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DAP12 Expression in Lung Macrophages Mediates Ischemia/Reperfusion Injury by Promoting Neutrophil Extravasation

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Neutrophils are critical mediators of innate immune responses and contribute to tissue injury. However, immune pathways that regulate neutrophil recruitment to injured tissues during noninfectious inflammation remain poorly understood. DAP12 is a cell membrane–associated protein that is expressed in myeloid cells and can either augment or dampen innate inflammatory responses during infections. To elucidate the role of DAP12 in pulmonary ischemia/reperfusion injury (IRI), we took advantage of a clinically relevant mouse model of transplant-mediated lung IRI. This technique allowed us to dissect the importance of DAP12 in tissue-resident cells and those that infiltrate injured tissue from the periphery during noninfectious inflammation. Macrophages in both mouse and human lungs that have been subjected to cold ischemic storage express DAP12. We found that donor, but not recipient, deficiency in DAP12 protected against pulmonary IRI. Analysis of the immune response showed that DAP12 promotes the survival of tissue-resident alveolar macrophages and contributes to local production of neutrophil chemoattractants. Intravital imaging demonstrated a trans-endothelial migration defect into DAP12-deficient lungs, which can be rescued by local administration of the neutrophil chemokine CXCL2. We have uncovered a previously unrecognized role for DAP12 expression in tissue-resident alveolar macrophages in mediating acute noninfectious tissue injury through regulation of neutrophil trafficking. The Journal of Immunology, 2015, 194: 000–000.

It is well established that neutrophils infiltrate acutely infected tissues where they play an important role in host defense. However, neutrophils also enter tissue during noninfectious processes where they contribute to tissue damage. Ischemia/reperfusion injury (IRI) is a clinically relevant noninfectious inflammatory insult that can affect a variety of tissues. Lung IRI is manifest during several clinical scenarios, including blood flow restoration after pulmonary artery embolism, cardiopulmonary bypass, and lung transplantation (1). Hallmarks of pulmonary IRI include alveolar damage, pulmonary edema, and hypoxemia (2). In the case of lung transplantation, where reperfusion of a lung transiently subjected to cold ischemia is an inherent component of this procedure, IRI is a critical determinant of short- and long-term morbidity and mortality (3). We and others have demonstrated that neutrophils are important mediators of pulmonary IRI (4, 5). Using intravital imaging we have shown that neutrophils rapidly infiltrate freshly perfused lung transplants, forming dynamic clusters within the graft tissue (5). Furthermore, we have reported that G-CSF–mediated emergency granulopoiesis after transplantation exacerbates lung injury and that neutrophil depletion improves graft function (6, 7). Although several cytokines, chemokines, and cell populations have been suggested to play a role in mediating trafficking of neutrophils into inflamed lung tissue, the precise cellular and molecular cues that regulate their migratory behavior are poorly understood.

In infectious models, data have been emerging that innate immune pathways can be regulated through the cell membrane–associated protein DAP12, which associates with several ligand-binding receptors (8). DAP12 and its associated receptors are expressed on a variety of myeloid cells. Investigations into the role of DAP12 have focused mainly on infectious immunity, where its role has been controversial, with some studies reporting inhibitory and others activating effects on inflammatory responses (8–12). However, the role of DAP12 in noninfectious inflammation remains largely unexplored.

In this study we have uncovered a role for DAP12 in exacerbating pulmonary IRI. The use of a transplant model allowed us to identify that DAP12 signaling in donor lung–resident alveolar macrophages promotes their survival and impacts local neutrophil chemokine production, which regulates extravasation of neutrophils into inflamed pulmonary tissue. Thus, we describe a clinically relevant pathway mediating neutrophil recruitment into acutely injured lungs that has the potential to be targeted therapeutically.

Materials and Methods

Mice
C57BL/6 (CD45.2⁺) and B6.SIL–Ppec–Pepc⁺BoyJ (CD45.1⁺) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Lysozyme M (LysM)–GFP mice were obtained from Klaus Ley (La Jolla Institute) and DAP12-deficient (DAP12 knockout [KO]) mice were from Marco Colonna (Washington University). Procedures were approved by the Institutional Animal Studies Committee. Animals received humane care in compliance with the guidelines of the Institutional Animal Care and Use Committee of Washington University, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.
with the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health and with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research.

**Surgical procedures**

Wild-type (WT) or DAP12KO lungs were transplanted into CD45.1+ B6 WT, DAP12KO, or LysM-GFP mice after 18 h of cold ischemia (5, 13, 14). Graft function was assessed as described previously (7). For examining neutrophils, 3 μg anti-Ly6G Ab (1A8) was injected i.v. 5 min before sacrifice (15). For selected imaging experiments, 1 × 106 bone marrow–derived macrophages (BMDM) were injected into the donor bronchi prior to transplantation. To generate BMDM, cells were flushed from B6 or DAP12KO femurs and cultured for 4 d in medium with 50 ng/ml M-CSF (PeproTech, Rocky Hill, NJ). Murine recombiant CXCL2 (500 ng) (R&D Systems, Minneapolis, MN) or sterile PBS was instilled into DAP12KO bronchi before implantation. Grafts were harvested 2 or 6 h after transplant (14).

