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Staphylococcus aureus Induces Type I IFN Signaling in Dendritic Cells Via TLR9

Dane Parker and Alice Prince

The importance of type I IFN signaling in the innate immune response to viral and intracellular pathogens is well established, with an increasing literature implicating extracellular bacterial pathogens, including Staphylococcus aureus, in this signaling pathway. Airway epithelial cells and especially dendritic cells (DCs) contribute to the production of type I IFNs in the lung. We were interested in establishing how S. aureus activates the type I IFN cascade in DCs. In vitro studies confirmed the rapid uptake of S. aureus by DCs followed promptly by STAT1 phosphorylation and expression of IFN-β. Signaling occurred using heat-killed organisms and in the absence of PVL and α-toxin. Consistent with the participation of endosomal and not cytosolic receptors, signaling was predominantly mediated by MyD88, TLR9, and IRF1 and blocked by cytochalasin D, dynasore, and chloroquine. To determine the role of TLR9 signaling in the pathogenesis of S. aureus pneumonia, we infected WT and Tlr9<sup>−/−</sup> mice with MRSA USA300. Tlr9<sup>−/−</sup> mice had significantly improved clearance of S. aureus from the airways and lung tissue. Ifnar<sup>−/−</sup> mice also had improved clearance. This enhanced clearance in Tlr9<sup>−/−</sup> mice was not due to differences in the numbers of recruited neutrophils into the airways, but instead correlated with decreased induction of TNF. Thus, we identified TLR9 as the critical receptor mediating the induction of type I IFN signaling in DCs in response to S. aureus, illustrating an additional mechanism through which S. aureus exploits innate immune signaling to facilitate infection. The Journal of Immunology, 2012, 189: 4040–4046.

**S** taphylococcus aureus is a common cause of pneumonia, the most frequent pathogen identified with health care-associated pneumonias (1, 2), as well as a major cause of superinfection and mortality after influenza (3–8). Analysis of the patterns of human staphylococcal infection and the use of murine models suggest that participation of type I IFN signaling, a major component of the host response to influenza and other respiratory viral infections, actually contributes to the pathology associated with staphylococcal pneumonia (9). *S. aureus* as well as several other bacterial pulmonary pathogens trigger type I IFN signaling (10–12). This is mediated by airway epithelial cells that can be activated directly by either intact bacteria or shed pathogen-associated molecular patterns (PAMPs) (10), as well as by dendritic cells (DCs), which account for a substantial amount of type I IFN signaling (13). Type I IFN signaling is generally initiated from intracellular receptors, those that would respond to intracellular pathogens such as viruses. As *S. aureus* persists intracellularly in both phagocytic and nonphagocytic cells (14), it is well positioned to activate type I IFN signaling.

The severity of *S. aureus* pneumonia is due to both expression of specific virulence factors as well as the nature of the host response that is activated. The epidemic USA300 strain of methicillin-resistant *S. aureus* is associated with especially severe pneumonia in humans that can be modeled in mice using a high bacterial inoculum; this results in 80% mortality by 24 h postinoculation in wild-type 129/SvEv mice but less than 10% mortality in Ifnar<sup>−/−</sup> mice (15). The excessive activation of proinflammatory signaling, mediated by airway epithelial cells as well as resident and recruited macrophages, T cells, and DCs, is thought to contribute to this pathology.

DCs have multiple roles in mucosal defense. They have signaling capabilities linking pathogen recognition and activation of T cells and function as a major source of the type I IFNs in viral infection (16). DCs are also phagocytic and appear to contribute to *S. aureus* clearance as was demonstrated using a CD11c-DTR depletion murine model of pneumonia (17). We were interested in establishing how DCs interact with *S. aureus* and their participation in type I IFN signaling in the host response to *S. aureus* pulmonary infection. In the studies detailed in this report, we demonstrate that DCs actively phagocytose *S. aureus* and activate gene expression in response to the recognition of staphylococcal DNA by TLR9. Consistent with the hypothesis that type I IFN signaling increases susceptibility to severe staphylococcal pneumonia, Tlr9<sup>−/−</sup> mice had improved staphyloccocal clearance from the lung without defects in the recruitment of phagocytes into the lung.

