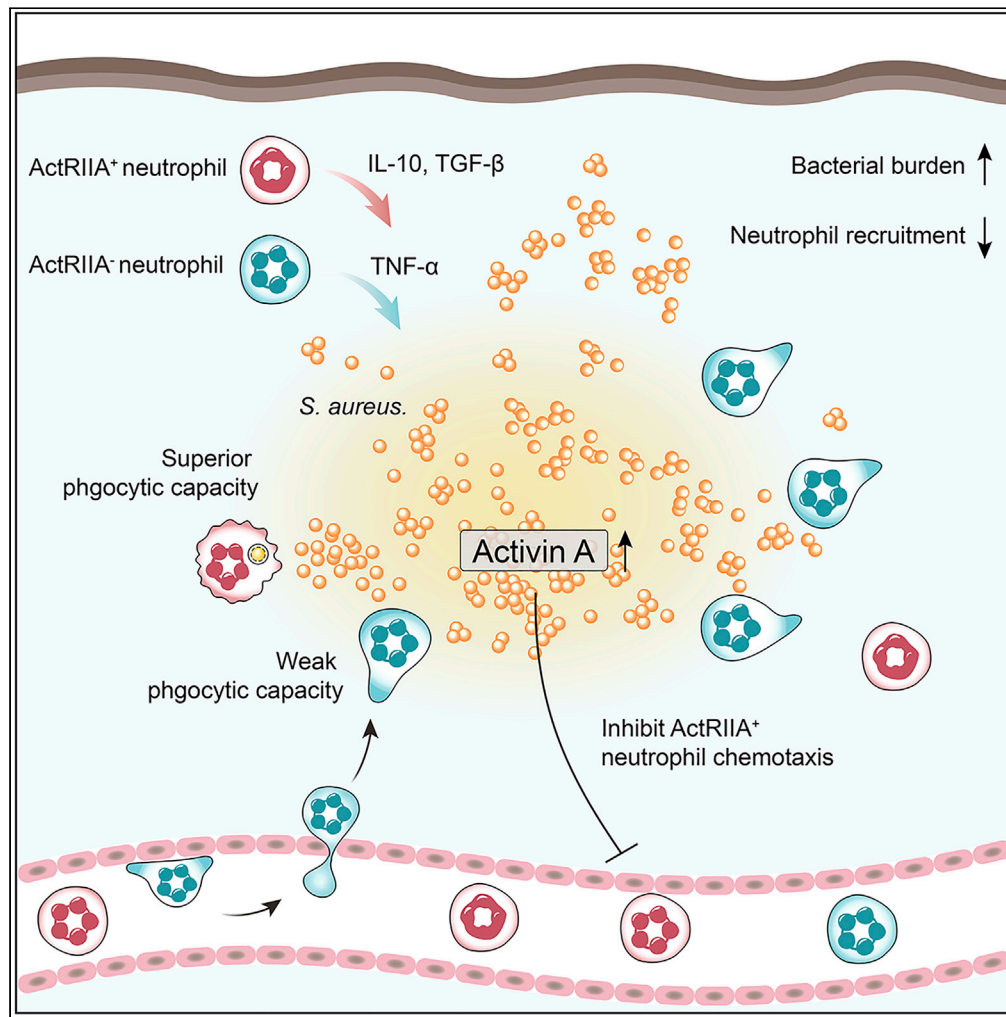**Article**

Activin A impairs ActRIIA+ neutrophil recruitment into infected skin of mice

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HIGHLIGHTS

A novel activin A-responsive subpopulation of neutrophils (ActRIIA+) was identified

ActRIIA+ neutrophils exhibit N2-like immunoregulatory properties

Activin A inhibits ActRIIA+ neutrophil recruitment to infected skin

Article

Activin A impairs ActRIIA+ neutrophil recruitment into infected skin of mice

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SUMMARY

Activin A levels are elevated during multiple severe infections and associated with an increased risk of death. However, the role of activin A in bacterial infection is still unclear. Here, we found that activin A levels were increased during *S. aureus* skin infection in mice. Administration of activin A increased the bacterial burden and promoted the spread of bacteria *in vivo*. Moreover, activin A inhibited neutrophil chemotaxis to N-formylmethionine-leucyl-phenylalanine via the type IIA activin receptor (ActRIIA) *in vitro* and impaired ActRIIA+ neutrophil recruitment to infection foci *in vivo*. Additionally, we identified a novel subpopulation of neutrophils, ActRIIA+ neutrophils, which exhibit superior phagocytic capacity compared to ActRIIA− neutrophils and possess an N2-like immunoregulatory activity via secreting IL-10 and TGF-β. Taken together, these findings indicate that activin A inhibits the recruitment of ActRIIA+ neutrophils to infected foci, leading to the impairment of bacterial clearance, and thus may hamper early infection control.

INTRODUCTION

Activin A is a member of the transforming growth factor β (TGF-β) family that participates in a broad spectrum of biological processes, including embryonic development, tumorigenesis, tissue remodeling, inflammation and the immune response (Bloise et al., 2019; Chen and Ten Dijke, 2016). Activin A binds to distinct type II receptors (ActRIIA or ActRIIB) on the surface of target cells (Bloise et al., 2019; de Caestecker, 2004). ActRII bound by activin A results in recruitment and phosphorylation of type I receptors and activates a number of downstream signaling proteins, such as SMAD2/3 or MAP kinases, and so on, which are important regulators of inflammation and infection control (Morianos et al., 2019; Li et al., 2013).

An increasing number of clinical studies have reported that activin A levels are markedly elevated during the various bacterial infections, including sepsis, meningitis and intra-amniotic infection, and so on (Ebert et al., 2006; Hardy et al., 2016; Lee et al., 2016; Michel et al., 2003; Petrakou et al., 2013; Rosenberg et al., 2012; Wilms et al., 2010). Higher levels of serum activin A have been observed in patients with sepsis compared to healthy volunteers, and activin A levels were correlated with disease severity in these patients, suggesting that activin A may serve as a potential diagnostic marker for sepsis severity (Lee et al., 2016). Currently, available studies investigating activin A in the context different infectious diseases have predominantly focused on assessing activin A expression levels, while the investigation of a functional role of activin A during infection has only been reported in a handful of papers. These include experimental animal models of endotoxin shock, where lipopolysaccharide (LPS) injection induced a rapid release of activin A into the circulation within one hour (Jones et al., 2000, 2007), and blockade of activin A action by its binding protein, follistatin (FST), could prevent pro-inflammatory cytokine production and LPS-induced mortality (Jones et al., 2007). However, the exact role of activin A during the infection remains a tantalizing question to be addressed.

Multiple lines of evidence indicate that activin A is a pleiotropic cytokine which plays pivotal roles in regulating immune responses. It exhibits different effects on a wide variety of immune cells, including macrophages, dendritic cells, mast cells, natural killer cells, T cells, and B cells, with both pro- or anti-inflammatory characteristics depending on the cellular context (Morianos et al., 2019; Li et al., 2013; Ma et al., 2020). Although *in vitro* experiments have uncovered neutrophils as an important source of activin A

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(Chen et al., 2011) and elevated activin A levels occur in diseases characterized by neutrophil activation, which suggest that a relationship between neutrophils and activin A may be anything but coincidental (Sideras et al., 2013), it was only recently that our studies demonstrated an expression of activin signaling components in neutrophils and that activin A can in fact modulate neutrophil function in an autocrine and paracrine manner (Qi et al., 2017; Xie et al., 2017). Moreover, elevated activin A levels and an impairment of neutrophil chemotaxis have previously been described in multiple inflammatory and infectious diseases, such as sepsis, chronic obstructive pulmonary disease, cancer, and diabetes (Bian et al., 2019; Brandau et al., 2011; Hoda et al., 2016; Tania et al., 2014; Trevelin et al., 2017; Yoshikawa et al., 2007; Zhang et al., 2016; Zhong et al., 2019). However, the relationship between these two phenomena has so far not been explored.

