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Resolution of Experimental Lung Injury by Monocyte-Derived Inducible Nitric Oxide Synthase


Although early events in the pathogenesis of acute lung injury (ALI) have been defined, little is known about the mechanisms mediating resolution. To search for determinants of resolution, we exposed wild type (WT) mice to intratracheal LPS and assessed the response at intervals to day 10, when injury had resolved. Inducible NO synthase (iNOS) was significantly upregulated in the lung at day 4 after LPS. When iNOS−/− mice were exposed to intratracheal LPS, early lung injury was attenuated; however, recovery was markedly impaired compared with WT mice. iNOS−/− mice had increased mortality and sustained increases in markers of lung injury. Adoptive transfer of WT (iNOS+/+) bone marrow–derived monocytes or direct adenoviral gene delivery of iNOS into injured iNOS−/− mice restored resolution of ALI. Irradiated bone marrow chimeras confirmed the protective effects of myeloid-derived iNOS but not of epithelial iNOS. Alveolar macrophages exhibited sustained expression of costimulating molecule CD86 in iNOS−/− mice compared with WT mice. Ab-mediated blockade of CD86 in iNOS−/− mice improved survival and enhanced resolution of lung inflammation. Our findings show that monocyte-derived iNOS plays a pivotal role in mediating resolution of ALI by modulating lung immune responses, thus facilitating clearance of alveolar inflammation and promoting lung repair. The Journal of Immunology, 2012, 189: 000–000.

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cute lung injury (ALI) remains an important clinical problem, affecting >190,000 patients annually in the United States, with an estimated 75,000 deaths/year (1). Despite extensive investigation of the underlying mechanisms in ALI pathogenesis, therapy remains mainly supportive, with only lung-protective ventilation offering survival benefits (2).

The pathogenesis of ALI is notable for the activation of inflammatory cells, including neutrophils and alveolar macrophages (AMs), with increased production of pro- and anti-inflammatory mediators. An acute exudative phase, lasting for up to 5–7 d, is manifest pathologically as diffuse alveolar damage, with protein-rich edema fluid, neutrophilic infiltration of the interstitium and airspaces, and variable amounts of hyaline membranes (3). Following the acute phase of ALI, some patients progress to a fibro-proliferative phase (4–6), which has been correlated with an increased risk for death (7). Although it has long been recognized that ALI may evolve through these stages, investigation has largely focused on defining and targeting early steps in the pathogenesis. Little attention has been devoted to identification of the mechanisms responsible for resolution of lung injury.

NO has been implicated in the pathophysiology of ALI in animals and humans (8–13). NO participates in the regulation of every organ system in normal and pathologic situations (14, 15). Although NO in the normal lung is produced by the constitutive isoforms of NO synthase (neuronal NO synthase [nNOS] and endothelial NO synthase [eNOS]), its production during inflammation is mainly due to the inducible isoform (inducible NO synthase [iNOS]) (16). In the lung, the major cells expressing iNOS are AMs, alveolar epithelial cells, and inflammatory infiltrating cells (17).

ALI and sepsis are associated with increased iNOS-derived NO (18–20). Several groups noted that deletion or inhibition of iNOS in mice is protective from LPS-induced mortality (21, 22), indicating a proinflammatory role for iNOS. Other investigators found that iNOS deletion had no effect (23). NO can contribute to microvascular injury, pulmonary edema, and neutrophilic infiltration in mouse models of sepsis (22, 23), with variable effects on mortality. However, NO can also have beneficial effects on the immune system (24), acting as an antimicrobial (25–28) or anti-inflammatory/immunosuppressive agent (29–32), and it can modulate signaling pathways, including NF-κB (33–36).

In contrast to the effect on acute responses, little is known about the impact of iNOS on the resolution of lung inflammation and injury. We examined the effects of iNOS deletion on the resolution of lung injury in a mouse model of LPS-induced ALI. We found that iNOS−/− mice had a biphasic response, with reduced severity of lung injury on day 1 after intratracheal (i.t.) LPS, but with a subsequent marked delay in ALI resolution, indicating a previously

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inundated role for iNOS in lung repair. The defect in resolution in iNOS−/− mice was abrogated by adoptive transfer of naïve CD11b+ iNOS−/− monocytes or direct adenoviral delivery of iNOS to the lung. Furthermore, epithelial-derived iNOS did not contribute significantly to the resolution of ALI. We found that macrophage-derived iNOS regulates CD86 expression, which actively participates in the regulation of lung inflammation. Monocyte-derived iNOS plays a pivotal role in the resolution of lung inflammation and lung repair.

Materials and Methods

Animals

Male C57BL/6 wild type (WT) mice, 8–10 wk old and C57BL/6 background iNOS−/− male mice and congenic CD45.1 male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed at the Johns Hopkins University Asthma and Allergy Center, and experiments were conducted under a protocol approved by the Johns Hopkins Animal Care and Use Committee.

Animal preparation

Using our published model (37), mice were anesthetized i.p. with ketamine/acepromazine (150/13.5 mg/kg) before exposure of the trachea. Escherichia coli LPS (055:B5 Sigma-Aldrich 1.2880; 3.75 μg/mouse weight) or sterile water (control) was then instilled i.t. (total volume 35 μl) via a 20-gauge catheter. At 1, 4, 7, and 10 d after instillation, groups of mice were anesthetized i.p. with ketamine/acepromazine and euthanized by exsanguination from the inferior vena cava. The lungs were perfused with 1 ml PBS, and bronchoalveolar lavage (BAL) was performed on the right lung; the left lung was processed for histology. BAL samples were routinely cultured to assess for bacterial infection.

