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TLR2 Stimulation Drives Human Naive and Effector Regulatory T Cells into a Th17-Like Phenotype with Reduced Suppressive Function

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Naturally occurring CD4+CD25+FOXP3+ regulatory T cells suppress the activity of pathogenic T cells and prevent development of autoimmune responses. There is growing evidence that TLRs are involved in modulating regulatory T cell (Treg) functions both directly and indirectly. Specifically, TLR2 stimulation has been shown to reduce the suppressive function of Tregs by mechanisms that are incompletely understood. The developmental pathways of Tregs and Th17 cells are considered divergent and mutually inhibitory, and IL-17 secretion has been reported to be associated with reduced Treg function. We hypothesized that TLR2 stimulation may reduce the suppressive function of Tregs by regulating the balance between Treg and Th17 phenotype and function. We examined the effect of different TLR2 ligands on the suppressive functions of Tregs and found that activation of TLR1/2 heterodimers reduces the suppressive activity of CD4+CD25hiFOXP3lowCD45RA+ (naive) and CD4+CD25hiFOXP3hiCD45RA− (memory or effector) Treg subpopulations on CD4+CD25−FOXP3−CD45RA+ responder T cell proliferation while at the same time enhancing the secretion of IL-6 and IL-17, increasing RORC, and decreasing FOXP3 expression. Neutralization of IL-6 or IL-17 abrogated Pam3Cys-mediated reduction of Treg suppressive function. We also found that, in agreement with recent observations in mouse T cells, TLR2 stimulation can promote Th17 differentiation of human T helper precursors. We conclude that TLR2 stimulation, in combination with TCR activation and costimulation, promotes the differentiation of distinct subsets of human naive and memory/effector Tregs into a Th17-like phenotype and their expansion. Such TLR-induced mechanism of regulation of Treg function could enhance microbial clearance and increase the risk of autoimmune reactions. The Journal of Immunology, 2011, 187: 000–000.

E ffective control mechanisms are required for immune responses to neutralize and eliminate pathogens without leading to autoimmunity. Naturally occurring CD4+CD25+ regulatory T cells (nTregs) play a crucial role in maintaining tolerance to self-antigens and preventing autoimmune diseases (1). nTregs are characterized by the constitutive expression of the α-chain of the IL-2 receptor, CD25, and the transcription factor FOXP3, which is associated with their development and function (2). Recent evidence indicates that TLRs expressed on Tregs can modulate their suppressive activity (3, 4). TLRs are essential receptors of the innate immune system that recognize pathogen-associated molecular patterns expressed by viruses and bacteria. Each member of the TLR family recognizes different microbial components (5). In particular, TLR2 recognizes bacterial lipopeptides, lipoteichoic acid from Gram-positive bacteria, and zymosan from yeast cell wall (5). TLR2 forms heterodimers with other TLRs, thus broadening the spectrum of its ligands. For example, TLR1/TLR2 heterodimers recognize tris-acylated lipopeptides, including the synthetic bacterial lipoprotein Pam3Cys-SK4 (6, 7), and TLR2/TLR6 heterodimers recognize bis-acylated lipopeptides such as mycoplasma lipopeptide and the synthetic mycoplasma lipopeptide-2 analogue fibroblast stimulating lipopeptide-1 (FSL-1) (7). TLRs are mainly expressed on APC, but their detection on T cell subsets, including nTregs, has provided insight into a new aspect of innate regulation of adaptive immunity (8, 9).

Previous reports in murine cells demonstrated that TLR2 ligation leads to reduced suppressive function of regulatory T cells (Tregs) (4, 10, 11). In human Tregs, various TLR2 ligands including the 60-kDa heat shock protein (HSP60), Pam3Cys, FSL-1, and Pam2Cys have been reported to differentially affect suppressive function by either augmenting or decreasing it (10, 12). Zanin-Zhorov et al. (12) showed that HSP60 increased Treg suppressive function, and its effects led to activation of signaling molecules PKC, PI3K, and p38. By contrast, recently Oberg et al. (10) showed that a mixture of synthetic lipopeptides including Pam3-Cys, FSL-1, and Pam2Cys enhances AKT phosphorylation and reduces Treg function. In fact, a reduced AKT phosphorylation and decreased activation of S6 and FOXO pathways is required for the suppressive function of Tregs (13).

In the presence of inflammatory cytokines, such as IL-1β and IL-6, both murine and human Tregs can be induced to produce IL-17,
which results in reduction of Treg suppressive function (14). This observation is consistent with the known reciprocal and mutually inhibitory regulation of Treg and Th17 cell development (15–17). Th17 cells are considered important effectors of protection against bacterial and fungal infections (18) but also mediators of tissue damage in experimental autoimmune diseases, such as experimental autoimmune encephalomyelitis and autoimmune arthritis (19, 20). Th17 T cells differentiate from naïve CD4+ lymphocytes in the presence of IL-6 and TGF (TGF-β) (21). Their differentiation also requires IL-21 for amplification and IL-23 for stabilization and terminal differentiation (22, 23). Committed Th17 cells produce effector cytokines including IL-17A (IL-17), IL-17F, IL-21, and IL-22 (3, 4). The impact of innate immune signals on Th17 function and autoimmunity is a subject of interest. In murine CD4+ T cells, TLR2 signaling promotes Th17 responses and facilitates the development of autoimmune inflammatory demyelination (24).

