Lung Natural Killer Cells Play a Major Counter-Regulatory Role in Pulmonary Vascular Hyperpermeability After Myocardial Infarction

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Rationale: Natural killer (NK) cells are lymphocytes of the innate immune system that play specialized and niche-specific roles in distinct organs.

Objective: We investigated the possible function of NK cells in the pathogenesis of congestive heart failure after myocardial infarction.

Methods and Results: Depletion of NK cells from mice had little effect on cytokine expression (tumor necrosis factor-α, interleukin [IL]-6, and IL-1β), neutrophil and macrophage infiltration into infarcted myocardium, or left ventricular remodeling after myocardial infarction. However, these mice exhibited severe respiratory distress associated with protein-rich, high-permeability alveolar edema accompanied by neutrophil infiltration. In addition, there were 20-fold more NK cells in the mouse lungs than in heart, and these cells were accumulated around the vasculature. CD107a-positive and interferon-γ-positive cell populations were unchanged, whereas IL-10–positive populations increased. Adoptive transfer of NK cells from wild-type mice, but not from IL-10 knockout mice, into the NK cell–depleted mice rescued the respiratory phenotype. IL-1β–mediated dextran leakage from a lung endothelial cell monolayer was also blocked by coculture with NK cells from wild-type mice but not from IL-10 knockout mice.

Conclusions: This study is the first to identify a critical role for lung NK cells in protecting lung from the development of cardiogenic pulmonary edema after myocardial infarction. (Circ Res. 2014;114:637-649.)

Key Words: adaptive immunity | inflammation | interleukin-10 | killer cells, natural | myocardial infarction

Left ventricular (LV) remodeling after myocardial infarction (MI) results in LV dilation, a major cause of congestive heart failure and sudden cardiac death. MI causes sterile inflammation, which is characterized by the recruitment and activation of immune cells of the innate and adaptive immune systems.1,3 These cells are likely to have cell type–specific functions in the time-course after MI that involves clearance of dead tissues, the reparative response, and adverse remodeling.1,3 Disruption of myeloperoxidase (MPO), released predominantly by neutrophils, decreases leukocyte infiltration and LV dilation, enhances ventricular function, and delays early death attributable to myocardial rupture,4 whereas macrophage depletion impairs wound healing and increases LV remodeling after MI.5,13 Dendritic cells are a potent immunoprotective regulator during the postinfarction healing process via their control of monocyte/macrophage homeostasis.6 Treg cells serve to protect against adverse ventricular remodeling and contribute to improved cardiac function after MI via inhibition of inflammation and direct protection of cardiomyocytes.7 γδT cells are the major source of interleukin (IL)-17 in the infarcted myocardium and function specifically in the late remodeling stages by promoting sustained infiltration of neutrophils and macrophages, stimulating macrophages to produce proinflammatory cytokines, aggravating cardiomyocyte death, and enhancing fibroblast proliferation and profibrotic gene expression via IL-17 production.3

Natural killer (NK) cells (belonging to the group of innate lymphoid cells) are defined as large granular lymphocytes and constitute the third kind of cells differentiated from the common lymphoid progenitor generating B and T cells.8 NK cells differ from NK T cells (NKT) phenotypically, by origin, and

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Nonstandard Abbreviations and Acronyms:

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage (BAL) fluid</td>
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<tr>
<td>CXCL-1</td>
<td>Chemokine (C-X-C motif) ligand 1</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria toxin</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage-associated molecular patterns</td>
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<tr>
<td>+dP/dt</td>
<td>Maximum rate of isovolumic pressure development</td>
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<tr>
<td>-dP/dt</td>
<td>Minimum rate of isovolumic pressure decay</td>
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<tr>
<td>EC</td>
<td>Endothelial cell</td>
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<tr>
<td>FS</td>
<td>Fractional shortening</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IL-1R1</td>
<td>Interleukin-1 receptor 1</td>
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<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
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<tr>
<td>LVEDP</td>
<td>Left ventricular end-diastolic pressure</td>
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<tr>
<td>LVEDD</td>
<td>Left ventricular end-diastolic diameter</td>
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<tr>
<td>LVESP</td>
<td>Left ventricular end-systolic pressure</td>
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<tr>
<td>MI</td>
<td>Myocardial infarction</td>
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<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
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<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
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<tr>
<td>NKDTR/EGFP</td>
<td>Transgenic mice that express enhanced green fluorescent protein (EGFP) and diphtheria toxin (DT) receptor under a functional NKp46 promoter</td>
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by respective effector functions. NK cells are cytotoxic lymphocytes critical to the innate immune system that can differentiate and mature in the bone marrow, lymph node, spleen, tonsils, and thymus before entering the circulation. In contrast to NKT cells, NK cells do not express invariant T-cell antigen receptors, Pan T marker CD3, or surface immunoglobulin (Ig) B cell receptors, but they usually express the surface markers CD16 (FcyRIII) and CD56 in humans, and NK1.1 in mice. NK cells play a highly important role in the killing of tumors and cells infected by viruses, and can have specialized and niche-specific functions in distinct organs.

In the present study, we sought to examine the roles of cardiac NK cells in the pathogenesis of LV remodeling after MI. Unexpectedly, depletion of NK cells from mice had little effect on LV remodeling after MI; however, these mice exhibited severe respiratory distress associated with protein-rich, high-permeability alveolar edema accompanied by neutrophil infiltration. Thus, we examined a mechanism for lung NK cells in protecting lung from the development of cardiogenic pulmonary edema after MI.

**Methods**

An expanded Materials and Methods section is available in the online Data Supplement.

**Results**

**Genetic Ablation of NK Cells Exacerbated Cardiogenic Pulmonary Edema After MI**

We used a mouse model of nonreperfused MI to examine the possible role of NK cells in the pathogenesis of cardiac remodeling after MI. A detailed temporal analysis of cellular infiltrate dynamics in the post-MI heart revealed an initial increase in the number of NK cells on day 3 after MI, to a peak on day 7 (Online Figure I). In this study, we took advantage of the NKDTR/EGFP transgenic mice that express enhanced green fluorescent protein (EGFP) and the diphtheria toxin (DT) receptor under a functional NKp46 promoter.9 The complete and selective ablation of NK cells from the peripheral blood and lungs of NKDTR/EGFP transgenic mice after DT treatment is shown in Online Figure IIA and IIB. Our previous studies have already proved that DT itself has no discernible cardiotoxic effects.6

There was no difference in LV fractional shortening (Figure 1A), LV end-diastolic diameter (Figure 1B), gross morphology of the hearts (Figure 1C), infarct size (Figure 1D), or hemodynamic parameters (heart rate, LV end-diastolic pressure) between the groups. However, NK cell depletion led to a significant increase in LV end-diastolic pressure (LVEDP) and LV end-systolic pressure (LVESP) (Figure 1B and D). The echocardiographic analysis of fractional shortening (FS) and left ventricular end-diastolic diameter (LVEDD) at day 7 after MI or sham operation (n=9–11) is shown. C, Azan staining of cardiac sections at day 7 after MI. Scale bar: 500 μm. D, Quantification of infarct size in the Azan-stained sections (n=9-11). NS indicates not significant. Statistical analysis was performed using Kruskal–Wallis tests followed by Bonferroni post hoc analysis.