**Materials and Methods**

**Bacteria and cell culture**

*S. aureus* USA300 strains FPR3757 (18) and LAC (19) were grown in Luria-Bertani broth at 37°C. No differences in *Ifnb* induction were observed between these strains (data not shown). Heat-killed preparations of *S. aureus* were obtained by heating cells at 65°C for 1.5 h. Bone marrow-derived dendritic cells (BMDCs) were cultured from wild-type and knockout mice as described previously (11). BMDCs were stimulated with *S. aureus* (multiplicity of infection [MOI] of 100) for 2 h. Cytokine studies on BMDCs for ELISA quantitation were conducted for 20 h with an MOI of 5. Cell lysate experiments were performed using exponential phase *S. aureus* resuspended at 5 × 10<sup>8</sup> CFU/ml. *S. aureus* suspensions were treated with 500 μg/ml lysostaphin (Sigma) for 10 min at 37°C before sonication, then DNase and RNase treatment as before (11). Experiments with cellular inhibitors were performed by preincubating the cells for 30 min prior to bacterial stimulation using cytochalasin D 20 μM (Sigma), dynasore 80 μM (20), and chloroquine 10 μM (Sigma). Stimulation of BMDCs for ELISA analysis was performed for 20 h at an MOI of 5.
RNA was isolated using the PureLink RNA mini kit (Life Technologies) followed by DNeasy treatment using DNAfree (Life Technologies). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed using Power SYBR Green PCR Master Mix in a StepOne Plus thermal cycler (Applied Biosystems). Samples were normalized to β-actin. Primers for mouse actin, Ifnb, and Mx1, KC, and Il6 have been described elsewhere. The following primers were used: Cxcl10, sense 5'-CGATGACGGGACCAGTGAGAATG-3' and antisense 5'-CTCAACAGATGAGGATCTG-3'; Tnf, sense 5'-ATGAGCACAGAAAGCATGATC-3' and antisense 5'-TACAGGCTGTAGCGGATGACG-3'; and antisense 5'-CAGAAGTGCTTGAGGTTGTTG-3'.

**Western hybridization**

BMDCs were lysed in RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate) with Halt protease and phosphatase single-use inhibitors (Thermo Scientific). Phosphorylation of transcription factors was detected using Abs to P-IRF3 (S396; Cell Signaling), P-IRF7 (S471/472; Cell Signaling), and p-STAT1 (Y701; Abcam) normalized to β-actin (Sigma), as transcription of total protein can be regulated. Protein separation, transfer, and detection has been described elsewhere.

**Microscopy**

Exponential phase bacteria were labeled with 100 μg/ml FITC in PBS with calcium and magnesium (Celloglo) for 20 min before three washes and incubation with BMDCs as described earlier. Fluorescence microscopy was performed using a Zeiss Observer Z1 inverted fluorescence microscope with AxioVision software (version 4.6.2.0; Zeiss).

**Mouse studies**

C57BL/6J, Tlr7−/−, and Ifi−/− mice were from The Jackson Laboratory (Bar Harbor, ME). All mice were on a C57BL/6J background. Six-week-old sex-matched mice were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine before intranasal inoculation with 2 × 10⁵ to 5 × 10⁶ CFU S. aureus USA300 (18). Mortality (including mice moribund that were euthanized) was data from infections with 1 × 10⁵ to 2 × 10⁶ CFU S. aureus USA300. Mice were euthanized 20 h later when bronchoalveolar lavage fluid (BALF) was obtained before lungs were extracted. Bacteria were enumerated from BALF and lung homogenate using serial dilution before plating on CHROMagar S. aureus plates at 37°C (Becton Dickinson). All mouse infections were performed under the guidelines of the Institutional Animal Care and Use Committee of Columbia University.

**FACS**

Analysis of cell populations in BALF was conducted as before. Cells were labeled with combinations of FITC-labeled anti-Ly-6G (Gr-1; RB6-8C5; eBioscience), PerCP-Cy5.5-labeled anti-CD11c (N418; eBioscience), allophycocyanin-labeled anti-MHC class II (I-A/I-E; eBioscience), PE-labeled anti-NK 1.1 (NKR-PIc, Ly-55; eBioscience), PE-labeled anti-CD4 (RM 4-5; eBioscience), and FITC-labeled anti-CD3 (eBio500A2; eBioscience). Neutrophils were defined as Ly6G+/MHCII− CD4−CD11b−, macrophages as CD11c+/MHCII+, and DCs as CD11c+/MHCIIhigh. Data were analyzed using FlowJo (version 10.2.5; TreeStar). Cytokine levels were quantified using ELISA: TNF, IFN-β, IL-6, CXCL10 (R&D Systems). The IFN-β ELISA was from PBL IFNSource.