Macrophage cell lines

An AM immortalized cell line (MH-S; American Type Culture Collection) was cultured in six-well plates until 80% confluence before experimental challenge. Peritoneal macrophages were lavaged from C57BL/6 WT mice (The Jackson Laboratory) 6 d after injection of 3 ml sterile thioglycollate (Sigma-Aldrich). Cells were allowed to adhere overnight in RPMI 1640 supplemented with 10% heat-inactivated low-LPS FBS and 1% penicillin-streptomycin/glutamine before use. Cells were harvested by gentle scraping and resuspended in a single-cell suspension for flow cytometric staining.

Analysis of BAL fluid

BAL fluid was obtained by cannulating the trachea with a 20-gauge catheter. The right lung was lavaged with two aliquots of 0.7 ml PBS without calcium. BAL fluid was centrifuged at 700 × g for 10 min at 4°C. The cell-free supernatants were stored at −80°C until further analysis. The cell pellet was diluted in PBS, and total cell number was counted with a hemocytometer after staining with Turk’s Stain Solution (Merck, Tokyo, Japan). Cell differentials (300 cells/sample) were counted on a cytocentrifuge preparation with Diff-Quik stain (Baxter Diagnostics, McGaw Park, IL). Total protein and albumin were measured in the cell-free supernatant using bichromatic acid assay (Pierce, Rockford, IL) and a mouse serum albumin ELISA kit (Alpha Diagnostic International, San Antonio, TX), respectively.

Measurement of cytokines

The levels of TNF-α, IL-10, MIP-2, and activated TGF (TGF-β1) were measured in BAL fluid by ELISA (R&D Systems, Minneapolis, MN).

Lung histology, immunofluorescence, and lung injury scoring

Lungs (n = 5/time point) were inflated to a pressure of 25 cm H2O using 1% low-melting agarose (Invitrogen, Carlsbad, CA) for histologic evaluation by H&E staining (38). iNOS expression was assessed as previously described (13). Left lungs were fixed in 10% formalin neutral buffer for 8–10 h, embedded in paraffin, and sectioned. Briefly, for lung immunofluorescence, frozen sections were fixed with 4% paraformaldehyde, permeabilized with Triton X-100 (Fisher), and subsequently blocked with 10% donkey serum. Tissue sections were then incubated with FITC-conjugated iNOS mAb (BD Biosciences) for 1 h and then washed three times with cold PBS. Coverslips were mounted using Fluoromount-G (Southern Biotech) and analyzed using a fluorescent microscope.

For lung injury scoring, two blinded investigators analyzed the samples and determined the levels of lung injury according to the semiquantitative scoring system outlined below. All lung fields (>20 magnification) were examined for each sample. Assessment of histological lung injury was performed using the following scoring: 1, normal; 2, focal (<50% lung section) interstitial congestion and inflammatory cell infiltration; 3, diffuse (>50% lung section) interstitial congestion and inflammatory cell infiltration; and 4, focal (<50% lung section) consolidation and inflammatory cell infiltration. The mean score was used for comparison between groups.

Immunoblotting

To assess the relative abundance of NO synthase isomers (iNOS, eNOS, and nNOS) during the acute and resolution phases of lung injury, we performed immunoblots of lung homogenates, as described (39). Thirty-two micrograms of total protein/sample in 1.5% (w/v) SDS was resolved on 8% SDS-PAGE gels; transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA); incubated with Abs to iNOS, total eNOS (BD Pharmingen), phosphorylated eNOS (Cell Signaling, Danvers, MA), and nNOS (BD Pharmingen); and detected using ECL Plus (Amersham, Piscataway, NJ). Equivalence of samples was confirmed using β-actin (Sigma-Aldrich) as a loading control. Relative band intensities were determined by densitometry using UN-SCAN-IT imaging software (Bio-Medience, Orem, UT).

Measurement of reactive oxygen species generation

Reactive oxygen species (ROS) were monitored by measurement of H2O2 generation, based on the oxidation of 2′,7′-dichlorodihydrofluorescein (Sigma-Aldrich) to fluorescein (DCF) or 2′,7′-dichlorodihydrofluorescein (DCF) dye to fluorescence (DCFH) by BAL. AMs were incubated with 100 μM DCF reagent for 20 min at room temperature. Lung tissue was homogenized using a Polytron homogenizer in 1 ml homogenization buffer containing 0.25 M sucrose, 1 mM EDTA, 1 mM sodium azide, and protease inhibitors (Complete Protease Inhibitors; Roche). One hundred microliters of lung homogenate was incubated with 100 μl Griess reagent for 20 min at room temperature. Absorbance was measured at 570 nm using a microreader, and NOx concentration was assessed against a sodium nitrite standard curve (0–100 μM analyzed in duplicate).

Phagocytic index

AMs were obtained from WT and iNOS−/− mice by BAL. AMs (105/well in 5% FCS) were allowed to adhere in eight-well tissue culture-treated plastic slides (Lab-Tek) for 30 min. The cells were washed gently, and latex microbeads labeled with FITC (1.7 mm in diameter; Polysciences) were added to the wells (8 × 105 beads/well). After 1 h of incubation at 37°C, the wells were washed gently with PBS, fixed with methanol at −20°C for 20 min, and washed extensively.

The cells were viewed by a blinded observer using a Nikon fluorescent microscope. The fraction of cells containing labeled beads and the phagocytic index (PI) were determined by counting ≥200 cells in each well in random high-power fields. PI was calculated as follows: PI = fraction of AM with beads × mean number of beads/positive AM. Three replicate wells were evaluated for each condition.