![Figure 1](http://circres.ahajournals.org/). Ablation of NK cells did not affect cardiac function or infarct size. NK cells were depleted by diphtheria toxin (DT) injection 1 day before surgery to induce myocardial infarction (MI) in the transgenic mice that express enhanced green fluorescent protein (EGFP) and the diphtheria toxin (DT) receptor under a functional NKp46 promoter (NKDTR/EGFP) mice. NK cells were then purified from wild-type and IL-10 knockout mice and transferred into the NKDTR/EGFP mice injected with DT. The echocardiographic analysis of fractional shortening (FS; A) and left ventricular end-diastolic diameter (LVEDD; B) at day 7 after the MI or sham operation (n=9–11) is shown.

**Figure 1.** Ablation of NK cells did not affect cardiac function or infarct size. NK cells were depleted by diphtheria toxin (DT) injection 1 day before surgery to induce myocardial infarction (MI) in the transgenic mice that express enhanced green fluorescent protein (EGFP) and the diphtheria toxin (DT) receptor under a functional NKp46 promoter (NKDTR/EGFP) mice. NK cells were then purified from wild-type and IL-10 knockout mice and transferred into the NKDTR/EGFP mice injected with DT. The echocardiographic analysis of fractional shortening (FS; A) and left ventricular end-diastolic diameter (LVEDD; B) at day 7 after the MI or sham operation (n=9–11) is shown.
Figure 2. Myocardial infarction (MI) induces pulmonary vascular hyperpermeability and neutrophil infiltration. Mice were subject to MI and euthanized at the indicated time points. **A**, The bronchoalveolar lavage (BAL) fluid (BALF) albumin, **B** total protein levels, and **C** total leukocyte number recovered from BALF were quantified at the indicated time points after MI. **D**, Flow cytometry analysis of alveolar macrophages (AM), dendritic cells (DC), CD11b+Gr-1+ neutrophils, and CD11b+Gr-1Int myeloid cells (Gr-1Int myeloid cells) in total lung tissue at the indicated time points after MI (n=4–6). **E**, Myeloperoxidase activity in lung tissue of C57BL6 mice (n=4–7). **F**, Left, BAL fluid from sham operation and MI at day 3 after MI. Red color indicates that red blood cells existed in the BALF. Right, Cytospin preparation of BALF from sham operation and MI. Scale bar: 20 μm. *P<0.05; **P<0.01 vs Sham group. Data in (A–E) were analyzed by Kruskal–Wallis tests with Dunn multiple comparison.
Figure 3.
Next, we examined the impact of NK-cell depletion on post-MI inflammation (Online Figure III). The expression of cytokines, tumor necrosis factor-α, IL-6, and IL-1β in the infarcted myocardium was comparable between the PBS- and DT-treated NKDTR/EGFP-transgenic mice, whereas IFN-γ expression was less in the DT-treated NKDTR/EGFP-transgenic mice than in the PBS-treated controls. Numbers of infiltrating CD45+ leukocytes, CD45+CD11b+ myeloid cells, CD45+CD11b+ F4/80+ macrophages, and Ly-6G+ myeloid cells (neutrophils) were comparable between the 2 mice groups.

A large MI causes an elevation in LVEDP. Here, although the change in LVEDP was comparable between the PBS-treated NKDTR/EGFP-transgenic mice (13.3±5.1 mm Hg, P<0.05 versus baseline) and DT-treated NKDTR/EGFP-transgenic mice (14.1±4.6 mm Hg, P<0.05 versus baseline), arterial oxygen desaturation was significantly more prominent in the latter group (Online Table I). In fact, DT-treated NKDTR/EGFP-transgenic mice exhibited respiratory distress during the early phase after MI (Online Movie). In turn, adoptive transfer of NK cells rescued the arterial oxygen desaturation in DT-treated NKDTR/EGFP-transgenic mice. These results tempted us to speculate that lung NK cells play a protective role against cardiogenic pulmonary edema.

Development of Protein-Rich Alveolar Edema After MI

Then, we examined the quantitative time-course change in the profiles of bronchoalveolar lavage (BAL) fluid after MI. Pulmonary edema occurring because of an imbalance in Starling hydrostatic forces is theoretically accompanied by low-protein concentration fluid accumulation in the lung. However, BAL albumin (Figure 2A) and protein (Figure 2B) levels were elevated, peaking at day 7 post-MI, whereas BAL cell number also progressively increased to a maximum value at day 7 post-MI (Figure 2C). Flow cytometric analysis of immune cells in the lung infiltrate (Online Figure IV) revealed a >2-fold increase in neutrophils by as early as 1 day post-MI that was sustained until day 7 after MI, whereas there was only a modest increase in alveolar macrophages (Figure 2D), and the number of dendritic cells gradually increased to a maximum value at day 14 post-MI. The neutrophil accumulation was associated with increased MPO activity in the bronchoalveolar lavage (BAL) fluid (Figure 2E), and polymorphonuclear neutrophils were conspicuous by light microscopy in the erythrocyte-containing BAL fluid obtained from mice at day 3 post-MI compared with day 3 postsham operation (Figure 2F). These results suggested that impaired gas exchange post-MI is not solely attributable to hemodynamic changes but is in part attributable to neutrophil infiltration of the lung that induces an inflammatory response and, hence, increased endothelial-alveolar permeability.

IL-1β Mediates Neutrophil Infiltration Into the Alveolar Space After MI

Next, we examined factors responsible for the substantial recruitment of blood neutrophils into the alveolar space. Previous studies implicated neutrophil adhesion to vascular endothelial cells in the pathogenesis of pulmonary vascular hyperpermeability and neutrophil infiltration, mediated via the interaction of CD11/CD18 and intercellular adhesion molecule-1 (ICAM-1), followed by the transmigration of neutrophils into alveolar spaces along the CXCL-1 chemotactic gradient. The IL-6–triggered positive feedback loop for nuclear factor-κB signaling would then act to amplify the inflammatory response.

