

# *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1

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*Staphylococcus aureus* is a major human pathogen that is associated with diverse types of local and systemic infection characterized by inflammation dominated by polymorphonuclear leukocytes. Staphylococci frequently cause pneumonia, and these clinical isolates often have increased expression of protein A, suggesting that this protein may have a role in virulence. Here we show that TNFR1, a receptor for tumor-necrosis factor- $\alpha$  (TNF- $\alpha$ ) that is widely distributed on the airway epithelium, is a receptor for protein A. We also show that the protein A–TNFR1 signaling pathway has a central role in the pathogenesis of staphylococcal pneumonia.

*Staphylococcus aureus* is an important pathogen associated with pneumonia and sepsis, particularly in patients in hospital<sup>1</sup>. These organisms cause primary pneumonias, often in infants and individuals with cystic fibrosis<sup>2</sup>, complicate influenza and other viral infections, and are the main cause of ventilator-associated pneumonia, a frequent problem in intensive care units<sup>1</sup>. It has long been recognized that staphylococci evoke an intense host response dominated by polymorphonuclear leukocytes (PMNs), although the molecular mechanisms by which PMNs are recruited into the lung are not completely understood<sup>2–4</sup>. The virulence of *S. aureus* is attributed to many factors: exo-products that cause tissue destruction, ligands (MSCRAMMS) that bind host cell matrix components, and several gene products that thwart immune clearance mechanisms<sup>5,6</sup>. Despite the morbidity and mortality associated with staphylococcal pneumonia, however, it is unclear how these organisms interact with mucosal cells to evoke the characteristic PMN response.

*S. aureus* strains isolated from individuals with airway infection show increased expression of surface proteins, particularly protein A<sup>7</sup>. Protein A interferes with opsonization by binding to the Fc portion of immunoglobulin<sup>8</sup>, and we postulated that it might also have a direct effect on the epithelial cells that line the respiratory tract, even in the absence of IgG. Here we have examined the interactions of *S. aureus* protein A and airway epithelial cells, which show that protein A has a chief role in the induction of pneumonia and the recruitment of PMNs into the airway through activation of TNFR1.

## RESULTS

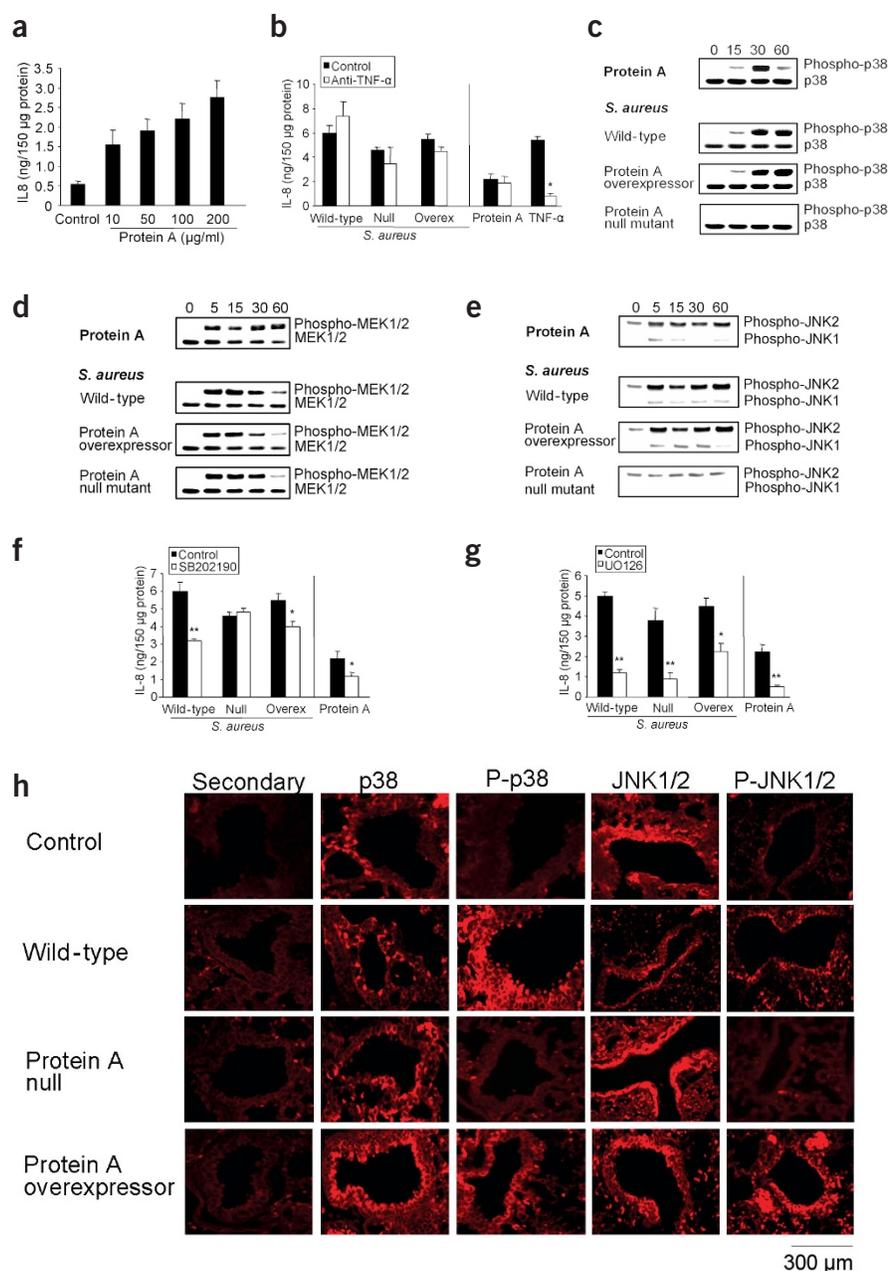
### Protein A induces IL-8 expression in epithelial cells

