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IFN Regulatory Factor 3 Contributes to the Host Response during Pseudomonas aeruginosa Lung Infection in Mice

Svetlana O. Carrigan,* Robert Junkins,* Yong Jun Yang,*‡ Adam MacNeil,* Christopher Richardson,*† Brent Johnston,*‡ and Tong-Jun Lin*†

Pseudomonas aeruginosa is a major opportunistic pathogen. However, host defense mechanisms involved in P. aeruginosa lung infection remain incompletely defined. The transcription factor IFN regulatory factor 3 (IRF3) is primarily associated with host defense against viral infections, and a role of IRF3 in P. aeruginosa infection has not been reported previously. In this study, we showed that IRF3 deficiency led to impaired clearance of P. aeruginosa from the lungs of infected mice. P. aeruginosa infection induced IRF3 translocation to the nucleus, activation of IFN-stimulated response elements (ISRE), and production of IFN-β, suggesting that P. aeruginosa activates the IRF3–ISRE–IFN pathway. In vitro, macrophages from IRF3-deficient mice showed complete inhibition of CCL5 (RANTES) and CXCL10 (IP-10) production, partial inhibition of TNF, but no effect on CXCL2 (MIP-2) or CXCL1 (keratinocyte-derived chemokine) in response to P. aeruginosa stimulation. In vivo, IRF3-deficient mice showed complete inhibition of CCL5 production and partial or no effects on production of other cytokines and chemokines in the bronchoalveolar lavage fluids and lung tissues. Profiling of immune cells in the airways revealed that neutrophil and macrophage recruitment into the airspace was reduced, whereas B cell, T cell, NK cell, and NKT cell infiltration was unaffected in IRF3-deficient mice following P. aeruginosa lung infection. These data suggest that IRF3 regulates a distinct profile of cytokines and chemokines and selectively modulates neutrophil and macrophage recruitment during P. aeruginosa infection. Thus, IRF3 is an integral component in the host defense against P. aeruginosa lung infection. The Journal of Immunology, 2010, 185: 3602–3609.

Lung infection with Pseudomonas aeruginosa is a major cause of death in immunocompromised individuals (1, 2). Major efforts have been made in dissecting host defense mechanisms involved in P. aeruginosa infection. TLRs are a family of pattern recognition receptors that are critical for cellular responses to microbial products (3). A number of TLRs have been implicated in P. aeruginosa infection in mice (4–6) and in humans (7–9), including TLR1, -2, -4, -5, and -6. To date, two major pathways have been described in TLR signaling, the MyD88 pathway leading to NF-κB activation and the Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) pathway leading IRF3/7 activation (10). We and others (4, 11) have shown that the MyD88 pathway is essential for host defense against P. aeruginosa lung infection. Global gene profiling revealed that the MyD88-dependent pathway regulates 21.5% of the genes activated though TLR4, whereas 74.5% of genes are controlled by MyD88-independent mechanisms (12). These data suggest that both MyD88-dependent and -independent mechanisms are required for the development of full host response to bacterial challenge. Consistent with this notion, we demonstrated that TRIF-dependent immune responses are also needed for the host defense against P. aeruginosa lung infection (13). Accordingly, both the MyD88 pathway and the TRIF pathway are likely to function coordinately to mediate host responses to P. aeruginosa infection.

TRIF not only activates the IFN regulatory factor (IRF) 3 and 7 transcription factors, it also mediates NF-κB activation through receptor-interacting protein 1 (RIP1) (14). Indeed, TRIF deficiency leads to impaired NF-κB activation in macrophages in vitro and in mouse lung in vivo following P. aeruginosa infection (13). NF-κB–regulated gene products, such as TNF, were significantly reduced in TRIF-deficient mice (13). Thus, the TRIF–RIP1–NF-κB pathway contributes to the effective immune response to P. aeruginosa infection. However, the contribution of the TRIF–IRF3/7 pathway in host defense against P. aeruginosa infection has not been reported previously.