The pulmonary circulation is continuously exposed to circulating damage-associated molecular pattern molecules released from injured myocardium and capable of triggering innate immunity. Therefore, we tested the involvement of damage-associated molecular pattern molecules in our system. Lung endothelial cells (lung-ECs) were treated with tissue homogenate prepared from sham-operated or MI hearts. Tissue homogenate from sham-operated hearts did not increase the expression levels of IL-6, ICAM-1, or chemokine (C-X-C motif) ligand 1 (CXCL-1) in lung-ECs. By contrast, tissue homogenate from MI hearts increased the expression levels of these genes (Figure 3A–C). These results suggested the involvement of factors synthesized post-MI, rather than preexisting heart constituents, in inducing lung inflammation. We speculated the involvement of IL-1β, because IL-1β is the primary initiator of pulmonary inflammation after liver injury in mice. In addition, we and others reported high levels of circulating IL-1β produced and maintained by fibroblasts after cardiac injury that is then available to activate innate cells via the activation of Toll-like receptor signaling and the inflammasome. As expected, the tissue homogenate prepared from MI hearts induced an elevation of IL-6, ICAM-1, and CXCL-1 expression in lung-ECs that was almost completely blocked by anti–interleukin-1 receptor 1 (IL-1R1) antibody. Similarly, increased adhesion after pretreatment of the lung-ECs with the post-MI tissue homogenate was strongly blocked by...
IL-1R1 antibody (Figure 3D and 3E). IL-1β is not a direct chemoattractant for neutrophils, but it mediates neutrophil recruitment through the synthesis of IL-6, ICAM-1, and CXCL-1.14 We confirmed that IL-1β stimulated the expression of IL-6, ICAM-1, and CXCL-1 in lung-ECs (Figure 3F–H) and neutrophil adhesion to lung-ECs (Figure 3I and 3J), both of which were blocked by IL-1R1 antibody-mediated neutralization. The increase in lung IL-6, ICAM-1, CXCL-1 expression after MI was also blocked by IL-1R1 antibody, and IL-1β stimulated lung IL-6, ICAM-1, and CXCL-1 expression in the absence of MI (Figure 3K–M). IL-1R1 antibody-mediated neutralization further stimulated an increase in lung IL-10 expression after MI although IL-1β alone had no effect on lung IL-10 expression in the absence of MI (Figure 3N). Finally, the neutrophil accumulation in lung was attenuated by IL-1R1 antibody after MI and stimulated by IL-1β in the absence of MI (Figure 3O and 3P).

Inflammation in the lung after MI could be caused by either cardiac injury or an elevation in lung capillary pressure.15 To exclude the confounding effects of an elevated LVEDP (=lung capillary pressure) resulting from the induction of a large MI, we examined lung inflammation in a myocardial ischemia-reperfusion model, in which infarct size was small, and LVEDP was not significantly elevated2 (Online Figure V). Results with this model revealed that even a small MI induced neutrophil accumulation in the lung, suggesting that cardiac injury alone suffices to induce inflammatory responses in the lung after MI. We also examined the lung inflammation after transverse aortic constriction (TAC), which results in much less cardiac inflammation compared with the MI model, but LVEDP elevated to the same degree as with a large MI model (permanent ligation). We found that TAC also induced neutrophil accumulation in the lung, confirming that both cardiac injury and elevated lung capillary pressure contribute to the neutrophil accumulation in the lung and that both circulatory and locally produced IL-1β could act as the primary initiator of pulmonary inflammation after MI.

Characterization of Lung NK Cells

The number of leucocytes in lung tissues gradually increased after MI, reaching significance at day 14 (Figure 4A), whereas the number of NK cells (Figure 4B) and the component ratio of NK cells (Figure 4C) increased at day 4 post-MI, and peaked at days 7 to 14. The number of NKT cells did not change during the time-course of this study (Figure 4D). A comparison of NK-cell numbers between lung and heart tissues showed substantial overlap between NKp46-positive cells and NK1.1-positive cells among CD45+ CD3− cells in peripheral blood, spleen, and lung (Figure 4F). NK-cell maturation is a 4-stage process as follows: CD11b+CD27lo → CD11b+CD27hi → CD11b+CD27hi → CD11b+CD27hi. In this study, >80% of the lung NK cell population comprised CD11b+CD27hi cells, implying that lung NK cells maintain a terminally mature phenotype compared with spleen NK cells (Figure 4G). The remaining 20% of lung NK cells were positive for CD107a, a marker of cytotoxic activity (Figure 4H and 4I), and not >1% of NK cells were positive for IFN-γ (Figure 4J and 4K). Neither the maturation status of NK cells nor the populations of CD107a-positive and IFN-γ-positive NK cells were changed after MI in lung and spleen.

Genetic Ablation of NK-Cell Worsen High-Permeability Pulmonary Edema After MI

Next, we examined the role of lung NK cells in post-MI lung inflammation. Indexes of high-permeability pulmonary edema, BAL albumin (Figure 5A), BAL protein (Figure 5B), BAL cell number (Figure 5C), lung wet/dry weight (Figure 5D), the thickness of the alveolar-capillary wall in the lung (Figure 5E and 5F), lung MPO activities (Figure 5G), and BAL IL-6 levels (Figure 5H) deteriorated in DT-treated NKDTR/EGFP-transgenic mice compared with PBS-treated NKDTR/EGFP-transgenic mice. Adaptive transfer of NK cells from WT mice thus rescued the worsened post-MI lung inflammation observed for DT-treated NKDTR/EGFP-transgenic mice.

Antibody Depletion of NK-Cell Worsen High-Permeability Pulmonary Edema After MI

To confirm our initial observations, we used 2 different antibodies, anti-NK1.1 and anti-asialo GM1, to deplete NK cells in vivo selectively and conditionally (Online Figure IIC; Online Figure VIA and VIB). Depletion of NK cells using either of these antibodies had no obvious effects on functional and anatomic parameters in the heart at day 7 post-MI (Figure 6A–C; Online Figure VIC–E). However, all high-permeability pulmonary edema indices worsened after NK-cell depletion by either anti-NK1.1 or anti-asialo GM1 antibodies as follows: BAL albumin (Figure 6D; Online Figure VIF), BAL protein (Figure 6E; Online Figure VIG), BAL cell number (Figure 6F; Online Figure VIH), lung wet/dry weight (Figure 6G; Online Figure VII), lung MPO activities (Figure 6H; Online Figure VII), the thickness of the alveolar-capillary wall in the lung (Figure 6I and 6J; Online Figure VIK and VIL), and BALF IL-6 levels (Figure 6K; Online Figure VIM).

Mechanism Underlying the Protection of Alveolar-Capillary Wall by NK Cells

We investigated the underlying mechanism of alveolar-capillary wall protection by NK cells in the setting of MI using NKDTR/EGFP-transgenic mice. DT-mediated depletion of NK cells further increased the expression of IL-1β, IL-6, CXCL-1, and ICAM-1 in the lungs at day 3 and day 7 post-MI (Figure 7A–D). Interestingly, an increase in the expression of IL-10 in the lung after MI was blunted in DT-treated NKDTR/EGFP-transgenic mice (Figure 7E) to half the level in the lung of PBS-treated NKDTR/EGFP-transgenic mice after MI. Furthermore, neutrophil infiltration in the lung was exaggerated in DT-treated NKDTR/EGFP-transgenic mice compared with PBS-treated NKDTR/EGFP-transgenic mice after MI (Figure 7F and 7G). We also examined the localization of NK cells in the lung (Online Figure VII). In healthy mice, NK cells were not associated with pulmonary vasculature; however, after MI, NK-cell accumulation was apparent around the vasculature.