Bone marrow isolation, adoptive transfer, and chimeras

iNOS−/− and CD45.1 mice were used to harvest bone marrow (BM) cells, obtained from femurs and tibias by flushing with 50 ml PBS through a 26-gauge needle. After centrifuging, RBCs were lysed, and the remaining cells in RPMI 1640 medium were counted. CD11b+ cells were isolated from the BM cells using magnetic bead separation with CD11b MicroBeads (Miltenyi Biotec, Auburn, CA). Magnetically retained CD11b+ cells were eluted, and the purity was checked using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The purity of CD11b+ BM cell fractions was always ≥99%. Purification of donor BM cells with magnetic
CD11b beads yielded isolated CD11b+ cells: ∼40% monocytes and 60% neutrophils. Each single, purified cell suspension (25–30×10^6 cells in 200 μl PBS) was adoptively transferred into iNOS−/− mice (n = 4–5) at the designated time after i.t. LPS via tail vein injection, and samples were harvested at days 2, 4, and 10 after i.t. LPS. Mice were separated into the following groups: WT without adoptive transfer, PBS 200 μl into iNOS−/− mice, WT BM into iNOS−/− mice, and iNOS−/− BM into iNOS−/− mice. Recipient mice, B6.SJL-Pippa Pep3b/BoyJ (congenic CD45.1 WT), and iNOS−/− mice (6–7 wk of age), were lethally irradiated with one exposure to 9 Gy (Gammaxcell 40; Atomic Energy of Canada, Ottawa, ON). Immediately after irradiation, 2 × 10^6 BM cells were transferred via retro-orbital injection. Mice were housed in microisolators for 8 wk before ALI challenge and fed autoclaved food and water containing 5 mM sulfamethoxazole and 0.86 mM trimethoprim. Reconstitution was assessed by flow cytometry.

Flow cytometry

Cells were first incubated with Fe-Block-2.4G2 (BD Pharmingen) Ab to block Fe-RIII/B before staining with a specific Ab. The following Abs were purchased from BD Pharmingen (San Diego, CA) and BioLegend: anti–annexin V–PE, anti–7–aminocoumarin D (7–AAD), anti–LY6G–FITC, anti–IA-1E–PE, anti–CD11b–allophycocyanin-cy780, anti–CD45.1–PE, anti–CD45.2–PerCy5.5, anti–CD14–FITC, anti–CD86–allophycocyanin, anti–CD80–PE, and anti–I–A–I–F–allophycocyanin; relevant isotype Abs were also purchased. Monocytes, AMs, and lymphocytes were gated with characteristic forward scatter/side scatter using a FACSaria instrument, with Cell Diva used for data acquisition (Becton Dickinson, San Jose, CA) and FlowJo used for analysis (Tree Star, San Carlos, CA).

Cell sorting

WT and iNOS−/− mice were sacrificed 4 d after i.t. LPS. A 20 gauge catheter was used to instill 1 ml dispase (Roche), followed by 0.5 ml 1% liquid agarose, which was solidified with ice. The lungs were removed and enzymatically digested with dispase (Roche), followed by 0.5 ml 1% collagenase with the addition of 1× PBS. The lungs were enzymatically digested until single cells were observed, and filtered through a 70-μm cell strainer (Becton Dickinson). CD11b–FSC− (monocytes) and CD11b+CD11c+ (lung epithelial cells) subsets were isolated by FACS using a Becton Dickinson FACSaria machine. After cytopsin, cells were fixed with methanol, permeabilized with Triton X, stained with mouse iNOS–FITC (BD Biosciences) for 1 h, and analyzed by fluorescent microscopy.

Adenoviral iNOS gene construct and delivery

To generate an adenovirus expressing red-shifted GFP-tagged iNOS (NOS2-gsGFP), we obtained the vector pCDNA3-lneo-iNOS-rsGFP from Dr. N.T. Eisen (Baylor College of Medicine, Houston, TX) and subcloned the cDNA for the tagged iNOS, and transferred iNOS–rsGFP into the shuttle vector pAdCMV-DEST-V5 (Invitrogen). Replication-defective virus was prepared by transfecting pAdCMV-DEST-NOS2-gsGFP into HEK293A cells. Extracts from cultures showing cytopathic effects were collected and purified using the Adenopure Filter system (Puresyn). Control virus was constructed similarly. Viral titers are determined in HEK293A cells by counting GFP+ cells after transfection. Virus infectivity is determined in HEK293A cells by counting GFP+ cells after transfection.

Statistical analysis

All values are reported as mean ± SEM. Parametric or nonparametric testing was performed as indicated. Results between groups were compared using the Mann–Whitney U test. Baseline and pre- and posttreatment data within a group were compared using repeated-measures one-way ANOVA (Fisher protected least-significant difference test). The survival curve was established with Kaplan–Meier survival analysis. A p value < 0.05 was used as the cut-off point for significance.

Results

Resolution of lung injury is impaired in iNOS−/− mice

We examined the response of WT and iNOS−/− mice to i.t. LPS over 10 d. Survival was significantly reduced in iNOS−/− mice, which was evident after day 5 (Fig. 1A). Surviving WT and iNOS−/− mice appeared ill, with lethargy, piloerection, and huddling behavior after i.t. LPS. These clinical signs resolved by day 5–6 in WT mice but persisted in iNOS−/− mice. In addition, although both WT and iNOS−/− mice lost weight after i.t. LPS, WT mice regained the lost weight by day 10, whereas weight loss was sustained in iNOS−/− mice (Fig. 1B). BAL protein (Fig. 1C) and albumin (Fig. 1D) increased in both groups to more than four to eight times the baseline levels by day 1. In WT mice, BAL protein and albumin peaked at day 4 and returned to baseline by day 10. In iNOS−/− mice, BAL protein and albumin continued to increase until day 7 and remained elevated at day 10, suggesting impaired resolution of alveolar capillary injury.

