JCI The Journal of Clinical Investigation

Staphylococcus aureus activates type I IFN signaling in mice and humans through the Xr repeated sequences of protein A

Francis J. Martin, … , Christian Schindler, Alice Prince

J Clin Invest. 2009;119(7):1931-1939. https://doi.org/10.1172/JCI35879.

Research Article **Infectious disease**

The activation of type I IFN signaling is a major component of host defense against viral infection, but it is not typically associated with immune responses to extracellular bacterial pathogens. Using mouse and human airway epithelial cells, we have demonstrated that *Staphylococcus aureus* activates type I IFN signaling, which contributes to its virulence as a respiratory pathogen. This response was dependent on the expression of protein A and, more specifically, the Xr domain, a short sequence–repeat region encoded by DNA that consists of repeated 24-bp sequences that are the basis of an internationally used epidemiological typing scheme. Protein A was endocytosed by airway epithelial cells and subsequently induced IFN-β expression, JAK-STAT signaling, and IL-6 production. Mice lacking IFN-α/β receptor 1 (IFNAR-deficient mice), which are incapable of responding to type I IFNs, were substantially protected against lethal *S. aureus* pneumonia compared with wild-type control mice. The profound immunological consequences of IFN-β signaling, particularly in the lung, may help to explain the conservation of multiple copies of the Xr domain of protein A in *S. aureus* strains and the importance of protein A as a virulence factor in the pathogenesis of staphylococcal pneumonia.

Staphylococcus aureus activates type I IFN signaling in mice and humans through the Xr repeated sequences of protein A

Francis J. Martin,[1] Marisa I. Gomez,[2] Dawn M. Wetzel,[2] Guido Memmi,[3] Maghnus O'Seaghdha,[4] Grace Soong,[2] Christian Schindler,[5] and Alice Prince[1,2]

[1]Department of Pharmacology and [2]Department of Pediatrics, Columbia University College of Physicians and Surgeons, New York, New York, USA.
[3]Department of Microbiology and Immunology, Dartmouth Medical School, Hanover, New Hampshire, USA.
[4]Department of Microbiology, The Moyne Institute of Preventative Medicine, Trinity College, Dublin, Ireland.
[5]Department of Microbiology, Columbia University College of Physicians and Surgeons, New York, New York, USA.

The activation of type I IFN signaling is a major component of host defense against viral infection, but it is not typically associated with immune responses to extracellular bacterial pathogens. Using mouse and human airway epithelial cells, we have demonstrated that *Staphylococcus aureus* activates type I IFN signaling, which contributes to its virulence as a respiratory pathogen. This response was dependent on the expression of protein A and, more specifically, the Xr domain, a short sequence–repeat region encoded by DNA that consists of repeated 24-bp sequences that are the basis of an internationally used epidemiological typing scheme. Protein A was endocytosed by airway epithelial cells and subsequently induced IFN-β expression, JAK-STAT signaling, and IL-6 production. Mice lacking IFN-α/β receptor 1 (IFNAR-deficient mice), which are incapable of responding to type I IFNs, were substantially protected against lethal *S. aureus* pneumonia compared with wild-type control mice. The profound immunological consequences of IFN-β signaling, particularly in the lung, may help to explain the conservation of multiple copies of the Xr domain of protein A in *S. aureus* strains and the importance of protein A as a virulence factor in the pathogenesis of staphylococcal pneumonia.

Introduction

Staphylococcus aureus has reemerged as a major human pathogen, and in 2005, infection due to methicillin-resistant (MRSA) strains alone caused more than 18,000 deaths in the US, many acquired in the community by normal hosts. Staphylococcal pneumonia contributed to more than 75% of these deaths (1). Such invasive infections indicate successful bacterial exploitation of the immune system. While *S. aureus* has many gene products that directly interact with diverse immune effectors, exactly how such devastating infections occur is not entirely clear. Local production of cytotoxin, such as the α-hemolysin (2), Panton-Valentine leukocidin (PVL) (3), and phenol soluble modulins (4), clearly contributes to pathology. However, none of these are uniformly expressed by these virulent isolates of *S. aureus* (5), suggesting that additional virulence factors must be important in the pathogenesis of these infections.

Staphylococcal protein A (SpA) is a conserved surface component of all *S. aureus* strains, consisting of an N-terminal IgG-binding domain, an Xr or short sequence–repeat region (SSR) encoded by variable numbers of 24-bp repeated DNA sequences, and a C-terminal anchor to the bacterial cell wall (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI35879DS1) (6). The amino terminus of SpA, the IgG-binding domain, activates tumor necrosis factor receptor 1 (TNFR1) (7) and EGFR (8), contributing substantially to the pathology induced by the inflammatory response evoked by *S. aureus*, and the SpA-TNFR1 interaction has been shown to be essential in the pathogenesis of staphylococcal pneumonia (7). The Xr region provides the basis of the *spa* typing system and is generated by deletion, duplication, and point mutation of the repeated DNA sequences (9). This provides sufficient diversity to differentiate strains involved in clinical outbreaks of infection from all over the world (10). However, despite their widespread conservation, the function of these repeated sequences has been entirely unclear.

To explore the biological role of the Xr domain of SpA, we translated the DNA sequences of the repeats present in the most frequent *spa* types and found that they encode highly conserved peptides (Supplemental Figure 1B) with homology to LMP1 of EBV, the major oncoprotein of EBV and a potent activator of type I IFN signaling (11). The type I IFN cascade is a major immune effector critical for the systemic response to viral infection (12) and the coordination of innate and adaptive immune responses (13). Mice lacking components of the type I IFN signaling system (*trif*$^{-/-}$ and *Ifnar*$^{-/-}$, where TRIF indicates TIR-domain-containing adapter-inducing interferon-β and IFNAR indicates IFN-α/β receptor 1) are highly susceptible to viral infection but are afforded significant protection from endotoxin-mediated death (14, 15). Similarly, type I IFN production has been shown to increase host susceptibility to the Gram-positive obligate intracellular organism *Listeria monocytogenes* (16). This cascade has been associated with both pro- and antiinflammatory signaling through the activation of STAT1, STAT2, and STAT3 (17). In a number of inflammatory conditions, IFN-β downregulates many genes, including matrix metalloproteinases, chemokines, and adhesion molecules (18). While type I

Authorship note: Francis J. Martin and Marisa I. Gomez contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: BAL, bronchoalveolar lavage; GST, glutathione-*S*-transferase; IFNAR, IFN-α/β receptor 1; PMN, polymorphonuclear leukocyte; SpA, staphylococcal protein A; SSR, short sequence–repeat region; TNFR1, tumor necrosis factor receptor 1; TRIF, TIR-domain-containing adapter-inducing interferon-β; Tyk2, tyrosine kinase 2.

Citation for this article: *J. Clin. Invest.* **119**:1931–1939 (2009). doi:10.1172/JCI35879.