In a previous study, we found that activin A can inhibit human neutrophil chemotaxis to N-formylmethio-nine-leucyl-phenylalanine (fMLP) *in vitro* (Xie et al., 2017). We therefore hypothesized that elevated activin A in the infectious diseases may impair neutrophil recruitment and bacterial clearance, hampering early infection control and exacerbating infections. Here, using a mouse model of cutaneous *S. aureus* infection, we found that activin A impaired neutrophil recruitment to infected foci and resulted in increased bacterial burden. Additionally, an activin A-responsive neutrophil subpopulation expressing ActRIIA was identified, which exhibited superior phagocytic capacity and potent immunoregulatory properties compared with other neutrophils. These data demonstrated that elevated activin A during bacterial skin infections resulted in a higher bacterial burden and exacerbated serious skin lesions via inhibition of ActRIIA$^+$ neutrophil recruitment to infected foci. The findings of this study may potentially be exploited for the development of new treatment strategies controlling bacterial infection.

RESULTS

Activin A decreases bacterial clearance during *S. aureus* skin infection

In order to determine whether activin A was elevated during the early skin infection, a mouse model of cutaneous infection was established by inoculating animals intradermally with various amounts of *S. aureus* (Figure 1A). We found that activin A levels were significantly increased in the sera in *S. aureus*-infected groups with 1x10^6 and 1x10^7 colony forming unit (CFU) compared with the control group (p < 0.05) and also elevated significantly in the skin tissues in three *S. aureus*-infected groups (p < 0.01) compared with the control group (Figures 1B and 1C). Additionally, activin A levels in infected foci, but not in the serum, were correlated with the amount of inoculated bacteria (r = 0.958, p < 0.01; Figures 1D and 1E).

To further investigate the impact of elevated activin A on bacterial burden in acute skin infections, mice were intradermally injected with both exogenous activin A and *S. aureus* (1 × 10^6 CFU), and histological features of the skin lesions and bacterial burden were evaluated 12 h after administration. H&E and immunofluorescence images showed that exogenous activin A aggravated tissue injury and promoted the spread of bacteria in the epidermal skin (Figures 1F and 1G). Moreover, treatment with exogenous 500 and 1000 pg/mouse activin A significantly increased bacterial counts in the lesioned skin compared with the control group (p < 0.05, Figure 1H). These findings suggested that elevated activin A in infected foci may lead to an impairment of bacterial clearance during cutaneous infections.

Activin A impairs neutrophil recruitment during cutaneous infections

During the early stages of an infection, neutrophils and macrophages are key phagocytic cells recruited to clear bacteria. To assess whether activin A affected the innate immune cell infiltration in cutaneous infection foci, we analyzed the populations of neutrophils (Ly6G$^+$CD11b$^+$) and macrophages (F4/80$^+$CD11b$^+$) collected from infected skin by flow cytometry. As shown in Figure 2A, numbers of neutrophils were significantly decreased in the infected skin of mice treated with activin A at 500 and 1000 pg/mouse compared with the control group (p < 0.01, p < 0.05), but there is no significant difference between activin A 250 pg/mouse and control group (p > 0.05), which was consistent with immunofluorescence observations (Figure 2C). Numbers of macrophages did not differ significantly between activin A treatment and control groups. These results indicate that activin A impairs neutrophil recruitment to cutaneous infection foci.

ActRIIA on neutrophils is necessary for the inhibitory effects of activin A on chemotaxis

A previous study has demonstrated that activin A exhibits an inhibitory effect on human neutrophil chemotaxis to fMLP (Xie et al., 2017). In the present study, using a transwell assay, we found that activin A
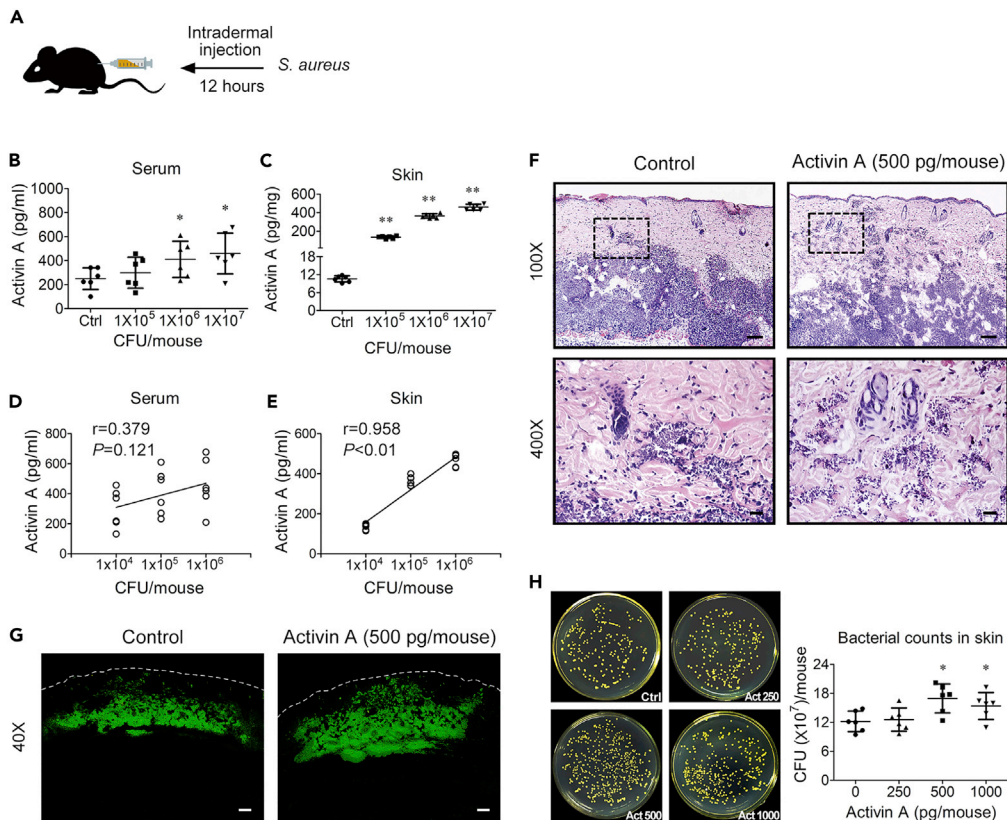
Figure 1. Activin A levels and bacterial clearance during *S. aureus* skin infection

(A) The mouse model of cutaneous infection was generated by intradermal injection of *S. aureus*.

(B and C) Levels of activin A in serum (B) and infected skin (C) of mice 12 h after *S. aureus* infection were examined using an enzyme-linked immunosorbent assay (ELISA).

(D and E) Correlation analysis of activin A levels in serum (D) and infected skin (E) with amounts of *S. aureus* was performed. Pearson coefficient tests were performed to assess statistical significance (p < 0.01).

(F) Representative H&E images of mice were shown 12 h after intradermally injected with recombinant activin A or PBS (as control) mixed with *S. aureus* (1 × 10^6 CFU). Scale bars, 100 μm (x100), 20 μm (x400).

