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Activin A inhibits activities of lipopolysaccharide-activated macrophages via TLR4, not of TLR2

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ABSTRACT

Activin A, a member of TGF-β superfamily, is involved in either pro-inflammatory or anti-inflammatory responses. Our previous studies have reported that lipopolysaccharide (LPS) can simulate activin A secretion from macrophage, and activin A can induce rest macrophage activation in mice, but inhibit the activities of the activated macrophages. However, the relationship of activin and LPS actions and their mechanism are not well characterized. In the present study, the results showed that both activin A and LPS promoted the phagocytic activities of mouse peritoneal macrophages *in vivo* and *in vitro*, but activin A inhibited the phagocytosis of LPS-activated macrophages. Simultaneously, the results revealed that activin A inhibited the Toll-like receptor 4 (TLR4) expression on LPS-activated mouse peritoneal macrophages *in vivo* and *in vitro,* whereas there was no obvious change of TLR2 expression. Moreover, the results showed that activin A obviously reduced the TLR4 mRNA and protein expressions in LPS-activated macrophage cell line RAW264.7 cells, and the inhibitory effect of activin A on the TLR4 expression was significantly attenuated in Smad3 knock-down RAW264.7 cells. Interestingly, LPS promoted the expression of activin type IIA receptor (ActRIIA) on mouse peritoneal macrophages *in vivo*, and also up-regulated ActRIIA and activin signal molecules Smad2, 3 mRNA expressions. These data suggest that activin A inhibits its LPS action on macrophages *in vivo* via suppressing TLR4 expression, and LPS further augments the negative feedback action of activin A via up-regulating activin signaling transduction.

1. Introduction

Activin A is a multifunctional growth and differentiation factor of the transforming growth factor-β (TGF-β) superfamily, also called immunological inhibitor restrictin-P [3,24]. Activin A is a critical controller of inflammation, immunity and fibrosis. Its expression levels are increased in several inflammatory diseases such as septicemia, inflammatory bowel disease, as well as rheumatoid arthritis [31,9]. Activin A as a pro-inflammatory factor is involved in the pathogenesis of fibrotic diseases [19,22,27]. Meanwhile, activin A also acts as a potential anti-inflammatory cytokine, and is involved in the regulation of acute phase response in inflammatory diseases [12].

Lipopolysaccharide (LPS), the Gram-negative bacterial wall component, is one of the most potent pathogen-derived inflammatory stimuli. It induces a massive release of cytokines and other inflammatory mediators in the infected host. It is now well understood that LPS mediates its effects through a member of the highly conserved Toll-like receptor (TLR) family, leading to the induction of numerous pro-inflammatory genes in most cell types. LPS also induces differentiation and maturation of monocytes/macrophages [29,4,7]. Previous studies have demonstrated that activin A can inhibit the function of LPS-activated macrophages *in vitro* and *in vivo*, and exert antagonistic effect on IL-6 and IL-1β [24,26,17]. The latest study reveals that activin A is a critical component of the inflammatory response, and it is rapidly released into the blood following a LPS-induced challenge in experimental animals [13], which raises the possibility that it plays a significant role in the acute phase response via inducing inflammatory mediators [29,26]. However, relationship of activin and LPS actions on macrophages and their mechanism are not well characterized.

In the present study, mouse peritoneal macrophages and mouse macrophage cell line RAW264.7 cells were used to investigate the mechanism of activin A and LPS action on macrophages, and it was found that activin A inhibited the activities of LPS-activated macrophages *in vivo* via down-regulating TLR4 and LPS further augmented the negative feedback action of activin A via

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up-regulating activin type IIA receptor (ActRIIA) expression. These data suggest that activin A may promote the rest macrophage activation to enhance the body's defense capabilities in the early acute phase response, but play anti-inflammatory roles in the late inflammatory response.

2. Materials and methods

2.1. Mice

Male Balbc mice (6 weeks of age, 18–20 g) were provided by Animal Center of Jilin University (Changchun, China) and all animal experiments were conducted in accordance with the Jilin University guidelines for the care and use of animals.