Based on the experiments thus far, we hypothesized that the alveolar-capillary barrier stabilization by NK cells was mediated through paracrine secretion of IL-10. To investigate
Figure 4. Characterization of lung natural killer cells. The absolute cell numbers of total lung CD45+ leukocytes (A) and NK cells (B), and the percentage of NK cells among total CD45+ leukocytes (C) and NKT cells (D) in the lung were quantified by flow cytometry analysis at the indicated time points after myocardial infarction (MI). *P<0.05; **P<0.01 vs. Sham group. Data in (A–D) were analyzed by Kruskal–Wallis tests with Dunn’s multiple comparison. E, The absolute cell numbers of NK cells in the heart and lung were determined by flow cytometry analysis at day 7 after MI (n=4–6). F, Flow cytometric measurement of NK1.1 and NKp46 on gated CD3− lymphocytes in the peripheral blood, spleen, and lung. G, Representative flow cytometry profiles of splenic and lung NK cells stained with anti-CD27 and anti-CD11b mAb from sham and MI mice. Experiments were performed 4 times. H, Representative flow cytometry analysis of CD107a expression levels in CD45+NKp46+CD3− NK cells from spleen and lung. I, Histograms representing the percentage of CD107a+ NK cells. Data were pooled from three independent experiments (n=3). J and K, Interferon-γ expression level in the NK cells from spleen and lung (n=3). NS indicates not significant; **P<0.01, ***P<0.001. Statistical analysis was performed by Mann–Whitney U tests (E, I, and K).
this, we examined IL-10 expression from the NK cells of IL-10–Venus reporter mice. Flow cytometry analysis revealed a higher proportion of IL-10–positive NK cells in lung than in spleen, and an increase in lung after MI from 9.8±0.6% in sham-operated lung to 17.6±0.8% in post-MI lung (Figure 8A and 8B). Consistent with this result, mRNA levels of IL-10 from sorted lung NK cells were significantly higher than those in splenic NK cells and mRNA levels of IL-10 from sorted lung and splenic NK cells increased in response to MI (Figure 8C). Immunohistochemistry analysis at day 7 post-MI clearly demonstrated that IL-10 is scarcely expressed on splenic NK cells but is frequently (≈50%) expressed on lung NK cells (Figure 8D and 8E).

Finally, we examined whether IL-10 produced by NK cells affects endothelial permeability. IL-10 suppressed both IL-1β–induced expression of IL-6, ICAM-1, and CXCL-1 in lung-ECs and IL-1β–induced neutrophil adhesion to lung-ECs (Online Figure VIIIA–E). Confluent monolayers of lung-ECs cultivated on Transwell filter inserts were treated with IL-1β, and the permeability of the monolayer to fluorescein isothiocyanate (FITC)-dextran was determined at several time points. IL-1β–mediated dextran leakage was blocked by IL-10 (Online Figure VIIIF). Next, NK cells sorted from wild-type and IL-10 knockout mice were added to the lower chamber. Notably, the IL-1β–mediated dextran leakage was blocked only by co-culture with NK cells sorted from wild-type mice and not by those cells sorted from IL-10 knockout mice (Figure 8F), confirming an effect on endothelial cell permeability.

Consistent with this, adoptive transfer of NK cells from IL-10 knockout mice failed to rescue the deteriorated lung...
Discussion

We dealt with an interesting concept, exploring an important and neglected topic: the pathogenesis of cardiogenic pulmonary edema after MI. Acute cardiogenic pulmonary edema after MI is thought to arise when abnormally high pulmonary capillary pressure induces the characteristic accumulation of low-protein fluid in the interstitial and alveolar spaces of the lung associated with this disorder. However, we found that nonreperfused murine MI causes protein-rich alveolar edema accompanied by neutrophil-predominant infiltration. Erythrocyte-containing BAL fluid was obtained from mice at day 3 post-MI, suggesting that alveolar-capillary barrier broke down. Therefore, we proposed the novel concept that impaired gas exchange post-MI is not solely attributable to hemodynamic changes but is in part attributable to cellular infiltration of the lung that prompts an inflammatory response and, hence, increased endothelial-alveolar permeability.

Although the underlying immunologic mechanisms of remote lung injury after sepsis\(^17\) and severe acute pancreatitis\(^18\) are beginning to emerge, little attention has been paid to the inflammatory mechanism behind these severe abnormalities in respiratory gas exchange after cardiogenic pulmonary edema. Our present data introduce the concept that IL-1\(\beta\) sensitizes lung-ECs to neutrophil adhesion, resulting in an increased chance of neutrophil trafficking from the intravascular environment into the interstitial and alveolar compartments. Experiments in small MI model (no LVEDP...
elevation/moderate myocardial inflammation) and TAC model (LVEDP elevation with mild myocardial inflammation) supported both the infarcted myocardium and pulmonary capillary ECs exposed to high microvascular pressure, which are the source of IL-1β.

The present study reveals a critical role for pulmonary NK cells in protecting lungs from the development of cardiogenic pulmonary edema after MI. It was surprising that NK cells play a minor effect on myocardial remodeling where inflammatory activation is marked, associated with >10-fold increases in NK cells number and >100-fold increases in myocardial leukocytes but is critical in a process involving much lower levels of inflammatory activation, in which NK cells and inflammatory leukocyte numbers seem to increase only by 30% to 40% in the lungs of infarcted animals. Notably, the number of NK cells in lung was 20-fold higher than that in heart.

The pulmonary NK cells, which have a CD11b high CD27low mature phenotype and express IL-10, translocate to the site of vascular inflammation after MI. The unique pulmonary environment promotes the development of NK cells with a lung-specific phenotype. For example, microbes entering the neonatal lung could specifically prime the resident NK cells, possibly explaining why lung NK cells have a more mature phenotype and express IL-10. Unlike in the case of K. Pneumoniates infection, there was no significant change in the frequency of CD11b-positive (a marker for degranulation of NK cells) or IFN-γ-positive NK cells in the lung in response to MI in this study. Instead, the frequency of lung IL-10–producing NK cells increased >2-fold after MI. IL-12 is known to induce IL-10 production from NK cells via a STAT4-dependent, T-bet–independent pathway, and MI induces a local and systemic cytokine storm, including IL-12, which may further promote NK cells expressing IL-10.

There is a bidirectional interaction between NK cells and neutrophils, whereby neutrophil stimulates translocation of NK cells to inflamed vasculature where neutrophils are accumulated. NK cells also induce neutrophil apoptosis via an NKp46– and Fas-dependent mechanism, and this may be involved in the resolution of acute inflammation. In the present study, we demonstrated an unreported interaction between NK cells and neutrophils via a paracrine secretion of IL-10. The representative flow cytometry dot plots of CD11b–Gr-1– neutrophils infiltrated into the lung at day 7 after MI. Statistical analysis was performed using Kruskal–Wallis tests followed by Bonferroni post hoc analysis. IL-10 has been shown to suppress nuclear factor-κB, P38 mitogen-activated protein kinases, and the cytokine mRNA-stabilizing protein 36-kDa RNA binding protein (HuR), as well as inhibit the production of numerous proinflammatory cytokines (tumor necrosis factor-α, IL-1β), IL-6, chemokines (CXCL-1, CXCL-2), and adhesion molecules (ICAM-1) that are known to mediate sequestration of neutrophils into the lung, neutrophil adhesion to ECs, and transendothelial migration of neutrophils. IL-10 also attenuates the inflammatory response by increasing the expression of anti-inflammatory proteins including IL-1 receptor antagonist and soluble tumor necrosis factor-α receptor, and by decreasing the expression of major histocompatibility complex and costimulatory molecules on antigen-presenting cells. P38 mitogen-activated protein kinases activation in neutrophils plays a key role in the migration of neutrophils into airways. Thus, IL-10 may...
directly suppress such migration by suppressing P38 mitogen-activated protein kinases activation in neutrophils.