We hypothesized that TLR2 regulates the balance between human Treg and Th17 phenotype based on the premises that 1) developmental pathways for Tregs and Th17 cells are thought to be linked but divergent and mutually inhibitory (25); 2) IL-17 production has been reported to be associated with reduced Treg function (14); 3) IL-6, which promotes the development of Th17 cells in the presence of TGF-β and other cytokines, such as IL-1β and IL-23 (26), is known to reduce Treg function (27); and 4) TLR2 stimulation promotes Th17 development in the mouse (24). We examined the effect of Pam3Cys and FSL-1 on the suppressive functions of Tregs and investigated the effect of TLR2 stimulation on IL-17-mediated control of human Tregs. We observed that the synthetic lipopeptide Pam3Cys significantly reduces the suppressive functions of CD4+CD25+CD127negFOXP3+ Tregs, whereas FSL-1 does not, suggesting a predominant role of the TLR1/2 rather than TLR2/6 heterodimer in modulating Treg functions. The same effect was observed on Tregs classified as naïve (FOXP3−CD45RA+) and effector (or memory, FOXP3hi CD45RA−) (14, 28, 29). In addition, we found that Pam3Cys induced Tregs to produce IL-17A/F and that neutralization of IL-6 abrogated Pam3Cys-mediated reduction of Treg suppressive activity. These data point to a mechanistic link between IL-6 production and reduced suppressive function of Tregs. Pam3Cys also led to increased production of IL-17, the neutralization of which reversed the inhibition of suppression induced by TLR2.

Our data are consistent with a model in which Tregs may be skewed toward a Th17 phenotype and effector function under inflammatory conditions. Such modulation of Treg phenotype and function may allow more effective protection against inflammation but may lead to increased susceptibility to autoimmunity reactions (3, 30).

Materials and Methods

Cell purification and sorting

Human PBMCs were isolated from whole blood by Ficoll density gradient centrifugation (Histopaque; Sigma-Aldrich, Dorset, U.K.). CD4+CD25+ and CD4+CD25- responder T cells (Tresps) were purified from PBMCs by depletion of non-CD4+ T cells followed by positive selection of the CD4+CD25+ fraction using MACS microbeads (Treg cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany) using suboptimal amounts of anti-CD25 beads to increase cell purity. In separate experiments, CD4+CD25hiCD127neg Tregs and CD4+CD25 Tregs were sorted from CD4+ T cells using a FACSaria cell sorter (BD Biosciences, San Jose, CA). Next, in light of the recent classification of human CD4+ T cell subpopulations by Miyara et al. (28, 31), we FACS-sorted CD45RA−CD25+ (Fraction Fr. I, naïve CD4+ T cells) and CD45RA+CD25− (Fr. VI, memory-like non-Tregs), and CD45RA−CD25+ (Fr. II, “memory-effector” or “activated” Tregs), CD45RA+CD25+ (Fr. III, “memory-like non-Tregs”), and CD45RA−CD25+ (Fr. IV, naive T cells) populations using a MoFlo XDP cell sorter (Beckman Coulter, High Wycombe, U.K.). The other fractions comprise “memory-like” FOXP3+CD45RA+CD4− non-Tregs (Fr. IV and V together) (28). Before sorting, CD4+ T cells were enriched from PBMCs using a CD4+ T cell isolation kit (Miltenyi Biotec). The study included 48 healthy subjects (mean age 45 y; range, 24–64 y) who had no history of autoimmune disease. Informed consent was obtained from all study participants. The study was approved by the University of Nottingham Research Ethics Committee.

Proliferation and suppression assays

Cells were cultured in 96-well U-bottom plates (Corning, Corning, NY) in RPMI 1640 medium (Sigma-Aldrich) supplemented with 5% FCS (Sigma-Aldrich). The suppressive functions of Tregs and the effect of TLR2 stimulation were studied in a coculture suppression assay. CD4+CD25 Tregs (10 × 10^5) were seeded at a constant number in coculture with CD4+CD25CD127neg T cells, and CD45RA-CD25+ cells (Fr. VI) were cocultured with CD45RA-CD25+ (Fr. I) or CD45RA-CD25++ (Fr. II) Tregs at 1:16, 1:8, and 1:4 Treg/Tresp ratios in the presence or absence of Pam3Cys or FSL-1 (EMC Microcollections, Tuebingen, Germany), both at 5 µg/ml. In separate experiments, CD4+CD25 Tresps were cocultured with CD4+CD25CD127neg Tresps in the absence or presence of neutralizing anti-IL-6, anti-IL-17, or Pam3Cys. Thus in summary, magnetic bead-purified CD4+CD25+ or FACS-sorted CD4+CD25RA-CD25+ T cells (Fr. VI) were used as Tresps, whereas magnetic bead-purified CD4+CD25+ T cells or FACS-sorted CD4+CD25CD127neg or CD45RA+CD25+ (Fr. II) T cells were used as Tregs as indicated in different experiments. Cells were cultured in RPMI 1640 medium supplemented with 5% FCS and incubated for 48 or 120 h at 37˚C and then pulsed with 1 µCi [3H]thymidine (PerkinElmer, Cambridge, U.K.) and incubated for the last 16 h of culture. Cells were harvested and assessed for thymidine incorporation using a liquid scintillation β counter (Top Count, Microplate Scintillation Counter; Packard, U.K.). Cell proliferation was expressed as cpm per well.

