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Low dose zymosan ameliorates both chronic and relapsing experimental autoimmune encephalomyelitis ☆

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ABSTRACT

Zymosan has previously been reported to have both pro-inflammatory and anti-inflammatory effects. Here we demonstrate that low dose zymosan prevented or reversed chronic and relapsing paralysis in EAE. In suppressing CNS autoimmune inflammation, zymosan not only regulated APC costimulator and MHC class II expression, but also promoted differentiation of regulatory T cells. Following adoptive transfer of zymosan-primed $CD4^{+}$ T cells, recipient mice were protected from EAE. In contrast, a MAPK inhibitor and a blocker of β-glucan, reversed the effects of zymosan. These results demonstrate that zymosan may be a promising beneficial agent for Multiple Sclerosis (MS).

1. Introduction

Zymosan, a yeast-derived β-glucan- and mannan-rich particle (Brown et al., 2010), has been shown to markedly reduce the phagocytic activity of DCs (Reis e Sousa et al., 1993), to inhibit production of specific pro-inflammatory molecules such as TNF-α, and to induce high levels of IL-10 production by macrophages and DCs (Saijo et al., 2003; Samarasinghe et al., 2006; Goodridge et al., 2007; Slack et al., 2007). MHC class II expression is central to immune regulation in T-cell-mediated autoimmune disease (Steinman et al., 1980, 1981; Bottazzo et al., 1983; Slavin et al., 2001). Zymosan prevented IFN-γ-inducible MHC class II expression on monocytes (Volk et al., 1986), suggesting that zymosan might inhibit antigen presentation to pro-inflammatory Th cells (Volk et al., 1986). In the *Volk et al.* study, it was observed that following digestion of phagocytosed zymosan particles, IFN-γ could not restore MHC class II antigen expression on cultured monocytes, suggesting that soluble yeast β-glucan may work in a different way than whole yeast β-glucan particles (WGPs) (Volk et al., 1986). Although resident macrophages were effectively activated by WGPs, the binding ability of WGPs and the levels of cytokine secretion in resident macrophages were significantly inhibited by soluble yeast β-glucan, but not by blockade of the zymosan glucan receptor dectin-1. These observations suggest that β-glucan recognition is necessary but in itself not sufficient to induce the inflammatory response of resident macrophages. In addition, soluble yeast β-glucan may use differential mechanisms for cytokine secretion in resident macrophages that may modulate both innate and adaptive immunity (Li et al., 2007).

Recent studies have shown that zymosan signaling via the TLR2 and dectin-1 pathway can regulate cytokine secretion by $CD11c^{+}$ $CD11b^{+}$ DCs and macrophages to induce immune tolerance (Dillon et al., 2006; Slack et al., 2007). These observations suggest that zymosan could be beneficial in MS. In this study, we examined the effect of zymosan on triggering of regulatory APCs and T cells, induction of immune tolerance, and amelioration and reversal of paralysis in the EAE model.

2. Materials and methods

2.1. Animals

Female SJL/J and C57BL/6 mice (8–12 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). MOG-specific T-cell receptor transgenic mice (2D2) were a generous gift from Dr. Vijay K. Kuchroo, (Harvard University). All mice were housed in Thomas Jefferson University animal care facilities. All work was performed in accordance with the Thomas Jefferson University guidelines for animal use and care. Mouse $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) and $PLP_{139–151}$ peptide (HCLGKWLGHPDKF) were purchased from Invitrogen (Carlsbad, CA).

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2.2. Microglia and macrophages

Microglia EOC 20 cells, derived from C3H/HeJ CH-2 k mice, were obtained from the American Type Culture Collection (ATCC) and were grown as recommended using DMEM media supplemented with 1 mM sodium pyruvate, 10% (v/v) fetal calf serum (FCS) and 20% (v/v) conditioned media as a source for mouse CSF-1. Microglia stained positively for $CD11b^+$ by fluorescence activated cell sorting (FACS). Primary macrophages (peritoneal exudate cells [PEC]) were harvested from immunized mice, 24 h after intraperitoneal injection with 1 ml of 3% (w/v) thioglycollate. PEC were cultured with media alone for 72 h, then activated with IFN-γ (100U/ml) or treated with media alone. PEC were 98% (w/v) $CD11b^+$ by FACS analysis.

