Efb released by intracellular *Staphylococcus aureus* suppresses host immunity by targeting TRAF3

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2 ***aureus* suppresses host immunity by targeting**
3 **TRAF3**

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ABSTRACT

Many pathogens secrete effectors to hijack intracellular signaling regulators in host immune cells to promote pathogenesis. However, the pathogenesis of *Staphylococcus aureus* (*S. aureus*) secretory effectors within host cells is unclear. Here, we report that intracellular *S. aureus* secretes fibrinogen binding protein (Efb) into the cytoplasm of macrophages to inhibit proinflammatory cytokine expression and suppress host immunity. Mechanistically, RING finger protein 114, a host E3 ligase, mediates K27-linked ubiquitination of Efb at lysine 71, which facilitates the recruitment of TNF receptor associated factor 3 (TRAF3). The binding of Efb to TRAF3 disrupts the formation of the TRAF3/TRAF2/cIAP1 complex, which mediates K48-ubiquitination of TRAF3 to promote degradation, resulting in suppression of the inflammatory signaling cascade. Additionally, the Efb K71R mutant loses the ability to inhibit inflammation and exhibits decreased pathogenicity. Therefore, our findings identify an unrecognized mechanism of *S. aureus* to suppress host defense, which may be a promising target for developing effective anti-*S. aureus* immunomodulators.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the most common bacterial strains causing hospital- and community-acquired pneumonia that is associated with significant mortality worldwide^{1,2}. It is estimated that ~85% of the human population carries or previously carried *S. aureus*³. The nasal cavity is the main site of *S. aureus* colonization in the human body⁴, providing a pathogen reservoir that significantly increases the chance of secondary *S. aureus* pulmonary infection. However, morbidity associated with *S. aureus* pneumonia is far below the bacterial carrying rate due to a powerful host immune system. Resident macrophages, as a class of important innate immune cells, are the first to encounter invading pathogens⁵. Besides killing the invading pathogens, macrophages can produce proinflammatory cytokines, chemokines, and lipid mediators that recruit other immune cells, such as neutrophils, monocytes, and dendritic cells, to orchestrate immune responses and fight infections⁶. Therefore, resident macrophages represent a critical defense line that *S. aureus* must overcome in order to propagate in the host⁷.

S. aureus secretes multiple effectors that functionally evade or inhibit host immune responses⁸. Previous pathogenic studies on *S. aureus* have mainly focused on the effect of its virulent factors on the cytomembrane and its receptors in innate immune cells. Several hemolysins, leukocidins, and phenol-soluble modulins have been identified to lyse cells by forming pores on the membranes of macrophages and other innate immune cells⁹. Other bacterial factors, including staphylococcal superantigen-like protein 3 and lipoylated E2 subunit of the pyruvate dehydrogenase complex, released from *S. aureus* can suppress macrophage activation through inhibiting Toll-like receptors activation^{10,11}. Recently, it has been demonstrated that *S. aureus* can invade and survive within macrophages and other host cells(Assis et al., 2017). Even in a hostile environment such as that in macrophages, *S. aureus* can develop specific countermeasures to evade the immune response. Numerous intracellular bacteria have been shown to weaken host immune defenses by secreting virulent factors capable of hijacking macrophage signaling pathways¹³. However, which effectors are secreted by intracellular *S. aureus* to manipulate the signaling pathways of macrophages and their underlying mechanisms remains unclear.

To inhibit the intracellular survival of invading pathogens, the inflammatory signaling pathway promotes the expression of various proinflammatory cytokines including tumor necrosis factor (TNF), interleukin 1 β (IL-1 β), IL-6, and IL-12^{14,15}. In the present study, we find that extracellular fibrinogen-binding protein (Efb) released by intracellular *S. aureus* inhibits proinflammatory cytokine expression in macrophages. Consistently, intracellular Efb also inhibits p65, p38, JNK, and ERK phosphorylation in macrophages. Previous studies suggested that extracellular Efb can block the function of C3b, inhibit the formation of platelet-leukocyte complexes, and bind fibrinogen to prevent neutrophil activation^{16,17}, but little is known about the role of intracellular Efb in modulating host signaling pathways. Therefore, we sought to investigate how intracellular Efb modulates the host signaling pathway and identify key target molecules in macrophages that are involved in this process. We demonstrate that intracellular *S. aureus* secretes Efb into the cytoplasm of macrophages to inhibit expression of proinflammatory cytokines and suppress host immunity by interacting with tumor necrosis associated factor 3 (TRAF3). Mechanistically, the interaction between Efb and TRAF3 requires K27-linked ubiquitination of Efb mediated by a host E3 ubiquitin ligase, RING finger protein 114 (RNF114).

RESULTS

Efb released by intracellular *S. aureus* inhibits host proinflammatory responses

To identify the anti-inflammatory components secreted by intracellular *S. aureus*, we tested mature chains of 78 secretory proteins of *S. aureus* on nuclear factor- κ B (NF- κ B) activation in HEK 293T cells using a dual-luciferase reporter gene assay (Extended Data Fig. 1a, b). Efb was one of the proteins shown to inhibit NF- κ B activation induced by TNF- α (Extended Data Fig. 1b). Efb was co-expressed with an NF- κ B reporting gene in HEK 293T cells to verify the inhibitory role of intracellularly expressed Efb on activation of the NF- κ B pathway (Extended Data Fig. 1c). Next, we found that adenoviral vector mediated intracellular overexpression of Efb strongly suppressed phosphorylation of p65 (Extended Data Fig. 1d) and mRNA levels of proinflammatory cytokines,

including *Tnf*, *Il1 β* , *Il6*, and *Il12p40* (Extended Data Fig. 1e), in peritoneal macrophage (PMs) stimulated with heat-killed *S. aureus*. These results suggest that intracellular Efb inhibits the transcription of proinflammatory cytokines likely through inhibiting the NF- κ B pathway.