Airway epithelial cells respond to many bacterial components by producing the PMN chemokine interleukin-8 (IL-8). Exposure of 1HAEo<sup>-</sup> epithelial cells to purified protein A resulted in a dose-

dependent induction of IL-8 (Fig. 1a). The effects of protein A on IL-8 expression were also studied by comparing responses induced by wild-type *S. aureus* strain RN6390, a protein A–null mutant and a strain that overexpresses protein A after induction with tetracycline<sup>9</sup>. All three strains induced significant amounts of IL-8 (Fig. 1b). The protein A–null mutant strain could still induce significant IL-8 because it expresses other proinflammatory agonists such as peptidoglycan and lipoteichoic acid. We excluded the possibility that IL-8 production was secondary to TNF- $\alpha$  production by showing that IL-8 production continued in the presence of a neutralizing antibody to TNF- $\alpha$  that inhibited 85% of the IL-8 produced in response to TNF- $\alpha$  (Fig. 1b).

Mitogen-activated protein kinases (MAPKs) are often involved in proinflammatory signaling, and protein A induced phosphorylation of MAPKs within 15–30 min of exposure (Fig. 1c–e). The protein A–null mutant did not activate either p38 or Jun amino-terminal kinases 1 and 2 (JNK1/2; Fig. 1c,e), whereas both the wild-type and the protein A overexpressor strain activated all three MAPKs (Fig. 1c–e). We confirmed this finding by testing the effects of specific MAPK inhibitors on the induction of IL-8 expression by wild-type and mutant strains and by purified protein A (Fig. 1f,g). Whereas IL-8 induction by wild-type *S. aureus*, the protein A overexpressor strain and purified protein A was significantly diminished in the presence of inhibitors of p38 and MAPK kinases 1 and 2 (MEK1/2;  $P < 0.05$ ,  $P < 0.01$ ; Fig. 1f,g), induction of IL-8 in response to the protein A–null mutant was decreased only in the presence of the MEK1/2 inhibitor UO126 (Fig. 1g), consistent with the fact that this mutant can induce phosphorylation of MEK1/2. These results indicate that although protein A can activate all three MAPKs, it is essential for activation of p38 and JNK1/2 by *S. aureus*. We confirmed the biological relevance of these findings in mice. Phosphorylated forms of both

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**Figure 1** Protein A induction of IL-8 in epithelial cells. **(a,b)** IL-8 was assayed by ELISA after exposure of 1HAE<sup>-</sup> cells to media alone (control), purified protein A or the *S. aureus* strains indicated. Overex, protein A overexpressor. Data are the mean  $\pm$  s.d. of six wells (\* $P < 0.05$ , \*\* $P < 0.01$ ). **(c–e)** Lysates from 1HAE<sup>-</sup> cells collected at various durations (min) after exposure to purified protein A or the *S. aureus* strains indicated were screened for phosphorylated (phospho-) and total p38 **(c)**, MEK1/2 **(d)** and JNK1/2 **(e)**. **(f,g)** IL-8 production was assayed by ELISA after stimulation of 1HAE<sup>-</sup> cells in the presence of inhibitors for p38 (SB202190) and MEK1/2 (U0126). **(h)** Mice were inoculated intranasally with the *S. aureus* strains indicated and lung sections were stained with antibodies specific for phosphorylated (P-) and total p38 and JNK1/2.

that mediates this response. Airway epithelial cells were screened for surface components that could bind protein A. The cells were surface-biotinylated, lysed and incubated with protein A-coated magnetic beads. After extensive washing, protein A-bound material was eluted and subjected to SDS-PAGE and western blotting with an antibody to biotin (Fig. 3a). One of the four proteins identified, which was particularly abundant, showed a molecular mass of roughly 55 kDa (Fig. 3a), which matches the size of TNFR1.

The signaling pathway activated by protein A shares the same MAPKs, as well as ATF-2 and NF- $\kappa$ B activation, that TNF- $\alpha$  uses to induce expression of inflammatory chemokines and cytokines such as IL-8, granulocyte-macrophage colony-stimulating factor and RANTES in epithelial cells<sup>11–13</sup>. TNF- $\alpha$  exerts its effects through two distinct receptors, TNFR1 and TNFR2, whose molecular masses are 55 kDa and 75 kDa, respectively<sup>14</sup>. Whereas TNFR2 is restricted to cells of hematopoietic origins, TNFR1 is ubiquitous and is expressed in epithelial cells<sup>15</sup>. Because protein A signaling resembles TNF- $\alpha$  signaling, we postulated that TNFR1 may function as the receptor for protein A.

We confirmed the presence of TNFR1 in epithelial cell lysates by western blotting (Fig. 3b). To determine whether the 55-kDa protein that bound to protein A-coated beads was TNFR1, we eluted the cellular proteins bound to the beads, separated them by SDS-PAGE and identified TNFR1 (Fig. 3b). The receptor was not detected in the eluate when epithelial cell lysates were incubated with protein G-coated beads as a control (Fig. 3b). Further evidence that the 55-kDa protein was functional TNFR1 was provided by competition binding studies. Cell lysates were incubated with TNF- $\alpha$  overnight before the addition of protein A-coated beads. TNF- $\alpha$  competed with protein A for binding to TNFR1, and the amount of receptor eluted from the protein A-coated beads was substantially less than that obtained in absence of TNF- $\alpha$  (Fig. 3b).