2.3. Histopathology and immunohistochemistry

Microglia cells were grown on cover slides and divided into three different groups. 1) IFN-γ (100 units /ml, 48 h) 2) IFN-γ plus zymosan (25 μg/ml) and 3) IFN-γ plus zymosan with addition of both U0126 (10 μM) and Laminarin (100 μg/ml) for 48 h. Slides were fixed with acetone and then labeled with mouse anti-MHC class II mAb (10–3.6) or mouse anti-MHC class II mAb (AF6-120.1, PharMingen), using the avidin-biotin technique (Vector Laboratories, Burlingame, CA). Staining was visualized by reaction with diaminobenzidine (DAB). To assess the infiltration of immune cells in the CNS, zymosan-treated and PBS-treated EAE mice were euthanized. The lumbar region of the spinal cord was removed on day 15 and stored in 10% buffered formalin. Paraffin-embedded 5 μm thick transverse sections of the spinal cord (six sections per mouse) were stained with haematoxylin and eosin (H&E) for infiltration of cells. Slides were assessed in a blind fashion for histopathological score, as documented previously (Li et al., 2008). Briefly, the level of inflammation was scored as follows: 1, a few inflammatory cells; 2, organization of perivascular infiltrates; and 3, increasing severity of perivascular cuffing with extension into the adjacent tissue.

2.4. Zymosan preparation and administration

Zymosan (Z4250, Sigma-Aldrich) was boiled for 30 min, washed extensively, and stored at −70 °C. Zymosan was analyzed for the presence of LPS by Associates of Cape Cod Inc. (East Falmouth, MA). A trace amount of LPS (0.136EU/μg) was detected. Both the MAPK inhibitor U0126 (Calbiochem) and/or the dectin-1 receptor inhibitor laminarin (*L. digitata* Sigma-Aldrich) were added prior to the addition of stimuli.

For zymosan treatment *in vivo*, mice were injected *i.p.* with 200 μl PBS or zymosan (100 μg/mouse) once daily.

2.5. EAE induction

EAE was induced in SJL/J mice by immunization with 100 μg $PLP_{139–151}$, and in C57BL/6 and 2D2 mice by immunization with 100 μg $MOG_{35–55}$. All peptides were dissolved in complete Freund's adjuvant (CFA) containing 4 mg/ml of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) as described previously (Li et al., 2008). On the day of immunization and 48 h later, C57BL/6 mice and 2D2 mice were injected with 100 ng of Bordetella pertussis toxin (BPT) in PBS. Mice were examined daily for clinical signs of EAE and scored as follows: 0, no paralysis; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hind limb and forelimb paralysis; 5, moribund or dead.

2.6. Flow cytometry

Monocytes were incubated with antibodies to murine CD11b, CD11c, CD3, CD4, and CD8a, MHC class II, CD40, CD80 and CD86 (BD Pharmingen, San Diego, CA). The absolute numbers for each population were calculated by multiplying the frequency of each population by the total number of cells isolated per treatment group.

For analysis of STAT4 and STAT6 signaling pathways, $2–3\times10^6$ cells from draining lymph nodes were stimulated *in vitro* with 25 μg/ml $MOG_{35–55}$ for 12 h, then washed with 3% FBS/PBS buffer. 0.5×10^6 cells were then incubated with mAbs (0.5 μg/sample) for cell surface proteins CD4 and CD8 (BD Biosciences, San Diego, CA). After 30 min, T cells were washed twice, followed by fixation in PBS/4% paraformaldehyde for 10 min and permeabilized with Permeabilization Buffer (BD Bioscience) for 5 min. Subsequently, cells were labeled with rabbit anti-phospho-STAT6 antibody (1:100 dilution) (Cell Signaling Technology Inc, Danvers, MA), goat anti-p-STAT4 antibody (Ser 721) (1:100 dilution) (Santa Cruz, Inc. Santa Cruz, CA), for 30 min at 4 °C, followed by the addition of secondary Cy3 conjugated anti-goat antibody (1:500 dilution) or Rhodamine conjugated anti-rabbit antibody (1:200 dilution) (Jackson ImmunoResearch Laboratories, West Grove, PA) for 20 min at 4 °C. After being washed twice, the stained cells were analyzed on a FACSAria flow cytometer with CellQuest software (BD Biosciences).