To validate the anti-inflammatory activity of physiological Efb in an infection model, we generated an *S. aureus* Newman strain with an Efb-deletional mutant (Δ Efb) and a complementation strain (Δ Efb+Flag-Efb). As shown in Extended Data Fig. 2a, western blot analysis with anti-Efb or anti-Flag antibodies and silver staining of polyacrylamide gel electrophoresis (PAGE) revealed Efb expression in the culture supernatant of the corresponding strains. Consistent with a previous report¹⁸, the absence of Efb did not affect *S. aureus* growth (Extended Data Fig. 2b).

Next, we found that macrophages infected with the Δ Efb strain significantly increased the mRNA levels of *Tnf*, *Il1 β* , *Il6*, and *Il12p40* in infected macrophages, while the Δ Efb+Flag-Efb strain restored the inhibitory effect of Efb (Fig. 1a, Extended Data Fig. 3a). However, the inhibitory effect of Efb on the expression of inflammatory cytokines was lost after stimulation with only the culture supernatants of *S. aureus* (Extended Data Fig. 3b) or non-contacting co-culture of macrophages and *S. aureus* in Transwells (Extended Data Fig. 3c). These data suggest that Efb released by intracellular *S. aureus*, not Efb released by extracellular *S. aureus*, exerts anti-inflammatory functions. In the control experiments, we found no difference in intracellular *S. aureus* loading among macrophages infected with Newman, Δ Efb, or Δ Efb+Flag-Efb strains for 6 h (Extended Data Fig. 3d, left, middle; GFP did not affect the expression of Efb in *S. aureus*, Extended Data Fig. 3d, right), indicating that the above observed differences in mRNA levels of inflammatory cytokines are Efb dependent. Compared to the Newman strain, the Δ Efb strain also exhibited accelerated phosphorylation of p65, p38, JNK, and ERK in macrophages (Extended Data Fig. 4c, d), suggesting that Efb may also inhibit the MAPK pathway in addition to NF- κ B pathway. These results provide further evidence that physiological Efb released by intracellular *S. aureus* suppresses *S. aureus*-triggered inflammatory responses in macrophages.

To investigate the role of Efb *in vivo*, we established a trachea cannula infection model by

challenging 6-week-old C57BL/6 mice with Newman, Δ Efb, or Δ Efb+Flag-Efb strains. At 24 h post infection, there was increased expression of TNF- α , IL-1 β , IL-6, and IL-12 in the lungs of mice infected with the Δ Efb strain compared to those infected with the Newman and Δ Efb+Flag-Efb strains (Fig. 1b). In agreement, the bacterial burden in the lungs of Newman and Δ Efb+Flag-Efb infected mice was significantly higher than that of Δ Efb infected mice (Fig. 1c). There were also more intact alveolar spaces, along with less infiltration of neutrophils and lymphocytes, in the lungs of Δ Efb infected mice (Fig. 1d, e). We also revealed that Δ Efb directly led to an increase in survival rates in lethal pneumonia (Extended Data Fig. 5a) and bacteremia (Extended Data Fig. 5b) models, as well as alleviated festering areas in the skin infection model (Extended Data Fig. 5c), compared to Newman and Δ Efb+Flag-Efb infected mice. Together, these results suggest that Efb released by intracellular *S. aureus* may act as an inhibitor of host inflammatory responses, making Efb an important factor for *S. aureus* infection.

Efb interacts with TRAF3

Immunofluorescence confocal microscopy showed that Efb was secreted by intracellular *S. aureus* into the cytoplasm of the macrophages and gradually accumulated over time during infection (Extended Data Fig. 6a). TLR signaling, especially TLR2, plays an important role in detecting *S. aureus* and inducing the expression of proinflammatory cytokines during infection^{10,19}. Therefore, using a co-immunoprecipitation assay, we screened Efb-interactive proteins from a list of key signaling molecules in the TLR pathway. TRAF3 appeared to be the only Efb associated protein (Extended Data Fig. 6a and Fig. 2a, b). Further experiments in Δ Efb+Flag-Efb infected macrophages demonstrated that Efb released by intracellular *S. aureus* interacted physiologically with TRAF3 (Fig. 2c, d and Extended Data Fig. 6c). The *in vitro* GST-pull down assay also demonstrated a direct interaction between purified Efb and TRAF3 (Extended Data Fig. 6d, e). In addition, the zinc finger domain of TRAF3 was responsible for the interaction (Figure 2e, f).

Efb inhibits host proinflammatory responses via stabilizing TRAF3

Next, we examined whether TRAF3 mediated the inhibition effects of Efb on host proinflammatory responses. We generated conditional knockout mice for TRAF3 (genotype: TRAF3^[flox/flox, Lyz2-Cre]) using CRISPR/Cas9-mediated genome editing and isolating the PMs from these mice. TRAF3 deficiency effectively abrogated Efb's inhibitory effects on the mRNA levels of *Tnf*, *Il1β*, *Il6*, and *Il12p40* in PMs (Fig. 3a). Furthermore, Efb's inhibitory effects were lost in lungs of TRAF3^[flox/flox, Lyz2-Cre] mice, but not in TRAF3^[flox/flox] mice (Fig. 3b). Consistent with this result, there was no significant difference in the pulmonary bacteria burdens and histopathology between TRAF3^[flox/flox, Lyz2-Cre] mice infected with the Newman and ΔEfb strains (Fig. 3c, d, e). These *in vitro* and *in vivo* results collectively indicate that Efb inhibits host inflammatory responses via TRAF3.