In response to viral infection, IRF3 and IRF7 are the central transcription factors regulating type I IFN expression (15). However, less is known about the role of IRF3/7 in bacterial infection. IRF3 appears to have disparate effects in different models of bacterial infection. Unlike the critical protective role of IRF3/7 in viral infection, IRF3-mediated responses appear to be detrimental in infections with the Gram-positive intracellular bacterium Listeria monocytogenes (16). Deficiency of IRF3 leads to improved clearance of L. monocytogenes (16). In contrast, IRF3-dependent responses were essential for the control of intracellular replication of Legionella pneumophila, a Gram-negative facultative intracellular bacterium, in lung epithelial cells (17). Accordingly, the functional significance of IRF3-mediated responses is dependent on the nature of the microbial pathogen.
In this study, we reported that the IRF3–ISRE–IFN pathway is activated by *P. aeruginosa* infection. Deficiency of IRF3 induced a specific pattern of impaired host response to *P. aeruginosa* infection in vitro and in vivo, leading to impaired bacterial clearance from the lung. Thus, IRF3 is an integral part of the host defense against *P. aeruginosa* infection.

**Materials and Methods**

**Mice**

IRF3-knockout (IRF3 KO) mice and TRIF-knockout (TRIF KO) mice on the C57BL/6 background were from Dr. S. Akira (Osaka University, Osaka, Japan) (16). C57BL/6 mice were purchased from Charles River (Saint Constant, Quebec, Canada). IRF3 KO mice were matched with C57BL/6 KO mice for age and sex. The protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with the guidelines of the Canadian Council on Animal Care (Halifax, Nova Scotia, Canada).

**Abs**

PE-conjugated rabbit anti-mouse TCRβ (IgG2, clone H57-958), PerCP-Cy5.5-conjugated rat anti-mouse CD19 (IgG2a, clone 1D3), APC-conjugated mouse anti-NK1.1 (IgG2a, clone PK136) and FC block (rat IgG2b, clone 2.4G2) were purchased from BD Biosciences (Mississauga, Ontario, Canada). PE-conjugated rat anti-mouse Ly6G (IgG2a, clone 1A8), APC-conjugated rat anti-mouse F4/80 (IgG2a, clone BM8), and APC-conjugated rat IgG2a isotype control (clone R53-95) were purchased from eBioscience (San Diego, CA).

**Bacterial preparation and macrophage activation**

*P. aeruginosa* strain 8821 (a gift from Dr. A. Chakrabarty, University of Illinois, Chicago, IL) is a mucoid strain isolated from a patient with cystic fibrosis (19). *Staphylococcus aureus* is a clinical isolate from a patient abscessed (provided by Dr. Andrew Issekutz, Izaak Walton Killam Health Center, Dalhousie University). *P. aeruginosa* and *S. aureus* were cultured in Luria-Bertani broth. Bacterial preparation, peritoneal macrophage collection, and activation were carried out as previously described (13). Un-otherwise specified, *P. aeruginosa* and *S. aureus* used in cell coculture assays were killed using an antibiotic mixture (50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml piperillin, 100 μg/ml ceftazidime, and 200 μg/ml gentemycin). *P. aeruginosa* strain 8821 at a multiplicity of infection (MOI) 100 elicits a strong cellular host response (13). Thus, this concentration has been selected in this study.

**Lung infection with *P. aeruginosa*, collection of lung and BALF samples, and flow cytometry**

Mice were intranasally infected with *P. aeruginosa* (1 × 10⁸ CFU/mouse). After 4 or 24 h, bronchoalveolar lavage fluid (BALF) was obtained by lavaging the lung with 3 × 1 ml PBS solution containing soybean trypsin inhibitor (100 μg/ml). The lung tissues were homogenized for detection of cytokines and chemokines, myeloperoxidase (MPO), and bacterial CFUs as described previously (4, 13).

For flow cytometric analysis of immune cells, lung lobes were minced and mechanically dispersed through a 100-μm stainless steel mesh. Cells were washed and centrifuged through an isotonic 33% Percoll gradient (GE Healthcare Biosciences, Baie d’Urfe, Quebec, Canada). Cell pellets were resuspended in NH₄Cl buffer to lyse erythrocytes, washed, and resuspended. Lung cells were incubated with BD FC block (BD Biosciences) for 10 min and stained with Abs to TCRβ, CD19, NK1.1, Ly6G, F4/80, Ly6G or rat IgG2a for 30 min. Cells were then analyzed using a two-laser Becton-Dickinson FACSCalibur flow cytometer (BD Biosciences) and WinList 5.0 software (Verity Software House, Topsham, ME).

