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Complement C5a exacerbates acute lung injury induced through autophagy-mediated alveolar macrophage apoptosis

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Intestinal ischemia has a high mortality and often causes acute lung injury (ALI), which is a serious complication, and is accompanied by high mortality up to 40%. An intense local and systemic inflammation occurs during intestinal ischemia/reperfusion (IR)-induced lung injury resulting from activation of immune responses. It has been reported that one component of complement, C5a, is indispensable for the full development of IR-induced lung injury, whereas the detailed molecular mechanism remains to be elucidated. In this study, we found that intestinal IR induced ALI-like symptoms, and C5a receptor (C5aR) expression was upregulated in alveolar macrophages, which are resident macrophages in lung tissue and are important in pulmonary homeostasis. C5a produced during lung injury binds to C5aR in alveolar macrophages, initiates downstream signaling that promotes autophagy, leading to apoptosis of alveolar macrophages. Using Mϕ-ATG5$^{-/-}$ mice, in which the atg5 is deficient specifically in macrophages and autophagy is inhibited, we confirmed that *in vivo* C5a interacting with C5aR induced autophagy in alveolar macrophages, which promoted alveolar macrophage apoptosis. Further study indicated that autophagy was induced through C5aR-mediated degradation of bcl-2. Taken together, our results demonstrated that C5aR-mediated autophagy induced apoptosis in alveolar macrophages, disrupting pulmonary homeostasis and contributing to the development of ALI. This novel mechanism suggests new therapeutic potential of autophagy regulation in ALI.
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During diverse clinical procedures, transient ischemia and reperfusion, known as ischemia/reperfusion (IR) clinically, are found in organs or tissues, and cause intense inflammation, both locally and systemically,[1,2] which in turn leads to various types of injury, even multiple organ failure, contributing to high mortality. Acute lung injury (ALI) is a common outcome of IR, and usually occurs in patients with intestinal ischemia, leading to high mortality of 60–80%.[3] In addition, ALI is a life-threatening complication associated with sepsis, pneumonia, trauma, and many other clinical conditions. Despite improvements in the management of critically ill patients, ALI mortality is approximately 40%, and survivors often do not return to a normal life.[4] During the IR process, ischemia initiates a local inflammatory response, by releasing pro-inflammatory factors and activating/attracting inflammatory cells, such as neutrophils, macrophages, and lymphocytes.[5] Oxidative stress resulting from ischemia also contributes to IR injury. Owing to the unique anatomic and physiological features, the lung is susceptible to IR injury through pro-inflammatory cytokines storm.[6] Only a few pharmacologic treatment options are available for IR-induced ALI, which work by inhibiting inflammation or anti-oxidative effects.[7] Obviously, more effort is needed to clarify the underlying pathophysiological mechanisms of ALI and find more efficient therapeutic methods.

Macrophages are believed to derive from hematopoietic stem cells and are distributed all over the body. Macrophages are of vital importance in immune homeostasis, tissue remodeling, and biological events. Alveolar macrophages are resident lung macrophages, and present the first line of encountering inhaled substances.[8] Alveolar macrophages have essential roles in maintaining pulmonary homeostasis, without pro-inflammatory effects.[9] More importantly, alveolar macrophages suppress excessive inflammation, putatively through the strong inhibition of local immune cells, such as T lymphocytes and DCs. For example, rodent alveolar macrophages render inhibition on T-cell activation in the presence of DCs *in vitro,* through multiple mechanisms, such as releasing the suppressive cytokines, transforming growth factor-β and interleukin-10 (IL-10).[8–12] If alveolar macrophages are depleted, the animals display stronger inflammatory responses to otherwise innocuous inhaled antigens.[13] During ALI, cytokines and chemokines produced by tissue macrophages recruit neutrophils to the injury sites,[14] but the neutrophil recruitment also affects alveolar macrophage activity.[15,16] IL-10 production is induced by macrophages after

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Abbreviations: ALI, acute lung injury; IR, ischemia/reperfusion; MBL, mannan-binding lectin; BAL, bronchoalveolar lavage; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling; 3-MA, 3-methyl adenine; C5aR, C5a receptor
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phagocytosis of apoptotic neutrophils, which in turn suppresses additional cytokine production and inflammation, affecting both pro-inflammatory and anti-inflammatory cellular components of ALI.[12] For these reasons, alveolar macrophages have attracted interest in studies on the mechanisms of ALI.[8–11]

Complements are key mediators of the first line in protecting hosts from pathogen invasions and have been shown to be involved in IR-induced inflammation. During the ignition and amplification stages, complement activation contributes to inflammation-mediated tissue injury,[1,2,17] which would be significantly diminished if complement factors were depleted.[18,19] The complement activation product, C5a, is essential for the full development of injury. C5a has the ability of chemotaxis[20] and it can also directly activate neutrophils and macrophages for chemokine production.[21] C5a receptor (C5aR) signaling is required for C5a to render its effects on the process, as blockade of C5aR signaling will have similar effects to depletion of C5a in the survival of animals with cecal ligation and puncture,[22] suggesting that intercepting C5a or C5aR signaling may provide a potential target for therapeutic treatment in inflammatory diseases.[23]

Although significant effort has been aimed at determining the mechanism of macrophages in ALI, the activity of C5aR on macrophages is unclear. This study aimed to clarify the role of C5aR in macrophage biology during ALI development, and found that elevated C5a induced C5aR signaling in alveolar macrophages, and contributed to autophagy-mediated apoptosis, thus exacerbating the ALI symptoms. This novel mechanism provides a potential role for autophagy regulation in ALI therapeutic applications.

