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STAT4 Is a Critical Mediator of Early Innate Immune Responses against Pulmonary Klebsiella Infection

Jane C. Deng,* Xianying Zeng,* Michael Newstead,* Thomas A. Moore,* Wan C. Tsai,† Victor J. Thannickal,* and Theodore J. Standiford2*

Bacterial pneumonia is a leading cause of morbidity and mortality in the U.S. An effective innate immune response is critical for the clearance of bacteria from the lungs. IL-12, a key T1 cytokine in innate immunity, signals through STAT4. Thus, understanding how STAT4 mediates pulmonary innate immune responses against bacterial pathogens will have important implications for the development of rational immunotherapy targeted at augmenting innate immunity. We intratracheally administered Klebsiella pneumoniae to wild-type BALB/c and STAT4 knockout (STAT4−/−) mice. Compared with wild-type controls, STAT4−/− mice had decreased survival following intratracheal Klebsiella administration, which was associated with a higher lung and blood bacterial burden. STAT4−/− animals also displayed impaired pulmonary IFN-γ production and decreased levels of proinflammatory cytokines, including the ELR+ CXC chemokines IFN-γ-inducible protein-10 and monokine induced by IFN-γ. Although total lung leukocyte populations were similar between STAT4−/− and wild-type animals following infection, alveolar macrophages isolated from infected STAT4−/− mice had decreased production of proinflammatory cytokines, including IFN-γ, compared with infected wild-type mice. The intrapulmonary overexpression of IFN-γ concomitant with the systemic administration of IFN-γ partially reversed the immune deficits observed in STAT4−/− mice, resulting in improved bacterial clearance from the blood. Collectively, these studies demonstrate that STAT4 is required for the generation of an effective innate host defense against bacterial pathogens of the lung. The Journal of Immunology, 2004, 173: 4075–4083.

Klebsiella pneumoniae is a major Gram-negative pathogen in patients with hospital- and community-acquired pneumonia. Even with appropriate antimicrobial therapy, however, the host may not be able to clear bacterial pathogens from the lung effectively if the innate immune response is impaired (1). Thus, effective innate immunity is essential to the resolution of bacterial pneumonia.

Among the cytokines important to pulmonary host defense in pneumonia is IL-12. IL-12 has been demonstrated to be a critical mediator of pulmonary host defense against K. pneumoniae, Chlamydia trachomatis, and Legionella pneumophila (2–4). Many of the effects of IL-12 are mediated through IFN-γ. In murine models of Klebsiella pneumonia, a deficiency in IFN-γ confers increased susceptibility to pulmonary K. pneumoniae infection (5, 6), which may be due in part to increased rates of progression from pneumonia to bacteremia. Collectively, the T1-type cytokines appear to play important roles in the clearance of Klebsiella and other bacterial pathogens from the lung.

A family of proteins, STATs, mediates the cellular responses to cytokine signaling. In particular, STAT4 appears to be a critical element in the IL-12 signaling pathway (7). Through gene targeted deletion, STAT4 has been shown to regulate the polarization of naive Th cells into Th1 cells. Knockout mice deficient in STAT4 (STAT4−/− mice) (3) have defects in Th1 differentiation due to a decreased ability to respond to IL-12, and as a result have decreased IFN-γ levels (8). Indeed, STAT4−/− mice have impaired resistance to several intracellular pathogens, as demonstrated in models of i.p. Babesia, cutaneous Leishmania major, and i.p. Toxoplasma gondii infections (9–11). STAT4 also appears to play a role in pulmonary immunity in a murine model of tuberculosis (12).

Reports regarding the necessity of STAT4 in generating an effective T1 response, however, are inconsistent. IFN-γ-secreting Th1 cells and CD8+ T cells can arise in the absence of STAT4 in vivo and in vitro (13, 14). In a T. gondii infection model, STAT4−/− mice were able to generate IFN-γ in response to IL-2 and IL-18, demonstrating a STAT4-independent mechanism of inducing IFN-γ (15). In a pulmonary influenza murine model, STAT4−/− mice were capable of mounting a virus-specific helper and cytotoxic type 1 T cell response to respiratory influenza infection (11). Furthermore, there is evidence that IL-12 can signal via a STAT4-independent, MAPK pathway in T cells (16) or alternatively via STAT1 and STAT5 (17).

