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NK cells regulate CXCR2$^+$ neutrophil recruitment during acute lung injury

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ABSTRACT

A critical step in the pathogenesis of acute lung injury (ALI) is excessive recruitment of polymorphonuclear neutrophils (PMNs) into the lungs, causing significant collateral tissue damage. Defining the molecular and cellular steps that control neutrophil infiltration and activation during ALI is therefore of important therapeutic relevance. Based on previous findings implicating the transcription factor Tbet in mucosal Th1-inflammation, we hypothesized a detrimental role for Tbet during ALI. In line with our hypothesis, initial studies of endotoxin-induced lung injury revealed a marked protection of *Tbet*$^{-/-}$ mice, including attenuated neutrophilia compared to WT counterparts. Surprisingly, subsequent studies identified natural killer (NK) cells as the major source of pulmonary Tbet during ALI. In addition, a chemokine screen suggested that mature Tbet$^+$ NK-cells are critical for the production of pulmonary CXCL1 and -2, thereby contributing to pulmonary PMN recruitment. Indeed, both NK-cell Ab depletion and adoptive transfer studies provide evidence for NK cells in the orchestration of neutrophil recruitment during endotoxin-induced ALI. Taken together, these findings identify a novel role for Tbet$^+$ NK-cells in initiating the early events of noninfectious pulmonary inflammation. *J. Leukoc. Biol.* 101: 471–480; 2017.

Introduction

ALI or ARDS is an inflammatory lung disease characterized by acute hypoxemic respiratory failure with bilateral pulmonary infiltrates, not attributable to left heart failure [1]. Common causes of ARDS include both direct (pneumonia, lung contusion, inhalational injury, and aspiration of stomach contents) or indirect (sepsis, trauma, and multiple blood transfusions) lung injury [2, 3]. Although the clinical presentation of ALI/ARDS is heterogeneous with 3 proposed subcategories based on the severity of hypoxemia (ranging from mild to severe), it causes significant morbidity and mortality in many critically ill patient populations [1, 3, 4]. There remains a pressing need to find new therapeutic approaches for the treatment of ALI and to provide a better understanding of the pathogenesis of ALI-associated lung inflammation [5]. In this regard, a predominant hallmark of ALI is the uncontrolled accumulation of innate and adaptive leukocyte subsets into different compartments of the lung tissue, in conjunction with cytokine release and an inflammatory process that potentiates tissue damage [6–8]. As such, novel therapeutic modalities that prevent the destructive inflammatory cascade are an attractive avenue for development.

A critical effector cell in both sterile and infectious ALI is the neutrophil [9]. Its role is most evident clinically, because, whereas ALI/ARDS is a heterogeneous condition, neutrophils are a pathologic hallmark of human disease and many preclinical murine models (reviewed in ref. 9). In patients with ALI, the number of neutrophils in the BAL fluid correlates with ALI severity [10–12]. Neutrophils become entrapped in pulmonary capillaries during ALI and instigate the characteristic changes in permeability and edema [13, 14]. However, to date, the cellular sources responsible for neutrophil recruitment remains to be fully defined.

Although the alveolar macrophage is a major first line of defense against pulmonary infections and responds to the presence of LPS, the contribution of other resident and infiltrating leukocyte subsets to the inflammatory neutrophilic cascade during ALI is not fully defined. For example, NK cells fine-tune immune responses as a result of contact and recognition of target cells or activating interactions with other immune cells, particularly macrophages and dendritic cells [15, 16]. During active inflammation, an extensive body of literature highlights the ability of NK cells and NK-cell–derived cytokines to regulate neutrophil activation [9, 17–19]. However, the extent to which NK cells or NK-cell crosstalk can control the initial recruitment of neutrophils is not well defined. In the present study, during the acute phase of ALI induced by intratracheal

Abbreviations: ALI = acute lung injury, ARDS = acute respiratory distress syndrome, BAL = bronchoalveolar lavage, KC = keratinocyte-derived chemokine, FMLP = *N*-Formylmethionyl-leucyl-phenylalanine, MPO = myeloperoxidase, WT = wild-type

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administration of LPS, NK cells were a predominant cellular subset within the BAL fluid. These NK cells displayed an activated phenotype with a marked expression of the transcription factor Tbet. Thus, we assessed the contribution of $Tbet^{+}$ NK cells to the pathogenesis of LPS-induced ALI in $Tbet^{-/-}$ mice. Identifying a protective affect after Tbet deficiency, we found a critical role for activated NK cells in the production of inflammatory cytokines, CXCL1, and CXCL2 and in neutrophil recruitment during ALI. Last, Ab-mediated depletion of NK cells and NK-cell adoptive transfer studies define a critical and previously unappreciated role for NK cells in the recruitment of neutrophils and perpetuation of lung inflammation during ALI.

MATERIALS AND METHODS

Mice

Wild-type (C57BL6/J), $Tbet^{-/-}$ mice (*B6.129S6-Tbx21tm1Glm/J*) and CD45.1 (B6.SJL-Ptprca Pep3b/BoyJ) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were kept in specific pathogen-free conditions, and fecal samples were negative for *Helicobacter* species, protozoa, and helminthes. Animal procedures were approved by the Institutional Animal Care and Use Committees at the University of Colorado Denver.

LPS lung injury

Age- (8–12 wk old) and gender-matched mice were anesthetized with pentobarbital (70 mg/kg) and LPS (5.0 μg/g body weight *Escherichia coli* 0111: B4, L4391; Sigma-Aldrich, St. Louis, MO, USA), or, as the control, PBS was administered intratracheally via a 22-gauge catheter. During ALI, mice were weighed daily to assess for disease severity [20]. After the indicated time points (1–5 d), mice under deep anesthesia were killed by exsanguination. BAL samples were centrifuged at 300 *g* for 5 min at 4°C to separate the BAL cells from the cell-free BAL fluid. Before obtaining pulmonary tissue, the pulmonary vascular system was flushed with 10 ml saline via the right ventricle [20].

In vivo NK-cell depletion studies

For the purpose of NK-cell depletion, WT mice (C57B6/J) at the age of 8–12 wk were matched in age, gender, and weight. NK-cell depletion was achieved by injection with 200 μg anti-NK1.1 (i.p.; clone PK136, BioXcell, West Lebanon, NH, USA) or IgG2a (clone C1.18.4, BioXcell) on d −3 and −1 before LPS intratracheal administration. Depletion efficiency was determined with flow cytometry.

Purification and adoptive transfer of splenic NK cells

CD49b $(DX5)^{+}$ NK cells were isolated from spleens of WT mice (C57BL/6J) via a 2-step process, using magnetic bead separation. In brief, spleens from WT mice (CD45.1; C57BL6/J) were mashed through a cell strainer (100 μm nylon mesh) and RBC lysis was performed with ammonium-chloride-potassium lysing buffer (Quality Biologic, Gaithersburg, MD, USA). Non-NK cells were depleted with an NK-cell isolation kit II (Miltenyi Biotec, Auburn, CA, USA) before positive selection of NK cells with CD49b (DX5) micro beads, per the manufacturer's instructions. After the NK-cell isolation procedure, the number of cells was assessed by using trypan blue staining. Flow cytometry analysis was performed to determine the percentage of NK cells. On average, an NK-cell purity of more than 85% was achieved when following this experimental protocol. For NK-cell transfer studies, NK-cell transfer was performed 1 hour before inducing ALI via LPS administration. For this purpose, 1.5×10^{6} $CD49b^{+}$ $(DX5)^{+}$ (CD45.1) NK cells/mouse were injected into $Tbet^{-/-}$ (CD45.2) recipient mice via the retro-orbital venous plexus. After 24 h, BAL, lung, and spleen were harvested, as described earlier. Transferred NK cells and the resultant impact on neutrophil recruitment were assessed by flow cytometry.

