NIH Public Access
Author Manuscript
J Neuroimmunol. Author manuscript; available in PMC 2014 January 15.

Published in final edited form as:
J Neuroimmunol. 2013 January 15; 254(1-2): 28–38. doi:10.1016/j.jneuroim.2012.08.013.

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).

Keywords

Zymosan; EAE/MS; macrophage/microglia; $CD11c^+CD11b^+$ DCs; Treg

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; Steinman et al. 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

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CONFLICT OF INTERESTS
The authors declare no competing financial interests.

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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

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).

Microglia and macrophages

Microglia EOC 20 cells, derived from C3H/HeJ CH-2k mice, were obtained from the American Type Culture Collection (ATCC) and were grown as recommended using DMEM media supplemented with 1mM 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 1ml 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.

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

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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.

Zymosan preparation and administration

Zymosan (Z4250, Sigma-Aldrich) was boiled for 30 minutes, 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.

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.

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, $2\sim3 \times 10^6$ cells from draining lymph nodes were stimulated *in vitro* with 25 μg/ml MOG_{35-55} for 12 hrs, 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).

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).

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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 (5μg/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.

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 96-well microtiter plates at a concentration of 5×10^6 cells/ml. Culture medium consisted of RPMI 1640 supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids (0.1 mM), penicillin (100U/ml), streptomycin (0.1 mg/ml), 2-mercaptoethanol (5×10^5 M) and 10% (v/v) FCS. Splenocytes from SJL/J or C57BL/6 mice were incubated for 72 h, while cultures from 2D2 mice were incubated for 48 h. Cultures were then pulsed for 18 h with 1 μCi per well of [^{3}H] thymidine before harvesting. Results are shown as mean of triplicates ± SEM.

Supernatants from splenocyte cultures and from cultured LNC were also used for cytokine analysis. Supernatants were collected at different times for measurement of cytokine levels: 48 h for IL-23, IFN-γ and IL-12 (p70), 72 h for IL-17 and TGF-β1, and 120 h for IL-4, IL-5 and IL-10. IL-4, IL-5, IL-10, IL-12(p70), IL-23 and IFN-γ levels were determined by using ELISA kits for the corresponding cytokines according to the manufacturer's protocols (PharMingen). TGF-β1 was measured by ELISA (R &D Systems). Results are shown as mean of triplicates ± SEM.

Isolation of $CD4^+CD25^+$, $CD4^+CD25^-$ T cell subpopulations and cytokine profile examination

Total T cells were enriched to >98% using a T cell negative selection kit (Miltenyi Biotec, Auburn, CA). $CD4^+$, $CD4^+CD25^-$, and $CD4^+CD25^+$ Treg cell subpopulations were enriched to >95% using a $CD25^+$ T cell isolation kit from Miltenyi Biotec according to the manufacturer's directions. Isolated cells were washed, stained with FITC-, PE-, and allophycocyanin-labeled appropriate Abs, and tested for purity by FACS before use. Enriched $CD4^+CD25^+$ cells were also analyzed for Foxp3 expression by FACS.

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$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from control and zymosan-treated mice were examined for cytokine profiles. Enriched T cell populations were cultured in the presence of 10 U/ml rIL-2 with and without anti-CD3 and anti-CD28 Abs (2 μg/ml each) with or without zymosan (50 μg/ml) for 48h. TGF-β1 and IL-10 levels were determined using ELISA kits.

Adoptive transfer

2D2 mice (five per group) were treated *i.p.* with either zymosan (100 μg/mouse/day) or PBS. After 7 days of treatment, mice were sacrificed. Splenocytes were isolated and cultured in the presence of MOG_{35-55} (10 μg/ml) for 48 h. Viable cells were removed by Ficoll gradient. $CD3^+$, $CD4^+$ or $CD8^+$ T cells were purified by high-affinity negative selection (R & D Systems) to greater than 95% purity. One million T cells were injected intravenously into naive 2D2 recipient mice. Twenty-four hours after adoptive transfer, recipient mice were immunized with MOG_{35-55} for EAE induction as previously described.

Statistical analysis

Data sets were tested for statistical significance using unpaired, 2-tailed, Student's t tests (for parametric data) and Mann-Whitney tests (for non-parametric data). Differences were considered significant if $p < 0.05$.

3. RESULTS

Zymosan treatment ameliorates chronic and relapsing experimental EAE

Zymosan treatment (100 μg/mouse) was administered at clinical onset in MOG_{35-55}-induced EAE in C57BL/6 mice, resulting in reduction of disease severity (Fig. 1a). When treatment was started during the acute phase, chronic EAE was also ameliorated (Fig. 1b). In $PLP_{139-151}$ induced relapsing-remitting EAE (Miller et al. 2010), zymosan administration prevented relapses when treatment began during the acute EAE phase (Fig. 1c) and reversed relapsing paralysis when administration was initiated at the onset of the first relapse (Fig. 1d). Zymosan also suppressed EAE in MOG-specific T-cell antigen receptor transgenic (2D2) mice (Fig. 1e), which develop fulminant EAE (Bettelli et al. 2003). After zymosan was discontinued, these mice did not develop fulminant EAE, whereas mice in the PBS-treated group succumbed to severe EAE. Thus, zymosan was effective in treatment at separate stages in three different EAE models. Histopathological analysis of the spinal cords revealed that zymosan-treated EAE in C57BL/6 mice resulted in fewer infiltrating inflammatory cells compared to PBS-treated EAE mice (Fig. 1f, g). Pathological scores of zymosan-treated EAE mice were decreased compared to PBS-treated EAE control groups (Fig. 1h).

In a pilot study we sought to ascertain the optimal dose of zymosan *in vivo*. In brief, zymosan at doses of 25, 100, 200, 500 μg/mouse ($n = 3$ in each group) or PBS was injected intraperitoneally (i.p.) once starting with day −1 of EAE induction. A zymosan dose of 100 μg/mouse was found to be the optimal dose based on our preliminary observation that lower doses failed to produce a significant delay in onset of EAE when treatment was initiated at 8 or 12 weeks of age. A higher dose of 200μg/mouse was less effective in the induction of tolerance, and 500 μg/mouse promoted EAE as reported by Veldhoen et al. 2006.