2.2. Reagents

Activin A was purchased from R&D Company (Minneapolis, MN, USA); LPS (lipopolysaccharide, from Escherichia coli 0111:B4) was obtained from Sigma (St. Louis, MO, USA); FluoSpheres® carboxylatemodified red fluorescent microspheres (1 μm diameter) were provided by Invitrogen (California, CA, USA); Monoclonal antibodies against mouse ActRIIA, ActRIIB, TLR2 and TLR4 were obtained from R&D Company and eBioscience company (San Diego, CA, USA); RPMI-1640 medium were provided by GIBCO (Grand Island, NY, USA); One step reverse transcription-PCR (RT-PCR) kit was purchased from Takara Biotechnology Co (Kyoto, Japan).

2.3. Assay of phagocytosis of mouse peritoneal macrophages in vitro

The rest mouse peritoneal macrophages were prepared as previously described [29] and seeded into 12-well tissue culture plates at a density of 5×10^5 cells/ml, and incubated in 1 ml of 2.5% FCS-RPMI 1640 medium in the presence or absence of activin A (5 ng/ml), LPS (0.5 μg/ml), and LPS (0.5 μg/ml) plus activin A (5 ng/ml) at 37 °C for 12 h in a humidified 5% CO_2 and 95% air incubator, respectively, followed by incubated with 1×10^7 microspheres/well for 1 h. Then the macrophages were rinsed with pH7.4, 0.01 mol/l phosphate-buffered saline (PBS). Phagocytosis ratio was analyzed by flow cytometry (New Jersey, USA, BD Calibur).

2.4. Assay of phagocytosis of mouse peritoneal macrophages in vivo

Mice were injected intraperitoneally with activin A (20 ng/ml), LPS (2 μg/ml), LPS (2 μg/ml) plus activin A (20 ng/ml) or 1 ml of PBS, respectively [32]. 12 h later, 500 μl of 1×10^8 microspheres were injected into the abdominal cavity for 1 h, and then the peritoneal cavity was injected with 1 ml PBS to harvest peritoneal macrophages. After washing macrophages, the phagocytosis ratio was analyzed by flow cytometry.

2.5. Flow cytometry

The cells were incubated with FITC-conjugated anti-mouse TLR2, TLR4, ActRIIA and ActRIIB antibodies or FITC-conjugated IgG as isotype control for 30 min at 4 °C, respectively. The FITC-labeled cells were analyzed by flow cytometry (BD Calibur). The data were collected and analyzed with Cell Quest software (BD Biosciences) to assess the percentage of fluorescence positive cells.

2.6. Real-time quantitative RT-PCR

Total RNA from macrophages was extracted using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, UK). The levels of ActRIIA, ActRIIB, Smad2 and Smad3 mRNA expression were examined by real-time quantitative RT-PCR as previously described [15]. The primers was designed as follows: Smad2 forward primer 5′-ATGGCCGTCTTCAGGTTTCACA-3′ and reverse primer 5′-ACTCTGTGGCTCAATTCCTGCT-3′; Smad3 forward primer 5′-CGGTC AAGAGCCTGGTCAAGA-3′ and reverse primer 5′-TTGAAGGCGAAC TCACACAGC-3′; ActRIIA forward primer 5′-ATTGGCCAGCATCC ATCTCTTG-3′ and reverse primer 5′-TGCCACCATCATAGACTAGATT C-3′; ActRIIB forward primer 5′-TGCTGAAGAGCGACCTCAC-3′ and reverse primer 5′-AGCAGGTCCACATTGGTGAC-3′; TLR4 forward primer 5′-CTTCATTCAAGACCAAGCCTTTC-3′ and reverse primer 5′-AACCGATGGACGTGTAAACCAG-3′. GAPDH forward primer 5′-GATTGTTGCCATCAACGACC-3′ and reverse primer 5′-GTGCAGGA TGCATTGCTGAC-3′.

2.7. Smad3 knock-down

To further confirm activin A action on macrophages, RAW264.7 cells were transfected with pGCsi-U6/Neo-Smad3 shRNA to knock-down activin signaling molecule Smad3 gene and with empty plasmid pGCsi-U6/Neo as control, respectively. The stable transformation was selected by G418, and then the cells were subcloned to collect the stably transfected RAW264.7 cells. The cells were further incubated in 2.5% FCS-RPMI 1640 medium in the presence or absence of activin A (5 ng/ml), LPS (0.5 μg/ml), and LPS (0.5 μg/ml) plus activin A (5 ng/ml) for 12 h, respectively, and the expression of TLR4 was examined by flow cytometry.

