Alveolar Epithelial STAT3, IL-6 Family Cytokines, and Host Defense during *Escherichia coli* Pneumonia

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While signal transducer and activator of transcription (STAT) 3 signaling has been linked to multiple pathways influencing immune function and cell survival, the direct influence of this transcription factor on innate immunity and tissue homeostasis during pneumonia is unknown. Human patients with dominant-negative mutations in the *Stat3* gene develop recurrent pneumonias, suggesting a role for STAT3 in pulmonary host defense. We hypothesized that alveolar epithelial STAT3 is activated by IL-6 family cytokines and is required for effective responses during gram-negative bacterial pneumonia. STAT3 phosphorylation was increased in pneumonic mouse lungs and in murine lung epithelial (MLE)-15 cells stimulated with pneumonic bronchoalveolar lavage fluid (BALF) through 48 hours of *Escherichia coli* pneumonia. Mice lacking active STAT3 in alveolar epithelial cells ($Stat3^{\Delta/\Delta}$) had fewer alveolar neutrophils and more viable bacteria than control mice early after intratracheal *E. coli*. By 48 hours after *E. coli* infection, however, lung injury was increased in $Stat3^{\Delta/\Delta}$ mice. Bacteria were cleared from lungs of both genotypes, albeit more slowly in $Stat3^{\Delta/\Delta}$ mice. Of the IL-6 family cytokines measured in lungs from infected C57BL/6 mice, IL-6, oncostatin M, leukemia inhibitory factor (LIF), and IL-11 were significantly elevated. Neutralization studies demonstrated that LIF and IL-6 mediated BALF-induced STAT3 activation in MLE-15 cells. Together, these results indicate that during *E. coli* pneumonia, select IL-6 family members activate alveolar epithelial STAT3, which functions to promote neutrophil recruitment and to limit both infection and lung injury.

Keywords: lung; neutrophils; STAT3; pneumonia; cytokines

Lung infections account for a tremendous burden of disease worldwide and are a leading cause of acute lung injury (1, 2). While *Streptococcus pneumoniae* is the most common agent in patients with community-acquired pneumonia (3), gram-negative rods such as *Escherichia coli* are a frequent cause of nosocomial pneumonia (4). Elimination of these and other pathogens from the lower respiratory tract is made possible by an effective innate immune response (5), which is necessary yet potentially dangerous to the infected host. For this reason, cytokine networks, neutrophil emigration, plasma extravasation, and other characteristics of acute inflammation must be precisely regulated to maintain tissue homeostasis.

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CLINICAL RELEVANCE

Our results indicate that alveolar epithelial STAT3 activation requires IL-6 family cytokines and mediates inflammation during pneumonia. This pathway is a plausible therapeutic target for improving host defense and/or preventing lung injury.

The STAT3 transcription factor influences both immunity and inflammatory injury, but the importance of STAT3 signaling during pneumonia is unknown. STAT3 activity has been attributed both inflammatory (6–9) and anti-inflammatory (10–12) roles. Likewise, the cytokine interleukin (IL)-6, which largely signals through STAT3 (13, 14), has also been described as both pro- (15–19) and anti-inflammatory (16, 20–22), depending on the biological context. During *E. coli* pneumonia, neutrophil recruitment and bacterial clearance are impaired in IL-6–deficient mice (15). While the mechanisms through which IL-6 functions during this infection were not determined, tyrosine 705-phosphorylated STAT3 (pSTAT3) content was reduced in the lungs of IL-6–deficient mice, suggesting that this pathway may be required for activation of innate host defense during gram-negative pneumonia. A role for STAT3 in pulmonary host defenses is also suggested by human patients with hyper-IgE syndrome, in which defective STAT3 activity results in recurrent lung infections (23–25).

IL-6 is but one member of a family of cytokines bearing its name, all of which signal through STAT3 (14). In the current study we hypothesized that alveolar epithelial STAT3 is activated by IL-6 family cytokines and is required for host defense and the prevention of lung injury during gram-negative pneumonia. We focused on alveolar epithelial cells for several reasons: (*1*) STAT3 is rapidly activated in this cell type during inflammatory responses to LPS (26), the major inflammatory stimulus of gram-negative bacteria; (*2*) alveolar epithelial cells produce cytokines and other inflammatory mediators required for host defense (27, 28); (*3*) activated STAT3 in alveolar epithelial cells is required to prevent lung injury in response to hyperoxia (10) and adenovirus exposure (12); and (*4*) STAT3 overexpression in alveolar epithelial cells is sufficient to induce pulmonary inflammation (9). To address our hypothesis, we identified factors required for alveolar epithelial STAT3 activation, and determined the outcome of bacterial pneumonia in mice lacking functional STAT3 in alveolar epithelial cells.

MATERIALS AND METHODS

Mice

Triple transgenic mice were bred as previously described (10) to generate colonies of control mice ($SP\text{-}CrtTA^{-/-}/(tetO)_7CMV\text{-}Cre^{tg/tg}/Stat3^{flx/flx}$) or $Stat3^{\Delta/\Delta}$ mice ($SP\text{-}CrtTA^{tg/-}/(tetO)_7CMV\text{-}Cre^{tg/tg}/Stat3^{flx/flx}$), which cannot express the Y705-phosphorylated, active form of STAT3 in their alveolar epithelial cells. For select experiments, $SP\text{-}CrtTA^{-/-}/$

(tetO)$_7$CMV-Cre$^{tg/tg}$ (rtTA−) mice were bred with *SP-CrtTA$^{tg/-}$/(tetO)$_7$CMV-Cre$^{tg/tg}$* (rtTA+) mice to generate colonies containing rtTA and Cre-recombinase mutations without a mutation in either *Stat3* allele. Results obtained from transgenic mice were compared with littermate controls. Food for breeders contained doxycycline (625 mg/kg) to induce Cre-recombinase–mediated STAT3 deletion. The differentiation pattern of cells bearing surfactant protein C promoter activity during lung development results in gene rearrangement within virtually all alveolar epithelial cells (both types I and II) using this doxycycline regimen (29). Progeny were not exposed to the doxycycline diet once weaned from their mothers at 3 weeks of age, reducing the possibility that doxycycline might be present during experiments. At the time of experimentation, mice were 6 to 9 weeks of age. Experiments with nontransgenic mice were performed using C57BL/6 mice. All experimental protocols were approved by the Harvard Medical Area Standing Committee on Animals.