In any given infection model, it is unclear whether STAT4 is more essential to modulating innate immune functions vs adaptive immune responses. Although STAT4 clearly affects Th1 differentiation and cytotoxic T cell functions (7, 14, 18–20), this molecule also appears to play a role in the augmentation of cytolytic function of NK cells, an important cell in innate immunity (8, 21). However, the effects of STAT4 on other cells important to innate immunity in the lung, such as macrophages and dendritic cells, are less well defined. Immature dendritic cells and resting monocytes do not express STAT4 (22). In these cell types, STAT4 expression is dependent upon maturation or activation (23). It has also been
reported that IL-12 does not activate STAT4 in murine macrophages or dendritic cells (24, 25).

In the lung, alveolar macrophages and dendritic cells are important early responders for the clearance of bacterial pathogens. It has previously been demonstrated that animals depleted of αβ T cells do not have increased mortality compared with control animals in murine *Klebsiella pneumonia* (26). Given that Th and cytotoxic T cells do not appear to be critical to the immune response against *K. pneumoniae*, we investigated whether or not STAT4 is necessary for mounting an effective innate immune response against pulmonary *Klebsiella* infection. Furthermore, as prior studies have focused predominantly on the role of STAT4 in cell-mediated immunity against intracellular pathogens, we wished to determine the importance of STAT4 in innate host defense against a clinically relevant extracellular pathogen, such as *Klebsiella*.

**Materials and Methods**

**Animals**

Female specific pathogen-free 6- to 8-wk-old STAT4−/− mice (backcrossed to the BALB/c background for 10 generations) were purchased from The Jackson Laboratory (Bar Harbor, ME). Sex- and age-matched wild-type (wt)3 BALB/c mice from The Jackson Laboratory were used as controls. All animals were housed in specific pathogen-free conditions within the University of Michigan animal care facility (Ann Arbor, MI) until the day of sacrifice.

**Bacterial inoculation**

*K. pneumoniae* strain 43816, serotype 2 (American Type Culture Collection, Manassas, VA), was used in our studies. *Klebsiella* was grown over-night in tryptic soy broth (Difco, Detroit, MI) at 37°C. The concentration of bacteria in broth was determined by measuring the absorbance at 600 nm, and then plotting the OD on a standard curve generated by known CFU values. The bacteria culture was then diluted to the desired concentration.

Animals were anesthetized with an i.p. ketamine and xylazine mixture. The trachea was exposed, and 30 μl of inoculum was administered intra- tracheally (i.t.) via a sterile 26-gauge needle. The skin incision was closed using surgical staples.

**Reagents**

Murine rIFN-γ (rmIFN-γ) (3) was purchased from R&D Systems (Minneapolis, MN). A recombinant type 5 adenovirus containing the murine IFN-γ cDNA (Adeno-IFN-γ) was a generous gift from J. Kolls (University of Pittsburgh School of Medicine, Pittsburgh, PA). We have previously shown that the i.t. administration of this adenovirus vector results in the intrapulmonary expression of IFN-γ in a time- and dose-dependent manner, leading to beneficial effects on pulmonary clearance of *L. pneumophila* (27). IFN-γ, IL-12, TNF-α, IFN-γ-inducible protein-10 (IP-10), monokine induced by IFN-γ (Mig), KC, and MIP-2 Abs used in ELISA were obtained from R&D Systems.

**Whole lung homogenization for CFU and cytokine analysis**

At designated time points, the mice were euthanized by CO2 inhalation. Before lung removal, the pulmonary vasculature was perfused by infusing 1 ml of PBS containing 5 mM EDTA into the right ventricle. Whole lungs were removed, taking care to dissect away lymph nodes. The lungs were then homogenized in 1 ml of PBS with protease inhibitor (Boehringer Mannheim, Indianapolis, IN). Homogenates were then serially diluted 1/5 with PBS and plated on blood agar to determine lung CFU. The remaining residual extracellular bacteria. After incubation for 18 h, the cell culture medium was replaced. Gentamicin was added to medium to kill any residual extracellular bacteria. After incubation for 18 h, the cell culture supernatant was removed for cytokine analysis.

**Blood**

Blood was collected in a heparinized syringe from the right ventricle at designated time points, serially diluted 1/2 with PBS, and plated on blood agar to determine blood CFU.