Previous studies demonstrated that K48-ubiquitination-induced degradation of TRAF3 is essential for activation of the NF-κB and MAPK pathways²⁰⁻²². Here we found that, compared to the Newman strain, the ΔEfb strain markedly reduced the amounts of TRAF3 in macrophages, suggesting that Efb may stabilize TRAF3 (Fig. 4a and Extended Data Fig. 7a). Consistently, both overexpressed and physiological Efb significantly reduced the K48-linked ubiquitination of TRAF3 in HEK293T cells and macrophages (Figure 4b, c, Extended Data Fig. 7b). The degradative ubiquitination of TRAF3 is typically mediated by cIAP1/2, and the modification effects require TRAF2 to act as a bridge between TRAF3 and cIAP1/2^{24,25}. We found that TRAF2 mediated K48-linked ubiquitination of TRAF3 in macrophages infected by *S. aureus* (Extended Data Fig. 7c), and Efb could prevent TRAF3 from interacting with TRAF2 and cIAP1 (Fig. 4d, e, Extended Data Fig. 7d). We further demonstrated that the zinc finger domain of TRAF3 was also responsible for its binding to TRAF2 (Extended Data Fig. 7e), suggesting that Efb could competitively bind TRAF3 and affect TRAF2 binding to TRAF3. Lysines 106 and 155 of TRAF3 were reported as the main K48-linked ubiquitination sites²³, and our results showed that Efb mainly inhibited the K48-linked ubiquitination of lysine 155 in the zinc finger domain of TRAF3 (Fig. 4f and Extended Data Fig. 7f). Taken together, these results suggest that Efb disturbs the TRAF3/TRAF2/cIAP1 complex, inhibits the K48-linked ubiquitination of TRAF3 at lysine 155, and prevents TRAF3 degradation in macrophages during *S. aureus* infections.

RNF114 mediated K27-linked ubiquitination of Efb

Several studies have suggested that bacteria can utilize the post-translational modification system of the host to modify their effectors for pathogenesis^{26,27}. Given that Efb is small in size (mature protein, 15kD) and contains 20 lysine residues that are common sites for ubiquitination, we investigated the ubiquitination of intracellular Efb. Plasmids expressing different ubiquitins (Ub, K6, K11, K27, K29, K33, K48 and K63) were co-transfected with Efb into HEK 293T cells. Co-immunoprecipitation results revealed multiple types of ubiquitination of Efb, with K27-linked polyubiquitination being most dominant (Extended Data Fig. 8a). Furthermore, we detected the endogenous K27-linked polyubiquitin conjugates of intracellular Efb in macrophages infected with the ΔEfb+Flag-Efb strain (Fig. 5a, Extended Data Fig. 8b), indicating that Efb is polyubiquitinated by K27 in host cells. We also carried out proteomics analysis on immunoprecipitated overexpressed Efb in HEK 293T cells using tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo) coupled online to UPLC (Supplementary Table 3). We identified a protein named RNF114 as an E3 ligase with K27-linked polyubiquitination activity²⁸. Further overexpression co-immunoprecipitation (Fig. 5b, c) and endogenous co-immunoprecipitation (Fig. 5d, Extended Data Fig. 8c) consistently demonstrated reproducible Efb interaction with RNF114. Overexpression of RNF114 also increased the K27-linked (but not other types), polyubiquitination of Efb in HEK 293T cells (Fig. 5e, Extended Data Fig. 9), and RNF114 KD by specific siRNA reduced the K27-linked polyubiquitination of Efb in macrophages (Fig. 5f). These results collectively suggest that RNF114 is an important ubiquitinase that mediates K27-linked polyubiquitination of Efb.

To determine which lysine residue of Efb was modified by K27-linked polyubiquitination, we constructed 20 Efb mutants by replacing lysine with arginine and co-transfected these mutants with a plasmid expressing K27 ubiquitin in HEK 293T cells. The immunoprecipitation results revealed that the K71R mutant significantly reduced in the K27-linked polyubiquitination of Efb (Extended Data Fig. 10a). Consistently, RNF114 did not promote the K27-linked ubiquitination of K71R mutant of Efb in HEK 293T cells (Fig. 5g). Next, we replaced all other Efb lysines with arginines, except lysine 71, and RNF114 still maintained the K27-linked polyubiquitination of Efb at lysine

71 (Extended Data Fig. 10b, c). Moreover, physiological K27-linked ubiquitination of the K71R mutant of Efb disappeared in MH-S (Fig. 5h). These results suggest that RNF114 can mediate K27-linked polyubiquitination of Efb at lysine 71.

Ubiquitination of Efb facilitates the stabilization of TRAF3

To investigate whether RNF114 is involved in suppressing TRAF3 signaling by Efb, we observed the enhancing effect of RNF114 on Efb inhibiting K48-linked ubiquitination of TRAF3 (Extended Data Fig. 11a). When compared to wild type Efb, the K71R mutant appeared to reverse the inhibition of Efb on the K48-linked ubiquitination of TRAF3 (Extended Data Fig. 11b). The results obtained from Δ Efb+Flag-Efb and Δ Efb+Flag-Efb K71R infected macrophages (Extended Data Fig. 11c, d, e) showed that the K71R-Efb mutant reduced the inhibitory effects on TRAF2 and cIAP1 mediated K48-linked polyubiquitination of TRAF3 at physiological levels. Furthermore, we found that Δ Efb infection in RNF114 KD macrophages resulted in an even lower level of proinflammatory cytokine expression compared to Newman infection (Extended Data Fig. 11f), suggesting that RNF114 is involved in the regulation of inflammatory cytokine production during *S. aureus* infection in macrophages independent of Efb. Together, these results suggest that RNF114 promotes K27-linked ubiquitination of Efb to facilitate TRAF3 stabilization.