Zymosan treatment suppresses MHC class II expression on microglia

Zymosan and PBS-treated EAE mice were examined for $CD11b^+CD45^{low}$ microglia MHC class II expression in the CNS. A significant reduction was observed in zymosan-treated EAE mice (Fig. 2a), consistent with a marked reduction in CNS microglia cells. Similar results were obtained for peritoneal macrophage MHC class II expression in zymosan-

treated mice (Fig. 2b). Thus, zymosan-induced MHC class II suppression correlated with suppression of clinical EAE. Zymosan also inhibited IFN-γ-inducible MHC class II expression on microglia *in vitro* (Fig. 2c, d). Moreover, suppression of IFN-γ-inducible MHC class II expression by zymosan was markedly reversed by addition of U0126 (Fig. 2e), to a lesser extent by Laminarin (Fig. 2f), and totally reversed by U0126 plus Laminarin *in vitro* (Fig. 2g). Similar results were obtained with immunohistochemical staining of culture microglia (Fig. 2h, i, j, k, l, m).

Zymosan treatment decreased encephalitogenic T-cell proliferation and cytokine production

As EAE is mediated by encephalitogenic T cells (Schulze-Topphoff et al. 2009), we examined whether T-cell activation and regulation was altered in zymosan-treated EAE mice. Compared with PBS-treated EAE mice, *in vivo* treatment with zymosan suppressed recall responses to the encephalitogenic myelin peptide used in all three models (Fig. 3a). Culture supernatants were examined for Th1 cytokines (IFN-γ, IL-12), Th17 (IL-17), Th2 cytokines (IL-4, IL-5) and Th2/iTreg (IL-10). As shown in Fig. 3b, zymosan treatment was associated with a reduction in secretion of IL-17 and IFN-γ. In contrast, secretion of IL-4, IL-5, and IL-10 was increased.

Jak/Stat signaling pathway analysis showed that *in vivo* zymosan treatment was associated with inhibition of STAT4 phosphorylation and induction of STAT6 phosphorylation (Fig. 3c). Activated (tyrosine-phosphorylated) signal transducer and activator of transcription STAT4 has a key role in Th1, Th17 lineage commitment (Darnell 1997). Conversely, STAT6 is required for Th2-dependent lineage commitment (Darnell 1997). Thus, zymosan treatment can induce Th2 lineage commitment *in vivo*.

Zymosan exerts immunomodulatory effects on $CD11c^+CD11b^+$ DCs and T cells

To determine whether zymosan has direct immunomodulatory effects on both $CD11c^+CD11b^+$ DCs and $CD4^+$ T cells, cells were purified and cultured from zymosan or PBS-treated mice. In a reciprocal manner, $CD11c^+CD11b^+$ DCs from zymosan or PBS-treated mice were cultured with purified $CD4^+$ T cells from zymosan or PBS-treated mice. Proliferation was inhibited using zymosan-treated $CD11c^+CD11b^+$ DCs or $CD4^+$ T cells (Fig. 4a). We also examined MOG_{35-55} specific T-cell activation after incubation of $CD11c^+CD11b^+$ DCs or T cells with zymosan. Similarly, proliferation was suppressed when $CD11c^+CD11b^+$ DCs or purified 2D2 naïve $CD4^+$ T cells were treated with zymosan *in vitro* (Fig. 4b). A Th2/Treg cytokine pattern was also observed when using either *in vivo* or *in vitro* zymosan-treated $CD11c^+CD11b^+$ DCs and T cells (Fig. 4c). These results confirmed that zymosan exerted immunomodulatory effects on $CD11c^+CD11b^+$ DCs and $CD4^+$ T cells. Since zymosan treatment suppressed MHC class II (signal 1) expression on microglia and macrophages (Fig. 2a, b), and inhibited secretion of IL-23 and IL-12 (Fig. 4c), we examined whether zymosan altered expression of co-stimulatory (signal 2) molecules on $CD11c^+CD11b^+$ DCs. Zymosan inhibited IFN-γ-inducible expression of CD40, CD80 and CD86 co-stimulatory molecules on $CD11c^+CD11b^+$ DCs as well as MHC class II on primary microglia and macrophages. These effects were reversed by U0126 and Laminarin (Fig. 4d).

Generation of $CD4^+CD25^+$ T cells by $CD11c^+CD11b^+$ DCs *in vitro*

To determine whether the $CD11c^+CD11b^+$ DC subset is capable of triggering differentiation of $CD4^+$ T cells with a regulatory phenotype, $CD11c^+CD11b^+$ DCs from spleens of zymosan-treated EAE mice were cultured with $CD4^+$ T cells obtained from zymosan-treated or PBS-treated EAE mice for 3 days, with or without MOG_{35-55}. When $CD11c^+CD11b^+$ DCs were cultured with $CD4^+$ T cells from zymosan-treated mice in the presence of

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MOG_{35-55} the proportion of $CD4^+CD25^+$ T cells increased from 1.3% to 28.7% after 3 days of culture (Fig. 4e). In addition, the generation of $CD4^+CD25^+$ T cells was more pronounced when $CD4^+$ T cells were obtained from zymosan-treated mice compared to PBS-treated EAE mice. These findings suggest that zymosan-treated $CD11c^+CD11b^+$ DCs may induce $CD4^+CD25^+Foxp3^+$Tregs. $CD4^+CD25^+$ T cells from zymosan-treated mice also expressed significant amounts of TLR2 compared to $CD4^+CD25^-$ T cells (Fig. 4f). Furthermore, activated $CD4^+CD25^+$ T cells with anti-CD3 and anti-CD28 Abs from zymosan-treated mice produced greater amounts of TGF-β and IL-10 than control $CD4^+CD25^+$ T cells (Fig. 4g). These results suggest that IL-10 and TGF-β may be major mediators for the enhanced suppression by $CD4^+CD25^+$ T cells in zymosan-treated mice. The expression of dectin-1 on $CD11c^+CD11b^+$ DCs treated with zymosan was higher than untreated cells in both *in vitro* and *in vivo experiments* (Fig. 5a, b).

