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*J Immunol* published online 13 June 2014
http://www.jimmunol.org/content/early/2014/06/13/jimmunol.1203334

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/06/13/jimmunol.1203334.4.DCSupplemental

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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 TLR1L:LPS, TLR2L:PGN, TLR5L:flagellin, TLR4L:LPS, and TLR8L:CL075 also blocked Treg suppression of CD4+ or CD8+ T cell proliferation, but not B cell proliferation. Besides CL079, TLR2L:PGN, CL075, and TLR9L:CpG-A, CpG-B, and CpG-C) were strong activators of NK cells. Importantly, we found that Pam3CSK4 could: 1) activate CD4+ 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 Tregs), 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. The Journal of Immunology, 2014, 193: 000–000.

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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 (TLR1L-9) (12). These TLRs are expressed by APCs, tumor cells, and both Teffs and Tregs (13–15). Recent studies using TLR agonists have shown that certain types of TLRs, expressed on different cells, display alternate functions. For instance: 1) on T cells, they function as costimulatory receptors to enhance TCR-induced Teff proliferation, survival, and cytokine production (16); 2) on suppressive Tregs, they can function to block Treg function (10, 17); and 3) on APCs, they induce autocrine maturation and secrete proinflammatory cytokines leading to the modulation of Teff and Treg function (18). Although these studies identified TLR ligands (TLRLs) that can reinvigorate Teff 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 TLR2 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 TLRLs (Pam3CSK4, LPS, flagellin, CL097, and CL075) were able to completely block nTreg suppression on CD4+ or CD8+ T eff cell proliferation. Analyzing the flagellin, CL097, and CL075) were able to completely block nTreg suppression on CD4+ or CD8+ T eff cell proliferation. Analyzing the complete data set, we found that the TLR7/8L:CL097 could simultaneously activate CD8+ T cells, B cells, and NK cells plus block Treg suppression on CD4+/CD8+ T and B cell proliferation. Furthermore, we found that TLR1/2L:Pam3CSK4 could work directly to: 1) stimulate CD4+ T cell proliferation, 2) inhibit the expansion of IL-10+ nTregs, 3) block the induction of IL-10+CD4+ Tregs (IL-10–producing type 1 Treg [Tr1]) from total CD4+ T cells; and 4) 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), polyinosinic-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), CpG-A (ODN2216), CpG-B (ODN2006), and CpG-C (ODN M362) were purchased from Invivogen (San Diego, CA). FMA, 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 against IL-2 and IL-6, and normal goat IgG control 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. After the spin, the cell pellet was resuspended, diluted with PBS, and centrifuged at 180 × 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 (Gemi) in RPMI 1640-GluMaX 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+CD25highCD127low nTregs in T cell medium. Proliferation of CD4+ T, CD8+ T, and B cells was monitored by CFSE dilution and cytokine production 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, 1.0, 2.5, 5.0 μg/ml); TLR5L:flagellin (ultrapure, 2, 20, 100 μg/ml); TLR2/6L:FSL (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, 1.0, 2.5, 5.0 μM). After 3–4 d in culture, the ability of the added TLRL to enhance T cell proliferation or reverse Treg suppression was analyzed based on the proliferation of CD4+ or CD8+ T cells and B cell proliferation in the presence of nTregs:Pam3CSK4 (10, 50, 100 ng/ml); PGN (1, 5, 10 μg/ml);}

FIGURE 1. Identification of TLRLs that enhance proliferation and/or block nTreg suppression of lymphocytes within PBMCs. (A) Proliferation of CD4+ and CD8+ T cells and B cells after 3.5-d culture of CFSE-labeled PBMCs stimulated with soluble anti-CD3 in the absence or presence of a TLRL; four concentrations were tested as described in Materials and Methods. White color bars and dark color bars indicate lowest and highest concentration, respectively. T and B cell proliferation were determined by CFSE dilution assessed by flow cytometry as described in Materials and Methods. (B) Proliferation of CD4+ and CD8+ T cells and B cells under culture conditions described in (A) with autologous CD4+ CD25highCD127+ nTregs added to the PBMCs at a 1:1 ratio of nTregs to effector cells (assuming that lymphocytes constitute ~40% of total PBMCs). Data are representative of independent experiments using PBMCs derived from n = 3 (Fig. 2A), n = 4 (Fig. 2B), and n = 5 (Fig. 2C).
poly(I:C) (0.1, 0.5, 1.0 µg/ml); LPS-SM (0.1, 0.25, 1.0 µg/ml); flagellin (20, 50, 100 ng/ml); FSL (10, 50, 100 ng/ml); CL097 (0.1, 0.5, 1.0 µg/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, PerCP-Cy5.5-CD11c, CD14, CD19, CD11c, CD56, CD303, and CD45RA, allophycocyanin-CD45RO Abs, and FITC-labeled lineage mixtures Abs against CD11c, CD14, CD16, CD19, CD56, CD303, and γδ-TCR (BD Biosciences, San Jose, CA, and Miltenyi Biotec, Auburn, CA; FITC-CD19; PE-CD127, PerCP-Cy5.5-TCR (BD Biosciences, San Jose, CA, and Miltenyi Biotec, Auburn, CA; FITC-CD3). Purification of T cell subsets and NK cells was achieved using EasySep human NK cell enrichment kit (Stemcell Technologies) according to manufacturer’s protocols and stained with allophycocyanin-CD56, PE-CD16, Pacific Blue-CD3 Abs, and FITC-labeled lineage mixture Abs against CD14, CD19, CD11c, and γδ-TCR. Pure CD4+ CD19- CD11c- γδ-TCR- CD3+ CD56+CD16+ NK cells were obtained by sorting on a FACSAria.

