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Exopolysaccharide from *Bacillus subtilis* induces anti-inflammatory M2 macrophages that prevent T cell-mediated disease

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Abstract

Commensal bacteria contribute to immune homeostasis in the gastrointestinal tract; however, the underlying mechanisms for this are not well understood. A single dose of exopolysaccharide (EPS) from the probiotic spore-forming bacterium *Bacillus subtilis* protects mice from acute colitis induced by the enteric pathogen *Citrobacter rodentium*. Adoptive transfer of macrophage-rich peritoneal cells from EPS-treated mice confers protection from disease to recipient mice. *In vivo*, EPS induces development of anti-inflammatory M2 macrophages in a TLR4-dependent manner, and these cells inhibit T cell activation both *in vitro* and in *C. rodentium*-infected mice. *In vitro*, the M2 macrophages inhibit both $CD4^+$ and $CD8^+$ T cells, and the inhibition of $CD4^+$ T cells is dependent on TGF-β, whereas inhibition of $CD8^+$ T cells is dependent on both TGF-β and PD-L1. We suggest that administration of *B. subtilis* EPS can be utilized to broadly inhibit T cell activation and thus control T cell-mediated immune responses in numerous inflammatory diseases.

Introduction

Trillions of bacteria live in homeostasis within the gastrointestinal tract and provide a variety of benefits to the host including digestion of food, synthesis of vitamins, and development and maintenance of the immune system. Disruption of the normal microbiota can contribute to a wide range of diseases, including inflammatory bowel disease, allergy, and diabetes (1). Although the benefits of probiotics and a healthy microbiota in disease prevention are well documented (2, 3), we have limited knowledge of the mechanisms by which bacteria exert these beneficial effects.

Several commensal bacteria have been shown to limit colitis through both induction and inhibition of immune responses (4–11). Although polysaccharide A (PSA) from *Bacteroides fragilis* is the best characterized (12, 13), a few other bacterial molecules including carbohydrates (14, 15), proteins (16, 17), and sphingolipids (18), have been identified as immune modulators. For most probiotics, however, the molecules that mediate protection are not known. We utilize the Gram (+) spore-forming probiotic, *Bacillus subtilis*, which protects mice from acute colitis induced by the enteric pathogen *Citrobacter rodentium* (19). Infection with this pathogen is characterized by diarrhea, colonic hyperplasia, mucosal

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infiltration of hematopoietic cells, and increases in chemokines and pro-inflammatory cytokines, similar to the pathology induced by enteropathogenic *Escherichia coli* in humans (20–23). Protection by *B. subtilis* is not due to decreases in pathogen colonization, nor to increases in epithelial barrier integrity. Instead, it appears that *B. subtilis* prevents inflammation by modulating the innate immune response (14).

By screening several *B. subtilis* mutants, we found that protection from *C. rodentium*-induced inflammation requires the *eps* locus, which encodes molecules responsible for the synthesis of exopolysaccharide (EPS) (19, 24). We purified EPS from *B. subtilis* and showed that intraperitoneal (i.p.) injection of this material protected mice from disease, indicating that EPS is the molecule responsible for protection (14). Whereas other probiotics and probiotic molecules require repeated treatments (6, 13, 25–27), sometimes over the course of weeks, *B. subtilis* and purified EPS prevent disease after only a single dose (14, 19). Our goal is to elucidate the mechanism by which *B. subtilis* EPS protects from inflammation caused by *C. rodentium* infection. Here, we identify the protective cells as M2 macrophages and show that they inhibit activation of both $CD4^+$ and $CD8^+$ T cell responses, and that the inhibition is mediated by TGF-β and PD-L1.

Materials and Methods

Mice and Reagents

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Loyola University Medical Center (Maywood, IL). Specific pathogen–free C57BL/6 and $TLR4^{-/-}$ founders were purchased from The Jackson Laboratory and bred in-house. Mice lacking MyD88 in myeloid cells were generated by crossing *Lyz2*-Cre transgenic mice to MyD88-floxed mice as described (49). Sterile standard chow and tap water were given to mice ad libitum.

All base media and supplements were from Life Technologies (Grand Island, NY). All antibodies were from Biolegend (San Diego, CA) unless otherwise indicated. The fluorescent antibodies used for flow cytometry include anti-CD16/32 (FC block), anti-F4/80 (BM8), anti-CD25 (PC61), anti-CD4 (GK1.5), anti-CD8 (53–6.7), anti-CD11b (M1/70), anti-CD44 (IM7), anti-IL4Rα (I015F8), anti-CD206 (C068C2), and sheep anti-mouse/human arginase1 (R&D systems). Enzyme-Linked Immunosorbent Assay (ELISA) reagents for quantitation of IFNγ, TNFα, and KC/CXCL1 were from R&D Systems; IL-17A, IL-2, IL-13 and TGF-β matched-pair reagents were from Biolegend. Clodronate and PBS liposomes were from VU medisch centrum (Amsterdam, The Netherlands). The anti-CD3ε (145-2C11) for *in vivo* experiments was from Leinco Technologies, Inc, (St. Louis, MO). The anti-CD3ε used for *in vitro* experiments was LEAF-purified anti-CD3ε (145-2C11, Biolegend).

The following neutralizing antibodies were used for *in vitro* assays: TGF-β inhibition, 5 μg/mL anti-TGF-β (1D11, R&D Systems) or equivalent concentration of LEAF-purified mouse IgG1 isotype control; PD-L2 inhibition, 3 μg/mL anti-PD-L2 (TY25) or isotype control LEAF-purified rat IgG2a; PD-L1 inhibition, 5 μg/mL anti-PD-L1 (10F.9G2) or isotype control LEAF-purified rat IgG2b. Experiments with blocking antibody included the

addition of 1 μg/mL LEAF-purified anti-CD16/32 (FcγRII/III) to all wells. For small molecule inhibitors, the following concentrations were used: Nor-NOHA (12 μM), exogenous L-arginine (2 mM), NS-398 (1 μM).

Purification of Exopolysaccharide

Exopolysaccharide was isolated from *B. subtilis* DS991 (Δ*sinRtasA* mutant), a strain that produces and secretes large amounts of EPS (24). The negative control, designated ΔEPS, DS5187 (*sinRtasAepsH* mutant), does not produce EPS and does not protect from *C. rodentium*-induced disease (14, 24). EPS was isolated from stationary phase supernatants of bacteria grown in Luria–Bertani (LB) or MSgg medium isolated by 50% EtOH precipitation at −20º C. The precipitate was pelleted (15,000xg, 4°C, 20 min), and resupended in 0.1 M Tris (pH 8.0) and samples were treated with DNase (67 mg/ml) and RNase (330 mg/ml) at 37°C for 2 h followed by proteinase K (40 mg/ml) digestion at 55°C for 3h. EPS was further purified by gel filtration on an S1000 column. Dialyzed EPS was quantified by dry weight and phenol sulfuric acid assay.

B. subtilis spore preparation

B. subtilis wild-type 3610, DS76 (*espH* mutant) were germinated via exhaustion as described previously (19). On the day of administration, *B. subtilis* spores were washed with ice-cold water, resuspended in 100 mL PBS, and administered to mice via oral gavage. Cells were isolated 5 days post-gavage for analysis.

Adoptive transfer studies

For adoptive transfer, peritoneal cells were isolated by lavage (with RPMI/50%FBS) from mice 3 days post-treatment with EPS (i.p.). Cells in the granulocyte and lymphocyte gates were FACS-sorted based on forward scatter (FSC) and side scatter (SSC) and injected i.p. into mice. For macrophage depletion studies, mice were injected i.p. with 200 μL clodronate-loaded or PBS-loaded liposomes (Stock 5 mg/mL). Four to 6 hr later, mice were treated with EPS, and then 3 days later, peritoneal cells were isolated by PBS lavage. By flow cytometry, less than 1% of the transferred cells were macrophages.

C. rodentium ATCC 51459 was cultured 16 hr in LB medium and washed once in PBS. An infectious dose (5×10^8 CFUs) was resuspended in 100 μL sterile PBS and administered to mice by oral gavage. Disease was assessed 11 days post-infection (dpi). Serum cytokine levels were assessed by ELISA and distal colons were collected and processed for histological analysis as described (19). To assess diarrhea, feces were examined and scored 1–4 (19): 1, no diarrhea (hard, dry pellets); 2, slightly soft stool (mild diarrhea); 3, very soft stool (moderate diarrhea); and 4, unformed stool (severe diarrhea).