Pneumonia

Mice were anesthetized by an intraperitoneal injection of ketamine (50 mg/kg)/xylazine (5 mg/kg). An angiocatheter was placed down the left bronchus, and mice received intratracheal administrations of 50 μl saline containing approximately 10^6 colony-forming units (CFU) *E. coli* (American Type Culture Collection # 19138; ATCC, Manassas, VA). The concentration of viable bacteria was estimated by optical density and subsequently verified by enumerating CFU from serial dilutions grown on 5% sheep blood agar plates. For histologic experiments, the instillate contained 1% colloidal carbon to visualize pulmonary deposition.

Lung Histology and Morphometry

After 24 or 48 hours of infection, mice were killed by halothane overdose and the heart was ligated to maintain pulmonary blood volume. Lungs were removed and instilled with 6% gluteraldehyde at 23 cm H_2O pressure for fixation. The percentage of alveolar airspace occupied by neutrophils or edema fluid was quantified by blinded morphometric analysis on hematoxylin/eosin-stained lung sections as previously described (30). In rare cases in which lung sections from *Stat3$^{\Delta/\Delta}$* or rtTA+ mice contained areas of emphysema-like airspace enlargement, these regions were excluded from morphometric analyses.

Bacteriology

Lungs were collected 24 and 48 hours after intratracheal *E. coli*, homogenized in 10 ml sterile H_2O, serially diluted, and grown overnight at 37°C on 5% sheep blood agar plates. Viable bacteria were determined by colony counts and expressed as total CFU per lung.

Cytokine Protein Measurement

Lungs were homogenized in H_2O containing the Roche Complete protease inhibitor cocktail (Roche, Indianapolis, IN), resuspended in lysis buffer (0.5% Triton X-100, 150 mM NaCl, 15 mM Tris-HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$), and incubated on ice for 30 minutes. After the incubation, lysates were cleared by centrifugation, and supernatants were collected for protein analyses. Cytokine concentrations were determined using enzyme-linked immunosorbent assay according to the protocols provided by the manufacturer (R&D Systems, Minneapolis, MN).

Real-Time RT-PCR

Cytokine and S100 mRNA levels were quantified in lung tissue using real-time RT-PCR. Left lung lobes were removed from mice at the indicated times after i.t. *E. coli* and preserved in RNA*later* solution (Qiagen, Valencia, CA). Total RNA was extracted and purified using the RNeasy Mini Kit and RNAse-free DNase set, respectively (Qiagen). Real-time RT-PCR was performed on 10 ng purified RNA using the iScript One-Step RT-PCR Kit for Probes (Bio-Rad, Hercules, CA) and the iCycler iQ Real-Time PCR detection system (Bio-Rad). The Beacon Designer software (Premier Biosoft International, Palo Alto, CA) was used to design primers and Taqman probes (Table 1). Probes were modified with 6-FAM (reporter dye) and Black Hole Quencher-1 (quencher dye) at the 5′ and 3′ ends, respectively. Values for each sample were normalized to the content of 18S rRNA and expressed as the fold induction compared with uninfected mice (15).

Lung Wet:Dry Weight Ratios

Mice were killed as above 48 hours after intratracheal *E. coli* and exsanguinated via the abdominal aorta. The left bronchus was ligated, and left lung lobes were removed and weighed immediately. After desiccation at 60°C, lungs were weighed again to determine wet:dry ratios.

Bronchoalveolar Lavage

At the indicated times after intratracheal *E. coli*, lungs were removed from killed mice and tracheas were cannulated with a 20-gauge, blunted stainless steel needle. Antibiotic-free DMEM supplemented with 10% FBS (1 ml) was instilled and withdrawn. Bronchoalveolar lavage fluid (BALF) was then centrifuged at 300 × *g* to remove cells followed by 16,100 × *g* to remove bacteria and other remaining particulate matter. Samples were stored at −20°C until use.

Cell Culture and Immunoblots

Murine lung epithelial (MLE)-15 cells were maintained as described previously (31). Cells were seeded in 24-well tissue culture plates (150,000 cells/well) and incubated overnight (37°C in a humidified atmosphere containing 5% CO_2), resulting in approximately 80% confluence. Where indicated, BALF was supplemented with neutralizing antibodies for IL-6, oncostatin M (OSM), and/or leukemia inhibitory factor (LIF) (10 μg/ml each; R&D Systems) and incubated for 1 hour at 37°C. Preliminary experiments were performed using recombinant cytokines to verify antibody efficacy at the selected concentrations. The same concentrations of isotype-matched nonspecific antibody were used for controls, such that the total immunoglobulin concentration (30 μg/ml) was equivalent in each sample. MLE-15 cells were washed once with PBS and stimulated for 10 minutes with 300 μl BALF. At the end of the incubation, cells were washed once with ice-cold PBS and lysed with 50 μl ice-cold lysis buffer (2% NP-40, 25 mM Tris pH 7.4, 50 mM NaCl, 0.5% Na deoxycholate, and 0.2% SDS) containing the Roche Complete