Beneficial effects of early systemic IL-10 administration on pulmonary neutrophil accumulation and lung edema after injury have been reported in various models. Intravenous administration of IL-10 protects against hepatic ischemia-reperfusion–induced lung injury by inhibiting lung nuclear factor-κB activation and the resulting pulmonary neutrophil accumulation and lung edema.11 In a porcine model of acute bacterial pneumonia, local expression of IL-10 suppressed lung edema and neutrophil invasion, resulting in significantly reduced lung damage.29 These findings suggested that the protective role of IL-10 on the alveolar-capillary barrier is universal. However, the impact of systemic IL-10 is determined by the timing of application and the underlying disease, whereby in states of sterile inflammation it may be beneficial but deleterious in infection.30 In this study, we demonstrated that post-MI pulmonary edema engaged NK cells in an IL-10–mediated immunoregulatory circuit that functions to alleviate pulmonary edema.

How does MI lead to neutrophil attraction in the lung but not in other organs? Such selectivity is most likely because of vascular bed heterogeneity and unique properties of the pulmonary circulation. Even under physiological conditions,
neutrophils must stop several times and change their shape to transverse the small pulmonary capillaries.31 This increases the transit time through the pulmonary capillary bed and, as a consequence, induces a significant 40- to 100-fold neutrophil accumulation in the lung.32 Activated neutrophils lose their ability to deform, mainly because of the intracellular polymerization of actin filaments. As a result, transition time through the pulmonary vasculature prolongs even further, ensuring sequestration into the lung. Using intravital microscopy, Schmidt et al17 recently demonstrated that sepsis-mediated acute lung injury is initiated by degradation of the pulmonary endothelium glycocalyx, leading to neutrophil adherence and inflammation. This glycocalyx forms an endothelial surface layer that acts as a barrier to circulating cells and large molecules. Endothelial glycocalyx degradation–associated neutrophil retention seems to be unique in lung, because lipopolysaccharide caused a rapid loss in the pulmonary endothelial surface layer but cremasteric endothelial surface layer thickness was unchanged.

In summary, we demonstrated the novel finding that NK cells play a counter-regulatory role in remote lung injury associated with MI via a paracrine secretion of IL-10 (Online Figure X). The alveolar macrophage and dendritic cells also increased in the lung in the late phase after MI, which may be involved in the chronic lung remodeling. This is consistent with previous finding, in which chronic heart failure induces lung fibrosis and macrophages infiltration.33 The hope is that identifying these major components of both proinflammatory and local counter-regulatory mechanisms against an inflammatory change in microvascular permeability in the lung may help to identify novel pharmacological targets for congestive heart failure on MI and in more general settings involving other pathogeneses.

Acknowledgments

We thank Dr Kiyoshi Takeda from Osaka University and Dr Akihiko Yoshimura from Keio University for kindly providing us the IL-10 knockout and IL-10–Venus reporter mice. We thank Y. Miyake and A. Itaya for technical assistance.

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Disclosures

None.

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What Is Known?
• Acute cardiogenic pulmonary edema arises when abnormally high pulmonary capillary pressure induces the characteristic accumulation of low-protein fluid in the interstitial and alveolar spaces of the lung.
• Natural killer (NK) cells play a highly important role in the killing of tumors and cells infected by viruses, and can have specialized and niche-specific functions in distinct organs.

What New Information Does This Article Contribute?
• Impaired gas exchange after myocardial infarction (MI) is not solely because of hemodynamic changes but is in part attributable to neutrophil infiltration of the lung that induces an inflammatory response and, hence, increased endothelial-alveolar permeability.
• Interleukin-1β is the primary initiator of pulmonary inflammation after MI in mice.
• Lung NK cells play a protective role against cardiogenic pulmonary edema via a paracrine secretion of interleukin-10.

Acute cardiogenic pulmonary edema after MI is associated with poor long-term outcomes. Here, we show that post-MI pulmonary edema engaged NK cells in an interleukin-10–mediated immunoregulatory circuit that functions to alleviate pulmonary edema. We found that depletion of NK cells does not affect cardiac inflammation and function after MI. However, depletion of NK-cell in mice led to severe respiratory distress associated with protein-rich, high-permeability alveolar edema accompanied by neutrophil infiltration. The number of NK cells in lung was 20-fold higher than that in heart. The pulmonary NK cells, which have a CD11b<sup>hi</sup> CD27<sup>+</sup> mature phenotype and express interleukin-10, translocate to the site of vascular inflammation after MI. NK cells are a major interleukin-10 source in the lung, and interleukin-10 secreted from lung NK cells alleviates the increased permeability of the inflamed alveolar-capillary barrier after MI.
Lung Natural Killer Cells Play a Major Counter-Regulatory Role in Pulmonary Vascular Hyperpermeability After Myocardial Infarction

Xiaoxiang Yan, Ahmed E. Hegab, Jin Endo, Atsushi Anzai, Tomohiro Matsuhashi, Yoshinori Katsumata, Kentaro Ito, Tsunehisa Yamamoto, Tomoko Betsuyaku, Ken Shinmura, Weifeng Shen, Eric Vivier, Keiichi Fukuda and Motoaki Sano

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Supplementary Material

Detailed Methods

Mice

NKDTR/EGFP transgenic mice and IL-10-Venus reporter mice in a C57BL/6 background were generated as described previously 1,2. IL-10 knockout mice (IL-10 KO mice) were purchased from The Jackson Laboratory. All mice were bred on the C57BL/6 background and 10 to 16-week-old male mice were used in this study. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the Keio University School of Medicine.

Induction of MI, IR and TAC

Mice were subjected to a permanent ligation of the left anterior descending artery (MI) or to a sham operation without ligation as described previously 3. In brief, mice were lightly anesthetized with diethyl ether, intubated, and then fully anesthetized with 1.0-1.5% isoflurane gas while being mechanically ventilated with a rodent respirator. The chest cavity was opened via left thoracotomy to expose the heart such that the left anterior descending coronary could be visualized by microscopy and permanently ligated with a 7-0 silk suture at the site of its emergence from the left atrium. Complete occlusion of the vessel was confirmed by the presence of myocardial blanching in the perfusion bed. Mice that died during recovery from anesthesia were excluded from the analysis. Sham-operated animals underwent the same procedure without coronary artery ligation.

To elucidate the role of IL-1 signaling in lung inflammation induced by MI, C57BL/6 mice were randomly assigned to 4 groups: 1) Sham group; 2) MI group; 3) MI mice injected IV with anti-IL-1RI antibody (200 mg/mouse, R&D Systems) 2 hours before induction of MI; 4) Sham mice injected with IV recombinant mouse-IL-1β (rIL-1β, 1 mg/mouse, R&D Systems). One day after MI, the lungs were removed for lung gene expression and flow cytometry analyses.

