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Authors for correspondence:

Xueling Cui

e-mail: cxl@jlu.edu.cn

Zhonghui Liu

e-mail: liuzh@jlu.edu.cn

†These authors contributed equally to this study.

Activin A regulates activation of mouse neutrophils by Smad3 signalling

Yan Qi^{1,2,†}, Jingyan Ge^{2,†}, Chunhui Ma¹, Na Wu¹, Xueling Cui² and Zhonghui Liu^{1,2}

¹Department of Immunology, and ²Key Laboratory of Neuroimmunology, College of Basic Medical Sciences, Jilin University, Changchun 130021, People's Republic of China

ZL, 0000-0001-5551-7603

Activin A, a member of the transforming growth factor beta superfamily, acts as a pro-inflammatory factor in acute phase response, and influences the pathological progress of neutrophil-mediated disease. However, whether activin A can exert an effect on the activities of neutrophils remains unclear. In this study, we found that the release of activin A was enhanced from neutrophils of mouse when stimulated with lipopolysaccharide. Furthermore, neutrophils were not only the source of activin A but also the target cells in response to activin A, in which canonical activin signalling components existed, and levels of ACTRIIA, SMAD3 and p-SMAD3 proteins were elevated in activin A-treated neutrophils. Next, the role of activin A was determined in regulation of neutrophils activities. Our data revealed that activin A induced O₂⁻ release and reactive oxygen species production, promoted IL-6 release, and enhanced phagocytosis, but failed to attract neutrophils migrating across the trans-well membrane. Moreover, we found that effect of activin A on IL-6 release from the peritoneal neutrophils of mouse was significantly attenuated by *in vivo* Smad3 knockdown. In summary, these data demonstrate that activin A can exert an effect on neutrophils activation in an autocrine/paracrine manner through Smad3 signalling, suggesting that activin A is an important regulator of neutrophils.

1. Introduction

Activins are members of the transforming growth factor beta (TGF-β) superfamily, and have pleiotropic roles in physiological and pathological processes including regulation of embryogenesis, induction of mesoderm, protection of neurons, tumorigenesis and control of immune response [1–7]. So far, at least three different forms of activin have been identified, including activin A, activin B and activin AB [8,9]. Activin A has received the most attention until now, in part because it may play a crucial role in regulation of immune cells function [10].

Like most other TGF-β family members, activins share homology and canonical Smad-mediated signalling pathway with TGF-β. They conduct signalling by binding to type II activin receptor (ACTRII), forming a ligand/ACTRII complex, then the complex recruits type I activin receptor (ACTRI) to phosphorylates the recruited receptor-SMADs (SMAD2 and SMAD3) [11,12]. Once phosphorylated, SMAD2/3 dissociate from the receptor, bind to SMAD4, then the complex transmits the signal into the nucleus and promotes gene transcription [13–15].

Neutrophils are the most abundant white cell type in circulation that is recruited to the inflammatory sites rapidly in response to infection, injury and repair [16–18]. They have been established as the key mediator of multiple disease such as sepsis, acute respiratory distress syndrome (ARDS), rheumatoid arthritis, inflammatory bowel disease and different types of tissue injury [19,20]. Coincidentally, the strong expression of activin A has also been found in these diseases [6,21,22]. Several studies have demonstrated that activin A levels are elevated during sepsis, and serum concentrations of activin A in