Results

Intestinal IR induces ALI-like disease in mice. To further research on the mechanism underlying ALI, we established intestinal IR-induced lung injury in this study. Breathing pattern changes are observed during the onset and progression of ALI, usually accompanied by decreased blood oxygenation.[24,25] In our study, intestinal IR caused a change in breathing pattern. This increase in breathing pattern was accompanied by ALI-induced lower blood oxygenation (Figure 1a), and more water in the lung, thus increasing lung water content (Figure 1b). Along with water leakage, more protein was present in the airways (Figure 1c), and these conditions became more serious with longer ischemia time (Figures 1a–c). In addition, more blood neutrophils

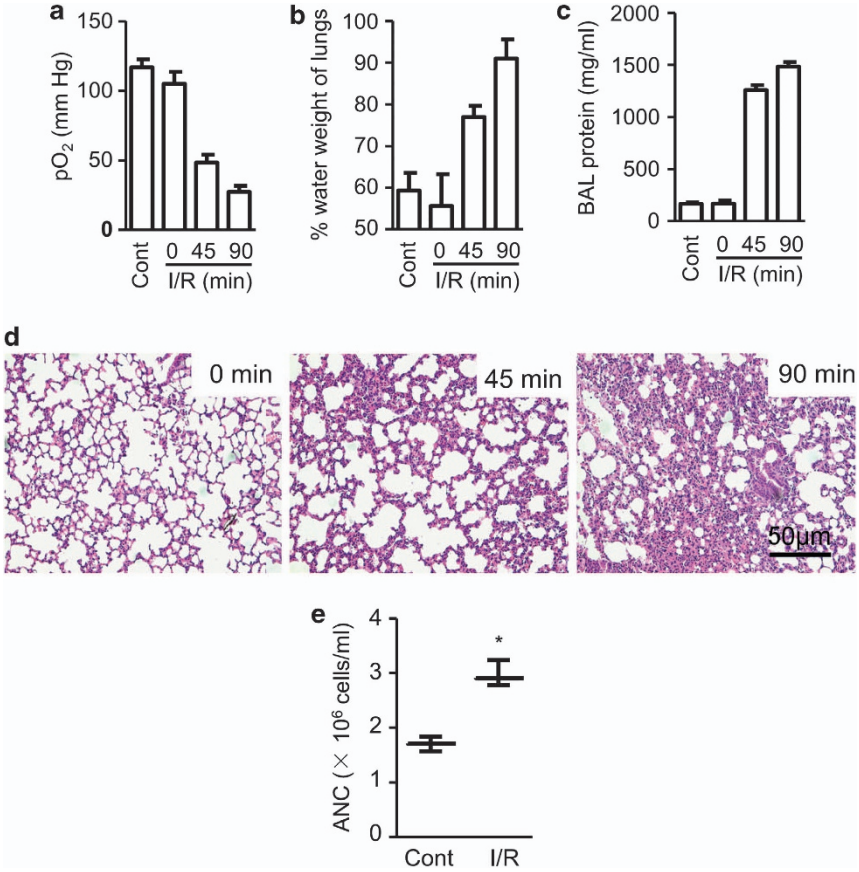

Figure 1 Intestinal ischemia/reperfusion induced ALI-like disease in mice. (**a**) Eight-week-old C57BL/6 male mice (six mice per group) were subjected to intestinal ischemia/reperfusion, and arterial blood pO_2 was detected 45 min later. Results are representative of three experiments. (**b**) Mice in **a** underwent lung removal, and water content percentage was measured. Data are representative of three experiments. (**c**) Mice as in **a** with BAL fluid collected, and protein content was measured. Data are representative of three experiments. (**d**) Representative H&E-stained lung sections from C57BL/6 male mice (six mice per group) subjected to intestinal ischemia/reperfusion. Lungs were harvested 45 or 90 min later. (**e**) Mice in **a** underwent blood collection before or 90 min after injury. Absolute neutrophil count (ANC) was measured, and data are the mean ± S.D. of three independent experiments. $^*P < 0.05$

infiltrated the lung (Figures 1d and e). Changes in breathing pattern were observed at 2 min after ischemia and correlated with increased lung water and the presence of shock and hemoconcentration, typical characteristics in ALI. During the development of ALI, endothelial cells rapidly separated from the underlying basement membrane (Figure 1d), as quickly as 5 min after ischemia, suggesting dysfunction of capillary walls. However, the observed capillary wall changes were not associated with neutrophil infiltrates or platelet thrombi, as no such events occurred in control mice. Both the increase in lung water and enhanced pause were fully resolved by 24 h. Thus, in our ALI model, we observed characteristic features such as the early presence of damage to alveolar capillary walls, then the presence of fluid and protein leakage into the lung parenchyma. Intestinal IR induced ALI, altered breathing patterns, neutrophilic alveolitis, and the formation of microthrombi.

Inhibition of complement decreases IR-induced lung injury. During ischemia followed by reperfusion, inflammation appeared because of tissue injury and oxidative stress, and both kidney and lung are the most commonly affected organs. IR-induced tissue injury, combined with inflammation, induces complement activation. Among the complement components, C5a has been shown to be involved in the tissue injury process, therefore, we next determined to detect the presence of C5a during ALI. In injured lung, inflammatory factors are released into the bronchoalveolar space, thus we collected bronchoalveolar lavage fluid (BALF) and tested for C5a. The results showed that C5a was markedly elevated in ALI compared with normal lung (Figure 2a). Lung tissue from mice subjected to IR and lung tissues from normal controls were sectioned, and these sections were immunohistochemically stained. As shown in Figure 2b, the injured lung showed significantly higher expression of C5a in lung tissue sections. In order to further confirm the contribution of C5a to the development of IR-induced lung injury, we administered C5a-neutralizing antibody i.v. to mice before IR. Our data demonstrated that C5a neutralization alleviated the IR-induced lung tissue injury, with less inflammatory cell infiltration (Figure 2c), and a drastic reduction in the pathological score (Figure 2d), confirming the contribution of C5a to ALI development. Taken together, these data demonstrated that C5a was essential for full development of ALI.