**Immunostaining**

Cold ischemic murine and human lungs were fixed in parafomaldehyde and stained with DAP12, CD68 (DA-11, Serotec, Raleigh, NC or FA-11, BioLegend, San Diego, CA), cleaved caspase-3 (Asp175, Cell Signaling Technology, Danvers, MA) and/or DAP12 (FL-113, Santa Cruz Biotechnology, Dallas, TX). CD68 staining in Suplementary Fig. 2 used an Ab directly conjugated to Alexa Fluor 488 whereas all other primary Abs were detected using Alexa Fluor–conjugated secondary Abs (Molecular Probes, Carlsbad, CA). Fluorescence microscopy was performed using a Leica DM5000 (Leica, Wetzlar, Germany) with a Retiga 200R CCD camera (Q-Imaging, Surrey, BC, Canada) interfaced with Q-Capture Pro (Q-Imaging). Images were globally adjusted using Adobe Photoshop (Adobe Systems, San Jose, CA). Human protocols were approved by the Institutional Review Board at Washington University.

**Flow cytometry**

Graft-infiltrating cells were assessed using CD45.2 (104), CD45.1 (A20), Gr1 (RB6-8C5), Ly6G (1A8), CD11c (N418), CD11b (M1/70), IA-1* (25-9-17), Siglec-F (E50-2440), CD46 (X54-57.1), and isotype control Abs (BD Biosciences, San Jose, CA; BioLegend, San Diego, CA; and eBioscience, San Diego, CA).

**Quantitative PCR**

Alveolar and interstitial macrophages as well as endothelial cells (CD31, clone MEC13.3) were sorted into TRIzol (Life Technologies, Grand Island, NY) using a Beckman Coulter MoFlo. RNA was isolated and converted into cDNA. cDNA (30 ng) was assessed for DAP12, CXCL1, CXCL2, IL-6, TNF-α, and β-2-microglobulin (Life Technologies on a Roche Light Cycler 480 using iTaq Universal Probes Supermix (Bio-Rad, Hercules, CA).

**In vitro cultures**

BMDM were harvested and 105 cells plated in 10 ml 20% M-CSF L cell–conditioned medium. On day 3, 5 ml L cell–conditioned medium was added. BMDM were harvested on day 4. B6 or B6 transplants lyses were added (1:20) and cultured for 17 h. To prepare lysates, lungs were harvested 2 h after transplant, homogenized in PBS (0.1 g/ml), and treated with three rounds of freeze/thawing in liquid nitrogen and a 37°C water bath. This solution was filtered using a 70-μm sieve, and the supernatant was used for the experiments. IL-6, TNF-α (eBioscience), CXCL-1, and CXCL-2 (R&D Systems) ELISAs were performed.

**Neutrophil transmigration assays**

Neutrophils were purified from WT and DAP12KO bone marrow using MACS negative selection. Cells were incubated with biotinylated Abs to Ter119 (TER-119), CD11c (N418), NK1.1 (PK136), CD3e (145-2C11), CD5 (53-7.3), CD4 (GR1.1), CD3 (53-6.7), B220 (RA3-6B2), CD19 (1D3), F4/80 (BM8), CD11S (AF589), IA/IE (2C9), and CD117 (2B8) followed by streptavidin-conjugated microbeads. After incubation and washing, cells were applied to an LS column, and neutrophils were collected in the flow-through. Media alone or supplemented with 10 or 100 ng/ml CXCL2 was added. BMDM were harvested on day 6. B6 transplant lysates were used for the experiments. IL-6, TNF-α (eBioscience), CXCL-1, and CXCL-2 (R&D Systems) ELISAs were performed.

**Two-photon microscopy**

We performed intravital two-photon (2P) microscopy (5, 16–18). First, 655-nm Q-dots (30 μl) (Life Technologies) were injected to label vessels. Mice were then anesthetized, grafts exposed, and imaging was performed with a custom 2P microscope using ImageWarp (A&B Software, New London, CT). Sequential z-sections (21, 2.5 μm each) were acquired yielding an imaging volume of 220 × 247.5 × 50 μm3. Analyses were performed with Imaris (Bitplane, Zurich, Switzerland). For each mouse, up to five lung areas at least 80 μm deep were analyzed for each of the three time points shown. Each datum point represents an independent measurement of one area of the lung, and the data have been pooled from three to four mice per experimental group.