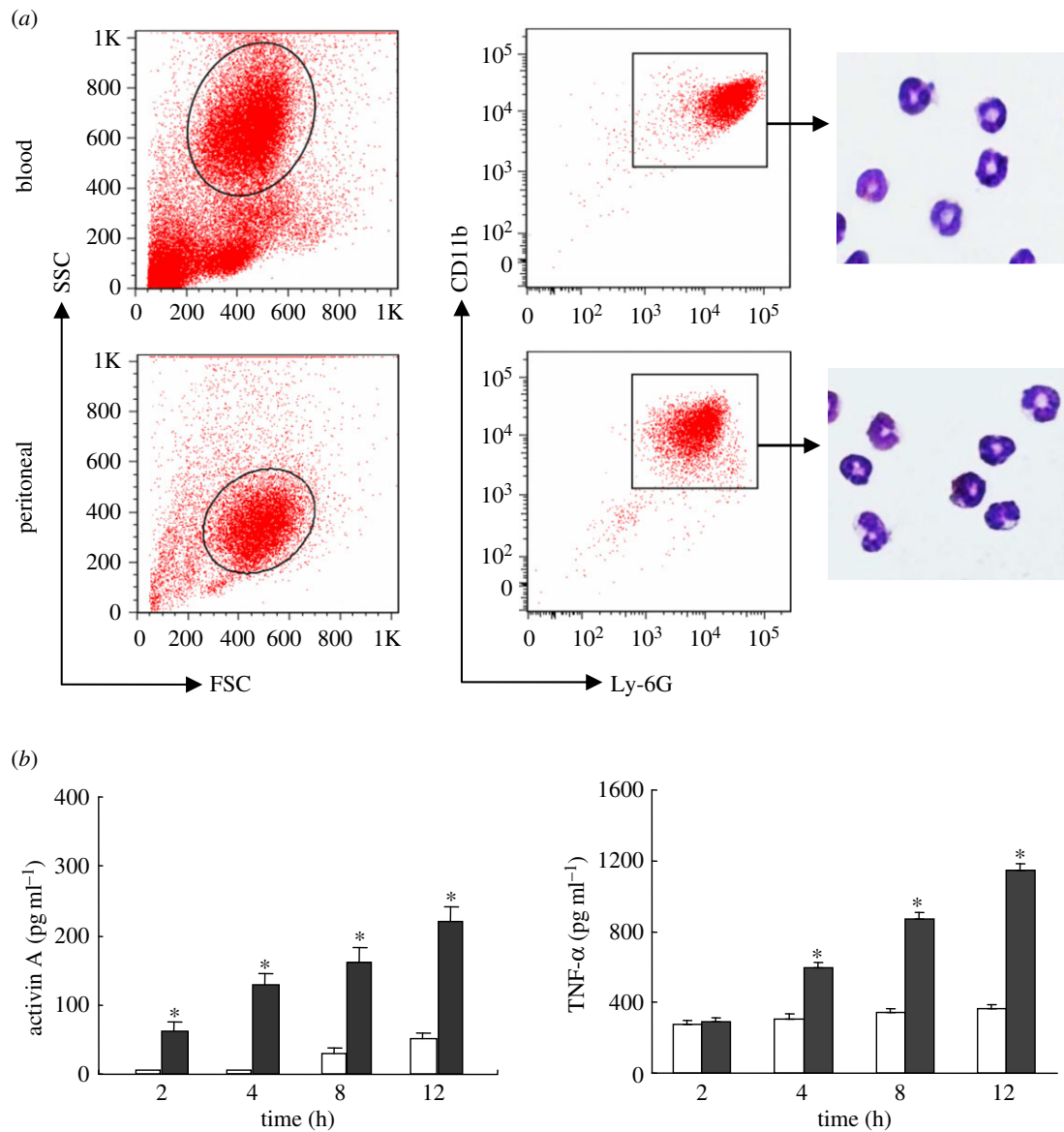


Figure 1. Release of activin A from mouse neutrophils treated with LPS. (a) CD11b⁺Ly-6G⁺ cells in peritoneal and peripheral blood cells of mouse were sorted by FACS, respectively, and assessed by cytology following diff-quick staining. (b) The levels of activin A and TNF- α in the supernatant of the cultured peritoneal neutrophils treated with 200 ng ml⁻¹ LPS were examined by ELISA. Open bar, culture medium control; filled bar, LPS.

Table 1. Primer sequences.

target	primers	sequences	products size (bp)	GenBank no.
<i>Gapdh</i>	sense	5'-GACTTCAACAGCAACTCCCACTC-3'	107	BC083149
	antisense	3'-TAGCCGTATTTCATTGCATACCAG-5'		
<i>Activin βA</i>	sense	5'-GAGAGGAGTGAAGTGTGCT-3'	514	NM_008380
	antisense	3'-ATGACTGTTGAGTGAAGGA-5'		
<i>ActRIIA</i>	sense	5'-ATTGCCAGCATCCATCTCTTG-3'	296	XM_123799
	antisense	3'-GCCACCATCATAGACTAGATTC-5'		
<i>ActRIIB</i>	sense	5'-TGCTGAAGAGCGACCTCAC-3'	544	NM_007397
	antisense	3'-AGCAGGTCCACATTGGTGAC-5'		
<i>Smad3</i>	sense	5'-CCAGCACACAATAACTTGA-3'	574	NM_016769
	antisense	3'-AGACACTGGAACAGCGGA-5'		

the patients who died from sepsis are higher than the survivors [23–25]. Additionally, the upregulated activin A is a pathogenic factor in the murine lung that causes a phenotype similar to ARDS [26]. Mounting evidence suggests that

activin A may influence the pathological progress of neutrophil-mediated disease [22,27].

Thus, it is meaningful to perform a search to investigate the relationship between neutrophils and activin A. However, we

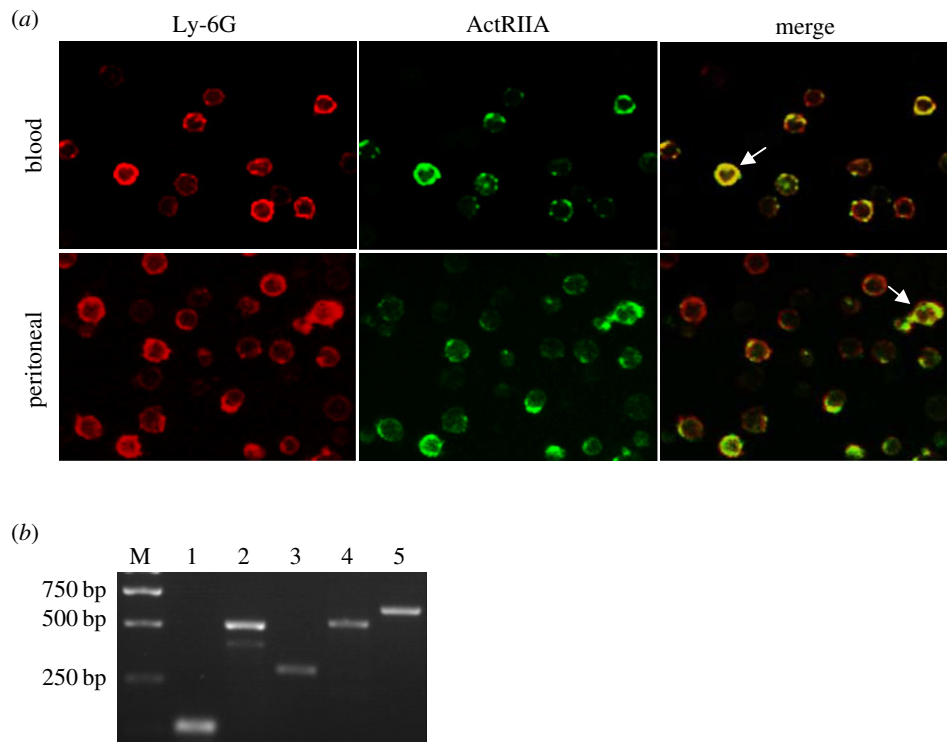


Figure 2. Expression of activin signalling components in mouse neutrophils. (a) Expression of ActRIIA on peritoneal or peripheral blood neutrophils was examined by dual immunofluorescent staining with anti-Ly-6G antibody (red) and anti-ActRIIA antibody (green). The yellow represented the superposition of ActRIIA and Ly-6G on neutrophils (merge). (b) The expression of *Actin β*, *ActRIIA*, *ActRIIB* and *Smad3* mRNA in the peritoneal neutrophils of mouse was examined by RT-PCR. M, molecular weight marker (bp); lane 1, *Gapdh* (107 bp); lane 2, *Actin β* (514 bp); lane 3, *ActRIIA* (296 bp); lane 4, *ActRIIB* (544 bp); lane 5, *Smad3* (574 bp). * $p < 0.01$, compared with control group.