Flow cytometry

For flow cytometry, cells were treated with anti-CD16/32 Fc Block and then stained with surface antibodies. Cells were analyzed on FACSCanto II or LSRFortessa flow cytometers; cell sorting was performed on a FACSAria cell sorter (BD Biosciences). Analyses were performed using FlowJo software (Tree Star, Ashland, OR) by first gating on single cells, and then analyzing each population as described.

In vitro and in vivo treatment with EPS

For *in vitro* treatment of peritoneal cells, total peritoneal cells were isolated by lavage with 5mL PBS and plated in 24-well plates for 2 hr to allow macrophages to adhere. Non-adherent cells were aspirated and washed away. The remaining cells were 80–90% $F4/80^+CD11b^+$ macrophages. Cells were treated for 16 hr with 1 μg/mL EPS purified from DS991 *B. subtilis* grown in MSgg medium. For intracellular cytokine analysis, cells were cultured for an additional 2 hr with Brefeldin A, and analyzed by flow cytometry or qRT-PCR. For *in vivo* treatment, mice were injected i.p. with EPS (100 μg) and 3 days later, peritoneal cells obtained by lavage as described (14). The level of TGF-β production in serum (1:100) was determined by ELISA after activating TGF-β with 5μL 1N HCL/100μL for 15 min at RT, followed by neutralization with 1N NaOH.

T cell proliferation assay

Splenocytes were labeled with 5 μM CellTrace Violet (Life Technologies) according to manufacturer's directions and cultured either alone (3×10^5 cells) or with total peritoneal cells (10^4) or purified macrophages (5×10^3) in 96-well flat-bottom tissue culture plates coated with 2 μg/mL anti-CD3. Three days later, non-adherent cells were collected, stained and analyzed by flow cytometry. For transwell experiments, 1×10^6 splenocytes were cultured in anti-CD3-coated 24-well plates with or without transwell inserts (Corning) containing 5×10^5 peritoneal cells.

In vitro T cell stimulation

Mesenteric lymph nodes were isolated from mice 7 days post-infection with *C. rodentium*. Cells (3×10^5) were stimulated with PMA (50 ng/mL) and Ionomycin (1 μg/mL) for 4 h. Cytokine production was assessed by ELISA.

Real-time quantitative PCR

RNA was isolated from flow cytometry–sorted $F4/80^+CD11b^+$ macrophages using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was prepared. PCR was performed on a C1000 thermal cycler with CFX96 real-time detection system (Bio-Rad, Hercules, CA) using the following primers (forward and reverse): *Arg1*, 5′-AGACCACAGTCTGGCAGTTG-3′ and 5′-CCACCCAAATGACACATAGG-3′; *Nos2*, 5′-CAGCTGGGCTGTACAAACCTT-3′ and 5′-CATTGGAAGTGAAGCGTTTCG-3′; *Ym-1*, 5’ – CATGAGCAAGACTTGCGTGAC-3’ and 5’ – GGTCCAAACTTCCATCCTCCA-3’; *FIZZ-1*, 5’ – TCCCAGTGAATACTGATGAGA-3’ and 5’ – CCACTCTGGATCTCCCAAGA-3’; *IL-12p40*, 5’ - GAAGTTCAACATCAAGAGCAGTAG-3’ and 5’ – AGGGAGAAGTAGGAATGGGG-3’; *Actb* (β-actin), 5′-GGCTGTATTCCCCTCCATCG-3′ and 5′-CCAGTTGGTAACAATGCCATGT-3′. Expression of each target gene was normalized to β-actin expression, and data are presented relative to $F4/80^+CD11b^+$ cells isolated from untreated mice.

Statistical analysis

Statistical significance was determined by unpaired two-tailed Student's *t* test unless otherwise indicated using Prism software (GraphPad Software; La Jolla, CA). A *p* value <0.05 was considered statistically significant with p<0.001 denoted as ***, p = 0.001 to 0.01 denoted as **, and p = 0.01 to 0.05 denoted as *. Not significant denoted as ns (p > 0.05).

Results

Requirement of macrophages for EPS-mediated protection

Previous studies showed that transfer of total peritoneal cells from EPS-treated mice into naïve mice protected recipients from development of *C. rodentium*-induced infectious colitis (14). To identify the cell-type that mediates protection, we FACS-purified cells from the lymphocyte and granulocyte gates from the peritoneal cavity of EPS-treated mice. These cells (3×10^4) were adoptively transferred to recipient mice −1, +1, and +3 days post-infection (dpi) with *C. rodentium*, and disease was assessed 11 dpi. The cells in the granulocyte gate protected recipient mice from disease, whereas lymphocytes did not, as evidenced by increased colonic crypt heights, loose stool, and increased levels of serum pro-inflammatory chemokine CXCL1 (Figure 1A & B). These data suggest that cells in the granulocyte gate, which are 90% macrophages, mediate protection by EPS. To test if macrophages traffic from the peritoneal cavity to inhibit disease, we labeled peritoneal cells from EPS-treated mice with CFSE and transferred them into recipient mice by i.p. injection. By flow cytometry, we found $CFSE^+F4/80^+$ macrophages in the mesenteric lymph node (MLN), a few in the spleen, and some cells were still present in the peritoneum (Figure 1C). These data suggest that macrophages traffic to the site of inflammation to prevent disease.

To test if macrophages are required for protection, we first injected mice with clodronate liposomes to deplete macrophages. Subsequently, mice were treated with EPS and 3 days later, we adoptively transferred peritoneal cells (6×10^4) to naïve recipients −1, +1, and +3 dpi with *C. rodentium*; disease was assessed 11 dpi. All mice that received macrophage-depleted peritoneal cells from EPS-injected mice had evidence of disease, whereas only 1 of 7 that received peritoneal cells from EPS-injected mice treated with PBS liposomes developed disease (Figure 1D & E). These data indicate that macrophages are required for EPS-mediated protection from *C. rodentium*-induced inflammation.

M2 macrophage induction by B. subtilis EPS

Macrophages polarize into pro-inflammatory M1 or anti-inflammatory M2 macrophages. We tested if EPS induces M2 macrophages by culturing peritoneal $F4/80^+$ cells overnight with EPS (1 μg/mL). By flow cytometry, we found upregulation of the M2 markers arginase-1 (Arg-1), CD206, IL-4Rα, and PD-L1 in EPS-treated cells (Figure 2A). These data indicate that EPS induces M2 macrophages *in vitro*. These cells also expressed increased levels of IL-13 and IL-4, cytokines required for induction of M2 macrophages (Figure 2A). We do not find increased expression of IL-13 or IL-4 in $CD3^+$ T cells or IgM^+ B cells cultured with EPS (data not shown).

To determine if EPS also induces an M2 macrophage phenotype *in vivo*, we examined peritoneal cells from mice 3 days after i.p. injection with EPS. We found increased M2 macrophage marker expression on F4/80$^+$CD11b$^+$ cells, whereas treatment with the negative control, ΔEPS, did not (Figure 2B & C). Additionally, we FACS-purified F4/80$^+$CD11b$^+$ macrophages from EPS-injected mice and found that the M2 macrophage transcripts *Ym-1* and *FIZZ-1* were upregulated 5-fold and 9-fold, respectively, compared to macrophages from ΔEPS-treated mice. No upregulation of the M1 macrophage markers *IL-12p40* or *Nos2* (iNOS) was found (Figure 2D). We conclude that *in vivo* administration of EPS induces cells with an M2 macrophage phenotype.

EPS protection from *C. rodentium*-induced disease requires TLR4, and peritoneal cells from TLR4$^{-/-}$ mice were unable to prevent disease following transfer to naïve WT mice (14). These findings suggest that EPS induction of M2 macrophages requires TLR4 signaling. After injecting TLR4$^{-/-}$ mice with EPS, we found no change in expression of the M2 markers Arg-1, CD206, IL-4Rα, or PD-L1 (Figure 2B & C). Similarly, little to no upregulation of *Ym-1* and *FIZZ-1* transcripts occurs in macrophages from EPS-injected TLR4$^{-/-}$ mice (Figure 2D). As expected, EPS also does not alter expression of the M1 macrophage markers, *IL-12p40* and *Nos2* (iNOS) (Figure 2D). These data show that the induction of M2 macrophages by EPS requires TLR4 signaling. Consistent with this finding, no M2 macrophages were found in the peritoneal cavity of EPS-injected myeloid-specific MyD88$^{-/-}$ mice (data not shown), which are not protected by EPS from *C. rodentium*-induced disease (14). These data suggest that polarization of M2 macrophages by EPS requires both TLR4 and MyD88 in myeloid cells.

