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*J Immunol* 2009; 183:4458-4466; Prepublished online 11 September 2009;
doi: 10.4049/jimmunol.0901465
http://www.jimmunol.org/content/183/7/4458

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Engagement of TLR2 Does not Reverse the Suppressor Function of Mouse Regulatory T Cells, but Promotes Their Survival

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TLRs are a class of conserved pattern recognition receptors that are used by cells of the innate immune system. Recent studies have demonstrated the expression of TLRs on both human and mouse T cells raising the possibility that TLRs play a direct role in adaptive immunity. TLR2 is activated primarily by bacterial wall components including peptidoglycan and lipoproteins. Several studies have shown that mouse regulatory T (Treg) cells express TLR2 and claimed that engagement of TLR2 by synthetic ligands reversed their suppressive function. In contrary, enhancement of Treg function was observed following engagement of TLR2 on human Treg. We have reexamined the expression and function of TLR2 on mouse Treg purified from Foxp3-GFP knock-in mice. TLR2 ligation by TLR2 agonist, the synthetic bacterial lipoprotein Pam3CSK4, enhanced the proliferative responses of both conventional T cells and Treg in response to TLR stimulation in the absence of APC. Treatment of Foxp3+ Treg with Pam3CSK4 did not alter their suppressive function in vitro or in vivo and did not reduce their level of Foxp3 expression. An additional effect of TLR2 stimulation of Treg was induction of Bcl-xL resulting in enhanced survival in vitro. Treatment of mice with the TLR2 agonist enhanced the Ag-driven proliferation of Treg in vivo, but did not abolish their ability to suppress the development of experimental autoimmune encephalomyelitis. Development of methods to selectively stimulate TLR2 on Treg may lead to a novel approaches for the treatment of autoimmune diseases. The Journal of Immunology, 2009, 183: 4458–4466.

Microbes are recognized by cells of the innate immune system in the host via pattern recognition receptors, with the TLR family the best characterized. Currently, 13 TLRs have been identified including 10 human TLRs (TLR1–10) and 12 murine TLRs (TLR1–9 and 11–13), which recognize different pathogen-associated molecular patterns (1). TLRs were originally thought to be expressed in cells involved in innate immunity such as macrophages, dendritic cells (DCs), epithelial cells, endothelial cells, as well as organ parenchymal cells (2, 3). However, more recent studies have shown that certain TLRs are also expressed in CD4+ and CD8+ T lymphocytes. Flow cytometric analysis indicates human TLR2, 3, 4, 5, and 9 proteins are expressed intracellularly in nonstimulated T cells (4). TLR1, 2, 3, 6, and 7 mRNA are readily detected in murine CD4+ T cells by RT-PCR (5, 6). Several studies have suggested that TLRs can directly modulate T cell functions as novel costimulatory receptors. For example, TLR2 is shown to regulate both murine and human CD4+ T cell functions (5, 7, 8). Poly(I:C) and CpG DNA enhance the survival, but not the proliferation of activated murine CD4+ T cells by up-regulating the expression of the antiapoptotic protein Bcl-xL and augmenting the activation of NF-kB (9).

Foxp3+ regulatory T (Treg) cells are critical for the maintenance of peripheral T cell tolerance and their depletion leads to organ-specific autoimmune diseases. Treg cells suppress CD4+ and CD8+ T lymphocytes via contact-dependent mechanisms and the secretion of suppressor cytokines (10–13). Although mouse Treg cells express TLR1, 2, 4, 5, 6, 7, and 8 mRNA (14), the function of TLRs on Treg cells is controversial. Engagement of either TLR4 (6) or TLR5 (15) has been shown to enhance Treg suppressive activity, while TLR2 ligation on mouse (5, 16) or TLR8 ligation on human Treg (17) cells are claimed to reverse Treg suppressive function. One of the major problems in the analysis of TLR function on Treg cells is the difficulty in obtaining highly purified populations of Foxp3+ Treg cells. Because TLRs are also expressed on monocytes, B cells, and DCs, an analysis of the effects of TLR engagement on Treg function must carefully exclude the presence of contaminating cells including Foxp3+ CD25+CD4+ and Foxp3+CD25−CD4+ T cells. We have reexamined the effects of the TLR2 agonist Pam3CSK4 on Foxp3− Treg cells. Unlike previous studies, we isolated highly purified populations of Treg cells by FACs sorting GFP+ cells from mice expressing a GFP−Foxp3 fusion protein (18). We demonstrate that freshly isolated Treg cells express only low levels of TLR2 mRNA that can be up-regulated by TCR stimulation and further increased upon TLR2 ligation. Contrary to previous studies (5, 16), we demonstrate that Treg cells pretreated and activated with Pam3CSK4 in vitro maintain Foxp3 expression and have normal suppressive activity when cocultured with TLR2−/− responder T cells. Using a model of experimental autoimmune encephalomyelitis (EAE) in TLR2−/− mice, we further conclude that administration of Pam3CSK4 in vivo does not alter the ability of wild-type (WT) Treg cells to modulate the induction of disease. The major effect of TLR2 ligation on Treg cells is to reduce their threshold for

1. These studies were supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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3. Abbreviations used in this paper: DC, dendritic cell; TLR, toll-like receptor; LTA-SA, lipoteichoic acid from Staphylococcus aureus; WT, wild type; FSC, forward scatter; SSC, side scatter; 7-AAD, 7-aminoactinomycin D; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein.
activation and significantly enhance their survival via up-regulating anti-apoptotic molecule Bcl-xL.