To induce cardiac ischemia reperfusion (IR), a slipknot was made around the left anterior descending coronary artery against PE10 tubing with a 7-0 silk suture. After 45 minutes of ligation, the ligature was released to allow reperfusion 4. Mice were subjected to left ventricular pressure overload produced by TAC (transverse aortic constriction) as previously described 5.

In vivo depletion of NK cells

NKDTR/EGFP transgenic mice were injected i.p. with 2 µg diphtheria toxin (Sigma-Aldrich) 1 day before MI. For the wild-type mice, NK-cell depletion was achieved by injecting 200 mg of anti-NK1.1 antibody (clone: PK136, Bio-X-Cell, NH, USA), 250 mg of anti-Asialo GM1 antibody (Wako Pure Chemical Industries, Osaka, Japan), or isotype controls i.p. into mice 1 day before MI. NK depletion was confirmed by flow cytometry in peripheral blood cells of mice receiving antibody treatment and compared to the isotype control group.

NK-cell enrichment and adoptive transfer

NK cells were isolated from the lungs of wild type or IL-10-deficient mice via negative selection by autoMACS (mouse NK cell isolation kit II, Miltenyi Biotec, Auburn, CA, USA), and then NK1.1+ CD3− NK cells were sorted by fluorescence-activated cell sorting (FACS). The cells were analyzed by flow cytometry to determine NK cell purity (more than 95% purity) (Online Figure IX A). On the day of MI induction, NK cells were injected intravenously into NKDTR/EGFP mice (~2 x 10⁶ cells/mouse) in which the NK cells had been depleted 1 day before MI. We confirmed that transferred NK cells labeled with CFSE were recruited into the lung (Online Figure IX B).

Cell preparation for flow cytometry

At each time point, mice were deeply anesthetized and intracardially perfused with 40 ml of ice-cold PBS to exclude blood cells. The heart was dissected, minced with fine scissors, and then enzymatically digested with a cocktail of type II collagenase (Worthington Biochemical Corporation, Likewood, NJ), elastase (Worthington Biochemical Corporation) and DNase I (Sigma, St. Louis, MO) for 1.5 hours at 37°C with gentle agitation. After digestion, the tissue was triturated and passed through a 70-µm cell strainer. Leukocyte-enriched fractions were isolated by 37–70% Percoll (GE
Healthcare) density gradient centrifugation as described elsewhere. Cells were removed from the interface and washed with RPMI-1640 cell culture medium for further analysis. Spleens were removed, homogenized, and then passed through a 70-µm nylon mesh in PBS. After addition of red blood cell lysis buffer (eBioscience) to exclude erythrocytes, the single cell suspension in PBS was refiltered through a 70-µm nylon mesh to remove connective tissue. To obtain single-lung-cell suspensions, lungs were perfused with cold PBS through the right ventricle, cut into small pieces, and digested for 1 hour at 37°C with a cocktail of type II collagenase, elastase, and DNase I for 1 hour at 37°C with gentle agitation. After digestion, the tissue was triturated and passed through a 70-µm cell strainer.

**Flow cytometric analysis**

To block nonspecific binding of antibodies to Fcγ receptors, isolated cells were first incubated with anti-CD16/32 antibody (2.4G2, BD Biosciences) at 4°C for 5 min. Subsequently, the cells were stained with a mixture of antibodies at 4°C for 20 min. For CD107a staining, we cultured cells in 10% RPMI with Golgistop (BD Pharmingen) for 4.5 h before cell-surface staining. Flow cytometric analysis and sorting were performed on a FACSariaIII instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Antibodies used for flow cytometry**

Anti-CD45-FITC (30F11.1, eBioscience), anti-CD11b-PerCP-Cy5.5, anti-CD11b FITC (M1/70, eBioscience), anti-CD3e-FITC, anti-CD3e-APC (145-2C11, eBioscience), anti-CD4-PE (GK1.5, eBioscience), anti-CD4-APC-H7 (GK1.5, BD Pharmingen), anti-NKp46-eFluor®450 (29A1.4, eBioscience), anti-CD27-PE-Cy7 (LG.7F9, eBioscience), anti-NK1.1-eFluor®450, anti-NK1.1-PE (PK136, eBioscience), anti-CD11c-APC, anti-CD11c-PE (N418, Biolegend), anti-MHC-II (I-A/I-E)-PE (M5/114.15.2, eBioscience), anti-Gr-1-APC, anti-Gr-1-Alexa Fluor488 (RB6-8C5, eBioscience), anti-CD107a-eFluor®660 (eBioscience), anti-IL-10-PE (JES5-16E3, BD Pharmingen), and anti-IFN-γ-APC (XMG1.2, eBioscience) antibodies were used for flow cytometric analysis in this study.

**Intracellular cytokine staining**

For surface and intracellular cytokine staining, single cells were restimulated for 4.5 hours with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 1 µg/ml ionomycin (Sigma-Aldrich) in the presence of Golgistop (Cytofix/Cytoperm Plus Kit with Golgistop, BD Biosciences). Surface staining was performed for 20 minutes with the corresponding mixture of fluorescently labeled antibodies. After fixation and permeabilization, the cells were incubated for 30 min at 4°C with anti-IFN-γ-APC (eBioscience).

**Quantitative Real-time PCR**

Total RNA samples from sorted cells, cultured cells, and lung tissue were prepared using RNeasy Mini Kit (Qiagen) or Trizol reagent (Invitrogen), according to the manufacturer's instructions. The First-strand cDNA synthesis kit (Invitrogen) was used for cDNA synthesis. Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems). Predesigned gene-specific primer and probe sets (Taqman Gene Expression Assays, Applied Biosystems) were used. The 18S ribosomal RNA was amplified as an internal control.

**Mouse BAL recovery, albumin, and total protein contents measurements**

To obtain BAL samples for analysis of albumin and total protein concentrations, we lavaged lungs with three 0.6-ml aliquots of cold PBS. RBCs in the lavage were lysed using RBC lysis buffer (eBioscience), and nucleated cells were counted by a hemocytometer. Cell differentials were performed on cytopsin preparations stained with Diff-Quick (Sysmex, Kobe, Japan). Total protein concentrations in BAL samples were determined using a commercially available Bio-Rad Protein Assay Kit (500-0006, Bio-Rad). Mouse albumin and IL-6 levels in BAL fluid were measured using mouse albumin (Bethyl Laboratories) and IL-6 (eBioscience) ELISA kits.
Wet/dry lung weight ratio

At the end of the experiment, lungs were weighed, dried, and reweighed. Wet/dry lung weight ratio was used as an index of accumulated lung water. For determination of lung dry weight, lung tissue was dried in an oven at 80°C for 48 h.