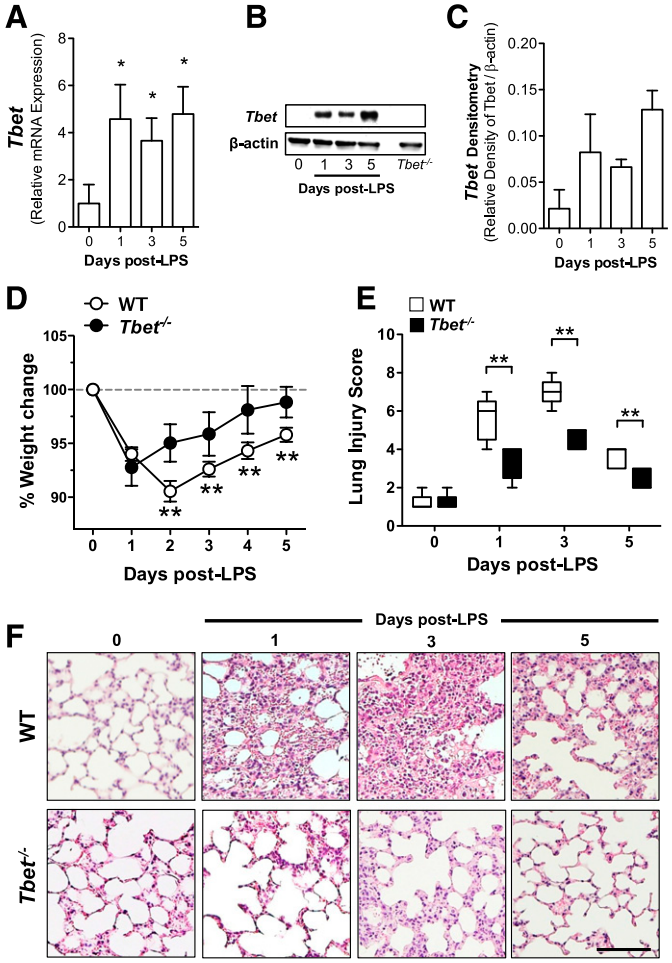

Figure 1. Increased Tbet expression during endotoxin-induced lung injury and attenuation of pathology in $Tbet^{-/-}$ mice. ALI was induced in C57BL/6J mice by intratracheal injection (5.0 μg/g body weight) of LPS (*Escherichia coli* 0111:B4). (A) Tbet mRNA was quantified by RT-PCR in whole-lung tissues on d 0, 1, 3, and 5 after LPS treatment. (B) Tbet protein was assessed in whole-lung tissues by using Western immunoblot analysis on d 0, 1, 3, and 5 after LPS treatment. (C) Tbet Western blot densitometry analysis. (D) Representative weight loss curve from WT and $Tbet^{-/-}$ mice from 0–5 d after treatment. (E) Lung injury histology score of WT and $Tbet^{-/-}$ mice from LPS treatment on d 0, 1, 3, and 5. (F) Representative micrographs from of H&E-stained lung tissue of LPS-treated WT and $Tbet^{-/-}$ mice at indicated post-LPS time points. Scale bar, 100 μm. Data are expressed as mean ± SEM ($n = 5$ mice/group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. age-matched WT counterpart.

Measurement of BAL fluid albumin content and MPO assay

To assess the degree of pulmonary edema during ALI, albumin content in the BAL was measured with a mouse albumin ELISA quantitation set (Bethyl Laboratories, Montgomery, TX, USA), according to the manufacturer's instructions. MPO is rapidly released by activated PMNs and was used as a marker of neutrophilic infiltration. MPO concentrations in the BAL were measured with a mouse MPO ELISA kit (Hycult Biotech, Plymouth Meeting, PA, USA) according to the manufacturer's instructions.

Quantification of CXCL1 and CXCL2 by ELISA

CXCL1 (KC) and CXCL2 (MIP-2) concentrations were measured by ELISA from tissues using specific Abs and standards (Duoset; R&D Systems, Minneapolis, MN,

USA) according to the manufacturer's instructions. Tissue protein concentrations were equalized by using a Bradford assay (Bio-Rad, Hercules, CA, USA).

Lung histology and lung injury scoring

After dehydration in ethanol gradients, perfused and formalin-fixed lungs were embedded in paraffin, sectioned at 5 μm, and stained with H&E. Lung samples were analyzed by researchers blinded to group assignments. Histologic lung injury was graded as previously described [20]. A composite scale (1–9); comprising of infiltration or aggregation of inflammatory cells in air space or vessel wall [1 = only wall, 2 = few cells (1–5 cells) in air space, 3 = intermediate, 4 = severe (air space congested)]; interstitial congestion and hyaline membrane [formation: 1 = normal lung, 2 = moderate (<25% of lung section), 3 = intermediate (25–50% of lung section), 4 = severe (>50% of lung section)]; hemorrhage: (0 = absent, 1 = present).

RNA isolation and RT-PCR

Total RNA was extracted from lung tissue by Qiazol Reagent (Qiagen Sciences, Germantown, MD, USA), followed by cDNA synthesis using the iScript cDNA Synthesis Kit (Qiagen) according to the manufacturer's instructions. Quantitative reverse transcriptase PCR (Qiagen) was performed to measure relative mRNA levels for various transcripts according to the manufacturer's instructions. The following murine Quantitect Primer Assays were used (Qiagen): β-actin (QT01136772), TNF-α (QT00104006), IL-1β (QT01048355), IL-6 (QT00098875), CXCL1 (QT00115647), CXCL2 (QT00113253), and IFNγ (QT01038821). All RT-PCR assays were standardized relative to β-actin levels.

Flow cytometry

Cells from the various compartments were incubated with fluorescently labeled anti-mouse Abs against CD4 (GK1.5), CD19 (6D5), and CXCR2 (TG11) (Biolegend, San Diego, CA, USA); CD3ε (145-2C11), CD8 (Ly-2), MHCII (M5/114.15.2), CD11b (M1/70), CD11c (N418), Ly6G (1A8), NK-1.1 (PK136), NKp46 (29A1-A), CD49b (DX5), Eomes (DAN1mag), GATA3 (TWAJ), NKg2D (CX5), GzmB (NGZB), CD27 (LG.7F9), CD69 ([^{1}H].2F3), CD62L (Mel14), and Ly6C (HK1.4) (eBioscience, San Diego, CA, USA); Tbet (04-46) (BD Bioscience, San Jose, CA, USA); and Blimp1 (N-20) (Santa Cruz Biotechnology, Dallas, TX, USA), or the corresponding isotype controls. Intracellular staining for indicated transcription factors and granzyme-B was performed with a FoxP3 staining kit (eBioscience), according to the manufacturer's instructions. Analysis was performed with a BD FACSCanto II (BD Biosciences). Further analyses were performed with FlowJo software (Tree Star Inc, Ashland, OR, USA).