(G) Representative fluorescence images of carboxyfluorescein succinimidyl ester (CFSE)-labeled bacteria (green) in the infected skin were represented. Scale bars, 200 μm (x40).

(H) Homogenized skin biopsies were plated on plates for bacterial counts.

All the data (mean ± SD that represents standard deviation) were obtained from 6 mice per group. Statistical significance was assessed using the Student's *t* test (*p < 0.05, **p < 0.01).

significantly inhibited mouse neutrophil transmigration induced by fMLP (p < 0.01; Figure 3A) and also significantly suppressed mouse neutrophil chemotaxis to CXCL8 (p < 0.05; Figures S1A and S1B). The inhibitory effect of activin A on neutrophil chemotaxis to fMLP was significantly attenuated by an anti-ActRIIA antibody (p < 0.01; Figure 3A) and activin-binding protein FST (p < 0.05; Figure S1C).

To further verify whether ActRIIA is necessary for the anti-chemotactic effect of activin A on neutrophils, we sorted ActRIIA positive and negative neutrophils by flow cytometry and assessed chemotaxis. ActRIIA$^+$ neutrophils exhibited a significantly weaker chemotactic activity to fMLP than ActRIIA$^-$ neutrophils (p < 0.01), and activin A significantly inhibited transmigration of ActRIIA$^+$ but not ActRIIA$^-$ neutrophils induced by fMLP (p < 0.05; Figure 3B). Meanwhile, *in vivo* studies also showed that the percentage of ActRIIA$^+$ neutrophils in infected skin tissues was significantly reduced in the group treated with activin A at 500 and 1000 pg/mouse compared with the control group (p < 0.01; Figures 3C and 3D). These data suggest that ActRIIA is required for inhibitory effect of activin A on neutrophil chemotaxis.
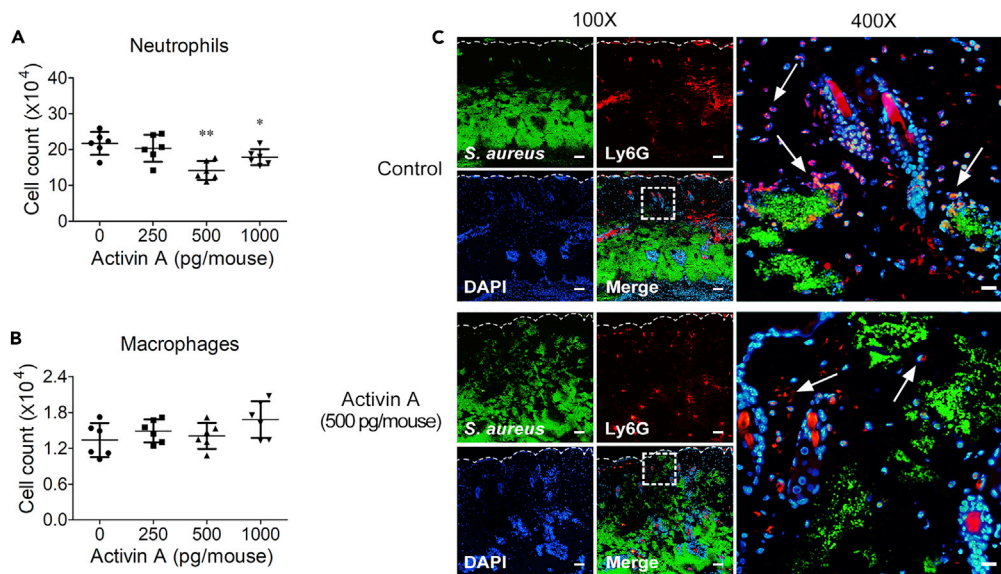
Figure 2. Impairment of neutrophil recruitment to infected skin by activin A

(A and B) CD11b$^+$Ly6G$^+$ neutrophils (A) and CD11b$^+$F4/80$^+$ macrophages (B) were examined by flow cytometry in infected skin of mice 12 h after intradermally injected with recombinant activin A or PBS (as control) mixed with *S. aureus* (1×10^6 CFU).

(C) Representative sections were stained for neutrophil-specific marker Ly6G (red), nuclei (DAPI, blue), and CFSE-labeled bacteria (green). The merged high magnification image (×400) showed infiltrated neutrophils (white arrows) at the infection site. Scale bars, 100 μm (x100), 20 μm (x400).

All the data (mean ± SD) were obtained from 6 mice per group. Statistical significance was assessed using the Student's *t* test (*p < 0.05, **p < 0.01).

An ActRIIA$^+$ neutrophil subpopulation is identified

As described above, ActRIIA$^+$ and ActRIIA$^-$ neutrophils displayed different chemotactic responses to fMLP. Accordingly, we hypothesized that the expression of ActRIIA may be useful to define novel neutrophil subpopulations. Neutrophils have historically been characterized by their distinct nuclear shape and buoyant density. Here, ActRIIA$^+$ and ActRIIA$^-$ neutrophils from peripheral blood were sorted by flow cytometry and stained with DAPI. We found that ActRIIA$^+$ neutrophils accounted for 35% of peripheral neutrophils (Figure 4A) and exhibited both an immature-like ring-shaped or mature-like segmented nuclear morphology, while most ActRIIA$^-$ neutrophils were mature-like neutrophils with segmented nuclei (Figure 4B).

Neutrophils could be classified on the basis of their density as low-density neutrophils (LDNs) and normal-density neutrophils (NDNs). LDNs are often considered to represent immature or degranulated neutrophils (Scapini et al., 2016). After density gradient centrifugation, we observed that ActRIIA$^+$ neutrophils accounted for 41.5%, and ActRIIA$^-$ neutrophils accounted for 58.5% in LDNs. Moreover, ActRIIA$^+$ neutrophils accounted for 9.5% of NDNs, while ActRIIA$^-$ neutrophils accounted for 90.5% (Figure 4C). These results implied that, unlike ActRIIA$^-$ neutrophils with mature morphology, ActRIIA$^+$ neutrophils are a heterogeneous population composed of both mature and immature cells. We analyzed the expression of molecular markers in the aforementioned neutrophil subpopulations by flow cytometry and found that ActRIIA$^+$ neutrophils expressed higher CD64 and TLR4 levels than ActRIIA$^-$ neutrophils. However, we did not find any significant differences in molecular markers between these two subpopulations apart from ActRIIA (Figure 4D).