C5a induces activation of alveolar macrophages. During IR-induced ALI, multiple inflammatory factors are released in local lesions. Several cytokines and chemokines were detected in BALF, and our data showed that TNF-α, IL-6,

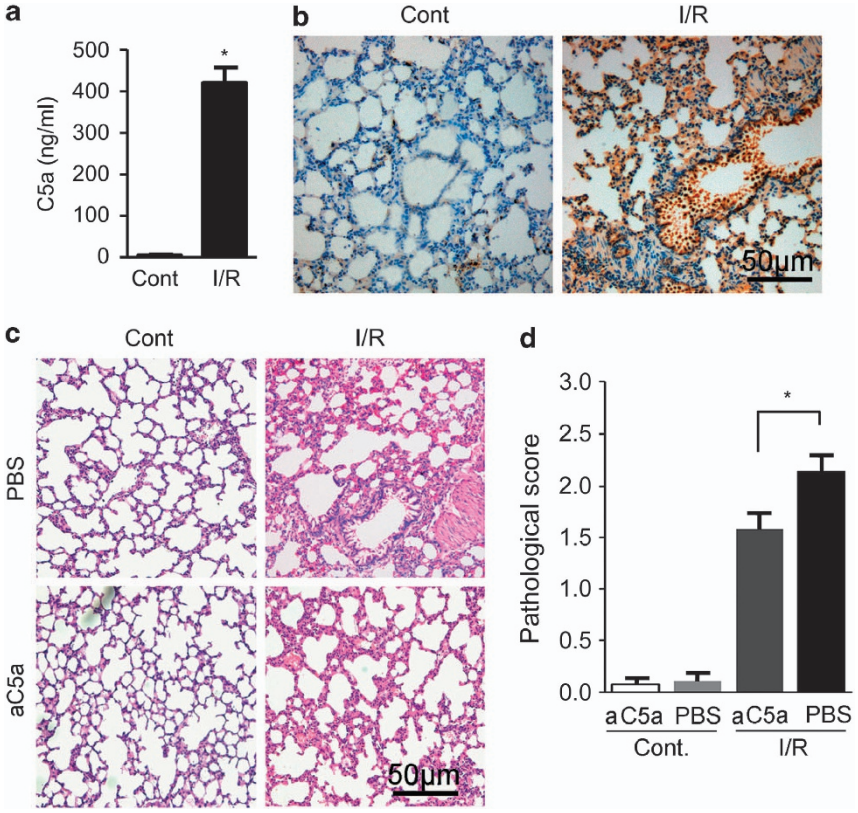

Figure 2 Inhibition of complement C5a decreases IR-induced lung injury. (**a**) Eight-week-old C57BL/6 male mice (six mice per group) were subjected to intestinal ischemia/reperfusion and BAL fluid was collected 45 min later. C5a was measured by ELISA. Data are from three independent experiments. (**b**) Lung samples fixed in 10% formalin and embedded in paraffin. Tissue blocks were sectioned at 5 μm for immunohistochemistry with C5a antibody. Morphologic examinations were by light microscopy. Data are representative of three experiments. (**c**) Before intestinal ischemia/reperfusion, C5a antibody (10 μg per mouse) was administrated intravenously. Lung samples were fixed and embedded in paraffin. Tissue blocks were sectioned at 5 μm and stained with hematoxylin/eosin. Morphologic examinations were by light microscopy. (**d**) The sections were analyzed by an experienced pathologist and were given pathological scores. Data are the mean ± S.D. of three independent experiments. $^*P<0.05$

and MCP-1 were significantly higher in injured lung than in normal organs (Figure 3a). These factors attract neutrophil infiltration, which were activated and released more inflammatory factors. Alveolar macrophages are essential for maintaining pulmonary homeostasis, and in this study, we attempted to characterize lung macrophage status. To further explore changes in alveolar macrophages, we separated alveolar macrophages from mouse lungs. C5a promotes the activation and phagocytosis of macrophages, thus we assumed that C5a released in lungs activated the alveolar macrophages. We identified macrophage activation status by FACS after staining with CD80, CD11b, CD11c, F4/80, and MHC II, and our results showed that CD80, CD11b, CD11c, F4/80, and MHC II expression were upregulated (Figure 3b), confirming the activation status of alveolar macrophages in injured lungs. We also cultured alveolar macrophages from injured and normal lungs, and C5a stimulation strongly enhanced the production of proinflammatory cytokines and chemokines such as TNF-α, IL-6, and MCP-1 that were released from the activated macrophages (Figure 3c). These cytokines are either essential for attracting neutrophils directly or activate alveolar epithelial cells to express adhesion molecules that improve neutrophil infiltration. C5a-induced activation was pronounced in ALI, which could be a hallmark of the disease process. In order to further confirm the essential role of C5a in ALI development, we constructed the IR-induced ALI model with C5aR$^{-/-}$ mice, and TNF-α, IL-6, and MCP-1 were detected in BALF from control and ALI mice. Our data demonstrated that there were no significant differences for IL-6 and MCP-1. As for TNF-α was increased in ALI mice, and was approximately 10-fold higher than in control mice (Figure 3a, lower panel),