Protection from EAE upon adoptive transfer of zymosan-primed $CD4^+$ T cells

To test whether zymosan-induced $CD3^+$ T cells could protect mice from EAE induction, we adoptively transferred T cells primed with zymosan *in vivo* into syngeneic recipient mice. Splenocytes were isolated from 2D2 mice treated with either zymosan or PBS, and cultured with MOG_{35-55}. Cytokine analysis confirmed a Th2/Treg bias for lymphocytes from zymosan-treated mice and an encephalitogenic T bias from PBS-treated EAE mice (data not shown).

Following adoptive transfer of $CD3^+$ T cells from zymosan-treated mice, recipient 2D2 mice were subjected to EAE induction. All of the recipient mice that received lymphocytes from PBS-treated mice developed severe EAE (Fig. 6a). In contrast, mice receiving donor lymphocytes from mice treated with zymosan were almost entirely protected from EAE. In a subsequent experiment, zymosan-treated donor T cells were separated into $CD4^+$ and $CD8^+$ subpopulations. $CD4^+$ T cells (Fig. 6b), but not $CD8^+$ T cells (Fig. 6c), protected recipient mice from EAE induction. The data from adoptive transfer studies provided evidence that zymosan treatment induced $CD4^+$ T cell-dominant immune responses that suppress clinical autoimmune disease.

We next investigated whether donor $CD4^+$ T cells induce immune modulation *in vivo*. Zymosan-treated $CD4^+$ T cells were associated with an increased frequency of $Foxp3^+$ Treg cells (Fig. 6d). Proliferation and secretion of proinflammatory cytokines IFN-γ, IL-12 and IL-17 were reduced in recipients of zymosan-treated $CD4^+$ T cells (Fig. 6e). Conversely, secretion of Th2 cytokines IL-4 and IL-10 was increased in comparison with recipients of control $CD4^+$ T cells.

4. DISCUSSION

Previous studies have shown that injection of ovalbumin plus zymosan induced regulatory APCs and immune tolerance, skewing the cytokine balance to a Th2/Treg type response (Dillon et al. 2006). In the present study, we demonstrate that zymosan alone was effective in the prevention and reversal of chronic and relapsing CNS autoimmune disease. We also show that treatment with zymosan suppressed MHC class II up regulation *in vivo*, inhibited upregulation of co-stimulatory molecules on $CD11c^+CD11b^+$ DC, and promoted differentiation of regulatory T cells, which were biologically active in inhibiting encephalitogenic T cell-mediated autoimmune disease.

$CD11c^+CD11b^+$ DCs have been shown to play critical roles in immune modulation (Zhang et al. 2004; Min et al. 2006; Tang et al. 2006; Li et al. 2008). Our data show that zymosan induced $CD11c^+CD11b^+$DC activation via TLR2 and dectin-1-dependent activation of ERK MAPK, which promotes IL-10 production. Furthermore, zymosan induced splenic $F4/80^+$

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macrophages to secrete TGF-β, another immunosuppressive cytokine (Dillon et al. 2006). In addition, we show that the splenocyte cytokine profile of zymosan-treated animals was changed compared to control animals, resulting in inhibition of IFN-γ, IL-12, IL-23 and IL-17 production and upregulation of IL-4, IL-5, IL-10 and TGF-β. Furthermore, macrophages treated with zymosan promoted axon regeneration in a transient manner (Gensel et al. 2009). These results suggest that zymosan affects immune response via multiple mechanisms.

Dectin-1 is a C-type lectin (Brown and Gordon 2001; Reid et al. 2004; Taylor et al. 2007) expressed on macrophages, DCs and neutrophils that mediates attachment and ingestion of zymosan. Dectin-1 recognition of zymosan has been reported to be dependent on β-glucan (Rogers et al. 2005; Goodridge et al. 2007). It has been shown that the production of IL-10 by DCs was reversed by Laminarin, a blocker of β-glucan (Dillon et al. 2006; Ferreira et al. 2007; Sonck et al. 2010). On the other hand, a microglia cell line treated with Laminarin showed a modest increase in MHC II expression compared to TLR2 blocker and reversed zymosan stimulation, suggesting that the effects of Laminarin may have been mediated in part by contaminants that triggered TLR2; dectin-1 likely does not have a major role in inducing zymosan stimulation signaling pathways in microglia. We have shown that upregulation of IL-10 and TGF-β in $CD11c^+CD11b^+$ DCs in EAE mice is induced by zymosan and mediated through the TLR2 and dectin 1 pathway. A recent report indicated that TLR2 and poly (ADP-ribose) polymerase 1 promote CNS neuroinflammation in progressive EAE (Farez et al. 2009). TLR2 agonists have been shown to promote Th17 differentiation *in vitro*, leading to more robust cellular proliferation and Th17 cytokine production (Reynolds et al. 2010). On the other hand, TLR2 deficiency has been shown to result in increased Th17 infiltrates in experimental brain abscesses (Nichols et al. 2009). However, recent studies have also shown that TLR2 engagement enhances natural Treg function (Zanin-Zhorov et al.2006) and helps to expand Treg numbers (Liu et al. 2006; Sutmuller et al. 2006). Manicassmy et al. showed that zymosan induces retinaldehyde dehydrogenase type 2 (Raldh2) expression in DCs via a mechanism dependent largely on TLR2- mediated activation of MAPK (Manicassmy et al. 2009). TLR2 signaling is crucial for zymosan-mediated induction of Treg cells, and suppression of Th1 and Th17 mediated autoimmunity *in vivo*. However, the zymosan effect was not tested on microglia and peritoneal macrophages, and it is not known if zymosan could also be beneficial in the treatment of relapsing EAE. These reports, and our observation that zymosan triggers secretion of significant amounts of IL-10, IL-4 and TGF-β in EAE mice prompted us to examine the effect of zymosan, which is known to bind to TLR2 and the receptor, dectin-1, in modulating EAE. TLR2 and dectin-1 receptors are also known to collaborate in activating NF-κB and NFAT, leading to the production of IL-10 (Gantner et al. 2003; Slack et al. 2007) indicating that profound disease modulation could be achieved using zymosan. Our observations suggest that the immune response induced through TLR2 and dectin-1 affects the pathogenicity of T cells, and that zymosan treatment leads to repopulation of nonpathogenic T cells.