**IRF3-red fluorescent protein expression and microscopy**

Mouse IRF3-red fluorescent protein (RFP) construct was generously donated by Dr. Karen Mossman (McMaster University, Hamilton, Ontario, Canada). NR8383 cells, a rat alveolar macrophage cell line (American Type Culture Collection, Manassas, VA), were electroporated (4 × 10⁶ cells in 100 μl) with 4 μg IRF3-RFP plasmid using the Amaxa Nucleofector Device (program U-023, Amaxa, Gaithersburg, MD). Transfection efficiency was 80% as determined by colony enumeration. After 24 h, cells were left untreated or exposed to *P. aeruginosa* 8821 at a MOI of 1:100 for 4–24 h. Cells were fixed in BD Perm/Fix buffer (BD Biosciences) according to the manufacturer’s instructions and cytocentrifuged onto glass slides. Cells were examined with a confocal laser scanning microscope (Zeiss LSM510, Zeiss, Toronto, Ontario, Canada).

**Luciferase assay for ISRE activities**

NR8383 alveolar macrophages (4 × 10⁵ cells) were cotransfected with pISRE-Luc plasmid (Strategene, La Jolla, CA) and RL-TK plasmid (Promega, Madison, WI) using the Amaxa Mouse T Cell Nucleofector Kit (VPA-100) with the Amaxa Nucleofector Device (program U-023, Amaxa). Post-transfection, cells were allowed to recover for 24 h. Cells were then challenged for various times (3, 4, 8, 12, or 24 h) with live *P. aeruginosa* (MOI 100), antibiotic-killed *P. aeruginosa* (MOI 100), LPS (10 μg/ml), polyinosinic-polycytidylic acid (polyIC; 100 μg/ml), or killed *S. aureus* (MOI 100). Firefly and Renilla activities were sequentially quantified using a dual-luciferase reporter assay system (Promega) in a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA).

**Cytokine and chemokine production**

The concentrations of CCL5 (RANTES), CXCL10 (IP-10), CXCL1 [keratinocyte-derived chemokine (KC)], TNF, CXCL2 (MIP-2), IL-1β, or IFN-β in the lung, BALF, or cell-free supernatants were determined by ELISA as previously described using Ab pairs from R&D Systems (Minneapolis, MN) (4, 13). Quantitative real-time RT-PCR analysis for IFN-β was performed using an ABI 7000 Sequence Detector System with Assays-on-Demand reagents containing FAM dye-labeled TaqMan MGB probes (Applied Biosystems, Foster City, CA). GAPDH was used as an endogenous reference. Data were analyzed using the relative standard curve method according to the manufacturer’s protocol. An average value of IFN-β expression after GAPDH normalization at the time point showing highest expression (*P. aeruginosa* infection for 4 h) was used as a calibrator to determine the relative levels of IFN-β under different conditions. PCR products were also separated on 2% agarose gel and stained with ethidium bromide.

**Bacterial internalization assay**

Peritoneal macrophages from IRF3-deficient or wild-type mice were seeded at 5 × 10⁵ cells/well in a six-well plate. Cells were infected with *P. aeruginosa* strain 8821; MOI 100 for 3 h at 37°C. Cultures were treated for 90 or 180 min with 50 μM penicillin, 50 μM streptomycin, 100 μg/ml piperillin, 100 μg/ml ceftazidime, and 200 μg/ml gentemycin to kill extracellular bacteria. Macrophages were washed and lysed in 0.1% Triton X-100 in sterile phosphate buffer. Lysates were diluted 1:5, streaked on Luria-Bertani plates, and incubated overnight at 37°C for colony counting.

**Statistics**

Data are presented as mean ± SEM of the indicated number of experiments. Statistical significance was determined by assessing means with ANOVA and the Tukey-Kramer multiple comparisons test or by using an unpaired *t* test. Differences were considered significant at *p* < 0.05.