know nothing about whether activin A can exert an effect on the activities of neutrophils until now. This study demonstrated for the first time that activin A could regulate neutrophil activities in an autocrine/paracrine manner.

2. Material and methods

2.1. Animals

Male C57BL/6 mice from 8 to 10 weeks were provided by the animal centre of Jilin University.

2.2. Reagents and antibodies

Activin A and APC-labelled mouse anti-ActRIIA antibody were purchased from R&D Company. PE-labelled rat anti-Gr-1 antibody and FITC-conjugated mouse anti-CD11b antibody were provided by eBioscience Company. The fluoSpheres carboxylate-modified red fluorescent microspheres (1 μm diameter) were supplied by Invitrogen. Superoxide detection kits and reactive oxygen species (ROS) assay kits were obtained from Beyotime Company.

2.3. Isolation of the neutrophils

Peripheral blood neutrophils of mouse were isolated by fluorescence-activated cell sorting analysis (FACS). Briefly, fresh blood of mice was incubated with 6% dextran T-70 (1:4) at room temperature for 20 min, and then the leucocyte-rich upper fraction was collected. The cells were stained with PE-conjugated anti-Ly-6G and FITC-conjugated anti-CD11b antibodies or with appropriate fluorochrome-conjugated

isotype IgG as control for 30 min. Neutrophils ($\text{CD11b}^+\text{Ly-6G}^+$) were sorted by FACS on BD FACSaria II.

The peritoneal neutrophils were prepared as described previously [23]. Briefly, 1 ml of 9% casein was injected into the peritoneal cavity per mouse, and after 24 h the same volume of casein was injected. Three hours later, peritoneal cells were collected and cultured in 10% fetal calf serum (FCS)-RPMI 1640 medium in 5% CO_2 at 37°C to remove the peritoneal macrophages [24]. One hour later, suspending cells were collected, and neutrophils ($\text{CD11b}^+\text{Ly-6G}^+$) were sorted by FACS. Neutrophils were evaluated by cytology following diff-quick staining.

2.4. Immunofluorescent staining

The type IIA receptor of activin (ActRIIA) and Ly-6G, a marker of mouse neutrophils, on neutrophils was examined by the dual immunofluorescent staining. In brief, the isolated neutrophils were incubated with rabbit anti-ActRIIA antibody for 60 min. After being washed with phosphate-buffered saline (PBS) three times, the cells were also incubated with FITC-conjugated goat anti-rabbit IgG antibody for 30 min, and then incubated with PE-conjugated anti-Ly-6G antibody for 30 min. Finally, the cells were observed under a fluorescence microscope.

2.5. RT-PCR

Total RNA from neutrophils was extracted using the TRIzol reagent according to the manufacturer's protocol (Invitrogen). PCR was performed using the one-step RT-PCR kit according to the manufacturer's instructions (Takara Biotechnology Co). PCR products were subjected to 1.5% agarose gel electrophoresis, and the specific bands were analysed