ActRIIA$^+$ neutrophils exhibit a higher bacterial clearance capacity than ActRIIA$^-$ neutrophils

To further elucidate the bacterial clearance capacity of these two novel neutrophil subpopulations, ActRIIA$^+$ and ActRIIA$^-$ neutrophils were incubated with bacteria *in vitro*. In the presence of ActRIIA$^+$ neutrophils, bacterial counts were significantly lower after 4 h of incubation (p < 0.05, Figure 5A) than in the presence of ActRIIA$^-$ neutrophils, indicating that ActRIIA$^+$ neutrophils exhibit a higher bacterial clearance
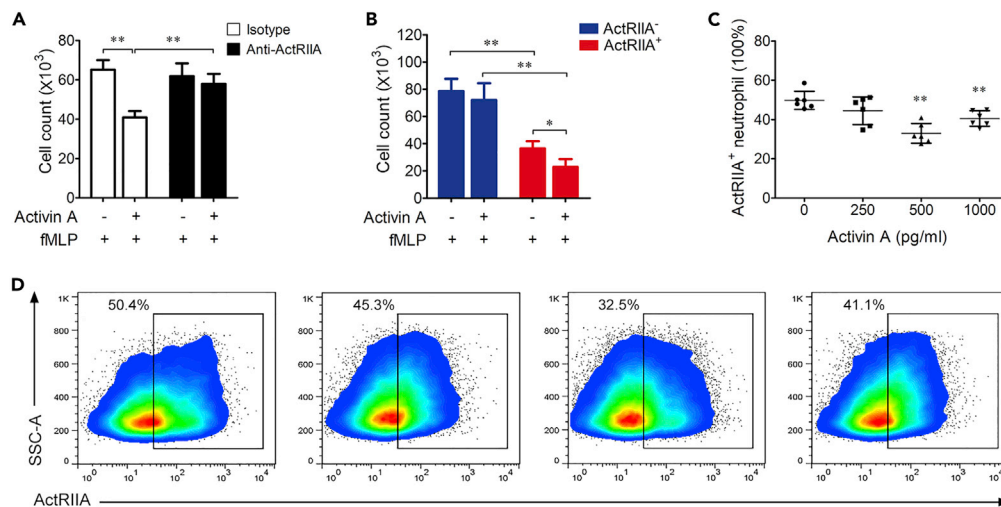
Figure 3. The inhibitory effect of activin A on neutrophil chemotaxis to fMLP via ActRIIA

(A) Neutrophils were pre-cultured with isotype IgG or anti-ActRIIA polyclonal antibody and allowed to migrate through the 3 μm pore transwell chamber toward activin A (5 ng/mL) and fMLP (100 nM).

(B) Migration of ActRIIA⁺CD11b⁺Ly6G⁺ and ActRIIA⁻CD11b⁺Ly6G⁺ neutrophils sorted, respectively, by flow cytometry were examined in the presence of activin A (5 ng/mL) and fMLP (100 nM).

(C and D) The percentage (C) and representative flow plots (D) of ActRIIA⁺CD11b⁺Ly6G⁺ neutrophils in the infected skin of mice inoculated with *S. aureus* pre-mixed with PBS (as control) or activin A were examined by flow cytometry. Neutrophils were gated on CD11b and Ly6G double positivity.

All the data (mean ± SD) were obtained from 6 mice. Statistical significance was calculated by Student's *t* test (*p < 0.05, **p < 0.01).

capacity than ActRIIA⁻ neutrophils. Next, we analyzed the percentage of phagocytic cells by flow cytometry using immunofluorescent microspheres. We found that ActRIIA⁺ neutrophils exhibited a significantly higher phagocytic activity than ActRIIA⁻ neutrophils (p < 0.01, Figure 5B), which was consistent with immunofluorescence observations (Figure 5C). As the respiratory burst is one of bacterial killing mechanisms of phagocytic cells, we evaluated differences in reactive oxygen species (ROS) production between the two subpopulations. Interestingly, although ActRIIA⁺ neutrophils displayed a higher bacterial clearance and phagocytic capacity than ActRIIA⁻ neutrophils, their levels of ROS production were significantly lower than those in ActRIIA⁻ neutrophils (p < 0.01, Figure 5D).

IL-10 and TGF-β are produced by ActRIIA⁺ neutrophils

Neutrophils have the potential to polarize toward a pro-inflammatory (N1) or an anti-inflammatory (N2) phenotype. We therefore characterized cytokine profiles in the supernatant of cultured neutrophils by enzyme-linked immunosorbent assay (ELISA). Without LPS stimulation, ActRIIA⁺ neutrophils produced significantly higher levels of IL-10, TGF-β, and TNF-α than ActRIIA⁻ neutrophils (p < 0.01, p < 0.05, p < 0.05). Following LPS challenge, ActRIIA⁺ neutrophils produced significantly lower levels of the pro-inflammatory cytokine TNF-α (p < 0.01), but higher levels of immunoregulatory cytokines IL-10 and TGF-β than ActRIIA⁻ neutrophils (p < 0.05, p < 0.01). Moreover, the ratio of IL-10/TNF-α and TGF-β/TNF-α in ActRIIA⁺ neutrophils was significantly higher than that in ActRIIA⁻ neutrophils (p < 0.01; Figure 6). These data indicated that the activin A-responsive ActRIIA⁺ neutrophil subpopulation may exhibit N2-like immunoregulatory activities after exposure to bacterial stimuli.

DISCUSSION

In the present study, a novel activin A-responsive subpopulation of neutrophils expressing the ActRIIA receptor was identified. ActRIIA⁺ neutrophils exhibited superior phagocytic capacity than ActRIIA⁻ neutrophils, as well as a potent immunoregulatory activity by secretion of IL-10 and TGF-β. In skin infection models, the levels of activin A were elevated in infected foci, and exogenous activin A promoted bacterial spread in the epidermal skin. Moreover, we found that activin A impaired the chemotaxis of ActRIIA⁺ neutrophils to fMLP *in vitro* and recruitment to infected foci *in vivo*. These data suggest that an elevation of
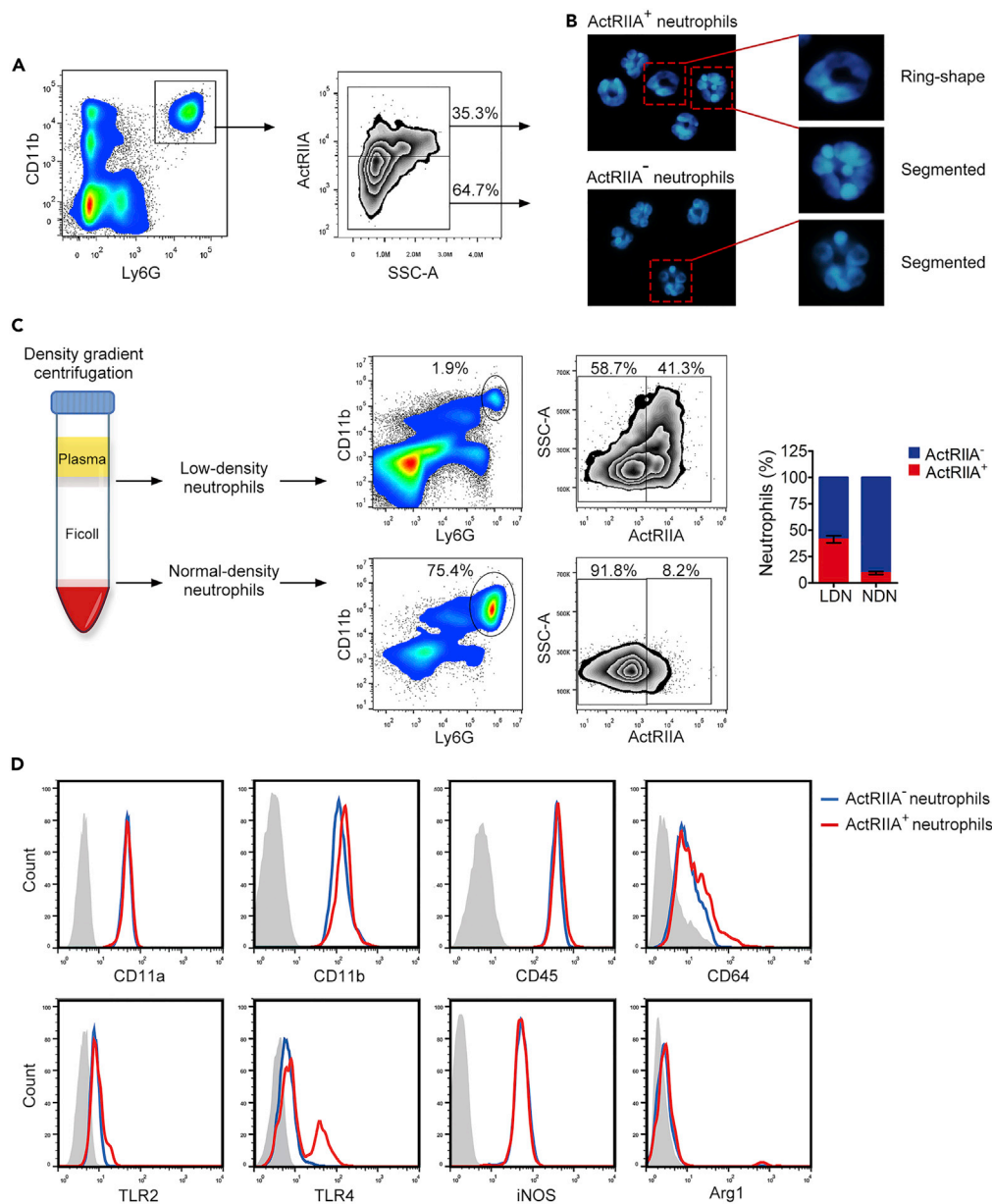
Figure 4. Morphology and density characterization of ActRIIA+ and ActRIIA− neutrophils