In selected flow cytometry-based proliferation assays, Fr. VI Tresps (<10^5 cells/ml) were labeled with CFSE (Invitrogen, Paisley, U.K.) then cocultured with unlabelled Fr. I or Fr. II Tregs at 1:16, 1:8, and 1:4 ratios in the presence or absence of Pam3Cys or FSL-1 (5 µg/ml) and cultured for 96 h. CFSE dilution of Tresps was determined using an LSRII flow cytometer (BD Biosciences). In all experiments, cells were cultured with plate-bound anti-CD3 (1 µg/ml; clone UCHT1) and anti-CD28 (1 µg/ml; clone CD28.2) (both from Beckman Coulter).

For preincubation experiments, purified Tregs or Tresps were incubated with or without Pam3Cys or FSL-1 (5 µg/ml) for 16 h. Tresps were also stimulated with plate-bound anti-CD3 and anti-CD28 Abs. After overnight culture, Tregs and Tresps were washed and incubated with freshly isolated Tregs or Tresps from the same donor, respectively, at 1:16, 1:8, and 1:4 Treg/Tresp ratios. Cocultures were then further incubated for 72 h and pulsed with [3H]thymidine for the final 16 h. [3H]thymidine incorporation was measured as described earlier. Cultures were set up in triplicate wells in 96-well plates (Corning).

Th17 polarization assays

CD4+ T cells or subpopulations including CD45RA-CD25+ (Fr. I), CD45RA-CD25++ (Fr. II), CD45RA-CD25+++ (Fr. III), and CD45RA+CD25+ (Fr. VI) were cultured on plates coated with 1 µg/ml anti-CD3 and anti-CD28 in IMDM medium (Sigma-Aldrich) supplemented with 5% AB serum (Invitrogen) in the absence or presence of a “Th17 differentiation cocktail” including 10 ng/ml IL-1β, 30 ng/ml IL-6, 10 ng/ml TGF-β (Invitrogen), 0.5 ng/ml IL-23 (R&D Systems, Abingdon, U.K.) or, alternatively, Pam3Cys or FSL-1 (5 µg/ml; EMC Microcollections). Neutralizing Abs to IFN-γ or IL-4 (R&D Systems) were added at 10 µg/ml in some experiments. In selected experiments, CD4+ T cells were cultured with Pam3Cys, Pam2Cys, and FSL-1 without TCR stimulation. Coculture with intracellular cytokines, T cells were incubated for 72 or 96 h then stained with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (250 ng/ml; Sigma-Aldrich) in the presence of brefeldin A (10 µg/ml; Sigma-Aldrich) for the last 4 h of culture. In all experiments, culture supernatants were collected before adding thymidine or stimulating with PMA/ionomycin/brefeldin A.

Flow cytometric analysis

Cells were freshly stained from isolation or culture for 48 or 72 h on plate-bound anti-CD3 and anti-CD28 in absence or presence of Pam3Cys, Pam2Cys, and FSL-1. In some experiments, then stimulated with PMA/ionomycin and brefeldin A for the last 4 h of culture. Cells were surface stained with anti-TLR1, anti-TLR2 (clone TL2.1), anti-TLR6, anti-CCR6, anti-IFN-γ (all from eBioscience, Hatfield, U.K.) or intracellularly stained with anti-
FOXP3 (PCH101; eBioscience), anti–TGF-β (R&D Systems), anti–IL-6, anti–IL-17 (eBioscience), anti–IL-22, anti–RORC, and anti–T-bet (R&D Systems) mAbs. Negative control staining was carried out by staining with the respective isotype Ab. Cells were analyzed by flow cytometry using an LSRII flow cytometer (BD Biosciences) and FlowJo software (version 7.2.5; Tree Star, Ashland, OR).

Measurement of cytokines in culture supernatants

Human IL-17A, IL-22, IL-6, and TGF-β were determined in the supernatants of Treg and Tresp cultures or cocultures using human cytokine multiplex kits (eBioscience). ELISA was used to analyze IL-17F (eBioscience).

Statistics

All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Paired or unpaired Student t test and Mann–Whitney U test were applied with significance levels set at p < 0.05.

