Engagement of TLR2 Reverses the Suppressor Function of Conjunctiva CD4⁺CD25⁺ Regulatory T Cells and Promotes Herpes Simplex Virus Epitope-Specific CD4⁺CD25⁻ Effector T Cell Responses

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PURPOSE. The authors recently reported that Foxp3⁺CD4⁺CD25⁺(Bright) “natural” regulatory T cells (nT_{reg} cells) are abundant in rabbit conjunctiva and suppress herpes simplex virus (HSV)-1-specific CD4⁺ and CD8⁺ effector T cells (T_{eff} cells). However, little is known about the overall regulatory mechanisms of these nT_{reg} cells. The authors investigate the regulation of conjunctiva-resident nT_{reg} cells through Toll-like receptors (TLRs) and their effect on ocular mucosal T_{eff} cell immunity.

METHODS. CD4⁺CD25⁺ nT_{reg} cells were purified from naive rabbit conjunctivas, and their TLR expression profile was determined. The effects of TLR engagement on nT_{reg} cell-mediated suppression of CD4⁺ T_{eff} cells were determined in vitro and in vivo.

RESULTS. The authors found that conjunctiva-resident nT_{reg} cells express high levels of TLR2 and TLR9; exposure to the TLR2 ligand lipoteichoic acid (LTA) led to the increased activation and proliferation of nT_{reg} cells, and the addition of autologous APCs further increased nT_{reg} cell expansion; in contrast, the TLR9 ligand CpG₂₀₀₇ inhibited the proliferation of nT_{reg} cells, and the addition of autologous APCs had no effect on such inhibition; nT_{reg} cells treated with LTA, but not with CpG₂₀₀₇, expressed IFN-γ and IL-10 mRNA, but not TGF-β; consistent with in vitro data, rabbits immunized by topical ocular drops of HSV-gD peptides + TLR2 ligand (LTA) displayed enhanced CD4⁺CD25⁻ T_{eff} cell immune responses when compared with HSV-gD peptides + TLR9 ligand (CpG₂₀₀₇).

CONCLUSIONS. Although conjunctiva-resident CD4⁺CD25⁺ nT_{reg} cells express high level of TLR2 and TLR9, their suppressive function is more significantly reversed after the administration of TLR2 ligand (LTA; $P < 0.005$) than of TLR9 ligand (CpG₂₀₀₇; $P > 0.005$). These findings will likely help optimize the topical ocular administration of immunotherapies. (*Invest Ophthalmol Vis Sci.* 2011;52:3321–3333) DOI:10.1167/iops.10.6522

Regulatory T cells (T_{reg}) constitute a phenotypically and functionally distinct αβ T cell population that represents 1% to 2% of peripheral mononuclear cells and 5% to 10% of all peripheral CD4⁺ T cells in both mice and humans.^{1,2} Naturally occurring CD4⁺CD25⁺ regulatory T cells (also known as nT_{reg}) have emerged as a dominant regulatory T cell subpopulation mediating peripheral adaptive immune tolerance and suppression of immunity against many pathogens.^{2–7} In general, most nT_{reg} cells constitutively express high-affinity IL-2Rα receptor (i.e., CD25) and the transcription factor forkhead/winged-helix transcription factor (Foxp3) molecules.^{8,9} In addition, in the resting state, they constitutively express several activation markers, such as glucocorticoid-induced receptor TNF-R (GITR), OX40 (CD134), and CTLA-4.^{10,11} The mucosal epithelia and underlying lamina propria contain large numbers of regulatory T cells that play an important role in maintaining mucosal homeostasis and defense against external pathogens.¹²

We recently described an abundance of “natural” Foxp3⁺CD4⁺CD25⁺(Bright) nT_{reg} cells in “nonimmune” rabbit conjunctiva, the main inductive site of the ocular mucosal immune system.¹ We demonstrated that conjunctiva-resident nT_{reg} cells suppress herpes simplex virus type 1 (HSV-1)-specific CD4⁺ and CD8⁺ effector T cells (T_{eff}). Converging evidence, from our laboratory and other laboratories, demonstrates that nT_{reg} cells have the potential to dampen the vaccine-induced, HSV-specific, T_{eff}-mediated immunity.^{13–16} Despite recent extensive studies on nT_{reg} cells, the molecular mechanism by which nT_{reg} cells mediate the suppression of pathogen-specific T cell immunity or dampen vaccine-induced T_{eff} cells remains poorly understood.

Recent discovery of Toll-like receptors (TLRs) has provided the link between the innate and adaptive immune systems.^{17–19} At least 13 TLRs that recognize a limited but conserved set of ligands from viral, bacterial, protozoan, and helminth pathogens have now been identified in mice and humans.^{20–23} It was initially thought that TLRs are primarily expressed by antigen-presenting cells (APCs) such as macrophages and dendritic cells and that interactions between microbial ligands and TLRs in these cells would indirectly result in

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the activation of effector T cells (T_{eff} cells). However, it has become evident that TLRs are also expressed by conventional $\alpha\beta$ T cells, regulatory T cells, and $\gamma\delta$ T cells and by natural killer T cells.^{21,24–26} A correlation between nT_{reg} cell-suppressive function and TLR expression has been documented.^{27–29} Although some TLR ligand-receptor interactions have been proposed to increase nT_{reg} cell suppressive capacity, others have been shown to limit such function.^{28,30–32} For example, TLR2 signaling temporally abrogates the suppressive phenotype of nT_{reg} cells and decreases Foxp3 expression.^{33,34} A direct involvement of TLR-5 on nT_{reg} cell immunosuppressive function has been demonstrated recently.²⁸ TLR9 ligand CpG DNA also modulates adaptive immune responses by inhibiting the suppressive effects of nT_{reg} cells in mice.³² However, the phenotypic and functional characterization of TLRs, particularly on conjunctiva-resident nT_{reg} cells, has not been reported.

In the present study, we examined the expression profile of TLRs on conjunctiva-resident nT_{reg} cells and assessed whether TLRs are functionally active by assessing whether stimulation of these nT_{reg} cells with TLR agonists, in the absence of APCs, would result in modification of their regulatory functions. We show that rabbit conjunctiva-resident $CD4^+CD25^+$ nT_{reg} cells express high levels of functional TLR2 and TLR9. Topical ocular immunization of rabbits with HSV-gD peptide T cell epitopes, together with a TLR2 ligand (LTA), reverses $CD4^+CD25^+$ nT_{reg} cell suppressive function. In contrast, topical ocular immunization of rabbits with the same epitopes delivered with a TLR9 ligand (CpG₂₀₀₇) resulted in only a slight effect on $CD4^+CD25^+$ nT_{reg} cell suppressive function. Our findings demonstrate that regulating conjunctiva $CD4^+CD25^+$ nT_{reg} cell function through TLR2 and TLR9 is possible and leads to the modulation of ocular mucosal HSV-specific $CD4^+CD25^-$ T_{eff} cell responses. The potential use of TLR agonists for steering ocular mucosal T cell responses in a topical ocular therapeutic vaccine strategy is discussed.

MATERIALS AND METHODS

Rabbits

For in vitro studies, healthy rabbit eyes with palpebral conjunctiva were purchased from Pel Freeze Biologicals (Rogers, AR). For in vivo studies, 7-week-old New Zealand White female rabbits (Harlan, Indianapolis, IN) were used for all experiments. Rabbits were maintained under specific pathogen-free conditions in the animal facilities of the University of California at Irvine.