**Statistical analysis**

Prism was used to analyze data. Paired t tests were used to compare results of experiments examining neutrophil numbers and their distribution by flow cytometry and macrophage numbers by flow cytometry where one WT and one DAP12KO lung transplant were performed in parallel to account for day-to-day variations (p < 0.05; ±SEM). Oxygen levels were analyzed with a one-way ANOVA with a Tukey posttest. Cell numbers and percentages for 2P were analyzed with two-way ANOVA and a Tukey posttest.

**Results**

**DAP12 deficiency in lung-resident cells ameliorates IRI**

To examine whether DAP12 plays a role in mediating acute lung injury, we used a transplant-mediated model of IRI that we have previously described (13, 14, 17, 19). In addition to providing a robust clinically relevant model of noninfected tissue injury, pulmonary transplantation allows for an assessment of contributions of lung-resident versus graft-infiltrating cells to injury. B6 WT or B6 DAP12KO lungs were transplanted into syngeneic WT recipients and assessed functionally 6 h after reperfusion. Transplantation of DAP12KO lungs resulted in significant amelioration of injury as evidenced by improved oxygen exchange in the graft (Fig. 1A). When we transplanted WT lungs into syngeneic DAP12KO hosts, however, graft function was significantly worse and comparable to that observed after transplantation of WT lungs into WT recipients. Eliminating DAP12 expression in recipients of DAP12KO grafts did not result in further improvement of graft function. Thus, DAP12 expression in lung-resident, but not graft-infiltrating, cells regulates acute ischemia/reperfusion-mediated lung injury following pulmonary transplantation.

**DAP12 is expressed in mouse and human lung grafts prior to transplantation**

Having established that DAP12 expression in donor lungs regulates transplant-mediated IRI, we next wanted to examine which graft-resident cells mediate this effect. Previous studies have shown that DAP12 can be expressed in a variety of bone marrow–derived cell types, including lymphocytes, macrophages, neutrophils, and dendritic cells and also to a lesser extent in stromal cells such as endothelial (20). We first analyzed gene expression of DAP12 in lung graft–resident cells. We transplanted WT CD45.2+ or DAP12KO CD45.2+ lungs into congenic WT CD45.1+ recipients and 2 h after reperfusion we sorted donor alveolar macrophages (21) (CD45.2+ CD45.1+ CD64+CD11c−Siglec-F−CD11b+) and endothelial cells (CD45.2+ CD45.1−CD14+CD11c+Tie2+CD31+) from lung graft tissue (Supplemental Fig. 1). Quantitative RT-PCR analysis revealed that DAP12 was expressed in WT alveolar macrophages, but expression levels of DAP12 in endothelial cells were very low (Fig. 1B). Expression levels of DAP12 in alveolar macrophages are not significantly different after transplantation compared with before transplantation (Supplemental Fig. 2A). To corroborate these findings we evaluated protein expression of DAP12 in lungs by immunostaining naive WT lungs as well as WT and DAP12KO lungs following 18 h of cold ischemia. Immunofluorescence microscopy demonstrated DAP12 expression in naive WT lungs (Supplemental Fig. 2B) and WT lung grafts (Fig. 1C) (with minimal background staining in DAP12KO), which predominantly colocalized with CD68, a member of the lysosome-associated membrane protein family. Expression of CD68 is largely...
restricted to tissue-resident macrophages, including lung macrophages, but it has not been observed in pulmonary dendritic cells (22). We did not observe DAP12 staining in stromal cells such as endothelium or airway epithelium. We next stained portions of human lungs following cold ischemic storage just prior to and several hours after their implantation into end-stage pulmonary failure patients. Similar to our findings in mice, we observed DAP12 staining that colocalized with CD68 (Fig. 1C). Again, stromal cells did not express DAP12. Thus, in both mice and humans lung-resident macrophages express DAP12 following cold ischemic storage.

**DAP12 regulates macrophage survival and production of inflammatory cytokines in vitro**

It is well established that cold ischemia and subsequent reperfusion induce tissue necrosis (23). Furthermore, endogenous substances that are released in the setting of IRI can trigger innate immune
responses that contribute to organ damage (23, 24). Our results thus far raised the possibility that DAP12 expression by macrophages plays an important role in regulating responses that are mediated by such endogenous ligands. To evaluate this further we stimulated BMDM with lysates that were generated from transplanted syngeneic B6 lung grafts 2 h after reperfusion. Incubation of cultured macrophages with lung lysates does not result in changes in DAP12 expression levels (Supplemental Fig. 2C). Compared to WT macrophages, a significantly higher percentage of DAP12KO macrophages stained positive for annexin V and 7-aminoactinomycin D regardless of the presence of lung lysates indicating enhanced cell death in the absence of DAP12 (Fig. 2A, 2B). DAP12KO macrophages secreted lower amounts of TNF-α, IL-6, and the neutrophil chemokines CXCL1 and CXCL2 in most experiments after stimulation with lung transplant lysates (Fig. 2C). Additionally, compared with WT, DAP12KO macrophages had lower baseline TNF-α but similar IL-6, CXCL1, and CXCL2 production without treatment. Our findings indicate that after stimulation with physiologically relevant endogenous substances, production of inflammatory cytokines and chemokines by macrophages is regulated by DAP12, which may be at least in part due to alterations in their survival.

