Stimulation of Mouse Dendritic Cells by Gum Arabic

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Key Words

Interferon γ • Interleukin 6 • Interleukin 12 • TNF α • Phagocytosis • MAPK • Erk

Abstract

Gum Arabic (GA), a nonabsorbable nutrient manufactured from the exudate of *Acacia senegal*, is composed of a complex polysaccharide. GA is used by the pharmaceutical and food industry as an emulsifier but may, at an appropriate dosage, modify intestinal transport. Dendritic cells (DCs) can protrude between epithelial cells and sense the composition of the lumen. As DCs are stimulated by bacterial polysaccharides, we hypothesized that GA may similarly stimulate DCs. To test that hypothesis, mouse DCs were treated with either LPS or GA and expression of maturation markers, phagocytotic activity, cytokine production and ability to stimulate CD4⁺ T cells in allogenic mixed leukocyte reaction (allo-MLR) was analyzed. As a result both LPS and GA increased the percentage of CD11c⁺CD86⁺, CD11c⁺MHCII⁺, CD11c⁺CD40⁺, CD54-expressing DCs and decreased their phagocytic activity. Both LPS and GA stimulated the production of IL-6, IL-10, IL12p70 and TNF α in a p38- and/or ERK-dependent manner. GA treatment led to an enhanced IL-10 secretion,

whereas LPS was more effective on IL-6 and IL-12p70 production. Both LPS- and GA-stimulated DCs enhanced CD4⁺ T cell proliferation but the profile of cytokines produced in allo-MLR was different. High levels of IL-10 and IL-6 were observed in the presence of GA-treated DCs, whereas IFN- γ and IL-12p70 production was similar with LPS- or GA-treated DCs. LPS upregulated p38 and transiently ERK1/2, while GA led to more sustained activation of ERK1/2, only. In conclusion, the observations reveal a powerful immunomodulatory effect of GA.

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Introduction

Gum Arabic (GA) is a water-soluble [1] polysaccharide based on branched chains of (1-3) linked β -D-galactopyranosyl units containing α -L-arabinofuranosyl, α -L-rhamnopyranosyl, β -D-glucuronopyranosyl and 4-O-methyl- β -D-glucuronopyranosyl units [2]. It is fabricated from gummy exudates of *Acacia Senegal* [3]. In the colon GA is fermented by microorganisms to short chain fatty acids [4]. It is considered one of the safest dietary fibers [5].

In Middle Eastern countries GA is used in the treatment of patients with chronic kidney disease [6]. GA increases fecal nitrogen excretion [7] and decreases

production of free oxygen radicals [6]. Intestinal GA fermentation leads to the formation of several degradation products including short-chain fatty acids [8]. Accordingly, GA treatment may enhance serum butyrate concentrations [9].

In the intestine, GA may modify the function of the reabsorbing epithelial cells [10]. At least in theory, GA may modify the function of intestinal dendritic cells (DCs), which are in direct contact with the intestinal lumen [11]. DCs are antigen-presenting cells involved in the initiation of both innate and adaptive immunity and thus play a pivotal role in the regulation of the immune response [12-15].

Many stimuli that initiate DC maturation induce phosphorylation of extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK. Three MAPK signaling pathways have been suggested to differentially regulate all aspects of phenotypic maturation, cytokine production, and functional maturation of DCs. Thus, p38 MAPK and ERK have both been shown to induce the IL-12 p40 subunit in DCs [16, 17]. IL-12 production by DC is critically important for the differentiation of IFN- γ -producing Th1 T lymphocytes. In LPS-stimulated murine DCs, ERK was also shown to be important for DC survival after maturation [18]. Thus, distinct maturation of DCs may be induced by modulating the balance of phosphorylation of the three MAPKs [19].

The present study explored, whether exposure of DCs to GA influences their function. To this end, bone marrow-derived mouse DCs have been left untreated or exposed to GA and the expression of surface markers, phagocytotic activity, cytokine release, CD4⁺ T cell proliferation and cytokine production in allo-MLR was determined. For comparison, the DCs were treated with bacterial LPS. Activity of MAPK was assessed in GA- and LPS-stimulated DCs.