Media, Reagents, and Antibodies

Cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FCS, 100 IU/mL penicillin, 100 μ g/mL streptomycin, 2 mM L-glutamine, and 1 mM Na-pyruvate at 37°C, 5% CO₂ incubator. Lipoteichoic acid (LTA) and phytohemagglutinin were purchased from Sigma. CpG₂₀₀₇ was purchased from Coley Pharmaceutical Group (Wellesley, MA). Anti-human TLR2-PE, TLR3-PE, TLR4-PE, TLR8-PE, and TLR9-PE monoclonal antibodies were purchased from Imgenex (San Diego, CA). Purified anti-human CD3 was purchased from R&D Systems (Minneapolis, MN). CFSE was purchased from Invitrogen (Carlsbad, CA). Anti-rabbit CD4, anti-rabbit CD25, and goat anti-mouse IgG-PE were purchased from Antigenix America (Melville, NY). Rabbit anti-mouse IgG-PE was purchased from Serotec (Raleigh, NC). Monoclonal anti-rabbit CD11b-PE, CD11b-FITC, CD11c-PE, and CD11c-FITC were purchased from Chemicon (Temecula, CA). Pam3CSK4 was purchased from Imgenex (catalog no. IMG-2201). PE-conjugated anti-human Foxp3 was purchased from eBioscience (San Diego, CA; catalog no. 12-4776-71). Purified anti-human Foxp3 was purchased from BioLegend (San Diego, CA; catalog no. 320101).

Purification of $CD4^+CD25^+$ and $CD4^+CD25^-$ Cells

Single cell suspension from healthy rabbit conjunctiva was prepared as previously described.¹ $CD4^+CD25^+$ nT_{reg} cells were purified from conjunctiva-derived single cell suspension by positive selection with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, 18 to 20 $\times 10^7$ single cell suspension in 2 mL MACS buffer (Miltenyi Biotec) was first incubated with 100 to 120 μ L anti-rabbit CD25-biotin followed by a second incubation with 360 μ L anti-biotin microbeads (Miltenyi Biotec) according to manufacturer's instructions. $CD25^+$ cells were subsequently eluted from the MACS column. The flow-through containing 9 to 10 $\times 10^7$ $CD25^+$ cells was collected and further purified by two successive incubations, the first with 100 μ L anti-rabbit CD4-FITC and the second with 100 μ L anti-FITC microbeads (Miltenyi Biotec) according to manufacturer's instructions. $CD4^+CD25^+$ cells were subsequently eluted from the MACS column. The flow-through containing $CD4^-CD25^-$ cells was collected and used as a source of APCs.

Purification of $CD11b^+$ Cells

Single cell suspension from healthy rabbit conjunctiva was prepared as described earlier.¹ Cells were plated in a T75 flask at a density of 10 $\times 10^6$ /20 mL culture media and were allowed to attach for 2 hours at 37°C in a 5% CO₂ incubator. Nonattached cells were removed by aspiration. Attached cells were gently washed two times with culture media, scraped, and collected in culture media. Trypan blue staining was used to check cell viability. The expression of $CD11b^+/CD11c^+$ cells in the attached cells was checked by staining with anti-rabbit CD11b-FITC or anti-rabbit CD11c-PE antibody. The purity of $CD11b^+$ cells was 95% as checked by flow cytometry (data not shown). The average yield of $CD11b^+/CD11c^+$ cells from 30 healthy conjunctivas was 20% (data not shown).

Intracellular Staining of TLRs

Purified $CD4^+CD25^+$ cells (0.24 $\times 10^6$ cells/100 μ L culture medium) were washed and suspended in FACS staining buffer (PBS [pH 7.4] containing 0.5% BSA, 0.02% sodium azide). Cells were stained with 1 μ L anti-rabbit CD25-biotin for 30 minutes at room temperature, washed, and further incubated with 1 μ L anti-streptavidin-Cy5 (Chemicon) for 30 minutes at 4°C in the dark. Cells were washed twice with FACS staining buffer. For intracellular staining, cells were fixed with fixation-permeabilization buffer (eBioscience) according to the manufacturer's instruction. Cells were then stained with 3 μ L anti-human TLR2-PE, TLR3-PE, TLR4-PE, TLR8-PE, and TLR9-PE for 30 minutes at 4°C in the dark. Cells were washed twice with FACS staining buffer and analyzed by flow cytometry (FACScan; BD Biosciences, San Jose, CA). The acquired data were analyzed by acquisition and analysis software (Cell Quest; BD Biosciences).

Intracellular Staining of Foxp3

Harvested $CD4^+CD25^+$ cells (0.5 $\times 10^6$ cells/assay) from a 96-well tissue culture plate were washed and suspended in FACS staining buffer (PBS [pH 7.4] containing 0.5% BSA, 0.02% sodium azide). Cells were surface stained with anti-rabbit CD4-FITC (10 μ L/assay) for 30 minutes at 4°C in the dark. For intracellular staining, cells were fixed as described and were stained with anti-human foxp3-PE (20 μ L/assay) for 30 minutes at 4°C in the dark. The rest of the procedure was same as described.

CFSE Labeling of $CD4^+CD25^+$ Cells

Purified $CD4^+CD25^+$ or $CD4^+CD25^-$ cells at a density of 1 $\times 10^6$ cells/mL PBS were labeled with a final concentration of 2.5 μ M CFSE for 10 minutes at 37°C according to the product instructions. The reaction was quenched by the addition of 5 vol culture media. Cells were washed three times with culture media before they were placed in culture.

Proliferation CD4⁺CD25⁺ T Cells

Freshly isolated and CFSE-labeled CD4⁺CD25⁺ nT_{reg} cells (5×10^4 cells/well in 200 μ L) were plated in a 96-well, flat-bottomed tissue culture plate in culture media. Cells were stimulated with either soluble anti-human CD3 (1 μ g/mL) alone or with a combination of soluble anti-CD3 (1 μ g/mL) or autologous APCs (1×10^5 APCs/well in 200 μ L) for 6 days at 37°C in a 5% CO₂ incubator. To test the effect of TLR ligands on the proliferation of CD4⁺CD25⁺ nT_{reg} cells, in a second set, CFSE-labeled CD4⁺CD25⁺ nT_{reg} cells (5×10^4 cells/well in 200 μ L) were incubated with either 10 μ g/mL LTA (ligand for TLR2) or 1 μ g/mL CpG₂₀₀₇ (ligand for TLR9) along with soluble anti-human CD3 (1 μ g/mL) only or with a combination of soluble anti-CD3 (1 μ g/mL) and autologous APCs (1×10^5 APCs/well in 200 μ L). Autologous APCs were treated with 1 μ g/mL mitomycin C for 45 minutes at 37°C and were washed five times before they were put into the culture. Cells were plated in triplicate and incubated for 6 days at 37°C in a 5% CO₂ incubator. Cells were harvested, washed twice with FACS staining buffer, stained with anti-rabbit CD4-PE or anti-rabbit CD25-PE, and analyzed with flow cytometry. The absolute number of proliferating cells was calculated using the formula (No. events in CD4⁺ proliferating cells) $\times$ (No. events in gated cells)/(No. total events acquired).

