Targeting of TLRs Inhibits CD4\(^+\) Regulatory T Cell Function and Activates Lymphocytes in Human Peripheral Blood Mononuclear Cells

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Targeting of TLRs Inhibits CD4+ Regulatory T Cell Function and Activates Lymphocytes in Human Peripheral Blood Mononuclear Cells

Kui Shin Voo,* Laura Bover,*† Megan Lundell Harline,*† Jinsheng Weng,† Naoshi Sugimoto,‡ and Yong-Jun Liu‡

Accumulating evidence suggests elements within tumors induce exhaustion of effector T cells and infiltration of immunosuppressive regulatory T cells (Tregs), thus preventing the development of durable antitumor immunity. Therefore, the discovery of agents that simultaneously block Treg suppressive function and reinvigorate effector function of lymphocytes is key to the development of effective cancer immunotherapy. Previous studies have shown that TLR ligands (TLRLs) could modulate the function of these T cell targets; however, those studies relied on cell-free or accessory cell-based assay systems that do not accurately reflect in vivo responses. In contrast, we used a human PBMC-based proliferation assay system to simultaneously monitor the effect of TLRLs on T cells (CD4+, CD8+, Tregs), B cells, and NK cells, which gave different and even conflicting results. We found that the TLR7/8L:CL097 could simultaneously activate CD8+ T cells, B cells, and NK cells plus block Treg suppression of T cells and B cells. The TLRLs TL1R1L:Pal, C5K4, TL1R5L:flagellin, TL1R4L:LP, and TL1R8L/TL1C:CL075 also blocked Treg suppression of CD4+ or CD8+ T cell proliferation, but not B cell proliferation. Besides CL097, TL2RL:PGN, CL075, and TL9RL:CpG-A, CpG-B, and CpG-C) were strong activators of NK cells. Importantly, we found that Pal,C5K4 could: 1) activate CD8+ T cell proliferation, 2) inhibit the expansion of IL-10+ naturally occurring FOXP3+ Tregs and induction of IL-10+ CD4+ Tregs (IL-10-producing type 1 Treg), and 3) block naturally occurring FOXP3+ Tregs suppressive function. Our results suggest these agents could serve as adjuvants to enhance the efficacy of current immunotherapeutic strategies in cancer patients.

Evidence from the literature suggests that these suppressive elements within the tumor microenvironment can be modulated by triggering signals from members of the TLR family (9, 10). TLRs belong to a family of conserved pattern recognition receptors that recognize unique molecular structures of pathogens to distinguish “infectious non-self” from “self” Ags (11), allowing them to sense and initiate innate and adaptive immune responses. To date, 10 functional TLRs have been identified in humans with 9 known agonists (TLRL1-9) (12). These TLRs are expressed by APCs, tumor cells, and both Tiffs and Tregs (13–15). Recent studies using TLR agonists have shown that certain types of TLRs, expressed on different cell types, display alternate functions. For instance: 1) on T cells, they function as costimulatory receptors to enhance TCR-induced Tiff proliferation, survival, and cytokine production (16); 2) on suppressive Tregs, they can function to block Tiff function (10, 17); and 3) on APCs, they induce autocrine maturation and secrete proinflammatory cytokines leading to the modulation of Tiff and Treg function (18). Although these studies identified TLR ligands (TLRLs) that can reinvigorate Tiff function and block Treg suppressive function, they showed conflicting results, probably because they relied on cell-free (plate-bound or beads conjugated with anti-CD3) or accessory cell-based experimental systems (soluble anti-CD3 plus monocytes, dendritic cells [DCs], or CD3-depleted PBMCs) that do not necessarily reflect the in vivo response. For instance, by using a DC-based proliferation system, Peng et al. (17) reported that only CpG-A could block Treg suppressive function, whereas other TLRLs had no effect. In contrast, by using a cell-free proliferation system, Nyirenda and colleagues (10) showed that a TL2L ligand blocked Treg function. Because responder T cells are likely to interact with different T cell subtypes and with APCs in vivo, we believe that the use of whole PBMCs, which contain most cell types found in vivo (CD4+, CD8+, γδ T cells, CD4+Tregs, CD8+Tregs, Th17...
cells, monocytes, myeloid DCs, and plasmacytoid DCs, among others), would result in mimicking the in vivo responses after TLRL stimulation.

