Article

A Critical Role of Activin A in Maturation of Mouse Peritoneal Macrophages *in vitro* and *in vivo*

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Activin A, a multifunctional factor of the transforming growth factor-beta (TGF-β) superfamily, is mainly produced by microglia and macrophages, and its anti-inflammatory and pro-inflammatory activities are both related to macrophage functions. However the direct effect of activin A on the rest macrophages *in vivo* remains unclear. In the present study, the results showed that activin A not only increased NO and IL-1β release, but also promoted phagocytic abilities of mouse peritoneal macrophages *in vitro* and *in vivo*, whereas it did not influence MHC I and MHC II expression. Moreover, we found that activin A significantly upregulated the expressions of CD14 and CD68, markers of mature macrophages, on the surface of macrophages *in vitro* and *in vivo*. These data suggest that activin A can induce primary macrophage maturation *in vitro* and *in vivo*, but may not trigger the acquired immune response *via* regulating expression of MHC molecules involved in presentation of antigen. *Cellular & Molecular Immunology*. 2009;6(5):387-392.

Key Words: activin A, macrophage, maturation, CD68, CD14

Introduction

Activin is a multifunctional factor of transforming growth factor-beta (TGF-β) superfamily (1-3). Homo- or hetero-dimerization of two inhibin β subunits (βA and βB) form three types of activins, activin A (βAβA), activin B (βBβB) and activin AB (βAβB). Activin A acts as a potential anti-inflammatory cytokine, and is involved in the regulation of acute phase response in inflammatory diseases (4-8). Meanwhile, activin A is also a pro-inflammatory factor, which is involved in the pathogenesis of fibrotic human diseases (8-10).

Activin A is mainly produced by microglia and macrophages and its anti-inflammatory and pro-inflammatory activities are both related to macrophage functions (5, 11-13). When induced by inflammatory stimuli, tissue resident macrophages become activated and release pro-inflammatory mediators, interleukin 1β (IL-1β), IL-6 and nitric oxide (NO) to initiate defense responses. At the same time, pinocytic and phagocytic activities of macrophages are enhanced greatly, and the expression of MHC molecules that are involved in presentation of antigen is increased. Previous studies have demonstrated that as an anti-inflammatory cytokine, activin A can inhibit the function of lipopoly-saccharide (LPS)-activated macrophages *in vitro* and *in vivo*, and exert antagonistic effect on IL-6 and IL-11 (13-16). However, the latest study reveals that insulin results in regulation of macrophage activity in response to endotoxin through the release of activin A (17), and our studies report that activin A can induce the production of inflammatory mediator NO from mouse peritoneal macrophages *in vitro* and activation of mouse macrophage cell line RAW264.7 cells directly (11, 18). However, effect of activin A on activation and maturation of rest macrophages *in vivo* are not well discovered.

In the present study, the effect of activin A on rest macrophages was investigated in mouse peritoneal macrophages. We found that activin A not only increased the production of NO and IL-1β, promoted phagocytic activities of mouse peritoneal macrophages *in vitro* and *in vivo*, but also enhanced the expressions of CD14 and CD68, which are markers of mature macrophages. These data suggest that activin A can directly induce primary macrophage maturation

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Abbreviations: TGF-β, transforming growth factor β; NO, nitric oxide; IL-1β, interleukin 1β; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay.

and activation *in vitro* and *in vivo*.

Materials and Methods

Reagents and antibodies

Recombinant human activin A (Act) was obtained from R&D (Minneapolis, MN 55413). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) were purchased from Sigma (St. Louis, MO, USA); IL-1β ELISA kit and mouse monoclonal antibody against CD14 were purchased from eBioscience (San Diego, CA, USA); and antibodies against MHCI, MHCII, CD68 were obtained from Serotec (Oxford, UK).

Culture of mouse peritoneal macrophages

Female C57BL/6 mice at 8 wk of age were obtained from the Laboratory Research Center of Jilin University (Changchun, China). Mouse peritoneal macrophages were obtained by lavaging the peritoneal cavity with 5 ml of ice-cold sterile RPMI 1640 medium, and cultured in a humidified incubator containing 5% CO_2 at 37°C in 10% fetal calf serum (FCS)-RPMI 1640 medium for 1 h (11). After incubation, the non-adherent cells were removed by washing with serum-free medium and the adherent cells were seeded in 12-well plates at a density of 1×10^6 cells/well and incubated in 5% FCS-RPMI 1640 medium in the presence or absence of activin A (0.8 - 10 ng/ml) for 24 h, respectively.