**Total lung leukocyte isolation**

Total lung leukocytes were isolated, as previously described (26, 28). Briefly, lung tissue was minced to a fine slurry in 15 ml of digestion buffer (RPMI 1640, 5% FCS, 1 mg/ml collagenase (Boehringer Mannheim), and 30 μg/ml DNase (Sigma-Aldrich, St. Louis, MO)). Lung slurry was enzymatically digested for 30 min at 37°C. Undigested fragments were further dispersed by drawing the solution up and down through the bore of a 10-ml syringe. The total lung cell suspension was pelleted, resuspended, and spun through a 20% Percoll gradient to enrich for leukocytes for flow analysis. Cell counts and viability were determined on a hemacytometer using trypan blue exclusion.

**Bronchoalveolar lavage (BAL) and cytopsins**

Mice were sacrificed at the designated time points for the performance of BAL. The trachea was exposed and intubated using a 1.7-mm-outer-diameter polyethylene catheter. BAL was performed by instilling PBS containing 5 mM EDTA in 1-ml aliquots. Total cell number was determined by counting on a hemacytometer. Cytocentrifugation slides (Cytospin 2; Thermo Shandon, Pittsburgh, PA) were prepared from BAL cells or lung digest leukocyte suspensions and stained with Diff-Quik (Dade Behring, Newark, DE) for cell differential.

**Alveolar macrophage isolation**

Mice were sacrificed at designated time points for the isolation of alveolar macrophages from BAL fluid. The BAL was centrifuged at 580 × g for 10 min, the supernatant was discarded, and the cell pellet was resuspended in RPMI 1640 with 5% FBS and antibiotics. The cell count was determined using a hemacytometer, and the cells were then diluted to a 106 cells/ml concentration. Trypan blue exclusion revealed that >95% of the cells were viable. The cells were cultured on a 24-well tissue culture plate (Costar, Cambridge, MA). After 1 h of incubation at 37°C in 5% CO2, the wells were washed of nonadherent cells and washed again, and then fresh medium was replaced. Gentamicin was added to medium to kill any residual extracellular bacteria. After incubation for 18 h, the cell culture supernatant was removed for cytokine analysis.

**Marine cytokine ELISAs**

Murine cytokines were quantitated using a modification of a double ligand method, as previously described (29). Standards were 0.5-log dilutions of recombinant cytokine from 1 pg/ml to 100 ng/ml. The ELISAs did not cross-react with other cytokines.

**Isolation and RT-PCR amplification of lung mRNA**

Whole lung was harvested at designated time points, immediately frozen in liquid nitrogen, and then stored at −70°C for RNA extraction. Total cellular RNA was isolated by homogenizing the tissue in 3 ml of TRIzol

3 Abbreviations used in this paper: wt, wild type; Adeno-IFN-γ, adenovirus containing murine IFN-γ cDNA; BAL, bronchoalveolar lavage; IP-10, IFN-γ-inducible protein-10; i.t., intratracheal; Mig, monokine induced by IFN-γ; rmIFN-γ, murine rIFN-γ.
reagent (Invitrogen Life Technologies, Gaithersburg, MD). Total RNA was determined by spectrometric analysis at 260 nm wavelength. Measurement of gene expression was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes for /H9252-actin and IFN-/H9253 were designed using Shortcat to Primer Express software (Applied Biosystems). The primers, placed in different exons, were confirmed not to amplify genomic DNA. Primer and probe nucleotide sequences (Applied Biosystems) were as follows: murine IFN-/H9253, forward primer, 5’-CTG CGG CCT AGC TCT GAG A-3’, reverse primer, 5’-CAG CCA GAA ACA GCC ATG AG-3’, and TaqMan probe, 5’-(FAM)-CAC ACT GCA TCT TGG CTT TGC AGC TC-(TAMRA)3’; murine /H9252-actin, forward primer, 5’-CTG CCT AGC TCT TGG CCT TGC AGC TC-(TAMRA)3’; murine /H9252-actin, forward primer, 5’-CCG AGC CTG GAT GGC TAC GT-3’, and TaqMan probe, 5’-(FAM)-TTT GAG ACC TTC AAC ACC CCA GCC A-(TAMRA)3’. Thermal cycling parameters used with the TaqMan One-Step RT-PCR Master Mix Reagents Kit were 30 min at 48°C, 10 min at 95°C, 40 cycles involving denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. Relative quantitation of IFN-γ mRNA levels was plotted as fold change compared with uninfected controls. All samples were performed in triplicate.