2.8. Statistical analysis

The data were expressed as means ± standard deviation (SD) and statistic analysis was performed with the software SPSS13.0. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Activin A affected the phagocytosis of mouse peritoneal macrophages in vitro and in vivo

The flow cytometry results showed that the phagocytosis ratio of activin A group and LPS group was higher than that of control group. However, activin A decreased the phagocytic activities of LPS-treated macrophages *in vitro* and *in vivo* (Fig. 1), and there was significant difference in phagocytosis ratio in LPS plus activin A group, compared with that in LPS group ($P < 0.01$). These data confirm that activin A can stimulate rest macrophage activation in mice, and inhibit activities of the activated macrophages as previously described.[13]

3.2. Activin A inhibited the expression of TLR4 on the LPS-treated peritoneal macrophages in vitro and in vivo

As LPS receptors on macrophages, TLRs can transfer the signal into cells, which results in activation of macrophages. In this study, we found that TLR4 expression on activin A plus LPS-treated mouse peritoneal macrophages *in vitro* and *in vivo* decreased obviously, compared with that on LPS-treated macrophages ($P < 0.01$), whereas there was no obvious change of TLR2 expression (Fig. 2). These data indicated that the inhibitory effect of activin A might be related to LPS-TLR4 pathway.

3.3. Smad3 knock-down attenuated the inhibitory effect of activin A on TLR4

To further confirm activin A action on macrophages via TLR4, mouse macrophage cell line RAW264.7 cells were used to