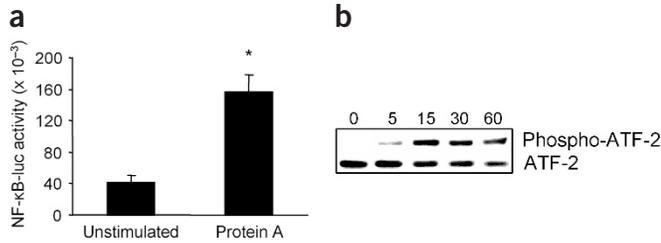
Soluble TNFR1 (sTNFR1) was also incubated with protein A- or protein G-coated beads, and after extensive washing protein A- or

p38 and JNK1/2 were abundant in epithelial cells lining the large airways of mice infected with either wild-type *S. aureus* or the protein A overexpressor strain, but not in those of mice infected with the protein A-null mutant (Fig. 1h).

Activation of MAPKs often stimulates the transcription factors NF- $\kappa$ B and AP-1, which mediate expression of IL-8 (ref. 10). The effect of purified protein A on NF- $\kappa$ B activity was tested on human airway cells transfected with an NF- $\kappa$ B-luciferase reporter construct. Protein A induced a 3.5-fold increase in NF- $\kappa$ B activation in epithelial cells as compared with unstimulated cells (Fig. 2a). Protein A also induced phosphorylation of ATF-2 (Fig. 2b), a component of the AP-1 transcription complex that is phosphorylated by p38 and JNK1/2 MAPKs.

#### Protein A recognizes TNFR1 on epithelial cells

Having identified the distal components of the epithelial signaling pathway stimulated by protein A, we looked for the surface receptor



**Figure 2** NF-κB and ATF-2 activation induced by protein A. (a) 1HAEo<sup>-</sup> cells were transiently transfected with an NF-κB reporter construct. After stimulation with purified protein A, luciferase activity was measured and compared with that of unstimulated control cells (\**P* < 0.05). One representative experiment out of three is shown. (b) Lysates from cells collected at various durations after exposure to purified protein A were screened for the presence of phosphorylated (phospho-) and total ATF-2 by western blotting.

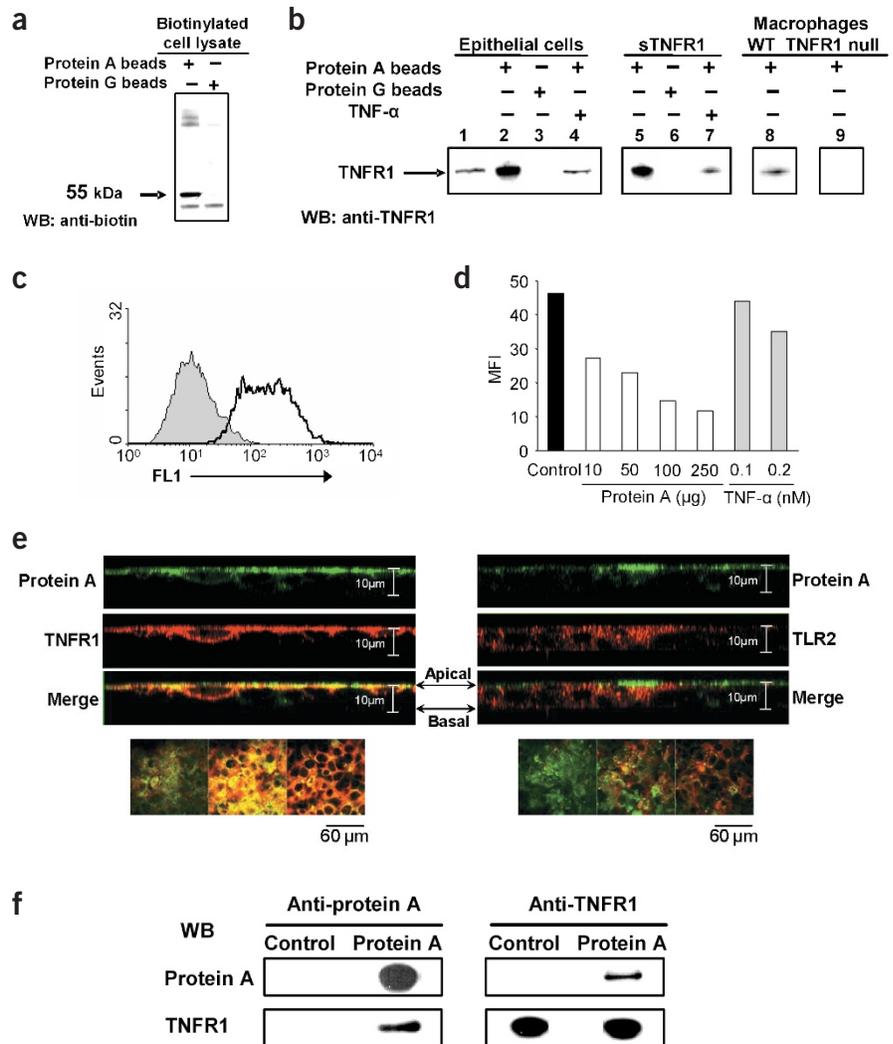
protein G-bound material was eluted and analyzed by western blotting. Whereas sTNFR1 bound to protein A, no binding to protein G-coated beads was observed (Fig. 3b). Competition binding studies were done by preincubating sTNFR1 with TNF-α before the addition of protein A-coated beads. TNF-α inhibited binding of protein A to sTNFR1 (Fig. 3b), confirming the interaction of this staphylococcal protein with the receptor for TNF-α. To confirm further that the 55-kDa band detected in the western blots corresponded to TNFR1, lysates of macrophages from wild-type and TNFR1-null mice were incubated with protein A-coated beads and processed as described above. The 55-kDa band was observed in pull-down assays of lysates from wild-type but not TNFR1-null cells (Fig. 3b).