Detection of NO levels

NO levels in the culture supernatant were determined by NO kit according to the manufacturer's protocol (NJJC, Nanjing, China). The absorbance was measured at 540 nm on an automated microtiter plate reader. Nitrite concentration was calculated by comparison with a standard curve generated with sequential dilutions of standard sodium nitrite.

Enzyme-linked immunosorbent assay for IL-1β

IL-1β levels were measured in the culture supernatant by using a commercial two-site mouse IL-1β enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol. The absorbance was then detected at 450 nm to evaluate the IL-1β levels.

In vitro assay of mouse peritoneal macrophage phagocytosis

To evaluate phagocytic capability of mouse macrophages, chicken red blood cells (cRBC) were used as antigen particles. Macrophages were treated with or without activin A (2 ng/ml or 5 ng/ml) in 5% FCS-RPMI 1640 medium at 37°C in a humidified incubator containing 5% CO_2 at 37°C for 24 h, and then macrophages were incubated in 5% FCS-RPMI 1640 medium containing 0.5% cRBC for 1 h. Macrophages were rinsed with pH7.4, 0.01 mol/L phosphate-buffered saline (PBS). After fixed with 4% paraformaldehyde, the cells were stained with Wright-Giemsa dye. Phagocytosed cRBC were examined with light microscopy and a minimum of 200 macrophages were counted in each well. The phagocytosis ratio and index of macrophages were calculated as follows: phagocytosis ratio (PR) = number of macrophages phagocyting chicken red blood cells / number of macrophages; phagocytosis index (PI) = number of chicken red blood cells phagocytosed by macrophages / number of macrophages (19).

***In vivo* assay of mouse peritoneal macrophage phagocytosis**

Mice were injected with activin A (10 ng/200 μl or 20 ng/200 μl) or 200 μl of PBS intraperitoneally (20). On the next day, 500 μl of 2% cRBC were injected into the abdominal cavity 30 min before the mice were sacrificed. Then the peritoneal cavity was washed with 1 ml of PBS under aseptical conditions to collect peritoneal macrophages. The macrophages were incubated on a slide at 37°C in a humidified incubator containing 5% CO_2 at 37°C for 30 min. After fixed with 4% paraformaldehyde solution, adherent cells were stained with Wright-Giemsa dye and observed through light microscopy. Both PR and PI were also calculated as described above.

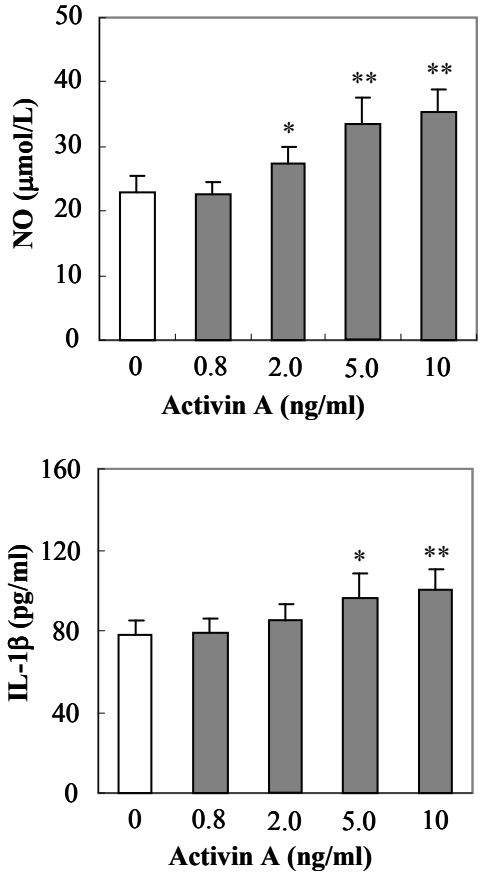

Figure 1. Assay of NO and IL-1β levels from cultured macrophages. Mouse peritoneal macrophages were incubated in the absence or presence of activin A (0.8 - 10 ng/ml). NO and IL-1β levels in the supernatant of cultured macrophages were examined. *$p < 0.05$, **$p < 0.01$, compared with control. All values are presented as mean ± SD of three independent experiments.