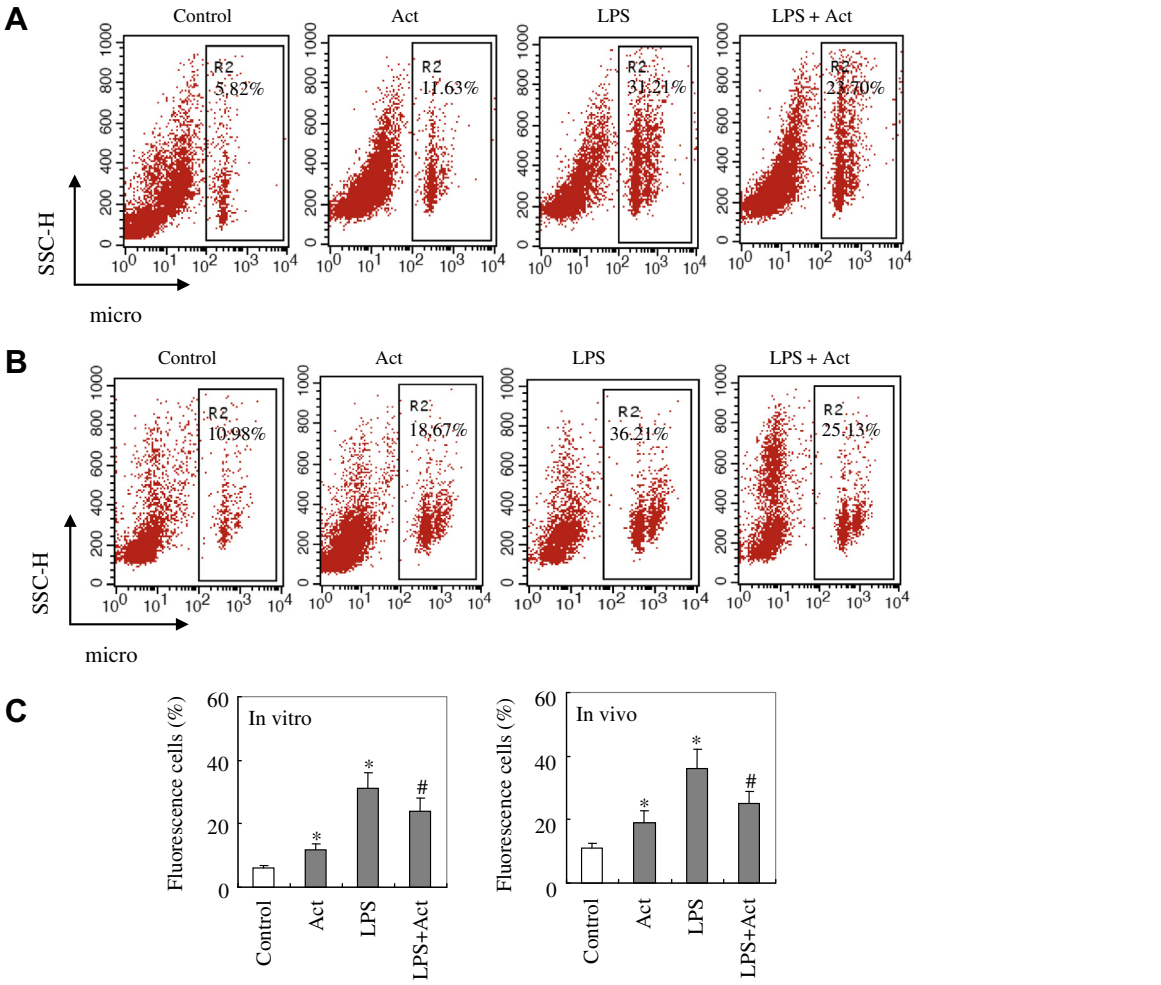

Fig. 1. Analysis of phagocytosis of mouse peritoneal macrophages *in vitro* and *in vivo*. (A) Phagocytosis of mouse peritoneal macrophages to microspheres *in vitro* was evaluated by flow cytometry 12 h after untreated (Control) or treated with activin A (Act), LPS (LPS), and LPS plus activin A (LPS + Act), respectively. (B) Phagocytosis of mouse peritoneal macrophages to microspheres *in vivo* was examined by flow cytometry 12 h after injected intraperitoneally with activin A, LPS, LPS plus activin A or 1 ml of PBS as control. (C) The graph represents the percent of positive fluorescence cells from three independent experiments. $^{*}P < 0.01$, compared with control. $^{\#}P < 0.01$, compared with LPS group.

investigate activin signaling molecule Smad3 knock-down on TLR4 expression. The results showed that TLR4 mRNA and protein expressions on LPS-stimulated RAW264.7 cells were evidently up-regulated, and the expression on activin A plus LPS-treated cells were remarkably decreased, compared with that in LPS group (Fig. 3A and B). Furthermore, Smad3 knock-down RAW264.7 cell line was established by stably transfected with pGCsi-U6/Neo-Smad3 shRNA (Fig. 3C), and the results showed that inhibitory effect of activin A on TLR4 expression was significantly attenuated in Smad3 knock-down RAW264.7 cells (Fig. 3D). These data further demonstrated that activin A inhibited TLR4 expression on macrophages.

3.4. LPS promoted the expression of ActRII and Smad2, 3 in macrophages in vivo