**DAP12 regulates expression of cytokines and chemokines by lung-resident macrophages during IRI in vivo**

Having shown that DAP12 impacts inflammatory responses by macrophages to endogenous substances in vitro, we next set out to examine how DAP12 regulates donor lung–resident macrophages in vivo. We transplanted WT CD45.2+ or DAP12KO CD45.2+ lungs into congenic CD45.1+ hosts and evaluated their abundance 2 h after reperfusion. We observed a significantly lower number of donor alveolar macrophages (CD45.2+CD11c−CD64−Siglec-F+CD11b+) (25) in the pulmonary graft when donor lungs lacked expression of DAP12 (Fig. 3A, 3B). Additionally, we performed immunostaining of cleaved caspase-3 and CD68 in WT and DAP12KO lungs that had been subjected to 18 h of cold ischemic storage. We did not observe colocalization of CD68 and cleaved caspase-3 in either experimental condition, raising the possibilities that DAP12KO macrophages die at earlier stages during cold ischemic storage or that signals associated with reperfusion may trigger the death of DAP12KO macrophages (Supplemental Fig. 3A). Consistent with published data, the abundance of alveolar macrophages in the lung was similar in WT and DAP12KO mice at baseline (data not shown) (26). We next flow cytometrically sorted donor macrophages from untreated and transplanted WT→WT and DAP12KO→WT lungs (Supplemental Fig. 1) and quantitatively analyzed gene expression of cytokines and chemokines that have been reported to contribute to acute lung injury. Compared to untreated controls, sorted alveolar macrophages from WT→WT transplanted lungs expressed significantly higher levels of IL-6, CXCL1, and CXCL2 (Supplemental Fig. 2D). In transplanted grafts, expression levels of TNF-α, CXCL1, and CXCL2 were significantly decreased in donor alveolar macrophages that were deficient for DAP12 (Fig. 3C). Because expression levels of chemokines and cytokines were normalized to β2-microglobulin, the observed decrease indicates a cell-intrinsic defect in expression of these genes rather than simply a decrease due to increased cell death. Thus, DAP12 regulates expression of cytokines and chemokines in lung-resident macrophages during pulmonary IRI.

**FIGURE 2.** DAP12KO BMDM have decreased survival and cytokine/chemokine production upon incubation with lung transplant lysate in vitro. (A) B6 WT and B6 DAP12KO BMDM viability following 17 h of incubation with B6 WT→B6 WT transplant lysate demonstrated as percentage annexin V+7-aminoactinomycin D (7AAD)+. Graph is representative of four separate experiments performed in duplicates with similar results. (B) Representative dot plots of B6 WT and B6 DAP12KO BMDM after 17 h of incubation with transplant lysate. (C) BMDM production of cytokines and chemokines as measured by ELISA following 17 h of incubation with B6 WT→B6 WT transplant lysate. IL-6, CXCL1, and CXCL2 were significantly decreased in DAP12KO BMDM in two of three independent experiments. Graph represents one experiment repeated independently three times performed at least in duplicate ± SEM. *p < 0.05, **p < 0.01 by two-way ANOVA.
Tissue infiltration of neutrophils into DAP12-deficient lungs is impaired

We and others have previously shown that neutrophils are critical mediators of IRI after lung transplantation (7). In fact, our group has previously reported that partial depletion of neutrophils results in significant improvement of graft function to a comparable degree to that observed when transplanting DAP12KO lungs (7). Because we have found that DAP12 expression in donor macrophages regulates production of neutrophil chemokines, we next set out to examine how DAP12 expression in lung grafts regulates neutrophil infiltration after transplantation. We first transplanted WT (CD45.2+) or DAP12KO (CD45.2+) lungs into congenic CD45.1+ hosts. Six hours after engraftment a similar number of recipient CD45.1+CD45.2+CD11c+Siglec-F+CD11b+ neutrophils had trafficked to WT and DAP12KO lungs (Fig. 4). To distinguish between intravascular and interstitial neutrophils, a PE-labeled neutrophil-specific Ab, anti-Ly6G, was injected i.v. 5 min prior to sacrifice as previously described (15). After processing of the tissue, cells were labeled with anti-Ly6G in allophycocyanin, allowing for differential labeling of intravascular neutrophils (PE+allophycocyanin+) and interstitial neutrophils (PE+allophycocyanin−). Significantly more neutrophils had undergone extravasation into DAP12-sufficient pulmonary grafts than into DAP12KO lungs (Fig. 4A, 4B). Of note, the vast majority of neutrophils in the native right lungs were located in the intravascular compartment (Supplemental Fig. 3B).