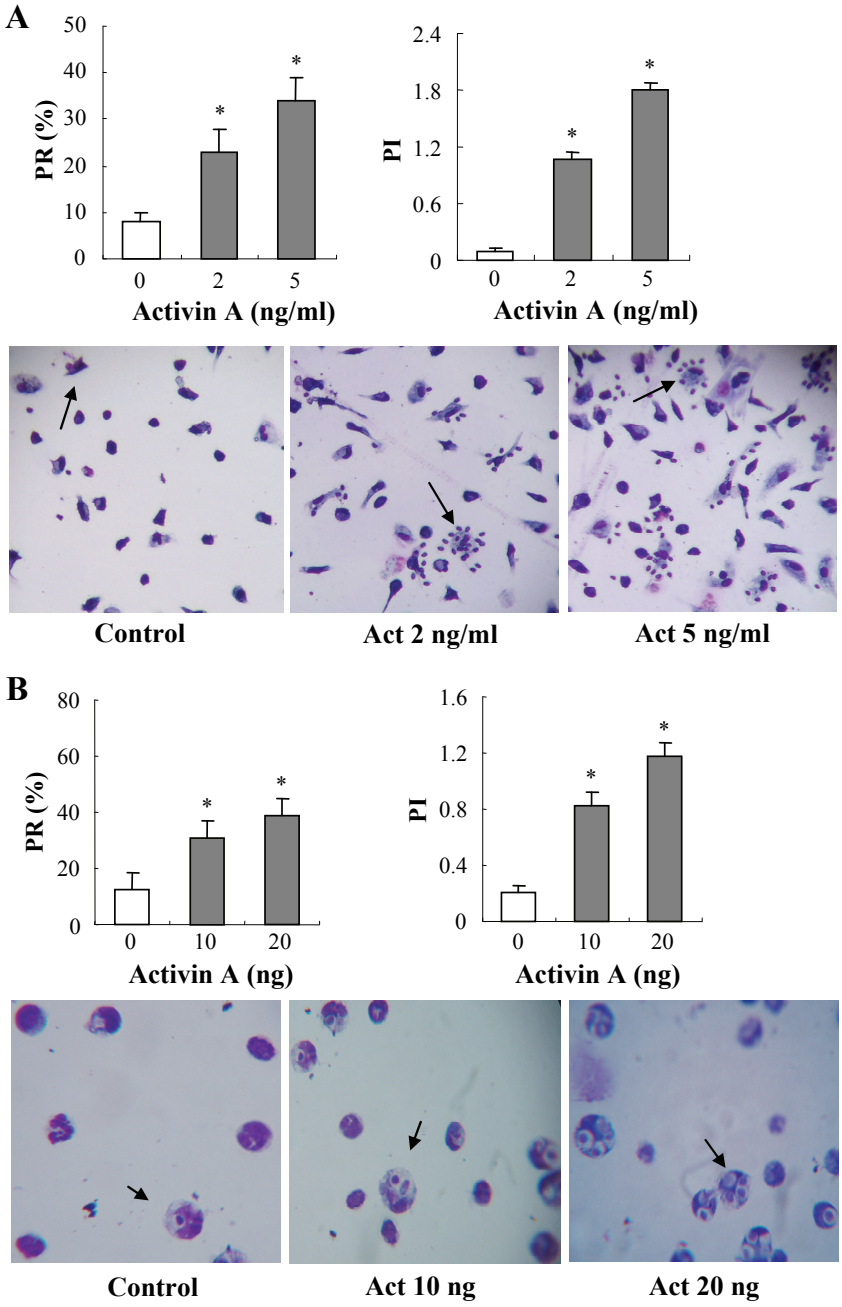

Figure 2. Analysis of phagocytosis of mouse peritoneal macrophages *in vitro* and *in vivo*. (A) Phagocytosis of mouse peritoneal macrophages to cRBC was evaluated after treated with or without activin A *in vitro*. (B) Phagocytosis of mouse peritoneal macrophages to cRBC was examined after treated with or without activin A *in vivo*. All values are presented as mean ± SD of three independent experiments. *$p < 0.01$, compared with control. Mouse peritoneal macrophages phagocytosing cRBC were stained with Wright-Giemsa dye and observed with light microscopy. The arrows represent the phagocytosed cRBC.