We obtained evidence that protein A binds to TNFR1 in intact epithelial cells by carrying out flow cytometry studies in which epithelial cells were incubated with fluorescent dye-conjugated protein A (Fig. 3c). Binding of fluorescent protein A was inhibited by the addition of increasing amounts of unlabeled protein A and to a lesser extent by TNF-α (Fig. 3d). Confocal microscopy showed that protein A and TNFR1 colocalized on the apical surface of polarized airway epithelial cells (Fig. 3e). TNFR1 was abundant on the apical surface of epithelial cells and accessible to protein A (Fig. 3e). By contrast, Toll-like receptor-2 (TLR2), which is expressed apically and also basolaterally, did not colocalize with protein A (Fig. 3e).

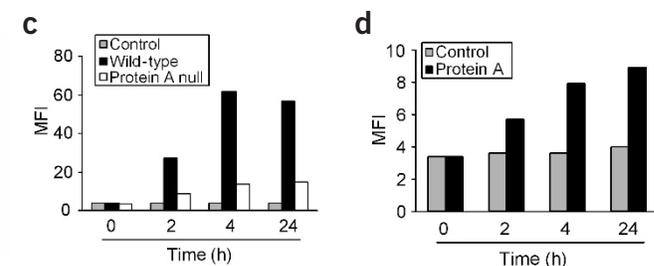
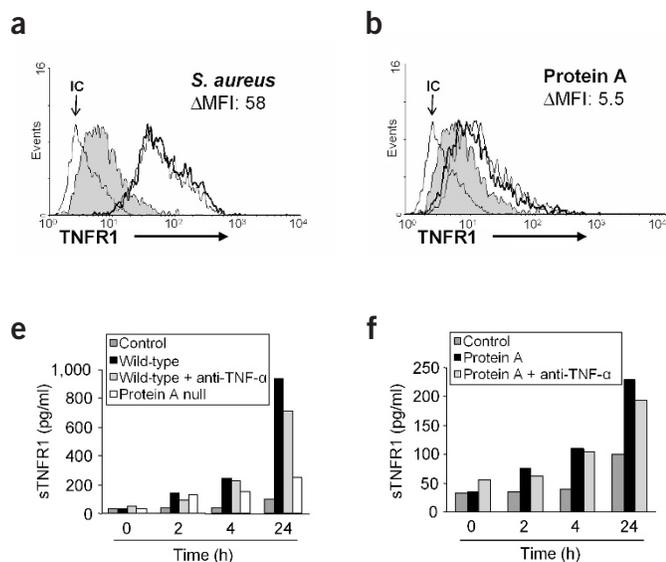
We confirmed the interaction between protein A and TNFR1 in coimmunoprecipitation studies. Epithelial cells were incubated with protein A or bovine serum albumin as a control, and lysates were immunoprecipitated with antibodies to TNFR1 or protein A. We detected protein A by western blotting in protein A-stimulated lysates captured with antibodies to either protein A or TNFR1 (Fig. 3f). TNFR1 was also detected in protein A-stimulated lysates captured with antibodies to protein A (Fig. 3f).

**Protein A induces mobilization and shedding of TNFR1**

TNF-α signaling is a dynamic process that is regulated by mobilization of TNFR1 to the cell surface and its shedding into the extracellular media<sup>16</sup>. To verify that TNFR1 signaling is actively involved in



**Figure 3** Recognition of protein A by TNFR1. (a,b) Pull-down assays of lysates from surface-biotinylated 1HAEo<sup>-</sup> cells or mouse macrophages (WT, wild-type), as well as sTNFR1, were analyzed by western blotting (WB) with antibodies specific for biotin (a) or TNFR1 (b). Lane 1, whole-cell lysate. (c) Epithelial cells were incubated with Alexa Fluor 488-conjugated protein A (green, FL1) and binding of protein A to the surface quantified by flow cytometry. Shadow histogram indicates autofluorescence. (d) Epithelial cells were incubated with Alexa Fluor 488-conjugated protein A in the presence or absence of protein A or TNF-α and analyzed by flow cytometry. One representative example of three is shown. MFI, mean fluorescence intensity. (e) Polarized epithelial cells were stained for TNFR1 (red) or TLR2 (red) and incubated with protein A (green). Apical colocalization (yellow) of TNFR1 and protein A is shown (merge). (f) Lysates from epithelial cells either unstimulated or stimulated with protein A were immunoprecipitated (WB) with the capture antibodies indicated and screened for protein A and TNFR1.