Suppression of CD4⁺CD25⁺ T_{eff} Cell Proliferation by CD4⁺CD25⁺ nT_{reg} Cells

To monitor the activation and proliferation of T_{eff} cells, freshly purified and CFSE-labeled CD4⁺CD25⁺ effector T cells (5×10^4 cells/well in 200 μ L) were stimulated with soluble anti-human CD3 antibody (1 μ g/mL) and autologous APCs (1×10^5 APCs/well in 200 μ L). To monitor the suppressed proliferation of T_{eff} cells influenced by nT_{reg} cells, in a second set, nonlabeled CD4⁺CD25⁺ nT_{reg} cells (10×10^4 /well in 200 μ L) were added together with CFSE-labeled CD4⁺CD25⁺ effector cells (5×10^4 cells/well in 200 μ L) and stimulated with soluble anti-human CD3 antibody (1 μ g/mL) and autologous APCs (1×10^5 APCs/well in 200 μ L). To monitor the effect of TLR ligands on this suppression assay, in a third set, nonlabeled CD4⁺CD25⁺ nT_{reg} cells (10×10^4 /well in 200 μ L) were added together with CFSE-labeled CD4⁺CD25⁺ effector cells (5×10^4 cells/well in 200 μ L) along with either 10 μ g/mL LTA (ligand for TLR2) or 1 μ g/mL CpG₂₀₀₇ (ligand for TLR9) and were stimulated as described with soluble anti-human CD3 antibody (1 μ g/mL) and autologous APCs (1×10^5 APCs/well in 200 μ L). Autologous APCs were always treated with 1 μ g/mL mitomycin C for 45 minutes at 37°C and were washed five times before they were put into the culture. Cells in all three sets were plated in triplicate and incubated for 6 days at 37°C in a 5% CO₂ incubator. Cells were harvested, washed twice with FACS staining buffer, and stained with anti-rabbit CD4-PE or anti-rabbit CD25-PE and analyzed by flow cytometry. The absolute number of proliferating cells was calculated using the formula (No. events in CD4⁺ proliferating cells) $\times$ (No. events in gated cells)/(No. total events acquired).

Ex Vivo Assay

Six New Zealand White female rabbits (two per group) were immunized ocularly (both eyes) with a mixture of four HSV-gD peptides (gD₁₄₆₋₁₇₉, gD₂₈₇₋₃₁₇, gD₄₉₋₈₂, and gD₂₀₀₋₂₃₄) along with either TLR ligand CpG₂₀₀₇ (group 1) or LTA (group 2), or not immunized (group 3), at an interval of 14 days. The amount of each HSV-gD peptide in the peptide mixture was 25 μ g and was suspended in 100 μ L sterile PBS. The amount of CpG₂₀₀₇ or LTA in the peptide mixture was 25 μ g/100 μ L peptide mixture. Each rabbit was immunized with 100 μ L (50 μ L/eye) peptide mixture. Ten days after the third immunization, the conjunctiva was removed from each rabbit eye, and a single cell suspension was prepared as described earlier.¹ CFSE-labeled, conjunctiva-derived, single cell suspensions were plated at a density of 3×10^5 cells in 200 μ L in a 96-well, round-bottomed tissue culture plate, stimulated with individual HSV-gD peptide (10 μ g/mL) or nonspecific positive control phytohemagglutinin (10 μ g/mL) and were incubated

for 5 days at 37°C in a CO₂ incubator. Cells were harvested and stained with anti-rabbit CD4-PE and analyzed by flow cytometry.

Cytokine Expression Quantification Using Real-Time PCR

Purified nT_{reg} cell lysate was suspended directly in RLT buffer (Qiagen, Valencia, CA). This lysate was then processed over RNeasy columns according to the manufacturer's instructions (Qiagen). The RNA was eluted from the silica column with 50 μ L DEPC-treated water. One microliter of the product was analyzed (2100 Bioanalyzer; Agilent, Wilmington, DE) using the nano-RNA protocol to verify both the quantity and the quality of the RNA. RNA obtained from purified nT_{reg} cells was converted to cDNA using a synthesis protocol (Sensiscript; Qiagen). For these, 1000 ng RNA was reverse transcribed in a 20- μ L reaction volume after wiping out the genomic DNA. Reactions were carried out for 30 minutes at 42°C, followed by 5 minutes at 95°C and cooling to 4°C. After synthesis, the cDNA from all sources was diluted to equivalent input RNA levels with water (10 ng input RNA/ μ L) and was stored at -20°C until use. cDNA samples were subjected to PCR using specific primers. Primers were designed with a software program (Primer 3 Internet; The Whitehead Institute, Cambridge, MA),³⁵ and their specificities were confirmed by a BLAST Internet software-assisted search of the nonredundant nucleotide sequence database (National Library of Medicine, Bethesda, MD). The following primers were used for cytokine detection: IFN- γ : sense, 5'-CATCAGATGTG-GCAAATGGT-3'; forward, 5'-ATCCACCGAAATTCGAGTCA-3'; IL-10: sense, 5'-AAAAGCTAAAAGCCCCAGGA-3'; forward, 5'-ATAGGATCG-GGAGCTGAGGT-3'; TGF- β : sense, 5'-TGCTTCAGCTCCACAGAGAA-3'; forward, 5'-CTTGCTGTACTGGGTGTCCA-3'.

All PCR reactions were carried out with 5 ng reverse-transcribed RNA, 200 nM each forward and reverse primer, and master mix (SYBR Green; Applied Biosystems, Foster City, CA) in a final volume of 25 μ L. Cycling parameters were 50°C for 2 minutes and 95°C for 5 minutes, followed by 45 cycles of 95°C for 30 seconds and 60°C for 90 seconds. Plates were read after each cycle, and a melting curve was generated after amplification. For quantitative real-time PCR, samples were normalized by GAPDH amplification and were amplified in triplicate using a thermal cycler (Opticon; MJ Research, Waltham, MA). PCR controls without reverse transcriptase (water control) or with normal human genomic DNA as a template were routinely negative. Cytokine expression were carried out using a 2- $\Delta\Delta$ CT method, as recently described.¹

Statistical Analysis

All analyses for statistically significant differences were performed with the Student's paired *t*-test. *P* < 0.05 was considered significant. All cultures were performed in triplicate, and error bars represented standard deviation.

RESULTS

Conjunctiva-Resident CD4⁺CD25⁺ T Cells Express High Levels of TLR2 and TLR9

CD4⁺CD25⁺ T cells were purified from normal rabbit conjunctiva by magnetic cell separation, as we described previously.¹ The expression of TLR2, TLR3, TLR4, TLR8, and TLR9 by conjunctiva-resident CD4⁺CD25⁺ T cells was determined by intracellular staining and analyzed by flow cytometry. As shown in Figure 1A, conjunctiva-derived CD4⁺CD25⁺ T cells expressed detectable amounts of all tested TLRs. Further analysis of mean fluorescence intensity (MFI) of each TLR revealed significantly higher expression levels of TLR2 and TLR9 compared with TLR3, TLR4, and TLR8 (*P* < 0.005; Fig. 1C). Given that no anti-rabbit TLR mAbs were commercially available, we used a mouse anti-human TLR IgG₁ mAb to detect the TLR expression profile in rabbits. The specificity of anti-human TLR mAbs to detect TLR in rabbit CD4⁺CD25⁺ T cells was verified

FIGURE 1. Intracellular staining of Toll-like receptors in CD4⁺CD25⁺ regulatory T cells purified from rabbit conjunctiva. (A) Purified CD4⁺CD25⁺ cells (0.66×10^5 per assay) were first surface stained with 1 μ L α rabbit CD25-biotin and then intra cellularly stained with 1 μ L α human TLR2-PE, TLR3 PE, TLR4-PE, TLR8-PE, TLR9-PE, as shown on each histogram. *Dotted line*: CD4⁺CD25⁺ cells only; *solid line*: cells stained with anti-human TLR-PE antibody. (B) Isotype control (*dotted line*) where CD4⁺CD25⁺ cells (0.66×10^5) were first stained with 1 μ L anti-rabbit CD25-biotin followed by staining with 1 μ L mouse IgG1 PE. (C, D) Level of TLRs in CD4⁺CD25⁺ and CD4⁺CD25⁻ cells as a function of MFI. * $P < 0.05$ when compared with the MFI of TLR2 and TLR9 expression in CD4⁺CD25⁺ and CD4⁺CD25⁻ populations.

by an isotype mAb control. Figure 1B shows the binding of mouse IgG₁-PE (isotype control) to CD4⁺CD25⁺ T cells under identical experimental conditions and overlaid on the binding of anti-human TLR2 to CD4⁺CD25⁺ T cells. No binding between mouse IgG₁-PE and CD4⁺CD25⁺ T cells was observed, which ruled out the possibility of any nonspecific binding between anti-human TLR-PE and CD4⁺CD25⁺ T cells.