TABLE 1. PRIMER AND PROBE SEQUENCES FOR REAL-TIME RT-PCR

Gene	Forward Primer	Reverse Primer	Taqman Probe
S100A8:	CTTGTTCAGAGAATTGGACATC	TGCCACACCCACTTTTATC	CCATCGCAAGGAACTCCTCGAAGT
S100A9:	AGGCTGTGGGAAGTAATTAAGAGG	CCAGAACAAAGGCCATTGAGTAAG	AGCCATGTGACTGCTGCCCAACCA
IL-6:	AGTTGCCTTCTTGGGACTGATG	CAGGTCTGTTGGGAGTGGTATC	AACCACGGCCTTCCCTACTTCACA
OSM:	CCCTATATCCGCCTCCAAAACC	GACTCTGTCCAGTGTGGTGTAC	CCTGACCTGAGAGCTGCCTGCACC
LIF:	TCTTCCCATCACCCCTGTAAATG	CTTGATCTGGTTCATGAGGTTGC	CCTGTGCCATACGCCACCCATGCC
IL-11:	CCCCAGACCAACCTGTGATC	AGTCACAGTCGAGTCTTTAACAAC	CGCACGGCCCAGTCCAAGGTCAG
CT-1:	GAGGCCAAGATCCGCCAGAC	TGCACGTATTCCTCCAGAAGTTG	ACAACCTTGCCCGCCTCCTGACCA
CNTF:	TGACTTCCATCAGGCAATACATAC	TTTCAGGGACCTTCTGTTCCAG	ACGCTCCAAGTTTCTGCCTTCGCC
NP:	TTCAGTGACCCCGGCTTCTC	GGACTGCCAGGATTCAGATAACTC	AGCTCCAGCTCAGCACCCTGCCTT
CLC:	TCAACGAGCCTGACTTCAATCC	GTAACACAGGAGGTGACTGTACG	TCTGGGTCAGCCGCAGCCTGTCAT
IL-27:	CCTACCAGCTCCTTCACTCC	TTAGGAATCCCAGGCTGAGC	TCCTGTCTCGGGCTGTTCGGGACC

Definition of abbreviations: CLC, cardiotrophin-like cytokine; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; LIF, leukemia inhibitory factor; NP, neuropoietin; OSM; oncostatin M.

Primer and probes were designed (listed 5′–3′) to amplify an 80– to 200–base pair region within the open reading frame of the following transcripts: S100A8, S100A9, IL-6, OSM, LIF, IL-11, CT-1, CNTF, NP, CLC, and IL-11.

protease inhibitor cocktail (Roche). Lysates were incubated on ice for 15 minutes and then cleared by centrifugation. Protein quantification and immunoblots were performed on supernatants as previously described (31, 32). Primary antibodies were directed against pSTAT3 or STAT3 irrespective of phosphorylation (Cell Signaling, Danvers, MA) and developed with a horseradish peroxidase–conjugated anti-rabbit polyclonal Ab (Cell Signaling). Relative densitometric units were determined using the Image J software (National Institutes of Health, Bethesda, MD). pSTAT3 densitometric units were normalized to that of total STAT3, and data were expressed as the percent pSTAT3 immunoreactivity obtained from control (0 h) BALF-stimulated MLE-15 cells.

For *in vivo* experiments, lungs were collected from killed mice at the indicated times after intratracheal *E. coli*, and left lobes were homogenized in 1 ml ice-cold lysis buffer (*see above*) using a rotor-stator homogenizer. Protein extractions and immunoblots were performed as described above.

Statistics

Statistical analyses were performed using GraphPad Prizm (GraphPad Software, San Diego, CA) or Statistica (StatSoft, Tulsa, OK). Data were presented as means ± SE for the number of samples identified in each figure. Real-time RT-PCR data were calculated as fold-induction and thus were presented as geometric means ± geometric SE. Comparisons were performed with a Student's *t* test, Mann-Whitney *U* test, or a one-way ANOVA followed by a Bonferroni *post hoc* analysis. When data did not pass Levene's test for homogeneity of variance they were log-transformed to fit requirements for ANOVA. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Lung and Alveolar Epithelial STAT3 Are Activated during *E. coli* Pneumonia

pSTAT3 levels were measured in total lung protein extractions as a determinant of STAT3 activity. After infection of mouse lungs with *E. coli*, pSTAT3 was detectable by 2 hours, peaked at 6 hours, and remained elevated through 48 hours (Figure 1A). To determine whether an *E. coli* exudate was capable of activating STAT3 in epithelial cells, we harvested cell/bacteria-free BALF from pneumonic mice 0 to 72 hours after intratracheal *E. coli* and stimulated the alveolar epithelial cell line MLE-15 (33) for 10 minutes. pSTAT3 increased in MLE-15 cells in response to pneumonic BALF compared with nonpneumonic BALF, peaking with 6 hours of infection and evident through 48 hours. MLE-15 cell pSTAT3 was not increased in response to direct *E. coli* stimulation (data not shown), suggesting that host-derived factors rather than trace amounts of bacteria were the cause of BALF-induced STAT3 activation. Thus, alveolar lining fluid contains mediators that activate STAT3 phosphorylation during *E. coli* pneumonia.

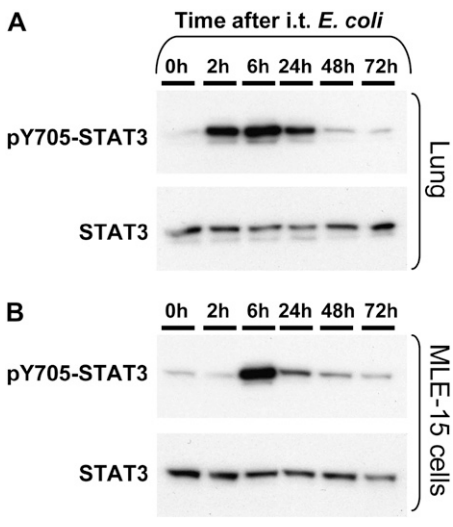

Figure 1. Signal transducer and activator of transcription (STAT)3 activation during pneumonia. STAT3 activation (pSTAT3 content) was determined in (*A*) lungs and (*B*) MLE-15 cells 0 to 72 hours after intratracheal *E. coli*. C57BL/6 mice were instilled with 10^6 colony-forming units (CFU) *E. coli*, and left lung lobes or bronchoalveolar lavage fluid (BALF) was collected at the indicated times. Representative images are shown for pSTAT3 and total STAT3 in (*A*) lungs or (*B*) MLE-15 cells stimulated with BALF for 10 minutes. Each *lane* represents results from lung tissue or BALF collected from a single mouse. Immunoreactive bands shown were approximately 90 kD in size. The two *rows* in each *panel* show the same membrane labeled first for pSTAT3 and the second, after stripping, for total STAT3. Data were generated with three mice at each time point.