Flow cytometric analysis

The expressions of MHC I, MHC II, CD14 and CD68 on the surface of mouse peritoneal macrophages were examined by flow cytometry. Macrophages were incubated with IgG at 4°C for 30 min to block Fc-receptor. The cells were washed twice with cold buffer and then incubated with FITC-conjugated anti-mouse MHC I, MHC II, CD68, and CD14 antibody or FITC-conjugated IgG as isotype control for 30 min at 4°C, respectively. The FITC-labeled cells were analyzed with flow cytometry (FACSort Vantage; BD, Franklin Lakes, NJ). The data were collected and analyzed with Cell Quest software (BD Biosciences) to assess the

percentage of fluorescence positive cells.

Analysis of mouse peritoneal macrophage proliferation

Mouse peritoneal macrophages were seeded into 96-well culture plates at a density of 2×10^5 cells/ml, and incubated in 200 μl of 5% FCS-RPMI 1640 medium in the presence or absence of activin A (2 - 10 ng/ml) at 37°C for 24 h, respectively. The viable cells were stained with MTT for 4 h. Media were removed and the formazan crystala was dissolved by adding 200 μl of dimethylsulfoxide (DMSO). Absorbance was then detected at 570 nm to express the cell viabilities (21).

Statistical analysis

Data were expressed with means ± SD and statistic analysis was performed by Student's *t* test. $p < 0.05$ was considered statistically significance.

Results

Activin A induces NO and IL-1β productions in mouse peritoneal macrophages

Macrophages have shown to be activated and secreted a variety of inflammatory mediators in response to inflammatory stimuli. In this study, we found that activin A increased NO and IL-1β productions significantly from mouse peritoneal macrophages in a dose-dependent manner (Figure 1). These data suggested that activin A might directly inspire activation of rest mouse peritoneal macrophages.

Activin A enhances phagocytic activities of mouse peritoneal macrophages in vitro and in vivo

Phagocytic activities are the most important functions of activated macrophages in innate immune response. To assess direct effect of activin A on macrophage phagocytosis, the phagocytic activities of macrophages to cRBC were examined *in vitro* and *in vivo*. As shown in Figure 2, Activin A promoted the phagocytic activities of mouse peritoneal macrophages greatly *in vitro* and *in vivo*. We also found that activin A-activated macrophages had obviously morphological changes from round cells to irregular cells and the activated macrophages with an irregular shape had phagocytic ability to cRBC (Figure 2A). The above results revealed that by promoting phagocytic activities of macrophages *in vivo*, activin A played critical roles in macrophage-mediated innate immune response.

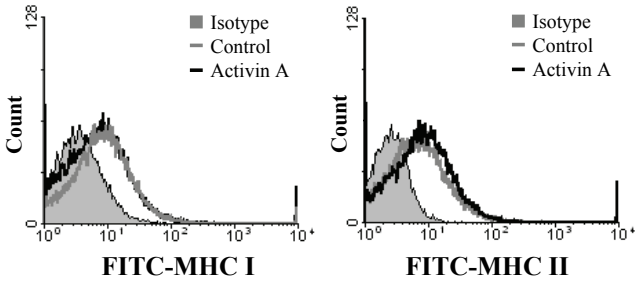

Figure 3. Flow cytometry assay of MHC I, MHC II on surface of mouse peritoneal macrophages. Mouse peritoneal macrophages were incubated overnight in the absence or presence of 5 ng/ml activin A *in vitro*. The cells were centrifuged, resuspended in 2% FCS-PBS, and incubated with 1 μg of FITC-conjugated anti-mouse MHC I or MHC II antibody for flow cytometry analysis. Isotype controls were analyzed in each case using FITC-conjugated IgG. Values (mean ± SD of three independent experiments) in the profile showed the percent of positive fluorescence cells. A representative experiment of the three performed is shown.