We also checked the TLR expression profile on CD4⁺CD25⁻ T_{eff} cells, which was copurified along with the magnetic separation of CD4⁺CD25⁺ nT_{reg} cells from rabbit conjunctiva. Figure 1D showed a significantly lower expression of TLR2 (MFI <50) in CD4⁺CD25⁻ T_{eff} cells compared with the TLR2 expression in CD4⁺CD25⁺ nT_{reg} cells (MFI >250) shown in Figure 1C. Interestingly, no significant differences in the expression level of TLR9 between these two cell populations (i.e., CD4⁺CD25⁺ nT_{reg} cells and CD4⁺CD25⁻ T_{eff} cells) were observed (Figs. 1C, 1D). This indicates the integrity of the CD4⁺CD25⁺ T_{reg} cell and CD4⁺CD25⁻ T_{eff} cell populations.

We next investigated whether the intracellular expression of TLR2 and TLR9 was comparable to that of APCs, an immune cell population known for high expression of TLRs. We tested the expression levels of TLRs in CD11b/c⁺ APCs isolated from healthy rabbit conjunctiva. Figure 2A shows the histogram representation of all tested TLRs in autologous CD11b/c⁺ APCs, and Figure 2B shows their corresponding MFI. Autologous CD11b/c⁺ APCs also expressed all tested TLRs, and the highest expression was observed for TLR9 (MFI ≥ 500). However, no significant difference in the expression level of TLR2 was observed in CD11b/c⁺ APCs or CD4⁺CD25⁺ nT_{reg} cells (Figs. 1B, 2B), indicating a similar TLR expression profile between nTreg cells and APCs in the conjunctiva compartment.

LTA (a TLR2 ligand) Induces the Proliferation of CD4⁺CD25⁺ nT_{reg} Cells

To determine whether TLR2 and TLR9 receptors expressed on CD4⁺CD25⁺ nT_{reg} cells were functional, we performed an in vitro proliferation assay for nT_{reg} cells in the presence and absence of their specific ligands: LTA and CpG₂₀₀₇ DNA, TLR2 and TLR9 ligands, respectively. CFSE-labeled CD4⁺CD25⁺ nT_{reg} cells were stimulated with either soluble anti-human CD3 or a combination of soluble anti-human CD3 and autologous APCs and were incubated for 6 days in the presence or absence of either LTA or CpG₂₀₀₇. The proliferation of CFSE-labeled CD4⁺CD25⁺ nT_{reg} cells was then analyzed by flow cytometry on gated CD4⁺ T cells. Figure 3A shows that the proliferation of CD4⁺CD25⁺ nT_{reg} cells was enhanced approximately two-fold after stimulation with soluble anti-human CD3 ($P < 0.005$). Interestingly, the addition of LTA further enhanced the overall proliferation of CD4⁺CD25⁺ nT_{reg} cells ($P < 0.005$). In contrast, a decrease (rather than an enhancement) in the absolute number of proliferating nT_{reg} cells was observed when CpG₂₀₀₇ was used.

Next we assessed whether the addition of autologous APCs would affect the proliferation of anti-CD3 stimulated nT_{reg} cells. Purified CD4⁺CD25⁺ nT_{reg} cells were stimulated in the presence of both soluble anti-CD3 and autologous APCs, in the presence of soluble anti-CD3 alone, or in the presence of media alone (control). As expected, the proliferation of nT_{reg} cells was enhanced twofold to threefold when incubated with both anti-CD3 and autologous APCs compared with anti-CD3 without autologous APCs (Fig. 3B vs. Fig. 3A). The addition of LTA to these APC/CD4⁺CD25⁺ cocultures, along with anti-human CD3, further enhanced the proliferation by a factor of 10-fold compared with media alone (without anti-human CD3 or APCs). However, the addition of CpG₂₀₀₇, significantly de-

FIGURE 2. (A) Intracellular staining of Toll-like receptors in CD11b⁺ APCs isolated from rabbit conjunctiva. CD11b⁺ cells (0.24×10^6 per assay) were first surface stained with 1 μ L anti-rabbit CD11b-FITC antibody and then intracellularly stained with 3 μ L anti-human TLR2-PE, TLR3-PE, TLR4-PE, TLR8-PE, TLR9-PE in each set. *Dotted line*: CD11b⁺ cells only; *solid line*: cells stained with anti-human TLR-PE antibody. (B) TLR level in CD11b⁺ cells as a function of MFI. * $P < 0.05$ compared with the MFI of TLR2 and TLR9 expression in the CD11b⁺ populations.

creased the overall proliferation of CD4⁺CD25⁺ nT_{reg} cells, even in the presence of APCs ($P < 0.005$). Together these results demonstrate that TLR2 and TLR9 expressed by conjunctiva residual nT_{reg} are functional. Although TLR2 ligand promoted nT_{reg} proliferation, TLR9 ligand inhibited nT_{reg} proliferation.

TLR2 Ligand LTA Induces IFN- γ and IL-10 mRNA Expression in Conjunctiva-Resident CD4⁺CD25⁺ nT_{reg} Cells

To determine whether conjunctiva-resident nT_{reg} cells in “anergic” and “stimulated” states are able to produce IFN- γ , IL-10, and TGF- β , we checked their expression profiles by real-time

PCR. Unfortunately, there are no kits available to detect rabbit cytokines and growth factor by ELISA. Conjunctiva-purified nT_{reg} cells were pretreated with LTA for 6 hours, followed by incubation with anti-CD3 and IL-2 for another 6 hours at 37°C. Cells were harvested, and mRNA expression of IFN- γ , IL-10, and TGF- β were measured by real-time PCR. Figure 4 shows that LTA-pretreated and anti-CD3/IL-2-stimulated nT_{reg} cells expressed 2.5-fold more IFN- γ (Fig. 4A) and IL-10 (Fig. 4B) mRNA than untreated nT_{reg} cell controls (i.e., α CD3⁺ IL-2 without LTA; $P < 0.05$). However, LTA pretreatment did not affect TGF- β (Fig. 4C) expression because its mRNA level remained unchanged compared with untreated nT_{reg} cell controls (i.e., α CD3⁺ IL-2 without LTA; $P > 0.05$). Treatment of

FIGURE 3. In vitro proliferation of CD4⁺CD25⁺ T_{reg} cells purified from rabbit conjunctiva. CFSE-labeled CD4⁺CD25⁺ T_{reg} cells were stimulated with 1 μ g/mL soluble α -CD3 (A) or α -CD3 + APCs (B) for 6 days at 37°C in a 5% CO₂ incubator, in the presence and absence of TLR ligand 2 (LTA) and TLR ligand 9 (CpG₂₀₀₇) at a concentration of 10 μ g/mL and 1 μ g/mL, respectively. Cells were harvested and stained with anti-rabbit CD4-PE or anti-rabbit CD25-PE and analyzed by flow cytometry. Values in each bar indicate the average $\pm$ SD of two wells. Each well has 5×10^4 CFSE-labeled CD4⁺CD25⁺ T_{reg} cells and with and without 1×10^5 APCs (mitomycin C treated), as designated in the figure.