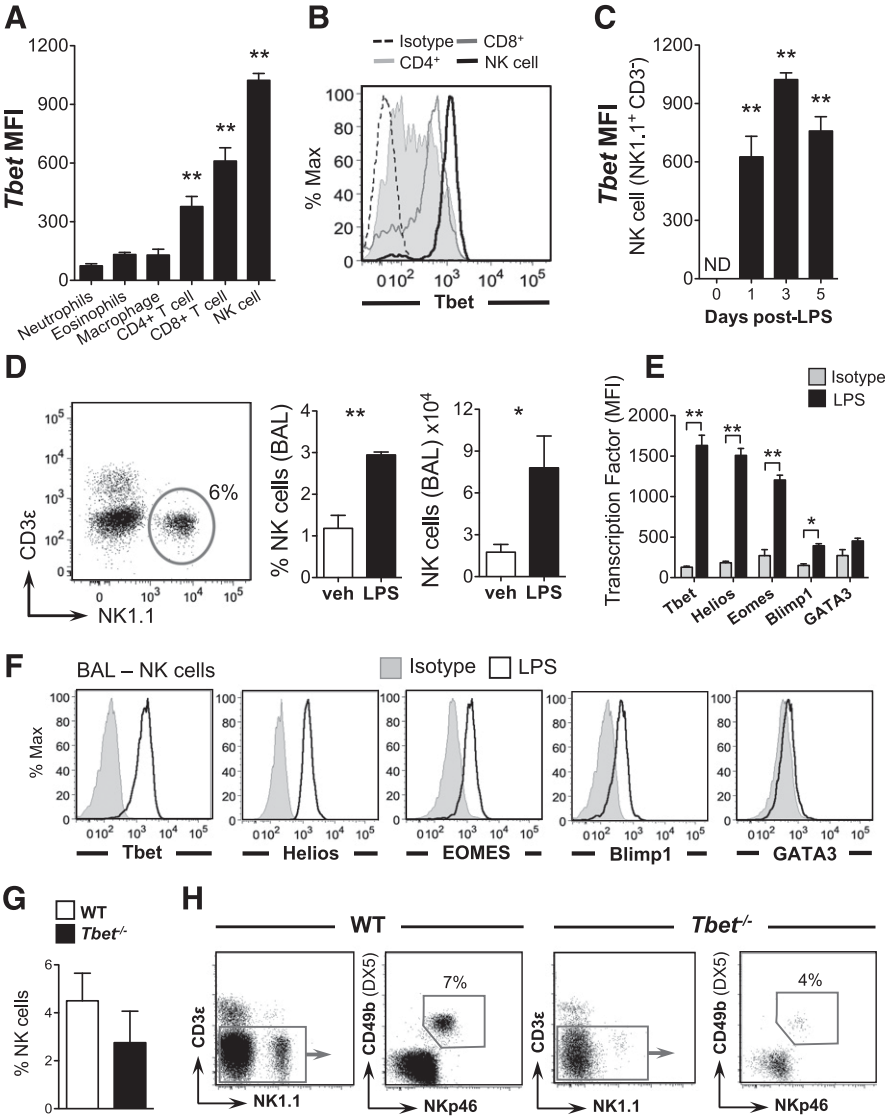

Figure 2. Increased Tbet expression during endotoxin-induced lung injury is attributable to NK-cell infiltration. ALI was induced in C57BL/6J mice by intratracheal injection (5.0 μg/g body weight) of LPS (*Escherichia coli* 0111:B4). (A) Flow cytometry analysis of Tbet MFI in leukocytes extracted from BAL 3 d after LPS challenge. (B) Representative flow cytometry plot of Tbet expression histograms in BAL CD4$^+$ and CD8$^+$ T cells and NK cells after LPS. (C) Tbet MFI was quantified in CD45$^+$CD3$^-$NK1.1$^+$ NK cells from BAL fluid on d 0–5 after LPS treatment. Data are expressed as means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ vs. d 0 (A vs. neutrophils; n = 3–5 mice/group). (D) Representative flow cytometry plot, percentage, and absolute number of CD45$^+$NK1.1$^+$CD3$^-$ NK cells from the BAL. Data are expressed as means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ vs. saline vehicle. (E) Further subanalysis was performed with flow cytometry assessing the cellular profile of NK-cell transcription factors from BAL of LPS-treated mice. (F) Representative flow cytometry histograms of NK-cell–expressed transcription factors in the BAL of LPS-treated mice. (G) The frequency of lung-infiltrating NK cells was assessed in the BAL fluid after LPS challenge by flow cytometry. (H) Representative flow cytometry plots of CD45$^+$CD3ε$^-$NK1.1$^-$NKp46$^+$ mature NK cells in the BAL after LPS challenge. Data are expressed as means ± SEM (n = 3 mice/group). $^*P < 0.05$, $^{**}P < 0.01$ vs. isotype control.

Western immunoblot analysis

To measure Tbet protein content, we disrupted lung tissue in T-Per Tissue Protein Extraction Reagent with Pierce Phosphatase and Protease Inhibitor (Thermo Fisher Scientific, Rockford, IL, USA). The protein was resuspended in reducing Laemmli sample buffer (Bio-Rad) and heated to 90°C for 5 min. Samples were resolved on a 10% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in TBST supplemented with 5% blotting-grade nonfat milk (Bio-Rad). The membranes were subsequently incubated with Tbet Ab (sc-21749; Santa Cruz Biotechnology) at a concentration of 1:100 overnight at 4°C. After three 10 min washes in TBST, the membranes were incubated in a goat anti-mouse secondary Ab at a concentration of 1:10,000 (31172; Thermo Fisher Scientific) for 1 h at room temperature. The wash was repeated, and proteins were detected by enhanced chemiluminescence (Thermo Fisher Scientific). To control for protein loading, blots were stripped and reprobed for β-actin with a mouse monoclonal anti-human β-actin primary Ab (CP01; EMD Millipore, Billerica, MA, USA) and a goat anti-mouse secondary Ab (401225; EMD Millipore).

Statistical analysis

All parametric data were compared by 2-way ANOVA with Bonferroni's post hoc test or *t* test, as appropriate. Comparison of nonparametric results between groups was performed with the Mann-Whitney *U* test. Values are expressed as means ± SEM or as medians (5–95 percentiles), as indicated. $P < 0.05$ denoted statistical significance. For all statistical analyses, Prism ver. 5.0 software (GraphPad Software, San Diego, CA, USA) was used.

RESULTS

Endotoxin-induced lung injury is attenuated in Tbet$^{-/-}$ mice

It has been reported that the transcription factor Tbet plays a central role in the control of Th1-mediated mucosal inflammation (reviewed in refs. 21–23); however, its specific function during pulmonary disease and preclinical models of ALI is limited. Therefore, we examined the role of Tbet during endotoxin-induced lung injury. Indeed, we observed that Tbet mRNA and protein were significantly elevated during the acute phase of intratracheal LPS-challenge in whole lung tissues from WT mice, 1–5 d after treatment (**Fig. 1A–C**). To address the functional role of Tbet in ALI, we performed studies in *Tbet*$^{-/-}$ mice. In contrast to their unremarkable phenotype at baseline, *Tbet*$^{-/-}$ mice showed a dramatically attenuated susceptibility to LPS-induced ALI, as displayed by attenuated weight loss during the recovery phase of intratracheal LPS challenge and a marked reduction in all histologic indices of lung injury, including reduced leukocyte accumulation, alveolar disruption, and alveolar congestion (Fig. 1D–F). Together, these genetic experiments indicated a detrimental role for the Tbet transcription factor during LPS-induced ALI.