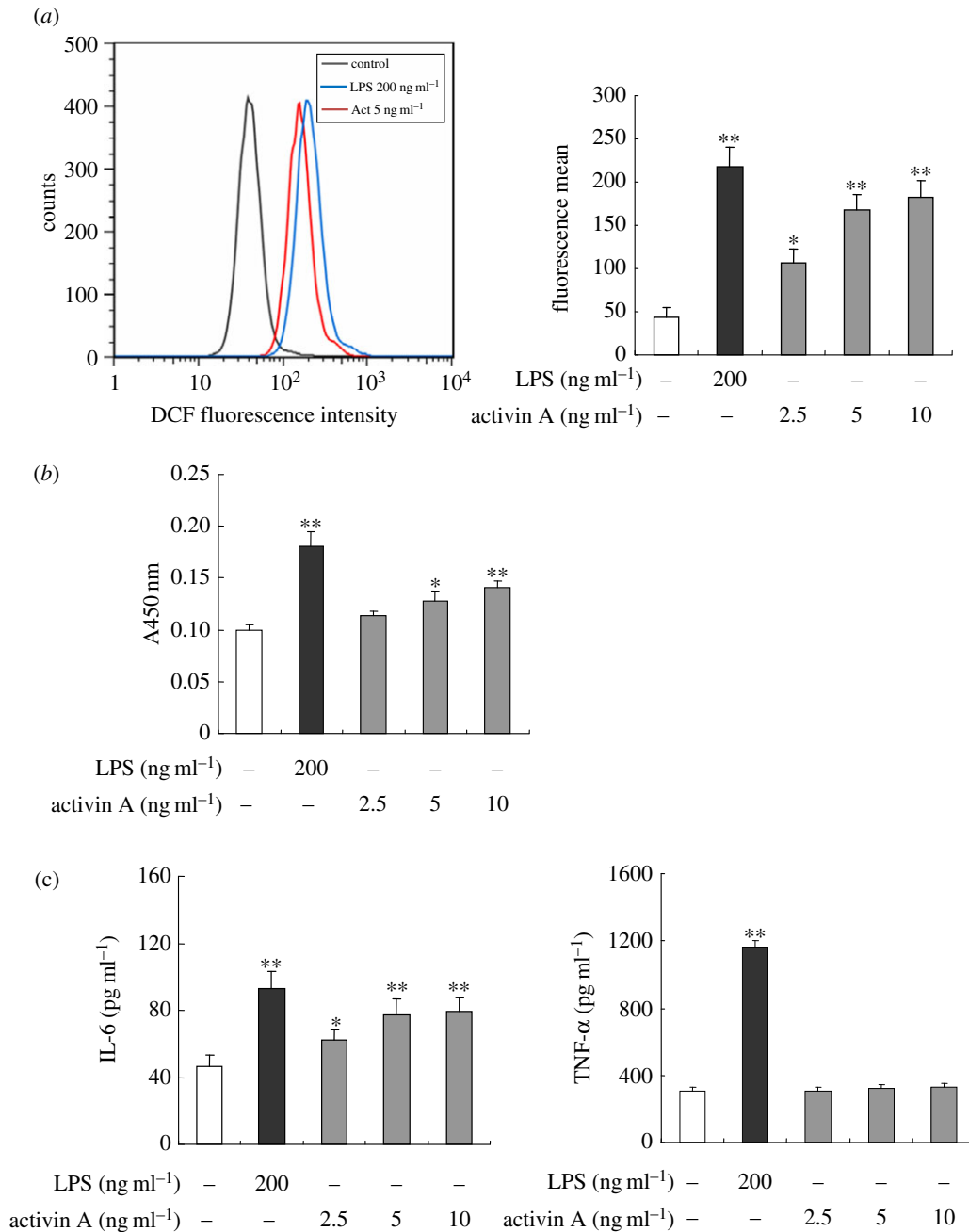


Figure 3. Effects of activin A on respiratory burst and IL-6 release of mouse neutrophils. (a) The peritoneal neutrophils of mouse were incubated for 30 min in the absence or presence of activin A and LPS, and then the intracellular ROS was detected using fluorescence probe DCFH-DA. Each bar represented the fluorescence mean from three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with control group. (b) O_2^- production was detected with superoxide detection kits. (c) The levels of IL-6 and TNF- α in the supernatant of the cultured neutrophils were assayed by ELISA. All values were presented as mean \pm s.d. of three independent experiments ($n = 6$). * $p < 0.05$, ** $p < 0.01$, compared with control group.

using ImageMaster VDS (Pharmacia Biotech Company). Primer sequences are available in table 1.

2.6. Detection of activin A

The peritoneal neutrophils of 1×10^6 /well were incubated in the absence or the presence of 200 ng ml⁻¹ lipopolysaccharide (LPS) for 2–12 h. The supernatants of the cultured neutrophils were collected, and activin A levels were detected using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (R&D).

2.7. Western blotting

The neutrophils of 2×10^6 /well were incubated in the absence or the presence of activin A for 2 h. The cells were lysed in

protein lysis buffer, and the lysate was cleared by centrifugation at 10 000 r.p.m. min⁻¹ for 20 min. The proteins in the supernatant were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was probed with anti-ACTRIIA, SMAD3, phosphorylated SMAD3 (p-SMAD3) and GAPDH antibodies, respectively. Finally, the labelled proteins were detected by chemiluminescence (ECL-Plus; Amersham Pharmacia Biotech).

2.8. Analysis of intracellular ROS

Intracellular ROS was detected by fluorescence probe DCFH-DA. DCFH-DA itself is without fluorescence, which can be hydrolysed by ester hydrolysis enzyme to DCFH inside the cells. ROS can oxidize DCFH without fluorescence into DCF with fluorescence. So the levels of intracellular ROS can be