**Screening of TLRs that directly enhance T cell proliferation**

Freshly sorted 1 × 10^5 T cells (CD4 naive, CD4 memory, CD4 nTregs, CD3+CD45RA-CD27+ CD8) were stimulated with plate-bound anti-CD3 (CD4 [2 µg/ml], CD8 [1 µg/ml]) in T cell medium in the absence or presence of single TLRs. The following TLRs were tested at five concentrations: Pam3CSK4, FSL, and flagellin (400, 200, 100, 25 ng/ml); poly(I:C) (5, 2.5, 1.25, 0.62, 0.315 µg/ml) and CpG-A, CpG-B, and CpG-C (5, 2.5, 1.25, 0.62, 0.315 µM); and LPS-SM, CL097, and CL075 (1, 0.5, 0.25, 0.125, 0.063 µg/ml). [3H]thymidine was added on the third day of culture, and cells were harvested after another 15 h of incubation. Proliferation of T cells was evaluated by thymidine incorporation.

**FIGURE 2.** Identification of TLRs 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 TLRs described in Materials and Methods. Lymphocyte proliferation was assessed by flow cytometry for CFSE dilution. Data points for each TLR were taken from one of the three TLRs 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 × 10^5/well) in the presence or absence of a TLR at different concentrations achieved by using four successive half dilutions: Pam3CSK4, FSL, and 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, CL097, and CL075 (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\textsuperscript{+} nTreg treatment with TLRLs

Freshly sorted CD4\textsuperscript{+}CD25\textsuperscript{high}CD127\textsuperscript{low} nTregs were cultured in T cell medium containing 5% human AB serum in the presence of each separate TLRL tested: Pam3CSK4, FSL, and flagellin (400 ng/ml); poly(I:C) (5 mg/ml); and LPS-SM, CL097, and CL075 (1 mg/ml) for 24 h. nTregs were then washed three times and cocultured with autologous CFSE (4 mM)-labeled PBMCs (1 \times 10^5 nTregs:2.5 \times 10^5 PBMCs) in the presence of 1.5 mg/ml anti-CD3 (OKT3). Proliferation of CD8\textsuperscript{+} T cells after 4 d of stimulation was monitored by CFSE dilution assessed by flow cytometry.

Stimulation of nTregs in the presence of Pam\textsubscript{3}CSK4 and flagellin

Freshly sorted 4 \times 10^5 nTregs were stimulated with plate-bound anti-CD3 (2 \mu g/ml) in T cell medium containing 10% FCS plus IL-2 (300 IU/ml) and soluble anti-CD28 (0.25 \mu g/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 \mu g/ml) for 6 h. During the last 4 h, brefeldin A-protein trafficking blocker (10 \mu g/ml) was added. The cells were stained with IL-2, IFN-\gamma, TNF-\alpha, or IL-10 Abs (eBioscience) using Caltag FIX and PERM kit (Invitrogen).

Generation of Tr1 from CD4\textsuperscript{+} T cells

A total of 2 \times 10^6 freshly isolated CD4\textsuperscript{+} T cells were cultured with irradiated (60 Gy) ICOSL-expressing CD32-L cells (8 \times 10^4), which were plated for 2 h and precoated with anti-CD3 (0.2 \mu g/ml) for another hour, in the presence of dexamethasone (5 \times 10^{-8} M) and 1a,25-dihydroxyvitamin D3 (1 \times 10^{-7} M; Life Technologies, Carlsbad, CA) in T cell medium containing 10% FCS plus IL-2 (50 IU/ml) and soluble anti-CD28 (0.2 \mu g/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 3. Identification of TLRLs that enhance proliferation of CD4\textsuperscript{+} T, CD8\textsuperscript{+} T and B cells without activation through TCR. Proliferation of CFSE-labeled nonstimulated PBMCs in the absence or presence of single TLRLs, 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 TLRL added.