Isolation and culture of primary mouse lung endothelial cells and bone marrow neutrophils

Mouse lung endothelial cells (MLEC) were isolated as described previously. Briefly, lungs were prepared and digested as described above, the resulting lung cells were then plated and cultured in DMEM/F12 supplemented with 20% FBS, 75 mg/ml ECGS; 20 U/ml heparin sulfate (Sigma-Aldrich), and 1% antibiotics at 37°C in a humidified atmosphere of 5% CO₂/95% air in tissue culture flasks or dishes coated with 1% gelatin. The following day, the medium was changed, and the cells were cultured for an additional 1-2 days. At this time, the cells from each lung were removed by trypsin and pooled into one suspension for the sorting. CD31⁺ endothelial cells were immunomagnetically isolated with rat anti-mouse CD31 (BD Pharmingen) and anti-rat IgG microbeads (Miltenyi Biotec) according to the manufacturer’s instructions (Miltenyi Biotec). The sorted cells were resuspended in DMEM/F12 complete medium, plated in gelatin-coated T-25 flasks, and subsequently split 1:2 at each passage. Endothelial identity was confirmed by cobblestone morphology, and the CD31 expression was determined by flow cytometric analysis. All cells were used at passages 3-5.

We isolated mature neutrophils from the bone marrow of C57BL/6 mice by centrifugation over 60%/72% Percoll gradients as previously described; 85% to 90% of the cells were mature neutrophils as determined by Diff-Quick staining (Sysmex, Kobe, Japan) and CD11b⁺Gr-1⁺ FACS analysis.

Neutrophil-endothelial cell adhesion assays

The mice were perfused with PBS transcardially. The left ventricular was removed, homogenized with RPMI-1640, and centrifuged at 15,000 r.p.m. for 5 min. The supernatant was made up to 1 ml with RPMI-1640 and used as the heart lysate.

Confluent mouse lung endothelial cell monolayers were stimulated with rIL-1β (50 ng/ml, R&D Systems) or heart lysate in the presence or absence of 10 µg/ml IL-1RI (IL-1 Receptor I, R&D Systems) antibody. After 6 hours of treatment, the gene expression in endothelial cells was quantified by quantitative PCR. Isolated neutrophils were labeled with 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Sigma-Aldrich) according to the manufacturer’s instructions (Sigma-Aldrich) and added to endothelial cell monolayers at a neutrophil-to-endothelial ratio of 10:1. After 1 hour of incubation at 37°C, non-adherent cells were removed by three gentle washes with PBS. Cells were fixed with 4% paraformaldehyde, and adherent cells were counted in five randomly chosen fields using fluorescence microscopy.

Measurement of Endothelial Leakage in vitro

A Transwell insert (0.4-µm pore) was coated with gelatin, and LECs were then seeded at a density of 2 × 10⁵/well in a final volume of 500 µl DMEM/F12 supplemented with 20% FBS, 75 µg/ml heparin sulfate (Sigma-Aldrich), and 1% antibiotics. Cells were cultured to confluence, and then NK cells sorted from wild-type and IL-10 knockout mice were added to the lower chamber. LECs were stimulated with rmIL-1β (50 ng/ml, R&D Systems) for 22 hours, then 2,000-kDa FITC-dextran (0.5 mg/ml, Sigma-Aldrich) was added into the top chamber and the cells were incubated for up to 60 minutes. In some experiments, 10 ng/ml IL-10 was added to the medium. The fluorescence intensity of the bottom chamber was analyzed on a fluorescence plate reader with filters appropriate for 485 nm and 535 nm excitation and emission, respectively.

MPO assay

MPO lung tissue level activity was determined as previously described. Optical density was measured at 460 nm with a spectrophotometer. One unit of enzyme activity was defined as 1.0 optical density (AU) min⁻¹ng protein⁻¹ at room temperature.

Heart infarct size determination
Heart tissue was fixed in formalin, embedded in paraffin, and cut into 5-µm-thick sections. Hematoxylin and eosin (H&E) and Azan staining were performed on paraffin-embedded sections to determine the morphological effects and infarct size, with the latter calculated as total infarct circumference divided by total LV circumference x 100, as described previously.5

**Morphological assessment of lung injury**

The mice were euthanized and lungs were fixed by instillation of 10% formaldehyde solution via the tracheal cannula at a pressure of 25 cmH2O. Lungs were then embedded in paraffin, sectioned at 5-µm thickness, and stained with hematoxylin and eosin. The histopathology was reviewed in a blinded manner with respect to which group or mouse was being reviewed, using a modified histological scoring system as previously described.9 Five easily identifiable pathological processes were scored on a scale of 0-4 as previously described: (a) alveolar congestion; (b) hemorrhage; (c) thickness of vascular wall; (d) leukocyte infiltration in airspace or the vessel wall; and, (e) thickness of the alveolar wall. A score of 0 represented normal lungs; 1, mild, < 25% lung involvement; 2, moderate, 25–50% lung involvement; 3, severe, 50–75% lung involvement; and 4, very severe, > 75% lung involvement. The overall score was based on the sum of all scores.

**Echocardiography**

Transthoracic echocardiography was performed with a Vevo 2100 instrument (VisualSonics) equipped with an MS-400 imaging transducer. Mice were kept awake without anesthesia during the echocardiographic examination to minimize data deviation, and heart rate was maintained at approximately 550–650 bpm in all mice. M-mode tracings were recorded through the anterior and posterior LV walls at the papillary muscle level to measure LV end-diastolic dimension (LVEDD) and LV end-systolic dimension (LVESD). LV fractional shortening (FS) was calculated according to the following formula: LV FS = [(LVEDD-LVESD)/LVEDD] × 100.

**Hemodynamics**

Cardiac catheterization studies were performed using a 1.4 French microtip catheter (SPR-671, Millar Instruments, Houston, TX) under sedation using 1.5% isoflurane inhalation with spontaneous respiration. LV end-systolic pressure (LVESP), maximum rate of isovolumic pressure development, and minimum rate of isovolumic pressure decay were measured using analysis software (PowerLab, AD Instruments). Ten sequential beats were averaged for each measurement.

**Immunofluorescence**

We performed immunofluorescence on paraffin-embedded sections from perfusion-fixed lungs from intact mice as described previously.10 Primary antibodies used were rabbit polyclonal antibody to CD31 (1:200, sc-1506R, Santa Cruz Biotechnology), rabbit polyclonal antibody to GFP (1:500, 598, MBL), and mouse monoclonal antibody to NK1.1 (1:200, clone PK136, Biolegend). The appropriate Alexa-Fluor-coupled secondary antibodies were used in double-staining experiments. Sections were counterstained with DAPI and analyzed by fluorescent microscopy with a Leica microscope.