Previous studies have reported that LPS can simulate activin A secretion from macrophage [26]. In the present study, we found that ActRIIA expression on LPS-stimulated mouse peritoneal macrophages *in vivo* was evidently up-regulated, whereas there was no obvious change of ActRIIB expression (Fig. 4A and B). Furthermore, the results showed that LPS promoted the expression of ActRIIA mRNA, and up-regulated the expression of Smad2, 3 mRNA in macrophages, but did not alter ActRIIB mRNA expression (Fig. 4C). The above results suggested that LPS might enhance the negative feedback actions of activin A via up-regulating ActRIIA-Smad signal transduction.

4. Discussion

Activin A as a multifunctional factor plays an important role in the early development of embryos, hematopoiesis, cell apoptosis, hormone releases from anterior pituitary cells, the acute phase response and neurotrophicity in the various systems [10,20,16,21,33]. Activin A can also directly regulate the development of monocyte/macrophages, antibody production from B cells, T cell type 2 subsets activation and immune responses [6,11]. It is well documented that activin A can induce rest macrophage activation in mice, and stimulate monocyte/macrophages from a range of species to produce many inflammatory mediators, such as IL-1β, NO, PGE2 and thromboxanes [29,17]. However, other studies have indicated that, when macrophages are in an activated state, activin A can play anti-inflammatory roles, such as inhibiting the conversion of the IL-1β precursor to its active form or suppressing the production of key pro-inflammatory mediators TNF-α, IL-6 and NO in LPS-activated macrophages [13,32,15,10,5,25,28,18].