Materials and Methods

Animals

Foxp3<sup>Cre</sup> mice were a gift from Dr. A. Rudensky (University of Washington, Seattle, WA) and bred at the National Institute of Allergy and Infectious Diseases (NIAID) contract facility at Taconic Farms and maintained on a mixed C57BL/6 × 129 background. TLR2<sup>−/−</sup> mice on the C57BL/6 background were purchased from The Jackson Laboratory and bred in our facility. OVA-specific TCR-transgenic OT-II mice (C57BL/6 background) and WT C57BL/6 were obtained from Taconic Farms. Female heterozygous B6.Cg-Foxp3<sup>+/−</sup> (scuffy) mice were purchased from The Jackson Laboratory and bred to C57BL/6 WT male mice to generate hemizygous male B6 (Cg-Foxp3<sup>+/−</sup>/scuffy) offspring. All animals used for this study were female, 6–8 wk of age. They were housed and handled according to National Institutes of Health institutional guidelines under an approved animal protocol.

Cell isolation and flow cytometry

APCs were purified from mouse spleen by magnetic sorting the CD90<sup>−</sup> fraction and then irradiated (3000 rad) before use. T cells were obtained from pooled mouse spleen and lymph nodes and the CD<sup>4</sup> fraction was first purified by an AutoMACS Cell Separator using anti-mouse CD4 beads (Miltenyi Biotec). The enriched CD<sup>4</sup> fractions were then further separated into conventional T or Treg populations by FACs sorting for the CD<sup>4</sup>CD25<sup>+</sup>, CD<sup>4</sup>CD45R<sup>−</sup>, and CD<sup>4</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> subsets (either the FACS Vantage Diva or FACSaria flow cytometer (BD Biosciences)). The postsort purity for each cell type was higher than 98% and the Foxp3 purity for Treg cells was higher than 95%.

Single-cell suspensions were stained using the following conjugated Abs according to the manufacturer’s protocol: PE or allophycocyanin anti-mouse CD4 (L3T4), PE anti-mouse CD25 (PC61.5), anti-CD45.2 (104), biotin anti-mouse CD45.1 (A20), PE-Texas Red-streptavidin, allophycocyanin, or Pacific Blue anti-mouse Foxp3 (FJK-16s), PE anti-V<sup>α2</sup> (B20.1) (all from eBioscience), and biotin anti-V<sup>β</sup>5<sup>+</sup> (MR 9-4; BD Pharmingen). For staining of Foxp3 or PE anti-mouse BCL-2 (BD Pharmingen), the cells were fixed and permeabilized using a fixation/permeabilization kit (eBioscience). Acquisition was done on an LSR II (BD Biosciences) equipped with a FACSDiva digital upgrade. Analysis was done using FlowJo software (Tree Star).

Cell cultures

T cells were stimulated with plate-bound anti-mouse CD3e mAb (clone 145-11; BD Pharmingen) at the concentrations shown in the figure legends. Unless indicated otherwise, TLR ligands were used at the following concentrations: poly(I:C) (2 μg/ml), CpG (10 μg/ml), CL097 (0.5 μg/ml), lipopolysaccharide from Staphylococcus aureus (LTA-SA; 0.5 μg/ml), Pam2CSK4 (50 μg/ml), and Pam3CSK4 (1 μg/ml). The recovered cells were then lysed with cell lysis buffer (30 mM Tris-Cl (pH 7.5), 150 mM NaCl, and 1% CHAPS). Total protein (50 μg) was resolved on 10% SDS-PAGE gels and transferred with Bcl-xL Ab (Cell Signaling Technology) and anti-mouse β-actin (Sigma-Aldrich). Bands were visualized with secondary HRP-conjugated Ab (Bio-Rad) and the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

In vivo Treg proliferation

Treg cells from OT-II transgenic mice were isolated by FACs purification of CD4<sup>+</sup>CD25<sup>+</sup> T cells. The postsort Foxp3 purity was higher than 90%. The Treg cells were then CFSE labeled (2 μM) and injected into TLR2<sup>−/−</sup> mice via the tail vein (1 × 10<sup>6</sup>/mouse). Twenty-four hours later, mice were immunized with OVA<sub>257-264</sub> peptide (50 μg) synthesized by the peptide synthesis facility, NIAID, in IFA or injected together i.p. with 100 μg of Pam3CSK4. Five days later, the draining lymph nodes from mice were taken and stained with allophycocyanin anti-CD4<sup>+</sup>, PE anti-V<sup>α2</sup>, and biotin anti-V<sup>β</sup>5<sup>+</sup> followed by PE-Texas Red-streptavidin. The cells were then fixed and permeabilized for staining with Pacific Blue anti-Foxp3 and analyzed by flow cytometry.