**Statistics**

Significance of data that followed a normal distribution was determined using a two-tailed Student t test, and for data that did not follow a normal distribution, a nonparametric Mann–Whitney U test was used. Dichotomous outcomes were assessed using the Fisher exact test. Statistics were performed with Prism software (GraphPad, La Jolla, CA).

**Results**

**Conserved components of S. aureus activate type I IFN signaling in BMDCs**

The virulence of the epidemic USA300 strains of S. aureus has been attributed to their activation of multiple host immune sig-
duced by 64% in comparison with wild-type (WT) controls ($p < 0.01$) (Fig. 1A). We also examined the transcription levels of two type I IFN-dependent genes, Cxcl10 (23) and Mx1 (24), in WT and Ifnar$^{-/-}$ BMDCs after S. aureus stimulation. Even at this early time point, both Cxcl10 and Mx1 were induced in WT BMDCs compared with unstimulated cells and reduced in Ifnar$^{-/-}$ cells (Fig. 1A) indicating the contribution of both direct activation of type I IFN signaling by bacterial ligands as well as substantial amplification by autocrine signaling.

To address the role(s) of specific staphylococcal virulence factors in Ifnb induction, we compared several isogenic mutants in the USA300 background. The α-toxin (Hla, encoded by hla) of S. aureus is important in numerous models of infection and has been shown to activate the NLRP3 inflammasome in monocytes (14, 25, 26). However, Hla was not required for Ifnb induction (Fig. 1C), nor was expression of the Panton Valentine Leukocidin (pvl-lukS/lukF mutant) (14, 27, 28) in these murine cells (Fig. 1C). The abundant surface protein, protein A (encoded by spa), has been shown to play a role in type I IFN signaling in epithelial cells and to contribute to invasion in various cell types (15, 29, 30) but was not required for Ifnb induction in BMDCs (Fig. 1C). We inferred from these data that a generally conserved PAMP of S. aureus is likely to be involved in the activation of type I IFN signaling. Consistent with this, we did not observe a decrease in signaling with heat-killed organisms (Fig. 1D), suggesting that the activation of the BMDCs was primarily a function of the DCs and not an active process initiated by the organism.

Ifnar$^{-/-}$ mice display improved clearance of S. aureus from the lung

The biological significance of staphylococcal induction of the type I IFN cascade has been previously reported based on experiments

FIGURE 3. Uptake of S. aureus is required for type I IFN production. (A–C) WT BMDCs were stimulated with S. aureus USA300 in the presence of (A) cytochalasin D (cytoD), (B) dynasore, or (C) chloroquine compared with a DMSO control ($n = 6$). (D) FITC-labeled S. aureus USA300 (green) visualized inside WT BMDCs; cell nuclei are stained with DAPI (blue). Graphs display means and SD and are representative of two independent experiments. $^* p < 0.05$.

FIGURE 4. TLR9 is the receptor for S. aureus DNA-induced type I IFN signaling. (A) BMDCs from WT and knockout mice were cultured with S. aureus for 2 h, and levels of Ifnb were detected using qRT-PCR. Data are from two independent experiments ($n = 6$). (B) Phosphorylation of IRF3 and IRF7 was detected using immunoblots from WT BMDCs stimulated with S. aureus USA300 for 2 h. β-Actin was used as a loading control. (C and D) BMDCs were incubated with S. aureus USA300 for 20 h and supernatants collected for (C) IFN-β and (D) TNF ELISA. (E) BMDCs were incubated with S. aureus lysates treated with DNase and RNase, and levels of Ifnb were quantitated with qRT-PCR. Shown is a representative of two independent experiments ($n = 3$). $^* p < 0.05$. UN, Unstimulated.
using a high inoculum of USA300 in Svev/129 Ifnar\(^{-/-}\) mice. These Ifnar\(^{-/-}\) mice have significantly reduced mortality in an acute pneumonia model of infection (15). To determine if C57BL/6J Ifnar\(^{-/-}\) are similarly protected from severe infection at lower doses of S. aureus, we infected mice intranasally with 10\(^7\) CFU of S. aureus USA300. Enumeration of bacteria from lung homogenates at 24 h postinoculation indicated an average of 20-fold (\(p < 0.05\)) fewer bacteria present than that of the WT control mice (Fig. 2A). The cellular populations comprising the inflammatory infiltrate in the BALF of the WT and Ifnar\(^{-/-}\) were similar, and aside from a slight increase in CD4\(^+\) cells (Fig. 2B), no compensatory changes in other cell types were observed (data not shown).