**Multiparameter flow cytometric analysis/intracellular cytokine staining**

Total lung leukocytes were isolated, as described above. Leukocytes were stained with the following cell surface markers: anti-CD4, anti-CD8, anti-βTCR (αβ T cell marker), anti-γδTCR, and anti-DX5 (NK cell marker).
using FITC- or PE-labeled Abs (BD Pharmingen, San Diego, CA). In addition, cells were stained with anti-CD45 Tricolor (Caltag Laboratories, South San Francisco, CA) to distinguish leukocytes from nonleukocytes. Cells were then stimulated with PMA and ionomycin for 3 h at 37°C. Cells were permeabilized using saponin and sodium azide and fixed with formaldehyde (Cytofix/Cytoperm kit; BD Pharmingen). Cells were collected on a FACSCalibur cytometer (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences). T cell subsets were analyzed after gating on CD45+ lymphocyte-sized cells, and then examining for FL-1 and FL-2 fluorescence expression.

**Statistical analysis**

Survival curves were compared using the log rank test. For other data, statistical significance was determined using the Mann-Whitney U test or unpaired t test. All calculations were performed using the Prism 3.0 software program for Windows (GraphPad, San Diego, CA).

**Results**

**STAT4−/− mice have increased mortality following i.t. Klebsiella administration**

The importance of STAT4 during acute Gram-negative bacterial pneumonia was assessed using BALB/c mice with a targeted disruption of the STAT4 gene (STAT4−/−). STAT4−/− and control wt animals were inoculated i.t. with an ~LD₅₀ dose of K. pneumoniae and survival was monitored. STAT4−/− animals had markedly decreased survival, with all animals dying within 10 days of infection (p < 0.01, Fig. 1). In contrast, appropriately 60% of infected wt mice survived long-term. This indicates that STAT4 is a critical element in the host immune defense against extracellular bacterial pathogens in the lung.

**STAT4−/− mice have increased lung and blood bacterial burden**

To elucidate possible mechanisms by which the absence of STAT4 resulted in increased lethality, wt and STAT4−/− animals were sacrificed at days 2 and 3 following i.t. Klebsiella inoculation, and their lungs and blood were harvested for CFU analysis. On day 2, STAT4−/− animals did not have significantly different lung or blood bacterial counts compared with wt animals. By day 3, however, wt animals were beginning to clear bacteria from the lung, whereas STAT4−/− animals displayed a progressive increase in lung bacterial burden (23-fold higher bacterial CFU compared with wt; Fig. 2a) and were unable to control bacteremic seeding (p < 0.05, Fig. 2b). All except 1 (9 of 10) of the STAT4−/− animals were bacteremic by day 3, as compared with only 50% (5 of 10) of wt animals (data not shown). Examination of lung histology on day 3 confirmed the presence of markedly increased numbers of bacterial organisms in the alveolar spaces of STAT4−/− mice, while wt animals exhibited neutrophilic inflammation in the airspaces with only occasional bacteria (Fig. 2c). No
appreciable differences were noted in lung histology between the STAT4−/− and wt animals at baseline. Thus, STAT4 appears to be essential for clearing bacteria from the lung and blood.

**STAT4−/− mice have impaired T1 cytokine production**

Experiments were performed to determine whether STAT4−/− animals had impaired IFN-γ production in the lung. At both days 1 and 3 following i.t. *K. pneumoniae* inoculation, STAT4−/− animals had reduced expression of IFN-γ mRNA in whole lung homogenates, as determined by TaqMan real-time PCR (Fig. 3a). The amount of IFN-γ protein detectable in total lung homogenates was also significantly decreased, although not completely abolished, in STAT4−/− animals, as compared with infected wt controls (p < 0.05, Fig. 3b). We also examined the expression of other cytokines involved in the innate immune response in the lung at baseline and 1 and 2 days post-*Klebsiella* administration. We observed minimal induction of inflammatory cytokines in BAL fluid at 1 day postbacterial challenge. By 2 days, however, we found significant induction of inflammatory cytokines in infected wt mice. In contrast, there was considerably less induction of IL-12 in infected STAT4−/− mice at 2 days compared with wt infected animals (p < 0.01, Fig. 4), suggesting that endogenously produced IFN-γ may positively regulate IL-12 synthesis in *Klebsiella* infection. In addition, levels of the IFN-γ-inducible ELR+ CXC chemokines, IP-10 and Mig, were significantly decreased in the STAT4−/− animals at 2 days following i.t. infection, as compared with their wt counterparts. Infected STAT4−/− mice also exhibited a trend toward reduced expression of other inflammatory cytokines at 2 days, including TNF-α, and the ELR+ CXC chemokines, KC and MIP-2, in both BAL fluid (Fig. 4, and data not shown) as well as lung homogenates (data not shown). Collectively, these studies indicate that STAT4−/− animals have impaired production of T1 cytokines, as well as selected other inflammatory cytokines.