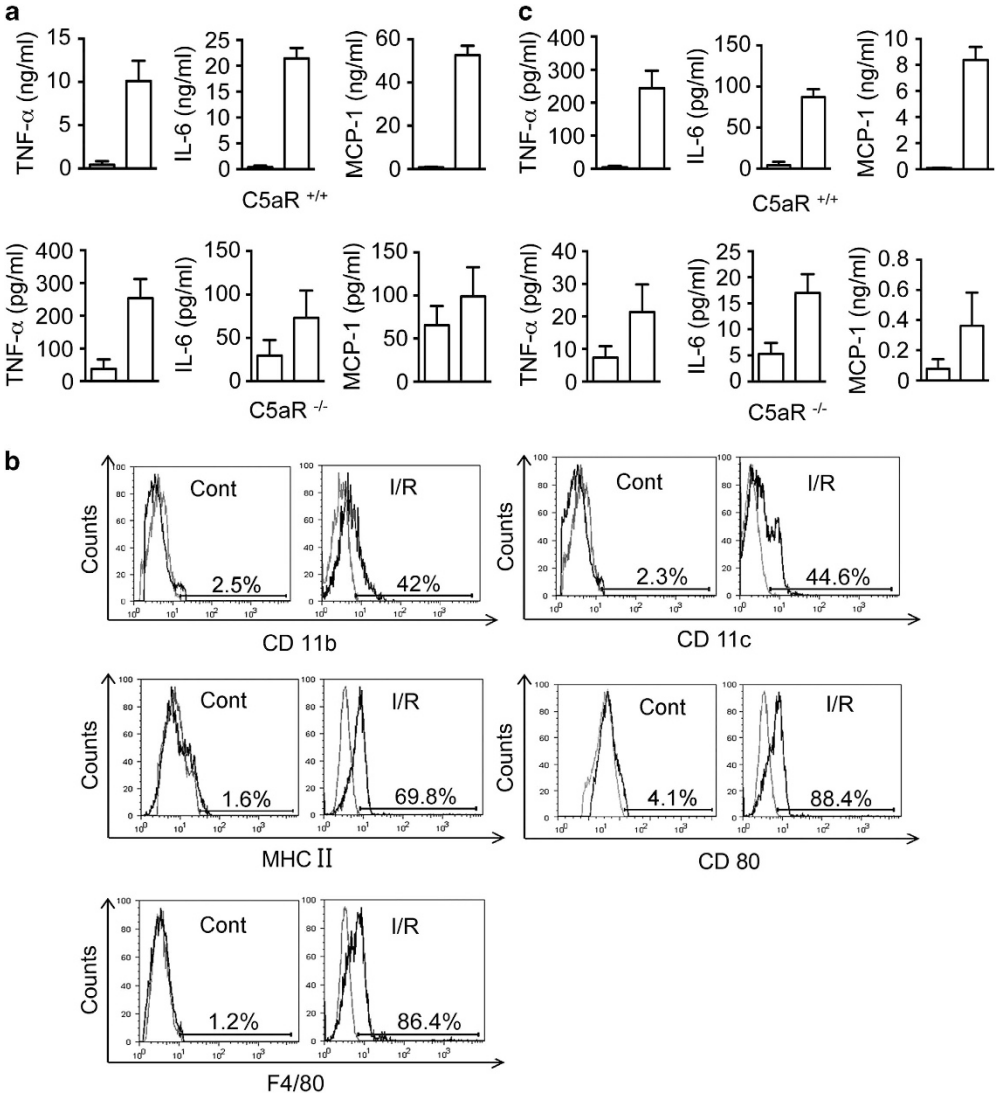

Figure 3 C5a production induces alveolar macrophage activation in acute lung injury. (**a**) Eight-week-old C57BL/6 male mice or C5aR$^{-/-}$ male mice (six mice per group) were subjected to intestinal ischemia/reperfusion and BAL fluid was collected 45 min later. TNF-α, IL-6, and MCP-1 were measured by ELISA. Data are from three independent experiments. (**b**) Alveolar macrophages prepared as described in Materials and Methods were stained with F4/80, CD11b, or CD80, and subjected to FACS analysis. (**c**) Alveolar macrophages were cultured, and supernatants were collected after 12 h. TNF-α, IL-6, and MCP-1 were measured by ELISA. Data are the mean $\pm$ S.D. of three independent experiments. $^{*}P<0.05$

and was more than 40-fold higher than that in wild-type mice. In addition, TNF-α, IL-6, and MCP-1 from alveolar macrophages of C5aR$^{-/-}$ mice showed no significant differences between control and ALI mice (Figure 3c, lower panel).

C5a induced autophagy in alveolar macrophages. As mentioned above, C5a promotes the activation of alveolar macrophages and the production of inflammatory factors. Autophagy has been shown to have a pivotal role in the regulation of cytokine production in macrophages, therefore we then focused our attention on the function of C5a in regulating autophagy of alveolar macrophages. Autophagy induction was assessed by monitoring LC3. In resting cells, LC3 is in an 18-kDa cytosolic LC3-I form. Following cell activation to induce autophagy, some LC3 is lipidated to LC3-II, which migrates as a 16-kDa protein as shown by SDS-PAGE. Alveolar macrophage lysates were analyzed for LC3-II by immunoblotting. LC3-II levels in alveolar macrophages from mice with ALI were significantly increased compared with control mice (Figure 4a). Alveolar macrophages cultured *in vitro* were stimulated with C5a, and then LC3 was analyzed by immunoblotting. Our data demonstrated enhanced autophagy in macrophages after stimulation with C5a (Figure 4b). When autophagosomes were formed, LC3-II was recruited from the cytosol to autophagosome membranes, which were seen as punctate dots by confocal microscopy. LC3-positive cells with punctate dots were counted to quantitate autophagy induction. Consistent with the immunoblot results (Figure 4a), LC-staining of frozen sections from different groups of mice models showed marked increases in autophagy in the lungs of ALI mice, whereas the administration of C5a antibody significantly inhibited the induction of autophagy in ALI mice (Figure 4c). Taken together, our results demonstrated that the presence of C5a promoted autophagy in alveolar macrophages.

Inhibition of autophagy decreases ALI. C5a-mediated autophagy in alveolar macrophages potentially contributed to ALI development, therefore in order to clarify whether this is the case *in vivo*, we established mice with Atg5-deficiency in macrophages (Mϕ-ATG5$^{-/-}$ mice). Atg5 is an indispensable regulator in the autophagy elongation stage, and loss of atg5 results in no cell of autophagy.[26,27] We used Mϕ-ATG5$^{-/-}$ mice to further investigate the role of alveolar macrophage autophagy in ALI. First, we demonstrated that atg5 expression was not detected in alveolar macrophages from Mϕ-ATG5$^{-/-}$ mice, and as expected, there was no visible autophagosome formation (Figure 5a). In addition, the inflammatory cytokine production in BALF was decreased, and lung injury was significantly alleviated in Mϕ-ATG5$^{-/-}$