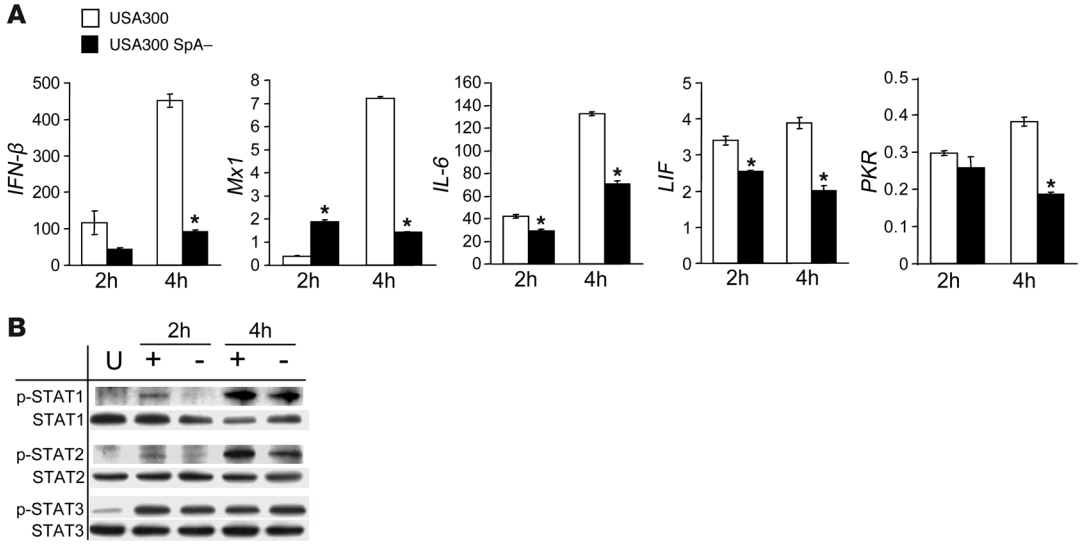

Figure 1

S. aureus strain USA300 induces type I IFN signaling in airway epithelial cells that is dependent on the expression of protein A. (**A**) Primary mouse airway epithelial cells were incubated with *S. aureus* USA300 or a SpA deletion mutant, and induction of *IFN*-β, *Mx-1*, *IL-6*, *LIF*, and *PKR* was detected by real-time PCR. Data are represented as fold increases over unstimulated controls and are representative of 3 independent experiments. **P* < 0.05 as compared with the *spa*-null strain, Student's *t* test. Error bars represent standard deviations. (**B**) p-STAT1, p-STAT2, and p-STAT3 were detected by immunoblot in primary airway epithelial cell cultures treated with USA300 (+) or *spa*-null (–). Corresponding total STATs are shown for loading controls.

IFN signaling can be activated by diverse microbial signals, a typical cascade involves TLR-mediated recognition of an intracellular microbial component, IRF3- and IRF7-mediated transcription of IFN-β, and, through an autocrine pathway, ligation of the IFN-α/β receptor IFNAR, which in turn stimulates tyrosine kinase 2 (Tyk2) and the JAK-STAT cascade, resulting in cell type–dependent immune responses, including IL-6 production (19).

Having previously found that SpA readily activates IL-6 production in airway cells (20), we postulated that *S. aureus* activates the type I IFN cascade and that SpA is involved. As the major mediators of the type I IFN cascade are well known, we focused on what we believe are the novel aspects of this hypothesis: how extracellular Gram-positive bacteria could induce signaling that has been previously associated with intracellular viral infection or mediated by LPS and TLR4 (15, 21, 22). We demonstrate that *S. aureus*, through the Xr domain of SpA, activates known components of the type I IFN cascade and that this contributes to the virulence of the organism as a respiratory pathogen.

Results

S. aureus USA300 activates type I IFN signaling in airway cells. S. aureus strain USA300 is a community-acquired methicillin-resistant strain now epidemic in the US (1). We tested the ability of this strain to induce type I IFN signaling in murine airway epithelial cells in primary culture and used a *spa*-null mutant to determine the contribution of SpA in this process. A sharp increase in *IFN*-β transcription was detected following exposure to USA300 but not the *spa* mutant (100-fold at 2 hours and >400 fold at 4 hours) (Figure 1A). Induction of IFN-α family members was not detected. Production of IFN-β–dependent gene products was also observed: USA300 induced a 7-fold increase in *Mx-1*, a prototypical type I IFN antiviral effector, a 130-fold increase in *IL-6*, and a 4-fold increase in *Lif*, a gene product that is important in host defense in the lung specifically (23). Induction of these IFN-dependent gene products was significantly attenuated in the absence of SpA (Figure 1A). Induction of *PKR* transcription, important in the induction of apoptosis and sensitization of macrophages against the effects of LPS (24), was not observed (Figure 1A).

Production of IFN-β is followed by an autocrine pathway consisting of activation of the common IFN-α/β receptor (IFNAR), phosphorylation of the transcription factors STAT1, STAT2, and STAT3 by JAK family kinases, and expression of more than 300 type I IFN-dependent genes (25). We compared the activation of this cascade in murine airway cells in response to USA300 with that in response to a *spa* mutant. Both STAT1 and STAT2 became phosphorylated after exposure to USA300 (required for induction of IFN-β–dependent gene products such as Mx-1), coincident with IFN-β production. This phosphorylation was less pronounced in cells exposed to the *spa*-null mutant. Tyrosine phosphorylation of STAT3 was induced by both strains, indicating the presence of SpA-independent signaling mechanisms (Figure 1B). These data indicate that *S. aureus* potently induces the type I IFN cascade in airway epithelial cells and that much of this response is dependent on expression of SpA.

Ifnar$^{-/-}$ *mice are protected from S. aureus–associated mortality*. The requirement for type I IFN signaling in the resolution of viral respiratory infections has been well documented (12). However *trif*$^{-/-}$ and *Ifnar*$^{-/-}$ mice are protected from the lethal affects of endotoxin (14, 15). To determine whether staphylococcal activation of type I IFN signaling has similar negative consequences, WT (129/SvEv) and *Ifnar*$^{-/-}$ mice were intranasally infected with *S. aureus* USA300. The *Ifnar*$^{-/-}$ mice were protected from lethal pneumonia with significantly reduced mortality, 10% as compared with 80% seen in the WT animals ($P = 0.0055$ by Fisher's exact test; Figure 2A).