Ubiquitination of Efb inhibits host pro-inflammatory responses

Next, the reconstitution experiments using Δ Efb infected macrophages demonstrated that wild-type Efb, but not the K71R-mutant strain, was able to restore inhibition of the infection-induced proinflammatory cytokine production (Fig. 6a, Extended Data Fig. 11c). To investigate the function of the K71R mutant on the innate immune response *in vivo*, we infected 6-week-old mice with the Newman, Δ Efb, Δ Efb+Flag-Efb, or Δ Efb+Flag-Efb K71R strains and evaluated the expression levels of proinflammatory cytokines in the lungs. Compared with Δ Efb infection, Δ Efb+Flag-Efb, but not Δ Efb+Flag-Efb (K71R), significantly suppressed proinflammatory cytokine production in the affected lungs (Fig. 6b). The bacterial load of Δ Efb and Δ Efb+Flag-Efb (K71R) strains in the

lungs was significantly lower than that of the Newman and Δ Efb+Flag-Efb strains (Fig. 6c). Consistently, histopathology results revealed more severe lung damage and more neutrophil and lymphocyte infiltration in the lungs of Δ Efb mice reconstituted with wild-type but not the K71R mutant Efb strains (Fig. 6d, e). These results suggest that the pathogenesis of *S. aureus* might rely on the K27-linked ubiquitination of Efb at its lysine 71 residue.

DISCUSSION

As a reservoir of *S. aureus*, macrophages play an important role in regulating the inflammatory responses that are critical for eliminating invading pathogens^{6,12}. To achieve a successful infection, *S. aureus* must overcome activation of the inflammatory pathways of macrophages. Secreting multiple effectors is a unique strategy that *S. aureus* has adopted to cope with environmental challenges⁹. However, whether and how the secreted effectors of intracellular *S. aureus* interfere with the inflammatory pathways of macrophages remains unclear. In the present study, we screened 78 secreted effectors on activation of the NF- κ B pathway. The results showed that more than half of the secretory proteins decreased NF- κ B activation. We conducted several bioinformatics and literature searches on these proteins, especially α -hemolysin (WP-000857483.1), serine protease SplE (WP-001038759.1), and cysteine protease staphopain A (WP-000827748.1). We found that the inhibitory effects of these three proteins were false positives due to the fact that overexpressing these proteins in HEK 293T cells leads to cell deformation or death (Extended Data Fig. 1f). Moreover, some of these proteins have effects when overexpressed in HEK 293T cells. However, when we constructed knockout strains to infect macrophages, there was no statistical difference between wild-type and the corresponding knockouts. Efb was the first protein we discovered that could inhibit both NF- κ B and MAPK, as well as inhibit *S. aureus*-induced expression of proinflammatory cytokines, including TNF α , IL-1 β , IL-6, and IL-12, *in vitro* and *in vivo*, respectively. In the early stages of infection, increased levels of TNF α and IL-1 β are critical for eliminating bacterium in human *S. aureus* bacteremia²⁹. IL-1 β has also been shown to be a key cytokine for the eradication of *S. aureus* in experimental models³⁰. Our results indicate that decreased bacterial burdens in lung tissues infected with Δ Efb could be attributed to elevated TNF-

α and IL-1 β levels. Thus, inhibiting host proinflammatory responses may be one way by which Efb contributes to the pathogenicity of *S. aureus*.

Although the post-translational modification system in bacteria is very simple in comparison to eukaryotes³¹, accumulating studies have shown that some bacteria effectors can be modified by the host post-translational modification system²⁶. Under certain conditions, some bacteria effectors can disturb host post-translational modification(Ding et al., 2019) to facilitate infection. Our present study found that a host E3 ligase, RNF114, ubiquitinates Efb of *S. aureus* to suppress host immunity. K27-linked ubiquitination serves a variety of functions, including enhancing protein–protein interactions, promoting proteasomal degradation, and providing binding platforms for DNA repair proteins³³. RNF114 has been reported as a host E3 ligase with K27-linked polyubiquitination activity with degradation effects in porcine and sea perch^{34,35}. However, our results showed that K27-linked ubiquitination of Efb by RNF114 did not lead to degradation of Efb but promoted the interaction between Efb and TRAF3.

Efb has been identified as an immune evasion effector, and its complement inhibitory effect depends on its C-terminal¹⁶. However, our results demonstrate that replacing lysine 71 with arginine in the N-terminal of Efb can eliminate its inhibitory effects *in vivo* and *in vitro*, suggesting that the inhibitory effects of Efb are independent of its complement effects *in vivo*. On the other hand, the immunosuppressive abilities of Efb were shown to be correlated with inhibiting the formation of platelet-leukocyte complexes and binding fibrinogen to prevent neutrophil activation, which depends on the N-terminal of Efb¹⁷. However, lysine 71 of Efb was not the key residue in the interaction between Efb and fibrinogen according to a previous report³⁶. Platelets have been reported to play an important role in inflammation³⁷. However, there is no detailed information as to which amino acid of Efb plays a key role in the interaction of Efb's N-terminal and platelets. Our results demonstrate that the direct inhibitory effect of Efb on proinflammatory cytokines was accomplished through interaction with TRAF3 in myeloid cells, including mature macrophages, monocytes, and granulocytes. However, it is still possible that our *in vivo* results were partly due to the interaction between Efb and platelets or other unknown mechanisms.

TRAF3 is a tri-faced immune regulator that has distinct roles depending on its targeted receptors, even within the same cell, and is also highly cell-type-dependent³⁸. TRAF3 has been reported to positively regulate type I interferon production while negatively regulating NF- κ B and MAPK pathways²⁰. Our results demonstrate that TRAF3 regulates *S. aureus*-induced activation of NF- κ B and MAPK pathway in macrophages. *S. aureus* infection induces the degradation of TRAF3 in macrophages. However, Efb can inhibit degradation by disturbing the K48-linked ubiquitination of TRAF3 in HEK 293T cells, PMs, and alveolar macrophages (MH-S). K48-linked ubiquitination of TRAF3 for degradation is accomplished by cIAP1/2, which requires the bridging effects of TRAF2. Our data indicate that the interaction between TRAF3 and TRAF2 was disturbed by Efb, and the inhibition on K48-linked ubiquitination of TRAF3 by Efb was accomplished by disrupting the TRAF3-TRAF2-cIAP1/2 complex.