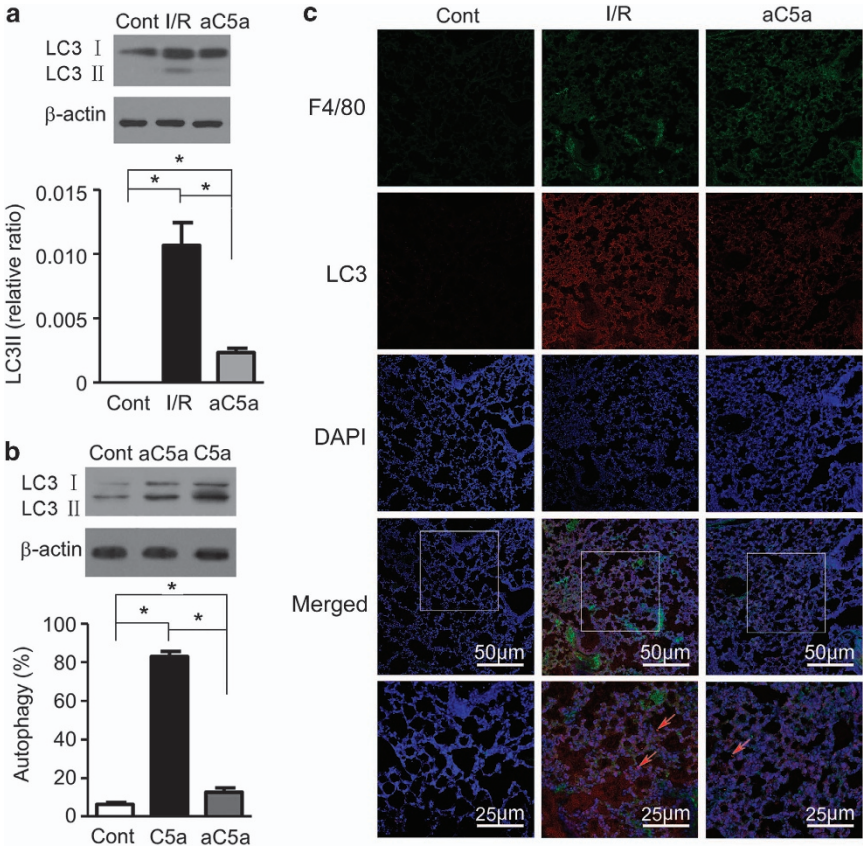

Figure 4 Autophagy increases in alveolar macrophages in acute lung injury. (**a**) Alveolar macrophages from normal mice, I/R-induced ALI mice, and ALI mice with C5a antibody (10 μg per mouse) administered intravenously were isolated and lysed. Lysates were analyzed by immunoblotting with LC3 antibodies for conversion of LC3-I to LC3-II. Band intensity was determined with ImageJ. (**b**) Alveolar macrophages from normal mice were treated as indicated and lysed. Lysates were analyzed by immunoblotting with LC3 antibodies for conversion of LC3-I to LC3-II. Band intensity was determined with ImageJ. (**c**) Frozen lung tissue sections stained with Alexa 488-F4/80, Alexa-594-LC3, and nucleus counterstained with DAPI. Data are the mean ± S.D. of three independent experiments. *$P<0.05$

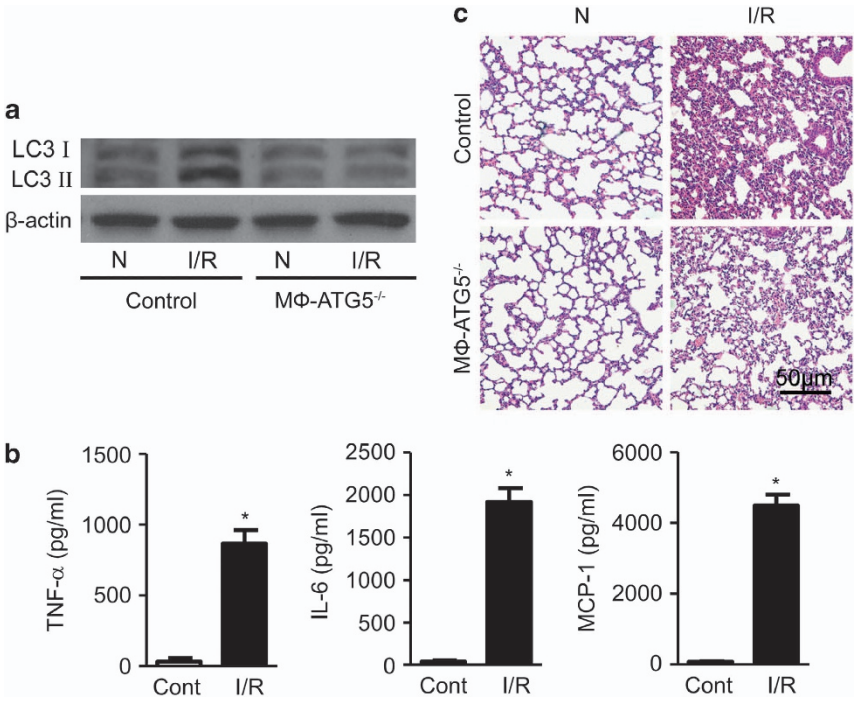

Figure 5 Inhibition of autophagy in alveolar macrophages decreases tissue injury in ALI mice. (**a**) ATG5$^{flox/flox}$ mice and Mϕ-ATG5$^{-/-}$ mice were used to establish the I/R-induced ALI model. Alveolar macrophages were isolated and lysed. Lysates were analyzed by immunoblotting with LC3 antibody. (**b**) BAL fluid was collected from ATG5$^{flox/flox}$ and Mϕ-ATG5$^{-/-}$ ALI mice. TNF-α, IL-6, and MCP-1 were measured by ELISA. (**c**) Lung samples were fixed and embedded in paraffin. Tissue blocks were sectioned at 5 μm and stained with hematoxylin/eosin. Morphologic examinations were by light microscopy. Quantitative data are the mean ± S.D. of three independent experiments. *$P < 0.05$

mice (Figures 5b and c), confirming the contribution of autophagy in alveolar macrophages to lung injury.

Alveolar macrophage apoptosis decreases after autophagy inhibition. Autophagy is an autonomous means of homeostasis, and has been shown to counter the induction of apoptosis, thus protecting the cell from death. However, autophagy could also cause cell death in harsh settings. To elucidate the role of autophagy in alveolar macrophage apoptosis in the ALI setting, we determined the apoptosis of alveolar macrophages from ALI mice compared with controls and Mϕ-ATG$^{-/-}$ mice. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to detect apoptosis, and the results indicated that apoptosis was strongly suppressed in Mϕ-ATG$^{-/-}$ mice (Figure 6a). We also used an alternative method, by administering 3-methyl adenine (3-MA), an autophagy inhibitor, via the tail vein in mice, and our data showed that alveolar macrophage autophagy was decreased, as evidenced by the quantitation of punctuate dots using confocal microscopy and by LC3 immunoblotting (Figures 6b and c). Accordingly, the apoptosis was inhibited to a similar degree. Taken together, these results demonstrated that autophagy in alveolar macrophages contributes to ALI by inducing apoptosis.