In vitro transmigration assays showed that WT and DAP12KO neutrophils migrated toward CXCL2 in a comparable manner (Supplemental Fig. 3C). Taken together, these results demonstrate that whereas neutrophils traffic to lungs during IRI regardless of their expression of DAP12, extravasation of neutrophils is impaired in the absence of DAP12.

Intravital imaging reveals a neutrophil extravasation defect in DAP12KO grafts that can be overcome by local neutrophil chemokine administration

To further evaluate how DAP12 expression in lung-resident cells regulates neutrophil behavior, we took advantage of our recently described method to image lungs in vivo by 2P microscopy (5). We

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**FIGURE 3.** Lower abundance of DAP12KO donor-derived macrophages and decreased expression of cytokines/chemokines following pulmonary IRI in vivo. (A) Representative dot plots of donor-derived alveolar macrophages (CD64+CD11c+Siglec-F+CD11b−) in graft tissue 2 h after B6 WT CD45.2→B6 WT CD45.1 or B6 DAP12KO CD45.2→B6 WT CD45.1 lung transplantation. Cells were first gated on CD45.2+CD45.1− cells as seen in Supplemental Fig. 1. (B) Graphical representation of donor macrophage numbers per milligram of tissue (n = 7/experimental group). *p < 0.05 by paired t test. (C) Expression of cytokines and chemokines in donor-derived, sorted alveolar macrophages (CD45.2+CD45.1−CD64+CD11c+Siglec-F+CD11b−) (n = 4/experimental group) was assessed by quantitative PCR. Results were normalized to β2-microglobulin and compared with the WT population in each experiment. *p < 0.05, **p < 0.01 by two-way ANOVA.
first transplanted WT lungs into syngeneic LysM-GFP mice that express GFP under the control of the LysM promoter (LysM-GFP) and started imaging the grafts 3 h after reperfusion. We and others have used these mice to track neutrophils in vivo (5, 18, 27). Quantum dots were injected to visualize the vasculature. Consistent with our previous report, we observed marked recruitment of neutrophils into these freshly reperfused WT lungs (5). During a 45-min imaging period we observed progressively increasing extravasation of neutrophils resulting in their tissue infiltration with formation of dynamic clusters (Fig. 5A, Supplemental Videos 1, 5). In contrast, whereas a similar number of neutrophils had migrated to DAP12KO lungs (Fig. 5B), neutrophils accumulated in the pulmonary vessels and did not migrate efficiently into the pulmonary interstitium (Fig. 5C, Supplemental Videos 2, 6). Dense accumulations of arrested neutrophils were found on vessel walls. Having shown that macrophages within lungs express DAP12, we next wanted to explore whether WT macrophages are able to restore extravasation of neutrophils in DAP12KO pulmonary grafts. For this purpose we administered WT BMDM into the donor bronchus of DAP12KO→LysM-GFP lung recipients immediately before reperfusion. When we imaged these grafts 3 h later, we observed a significantly increased level of neutrophil extravasation compared with DAP12KO grafts (Fig. 5A, 5C, Supplemental Videos 3, 7). Administration of DAP12KO BMDM into the airways of DAP12KO grafts resulted in enhanced neutrophil extravasation compared with untreated DAP12KO grafts. However, compared with DAP12KO grafts that received WT BMDM, neutrophil extravasation was significantly decreased after administra-