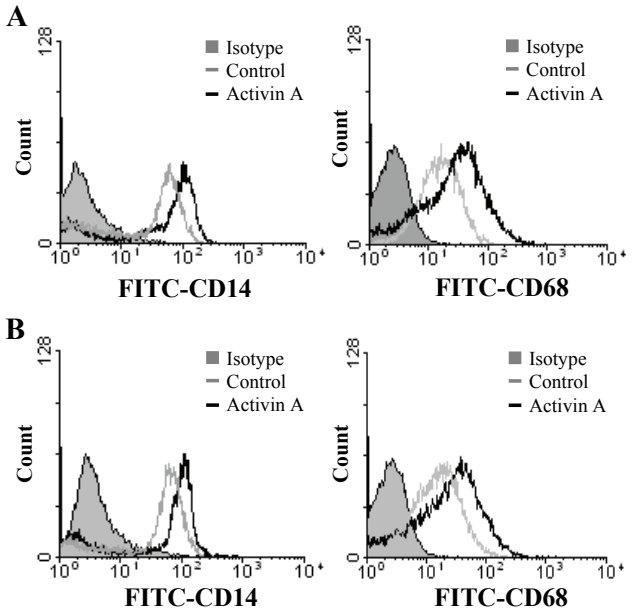

Figure 4. Detection of CD14 and CD68 expressions on surface of mouse peritoneal macrophages by flow cytometry *in vitro* and *in vivo*. (A) *In vitro* CD14 and CD68 expressions on mouse peritoneal macrophages were evaluated by flow cytometry. Mouse peritoneal macrophages were incubated overnight in the absence or presence of 5 ng/ml activin A, and isotype controls were analyzed in each case using FITC-conjugated IgG. (B) *In vivo* CD14 and CD68 expressions on mouse peritoneal macrophages were examined by flow cytometry. Mice were injected with 10 ng activin A/200 μl PBS or 200 μl PBS intraperitoneally. On the next day, the peritoneal cavity was washed with 2 ml of PBS to collect peritoneal macrophages. A representative experiment of the three performed is shown.

Activin A does not influence the expressions of MHC I and MHC II molecules on mouse peritoneal macrophages

MHC I and MHC II molecules are important surface molecules of macrophages, and involved in presentation of endogenous and exogenous antigen, respectively. To explore effect of activin A on antigen presentation by macrophages, the expression of MHC I and MHC II on surface of mouse peritoneal macrophages were examined by flow cytometry. The results revealed that activin A application did not influence MHC I and MHC II expressions on mouse

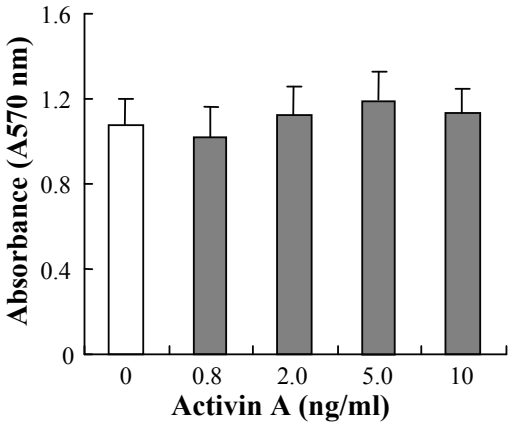

Figure 5. Proliferation assay of mouse peritoneal macrophages. Mouse peritoneal macrophages in 96-well culture plates were incubated for 24 h in the absence or presence of activin A (0.8 - 10 ng/ml). The cells were stained with MTT for 4 h. Media were removed and the formazan crystala was dissolved by adding dimethylsulfoxide. Absorbance was detected at 570 nm to express the cell viabilities.

peritoneal macrophages (Figure 3). These data indicated that activin A may have no effect on antigen presenting ability of mouse macrophages.