Alveolar Epithelial STAT3 Contributes to Early Inflammation and Host Defense

To determine the function of alveolar epithelial STAT3 activation during *E. coli* pneumonia, we instilled bacteria into the lungs of $Stat3^{\Delta/\Delta}$ and control mice. $Stat3^{\Delta/\Delta}$ mice express a truncated form of STAT3 that lacks exon 21, the region encoding the tyrosine residue (Y705) requisite for STAT3 activation, in their alveolar epithelial cells (10). After 24 hours of *E. coli* pneumonia, $Stat3^{\Delta/\Delta}$ mice had significantly fewer emigrated neutrophils in their airspaces compared with control mice (Figure 2A). Circulating neutrophil counts did not significantly differ between control ($7.95 \times 10^5 \pm 0.98 \times 10^5$ cells/ml) and $Stat3^{\Delta/\Delta}$ mice ($6.35 \times 10^5 \pm 1.11 \times 10^5$ cells/ml), and hence did not likely contribute to the differences observed in the airspaces. Decreased neutrophil recruitment in the lungs was associated with and perhaps a cause

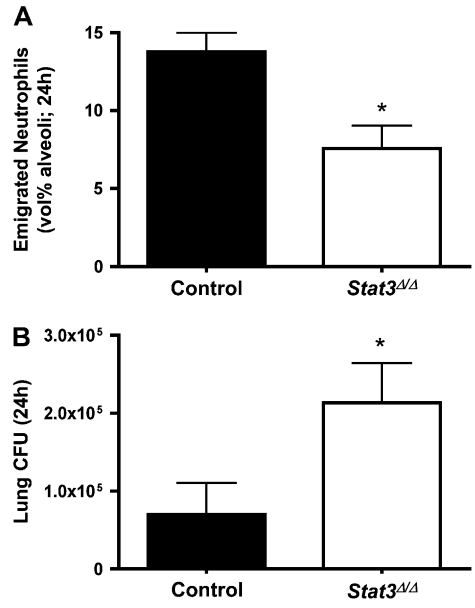

Figure 2. Alveolar epithelial STAT3 and host defense at 24 hours. (*A*) Alveolar neutrophils and (*B*) lung bacterial clearance were measured 24 hours after intratracheal *E. coli* in the presence and absence of functional alveolar epithelial STAT3. Alveolar neutrophil counts (*A*) were determined in control or $Stat3^{\Delta/\Delta}$ mice by morphometric analysis of histologic lung sections. Viable lung *E. coli* (*B*) were quantified by colony counts on 5% sheep blood agar plates. Neutrophil data are presented as means ± SE (n = 9–11) of the percentage of alveolar airspace occupied by neutrophils. Bacteriology data are expressed as means ± SE (n = 5–10) of total lung CFU. *Statistically significant compared with control mice ($P < 0.05$).

of increased lung bacterial burdens in *Stat3*$^{\Delta/\Delta}$ mice (Figure 2B). These data indicate that the inability to activate STAT3 in alveolar epithelial cells compromised innate immune pulmonary host defense during this early phase of pneumonia.

Expression of Multiple Inflammatory Mediators Is Not Dependent on Alveolar Epithelial STAT3 Activation

Since neutrophil emigration was significantly impaired in *Stat3*$^{\Delta/\Delta}$ mice during pneumonia, we determined whether the expression of early response cytokines and neutrophil chemoattractants is dependent on alveolar epithelial STAT3 activation. CXC ELR+ chemokines and S100 proteins mediate neutrophil migration (34–36), are expressed by alveolar epithelial cells (28, 37), and are regulated at least in part by STAT3 (7, 38). In addition, the early response cytokines TNF-α and IL-1β are required for maximal pulmonary inflammation during *E. coli* pneumonia (39). The CXC chemokines macrophage inflammatory protein-2 and LPS-induced CXC chemokine (LIX) were not diminished by alveolar epithelial STAT3 deficiency, nor were levels of TNF-α and IL-1β (Figure 3A). In fact, LIX and IL-1β levels were significantly increased at 6 hours in *Stat3*$^{\Delta/\Delta}$ mice. Similarly, S100A8/9 mRNA expression was strongly induced in response to *E. coli* in both *Stat3*$^{\Delta/\Delta}$ and control mice, with no significant effect of genotype (Figure 3B). Therefore, neutrophil recruitment was decreased by the deficiency of STAT3 activation in alveolar epithelial cells by a mechanism other than the induction of the inflammatory mediators measured.

Alveolar Epithelial STAT3 Prevents Lung Injury

Neutrophil recruitment and bacterial counts were examined 48 hours after infection. Contrasting with the findings at 24 hours, neutrophil recruitment was unaffected or perhaps slightly increased ($P = 0.055$) by epithelial STAT3 deficiency at this later time point (Figure 4A). Numbers of viable *E. coli* remained elevated in *Stat3*$^{\Delta/\Delta}$ mice 48 hours after *E. coli* infection (Figure 4B). While bacteria were less effectively cleared from the lungs of *Stat3*$^{\Delta/\Delta}$ mice at both 24 and 48 hours, neutrophilic inflammation was restored at the later time point.