In this study, we used PBMCs that contained all T cell subtypes and APCs as accessory cells for our proliferation/suppression assays (19). We found that five of the nine known TLRs (Pam3CSK4, LPS, flagellin, CL097, and CL075) were able to completely block nTreg suppression on CD4+ or CD8+ Teff cell proliferation. Analyzing the flagellin, CL097, and CL075) were able to completely block nTreg suppressive function. Our results suggest the potential use of these agents as adjuvants to enhance the efficacy of therapeutic vaccines and other immunotherapeutic strategies in cancer patients.

Materials and Methods

Reagents and cell lines

TLRLs—Pam3CSK4, lipoteichoic acid (LTA), peptidoglycan (PGN), polynosinic-polycytidylic acid [poly(I:C)], CL075, CL097, LPS-SM, flagellin, CpG-A (ODN2216), CpG-B (ODN2006), and CpG-C (ODN M362)—were purchased from Invivogen (San Diego, CA); PMA, ionomycin (ION), and brefeldin A were purchased from Sigma-Aldrich (St. Louis, MO). ELISA kits for IL-2, IL-10, TNF-α, and IL-6 and neutralizing Abs were purchased from R&D Systems (Minneapolis, MN). FCS and human serum were purchased from Gemini (Manhattan, NJ). CFSE was purchased from Invitrogen (Grand Island, NY).

PBMC-based proliferation/Treg suppression assays/cytokine production

Adult blood buffy coat samples from healthy donors were obtained from the Gulf Coast Regional Blood Center in Houston, TX (Human Research Protocol LAB-03-0390- MDACC). Human PBMCs were isolated from the adult blood buffy coats by Ficoll-Paque (GE Healthcare, Waukesha, WI) density gradient centrifugation according to manufacturer’s procedures. In brief, a diluted suspension of the buffy coat was layered over 15 ml Ficoll-Paque and centrifuged at 1100 × g for 15 min at 20°C without the brake. The mononuclear cell layer containing lymphocytes, monocytes, and thrombocytes was then transferred to a new 50-ml conical tube, filled with PBS, and centrifuged at 260 × g for 8 min. The cell pellet was washed one more time with PBS by spinning at 625 × g for 7 min. Cells were then resuspended with PBS and labeled with 3 μM CFSE. Next, 2.5 × 10^5 CFSE-labeled PBMCs were stimulated with soluble anti-CD3 (1 μg/ml) in T cell medium containing 10% human AB serum (Gemini) in RPMI 1640-GlutaMAX plus 1% penicillin-streptomycin. After 3.5 d of culturing, the PBMCs were stained with allophycocyanin-Cy7-CD4, Pacific Blue-CD8, and PE-CD19 Abs. B cells were identified by gating on CD4 CD8 CD19+ cells. To determine the Treg suppressive activity, 2.5 × 10^5 CFSE-labeled PBMCs were stimulated with soluble anti-CD3 (1 μg/ml) in the presence of autologous 1 × 10^5 CD4+CD25-CD127low/nTregs in T cell medium. Proliferation of CD4+ T, CD8+ T, and B cells was monitored by CFSE dilution assessed by flow cytometry. For evaluation of cytokine production, 2.5 × 10^5 PBMCs were stimulated with soluble anti-CD3 (1 μg/ml) for 3.5 d. Supernatants were collected and analyzed for IL-10, IL-6, IFN-γ, and TNF-α production.