(A) ActRIIA+ and ActRIIA−CD11b+Ly6G+ neutrophils from peripheral blood were sorted by flow cytometry.

(B) Nuclear morphology of the two subpopulations was analyzed by fluorescence microscopy (DAPI staining). Neutrophils exhibited a ring-shaped or segmented nuclear morphology.

(C) Ficoll-Paque plus was used to separate LDN and NDN from mouse peripheral blood (left panel). Percentages of ActRIIA+ and ActRIIA−CD11b+Ly6G+ neutrophils in LDN and NDN were analyzed by flow cytometry (middle and right panel). All the data were obtained from 3 experiments.

(D) Phenotypic characterization of ActRIIA+ and ActRIIA−CD11b+Ly6G+ neutrophils was examined by flow cytometry. ActRIIA+ and ActRIIA−CD11b+Ly6G+ peripheral neutrophils were gated as shown in (A). Expression of CD11a, CD11b, CD45, CD64, TLR2, TLR4, iNOS, and Arg1 in ActRIIA+ and ActRIIA− CD11b+Ly6G+ neutrophils was shown in the histograms. Red lines depict ActRIIA+ neutrophils; blue lines depict ActRIIA− neutrophils; gray-filled lines depict FMO control.
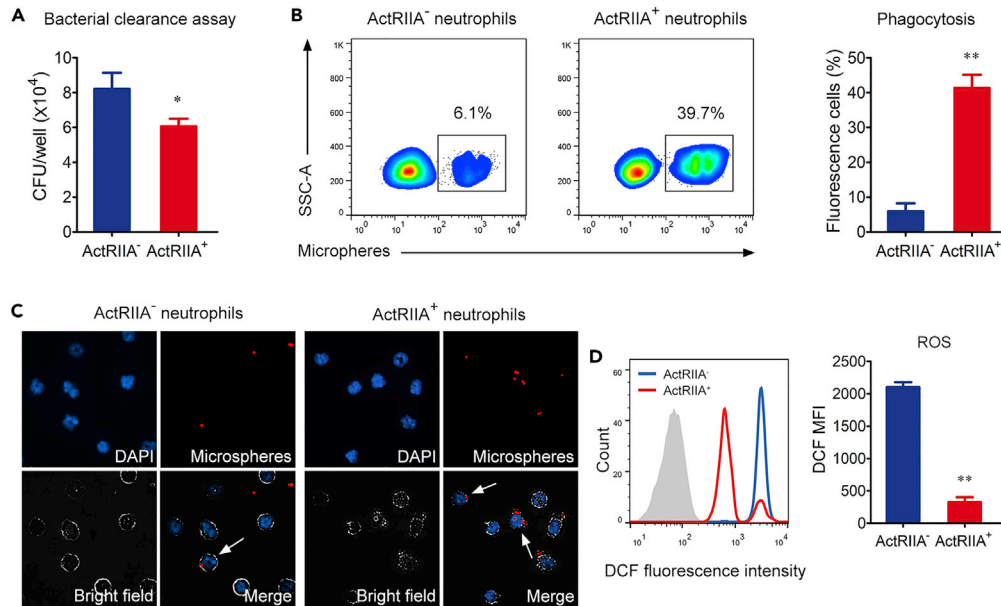
Figure 5. Assay of bacterial clearance and phagocytic capacity of ActRIIA$^+$ and ActRIIA$^-$ neutrophils

(A) ActRIIA$^+$ and ActRIIA$^-$CD11b$^+$Ly6G$^+$ neutrophils (5x10^5 cells/well) were incubated with *S. aureus* (1 × 10^5 CFU/well) for 4 h; diluted aliquots were spread on agar plates for overnight incubation and after which CFU were counted. The CFU reflected the bacterial clearance capacity of neutrophils.

(B) The phagocytic activity of neutrophils was analyzed by flow cytometry after incubation with fluorescent microspheres (red).

(C) Representative fluorescence images of phagocytosis (white arrows) by ActRIIA$^+$ and ActRIIA$^-$ neutrophils were shown, in which it was nuclei (DAPI, blue), microspheres (red), and brightfield.

(D) ROS generation was examined by flow cytometry and quantified as MFI.

All the data (mean ± SD) were obtained from 3 experiments. Statistical significance was assessed using the Student's *t* test (*p < 0.05, **p < 0.01).

activin A during bacterial skin infections in mice may result in a higher bacterial burden and exacerbated skin lesions by inhibiting recruitment of ActRIIA$^+$ neutrophils with superior phagocytic capacity to infected foci.

Neutrophils, also referred to as polymorphonuclear leukocytes, are the first responders to injury and infection and are recruited to infection sites within 4 h (Kim et al., 2008). Neutrophil recruitment toward chemoattractants is crucial for early bacterial clearance (Nemeth et al., 2020). Interestingly, an impairment of neutrophil chemotaxis has been observed in multiple inflammatory and infectious diseases, and these disorders are also characterized by elevated activin A levels (Lee et al., 2016; Tania et al., 2014; Yoshikawa et al., 2007; Zhang et al., 2016). Although higher levels of activin A is correlated with the severity of inflammatory and infectious disease (Lee et al., 2016), the role of activin A in bacterial clearance of cutaneous tissues remains unknown. In this study, we established a model of cutaneous infection, in which mice were intracutaneously inoculated with different amounts of *S. aureus*. Our study showed that activin A levels in the serum and infected skin tissues of mice were significantly increased following skin infection with *S. aureus* and that elevated levels of activin A in infected foci were positively associated with the amount of inoculated bacteria. Moreover, mice that had been intradermally injected with *S. aureus* and exogenous activin A exhibited an enhanced spread of bacteria in the epidermal skin and an increased bacterial burden in the infected skin as observed by conventional colony counting. These data suggested that the elevated activin A levels in infected foci might lead to an impairment of bacterial clearance during the cutaneous infections.