Results

TLR2 stimulation reduces the suppressive functions of Tregs

Naturally occurring Tregs, as defined by a CD4+CD25+ profile, have been shown to play a crucial role in maintaining balanced immune responses (1, 32). Recent evidence suggests that TLRs are involved in controlling Treg functions (3, 4). Previous reports in murine and human cells demonstrated that TLR2 ligation leads to reduced suppressive function of Tregs (4, 10, 11), even when combinations of various TLR2 agonists are used (10). We examined the effect of Pam3Cys and FSL-1 separately on the suppressive functions of magnetic bead-isolated and FACS-sorted Tregs. Pam3Cys significantly reduced the suppressive functions of magnetic bead-isolated CD4+CD25+ Tregs, as determined using thymidine incorporation. In contrast, Treg suppression was
not significantly reduced by FSL-1 (Fig. 1A). Pam3Cys also significantly reduced the suppressive activity of FACS-sorted, highly purified CD4⁺CD25⁺CD127⁻FOXP3⁺ Tregs (Fig. 1B, 1C). Based on the expression of CD25 and CD45RA, human CD4⁺ T cells have been classified into six subpopulations, Fr. I–VI (28) (Fig. 1D). Fr. I, II, and III are FOXP3⁺, and the degree of FOXP3 protein expression is proportional to CD25 expression (Fig. 1E) (28). Fr. I and II are highly suppressive when cocultured with Tresps (Fr. VI), whereas Fr. III cells are non-suppressive and produce higher levels of IL-17 than all the other fractions (28). We examined the effect of Pam3Cys and FSL-1 on the suppressive functions of Fr. I and II Tregs on the proliferation of Fr. VI Tresps. Fr. III cells were not used as Tregs because they are considered nonsuppressive and produce higher baseline amounts of IL-17 (28). We found that Pam3Cys but not FSL-1 reduced the suppressive functions of both Fr. I and Fr. II when cocultured with Fr. VI (Fig. 1F, 1G). These data demonstrate significant inhibition of the highly suppressive human “naive-like” (resting, Fr. I) and “memory-like” (activated, Fr. II) Tregs by TLR2 stimulation.

**Biological effects of Pam3Cys are predominantly exerted on Tregs**

Another aspect of our investigation was to discriminate between TLR2 agonist effects on Tregs and Tresps respectively, to determine whether they act directly on either or both cell populations. On the basis of the higher expression of TLR2 on Tregs (10), we hypothesized a predominant effect of Pam3Cys on these cells. For this purpose, we performed preincubation assays. CD4⁺CD25⁺ Tregs or CD4⁺CD25⁻ Tresps were preincubated with Pam3Cys or FSL-1 in separate wells overnight and washed before coculture with freshly isolated Tresps and Tregs from the same donor, respectively. Preincubation of Tregs with Pam3Cys led to significant reduction of their suppressive activity (Supplemental Fig. 1A). By

![FIGURE 2. Surface expression of TLR2, TLR1, and TLR6 by subpopulations of CD4⁺ T cells. CD4⁺-enriched cells were stained for CD45RA, CD25, TLR2, TLR1, TLR6, and FOXP3 expression. A, The expression of TLR2, TLR1, TLR6, and FOXP3 by Fr. I–VI was determined (Fr. I–VI were defined by the expression of CD45RA and CD25, see Fig. 1D). B–D, The percentage of cells expressing TLR2, TLR1, and TLR6 by Fr. I–VI was compared between different donors. Data obtained from five to eight subjects is shown.](http://www.jimmunol.org/)

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contrast, there was minor reduction of Treg suppression of Tresp proliferation when Tresps were precultured with Pam3Cys (Supplemental Fig. 1B).

Because overall suppression of Tresp proliferation was lower when Tregs were precultured in the presence or absence of Pam3Cys before adding freshly isolated Tresps than in the reciprocal condition, we wanted to assess whether Pam3Cys caused apoptotic death of either cell population. Tregs and Tresps were cultured separately or cocultured in the conditions described earlier in the presence or absence of Pam3Cys or FSL-1. Staining with annexin V and 7-aminoactinomycin D showed a trend to higher apoptosis at tested Pam3Cys or FSL-1 concentrations in single cultures of Tregs compared with Tresps, which was not statistically significant (Supplemental Fig. 1C). No differences in apoptosis were observed when Tregs and Tresps were cultured together (Supplemental Fig. 1D), with or without Pam3Cys or FSL-1. These data suggest that precultivation of Tregs cultured alone (with or without Pam3Cys or FSL-1) partly reduces their viability. However, apoptosis does not account for the observed modulation of Treg function by Pam3Cys.