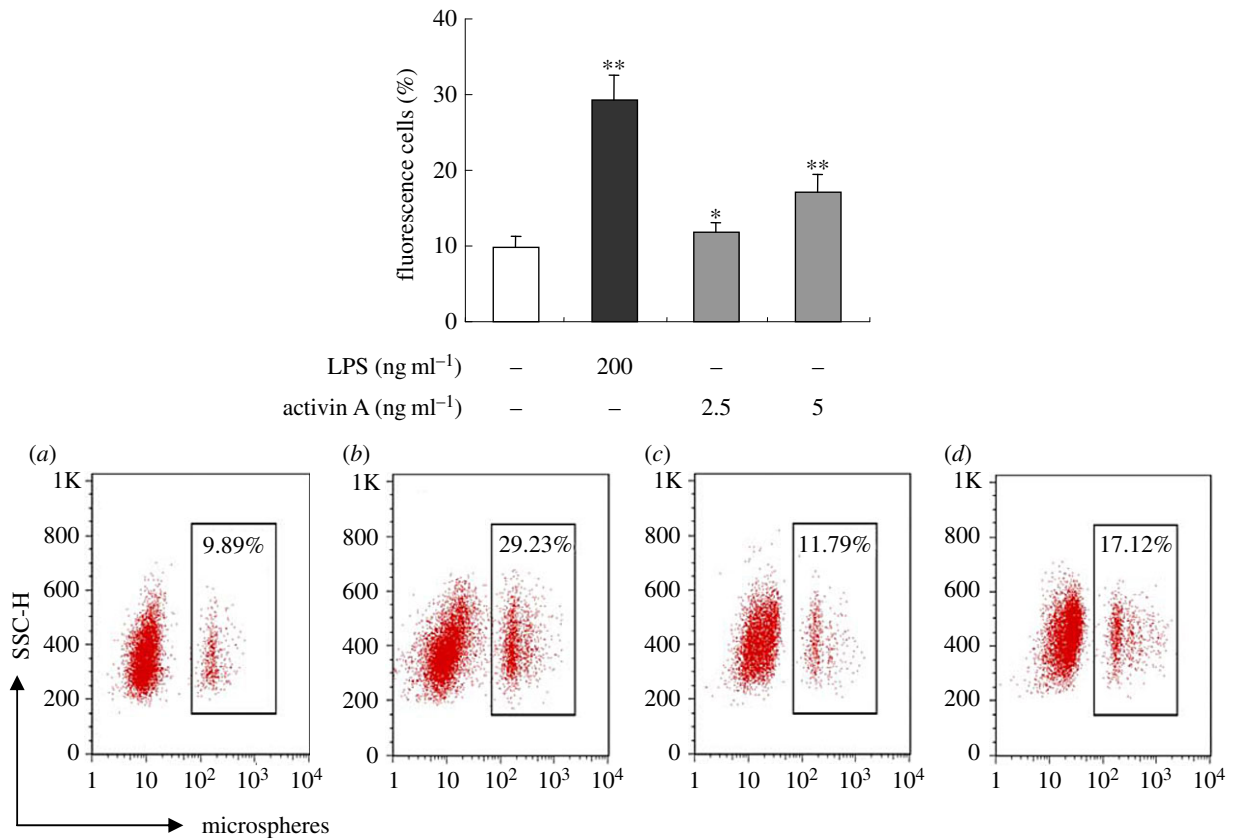


Figure 4. Effects of activin A on phagocytosis of mouse neutrophils. The peritoneal neutrophils of mouse were incubated in the absence or the presence of activin A and LPS for 12 h, and the phagocytic abilities of neutrophils to microspheres with red fluorescence were evaluated by flow cytometry. A representative experiment of the three performed is shown with (a) culture medium control, (b) 200 ng ml⁻¹ LPS, (c) 2.5 ng ml⁻¹ activin A and (d) 5 ng ml⁻¹ activin A. The graph represents the phagocytic capabilities of neutrophils to microspheres with red fluorescence from three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with control group.

detected by measuring fluorescence intensity of DCF. The isolated neutrophils were incubating with DCFH-DA in a humidified incubator containing 5% CO₂ at 37°C for 20 min. After incubation, 0–10 ng ml⁻¹ activin A or 200 ng ml⁻¹ LPS were added, and the increase of fluorescence was measured by flow cytometry.

2.9. Detection of superoxide

Superoxide (O₂⁻) that can decompose water-soluble tetrazolium salt WST-1 was detected with superoxide detection kits (Beyotime, China). Briefly, the neutrophils were stimulated with activin A or LPS in 5% CO₂ at 37°C for 12 h. After discarding the supernatant, the cells were incubated with WST-1 in dark at 37°C for 5 min. Absorbance was detected at 450 nm by an automated microtitre plate reader.

2.10. Detection of IL-6 and TNF-α

The supernatants of the cultured neutrophils in the presence or absence of activin A were collected, and levels of interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) were detected by ELISA kit according to the manufacturer's protocol (eBioscience).

2.11. Analysis of phagocytosis of neutrophils

The neutrophils of 1 × 10⁶/well were treated with activin A or LPS in 5% FCS/RPMI 1640 medium in 5% CO₂ at 37°C for 12 h, and then carboxylate-modified fluorescent microspheres with

red fluorescence were added for 1 h. Neutrophils were rinsed with PBS, and then the ratio of phagocytosis was examined with flow cytometry.

2.12. Neutrophil chemotaxis assay

Neutrophil chemotaxis was determined using trans-well chambers (3 μm pore size, Corning). Briefly, the neutrophils were incubated with 1 μmol l⁻¹ CFSE at 37°C for 10 min. The neutrophils (1 × 10⁶/well) labelled by CFSE with green fluorescence were loaded into the upper chamber and the lower chamber was full of 5% FCS-RPMI 1640 medium containing activin A or FMLP (Sigma) as positive control. The cells were cultured at 37°C in 5% CO₂ for 45 min. The number of cells that migrated to the underside of the membrane was counted under inverted fluorescence microscope.

2.13. Smad3 knockdown

Smad3 was knocked down *in vivo* with pGCsi-U6/Neo-Smad3 shRNA as described previously [28]. Briefly, 1 ml of 9% casein was injected into peritoneal cavity per mouse. After 24 h, the equivalent volume of casein was injected. 2 h later, each mouse was injected intraperitoneally with 3 μg pGCsi-U6/Neo-Smad3 shRNA-lipofectamine 2000 reagent complex in accordance with the manufacturer's protocol (Invitrogen) or with 3 μg pGCsi-U6/Neo-lipofectamine 2000 reagent complex as empty plasmid control. After 12 h, neutrophils were isolated from peritoneal cells and incubated in the presence or absence