Induction of scurfy-like disease in RAG<sup>−/−</sup> mice

Male RAG<sup>−/−</sup> mice (five per group) were reconstituted with 5 × 10<sup>6</sup> total lymph node and spleen cells from 7-day-old scurfy mice alone or cotransferred with either 1 × 10<sup>6</sup> Treg cells. Histopathological evaluation was performed as previously described (19).

Induction of EAE

Mice (five per group) were immunized s.c. with myelin oligodendrocyte glycoprotein (MOG<sub>35-55</sub>) peptide in CFA. Pertussis toxin (200 ng) was injected i.p. on days 0 and 2 to induce EAE. CD4<sup>+</sup> Foxp3<sup>+</sup> Treg cells (1 × 10<sup>6</sup>) were transferred into TLR2<sup>−/−</sup> mice 24 h before immunization. Mice were treated with Pam3CSK4 (20 μg, i.p.) on days −1, 1, 3, and 5. Mice were then monitored daily for disease until day 30. Clinical assessment of EAE was performed according to the following criteria: 0, no disease; 1, tail paralysis; 2, hind leg weakness; 3, full hind leg paralysis; 4, complete hind limb paralysis plus front limb paralysis; and 5, death. The data shown are mean clinical scores of each group.

Results

TLR2 mRNA is selectively expressed in activated Treg and its expression is enhanced by Pam3CSK4 stimulation

A previous study (16) suggested that CD4<sup>+</sup>CD25<sup>+</sup> Treg cells expressed TLR2 protein as determined by flow cytometry. To more accurately characterize TLR2 expression in Treg cells, we sorted Foxp3<sup>+</sup> T cells from Foxp3-GFP knock-in mice. We first attempted to examine TLR2 protein expression by flow cytometry, but could not observe a positive signal above control staining using commercially available mAbs (data not shown). Levels of TLR2 mRNA were analyzed by reverse transcription-PCR (RT-PCR) and quantified by real-time PCR. Total RNA was extracted from cells (1 × 10<sup>6</sup>/sample) using RNeasy Plus Kit (Qiagen). cDNAs were generated from 1 μg of total RNA using iScript II reverse transcriptase (Invitrogen). The resulting cDNA was subjected to a 14-cycle PCR amplification using the TaqMan Universal PCR Master Mix (Invitrogen) in triplicate on the Applied Biosystems PRISM 7900 Sequence Detector System. The ready-made primer and probe sets for TLR2 were ordered from Applied Biosystems (catalog no. Thr2; Mn00442346_m1). The amount of TLR2 mRNA expression was normalized to the 18S RNA and calculated according to the comparative cycle threshold method as described by Applied Biosystems.

Analysis of apoptosis by flow cytometry

Cells were washed in PBS and resuspended at a concentration of 1 × 10<sup>6</sup> cells/ml in Annexin V Binding Buffer (BD Pharmingen) and stained with Annexin V<sup>FITC</sup> (BD Pharmingen) and 7-aminoactinomycin D (7-ADD; Sigma-Aldrich), according to the manufacturer’s protocol. Alternatively, apoptosis was also quantified by a reduction in forward scatter (FSC) and an increase in side scatter (SSC) as determined by flow cytometry.

The proliferation of GFP<sup>+</sup> Treg cells (1 × 10<sup>6</sup>) were left unstimulated or stimulated for 24 or 48 h with plate-bound anti-CD3 (0.5 μg/ml) and IL-2 (100 U/ml) in the presence or absence of Pam3CSK4 (1 μg/ml). The recovered cells were then lysed with cell lysis buffer (30 mM Tris (pH 7.5), 150 mM NaCl, and 1% CHAPS). Total protein (50 μg) was resolved on 10% SDS-PAGE gels and transferred to ImmunoBlot polyvinylidene fluoride membranes (GE Osmonics Labstore). Blotting was performed with Bcl-xL Ab (Cell Signaling Technology) and anti-mouse β-actin (Sigma-Aldrich). Bands were visualized with secondary HRP-conjugated Ab (Bio-Rad) and the SuperSignal West Pico Chemiluminescent Substrate (Pierce).
Effects of TLR agonists on the proliferation of GFP⁺ Treg cells in vitro

