-18 was critical for lung inflammation, whereas IL-1\textbeta was more responsible for the system effect of sepsis.

Lung inflammation predisposed autophagy-deficient mice to increased mortality from sepsis and led to enhanced lung fibrosis in response to fibrotic agent. Importantly, our findings were shown in mice deficient in either Atg7 or Atg5. Thus, they strongly suggest that the findings are due to autophagy deficiency and not due to deficiency of autophagy-independent functions of autophagy proteins. In addition, the reproduction of similar phenotype in Atg\textsuperscript{7\textmu} mice rederived in high barrier facility confirmed that lung inflammation in these mice was not due to occult infection predisposed to by autophagy deficiency.

Inflammatory lung injury contributes to the underlying etiology of many lung diseases. In its acute form, lung injury is often associated with sepsis and carries a high mortality rate (29). Other less acute forms of lung injury are thought to contribute to a variety of lung diseases, including interstitial lung diseases, pneumonia, asthma, chronic obstructive pulmonary disease, cystic fibrosis, and lung cancer. In our study, mice with conditional deletion of Atg7 or Atg5 in cells of myeloid origin presented with marked recruitment of inflammatory cells to the lungs. Macrophages, lymphocytes, and neutrophils accumulated into the alveolar spaces and lung parenchyma. Furthermore, airway epithelia underwent mucus cell metaplasia, an important feature of lung remodeling. Moreover, lung tissues accumulated much higher amounts of the extracellular matrix protein collagen, another feature that manifests during lung fibrosis and remodeling. In older autophagy-deficient
mice, there was increase in collagen in BAL, a feature suggestive of a progressive phenotype. Intraperitoneal injection of LPS induced lung inflammation that was much more enhanced and was associated with collagen deposition in autophagy-deficient mice. Lung inflammation in Atg7 knockout mice was enhanced after bleomycin challenge. In response to bleomycin, lungs from Atg7 knockout mice showed an amplified inflammation, as well as increase in fibrotic mediators, collagen deposition, and fibrosis.

Our data indicate that lung inflammation was primarily mediated by IL-1β, as indicated by our neutralization results. Despite failure of the IL-1R antagonist to neutralize the lung inflammation at basal level of autophagy deficiency, Anakinra treatment was effective in antagonizing morbidity and mortality induced by systemic administration of LPS. The neutralization studies suggest that the two cytokines have distinct roles in inflammation. Our data indicate that IL-1β mediates the sterile lung inflammation observed at baseline in Atg7 knockout mice, whereas IL-1β effect was more pronounced in sepis. The underlying mechanisms of such roles might be explained by the fact that IL-1β is constitutively expressed in macrophages, whereas IL-1β requires transcriptional activation by NF-kB (14). Thus, IL-1β might represent a therapeutic target in lung inflammation associated with sepsis.

Lung inflammation in Atg7 knockout mice was also evident in airway epithelial cells. Prior studies found that myeloid cell–specific deletion of IκBα resulted in NF-κB activation in both myeloid cells and epithelial cells with intact IκBα (24). IL-1β or IL-18 secreted by macrophages can bind to their cognate receptors expressed on epithelial cells and lead to transcriptional activation of downstream signals, such as NF-κB or MAPKs, and the subsequent expression of more IL-1β/IL-18 as well as other proinflammatory cytokines and chemokines. IL-1β/IL-18 can also lead to recruitment of neutrophils and lymphocytes, which can interact with airway epithelial cells and amplify the inflammation. The finding of mucus cell metaplasia in lungs of Atg7 knockout mice is most likely the consequence of the interaction between myeloid cells and epithelial cells. It has been shown previously that human IL-1β expression in Clara cells leads to mucus cell metaplasia in mice (30). Recently, IL-18 was found to induce similar pathology via IL-17, IFN-γ, and IL-13 (31). Neutrophils release several inflammatory and oxidative mediators that can lead to lung tissue damage and features of remodeling, including goblet cell metaplasia. Hence, mucus cell metaplasia in Atg7 knockout mice could be caused by one or a combination of several inflammatory mediators.

Another important finding in our study is the increase of basal levels of TGF-β and IL-6 in the BAL of Atg7 knockout mice. TGF-β has an important role in epithelial to mesenchymal transition and in the pathogenesis of pulmonary fibrosis. TGF-β, together with IL-1β and IL-6, induces TH-17 lineage development. IL-17 is a strong neutrophil attractant implicated in the induction of inflammatory responses and lung damage in response to bleomycin, partially by increasing TGF-β (32). In the context of pulmonary fibrosis, a recent report suggested that autophagy might be deficient in subjects with the disease (33). In summary, our study indicated that autophagy in myeloid cells was required to maintain normal lung homeostasis and to prevent excessive inflammatory responses. This study provides the groundwork for further understanding of the role of autophagy in human diseases.

Acknowledgments
We thank members of the Eissa Laboratory for useful discussions and technical assistance and Joel M. Sederstrom and Dr. Shixia Huang for expert assistance. Atg7 knockout and Atg6 knockout mice were gifts of Dr. M. Kmoato (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and RIKEN BioResource, respectively.