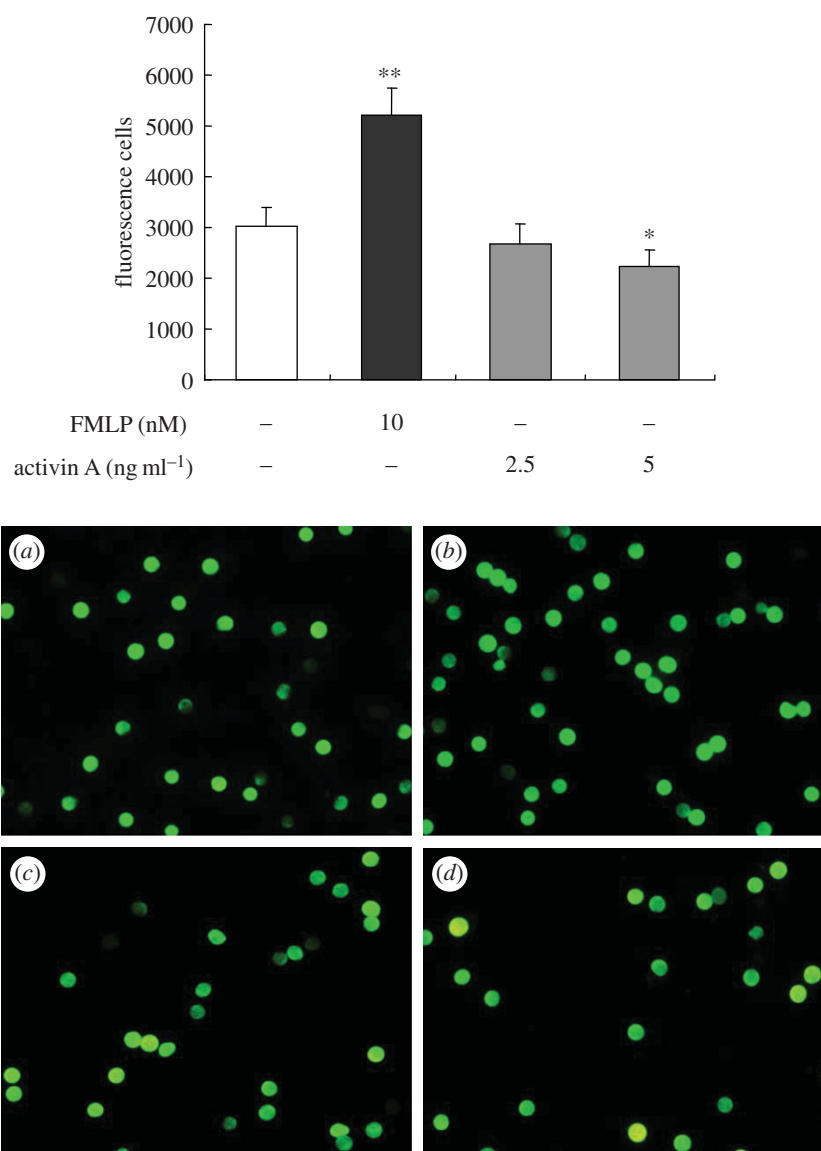


Figure 5. Assay of chemotaxis of mouse neutrophils treated with activin A. The migration of neutrophils labelled by CFSE with green fluorescence was determined using trans-well chambers and the number of cells that migrated to the underside of the membrane was counted under inverted fluorescence microscope. A representative experiment of the three performed is shown with (a) culture medium control, (b) 10 nmol l⁻¹ FMLP, (c) 2.5 ng ml⁻¹ activin A and (d) 5 ng ml⁻¹ activin A. The graph represents the migrated neutrophils from three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with control group.

of activin A for 12 h, and then the supernatants were collected and IL-6 levels were examined by ELISA.

2.14. Statistical analysis

All data are expressed as means \pm s.d. The data were analysed using a Student's *t*-test, and values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. LPS promotes the release of activin A from neutrophils

CD11b and Ly-6G are often used in combination to identify mouse neutrophils, thus we sorted CD11b⁺Ly-6G⁺ cells from peritoneal or peripheral blood cells with greater than 99.5% purity by FACS. The isolated cells which stained with diff-quick staining reagent had the typical neutrophil feature of the ring- and lobe-shaped nuclei (figure 1a). We

know that LPS can induce neutrophil activation, and TNF- α can stimulate human neutrophils to release activin A [29,30]. In this study, neutrophils were stimulated with LPS as agonist, and TNF- α and activin A production were measured by ELISA. The result showed that LPS not only promoted the release of TNF- α from neutrophils, but also enhanced the production of activin A (figure 1b).

3.2. Canonical activin signalling components exist in mouse neutrophils

To investigate whether neutrophils are able to sense activin A, we examined the expression of activin receptor by immunofluorescent staining. The results showed that Ly-6G and ACTRIIA were co-expressed on the isolated peritoneal and peripheral blood neutrophils (figure 2a). Next, the mRNA expressions of activin signalling components were measured in peritoneal neutrophils by RT-PCR. The results revealed that not only *Activin β A*, but also *ActRIIA*, *ActRIIB* and *Smad3* mRNA were expressed in peritoneal neutrophils of mouse (figure 2b). Thus, canonical activin signalling

components exist in mouse neutrophils, indicating that activin A may act on neutrophils.

3.3. Activin A modulates activities of mouse neutrophils

Neutrophils play a critical role in innate defence via their primary functions, such as respiratory burst and the release of the pro-inflammatory cytokines [31,32]. To investigate whether neutrophils can change their behaviour in response to activin A, we first assessed the effects of activin A on respiratory burst of neutrophils. The respiratory burst generates ROS from neutrophils, which is responsible for killing the invading microbes. In this study, we found that activin A induced O_2^- release and ROS production (figure 3*a,b*). Then, we evaluated the effects of activin A on IL-6 and TNF- α release from neutrophils. As shown in figure 3*c*, activin A promoted production of IL-6 in mouse neutrophils, but did not alter levels of TNF- α .

Neutrophils also have phagocytic activities to kill the invading bacteria and other foreign matter. Thus, the ability of phagocytosis of neutrophils was examined by flow cytometry. Our data revealed that LPS as positive control could obviously promote phagocytosis of neutrophils to microspheres with red fluorescence, compared with a control group, and activin A also significantly enhanced phagocytic ability of neutrophils to microspheres with red fluorescence (figure 4).

Finally, chemokines are known to regulate neutrophil recruitment to sites of infection or injury [26,27]. Two-dimensional trans-well chambers were used to examine whether activin A could affect neutrophil chemotaxis *in vitro*. We found that the chemoattractant FMLP attract neutrophils migrating to the lower chamber, whereas addition of activin A to the lower chamber reduced the number of neutrophils that migrated through the membrane (figure 5). Collectively, these data suggest that activin A is an important mediator in the regulation of neutrophil activation, but is not a potential chemoattractant for neutrophils.