Screening of TLRLs that block nTreg suppression of lymphocytes

TLRLs were screened using PBMC-based proliferation/suppression assays. The following TLRLs were tested at four different concentrations for T cell proliferation according to the manufacturer’s specification and our preliminary data: TLR1/2L:Pam3CSK4 (2, 10, 50, 100 ng/ml); TLR2L:PGN (0.5, 1, 5, 10 μg/ml) and LTA (0.1, 0.25, 0.5, 1.0 μg/ml); TLR3L:poly(I:C) (0.1, 0.5, 1, 5 μg/ml); TLR4L:LPS-SM (0.25, 1, 2.5, 5 μg/ml); TLR5L:flagellin (ultrapure, 2, 20, 50, 100 ng/ml); TLR7/8L:FLS (2, 10, 50, 100 ng/ml); TLR7/8L:CL075 and CL097 (0.1, 0.5, 1, 5 μg/ml); TLR9L:CpG-A (ODN2216), CpG-B (ODN2006), and CpG-C (ODN M362: 0.25, 1, 2.5, 5.0 μM). After 3–4 d in culture, the ability of the added TLRLs to enhance T cell proliferation or reverse Treg suppression was analyzed based on the proliferation of CD4 or CD8 T cells by CFSE dilution assays. The following TLRLs were tested at three concentrations for CD4+ and CD8+ T cells and B cell proliferation in the presence of nTregs:Pam3CSK4 (10, 50, 100 ng/ml); PGN (1, 5, 10 μg/ml);
nTregs (CD4+CD25highCD127low [top 4–7%]). NK cells were enriched (0.25, 1.0, 2.5 and CpG-A (ODN2216), CpG-B (ODN2006), and CpG-C (ODN M362) (BD Immunocytometry Systems, San Jose, CA). CD4+FITC lineage negative CA; FITC-CD303). Stained cells were sorted on a FACSAria cell sorter.
gd mixture Abs against CD11c, CD14, CD16, CD19, CD56, CD303, and CD45RA, allophycocyanin-CD45RO Abs, and FITC-labeled lineage allophycocyanin-Cy7-CD4, PE-Cy7-CD25, PE-CD127, PerCP-Cy5.5-TCR (BD Biosciences, San Jose, CA, and Miltenyi Biotec, Auburn, CA; FITC-TCR). Freshly sorted 1 × 10^5 T cells (CD4 naive, CD4 memory, CD4 nTregs, CD3+CD45RA+CD27+ CD8) were stimulated with plate-bound anti-CD3 and activate NK cells. Consistently block nTreg suppression of lymphocytes and activate NK cells. Poly(I:C) (0.1, 0.5, 1.0 μg/ml); flagellin (20, 50, 100 ng/ml); FSL (10, 50, 100 ng/ml); and CpG-A (ODN2216), CpG-B (ODN2006), and CpG-C (ODN M362) (0.25, 1.0, 2.5 μM).

**Purification of T cell subsets and NK cells**

CD4+ T cells were enriched from peripheral blood buffy coat samples using a CD4 T cell isolation kit (Stemcell Technologies, Vancouver, B.C.) according to manufacturer’s procedures. CD4+ T cells were stained with allophycocyanin-Cy7-CD4, PE-Cy7-CD25, PE-CD127, PerCP-Cy5.5-CD45RA, allophycocyanin-CD45RO Abs, and FITC-labeled lineage mixture Abs against CD11c, CD14, CD16, CD19, CD56, CD303, and γδ-TCR (BD Biosciences, San Jose, CA, and Miltenyi Biotec, Auburn, CA; FITC-TCR). Stimulated cells were sorted on a FACSaria cell sorter (BD Immunocytometry Systems, San Jose, CA). CD4+FITC lineage negative cells were sorted into three fractions: 1) naive T (CD4+CD25lowCD45RA+); 2) memory T (CD4+CD25lowCD45RA-); and 3) nTregs (CD4+CD25highCD127low [top 4–7%]).

**Table I. Summary of TLRLs that enhance T and B cell proliferation, block nTreg function, and activate NK cells in two different proliferation systems**

<table>
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<th>CD8+ Proliferation</th>
<th>B Proliferation</th>
<th>PBMC System</th>
<th>Three Components System</th>
<th>Blocking Treg/CD4+ Suppression</th>
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**FIGURE 2.** Identification of TLRLs that consistently block nTreg suppression of lymphocytes and activate NK cells. (A-C) Relative proliferation of CD4+ and CD8+ T cells and B cells under conditions described in Fig. 1 using three concentrations of TLRL described in Materials and Methods. Lymphocyte proliferation was assessed by flow cytometry for CFSE dilution. Data points for each TLRL were taken from one of the three TLRLs concentration tested that yielded the highest lymphocyte proliferation. Error bars represent means ± SEM. Statistical significance between treatment groups (p < 0.05 compared with responder cells plus nTregs) is indicated by brackets. The p values were calculated by paired t test. Data are representative of independent experiments using PBMCs derived from four to five donors. (D) Relative percentage of 69+ NK cells after 1-d culture of PBMCs (0.25 × 10^6) and autologous CFSE-labeled NK cells (1 × 10^6/well) in the presence or absence of a TLRL at different concentrations achieved by using four successive half dilutions: Pam, Clostridium, flagellin (400 to 50 ng/ml); poly(I:C) (5 to 0.63 μg/ml); and CpG-A, CpG-B, and CpG-C (5 to 0.63 μM); LPS, Clostridium, and flagellin (1 to 0.125 μg/ml). NK cell activation was evaluated by the expression of CD69 activation markers by flow cytometry analyses. Data are representative of two donors.
FOXP3+nTreg treatment with TLRs