FIGURE 4. Quantification of IFN- γ (A, D), IL-10 (B, E), and TGF- β (C, F) expression in rabbit conjunctiva-purified CD4⁺CD25⁺ cells using real-time PCR. One million nT_{reg} cells were plated in a six-well tissue culture plate and were pretreated with LTA (10 μ g/mL; A–C) or CpG (1 μ g/mL; D–F) for 12 hours, followed by stimulation for another 12 hours in the presence of anti-CD3 (1 μ g/mL) and IL-2 (20 U/mL) at 37°C. RNA was isolated from harvested cells and was quantified. An equal amount of RNA was reverse transcribed. cDNA was amplified using real time PCR and specific primers for rabbit IFN- γ , IL-10, and TGF- β . *GAPDH* was used as a housekeeping gene. RNA quantification was calculated using the comparative Ct method, also known as the 2^{-ΔΔCt} method, where ΔΔCt = ΔCt sample – ΔCt reference (none). Here, ΔCt sample is the Ct value for any sample normalized to the endogenous housekeeping gene, and ΔCt reference (none) is the Ct value for the calibrator also normalized to the endogenous housekeeping gene.

nT_{reg} cells with LTA alone, without CD3⁺ IL-2, was unable to induce significant levels of IFN- γ , IL-10, or TGF- β mRNAs compared with untreated nT_{reg} cells (medium). These results suggest that LTA-TLR2 interaction might transform nT_{reg} cells from an anergic state to an active state. Unlike LTA, CpG₂₀₀₇ treatment has no effect on cytokine production by nT_{reg} cells (Figs. 4D–F).

Treatment with the TLR2 Ligand LTA Significantly Reverses the Immunosuppressive Function of CD4⁺CD25⁺ nT_{reg} Cells

We next assessed the effect of TLR2 and TLR9 ligands on the immunosuppressive function of conjunctiva-resident nT_{reg} cells. In a conventional in vitro suppression assay, purified CD4⁺CD25⁺ T_{eff} cells were first labeled with CFSE and then incubated with purified nonlabeled CD4⁺CD25⁺ nT_{reg} cells in the presence or absence of either TLR2 (LTA) or TLR9 (CpG₂₀₀₇) ligands. As shown in Figure 5A, the addition of CD4⁺CD25⁺ nT_{reg} cells at a ratio of 1:2 to CD4⁺CD25⁺ T_{eff} cells resulted in a significant suppression of CD4⁺CD25⁺ T_{eff} cell proliferation ($P < 0.005$). The results presented in Figure 5A were also expressed as the percentage of proliferation in Figure 5B. This CFSE-labeled suppression assay result confirmed our previous observation¹ that conjunctiva-resident nT_{reg} cells can suppress the proliferation of CD4⁺CD25⁺ T_{eff} cells. Here we observed approximately 92% suppression of the proliferation of CD4⁺CD25⁺ T_{eff} cells in the presence of nT_{reg} cells. Interestingly, when LTA was added in the T_{eff} and nT_{reg} coculture cells, the suppressive effect of nT_{reg} cells was reversed, and the CD4⁺CD25⁺ T_{eff} cells regained their proliferation efficiency by an average of 30%. However, under identical conditions, the addition of CpG₂₀₀₇ to the T_{eff} and nT_{reg} coculture cells reversed the suppression effect by only 10%. This result suggests that TLR2 ligand has the potential to

reverse the suppressive property of CD4⁺CD25⁺ nT_{reg} cells. Under these experimental conditions, LTA was more efficient than CpG₂₀₀₇ in reversing the suppressive function of CD4⁺CD25⁺ nT_{reg} cells.

TLR2 Ligand LTA Enhances the Immunogenicity of HSV-1 gD-Derived Peptide Antigen Delivered Topically to the Conjunctiva

Next we compared the adjuvant effect of TLR2 and TLR9 ligands on topical ocular mucosal immunogenicity of the HSV-1 gD peptide epitope. Rabbits were immunized by topical application of a mixture of four immunodominant CD4⁺ T cell peptide epitopes (gD_{144–179}, gD_{287–317}, gD_{49–82}, gD_{332–358}) selected from HSV-1 gD,^{36,37} along with either the TLR2 ligand LTA or the TLR9 ligand CpG₂₀₀₇ as immunoadjuvants. The control group received saline alone, LTA alone, or CpG₂₀₀₇ alone (Mock). After the third immunization, conjunctivas were harvested from each immunized and mock-immunized rabbit group. Single cell suspension was labeled with CFSE, and the proliferation of CD4⁺CD25⁺ T_{eff} cells was monitored following stimulation with the individual HSV gD peptide (i.e., gD_{144–179}, gD_{287–317}, gD_{49–82}, or gD_{332–358}). The cells were analyzed by flow cytometry on gated CD4⁺CD25⁺ T_{eff} cells. Figure 6 shows a comparative analysis of the proliferation of CD4⁺ T cells obtained from gD peptides + LTA versus gD peptides + CpG₂₀₀₇-immunized rabbits. Significant CD4⁺CD25⁺ T_{eff} cell proliferation was obtained against gD_{287–317} and gD_{49–82}-immunodominant peptide epitopes when LTA was used as an immunoadjuvant compared with when CpG₂₀₀₇ was used as an immunoadjuvant ($P < 0.005$). However, no significant difference in T cell proliferative responses against gD_{144–179} and gD_{49–82} peptides were detected in the CpG₂₀₀₇ and LTA groups.

FIGURE 5. In vitro suppression of CD4⁺CD25⁻ effector cells by CD4⁺CD25⁺ nT_{reg}. Conjunctiva-purified CD4⁺CD25⁻ effector cells (5×10^4 cells) were labeled with CFSE and stimulated with soluble α human CD3 (1 μ g/mL) and mitomycin C (50 μ g/mL)-treated autologous APCs (1×10^5 cells) for 5 days at 37°C. For the suppression assay, CFSE-labeled CD4⁺CD25⁻ effector cells (5×10^4 cells) were mixed with nonlabeled CD4⁺CD25⁺ cells (10×10^4 cells) in culture medium containing soluble α human CD3 (1 μ g/mL) and autologous APCs (1×10^5 cells) and were incubated in the presence and absence of LTA (10 μ g/mL) and CpG₂₀₀₇ (1 μ g/mL) for 5 days at 37°C. Cells were harvested and stained with α rabbit CD4-PE and analyzed by flow cytometry. (A, dotted line) Absolute number of CD4⁺CD25⁻ effector cells in the absence of anti-human CD3 and autologous APCs. (B) Percentage of T_{eff} cells calculated from (A). Values in each bar indicate the average $\pm$ SD of two wells. (C) Histogram that represents nonstimulated CFSE-labeled CD4⁺CD25⁻ T_{eff} cells (dashed lines) overlaid with histograms (bold lines) that represent CFSE-labeled CD4⁺CD25⁻ T_{eff} cells stimulated with (a) an anti-CD3⁺ mAb alone or with (b) an anti-CD3⁺ mAb in the presence of untreated CD4⁺CD25⁺ T_{reg} cells, (c) an anti-CD3⁺ mAb in the presence of LTA-treated CD4⁺CD25⁺ T_{reg} cells, or (d) an anti-CD3⁺ mAb in the presence of CpG-treated CD4⁺CD25⁺ T_{reg} cells.