NK cells represent a major source for pulmonary Tbet during LPS-induced ALI

Based on the above studies showing a detrimental role for pulmonary Tbet during LPS-induced ALI, we next set out to define the leukocyte subsets responsible for pulmonary Tbet increases during ALI. For this purpose, we screened the lungs of WT mice by flow cytometry to assess the relative expression of Tbet in different leukocyte populations. These studies revealed that at the peak of pulmonary inflammation, NK cells represented the predominant source of Tbet in ALI compared to a modest number of CD4$^+$ and CD8$^+$ T cells (**Fig. 2A, B**). Of note, Tbet was virtually unexpressed in neutrophils, eosinophils, and macrophages (Fig. 2A). Tbet$^+$ NK cells were essentially undetectable in the lungs of control mice that received normal saline instead of LPS (Fig. 2C). Furthermore, a marked influx of NK cells was observed in the BAL fluid from lungs following LPS challenge (Fig. 2D). Assessment of relevant NK-cell–expressed transcription factors showed a predominant expression of Tbet in NK cells from this population (Fig. 2 D–F). Together, these experiments suggest that infiltrating pulmonary NK cells represent the major source of Tbet in the lungs of mice experiencing lung inflammation after LPS installation.

Reduced NK cell frequency and inflammatory cytokines during ALI in *Tbet*$^{-/-}$ mice

Based on the above studies suggesting that NK cells represent a major source of pulmonary Tbet during LPS-induced ALI, we subsequently assessed the frequency of NK cells in *Tbet*$^{-/-}$ mice. In line with previous reports suggesting that Tbet regulates NK-cell maturation [24, 25], we observed a significant reduction in the number of NK cells in the lungs of LPS-treated animals (Fig. 2G, H). Furthermore, this reduction of infiltrating NK cells

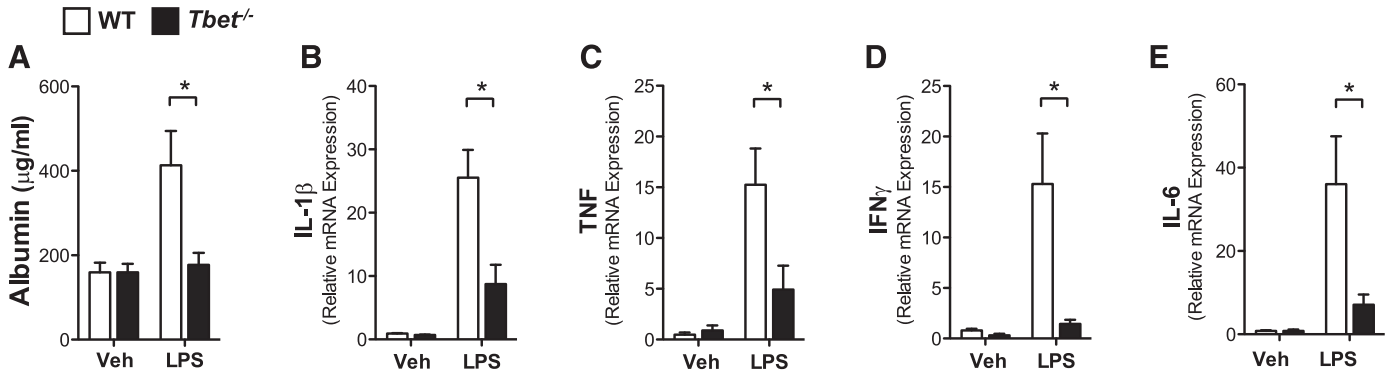

Figure 3. Attenuated inflammatory cytokine expression in Tbet$^{-/-}$ mice during endotoxin-induced lung injury. ALI was induced in C57BL/6J (WT) and *Tbet*$^{-/-}$ mice by intratracheal injection (5.0 μg/g body weight) of LPS (*Escherichia coli* 0111:B4). (A) Albumin was measured by ELISA from BAL fluid. RT-PCR was used to assess the relative expression of (B) IL-1β, (C) TNF, (D) IFNγ, and (E) IL-6 in whole lung tissues after LPS challenge. Data are expressed as means ± SEM (n = 4–8 mice/group). *$P < 0.05$, **$P < 0.01$ vs. indicated counterparts.

correlated with lower albumin leakage in the BAL fluid (**Fig. 3A**) of *Tbet*$^{-/-}$ mice compared to WT controls. Similarly, we observed reduced transcript levels of the proinflammatory cytokines IL-1β, TNFα, IL-6, and IFNγ in *Tbet*$^{-/-}$ lungs after application of LPS (Fig. 3B–E). The collective data imply a critical role for the transcription factor Tbet in controlling lung inflammation during LPS-induced ALI and imply that NK cells are an additional major source of pulmonary Tbet levels in this process.

Reduced CXCR2 chemokines and neutrophil recruitment in Tbet$^{-/-}$ mice during endotoxin-induced lung injury

As critical regulators of the innate immune response, NK cells produce a wide array of chemokines after activation [26]. In a further attempt to understand the role of Tbet-positive NK cells and the afforded protection of *Tbet*$^{-/-}$ mice during ALI, we next assessed the expression profile of murine chemokines in whole-lung tissue at 1 d after LPS challenge, to define the early functional roles of Tbet^{+} NK cells in lung inflammation. Although LPS induced the expression of multiple chemokines, including CCL2, CCL3, CCL5, CCL28, and CXCL9, their pulmonary expression levels were similar in WT and *Tbet*$^{-/-}$ mice (**Fig. 4A, B**). In contrast, lungs of *Tbet*$^{-/-}$ mice after LPS challenge displayed a significant reduction in the expression of the neutrophil chemotactic cytokines, including CXCL1 (KC) and CXCL2 (MIP-2) (Fig. 4B). This result corresponded with a marked reduction of infiltrating neutrophils, as measured by MPO expression (Fig. 4C) and by flow cytometry of BAL fluid (Fig. 4D, E). Of note, although the neutrophilic chemokines CXCL1 and -2 were repressed in LPS-treated *Tbet*$^{-/-}$ mice, the expression of the CXCR2 receptor was comparable on *Tbet*$^{-/-}$ neutrophils compared with WT counterparts (Fig. 4F). Both CXCL1 and -2 are known to play a central role in neutrophil recruitment into the lung during ARDS, both in animal and clinical studies [27]. Thus, Tbet-deficiency appears to selectively inhibit neutrophil recruitment by regulating the expression of CXCR2 chemokines during ALI.