3.4. Activin A enhanced phosphorylation of SMAD3 in neutrophils

Activin A combines activin receptors and activates the downstream signalling molecules SMAD3. In this study, the results revealed that ACTRIIA, SMAD3 and p-SMAD3 levels increased obviously in neutrophils stimulated by activin A (figure 6), suggesting that activin A may act as a regulator of neutrophils via ACTRIIA-SMAD3 signalling.

3.5. *Smad3* knockdown attenuated the effect of activin A on IL-6 release

To confirm *Smad3* signalling mediated activin A action, the peritoneal neutrophils were transfected with pGCsi-U6/Neo-GFP-*Smad3* shRNA *in vivo* to knock down *Smad3* gene expression (figure 7*a,b*). Additionally, the results of this study revealed that the effect of activin A on IL-6 release was significantly weakened after knockdown of *Smad3* gene in neutrophils (figure 7*c*). These findings further demonstrated that activin A as regulator could activate neutrophils through *Smad3* signalling.

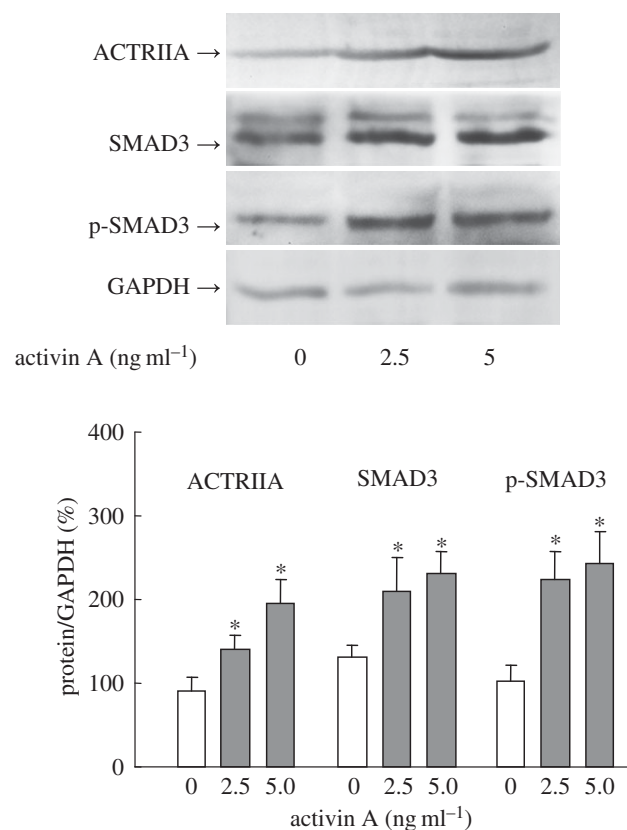


Figure 6. Expression of ACTRIIA and SMAD3 in mouse peritoneal neutrophils treated with activin A. The levels of ACTRIIA, SMAD3 and p-SMAD3 proteins in activin A-treated neutrophils were examined by western blotting. The graph represents relative protein levels from triplicate determinations. * $p < 0.01$, compared with control group.

4. Discussion

In this study, we found that activin A could be produced by LPS-stimulated neutrophils. More than that, activin A was able to regulate the functions of neutrophils, such as respiratory burst, IL-6 release, phagocytosis and migration. Furthermore, the effect of activin A on IL-6 release was significantly weakened after knockdown of *Smad3* in neutrophils. These data provide the evidence that activin A can regulate neutrophil activation in an autocrine/paracrine manner via *Smad3* signalling.

Upregulated expression of activin A has been observed in various acute and chronic inflammations. The production of activin A can be stimulated by pro-inflammatory cytokines such as LPS, TNF- α and IL-1 β [33–35]. The main source of activin A under inflammatory stimuli remains indeterminate, but various immune cells including monocytes, macrophages, mastocytes and neutrophils may be potential candidates [29,34,36–38]. These previous studies have reported that the cultured neutrophils secreted activin A *in vitro* when stimulated by TNF- α . Although it has also been claimed that LPS itself is insufficient to elicit activin A release from neutrophils *in vitro*, it significantly induced activin A mRNA expression [29,34]. In this study, we found that LPS were able to promote the release of activin A from neutrophils, further confirming that inflammatory stimuli, such as LPS and TNF- α can induce the release of activin A from neutrophils [29,34].

To investigate whether neutrophils are able to respond to activin A, we first examined the expression of activin