C5a interacting with C5aR induced Bcl-2 degradation. C5aR is expressed in multiple cell types, and binding with C5a initiates the downstream signaling cascades. Under pathological conditions, C5aR is upregulated. Our data showed that C5aR was markedly upregulated in alveolar macrophages in ALI mice (Figure 7a). Alveolar macrophages from ARDS patients, a serious ALI condition, also showed increased autophagy, and higher expression of C5aR (Figure 7b). When mice were pretreated with C5aR antibody, autophagy was decreased in alveolar macrophages as seen in lung frozen sections from ALI mice (Figure 7c). *In vitro* culturing of alveolar macrophages showed that after C5a treatment, autophagy increased significantly, whereas C5a antibody and C5aR antibody reversed this increase in autophagy (Figure 7d). Beclin 1, combined with vps15 and vps34, comprise the phosphoinositide 3 kinase complex, and have an essential role in autophagy. To further investigate the mechanism of C5aR in the regulation of autophagy, we explored Beclin 1 expression and complex I formation in alveolar macrophages, and our results showed no change in Beclin 1 expression. As Bcl-2 associates with Beclin 1 through the BH3 domain to inhibit induction of autophagy, we also detected Bcl-2 expression, and found that C5aR binding of C5a led to Bcl-2 degradation, which released the inhibition of autophagosome formation (Figure 7e). Collectively, our results showed that C5a interacted with C5aR and induced autophagy by promoting degradation of Bcl-2 from Beclin 1, thus contributing to autophagy and subsequent apoptosis of alveolar macrophages.

Discussion

Intestinal IR induces pulmonary morphological pathology and elevated local cytokine production consistent with ALI.[15,28]

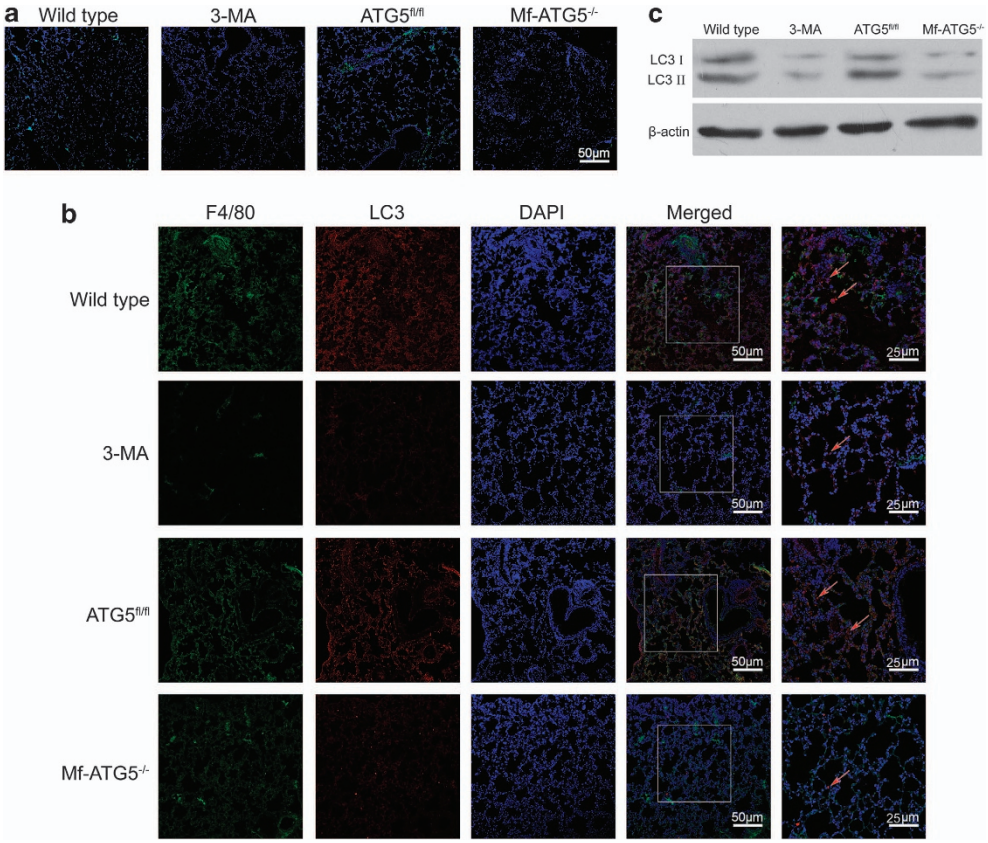

Figure 6 Apoptosis of alveolar macrophages decreases after inhibition of autophagy. (**a**) Wild-type mice were administered 3-MA (10 mM) one day before intestinal ischemia/reperfusion, and $ATG5^{flox/flox}$ mice as well as $M\phi\text{-}ATG5^{-/-}$ mice were used to establish the I/R-induced ALI model. Frozen lung tissue sections were generated for TUNEL analysis, and nucleus counterstained with DAPI. (**b**) Frozen lung tissue sections were stained with Alexa 488-F4/80, Alexa-594-LC3, and nucleus counterstained with DAPI. (**c**) Alveolar macrophages were isolated and lysed. Lysates were analyzed by immunoblotting with LC3 antibody. Quantitative data are the mean ± S.D. of three independent experiments. $^*P < 0.05$

During the disease progression, complement is activated producing complement components such as C3a and C5a.[20,29,30] Complement cascades are necessary for the initiation and amplification of tissue injury.[7,21,31] Our study showed that C5aR expression was upregulated in alveolar macrophages after ALI induced by intestinal IR. Binding of C5a induced autophagy and subsequent apoptosis in alveolar macrophages, contributing to pulmonary insult in ALI.

The complement system is a key in innate immunity, but overwhelming activation of complement can lead to severe tissue injury.[1] Complement is involved in diverse processes, such as angiogenesis, inflammation, tissue injury, and regeneration.[21] Three distinct pathways initiate the complement cascade, and all lead to the activation of C3, which is essential for the production of C5a in animal models of reperfusion injury. C5a is required for neutrophil infiltration, in addition to the full development of IR-induced injury in an animal model of lung injury. C5a directly activates neutrophils and macrophages for proinflammatory and chemokine production,[20,22,32] but the effects of C5a on alveolar macrophages remain to be clarified. In this study, we found that in ALI, C5a activated alveolar macrophages, based on increased expression of MHC class II molecules and CD11b expression in alveolar macrophages, suggesting the sensitive state of alveolar macrophages in pathological conditions.