Adoptive transfer of NK cells into Tbet$^{-/-}$ mice drives neutrophil recruitment and associated inflammation during endotoxin-induced lung injury

To determine directly whether NK cells are involved in the control of neutrophil recruitment via induction of CXCR2

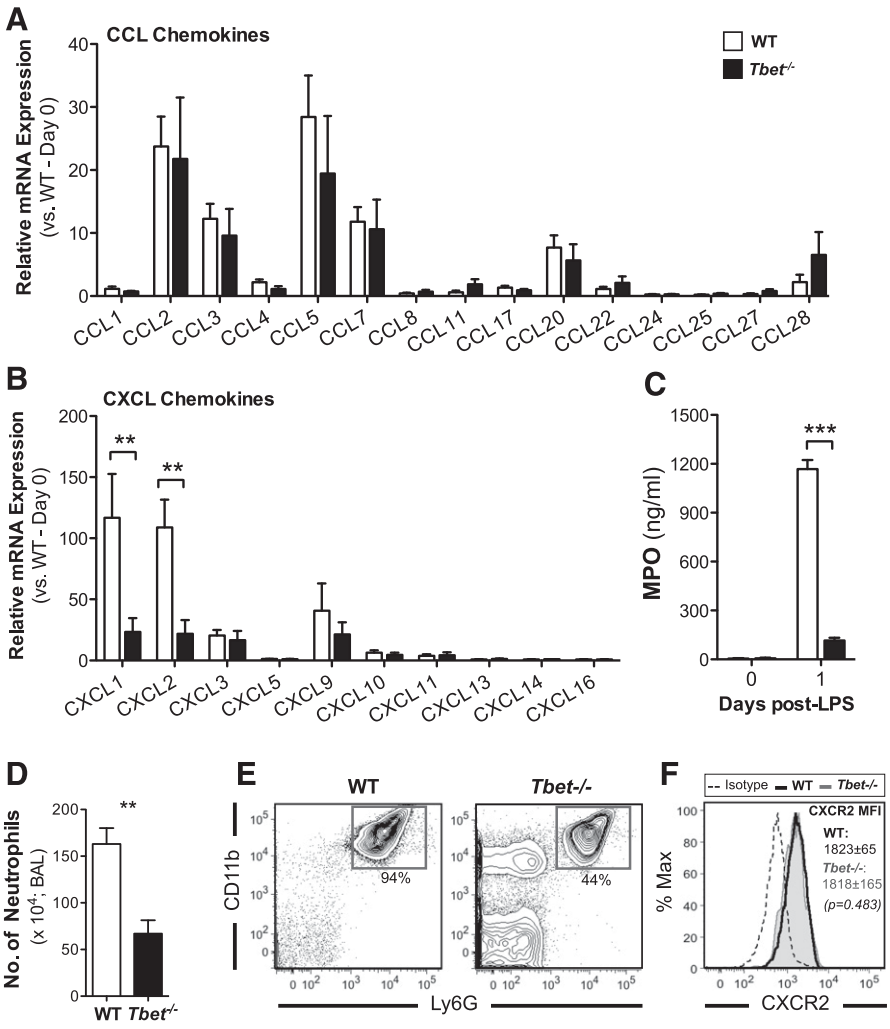

Figure 4. Abrogated neutrophil recruitment and chemokine profile in Tbet$^{-/-}$ mice during endotoxin-induced lung injury. ALI was induced in C57BL/6J (WT) and *Tbet*$^{-/-}$ mice by intratracheal injection (5.0 μg/g body weight) of LPS (*Escherichia coli* 0111:B4). Chemokine expression profiles were assessed from whole-lung tissue 1 d after LPS treatment. (A) CCL chemokines. (B) CXCL chemokines. (C) MPO was quantified by ELISA in BAL fluid 1 d after LPS treatment. (D) The number of lung infiltrating neutrophils was assessed in the BAL fluid after LPS challenge by flow cytometry. (E) Representative flow cytometry plots of CD45^{+} MHCII^{-}Ly6G^{+}CD11b^{+} neutrophils in the BAL after LPS challenge. (F) Representative flow cytometry histograms of CXCR2 expression on neutrophils in the BAL after LPS challenge. Data are expressed as means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. WT counterparts (n = 3–8 mice/group).

chemokines, we purified mature NK cells from the spleens of WT mice and adoptively transferred them into NK-cell–deficient *Tbet*$^{-/-}$ recipients. Mice were then exposed to an intratracheal LPS-challenge, as before. We chose to assess the impact of NK transfer at 24 h after the challenge, because this is the peak of neutrophil influx in this model. Although *Tbet*$^{-/-}$ mice were protected from lung injury after LPS challenge, the transfer of WT NK cells drove lung pathology similar to that in WT mice and directly implicated NK cells in the pulmonary inflammation associated with endotoxin exposure during ALI (**Fig. 5A–C**). Transferred NK cells were tracked with the use of congenic markers by flow cytometry (CD45.1) and were retrieved from the spleen, lung tissues, and BAL fluid (Fig. 5D, E). Of note, *Tbet*$^{-/-}$ mice presented with attenuated albumin leakage and inflammatory cytokine production, but the transfer of WT NK cells induced increased edema and expression of IL-1β, TNFα, IFNγ, and IL-6, comparable to that in WT mice (Fig. 5F–J).

Last, we assessed the direct role of NK cells in driving neutrophil recruitment during pulmonary inflammation. *Tbet*$^{-/-}$ mice presented with attenuated neutrophilia, but the adoptive transfer of WT NK cells induced the pulmonary expression of CXCL1 and -2 mRNA and protein, in addition to recruiting neutrophils into the bronchoalveolar spaces. This process reversed the protective phenotype observed in *Tbet*$^{-/-}$ mice and resulted in lung disease, which was comparable to that in WT mice after LPS challenge (**Fig. 6**). Thus, NK cells directly contribute to the acute lung disease associated with localized endotoxin challenge by orchestrating the expression of neutrophil chemokines and recruitment.

Antibody depletion of NK cells reduces neutrophil recruitment and attenuates endotoxin-induced lung injury

Based on our findings indicating a functional role of Tbet^{+}NK cells in pulmonary recruitment of PMNs and concomitant lung injury after LPS challenge, we subsequently attempted to explore these findings in a therapeutic approach. We hypothesized that depletion of NK cells would reduce pulmonary Tbet levels and consequently attenuate endotoxin-induced ALI. To address this hypothesis, we subsequently performed NK-cell depletion studies of LPS-induced ALI. Wild-type mice were treated with anti-NK1.1 mouse Ab (200 μg/mouse, i.p.; d −3 and −1) treatment before intratracheal application of LPS (**Fig. 7A**). To confirm successful depletion of NK cells, depletion efficiency was assessed by flow cytometry of cells from the lungs, spleen, and liver by assessing the frequency of CD45^{+}, CD3ε^{-}, NK1.1^{+}, NKp46^{+} DX5^{+} NK cells (Fig. 7B, C). Indeed, 80% of pulmonary NK cells were depleted after anti-NK1.1 treatment, as compared to control IgG2a. Of note, anti-NK1.1 treatment also depleted liver and splenic NK-cell pools (spleen; 81%; liver; 94%) (Fig. 5C). Consistent with the findings in *Tbet*$^{-/-}$ mice of attenuated neutrophilia and protection during LPS-induced ALI, we observed that NK-cell