2.7. Purification of $CD11c^+CD11b^+CD8\alpha^-$ DC subsets and co-culture with $CD4^+$ T cells

$CD11c^+CD11b^+CD8\alpha^-$ DCs from spleen were prepared as previously described (Schlecht et al., 2006). Briefly, MNCs from spleen of mice were incubated with anti-mouse CD11c-coated magnetic beads (Miltenyi Biotec, Auburn, CA) and then subjected to positive selection through MACS separation columns. Cells selected on the basis of CD11c expression routinely consisted of >90% viable DCs. The enriched DCs were stained with FITC-conjugated CD11c, PE-conjugated CD11b, and APC-conjugated CD8α and sorted into $CD11c^+CD11b^+CD8\alpha^-$ DCs with a FACSAria (BD Biosciences, San Jose, CA).

Purification of $CD11c^+CD11b^+CD8\alpha^-$ DCs co-cultured with $CD4^+$ T cells to evaluate the effect of zymosan. For *in vivo* experiments, 2D2 mice were injected with either PBS or zymosan (100 μg /mouse/d) intraperitoneally (*i.p.*) for 10 days, and their $CD11c^+CD11b^+$ DCs and $CD4^+$ T cells were isolated and separately cultured in the presence of $MOG_{35–55}$ (25 μg/ml). In a reciprocal manner, zymosan-treated or untreated $CD11c^+CD11b^+$ DCs (1×10^4 per well) were cultured with zymosan-treated or untreated $CD4^+$ purified naïve T cells (10^5 per well) and stimulated with $MOG_{35–55}$ (25 μg/ml) or anti-CD3mAb (5 μg/ml). For the *in vitro* study, splenic $CD11c^+CD11b^+$ DCs (1×10^4 /ml) from 2D2 mice were cultured in the presence of zymosan (25 μg/ml) or media alone for 4 h at 37 °C. Separately, purified $CD4^+$ naive T cells (1×10^5 /ml) from 2D2 mice were cultured in the presence of zymosan (25 μg/ml) or media for 4 h. $CD11c^+CD11b^+$ DCs and T cells were then washed three times by centrifugation. $CD11c^+CD11b^+$ DCs were irradiated (28 GY) and $CD11c^+CD11b^+$ DCs and T cells were counted.

In vitro induction of $CD4^+CD25^+$ T cells by $CD11c^+CD11b^+$ DCs from zymosan- or PBS-treated control EAE mice were also evaluated in the presence or absence of MOG_{35-55} (25 μg/ml). $CD11c^+CD11b^+$ DCs were purified by FACS from zymosan-treated mice on day 21 post injection. These cells (1×10^4/ml)were co-cultured with $CD4^+$ T cells (1×10^5 ml) of zymosan- or PBS-treated control EAE mice in the presence or absence of $MOG_{35–55}$ (10 μg/ml). Seventy-two hrs after culture, the proportion of $CD4^+CD25^+$ T cells was examined by flow cytometry. Enriched CD4 + CD25 + T cells were analyzed for Foxp3 expression by FACS.

2.8. Antigen-specific T-cell proliferation and cytokine analysis

Splenocytes and peripheral lymph nodes were isolated from zymosan- or PBS-treated mice and cultured *in vitro* with the specific encephalitogenic peptide ($PLP_{139–151}$, $MOG_{35–55}$) used for the immunization or with concanavalin A (positive control). Cells were cultured in