**Figure 4** TNFR1 mobilization and shedding. (a–d) Histograms show TNFR1 expression at 24 h.  $\Delta$ MFI, change in mean fluorescence intensity in response to stimulation. (a, b) IC, indicates secondary antibody, shadow histogram indicates unstimulated cells, thick line indicates *S. aureus* or protein A–stimulated cells, thin line indicates cells stimulated in the presence of blocking antibody to TNF- $\alpha$ . One representative experiment of three is shown. (e, f) 1HAEo<sup>-</sup> cells were stimulated with wild-type or protein A–null *S. aureus* or purified protein A in the presence or absence of TNF- $\alpha$  blocking antibody, and sTNFR1 was measured by ELISA. One representative experiment of three is shown.

epithelial responses to protein A, we monitored the surface expression and shedding of TNFR1. After exposure to either *S. aureus* or protein A for 24 h, expression of TNFR1 increased (18- and 2.6-fold, respectively) on the surface of epithelial cells (Fig. 4a–d). Shedding of TNFR1 into the culture media was observed as early as 2 h after stimulation with either *S. aureus* or purified protein A, and had increased by 28- and 6.8-fold after 24 h of stimulation with *S. aureus* or protein A, respectively (Fig. 4e, f). Expression of TNFR1 on the surface was similar between 4 h and 24 h of stimulation (Fig. 4c, d). Purified protein A induced less TNFR1 mobilization and shedding as compared with protein A expressed by intact bacteria.

We confirmed the role of protein A in stimulating both surface expression and shedding of TNFR1 by testing the effects of the protein A–null *S. aureus* strain. This strain neither mobilized TNFR1 to the epithelial surface nor induced shedding into the extracellular media (Fig. 4c, e). Mobilization of TNFR1 to the surface (Fig. 4a, b) and its shedding (Fig. 4e, f) were observed when cells were stimulated with *S. aureus* or protein A in the presence of a neutralizing antibody to TNF- $\alpha$ , confirming that this response was not secondary to endogenous TNF- $\alpha$  production.

### Protein A induces inflammation through TNFR1

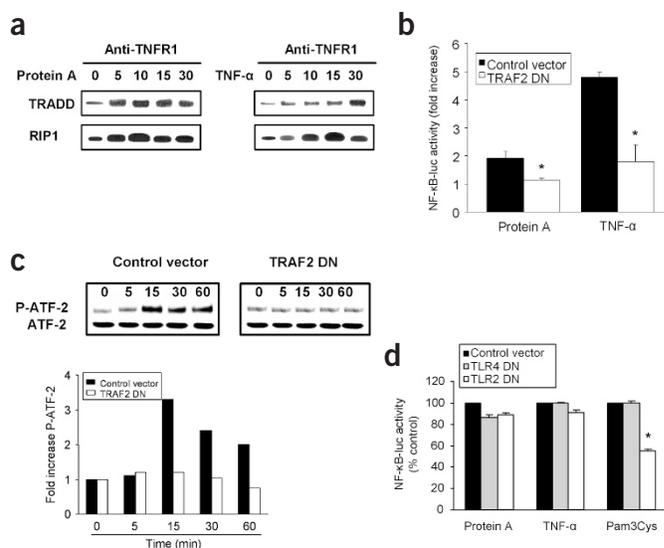
Having established that TNFR1 is the surface receptor for protein A, we predicted that protein A binding would mimic TNF- $\alpha$  activation of airway cells. Binding of TNF- $\alpha$  to TNFR1 induces receptor trimerization and recruitment of the TNFR1-associated death domain protein (TRADD), which in turn functions as a platform to recruit TNF-receptor-associated factor 2 (TRAF2) and receptor interacting protein 1 (RIP1). TRAF2 has a central role in early responses to TNF- $\alpha$

that lead to activation of I $\kappa$ B kinase and MAPK (JNK and p38) activation<sup>11,17</sup>. Protein A–induced recruitment of TRADD and RIP1 to TNFR1 within 5 min of stimulation was shown by coimmunoprecipitation (Fig. 5a). Protein A–induced NF- $\kappa$ B activity was also inhibited by 42% by expression of the TRAF2 dominant-negative mutant, as compared with control vector ( $P < 0.05$ ; Fig. 5b). Phosphorylation of ATF-2 induced by protein A was completely inhibited in cells expressing dominant-negative TRAF2 as compared with control vector (Fig. 5c), as would be expected for TNF- $\alpha$  activation.

To rule out the possibility that inflammatory responses induced by protein A were due to trace contamination of TLR2 or TLR4 agonists, NF- $\kappa$ B activity was determined in the presence of dominant-negative TLR2 and TLR4 mutants, neither of which had an inhibitory effect on protein A–induced NF- $\kappa$ B activity (Fig. 5d).

### Protein A signals through TNFR1 *in vivo*

We confirmed the importance of protein A–TNFR1 signaling in the pathogenesis of *S. aureus* pneumonia in mice. We first compared the



**Figure 5** Protein A signaling through TNFR1. (a) Anti-TNFR1 pull-down assays of lysates from cells stimulated with protein A or TNF- $\alpha$  were screened for TRADD and RIP1 by western blotting. (b) NF- $\kappa$ B activity. Data are the mean of three wells and one representative experiment of three is shown ( $*P < 0.05$ ). (c) Phosphorylation of ATF-2 after protein A stimulation was measured by western blotting. Densitometric analysis were done and standardized against actin expression. Bars show the increase in volume at the different time points. (d) NF- $\kappa$ B activity in response to protein A, TNF- $\alpha$  or Pam3Cys (positive control for TLR2DN) was determined ( $*P < 0.05$ ).