**TLR2 is highly expressed by CD45RA^−CD25^++ (naive) and CD45RA^−CD25^+++ (effector) Tregs**

TLR2 expression has been reported on CD4^+^ T cells (10, 33). We first compared the surface expression of TLR2, TLR1, and TLR6 on magnetic bead-sorted CD4^+^CD25^+^ Tregs and CD4^+^CD25^- Tresps. We observed that Tregs expressed higher density of TLR2 compared with that of Tresps. Both Tregs and Tresps expressed TLR1 and TRL6 at lower density than that of TLR2 (data not shown). Next, we examined the expression of TLR2, TLR1, and TLR6 by T cell fractions I–VI as defined earlier. Fr. I and II expressed higher density of TLR2 compared with Fr. IV, V, and VI (Fig. 2). Fr. III expressed intermediate levels of TLR2 and very low levels of its heterodimeric partners TLR1 and TRL6, Fr. IV, V, and VI expressed very low levels of all the three TLRs tested. Of note, Fr. I, II, and III expressed lower levels of TLR1 and TRL6 compared with levels of TLR2. These observations suggest that Fr. I–II (Tregs) and III (FOXP3^low^ non-Tregs) are the main target of TLR2 agonists.

**TLR2 stimulation enhances IL-6 and TGF-β expression by both naive and effector Tregs**

To understand further the mechanisms by which Pam3Cys reduced the suppressive functions of Tregs, we first studied the effects of Pam3Cys on IL-6 and TGF-β expression. We hypothesized that IL-6 production might be involved in the loss of regulatory function induced by TLR2 activation because IL-6 is known to reduce Treg function (27). Magnetic bead-isolated Tregs and Tresps were cultured separately with plate-bound anti-CD3 and anti-CD28 in the absence or presence of Pam3Cys, FSL-1, or a Th17 polarization mixture. As expected, these cell conditions led to the differentiation of Th17 cells (Fig. 4). In parallel cultures, stimulation of plate-bound anti-CD3/CD28-activated cells with Pam3Cys induced equivalent or higher IL-17 and IL-22 intracellular expression compared with the standard Th17 polarization mixture and FSL-1 (Fig. 4A). As in previous experiments, IFN-γ expression was low in all conditions (Fig. 4A). Consistent with intracellular staining data, cytokine analysis of culture supernatants revealed that treatment with Pam3Cys (p = 0.014) or Th17 polarization mixture (p = 0.03) significantly enhanced IL-17A, IL-17F, and IL-22 production compared with that by untreated cells (Fig. 4B). In contrast, FSL-1 did not enhance IL-17A but tended to increase IL-17F and IL-22 levels. In the absence of anti-CD3 and anti-CD28, TLR2 stimulation was not sufficient to drive Th17 differentiation (data not shown). Together, these data show that TLR2 stimulation can promote the expansion of Th17 cells in combination with TGF-β.

**FIGURE 3.** Expression of IL-6 and TGF-β by subpopulations of CD4^+^ T cells. FACSSorted CD4^+^CD45RA^−CD25^++ (Fr. I), CD4^+^CD45RA^−CD25^+++ (Fr. II), CD4^+^CD45RA^−CD25^++ (Fr. III), and CD4^+^CD45RA^−CD25^− (Fr. VI) cells were cultured separately for 4 h or for 72 h on plate-bound anti-CD3 and anti-CD28 (1 μg/ml) in the absence or presence of Pam3Cys or FSL-1 (5 μg/ml). A and B, Percentages of IL-6^−^ and TGF-β^−^ cells in the absence or presence of Pam3Cys or FSL-1 (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001.
cells and/or their differentiation from T helper precursors in the presence of TCR stimulation, with a predominant role of the TLR1/2 heterodimer.

**TLR2 stimulation enhances IL-17 production by human naive and effector Tregs**

To test the hypothesis that the simultaneous induction of IL-6 and TGF-β by Pam3Cys may induce Th17 skewing of human Tregs, we first studied the effect of TLR2 stimulation on IL-17 expression by FACS-sorted CD4⁺CD25⁺CD127negFOXP3⁺ Tregs and CD4⁺CD25⁺ Tresp (see Fig. 1B). The expression of IL-17 as well as IL-22, RORC, CCR6, IFN-γ, and T-bet was assessed in the two cell populations before and after culture for 72 h in the absence or presence of Pam3Cys or FSL-1 (5 µg/ml) on plate-bound anti-CD3 and anti-CD28 (1 µg/ml). We found a significant increase of CD4⁺CD25⁺CD127negFOXP3⁺ Tregs expressing IL-17 (Fig. 5).}

**FIGURE 4.** TLR2 stimulation enhances IL-17A production by human CD4⁺ T cells. A, CD4⁺-enriched T cells were intracellularly stained for IL-17, IL-22, and IFN-γ after culture on plate-bound anti-CD3 and anti-CD28 for 72 h in the absence or presence of a Th17 mixture of cytokines and Abs (IL-1β, IL-6, IL-23, TGF-β, and neutralizing anti-IL-4 and anti-IFN-γ), Pam3Cys, or FSL-1 (5 µg/ml). Cells were stimulated with PMA/ionomycin in the presence of brefeldin A during the last 4 h of culture. One representative plot is shown of seven experiments. B, The secretion of IL-17A, IL-17F, and IL-22 was examined in supernatants collected before stimulation with PMA/ionomycin and brefeldin A (n = 7). *p < 0.05.