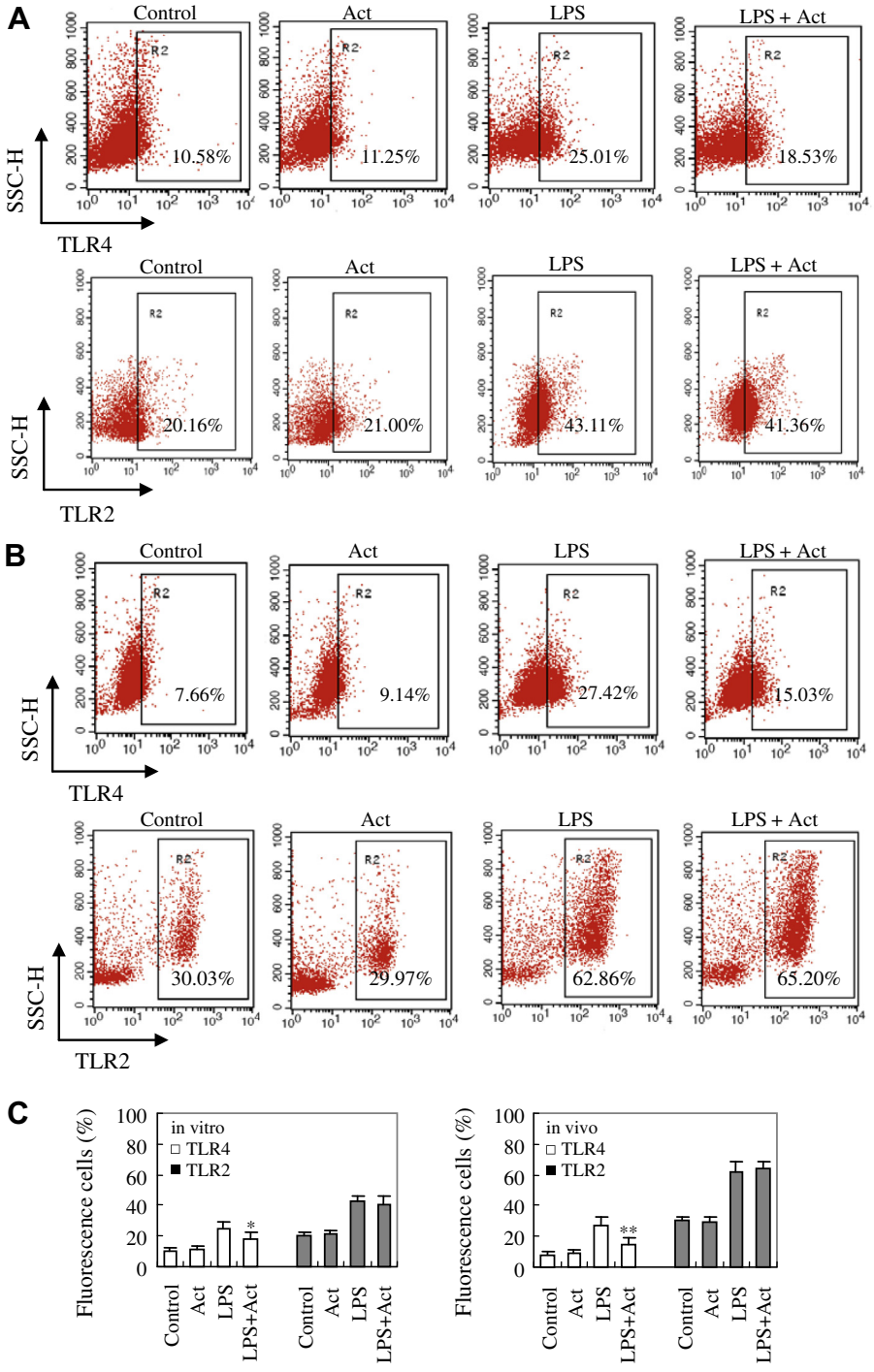

Fig. 2. Detection of TLR2 and TLR4 expressions on mouse peritoneal macrophages by flow cytometry *in vitro* and *in vivo*. (A) TLR2,4 expressions on mouse peritoneal macrophages *in vitro* were examined by flow cytometry 12 h after untreated or treated with of activin A and LPS, respectively. (B) TLR2,4 expressions on mouse peritoneal macrophages *in vivo* was examined by flow cytometry 12 h after injected intraperitoneally with activin A, LPS, LPS plus activin A or 1 ml of PBS. (C) The graph represents the percent of positive fluorescence cells from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with LPS group.