Because other TLR ligands have been reported to have direct effects on Treg cells (6, 7, 15–17), we next compared several different TLR agonists for their capacity to modulate Treg proliferation. When Treg cells were stimulated with soluble anti-CD3 in the presence of irradiated T-depleted splenocytes or with plate-bound anti-CD3 in the absence of APC, none of the TLR agonists, including poly(I:C) (TLR3), CpG (TLR9), CL097 (TLR7/8), and Pam3CSK4 (TLR2/TLR1) were able to stimulate the proliferation of the Treg cells (Fig. 2, A and B). Since the proliferation of Foxp3⁺ Treg cells can be induced by TCR stimulation in the presence of exogenous IL-2, we also tested the effects of the TLR agonists on Treg cells in response to stimulation with a suboptimal concentration of anti-CD3 in the presence of IL-2 and APC (Fig. 2C). Slight enhancement (−2-fold) of Treg proliferation was observed when CpG and CL097 were added, while Pam3CSK4 increased Treg proliferation −4-fold. To determine whether the TLR agonists were acting on the APC or on the Treg cells, we stimulated the Treg cells in the absence of APC with plate-bound anti-CD3 and IL-2 (Fig. 2D). Under these conditions, a significant enhancement of T cell proliferation (−8-fold) was only seen in the presence of Pam3CSK4. Taken together, it appears that the enhancement of proliferation induced by CpG and CL097 is mediated indirectly via acting on the APC, while the synthetic TLR2 agonistic Pam3CSK4 is able to enhance Treg proliferation by directly acting on Treg cells.

The costimulatory effects of Pam3CSK4 were most prominent when the Treg cells were stimulated with a low concentration of anti-CD3 in the presence of IL-2 and no enhancement of proliferation was observed at the higher concentration (Fig. 2E). Pam3CSK4 also appeared to directly enhance the proliferation of conventional GFP⁻ T cells stimulated with a low concentration of anti-CD3 in the absence of exogenous IL-2 (Fig. 2F). Thus, TLR2 ligation on both Treg and conventional T cells results in a costimulatory signal and appears to lower the threshold for TCR activation.

Engagement of TLR2/TLR1 heterodimers preferentially results in costimulation of Treg proliferation

Pam3CSK4 is a synthetic tripalmitoylated lipopeptide that mimics the acylated amino terminus of bacterial lipoprotein. Recognition of this synthetic TLR ligand is mediated by the TLR2-TLR1 heterodimer complex through their cytoplasmic domains (21). TLR2 homodimer or TLR2/TLR6 heterodimer can recognize other microbial components (2, 3). We therefore compared the ability of different TLR2 complex ligands to costimulate the proliferation of Treg cells induced by plate-bound anti-CD3 and IL-2 (Fig. 3). LTA-SA (TLR2/TLR2 homodimer) and the synthetic ligand Pam2CSK4 (TLR6/TLR2 heterodimer) only slightly enhanced GFP⁺ Treg proliferation (−2-fold). In contrast, Pam3CK4 induced a dose-dependent, 4-fold increase in Treg proliferation, suggesting that signal transduction via the TLR2/TLR1 heterodimer results in the most effective costimulus for Treg proliferation.

Pam3CSK4 stimulation does not reduce Foxp3 expression and does not alter the suppressive activity of Treg in vitro

Because TLR2 engagement on Treg cells enhances their proliferation in vitro, it is important to clarify whether TLR2 agonists modulate Treg function. Several reports have claimed that engagement of TLR2 on Treg cells transiently reverses their suppressive function secondary to reduced Foxp3 expression (5, 16). We

are indicative of protein expression, as TLR proteins are tightly regulated by ubiquitin-mediated degradation (20). We therefore determined the level of TLR2 mRNA by RT-PCR. TLR2 mRNA levels were compared with the levels expressed in epithelial cells from TLR2⁻/⁻ mice as a negative control to levels in bone marrow-derived macrophages from WT mice as a positive control. Macrophages derived from WT mouse bone marrow following culture in M-CSF and IL-3 served as a positive control population. The TLR2 mRNA level in TLR2⁻/⁻ epithelial cells was set at 1.0, and all other normalized mRNA levels were plotted relative to that value. B. The TLR2 mRNA level in unstimulated GFP⁺ T cells was set at 1.0, and all other normalized mRNA levels were plotted relative to that value. Each point represents the mean ± SEM of triplicate samples. Similar results were seen in one other experiment. KO, Knockout.

FIGURE 1. Expression of TLR2 mRNA in GFP⁺ Treg cells and GFP⁻ T cells. A, FACS-sorted GFP⁺ T cells or GFP⁻ Treg cells (5 × 10⁶) were either unstimulated or activated by plate-bound anti-CD3 in the absence or presence of Pam3CSK4 for 16 h. IL-2 was added to Treg cultures. Total cellular RNA was isolated and TLR2 mRNA expression was measured by real-time PCR and normalized to β-actin mRNA levels. Epithelial cells from TLR2⁻/⁻ mice served as negative control. Macrophages derived from WT mouse bone marrow following culture in M-CSF and IL-3 served as a positive control population. The TLR2 mRNA level in TLR2⁻/⁻ epithelial cells was set at 1.0, and all other normalized mRNA levels were plotted relative to that value. B. The TLR2 mRNA level in unstimulated GFP⁺ T cells was set at 1.0, and all other normalized mRNA levels were plotted relative to that value. Each point represents the mean ± SEM of triplicate samples. Similar results were seen in one other experiment. KO, Knockout.