**FIGURE 4.** Recipients of DAP12KO lungs have decreased graft infiltration of neutrophils. (A) Representative dot plots of neutrophil gating in B6 WT CD45.2→B6 WT CD45.1 lung grafts (n = 7/experimental group). Neutrophils were gated on recipient CD45.2+CD45.1+ cells and identified as Ly6G+Gr1+. These cells were CD11b+ and had high side scatter. (B) Representative dot plot showing percentage of extravasated neutrophils in B6 WT CD45.2→B6 WT CD45.1 or B6 DAP12KO CD45.2→B6 WT CD45.1 and assessed based on their staining for PE-labeled Ly6G, which was injected i.v. into recipient mice prior to sacrifice. Intravascular neutrophils were Ly6G-PE+Ly6G-allophycocyanin whereas interstitial neutrophils were Ly6G-PE Ly6G-allophycocyanin. (C) Graphical representation of absolute numbers of neutrophils (left) and neutrophil extravascular (EV) versus intravascular (IV) distribution (right). Graphs represent seven separate experiments. **p < 0.01 by paired t test.
As we observed reduced expression of the neutrophil chemokine CXCL2 in DAP12KO macrophages in vitro and in vivo, we next set out to examine whether CXCL2 plays a role in neutrophil trafficking within reperfused lungs. We transplanted DAP12KO lungs into syngeneic LysM-GFP hosts and instilled recombinant CXCL2 or PBS into the donor bronchus immediately prior to reperfusion (Supplemental Videos 1–8). CXCL2 administration resulted in significantly enhanced extravasation of neutrophils compared with untreated or PBS-treated DAP12KO lungs (Figs. 5A, 5C, 6D–F, Supplemental Videos 4, 8). Neutrophil behavior in these grafts was comparable to that observed in WT and DAP12KO grafts that received WT BMDM or recombinant CXCL2 endobronchially immediately prior to reperfusion. Of note, consistent with our flow cytometric assessment (Fig. 4C), the neutrophil density was similar in WT and DAP12KO graft tissue, indicating that DAP12 expression in lungs does not impact neutrophil trafficking to the reperfused grafts (Fig. 5B). Thus, our data show that DAP12 expression by pulmonary resident cells regulates transendothelial migration of neutrophils into inflamed lungs. Furthermore, extravasation of neutrophils in DAP12KO lungs can be reconstituted to levels observed in WT grafts through administration of WT macrophages or a neutrophil chemokine.

Discussion
Our work has identified a novel role for DAP12 in mediating acute noninfectious lung injury. To our knowledge, this is the first study to describe that DAP12 expression in lung-resident cells regulates...
FIGURE 6. Treatment of DAP12KO lung grafts with DAP12KO BMDM or PBS. (A and D) Intravital 2P microscopy was initiated 3 h after transplantation into LysM-GFP hosts. Snapshots were taken of time points immediately after start of imaging and 45 min later showing B6 DAP12KO grafts that received DAP12KO B6 BMDM (A) or PBS (D) endobronchially immediately prior to reperfusion. Green shows LysM-GFP+ neutrophils; red, nontargeted quantum dots; blue, second harmonic generation signal. Scale bar, 50 μm. (B and E) Graphical representation of neutrophil numbers (per mm³) at 0, 30, and 45 min after initiation of imaging (n = 3–4 transplants/experimental group with two to five areas analyzed per transplanted lung). No statistically significant differences were detected between the four experimental groups at any time point. (C and F) Graphical representation of percentage of extravasated neutrophils at 0, 30, and 45 min after initiation of imaging (n = 3–4 transplants/experimental group with two to five areas analyzed per transplanted lung). Each dot in (B), (C), (E), and (F) represents a separate area of the lung graft (0 min, DAP12KO versus DAP12KO plus DAP12KO BMDM, DAP12KO plus WT BMDM versus DAP12KO plus DAP12KO BMDM, p < 0.05; 30 min, B6 versus DAP12KO plus DAP12KO BMDM, DAP12KO versus DAP12KO plus DAP12KO BMDM, DAP12KO plus WT BMDM versus DAP12KO plus DAP12KO BMDM, p > 0.05; 45 min, B6 versus DAP12KO plus DAP12KO BMDM, DAP12KO versus DAP12KO plus DAP12KO BMDM, DAP12KO plus WT BMDM versus DAP12KO plus DAP12KO BMDM, p < 0.05) (0 min, B6 versus DAP12KO plus PBS, DAP12KO plus CXCL2 versus DAP12KO plus PBS, p < 0.05; 30 min, B6 versus DAP12KO plus PBS, DAP12KO plus CXCL2 versus DAP12KO plus PBS, p < 0.05; 45 min, B6 versus DAP12KO plus PBS, DAP12KO plus CXCL2 versus DAP12KO plus PBS, p < 0.05). Data were analyzed with a two-way ANOVA with a Tukey posttest.
transendothelial migration of neutrophils during inflammation. Extending previous observations, we found that DAP12 regulates the survival of macrophages and their production of inflammatory molecules (26). Neutrophil migration out of the vasculature and into DAP12-deficient inflamed lungs was restored to levels observed after transplantation of WT grafts after intratracheal administration of CXCL2 or WT macrophages. Because DAP12 is expressed on macrophages, which reside in human lungs prior to transplantation, our observations are of potential clinical relevance in the setting of transplant-mediated IRI and other forms of acute lung injury.