Materials and Methods

Gum Arabic

Gum Arabic (GA) in powder form was provided as a generous gift from Dar Savanna Ltd., Khartoum, Sudan (www.ssgums.com). The fully natural extract powder with a particle size less than 210 μ m was produced mechanically from wildy grown *Acacia Senegal* tree. The quality conforms to the food and pharmaceutical requirements of the Food and Agriculture Organization of the United Nations (FAO), British pharmacopeia (BP), United States pharmacopeia (USP) and Joint FAO/WHO Expert Committee On Food Additives (JECFA).

The degree of contamination of GA with endotoxin was checked by Limulus Amoebocyte Lysate (LAL) Assay by

Hyglos Technologies GmbH (Regensburg, Germany, Endotoxin Detection Service). The 0.5% solution of GA used in this study contains 156.5 pg/ml endotoxin. At this concentration LPS did not induce any of the effects described here.

Culture of bone marrow dendritic cells

Dendritic cells were isolated from femurs and tibias of 8-12 weeks old C57/BL6 mice. Cells were then plated at a density of 2×10^6 cells/10 ml in 60-mm dish and cultured for 8 days in RPMI 1640 (GIBCO, Carlsbad, Germany) containing: 10% FCS, 1% penicillin/streptomycin, 1% glutamine, 1% non-essential amino acids and 0.05% β -mercaptoethanol. Cultures were supplemented with GM-CSF (35 ng/mL, Preprotech, Tebu-bio, Rocky Hill, NJ). Cultures were fed with fresh medium containing GM-CSF every 3 days. Most (>80%) of the cells expressed CD11c, which is a marker for mouse DCs. Experiments were performed on DCs at days 8-9. All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities.

Immunostaining and flow cytometry

Surface expression of CD11c, CD86, MHCII, CD54 and CD40 was determined by FACS analysis. To this end, cells (4×10^5) were incubated in 100 μ l FACS buffer (phosphate buffered saline (PBS) plus 0.1% FCS) containing fluorochrome-conjugated antibodies at a concentration of 10 μ g/ml. A total of 2×10^4 cells were analyzed. The following antibodies (all from BD Pharmingen, Heidelberg, Germany) were used for staining: FITC-conjugated anti-mouse CD11c, clone HL3 (Armenian Hamster IgG₁, λ 2), PE-conjugated anti-mouse CD86, clone GL1 (Rat IgG_{2a}, κ), PE-conjugated rat anti-mouse I-A/I-E, clone M5/114.15.2 (IgG2b, κ), PE-conjugated anti-mouse CD40 clone 3E2 (Rat IgG_{2a}), and PE-conjugated anti mouse ICAM-1 (CD-54), clone 3E2 (Armenian Hamster IgG1, κ). After incubating with the antibodies for 60 min at 4°C, the cells were washed twice and resuspended in FACS buffer for flow cytometry analysis.

DC phagocytosis assay

DCs (10^6 cells/ml) were suspended in prewarmed serum-free RPMI 1640 medium, pulsed with FITC-conjugated dextran (Sigma-Aldrich, Taufkirchen, Germany) at a final concentration of 1 mg/ml and incubated for 3h at 37°C. Uptake was stopped by adding ice-cold PBS. Then the cells were washed three times with ice cold PBS supplemented with 5% FCS and 0.01% sodium azide before FACS analysis. DCs were analyzed for the uptake of FITC-dextran.

Cytokine quantification in cell supernatants

DCs were stimulated with *Escherichia coli* lipopolysaccharide (LPS, 200 ng/ml, Sigma Aldrich, Taufkirchen, Germany) or Gum Arabic (0.5%) in the absence or in the presence of p38 inhibitor (SB203580, 10 μ M, Sigma Aldrich) or ERK inhibitor (U0126, 10 μ M, Calbiochem) for 24 h. Cell culture supernatant was collected and stored at -20°C until use for ELISA. IL-12p70, IL-6, IL-10, and TNF α concentrations in DC culture supernatants were determined by using OptEIA ELISA kits (BD Pharmingen) according to the manufacturer's protocol.