**FIGURE 5.** TLR2 stimulation enhances IL-17 production and reduces FOXP3 expression by subpopulations of CD4⁺ T cells. FACS-sorted CD4⁺ T cells were cultured separately for 4 h or for 72 h on plate-bound anti-CD3 and anti-CD28 in the absence or presence of a Th17 mixture of cytokines and Abs (10 ng/ml IL-1β, 30 ng/ml IL-6, 10 ng/ml IL-23, 0.5 ng/ml TGF-β, and neutralizing anti-IL-4 and anti-IFN-γ; 10 µg/ml), Pam3Cys, or FSL-1 (5 µg/ml). Cells were stimulated with PMA/ionomycin in the presence of brefeldin A during the last 4 h of culture. Percentages of IL-17 (A), RORC (B), FOXP3 (C), and IL-17⁺ FOXP3⁺ (D) are shown (n = 6). *p < 0.05, **p < 0.01.
22, CCR6, and RORC after culture with Pam3Cys but not FSL-1 (Supplemental Fig. 4A, 4B). Notably, TLR2 stimulation did not significantly affect the Th1 signature cytokine IFN-γ and the Th1 transcription factor T-bet.

Next, we investigated the effect of TLR2 stimulation on IL-17 production by subpopulations of CD4+ T cells according to the described classification (28). We examined IL-17, FOXP3, and RORC expression in each FACS-sorted population after 4-h culture and after 5 d of culture. We found that Fr. III expressed the highest baseline levels of IL-17A compared with those of Fr. I, Fr. II, or Fr. VI (Fig. 5A, Supplemental Fig. 5), as previously reported (28). After 5 d of culture, there was enhanced IL-17 production by purified Fr. I, Fr. II, and Fr. III in the presence of Pam3Cys but not FSL-1 (Fig. 5A, Supplemental Fig. 5). Of note is that IL-17 production by Pam3Cys-treated Fr. I and II was comparable with that of cells treated with Th17 mixture of Abs and cytokines. The addition of Pam3Cys or Th17 mixture of Abs did not enhance IL-17 production by Fr. VI. IL-17 secretion by Pam3Cys-stimulated Fr. I, II, and III was associated with significantly increased expression of the transcription factor RORC (Fig. 5B), a master regulator of Th17 development (36). Furthermore, we observed reduced FOXP3 expression by Pam3Cys-treated Tregs, indicating

**FIGURE 6.** Cytokine production by cocultures and monocultures of Tregs and Tresps. Supernatants collected from FACS-sorted CD4+ CD45RA+CD25++ (naive Tregs, Fr. I), CD4+ CD45RA-CD25+++ (effector Tregs, Fr. II), CD4+CD45RA-CD25++ (memory non-Tregs, Fr. III), and CD4+CD45RA-CD25- (naive Tresps, Fr. VI) cells cultured in the indicated conditions in the absence or presence of Pam3Cys or FSL-1 were analyzed for IL-17A, IL-17F, IL-6, and IL-22 production as indicated in Materials and Methods. A, Fr. I Tregs: Fr. VI Tresp cocultures; B, Fr. II Tregs: Fr. VI Tresp cocultures; C, monocultures of Fr. I, II, III, and VI cells. Data represent average concentrations ± SE of the indicated cytokines in six different subjects. *p < 0.05, **p < 0.01.
an inverse relationship between RORC and FOXP3 expression (Fig. 5C, Suplemental Fig. 6). In addition, the percentage of FOXP3+ Tregs expressing IL-17 increased significantly (Fig. 5D). These data were consistent with our observation of enhanced expression of CCR6, a chemokine receptor preferentially expressed on Th17 cells (37), by CD4+CD25+CD127negFOXP3+ Tregs (Supplemental Fig. 4).

To establish the significance of these findings in our coculture system, we measured levels of IL-17A, IL-17F, IL-22, and IL-6 in the supernatants of Tregs and Tresps cocultured at the ratios used in suppression assays. In addition, these cytokines were analyzed in supernatants collected from single cultures or cocultures of Fr. VI (Tresps) with Fr. I or II (Tregs). We found that Pam3Cys significantly increased the production of IL-17A, IL-17F, IL-22, and IL-6 in cocultures of Tregs and Tresps (Fig. 6A, 6B) and that each of these cell types, except for Tresp monocultures (Fig. 6C), can be a source of such cytokines. Together, these data indicate that TLR2 stimulation enhances the production of Th17-type cytokines in human naive and effector Tregs, as well as in “memory non-Tregs” (Fr. III).