Models employing ex vivo perfused rabbit and mouse lungs have suggested that pulmonary macrophages contribute to IRI (28, 29). Depletion of macrophages in isolated perfused mouse lungs through intratracheal administration of clodronate resulted in reduced expression of TNF-α, CXCL2, and the monocyte chemoattractant MCP-1. Others reported that global depletion of macrophages through i.p. administration of clodronate or elimination of CD11c+ cells with diphtheria toxin in CD11c-DTR mice attenuated IRI after transient ligation of the pulmonary vessels (30). Such treatment regimens resulted in reduced expression of proinflammatory cytokines and neutrophil chemokines in the injured lung and decreased neutrophil infiltration on histological evaluation. The authors inferred that alveolar macrophages orchestrated early inflammatory events in the setting of lung IRI, possibly through damage-associated molecular pattern (DAMP)-triggered TLR4 signaling. However, the experimental system did not allow the investigators to differentiate between lung-resident cells and cells that infiltrate the injured lung from the periphery.

The vascularized lung transplant model used in the present study, originally developed by our group, has several important advantages (13, 14, 17, 19). First, it represents a robust model of noninfectious pulmonary inflammation that mimics IRI after lung transplantation in humans. Similar to graft dysfunction following lung transplantation in humans, IRI results in an impairment of oxygen exchange in the murine model. Second, by using gene-deficient mice as donors or recipients, this model allows for an assessment of the contribution of lung-resident versus graft-infiltrating cells to lung injury. Such a differentiation may not be feasible in other models of inflammation, such as respiratory infections, chemical injury, or transient vascular occlusion, where both tissue-resident and graft-infiltrating cells share the same genotype (31). A previous study demonstrated that DAP12 expression on macrophages regulates their chemotaxis toward CXCL2 in smoke-exposed murine lungs (32). Furthermore, expression of DAP12 in neutrophils has been suggested to contribute to their adhesion to fibronectin (33). Based on these observations, we expected DAP12 deficiency in recipients to result in attenuation of IRI, because graft-infiltrating leukocytes mediate tissue damage. However, we were surprised that DAP12 expression did not regulate neutrophil chemotaxis and that recipient DAP12 expression did not impact graft function.

Previous studies have described that DAP12 signaling can regulate myeloid responses after TLR stimulation (34–36). These studies have yielded conflicting results, with some reports showing potentiation and others attenuation of inflammatory responses. For example, DAP12KO animals are more resistant to septic shock, induced by either i.p. injection of LPS or cecal ligation and puncture, than are WT mice (36). The increased survival of DAP12KO mice was associated with lower systemic levels of inflammatory mediators. Interestingly, recruitment of macrophages and neutrophils to sites of inflammation was comparable between WT and DAP12KO mice. These results were extended by a recent study using a model of dengue virus–triggered shock, which showed that activation of the DAP12-associated receptor MDL-1 on myeloid cells enhanced the production of TNF-α and NO (37). MDL-1-deficient mice were protected from death, providing further evidence that DAP12 can have deleterious consequences. In contrast, DAP12 deficiency in tissue-infiltrating macrophages results in higher production of inflammatory cytokines in response to stimulation with TLR agonists (34, 35, 38).

Variations in functional outcomes of DAP12 signaling have been attributed to engagement of distinct DAP12-associated receptors. It has been suggested that TREM2 mediates inhibitory signals via DAP12, whereas TREM-1 and MDL-1 enhance inflammatory responses. Furthermore, the quality of DAP12-mediated responses may be impacted by ligand avidity. Several DAMPs have been identified that are released during noninfectious tissue injury such as fractionated hyaluronic acid, HMGB-1, heat shock proteins, and haptoglobin (23, 24). These DAMPs can activate the innate immune system through stimulation of TLR2 and TLR4. It will be important to identify the DAP12-associated receptors and endogenous ligands that activate DAP12 signaling in the context of noninfectious inflammation such as IRI.

Recruitment of neutrophils and other leukocytes into inflamed tissues proceeds through a multistep cascade of events (39). Migrating cells are captured on vascular endothelial cells and undergo selectin-dependent rolling. Firm adherence to vessel walls, mediated through adhesion molecules and neutrophil chemokines expressed on endothelial cells, is followed by cells crossing the endothelial barrier. Molecular cues that regulate this process vary between tissues (40), but relatively little is known about their trafficking requirements within the lung. Our recently developed approaches to image murine lungs by intravital 2P microscopy (5, 17, 41) revealed that neutrophils, which infiltrate reperfused DAP12KO lung grafts, slow down, roll along vessel walls, and finally adhere to pulmonary vessels, but they cannot migrate into the tissue. In the present study we show that CXCL2 alone can induce transendothelial migration of neutrophils in inflamed lungs, extending observations previously made in other inflammatory models (42, 43). We found that administration of DAP12KO BMDM into DAP12KO grafts resulted in more neutrophil extravasation relative to untreated DAP12KO grafts. We speculate that this effect was due to higher overall numbers of macrophages within DAP12KO grafts that received DAP12KO BMDM. However, neutrophil extravasation under these conditions was significantly less when compared with DAP12KO grafts that received WT BMDM.