Although CD25 expression can be unregulated by activated $CD4^+$ T cells as well as Treg cells, the results in our study indicate that the $CD4^+CD25^+$ T cells expanded by exposure to $CD11c^+CD11b^+$ DCs are Treg cells, due to Foxp3 expression and inhibition of proliferation of $MOG_{35\text{-}55}$-reactive $CD4^+$ T cells *in vitro*. Tregs from treated mice secreted markedly higher amounts of TGF-β and IL-10 upon CD3 ligation and demonstrated an enhanced ability to suppress effector T cells compared with Tregs from untreated mice. These observations emphasize not only a positive role of innate immune response through receptors on natural Treg function, but also the importance of innate immunity through these receptors in maintaining peripheral tolerance.

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It is now known that mutually dependent steady state functions of APCs and Tregs are important to maintain peripheral T cell tolerance (Novak and Bieber 2008; Yamazaki and Steinman 2009; Fritzsching et al. 2009). Two major defects in the immune system that have been widely reported in EAE are Ag-presenting function and Treg properties (Slavin et al. 2001; Wang et al. 2008). Our observation that DCs and macrophages from zymosan-treated mice produce significant amounts of IL-4, IL-5, IL-10 and TGF-β indicates that these factors could be responsible, at least in part, for the alteration of T cell phenotype and function.

Several studies have shown that the dose of zymosan and the time of its administration can result in different immune reactions (Pillemer and Ross 1955; Martin et al. 1964). Zymosan is more effective at low doses than at high doses for host defense (Bradner et al. 1958; Karumuthil-Melethil et al. 2008). Recent studies have shown that low-dose zymosan induced regulatory APCs and immune tolerance (Dillon et al. 2006; Karumuthil-Melethil et al. 2008), but high-dose zymosan induced inflammatory disease, such as arthritis (Keystone et al. 1977) and EAE (Veldhoen et al. 2006). Similar to mycobacterium, high-dose zymosan induced Th17 cells and initiated EAE, but the disease was transient (Veldhoen et al. 2006). Although the mechanism is not known, recent studies have demonstrated that innate immune deficiency may result in chronic inflammation (Lalande and Behr 2010). TLR2 and dectin-1 mediate macrophage activation by innate immunity to mycobacterium (Yadav and Schorey 2006; Shin et al. 2008) while Th17 cells may link innate and adaptive immunity (Stockinger et al. 2007; Perry et al. 2011). Our observations indicate that protection from EAE through low-dose zymosan treatment could involve an innate immune response induced through receptors such as TLR2 and dectin-1. These observations also suggest that activation of diverse DC and macrophage subsets and production of pro- or anti-inflammatory cytokines in response to zymosan administration are time- and dose-dependent.

MS is a multiphasic disease (Steinman 2000). CNS inflammation and demyelination characterize the early relapsing-remitting phase, whereas neuronal loss and atrophy occur in the chronic ‘secondary progressive’ phase (Rasmussen et al. 2007). Our results suggest that zymosan may have therapeutic effects in the inflammatory phase. Zymosan is an attractive candidate for prophylactic treatment in patients who have experienced a single demyelinating event, a ‘clinically isolated syndrome’, and are at risk of recurrences or conversion to clinically definite MS. As zymosan has different mechanisms of action from currently approved MS treatments, it may be useful in combination with other therapies. Our results also provide a rationale for testing zymosan in other organ-specific $CD4^+$ T-mediated autoimmune diseases, including diabetes and rheumatoid arthritis.

In summary, zymosan was proven to effectively inhibit disease in the EAE murine model of MS. It therefore represents a new active, immunoregulatory drug with potential for the treatment of MS and other severe autoimmune diseases.

Acknowledgments

This work was funded by NIH grants R01NS40085, R01AT005322, R01NS046782, and R01NS048435.

We thank Hui Xu for critical review of this manuscript and Katherine Regan for editorial assistance.

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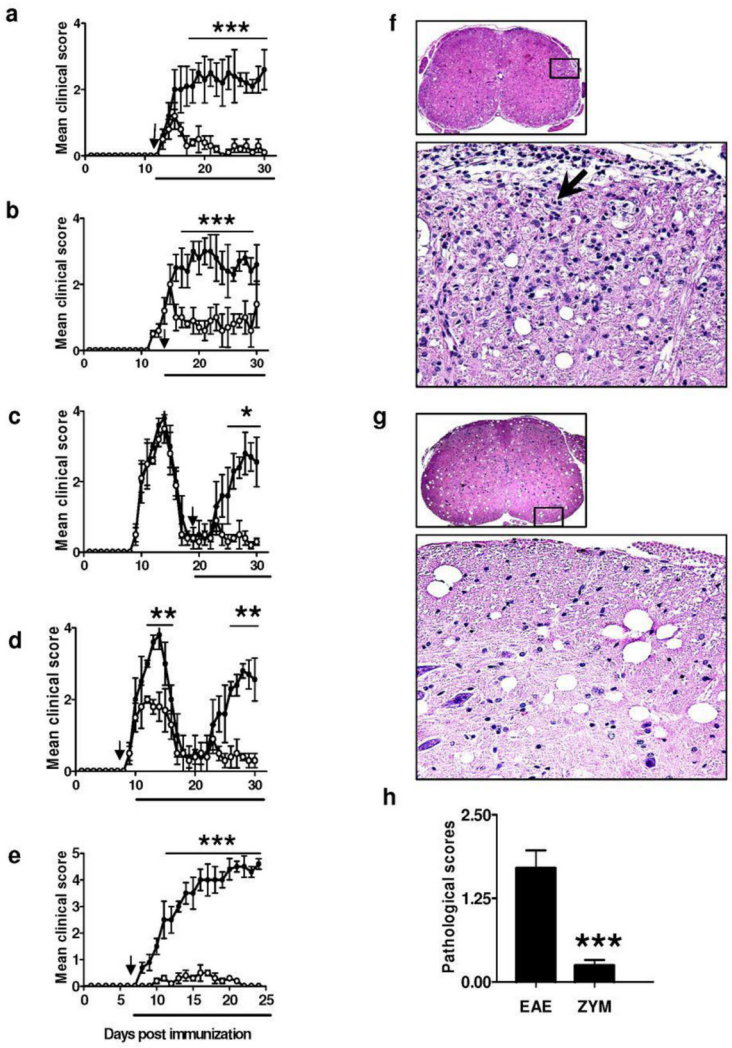