**TLR2 stimulation promotes a Th17 shift in naive and effector Tregs cocultured with Tresps**

To establish the relative contribution of TLR2 stimulation in IL-17+ Treg expansion as opposed to differentiation of IL-17+ Tregs into a Th17-like phenotype, naive (Fr. I) and memory-effector (Fr. II) Tregs were cocultured with CFSE-labeled Tresps (Fr. VI). After 96-h culture, cells were stained for intracellular IL-17 and analyzed by flow cytometry. In agreement with [3H]thymidine incorporation experiments (Fig. 1), CFSE dilution assays showed a clear reduction in the suppressive activity of both naive (Fr. I) and effector (Fr. II) Tregs in the presence of Pam3Cys but not...
FSL-1. Of note, we observed a significant shift of CFSE-unlabeled Fr. I (Fig. 7) and Fr. II (Fig. 8) Tregs to a Th17-like phenotype in the presence of Pam3Cys. Furthermore, a smaller shift of CFSE-labeled CD25+CD45+ Tregs (Fr. VI) toward IL-17 production was also observed in cocultures with both Fr. I (Fig. 7) and Fr. II (Fig. 8) Tregs. By contrast, FSL-1 did not significantly affect the IL-17 phenotype of Tregs or Tresps. Thus, although we cannot rule out that the proliferation of small percentages of preexisting IL-17+ Tregs occurs, these data are consistent with a TLR2-driven shift of the majority of Tregs toward a Th17-like phenotype.

**TLR2-induced reduction of Treg suppression is reversed by anti–IL-6 and anti–IL-17**

Our next aim was to investigate the functional role of IL-6, known to inhibit Treg function (27), in TLR2-induced reduction of Treg function. Cells were incubated either in monoculture or in coculture at different ratios in the absence or presence of neutralizing anti–IL-6. As previously observed (Fig. 1), Pam3Cys reduced the suppressive functions of Tregs at 1:16, 1:8, and 1:4 Treg/Tresp ratios ($p = 0.01, 0.03, 0.014$, respectively; Fig. 9A). Neutralization of IL-6 abrogated Pam3Cys-mediated reduction of Treg suppression at the indicated Treg/Tresp ratios ($p = 0.013, 0.011$, and 0.016, respectively). Anti–IL-6 alone did not significantly affect Treg suppressive function (Fig. 9A).

To test further the role of IL-6 in Treg function, we measured the expression of IL-17 and IL-22 by Tregs stimulated with Pam3Cys in the presence or absence of neutralizing anti–IL-6 Ab. IL-22, an IL-10 family member, is highly expressed by Th17 cells, and its expression can be induced by TGF-β signaling in the presence of IL-6 and other proinflammatory cytokines (38). Both IL-22 and IL-17 were induced by Pam3Cys in Treg monocultures, and IL-6 neutralization reduced their induction (Fig. 9B). These data suggest
that IL-6 induced by TLR2 stimulation contributes to the reduction of Treg suppressive function.

We then directly addressed the functional role of IL-17 in our system. Tregs and Tresps that were FACS-sorted as described in Fig. 1B were incubated either in monoculture or in coculture at different ratios in the absence or presence of neutralizing anti–IL-6 Abs, leading to increased responder T cell proliferation. We provide evidence that T cell-derived IL-6 contributes to such effect and that reduced suppressive activity is observed in association with a shift of Treg populations into a “Th17-like” phenotype marked by increased expression of RORC and decreased expression of FOXP3 (Fig. 10).

Discussion

We studied the effect of TLR2 stimulation on the suppressive function of human Treg populations and found that the suppressive activity of both naive and effector Tregs is inhibited by the TLR2 ligand Pam3Cys, leading to increased responder T cell proliferation. We provide evidence that T cell-derived IL-6 contributes to such effect and that reduced suppressive activity is observed in association with a shift of Treg populations into a “Th17-like” phenotype marked by increased expression of RORC and decreased expression of FOXP3 (Fig. 10).
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FIGURE 10. Model of reduced suppressive function of Tregs and Th17 development after TLR2 stimulation. This model emphasizes the role of TLR2 in 1) the differentiation of Tregs toward a Th17-like phenotype (dotted arrow) with reduced suppressive function and 2) the production of IL-6 leading to inhibition of the suppressive functions of Tregs (solid line).