The emergence of multi-drug resistant strains and the lack of a vaccines has made *S. aureus* a global concern. Immune evasion has been proven to be the main pathogenesises for *S. aureus* infection⁹, and is also one of the main challenges for vaccine development³⁹. We identified that K27-linked ubiquitination of Efb by RNF114 binding to TRAF3 disrupts the formation of the TRAF3/TRAF2/cIAP1 complex and prevents K48-ubiquitination mediated TRAF3 degradation, resulting in suppressed proinflammatory cytokine production *in vivo* and *in vitro*. The present findings identify a previously unrecognized mechanism that *S. aureus* uses to suppress host immunity (Extended Data Fig. 12). In addition, the Efb-RNF114 interface may be a promising target for the development of effective anti-*S. aureus* treatments.

Methods

Bacterial strains and cells

Bacterial strains adopted in the present study are described in Supplementary Table 1. The *E. coli*

strains DH5 α and BL21 (Tiangen Biotech, China) were grown in LB medium. When required, the antibiotics ampicillin (100 μ g/ml) (Sanggon Biotech) or kanamycin (50 μ g/ml) (Sanggon Biotech) were used for the *E. coli* strain selection. The *S. aureus* strains were grown in trypticase soy broth (OXOID, CM0129B). Chloramphenicol (25 μ g/ml) (Sanggon Biotech) was used for the selection of *S. aureus* strains when required. Unless noted, all bacteria were grown at 37°C in a shaking incubator at 200 rpm in tubes kept at a 45° angle.

HEK 293T cells (CRL-3216), obtained from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone) supplemented with 10% (v/v) fetal bovine serum (FBS, HyClone) and 100 U/ml penicillin and streptomycin (HyClone). MH-S cells (CRL-2019), obtained from the American Type Culture Collection, were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone) supplemented with 10% FBS. PMs were obtained from 6-week-old wild type or mutant C57BL/6J mice as follows: Mutant mice and their wild-type littermates were injected with 2 ml Thioglycolate Broth (4%) intraperitoneally. Three days later, the peritoneal lavage fluid was collected from the mice and washed with PBS three times. PMs were grown in DMEM supplemented with 10% FBS.

Plasmids, reagents and antibodies

Expressing plasmids were constructed by inserting a synthetic gene segment in the vector, the names of which are listed in Supplementary Table 1. The following antibodies were used for western blot, immunoprecipitation, or immunofluorescence assays: anti-TRAF3 (PA5-20165, Invitrogen; ab36988, Abcam; sc6933, Santa Cruz), anti-TRAF2 (4724, Cell signaling technology, CST), anti-Flag (F1804, Sigma-Aldrich), anti-HA (3724, CST), anti-Myc (2040, CST), anti-phospho-p65 (3033, CST), anti-phospho-p38 (9215, CST), anti-phospho-Erk1/2 (9101, CST), anti-phospho-JNK (4668, CST), anti-GFP (2956, CST), anti-K27 (ab181537, Abcam), anti-K48 (8081, CST), anti-cIAP1 (ab2399, Abcam), anti-RNF114 (ab97303, Abcam), Alexa Fluor Plus 488 conjugated anti-mouse IgG (A32723, Invitrogen), Alexa Fluor Plus 555 conjugated anti-mouse IgG (A32727, Invitrogen), Alexa Fluor Plus 647 conjugated anti-rabbit IgG (A32733, Invitrogen); Anti-Efb was

generated by immunization of rabbits with the protein of Efb, anti-GST (CW0085M, Cwbio), anti-His (CW0083M Cwbio); anti-GAPDH (G9545, Sigma-Aldrich), anti-Flag M2 Affinity Gel (A2220, Sigma-Aldrich), anti-HA Magnetic Beads (88836, Thermo Fisher), anti-TRAF3 agarose beads (sc-6933 AC, Santa Cruz), and anti-rabbit IgG (5127, CST), anti-mouse IgG (96714, CST). For western blot assays, all primary antibodies were diluted at 1:1,000 and secondary antibodies were diluted at 1:5,000. For immunofluorescence, monoclonal mouse anti-Flag M2 antibody was used at 1:500 dilution and rabbit anti-TRAF3 at 1:200 dilution. Corresponding Alexa Fluor Plus 488 labelled anti-mouse IgG, Alexa Fluor Plus 555 labelled anti-mouse IgG or Alexa Fluor Plus 647 labelled anti-rabbit IgG were used as at 1:200 dilution. DAPI Stain Solution for nuclear strain was from Sangon Biotech (E607303).

Construction of *S. aureus* strains

The pBT2 vector (provided by X. Rao, Army Medical University, Chongqing) was used to generate the *S. aureus* Newman strain with a deletion of the gene encoding Efb (Δ Efb) using an allelic replacement strategy as previously described⁴⁰. The primers for the construction of the Efb knockout vector are listed in Supplementary Table 2. The deletion of Efb was confirmed by PCR and Sanger sequencing. The pLI50 vector (provided by X. Rao, Army Medical University, Chongqing) was used to complement the Δ Efb strain with the wild-type Efb or Efb K71R gene driven by the original promoter of Efb. The expression of Efb or its mutants in the supernatant of *S. aureus* was examined by immunoblot assays.

Transfection and confocal microscopy

HEK 293T cells were transiently transfected using PEI (23966-2; Polysciences) according to instructions of the manufacturer. PMs were transduced or transfected with adenovirus (Hanbio) or jetMSENSENGER (150-07, Polyplus). MH-S cells were transiently transfected using the INVI DNA RNA Transfection Reagent (IV1216100, Invigentech). Confocal microscopy was performed as described previously⁴¹.

Luciferase assay

HEK 293T cells were transiently transfected with pNF- κ B-luc, pRL-TK and the indicated plasmids for 24 h. After TNF- α (210-TA, R&D) stimulation, the dual-luciferase reporter assay system (RG028, Beyotime) was used to detect luciferase activity according to the instruction of the manufacturer.