TLR2 Ligand Pam3CSK4, but Not LTA, Affects Foxp3 Expression in Conjunctiva-Resident CD4⁺CD25⁺ nTreg Cells

We investigated whether treatment with TLR2 ligands (LTA and Pam3CSK4) or TLR9 ligand (CpG) would affect Foxp3 expression in rabbit conjunctiva CD4⁺CD25⁺ Treg cells. A slight increase in Foxp3 expression was detected, by both flow cytometry (Figs. 7A, 7B) and Western blot analysis (Fig. 7C), after CpG treatment. However, no significant changes in the Foxp3 level were observed after LTA treatment. Interestingly, treatment of rabbit conjunctiva CD4⁺CD25⁺ Treg cells with a different TLR2 ligand, Pam3CSK4, induced a significant decrease in the level of Foxp3 ($P < 0.005$).

DISCUSSION

It was initially thought that TLRs are primarily expressed by APCs, such as macrophages and dendritic cells, and that interactions between microbial ligands and TLRs expressed by APCs

would indirectly result in activation of effector T cells. However, it has now become evident that TLRs are also expressed by various T cell subsets, such as conventional $\alpha\beta$ T_{eff} cells, T_{reg} cells, and $\gamma\delta$ T cells and by natural killer T cells.³⁸ Importantly, it appears that at least in some of these T cell subsets, TLRs are functionally active because stimulation of these cells with TLR agonists in the absence of APCs results in the exertion of effector or regulatory functions of T cells. In the present study, we provided evidence for the dominating expression of TLR2 and TLR9, over other TLRs, by conjunctiva-resident nT_{reg} cells and demonstrated their functional relevance with respect to ocular mucosal T cell immunity. To our knowledge, this is the first report describing the phenotypic expression and functional property of TLRs by conjunctiva-resident T cells.

Most data on mucosal T_{reg} cells originated from mouse models (see Ref. 12 for review). Because of the obvious ethical and practical considerations in studying human ocular mucosal T_{reg} cells, a critical question has been which species would be the most appropriate animal model to investigate conjunctiva T_{reg} cells.^{39,40} Instead of mice we elected rabbit as the working

FIGURE 6. Six rabbits (two per group) were immunized ocularly three times at an interval of 14 days with a mixture of four HSV-gD peptide (gD₁₄₄₋₁₇₉, gD₂₈₇₋₃₁₇, gD₄₉₋₈₂, and gD₃₃₂₋₃₅₈) mixed with either CpG₂₀₀₇ (group 1) or LTA (group 2) as a topical ocular mucosal immuno-adjuvants. The control group received saline alone, LTA alone, or CpG₂₀₀₇ alone (*mock*, group 3). Ten days after the third immunization, rabbits were euthanized, upper and lower conjunctivas were harvested from each rabbit, and lymphocytes were isolated. Lymphocytes were labeled with CFSE and stimulated with HSV-gD peptide (gD₁₄₄₋₁₇₉, gD₂₈₇₋₃₁₇, gD₄₉₋₈₂, or gD₃₃₂₋₃₅₈)-pulsed autologous APCs for 5 days at 37°C in a CO₂ incubator. Cells were harvested and stained with anti-rabbit CD4-PE, and their proliferation was analyzed by flow cytometry. (A) Absolute number of CFSE-labeled proliferating CD4⁺ cells under the marked conditions. (B) Dot plot representation with the percentage of CFSE-labeled proliferating CD4⁺ cells.

animal model, bearing in mind that numerous similarities exist between rabbit and human ocular mucosal tissues⁴⁰⁻⁴²; several T cell-mediated ocular surface diseases, including herpetic conjunctivitis and recurrent corneal herpetic stromal keratitis (HSK), have been reported to be similar in rabbits and humans, making it the most relevant animal model for exploring these human eye disorders⁴³⁻⁴⁵; compared with mice, rabbit conjunctiva-associated lymphoid tissue (CALT) more closely resembles human CALT⁴⁶⁻⁴⁸; microanatomy and immunohistologic studies indicate that rabbit conjunctival mucosa is comparable to that of humans and has a typical follicular ultrastructure with an abundance of conjunctival lymphoid follicles, whereas no lymphoid tissue was identified in mice and rats⁴⁸⁻⁵²; from a practical standpoint, unlike mice, rabbits have a relatively large conjunctival surface, offering abundant MALT for in vitro studies; finally, the recent availability of many monoclonal and polyclonal antibodies specific to rabbit immune cell CD markers, cytokines, and growth factors provides useful immunologic tools for an unprecedented phenotypic and functional analysis of rabbit T cell repertoire and function.

Rabbit conjunctiva-purified CD4⁺CD25⁺ T_{reg} cells display an increased level of TLR expression, especially TLR2 compared with autologous CD4⁺CD25⁻ T_{eff} cells, suggesting that the expansion and suppression function of T_{reg} cells may be closely regulated by TLR2 ligands. In general, T_{reg} cells express

higher levels of TLR2, TLR4, TLR5, TLR7/8, and TLR10 than conventional CD4⁺CD25⁻ T_{eff} cells.^{28-31,34,38,53,54} TLR10 is constitutively expressed at both mRNA and protein levels in T_{reg} cells but not in T_{eff} cells.⁵⁵ On the other hand, TLR3 is not detectable in T_{reg} cells, whereas it is expressed by T_{eff} cells.^{38,56} Several recent reports indicate direct sensing of “danger” signals by T cells (instead of APCs). This direct recognition of TLR ligands may play a role in T cell homeostasis and effector function. In particular, a crucial role of TLR2 activation in the functional properties of T_{reg} cells has been demonstrated in a series of reports showing that TLR2 ligand temporarily abrogates the immunosuppressive effects of murine T_{reg} cells and reduces the level of Foxp3 expression in T_{reg} cells and activates the expansion of dysfunctional T_{reg} cells.^{30,34,38} Here we found that engagement of TLR2 on rabbit conjunctival T_{reg} cells with its specific ligand, LTA, induced their proliferation (Fig. 3) and reversed their immunosuppressive function (Fig. 5). Questions might arise whether this downregulation of immunosuppression effect is due to an engagement of LTA on T_{reg} cells or a direct effect on T_{eff}. Although we cannot rule out the latter possibility, the probability is low because autologous rabbit T_{eff} cells express fivefold to sixfold less TLR2 than T_{reg} cells (Fig. 1D). Furthermore, in a separate experiment, we have shown that LTA does not affect the proliferation of CD4⁺CD25⁻ T_{eff} cells in the presence of soluble anti-CD3 and