Fig. 1. Effect of LPS and GA treatment on MHCII, CD86 and CD40 surface expression in mouse bone marrow derived dendritic cells (DCs). A., B., C. Original dot plots of CD11c⁺MHCII⁺ (A), CD11c⁺CD86⁺ (B), CD11c⁺CD40⁺ (C) in DCs cultured for 24 h without (control, 1st panel) or with LPS (200 ng/ml, 2nd panel) or Gum Arabic (GA, 0.5%, 3rd panel). Numbers depict the percentage of cells in the respective quadrants, acquired within the dead cell gate. D., E., F. Arithmetic means $\pm$ SEM (n = 6-7) of MHCII (D), CD86 (E) and CD40 (F) expression in DCs cultured without (control, white bar) or with LPS (200 ng/ml, dotted bar) or GA (0.5%, black bars) for 24 h. *** (p<0.001) show differences from untreated (control) DCs, ANOVA.

Immunoblotting

DCs (2×10^6 cells) were left unpulsed (control) or pulsed with LPS or Gum Arabic for 10, 60 or 120 min and then snap-frozen in dry-ice ethanol bath. Cell pellets were thawed on ice and washed twice with PBS, then solubilized in lysis buffer (Pierce) containing protease inhibitor cocktail (Sigma-Aldrich, Taufkirchen, Germany). Samples were stored at -80°C until use for Western blotting. Cell lysates were separated by 10% SDS-PAGE and blotted on nitrocellulose membranes. The blots were blocked with 5% nonfat-milk in triethanolamine-buffered saline (TBS) and 0.1% Tween-20. Then the blots were probed overnight with anti-phospho (p)-ERK, anti-ERK, anti-phospho SAPK/JNK, anti-SAPK/JNK (Cell Signaling), anti p-p38, anti-p38 (Santa Cruz, Heidelberg, Germany) antibodies diluted in 5% milk in PBS and 0.1% Tween-20, washed 5 times, probed with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature, and washed final 5 times. Antibody binding was detected with the enhanced chemiluminescence kit (Amersham, Freiburg, Germany). Densitometer scans of the blots were performed using Quantity One (BioRad, Munich, Germany).

Isolation of CD4⁺ T cells from the spleen

Spleens from 8- to 12-week-old naive Sv129J mice were removed aseptically, cut into pieces, a single-cell suspension of splenocytes was obtained through a cell strainer. All spleen cell preparations were resuspended in lymphocyte isolation buffer (supplemented with 0.5% bovine serum albumin and 2

mM EDTA) and kept on ice. CD4⁺ T cells from single-cell suspensions were isolated by the negative selection column method using the CD4⁺ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol.

Allogenic mixed leukocyte reaction

Allogenic mixed leukocyte reaction was performed using CD4⁺ T cells from spleens of Sv129J mice. The CD4⁺ T cells were washed twice with PBS and then labeled with a 10- μM solution of 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) in PBS (10^7 cells/10ml PBS total) and incubated at $37^\circ\text{C}/5\% \text{CO}_2$ for 10 min. Cells were washed and centrifuged to remove excessive CFSE. CFSE-labeled CD4⁺ cells were cultured in 96-well plates alone or with DCs from bone marrow of C57BL/6 mice (1:10DCs to T cells, 10^5 cells/100 μl) at $37^\circ\text{C}/5\% \text{CO}_2$ for 5 days. Control, GA (0.5%, 24 h)- or LPS (200 ng/ml, 24 h)-pretreated DCs were used. Proliferation of CD4⁺ T cells was assessed by FACS analysis. To evaluate cytokine production (IL-12p70, IL-6, IL-10, and IFN- γ), the culture supernatants were collected at day 5 and subjected to ELISA.

Statistics

Data are provided as means $\pm$ SEM, *n* represents the number of independent experiments. Differences were tested for significance using ANOVA, as appropriate. *P*<0.05 was considered statistically significant.

Fig. 2. Effect of LPS and GA treatment on CD54 surface expression in DCs. A. Representative FACS histograms depicting the expression of CD54 in DCs cultured for 24 h without (control, 1st panel) or with LPS (200 ng/ml, 2nd panel) or Gum Arabic (0.5%, 3rd panel). B: Arithmetic means $\pm$ SEM (n = 6 each) of CD54 expression in DCs cultured without (control, white bar) or with LPS (200 ng/ml, dotted bar) or Gum Arabic (0.5%, black bars) for 24 h. *** (p<0.001) significant differences from untreated (control) DCs, ANOVA.