Activin A upregulates CD68 and CD14 expressions on mouse peritoneal macrophages

In the present study, we further examined the expressions of CD68 and CD14, surface markers of mature macrophages, on mouse peritoneal macrophages by flow cytometry, and found that their expressions were up-regulated on mouse peritoneal macrophages when treated by activin A *in vitro* and *in vivo* (Figure 4). These results suggested that activin A might promote the activation and maturation of mouse macrophages *in vivo*.

Activin A has no effect on the proliferation of mouse peritoneal macrophages

To further study the roles of activin A on regulation of activities of mouse peritoneal macrophages, proliferation of mouse peritoneal macrophages was examined by MTT colorimetric method. The results revealed that there was no significant difference in proliferation of mouse peritoneal macrophages after 24 h treatment with activin A (Figure 5). These findings suggest that activin A induces the activation and maturation of mouse peritoneal macrophages, but do not influence macrophage proliferation.

Discussion

Activin A plays a critical role in many physiological processes, including regulation of secretion of follicle-stimulating hormone (FSH) from anterior pituitary, differentiation of erythroblasts and embryonic development (22-24). Our previous studies have reported that activin A can promote inflammatory mediator NO production from mouse peritoneal macrophages *in vitro* and induce the activation of mouse macrophage cell line RAW264.7 cells directly (11, 18), which suggest that activin A may contribute to macrophage activation in the early stage of inflammation.

Activated macrophages secrete several inflammatory mediators including IL-1β, IL-6 and NO, which may result in tissue damage and systemic inflammation. In this study, we found that activin A increased not only NO release, but also IL-1β production from mouse peritoneal macrophages. In addition to secreting inflammatory mediators, macrophages can also phagocytose large antigen particles, including various pathogenic microorganism, apoptotic cells and tumor cells. We found that both the percentage of phagocytic macrophages and the average number of ingested cRBC were increased after treatment with activin A *in vitro* and *in vivo*. Our results reveal a regulatory role of activin A in the phagocytic process and provide further evidence of activin A-induced host resistance against invading microbes, as well as suggest that activin A is a critical direct activator for mouse rest peritoneal macrophages.

Macrophages play an essential role in the activation of the adaptive immune system *via* processing and presenting antigens. Although we have found that activin A up-regulated MHC II expression on mouse macrophage cell line RAW264.7 cells (18), our data in the present study showed that activin A did not alter MHC I and MHC II expressions on surface of mouse peritoneal macrophages. The reason for the discrepancy could be the different sources of macrophages, and macrophage-like cell line RAW264.7 cells may not reflect all the features of macrophages. These data indicate that activin A may not affect the antigen presenting activity of mouse macrophages *in vivo*.

CD68 and CD14 are cell membrane molecule markers for monocytes/macrophages (25, 26), highly expressed on activated and mature monocyte/macrophage surface, and CD14 acts as one component of CD14/TLR4/MD2 receptor complex which binds the LPS of gram negative bacteria and facilitates destruction of microbes and induction of various cytokines secretion involved in triggering a wide array of immune responses. We found that activin A significantly increased CD14 and CD68 expression on surface of mouse peritoneal macrophages. Meanwhile, there was no significant change in peritoneal macrophage proliferation after treated with activin A. These data suggest that activin A can induce the activation and maturation of mouse peritoneal macrophages, but do not influence cell proliferation.

Macrophages are present in all tissues of human body where they normally assist in guarding against invading pathogens and regulate tissue remodeling. However, they are also known to accumulate in large numbers in inflammation sites, and might cause tissue injury. Our previous studies have found that activin A *in vitro* and in vivo plays an important role in down-regulating the activation of LPS-induced mouse peritoneal macrophages, suggesting activin A may have the potential to inhibit over-inflammatory response in the late stage of inflammation (11, 20). Our present study

provides further insight toward the potential roles that activin A plays directly in the regulation of rest macrophage activation and maturation in the early stage of inflammation response.

Taken together, activin A may play important regulatory roles in mouse peritoneal macrophages by promoting the maturation and activation of mouse rest macrophages in the early phage of inflammation and inhibiting the over-activation of activated macrophages in the late stage of inflammation. Thus, activin A might have the potential for treatment of macrophage-mediated inflammatory diseases through dual modulation of macrophage functions.

Acknowledgments

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