Immunoprecipitation and immunoblot assays

Immunoprecipitation and immunoblot assays were performed as previously described. Briefly, HEK 293T cells were transiently transfected with plasmids using PEI. After 48 h, culture supernatants were removed at corresponding times, and the cells were washed three times with PBS. Cells were lysed in cell lysis buffer (Beyotime) supplemented with 1% protease inhibitor cocktail (4693116001, Roche). After centrifugation, the supernatants of cell lysates were incubated with indicated gels at 4°C overnight. For endogenous immunoprecipitations, PMs and MH-S were infected with *S. aureus* for the indicated times. The cell lysates were subsequently incubated with indicated gels at 4°C overnight. After being centrifuged, the gels were then washed three times with cell lysis buffer and boiled with 1× SDS loading buffer. Equivalent amounts of total proteins were separated by SDS-PAGE and electro-blotted onto PVDF membranes. The membranes were then probed with antibodies, followed by exposure conducted using an ECL reagent (32209 or 34095, Thermo Fisher).

Real-time PCR analysis

RNA preparation and qPCR analysis were performed as described previously (Yan et al., 2012b) using gene-specific primers (Supplementary Table 2).

Mice infection model

Female specific-pathogen free C57BL/6 mice were purchased from Beijing HFK Bioscience CO.,

LTD. Traf3^[flox/flox] and Traf3^[flox/flox, Lys2-Cre] mice were purchased from Cyagen Biosciences. All animal experiments were reviewed and approved by the Animal Experiment Administration Committee of Army Medical University and were conducted in accordance with governmental guidelines and institutional policies for the Care and Use of Laboratory Animals. Six-week-old C57BL/6 mice were divided randomly into cages and infected by intratracheal administration with 2×10^8 CFUs or 6×10^8 CFUs of different *S. aureus* strains in 20 μ l PBS for 24 h or a few days. After the mice were sacrificed at the indicated times, the lungs were collected and homogenized in 1 ml of PBS for ELISA and CFU assays. *S. aureus* burden was determined by plating serial dilutions of each tissue homogenate on TSA plates, which were incubated at 37°C. Colonies were counted after 12 h of incubation. For histology, lungs were removed and fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (H&E) according to standard protocols. Imaging was performed using microscopy (ECLIPSE 80i, Nikon). For skin and blood infection model, the doses of *S. aureus* were 2×10^8 and 1×10^8 CFUs. All mice were age- and sex-matched in each experiment. The sample size was determined based on data from pilot experiments. For *in vitro* *S. aureus* infections, macrophages were infected with a single-cell suspension of bacteria at an moieties of infection (MOI) of 25.

Competing interests

The authors declare no competing interests.

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Fig. 1 Efb inhibits host proinflammatory responses. **a**, Quantitative polymerase chain reaction (qPCR) analysis of *Tnf*, *Il1b*, *Il6*, and *Il12p40* mRNA from alveolar macrophage (MH-S) infected with Newman, Δ Efb, or Δ Efb+Flag-Efb for indicated times (MOI = 25). **b**, ELISA quantification of $\text{TNF-}\alpha$, $\text{IL-1}\beta$, IL-6 , and IL-12 levels in lung tissue homogenized in 1 ml PBS 24 h after infection with Newman, Δ Efb, or Δ Efb+Flag-Efb. C57BL/6 mice were infected by intratracheal administration with the test strains (2×10^8 colony-forming units (CFUs) per mouse) for the indicated times. **c**, Quantification of the bacterial CFU of lung tissue homogenates obtained in **b**. **d**, **e**, Histopathology of lung tissues was assessed in sections stained with H&E from mice infected for 24 h; scale bars, 1,000 μm (top) and 200 μm (bottom), the boxed areas at the top are enlarged below. Student's two-tailed unpaired t-test (**a**, **b**, **d**) or Mann-Whitney U test (**c**) were used for statistical analysis, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Data are representative of three experiments with at least three independent biological replicates. The bars show the mean and s.d. of $n = 3$ (**a**), $n = 9$ (**b**, **c**) or $n = 5$ (**d**) per group.

Fig 2 Efb interacts with TRAF3. **a, b**, Immunoblots of whole cell lysates (WCL) and immunoprecipitation (IP) products of WCL from HEK 293T cells transfected with indicated plasmids. **c**, Immunoblots of WCL and IP products of WCL from MH-S infected with ΔEfb + Flag-Efb for indicated times (MOI = 2.5). **d**, Immunofluorescence assay of MH-S infected with ΔEfb + Flag-Efb for 4 h (MOI = 2.5), Scale bars, 2 μm. **e**, Truncation of TRAF3. **f**, Immunoblots of WCL and IP products of WCL from HEK 293T cells transfected with indicated plasmids; WT, full-length TRAF3. Data are representative of three experiments with at least three independent biological replicates.

Fig. 3 Efb inhibits host proinflammatory responses via TRAF3. **a**, qPCR analysis of *Tnf*, *Il1b*, *Il6*, and *Il12p40* mRNA of PMs infected with Newman, ΔEfb (MOI = 25) for 4 h; wildtype PMs (WT) were isolated from TRAF3^[flox/flox] mice; TRAF3 knockout (KO) PMs were isolated from TRAF3^[flox/flox, Lyz2-Cre] mice. **b**, ELISA quantification of TNF-α, IL-1β, IL-6, and IL-12 levels in lung tissues from TRAF3^[flox/flox] (Flox) or TRAF3^[flox/flox, Lyz2-Cre] (TRAF3 CKO) mice, homogenized in 1 ml PBS and infected with Newman, ΔEfb for 24 h (2×10^8 CFUs per mouse). **c**, Quantification of the bacterial colony-forming unit (CFU) of lung tissue homogenates obtained in **b**. **d**, **e**, Histopathology of lung tissues was assessed in sections stained with H&E from mice infected for 24 h; scale bars, 1,000 μm (top) and 200 μm (bottom), the boxed areas at the top are enlarged below. Student's two-tailed unpaired t-test (a, b, d) or Mann-Whitney U test (c) were used for statistical analysis, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Data are representative of three

experiments with at least three independent biological replicates. The bars show the mean and s.d.
of n = 3 (a), n = 9 (b, d) or n = 5 (e) mice per group.