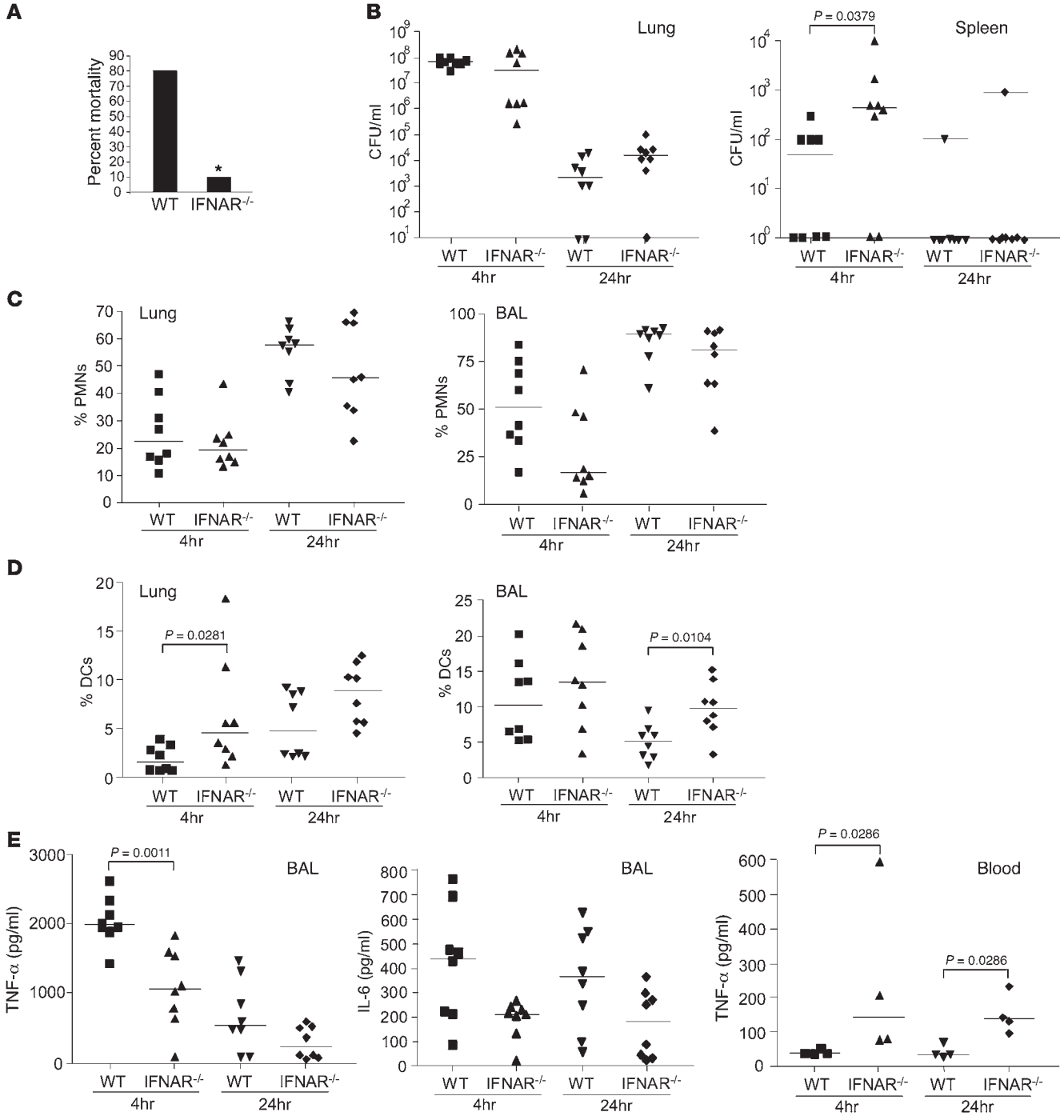

Figure 2

Type I IFN signaling increases susceptibility to staphylococcal respiratory infection. Adult WT or *Ifnar*$^{-/-}$ mice were intranasally infected with (**A**) 5 × 10^8 CFUs of *S. aureus* USA300 and mortality determined at 24 hours. **P* = 0.0055, Fisher's exact test; *n* = 10 mice per group. Mice were infected with 5 × 10^7 CFUs of *S. aureus* USA300 and at 4 and 24 hours after infection (**B**), bacterial loads were determined in lungs and spleens and lung suspensions and cells obtained by BAL fluid stained and analyzed by flow cytometry to enumerate (**C**) $CD45^+Gr1^+$ PMNs and (**D**) $CD11c^+CD11b^+CD45^+$ DCs. Data are represented as percentage of total CD45 population. (**E**) TNF-α and IL-6 were detected in BAL fluid and blood by ELISA. Each point represents 1 animal; horizontal lines represent median values.

Using a 10-fold lower sublethal inoculum, we were able to compare the immune responses to USA300 in mice. There were no differences in either the levels of infection (Figure 2B) or the numbers of polymorphonuclear leukocytes (PMNs) recruited into the lungs of the *Ifnar*$^{-/-}$ and WT mice at either 4 or 24 hours after inoculation (Figure 2C). Although there was slightly more bacteremia found at 4 hours in the *Ifnar*$^{-/-}$ animals (Figure 2B), this was less than a log difference, which was gone at 24 hours.

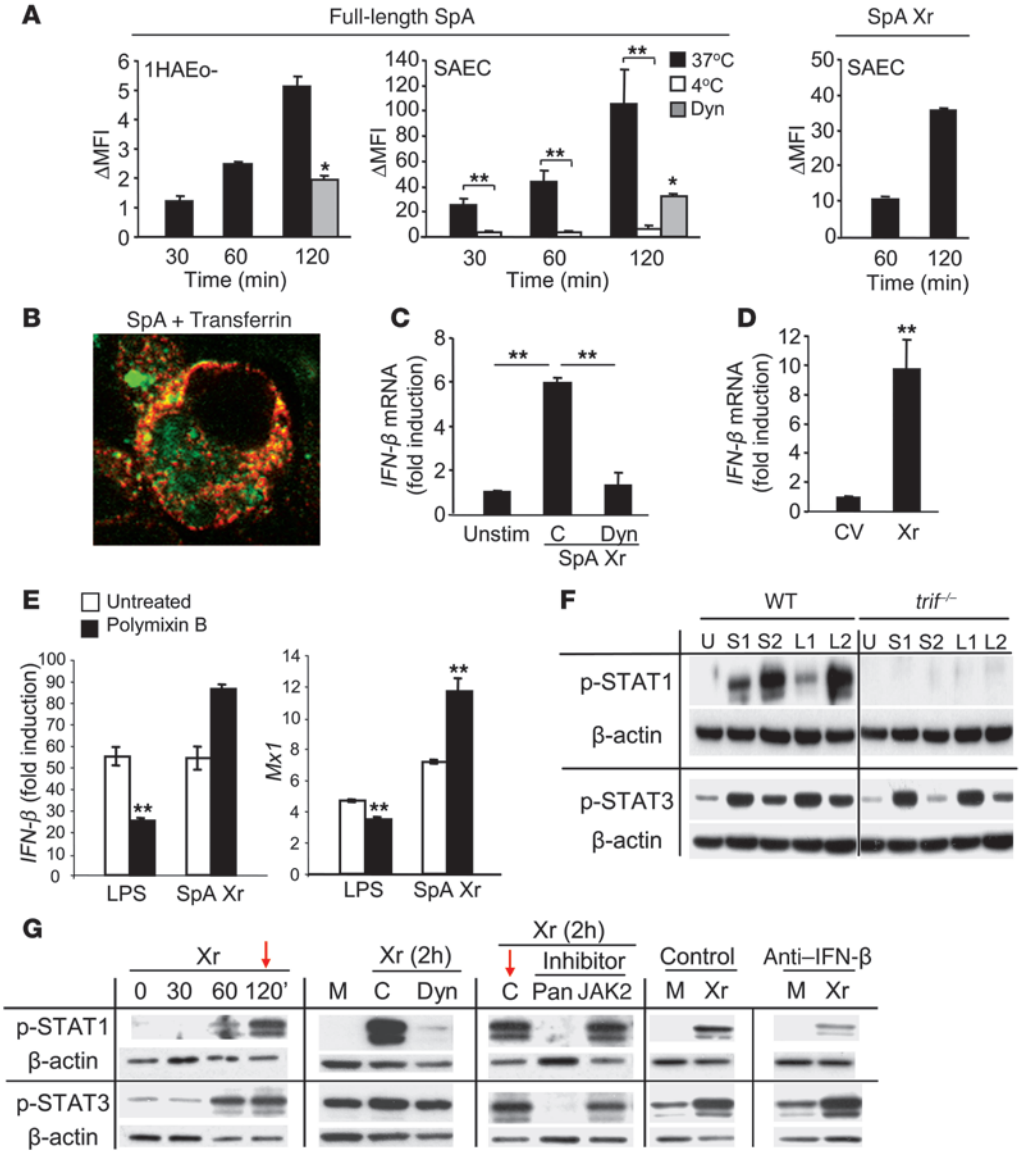

Figure 3

SpA is internalized and induces type 1 IFN signaling in airway epithelial cells. (**A**–**E**) Airway cells were incubated with SpA or SpA Xr and (**A**) internalization monitored by flow cytometry (ΔMFI, change in mean fluorescence intensity) or (**B**) by confocal microscopy for SpA (green) and transferrin (red) at 1 hour (original magnification, ×80). (**C**) SpA-induced *IFN*-β production was measured at 2 hours by real-time PCR in the presence of Dynasore (Dyn), (**D**) in response to transfection of an SpA Xr plasmid (Xr) or empty vector (CV), and (**E**) in the presence of 50 μg/ml polymixin B. (**F**) STAT1 and STAT3 phosphorylation in response to SpA (S) or LPS (L) after 1 and 2 hours was monitored in murine nasal epithelial cells from WT or *trif*$^{-/-}$ mice. (**G**) STAT1 and STAT3 phosphorylation was monitored in airway cells in response to SpA Xr (30 min., $n = 2$; 60 and 120 min., $n = 3$), in the presence of Dyn ($n = 3$), pan-JAK inhibitor (Pan, $n = 3$), JAK2 inhibitor (JAK2, $n = 3$), or neutralizing antibody to IFN-β ($n = 2$). Thin vertical lines between bands indicate data spliced together from original blot. Red arrows indicate that the same bands from the 120 min. Xr treatment were used as the control for pan-JAK and JAK2 inhibitor treatments. U, M, or Unstim. indicates unstimulated control. Unless otherwise indicated, data shows mean values of triplicate samples from 1 representative experiment out of 3, and error bars indicate standard deviations. *$P < 0.001$; **$P < 0.05$.