Results

GA enhances bone marrow-derived DC differentiation and maturation

At day 8 the cultures of mouse DCs were supplemented with either LPS (200 ng/ml) or GA (0.5%) and stained 24 h afterwards for MHC class II, costimulatory molecule CD86 and CD40 (Fig. 1) and the adhesion molecule CD54 (ICAM-1) (Fig. 2). Both LPS and GA increased the percentage of CD11c⁺MHCII⁺ (Fig. 1A, D), CD11c⁺CD86⁺ (Fig. 1B, E), CD11c⁺CD40⁺ (Fig. 1C, F) and CD54 expressing cells (Fig. 2), indicating that, similar to LPS, GA stimulates maturation and differentiation of DCs.

GA decreases phagocytic capacity in DCs

DC maturation is characterized by the loss of phagocytic capacity and LPS-induced maturation of DCs is known to down-regulate the FITC- dextran uptake [20]. Accordingly, the phagocytic capacity of DCs untreated or stimulated with LPS (200 ng/ml, 24 h) or GA (0.5%, 24 h) was analyzed. As shown in Fig. 3, FITC-dextran uptake was similarly impaired by GA and LPS, confirming that GA indeed promoted maturation of DCs.

Fig. 3. LPS and GA decrease phagocytic capacity of DCs. A. Representative FACS histograms depicting the percentage of the uptake of FITC-dextran after 3 h in DCs cultured for 24 h without (control, 1st panel) or with LPS (200 ng/ml, 2nd panel) or Gum Arabic (0.5%, 3rd panel). B. Bar diagram representing mean percent ($\pm$ SEM; n=4) of FITC-dextran uptake by DCs cultured without (control, white bars) and with LPS (200 ng/ml, dotted bar) or Gum Arabic (0.5%, black bars) for 24 h. *** (p<0.001) significant differences from untreated (control) group, ANOVA.

Stimulation with GA leads to upregulation of extracellular signal-regulated kinase (ERK1/2) in DCs

LPS stimulation has been shown to activate mitogen-induced protein kinases (MAPKs) in DCs [20, 21]. To gain insight into GA-induced signaling, we examined phosphorylation (and thus activation) of ERK1/2, p38 and SAPK/JNK kinases following stimulation with either LPS or GA (Fig. 4). LPS stimulation fostered p38 and ERK1/2 activation, while GA strongly stimulated ERK1/2 phosphorylation.

GA enhances cytokine secretion in DCs

Cytokine production was analyzed in DCs stimulated with LPS (200 ng/ml) or GA (0.5%) for 24 h. As illustrated in Fig. 5, both LPS and GA stimulated the secretion of IL-10, IL12p70, IL-6 and TNF α . GA-stimulated DCs secreted more IL-10 than LPS-stimulated cells (Fig. 5A) and in contrast, the effect of LPS on IL-12p70 (Fig. 5B) and IL-6 (Fig. 5C) production was significantly more pronounced than the effect of GA. Inhibitors of ERK (U0126) and p38 (SB203580) were used to examine the impact of ERK and p38 MAPKs on cytokine production. While LPS-induced cytokine production was sensitive to

Fig. 4. GA enhances the phosphorylation of ERK1/2 in DCs. DCs were stimulated with LPS (200 ng/ml) or GA (0.5%) for the indicated time: 10, 60, 120 min or left untreated (control). Protein extracts were analyzed by Western blotting using antibodies directed to phosphorylated (p) p38 (p-p38), ERK1/2 (p-ERK1/2) and SAPK/JNK (p-SAPK/JNK). Equal protein loading was controlled by anti-p38, anti-ERK1/2 and anti-SAPK/JNK antibodies. One representative experiment out of three is shown. B. Arithmetic means $\pm$ SEM (n = 3) of the abundance of phosphorylated (p) ERK1/2 (1st panel), p38 (2nd panel) and SAPK/JNK (3rd panel) as the ratio to total ERK1/2, p38 and SAPK/JNK respectively at 0 (control), 10, 60 and 120 min of incubation with GA (0.5%, black bars) or LPS (200 ng/ml, hatched bars). * (p<0.05) and *** (p<0.001) indicate significant differences from control, ANOVA.