Anti-inflammatory response induced by B. subtilis EPS

Much of the pathology associated with *C. rodentium* is driven by hyper-inflammatory CD4$^+$ T cell responses (20, 21). We hypothesize that EPS-induced M2 macrophages prevent disease by inhibiting T cell activation. To test this possibility, we FACS-purified peritoneal macrophages (F4/80$^+$CD11b$^+$) from EPS-treated mice or ΔEPS-treated mice and tested if they inhibited T cell proliferation. Peritoneal macrophages from EPS- (or ΔEPS-) treated mice were co-cultured with anti-CD3 stimulated splenocytes labeled with CellTrace Violet. As determined by flow cytometry, macrophages from EPS-treated mice inhibited the proliferation of not only CD4$^+$ T cells (Figure 3A), but also CD8$^+$ T cells (Figure 3B), indicating that EPS-induced M2 macrophages broadly inhibit T cell responses. In addition, these macrophages decreased T cell activation, as measured by expression of CD25 and CD44 (Figure 3A & B, right). As a control, macrophages from ΔEPS-treated mice did not inhibit T cell activation or proliferation (Figure 3A & B). The inhibition of T cell responses by F4/80$^+$CD11b$^+$ cells was specific because F4/80$^-$ peritoneal cells (CD3$^+$ T cells and IgM$^+$ B cells) did not inhibit T cell responses (Figure 3C). As expected, peritoneal cells from EPS-treated TLR4$^{-/-}$ or myeloid MyD88$^{-/-}$ mice, which do not generate M2 macrophages, also did not inhibit T cell proliferation (Figure 3D & E). We conclude that EPS-induced anti-inflammatory M2 macrophages inhibit T cell activation and proliferation *in vitro*. Similar to EPS, administration of *B. subtilis* spores (by oral gavage) resulted in the generation of peritoneal cells that inhibited T cell responses, whereas peritoneal cells from mice gavaged with EPS-deficient *epsH B. subtilis* spores did not inhibit T cell proliferation

(Figure 3F). We conclude that both *B. subtilis* and EPS induce systemic anti-inflammatory responses.

To determine if EPS reduces T cell responses during *C. rodentium* infection, we assessed IL-2 production in the MLN of mice pretreated with EPS. Seven days post-infection, we found that MLN cells from infected mice treated with EPS had reduced IL-2 production compared to mice infected with *C. rodentium* alone (Figure 4A, left). To directly test if EPS-induced M2 macrophages alter T cell responses during *C. rodentium* infection, we purified F4/80$^+$ peritoneal macrophages from EPS-treated mice and transferred them into recipient mice infected with *C. rodentium*. Seven days post-infection, we assessed T cell cytokine production by MLN cells. Infected mice that received F4/80$^+$ macrophages had reduced IL-2 production compared to mice that received F4/80$^-$ peritoneal cells from EPS-treated mice (Figure 4A, right). These data suggest that EPS-induced M2 macrophages prevent *C. rodentium*-induced inflammation by inhibiting T cells.

To further test if EPS inhibits T cell responses *in vivo*, we induced M2 macrophages by administering EPS and then 3 days later, injected the mice with anti-CD3 (0.25 mg/kg body weight) to induce potent T cell activation. We expected decreased production of TNF-α, IFN-γ, and IL-2 in EPS-treated mice compared to untreated mice. Indeed, we found a two-fold decrease in production of these cytokines in serum of EPS-injected mice compared to untreated mice (Figure 4A). We tested if EPS inhibited a specific T cell subset by measuring levels of Th1, Th17, and Th2 inflammatory cytokines, IFN-γ, IL-17A and IL-13, respectively, in cultures of splenocytes from EPS- or ΔEPS-treated mice stimulated with anti-CD3. The secretion of each of the cytokines was decreased in EPS-treated mice compared to ΔEPS-treated mice (Figure 4B), indicating that EPS induces a systemic anti-inflammatory response that broadly suppresses Th1, Th17, and Th2 cytokine production.

Requirement of TGF-β and PD-L1 for EPS-induced M2 macrophage inhibition of T cell proliferation

M2 macrophages inhibit T cell responses by multiple factors including Arg-1, PGE_2, IL-10, PD-L1/2 and TGF-β (28–30). When separated from T cells in a transwell, the M2 macrophages did not inhibit T cell proliferation, indicating that cell-to-cell contact is required for inhibition (Figure 5A & B). To identify the molecule(s) produced by EPS-induced M2 macrophages that prevents T cell activation, we added inhibitors to the T cell inhibition assay. Whereas only 14% of CD4$^+$ T cells proliferated in cultures with peritoneal cells from EPS-treated mice, proliferation was greatly increased by the addition of anti-TGF-β (69% CD4$^+$ T cells) (Figure 5C), indicating that EPS-induced M2 macrophages produce TGF-β, which inhibits CD4$^+$ T cell proliferation *in vitro*. In contrast, inhibition of Arg-1 activity by Nor-NOHA, or addition of exogenous L-arginine, did not restore T cell proliferation (Figure 5D), even though we find increased expression of Arg-1 in EPS-induced M2 macrophages (Figure 2A & B). Further, the addition of NS-398, a COX2 inhibitor that prevents PGE_2 production, or addition of neutralizing antibodies to PD-L2, PD-L1, and IL-10 did not restore CD4$^+$ T cell proliferation (Figure 5D), suggesting that TGF-β is responsible for the inhibitory effect of M2 macrophages on CD4$^+$ T cells.

Consistent with this conclusion, we found a small, but significant increase in levels of total TGF-β in serum from EPS-treated mice 3 days after treatment (Figure 5E).

Anti-TGF-β also partially restored $CD8^+$ T cell proliferation, as did neutralizing anti-PD-L1 antibody (Figure 5F). The addition of both antibodies resulted in complete restoration of $CD8^+$ T cell proliferation (Figure 5F), indicating that both TGF-β and PD-L1 contribute to the inhibition of $CD8^+$ T cells. Neutralizing anti-IL-10 did not restore proliferation of $CD8^+$ T cells. We conclude that the EPS-induced M2 macrophages inhibit T cells through TGF-β and PD-L1, in a cell contact-dependent manner.

Induction of Regulatory T Cells by B. subtilis EPS

Naïve $CD4^+$ T cells polarize into effector T cell subsets according to environmental conditions. In the presence of TGF-β, $CD4^+$ T cells upregulate expression of Foxp3 and become inducible T regulatory cells (iTreg) (31). Since M2 macrophages produce TGF-β, we hypothesize that these could also induce Treg cells. We examined Foxp3 expression in peritoneal $CD4^+CD25^+$ T cells 3 days post EPS-treatment, and found an increase in $Foxp3^+$ cells in EPS-treated mice compared to untreated mice (Figure 6A & B). Further, peritoneal cells from EPS-treated mice induced more $CD4^+CD25^+Foxp3^+$ T cells in IL-2-containing co-cultures of peritoneal cells with anti-CD3-stimulated splenocytes, compared to co-cultures from untreated mice (Figure 6C & D). Together, these data suggest that EPS has the capacity to induce Treg cells, presumably through TGF-β produced by the M2 macrophages.

Discussion

Studies of bacterial products that modulate the immune system have, until recently, focused primarily on pathogenic molecules that elicit pro-inflammatory responses or contribute to evasion of the immune system. These studies identified host pattern recognition receptors, such as TLRs and NODs and their cognate ligands, e.g., LPS, lipoteichoic acid and flagellin, along with the downstream signaling pathways. Less understood are the mechanisms by which commensal bacteria and commensal-derived products circumvent pro-inflammatory responses, allowing select bacteria to live in homeostasis with the host. We previously showed that a single oral dose of the probiotic *B. subtilis* or a single i.p. injection of *B. subtilis* EPS prevents inflammatory responses against the enteric pathogen, *C. rodentium*. Here, we showed that *B. subtilis* EPS induces M2 macrophages that broadly inhibit *in vitro* and *in vivo* T cell responses through both TGF-β and PD-L1.

