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Activin A down-regulates the phagocytosis of lipopolysaccharide-activated mouse peritoneal macrophages *in vitro* and *in vivo*

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ABSTRACT

Activin A is a multifunctional factor of the transforming growth factor-beta (TGF-β) superfamily and acts as an anti-inflammatory cytokine produced by microglia and macrophages. In this study, we investigated the regulatory effect and possible mechanism of activin A on activation of lipopolysaccharide (LPS)-induced mouse peritoneal macrophages. The results showed that activin A could decrease NO release in LPS-activated mouse peritoneal macrophages, and suppressed phagocytosis and pinocytosis of mouse peritoneal macrophages stimulated by LPS *in vitro* and *in vivo*. Furthermore, activin A remarkably inhibited the expressions of CD14 and MHC II on LPS-induced mouse peritoneal macrophages, but had no significant effect on the expression of MHC I and the proliferation of mouse peritoneal macrophages. These findings suggest that activin A can down-regulate inflammatory mediator production and phagocytosis of LPS-activated macrophages *via* suppressing CD14 expression, and may influence the presentation of exogenous antigens *via* inhibiting MHC II expression. Thus, activin A might have the potential for treatment of macrophage-mediated inflammatory diseases through modulating both innate and adaptive immune responses.

1. Introduction

Monocytes/macrophages have an important role in innate immune defence against microbial infections, which involve phagocytic activities and secretion of pro-inflammatory mediators [1–3]. Thus, their over-activation may cause inflammatory disorders. Macrophages also play a critical role in acquired immune response *via* its antigen-presenting activity. Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria and acts as one of the most powerful activators of rest macrophages *via* binding the CD14/toll-like receptor 4 (TLR4)/MD2 receptor complex, which not only promotes overproduction of pro-inflammatory mediators and phagocytosis, but also induces differentiation and maturation of monocytes/macrophages [2–5].

Activin is a multifunctional factor of transforming growth factor-beta (TGF-β) superfamily [6–8]. There are three types of activins, formed by homo- or hetero-dimerization of two inhibin β subunits (βA and βB), activin A (βAβA), activin B (βBβB) and activin AB (βAβB). Activin A (ActA) acts as an antagonist of interleukin 6 and interleukin 11 [9], which can be produced by macrophages, and is involved in the acute phase response in inflammatory diseases [10–13]. Recently our studies have demonstrated that activin A can inhibit the production of another inflammatory mediator, nitric oxide (NO) in mouse peritoneal macrophages [2] and pinocytosis of LPS-activated macrophage cell line RAW264.7 cells *in vitro* [14]. However, the *in vivo* regulatory effects of activin A as anti-inflammatory factor on phagocytosis of macrophages are still unclear.

Here, we investigated effects of activin A on phagocytosis of mouse peritoneal macrophages, MHC I and MHC II expressions on surface of macrophages stimulated by LPS. Synchronously, LPS signal-associated molecule, CD14 expression on surface of macrophages was also examined by flow cytometry.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant human activin A was obtained from R&D (Minneapolis, MN 55413). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) and LPS (lipopolysaccharide, from *Escherichia coli* 0111:B4) were purchased from Sigma (St. Louis, MO, USA). FITC-conjugated mouse monoclonal antibodies against MHC I and MHC II were purchased from AbD Secrotec (Oxford, OX5 1GE, UK) and mouse monoclonal antibody against CD14 was obtained from BD Biosciences (San Jose, CA, USA).

2.2. Animals

Female C57BL/6 mice, 8 weeks old, were obtained from the Laboratory Research Center of Jilin University (Changchun, China).

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2.3. Isolation and culture of macrophages

Mouse peritoneal macrophages were obtained by lavaging the peritoneal cavity with 5 mL of ice-cold sterile RPMI 1640 medium and cultured in 10% fetal calf serum (FCS)-RPMI 1640 medium at 37 °C for 1 h in a humidified 5% CO_2 and 95% air incubator [2]. After incubation, non-adherent cells were removed by washing with serum-free medium. Adherent cells were seeded in 12-well tissue culture plates at a density of 1×10^6 cells per well and incubated in 5% FCS-RPMI 1640 medium in the presence or absence of LPS (0.25–1.0 μg/mL) and LPS (0.5 μg/mL) plus activin A (0.4–10 ng/mL) overnight, respectively.

2.4. Detection of nitric oxide

NO levels in the culture supernatant of mouse peritoneal macrophages were determined by NO kit according to the manufacturer's protocol (NJJC, Nanjing, China). The absorbance at 540 nm in each well was measured with an automated microtiter plate reader (Bio-Rad Laboratories, Hercules, CA, USA). The nitrite concentration was determined by a standard curve generated with serial dilutions of a standard solution of sodium nitrite.