**Statistics**

Values are presented as mean ± SEM. Comparisons between groups were made using a Mann-Whitney U test, whereas data among multiple groups were compared using either the Kruskall-Wallis test with Dunn’s multiple comparison test or Bonferroni post hoc analysis, or two-way ANOVA followed by Tukey’s post hoc analysis, as appropriate. A value of P < 0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism 5.0 (Graph Pad Prism Software Inc, San Diego, CA, USA) and SPSS 15.0 for Windows (SPSS, Inc, Chicago, IL, USA).
References


Online Figures

Online Figure I. Time course changes of NK cells and NKT cells in the heart after MI
(A) Gating strategy for infiltrating NK and NKT cells in the infarcted heart. (B through D) Temporal dynamics of the CD45+ leukocytes, NK and NKT cells in the permanent MI heart. Quantities represent absolute number of cells per heart (n = 4-7, each). **P < 0.01 vs. sham. Data were analyzed by Kruskall-Wallis tests with Dunn’s multiple comparison.
Online Figure II. Validation of NK cells depletion in the NKDTR/EGFP and wild-type mice

(A) Flow cytometry analysis of NK cells in the peripheral blood mononuclear cells in the NKDTR/EGFP mice treated with or without DT. (B) Lung EGFP⁺ NK cells were depleted in the NKDTR/EGFP mice at day 7 after DT injection. (C) Flow cytometry analysis of NK1.1⁻CD3⁻ and NKp46⁺CD3⁻ NK cells in the peripheral blood mononuclear cells at day 2 after anti-NK1.1 antibody injection.
Online Figure III. Ablation of NK cells did not affect cardiac inflammation or immune cell infiltration

(A) The mRNA levels of TNF-α, IL-6, IL-1β, and IFN-γ were determined in the heart at day 3 after MI (n = 4-6). NS, not significant, *P < 0.05. Data were analyzed by Mann-Whitney U tests. (B) Representative flow cytometry dot plots of CD45+ leukocytes, CD11b+F4/80+ macrophages, and CD11b+Ly-6G+ neutrophils infiltrated into the heart at day 2 after MI (n = 3-4). (C) Leukocytes and myeloid cells infiltrated into the heart were analyzed by flow cytometry. NS, not significant. Statistical analysis was performed using Kruskall-Wallis tests followed by Bonferroni post hoc analysis.
Online Figure IV. Gating strategy of lung immune cells used in the present study

(A) Gating strategy for lung NK cells, T cells, and NKT cells. (B) Gating strategy for lung alveolar macrophage (AM), conventional dendritic cells (cDC), CD11b'Gr-1' neutrophils, and Gr-1int myeloid cells.
Online Figure V. Both myocardial inflammation and elevation in lung capillary pressure contribute to neutrophil accumulation in the lung.

At day 1 after the operation, (A) CD45+ leukocytes infiltrated into the heart were analyzed by flow cytometry (n = 3-4); the mRNA levels of TNF-α (B) and IL-1β (C) were determined (n = 4-6); (D) LVEDP was examined (n = 4-6); and (E, F) CD11b^Gr-1^ neutrophils infiltrated into the lung were analyzed by flow cytometry (n = 4-6). NS, not significant; *P < 0.05; **P < 0.01 vs. Sham group; *P < 0.05, **P < 0.01. Statistical analysis was performed using the Kruskall-Wallis test followed by Bonferroni post hoc analysis.
Online Figure VI. Depletion of NK cells with anti-ASGM1 antibody aggravates pulmonary vascular hyperpermeability and injury induced by MI

(A) Representative flow cytometric dot plots of NK cells in the peripheral blood mononuclear cells after anti-ASGM1 antibody administration. (B) Time course changes of NK cells and T cells in the peripheral blood after anti-ASGM1 antibody administration. NK cells were depleted with anti-ASGM1 antibody 1 day before MI in the wild type mice. Echocardiography and BALF recovery were done at day 7 after MI. Echocardiographic analysis of fractional shortening (FS) (C) and left ventricular end diastolic diameter (LVEDD) (D) at day 7 after MI or sham operation (n = 7-8). (E) Infarct size determined with Azan staining of sections (n = 7-8). The BALF albumin (F), total protein levels (G), and total leukocyte number (H) recovered from BALF and lung wet/dry weight ratio (I) were quantified at day 7 after MI (n = 7-9). (J) MPO activity in the lung tissues was determined at day 7 after MI (n = 6). (K) Histological score was quantified. (L) Histological sections of H&E-stained lung tissue at day 7 after MI (n = 7-8). Scale bar: 100µm. (M) BALF IL-6 protein levels were determined at the indicated time points after MI (n = 5-6). NS, not
significant; *$P < 0.05$; **$P < 0.01$. Statistical analysis was performed using Kruskall-Wallis tests followed by Bonferroni post hoc analysis.
Online Figure VII. Location of NK cells in the lung
Lung sections were stained with GFP (red) and CD31 (green) antibodies in the NKDTR/EGFP mice at day 7 after MI. Scale bar: 50 µm.
Online Figure VIII. IL-10 suppressed IL-1β-induced neutrophil adhesion to lung-ECs and increased permeability

Lung endothelial cells (LECs) were stimulated with IL-1β in the presence or absence of IL-10 for 6 hours, and then gene expression levels of IL-6 (A), ICAM1 (B), and CXCL1 (C) were determined by quantitative RT-PCR. (D) Representative photomicrograph of neutrophils adhering to LECs. Scar bar: 100 µm. (E) Neutrophil adhesion on LECs pretreated with IL-1β in the presence or absence of IL-10 was determined by fluorescence microscopy (experiments were performed 4 times). *P < 0.05; **P < 0.01; ***P < 0.001. Data were analyzed using the Kruskall-Wallis test followed by Bonferroni post hoc analysis. (F) Confluent monolayers of LECs were cultured on the Transwell inserts and stimulated with IL-1β in the presence or absence of IL-10 for 24 hours, then fluorescein isothiocyanate (FITC)-dextran was added into the top chamber and the cells were incubated for up to 60 minutes. The fluorescence intensity of the bottom chamber was analyzed on a fluorescence plate reader with filters appropriate for 485 nm and 535 nm excitation and emission, respectively. **P < 0.01 vs. Control group; ***P < 0.001 vs. IL-1β-treated groups. Statistical analysis was performed using the Kruskall-Wallis test followed by Bonferroni post hoc analysis.
Online Figure IX. NK cells sorting strategy

(A) The representative flow cytometry dot plots of lung NK1.1^CD3^- NK cells before and after sorting. (B) Transferred CFSE labeled NK cells were recruited into the lung.
Online Figure X. Schematic model of MI-induced pulmonary edema and protective role of NK cells
As shown in the diagram, 1) MI induces a storm of pro-inflammatory cytokines in the circulation, such as IL-1β, that could activate pulmonary ECs (producing IL-6, CXCL1, and ICAM1) and neutrophil infiltration, leading to hyperpermeability and pulmonary edema, in synergy with elevated pressure induced by heart failure; 2) abundant NK cells resident in the lung move toward the vascular walls, mediating IL-10 production and suppressing EC activation and neutrophil adhesion, thus attenuating hyperpermeability and pulmonary edema.
Online Video

Online Video. DT-treated (Online Video I) NKDTR/EGFP-transgenic mice exhibited more severe respiratory distress than PBS-treated mice (Online Video II) following MI
Online Table I. Hemodynamic Data and Arterial Blood Gas Analysis at 7 Days after MI in the NKDTR/EGFP mice

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<tr>
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<td>PaCO2 (mmHg)</td>
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Abbreviations: MI, myocardial infarction; BW, body weight; HW, heart weight; LW, lung weight; HR, heart rate; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; PaO₂, arterial oxygen tension; PaCO₂, arterial carbon dioxide tension. Results are presented as mean ± SEM. *P < 0.05 vs. corresponding sham group; †P < 0.05 vs. PBS MI group; ‡P < 0.05 vs. DT MI group (two-way ANOVA followed by Tukey’s post hoc analysis).