During the early infection, neutrophils and macrophages are essential phagocytic cells for bacterial clearance (Ley et al., 2018). Thus, we examined the number of CD11b$^+$Ly6G$^+$ neutrophils and F4/80$^+$CD11b$^+$ macrophages in the infected skin by flow cytometry. We found that activin A reduced the number of
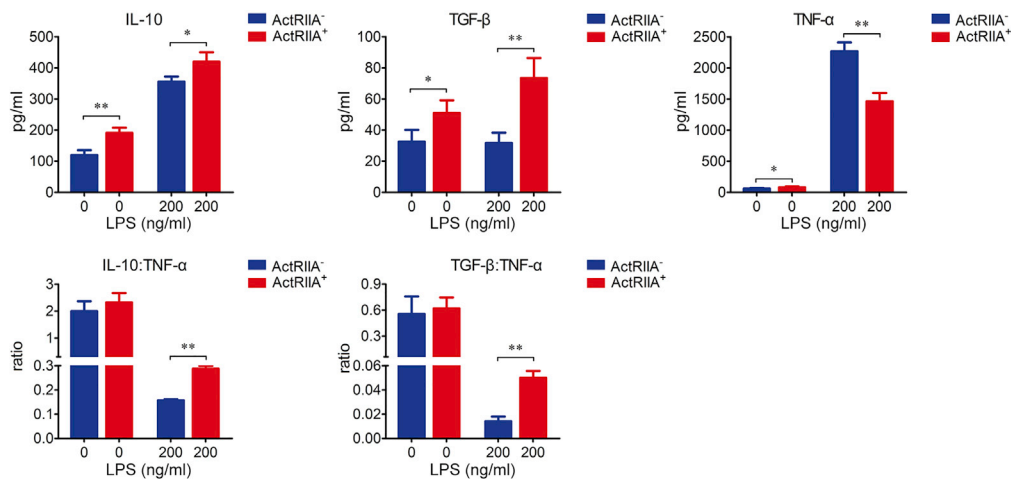
Figure 6. Production of cytokines by ActRIIA$^+$ and ActRIIA$^-$ neutrophils

ActRIIA$^+$ and ActRIIA$^-$CD11b$^+$Ly6G$^+$ neutrophils were treated with LPS or PBS for 24 h, and the levels of cytokines in the supernatant of cultured cells were measured by ELISA. All the data (mean $\pm$ SD) were obtained from 3 experiments. Statistical significance was calculated by Student's t test (*p < 0.05, **p < 0.01).

neutrophils, but not macrophages, in the infected skin, and immunofluorescence similarly showed that, after administration of activin A, fewer neutrophils were present in the infected skin. Neutrophil chemotaxis toward chemoattractants is critical for the recruitment to infection sites (Tecchio and Cassatella, 2016). Our previous studies have shown that activin A can regulate the activation of neutrophils and macrophages but inhibit human neutrophil chemotaxis to fMLP (Qi et al., 2017; Xie et al., 2017). To verify the inhibitory effect of activin A on neutrophil chemotaxis, here we used a polyclonal antibody against ActRIIA and activin-binding protein FST to block the action of activin A and found that the inhibitory effect of activin A on neutrophil chemotaxis to fMLP was attenuated. These findings indicate that activin A impairs the recruitment of neutrophils to cutaneous infection foci. Inflammation is a double-edged sword. The immune system must be restrained to prevent excessive inflammation and tissue damage (Poon and Farber, 2020). It is well known that many types of immune cells are capable of self-terminating in the later stages of infection. For example, Treg cells release TGF-β that inhibit T-cell proliferation (Kanamori et al., 2016), macrophages secret activin A to induce M2 polarization and produce IL-10 to limit the activity of macrophages (Ogawa et al., 2006; Atri et al., 2018). During the early infection, large numbers of neutrophils are recruited from the blood, but the flux will be faded off after several days. Few studies in the literature have reported about the neutrophil self-limiting. Since neutrophils are a major source of activin A, neutrophils may regulate its own tissue infiltration by secreting activin A, establishing a negative feedback regulation to prevent excessive inflammation. Therefore, the role of activin A in infectious diseases may vary from beneficial to harmful, depending on the stage of inflammation.

Heterogeneous populations of neutrophils with diverse phenotypes and functions have been described previously (Silvestre-Roig et al., 2019). Herein, we surprisingly found that about 35% of peripheral neutrophils expressed ActRIIA. We sorted ActRIIA$^+$ and ActRIIA$^-$ neutrophils by flow cytometry and found that activin A significantly inhibited the chemotaxis of ActRIIA$^+$ neutrophils but not ActRIIA$^-$ neutrophils. Additionally, the data showed that there was a significant difference in chemotaxis between two subpopulations. ActRIIA$^+$ neutrophils exhibited weaker chemotactic activity than ActRIIA$^-$ neutrophils. Neutrophil populations are typically characterized by their distinct nuclear shape and buoyant density (Pillay et al., 2013; Sagiv et al., 2015; Silvestre-Roig et al., 2019). Our study revealed that a fraction of ActRIIA$^+$ neutrophils had a ring-shaped nuclear morphology, exhibiting an immature-like neutrophil morphology, while others appeared segmented, which is typically a mature morphology. By contrast, most ActRIIA$^-$ cells exhibited a segmented nuclear morphology. LDNs are usually referred as "suppressive neutrophils" containing immature cells (Sagiv et al., 2015). The proportion of ActRIIA$^+$ neutrophils was significantly higher in LDNs than that in NDNs. These findings implied that, unlike ActRIIA$^-$ neutrophils which resembled mature cells, ActRIIA$^+$ neutrophils belonged to a heterogeneous population composed of both mature and immature neutrophils. We furthermore assessed molecular markers on ActRIIA$^+$ and ActRIIA$^-$ neutrophils, but did not detect significantly different phenotypic characteristics of these subpopulations.

Finally, we examined whether ActRIIA$^+$ and ActRIIA$^-$ neutrophil subpopulations exhibited different properties in response to bacteria. ActRIIA$^+$ neutrophils displayed higher capacity for bacterial clearance and phagocytic activity, although their levels of ROS production were lower than those in ActRIIA$^-$ neutrophils. ActRIIA$^+$ neutrophils may engulf bacteria directly rather than eliminate them within cells. In addition, similar to M1/M2 macrophages, neutrophils can also polarize toward pro-inflammatory (N1, similar to "M1") or immunoregulatory (N2, similar to "M2") subtypes via release of IL-10 and TNF-α following a bacterial challenge *in vitro* (Gideon et al., 2019; Scalerandi et al., 2018). In this study, we found that ActRIIA$^+$ neutrophils released less TNF-α, but more IL-10 and TGF-β after LPS stimulation, than ActRIIA$^-$ neutrophils, indicating that different subsets of neutrophils were dominant in secreting pro-inflammatory or immunoregulatory cytokines. ActRIIA$^+$ neutrophils appear to take on an "N2-like" subtype, responsible for production of immunoregulatory cytokines. Moreover, during cutaneous infections, activin A reduced the percentage of ActRIIA$^+$ neutrophils in the lesional skin. Therefore, it is possibly reasonable to speculate that activin A inhibits recruitment of ActRIIA$^+$ neutrophils with superior phagocytic capacity to infected foci, which may result in a higher bacterial burden and more aggressive inflammation.