Previously, we observed a similar extravasation defect of neutrophils with blood monocyte depletion (5). These similarities raise several possibilities. DAP12 expression by lung-resident macrophages could regulate trafficking of monocytes, which then facilitates neutrophil transendothelial migration. Our finding that neutrophil extravasation can be induced with intratracheal CXCL2 would argue against this possibility. We rather speculate that blood monocytes and lung-resident macrophages synergistically regulate distinct steps that enable transendothelial neutrophil migration. One possible scenario is that graft-infiltrating monocytes induce DAP12-dependent expression of neutrophil chemokines by lung-resident macrophages.

In conclusion, our work has revealed that DAP12 on lung-resident macrophages mediates noninfectious tissue injury through facilitating the transendothelial migration of neutrophils. In the clinical setting, transplant-mediated IRI has a detrimental impact on both short- and long-term outcomes (3). As we have demonstrated that graft-infiltrating neutrophils can potentiate alloimmune responses, DAP12 expression in donor organs may provide a link between innate and adaptive immune responses, thereby impacting graft rejection (6). The recently developed approach to perfuse lungs ex vivo prior to transplantation may provide an opportunity to improve outcomes by targeting DAP12 in donor organs (44).
References


DAP12 on lung macrophages mediates ischemia reperfusion injury by promoting neutrophil extravasation


Online Data Supplement
Supplementary Movie 1. Neutrophil trafficking 3 hours after reperfusion in B6 LysM-GFP recipient of B6 WT lung.

Supplementary Movie 2. Neutrophil trafficking 3 hours after reperfusion in B6 LysM-GFP recipient of B6 DAP12KO lung.

Supplementary Movie 3. Neutrophil trafficking 3 hours after reperfusion in B6 LysM-GFP recipient of B6 DAP12KO lung that received WT BMDM endobronchially.

Supplementary Movie 4. Neutrophil trafficking 3 hours after reperfusion in B6 LysM-GFP recipient of B6 DAP12KO lung that received recombinant CXCL2 endobronchially.

Supplementary Movie 5. Neutrophil trafficking 3 hours and 45 minutes after reperfusion in B6 LysM-GFP recipient of B6 WT lung.

Supplementary Movie 6. Neutrophil trafficking 3 hours and 45 minutes after reperfusion in B6 LysM-GFP recipient of B6 DAP12KO lung.

Supplementary Movie 7. Neutrophil trafficking 3 hours and 45 minutes after reperfusion in B6 LysM-GFP recipient of B6 DAP12KO lung that received WT BMDM endobronchially.

Supplementary Movie 8. Neutrophil trafficking 3 hours and 45 minutes after reperfusion in B6 LysM-GFP recipient of B6 DAP12KO lung that received recombinant CXCL2 endobronchially.
Supplemental Figure 1. Gating strategy for sorting alveolar macrophages and endothelial cells. For sorting cells for qRT-PCR and for macrophage analysis alveolar macrophages were defined as CD45.2+CD45.1+CD64+CD11c+SiglecF+CD11b− and endothelial cells were defined as CD45.2+CD45.1−CD31+. 
Supplemental Figure 2. WT and DAP12KO macrophage characterization. A. Alveolar macrophages were sorted from either untreated lungs (pooled from five animals) or transplanted WT→WT lung grafts (n=4) two hours after reperfusion. DAP12 expression was measured by qRT-PCR and compared to β2Microglobulin. B. Untreated WT and 18 hour cold Ischemic storage lungs were stained for DAP12 (red), CD68 (green), and DAPI (blue). Arrowheads mark cells double positive for both the macrophage-specific marker CD68 and DAP12. Scale bar represents 50μm. C. WT BMDM were cultured without and with lung transplant lysate. DAP12 gene expression was analyzed by qRT-PCR and compared to β2Microglobulin. D. Sorted macrophages from untreated WT or transplanted WT were analyzed for expression of cytokines and chemokines. Results were normalized to β2Microglobulin and compared to the untreated control pooled from five different mice.
Supplemental Figure 3. Neutrophil characterization and cleaved caspase 3 analysis. A. WT or DAP12KO lungs were left untreated or subjected to 18 hours of cold ischemic storage. Frozen sections were stained with CD68 (green) to identify macrophages, cleaved caspase 3 (red), and DAPI counterstain. Scale bar represents 50 μm. B. Dot plot of native right lung after B6 WT CD45.2+→B6 CD45.1+ transplantation. Neutrophils were analyzed for intravascular vs. extravascular distribution based on staining for Ly6G-PE as described in Materials and Methods. C. Neutrophil transmigration in response to 0, 10, or 100 ng/ml CXCL2. Neutrophils (1E6) were added to the upper chamber of a 3 μm transwell with chemokine in the lower chamber. After two hours, numbers of migrated cells were counted. Data were analyzed with two-way ANOVA. Graph is representative of 3 independent experiments performed in triplicate with similar results.