Figure 1. Zymosan treatment inhibits or reverses chronic and relapsing EAE

Zymosan ameliorated MOG $_{35-55}$-induced EAE in C57BL/6 mice when administered at (**a**) EAE onset (within one day of initial symptoms) or (**b**) after acute EAE was established. (**c**) Relapsing EAE in SJL/J mice was ameliorated when Zymosan treatment began at the onset of the first relapse. (**d**) Zymosan prevented relapsing EAE induced by immunization of SJL/J mice with $PLP_{139-151}$. (**e**) Limited EAE development in 2D2 mice after Zymosan treatment. Number of mice per group in each experiment: **a** (n=10), **b** (n=15), **c** (n=10), **d** (n=10), **e** (n=10). In **a–e** mice were scored and randomized immediately to receive either PBS (filled circles), or 100 μg/mouse/day zymosan (clear circles). Solid lines in each panel indicate zymosan treatment, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ when compared with PBS control groups. (**f**) and **(g)** represent the infiltration of leukocytes into the CNS of C57BL/6 mice after intraperitoneal (*i.p.*) injection of PBS and zymosan respectively. On 30d *p.i.*, spinal cords were harvested after extensive perfusion, and 5 μm sections were stained with H&E. Infiltration visualized at ×40, and ×200 magnifications in the white matter. (**h**) Mean scores of inflammation ± SD in the spinal cord of EAE C57BL/6 mice after zymosan or PBS-treatment. Three independent experiments are shown (n =10 per group). ***, $p < 0.001$. Error bars represent means ± SD.

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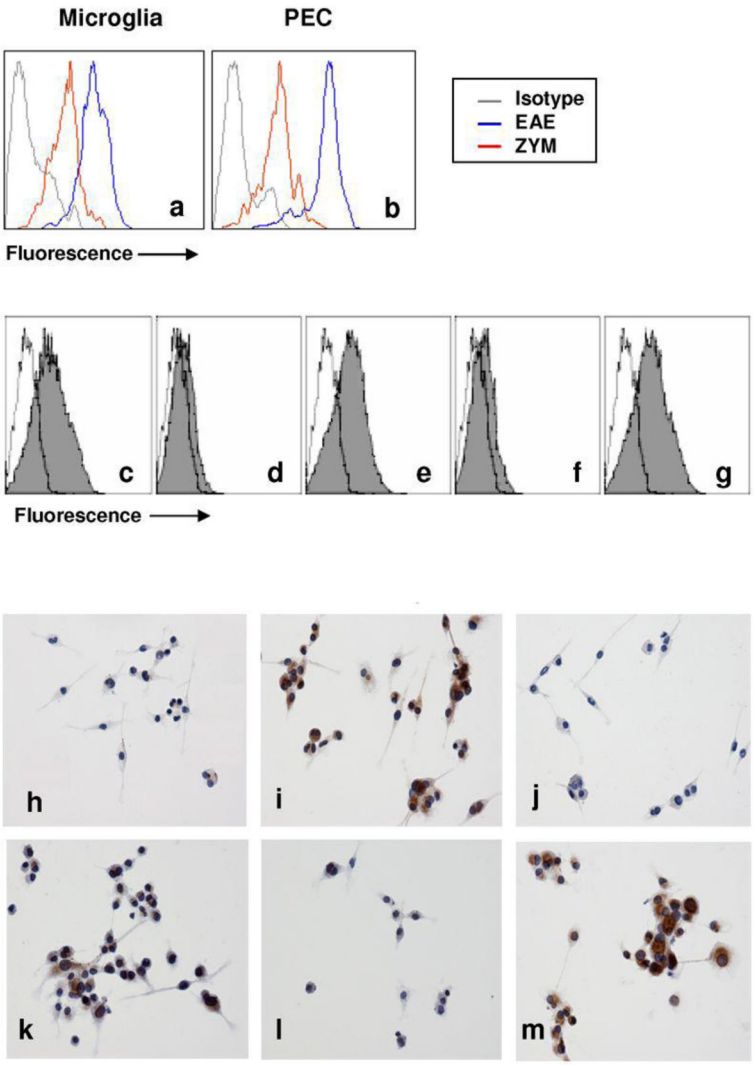

Figure 2. Zymosan treatment suppresses MHC class II expression on microglia

(**a**) Flow cytometric analysis indicated that MHC class II expression was downregulated in the $CD11b^{+}CD45^{low}$ microglia of the CNS in C57BL/6 EAE mice treated with zymosan. (**b**) Zymosan also inhibits MHC class II expression in peritoneal exudate cells (PEC) of C57BL/6 EAE mice. (**c**) Flow cytometric analysis of cell-surface MHC class II molecules on untreated or IFN-γ-treated (100 units/ml, 48 h) EOC 20 microglia. Filled histograms represent group treated *in vitro* with IFN-γ, while clear histograms represent the isotype control. (**d**) Zymosan (25 μg/ml) inhibited IFN-γ-inducible MHC class II expression, whereas (**e**) U0126 (10 μM), (**f**) Laminarin (100 μg/ml), and (**g**) U0126 (10 μM) plus Laminarin (100 μg/ml) reversed inhibition by zymosan. Immunohistochemical staining for MHC class II molecules on untreated (**h**) or microglia that received *in vitro* IFN-γ treatment (**i**). Suppression of IFN-γ-induced MHC class II expression on cultured microglia by zymosan (**j**) was largely reversed by the addition of U0126 (10 μM) (**k**), to a lesser extent by Laminarin (100 μg/ml) (**l**), and was totally reversed by U0126 (10 μM) plus Laminarin (100 μg/ml) (**m**). Original magnification ×160.