Freshly sorted CD4+CD25highCD127low nTregs were cultured in T cell medium containing 5% human AB serum in the presence of each separate TLR ligand: Pam3CSK4, FSL, and flagellin (400 ng/ml); poly(I:C) (5 μg/ml); CpG-A, CpG-B, and CpG-C (2.5 μM); and LPS-SM, CL097, and CL075 (1 μg/ml) for 24 h. nTregs were then washed three times and cocultured with autologous CFSE (4 μM)-labeled PBMCs (1 × 10⁶ nTregs:2.5 × 10⁵ PBMCs) in the presence of 1.5 μg/ml anti-CD3 (OKT3). Proliferation of CD8+ T cells after 4 d of stimulation was monitored by CFSE dilution assessed by flow cytometry.

Stimulation of nTregs in the presence of Pam3CSK4 and flagellin

Freshly sorted 4 × 10⁶ nTregs were stimulated with plate-bound anti-CD3 (2 μg/ml) in T cell medium containing 10% FCS plus IL-2 (300 IU/ml) and soluble anti-CD28 (0.25 μg/ml) in a 24-well tissue culture plate in the presence or absence of 50 ng/ml Pam3CSK4 or flagellin for 7 d. Expanded nTregs were either stained with a FOXP3 Ab or restimulated with PMA (50 ng/ml) and ION (2 μg/ml) for 6 h. During the last 4 h, brefeldin A-protein trafficking blocker (10 μg/ml) was added. The cells were stained with IL-2, IFN-γ, TNF-α, or IL-10 Abs (eBioscience) using Caltag FIX and PERM kit (Invitrogen).

FIGURE 3. Identification of TLR ligands that enhance proliferation of CD4+ T, CD8+ T and B cells without activation through TCR. Proliferation of CFSE-labeled nonstimulated PBMCs in the absence or presence of single TLR ligands, at a fixed concentration. (A and B) T and (C) B cell proliferation was determined by CFSE dilution assessed by flow cytometry as described in Materials and Methods. Data are representative of experiments using PBMCs derived from six donors plotted individually. Brackets indicate significance compared with the control with no TLR ligand added.

Generation of Tr1 from CD4+ T cells

A total of 2 × 10⁶ freshly isolated CD4+ T cells were cultured with irradiated (60 Gy) ICOSL-expressing CD32-L cells (8 × 10⁶), which were plated for 2 h and precoated with anti-CD3 (0.2 μg/ml) for another hour, in the presence of dexamethasone (5 × 10⁻⁸ M) and 1α,25-dihydroxyvitamin D3 (1 × 10⁻¹⁷ M; Life Technologies, Carlsbad, CA) in T cell medium containing 10% FCS plus IL-2 (50 IU/ml) and soluble anti-CD28 (0.2 μg/ml) for 7 d in a 48-well tissue culture plate. Expanded T cells were restimulated with PMA/ION as described earlier. Intracellular staining was performed on cells with Alexa Fluor 647–IL-10 Ab (clone JES3-9D7; eBioscience) using Caltag FIX and PERM kit (Invitrogen).

FIGURE 4. Cytokine production by PBMCs activated through TCR stimulus. PBMCs were stimulated with soluble anti-CD3 mAb. Supernatants were collected after 3.5 d and analyzed for (A) IL-10, (B) IFN-γ, (C) IL-6, and (D) TNF-α production with R&D Systems ELISA kits for each cytokine. Error bars represent means ± SEM. Statistical significance between treatment groups (p < 0.05 compared with nontreated group, medium alone) is indicated by brackets. The p values were calculated by unpaired t test. Data are from PBMCs derived from 9–10 donors plotted individually.
Statistical analysis

Statistical difference between experimental groups was determined by paired or unpaired t test or two-way ANOVA test using Prism software (GraphPad Software).