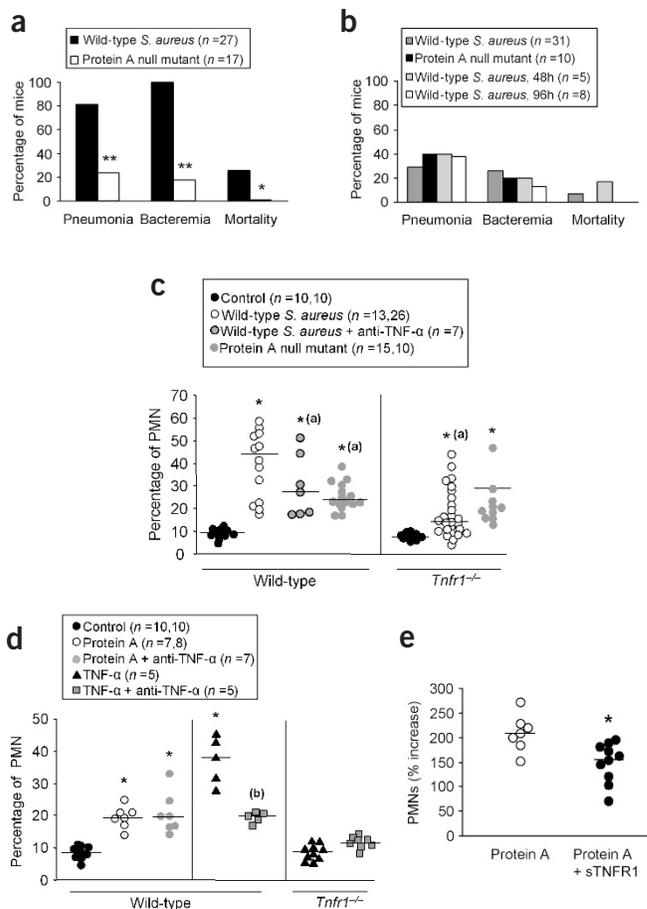
virulence of wild-type *S. aureus* and the protein A–null mutant in C57BL/6 mice. Mice infected intranasally with wild-type *S. aureus* had a significantly higher incidence of pneumonia and bacteremia ( $P < 0.001$ ) than did mice infected with the protein A mutant (Fig. 6a). Whereas 23% of the mice inoculated with wild-type *S. aureus* died, no mortality was observed among mice inoculated with the protein A–null mutant (Fig. 6a). TNFR1-null mice had significantly less pneumonia, bacteremia and mortality associated with *S. aureus* than did the wild-type mice (Fig. 6b). Although a slight increase in pneumonia and mortality was observed in these mice 48 h after infection, these differences were not statistically significant even when infection was allowed to continue for 96 h (Fig. 6b). The protein A–null mutant was attenuated similarly in TNFR1-null and wild-type mice (Fig. 6a,b). Thus, lack of either protein A or TNFR1 expression resulted in reduced bacterial virulence in a mouse model of pneumonia.

TNFR1-null mice reportedly show increased susceptibility to bacterial infection<sup>14</sup>. We postulated that a lack of TNFR1-dependent PMN recruitment may prevent morbidity caused by the pathological consequences of PMN accumulation in the airways. The inflammatory responses of wild-type and TNFR1-null mice to intranasally administered wild-type *S. aureus*, protein A–null *S. aureus* and purified protein A were compared. Wild-type mice responded to wild-type *S. aureus* (Fig. 6c) and protein A (Fig. 6d) with a significant recruitment of PMNs into lungs ( $P < 0.05$ ). Although the protein A–null mutant induced PMN mobilization as compared with control non-infected mice ( $P < 0.001$ ), the lack of protein A expression was associated with significantly diminished inflammatory responses as compared with wild-type *S. aureus* ( $P < 0.05$ ).

TNFR1-null mice did not recruit PMNs into the lungs in response to protein A (11.62% versus 9.16% in control non-infected mice,  $P > 0.5$ ; Fig. 6d). During *S. aureus* infection with either wild-type or protein A–null bacteria, TNFR1-null mice did not recruit as many PMNs as did wild-type mice ( $P < 0.001$ ; Fig. 6c). However, the number of PMNs was still significantly higher in TNFR1-null mice infected with *S. aureus* than in control non-infected mice ( $P < 0.05$ ; Fig. 6c). The percentage of PMNs detected in lungs of TNFR1-null mice infected with *S. aureus* did not change over time (median at 96 h, 15.35%). The protein A–null mutant and wild-type *S. aureus* induced the recruitment of equivalent numbers of PMNs into the lungs of TNFR1-null mice (Fig. 6c).

To exclude the possibility that TNFR1-null mice had less PMN recruitment because there were unable to respond to TNF- $\alpha$ , we carried out control experiments in which wild-type mice were treated with a neutralizing antibody to TNF- $\alpha$  before inoculation with *S. aureus* or protein A. Neutralization of TNF- $\alpha$  did not impair PMN recruitment induced by protein A (Fig. 6d), but it did decrease the numbers of PMNs recruited by intranasal administration of TNF- $\alpha$ , indicating that TNF- $\alpha$  neutralization was effective. The effect of TNF- $\alpha$  neutralization on PMN recruitment induced by *S. aureus* was also quantified (Fig. 6c). Although PMN mobilization was slightly decreased, this could be attributed to an impairment in signaling pathways not mediated by protein A.

To evaluate whether administration of sTNFR1 could block inflammatory responses induced by staphylococcal protein A, wild-type mice were inoculated intranasally with protein A in combination with sTNFR1. Administration of sTNFR1 resulted in significantly diminished PMN recruitment into the lungs as compared with protein A alone ( $P < 0.01$ ; Fig. 6e), confirming the specific interaction between protein A and TNFR1 and showing that soluble TNF receptor can neutralize inflammatory responses induced by staphylococcal protein A.