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Pam3CSK4 stimulation does not reduce Foxp3 expression and does not alter the suppressive activity of Treg in vitro

Because TLR2 engagement on Treg cells enhances their proliferation in vitro, it is important to clarify whether TLR2 agonists modulate Treg function. Several reports have claimed that engagement of TLR2 on Treg cells transiently reverses their suppressive function secondary to reduced Foxp3 expression (5, 16). We
initially examined the influence of the TLR2 agonist on Foxp3 expression by flow cytometric analysis. GFP−/H11001 Treg cells were expanded for 3 days with T-depleted spleen cells and soluble anti-CD3 (1 µg/ml) (A) or with plate-bound anti-CD3 (5 µg/ml) (B) in the presence of the following TLR agonists: poly(I:C) (TLR3), CpG (TLR9), TLR7/8 (CL097), and Pam3CSK4 (TLR2-TLR1). [3H]Tdr incorporation was determined during the last 6 h of culture. Results are expressed as the mean ± SEM of triplicate cultures.

To determine whether Pam3CSK4 treatment modulates the levels of antiapoptotic proteins in Treg cells, the intracellular expression of Bcl-2 was first examined by flow cytometry, but no differences in the levels of Bcl-2 were seen in cells cultured with or without Pam3CSK4 during the indicated time periods (Fig. 5B). However, marked enhancement of Bcl-xL protein expression was observed both after 24 and 48 h of stimulation when the Treg cells were cultured in the presence of Pam3CSK4 (Fig. 5C). Therefore, TLR2 ligation potentiates Treg cell survival and is associated with the up-regulation of antiapoptotic protein Bcl-xL.

**Pam3CSK4 improves Treg cell survival and induces the antiapoptotic protein Bcl-xL, but not Bcl-2**

As an initial approach to determining the mechanism of action of Pam3CSK4 on Treg function, we stimulated Treg cells with a low concentration of plate-bound anti-CD3, IL-2, and Pam3CSK4. After 7 days of culture, an increased percentage of viable cells was noted on FSC/SSC analysis of recovered cells in cultures containing Pam3CSK4 (Fig. 5A, top panel). Similarly, the percentage of 7-AAD and annexin V double-negative cells increased from 11 to 25% when the cells were cultured in the presence of Pam3CSK4 (Fig. 5A, bottom panel). All of the gated live cells maintained GFP-Foxp3 expression and no difference in the level of Foxp3 expression was seen when the cells were cultured with Pam3CSK4 (Fig. 5A, middle panel).

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**Pam3CSK4 enhances the proliferation of Ag-specific Treg cells in vivo**

To address the influence of TLR2 engagement on Treg proliferation in vivo, we transferred CFSE-labeled Treg cells purified from OT-II TCR-transgenic mice to nontransgenic TLR2−/− mice and then immunized the recipients with their cognate peptide in IFA. The mice were simultaneously injected i.p. with Pam3CSK4. As previously reported (22), TCR stimulation alone can induce vigorous
proliferation of Treg cells in vivo and treatment of the mice with Pam3CSK4 appeared to further enhance Treg proliferation (Fig. 6A) as measured by increased dilution of CFSE and in the absolute recovery of Foxp3+ T cells (Fig. 6B). Thus, engagement of TLR2 also costimulates Treg proliferation in vivo in the absence of exogenous cytokines.

Pam3CSK4-pretreated Treg cells efficiently prevent the development of autoimmune disease when cotransferred with scurfy cells into RAG−/− mice

To test whether TLR2 engagement alters the capacity of Treg cells to prevent the development of autoimmune disease, we used a previously characterized model (19) where T cells from scurfy mice are transferred into RAG−/− mice. Approximately 4 wk after transfer of total lymph node and spleen cells from 7-day-old scurfy to RAG−/− mice, all of the recipients developed lymphospleno-megaly, skin inflammation, and marked lymphocyte infiltration into the skin and liver. Cotransfer of natural Treg cells or TGF-β-induced Treg cells (19) prevents most of the manifestations of disease. Treg cells were precultured with anti-CD3 and IL-2 for 5 days in the presence or absence of Pam3CSK4 and cotransferred with scurfy T cells into RAG−/− recipients. Treg cells that had been pretreated with Pam3CSK4 were as efficient as nontreated Treg cells in suppressing both skin and liver disease 4 wk after transfer (Fig. 7).