Lung histology at 48 hours demonstrated alveolar flooding that was particularly pronounced in the *Stat3*$^{\Delta/\Delta}$ mice (Figures 5A and 5B). Morphometric quantification confirmed that a significantly greater amount of the alveolar airspace volume was filled with edema fluid in lungs collected from STAT3-deficient mice compared with controls at 48 hours (Figure 5C). In separate experiments, wet:dry weight ratios were determined as an independent measure of pulmonary edema. Although the difference between genotypes was relatively modest, wet:dry ratios were significantly increased in *Stat3*$^{\Delta/\Delta}$ mice compared with controls 48 hours after intratracheal *E. coli* (Figure 5D). While we did not perform a formal survival study using *Stat3*$^{\Delta/\Delta}$ mice, only one of five lived through 72 hours of infection in a pilot study, whereas seven of nine control mice survived. Together, these data demonstrate that STAT3 in the alveolar epithelium is required for prevention of lung injury during *E. coli* pneumonia.

Since vascular endothelial growth factor (VEGF) can be both STAT3 dependent (40) and cytoprotective in the lung (41, 42), we determined its expression in control and *Stat3*$^{\Delta/\Delta}$ mice during pneumonia. Lung VEGF content was unaffected by STAT3 deficiency at the time points analyzed (Figure 6), and, therefore, does not likely contribute to the lung injury present in *Stat3*$^{\Delta/\Delta}$ mice.

Changes in Host Defense and Lung Injury Are a Direct Result of STAT3 Deficiency

Rearrangement of the *Stat3* gene in *Stat3*$^{\Delta/\Delta}$ mice is mediated by expression of reverse tetracycline-transactivator (rtTA) and Cre-recombinase in lung epithelial cells. To control for the potential detrimental effects of these two products (43, 44), experiments

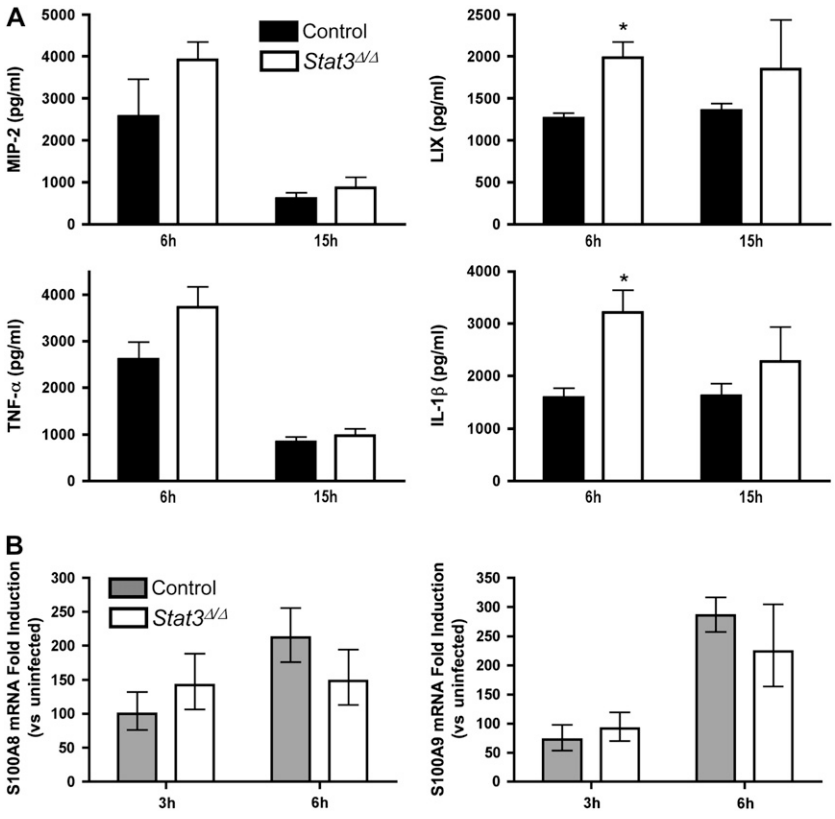

Figure 3. Alveolar epithelial STAT3 and lung inflammatory mediators. Lung ELR+ CXC chemokines, early response cytokines, and S100 expression were quantified after intratracheal *E. coli* in the presence and absence of alveolar epithelial STAT3. Macrophage inflammatory protein-2, LPS-induced CXC chemokine, TNF-α, and IL-1β protein levels (*A*) were measured in lung homogenate by enzyme-linked immunosorbent assay 6 and 15 hours after intratracheal *E. coli,* and expressed as means ± SE pg/ml (n = 4–8). S100A8/9 mRNA (*B*) was determined by real-time RT-PCR 3 and 6 hours after intratracheal *E. coli.* Fold-induction values (versus uninfected mice) are expressed as geometric means ± geometric SE (n = 5–10). *Statistically significant compared with control mice ($P < 0.05$).

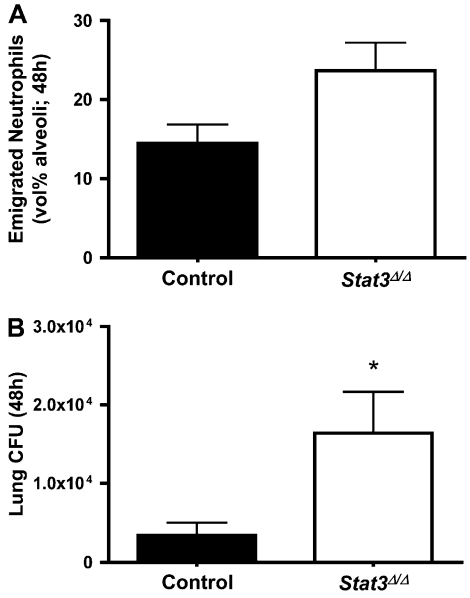

Figure 4. Alveolar epithelial STAT3 and host defense at 48 hours. Alveolar neutrophils (*A*) and lung bacterial clearance (*B*) were measured 48 hours after intratracheal *E. coli* in the presence and absence of alveolar epithelial STAT3. Alveolar neutrophil counts (*A*) were determined in control or $Stat3^{\Delta/\Delta}$ mice by morphometric analysis of histologic lung sections. Neutrophil data are presented as means ± SE (n = 9–11) of the percentage of alveolar airspace occupied by neutrophils. Bacteriology data are expressed as means ± SE (n = 5–10) of total lung CFU. *Statistically significant compared with control mice ($P < 0.05$).