Macrophages can secrete inflammatory factors and phagocytose large antigen particles, including various pathogenic microorganism, apoptotic cells and tumor cells, etc. Macrophages lyse and digest the phagocytosed microbes inside the phagolysosome via oxygen-dependent or oxygen-independent mechanisms. Thus macrophages play an important role in innate immune responses [30,14]. Our previous studies reported that activin A stimulated rest mouse peritoneal macrophages activation *in vitro* and *in vivo*, and promoted pinocytosis and phagocytosis of macrophage [29,26,25], whereas it had been observed that activin A significantly inhibited the phagocytosis of LPS-activated macrophages *in vitro* and *in vivo* [13,32]. However, it is not well characterized that activin A inhibits over-activation of macrophages by what mechanism.

It is now understood that the effect of LPS is mediated through a member of the highly conserved Toll-like receptor (TLR) family, among which is TLR4, leading to the induction of numerous pro-inflammatory genes in most cell types,

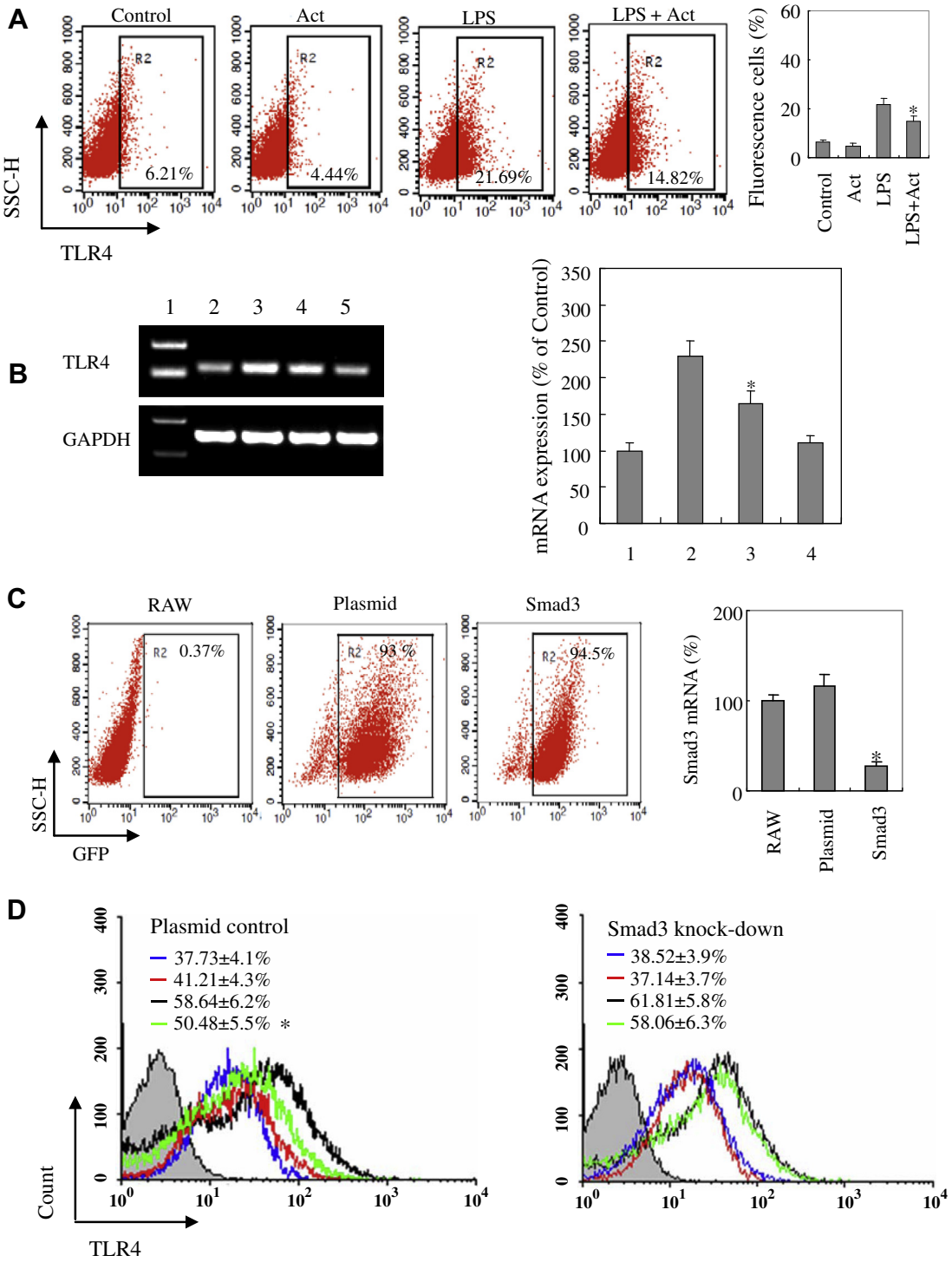

Fig. 3. Assay of TLR4 expression on Smad3 knock-down RAW264.7 cells. (A) TLR4 expression on RAW264.7 cells was detected by flow cytometry in the presence or absence of activin A and LPS, respectively. The graph represents the percent of positive fluorescence cells from three independent experiments. (B) The levels of TLR4 mRNA expression in RAW264.7 cells were analyzed by real-time quantitative RT-PCR. Lane1, molecular weight; lane2, culture medium control; lane3, LPS; lane3, LPS plus activin A; lane4, activin A. The graph represents TLR4 mRNA expressions in RAW264.7 cells from three independent experiments. The mRNA levels in control group were adjusted to 100%. $^{*}P < 0.01$, compared with LPS group. (C) The stably transfected RAW264.7 cell line was established. The percent of fluorescence cells was evaluated by flow cytometry in RAW264.7 cells (RAW), RAW264.7 cells transfected stably with empty plasmid pGCsi-U6/Neo-GFP (Plasmid), and transfected stably with pGCsi-U6/Neo-GFP-Smad3 shRNA (Smad3). The graph represents Smad3 mRNA expression in RAW264.7 cells group from three independent experiments. The mRNA levels in control group were adjusted to 100%. $^{*}P < 0.01$, compared with plasmid control. (D) TLR4 expressions on plasmid control or Smad3 knock-down RAW264.7 cells were examined by flow cytometry 12 h after incubated in the absence (—) or presence of activin A (—), LPS (—) and LPS plus activin A (—), respectively. Value in each profile showed the percent of positive fluorescence cells from three independent experiments. $^{*}P < 0.01$, compared with LPS group.

particularly macrophages [1]. The previous studies have demonstrated that LPS induces the release of activin A through TLR4 pathway [13,8]. In the present study, we investigated the effect of activin A on TLRs expressions of LPS-activated macrophages. As previously described, the results showed that both activin A and LPS promoted the phagocytic activities of mouse peritoneal