Our finding that the TLR1/2 agonist Pam3Cys reduces the suppressive functions of Tregs is in agreement with two previous findings in murine (4, 11) and, most recently, human (10) cells. In the mouse, Summler et al. and Liu et al. independently demonstrated that TLR2 ligation leads to reduced Treg suppressive function and proliferative expansion of TLR2-stimulated Tregs (4, 11). Controversy remained, however, as Chen et al. (39) found that TLR2 stimulation of murine Tregs did not reduce their suppressive function but enhanced their survival by inducing the antiapoptotic protein Bcl-xL. In human Tregs, a mixture of synthetic lipopeptides including Pam3Cys, FSL-1, and Pam2Cys enhanced AKT phosphorylation and reduced Treg suppressive function (10). Human Tregs have also been reported to respond to HSP60 in a TLR2-dependent manner, leading to an increase in their suppressive function, mediated by cell–cell contact mechanisms and the secretion of TGF-β and IL-10 (12). In agreement with Oberg et al. (10), we found that pretreatment of Tregs before coculture with Tresps significantly affected Treg functions, whereas pretreatment of Tresps before cocultures with Tregs was not effective. The observation that, in contrast to the TLR1/2 agonist Pam3Cys, the TLR2/6 agonist FSL-1 did not significantly affect Treg function is in partial agreement with the cited Oberg et al. study (10). In contrast to their study, however, we found little variability in the response of different normal subjects to TLR1/2 and TLR2/6 stimulation and were thus able to study the biological effects of individual TLR2 agonists instead of using them as mixtures. Predominant effects of TLR2 agonists on Tregs are also consistent with the significantly higher expression of TLR2 and its heterodimeric partner TLR1 on Treg populations compared with that on Tresps. We also wanted to compare the potency of highly purified (≥98%) CD4+CD25hiTregs (FACS-sorted as either CD4+CD25hiCD127neg or CD4+CD45RA−CD25++ and CD4+CD45RA−CD25+++ with magnetic bead-purified, CD4+CD25+nu cells (≥95% pure) in suppressing CD4+CD25neg responder cells. Although bead-purified Tregs are known to contain nonsuppressive T cells (40), the degree of suppression exerted by bead-purified and by FACS-sorted CD4+CD25hiCD127neg Treg populations was comparable. Differences between our findings and those reported by Oberg et al. (10) on the one hand and Zanin-Zhorov et al. (12) on the other may be due to methodological differences, including the use of different TLR2 stimuli. Neither previous study specifically focused on the role of Th17-type cytokines in response to TLR2 stimulation.

Multiple mechanisms for how Tregs suppress Tresps have been reported (41). Among cytokine mediators of Treg suppression, TGF-β and IL-10 have been well characterized and shown to inhibit proliferation and/or cytokine production by effector/responder T cells. Membrane-bound TGF-β can mediate Treg suppression in a cell contact-dependent fashion, which can be reversed by high concentrations of anti-TGF-β (42, 43). In view of the inhibitory function of TGF-β, its increased expression by Tregs (and, to a lesser extent, Tresps) in response to TLR2 stimulation was unexpected. Because such upregulation of TGF-β was accompanied by an increase in IL-6, we hypothesized that the simultaneous induction of IL-6 and TGF-β might be involved in driving Tregs to a Th17 phenotype. This is consistent with current models whereby IL-6, in combination with TGF-β, can drive Th17 cell differentiation in both mouse (25) and (with IL-1β and IL-23) human (44) T cells. Moreover, a fraction of human CD45RA−FOXP3− memory Tregs has been reported to constitutively express the Th17 lineage-specific transcription factor RORC and to secrete IL-17 (29).

We found that in the presence of TCR activation, Pam3Cys induced the production of IL-17A, IL-17F, and IL-22 in CD4+ T cells, with potency comparable with a Th17 differenti-
The observed shift toward IL-17 production in both naïve (Fig. 7) and effector Treggs (Fig. 8) cocultured with Tresps in the presence of Pan3Cys suggests that a drive toward a Th17-like phenotype is predominant over the expansion of preexisting, small populations of IL-17+FOXP3+ Tregs. Such a TLR2-driven Th17 conversion would be consistent with recent observations in murine Tregs (24). The fact that IL-6 neutralization abrogated TLR2-induced inhibition of Tregs indicates a mechanistic role for this cytokine in mediating TLR2 effects and is consistent with its known inhibitory function on Treg suppressive activity (27). Of note, because our experimental paradigm does not include APCs, it is T-cell-derived IL-6 that directly or indirectly influences Tregs, and possibly Tresps, function (45). Further studies will be required to establish the relative importance of autocrine and paracrine effects of IL-6 and the possible involvement of IL-6/IL-6R "trans-signaling" (46). Similar to the effect of anti–IL-6, and perhaps more surprisingly, neutralization of IL-17 also blocked TLR2-induced reduction of Treg function and the induction of its own intracellular accumulation and the production of IL-22. Whether IL-17 directly and principally acts on Treg or Tresps populations remains to be established. In our experimental system, Tregs may differentiate into a Th17-like phenotype with reduced suppressive function but may also produce sufficient IL-6, IL-17, and IL-22 in response to TLR2 stimulation to abrogate the function of other Tregs (Fig. 10). Together, our data suggest a mechanistic link between loss of Treg suppressive activity and the phenotypic change to a Th17-like profile.

We conclude that TLR2 regulates Treg function by inducing Tregs to produce and secrete IL-6, which acts on Tregs to reduce their suppressive function, and by promoting an IL-17–secreting phenotypic shift, which may well be reversible as suggested by others (14). TLR2 activation by pathogens may also expand pre-existing circulating memory-like IL-17+FOXP3+ Tregs (29). We can speculate that such mechanisms would enable populations of circulating Tregs to adapt their functions to more effective responses to infections. In case of chronic activation of these pathways, one could envision an increased risk of autoimmunity.

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Disclosures

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References