In conclusion, the data presented in the current study suggested that ActRIIA is critical for inhibitory effects of activin A on neutrophil chemotaxis and revealing a novel neutrophil subpopulation. ActRIIA$^+$ neutrophils exhibit superior phagocytic capacity and immunoregulatory properties than ActRIIA$^-$ neutrophils. Elevated levels of activin A impaired ActRIIA$^+$ neutrophil recruitment to invasion foci during early stages of skin infection, which may contribute to defects in bacterial clearance. Hence, this study provides novel insights for the development of potential therapeutic strategies for controlling bacterial infection.

Limitations of the study

In this study, we found that the administration of exogenous activin A impaired ActRIIA$^+$ neutrophil recruitment into infected foci, whereas the endogenous activin A could also be produced by neutrophils and other cells during the infection. Our study is limited by neutralization of endogenous activin A, which makes it difficult to investigate the relative contribution of autocrine as opposed to paracrine activin A action in regulating neutrophil responses. It has been reported that activin A levels in patients with sepsis predict the risk of death. However, it is still unclear whether ActRIIA$^+$ neutrophils can serve as a prognostic indicator, correlating with the severity of infection, and therefore, further clinical studies will be required.

Resource availability

Lead contact

Further information should be directed and will be fulfilled by the lead contact, Zhonghui Liu (liuzh@jlu.edu.cn).

Material availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate data sets/code.

METHODS

All methods can be found in the accompanying Transparent methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2021.102080.

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Supplemental Information

Activin A impairs

ActRIIA⁺ neutrophil recruitment

into infected skin of mice

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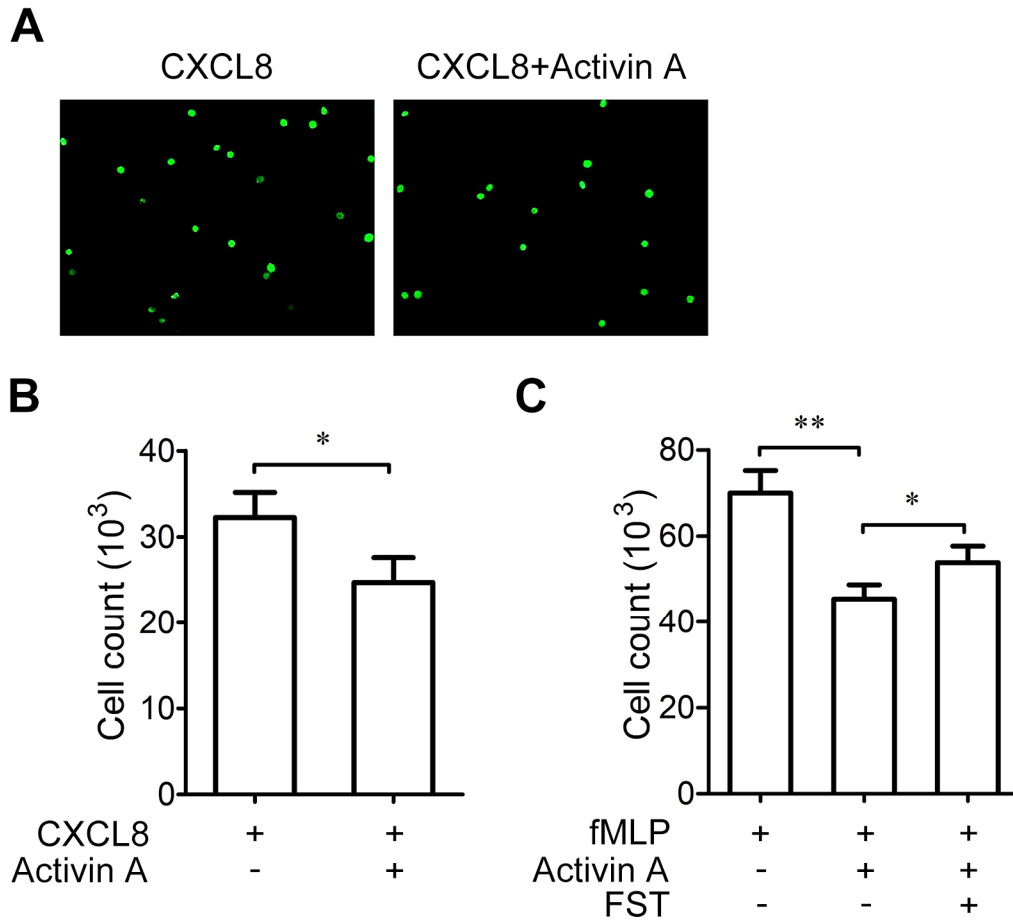
SUPPLEMENTAL DATA ITEMS

Figure S1. The inhibitory effect of activin A on neutrophil chemotaxis, Related to Figure 3

The chemotaxis of neutrophils labelled by CFSE was examined using a transwell assay. Activin A (5 ng/ml), CXCL8 (200 ng/ml), fMLP (100 mM) or FST (10 ng/ml) were added into the lower chamber to initiate migration. All the data (mean ± SD) were obtained from 3 experiments. Statistical significance was assessed using the Student's *t* test (*$P<0.05$, **$P<0.01$).

Transparent Methods

Staphylococcus aureus strain and preparation

The *S. aureus* strain ATCC 25925 used in all experiments was kindly provided by Hongyan Shi and Huimei Yu (Department of Pathogen Biology, Jilin University). *S. aureus* ATCC25925 was cultured on tryptic soy agar (TSA) plates (tryptic soy broth [TSB] plus 1.5% bacto agar), and grown overnight in a 37°C bacterial incubator. Single colony was picked from the culture plate and cultured in TSB at 37°C in a shaking incubator (240 rpm) overnight. The bacterial culture was diluted 1:50 for 2 h to reach mid-logarithmic phase bacteria. The concentration of bacterial suspensions was adjusted to 1×10^6-1×10^8 CFU/ml by correlation with absorbance (A600), and the CFU/ml was verified by plating.

Mouse model of cutaneous *S. aureus* infection

All procedures were approved by the Animal Ethics Committee of Jilin University. The skin infection experiment was performed similarly as described previously (Dokoshi et al., 2018). In brief, the back of C57BL/6 male mice (aged 8-10 weeks) was shaved using a chemical hair remover and animals were subsequently subcutaneously injected with 100 µl of midlogarithmic growth phase *S. aureus* (1×10^5 to 1×10^7 CFU of bacteria) using a 27-gauge insulin syringe. For additional exogenous activin A treatment, 1×10^6 CFU of *S. aureus* and 250-1000 pg recombinant activin A (R&D Systems) in PBS were previously mixed and the mice were subcutaneously injected with this mixture.

ELISA

Mice were sacrificed 12 h after infection with different amounts of *S. aureus*. For detection of activin A levels in the serum, blood was collected by a cardiac puncture and serum was isolated by centrifugation. For detection of activin A levels in the skin, 10 mm punch biopsies from the center of the injection site were collected and weighed. The specimen were homogenized (TissuePrep homogenizer, Gering Scientific Instruments) using Tissue Extraction Reagent I (Invitrogen) and homogenates were centrifuged at 10,000 rpm for 10 min. The supernatant was collected for activin A analysis using the enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (R&D Systems).

For the analysis of production of IL-10, TNF-α, and TGF-β by neutrophils, the ActRIIA$^+$ and ActRIIA$^-$ peritoneal neutrophils (1×10^6 cell/well) were sorted by flow cytometry and incubated

in the absence or the presence of 200 ng/ml LPS for 24 h, and then the culture supernatants were collected for activin A analysis by ELISA (R&D Systems).