Autophagy is a constitutive regulatory means of cellular homeostasis involved in diverse physiological and pathological events.[33–38] Autophagy in macrophages is important for maturation, activation, polarization, and regulation of cytokine production.[39–43] Macrophages express various complement receptors that bind pathogens opsonized with complement components. Our data indicated that under inflammatory conditions, C5aR was upregulated in alveolar macrophages, which could increase phagocytosis capabilities. Binding of C5a initiated Bcl-2 degradation, which released the inhibition of Beclin 1, a key regulator in autophagy. This process contributed to autophagy in alveolar macrophages. C5a also interacts with the C5L2 receptor to function,[44] and our data did not exclude that possibility; however, C5aR may be more important in this biological event, as blocking C5aR with blocking antibody almost prevented the C5a activities completely.

Of the multiple connections between apoptosis and autophagy, some are paradoxical; the two phenomena jointly determine cell fate.[45,46] Autophagy protects against cell death during starvation, growth factor withdrawal, and neurodegeneration, but is also a critical contributing factor for certain types of cell death.[47,48] Crossregulation between the evolutionarily conserved apoptosis and autophagy is complex. In ALI, C5aR-mediated autophagy induction of alveolar macrophage apoptosis could be a strategy to prevent an exaggerated

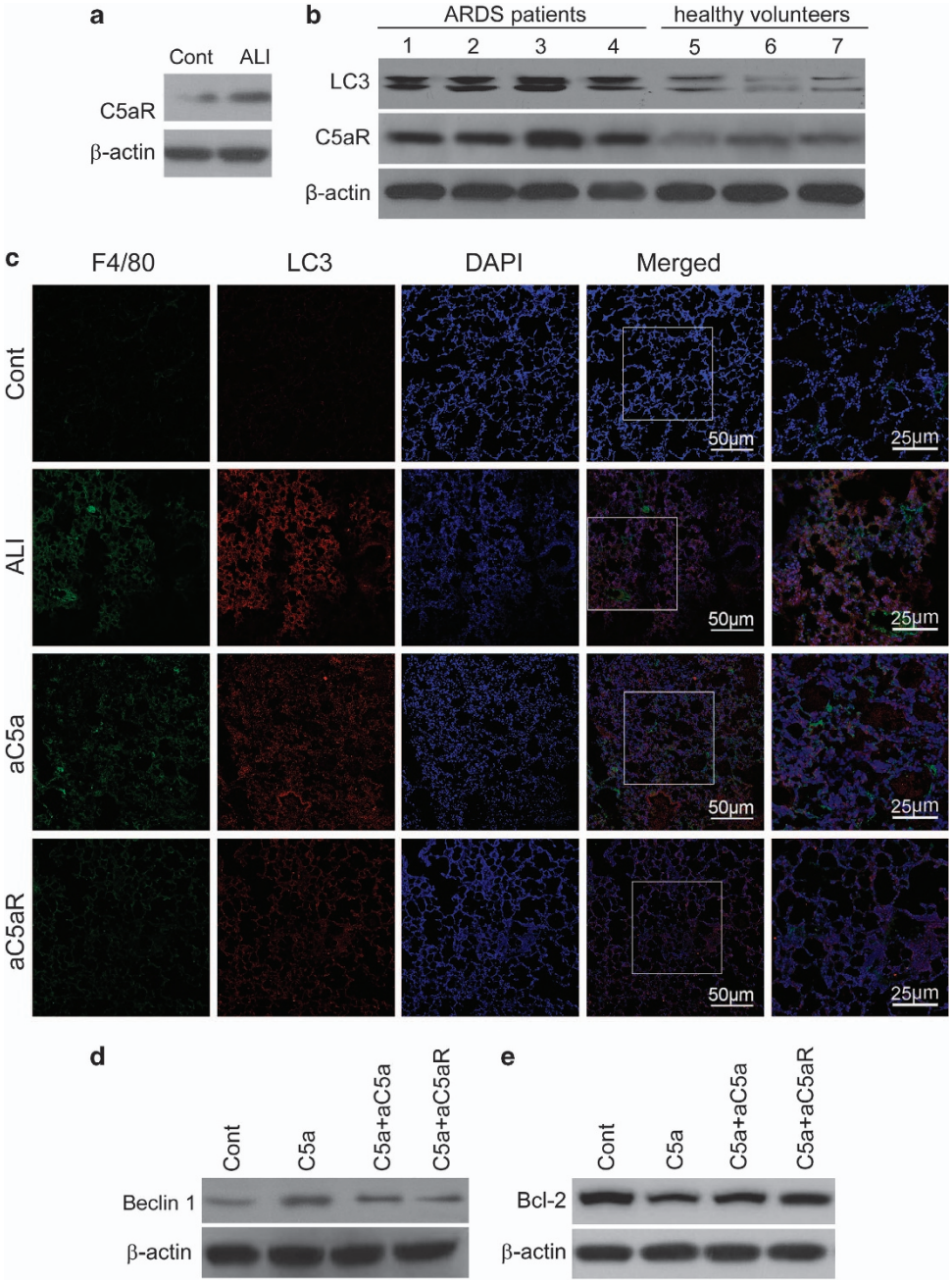

Figure 7 C5a induces alveolar macrophage autophagy through Bcl-2 degradation. (**a**) Alveolar macrophages were isolated from normal or ALI mice and lysed. Lysates were analyzed by immunoblotting with LC3 antibody. (**b**) Alveolar macrophages were isolated from ARDS patients and volunteers and lysed. Lysates were analyzed by immunoblotting with LC3 antibody and C5aR antibody. (**c**) Normal mice, ALI mice, and ALI mice pretreated intravenously with C5a antibody or C5aR antibodies were killed and frozen lung tissue sections were obtained and stained with Alexa 488-F4/80, Alexa-594-LC3, and nucleus counterstained with DAPI. (**d**) Alveolar macrophages were isolated from mice and lysates were analyzed by immunoblotting with Beclin 1 antibody. (**e**) Alveolar macrophages were isolated from mice and lysates were analyzed by immunoblotting with Bcl-2 antibody. Quantitative data are the mean ± S.D. of three independent experiments. $^{*}P<0.05$

proinflammatory presence that might occur during the death of other cells that release proinflammatory cytokines. However, apoptosis of alveolar macrophages affected lung homeostasis, making the lungs susceptible to severe inflammation. We found that alveolar macrophage apoptosis was largely blocked when autophagy was inhibited following 3-MA administration. In order to further explore the role of autophagy in alveolar macrophages, we bred Mϕ-ATG5$^{-/-}$ mice, in which atg5 is deficient specifically in macrophages. Using these mice in the ALI model, we highlighted the contribution of C5a-induced autophagy in lung injury, which provide an alternative method of alleviating ALI symptoms through inhibition of autophagy in alveolar macrophages.