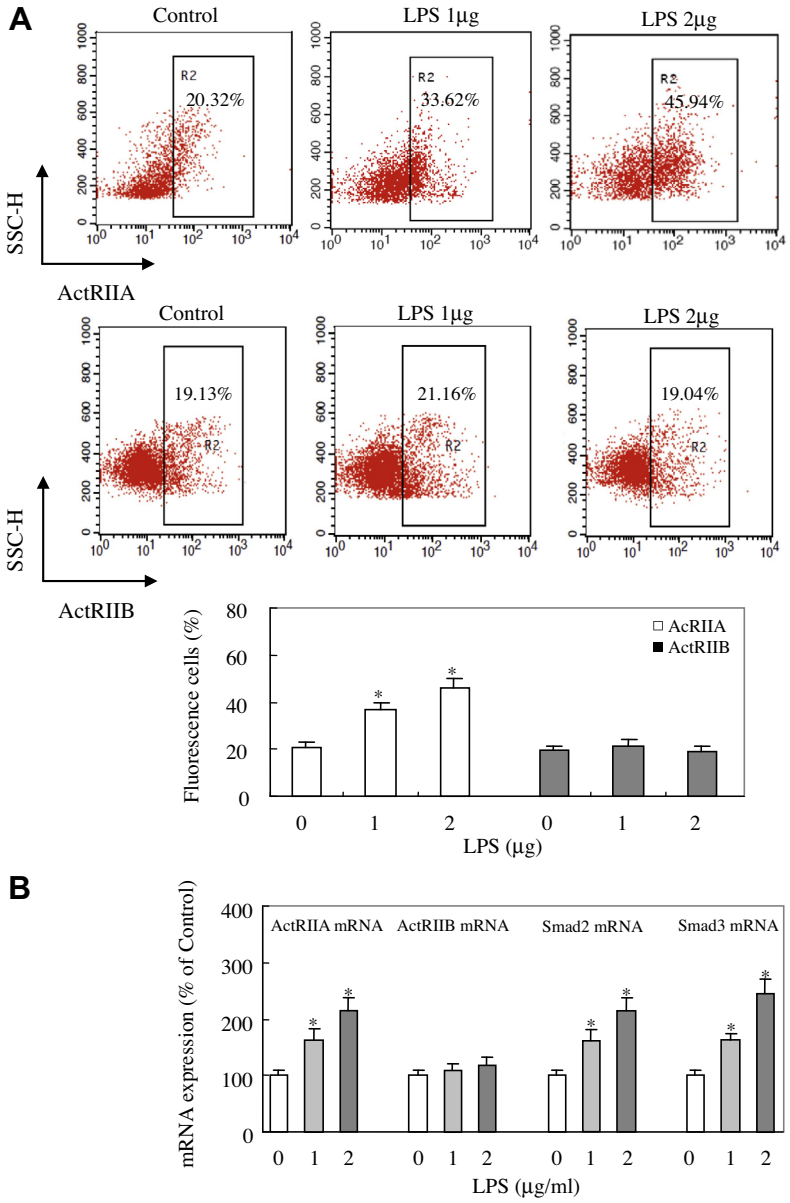

Fig. 4. Assay of ActRII and Smad2,3 expressions in macrophages. (A) ActRIIA and ActRIIB expressions on the surface of mouse peritoneal macrophages were examined by flow cytometry 12 h after injected mice intraperitoneally with LPS (1 μg, 2 μg/ml), or 1 ml of PBS. The graph represents the percent of positive fluorescence cells from three independent experiments. (B) ActRIIA,IIB and Smad2,3 mRNA expressions in mouse peritoneal macrophages were analyzed by real-time quantitative RT-PCR. The mRNA levels in control group were adjusted to 100%. All values were expressed as mean ± SD from three independent experiments. $^{*}P < 0.01$, compared with control.

macrophages *in vivo* and *in vitro*, respectively, but activin A inhibited the phagocytosis of LPS-activated macrophages. Simultaneously, the results revealed that activin A inhibited the Toll-like receptor 4 (TLR4) expression on LPS-activated mouse peritoneal macrophages *in vivo* and *in vitro,* whereas did not alter the obvious change of TLR2 expression. Moreover, it was also found that activin A could obviously reduce the expression of TLR4 mRNA and protein on LPS-induced RAW264.7 cells. These findings indicated activin A might play an inhibitory role in LPS-activated macrophages by suppressing LPS-TLR4 pathway.

Activin signaling is transmitted primarily via type II receptors (ActRII) and type I receptor (ActRI). In the case of TGF-β or activin, type I receptors alone display essentially negligible affinity for ligand. Activin binds directly to the type II receptor that can recruit type I receptor and the activation of ActRI propagates the signal through a cascade response elicited by Smad proteins into the nucleus [23,2]. Activin shares their intracellular signaling pathway via Smad2,3 with other members of the TGF-β superfamily [8], Although the previous studies have reported that LPS can simulate activin A production from macrophage, whether LPS can alter activin A signal transduction to affect activin action *in vivo* is still unclear. Interestingly, we found that LPS obviously up-regulated ActRIIA expression on mouse peritoneal macrophages *in vivo*, whereas did not alter ActRIIB expression on macrophages. Simultaneously, the results showed that LPS also up-regulated the expressions of ActRIIA and Smad2,3 mRNA. Moreover, the results revealed that inhibitory effect of activin A on the TLR4 expression was significantly attenuated in Smad3 knock-down mouse macrophage line RAW264.7 cells. These data suggested that LPS might further augment the negative feedback regulatory function of activin A on the activated macrophages via up-regulating activin signal transduction.

Taking together, these data suggest that activin A plays an important negative role in regulation of the activated mouse macrophage *in vivo* by suppressing LPS-TLR4 pathway, and LPS itself may further augment the negative feedback regulatory function of activin A via stimulating avtivin A release from macrophages [26,13] and activating activin signal transduction. Thus, as a pro-inflammatory factor, activin A can stimulate rest macrophage activation in an early acute response, which enhances the body's defense capabilities, and as an anti-inflammatory factor, activin A may play anti-inflammatory roles via inhibiting the activities of activated macrophages in a late inflammatory response.

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