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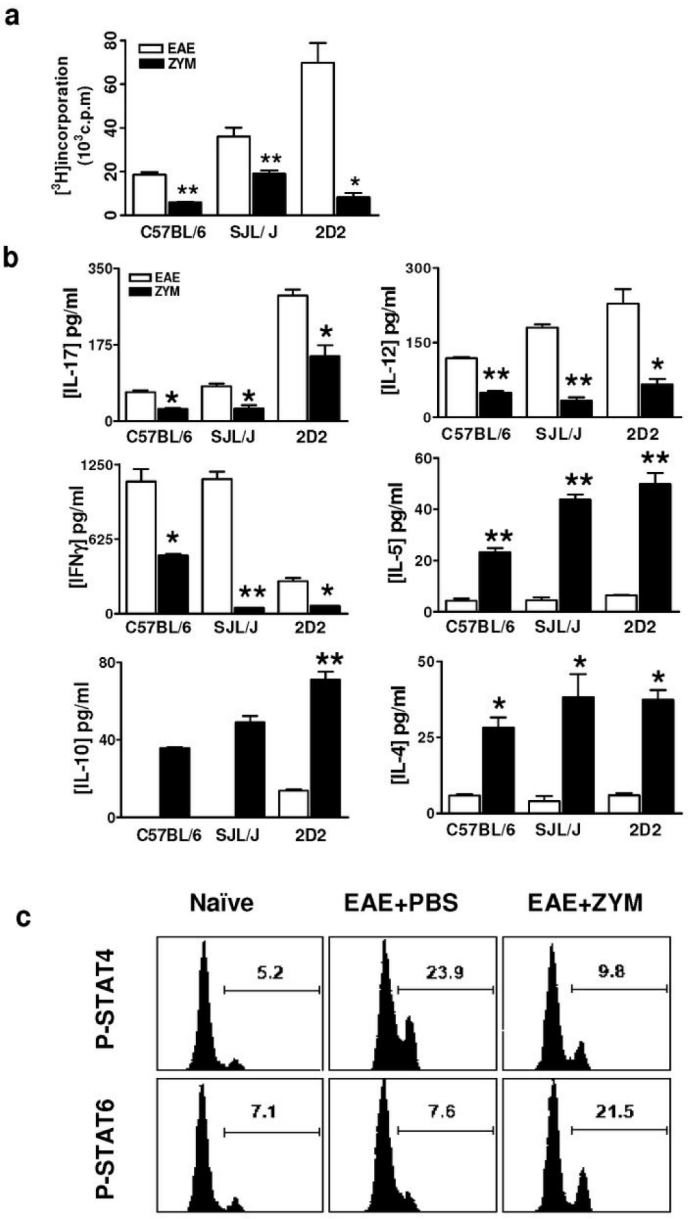

Figure 3. Zymosan treatment decreased T cell proliferation and cytokine production

(**a**) *In vivo* zymosan treatment of MOG_{35-55}-immunized C57BL/6 mice, $PLP_{139-151}$-immunized SJL/J mice and MOG_{35-55}-immunized 2D2 mice suppressed recall splenic proliferative responses. Splenocytes were isolated from mice treated with either PBS or zymosan (100 μg/mouse/day) *in vivo*, after 12d *p.i.* (**b**) *In vivo* zymosan treatment suppressed secretion of IL-17 (72h), IL-12 (48h), IFN-γ (48h), whereas secretion of IL-4 (120h), IL-5 (120h) and IL-10 (120h) was significantly increased. Open bars and filled bars represent data from PBS-treated and zymosan-treated mice respectively. Splenocytes were isolated from mice treated with either PBS or zymosan (100 μg/mouse/day) *in vivo*, after 10d *p.i.* *, $p < 0.05$; **, $p < 0.01$. Number of mice in each group: C57BL/6 mice (n=10), SJL/J mice (n=10), 2D2 mice (n=10). Error bars represent means ± SD. (**c**) Flow cytometric analysis indicated that *in vivo* zymosan treatment of MOG_{35-55}-immunized C57BL/6 mice suppressed STAT4 phosphorylation and promoted STAT6 phosphorylation by T cells. At

day 18 *p.i.*, 3×10^6 lymph node cells from mice were stimulated *in vitro* with MOG_{35-55} (25 g/ml) for 12 hrs. Cells were harvested and incubated with antibodies against p-STAT4 and p-STAT6, in combination with CD3 antibodies. One representative data set of 3 experiments is shown (total n =15 in each group).

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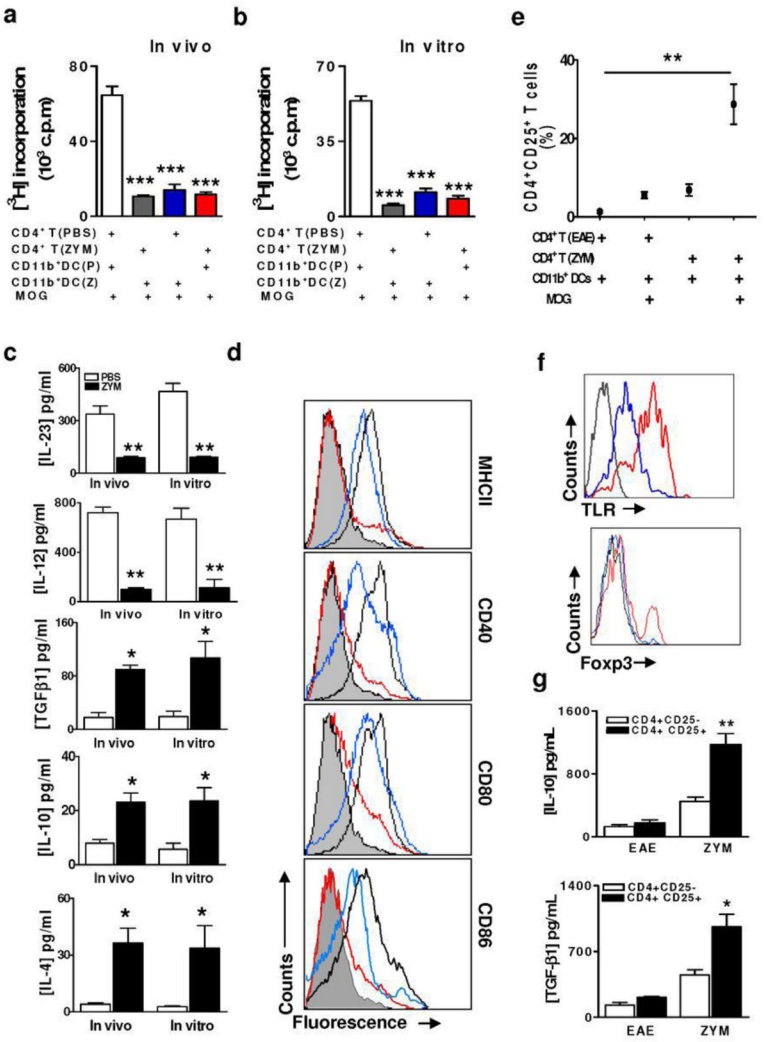