Results

Identification of TLRLs that promote T cell proliferation, block nTreg function, and activate NK cells in a PBMC-based proliferation system

Responder T cells and CD4+FOXP3+ Tregs are likely to interact with different T cell subtypes and with APCs in vivo. Therefore, to evaluate the ability of TLRLs to enhance T cell proliferation and block Treg suppression function, we used a PBMC-based proliferation assay system (19), which contains most cell types found in vivo. First, we evaluated lymphocyte proliferation using CFSE-labeled PBMCs polyclonally stimulated with anti-CD3 mAb in the presence or absence of the detailed TLRL, each tested at four different concentrations. Then, to evaluate the ability of the TLRLs to block Treg suppression of the induced proliferation, syngeneic CD4+CD127low CD25high nTregs were added at a 1:1 (lymphocyte/nTreg) ratio under the same culture conditions. After 3.5 d of culture, the proliferation of CD4+ T, CD8+ T, and B cells was assessed by CFSE dilution, as described in Materials and Methods, and found to have undergone five to six rounds of cell division (Supplemental Fig. 1). In the absence of nTregs, TLR1/2L: Pam3CSK4, TLR4L:LPS, and TLR5L:flagellin were the most potent ligands to enhance TCR-mediated CD4+ T cell proliferation, whereas the remaining ligands tested were ineffective (Fig. 1A, upper panel, and Table I). We also found that TLR7/8L:CL097 potently stimulated CD8+ T cell proliferation in addition to Pam3CSK4, LPS, and flagellin (Fig. 1A, middle panel, and Table I). In contrast, only TLR7/8L:CL097 and TLR9Ls:CpG-B and CpG-C were able to potently stimulate B cell proliferation (Fig. 1A, lower panel). In the presence of autologous nTregs, however, we found that TLR2/6L:FSL and TLR7/8L:CL097 that did not enhance CD4+ T cell proliferation were able to block nTreg suppression of CD4+ T cell proliferation (Fig. 1B, upper panel, and Table I). When CD8+ T cell proliferation was evaluated, Pam3CSK4, LPS, and CL097 simultaneously exerted both functions of activation of proliferation and inhibition of suppression mediated by Tregs (Fig. 1B, middle panels, and Table I). We then expanded our studies to multiple donor PBMCs and found that TLR7/8L:CL097 could consistently block nTreg suppression of all CD4+ T and CD8+ T and B cell proliferation (Fig. 2A–C). Other TLRLs such as Pam3CSK4, flagellin, LPS, and TLR8/7L:CL075 only blocked Treg suppression of CD4+ T and CD8+ T cell proliferation (Fig. 2A, 2B, Table I) but did not affect B cell proliferation (Fig. 2C). Of note, we found that FSL could block nTreg suppression of CD4+, but not CD8+ T cell proliferation (Fig. 2A, 2B). Besides CL097, CpG-B and CpG-C were the most potent in blocking Treg suppression of B cell proliferation. Because NK cells were shown to kill tumor targets in vitro and in vivo (20), we sought to determine the effect of TLRLs in activating NK cells. CFSE-labeled, freshly sorted NK cells were added to autologous PBMCs and stimulated with a TLRL for 1 d. Afterward, NK cell activation was determined by the expression of CD69 on CFSE+ cells. Fig. 2D shows that PGN, CL097, CL075, and CpG-A, CpG-B, and CpG-C were strong activators of NK cells. Because a small percentage of lymphocytes is already activated in vivo, we sought to determine whether the responses to TLRLs by lymphocytes in a total PBMC proliferation system differed from lymphocytes in a three-component system, in which CD3-depleted PBMCs, used they need this stimulus to activate proliferation on CD8+ T cells (Fig. 3A, 3B). On the contrary, 6 of 11 TLRLs increased B cell proliferation in the absence of anti-CD3 (Fig. 3B), compared with only three active TLRLs when PBMCs were polyclonal activated. We next sought to evaluate the proinflammatory and anti-inflammatory cytokines that are induced by TLRLs in the polyclonal activated TCR PBMC-based system. We found that the majority of the 11 TLRLs could induce anti-inflammatory IL-10 secretion except Pam3CSK4, FSL, poly(I:C), and flagellin (Fig. 4A). Of the three proinflammatory cytokines tested, only IL-6 and TNF-α responded to TLRL stimulation (Fig. 4B–D). We found that CpG-A, CpG-B, and CpG-C, and poly(I:C) did not induce IL-6 and TNF-α (Fig. 4C, 4D). In addition, flagellin and CL097 also failed to induce TNF-α. Taken together, these results suggest that Pam3CSK4, FSL, and flagellin are better agonists that only induce proinflammatory cytokines.