**Results**

**IRF3 deficiency leads to impaired clearance of *P. aeruginosa* from the lung**

To determine whether IRF3-dependent immune responses in the airways have an effect on the clearance of *P. aeruginosa* from the lung, the lung tissues from IRF3-deficient and wild-type mice were collected for the detection of viable bacteria by CFU counting after intranasal administration of *P. aeruginosa* strain 8821 (1 × 10⁸ CFU/mouse). At the early time points (4 and 8 h), there was a significant bacterial load in the lungs of IRF3-deficient and wild-type mice, with similar numbers of bacteria in each group. Postinfection for 24 h, CFU counts in the lung tissues from IRF3-deficient mice were 3.6-fold higher than wild-type mice (Fig. 1). These results suggest that IRF3 is required for the optimal clearance of *P. aeruginosa* from the lung.

**P. aeruginosa infection activates IRF–ISRE–IFN pathway**

IRF3 is primarily involved in viral infections. IRF3 induces transcription through activation of ISRE sites. To examine whether *P. aeruginosa* infection stimulates ISRE activity, the alveolar macrophage cell line NR8383 was transfected with an ISRE luciferase
plasmid and stimulated with live _P. aeruginosa_ (MOI 100) or killed _P. aeruginosa_ (MOI 100) for 3 h. LPS and polyI:C were used as controls. Significant _P. aeruginosa_-induced ISRE activation was seen in cells treated either with live or with killed _P. aeruginosa_. Levels of _P. aeruginosa_-induced ISRE activation were similar to those induced by LPS or polyI:C stimulation (Fig. 2A). We further carried out time-course experiments by stimulating NR8383 cells with killed _P. aeruginosa_ for various times (4–24 h). _P. aeruginosa_-induced ISRE activation was observed over 4–24 h. Unlike the steady increase of ISRE activity observed in LPS-treated cells, _P. aeruginosa_-induced ISRE activation remained constant over 24 h. The Gram-positive bacteria _S. aureus_ (MOI 100) was used as a control. No ISRE activity was detectable in _S. aureus_-treated cells (Fig. 2B).

IRF3 is a transcription factor that is transported into nucleus upon activation. To determine whether _P. aeruginosa_ infection induces IRF3 nuclear translocation, NR8383 cells were infected with IRF3-RFP and stimulated with _P. aeruginosa_ (MOI 100) for various times (4–24 h). The nuclear localization of IRF3-RFP was visualized by confocal microscopy. _P. aeruginosa_ infection induced a significant increase of IRF3 translocation from the cytosol into the nucleus (Fig. 2C, 2D).

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**FIGURE 1.** Impaired clearance of _P. aeruginosa_ from the lungs of IRF3-deficient mice. IRF3-deficient (IRF3 KO) and wild-type mice were intranasally challenged with _P. aeruginosa_ (mucoid strain 8821, $1 \times 10^9$ CFU/mouse). After 4, 8, 24 or 48 h, the right lung was collected, homogenized, and plated for colony counting. Data are the mean ± SEM of 12 mice per group. *p < 0.05 compared with wild-type group.

**FIGURE 2.** IRF pathway activation by _P. aeruginosa_ (Psa) infection. _A_. NR8383 rat alveolar macrophage cells were transfected with an ISRE luciferase reporter or control plasmids. Cells were stimulated for 3 h with Psa-Live (MOI 1:100), Psa-Dead (MOI 1:100), 10 μg/ml LPS, 100 μg/ml polyI:C, or left untreated (NT). ISRE activity was measured relative to the control luminescence and presented as fold increase compared with the NT group. Each bar is the mean increase ± SEM of three independent experiments. **p < 0.001 compared with no treatment.** _B_. NR8383 cells posttransfection with the ISRE reporter plasmid were stimulated with Psa (MOI 100), LPS (10 μg/ml), or _S. aureus_ (MOI 100) for 4–24 h. ISRE activity was determined. Data are the mean ± SEM of three independent experiments. **p < 0.01 compared with NT group.** _C_. NR8383 macrophages were transfected with IRF3-RFP by electroporation. After 24 h, cells were exposed to Psa (MOI 1:100) for 18 h or left untreated (NT) and examined by confocal microscopy. _D_. NR8383 macrophages posttransfection with IRF3-RFP were stimulated with Psa, LPS, or _S. aureus_ for 4–24 h. Cells with IRF3-RFP nuclear localization were enumerated and presented as percent nuclear localization. Data are the mean ± SEM of three independent experiments. **p < 0.01 compared with no treatment (NT) group.** _E_. Peritoneal macrophages were isolated from TRIF KO or wild-type mice. Cells were then transfected with IRF3-RFP by electroporation. After 24 h, cells were exposed to Psa (MOI 1:100) or 10 μg/ml LPS for 4 h or left untreated (NT) and examined by confocal microscopy. Cells with IRF3-RFP nuclear localization were enumerated and presented as percent nuclear localization. Each bar is the mean ± SEM of three independent experiments. Three hundred cells were counted on each slide from individual experiments. *p < 0.05; **p < 0.01. Psa-Dead, killed _P. aeruginosa_; Psa-Live, live _P. aeruginosa_.