In summary, our results shed light on the role of C5a-mediated alveolar macrophage autophagy inducing apoptosis, and identified potential therapeutic targets for ALI.

Materials and Methods

Reagents and antibodies. All antibodies for flow cytometry analysis were obtained from BioLegend (San Diego, CA, USA) unless otherwise specified. Conjugated antibodies FITC-F4/80, FITC-LC3, and PE-LC3 were purchased from Novus Biologicals (Littleton, CO, USA). LC3 and Beclin 1 antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). The anti-mouse C5aR antibody was obtained from OriGene (Rockville, MD, USA). F4/80 and LC3 were labeled with Alexa 488 and 594 (Molecular Probes, Eugene, OR, USA), respectively, according to the manufacturer's instructions. ELISA kits for C5a, TNF-α, IL-6, and MCP-1 were purchased from R&D System (Minneapolis, MN, USA).

Experimental model. Eight-week-old C57BL/6 male mice were used for intestinal IR. The animals were weighed and anesthetized intraperitoneally with 0.1 mg/g mouse weight of ketamine and 0.01 mg/g mouse weight of xylazine. IR gut injury results in severe inflammation, apoptosis, and remote organ damage, including ALI. A medial laparotomy was performed, and the intestines were carefully moved, allowing access to the superior mesenteric artery. The superior mesenteric artery was clamped using a vascular clamp (Fine Surgical Instruments Inc., Hempstead, NY, USA). The vascular clamp was released after 90 min to allow reperfusion.

ATG5$^{flox/flox}$ mice were established and bred by Usun Biotech (Shanghai, China), and mice expressing *Cre* recombinase, under the control of the macrophage-specific lysozyme M promoter (Lyz-Cre mice), were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). ATG5$^{flox/flox}$ mice were bred with Lyz-Cre mice to achieve macrophage ATG5-deficient mice (Mϕ-ATG5$^{-/-}$ mice). ATG5$^{flox/flox}$ mice were used as controls paralleled with Mϕ-ATG5$^{-/-}$ mice. C5aR$^{-/-}$ mice were purchased from the Jackson Laboratory. All mice were maintained in specific pathogen-free conditions. All experiments were conducted in accordance with the guidelines, and under approval from the Animal Care and Usage Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine.

Histopathology. Lung samples were fixed in 10% formalin and embedded in paraffin. Tissue blocks were sectioned to 5 μm, transferred to glass slides, and stained with hematoxylin/eosin. Morphologic examinations were performed using light microscopy, and lung injury was analyzed by an experienced investigator blinded for absent, mild, moderate, or severe injury (score 0–3) based on the presence of exudates, hyperemia and congestion, neutrophilic infiltrates, intra-alveolar hemorrhage and debris, and cellular hyperplasia.

Cell culture and transfection. RAW 264.7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. RAW 264.7 cells were transfected with TransIT-Jurkat (Mirus Bio, Madison, WI, USA), according to the manufacturer's instructions.

Determination of C5a in murine bronchoalveolar lavage (BAL) fluid by ELISA. C5a in murine BAL was determined by sandwich ELISA (R&D Systems) according to the manufacturer's instructions. Recombinant murine C5a (R&D Systems) was used as a protein standard.

Immunoblotting. Cells with or without treatment were collected and lysed in lysis buffer RIPA (Beyondtime, Shanghai, China). After brief vortexing and rotation, cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% fat-free milk in PBS for 30 min and incubated with appropriate antibody in PBS with 0.5% fat-free milk for 2 h. After washing in PBST, membranes were incubated for 1 h with HRP-conjugated secondary antibody. Bands were detected with ECL plus detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Fluorescence confocal microscopy. Cells grown on coverslips were fixed in 4% paraformaldehyde at room temperature for 15 min. Coverslips were incubated with the indicated primary and conjugated secondary antibodies. For fluorescence analysis, cells were visualized on an Olympus Fluoview confocal microscope with appropriate emission filters (Olympus, Tokyo, Japan).

Autophagy analyses. Autophagy was analyzed by immunoblotting or fluorescence microscopy, as described previously.[49] Briefly, cell lysates were immunoblotted with anti-LC3 antibody to monitor LC3-II generated during the formation of autophagosomes.

Apoptosis assay. Tissue samples were dewaxed, incubated with proteinase K, and stained using a TdT *In Situ* Apoptosis Detection Kit-DAB TUNEL-based Apoptosis Detection Assay (R&D Systems) according to the manufacturer's instructions.

Pulmonary leukocyte isolation. Animals were euthanized by approved protocols, and cells from individual mice were collected for flow cytometry. BAL was collected, and cells were dispersed by repetitive suction through a 10-ml syringe and centrifuged at 1100 r.p.m. for 10 min. Pellets were resuspended in 1 ml sterile ddH_2O to lyse red blood cells, and recentrifuged. Pellets were resuspended in 5 ml of complete medium or PBS. In addition, we obtained BALF macrophages from four ARDS patients and three healthy volunteers. Written informed consent documents were signed by the patients. The research was approved by the Ethics Committee of Shanghai Ninth People's Hospital affiliated to Shanghai Jiaotong University School of Medicine.

Flow cytometric analysis. Both BAL cells and lung leukocytes were assessed using flow cytometric analysis. BAL cells (50 000 cells) in 100 μl flow assay buffer were incubated with FITC-conjugated goat anti-rabbit IgG or stained with the other indicated antibodies (BioLegend). Cells were washed again, resuspended in 3% paraformaldehyde and analyzed using a FACS Caliber flow cytometer (BD Bioscience, Mount View, CA, USA).

Statistics. The two-tailed Student's *t*-test or one-way ANOVA were used for statistical analyses. A P value less than 0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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