During *C. rodentium* infection, mice display increased expression of the Th17 cytokine, IL-17A, in Peyer's patches (23), and Th1 cytokines, IL-12, IFN-γ and TNF-α, in the colon (20, 21). These cytokines lead to increased production of chemokines that recruit innate immune cells to the site of infection, exacerbating disease. EPS-induced M2 macrophages efficiently block this complex immune response to *C. rodentium* infection and also reduce $CD4^+$ and $CD8^+$ T cell responses after mice are injected with anti-CD3. Increases in M2 macrophages are found both in the peritoneum and spleen after i.p. or i.v. injection of EPS, as well as after oral administration of *B. subtilis* spores (data not shown). We also find donor M2 macrophages in the spleen and mesenteric lymph node in mice receiving adoptive transfer of peritoneal cells from EPS-treated mice, further indicating that injection of EPS

induces M2 macrophages that can migrate throughout the body. Because EPS and *B. subtilis* can elicit an anti-inflammatory response when administered through several different routes, we think that EPS and/or *B. subtilis* are good candidates as therapeutic agents for treating inflammatory diseases.

Mechanism of EPS induction of M2 macrophages

EPS does not protect *TLR4*$^{-/-}$ mice from disease caused by *C. rodentium*, and we showed that M2 macrophages did not develop in these mice or in mice defective in MyD88 signaling in myeloid cells. The dependency on TLR4 is not likely due to LPS because *B. subtilis* is Gram (+) and produces little to no LPS. In contrast to other bacterial polysaccharides, notably PSA from *B. fragilis* that signals through TLR2, EPS is unlikely to function through TLR2 because EPS induces M2 macrophages in TLR2$^{-/-}$ mice (data not shown). We hypothesize that EPS utilizes a co-receptor of the TLR4 signaling complex, similar to LPS, which binds to the co-receptors CD14 and MD2 (32). In addition, EPS could bind C-type lectins or scavenger receptors, which serve as carbohydrate-binding pattern recognition receptors, and can associate with TLR4 (33–36). EPS binding to these receptors could upregulate M2 macrophage-inducing transcription factors such as STAT6, IRF4 and peroxisome proliferator-activated receptor gamma (PPAR-γ), which regulate transcription of arginase-1 and CD206 (37, 38). We hypothesize that EPS binding to TLR4 co-receptors directly modifies macrophages and induces M2 macrophage-specific transcription factors. The upregulation of M2 macrophage markers on EPS-treated primary macrophages (Figure 2A) and on RAW264.7 macrophages (data not shown) will allow us to directly test this hypothesis.

Immune Modulation by B. subtilis EPS and Other Probiotics

M2 macrophages mediate an anti-inflammatory response not only by TGF-β and PD-L1, but also by arginase-1, IL-10, and PD-L2 (29, 30, 39, 40). Such molecules protect from colitis, promote tissue repair and metabolic homeostasis, and provide protective immunity to helminth infections (41). Even though EPS-induced M2 macrophages produce multiple anti-inflammatory molecules, in our *in vitro* co-cultures of EPS-induced M2 macrophages and proliferating T cells, we find restoration of CD4$^+$ T cell proliferation only by interfering with TGF-β. In contrast, CD8$^+$ T cell restoration occurs through PD-L1, as well as TGF-β. CD8$^+$ T cells are more sensitive to PD-1 ligation (42), which may explain that PD-L1 blockade increases proliferation of CD8$^+$ T cells, but not of CD4$^+$ T cells. Inhibition of other molecules including arginase-1, PGE_2, PD-L2 and IL-10 had no effect on T cell inhibition. We conclude that TGF-β and PD-L1 are the main inhibitory factors of the EPS-induced M2 macrophages. Although PD-L1 predominantly inhibits T cells, TGF-β is a pleiotropic cytokine that acts on virtually all cell-types. Here, we focus mainly on inhibition of T cells by EPS, but this does not exclude the potential to regulate a variety of cells and immune responses, given the multi-faceted nature of TGF-β signaling. In certain circumstances, TGF-β promotes wound healing, which is another mechanism whereby EPS may help to resolve *C. rodentium*-induced inflammation.

Several commensal bacteria other than *B. subtilis* prevent inflammatory diseases by modulating the immune response (Table I). The beneficial effects of commensals are

described mostly for models of inflammatory bowel disease (IBD), where, with a few exceptions (4, 11, 15), protection appears to be mediated, in large part, by TLR2 signaling, iTreg cells, and IL-10. For example, *B. fragilis*, *Bifidobacteria infantis*, and *Clostridia* species induce Treg cells that ameliorate disease in chemically-induced colitis models (8, 10, 12, 13) and VSL#3 and *Bifidobacterium breve* induce IL-10-producing Tr1 cells (6, 43). Although we find a slight increase in Treg cells after administration of *B. subtilis* EPS, we do not know if they contribute to protection. The probiotic *Clostridium butyricum* mediates protection from dextran sulfate-induced colitis in a Treg-independent manner, through TLR2-dependent, IL-10–producing F4/80^{+}CD11b^{+}CD11c^{int} macrophages (9). In contrast, *B. subtilis* EPS-induced M2 macrophages mediate protection through TLR4 signaling and do not produce detectable levels of IL-10 (data not shown). We conclude that *B. subtilis* EPS induces an anti-inflammatory response distinct from that of all previously described probiotics. With the exception of *B. subtilis* EPS, *B. fragilis* PSA and sphingolipids, and *Faecalibacterium prausnitzii* MAM protein (5, 14, 16–18), most of the bacterial molecule(s) responsible for protection have not yet been identified or purified. Undoubtedly, additional molecules from a large number of commensal bacteria will be found to regulate immune responses.

Potential of Probiotics to Treat Human Diseases

In addition to the probiotics indicated in Table 1, several others are known to limit diseases, although the mechanisms of immune modulation are unknown (25–27, 44). While most known probiotics target gastrointestinal disease, many probiotics will likely be useful for treating or preventing other inflammatory diseases, including diabetes, allergy and experimental autoimmune encephalomyelitis (EAE) (7, 45–47). In fact, SFB-induced Th17 cells prevent the spontaneous development of type-1 diabetes in NOD mice (48). *Clostridia* species alter innate lymphoid cells and prevent development of food allergy (7), and *B. fragilis* PSA prevents development of EAE (46). As our understanding of the mechanisms by which specific commensal bacteria modulate the immune system increases, we will likely identify more diseases for which these probiotics will be beneficial.

This study highlights a unique mechanism by which a Gram (+) commensal exopolysaccharide induces an anti-inflammatory environment, as modeled in Figure 7. EPS from *B. subtilis*, and presumably other organisms, induces the generation of anti-inflammatory M2 macrophages in a TLR4-dependent manner. These M2 macrophages produce multiple immune inhibitory molecules, including TGF-β, PD-L1 and arginase-1, all of which inhibit T cell responses and prevent inflammatory diseases. Understanding the mechanisms by which EPS and other molecules from commensal organisms regulate the immune system will lead to new rationally-designed therapeutics for inflammatory diseases.

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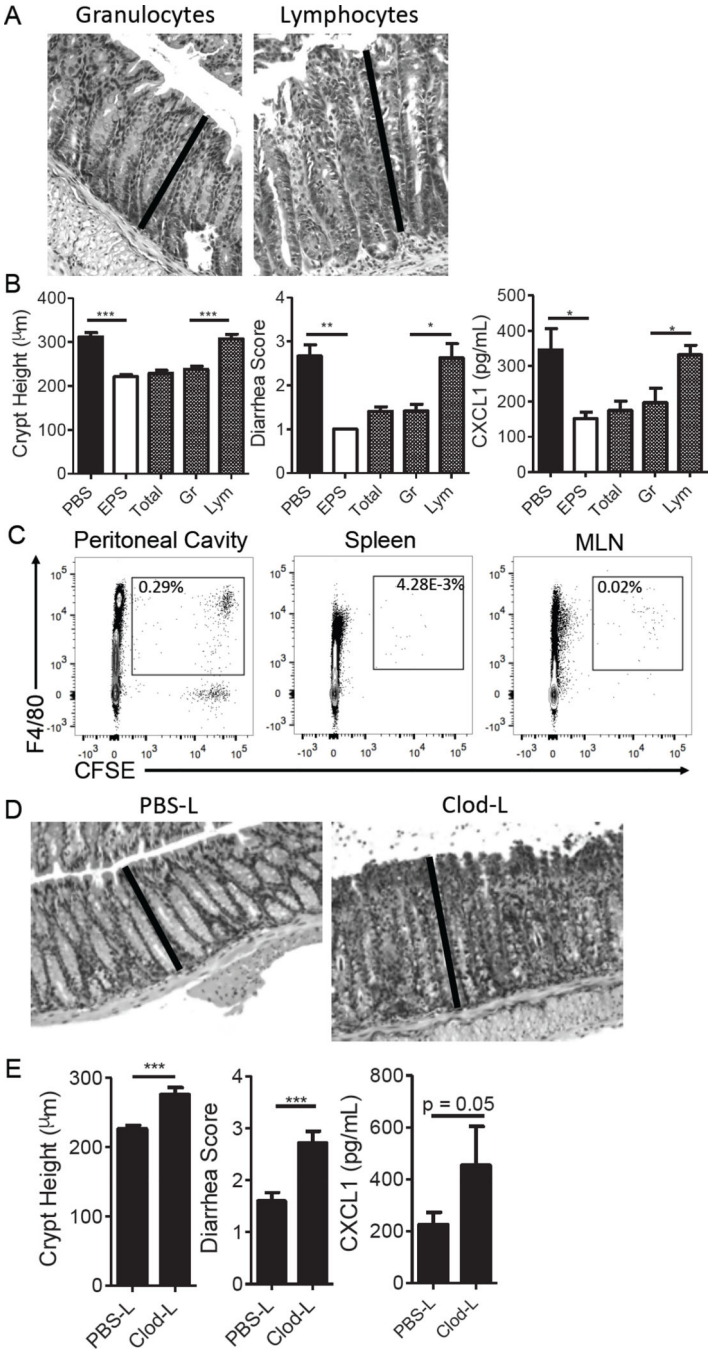