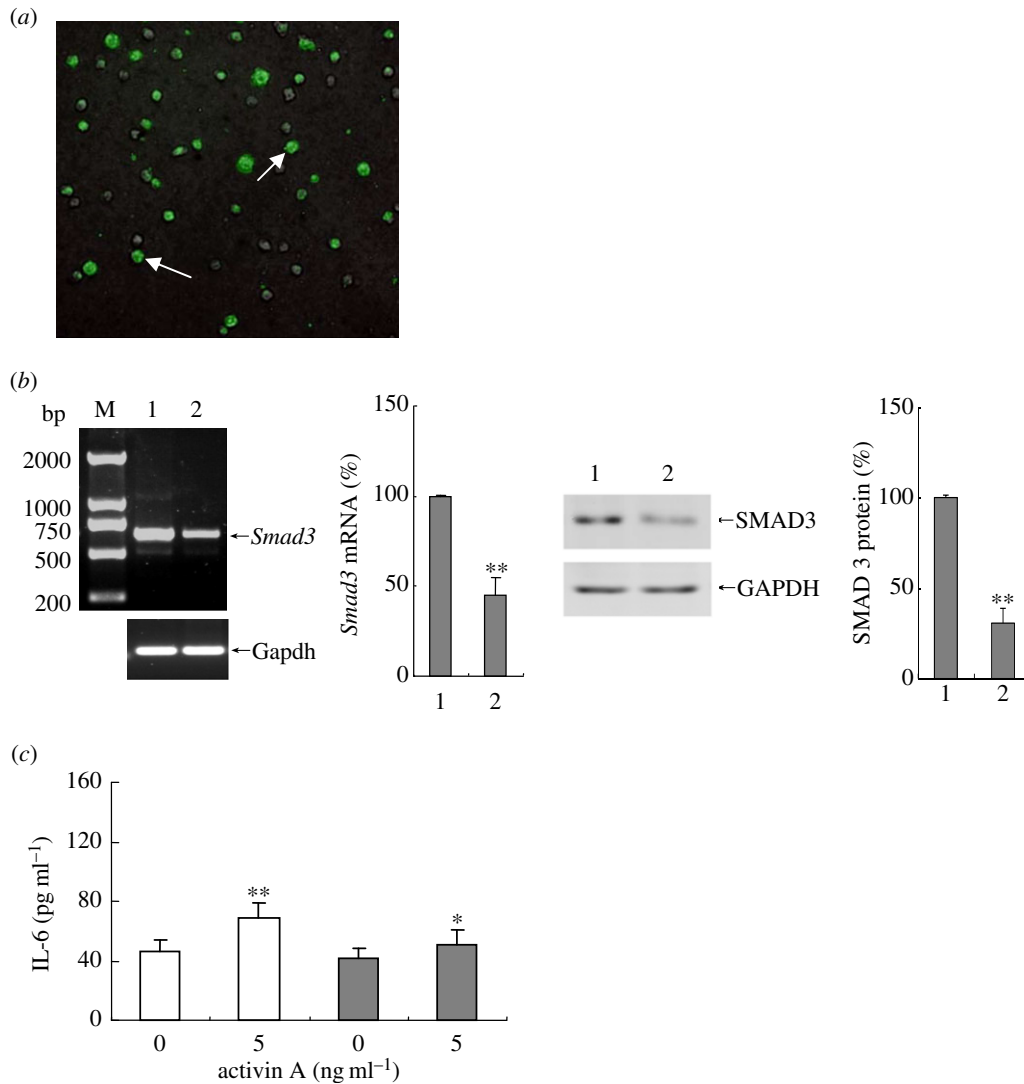


Figure 7. Assay of IL-6 production from *Smad3* knockdown neutrophils. (a) The isolated peritoneal neutrophils transfected *in vivo* with pGCSI-U6/Neo-GFP-*Smad3* shRNA (green) were observed under fluorescence microscope. (b) Expression of *Smad3* mRNA and SMAD3 protein in neutrophils transfected with pGCSI-U6/Neo (plasmid control) and pGCSI-U6/Neo-*Smad3* shRNA (*Smad3* shRNA) was examined by RT-PCR and western blotting. M, molecular weight marker (bp); lane 1, plasmid control; lane 2, *Smad3* shRNA. The graph represents the relative *Smad3* mRNA and SMAD3 protein levels from three independent experiments. ** $p < 0.01$, compared with plasmid control. (c) IL-6 levels in the supernatant of the cultured plasmid control (open bar) and *Smad3* knock-down (filled bar) neutrophils were examined by ELISA. ** $p < 0.01$, compared with 0 ng ml⁻¹ activin A plasmid control group; * $p < 0.05$, compared with 5 ng ml⁻¹ activin A plasmid control group.

signalling components. The results showed that not only activin receptor, but also other canonical activin signalling components, exist in mouse neutrophils. In addition, expressions of ACTRIIA, SMAD3 and p-SMAD3 proteins were elevated in activin A-treated neutrophils. Taken together, these data demonstrate that neutrophils not only produced activin A, but also were the target cells in response to activin A.

Next, to determine the role of activin A in regulation of neutrophil activities, respiratory burst, release of the pro-inflammatory cytokines and phagocytic ability of neutrophils were further analysed. We found that activin A induced O₂⁻ release and ROS production, enhanced phagocytosis, and promoted IL-6 release in mouse neutrophils. Moreover, *Smad3* knockdown significantly attenuated effect of activin A on IL-6 release in neutrophil. These data indicate that activin A may induce neutrophil priming and play an important role in neutrophil activation via *Smad3* signalling.

Interestingly, activin A is not a potential chemoattractant for neutrophils and addition of activin A to the lower

chamber reduced the number of neutrophils that migrated through the membrane. Neutrophils migrate from the bloodstream to sites of infection or injury to kill the invading bacteria and other foreign matter, but the highly destructive capacity of neutrophils can also raise the potential to damage the host is healthy tissues [39,40]. Activin A has been generally accepted as a pro- and anti-inflammatory mediator depending on both cellular context and stage of disease [36,41–44]. It is possible that activin A plays a dual role in regulating neutrophil functions. We suppose that, on the one hand, activin A may induce the activation of neutrophils in the early phase of inflammation; on the other hand, the high level of activin A enriched at inflammatory sites might stop the development of inflammation by restricting the migration of neutrophils in the late phase of inflammation. But our study was limited to knowing the effects of activin A *in vivo*, so more comprehensive scrutiny of the effect of activin A in neutrophil-mediated disease, such as sepsis, ARDS, rheumatoid arthritis and different types of tissue injury, is highly warranted.

In summary, these data demonstrate that activin A can exert an effect on neutrophil activation in an autocrine/paracrine manner through Smad3 signalling, suggesting that activin A is an important regulator of neutrophils.

Ethics. All animal experiments were conducted according to the Jilin University guidelines for the care and use of animals.

Authors' contributions. Y.Q., J.G. and C.M. performed experiments and analysed data; Y.Q. and N.W. carried out the statistical analyses;

X.C. and Z.L. participated in the design of the study; J.G., X.C. and Z.L. drafted the manuscript.

Competing interests. We declare we have no competing interests.

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