The *Ifnar*$^{-/-}$ mice had significantly more CD11c^{+} DCs both in lung homogenates and bronchoalveolar lavage (BAL) fluid, which persisted from 4 to 24 hours (Figure 2D). At the same time points, the *Ifnar*$^{-/-}$ mice had significantly less TNF-α, and a trend toward less IL-6 in the BAL fluid compared with the WT animals (Figure 2E). Systemic levels of TNF-α were minimal in both groups and at least an order of magnitude less than those detected in the BAL fluid (Figure 2E). These findings suggest that the major impact of type I IFN signaling in response to *S. aureus* is in the lung and involves the DC population and immune regulation.

The Xr domain of SpA activates IFN-β signaling. SpA has already been shown to be critical in the pathogenesis of pneumonia in a mouse model of infection (7). We next analyzed the contribution of each of the SpA subdomains in the activation of type I IFN responses

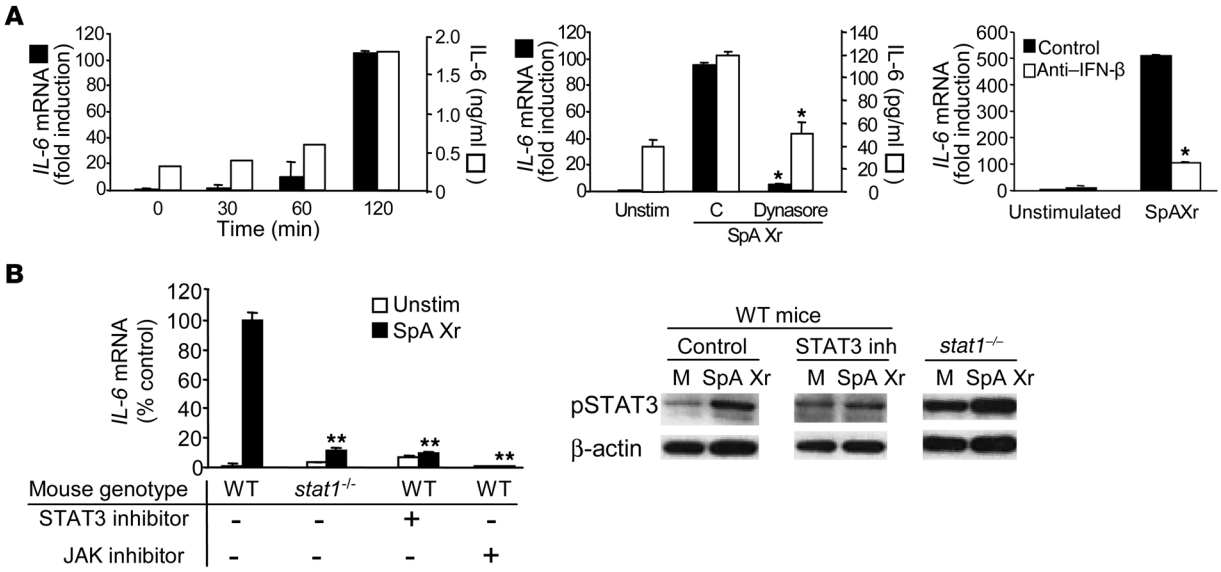

Figure 4

SpA Xr induces IFN-β– and STAT3-dependent IL-6 production. (**A**) Airway epithelial cells in primary culture were stimulated with SpA Xr for the times indicated or for 2 hours in the absence or presence of dynasore, and *IL-6* induction was detected by real-time PCR (black bars, left *y* axis) or ELISA (white bars, right *y* axis). Untreated cells or cells stimulated with SpA Xr were incubated with IFN-β–neutralizing antibody, and *IL-6* induction was measured by real-time PCR. *IL-6* mRNA production is expressed as the fold induction compared with that of unstimulated cells. (**B**) Airway epithelial cells from WT or *Stat1*$^{-/-}$ mice were stimulated with SpA Xr in the absence (control) or presence of the inhibitors indicated, and *IL-6* production was measured by real-time PCR. *IL-6* induction is expressed as a percentage of that induced by SpA Xr in cells from WT mice. (**A** and **B**) Data represent the mean and SD of 3 wells from 1 representative experiment out of 3. **P* < 0.05; ***P* < 0.001, Student's *t* test performed on the raw data. Immunoblots demonstrating phosphorylation of STAT3 in airway cells from *Stat1*$^{-/-}$ mice and inhibition in the presence of STAT3 inhibitor are shown in **B**.

in airway epithelial cells. The domains of SpA were subcloned to determine whether the Xr domain is both necessary and sufficient to activate the type I IFN response: namely, endocytosis of the ligand, IFN-β transcription, and JAK-STAT activation (Figure 3).

To determine whether SpA is endocytosed, we incubated fluorescently labeled full-length SpA or a construct consisting of the Xr domain from strain Newman (which is virtually identical to the SpA of USA300) with human airway epithelial cells in primary culture and cell lines; after repeated washing and trypsin treatment to remove surface-associated SpA, we assayed for intracellular SpA (Figure 3A). Substantial internalization was detected by 1 hour of incubation and was significantly inhibited in cells maintained at 4°C or treated with the dynamin inhibitor dynasore (26), suggesting an endocytic process. Confocal imaging was consistent with accumulation of SpA in endosomes, as it colocalized extensively with transferrin (Figure 3B). Translocation of internalized SpA to the cytosol was assessed by incubating airway epithelial cells with a SpA–β-lactamase fusion protein and determining cytosolic β-lactamase activity using a previously described reporter system (27). SpA did not translocate from the vesicles into the cytosol over the period of time studied (30 minutes to 4 hours) (data not shown).

Incubation of airway cells with SpA Xr for 2 hours induced *IFN*-β transcription; this induction of *IFN*-β transcription in response to SpA Xr was significantly blocked by dynamin inhibition (Figure 3C). Since these constructs were synthesized as overexpressed proteins in *Escherichia coli*, several control experiments were performed to exclude the possibility that contaminating LPS was affecting our experiments. As one control, we transfected airway cells with a plasmid expressing SpA Xr or a vector control and documented a 10-fold increase in *IFN*-β mRNA production as compared with that induced by the empty vector (Figure 3D). In addition, the recombinant Xr protein was pretreated with polymyxin B, which blocked LPS-induced *IFN*-β and *Mx-1* production but not that stimulated by SpA Xr (Figure 3E).

The adaptor protein TRIF has been shown to be essential in coupling pattern recognition receptors to IFN-β signaling. We predicted that SpA-mediated activation of the pathway would be similarly TRIF dependent. STAT1 phosphorylation in response to either LPS or SpA was not observed in epithelial cells from *trif*$^{-/-}$ mice as compared with those from the WT controls (Figure 3F). STAT3 phosphorylation was only partially reduced in these animals, indicating that there are non-TRIF and non–IFN-β–dependent pathways that elicit STAT3 activation. Thus, it appears that major components of the IFN-β pathway are activated both by LPS and SpA, although the specific receptor(s) and adaptor proteins remain to be identified.