Figure 4. Zymosan has immunomodulatory effects on both CD11c$^+$CD11b$^+$ DCs and T cells

MOG$_{35-55}$-specific T-cell proliferation was examined using PBS or zymosan-treated CD11c$^+$CD11b$^+$ DCs with either PBS-treated or zymosan-treated CD4$^+$ T cells, either *in vivo* or *in vitro*. (**a**) Proliferative responses were reduced using either CD11c$^+$CD11b$^+$ DCs or T cells isolated from zymosan-treated mice. **(b)** *In vitro* zymosan treatment of either CD11c$^+$CD11b$^+$ DCs or T cells also suppressed proliferation. (**c**) Inhibition of proliferation by either *in vivo* (**a**) or *in vitro* (**b**) treatment of CD11c$^+$CD11b$^+$ DCs and T cells corresponded with a Th2/Treg cytokine pattern of secretion when comparing the PBS (clear bars) and zymosan-treated groups (solid bars). (**d**) *In vitro* zymosan treatment (25 μg/ml) of primary CD11c$^+$CD11b$^+$ DCs inhibited IFN-γ-induced expression of MHC class II molecules and co-stimulatory molecules, while U0126 (10 μM) and Laminarin (100 μg/ml) reversed inhibition as measured by flow cytometric analysis. Untreated CD11c$^+$CD11b$^+$ DCs (grey area), IFN-γ (100 units/ml, 48 h, black line), IFN-γ and zymosan (25 μg/ml, red line), IFN-γ and zymosan (25 μg/ml) plus U0126 (10 μM) and Laminarin (100 μg/ml, blue line). (**e**) *In vitro* induction of CD4$^+$CD25$^+$ T cells by CD11c$^+$CD11b$^+$ DCs from zymosan or PBS-treated EAE control mice in the presence or absence of MOG$_{35-55}$. (**f**) Expression of Foxp3 and TLR2 protein determined by flow cytometry on CD4$^+$CD25$^+$, CD4$^+$CD25$^-$ T cells stimulated by CD11c$^+$CD11b$^+$ DCs from zymosan-treated EAE mice on day 9 *p.i.*, in

the presence or absence of MOG_{35-55}. $CD4^+CD25^+$ T cells (red lines), $CD4^+CD25^-$ T cells (blue line). Corresponding isotype controls are indicated by dotted lines. Representative results from two experiments are shown. (**g**) $CD4^+CD25^+$ T cells from zymosan-treated mice secreted greater amounts of IL-10 and TGF-β1 compared to $CD4^+CD25^+$ T cells from PBS-treated mice on day 9 *p.i.* A two-tailed paired *t* test was performed to compare cytokine secretion by T cells from control and zymosan-treated mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Error bars represent means± SD.

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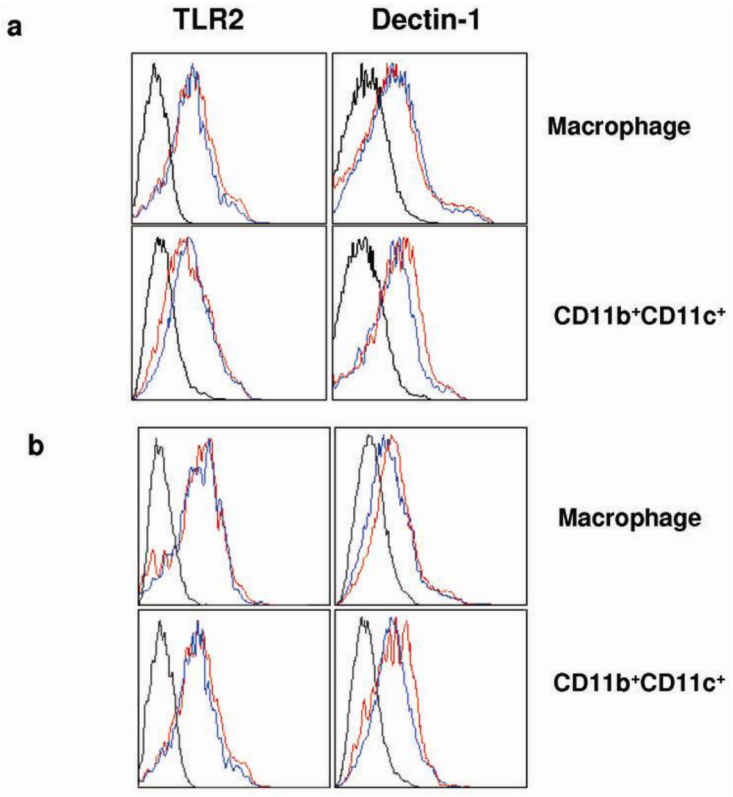

Figure 5. TLR2 and dectin-1 expression on macrophages and CD11c$^+$CD11b$^+$ DCs

(a) Freshly isolated splenic CD11c$^+$ CD11b$^+$ DCs and macrophages (1×10^6 cells/ml) were left untreated or exposed to zymosan (50 μg/ml) for 48 h. Cells were obtained from 8-wk-old EAE mice on day 10 *p.i.* Cells were stained using fluorochrome-labeled anti-mouse TLR2, or dectin-1 Abs. CD11c$^+$ CD11b$^+$ DC population and macrophages were gated for the graphs shown. Representative isotype control Ab staining histograms (black-outlined) that are overlaid with marker-specific staining histograms of untreated (blue-outlined) and zymosan-treated (red-outlined) cells are shown. (b) Eight-week-old female EAE mice were treated *i.p.* with zymosan (100 μg/mouse/day) or PBS control. Splenic CD11c$^+$ CD11b$^+$ DCs and macrophages obtained from these mice on day 7 were tested for TLR2 or dectin-1 Abs. Isotype control (black-outlined), untreated (blue-outlined) and zymosan-treated (red-outlined) mice are shown.