Pam3CSK4 treatment does not attenuate WT Treg-mediated modulation of EAE in TLR2−/− mice

One problem with the interpretation of the scurfy transfer study is that Treg cells were pretreated with the TLR2 ligand and may have recovered their suppressor function after transfer. To determine whether direct TLR2 signaling in vivo can modulate Treg function, we used a model of Treg-mediated suppression of EAE originally described by Kohm et al. (23), where supplementation of normal mice with polyclonal Treg cells before disease induction modulates the severity of disease. TLR2−/− mice were reconstituted with GFP+ Treg cells 1 day before

FIGURE 3. Effect of different TLR2 complex agonists on the proliferation of GFP+ Treg cells. FACS-sorted GFP+ Treg cells (5 × 10⁴) were cultured with plate-bound anti-CD3 (5 μg/ml) and IL-2 for 3 days in the presence or absence of A, TLR2/TLR2 agonist LTA-SA; B, TLR2/TLR6 agonist Pam2CSK4, and C, TLR2/TLR1 agonist Pam3CSK4. Proliferation was measured as in Fig. 1. All experiments were performed at least three times.

FIGURE 4. Pam3CSK4 stimulation does not alter Foxp3 expression or Treg suppressive activity. A, FACS-sorted GFP+ Treg were stimulated with plate-bound anti-CD3 and IL-2 for 3 days in the presence or absence of Pam3CSK4. Flow cytometric analysis of GFP expression (right panel) and Foxp3 (left panel) was performed after the cells were rested in the presence of IL-2 alone for 2 days. MFI, Mean fluorescence intensity. B, CD4+ GFP+ responder T cells and T-depleted spleen cell (5 × 10⁴ of each) from TLR2−/− mice were cocultured with the indicated number of WT GFP+ Treg cells and soluble anti-CD3 in the absence or presence of Pam3CSK4. T cell proliferation was assayed as in Fig. 1. C, FACS-sorted GFP+ Treg cells from WT mice were stimulated with plate-bound anti-CD3 and IL-2 for 3 days in the presence or absence of Pam3CSK4. The indicated number of preactivated Treg cells was tested for suppressive activity as in B in the presence or absence of Pam3CSK4. Experiments were repeated at least three times.
EAE induction and then were treated with four injections of Pam3CSK4. TLR2−/− mice that were not reconstituted with Treg cells all developed severe EAE. In contrast, mice reconstituted with Treg cells had a marked reduction in disease severity (Fig. 8). Treatment of the reconstituted mice with Pam3CSK4 had no effect on the ability of the transferred Treg cells to modulate the induction of disease. Thus, brief treatment of mice with a TLR2 agonist does not reverse the suppressive function of Treg cells.

**Discussion**

Most studies on TLR signaling have been focused on their function in cells of the innate immune system. Studies from a number of groups (4, 6, 14, 24) have demonstrated that TLRs can be

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**FIGURE 5.** Pam3CSK4 augments Treg survival and induces Bcl-xL but not Bcl-2 expression. A, FACS-sorted GFP+ Treg cells were cultured with plate-bound anti-CD3 (0.4 μg/ml) and IL-2 for 7 days in the absence (left panel) or presence (right panel) of Pam3CSK4. The cells were then analyzed by flow cytometry. FSC/SSC plots and the percentage of live cells (high FSC) are indicated (top panel). Gated live cells were analyzed for GFP expression (middle panel). Viability of the total cell population was measured as the percentage of 7-AAD and annexin V excluding cells (bottom panel). Percentage of live (7-AAD− annexinV−) Treg cells were calculated by combined data from three independent experiments (below). Values of p were calculated using the Mann-Whitney U test. B, Treg cells were stimulated with plate-bound anti-CD3 (0.4 μg/ml) and IL-2 in the absence (left panel) or presence (right panel) of Pam3CSK4 for the indicated times. Bcl-2 and GFP expression was determined by flow cytometry. C, Treg cells were stimulated as in B for the indicated times. The cells were then lysed and analyzed by Western blot for mouse Bcl-xL (top panel). β-Actin (bottom panel) was used as the loading control. All data are representative of three different experiments with similar results.
Fusing picture has emerged from the analysis of the function of TLRs on both human and mouse Treg cells. Some studies (5, 7, 16) have shown that engagement of TLR2 on mouse Treg cells or TLR5 on human Treg cells results in enhancement of their suppressive functions, while other studies have claimed that engagement of TLR2 on mouse Treg cells (5, 16) of TLR8 in human Treg cells (17) reverses Treg suppressive function. Two major problems exist in the interpretation of these studies. First, a consensus view (26) has yet to emerge as to the mechanisms used by Treg cells to suppress their targets in vivo or in vitro. It is therefore difficult to determine how TLRs modulate suppression either positively or negatively. Second, since the TLRs are widely expressed on both conventional T cells and innate immune cells, contamination of the Treg preparation with non-Treg cells may have been responsible for the differences between the studies.