were performed in mice with or without rtTA transgenes (rtTA+ or rtTA−, respectively) but lacking *lox*P insertions in *Stat3* alleles. The rtTA+ mice expressed both rtTA and Cre-recombinase, whereas rtTA− mice expressed little (if promoter leakage) or none of either transgene. During *E. coli* pneumonia, differences between rtTA− and rtTA+ mice were not observed in neutrophil emigration (24 h; 14 ± 1 versus 15 ± 1 [vol% alveoli]), bacterial killing (24 h; 56 ± 80 versus 120 ± 76 CFU/lung [× 10^6]), or lung liquid content (48 h; 4.7 ± 0.1 versus 4.5 ± 0.3 wet:dry ratio). Because the expression of rtTA and Cre-recombinase failed to impact these parameters, we conclude that the phenotypes identified above in $Stat3^{\Delta/\Delta}$ mice result specifically from the deficiency of STAT3 in alveolar epithelial cells rather than an indirect response to upstream transgene expression.

Alveolar Epithelial STAT3 Is Activated by IL-6 Family Cytokines during *E. coli* Pneumonia

The STAT3 signaling pathway is elicited by diverse cytokines, including all members of the IL-6 family (14). With the exception of IL-6 itself, which has been identified as a necessary component of pulmonary host defense (15, 18), the expression patterns of other IL-6 family cytokines have not been determined during bacterial pneumonia. We measured mRNA for the known IL-6 family cytokines to identify candidate STAT3-activating cytokines during *E. coli* pneumonia. Lungs were collected for RNA analysis between 0 and 72 hours of infection to associate changes in STAT3 activation (Figure 1) with changes in STAT3-signaling cytokines. Of the cytokines analyzed, IL-6, OSM, LIF, and IL-11 mRNAs were induced in response to intratracheal *E. coli* (Figure 7A), with no significant changes detectable in the other five cytokines analyzed (CT-1, CNTF, NP, CLC, and IL-27; data not shown). IL-6 expression was highest among the four cytokines (> 7,000-fold), followed in order by OSM (> 300-fold), LIF (> 15-fold), and IL-11 (> 5-fold). Since the kinetics of STAT3 activation (Figure 1) correlated with cytokine mRNA induction, these data implicate IL-6, OSM, LIF, and IL-11 as potential mediators of lung STAT3 signaling during pneumonia.

To determine contributions of IL-6 family members to alveolar epithelial STAT3 activation, MLE-15 cells were stimulated with BALF in the absence or presence of neutralizing antibodies for IL-6, OSM, and LIF, the three most highly expressed cytokines identified above. Consistent with our findings in Figure 1B, BALF from mice exposed to *E. coli* for 24 hours strongly stimulated STAT3 phosphorylation in MLE-15 cells (Figures 7B and 7D). Neutralization of IL-6 or OSM alone did not significantly affect BALF-induced STAT3 phosphorylation. LIF neutralization significantly diminished STAT3 activation in response to 24-hour BALF. Antibody combinations revealed an additional contribution of IL-6 but not OSM to MLE-15 cell STAT3 signaling, such that neutralization of both LIF and IL-6 resulted in significantly less pSTAT3 than did LIF blockade alone. In fact, STAT3 activation induced by 24-hour BALF was virtually abolished by the combined inhibition of both IL-6 and LIF, with densitometric values not significantly different from those measured in response to 0 hours control BALF. After stimulation with 48-hour BALF (Figures 7C and 7E), pSTAT3 levels were

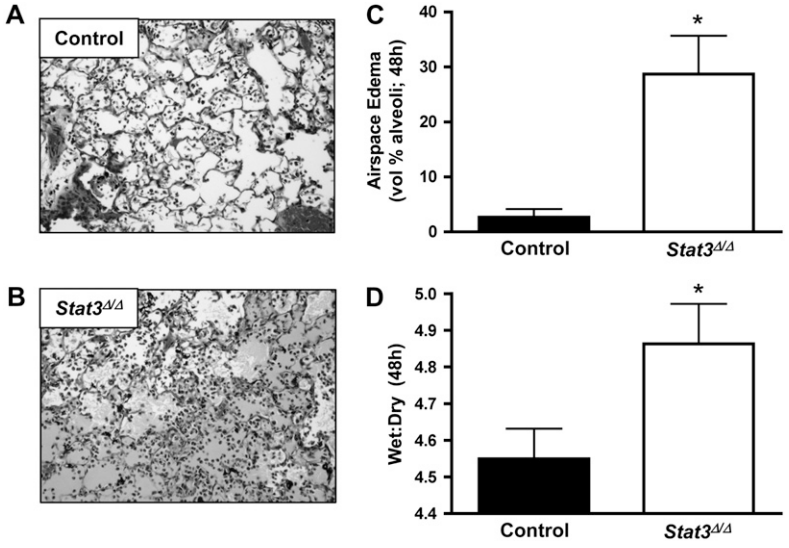

Figure 5. Alveolar epithelial STAT3 and lung injury. Pulmonary edema was examined 48 hours after intratracheal *E. coli* in the presence and absence of alveolar epithelial STAT3. Representative images are shown of pneumonic lung sections from (*A*) control or (*B*) $Stat3^{\Delta/\Delta}$ mice. Alveolar edema was quantified by morphometric analysis of lung sections (*C*) and data expressed as means ± SE (n = 7–8) of the percentage of alveolar space occupied by edema fluid. Pulmonary edema was also measured and expressed as means ± SE (n = 8–11) wet:dry (*D*). *Statistically significant compared with control mice ($P < 0.05$).