Figure 1. Assessment of *C. rodentium*-induced disease following adoptive transfer of peritoneal granulocytes, lymphocytes and macrophage-depleted cells from EPS-treated mice

A. H&E stained sections of the distal colon on day 11 post *C. rodentium* infection. Cells (3×10^4) from EPS-treated mice were transferred on days −1, +1, and +3 relative to *C. rodentium* infection. Black bar indicates crypt height. 200X magnification. **B**. Crypt height (left), diarrhea score (center), serum CXCL1 (right) (■) = PBS-injected mice, no cell transfer (− control); (2610) EPS-injected mice, no cell transfer (+ control); (▩) = cells transferred from EPS-injected mice, total unsorted (total), granulocytes (Gr), lymphocytes

(Lym). Data are from 2 independent experiments; N = 5–7 mice total per treatment. **C**. Peritoneal cells were isolated from EPS-treated mice 3 dpt, labeled with CFSE and transferred into recipient mice. $F4/80^{+}CFSE^{+}$ cells in the peritoneum, spleen and MLN were assessed by flow cytometry 24 h post-transfer. Data are representative of 3 independent experiments. **D & E**. Mice were treated with clodronate liposomes (Clod-L) or PBS liposomes (PBS-L) 4–6 hr before i.p. injection with EPS (100 μg); peritoneal cells (6×10^{4}) were transferred on days −1, +1 and +3 relative to *C. rodentium* infection. (D) H&E stained sections of the distal colon. Black bar indicates crypt height. 200X magnification. (E) Crypt height (left), diarrhea score (center), and serum CXCL1 (right) were measured 11 dpi. Data are from 2 independent experiments with N= 7–9 mice total per treatment.

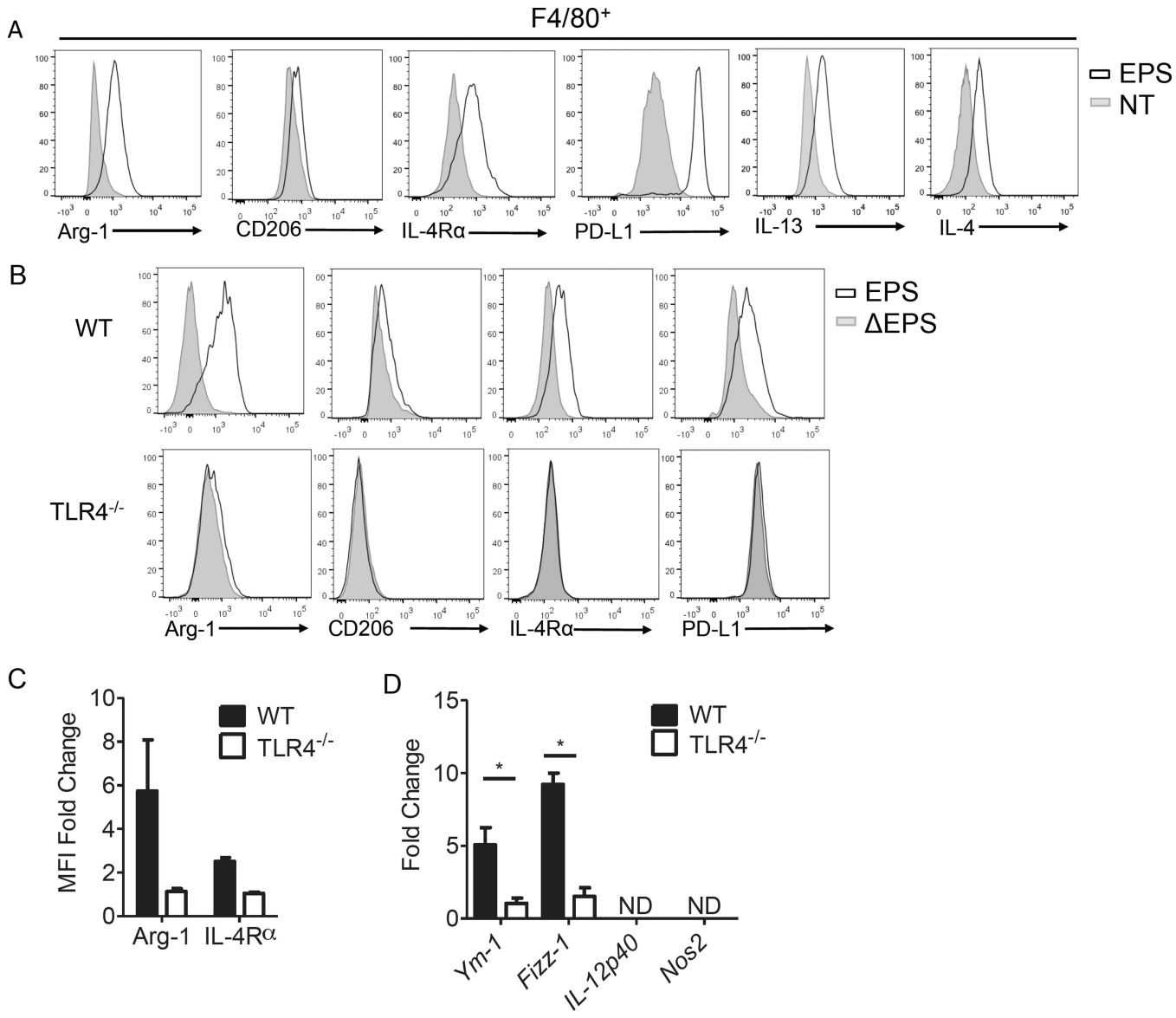

Figure 2. EPS-induced changes in phenotype of peritoneal macrophages *in vitro* and *in vivo*

A. Peritoneal macrophages were incubated 16 h with EPS *in vitro* and analyzed by flow cytometry. M2 marker expression (Arg-1, CD206, IL-4Rα, and PD-L1) and M2 cytokine expression (IL-13 and IL-4) by F4/80^{+}CD11b^{+} peritoneal macrophages. Data are representative of 3 independent experiments. **B-D**. WT and TLR4$^{-/-}$ mice were injected with EPS and 3 days later peritoneal cells were examined by flow cytometry and qRT-PCR. **B**. Representative flow cytometric profiles of M2 macrophage marker expression (Arg-1, CD206, IL-4Rα, and PD-L1) on F4/80^{+}CD11b^{+} cells from WT (top) or TLR4$^{-/-}$ mice (bottom). **C**. Fold change in mean fluorescence intensity (MFI) of Arg-1 and IL-4Rα on F4/80^{+}CD11b^{+} cells from WT (■) and TLR4$^{-/-}$ (□) mice (compared to mice injected with ΔEPS). Average of 4 independent experiments; N = 10 mice total per treatment. **D**. Fold change by qRT-PCR of *Ym-1, FIZZ-1, IL-12p40* and *Nos2* expression in F4/80^{+}CD11b^{+} cells in WT (■) and TLR4$^{-/-}$ (□) mice injected with EPS (compared to mice injected with

ΔEPS) (average of 4 independent experiments, N = 7 mice total per group). ND = not detectable.