To avoid the problems encountered when Treg cells are isolated based on CD25 expression, we have reexamined the effects of TLR expression on murine Treg cells using highly purified Treg cells isolated from mice expressing enhanced GFP under the control of the Foxp3 promoter. We initially tested TLR2, TLR3, TLR7/8, and TLR9 agonists to determine whether they could enhance the proliferation of Treg cells in culture when stimulated with anti-CD3. No changes were seen in the presence of anti-CD3 alone, but TLR2, TLR7/8, and TLR9 agonists all augmented the proliferative responses of Treg cells in the presence of soluble anti-CD3 and APC, while significant enhancement of proliferation to plate-bound anti-CD3 and IL-2 was only seen with the TLR2 agonist Pam3CSK4. We concluded from these studies that the augmentation of the Treg response by TLR7/8 and TLR9 agonists was likely to be indirect and mediated via the APC, whereas TLR2 stimulation had a direct effect on the Treg cells. We therefore focused our studies on the potential role of TLR2 in modulation of Treg function. Augmentation of proliferation was associated with enhanced Treg cell survival in culture and enhanced expression of the anti-apoptotic protein Bcl-xL. A similar increase in Bcl-xL, but not Bcl-2 levels, was seen when conventional CD4+ T cells were treated with poly(I:C) or CpG DNA (9).

In contrast to previous studies, we were unable to detect TLR2 expression on the cell surface using the commercially available anti-TLR2 Abs. Low levels of TLR2 mRNA expression were expressed by conventional CD4+ and CD8+ T cells, as well as by Foxp3+ Treg cells. Stimulation of TLRs on conventional T cells in all studies results in enhancement of T cell activation and prolongation of TCR survival in vitro and in vivo. In most, but not all studies (25), T cells require activation as a prerequisite to TLR responsiveness. This requirement for prior TCR activation would prevent nonspecific activation of T cells by TLR ligands. A confusing picture has emerged from the analysis of the function of TLR2 on mouse Treg cells (5, 16) of TLR8 in human Treg cells (17). The total vs proliferating OT-II Foxp3+ cell counts in draining lymph nodes from TLR2−/− mice were calculated using CFSE profiles. Data are representative of two different experiments with similar results.
detected in unactivated Foxp3− T cells, while TLR2 mRNA could not be detected in freshly isolated Foxp3+ T cells. Following TCR activation, a modest increase in TLR2 mRNA expression was seen both in Foxp3− and Foxp3+ T cells, but the level of TLR2 mRNA was augmented 10-fold in the Foxp3+ T cells in the presence of the TLR2 agonist, suggesting a positive feedback on TLR2 expression by stimulation in the presence of the TLR2 agonist. A similar positive effect of a TLR agonist on TLR expression was observed (27) when human γδ T cells were stimulated with poly(I:C). It has also been reported that TLR2 transcription is increased during infection with *Pneumocystis carinii* where the host utilizes TLR2 to recognize major surface glycoprotein of the infecting organism (28). Although TLR2 can exist as a homodimer or heterodimerize with TLR1 and TLR6, the TLR2-TLR1 complex appears to be the most critical for costimulation of Treg proliferation, as significant enhancement of proliferation was only seen with the TLR2/TLR1 ligand, Pam3CSK4, and not with the TLR2/TLR6 ligand, Pam2CSK4, or with the natural ligand for TLR2/TLR6 homodimers, LTA- SA. It will therefore be interesting to determine whether activation of the TLR2-TLR1 heterodimeric complex on Treg cells can modulate immune responses to different microbial pathogens.

Both of the previous studies (5, 16) on the function of mouse Treg cells also reported augmentation of Treg proliferation in the presence of TCR activation and Pam3CSK4. They concluded from this observation that the enhanced proliferative responses of the Treg cells should result in abrogation of their suppressive ability. However, abrogation of Treg suppressive function was not seen under conditions where Treg cells are actively proliferating. Addition of a high concentration of IL-2 to cocultures of Treg cells and responders masks the suppressive effects of Treg cells on responder cell proliferation, but does not reverse the capacity of the Treg cells to inhibit the production of IL-2 or IFN-γ by the responder T cells (29, 30). Proliferation of Treg cells can also be induced in the presence of activated bone marrow-derived DCs and inhibition of responder T cell proliferation is also not seen under those conditions. However, suppression of IL-2 production by the responder cells is not reversed (31).