Differential response to TLRLs by lymphocytes in PBMCs versus a three-component proliferation system

Because of conflicting results reported in the literature that used a non–PBMC-based proliferation assay system (10, 17), we sought to determine whether the responses to TLRLs by lymphocytes in a total PBMC proliferation system differed from lymphocytes in a three-component system, in which CD3-depleted PBMCs, used...
as APCs, CFSE-labeled CD4\(^+\) memory Teffs, used as responders, and nTregs were cocultured together. Experiments were performed as previously described, and all three cell components were isolated from the same donor. Interestingly, we found that Pam\(_3\)CSK4, PGN, flagellin, and CpG-A potently inhibited nTreg suppression of CD4\(^+\) T cell proliferation, whereas CL097 and CL075 were ineffective (Fig. 5, Table I). These conditions gave different results from our PBMC-based proliferation assay system where CL097 and CL075 were effective, but PGN and CpG-A were ineffective, suggesting that some missing component(s) of the total PBMC system might differentially modulate the lymphocyte response to TLRL.

**Pam\(_3\)CSK4 and flagellin act directly on T cells to promote proliferation**

Because the majority of TLRs, with the exception of TLR9, are expressed on T cells (14, 21, 22), we asked whether these ligands could act directly on T cell subtypes to enhance TCR-mediated proliferation. Freshly sorted, naive CD4\(^+\) and CD8\(^+\) T cells were stimulated with anti-CD3 in the absence or presence of single TLRLs. After 4 d of culture, the proliferation of T cells was monitored by CFSE dilution. From the three donors of T cells tested, and in the absence of IL-2, the TLRLs Pam\(_3\)CSK4, FSL, LPS, and flagellin were able to enhance anti-CD3-stimulated proliferation of naive CD4\(^+\) T cells but not CD8\(^+\) T cells (Fig. 6A, 6B). Taking into account that Pam\(_3\)CSK4 and flagellin lack the undesirable septic shock adverse effects associated with LPS in vivo (23) and have the ability to consistently block nTreg function (Table I), we next extended our studies to naive, memory, and regulatory CD4\(^+\) T cell subsets. Fig. 6C, 6D show that, in the absence of IL-2, Pam\(_3\)CSK4 and flagellin could enhance TCR-mediated proliferation of naive and memory CD4\(^+\) T cells in a dose-dependent manner, but did not enhance nTreg proliferation (data not shown); meanwhile, CpG-A and CpG-B had no effect on the tested cells. However, in the presence of a high IL-2, Pam\(_3\)CSK4 and flagellin were able to enhance nTreg proliferation, whereas CpG-B decreased it (Fig. 6E). These results suggest that anti-CD3 stimulation is sufficient for Pam\(_3\)CSK4 and flagellin to enhance CD4\(^+\) T cell proliferation directly without the presence of APCs. In contrast, in concert with TCR stimulation, the TLR9 ligands CpG-A and CpG-B inhibited CD4\(^+\) T cell and nTreg proliferation in a dose-dependent manner.