On day 1 after i.t. LPS, BAL total cell count was lower in iNOS−/− mice than in WT mice, whereas it was higher in iNOS−/− mice on days 7 and 10 (Fig. 1E). BAL macrophages increased in both groups, but they remained higher in iNOS−/− mice on days 7 and 10 (Fig. 1F). Lymphocytes increased similarly in both WT and iNOS−/− mice at day 4 and remained elevated through day 10 (Fig. 1F). BAL neutrophil numbers were significantly lower in iNOS−/− mice than in WT mice on day 1 after i.t. LPS (Fig. 1F), but the neutrophil number was increased in iNOS−/− mice on days 7 and 10. In WT mice, neutrophils were nearly undetectable in the alveolar compartment by day 10 after i.t. LPS. The pattern of histological changes in the lung was consistent with patterns for BAL protein and cells (Fig. 1G). Specifically, interstitial thickening and cellular infiltration were worse in WT mice at day 1 compared with iNOS−/− mice. However, at day 7 the histological changes were more pronounced in iNOS−/− mice than in WT mice. By day 10, lung histology appeared normal in the WT mice, yet histological changes persisted in iNOS−/− mice. Blinded lung injury scoring confirmed a biphasic injury response in iNOS−/− mice; despite an attenuated early injury, iNOS−/− mice displayed a significant impairment in the resolution of lung inflammation (Fig. 1H). These observations suggest an important role for iNOS in the resolution of lung injury.

iNOS expression and source during ALI

We sought to examine the patterns of NO generation in lung and BAL fluid using the Griess reaction, which measures total nitrate and nitrite (NOx). Both BAL (Fig. 2A) and lung NOx (Fig. 2B) were significantly reduced in iNOS−/− mice compared with WT mice at all time points. In WT mice, NOx production peaked at day 4 after i.t. LPS in both BAL and lung homogenate. Protein immunoblots revealed that iNOS had increased 5-fold in the lung of WT mice on day 4 after i.t. LPS, but it had returned to baseline levels by day 7 (Fig. 2C, 2D). iNOS protein was not detectable in iNOS−/− mice. The pattern of expression of total eNOS and phosphorylated eNOS was not different between WT and iNOS−/− mice at any time point (Supplemental Fig. 1A). NOS labeling was higher in iNOS−/− mice at day 4 after LPS and was similar to WT mice at all other time points. iNOS labeling was evident by immunofluorescence in alveolar cells only from WT mice on day 4 after i.t. LPS. No iNOS labeling was detected in injured iNOS−/− mice (Supplemental Fig. 1B). To better define cellular sources for iNOS during lung inflammation, we harvested lungs from WT and iNOS−/− mice 4 d after i.t. LPS. Lungs were minced and enzymatically digested to obtain a single-cell suspension. Lung cells were surface stained with markers for macrophages (CD11b) and epithelial cells (CD326) and subsequently sorted using a FACSaria cytometer. WT CD11b+CD326+ macrophages showed intense iNOS labeling by fluorescent microscopy, whereas iNOS−/− macrophages did not show any labeling. WT CD326+CD11b+ epithelial cells also labeled positive for iNOS; in contrast, iNOS−/− epithelial cells did not show any labeling (Fig. 2E). These data
indicate that iNOS is upregulated in inflammatory macrophages and lung epithelial cells during LPS-induced lung inflammation.

**Alveolar inflammatory milieu is altered in iNOS$^{-/-}$ mice**

To begin examining the mechanisms that might contribute to the difference in LPS response in iNOS$^{-/-}$ mice, we measured select BAL cytokines in the two groups. The proinflammatory cytokine TNF-α was elevated in both WT and iNOS$^{-/-}$ groups on day 1 after LPS (Fig. 3A), but it was greater in iNOS$^{-/-}$ mice on days 4, 7, and 10. The neutrophil chemokine MIP-2 was not different between WT and iNOS$^{-/-}$ mice on day 1 after i.t. LPS; however, it was greater in iNOS$^{-/-}$ mice on day 4 (Fig. 3B). Active TGF-β1 increased similarly in both groups on day 4 after i.t. LPS, but it only remained elevated on days 7 and 10 in iNOS$^{-/-}$ mice (Fig. 3C). The anti-inflammatory cytokine IL-10 increased in both groups on day 1 after i.t. LPS and was not different between the two groups at any time (Fig. 3D).

**Alveolar neutrophil clearance is impaired in iNOS$^{-/-}$ mice after ALI**

The persistent elevation of neutrophils in iNOS$^{-/-}$ mice on days 7 and 10 after i.t. LPS was striking, in contrast to WT animals in which alveolar neutrophils were almost completely cleared. iNOS$^{-/-}$ mice displayed higher levels of neutrophil chemokine MIP-2 (Fig. 3B) by day 4 and had persistent disruption of their alveolar capillary membrane (elevated BAL albumin; Fig. 1D),
suggesting roles for barrier disruption and chemokine gradients in neutrophil recruitment. We also evaluated neutrophil apoptosis and macrophage efferocytosis as potential contributors to the increase in alveolar neutrophils. To determine whether changes in apoptosis may contribute to the differences in neutrophil number, we harvested BAL cells at intervals after i.t. LPS and assessed annexin V and 7-AAD labeling of neutrophils by flow cytometry. On day 1 after LPS, apoptosis (annexin V+, 7-AAD<sup>-</sup>) (Fig. 4) was not different between the groups. By day 4, apoptosis was significantly greater in neutrophils of WT mice compared with iNOS<sup>−/−</sup> mice, and it remained elevated at day 7. By day 10, neutrophil apoptosis was similar between the two groups.