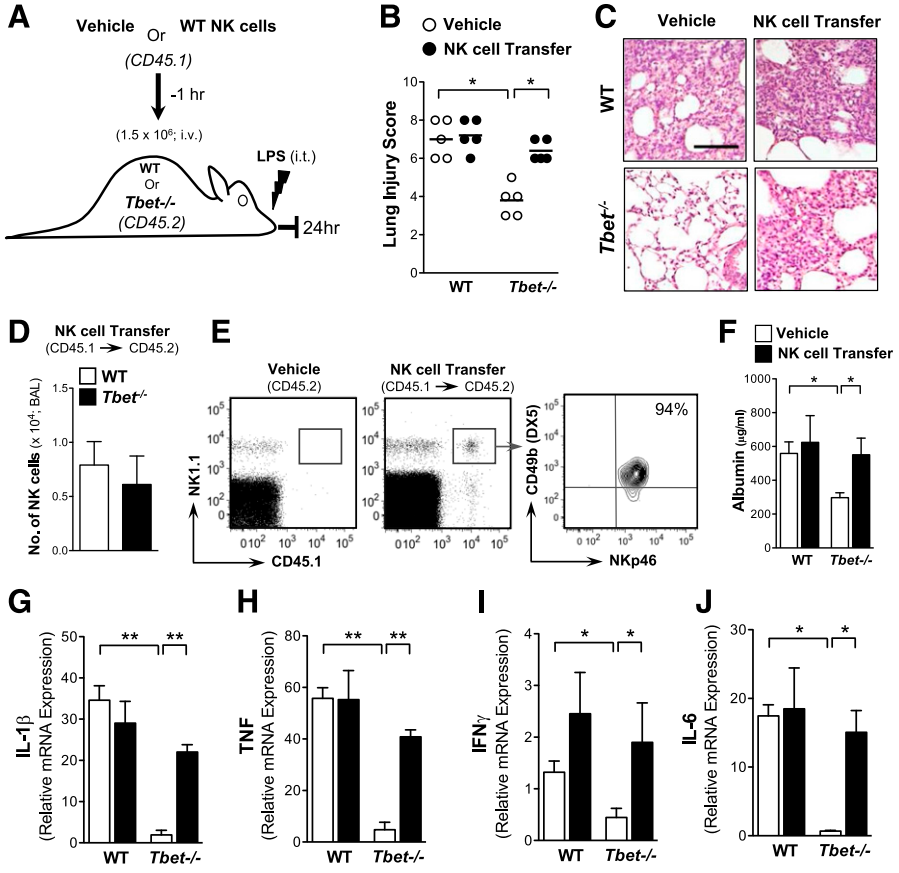

Figure 5. Adoptive transfer of WT NK cells exacerbated endotoxin-induced lung injury in Tbet$^{-/-}$ mice. NK cells were purified from spleens of C57BL/6J (WT) mice by magnetic separation and adoptively transferred (1.5×10^6; i.v.) into C57BL/6J (WT) or *Tbet*$^{-/-}$ mice 1 h before the induction of LPS-lung injury (5.0 μg/g body weight; *Escherichia coli* 0111:B4). Recipient mice were euthanized 24 h after LPS challenge. (A) Schematic outline of NK-cell adoptive transfer regimen. (B) Lung injury histology score of NK-cell recipient mice after LPS treatment. (C) Representative H&E micrographs from lung tissue of LPS-treated vehicle and NK-cell transferred mice, Scale bar, 100 μm. (D) The absolute number of NK cells was quantified in the BAL fluid by flow cytometry. (E) Representative flow cytometry plots of adoptively transferred NK cells in BAL. (F) Albumin was measured by ELISA from BAL fluid. Relative expression of mRNA was quantified by RT-PCR in whole-lung tissue for IL-1β (G), TNF (H), IFNγ (I), and IL-6 (J). Data are expressed as means ± SEM and are representative of 2 independent experiments ($n = 5$ mice/group). *$P < 0.05$, **$P < 0.01$ vs. indicated vehicle counterpart.

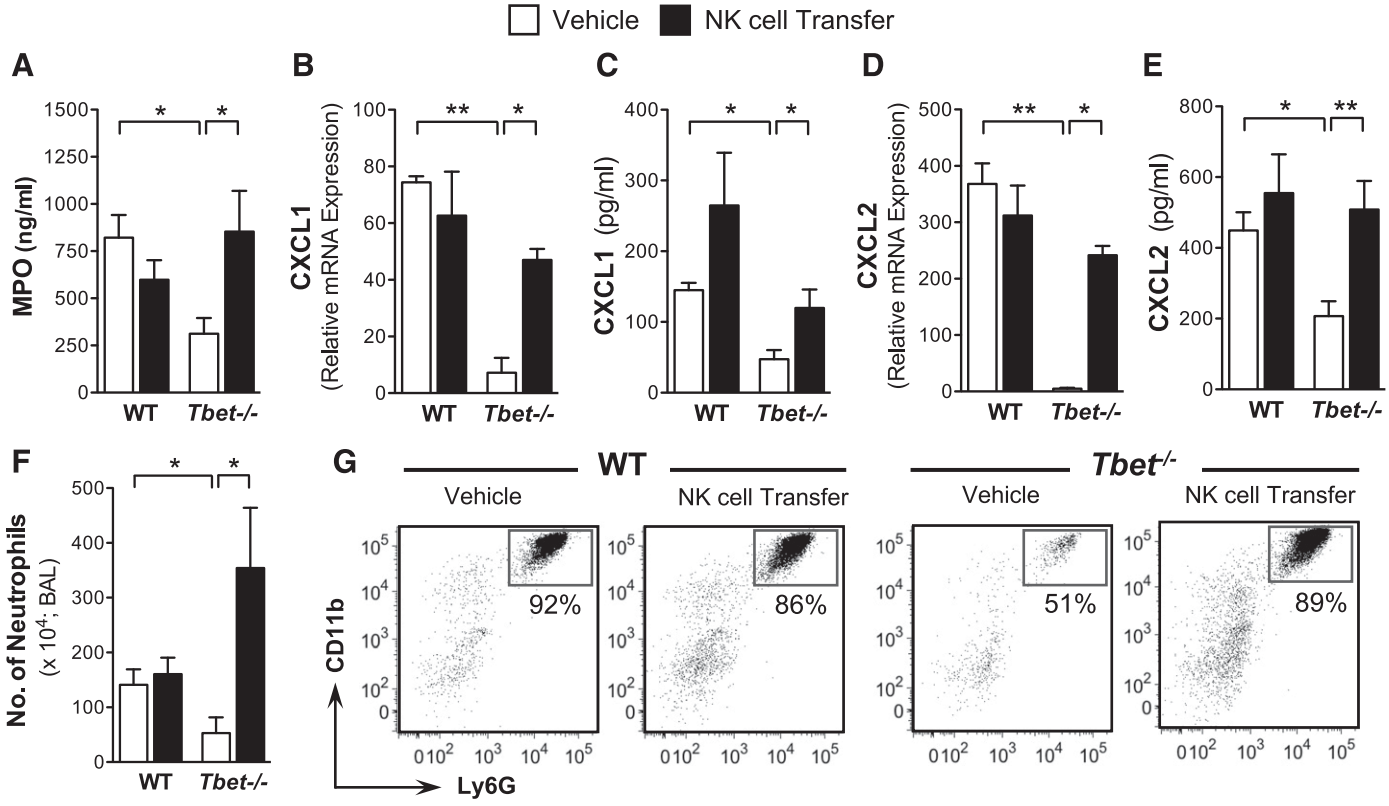

Figure 6. Adoptive transfer of WT NK cells into Tbet$^{-/-}$ mice drives neutrophil chemokines and recruitment during endotoxin-induced lung injury. NK cells were purified from spleens of C57BL/6J (WT) mice by magnetic separation and adoptively transferred (1.5×10^6; i.v.) into C57BL/6J (WT) or Tbet$^{-/-}$ mice 1 h before the induction of LPS-lung injury (5.0 μg/g body weight; *Escherichia coli* 0111:B4). Recipient mice were euthanized 24 h after LPS challenge. (A) MPO was quantified in the BAL fluid by ELIS (A) CXCL1, and mRNA (B) was quantified by RT-PCR in whole lung tissue. (C) CXCL1 protein was quantified by ELISA in BAL. (D) CXCL2 mRNA was quantified by RT-PCR in whole lung tissue. (E) CXCL2 protein was quantified by ELISA in BAL. (F) The absolute number of neutrophils was quantified in the BAL fluid by flow cytometry. (G) Representative flow cytometry plots of neutrophils in BAL. Data are expressed as means ± SEM and are representative of 2 independent experiments (n = 5–8 mice/group). *$P < 0.05$, **$P < 0.01$ vs. indicated vehicle counterpart.