Following IFN-β production, IFNAR was stimulated through an autocrine pathway, activating JAK-STAT signaling to initiate gene expression, which we monitored as STAT phosphorylation by the epithelial cells (28). Human airway epithelial cells incubated with the SpA Xr domain exhibited dynamin-dependent tyrosine phosphorylation of STAT1 and STAT3, mediated by activation of JAK1 or Tyk2 but not JAK2 (Figure 3G), although considerable amounts of constitutive phospho-STAT3 were present in unstimulated cells. Anti–IFN-β inhibited STAT1 but not STAT3 phosphorylation (consistent with the signaling through the gp130 receptors that utilize STAT3). STAT3 phosphorylation (Ser727) was detected in cells transfected with a vector encoding

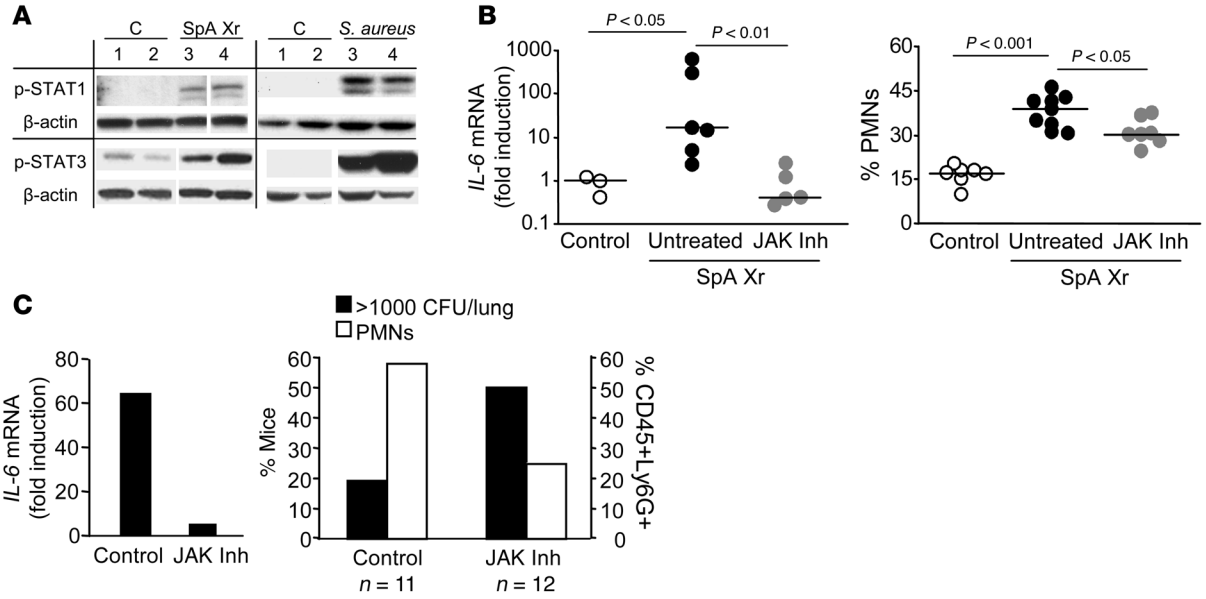

Figure 5

SpA Xr activates STAT1/3 in vivo. (**A**) C57BL/6 mice were intranasally inoculated with SpA Xr, *S. aureus,* or PBS (control). 4 hours later, p-STAT1 (Tyr701), p-STAT3 (Tyr705), and β-actin were detected in lung lysates by immunoblot. Data from 2 representative mice out of 6 are shown for both left quadrants and bottom right quadrant. Data from 2 representative mice out of 3 are shown for top right quadrant. The thin vertical line between bands within a group indicate data spliced from the original blot. (**B**) *IL-6* mRNA in the lung of SpA Xr–treated mice was detected by real-time PCR, and the percentage of PMNs among total leukocytes in the lung was determined by flow cytometry following intranasal inoculation of SpA Xr in untreated or JAK inhibitor–treated mice (Inh). Each dot represents an individual mouse, and horizontal lines show the median value in each group. (**C**) *IL-6* mRNA in the lung of *S. aureus*–infected mice was detected by real-time PCR. The median values of the percentage of PMNs corresponding to those mice are shown.

full-length SpA or the Xr domain but not in airway cells expressing the SpA N terminus, indicating specificity for the Xr domain (Supplemental Figure 2A).

IL-6 production is a major product of JAK-STAT signaling, and epithelial IL-6 production contributes substantially to the pathophysiology associated with staphylococcal infection. Cells in primary culture produced both *IL-6* mRNA and protein in response to the Xr domain of SpA in a dynamin-dependent fashion, which was blocked by exposure to anti–IFN-β (Figure 4A). Cells transfected with SpA Xr also produced *IL-6* (Supplemental Figure 2B). The involvement of JAK-STAT signaling in IL-6 production was documented by monitoring responses in primary airway cells from *Stat1*$^{-/-}$ mice or WT primary mouse cells treated with a STAT3 inhibitor (Figure 4B). The *Stat1*$^{-/-}$ cells and those treated with the STAT3 inhibitor did not produce *IL-6* mRNA in response to Xr exposure (Figure 4B). STAT3 phosphorylation was intact in cells from the *Stat1*$^{-/-}$ animals in response to SpA Xr but substantially diminished in animals treated with the STAT3 inhibitor (Figure 4B). These experiments indicate that both STAT1 and STAT3 are phosphorylated in response to SpA Xr and that both contribute to the production of IL-6.

To further document that IFN-β signaling occurs in vivo in response to staphylococcal infection, we demonstrated the phosphorylation of STAT1 and STAT3 in lung lysates from mice intranasally inoculated with either *S. aureus* strain Newman or with the SpA Xr peptide alone (Figure 5A). Phosphorylation of STAT3 at Ser727 was also activated in vivo in mice infected with WT *S. aureus* but not a protein A–null mutant (Supplemental Figure 3). *IL-6* mRNA was significantly increased in the SpA Xr–treated mice and correlated with recruitment of PMNs into the lung. Both *IL-6* mRNA and PMNs were decreased in mice treated with the pan-JAK inhibitor (Figure 5B). Mice treated with JAK inhibitor prior to and during *S. aureus* infection had much less *IL-6* production than vehicle-treated animals (Figure 5C). The JAK inhibitor also appeared to affect bacterial clearance and PMN recruitment, but the effects were not statistically significant (Figure 5C).

Discussion

These studies indicate that extracellular Gram-positive bacteria activate the type I IFN cascade effectively and that this contributes to the morbidity and mortality associated with *S. aureus* respiratory infection. Protein A, already established as a major virulence factor in the pathogenesis of pneumonia by virtue of its ability to activate TNF signaling (7), also appears to be critical in the activation of IFN-β. It has been previously shown that protein A induces type I IFN in lymphocytes (29). The Xr domain of SpA, which consists of multiple 24-bp repeats of highly conserved peptides, appears to be both necessary and sufficient for IFN-β activation in airway epithelial cells. As protein A is abundantly shed from the surface of growing staphylococci, it seems highly plausible that this protein, even in the absence of intact organisms, is sufficient to activate the epithelial mucosa. This is of particular importance in the lung, where resident alveolar macrophages do not produce bioactive IFN-β (30). Note that the induction of IFN-β is in addition to other SpA functions, such as IgG-Fc binding that thwarts opsonization, binding to von Willebrand factor (31), and activation of TNFR1 and EGFR, all of which have been mapped to the N-terminal IgG-binding region (8).

Many details of the SpA-activated IFN-β signaling cascade remain to be defined. While we demonstrate dynamin-dependent endocytosis of SpA and the involvement of the TRIF adaptor, the specific intracellular receptor, perhaps a TLR, remains to be identified. The downstream components of a typical IFN-β cascade previously shown to be activated by LPS are similarly stimulated by *S. aureus*. Whereas STAT3 activation seems to be independent of the cascade initiated by IFN-β, it is required for the downstream effects of IFN-β–induced STAT1, such as IL-6 production.