We also examined AM phagocytosis using BAL macrophages harvested at intervals after i.t. LPS. Phagocytosis by AMs from iNOS<sup>−/−</sup> mice was significantly lower than macrophages from WT mice on days 1 and 4 after i.t. LPS (Supplemental Fig. 1C). The PI was not different in the two groups on days 7 and 10 after i.t. LPS.

The absence of iNOS leads to sustained AM activation after LPS

The marked delay in the resolution of lung inflammation in iNOS<sup>−/−</sup> mice, coupled with the persistent elevation of BAL TNF-α, led us to examine phenotypic differences between AMs in WT and iNOS<sup>−/−</sup> mice. Using multiparameter flow cytometry, we observed that CD11b<sup>+</sup>CD11c<sup>−</sup> AMs in WT and iNOS<sup>−/−</sup> mice exhibited a similar expression of accessory and costimulatory molecules CD40, CD86, and MHC class II (MHC-II) (Fig. 5A). Cell surface expression of costimulatory molecule CD80 (data not shown) and TLR2 and TLR4 was similar between the two groups at all intervals after i.t. LPS (Fig. 5A). The mean fluorescence intensity (MFI) of macrophage cell surface molecules was compared between WT and iNOS<sup>−/−</sup> mice (<i>n</i> = 4 animals/interval/group) (Fig. 5B) and reinforces the differences noted in the graphs (Fig. 5A).

We also sought to determine whether CD11b<sup>+</sup>CD11c<sup>+</sup> monocytes were functionally different between the groups. Intracellular production of ROS, as measured by DCF, was elevated in both
WT and iNOS<sup>−/−</sup> monocytes at day 1, but it was significantly higher in iNOS<sup>−/−</sup> monocytes by day 4 (Fig. 5C). We also assessed thioglycollate-elicited peritoneal macrophages (1 × 10<sup>6</sup> cells/well) from WT and iNOS<sup>−/−</sup> mice following stimulation with LPS (100 ng/ml). Supernatant TNF-α was higher in the LPS-activated WT macrophages compared with iNOS<sup>−/−</sup> macrophages at 6 and 24 h after LPS. In contrast, by 48 h, WT macrophages exhibited a significant reduction in TNF-α secretion, whereas iNOS<sup>−/−</sup> macrophages had persistently elevated levels of TNF-α, comparable to their 24-h secretion profile (Fig. 5D). MIP-2 levels were similar in both groups at every interval (Supplemental Fig. 1D).

These findings indicate a role for iNOS in modulating AM phenotype and function.

**Delivery of iNOS restores resolution of ALI in iNOS<sup>−/−</sup> mice**

Our findings suggest that iNOS plays an important role in the resolution of lung injury and that AMs are a potential cellular source of iNOS. To further assess a potential role for monocyte/
macrophages in this response, we performed adoptive transfer of BM-derived CD11b+ monocytes into iNOS−/− mice and examined the effect on the resolution of lung injury. iNOS−/− mice receiving tail vein injections of PBS or iNOS−/− BM-derived CD11b+ cells exhibited delayed resolution of lung injury and decreased survival. In contrast, iNOS−/− mice receiving WT iNOS+/+CD11b+ BM-derived cells delivered 24 h after i.t. LPS had improved survival (Fig. 6A). Flow cytometry confirmed that following adoptive transfer, congenic donor BM monocytes (CD45.1+) trafficked to the alveolar compartment and lung interstitium after LPS-induced ALI (Supplemental Fig. 2A). Donor CD45.1+F4/80+CD11b+ macrophages peaked in the alveolar and interstitial compartment at day 4 after adoptive transfer and injury (data not shown).

iNOS−/− mice receiving BM-derived iNOS+/+CD11b+ cells had higher NOx levels in the BAL (Fig. 6B) and lung homogenate on day 4 after adoptive transfer than did iNOS−/− mice receiving PBS or iNOS−/− CD11b+ cells (Supplemental Fig. 2B), confirming that adoptive transfer of WT CD11b+ cells led to increased local NO production in the alveolar space. iNOS−/− mice receiving iNOS+/+CD11b+ cells exhibited complete resolution of histological injury by day 10 (Fig. 6C, 6D). BAL protein (Fig. 6E), total cell count (Fig. 6F), and BAL neutrophils (Fig. 6G) were elevated on day 10 in iNOS−/− mice receiving PBS or iNOS−/− CD11b+ cells, but they were reduced nearly to WT levels following transfer of iNOS+/+CD11b+ cells. Mice receiving PBS or iNOS−/− BM cells had elevated BAL TNF-α levels through day 10 after i.t. LPS, whereas transfer of BM iNOS+/+CD11b+ cells resulted in a reduction of BAL TNF-α to WT levels (Fig. 6H).

To test the potentially therapeutic effects of iNOS, we challenged iNOS−/− mice with i.t. LPS; after 48 h of established injury, animals were assigned to one of two treatment modalities: pharyngeal aspiration of a single dose of adenovirus encoding GFP driven by a CMV promoter (Ad5CMV-GFP) or an adenovirus encoding the human iNOS tagged to GFP (Ad5CMV-hiNOS-GFP). We measured outcomes at day 7 after i.t. LPS. Direct lung transgene delivery was confirmed by flow cytometry. Differential alveolar cell transduction of adenovirus was detected by GFP fluorescence (Supplemental Fig. 2C; macrophages > neutrophils.