**Pam\(_3\)CSK4 acts directly on nTregs to block suppressive activity**

To evaluate whether TLRLs act directly on nTregs to inhibit their suppressive activity, we treated nTregs with each TLRL for 24 h, washed out, and then cultured them with autologous CFSE-labeled PBMCs in the presence of anti-CD3. After 4 d of culture, CD8\(^+\) T cell proliferation was monitored by CFSE dilution assessed by flow cytometry. We found that only Pam\(_3\)CSK4 could consistently inhibit nTreg suppressive activity on CD8\(^+\) T cell proliferation, whereas the eight other TLRLs tested were ineffective (Fig. 7A). To investigate the mechanisms mediated by Pam\(_3\)CSK4 in reversing nTreg function, we stimulated nTregs with anti-CD3 and anti-CD28 with a high amount of IL-2 and in the presence or absence of the TLRL for 7 d. The nTregs were either stained for the Treg-specific marker FOXP3 or restimulated with PMA/ION to detect IL-2 and IL-10 cytokine expression. We found that treatment of nTregs with either Pam\(_3\)CSK4 or control flagellin did not result in a significant change in the percentage FOXP3\(^+\) cells (Fig. 7B). However, in the absence of IL-2, Pam\(_3\)CSK4 and flagellin could enhance TCR-mediated proliferation of naive and memory CD4\(^+\) T cells in a dose-dependent manner, but did not enhance nTreg proliferation (data not shown); meanwhile, CpG-A and CpG-B had no effect on the tested cells. However, in the presence of a high IL-2, Pam\(_3\)CSK4 and flagellin were able to enhance nTreg proliferation, whereas CpG-B decreased it (Fig. 6E). These results suggest that anti-CD3 stimulation is sufficient for Pam\(_3\)CSK4 and flagellin to enhance CD4\(^+\) T cell proliferation directly without the presence of APCs. In contrast, in concert with TCR stimulation, the TLR9 ligands CpG-A and CpG-B inhibited CD4\(^+\) T cell and nTreg proliferation in a dose-dependent manner.
Representative flow cytometry analyses are shown to evaluate the expression of FOXP3 (Pam3CSK4 because flagellin was ineffective. We note that the high induction of Tr1 from total CD4+ T cells. We stimulated CD4+ for 7 d as described in Materials and Methods. Error bars represent means ± SEM. Statistical significance between treatment groups (p < 0.05 compared with no treatment) is indicated by brackets. The p values were calculated by paired t test. (B and C) Freshly sorted nTregs were stimulated with anti-CD3 and anti-CD28 plus a high concentration of IL-2 in the presence or absence of Pam3CSK4 or flagellin for 7 d as described in Materials and Methods. Intracellular staining was then performed to evaluate the expression of FOXP3 (B) or IL-2 and IL-10 (C). Representative flow cytometry analyses are shown on upper panels. Data were taken from four donors. (D) Total CD4+ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of vitamin D3, dexamethasone, and ICOSL/CD32L cells. Each TLRL was tested at given increasing concentrations: Pam3CSK4 and flagellin (0, 50, 100, 500, 1000 ng/ml), and CpG-A (0, 0.5, 1, 2.5, 5 μM). Numbers 1 and 5 on x-axis indicate lowest and highest concentration, respectively. After 7 d, T cells were restimulated with PMA/ION and cells were subjected to intracellular staining for IL-10 expression. Results are from two independent experiments, with SD of the mean shown as error bars. Statistical significance between treatment groups (p < 0.05 compared with flagellin) is indicated by brackets. The p values were calculated by two-way ANOVA test.

FIGURE 7. Pam3CSK4 directly inhibits nTreg function and shuts down IL-10-producing Tregs. (A) Loss of nTreg function after Pam3CSK4 treatment. Freshly sorted nTregs were cultured in the presence of a TLRL, 9 tested, for 24 h, washed, and then cocultured with autologous CFSE-labeled PBMCs (1:2.5, nTregs:PBMCs) in the presence of 1.5 μg/ml OKT3. CD8+ T cell proliferation was determined by CFSE dilution assessed by flow cytometry as described in Materials and Methods. Statistical significance between treatment groups (p < 0.05 compared with no treatment) is indicated by brackets. The p values were calculated by paired t test. (B and C) Freshly sorted nTregs were stimulated with anti-CD3 and anti-CD28 plus a high concentration of IL-2 in the presence or absence of Pam3CSK4 or flagellin for 7 d as described in Materials and Methods. Intracellular staining was then performed to evaluate the expression of FOXP3 (B) or IL-2 and IL-10 (C). Representative flow cytometry analyses are shown on upper panels. Data were taken from four donors. (D) Total CD4+ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of vitamin D3, dexamethasone, and ICOSL/CD32L cells. Each TLRL was tested at given increasing concentrations: Pam3CSK4 and flagellin (0, 50, 100, 500, 1000 ng/ml), and CpG-A (0, 0.5, 1, 2.5, 5 μM). Numbers 1 and 5 on x-axis indicate lowest and highest concentration, respectively. After 7 d, T cells were restimulated with PMA/ION and cells were subjected to intracellular staining for IL-10 expression. Results are from two independent experiments, with SD of the mean shown as error bars. Statistical significance between treatment groups (p < 0.05 compared with flagellin) is indicated by brackets. The p values were calculated by two-way ANOVA test.