Ex vivo CFU quantification

Ex vivo CFU quantification was carried out similarly as described previously (Dillen et al., 2018). In brief, the entire 10 mm punch skin specimens from the infected skin of mice were homogenized (TissuePrep homogenizer, Gering Scientific Instruments) in PBS. Skin homogenates were serially diluted, cultured on TSA plates overnight at 37°C in bacterial incubator, and _ex vivo_ CFU were counted.

Skin cell preparation and flow cytometry

Skin single-cell suspensions were prepared as described previously (Bitschar et al., 2019). Briefly, the entire 10 mm punch skin specimens from the infected skin of mice were cut into small pieces and digested in RPMI 1640 medium with 0.25% Liberase TL (Roche) and 0.01% DNase I (MilliporeSigma) at 37°C for 2 h. Samples were then passed through a 40 μm cell strainer (Corning) to obtain a single-cell suspension. The cell suspensions were stained with anti-mouse Ly6G-PE (clone: 1A8, BD Biosciences), CD11b-PerCP-Cy5.5 (clone: M1/70, BD Biosciences), F4/80-FITC (clone: BM8, ebioscience) and ActRIIA-APC (cat: FAB340A, R&D Systems). All flow cytometric analyses were carried out on the BD FACSAria II flow cytometer (BD Biosciences) and the results were analyzed using FlowJo Version 10.

H&E and immunofluorescence staining

S. aureus (1×10^6 CFU) were labelled with the green fluorescent dye CFSE (Invitrogen) and injected into mice subcutaneously. Mice were sacrificed 12 h after infection, punch biopsies taken, and the entire 10 mm punch skin specimen was collected, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at a thickness of 5 μm on a Microtome (Microm HM355; Carl Zeiss). Sections were stained with hematoxylin & eosin (H&E) and imaged using microscope (Axio Imager 2, Carl Zeiss). For immunofluorescence images, sections were blocked for 1 h at room temperature with 2% BSA in PBS and stained overnight at 4°C with anti-mouse Ly6G-PE antibody (clone: 1A8, BD Biosciences). After washed with PBS three times, sections were counterstained with DAPI (Beyotime Institute of Biotechnology) for 15 min and imaged on a microscope (Axio Imager 2, Carl Zeiss).

Neutrophil isolation and chemotaxis assay

We noticed that the red blood cell (RBC) lysis buffer affected neutrophil viability and chemotaxis during our preliminary experiments (not shown). Thus, peritoneal neutrophils were collected avoiding RBC lysis in this study. Peritoneal neutrophils were prepared as described previously (Qi et al., 2017). Briefly, 1 ml of 9% casein was injected into the peritoneal cavity per mouse and injection was repeated after 24 h. Peritoneal cells were collected 3 h after the last injection. Neutrophils were isolated by either magnetic beads (Mouse Neutrophil Enrichment Kit, Stemcell Technologies) or sorted by flow cytometry. The sorted neutrophils were used for the chemotaxis assay, bacterial clearance assay, and ELISA.

The neutrophil chemotaxis assay was performed in transwell chambers (3 μm pore size, Corning). Neutrophils (1×10^6 cells/well) were purified by magnetic cell sorting (MACS), pre-cultured with 10 μg/ml isotype IgG or anti-ActRIIA antibody (R&D Systems), and then loaded into the upper chamber. RPMI-1640 medium with or without 5 ng/ml activin A (R&D system) and 100 mM fMLP (Sigma-Aldrich) was added into the lower chamber and cells were cultured for 1 h at 37°C. The number of cells that migrated into the lower chamber was quantified by flow cytometry.

Separation of LDN and NDN

Whole blood from normal healthy mice was stained with anti-mouse Ly6G-PE (clone: 1A8, BD Biosciences), CD11b-FITC (clone: M1/70, BD Biosciences), and ActRIIA-APC (cat: FAB340A, R&D Systems). The stained blood samples were diluted 1:5 in 0.5 % BSA/PBS to be added on the layer of Ficoll-Paque Plus (1.077 g/mL, Stemcell Technologies), and then the mixed samples were centrifuged at 400 g for 30 min at room temperature with no brake, as described previously (Sagiv et al., 2015). Low-density neutrophils (LDNs) were collected from the plasma-1.077 interface. Normal-density neutrophils (NDNs) were collected from granulocyte-erythrocyte pellet, and RBCs were eliminated using a FACS lysing solution (BD Biosciences). The samples were suspended for flow cytometry analysis.

Phenotypic analysis of neutrophils

For analysis of neutrophil surface molecule expression, whole blood from normal healthy mice was stained with the following antibodies: anti-mouse Ly6G-BV421 (clone: 1A8, BD Biosciences), CD11b-PE (clone: M1/70, BD Biosciences), ActRIIA-APC (cat: FAB340A, R&D Systems), CD11a-FITC (clone: M17/4, BD Biosciences), CD45-FITC (clone: RA3-6B2, BD Biosciences), CD64-FITC (clone: X54-5/7.1.1, BD Biosciences), TLR2-FITC (clone: 6C2,

ebioscience), and TLR4-FITC (clone: MTS510, BD Biosciences). For analysis of intracellular neutrophil iNOS and Arg1 production, peripheral blood samples were stained with extracellular markers Ly6G-BV421 (clone: 1A8, BD Biosciences), CD11b-PE (clone: M1/70, BD Biosciences) and ActRIIA-APC (cat: FAB340A, R&D Systems), fixed and permeabilized using the Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific), and finally stained with anti iNOS-FITC (cat: 610331, BD Biosciences) and Arg1-FITC (cat: IC58689, R&D Systems) antibodies, respectively. All flow cytometric analyses were carried out on the BD FACSAria II flow cytometer (BD Biosciences) and the results were analyzed using FlowJo Version 10.

Bacterial clearance assay

ActRIIA$^+$ and ActRIIA$^-$ peritoneal neutrophils were sorted by flow cytometry. For the bacterial clearance assay, ActRIIA$^+$ and ActRIIA$^-$ neutrophils (5×10^5 cells /well) were incubated with *S. aureus* at a multiplicity of infection (MOI) of 5:1 for 4 h, samples with viable bacteria were diluted and cultured on TSA plates overnight at 37°C in bacterial incubator, and the CFU were counted.

Neutrophil phagocytosis

Carboxylate-modified fluorescent microspheres (Nile Red, Thermo Fisher) were diluted in RPMI-1640 medium and added to neutrophils (1×10^6 cells/ml) sorted by flow cytometry at a particle:cell ratio of 20:1. Following incubation for 45 min at 37°C, neutrophils were collected for analysis of the phagocytic capability using flow cytometry. The phagocytic ability was quantified as the percent of phagocytic cells per total neutrophils.

Detection of intracellular ROS

Neutrophils purified by MACS were cultured in RPMI-1640 medium containing 25 μM DCFDA (Sigma) for 20 min at 37 °C and stimulated with 100 nM PMA (Sigma) for a further 15 min. Following 3 washing steps with cold PBS to remove excess DCFDA, the cells were stained with Ly6G-BV421 (clone: 1A8, BD Biosciences), CD11b-PE (clone: M1/70, BD Biosciences) and ActRIIA-APC (cat: FAB340A, R&D Systems), and examined by flow cytometry. ROS production was represented as the mean fluorescence intensity (MFI).

Statistical Analysis

Statistical analysis, including Pearsons coefficient test and 2-tailed unpaired t tests, were carried out using Graph Prism 5.0. Data are expressed as mean $\pm$ SD, and values of $P<0.05$ were considered statistically significant.

Supplemental References

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