**Phagocytosis and endosomal acidification are required for signaling**

The activation of type I IFN signaling cascades is often initiated by intracellular receptors that respond to ligands that are either endocytosed or available in the cytosol (31). As DCs are phagocytic, we investigated the role of uptake of intact S. aureus as a requirement for type I IFN activation. In the presence of cytochalasin D, an inhibitor of actin polymerization, induction of Ifnb was reduced by 96% (\(p < 0.001\)) (Fig. 3A). The involvement of dynamin-mediated endocytosis was addressed by treating the BMDCs with dynasore, which inhibits the GTPase activity of dynamin (20). Incubation of BMDCs with dynasore led to a 92% decrease (\(p < 0.05\)) in Ifnb induction in response to S. aureus (Fig. 3B). The subsequent involvement of endocytic processing was tested by treating the BMDCs with chloroquine, which prevents endosomal acidification and interferes with TLR signaling (32–34). In response to S. aureus, chloroquine-treated BMDCs induced 96% less Ifnb (\(p < 0.05\)) than the untreated controls (Fig. 3C).

**TLR9 senses S. aureus DNA to induce type I IFN signaling**

On the basis of our observation that host factors, endocytosis, and endosomal acidification were necessary for IFN-\(\beta\) signaling, we postulated that endosomal TLRs were involved in staphylococcal recognition. Type I IFN signaling can be mediated by either MyD88- or TIR domain-containing adaptor inducing IFN-\(\beta\)–

**FIGURE 5.** TLR9 knockout mice have improved clearance of S. aureus from the airways. Mice were inoculated intranasally with S. aureus USA300 and the response to infection assessed 24 h later. (A and B) Bacteria were enumerated from (A) BALF and (B) lung homogenate. (C) Cell populations were determined using FACS analysis from bronchoalveolar lavage samples. (D) WT and Tlr9\(^{-/-}\) mice were infected with 1 \(\times\) 10\(^8\) to 2 \(\times\) 10\(^8\) CFU and assessed for mortality 20 h later (\(n = 8\), WT; \(n = 9\), Tlr9\(^{-/-}\)). Each point represents a mouse. Lines display median values. *\(p < 0.05\).
dependent pathways. The ability of *S. aureus* to activate *Ifnb* in *MyD88*<sup>−/−</sup> BMDCs was decreased on average by 75% (*p < 0.05*) compared with WT cells (Fig. 4A), whereas *Tlr9*<sup>−/−</sup> cells were not affected as greatly. At the same time, we did not see a decrease in *Ifnb* induction using a mutant lacking a known receptor for *S. aureus* TNFR1 (35) (Fig. 4A). MyD88-dependent type I IFN signaling occurs through TLR7 and TLR9 in mice (36). TLR9 is located in the endosome and responds to CpG DNA, a likely product of staphylococcal degradation. The ability of *S. aureus* to activate *Ifnb* in a null background of TLR7 was unimpaired, but there was a significant (82% decrease, *p < 0.05*) decrease in signaling associated with the *Tlr9*<sup>−/−</sup> cells (Fig. 4A). As would be expected for MyD88-dependent TLR9 signaling, we saw significant decrease in *Ifnb* induction using *Irf1*<sup>−/−</sup> BMDCs (37, 38), as well as induction of IRF7 phosphorylation in response to *S. aureus*, as opposed to only a minor amount of IFR3 phosphorylation (Fig. 4B) (10). Consistent with the RNA data, we observed a significant reduction in IFN-β levels in *MyD88*<sup>−/−</sup> and *Tlr9*<sup>−/−</sup> BMDCs (88%, *p = 0.002; 66%, *p = 0.009*, respectively) (Fig. 4C). This contrasted with TNF levels, which were also significantly reduced in *MyD88*<sup>−/−</sup> BMDCs (87%, *p = 0.007) but only reduced by 27% in *Tlr9*<sup>−/−</sup> BMDCs (*p = 0.0149*) (Fig. 4D). The role of TLR9 (39) in sensing staphylococcal DNA was confirmed by incubating BMDCs with lysates of *S. aureus* USA300 treated with DNase and RNase. Stimulation of BMDCs with lysate treated with DNase resulted in a 90% decrease (*p < 0.001*) in signaling compared with the untreated lysates (Fig. 4E), whereas treatment of lysate with RNase had no significant effect on *Ifnb* levels (Fig. 4E).