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IRF3 functions in the downstream of TRIF (15). Previously, we found that the TRIF-dependent pathway is involved in the *P. aeruginosa*-induced host response (13). To determine whether *P. aeruginosa* induces IRF3 nuclear translocation through activation of TRIF, peritoneal macrophages from TRIF KO and wild-type mice were transfected with TRIF-RFP and then exposed to *P. aeruginosa* or LPS for 4 h. Cells with IRF3 nuclear translocation were enumerated. Macrophages from TRIF KO mice showed significantly reduced IRF3 nuclear translocation following *P. aeruginosa* or LPS stimulation (Fig. 2E). This finding suggests that *P. aeruginosa* induces IRF3 activation downstream of TRIF.

*P. aeruginosa*-induced activation of the IRF–ISRE–IFN pathway was further investigated in vivo using *IRF3*-deficient mice. Wild-type and *IRF3*-deficient mice were intranasally infected with *P. aeruginosa* (*10⁶ CFU/mouse*). Lung and BALF were collected to examine IFN-β levels. A significant increase of IFN-β mRNA expression was observed in wild-type lungs 4 h after *P. aeruginosa* infection. The level of IFN-β mRNA returned close to basal levels by 24 h after *P. aeruginosa* infection (Fig. 3A, 3B). Increased IFN-β protein was observed in the BALF of wild-type mice after 4 h *P. aeruginosa* infection (Fig. 3C). Importantly, *P. aeruginosa*-induced IFN-β expression and protein levels were dramatically reduced in *IRF3*-deficient mice (Fig. 3A–C). These data demonstrate that *P. aeruginosa* induced IFN-β is dependent on IRF3. Taken together, these results show that *P. aeruginosa* infection induces IRF3–ISRE–IFN pathway activation.

To determine whether IFN-β contributes to the host response to *P. aeruginosa* lung infection, *IRF3*-deficient mice were given recombinant mouse IFN-β (*10,000 U/mouse, i.p.*) immediately after lung infection. Bacterial clearance and neutrophil infiltration (MPO) were determined 24 h later. Interestingly, reconstitution of *IRF3*-deficient mice with IFN-β did not affect bacterial clearance or neutrophil infiltration (Supplemental Fig. 1). These results suggest that IFN-β is not the sole factor responsible for the host defense against *P. aeruginosa* infection.

**IRF3 deficiency leads to selective impairment in cytokine and chemokine production by macrophages stimulated in vitro with *P. aeruginosa***

The transcription factor IRF3 regulates various biologically important genes such as CCL5 (RANTES) and CXCL10 (IP-10) in addition to IFN-β (15, 20). To determine whether IRF3 plays a role in *P. aeruginosa*-induced production of inflammatory cytokine and chemokines, peritoneal macrophages from wild-type and *IRF3*-deficient mice were treated with *P. aeruginosa* strain 8821 (MOI 100) for 24 h. LPS and polyI:C were used as controls. Cell-free supernatants were collected for ELISA measurement of cytokines and chemokines. *P. aeruginosa* stimulation induced a significant production of CCL5 (RANTES) by wild-type macrophages. In contrast, *P. aeruginosa* induced little CCL5 (RANTES) production in peritoneal macrophages from *IRF3*-deficient mice (Fig. 4A).