**FIGURE 6.** Adoptive transfer of WT monocytes restores resolution of ALI in iNOS−/− mice. iNOS−/− mice were challenged with i.t. LPS; 24 h after injury they were given PBS (sham) or BM-derived CD11b+ monocytes isolated from iNOS+/+ or iNOS−/− mice by tail vein injection. (A) Survival curves for designated groups (n = 8–10/group). (B) BAL NOx levels 4 d after i.t. LPS after designated transfers into iNOS−/− mice. Mean ± SEM. Lung H&E-stained sections (×20 for panoramic view, ×100 for insets) (C) and histopathological lung injury scoring (D) were assessed 10 d after i.t. LPS (n = 6/group). BAL fluid protein (E), BAL fluid total cell counts (F), BAL fluid neutrophils (as a percentage of total BAL cells) (G), and BAL fluid TNF-α levels (H) were measured 10 d after i.t. LPS are shown after designated transfers into injured iNOS−/− mice. *p < 0.05 versus PBS to iNOS−/−, ***p < 0.05 versus WT BM to iNOS−/−.
> lymphocytes). In contrast, unstimulated animals had no transduction of AMs (data not shown), whereas lung parenchyma was effectively transduced in the absence of lung inflammation. Epithelial transduction increased when inflammation was present (Supplemental Fig. 2D). We found that in vivo transgene delivery of iNOS given as rescue therapy (48 h after LPS) produced significant transduction of AMs and drove resolution of lung inflammation in iNOS−/− mice. Furthermore, delivery of adeno-hiNOS-GFP downregulated the expression of macrophage costimulatory molecules CD86 and CD40 (Fig. 7C). In contrast, adeno-GFP had no effect on these cellular and molecular targets, and it did not alter the sustained expression of macrophage CD86 and CD40. In summary, direct delivery of iNOS to the air space rescued injured iNOS−/− mice and promoted resolution of ALI.

**Myeloid, but not epithelial-derived iNOS modulates resolution of lung injury**

We showed that iNOS is upregulated in inflammatory alveolar and lung epithelial cells during inflammation in WT animals. To evaluate the relative contributions of myeloid and epithelial-derived iNOS, we performed irradiated BM chimeras between congeneric WT (CD45.1) and iNOS−/− mice. Transfer of WT BM into irradiated WT mice was used as control; this group achieved complete resolution of i.t. LPS-induced lung injury by day 11. We noticed that, in irradiated animals, the injury response was shifted after i.t. LPS, as evidenced by their peak weight loss ∼6 d after LPS compared with 4 d in nonirradiated animals (data not shown). Similarly, WT BM transfer into irradiated iNOS−/− mice achieved almost complete resolution of ALI, as evidenced by the reduction in BAL total cells and protein (Fig. 8A). These mice had myeloid iNOS but no epithelial-derived iNOS. In marked contrast, the transfer of iNOS−/− BM into irradiated WT mice resulted in sustained alveolar lung inflammation even 11 d after i.t. LPS, with elevated BAL cell counts and protein, as well as persistent histological lung injury (Fig. 8). This group had epithelial iNOS but no myeloid iNOS.

These results are consistent with our adoptive-transfer experiments using BM-derived monocytes and indicate that epithelial-derived iNOS does not play an important role in the resolution of experimental lung injury after LPS.

**iNOS regulates CD86 expression to regulate lung inflammation**

We sought to determine whether sustained CD86 expression in macrophages contributed to the persistent lung inflammation seen in injured iNOS−/− mice. To avoid attenuating the initial inflammatory response to i.t. LPS and, thus, altering the primary injury, we administered i.p. injections of monoclonal anti-CD86 (250 μg/mouse/dose; Bio X Cell) or isotype Ab (rat IgG; Sigma-Aldrich) 2 d after LPS exposure and again on days 4 and 6. Seven days after i.t. LPS, survival was improved by 33% in anti-Cd86–treated iNOS−/− mice (Fig. 9A, 9B versus 60% in isotype-treated group). BAL protein (Fig. 9B) and total cell counts (data not shown) were markedly attenuated in the anti-Cd86 group. Lung histology (Fig. 9C) showed enhanced resolution of lung inflammation in iNOS−/− mice that received anti-CD86 compared with the isotype-treated mice (Fig. 9C, 9D). We confirmed blockade of CD86 of mononuclear cells in the lung by flow cytometry (data not shown).

To confirm that iNOS modulates macrophage CD86 expression, we challenged an AM cell line (MH-S) with LPS in the presence or absence of a specific iNOS inhibitor (1400W); we analyzed cell surface expression for CD86 by flow cytometry at 24 and 48 h. Density plots showed that, although CD86 expression on the cell surface of CD11b+ macrophages was attenuated 24 h after LPS in the iNOS-inhibited (1400W) cells, CD86 expression was higher in the same group when analyzed at 48 h (Fig. 9E, 9F). To evaluate the ex vivo macrophage response, we isolated and plated naive peritoneal macrophages from WT and iNOS−/− mice and then stimulated them with LPS. A group of WT macrophages was treated with 1400W. Cells were surface stained for CD86 expression at specific intervals. iNOS−/− and WT macrophages treated with 1400W had higher expression of CD86 at 24 h compared with WT macrophages (Fig. 9G). iNOS−/− macrophages had sustained CD86 expression after 48 h of LPS stimulation, whereas the WT and WT 1400W–treated cells returned back to baseline expression. Our findings support iNOS as a key regulator of macrophage CD86 expression during lung inflammation.

**Discussion**

NO has been implicated in the pathogenesis of lung inflammation and injury, principally as having a deleterious role in early injury. The role of iNOS as a proinflammatory mediator in the lung has been described in numerous animal models, including OVA-induced airway inflammation, carrageenan-induced pleuritis (40), ventilator-induced lung injury (41), direct infectious or LPS-induced ALI (12, 42, 43), and sepsis or indirect LPS-induced ALI (10, 19, 21, 22). Consistent with reports of the proinflammatory role of iNOS (44), we found, in our established model of i.t. LPS-induced lung injury, that the absence of iNOS attenuated early injury. In marked contrast, the absence of iNOS significantly delayed resolution of lung inflammation. Our findings reinforce the need to distinguish early from late responses when seeking a role for a specific mediator or pathway during an inflammatory response.