**STAT4−/− mice do not have impaired leukocyte recruitment**

We next examined the total number of leukocytes in the lung at 2 and 3 days following i.t. *Klebsiella* inoculation. The total numbers of lung leukocytes were not significantly different between wt and STAT4−/− animals. Furthermore, there were no significant differences in the numbers of specific leukocyte populations, including neutrophils and mononuclear phagocytes (data not shown). To determine whether STAT4−/− animals had differences in resident or recruited airspace leukocytes compared with wt animals, the animals were lavaged at baseline and at 1 and 2 days following infection. Similarly, no significant differences in total or specific leukocyte numbers were appreciated at baseline or at 1 and 2 days post-*K. pneumoniae* administration (Fig. 5, and data not shown). Thus, compared with their wt counterparts, STAT4−/− mice did not appear to have substantial differences in the resident lung leukocyte population or defects in leukocyte recruitment to the lung following pulmonary bacterial challenge.

Using flow cytometric analysis of total lung leukocytes, we also investigated whether there were differences in NK cell and specific T cell populations in the lung between STAT4−/− mice and wt controls during the course of *K. pneumoniae*. At 2 and 3 days following i.t. *Klebsiella* administration, STAT4−/− and wt mice did not have significantly different numbers of NK cells (DX5⁺), CD4⁺ T cells, CD8⁺ T cells, αβ⁺ T cells, or γδ⁺ T cells (γδTCR⁺) (data not shown).

We next wished to determine whether there were differences in the ability of resident or recruited cells to produce IFN-γ. Using intracellular cytokine staining for IFN-γ, we observed that the number of IFN-γ⁺ cells was decreased in the lungs of *Klebsiella*-infected STAT4−/− animals. In particular, STAT4−/− animals had decreased numbers of IFN-γ⁺ NK cells (DX5⁺ IFN-γ⁺), CD4⁺ IFN-γ⁺, CD8⁺ IFN-γ⁺, and γδTCR⁺ IFN-γ⁺ (γδ T cell marker) cells on day 2 following i.t. *Klebsiella* infection compared with wt animals (Fig. 6). Thus, while STAT4−/− animals do not have defects in pulmonary NK or T cell accumulation following bacterial infection, these cells appear to produce decreased amounts of IFN-γ in the absence of STAT4.

**Alveolar macrophage function is compromised in STAT4−/− animals following i.t. Klebsiella inoculation**

The alveolar macrophage plays a major role in pulmonary innate immunity against bacterial pathogens, either by direct ingestion and killing of bacteria or by producing cytokines that can amplify the inflammatory response. To determine whether there are functional differences in alveolar macrophages isolated from STAT4−/− animals compared with wt controls, we obtained BAL cells from STAT4−/− and wt animals at baseline (day 0) and 2 days following i.t. *Klebsiella* administration to examine the production of proinflammatory cytokines from these cells ex vivo. Resting alveolar macrophages isolated from uninfected animals did not produce appreciable levels of inflammatory cytokines. However, alveolar macrophages isolated from wt infected mice produced substantial quantities of cytokines when cultured ex vivo, whereas alveolar macrophages isolated from infected

**FIGURE 5.** Cell counts and differentials from BAL fluid. The wt and STAT4−/− animals underwent bronchoalveolar lavage at baseline, 1 and 2 days after i.t. *Klebsiella* administration, n = 3–5 per group; data are representative of 2 separate experiments.
STAT4−/− mice had a marked decrease in their ability to produce inflammatory cytokines ex vivo (Table I). Importantly, alveolar macrophages from i.t. Klebsiella-inoculated wt animals, but not STAT4−/− mice, were capable of producing appreciable quantities of IFN-γ. Collectively, these results demonstrate that alveolar macrophages from STAT4−/− mice are incapable of mounting a significant inflammatory response to Klebsiella pulmonary infection.