**Figure 6** Protein A induces inflammatory responses through TNFR1. (a,b) The percentage of wild-type (a) or TNFR1-null (b) mice that developed pneumonia, developed bacteremia or died is shown ( $*P < 0.05$ ,  $**P < 0.001$ ). (c,d) Short horizontal lines indicate the median values of each group ( $*P < 0.05$  versus control mice;  $^aP < 0.05$  versus wild-type mice inoculated with wild-type *S. aureus*,  $^bP < 0.05$  versus wild-type mice inoculated with TNF- $\alpha$ ). (e) The increase in the percentage of PMNs for each individual mouse is compared with the median PMN value in control mice. Short horizontal lines indicate the median values of each group ( $*P < 0.01$ ).

## DISCUSSION

By virtue of its ability to activate TNFR1 and to induce TNF- $\alpha$ -like responses, *S. aureus* protein A is a principal staphylococcal proinflammatory factor in the lung. The contribution of other *S. aureus* virulence factors has been well characterized, including the systemic responses associated with exotoxins that act as superantigens<sup>6</sup>, skin and soft tissue infections associated with the production of MSCRAMMS, and tissue destruction caused by proteases and hemolysins<sup>5,18</sup>. The role of protein A in pathogenesis is, however, less well established.

Because protein A is a major surface protein present in almost all strains of *S. aureus*, especially respiratory isolates<sup>7</sup>, we considered that it is likely to have a specific role in pulmonary infection. Other receptors for protein A, such as von Willebrand factor<sup>19</sup>, the platelet protein gC1qR/p33 (ref. 20) and the Fc fragment of IgG, have been reported but are not responsible for the main host response to the organism—that is, the accumulation of PMNs. The widespread distribution of TNFR1 and its accessibility on the surface of the respiratory epithelium make it an attractive candidate for mediating the host response

to staphylococci in the airway. Protein A-deficient mutants are less virulent in murine models of peritonitis, subcutaneous infections and arthritis<sup>21</sup>, and it seems likely that protein A-TNFR1 signaling is also induced in tissues other than the lung that express TNFR1.

An additional feature of the TNFR1 response is its 'autoregulation'. Not only does protein A or *S. aureus* induce mobilization of TNFR1 to the epithelial surface, the ligand induces active shedding of the receptor. Loss of TNFR1 from the apical plasma membrane is mediated by TACE, the TNF- $\alpha$  converting enzyme<sup>16,22,23</sup>. Shed TNFR1 neutralizes available TNF- $\alpha$ , as well as protein A, to diminish continuous TNFR1 signaling. Thus, activation of the TNFR1 pathway not only stimulates mobilization of PMNs but also provides a mechanism by which to regulate protein A-induced PMN recruitment.

Our data focus on the role of protein A-TNFR1 signaling in the airway and its relevance to the pathogenesis of staphylococcal pneumonia, an important clinical entity. Recruitment of neutrophils to the lung is crucial to eradicate respiratory pathogens; however, recruitment and activation of PMNs are not innocuous to the host. Inflammation is detrimental to the main function of the airway—that is, maintaining an open conduit for gas exchange—much more than it is to the functions of other mucosal surfaces. From our data, we conclude that protein A signaling through TNFR1 is the chief pathway through which *S. aureus* induces pneumonia. Protein A-induced mobilization of PMNs functions to clear bacteria from the airway, but at the cost of PMN-associated epithelial damage and respiratory compromise. TNF- $\alpha$  receptor antagonists, which are currently used for other clinical indications<sup>24</sup>, may have an application in modulating airway inflammation induced by *S. aureus*. This approach may be appropriate in cystic fibrosis, where staphylococcus-induced inflammation is a main cause of clinical symptoms<sup>25</sup>. This signaling pathway may provide an additional target by which to modulate the host immune response to this Gram-positive pathogen.

## METHODS

**Cell lines, bacterial strains and reagents.** Human airway epithelial cell lines, 1HAEO<sup>-</sup> and 16HBE (D. Gruenert, University of Vermont, Burlington, Vermont), were grown as described<sup>4,26</sup>. Protein A-null or protein A overexpressor mutants of *S. aureus* were constructed on strain RN6390 (ref. 9) and grown in the presence of chloramphenicol (10  $\mu$ g/ml) to an optical density of 0.5, and tetracycline (250 ng/ml) was added for 5 h to induce protein A expression. We obtained purified protein A from Sigma.

**IL-8 assays.** 1HAEO<sup>-</sup> cells, weaned from serum for 24 h, were exposed to *S. aureus* (10<sup>8</sup> colony-forming units; CFU) for 60 min or to purified protein A (200  $\mu$ g/ml) or TNF- $\alpha$  (50 ng/ml; R&D Systems) for 20 h. MAPK inhibitors SB202190 (6  $\mu$ M) and UO126 (10  $\mu$ M) (Calbiochem) or anti-TNF- $\alpha$  neutralizing antibody (10  $\mu$ g/ml; R&D Systems) were added for 60 min before and during stimulation. We measured IL-8 in the supernatant by enzyme-linked immunosorbent assay (ELISA)<sup>4</sup>.