In this model, lung injury progressed and peaked on day 4 after LPS. At that time we observed an induction of iNOS and NOx in the alveolar space, after which resolution of lung inflammation proceeded. As described by other investigators (45), we believe that early proinflammatory events are important for induction of repair mechanisms. Repair was significantly delayed in the absence of iNOS. Similarly, we recently described a pivotal role for alveolar regulatory T cells (Tregs) in the resolution of experimental lung injury, which peaked on days 4–7 after ALI (37). Potential interactions between iNOS and Tregs in this response are the subject of ongoing investigation.

The role of iNOS in inflammation and infection can vary, depending on the setting, the organ involved, the cell type, and/or the stage of the inflammatory response, ultimately enhancing inflammation or abrogating it (46). Kristof et al. (10) found that iNOS−/− mice displayed attenuated ALI in a murine model of septic shock. Similarly, Shanley et al. (44) found that exogenous
NO increased inflammation and vascular leak and that the absence of iNOS was associated with an attenuated acute inflammatory response to i.t. endotoxin. However, a few reports indicate a more complex role for iNOS in injury responses (sequestration, adhesion, and activation of neutrophils in the lung endothelium) (47). For example, iNOS-deficient mice are resistant to LPS-induced hypotension, but they suffer as much LPS-induced liver damage as do their WT counterparts (22).

We observed a delay in epithelial–endothelial barrier repair in iNOS−/− mice compared with WT mice, as evidenced by the persistently elevated alveolar protein and albumin. Both iNOS expression and the production of TGF-β play critical roles in vascular function during inflammatory responses; however, an imbalance can lead to pathology (48). In WT mice, active alveolar TGF-β peaked at day 4, concurrent with maximal iNOS expression, and then decreased to baseline levels on subsequent days.

FIGURE 8. Myeloid, but not epithelial-derived, iNOS modulates the resolution of lung injury. Irradiated BM chimeras were generated between congenic WT (CD45.1) and iNOS−/− mice. WT BM to WT mice were used as irradiated controls. Eight weeks postirradiation, mice were challenged with i.t. LPS and followed for 11 d. (A) BAL fluid protein and total cell counts were measured in designated groups. (B) Lung H&E-stained sections are shown for designated groups 11 d after i.t. LPS (original magnification ×20; insets, ×100). (C) Lung injury scoring was done for low-power (×20) lung histopathological sections. † p < 0.05, WT BM to WT.

FIGURE 9. iNOS regulates CD86 expression to modulate lung inflammation. iNOS−/− mice were challenged with i.t. LPS; 48 h after injury they received either isotype Ab (rat IgG) or anti-mouse CD86 (clone GL-1) on days 2, 4, and 6 after ALI. Survival (A) and BAL protein (B) were evaluated in iNOS−/− mice after i.t. LPS. (C) Histological sections were stained with H&E (original magnification ×20). (D) Histopathological mean lung injury scores from low-power (×20) sections (n = 4–6 animals/group). The AM cell line (MH-S) was challenged with media or LPS (100 ng/ml) in the presence of DMSO or specific iNOS inhibitor 1400W (25 mM) at designated intervals. (E) Flow cytometry density plots show the relative expression of surface CD86 at designated intervals after LPS. MFI for CD86 expression was measured in MH-S cells (F) and resident peritoneal macrophages (G) and compared among groups. Data are mean ± SEM. †,* p < 0.05, log-rank test (A) and unpaired Student t test (B, D–F).
In contrast, iNOS−/− mice had persistently elevated levels of TGF-β, perhaps reflecting a negative-feedback loop between iNOS and TGF-β (49). Although TGF-β can act as a potent anti-inflammatory and reparative mediator, uncontrolled and sustained elevation of TGF-β can lead to increases in alveolar epithelial permeability (50) and fibrotic complications (51). This is supported by the study by Hochberg et al. (52), who noted increased fibrotic complications with markedly elevated kidney TGF-β levels after unilateral ureteral obstruction in iNOS−/− mice compared with WT mice. Furthermore, although similar levels of alveolar collagen were present in WT and iNOS−/− mice by day 4, the latter group had markedly elevated collagen levels at day 7, whereas the levels in WT mice had returned to baseline (B.T. Garibaldi, unpublished observations), supporting a role for iNOS in the fibroproliferative response after ALI.

Apoptosis of inflammatory cells is fundamental to the resolution of inflammation, and failure to clear inflammatory cells leads to excessive tissue damage and sustained injury (45, 53). In contrast to the complete alveolar neutrophil clearance in WT mice, alveolar neutrophilia persisted in iNOS−/− mice. Several factors may contribute to the sustained elevation of alveolar neutrophils in the absence of iNOS. MIP-2, a potent neutrophil chemokine, was higher by day 4 in the BAL fluid from iNOS−/− mice; together with the persistently damaged alveolar-endothelial barrier seen in later stages in iNOS−/− mice, likely contributed to the elevated number of neutrophils. In addition, neutrophil apoptosis and efferocytosis were both impaired in iNOS−/− mice; NO can induce apoptotic neutrophil death (54). We cannot exclude that other factors contribute to persistent alveolar neutrophilia in iNOS−/− mice (e.g., production of neutrophil chemokine KC and/or LIX by a persistently injured alveolar epithelium or enhanced Th17 responses, which can be involved in LPS-induced lung inflammation) (55).