Similarly, *P. aeruginosa*-induced CXCL10 (IP-10) production was significantly impaired in *IRF3*-deficient macrophages compared with wild-type macrophages (Fig. 4B). However, CXCL1 (KC), TNF, and MIP-2 production by *IRF3*-deficient macrophages were not significantly altered (Fig. 4C–E), suggesting a selective effect of IRF3 on *P. aeruginosa*-induced cytokine/chemokine production.
IRF3 deficiency leads to selective impairment of cytokine and chemokine production in the airways following P. aeruginosa lung infection in vivo

To determine a role for IRF3 in the development of the innate immune response to P. aeruginosa lung infection in vivo, IRF3-deficient mice were infected with P. aeruginosa strain 8821 at the concentration of 1 × 10^8 CFU/mouse for 24 h. BALF and lung tissues were collected for the determination of CCL5 (RANTES), CXCL10 (IP-10), CXCL1 (KC), CXCL2 (MIP-2), TNF, and IL-1β. P. aeruginosa-induced CCL5 (RANTES) production was completely inhibited in IRF3-deficient mice, suggesting an essential role of IRF3 in production of this chemokine in vivo (Fig. 5A). It is noteworthy that unlike the complete inhibition of CXCL10 (IP-10) A of IRF3 in production of this chemokine in vivo (Fig. 5A). It is noteworthy that unlike the complete inhibition of CXCL10 (IP-10) production in IRF3-deficient macrophages in vitro, P. aeruginosa-induced CXCL10 (IP-10) production in the lung was only moderately reduced in IRF3-deficient mice (Fig. 5B). The mechanism of this difference is unclear and may be due to the different requirements for IRF3 in different cell types (15). Furthermore, IRF3-deficient mice produced similar levels of CXCL1 (KC), CXCL2 (MIP-2), TNF, and IL-1β in response to P. aeruginosa infection (Fig. 5C–F). A protein array assay (catalog number AAM-INF-1, Raybiotech, Norcross, GA) examining 40 cytokines and chemokines was also carried out using uninfected or P. aeruginosa-infected lung tissues from wild-type and IRF3-deficient mice. Most of the cytokines and chemokines were largely unaffected by IRF3 deficiency (data not shown). This is in contrast to MyD88 deficiency, which leads to dramatic impairment of MIP-2, TNF, and IL-1β production in the lung (4). Together, these data suggest that MyD88 and IRF3 regulate different subsets of cytokines and chemokines in response to P. aeruginosa lung infection.

IRF3 is required for the recruitment of neutrophils and macrophages but not other immune effector cells into the lung following P. aeruginosa infection

Multiple immune effector cells are involved in the host defense against P. aeruginosa lung infection. These include neutrophils (1), macrophages (21, 22), NKT cells (NKTs) (23), NK cells (24), dendritic cells (25), T cells (26, 27), and B cells (28, 29). We attempted to examine whether IRF3 regulates immune cell infiltration into the lung in response to P. aeruginosa infection. Histological observation of the lung did not reveal major differences between IRF3-deficient and wild-type mice (Fig. 6).

MPO assays were also used to determine neutrophil recruitment into the airways. Decreased MPO activity was seen in the BALF from IRF3-deficient mice after 24-h P. aeruginosa infection (Fig. 7A). Similarly, flow cytometric analysis revealed a decrease in Ly6Ghi neutrophils in the BALF of IRF3-deficient mice (Fig. 7B). However, no statistical differences in MPO activity or Ly6Ghi cells were observed in the lung tissues of IRF3-deficient and wild-type mice (Fig. 7C, 7D).

To examine macrophage recruitment into the airways, F4/80 Ab was used as a macrophage marker. F4/80+ cells were decreased in the BALF of IRF3-deficient mice following P. aeruginosa infection (Fig. 8A). However, the number of F4/80+ cells in the lung was similar between IRF3-deficient and wild-type mice (Fig. 8B). These data suggest that IRF3 is needed for the recruitment of neutrophils and macrophages into the airways in response to P. aeruginosa lung infection. The phagocytosis and bacterial killing activity of macrophages from IRF3-deficient mice were also evaluated. Macrophages were incubated with P. aeruginosa to allow the phagocytosis of bacteria. Subsequently, cultures were treated with antibiotics to remove extracellular bacteria, and the number of intracellular bacteria was quantified in lysed macrophages. No difference in bacterial numbers was observed between IRF3-deficient macrophages and wild-type macrophages (Fig. 8C). Thus, IRF3 deficiency has no effect on macrophage phagocytosis and bacterial killing.