Fig. 4 Efb stabilizes TRAF3. **a**, Immunoblots of whole cell lysates (WCL) from MH-S infected with Newman and ΔEfb (MOI=25). **b**, Immunoblots of WCL and immunoprecipitation (IP) products of WCL from HEK 293T cells transfected with indicated plasmids. Ub, wild-type ubiquitin; K48, Ub with a single 48 lysine residue left; K63 Ub with a single 63 lysine residue left. **c**, Immunoblots of IP products of WCL from MH-S infected with Newman and ΔEfb (MOI=25). **d**, Immunoblots of WCL and IP products from HEK 293T cells transfected with indicated plasmids. **e**, Immunoblots of IP products from MH-S infected with Newman and ΔEfb (MOI=25). **f**, Immunoblots of WCL and IP products from HEK 293T cells transfected with indicated plasmids. K/R, replace all lysine of TRAF3 with arginine; R106K, replace 106 arginine of K/R with lysine; R155K, replace 155 arginine of K/R with lysine. R106/155K, replace 106 and 155 arginine of K/R with lysine. Data are representative of three experiments with at least three independent biological

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Fig. 5 Efb is K27-ubiquitinated at K71 by host RNF114. **a**, Immunoblots of immunoprecipitation (IP) products of whole cell lysates (WCL) from MH-S infected with ΔEfb+Flag-Efb for indicated times (MOI = 2.5). **b**, **c**, Immunoblots of WCL and IP products from WCL of HEK 293T cells transfected with indicated plasmids. **d**, Immunoblots of IP products of WCL from MH-S infected with ΔEfb+Flag-Efb for indicated times (MOI. = 25). **e**, Immunoblots of WCL and IP products from HEK 293T cells transfected with indicated plasmids. **f**, Immunoblots of WCL and IP products from MH-S or RNF114 knockdown (KD) MH-S infected with ΔEfb + Flag-Efb for indicated times (MOI = 25). **g**, Immunoblots of WCL and IP products from HEK 293T cells transfected with indicated plasmids. K71R, replace 71 lysine of Efb with arginine. **h**, Immunoblots of WCL and IP

products from MH-S infected with Δ Efb + Flag-Efb or Δ Efb + Flag-Efb K71R for indicated times (MOI = 2.5). Data are representative of three experiments with at least three independent biological replicates.

Figure 6. Efb inhibits host immunity depending on K27-ubiquitination. **a**, qPCR analysis of *Tnf*, *Il1b*, *Il6*, and *Il12p40* mRNA from MH-S infected with Newman, Δ Efb, Δ Efb+Flag-Efb or Δ Efb+Flag-Efb K71R for indicated times (MOI = 2.5). **b**, ELISA quantification of TNF- α , IL-1 β , IL-6, and IL-12 levels in lung tissues homogenized in 1 ml PBS, infected with Newman, Δ Efb, Δ Efb+Flag-Efb or Δ Efb+Flag-Efb K71R (2×10^8 CFUs per mouse) for 24 h. **c**, Quantification of the bacterial colony-forming unit (CFU) in lung tissue homogenates obtained in **b**. **d**, **e** Histopathology of lung tissues was assessed in sections stained with H&E from mice infected for 24 h; scale bars, 1,000 μ m (top) and 200 μ m (bottom), the boxed areas at the top are enlarged below. Student's two-

tailed unpaired t-test (a, b) or Mann-Whitney U test (c) were used for statistical analysis, * $P < 0.05$,
** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Data are representative of three experiments with at
least three independent biological replicates. The bars show the mean and s.d. of $n = 3$ (a), $n = 9$ (b,
c) or $n = 5$ (d) per group.

Extended Data Fig. 1 Efb inhibits NF-κB activation. **a**, Vector construction methods. **b**, Luciferase assay of HEK 293T cells transfected using NF-κB luciferase reporters with 78 *S. aureus* secreted effector-encoding plasmids for 24 h. **c**, Luciferase assay and related immunoblot of HEK293T cells transfected using NF-κB luciferase reporters encoding plasmids with or without Efb-encoding plasmids for 24 h. **d**, Immunoblot of whole cell lysates (WCL) of PMs transduced

using control adenovirus (ADV) or Efb-encoding ADV for 48h, and then stimulated by hot killed *S.*
aureus (HS, MOI = 25) for 6 h. **e**, qPCR analysis of *Tnf*, *Il1b*, *Il6*, and *Il12p40* mRNA from PMs
transduced with control ADV or Efb-encoding ADV for 48 h and then stimulated with heat killed *S.*
aureus (HS, MOI = 25) for 6 h. **f**, Cell morphology of HEK 293T cells. Student's two-tailed unpaired
t-test (b, c, e) were used for statistical analysis, *P < 0.05, **P < 0.01, ***P < 0.001, and
****P < 0.0001. Data are representative of three experiments with at least three independent
biological replicates. The bars show the mean and s.d. of n = 3 (b, c, e).

Extended Data Fig. 2 Validation of Efb mutant strains. **a**, Immunoblot and silver staining of culture supernatant of Newman, Δ Efb, and Δ Efb+Flag-Efb for 12 h. **b**, Growth curve of Newman, Δ Efb, and Δ Efb+Flag-Efb. Data are representative of three experiments with at least three independent biological replicates.