Since TLR2 is expressed by both responder T cells and APCs, analysis of the effects of TLR2 engagement on Treg suppressive ability is only possible in assays using WT Treg and responder T cells/APC from TLR2−/− mice. In contrast to previous studies, the addition of Pam3CSK4 to these cocultures had no effect on Treg-mediated suppression of proliferation over a broad range of Treg cell:responder ratios. Previous studies also claimed that overnight exposure of Treg cells to a TLR2 agonist also abrogated their suppressive capacity, while in our studies continuous exposure of the Treg cells to the agonist for 3 days had no effect on their ability to suppress when they were added immediately to cocultures in the absence of a rest period or even when the agonist was also added to the cocultures. There are several potential reasons for the differences between our results and those reported previously. First, as noted above, Treg cells in our studies were purified based on Foxp3 expression and were therefore much less likely to be contaminated with CD4+ CD25− Foxp3+ T effector cells. In our hands, the TLR2 agonist markedly enhanced the proliferation of conventional T cells. Second, in both the studies of Sutmuller et al. (16) and Liu et al. (5), very low levels of proliferation were observed in cultures of responder cells alone, high ratios (1:1 or 1:2) of Treg cells to responder cells were tested, and the magnitude of the reversal of suppression (30−40%) was not impressive. Liu et al. (5) also claimed that the transient increase in Foxp3 mRNA induced by TCR activation of Treg cells was blocked by exposure to the TLR2 agonist, but did not analyze Foxp3 protein expression. In our hands, culture of the Treg cells for several days in the continuous presence of Pam3CSK4 has absolutely no effect on the expression of Foxp3 protein expression.

Conclusions based solely on in vitro studies with Treg cells must be interpreted with caution, as Treg function in vivo may be mediated by different mechanisms. Treatment of mice with Pam3CSK4 resulted in a moderate increase in the proliferation of Ag-specific Treg cells in response to immunization with Ag. Similar results were seen in the studies of Sutmuller et al. (16) although they did not observe significant proliferation in the presence of Ag alone. It should be noted that Treg suppression of the expansion of Ag-specific T cells in vivo may be accompanied by expansion of the Treg cells (32). Thus, proliferation of Treg cells in vivo as in vitro does not indicate abrogation of Treg suppressive capacity. We used two different models to determine whether TLR2 engagement inhibited Treg function in vivo. The first model was similar to the one used by Liu et al. (5) and involved pretreatment of the Treg cells with Pam3CSK4 before transfer in vivo. One of the most sensitive assays for Treg function in vivo is the ability of Treg cells to suppress the transfer of a global autoimmune syndrome by scurfy T cells to RAG−/− mice (19). Treg cells expanded and treated with Pam3CSK4 were as suppressive as Treg cells expanded in the absence of the TLR2 ligand. In contrast, Liu et al. (5) claimed that a brief exposure to Pam3CSK4 delayed the ability of Treg cells to treat inflammatory bowel disease and completely inhibited the ability of the animals to clear *Leishmania major*. We also used the system originally described by Kohm et al. (23) in which supplementation of normal mice with Treg cells from WT mice modulates the induction of EAE induced by immunization with a MOG peptide. When TLR2−/− recipient mice were supplemented with WT Treg cells and treated with multiple injections of Pam3CSK4 during the induction of EAE, the decrease in disease severity was identical to that seen in mice not treated with the TLR2 ligand. The treatment regimen used was very similar to the one used by Sutmuller et al. (16) in their studies of mice infected with *C. albicans*. However, they observed an enhancement of the magnitude of colonization of the mouse with *Candida albicans* when WT Treg cells were transferred to TLR2−/− mice and abrogation of this enhancement when the mice were treated with Pam3CSK4. Again, it is very difficult to determine the reasons for the differences between these results. Our studies used two well-characterized models of Treg-mediated modulation of autoimmune disease, while the other studies used models of immune responses to pathogens. In any case, it appears that TLR2 stimulation does not reproducibly modulate Treg function in vivo.

One of the most interesting questions raised by these studies is the significance of expression of TLRs on Treg cells. Taken together, our studies and those of Crellin et al. (15) and Zamin-Zhorov et al. (7) indicate that stimulation of Treg cells via TLR2 or TLR5 either enhances their suppressor function or their expansion and survival. Superficially, enhancement of Treg function should be deleterious to the immune response to a TLR ligand-expressing pathogen. In many respects, one would have predicted that TLR ligands should actually abrogate Treg function in response to pathogens as suggested by some studies. Alternatively, as proposed by Crellin et al. (15), early in the immune response, the TLR signal delivered to APC results in cytokine production that together with the direct action of the TLR ligands on responder T cells renders them resistant to suppression. The simultaneous stimulation of Treg cells with the TLR ligands would both result in enhancement of Treg viability and proliferation. When the acute response to the pathogen begins to subside, TLR-mediated...
enhanced Treg function can then play a critical role in the prevention of immune pathology or in maintaining low levels of pathogens that are needed for maintenance of immunologic memory (33). Further detailed analysis of the different signaling pathways modulated by TLR ligands in Treg cells compared with T effector cells may offer insights to the development of agents that can be used to selectively expand Treg cells in culture for use in cellular biotherapy or to enhance Treg function in vivo as a component of the treatment of autoimmune disease.

Acknowledgments

We thank the NIAID Flow Cytometry Section, particularly Bishop Hague and Elina Stregevsky for cell sorting.

Disclosures

The authors have no financial conflict of interest.

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