**Western blotting.** Cells were lysed with 60 mM *n*-octyl  $\beta$ -D-glucopyranoside plus fresh protease inhibitors on ice. We used primary antibodies to phosphorylated or total p38, MEK1/2, JNK1, JNK2 or ATF-2 (Cell Signaling Technology), antibodies to TNFR1, TRADD or RIP1 (Santa Cruz Biotech) and antibody to protein A (Sigma).

**Activation of NF- $\kappa$ B.** 1HAEO<sup>-</sup> cells grown to 50–70% confluence were transiently transfected with pNF- $\kappa$ B-luciferase (0.1  $\mu$ g; Stratagene) in the presence of TRAF2 (0.08  $\mu$ g), TLR2 (0.08  $\mu$ g) or TLR4 (0.05  $\mu$ g) dominant-negative constructs or a control vector by using FuGENE 6.0 (Roche). After 16 h, cells were weaned from serum for 24 h and stimulated with protein A (200  $\mu$ g/ml), TNF- $\alpha$  (50 ng/ml) or Pam3Cys (5  $\mu$ g/ml) for 6 h. Luciferase assays were done as described<sup>4</sup>.

**Protein A binding and signaling studies.** For biotinylation, 1HAEO<sup>-</sup> cells were washed with PBS, incubated for 30 min with 1 mg/ml of EZ-Link Sulfo-NHS-LC-Biotin (Pierce) and washed to remove and to quench the biotin. For binding, cell lysates (1 mg of protein) or sTNFR1 (2 ng in 500  $\mu$ l; R&D Systems) were incubated with 50  $\mu$ l of protein A- or protein G-coated microbeads (Miltenyi Biotec) on ice for 2 h. In competition experiments, TNF- $\alpha$  (2 ng) was added overnight at 4  $^{\circ}$ C.

To isolate peritoneal macrophages, cells were obtained after peritoneal lavages with RPMI 1640 medium plus 10% fetal bovine serum, washed twice and plated on plastic to select adherent cells. For coimmunoprecipitation, lysates (1 mg) from cells stimulated with bovine serum albumin (control) or protein A (200  $\mu$ g/ml) were incubated with rabbit antibody to TNFR1 (Santa Cruz Biotech) or monoclonal antibody to protein A (Sigma) plus 50  $\mu$ l of protein G-coated magnetic beads. The  $\mu$ -MACS separation columns (Miltenyi Biotec) were washed with 20 mM Tris buffer and the eluted sample was used for western blotting.

**Confocal microscopy.** We grew polarized 16HBE cells on Transwell-Clear filters (Corning-Costar) with an air-liquid interface. Cells were fixed with 4% paraformaldehyde, blocked with 5% normal serum and stained with rabbit polyclonal antibodies to TNFR1 or TLR2 (Santa Cruz Biotech) for 1 h. Alexa Fluor 594-conjugated antibody (Molecular Probes) and Alexa Fluor 488-conjugated protein A (Molecular Probes) were added for 1 h. After being washed, filters were removed and mounted with Vectashield (Vector Laboratories) onto glass slides.

**Flow cytometry and sTNFR1 detection.** Cells were washed and stained with antibody to TNFR1 (Santa Cruz Biotech), followed by Alexa Fluor 488-conjugated antibody (Molecular Probes) and analyzed with a FACSCalibur using Cell Quest software (Becton Dickinson). We detected sTNFR1 by DuoSet ELISA (R&D Systems). For protein A binding, cells were incubated with 5  $\mu$ g of Alexa Fluor 488-conjugated protein A (Molecular Probes) in the presence or absence of non-labeled protein A or TNF- $\alpha$  in a total volume of 200  $\mu$ l.

**Immunohistochemistry.** Paraffin lung sections were deparaffinized by successive washes with xylene, 100% ethanol, 95% ethanol and 70% ethanol. Sections were stained with antibodies to phosphorylated or total p38 or JNK1/2 (Cell Signaling Technologies) followed by Alexa Fluor 594-conjugated antibody (Molecular Probes).

**Mouse model.** C57BL/6 and C57BL/6-Tnfrsf1a<sup>tm1Imx</sup> (TNFR1-null; Jackson Laboratories) mice aged 7–10 d were inoculated intranasally with 2  $\times$  10<sup>8</sup> CFU of *S. aureus* (wild-type or the protein A-null mutant) in 10  $\mu$ l of PBS<sup>27</sup> and killed 16 h later with pentobarbital. Pneumonia was defined as the recovery of more than 10<sup>2</sup> CFU per lung, and bacteremia was defined as the presence of staphylococci in the spleen. In other experiments, C57BL/6 and C57BL/6-Tnfrsf1a<sup>tm1Imx</sup> mice were inoculated with protein A (50  $\mu$ g), TNF- $\alpha$  (20 ng), PBS (control) or protein A (50  $\mu$ g) in combination with sTNFR1 (R&D Systems). Neutralizing antibody to TNF- $\alpha$  (50  $\mu$ g, clone MP6-XT3; eBioscience) was administered intraperitoneally 1 h before *S. aureus*, protein A or TNF- $\alpha$  inoculation<sup>28</sup>.

For neutrophil detection, lung cell suspensions were double stained with phycoerythrin-labeled anti-CD45 and fluorescein isothiocyanate (FITC)-labeled anti-Ly6G antibodies (PharMingen). Irrelevant, isotype-matched antibodies were used as a control. Cells were gated on the basis of their forward scatter and side scatter profile and analyzed for the expression of both CD45 and Ly6G. Mice protocol number AAAA1718 was approved by the Institutional Animal Care and Use Committee at Columbia University.

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## COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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