Immunomodulatory Effects of ZYM-201 on LPS-stimulated B Cells

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ZYM-201 is a methyl ester of triterpenoid glycoside from Sanguisorba officinalis which has been used for treatment of inflammatory and metabolic diseases. In this study, immunomodulatory effects of ZYM-201 on B cells were examined in vitro and in vivo. When splenocytes were activated with lipopolysaccharide (LPS), the major population which had shown an increase in cell numbers was B cells. However, when the B cells were treated with ZYM-201 after LPS activation, their cell numbers and the expression of major costimulatory molecules, CD80 and CD86, were decreased. Furthermore, the effect of LPS, which induces activation of NF-κB, was abolished by ZYM-201: LPS-stimulated B cells showed decrease of phosphorylation after treatment of ZYM-201. The same results were shown in vivo experiments. These results suggest that ZYM-201 may play a role in the modulation of inflammatory responses through inhibiting NF-κB activation and downregulating the expression of costimulatory molecules on B cells.

Keywords: ZYM-201, B cell, Inflammation, LPS

INTRODUCTION

Sanguisorba officinalis L. (Rosaceae), a well known traditional medicinal plant in East Asia, is used for treatment of inflammatory and metabolic disease (1). Recent studies have been reported that various extracts of S. officinalis have medicinal effects such as anti-allergic, anti-cancer, anti-thrombin, anti-wrinkle, and neuroprotective properties (1-7). The root of S. officinalis includes various saponin components including triterpenoids and their glycosides, gallic acid, and disaccharide (8,9). ZYM-201 is a modified compound of ziyuglycoside, one of triterpene glycoside originated from the root of S. officinalis, through deglycosidation and esterification (10). Previous studies have shown that ZYM-201 plays a role in inhibiting tissue factor activity, tumor necrosis factor (TNF)-α production and modulating hyperlipidemic conditions (10-12).

LPS is the major component of the outer membrane of Gram-negative bacteria and commonly used to study inflammation because it initiates acute inflammatory responses in animals (13,14). The binding of LPS to Toll-like receptor 4 triggers signal transduction pathways and finally induces phosphorylation and translocation of nuclear factor (NF)-κB (15) leading to regulating the expression of a large number of genes which have important functions in immune responses (16). The stimulation of B cells by LPS induces proliferation and upregulates the expression of CD80 and CD86, which results in enhancing their antigen-presenting capacity (15,17-19). Increased expression of these costimulatory molecules is associated with activation of NF-κB (20,21).

Since ZYM-201 has been reported to inhibit the production of TNF-α, we investigated the effects of ZYM-201 on the activation of lymphocytes in LPS-induced inflammation. We found that the activation of LPS-stimulated B cells was abolished by ZYM-201 through inhibiting NF-κB activation and downregulating costimulatory molecules.

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Abbreviations: LPS, lipopolysaccharide; NF-κB, nuclear factor κB; MFI, mean fluorescence intensity
**MATERIALS AND METHODS**

**Preparation of cells**
All experiments were performed in accordance with the approval of Soongsil University Institutional Animal Care and Use Committee. 6-week-old C57BL/6 mice were purchased from Young Bio (Seongnam, Korea). To make cell suspensions, extracted spleens from mice were cut into small fragments and crushed between gauze. After depletion of red blood cells with Gey’s solution, the cell suspensions were cultured in RPMI1640 (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin (GIBCO).

**Chemicals**
ZYM-201 is a methyl ester of triterpenoid glycoside and modified compound through deglycosidation and esterification from ziya-glycoside, which is an originated component from the root of *S. officinalis* (10). Extraction, modification and purification of ZYM-201 were performed as previously described (10).

**Flow cytometry**
Splenocytes were cultured with 1 μg/ml LPS for 24 hours and/or ZYM-201 (50 or 100 μM) was added 6 hours after LPS treatment. mAbs for CD80 (clone 16-10A1), CD86 (clone GL1), and B220 (clone RA3-6B2) were purchased from eBioscience (San Diego, CA).