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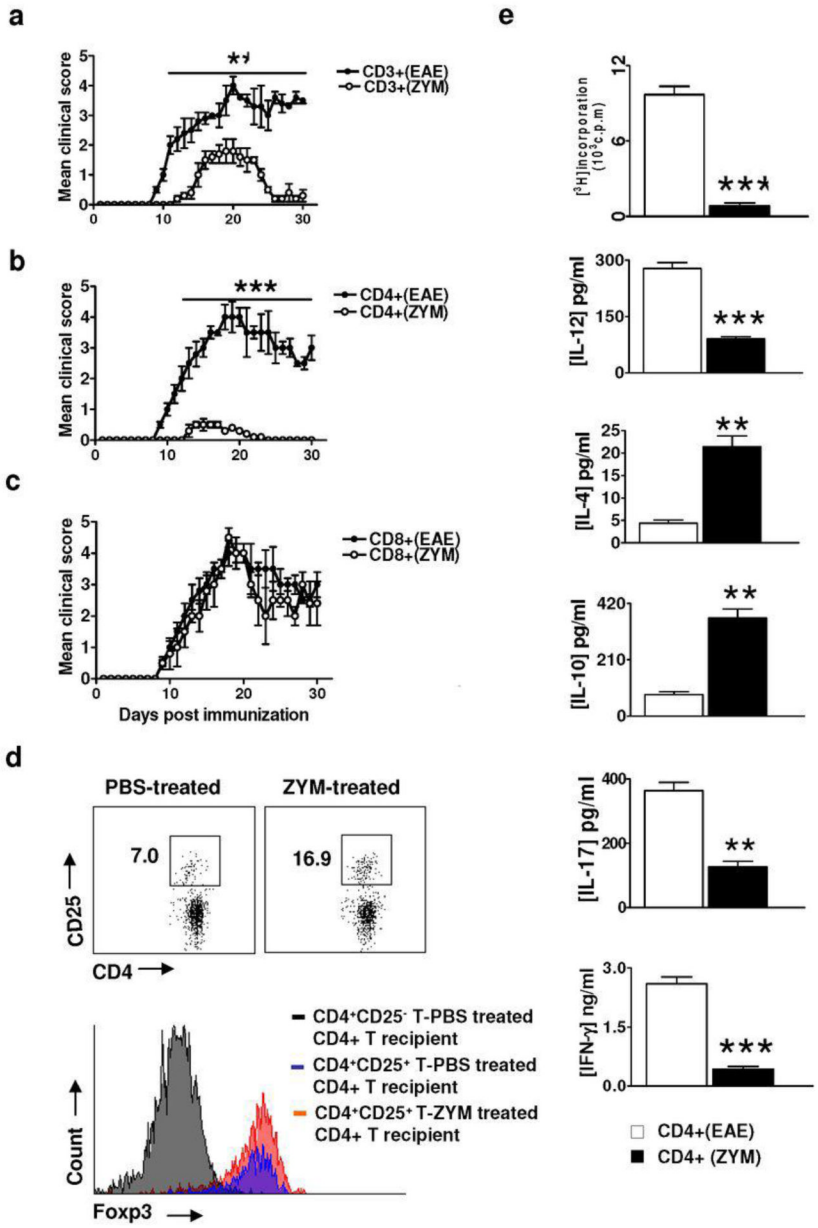

Figure 6. Adoptive transfer of zymosan-treated T cells prevents induction of EAE in recipient mice

Purified $CD3^+$ T cells (**a**) and $CD4^+$ T cells (**b**), but not $CD8^+$ T cells (**c**) from zymosan-treated donor mice protected recipient mice from EAE induction. Donor 2D2 mice were treated with either PBS, or zymosan (100 μg/mouse/day) *i.p.* At day 7 *p.i.*, splenocytes were isolated and cultured in the presence of MOG_{35-55} for 48 h. $CD3^+$ T cells were isolated and injected intravenously into recipient 2D2 mice (10^7 T cells per mouse) (**a**). One day later, recipient mice were immunized with 100 μg MOG_{35-55} for EAE induction. Recipient mice from PBS- or zymosan-treated cells were examined and scored for clinical EAE daily. In a separate experiment, $CD4^+$ (**b**) and $CD8^+$ (**c**) T cells were purified and tested in a similar manner. Solid circles represent data for recipients from the PBS-treated donor. Clear circles represent data for recipients from the zymosan-treated donor. (**d**) Splenocytes were isolated 13 d after adoptive transfer of zymosan- or PBS-treated $CD4^+$ T cells. Zymosan-treated

$CD4^+$ T cell expansion of $CD4^+CD25^+FoxP3^+$ cells in recipient mice. The percentage of $CD4^+CD25^+$ T cells within the population of $CD4^+$ T cells is indicated. (**e**) Zymosan-treated $CD4^+$ T cells showed decreased proliferation compared to control, indicating a Th2 bias and reduced development of IL-17-secreting T cells in recipient mice with established EAE. Evaluated for cytokine secretion upon restimulation with MOG_{35-55} (20 μg/ml). *, $p < 0.05$;**, $p < 0.01$; ***, $p < 0.001$ when compared with PBS-treated EAE controls. Results represent one of three similar experiments (total n=15 per group). Error bars represent means ± SD.

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