Extended Data Fig. 3 Efb inhibits host proinflammatory responses. **a**, qPCR analysis of *Tnf*, *Il1b*, *Il6*, and *Il12p40* mRNA from PMs infected by Newman, ΔEfb, or ΔEfb+Flag-Efb for indicated times (MOI = 25). **b**, qPCR analysis of *Tnf*, *Il1b*, *Il6*, and *Il12p40* mRNA from PMs stimulated by culture supernatant of Newman, ΔEfb, or ΔEfb+Flag-Efb. **c** qPCR analysis of *Tnf*, *Il1b*, *Il6*, and *Il12p40* mRNA from PMs infected by Newman, ΔEfb, or ΔEfb+Flag-Efb for indicated times using non-contact co-culture methods (Transwell 0.4 μm, MOI= 25). **d**, Intracellular survival of Newman, ΔEfb, or ΔEfb+Flag-Efb (scale bars, 100 μm, left, middle). Immunoblots of culture supernatants of Newman, ΔEfb, or ΔEfb+Flag-Efb expressed GFP for 12 h (right). Student's two-tailed unpaired t-test (d, e, f, g) were used for statistical analysis, *P < 0.05, **P < 0.01, ***P < 0.001, and

****P < 0.0001. Data are representative of three experiments with at least three independent biological replicates. The bars show the mean and s.d. of n = 3 (d, e, f, g).

Extended Data Fig. 4 Efb inhibits proinflammatory signaling pathways of macrophages. a, b
 Immunoblot of MH-S, PMs infected by Newman or ΔEfb for indicated times (MOI = 25). Data are
 representative of three experiments with at least three independent biological replicates.

Extended Data Fig. 5 Efb is an important effector for *S. aureus* infection. **a**, Survival rates of 6-week-old mice infected by intratracheal administration with roughly 6×10^8 CFUs per mouse of Newman, Δ Efb, or Δ Efb+Flag-Efb. **b**, Survival of 6-week-old mice infected by tail vein injection with roughly 2×10^8 CFUs per mouse of Newman, Δ Efb, or Δ Efb+Flag-Efb. **c**, Skin lesion size of 6-week-old mice infected by subcutaneous injection with roughly 1×10^8 CFUs per mouse of Newman, Δ Efb, or Δ Efb+Flag-Efb. Gehan-Breslow-Wilcoxon test (a, b, $n=8$) and Student's two-tailed unpaired t-test (c, $n=5$) were used for statistical analysis, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. Data are representative of three experiments with at least three independent biological replicates. The bars show the mean and s.d. of $n=3$ (c).

Extended Data Fig. 6 Efb interacts with TRAF3. **a**, Immunofluorescence assay of MH-S infected with Newman and Δ Efb + Flag-Efb expressed GFP for indicated times (MOI = 25), Scale bars, 2 μ m. **b**, Immunoblots of whole cell lysates (WCL) and immunoprecipitation (IP) products from HEK 293T cells transfected with indicated plasmids. **c**, Immunoblots of WCL and IP products from PMs infected with Δ Efb + Flag-Efb for indicated times (MOI = 2.5). **d**, **e**, Precipitation assay of purified

854 TRAF3 and purified Efb.

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Extended Data Fig. 7 Efb stabilizes TRAF3. **a**, Immunoblots of lysates from PMs infected with Newman and Δ Efb for indicated times (MOI = 25). **b**, Immunoblots of immunoprecipitation (IP) products of whole cell lysates (WCL) from PMs infected with Newman and Δ Efb for indicated times (MOI = 2.5). **c**, Immunoblots of WCL and IP products from lysates of MH-S infected with Newman and Δ Efb for indicated times (MOI = 2.5). **d**, Immunoblots of IP products from PMs infected with Newman and Δ Efb for indicated times. **e**, Immunoblots of WCL and IP products from HEK 293T cells transfected with indicated plasmids. **f**, Immunoblots of WCL and IP products from lysates of TRAF3 KO PMs (transfected with TRAF3 or TRAF3K155R mRNA) infected with Newman and Δ Efb for indicated times (MOI = 2.5).

Extended Data Fig. 8 Efb is K27-ubiquitinated by host RNF114. **a**, Immunoblots of immunoprecipitation (IP) products from lysates of HEK 293T cells transfected with indicated plasmids. **b**, Immunoblots of IP products from whole cell lysates (WCL) of PMs infected with Δ Efb+Flag-Efb for indicated times (MOI=25). **c**, Immunoblots of immunoprecipitation (IP) products culture supernatant Δ Efb+Flag-Efb strain and WCL of MH-S.

Extended Data Fig. 9 RNF114 cannot promote K6, K11, K29, K33, K48, K63 linked polyubiquitination of Efb. a, b, c, d, e, f, Immunoblots of whole cell lysates (WCL) and immunoprecipitation (IP) products of WCL of HEK 293T cells transfected with indicated plasmids.

Extended Data Fig. 10 RNF114 promotes K27-ubiquitination of Efb at K71. **a, b, c,** Immunoblots of whole cell lysates (WCL) and immunoprecipitation (IP) products of WCL of HEK 293T cells transfected with indicated plasmids. K71R, replace 71 lysine of Efb with arginine. R71K, replace 71 arginine of mutant of Efb (replace all lysine with arginine) with lysine.

Extended Data Fig. 11 Ubiquitinated Efb facilitates TRAF3 stabilization. **a, b**, Immunoblots of whole cell lysates (WCL) and immunoprecipitation (IP) products of WCL of HEK 293T cells transfected with indicated plasmids. K71R, replace 71 lysine of Efb with arginine. **c**, Immunoblots

of culture supernatant of *S. aureus* for 12 h. **d, e**, Immunoblots of IP products of WCL from PMs or MH-S infected with Δ Efb + Flag-Efb or Δ Efb + Flag-Efb K71R for indicated times. **f**, qPCR analysis of *Tnf*, *Il1b*, *Il6*, and *Il12p40* mRNA from control or RNF114 KD MH-S infected by Newman and Δ Efb for indicated times (MOI = 2.5). Student's two-tailed unpaired t-test (**f**) were used for statistical analysis, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Data are representative of three experiments with at least three independent biological replicates. The bars show the mean and s.d. of n = 3 (**f**).

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991 **Extended Data Fig. 12** RNF114 mediated K27-linked ubiquitination (K27ub) of Efb suppresses
 992 the TLR2/TRAFF3-mediated expression of proinflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-
 993 12) by disturbing formation of the TRAF3/TRAFF2/cIAP1 complex.

Supplementary Files

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