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-\gamma, (C) IL-6, and (D) TNF-\alpha production with R&D Systems ELISA kits for each cytokine. Error bars represent means \pm 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, TLRL1/2L: Pam3CSK4, TLRL4L:LPS, and TLRL5L: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 TLRL7/8L: CL097 potently stimulated CD8+ T cell proliferation in addition to Pam3CSK4, LPS, and flagellin (Fig. 1A, middle panel, and Table I). In contrast, only TLRL7/8L:CL097 and TLRL9Ls: 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 TLRL2/6L:FSL and TLRL7/8L: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 TLRL7/8L: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 TLRL8/7L: 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

FIGURE 5. Identification of TLRLs that block nTreg suppression of CD4+ T cell proliferation using a CD3-depleted, PBMC-based proliferation assay system. Experiments were performed as in Fig. 1 except that CD3-depleted PBMCs were used as APCs and CD4+ T effifs were used as responder cells. All cells were derived from the same donor. Anti-CD3 was used at a 0.3-μg/ml concentration. (A) Representative data showing the effects of TLRLs on nTreg suppression of CD4+ T cell proliferation. Each TLRL was tested at four different concentrations as described in Materials and Methods. White color bars and dark color bars indicate lowest and highest concentration, respectively. (B) Experiments were performed as described in Materials and Methods using three concentrations of TLRL. The ratio of nTregs to effector cells was 1:2. T cell proliferation was assessed by flow cytometry for CFSE dilution. 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.
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 Pam₃CSK₄, 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.

**Pam₃CSK₄ 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 Pam₃CSK₄, 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 Pam₃CSK₄ 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, Pam₃CSK₄ 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, Pam₃CSK₄ 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 Pam₃CSK₄ 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.

**Pam₃CSK₄ 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 Pam₃CSK₄ 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 Pam₃CSK₄ 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/Ionomycin to detect IL-2 and IL-10 cytokine expression. We found that treatment of nTregs with either Pam₃CSK₄ or control flagellin did not result in a significant change in the percentage FOXP3⁺ cells (Fig. 7B). However, in the presence of IL-2, Pam₃CSK₄ 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, Pam₃CSK₄ 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 Pam₃CSK₄ 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.

**FIGURE 6.** Pam₃CSK₄, FSL, LPS, and flagellin act directly on CD4⁺ T cells but not on CD8⁺T cells to enhance cell proliferation. (A and B) CD3-stimulated proliferation of freshly sorted, naive CD4⁺ and CD8⁺ T cells in the absence or presence of a TLRL; five concentrations tested as described in Materials and Methods. White color bars and dark color bars indicate lowest and highest concentration, respectively. (C–E) Dose-dependent enhancement of CD3-stimulated CD4⁺ naive, memory, and Treg proliferation by Pam₃CSK₄ and flagellin. The TLR9Ls CpG-A and CpG-B served as negative controls. The nTregs were stimulated in the presence of a high amount of IL-2 (300 IU/ml). TLRLs were tested at five concentrations: Pam₃CSK₄ and flagellin (0, 1, 10, 50, 100 ng/ml) and CpG-A and CpG-B (0, 0.5, 1, 2.5, 5 mM). Numbers 1 and 5 on x-axis indicate lowest and highest concentration, respectively. (A and B) [³H]thymidine was added on the third day of culture and cells were harvested after another 15 h of incubation. Proliferation of T cells was evaluated by thymidine incorporation. (C–E) T cell proliferation was assessed by flow cytometry for CFSE dilution. Each of the data points was performed in duplicates. Data are representative from two donors that yielded similar results.
The decrease in the expansion of IL-10+ Tregs was specific to Pam3CSK4, LPS, flagellin, CL097, and CL075. 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 T eff function in humans.

Collectively, we have identified six TLRL-specific ligands, Pam3CSK4, LPS, flagellin, CL097, CL075, and CpG-B or CpG-C that provide a new function of the TLR1/2 stimulation pathway.
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.

**Acknowledgments**

We thank Karen Ramirez and Zhiwei He for cell sorting and support and Melissa Wentz for careful reading of the manuscript.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplementary Figure 1. Percent cell proliferation calculation. Percent proliferation in a typical PBMC-based proliferation/Treg suppression assay is expressed as the percentage of a specific small lymphocytes (CD4 T cells, CD8 T cells, or B cells) in PBMCs that undergone proliferation monitored by CFSE dilution assessed by flow cytometry. In each treatment after 4 days of culture, cells have undergone at least 5 cycles of cell division.
Supplementary Figure 2. Percent IL-10+ T cells before and after 7 day cell culture. IL-10+ T cells were stained for IL-10 using freshly sorted nTregs or nTregs cultured for 7 days on CD32a-L cells.
**Supplementary Figure 3.** No TLRLs that could consistently stimulate CD4⁺ T or CD8⁺ T cell proliferation in the presence of FluA. CFSE-labeled PBMCs (0.25 x 10⁶) were stimulated for 6 days in the presence of TLRLs as described in methods and 1.0 pfu/cell FluA [Advanced Biotechnologies, human Influenza A/PR/8(H1N1)]. Cells were stained with anti-CD4 and anti-CD8 mAbs and T cell proliferation was assessed by CFSE dilution. Data are representative of 3 donors.
Supplementary Figure 4. No TLRLs that could consistently stimulate CD4+ T or CD8+ T cell proliferation in the presence of CMV peptides. CFSE-labeled PBMCs (0.25 x 10^6) were stimulated for 6 days in the presence of TLRLs as described in methods and 30 nmol/ml CMV peptides [Miltenyi, Peptivator CMV pp65 peptide]. Cells were stained with anti-CD4 and anti-CD8 mAbs and T cell proliferation was assessed by CFSE dilution. Data are representative of 2 donors.