We examined the transcription levels of several genes to monitor the contribution of TLR9 to the induction of other gene products. Apart from *Cxcl10*, a gene known to be regulated by type I IFN signaling and associated with lung pathology (40), no other significant changes were observed (Fig. 4F).

**TLR9 mice have improved outcome in a mouse pneumonia model**

SvEv/129 *Ifnar*<sup>−/−</sup> mice have improved outcome in response to *S. aureus* pneumonia (15), an observation we confirmed in the C57/B6 background (Fig. 2). To determine the role that TLR9-mediated type I IFN signaling plays in the overall host response to *S. aureus* infection, we compared the responses of WT and *Tlr9*<sup>−/−</sup> mice in our model of acute *S. aureus* pneumonia. After 24 h of infection, *Tlr9*<sup>−/−</sup> mice had improved clearance of *S. aureus* from both the BALF (36% reduction, *p < 0.05*, Fig. 5A) and lung homogenate (39% reduction, *p < 0.05*, Fig. 5B). We did not observe major changes to the overall cellular composition of BALF (Fig. 5C), with the exception of a 2-fold (*p < 0.05*) increase in macrophage numbers. At higher inocula, less mortality (11 versus 78%) was observed in *Tlr9*<sup>−/−</sup> mice compared with WT controls (Fig. 5D). There were similarly minimal consequences of TLR9 signaling on cytokine production in vivo (Fig. 6). TNF levels were reduced by 35% (*p < 0.05*) in infected *Tlr9*<sup>−/−</sup> compared with WT mice (Fig. 6), whereas the other proinflammatory cytokines were unchanged. The improved clearance of *Tlr9*<sup>−/−</sup> mice and concomitant decrease in TNF is consistent with the negative impact of TNF signaling on *S. aureus* pulmonary infection (35).

**Discussion**

DCs are important participants in the innate immune responses to inhaled bacterial pathogens. The immature DCs in the lung are avidly phagocytic (41) prior to maturation and the acquisition of surface markers associated with the ability to traffic to local lymph nodes. Although the importance of DCs in communicating with T cells is well established, their role in the pathogen recognition is less well characterized. In the in vitro data presented, we observed that DCs readily take up *S. aureus* through a classical endocytic process that results in the activation of TLR9 signaling. This observation is consistent with previous studies in which mice depleted of CD11c<sup>+</sup> cells, primarily DCs but also some macrophage populations, had diminished clearance of *S. aureus* from the lung (17). Although neutrophils are primarily responsible for the clearance of pathogens such as *S. aureus* from the airway, it appears that pulmonary DCs also contribute to bacterial uptake early in infection.

Perhaps the more significant consequence of DC uptake of *S. aureus* is the activation of TLR9 signaling and production of type I IFNs. DNA is sensed by a number of both endosomal and cytosolic receptors activating the expression of type I IFN signaling (42). This is a rapid response to endocytosed staphylococci, with IRF7 and STAT1 phosphorylation detected within 2 h of exposure, indicating that DCs are efficient in degrading the ingested organisms. In contrast to other mechanisms of immune activation such as the NLRP3 inflammasome (25), staphylococcal induction of type I IFN signaling did not require participation of α-toxin or other toxins but is mediated by bacterial DNA. Endocytosis and induction of IFN-β expression appears to be accomplished primarily by the DC, as heat-killed organisms are equally stimulatory as viable bacteria. Although a protein A mutant was not diminished in its stimulatory capacity for *Ifnb*, SpA is abundantly shed by organisms during growth and can activate signaling through TNF receptor 1 and IRF1, which can be activated by TNF as well. IRF1 has been shown to be involved in TLR9 signaling in myeloid DCs (37, 38). *Streptococcus pneumoniae*, another common pulmonary pathogen, also activates the production of type I IFNs. However, the signaling pathway activated in response to these organisms requires expression of the pore-forming toxinn
应有的分子信号通路，它们是免疫系统对抗病毒的关键。特别是作为流感在人类中的并发症（8）。研究显示，在流感感染的肺部，如I型干扰素级联反应的减弱，是流感导致的肺部炎症和损伤的原因（35）。正如这些实验中所示，提高TNF的水平也与观察到的结果呈逆向相关。这些结果进一步有助于解释流感导致的肺部损伤和死亡。