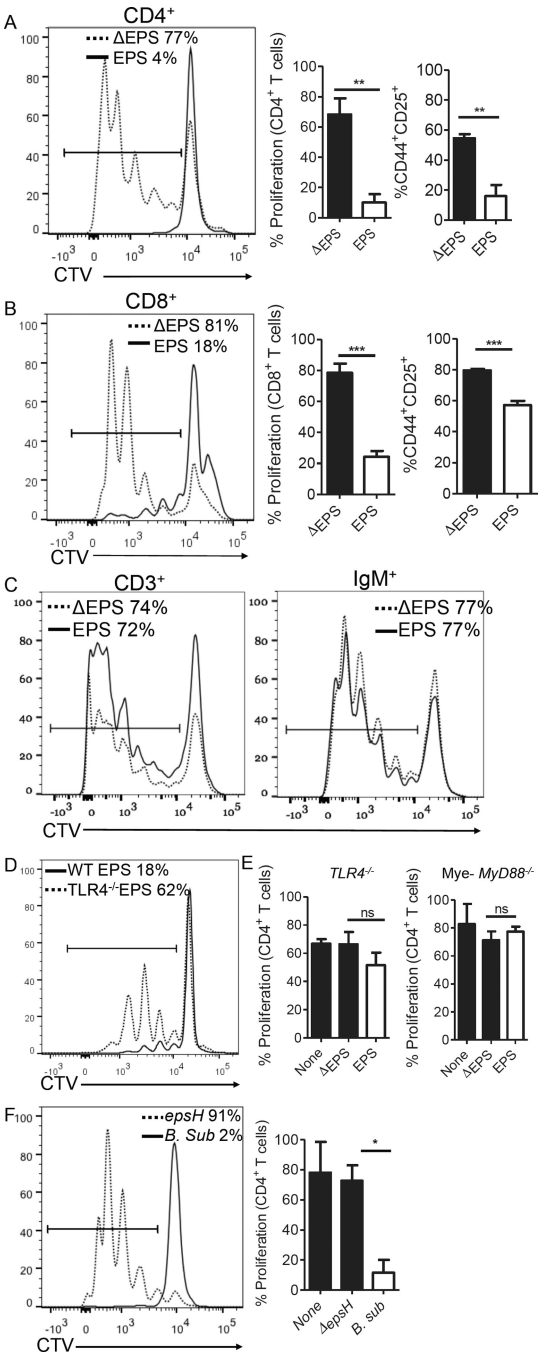

Figure 3. Inhibition of anti-CD3-stimulated T cell proliferation by peritoneal cells from EPS and *B. subtilis*-treated mice

CTV-labeled spleen cells were stimulated with anti-CD3 and cultured with F4/80$^+$CD11b$^+$ cells from EPS- or ΔEPS-treated mice. **A**. (Left): Proliferation of CD4$^+$ T cells; Horizontal bar = % proliferating cells; (center) % proliferating CD4$^+$ T cells; and (right) % activated CD4$^+$ T cells (CD44$^+$CD25$^+$) in 3 independent experiments; N = 7 mice total per group. **B**. (Left): Proliferation of CD8$^+$ T cells; Horizontal bar = % proliferating cells; (center) % proliferating CD8$^+$ T cells; and (right) % activated CD8$^+$ T cells (CD44$^+$CD25$^+$) in 3

independent experiments; N = 7 mice total per group. **C**. Proliferation of $CD4^+$ T cells co-cultured with peritoneal $CD3^+$ T cells (Left) or IgM^+ B cells from EPS- or ΔEPS-treated mice. Horizontal bar = % proliferation. **D**. Proliferation of $CD4^+$ T cells cultured with peritoneal cells from EPS-treated *TLR4*$^{-/}$ or EPS-treated WT mice. Horizontal bar = % proliferating cells. **E**. (Left) % proliferating $CD4^+$ T cells in co-cultures with peritoneal cells from *TLR4*$^{-/-}$ mice and (right) myeloid (mye) $MyD88^{-/-}$ mice in 4 independent experiments; none = splenocytes alone. N = 9 mice total per group. **F**. Proliferation of $CD4^+$ T cells cultured with peritoneal cells from mice gavaged with WT or *epsH B. subtilis* spores. (Left) Horizontal bar = % proliferating cells; (right) % proliferating $CD4^+$ T cells in 3 independent experiments; none = splenocytes alone. N = 5 mice total per group.

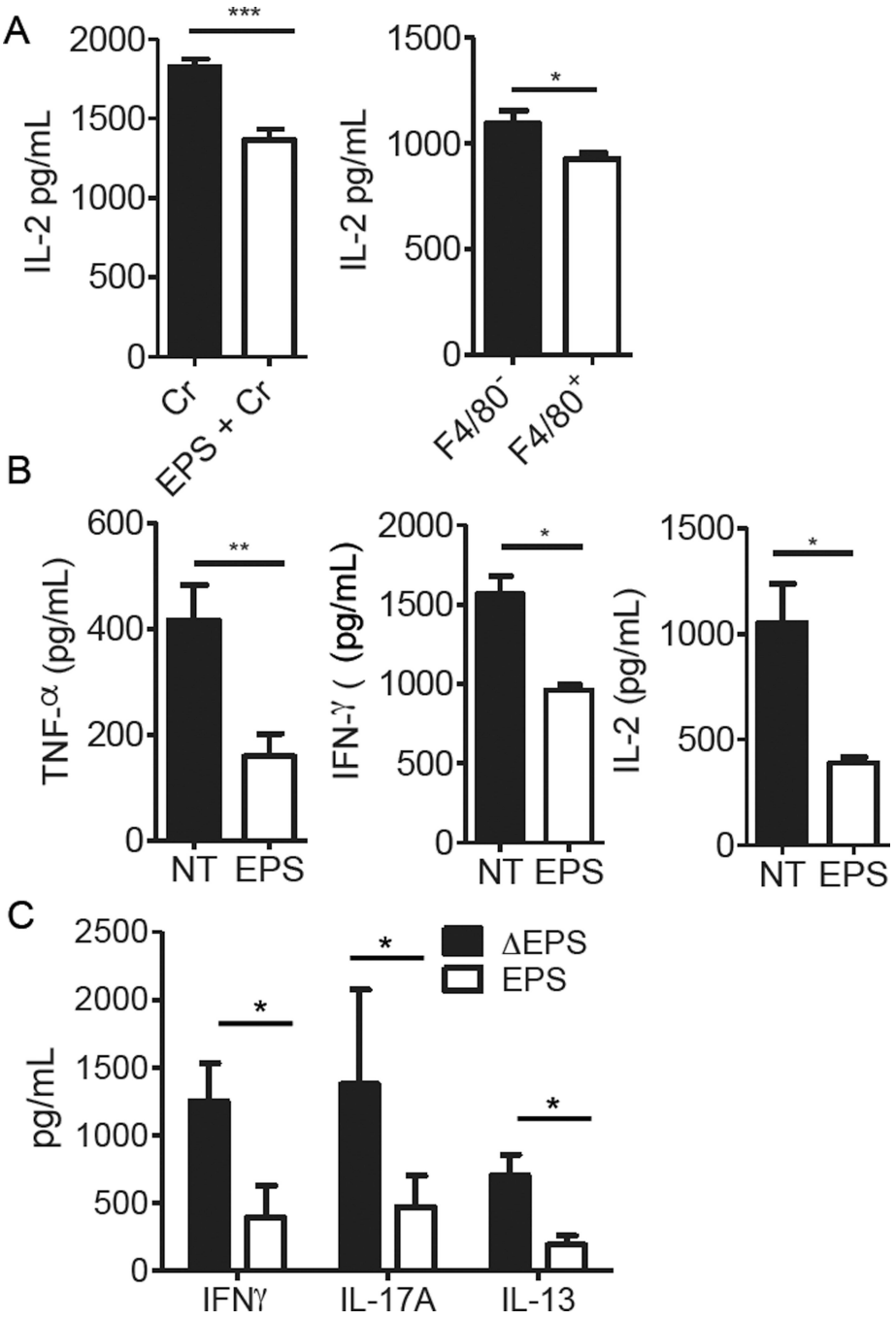

Figure 4. Inhibition of T cell responses in EPS-treated mice

A. (Left) Quantification of IL-2 by ELISA of PMA- and ionomycin-stimulated cells from the MLN of *C. rodentium*-infected mice either untreated, or treated with EPS. (Right) Quantification by of IL-2 by ELISA of PMA- and ionomycin-stimulated MLN cells from *C. rodentium*-infected mice that received adoptive transfer of F4/80^{+} or F4/80 peritoneal cells from EPS-treated mice. Data are representative of 2 independent experiments with N = 7–10 mice total per group. **B**. Quantification by ELISA of serum TNFα, IFN-γ and IL-2 in EPS-treated or untreated (NT) mice 2 h post-i.p. injection of anti-CD3. Average of 3 independent

experiments with N = 8 mice total per group. **C**. Quantification by ELISA of IFNγ, IL-17A, and IL-13 in culture supernatants of anti-CD3-stimulated splenocytes from EPS- or ΔEPS-treated mice. Average of 4 independent experiments with N = 8 mice total per group.