To examine the recruitment of B cells, NK cells, αβT cells, and NKTs, cells harvested from the BALF and lung tissues were stained with Abs to CD19, NK1.1, and TCRβ. Cells that are double positive for NK1.1 and TCRβ were identified as NKT-like cells. Flow cytometric analysis showed that there were no differences in B cell,
NK cell, αβT cell, and NKT-like cell numbers in the BALF of wild-type mice and IRF3-deficient mice with or without P. aeruginosa infection (data not shown). Similarly, no differences in these immune cells were observed between wild-type and IRF3-deficient mice in the lung tissues from uninfected or P. aeruginosa infection mice (Fig. 9).

Discussion

P. aeruginosa is an opportunistic pathogen that plays a major role in the deterioration of lung function in patients with cystic fibrosis and immune-compromised individuals. Airway infection with P. aeruginosa triggers both acute and chronic inflammatory responses in humans, including elevated production of a wide range of cytokines and chemokines in the lung (1). It has been postulated that P. aeruginosa-mediated lung injury is orchestrated by cytokines and chemokines. An important advance toward understanding the disease state in P. aeruginosa lung infection would be to fully elucidate the specific signaling pathways that are responsible for the production of specific cytokines and chemokines. In this study, we demonstrated for the first time that the IRF3-dependent pathway is specifically required for P. aeruginosa-induced production of IFN-β and the chemokine CCL5 (RANTES), but has limited effects on other cytokines and chemokines, such as CXCL2 (MIP-2), TNF, or IL-1. Importantly, IRF3 deficiency leads to impaired clearance of P. aeruginosa from the lung. This finding suggests that IRF3 is an integral component of host defense mechanisms in P. aeruginosa lung infection.

A number of TLRs have been implicated in P. aeruginosa infection in vitro and in vivo. Major TLR signaling pathways have been identified, including the MyD88 pathway leading to NF-κB activation, the TRIF–RIP1 pathway leading to NF-κB activation, and the TRIF–IRF pathway leading to transcription factor IRF3/7 activation. Previously, we and others (4, 11) have demonstrated that the MyD88-dependent TLR signaling pathway is essential for P. aeruginosa-induced production of NF-κB–regulated cytokines and chemokines. Recently, we further demonstrated that deficiency of TRIF leads to impaired NF-κB activation and...
impairment of production of NF-κB-regulated genes, such as TNF (13), suggesting a role of the TRIF–IRF1–NF-κB pathway in P. aeruginosa infection. In this study, we demonstrated the involvement of IRF3 in P. aeruginosa infection. Accordingly, development of full immune response to P. aeruginosa infection likely requires coordinated activation of the MyD88 pathway, the TRIF–IRF1 pathway, and the TRIF-IRF3 pathway.

The biological role of IRF3 is primarily associated with viral infections (15). This is because of the essential role of IRF3 in the regulation of IFN-α and -β, which are critical in the host defense against virus. We provide compelling evidence demonstrating that the IRF3–ISRE–IFN-β pathway is activated in response to P. aeruginosa infection. To determine the contribution of IFN-β in host response to P. aeruginosa infection, IRF3-deficient mice were given rIFN-β. No difference in bacterial clearance between IRF3-deficient and wild-type mice was found. These results suggest that IFN-β is not the sole factor responsible for the host defense against P. aeruginosa infection. It is important to note that IRF3 is a transcription factor that regulates multiple genes (15, 20). The promoter of CCL5 (RANTES) also contains an IRF3 binding site. The complete inhibition of P. aeruginosa-induced CCL5 (RANTES) production in vitro and in vivo in the absence of IRF3 suggests that CCL5 (RANTES) production is dependent on IRF3 activation during P. aeruginosa infection. In addition to IFNs and CCL5 (RANTES), IRF3 also regulates other genes (20, 30). A range of genes was identified to be regulated by IRF3 in a tetracycline-inducible expression system expressing a constitutively active form of IRF-3 (20). We found that IRF3 deficiency also leads to reduced production of CXCL10 (IP-10) following P. aeruginosa infection. Accordingly, IRF3 contributes to the host defense against P. aeruginosa infection likely through regulating the collective effects of multiple mediators including CCL5 (RANTES), IFN-β, and other IRF3-regulated genes.