Fig. 5. Effect of MAPK inhibitors SB203580 and U0126 on the formation of IL-10, IL-12p70, IL-6 and TNF- α in GA- and LPS-treated DCs. Concentrations of IL-10 (A), IL-12p70 (B), IL-6 (C) and TNF- α (D) in the supernatants of DCs cultured without (control, white bars) or with LPS (200 ng/ml) or GA (0.5%) in the absence (white bars) or in the presence of p38 inhibitor SB203580 (10 μ M, dotted bar) or ERK inhibitor U0126 (10 μ M, striped bars) for 24 h. The cytokine concentrations were determined by ELISA. Arithmetic means $\pm$ SEM were calculated from 6-12 independent experiments, each reflecting the mean of a duplicate measurement. * (p<0.05) and *** (p<0.001) significant differences from untreated (control) group, # (p<0.05), ## (p<0.01) and ### (p<0.001) significant differences from GA- or LPS-treated DCs in the absence of the inhibitors, §§§ (p<0.001) significant differences between GA- and LPS-treated DCs, ANOVA.

ERK- and p38-inhibitors, GA-stimulated secretion of IL-10 and TNF α was significantly inhibited only by the ERK inhibitor U0126 (Fig. 5).

GA enhances the proliferation of CD4⁺ T cells

We examined the ability of GA-treated DCs to stimulate CD4⁺ T cells in allogeneic mixed leukocyte reaction (allo-MLR). CFSE-labeled CD4⁺ T cells from the spleen

of Sv129 mice were cocultured with control, LPS (200 ng/ml, 24 h)- or GA (0.5%, 24 h)- stimulated DCs from C57BL/6 mice at a DC : T cell ratio of 1:10 for 5 days and proliferation activity was then evaluated (Fig. 6). No proliferation was observed in the cultures of T cells alone. Proliferation of T cells co-cultured with LPS- or GA-stimulated DCs was significantly enhanced, if compared to control DCs (Fig. 6).

Fig. 6. Effect of LPS and GA treatment on proliferation of CD4⁺ T cells. A. Representative FACS histograms depicting the proliferation of CD4⁺ T cells co-cultured either without (T cells alone, 1st panel) or with DCs untreated (control, 2nd panel) or treated for 24 h with LPS (200 ng/ml, 3rd panel) or Gum Arabic (GA, 0.5%, 4th panel). B. Arithmetic means $\pm$ SEM (n = 3 each) of the proliferation of CD4⁺ T cells co-cultured with DCs untreated (control, white bar) or treated with LPS (200 ng/ml, dotted bar) or GA (0.5%, black bars) for 24 h. *** (p<0.001) significant differences from untreated (control) DCs, ANOVA.

Ability of GA-stimulated DCs to induce cytokine production in allo-MLR

We next evaluated the Th1/Th2-cytokine production in the culture of allo-MLR. GA- or LPS-treated DCs were cocultured with splenic CD4⁺ T cells at a DC : T-cell ratio of 1:10 for 5 days and levels of Th1 (IFN- γ , IL-12p70) and Th2 (IL-6, IL-10) cytokines were analysed. Negligible production of these cytokines was observed in cultures of CD4⁺ T cells or each DC type alone. As illustrated in Fig. 7, both LPS- and GA-stimulated DCs led to cytokine production in allo-MLR. High production of IFN- γ and IL-12p70 was observed in the cultures of T cells stimulated with either LPS- or GA-treated DCs (Fig. 7C, D). However, GA-treated DCs were much more powerful in stimulating IL-6 and IL-10 production than LPS- treated DCs (Fig. 7A, B), indicating that GA can lead to a predominant Th2 response.

Discussion

The present study reveals a completely novel effect of gum arabic (GA), i.e. its ability to modify the maturation of dendritic cells (DCs). Similar to bacterial LPS, GA exposure leads to the upregulation of several maturation markers, such as CD86 [22] CD54 [23] and CD40 [24, 25]. Low expression levels of CD86 and CD40 are characteristic of a tolerogenic DC phenotype [24]. GA exposure further leads to upregulation of MHC II, which is critically important for antigen presentation [26].