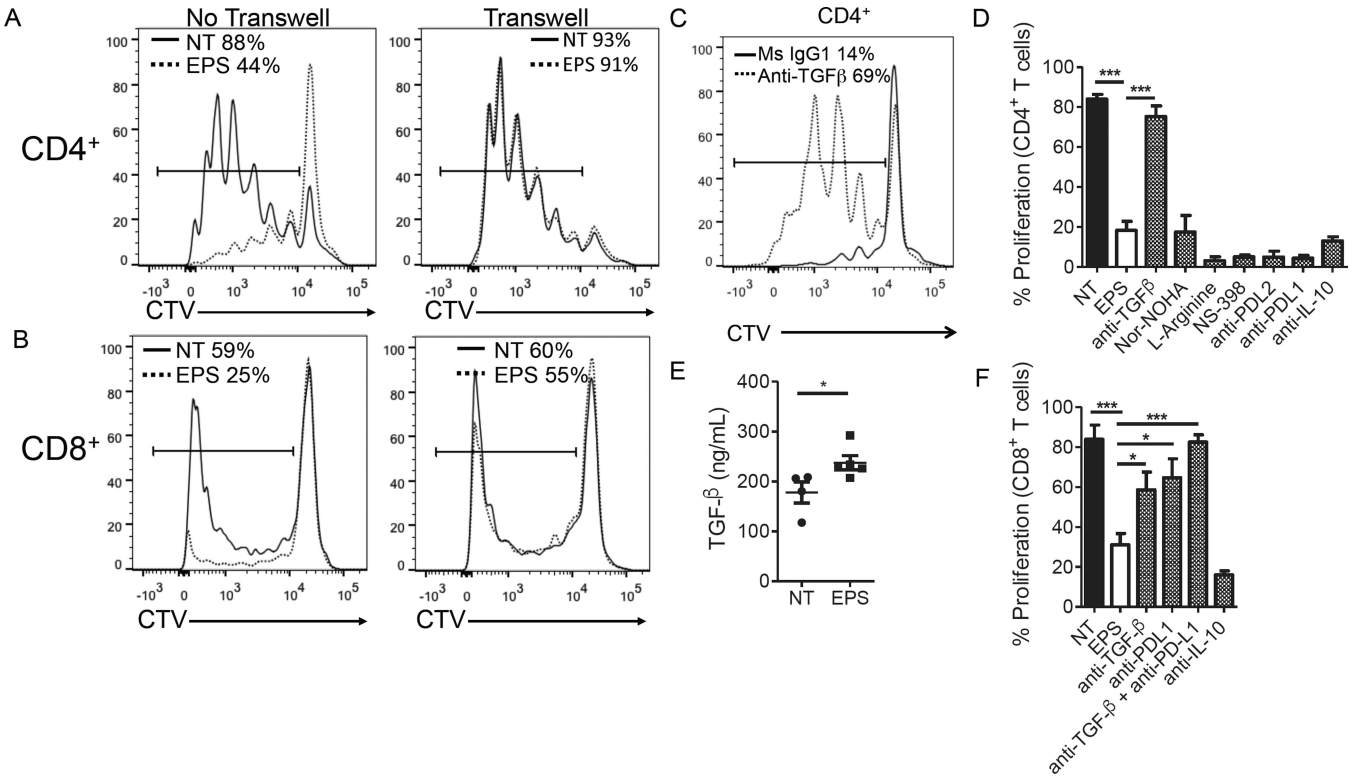

Figure 5. Restoration of T cell proliferation by inhibitors of M2 macrophage function

A & B Proliferation of $CD4^+$ (A) or $CD8^+$ (B) T cells co-cultured with peritoneal cells from untreated (NT)- or EPS-treated mice in direct contact (no transwell, left) or with peritoneal cells in a transwell insert (right). Horizontal bar = % proliferation. Representative of three independent experiments. **C**. Proliferation of $CD4^+$ T cells of anti-CD3 stimulated splenocytes cultured with peritoneal cells from EPS-treated mice in the presence of neutralizing anti-TGF-β antibody or mouse (Ms) IgG1 isotype control. Horizontal bar = % proliferation. Representative of three independent experiments. **D**. % Proliferation of $CD4^+$ T cells cultured with peritoneal cells from EPS-treated mice and containing inhibitors of M2 macrophage function. (■) NT peritoneal cells alone (negative control); (□) peritoneal cells from EPS-treated mice (positive control); (▩) cultures with inhibitors of M2 macrophages, anti-TGF-β, Nor-NOHA, L-Arginine, NS-398, anti-PD-L2, anti-PD-L1, and anti-IL-10. Data are from 3 independent experiments each, N = 6 mice total per group. **E**. Quantification by ELISA of total TGF-β in serum 3 days after EPS-treatment or no treatment (NT). N= 4–5 mice total per group. **F**. % Proliferation of $CD8^+$ T cells cultured with peritoneal cells from EPS-treated mice containing inhibitors of M2 macrophage function. (■) NT peritoneal cells alone (negative control): (□) peritoneal cells from EPS-treated mice (positive control); (▩) Cultures with inhibitors of M2 macrophages, as in D. D & F. Statistical significance determined by one-way ANOVA in combination with Bonferroni's test for multiple comparisons. $P < 0.0001$ by ANOVA for D & F.