depletion improved histologic tissue injury during ALI, showing less influx of leukocytes and congestion (Fig. 7D, E) on post-LPS challenge. Similarly, NK-cell depletion attenuated pulmonary edema and albumin leakage, decreased CXCL1 and -2 mRNA expression and concomitant neutrophilia in the lung (Fig. 7F–I). As with attenuated neutrophil recruitment and myeloid inflammation in *Tbet*$^{-/-}$ mice, NK-cell depletion with anti-NK1.1 treatment significantly abrogated the pulmonary expression of the proinflammatory cytokines IL-1β, TNFα, IFNγ, and IL-6 (Fig. 7J–M). Taken together, these results demonstrate that NK-cell depletion mimics the protective phenotype of *Tbet*$^{-/-}$ mice with reduced neutrophilia and associated lung inflammation during endotoxin-induced ALI.

DISCUSSION

Regardless of the inflammatory stimuli, a myriad of clinical and experimental data have implicated activated neutrophils in the pathogenesis of ALI. Although neutrophil activation is vital for host defense, ALI is characterized by tissue damage associated with neutrophil release of cytotoxic agents, such as cationic polypeptides, cytokines, reactive oxygen species and proteases (reviewed in ref. 9). Thus, understanding the cellular regulation of neutrophil recruitment and activation at mucosal surfaces has the potential for novel therapeutic interventions in ALI. One candidate leukocyte subset that can control neutrophil activation is the NK cell. NK cells optimize immune responses via contact and recognition of target cells [15, 16]. However, the extent to which NK cells or NK-cell crosstalk can control the initial recruitment of neutrophils is not fully defined. In this study, using a model of endotoxin-induced noninfectious ALI, we showed that *Tbet*$^{-/-}$ mice (which lack mature NK cells) or Ab depletion of NK cells markedly attenuated neutrophilic inflammation, CXCR2 chemokines and the acute-phase response–associated lung disease. Adoptive transfer studies using mature NK cells into *Tbet*$^{-/-}$ mice re-established neutrophilic inflammation with concomitant CXCL1 and -2 expression. Thus, our data highlight a critical and previously unappreciated role for NK cells as regulators of neutrophil recruitment to the inflamed lung.

The innate and adaptive immune responses are exquisitely controlled by a series of master transcription factors, which dictate both cellular responses and the resulting inflammatory phenotypes. The transcription factor Tbet (encoded by *Tbx21)* is critical for driving cellular immunity and Th1 responses;

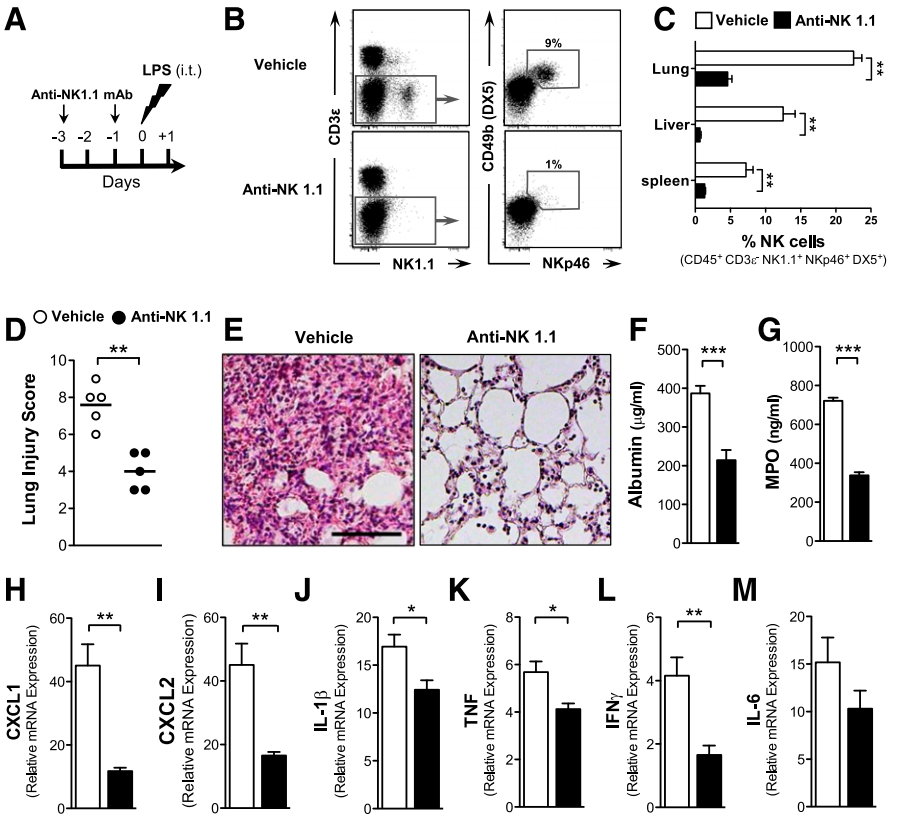

Figure 7. Antibody depletion of NK cells attenuates endotoxin-induced lung injury. C57BL/6J mice received 2 doses of anti-NK-1.1 (200 μg i.p.; clone PK136) or IgG2a (clone C1.18.4) on d −3 and −1 before induction of ALI with LPS (intratracheal.; 5.0 μg/g body weight; *Escherichia coli* 0111:B4) administration. (A) Schematic outline of Ab depletion regimen. (B) Representative flow cytometry plots assessing depletion efficiency of NK cells from the lungs of vehicle or anti-NK1.1-treated mice. (C) The frequency of $CD45^+CD3\varepsilon^-NK1.1^+$ $NKp46^+DX5^+$ NK cells in the lung, spleen, and liver of treated mice was assessed by flow cytometry. (D) Lung injury histology score of vehicle and anti-NK1.1-treated mice after LPS treatment. (E) Representative micrographs of H&E-stained lung tissue of LPS-, vehicle-, and anti-NK1.1-treated mice at indicated time points, Scale bar, 100 μm. (F) Albumin was measured by ELISA from BAL fluid. (G) MPO was quantified in the BAL fluid by ELIS (A). Relative expression of mRNA was quantified by RT-PCR in whole lung tissue for CXCL1 (H), CXCL2 (I), IL-1β (J), TNF (K), IFNγ (L), and IL-6 (M). Data are expressed as means ± SEM and are representative of 2 independent experiments ($n = 5$ mice/group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. indicated vehicle counterpart.

however, our understanding of how Tbet dictates the mucosal immune response to lung inflammation is incomplete. With respect to understanding the functional requirements for activated NK cells, it has been documented that Tbet instructs maturation signals for NK-cell subsets [24, 25, 28]. Specifically, $Tbet^{-/-}$ mice exhibit reduced numbers of NK cells in the spleen, liver, and peripheral blood and display impaired cytotoxicity and IFN-γ production after infection [28, 29]. NK cells have a significant role in bridging the innate and adaptive arms of the immune response, but they have predominantly been investigated for their roles in cancer immunosurveillance and fighting infections. Currently, the direct involvement of mature, $Tbet^+$ NK cells in the initiation of the mucosal inflammation during sterile ALI remains to be defined.