Treatment with GA further stimulates the formation of the interleukins 6 (IL-6), 10 (IL-10) and 12 (IL-12p70) as well as TNF α . The formation of those cytokines can

Fig. 7. Effect of GA-stimulated DCs on cytokine production in allo-MLR with CD4⁺ T cells. Levels of IL-10 (A), IL-6 (B), IFN- γ (C) and IL-12p70 (D) in the supernatants on day 5 of splenic CD4⁺ T cells co-cultured with DCs untreated (control, white bar) or pretreated with LPS (200 ng/ml, dotted bar) or GA (0.5%, black bars) for 24 h. The cytokine concentrations were determined by ELISA. Arithmetic means $\pm$ SEM were calculated from 3 independent experiments, each reflecting the mean of a duplicate measurement. * (p<0.05), ** (p<0.01) and *** (p<0.001) significant differences from untreated (control) group, ### (p<0.001) significant differences between GA and LPS, ANOVA.

also be stimulated by LPS. Nevertheless, the effects of LPS and GA are markedly different, i.e. GA has a particularly strong effect on IL-10 and IL-6 formation. The cytokines are important regulators of the immune response [27–29]. Notably, IL-10 is a negative feedback inhibitor

of exuberant T cell responses [29]. High amounts of IL-10 may lead to tolerance since IL-10 can suppress T cells or lead to regulatory T cell differentiation; IL-10 can also act on DCs to decrease their function or render them tolerogenic [14]. Thus, GA may, unlike LPS, exert anti-inflammatory effects. DCs from lamina propria of the small intestine from ovalbumin-fed mice also express significant levels of mRNA for IL-10, but not IL-12, and likewise may contribute to immune homeostasis in the gut [30].

Treatment of DCs with GA may result in the augmentation of the Th2 response presumably through enhanced IL-10 production by DCs. In the allo-MLR system with T cells and GA-pretreated DCs high levels of IL-10 and IL-6 were observed.

The *in vivo* effects of GA on the immune system may involve additional mechanisms beyond its direct effect on DCs. GA may influence the immune response by decreasing the plasma concentration of $1,25(\text{OH})_2\text{D}_3$ [10], a hormone produced in activated DCs and macrophages [31–33]. $1,25(\text{OH})_2\text{D}_3$ can act directly on T cells, though antigen-presenting cells, and in particular DCs, appear to be primary targets for its tolerogenic properties [34]. $1,25(\text{OH})_2\text{D}_3$ inhibits the differentiation and maturation of DCs as well as their capacity to secrete the Th1-polarizing cytokine IL-12 [35–38]. GA may further lead to the formation of short-chain fatty acids [8] with subsequent increase of serum butyrate concentrations [9]. Butyrate has in turn been shown to inhibit the functional differentiation of dendritic cells [39–41].

Our data demonstrate that stimulation by GA resulted in an increased activity of extracellular signal-regulated kinase ERK1/2. Enhanced activity of this MAPK fits well the observed high-level secretion of IL-10 by GA-stimulated DCs, since high levels of ERK phosphorylation

has been shown to be associated with IL-10 production [42]. The differential activation of the ERK1/2 and p38 pathways may play a role in the differential production of IL-10 and IL-12p70 by GA- and LPS-stimulated DCs, respectively. Increased ERK activation may be responsible for the preferential expression of IL-10 upon GA-treatment. Alternatively, the impaired IL-12p70 production may be attributed to the suppressed p38 pathway.

In conclusion, the present study reveals that GA is a powerful stimulator of DC maturation, and cytokine formation. Thus, GA is a novel nutrient with immunoregulatory potency.

Abbreviations

allo-MLR (allogenic mixed leukocyte reaction); CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester); DCs (Dendritic cells); EDTA (ethylenediamine-tetraacetic acid); ERK (extracellular signal-regulated kinase); FACS (Fluorescence-activated cell sorting); FCS (fetal calf serum); GA (Gum Arabic); GM-CSF (Granulocyte-macrophage colony-stimulating factor); IFN (Interferon); IL (interleukin); LPS (lipopolysaccharide); MAPK (mitogen-induced protein kinases); MHCII (Major Histocompatibility Complex class II); PBS (phosphate buffered saline); TBS (triethanolamine-buffered saline); TNF (Tumor necrosis factor).

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