The immune deficits in STAT4−/− animals are partially reversed by IFN-γ

Given that STAT4−/− animals displayed considerable impairment in IFN-γ production during pneumonia, we attempted to reverse the immune deficits seen in STAT4−/− animals by augmenting IFN-γ production and/or by administering exogenous IFN-γ to STAT4−/− animals. To achieve this, we administered i.t. a replication-deficient adenovirus containing the murine IFN-γ cDNA to transiently express IFN-γ in the lung. To augment the systemic effects of IFN-γ, we concurrently administered rmIFN-γ daily s.c. for 3 days. The coadministration of i.t. adenovirus vector expressing IFN-γ and s.c. rmIFN-γ (500 ng) concurrent with i.t. Klebsiella failed to alter bacterial counts in lungs of STAT4−/− animals at 3 days postbacterial challenge, as compared with infected controls. However, treatment of STAT4−/− mice with IFN-γ did result in a significant reduction in blood K. pneumoniae CFU (28-fold, p < 0.01) at that time point (Fig. 7).

Discussion

Bacterial pneumonia is the leading cause of death from infectious disease in the U.S. (30). Significant impediments to the effective treatment of this disease are the growing numbers of immunocompromised patients and the increasing rates of bacterial resistance. Although antimicrobial therapy will most likely continue to be an essential component of treatment for pneumonia, immunotherapy targeted at augmenting the host immune response may provide an added advantage, particularly among patients who have impaired immunity or who are infected with resistant pathogens. Because T1 cytokines are an integral component of the host response to bacterial pathogens in the lung, a better understanding of the downstream events of T1 cytokine signaling may ultimately facilitate the development of more specific and effective forms of immunotherapy.

Table I. Cytokine production from alveolar macrophages isolated from uninfected and infected wt and STAT4−/− mice

<table>
<thead>
<tr>
<th></th>
<th>wt, Uninfected</th>
<th>STAT4−/−, Uninfected</th>
<th>wt + Klebsiella</th>
<th>STAT4−/− + Klebsiella</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>0.04 ng/ml ± 0.001</td>
<td>0.04 ng/ml ± 0.004</td>
<td>1.99 ng/ml ± 0.4</td>
<td>0.42 ng/ml ± 0.03b</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5 pg/ml ± 0.5</td>
<td>5 pg/ml ± 0.3</td>
<td>153 pg/ml ± 35</td>
<td>5 pg/ml ± 0.17b</td>
</tr>
<tr>
<td>IL-12</td>
<td>6 pg/ml ± 1</td>
<td>6 pg/ml ± 0.5</td>
<td>47 pg/ml ± 9</td>
<td>13 pg/ml ± 2b</td>
</tr>
<tr>
<td>IP-10</td>
<td>9 pg/ml ± 0.5</td>
<td>9 pg/ml ± 0.2</td>
<td>309 pg/ml ± 61</td>
<td>30 pg/ml ± 4b</td>
</tr>
<tr>
<td>Mig</td>
<td>0.3 ng/ml ± 0.01</td>
<td>0.3 ng/ml ± 0.01</td>
<td>2.8 ng/ml ± 0.5</td>
<td>0.3 ng/ml ± 0.004</td>
</tr>
<tr>
<td>KC</td>
<td>50 pg/ml ± 1</td>
<td>49 pg/ml ± 1</td>
<td>701 pg/ml ± 95</td>
<td>209 pg/ml ± 12b</td>
</tr>
<tr>
<td>MIP-2</td>
<td>0.6 ng/ml ± 0.003</td>
<td>0.3 ng/ml ± 0.1</td>
<td>1.3 ng/ml ± 0.2</td>
<td>0.8 ng/ml ± 0.1</td>
</tr>
</tbody>
</table>

a Data shown represent the mean ± SEM of three to five mice.
b Value of p ≤ 0.01 compared with wt counterparts by unpaired t test.
c Value of p < 0.05 compared with wt counterparts by unpaired t test.
STAT proteins are part of the signaling pathway of several cytokines, which are important immune mediators against microbes. STAT4 has been demonstrated to mediate many of the functions of IL-12, including increases in IFN-γ production, stimulation of NK cell cytolytic function, and Th1 differentiation and proliferation. In the present study, we have demonstrated that STAT4 is essential for the generation of a protective innate immune response against *K. pneumoniae* in the lung. STAT4-/- animals had impaired pulmonary and blood *Klebsiella* clearance, compared with wt controls, accounting for the decreased survival seen following i.t. *Klebsiella* infection in these animals. Although STAT4 was previously thought to be expressed only in T and NK cells, its expression has now been described in activated macrophages and mature dendritic cells. Thus, while αβ T cells do not appear to be essential in the pulmonary clearance of *Klebsiella* (26), STAT4 appears to be critical in order for other cell types to develop effective immune functions. Leukocyte recruitment, however, is not impaired in animals lacking STAT4, as STAT4-/- mice had comparable numbers of neutrophils and mononuclear cells in the lung as wt animals both at baseline and following infection.