FIGURE 7. Foxp3 expression on rabbit conjunctival Treg by TLR2 and TLR9 ligands. Rabbit conjunctiva-purified $CD4^+CD25^+$ Treg cells (0.5×10^6 cells) were either left untreated or stimulated overnight with anti-human CD3 ($1 \mu\text{g/mL}$) $\pm$ treatment with LTA ($10 \mu\text{g/mL}$), CpG ($1 \mu\text{g/mL}$), or Pam3CSK4 (50 ng/mL). TLR ligand treatments and anti-CD3 stimulations were performed together in a 96-well, flat-bottomed tissue culture plate at $37^\circ\text{C}/5\% \text{ CO}_2$ incubator for overnight. (**A**, **B**), Cells were harvested and surface stained with anti-rabbit CD4-FITC ($10 \mu\text{L}/\text{assay}$) followed by intracellular staining with anti-human Foxp3-PE ($20 \mu\text{L}/\text{assay}$) and were analyzed by FACS. (**C**) Cells from each well (identical to those described) were lysed with $100\text{-}\mu\text{L}$ lysis buffer (RIPA with PI cocktail). Equal volumes ($20 \mu\text{L}$) of denatured cell lysates containing $\sim 1 \times 10^5$ cells were loaded in each lane. Details of the Western blot procedure have been previously described.¹ (**A**, dot plot) MFI of $CD4^+$ /Foxp3-positive cells for rabbit conjunctiva-purified Treg cells (*top*) and human PBMC-purified Treg cells (*bottom*). Human PBMC-purified Treg cells were used as a positive control in this assay. (**B**, overlapped histograms) Expression of Foxp3 in Treg by FACS. (**C**) Foxp3 protein in rabbit conjunctiva-purified $CD4^+CD25^+$ Treg cells detected by Western blot analysis.

autologous APCs (data not shown). In contrast to TLR-2, the expression level of TLR9 on both T_{reg} and T_{eff} cells is more or less similar (Figs. 1C, 1D). In the T_{reg} cell proliferation assay, when TLR9 is engaged with the specific ligand CpG, we observed a significant inhibition of T_{reg} cell proliferation (Fig. 3). However, in the suppression assay (Fig. 5), CpG reversed the immunosuppressive capability of T_{reg} cells, but at lesser extent than LTA. Whether this reduced effect of CpG in the suppression assay was caused by a simultaneous engagement of TLR9 on both T_{reg} or T_{eff} cells remains to be determined. Nevertheless, in a separate experiment, we have shown that CpG did not affect the proliferation of $CD4^+CD25^-$ T_{eff} cells in the presence of soluble anti-CD3 and autologous APCs, thus excluding a direct effect on T_{eff} cells (data not shown). Our results are consistent with recent finding by Chiffolleau et al.⁵⁷ showing that triggering TLR9 on human T_{reg} cells inhibits their immunosuppressive function but does not induce their proliferation or the downregulation of their Foxp3 expression. In that study the loss of suppression was in part due to the effect

of TLR9 ligand on T_{eff} cells, but it is not clear whether TLR9 ligand directly influences T_{reg} cells.

In this study we found that conjunctiva-resident $CD4^+CD25^+$ nT_{reg} cells express relatively high levels of TLR2 (MFI ~ 260), a moderate level of TLR9 (MFI ~ 115), and very low levels of TLR3, TLR4, and TLR8 (MFI < 50). In parallel, we checked the expression profile of the same set of TLRs in the copurified fraction of $CD4^+CD25^-$ T_{eff} cells and found extremely low levels (MFI < 50) for TLR3, TLR4, and TLR8 and a relatively higher level of TLR9 (MFI ~ 88) expression. This indicated that, with the exception of TLR9, the other TLRs expression on T_{eff} cells was statistically insignificant in the ocular mucosal conjunctiva. To determine whether the expression level of TLRs on conjunctiva-resident nT_{reg} cells was comparable with other immune cells known for high TLR expression, we evaluated the TLR expression profile in autologous APCs. Our data show that conjunctiva-resident $CD11b^+$ APCs follow similar expression patterns for TLR2 (MFI ~ 260) and low levels for TLR3, TLR4, and TLR8. However, the TLR9

level was extremely high and was expressed four to five times more (MFI ≥ 500) than the expression of TLR9 (MFI ≥ 100) on nT_{reg} cells. It is interesting that though a differential level of TLR9 expression was observed in two different immune cells, such as CD11b⁺ APCs and nT_{reg} cells of same conjunctival origin, the TLR2 expression level remained similar in both types of cells. This suggests that TLR2 is a more conserved type of toll receptor within the immune cells present in the mucosal conjunctiva tissue. However, in humans and mice, TLR4, TLR5, and TLR8, but not TLR2, are preferentially expressed on CD4⁺CD25⁺ T_{reg} cells.^{28–31,34,38,53,54} This highlights a striking phenotypic difference between mucosa and non-mucosa nT_{reg} cells. A similar observation of TLR2 and TLR9 expression in ocular epithelial cells was reported by Chang et al.⁵⁸ To our knowledge, there are thus far two reports of TLR2 expression on CD4⁺CD25⁺ T_{reg} cells in humans and mice.^{30,33} Although TLR expression patterns and magnitudes varied within the tissue and species,^{59,60} in rabbit, the selective high expression of TLR2 on conjunctiva-resident nT_{reg} cells is certainly a unique situation.

It has been shown that TLR ligands influence the immunosuppressive function of nT_{reg} cells either through a direct interaction with TLRs specifically expressed on T_{reg} cells³¹ or an indirect mechanism mediated by dendritic cells.⁶¹ In this study, TLR2 ligand LTA induced in vitro the proliferation of conjunctiva-resident T_{reg} cells in the presence of APCs and partially inhibited the immunosuppressive function of nT_{reg} cells. LTA alone failed to induce proliferation of T_{reg} cells. Interestingly, under identical experimental conditions, despite the moderate level of TLR9 expression on conjunctiva-resident T_{reg} cells, we found a strong inhibitory effect on T_{reg} cell proliferation and immunosuppressive function in vitro in the presence of the TLR9 ligand CpG₂₀₀₇. Accordingly, our in vivo data reflected that reducing T_{reg} cell proliferation by CpG₂₀₀₇ increased the proliferation of rabbit ocular CD4⁺ T_{eff} cells specific to HSV-1 gD peptides. In contrast to CpG₂₀₀₇, treatment of rabbit eyes by the TLR2 ligand (LTA) increases (i) the proliferation of T_{reg} cells, (ii) the mRNA level of IL-10 and IFN- γ and (iii) partially abrogates T_{reg} cell immunosuppressive function. This suggests that although LTA increases the pool of the T_{reg} population, those newly proliferated T_{reg} cells are functionally different from the original ones. These LTA-proliferated Treg cells not only lost their suppressive role by 30% (Fig 5B), they also expressing higher levels of IL-10 and IFN- γ mRNA, which is not conventional for nT_{reg} cells. A similar effect was reported by Suttmüller et al.^{30,33} The TLR2 ligand Pam3Cys-SK4 forced T_{reg} cells into the proliferative pathway, which was paralleled by a transient loss of suppressive activity, and they regained their suppression once cells were back in the resting phase.^{30,33} One possible explanation for the loss of suppression by LTA would be the higher production of IFN- γ by nT_{reg} cells when stimulated in the presence of LTA/anti-CD3/IL-2 (Fig. 4A). We have also detected higher expression of TGF- β in nT_{reg} cultures stimulated with LTA/anti CD3/IL-2 (Fig. 4C), thus confirming previous reports.⁶² However, controversial data about the role of TGF- β in nT_{reg} cells have also been reported.⁶³

The most prominent characteristics of nT_{reg} cells are their nonresponsiveness toward TCR stimulation in the presence of anti-CD3. However, our in vitro data (Fig. 3A) showed that the proliferation of nT_{reg} cells was slightly increased by anti-CD3 irrespective of the presence of either LTA or CpG or the addition of APCs in the culture. This suggested that nT_{reg} cells might not be equally anergic in all tissues and species. HSV infections occur at the ocular mucosal tissues and lead to self-limiting primary disease.⁶⁴ Foreign HSV antigens promote immune responses when coadministered with TLR ligands, which have been exploited as immune adjuvants promoting

protective T cells and antibody responses.^{65,66} Here we compared the potential efficacy of LTA and CpG as candidate immune adjuvants in the rabbit eye model and showed that although both CpG₂₀₀₇ and LTA are capable of inducing local CD4⁺ T_{eff} cell responses, these responses were better in the presence of LTA. Collectively, these results strongly suggest that TLR2 signals contribute to the activation and expansion of nT_{reg} cells (Fig. 3) and interfere with their suppressive activity (Fig. 5). Consequently, this led to an increase the magnitude of HSV-gD-peptide-specific T_{eff} cell responses in vivo (Fig. 6). As suggested by Conroy et al.⁶⁷ the nT_{reg} cell function is more like a “double-edged sword” that can lead to an outcome that is context dependent. Under these circumstances, greater understanding of the modulation and regulation of T_{reg} cells in the rabbit ocular immune system is critical to understand their complete biological role.