Type I IFN signaling appears to have an important role in the pathogenesis of staphylococcal pneumonia: *Ifnar*$^{-/-}$ mice had dramatically increased survival as compared with WT mice infected with *S. aureus* USA300. As has been shown in murine models of respiratory viral infection, potent type I IFN stimulation causes significant loss of CD11c^{+} cells from the lung and appears to have an important role in immunoregulation in the airways (32, 33). The WT mice had significantly more TNF-α in their airways than *Ifnar*$^{-/-}$ mice, and we have previously demonstrated that TNF-α signaling is associated with substantial pulmonary pathology in response to *S. aureus* (7). Unexpectedly, neither PMN accumulation in the airways nor sepsis was associated with the increased mortality of the WT mice. Several reports have documented that DCs and macrophages are highly sensitive to the effects of the type I IFNs. Consistent with our findings, in the absence of type I IFNs, recruited DCs are retained within the lung (34) and there is a failure to prime macrophages to secrete TNF-α (35). Exactly which component of the IFN-β cascade activated by *S. aureus* is directly responsible for the increased mortality observed in the mouse model is not yet known but could represent a target for therapeutic intervention.

The central role of the type I IFN in the host defense against viral infection has been well documented and is implicated in the pathogenesis of several bacterial infections (16, 23, 36). The many consequences of this cascade and its importance in linking the innate and adaptive immune response are the subject of numerous reviews (12, 22). Exactly how bacterial products activate type I signaling remains to be established for most pathogens. Nor is it clear whether the type I IFN responses are beneficial for the host or the pathogen. TLR4 signaling initiated by LPS is clearly a major activator of type I IFNs; both IFN-β– and Tyk2-null mice are resistant to endotoxemia (21). In the case of *Bacillus anthracis,* type I IFN signaling also appears to convey a protective response to the host. For other Gram-positive pathogens, *Streptococcus pneumoniae* and Group B streptococci, induction of type I signaling was important for bacterial clearance, and in these studies, in contrast with our findings, *Ifnar*$^{-/-}$ mice had persistent bacteremia and could not control the infection (35). More recent studies suggest that Group B streptococcus DNA is responsible for induction of type I IFN in mouse macrophages, although the proximal portions of this signaling system and the specific receptors involved have yet to be identified (36).

Our data indicate that *S. aureus* similarly activates type I IFNs and that this has a detrimental effect on the ability of the host to handle *S. aureus* pulmonary infection. The local induction of type I IFN in response to influenza similarly might create a milieu conducive to staphylococcal superinfection, a major cause of postinfluenza mortality (37). While we have not identified all of the components of the pathway, *S. aureus* protein A, which interacts with several eukaryotic receptors, is the ligand involved. Moreover, the heterogeneous Xr region or SSR of protein A is required for type I IFN activation. As the Xr region is a site of active mutation, it is tempting to speculate that the impetus for deletion and duplication in the Xr domain is in some ways related to the immune pressure exerted by type I IFN effectors. Activation of type I IFNs in the lung enhances susceptibility to *S. aureus* infection, and this well-characterized pathway could provide an attractive target for immunomodulatory therapy for pneumonia.

Methods

Bacteria and recombinant proteins. *S. aureus* 6390, USA300, and clinical isolates were grown in Casamino Acids–yeast extract–glycerophosphate (CYGP) broth, and *E. coli* was grown in Luria-Bertaini (LB) broth. The *spa* deletion mutant was generated with in-frame deletion of the target gene by allelic replacement, using the temperature-sensitive plasmid pMAD. USA300 is erythromycin resistant; therefore, we built a modified pMAD vector by cloning the kanamycin resistance gene from pFOU into the NaeI site of pMAD. In brief, about 1 kb PCR products upstream and downstream of targeted sequences were generated and ligated by gene splicing by overlap extension. The resulting 2-kb product was digested, gel purified, cloned into pMAD using the same restriction sites, and transformed into *E. coli*. Colony PCR was performed on *E. coli* transformants. Plasmids from positive clones were verified by digestion analysis and then used to transform *S. aureus* RN4220, selecting for kanamycin-resistant and blue colonies at 30°C. Plasmid from RN4220 was sequenced and used to transform *S. aureus* strain USA300, for which the gene was to be deleted. The process of allelic replacement was described previously (38). Chromosomal deletion was verified by PCR and DNA sequencing. The resulting deletion strain was devoid of all protein A sequence and exhibited growth characteristics similar to those of the parental strain.

The full-length *spa* and the fragment encoding the C-terminal region of SpA (SpA Xr) from *S. aureus* Newman have been previously cloned into pGEX-KG (39). Glutathione-*S*-transferase (GST) fusion recombinant proteins harboring 1 and 8 SSRs were similarly generated by cloning the C-terminal region of SpA from clinical isolates (*spa* type 515 and 131, respectively; Supplemental Table 1) into pGEX-KG. For expression of recombinant SpA or SpA Xr, overnight cultures of *E. coli* BL21 containing the p-GEX-KG–SpA constructs were inoculated into fresh medium and grown to an OD_{600} of 0.5. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM, and the culture was grown for a further 6 hours. Cells were harvested by centrifugation at 8,000 *g* for 10 minutes in a Sorvall GS-3 rotor. The pellet was resuspended in PBS containing protease inhibitors (Roche), lysozyme (200 μg/ml), and DNase I (3 μg/ml). Cells were lysed by repeated freeze/thaw cycles. Cell debris was removed by centrifugation. Recombinant proteins expressed from pGEX-KG contained an N-terminal GST fusion of 26 kDa. The GST fusion proteins were purified using the MicroSpin GST purification module (Amersham Biosciences; GE Healthcare) and dialyzed against PBS.

Cell lines and primary cultures. 1HAEo^{-} and 16HBE, human airway epithelial cell lines (D. Gruenert, California Pacific Medical Center Research Institute, San Francisco, California, USA), were grown as previously detailed (40, 41). Primary small airway epithelial cells (SAEC; Lonza) were grown in a polarized fashion on semipermeable supports and used between passage 2 and 3. Mouse airway epithelial cells, which have very low endogenous activation of STATs, were used throughout the study unless indicated. Cells were isolated from the nasal septum from indicated strains of adult mice and grown in primary culture in a polarized fashion as previously described (42). *trif*$^{-/-}$ mice were a gift of Ira Tabas at Columbia University.

The effect of biochemical inhibitors was tested by pretreating the cells for 30 minutes with dynasore (80 μM, synthesized by H. Pelish and provided by T. Kirchhausen, Harvard University, Boston, Massachusetts, USA), 25 μM JAK inhibitor I (Pan JAK inhibitor, Calbiochem), 50 μM JAK2 inhibitor (Calbiochem), and 20 μM STAT3 inhibitor (Calbiochem). During stimulation, inhibitors were replenished in the medium. To block the effects of contaminating LPS, recombinant proteins were incubated with polymixin B sulfate (50 μg/ml; Sigma-Aldrich) for 1 hour prior to stimulation. For depletion of IFN-β and IL-6, respective neutralizing antibodies were used at 2×10^4 U/ml (R&D Systems).

Internalization studies. For flow cytometry, cells were incubated with Alexa Fluor 488–conjugated full-length protein A (Molecular Probes; Invitrogen) or Alexa Fluor 488–conjugated SpA Xr (Protein Labeling Kit; Molecular Probes, Invitrogen) for various periods of time at 37°C or 4°C. Cells were washed 3 times and trypsin added for 30 minutes to remove surface-bound protein A. Cells were washed, and the remaining intracellular fluorescence was measured by flow cytometry (FACSCalibur, BD). For confocal microscopy, 16HBE cells were grown on Transwell-Clear filters (Costar; Corning) with an air-liquid interface to form polarized monolayers. Cells were incubated with Alexa Fluor 488–conjugated protein A (Molecular Probes; Invitrogen) and Alexa Fluor 546–conjugated transferrin (Molecular Probes; Invitrogen) for 1 hour. After washing, cells were fixed with 4% paraformaldehyde, and filters were removed from wells and mounted with VECTASHIELD (Vector Laboratories) onto glass slides.