During active inflammation an extensive body of literature highlights the ability of NK cells and NK-cell–derived cytokines to regulate neutrophil activation. Specifically, NK cells activated by FMLP and IL-2, -15, -18 and -21 potentiate neutrophil-intrinsic ROS production and phagocytic potential [17, 18]. Furthermore, it is now apparent that there are multiple subsets of NK cells defined by their activation state and anatomic location [24, 29, 30]. This finding is particularly evident for the $CD27^{low}$ $KLRG1^+$ subset required for antitumor immunity. It was demonstrated that although $CD27^{low}$ and $CD27^{high}$ NK-cell subsets display altered activation and chemotactic responses, the predominant resident NK cell in the murine lung was $CD27^{low}$ [31]. Our phenotypic analysis of activated $Tbet^+$ NK cells in the lungs of LPS-treated mice were positive for activation markers, such as CD69, NKG2D, CD27, and CD49b (DX5), indicating an infiltrating population instead of resident cells.

Aberrant neutrophil maturation and recruitment can exhibit profound multiorgan inflammation and tissue damage. When critical regulators of granulopoiesis are lost, such as NFκB ($Crel^{-/-}Nfκb1^{-/-}Rela^{+/-}$) or the micro-RNA miR-223 ($miR223^{-/y}$), mice develop a multitude of pathologic responses, including skin, intestine, and lung inflammation [32, 33]. Alternatively, the depletion or blockade of neutrophil recruitment has been efficacious in a multitude of preclinical models of sterile ALI including LPS-, hyperoxia-, ozone-, and ventilator-induced injuries [34–37]. Clinically, the blockade of neutrophil recruitment during sterile inflammation with an oral CXCR2 antagonist has shown therapeutic promise in healthy volunteers challenged with aerosolized LPS [38]. It is worth noting, however, that neutrophil blockade during infectious ALI would have detrimental results, because neutrophils are critical for clearing bacteria. This role is most evident in the requirement of neutrophils for the containment of multidrug-resistant *Klebsiella pneumonia* in the lung or intestinal bacterial burden during colitis [39, 40]. Depletion of NK cells during experimental DSS-colitis is deleterious because of increased neutrophilia and suppressive effects of NK cells on colonic neutrophils via inhibitory NKG2A receptors *in situ* [41]. A critical role for NK cells in controlling bacterial dissemination was further revealed by the same group during enteric infection with *Citrobacter rodentium* [42]. Thus, the role for NK regulation of neutrophilic inflammation and recruitment appears to be context, tissue, and stimuli dependent.

In mice, the chemokines CXCL1 and -2 are critical for neutrophil recruitment, but an outstanding question remains as to the cellular source of CXCL1 and CXCL2 in

endotoxin-inducted lung injury [43]. Previous reports have identified independent expression profiles for both chemokines in steady state conditions, with constitutive expression of CXCL2 from bone-marrow–derived $CD11b^+$ $GR1^+$ granulocytes, whereas CXCL1 was expressed in peripheral tissues of adult mice, such as the spleen, Peyer's patches, and gut epithelium [44]. Macrophages specifically upregulate CXCL2 after stimulation with whole bacteria or bacterial cell wall components, such as LPS [45, 46]. CXCL2 induction has also been observed in epithelial cells, vascular endothelial cells, mast cells, and neutrophils [47–50]. However, although alveolar macrophage are the likely first responders to intratracheal LPS via TLR4 activation and can upregulate CXCL1/CXCL2, they do not express Tbet and are unlikely to be the effector cell responsible for the reduced CXCL1/CXCL2 observed in $Tbet^{-/-}$ mice. Recent data have reported the ability of human NK cells to respond to LPS with the production of IFNγ, but the presence of NK-cell intrinsic TLR4 is debatable and only detected only at the level of mRNA [51, 52] or by intracellular flow cytometry [53]. Alternatively, an indirect method of NK-cell activation by LPS has been favored. In the LPS-induced model of ALI, the primary target cell for LPS stimulation would be alveolar macrophages [54], with the concomitant activation and cytokine cascade stimulation of resident or infiltrating NK cells by IL-12, IL-18, B7, and NKG2D ligands. In addition, as inflammatory cytokines such as IL-6, TNF, and IL-1β are potent inducers of neutrophil chemokines [55, 56], attenuated production of these cytokines in $Tbet^{-/-}$ mice may further prevent an LPS-driven cytokine feedback loop for neutrophil recruitment.

Our data point toward NK cells as the predominant expressers of Tbet during the acute phase of LPS challenge, and they are markedly depleted from the lungs of $Tbet^{-/-}$ mice. However, understanding of how the activation state and responsiveness of NK-cell subsets integrate neutrophil chemotactic signals is incomplete. One possible explanation is via the Tbet-responsive cytokine IFNγ. In the lung, a major source of IFNγ during acute-phase responses are NK cells and the treatment of mice with recombinant IFNγ-augmented CXC chemokine release after pulmonary LPS or *Pseudomonas aeruginosa* challenge [57]. In addition, T-cell-dependent IFNγ drives neutrophilic airway inflammation after endotoxin challenge in an ovalbumin-specific transgenic model [58]. Collectively, these results correlate with our data, whereby LPS-induced ALI in either $Tbet^{-/-}$ or anti-NK1.1 Ab–depleted mice display reduced IFNγ, CXCL1, CXCL2, and neutrophil recruitment. After intratracheal LPS, the maximum lung expression of CXCL1 and CXCL2 was observed after 24 h and, these were also the only 2 chemokines to be reduced in $Tbet^{-/-}$ mice at this time point. Although $Tbet^{-/-}$ is a whole-body gene-deficient mouse and chemokine responses may be representative of an altered immune profile by resident lung immune cells at baseline, our adoptive transfer and NK-cell Ab depletion studies support the major role played by NK cells in regulating CXCL1 and -2 during the acute phase of LPS lung injury.

The current array of immunosuppressive and anti-inflammatory drugs carry a multitude of complications associated with infections and generalized immunosuppression. There is now a heightened interest in identifying novel cellular targets and specific stimuli (e.g., damage-associated molecular patterns) that stratify sterile vs. infectious inflammation and associated tissue pathology. This dichotomy has not been completely elucidated for the different variants of ALI, but a further understanding of how NK-cell signaling regulate sterile vs. infectious pulmonary inflammation warrants ongoing investigation.

AUTHORSHIP

S.H., H.E., K.B., F.V., and E.N.M. acquired the data and performed the data analysis. L.G.-M. and H.E.K. designed the study and its interpretation. S.H., H.E., K.B., F.V., L.G.-M., H.E.K., and E.N.M edited the manuscript. S.H. and E.N.M. conceived of and designed the research, drafted the article, and had final approval of the manuscript.

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DISCLOSURE

The authors declare no conflicts of interest.

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