AMs play a critical role in the pathogenesis of ALI (56, 57). We observed similar number, phenotype, and function of AMs in WT and iNOS−/− mice during early lung injury. In marked contrast, during the resolution stage (>4 d after i.t. LPS), iNOS−/− animals had increased AM numbers, with significant differences in phenotype and function compared with WT macrophages. Macrophage cosignaling molecules (CD86, CD40) and MHC-II were increased in macrophages and other APCs, which direct, modulate, and fine-tune T cell responses (58, 59). Emerging data suggest that cosignaling molecules may regulate innate immune responses, best described in the context of Ag-specific models. For instance, genetic deletion of the cosignaling molecule CD40 in mice decreased susceptibility to direct injury with LPS and polymicrobial sepsis (60, 61). Additionally, cosignaling molecules (CD40 and CD80/86) regulate inflammation in a model of polymicrobial sepsis, and humans with septic shock have higher monocyte expression of CD40 and CD80 compared with patients with sepsis or healthy controls (62). Consistent with our findings, Shi et al. (32) described that, in a model of autoimmune myasthenia gravis, iNOS−/− macrophages had a higher expression pattern of CD40 and MHC-II compared with WT, as well as sustained production of proinflammatory TNF-α and ROS.

During lung inflammation, iNOS was reported to originate from inflammatory cells (neutrophils, macrophages) or epithelial cells (63). Although we cannot exclude a potential role for epithelial-derived iNOS during the resolution of lung inflammation, our adoptive-transfer experiments support a central role for macrophage-derived iNOS in this model. BM-derived CD11b+iNOS+ monocytes trafficked to the inflamed lung and alveolar compartments following adoptive transfer. CD11b+iNOS+/+, but not CD11b−iNOS−/−, BM-derived monocytes restored the resolution of ALI, even when delivered 24 h after established injury, suggesting a potential rescue therapy. Similarly, delivery of iNOS into the lung via adenovirus restored resolution. Importantly, adenoviral iNOS was readily taken up by macrophages in the inflamed alveolus, whereas transduction of the resident AMs in the uninjured lung was minimal. In contrast, the lung epithelium was readily transduced in normal and inflamed conditions. To dissect the role of cellular iNOS in driving the resolution of experimental lung inflammation, we created irradiated chimeras that showed that the myeloid-derived iNOS was sufficient to restore the normal lung repair. Lung epithelial iNOS had no effect in mediating the resolution of ALI. Although BM chimeras between WT and iNOS−/− were described in murine models of sepsis-induced lung injury (64, 65), these studies identified that myeloid-derived iNOS contributed to pulmonary oxidant stress and microvascular leak, whereas parenchymal-derived iNOS had no apparent effect. Our results appeared to contradict these reports, although there are two main differences. First, our model of ALI is direct, with a significant epithelial injury component, followed by an influx of inflammatory cells; other investigators reported results from indirect models of ALI in which there is no or modest alveolitis.

Second, the models reported focused on early events after ALI (hours); we focused on later stages, during the resolution and repair of ALI. Although inhaled NO has been tested in patients with ALI, and transient improvements were shown in physiological parameters, such as oxygenation and pulmonary vascular resistance, four multicenter, randomized, placebo-controlled trials failed to show a therapeutic role for inhaled NO in patients with acute respiratory failure (66–68). We find that upregulation of iNOS and NOx is tightly regulated following LPS-induced lung injury, so that location, cellular source, timing, and duration of increased NO may be critical to its overall effect and, therefore, may not be adequately reproduced through continuous inhaled delivery of NO. Further definition of the differences between inhaled NO and the upregulation of cell-based iNOS/NO may provide important mechanistic insights that could be leveraged for the design of therapy.

Although iNOS-derived NO has a diverse range of immunological effects, we found the sustained expression of AM cosignaling molecule CD86 in the absence of iNOS to be a prominent feature. We considered several factors with regard to the timing of CD86 Ab-mediated blockade. First, we wanted to intervene when ALI was fully established and, thus, prove that our intervention can be used as rescue therapy, which is directly relevant to strategies for therapy in most patients with ALI. Second, we sought to avoid attenuating the early inflammatory response with pretreatment or early intervention, because the focus of this line of investigation is on mechanisms of resolution. Our laboratory is pursuing subcellular mechanisms of iNOS-mediated CD86 regulation. Sustained CD86 expression could also be explained by enhanced APC survival and responsiveness to TLR agonist seen in the absence of iNOS, as described recently (69). Sustained macrophage CD86 expression, in conjunction with high MHC-II expression observed in iNOS−/− mice, could potentially lead to enhanced costimulation of Th1 responses from CD4+ cells, which were abundant by days 4–7 after alveolar inflammation and contribute to persistent lung immunopathology. In addition to the persistent CD86 expression in iNOS−/− monocytes, we observed sustained upregulation of CD40 expression. Its regulation by iNOS and its role in the modulation of lung inflammation need further evaluation. We were unable to use Ab-mediated blockade, because available murine Abs are agonistic for CD40. We cannot exclude a synergistic role for sustained macrophage CD86 expression in iNOS-mediated mod-
ulation of lung inflammation, although blockade of CD86 was sufficient to restore the resolution of ALI.

ALL continues to be a major clinical problem, with significant annual morbidity and mortality, for which therapy is largely supportive. In this article, we demonstrate a critical role for macrophage-derived iNOS in promoting the resolution of lung injury. iNOS has differential effects depending on the stage of injury, with a predominant early proinflammatory effect and a reparative, anti-inflammatory late-phase effect. The late effects are mediated, at least in part, by AM activation and modulation of CD86 expression, but they may occur via effects on other pathways, including Tregs, lipid mediators, and Th17 responses, among others. The surprising identification of iNOS as a determinant of resolution of lung injury provides new insights into the resolution of inflammation and may create opportunities for the development of new approaches to therapy.

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Disclosures

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