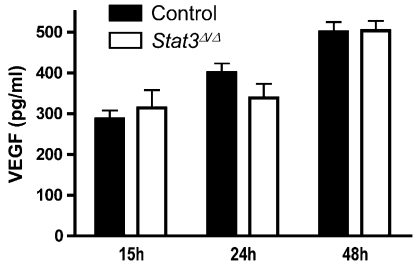

Figure 6. Alveolar epithelial STAT3 and vascular endothelial growth factor (VEGF). Lung VEGF levels were determined 15, 24, and 48 hours after intratracheal *E. coli* in the presence and absence of functional alveolar epithelial STAT3. Protein concentrations were measured in lung homogenate by enzyme-linked immunosorbent assay and expressed as means ± SE pg/ml (n = 3–7).

modestly affected by LIF and IL-6 neutralization, but changes in densitometric values did not reach statistical significance despite consistent patterns observed over three separate experiments. Considerable pSTAT3 immunoreactivity was detected in MLE-15 cells after stimulation with 48-hour BALF even in the presence of all three blocking antibodies. Taken together, LIF and IL-6 in alveolar lining fluid are necessary for early alveolar epithelial STAT3 activation. However, additional STAT3-activating cytokines are important for this process at later time points.

DISCUSSION

Alveolar epithelial STAT3 was required early (24 h) for maximal neutrophil recruitment and bacterial killing during *E. coli* pneumonia. Later in the infection (48 h), STAT3 prevented alveolar edema and lung injury. In addition, our *in vitro* findings suggest that IL-6 family cytokines, particularly LIF, in alveolar lining fluid are necessary for STAT3 activation. We conclude from these data that STAT3 activity in alveolar epithelial cells is downstream of IL-6 family cytokine expression and functions to both promote innate host defense and limit inflammatory injury during pneumonia.

The phenotype of $Stat3^{\Delta/\Delta}$ mice at 24 hours of infection was similar to that of IL-6–deficient mice with *E. coli* pneumonia, including decreased neutrophil recruitment and bacterial clearance (15). IL-6 deficiency transiently decreased total lung pSTAT3 content (15), and the present data demonstrate a transient role for IL-6 in activation of STAT3 in BALF-stimulated MLE-15 cells. Together, these studies implicate alveolar epithelial STAT3 as one avenue through which IL-6 promotes innate immunity. It is likely, however, that IL-6 signals through other cell types as well, such that deletion of STAT3 in only alveolar epithelial cells understates the full contribution of IL-6 to innate immunity in the lungs. The mechanisms, however, through which STAT3 signaling promotes inflammation at this stage of infection remain unknown. STAT3 has been shown to influence the expression of multiple inflammatory mediators (7, 9), yet our current results indicate no contribution of STAT3 on the expression of CXC chemokines, early response cytokines, or S100 proteins during *E. coli* pneumonia. Future research will be necessary to elucidate the factors that link IL-6 family-induced STAT3 activity to alveolar neutrophil emigration.

At 48 hours, the deficiency of STAT3 in alveolar epithelial cells resulted in exacerbated lung injury as measured by alveolar flooding and lung liquid accumulation. Although bacterial burdens were increased in $Stat3^{\Delta/\Delta}$ mice compared with controls at