**Western blot**
Splenocytes were positively enriched by using FITC-conjugated B220 mAbs and anti-FITC microbeads according to the manufacturer’s instructions (Miltenyi Biotec Ltd., Bergisch Gladbach, Germany). B220⁺ cells were cultured with 1 μg/ml LPS and/or 100 or 200 μM ZYM-201 for 30 minutes, and lysed in RIPA buffer (Sigma-Aldrich, St.Louis, MO, USA) supplemented with protease inhibitor cocktail tablets and PhosSTOP (Roche, Mannheim, Germany). Cell lysates were separated using SDS-PAGE and transferred on to polyvinylidene difluoride membrane (Millipore, Darmstadt, Germany). Proteins were visualized by enhanced chemiluminescence (Bio-Rad, Hercules, CA, USA) and x-ray films (AGFA, Düsseldorf, Germany) were used for detection.

**RESULTS AND DISCUSSION**

To investigate the effects of ZYM-201 on the cell number and activation of LPS-stimulated lymphocytes, cells from mouse spleens were cultured with 50, or 100 μM ZYM-201 after LPS treatment (Fig. 1A and 1B). The number of lymphocytes was increased from 100% (control) to 157% by LPS treatment, but decreased in the presence of ZYM-201 in a dose dependent manner: 142% with 50 μM ZYM-201 and 127% with 100 μM ZYM-201 (Fig. 1A). In particular, the number of B cells compared to other cell populations showed a big increase from 100% (control) to 185% by LPS treatment. However, this effect was abolished in the presence of ZYM-201: B cell numbers were decreased to 152% with 50 μM ZYM-201 and 132% with 100 μM ZYM-201 (Fig. 1B). In addition, the expression of CD80 and CD86 on LPS-stimulated B cells was downregulated in the presence of ZYM-201: mean fluorescent intensity (MFI) of CD80 expression on LPS-stimulated B cells was downregulated from 330 to 222 followed by incubation with ZYM-201, and MFI of CD86 expression was also downregulated from 2410 to 1929 with ZYM-201 (Fig. 1C). These data indicate that ZYM-201 downregulates the activation of LPS-stimulated B cells. Because the upregulation of CD80 and CD86 costimulatory molecules is related with activation of NF-κB, we examined levels of NF-κB p65 phosphorylation on serine 536 (22) in B cells by western blot (Fig. 1D). Levels of NF-κB p65 phosphorylation in B cells were markedly increased by LPS stimulation, but dramatically decreased in the presence of ZYM-201 indicating that ZYM-201 has an inhibitory effect on LPS-stimulated B cell activation through inhibiting NF-κB phosphorylation and downregulating costimulatory molecules on B cells.
To confirm whether ZYM-201 has an inhibitory effect on LPS-stimulated B cell activation in vivo, cells from mice that had been injected with LPS and subsequently fed ZYM-201 twice were compared with those from mice that had not (Fig. 2). The numbers of lymphocytes and B cells were increased from 100% (control) to 138% and 143%, respectively, by LPS injection. Consistent with in vitro experiments, this effect was abolished after feeding ZYM-201; the numbers of lymphocytes and B cells were decreased to 92% and 90%, respectively, by ZYM-201 (Fig. 2A and 2B). Furthermore, the expression of costimulatory molecules on B cells in vivo showed the same pattern to that in vitro; the expression of CD80 and CD86 on LPS-stimulated B cells was downregulated after feeding ZYM-201 (Fig. 2C).

Figure 2. \textit{in vivo} analysis of ZYM-201 effects on B cells of LPS-injected mice. Mice were intraperitoneally injected with LPS (1 mg/kg) and/or orally administrated with ZYM-201 (20 mg/kg). Twenty-four hours after LPS injection, spleens were taken. (A) and (B) Relative percentages of lymphocytes and B cells. The numbers of lymphocytes and B220$^+$ cells were calculated by using SPHEROTM AccuCount Blank Particles and the percentage of the cells was determined by comparison to control (100%). Error bars show standard deviation of four independent experiments. **p < 0.01; ***p < 0.005. (C) Flow cytometric analysis of CD80 and CD86 expression on B cells cultured with LPS and/or ZYM-201 (50 μM). Filled histograms show isotype-matched control Abs and numbers above bracketed line shows the percentage of positive cells for each Ab staining. Histograms are representative and mean fluorescence intensity (MFI) shown below each histogram is the average of four independent experiments.
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