Discussion

A variety of in vitro proliferation assay systems have been used to study the effect of TLRLs in blocking human CD4+ Treg suppression of Teff proliferation. Some systems involve the stimulation of Teffs and CD4+ Tregs with soluble anti-CD3 in the presence of accessory cells such as DCs (17) or CD3-depleted PBMCs (24), whereas other systems include the stimulation of T cells without accessory cells but in the presence of anti-CD3/CD28-coupled beads or plate-bound anti-CD3 plus soluble anti-CD28 (21). Conflicting results have often been reported, probably because of the use of these different in vitro proliferation assays. Therefore, to clarify these ambiguous results, we believe that the use of a human total PBMC culture system would better resemble the lymphocyte environment in vivo and yield findings relevant to the clinic. In fact, by using this PBMC-based proliferation system, we found that six TLR-specific ligands, TLR1/2:L.Pam3CSK4, TLR4:L.PPS, TLR5:flagellin, TLR7/8:CL097, TLR8/7:CL075, and TLR9:L.Cpg-B or Cpg-C, potent inhibits Treg suppression of either CD4+ T or CD8+ T cell, or B cell proliferation, and, in the case of CL097, the three cell types simultaneously. The most remarkable difference was that LPS, CL097, and CL075 were able to block nTreg suppression of CD4+ T cell proliferation in the PBMC-based assay system, but not in the CD3-depleted PBMC-based (three components) assay system. Conversely, PGN and Cpg-A were able to block nTreg suppression of CD4+ T cell proliferation only in the CD3-depleted PBMC assay system. Noteworthy, although CL097 failed to enhance CD4+ T cell proliferation in the PBMC-assay system, in the presence of nTregs, it was able to completely abrogate nTreg suppression of CD4+ and CD8+ T cells and B cells. We made attempts to evaluate the ability of TLRs to enhance T cell proliferation in the presence of CMV-specific peptides or attenuated flu virus. Our results suggest that the effects varied wildly among donors (Supplemental Figs. 3 and 4). We reasoned that the unpredictable effect could be because of the presence of a significant percentage of activated T cells in PBMCs that responded to TLRL stimulation in the absence of CMV peptides or flu Ags. Nonetheless, taken together, our results suggest that the cell environment where T cells and nTregs become activated determines whether a certain TLRL is able to block nTreg function.

Our results suggest that Pam3CSK4 could inhibit human Treg function by multiple mechanisms. We found that Pam3CSK4 could: 1) block induction of IL-10+Tr1 cells from CD4+ T cells, 2) directly inhibit expansion of IL-10+nTregs, and 3) directly inhibit nTreg suppression of CD8+ T cell proliferation. These findings are consistent with our inability to induce IL-10 cytokine secretion from PBMCs, and a recent report from Nyirenda and colleagues (10) showing that stimulation by Pam3CSK4 drives human naive and effector nTregs into a Th17-like phenotype with reduced suppressive activity on CD4+CD25+FOXP3+CD45RA+ responder T cell proliferation. However, these results are in stark contrast with a recent murine study in which Pam3CSK4 potently promoted expansion of IL-10-producing T cells (25), suggesting species-specific responses to Pam3CSK4. Interestingly, we further showed that Pam3CSK4 could directly enhance proliferation of naive and memory CD4+ T cells, but not nTregs, in a dose-dependent manner in the absence of IL-2, a likely scenario in the tumor microenvironment. Furthermore, both Pam3CSK4 and flagellin could simultaneously stimulate the release of IL-6- and/or TNF-α cytokines from PBMCs. Together, these results suggest that Pam3CSK4 or its derivatives might be useful to reinvigorate Teff function in humans.

Collectively, we have identified six TLR-specific ligands, Pam3CSK4, LPS, flagellin, CL097, CL075, and Cpg-B or Cpg-C.
that inhibit Treg suppression of either CD4+ T or CD8+ T cell, or B cell proliferation. Our results suggest that these agents could serve as adjuvants to enhance the efficacy of current immunotherapeutic strategies in cancer patients. However, because of the different cell types present in the tumor microenvironment, one TLR agonist may trigger either positive or negative signals from different cells, making difficult to predict what the outcome might be in patients receiving TLRL adjuvant treatment. Therefore, further studies are needed to determine which TLR agonists, whether used alone or in combination with other reagents, are best suitable for stimulating long-term beneficial immune responses in the tumor environment, which could potentially lead to improvement of the cancer treatment.

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References