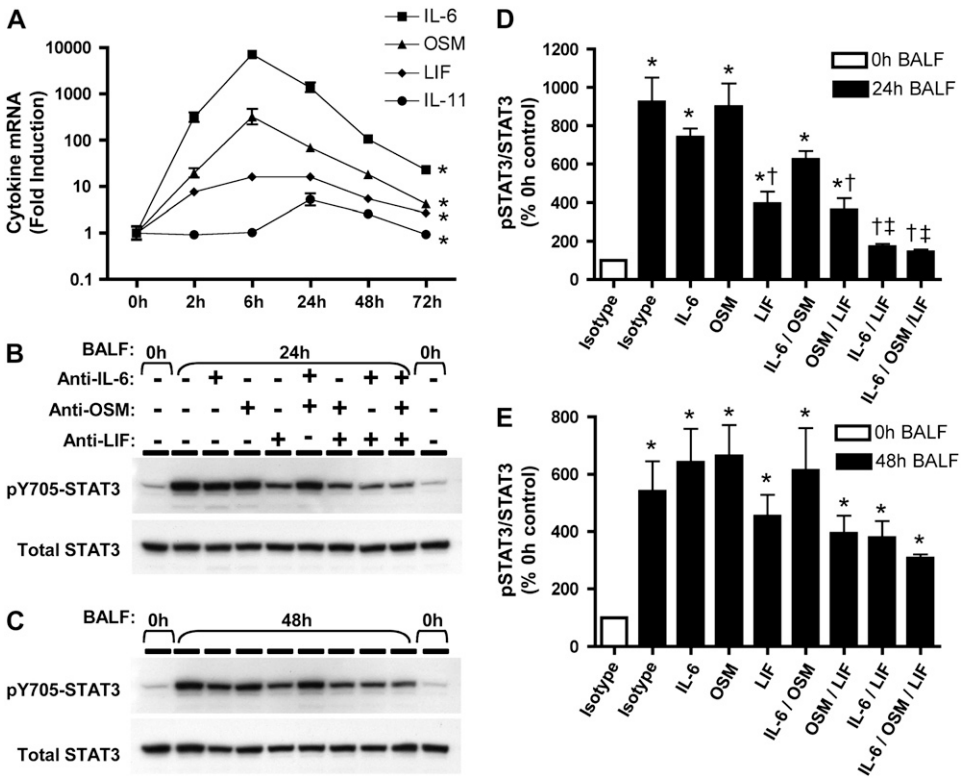

Figure 7. IL-6 family cytokines and pneumonia. IL-6 family mRNA in the lungs (*A*) was measured using real-time RT-PCR for IL-6, oncostatin M (OSM), leukemia inhibitory factor (LIF), and IL-11 mRNA. Pneumonic lung lobes were harvested from C57BL/6 mice 0 to 72 hours after intratracheal *E. coli*, and fold-induction values (versus uninfected mice) are expressed as geometric means ± geometric SE (n = 5–6). *Statistically significant effect of infection on mRNA levels for all shown ($P < 0.05$). IL-6 family-dependent STAT3 activation in MLE-15 cells (*B–E*) was measured using immunoblots. MLE-15 cells were stimulated for 10 minutes with BALF collected 0, 24 (*B/D*), or 48 hours (*C/E*) after intratracheal *E. coli*, in the absence and presence of neutralizing antibodies for IL-6, OSM, and/or LIF. Representative images from one of three separate experiments are shown for pSTAT3 and total STAT3 immunoreactivity in MLE-15 cell protein extracts in response to pooled BALF collected at (*A*) 24 and (*B*) 48 hours. Immunoreactive bands shown were approximately 90 kD in size. The two *rows* in *panels B* and *C* show the same membrane labeled first for pSTAT3 and the second, after stripping, for total STAT3. pSTAT3 immunoreactivity was quantified by densitometry (*D–E*) and normalized to that of total STAT3. pSTAT3/STAT3 ratios are expressed as a percentage of the value determined for MLE-15 cells stimulated with control BALF (0 h BALF) and expressed as means ± SE (n = 3). *Statistically significant compared with cells stimulated with 0 hours of BALF and isotype control IgG ($P < 0.05$). †Statistically significant compared with cells stimulated with pneumonic BALF and isotype control IgG. ‡Statistically significant compared with cells stimulated with pneumonic BALF and anti-LIF ($P < 0.05$).

this later time point, the CFU values observed at 48 hours represent less than 2% of the original *E. coli* inoculum. Therefore, exaggerated lung injury at 48 hours likely resulted from dysregulated inflammation or epithelial integrity rather than overwhelming infection. The results at 48 hours also indicate the possibility that STAT3 influences host defense independent of its early contribution to neutrophil recruitment (at 24 h), since bacterial burdens were higher in $Stat3^{\Delta/\Delta}$ mice despite normal or perhaps increased emigrated neutrophils. While neutrophil recruitment is a critical determinant of bacterial clearance in the lower respiratory tract, it is also possible that other bactericidal factors are downstream of STAT3 activation in the epithelium.

STAT3 activity has diverse biological consequences, but perhaps the most consistent role is tissue protection (10, 12, 45). Many mechanisms are possible, including but by no means limited to regulation of apoptosis (46), surfactant production (47), VEGF expression (40), and heme oxygenase-1 expression (48). STAT3 in alveolar epithelial cells helps prevent lung injury after hyperoxia or adenovirus administration via mechanisms that include the expression of surfactant protein-B and possibly the anti-apoptotic protein Bcl-x_L (10, 12). The overexpression of a constitutively active form of STAT3 in the airway epithelium protects mice from hyperoxic lung injury, in part due to decreased expression of matrix metalloproteinases 9 and 12 (11). VEGF is also expressed in response to STAT3 activation (40) and can be cytoprotective in the lungs (41, 42), but our current data suggest that this growth factor was not responsible for the phenotype observed in $Stat3^{\Delta/\Delta}$ mice. STAT3 target genes important during pneumonia may include those identified above as well as others. The net effects of STAT3 activity in the alveolar epithelium of infected lungs are to increase bacterial clearance and limit lung injury.

It was recently shown that dominant-negative mutations in the *Stat3* gene in humans result in the hyper-IgE syndrome (24, 25). Recurrent pneumonias are a hallmark of this disease, and lung infection is either directly or indirectly the cause of death in these patients (23). The present results establish a causal link between STAT3 and host responses to bacteria in the lungs. Our current data indicate that STAT3 deficiency in the alveolar epithelial cells reduces both innate immune responsiveness and tissue protection during pneumonia, which suggests that defective functions in these cells may contribute to the pathogenesis of hyper-IgE syndrome.

Because alveolar epithelial STAT3 has such important functions during pneumonia, we sought to determine upstream factors contributing to its activation. Multiple cytokines and growth factors stimulate STAT3 signaling. We focused on IL-6 family members due to the importance of IL-6 during pneumonia (15, 18) and the shared requisite use of the gp130/STAT signaling pathway by this family of cytokines (49). Alveolar epithelial cells express gp130 and are responsive to IL-6 family cytokines (26, 47, 50). Little is known about IL-6 family members other than IL-6 during pneumonia, but OSM and LIF are increased in serum and BALF of patients with pneumonia and acute lung injury (51, 52). We observed increased lung mRNA for IL-6, OSM, LIF, and IL-11 after intratracheal *E. coli*. The combined blockade of both IL-6 and LIF completely inhibited STAT3 phosphorylation induced by BALF from mice infected 24 hours previously, suggesting these two cytokines as essential to activating alveolar epithelial STAT3 early during pneumonia. Despite its comparatively lower level of mRNA induction, LIF was the most essential of the IL-6 family cytokines in BALF for activating STAT3 phosphorylation in MLE-15 cells, supporting a role for LIF in particular during pneumonia.

As with STAT3, LIF has protective effects during inflammation. Hyperoxia-induced lung injury, which is exacerbated in the absence of alveolar epithelial STAT3 (10), is ameliorated when LIF is overexpressed in the lungs (53). Similarly, intratracheal administration of recombinant LIF protects rats from hyperoxia-induced lung injury (54) and inhibits LPS-induced pulmonary inflammation (55). The roles of endogenous LIF are less clear. However, increased susceptibility to endotoxemia-induced shock in LIF-deficient mice again suggests a protective effect of this IL-6 family member during inflammation (56). The regulation and function of LIF in pneumonic lungs is an important direction for future research.

Together these results indicate a critical role for STAT3 in alveolar epithelial cells during pneumonia. Early during infection, epithelial STAT3 contributes to neutrophil recruitment, whereas later it serves a role in protection from lung injury. During *E. coli* pneumonia, both IL-6 and LIF activate STAT3 phosphorylation in alveolar epithelial cells. Variation among patients in the LIF/IL-6:STAT3 signaling pathway may influence both infection and lung injury during pneumonia.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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