β-lactamase TEM-1 reporter assay. GST-SpA was cloned into the pCX241 vector, and the resulting GST-SpA–TEM-1 fusion protein was purified as described above and used to stimulate airway epithelial cells. At different time points (30 minutes; 1, 2, 3, and 4 hours) cells were loaded with CCF2/AM (Invitrogen) and the percentage of blue fluorescent cells was determined by microscopy. Fluorescence was also quantified using a fluorescence reader as previously described (26).

Real-time PCR. RNA was isolated using the QIAGEN RNeasy Mini Kit and subjected to DNase treatment per the manufacturer's instructions (QIAGEN). cDNA was made from 1 μg of RNA using the iScript Synthesis Kit (Bio-Rad). For quantitative real-time PCR, amplification was performed with Power SYBR Green Master Mix in a Step One Plus Thermal Cycler (Applied Biosystems). The following primers were used for amplification: mouse IFN-β, 5′-AGACTATTGTTGTACGTCTCC-3′ and 5′-CAGTAATAGCTCTTCAAGTGG-3′; mouse Mx-1, 5′-TGTGCAGGCACTATGAGGAG-3′ and 5′-ACTCTGGTCCCCAATGACAG-3′; mouse PKR, 5′-GCACCGGGTTTTGTATCGA-3′ and 5′-GGAGCACGAAGTACAAGCGC-3′; mouse IL-6, 5′-TGATGCACTTGCAGAAAACAA-3′ and 5′-GGTCTTGGTCCTTAGCCACTC-3′; mouse LIF, 5′-TCTTCCCATCACCCCTGTAAATG-3′ and CTTGATCTGGTTCATGAGGTTGC-3′; mouse actin, 5′-CCTTTGAAAAGAAATTTGTCC-3′ and 5′-AGAAACCAGAACTGAAACTGG-3′; human IFN-β, 5′-CTTGGATTCCTACAAAGAAGC-3′ and 5′-CATCTCATAGATGGTCAATGC-3′; human IL-6, 5′-AAGAGTAACATGTGTGAAAGC-3′ and 5′-CTACTCTCAAATCTGTTCTGG-3′; and human actin, 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CGGTTGGCCTTGGGGTTCAGGGGGG-3′. Forty cycles were run with denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 60°C for 45 seconds. β-Actin was used as control for standardization.

Intracellular expression of protein A. 1HAEo$^-$ cells were transfected by nucleofection according to manufacturers instructions (Amaxa) with a pCMV-FLAG vector encoding FLAG fusions of full-length SpA, the N-terminal region of SpA (containing the 5 IgG-binding domains) or the C-terminal region of SpA (SpA Xr). For confocal microscopy, 48 hours after transfection, cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and stained for FLAG and phospho-STAT3 (Ser727; Cell Signaling Technology).

Western blot. Cells were lysed using 60 mM *n*-octyl-β-D-glucopyranoside in TBS (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.8) containing Complete Mini Protease Inhibitor Tablets (Roche), 1 mM sodium orthovanadate, 100 mM sodium fluoride, and 20 μM GM6001. Proteins were separated on 4%–12% Bis-Tris NuPAGE gels (Invitrogen), transferred to PVDF Immobilon-P Membrane (Millipore), and blocked with 5% milk in TBST (Tris-buffered saline plus Tween) (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween) for 1 hour at room temperature. Immunodetection was performed using p-STAT1 (Y701; Cell Signaling Technology), p-STAT2 (Y689; Millipore), p-STAT3 (Y705; Cell Signaling Technology), STAT1 (Cell Signaling Technology), STAT2 (rabbit polyclonal obtained from C. Schindler at Columbia University), STAT3 (Cell Signaling Technology), and β-actin (Sigma-Aldrich) antibodies followed by secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology Inc.).

IL-6 detection. 1HAEo$^-$ cells, weaned from serum for 24 hours, or mouse primary airway epithelial cells were exposed to the stimulus indicated, and IL-6 in the supernatant was measured by ELISA. IL-6 antibody pairs (Pierce Endogen; Thermo Scientific) were used for detection of human IL-6 (1HAEo$^-$ cells), and IL-6 DuoSet (R&D Systems) was used for detection of mouse IL-6.

Mouse studies. C57BL/6 mice (7 to 10 days old) were intranasally inoculated with 10 μl of *S. aureus* (10^8 CFUs), an *S. aureus* protein A–null strain (10^8 CFUs) (43), the C-terminal region of SpA (SpA Xr, 50 μM), or PBS (control). 6- to 8-week-old *Ifnar*$^{-/-}$ or 129/SvEV mice were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine and intranasally inoculated with 10^7 or 10^8 CFUs *S. aureus* strain USA300. Mice were sacrificed at 24 hours after bacterial infection by overdose of pentobarbital. BAL fluids were obtained by instillation of 3 × 1 ml HBSS into the trachea of euthanized animals and centrifugation at 180 *g* for 10 minutes. For immune cell identification, cell suspensions were obtained from lung homogenates or BAL fluid and red cells were lysed. Remaining cells were suspended in PBS containing 10% normal mouse serum and incubated for 30 minutes at 37°C. Cells were then stained with combinations of PE-labeled anti-CD45, FITC-labeled anti-Ly6G (BD Biosciences — Pharmingen), PE-Cy5.5–labeled anti-CD11b (eBioscience), and APC-labeled anti-CD11c (eBioscience) in the presence of mouse Fc block (2.4G2; BD Biosciences — Pharmingen) and normal mouse serum. Negative control samples were incubated with irrelevant, isotype-matched antibodies. Cells were gated based on their forward scatter/side scatter profile and analyzed for the double expression of CD45 and Ly6G for PMNs. DCs were identified by double staining for CD11c and CD11b within the CD45$^+$ population. For IFN-β and IL-6 mRNA quantification, mouse lungs were obtained at 16–18 hours after inoculation and stored in RNAlater (QIAGEN). Bacterial counts in lungs and spleens were determined by plating serial dilutions on CYGP-agar plates. IL-6 and TNF-α in the BAL fluid were detected by sandwich ELISA (R&D Systems and eBioscience, respectively). All animal studies were approved by the Institute of Comparative Medicine at Columbia University.

Immunofluorescence staining of mouse lung sections. Mouse lungs were inflated with and fixed overnight in 4% paraformaldehyde before embedding in paraffin. 5 μM serial sections were deparaffinized in xylenes, rehydrated, subjected to antigen retrieval, permeabilized in 0.3% Triton X-100, and blocked in 5% normal goat serum before staining with antibodies to p-STAT3 (S727) or total STAT3. Primary antibodies were revealed by Alexa Fluor 647–conjugated secondary antibodies (Invitrogen). Imaging was performed on a Zeiss LSM 510 META scanning confocal microscope and analyzed using LSM Image Browser software (version 4.2).

Statistics. Samples with normal distribution were analyzed using a 2-tailed Student's *t* test. Samples that did not follow normal distributions were analyzed with the nonparametric Mann-Whitney test. Mortality studies were subjected to Fisher's exact test. *P* values less than 0.05 were considered significant.

Acknowledgments

This work was supported by NIH grant 1R01HL079395-01A2 to A. Prince. The authors would like to express their gratitude to Carolyn Lee and Courtney Plumlee at Columbia University for their assistance with this research.

Received for publication October 29, 2008, and accepted in revised form April 1, 2009.

Address correspondence to: Alice Prince, Columbia University Department of Pediatrics, 650 West 168th Street; BB4-416, New York, New York 10032, USA. Phone: (212) 305-4193; Fax: (212) 342-5728; E-mail: asp7@columbia.edu.

Marisa I. Gomez's present address is: Department of Microbiology, University of Buenos Aires, Buenos Aires, Argentina.