2.5. In vitro pinocytosis assay of mouse peritoneal macrophages

Peritoneal macrophages plated to 96-well culture plates were incubated in 5% FCS-RPMI 1640 medium in the presence or absence of LPS (0.5 μg/mL) and LPS (0.5 μg/mL) plus activin A (2 or 5 ng/mL) at 37 °C for 24 h in a humidified 5% CO_2 and 95% air incubator, respectively. Culture media were removed and 200 μl/well of 0.7% neutral red was added. Media were discarded after incubation for 1 h. The macrophages were washed twice with pH 7.4, 0.01 mol/L phosphate-buffered saline (PBS) and then lysed in 200 μL of lysis solution (1:1 of 0.1 mol/L acetic acid and 100% ethanol) at 4 °C overnight. Absorbance was measured at 490 nm [15].

2.6. Assay of mouse peritoneal macrophage phagocytosis

2.6.1. In vitro

To evaluate phagocytosis of mouse peritoneal macrophages, chicken red blood cells (cRBC) were used as antigen particles. Macrophages were treated with or without LPS (0.5 μg/mL) and LPS (0.5 μg/mL) plus activin A (2 or 5 ng/mL) in 5% FCS-RPMI 1640 medium at 37 °C for 24 h in a humidified 5% CO_2 and 95% air incubator, respectively. Then 1% cRBC were added and incubated for 1 h. The macrophages were rinsed with PBS and fixed with 4% paraformaldehyde. The cells were stained with Wright-Giemsa dye for 3 min and rinsed with PBS. Phagocytosed cRBC were examined with light microscopy and a minimum of 200 macrophages were counted in each well. The phagocytosis ratio (PR) and index (PI) of macrophages were calculated as follows: PR = number of macrophages phagocytosing cRBC/number of macrophages; PI = number of cRBC phagocytosed by macrophages/number of macrophages [16].

2.6.2. In vivo

After injected with 0.5 mL of PBS into the abdominal cavity for 2 h, mice were injected with 0.2 mL of PBS containing LPS (1 μg) or LPS (1 μg) plus activin A (10 ng or 20 ng) into the abdominal cavity, and with alone PBS as negative control. 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. 1×10^5 macrophages/mL were put on glass slides and cultured at 37 °C for 30 min in a humidified 5% CO_2 and 95% air incubator. The slides were washed with PBS. The cells were fixed with 4% paraformaldehyde for 10 min and stained with Wright-Giemsa dye. Phagocytosed cRBC were examined with light microscopy. The PR and PI were calculated.

2.7. Flow cytometric analysis

The expressions of MHC I, MHC II and CD14 on surface of mouse peritoneal macrophages were examined by flow cytometry using anti-mouse MHC I, MHC II and CD14 antibodies, respectively. 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 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.

2.8. Proliferation assay of mouse peritoneal macrophages

Mouse peritoneal macrophages were seeded into 96-well tissue 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 LPS (0.5 μg/mL) and LPS (0.5 μg/mL) plus activin A (5 or 10 ng/mL) at 37 °C for 24 h in a humidified 5% CO_2 and 95% air incubator, 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 detected at 570 nm to express the cell viabilities [17]. To directly assay macrophage proliferation, the viabilities of macrophages were further detected by Trypan Blue exclusion method, and the viable cells were counted for each sample under the high magnification using haemocytometer chamber.

2.9. Statistical analysis

The data were expressed as means ± standard deviation (SD) and statistic analysis was performed by Student's *t*-test. $p < 0.05$ was considered statistic significance.

3. Results

3.1. Activin A decreased NO production in LPS-activated mouse peritoneal macrophages

NO is one of inflammatory mediators produced by macrophages. Our previous study has reported that activin A can inhibit NO production in mouse peritoneal macrophages induced by LPS [2]. The present results also revealed that LPS increased levels of NO secretion in mouse peritoneal macrophages, whereas activin A could reduce NO production in LPS-activated macrophages in a dose-dependent manner (Fig. 1). There was significant decrease of NO level in LPS (0.5 μg/mL) plus activin A (2 and 10 ng/mL) group ($p < 0.05$ and $p < 0.01$), compared with that in LPS (0.5 μg/mL) group. The data indicated that the activin A inhibited activity of LPS-induced mouse peritoneal macrophages in this study.

3.2. Activin A suppressed phagocytosis and pinocytosis of mouse peritoneal macrophages simulated by LPS in vitro

Both phagocytic and pinocytic activities are the most important functions of macrophages in innate immune response. To further assess effects of activin A on activities of mouse peritoneal macrophages stimulated by LPS, phagocytosis and pinocytosis were examined *in vitro*. We found that LPS promoted the phagocytosis