NF-κB is a master transcription factor that controls a range of cytokines and chemokines. Previously, we showed that TRIF deficiency or MyD88 deficiency leads to reduced NF-κB activation following P. aeruginosa infection (4, 13), suggesting a role for the MyD88–NF-κB pathway and the TRIF–IRF1–NF-κB pathway in P. aeruginosa infection. In an effort to determine whether NF-κB plays a role in IRF3-mediated immune response, lung tissues from IRF3-deficient mice after P. aeruginosa infection were analyzed by electrophoresis mobility shift assay for NF-κB. From this assay, NF-κB activation was enhanced in the lungs of IRF3-deficient mice following P. aeruginosa infection (data not shown).

We reasoned that this enhanced NF-κB activation is likely due to a compensatory mechanism when IRF3 is absent.

Previous studies have demonstrated that multiple immune effectors cells are recruited into the airways and participate in the host defense against P. aeruginosa infection. Major efforts have been made in identifying mechanisms involved in the regulation of recruitment of specific cell types. We showed that neutrophil and macrophage recruitment into the airways was reduced in IRF3-deficient mice, whereas T cells, B cells, NK cells, and NKTs were unaffected by IRF3 deficiency. Thus, IRF3 appears to selectively regulate neutrophil and macrophage infiltration during P. aeruginosa infection.

IRFs constitute at least nine members of transcription factors that possess structural similarities (15). Recent studies have established the essential and distinct roles of these transcription factors in the host defense against microbial infections (15). Interestingly, IRF1 transcript was upregulated in human epithelial cells in response to P. aeruginosa infection (31). In mice, IRF1 deficiency led to reduced TNF and IL-1 production in response to P. aeruginosa LPS and exotoxin A, but not phospholipase C stimulation (32), suggesting a role of IRF1 in P. aeruginosa infection. Structurally, IRF1 is closely related to IRF2, whereas IRF3 is closely related to IRF7 (15). Although limited information is available on IRF1 and IRF3 interactions in TLR signaling, IRF1 appears to be able to pair with IRF3 or IRF7 and upregulate gene transcription (33).

We also found that IRF7 is upregulated in the lung in mice following P. aeruginosa infection (data not shown). Thus, it is possible that multiple IRF family members may interplay and regulate the host response to P. aeruginosa lung infection.

In summary, we showed that IRF3 deficiency leads to impaired clearance of P. aeruginosa from the lung in mice. We provided compelling evidence showing that the IRF3–ISRE–IFN-β pathway is activated by P. aeruginosa infection. IRF3 deficiency leads to a complete inhibition of CCL5 (RANTES) and IFN-β production in vitro and in vivo and a partial reduction or no effects on several other cytokines and chemokines. Neutrophil and macrophage recruitment into the airways was selectively reduced in IRF deficient mice. These data suggest that IRF3-dependent immune responses are integral components of host defense against P. aeruginosa lung infection.

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Disclosures

The authors have no financial conflicts of interest.

References


**Supplementary Fig S1.** IFNβ reconstitution did not affect bacterial clearance and neutrophil recruitment in IRF3-deficient mice. IRF3-deficient (IRF3 KO) mice were inoculated intranasally with *P. aeruginosa* (strain 8821, 1×10⁹ CFU/mouse, Psa). Immediately after infection, mice were injected with recombinant murine IFNβ (10,000 units/mouse in 100μl of PBS, i.p.). Control mice received PBS alone. Twenty four hours later BALF and lung tissues were collected for the determination of bacterial load (A) and myeloperoxidase (MPO) activities (B). Data are the mean ± SEM of 6-7 mice per group.