It has been shown that though TLR2, TLR8, or TLR9 ligation abrogates or reverses the immunosuppressive function of CD4⁺CD25⁺ T_{reg} cells, TLR2, TLR4, or TLR5 ligation enhances the suppressive capacity of CD4⁺CD25⁺ T_{reg} cells.⁶⁸ Leu et al.³³ recently showed that in the presence of a TLR2 agonist, such as the synthetic bacterial lipoprotein Pam3Cys-SK4, there was marked expansion of CD4⁺CD25⁺ T_{reg} cells, but their immunosuppressive function was temporarily abrogated. This study suggests that the downregulation of Foxp3 could be a putative mechanism for the abrogation of T_{reg} cell-mediated suppression.^{33,69} Treatment of CD4⁺CD25⁺ T_{reg} cells with heat shock protein 60 (the in vivo ligand of TLR2) suppressed target T_{eff} cells both by cell-cell contact mechanisms and by the secretion of cytokines such as TGF- β and IL-10.⁷⁰ TLR2 activation on T_{reg} cells using the synthetic ligand PAM3Cys, in combination with IL-2 and TCR triggering, can induce T_{reg} proliferation and results in temporary loss of suppression. On removal of the TLR2 ligand, the T_{reg} cells regained their immunosuppressive function.^{27,30,71,72} This discrepancy could possibly be explained by the nature of the TLR ligands used (PAM3Cys is an exogenous TLR1/2 ligand and hsp60 is an endogenous TLR2 ligand), the concentrations of the ligands used, or differences in the ways endogenous and exogenous ligands interact with TLR2. TLR8 also directly reverses the immunosuppressive function of CD4⁺CD25⁺ T_{reg} cells.²⁹ TLR8 is strongly and preferentially expressed on human T_{reg} cells compared with human T_{eff} cells.²⁹ Triggering TLR8 on T_{reg} cells resulted in the specific abrogation of suppression without affecting T_{reg} cell proliferation, though no effects on human T_{eff} cells were observed.²⁹ Applying siRNA technology to knockdown TLR8 on T_{reg} cells completely blocked the effect, demonstrating a crucial role for TLR8.²⁹

Several studies, using mouse- or human-derived CD4⁺CD25⁺ Treg, pointed to an interplay between TLRs and Foxp3.⁷³ In humans, the engagement of TLR5 by its ligand flagellin leads to the upregulation of Foxp3 expression in Treg and consequently enhances their suppressive activity.^{28,74,75} In contrast, TLR9 engagement by CpG did not affect Foxp3 expression in Treg although it abrogated their suppressive function.^{32,57,76} Although the effect of most TLR engagement on Foxp3 expression and Treg-suppressive function appears to be settled for many TLRs, conflicting results have been reported in mice on the effect of TLR2 engagement on Foxp3 expression on Treg. Some studies showed that Foxp3 expression was downregulated after TLR2 ligation by the synthetic bacterial triacylated lipopeptide Pam₃CSK4.^{30,33} Chen et al.⁷⁷ recently challenged this report by showing that treatment of Treg with Pam₃CSK4 neither affected the level of Foxp3 expression on Treg cells nor abolished its suppressive function. This discrepancy could be explained by the use of different TLR2 ligands in each study that might lead to the use of different heterodimers, the possibility that there are species-specific differences in the

control of Treg cell activity by same TLR2 ligands, and the fact that differences might be linked to the origin of Treg in each study (i.e., peripheral vs. mucosal-derived Treg). In contrast to the murine system, TLR2 ligand-mediated abrogation of human Treg function was not associated with a downregulation of Foxp3.⁷³ Nevertheless, our data (Fig. 7) showed some increase in Foxp3 expression when TLR9 was engaged with CpG. However, no significant changes in Foxp3 expression were observed when TLR2 was engaged with LTA. Interestingly, the engagement of TLR2 with a different ligand Pam3CSK4 significantly decreased the Foxp3 expression on rabbit Treg.

In humans, the LTA ligand engages the TLR2/TLR6 combination, whereas Pam3Cys engages TLR2/TLR1.⁷⁸ In mice, the Pam3CSK4 ligand engages either the TLR2/TLR1 combination or TLR2 independently of TLR1. Furthermore, Hajjar et al.⁷⁹ reported on the recognition of triacylated lipopeptides in a TLR2/TLR1-dependent fashion in human cells. In the presence of the Pam3CSK4 ligand, a stable heterodimer is formed between TLR2 and TLR1.⁸⁰ Therefore, though both LTA and Pam3CSK4 are ligands for TLR2, the former engages TLR2/TLR6 and the latter engages TLR2/TLR1 combinations. Nevertheless, recent data by Carpenter and colleagues⁸¹ recently showed that engagement of either TLR2/TLR1 or TLR2/TLR6 combinations activate the same NF- κ B downstream signal pathway. To our knowledge, the present study is first to report the expression and functional engagement of any TLR on rabbit Treg cells. We demonstrated that engagement of TLR-2 on rabbit Treg cells with LTA and Pam3CSK4 differentially affects the expression of Foxp3 (Fig. 7), suggesting that, as with human Treg cells, these ligands might interact with either TLR2/TLR1 or TLR2/TLR6 combination. Given that most of the TLR data come from mice and humans, there is a possibility that LTA and Pam3CSK4 ligands might have different effects on rabbit TLR2/TLR-1 and TLR2/TLR-6 combinations and downstream intracellular pathways. This will be the subject of future reports.

In conclusion, the results reported here suggest a link between the TLR2 and its exogenous ligand LTA and how nT_{reg} cells in ocular mucosal tissue regulate cellular immunity in response to viral antigen. These findings might be useful in clinical settings to enhance the efficacy of immunotherapy directed toward infectious diseases. CD4⁺ T cell help is required for the generation of primary CTL responses and the promotion of protective CD8⁺ memory T cell development. Recent studies^{82,83} demonstrated a previously unappreciated role of CD4 help in mobilizing effector CD8⁺ CTL to the peripheral sites of infection. Our results reveal a previously unappreciated role of CD4⁺CD25⁺ nT_{reg} cells in interfering with CD4⁺CD25⁺ T_{eff} cells at the ocular mucosal sites of herpes infection, where they might help to eliminate herpes-infected cells. Ongoing and future research will further delineate the mechanisms involved in interactions between these two subsets of T cells and innate receptors and will pave the way for developing better prophylactic or therapeutic strategies for control of infectious, allergic, and autoimmune ocular diseases.

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