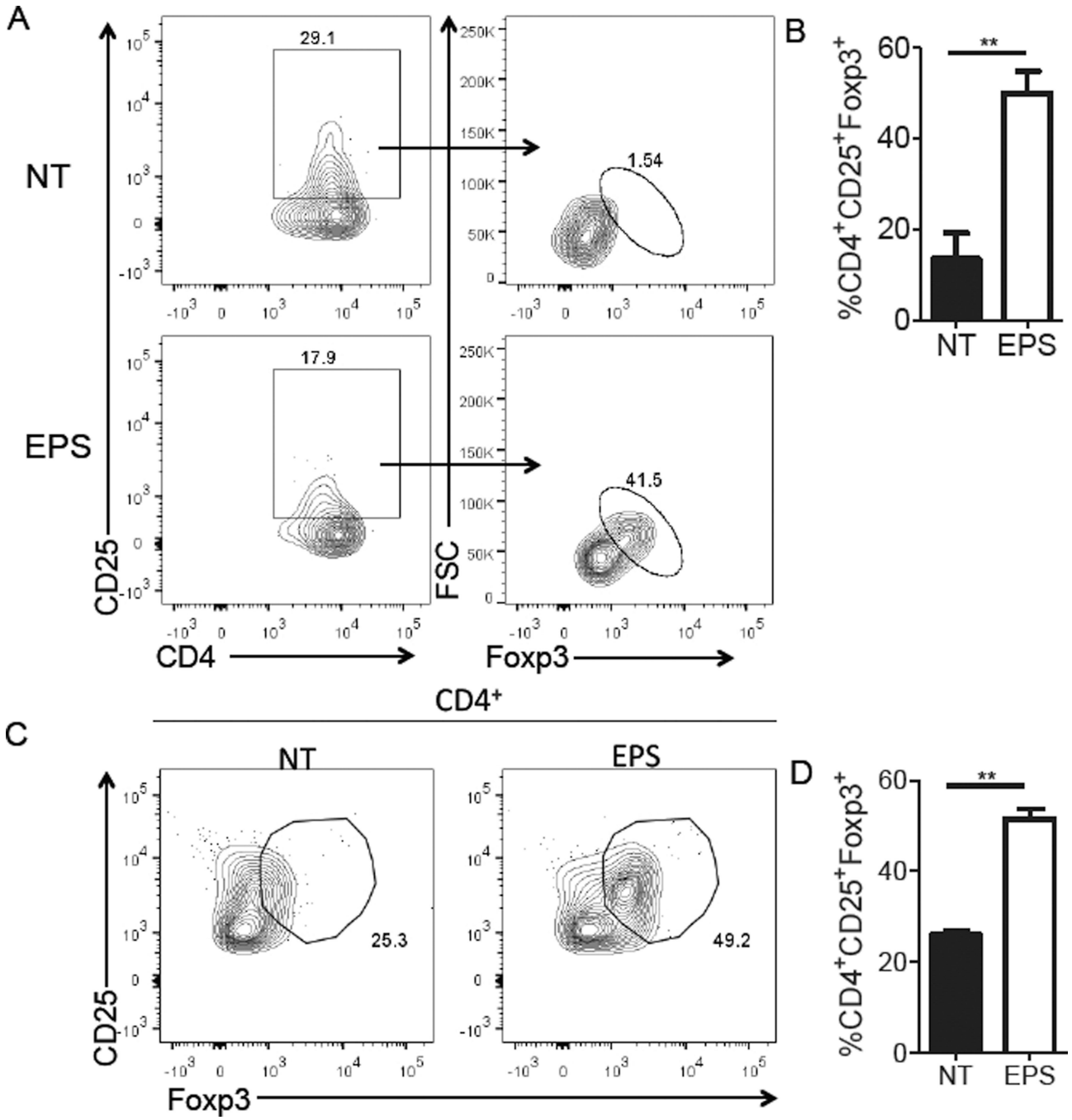

Figure 6. Induction of Treg cells by EPS

A. Representative example of Foxp3 expression in $CD4^+CD25^+$ T cells in the peritoneal cavity of untreated (NT, top) or EPS-treated (EPS, bottom) mice. **B**. % $CD4^+CD25^+Foxp3^+$ cells in 3 independent experiments with N = 6 mice total per group. **C**. $CD4^+CD25^+Foxp3^+$ T cells in co-cultures of anti-CD3 stimulated splenocytes, IL-2 (50 ng/mL), and peritoneal cells from EPS-treated or untreated (NT) mice. **D**. % $CD4^+CD25^+Foxp3^+$ cells in 3 independent experiments with N = 4 mice total per group.

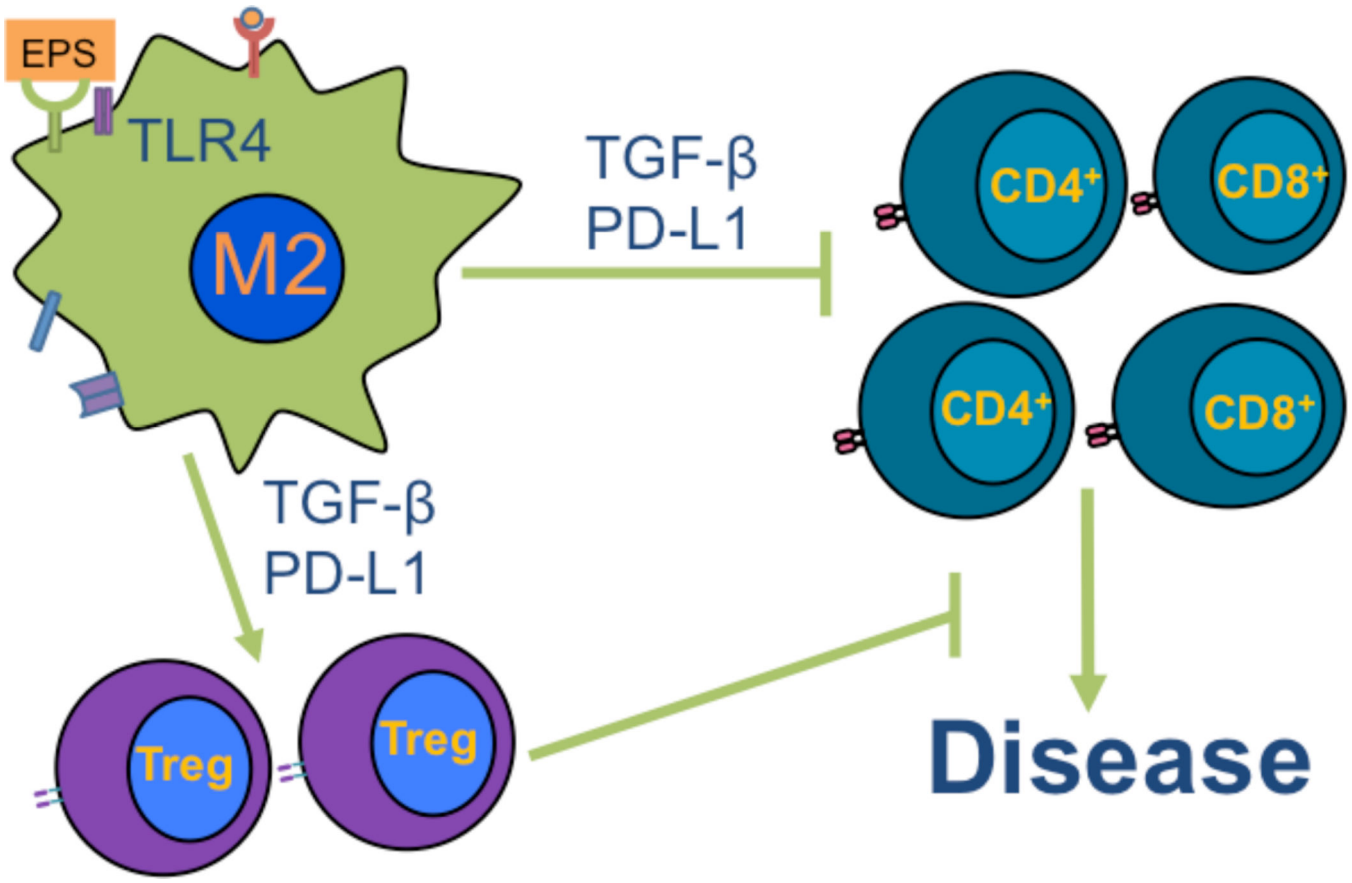

Figure 7. Model for EPS modulation of immune responses

B. subtilis and purified EPS induce M2 Macrophages which inhibit $CD4^+$ and $CD8^+$ T cells through production of TGF-β and PD-L1 and potentially through induction of regulatory T cells.

Table I

Commensal Bacteria with Beneficial Immunomodulatory Effects on Inflammatory Diseases

Bacteria	Elicited Immune Response	Bacterial Molecule Responsible for Protection	Inflammatory Disease	Ref
Bacillus subtilis	TLR4-dependent induction of M2 macrophages	Exopolysaccharide (EPS)	*C. rodentium*-induced colitis	14, 19
Bacteriodes fragilis	TLR2-dependent induction of iTreg by IL-10$^+$ DCs	Polysaccharide A (PSA)	*H. hepaticus*-induced colitis, TNBS-induced colitis, EAE	5, 12, 13, 46
Bifidobacterium spp.	Induction of Tr1 and Treg cells; reduction of *B. breve* specific antibody production	Possibly Exopolysaccharide	*C. rodentium*-induced colitis	6, 15
Bacteriodes fragilis	Negative regulation of iNKT cells in neonatal mice	Sphingolipid GSL-Bf717	Oxazolone-induced colitis	18
Faecalibacterium prausnitzii	Inhibition of NF-κB in intestinal epithelial cells	Anti-inflammatory protein MAM	DNBS-induced colitis	16, 17
Clostridium butyricum	TLR2-dependent induction of IL-10$^+$ F4/80$^+$CD11b$^+$CD11c^{int} cells	n.d.	DSS-induced colitis	9
Clostridia spp.	Induction of IL-10$^+$ Treg; upregulates IL-22 by ILC	n.d.	DSS-induced colitis, food allergy	7, 8, 45
Lactobacillus spp.	Reduction of pro-inflammatory mucosal cytokines	n.d.	*H. hepaticus*-induced IBD	25
Segmented filamentous bacteria	Induction of Th17 cells; IgA production	n.d.	*C. rodentium*-induced colitis, T1D	11, 48
VSL#3	Induction of TNF-α production by epithelial cells; IL-10$^+$ TGF-β$^+$ T cells	n.d.	Chronic CD-like ileitis (SAMP mice), TNBS-induced colitis	4, 43

Abbreviations: CD- Crohn's Disease; DCs- Dendritic cells; DSS- Dextran sulfate sodium; DNBS- Dinitrobenzene sulfonic acid; EAE- Experimental autoimmune encephalocyelitis; GIT- Gastrointestinal tract; iTreg- Inducible regulatory T cells; IBD- Inflammatory bowel disease; ILC- Innate Lymphoid Cell; MAM- Microbial anti-inflammatory molecule; n.d.- not determined; TNBS- 2,4,5-Trinitrobenzenesulfonic acid; T1D- Type 1 Diabetes