The absence of STAT4 leads to diminished, albeit not a complete lack of IFN-γ production. Decreased levels of IFN-γ are associated with impaired host responses in *Klebsiella*, as well as several other pulmonary bacterial infections (5, 31–33). Our results demonstrate that STAT4-/- animals have fewer numbers of IFN-γ+ NK cells, CD4+ cells, and CD8+ cells, which are believed to be the primary sources of IFN-γ in the lung. This is most likely attributable to decreased IL-12 responsiveness in the absence of STAT4, as IL-12 is known to be a potent inducer of IFN-γ production in these cells (34–37). Another possible explanation for the reduced accumulation of IFN-γ-producing cells is the impaired pulmonary production of the ELR− CXC chemokines, IP-10 and Mig, in STAT4-/- mice. IP-10 and Mig are chemotactic factors for Th1 cells (38–41). Thus, although the total numbers of T cells and T cell subsets appear to be similar in the presence and absence of STAT4 during the course of pulmonary bacterial infection, fewer IFN-γ-producing T cells are present in the lung, either due to decreased activation in the lung or impaired recruitment of activated T cells in STAT4 knockout animals.

Interestingly, in our model, the immune deficits created by the absence of STAT4 were partially reconstituted by systemic administration of exogenous IFN-γ combined with the transient expression of IFN-γ in the lung using adenoviral gene therapy. Specifically, treatment of STAT4-/- animals with IFN-γ led to reductions in blood bacterial burden, but did not affect pulmonary bacterial CFU. The most plausible explanation for the incomplete lack of immune functions is that we were unable to adequately replicate the intrapulmonary physiologic levels, timing, and site of IFN-γ production in STAT4-/- animals to that which occurs in wt animals during pulmonary *Klebsiella* infection. However, these results may also suggest that the lack of STAT4 has broader effects beyond simply resulting in a deficiency in IFN-γ. It may be that other downstream activating molecules such as IL-12 and Mig, which are decreased in STAT4-/- mice during bacterial pneumonia, are actually the critical response elements, rather than IFN-γ per se. Alternatively, STAT4-/- mice may have defective IFN-γ responsiveness, possibly due to decreased IFN-γ receptor expression or signaling. To date, the regulation of IFN-γ receptor expression by STAT4 has not been reported. Further studies are needed to explore these possibilities. Our findings are compatible with a previous report that exogenous IFN-γ administration only partially restored immunity in STAT4-/- animals against infection with *T. gondii* (15). Thus, while IFN-γ is necessary to host defense against *Klebsiella* in the lung, it appears to be insufficient in terms of fully reversing the immune defects exhibited by STAT4-/- mice.

In our model of murine pneumonia, we have highlighted the importance of alveolar macrophages to host defense. Our laboratory has demonstrated that depletion of alveolar macrophages leads to a dramatic decrease in survival and pulmonary bacterial clearance following i.t. *Klebsiella* administration (42). Other investigators have likewise established the essential role that alveolar macrophages play in clearing bacterial pathogens from the lung via their phagocytic and bactericidal functions (43–47). In this study, we demonstrate yet another potentially important function of alveolar macrophages in innate immunity: as an early source of cytokines following exposure to i.t. *Klebsiella* (500 ng on days 0, 1, and 2), STAT4-/- animals had partially restored immunity in STAT4-/- mice during bacterial pneumonia, which appears to be insufﬁcient in terms of fully reversing the immune defects exhibited by STAT4-/- mice.
from the lung, this defect in alveolar macrophage function is another potential mechanism underlying the impaired pulmonary clearance of Klebsiella in the absence of STAT4.

Collectively, the results of these studies indicate that STAT4 is an integral component of the host innate immune response against a major Gram-negative bacterial pathogen in the lung. STAT4 has clearly important effects on pulmonary bacterial clearance, T1 cytokine production, and effective alveolar macrophage function. Further studies are required to better elucidate the exact mechanisms by which alveolar macrophage function is impaired, and the significance of other events that are downstream of STAT4 activation.

References