1. Klevens, R.M., et al. 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA.* **298**:1763–1771.
2. Wardenburg, J.B., Bae, T., Otto, M., Deleo, F.R., and Schneewind, O. 2007. Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. *Nat. Med.* **13**:1405–1406.
3. Labandeira-Rey, M., et al. 2007. *Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia. *Science.* **315**:1130–1133.
4. Wang, R., et al. 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat. Med.* **13**:1510–1514.
5. Lowy, F.D. 2007. Secrets of a superbug. *Nat. Med.* **13**:1418–1420.
6. Foster, T.J. 2005. Immune evasion by staphylococci. *Nat. Rev. Microbiol.* **3**:948–958.
7. Gomez, M.I., et al. 2004. *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nat. Med.* **10**:842–848.
8. Gomez, M.I., Seaghdha, M.O., and Prince, A.S. 2007. *Staphylococcus aureus* protein A activates TACE through EGFR-dependent signaling. *EMBO J.* **26**:701–709.
9. Shopsin, B., et al. 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *J. Clin. Microbiol.* **37**:3556–3563.
10. Harmsen, D., et al. 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. *J. Clin. Microbiol.* **41**:5442–5448.
11. Shair, K.H., et al. 2007. EBV latent membrane protein 1 activates Akt, NFκB, and Stat3 in B cell lymphomas. *PLoS Pathog.* **3**:e166.
12. Garcia-Sastre, A., and Biron, C.A. 2006. Type 1 interferons and the virus-host relationship: a lesson in detente. *Science.* **312**:879–882.
13. de Weerd, N.A., Samarajiwa, S.A., and Hertzog, P.J. 2007. Type I interferon receptors: biochemistry and biological functions. *J. Biol. Chem.* **282**:20053–20057.
14. Jeyaseelan, S., et al. 2007. Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF)-mediated signaling contributes to innate immune responses in the lung during *Escherichia coli* pneumonia. *J. Immunol.* **178**:3153–3160.
15. Mahieu, T., et al. 2006. The wild-derived inbred mouse strain SPRET/Ei is resistant to LPS and defective in IFN-β production. *Proc. Natl. Acad. Sci. U. S. A.* **103**:2292–2297.
16. O'Connell, R.M., et al. 2004. Type I interferon production enhances susceptibility to *Listeria monocytogenes* infection. *J. Exp. Med.* **200**:437–445.
17. Benveniste, E.N., and Qin, H. 2007. Type I interferons as anti-inflammatory mediators. *Sci. STKE.* **2007**:pe70.
18. Zhao, X., Nozell, S., Ma, Z., and Benveniste, E.N. 2007. The interferon-stimulated gene factor 3 complex mediates the inhibitory effect of interferon-β on matrix metalloproteinase-9 expression. *FEBS J.* **274**:6456–6468.
19. Honda, K., and Taniguchi, T. 2006. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat. Rev. Immunol.* **6**:644–658.
20. Gomez, M.I., et al. 2005. Bacterial induction of TNF-α converting enzyme expression and IL-6 receptor α shedding regulates airway inflammatory signaling. *J. Immunol.* **175**:1930–1936.
21. Karaghiosoff, M., et al. 2003. Central role for type I interferons and Tyk2 in lipopolysaccharide-induced endotoxin shock. *Nat. Immunol.* **4**:471–477.
22. Uematsu, S., and Akira, S. 2007. Toll-like Receptors and Type I Interferons. *J. Biol. Chem.* **282**:15319–15323.
23. Quinton, L.J., et al. 2008. Alveolar epithelial STAT3, IL-6 family cytokines, and host defense during *Escherichia coli* pneumonia. *Am. J. Respir. Cell Mol. Biol.* **38**:699–706.
24. Hsu, L.-C., et al. 2004. The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4. *Nature.* **428**:341–345.
25. Der, S.D., Zhou, A., Williams, B.R., and Silverman, R.H. 1998. Identification of genes differentially regulated by interferon α, β, or γ using oligonucleotide arrays. *Proc. Natl. Acad. Sci. U. S. A.* **95**:15623–15628.
26. Macia, E., et al. 2006. Dynasore, a cell-permeable inhibitor of dynamin. *Dev. Cell.* **10**:839–850.
27. Charpentier, X., and Oswald, E. 2004. Identification of the secretion and translocation domain of the enteropathogenic and enterohemorrhagic *Escherichia coli* effector Cif, using TEM-1 β-lactamase as a new fluorescence-based reporter. *J. Bacteriol.* **186**:5486–5495.
28. Murray, P.J. 2007. The JAK-STAT Signaling Pathway: Input and Output Integration. *J. Immunol.* **178**:2623–2629.
29. Smith, E.M., Johnson, H.M., and Blalock, J.E. 1983. *Staphylococcus aureus* protein A induces the production of interferon- α in human lymphocytes and interferon-α/β in mouse spleen cells. *J. Immunol.* **130**:773–776.
30. Punturieri, A., et al. 2004. Specific engagement of TLR4 or TLR3 does not lead to IFN-β-mediated innate signal amplification and STAT1 phosphorylation in resident murine alveolar macrophages. *J. Immunol.* **173**:1033–1042.
31. Hartleib, J., et al. 2000. Protein A is the von Willebrand factor binding protein on *Staphylococcus aureus*. *Blood.* **96**:2149–2156.
32. Beyer, M., et al. 2004. Sustained increases in numbers of pulmonary dendritic cells after respiratory syncytial virus infection. *J. Allergy Clin. Immunol.* **113**:127–133.
33. Wang, H., et al. 2006. Local CD11c$^+$ MHC class II$^-$ precursors generate lung dendritic cells during respiratory viral infection, but are depleted in the process. *J. Immunol.* **177**:2536–2542.
34. GeurtsvanKessel, C.H., et al. 2008. Clearance of influenza virus from the lung depends on migratory langerin$^+$CD11b$^-$ but not plasmacytoid dendritic cells. *J. Exp. Med.* **205**:1621–1634.
35. Mancuso, G., et al. 2007. Type I IFN signaling is crucial for host resistance against different species of pathogenic bacteria. *J. Immunol.* **178**:3126–3133.
36. Charrel-Dennis, M., et al. 2008. TLR-independent type I interferon induction in response to an extracellular bacterial pathogen via intracellular recognition of its DNA. *Cell Host Microbe.* **4**:543–554.
37. Morens, D.M., Taubenberger, J.K., and Fauci, A.S. 2008. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *J. Infect. Dis.* **198**:962–970.
38. Arnaud, M., Chastanet, A., and Debarbouille, M. 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Appl. Environ. Microbiol.* **70**:6887–6891.
39. Gomez, M.I., O'Seaghdha, M., Magargee, M., Foster, T.J., and Prince, A.S. 2006. *Staphylococcus aureus* protein A activates TNFR1 signaling through conserved IgG binding domains. *J. Biol. Chem.* **281**:20190–20196.
40. Ratner, A.J., et al. 2001. Cystic fibrosis pathogens activate Ca2$^+$-dependent mitogen-activated protein kinase signaling pathways in airway epithelial cells. *J. Biol. Chem.* **276**:19267–19275.
41. Rajan, S., et al. 2000. *Pseudomonas aeruginosa* induction of apoptosis in respiratory epithelial cells: analysis of the effects of cystic fibrosis transmembrane conductance regulator dysfunction and bacterial virulence factors. *Am. J. Respir. Cell Mol. Biol.* **23**:304–312.
42. Antunes, M.B., et al. 2007. Murine nasal septa for respiratory epithelial air-liquid interface cultures. *Biotechniques.* **43**:195–196, 198, 200.
43. O'Brien, L., et al. 2002. Multiple mechanisms for the activation of human platelet aggregation by *Staphylococcus aureus*: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A. *Mol. Microbiol.* **44**:1033–1044.