

In Vitro Expanded CD4⁺CD25⁺Foxp3⁺ Regulatory T Cells Maintain a Normal Phenotype and Suppress Immune-Mediated Ocular Surface Inflammation

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PURPOSE. To determine whether in vitro expanded CD4⁺CD25⁺Foxp3⁺ regulatory T cells can suppress immune-mediated ocular surface inflammation in a mouse model of dry eye.

METHODS. C57BL/6 or BALB/c mice were exposed to a dry, desiccating environment produced by maintaining low humidity (<40%), injections of scopolamine, and air flow produced by a fan. CD4⁺CD25⁺ regulatory T cells were isolated and expanded in vitro in the presence of rmlL-2 and beads coated with anti-CD28 and anti-CD3. In vitro expanded regulatory T cells were phenotypically compared with freshly isolated regulatory T cells by flow cytometry and immunofluorescence. T-cell-deficient nude mice were reconstituted with CD4⁺ T-effector cells from donor mice exposed to a desiccating environment for 5 days, in combination with or without freshly isolated or in vitro expanded regulatory T cells. Tear cytokine levels were determined by a multiplex bead-based immunoassay.

RESULTS. In vitro regulatory T cells maintained normal levels of CD4⁺, CD25⁺, and intracellular Foxp3⁺, as determined by flow cytometry and immunohistochemistry. Freshly isolated and in vitro regulatory T cells were titrated in the presence of CD4⁺ pathogenic T cells (CD4⁺Path T cells) in reconstitution experiments and most efficiently ablated tear cytokine levels and conjunctival cellular infiltration at a ratio of 1:1 (T Regs: CD4⁺Path).

CONCLUSIONS. Regulatory T cells expressed CD4⁺, CD25⁺, and intracellular Foxp3⁺ at normal levels and retained their inhibitory function after in vitro expansion, providing a useful tool to determine the mechanism regulatory T cells use to sustain a homeostatic environment on the ocular surface. (*Invest Ophthalmol Vis Sci.* 2008;49:5434–5440) DOI:10.1167/iovs.08-2075

The importance of regulatory T cells in suppressing inappropriate inflammation has become increasingly clear over the past several years. These T cells can inhibit a wide variety

of autoimmune and inflammatory diseases, but determination of the mechanism of this suppression was limited because of the low percentage of regulatory T cells present in vivo. CD4⁺CD25⁺Foxp3⁺ regulatory T cells make up 5% to 10% of the mouse CD4⁺ T-cell population.^{1–5} Because of this low cell number and the inherent cell loss in the isolation process, mouse regulatory T cells were expanded in vitro to examine the mechanisms used by the cells to maintain an immune-tolerant environment on the ocular surface.

Dry eye affects >10% of the population within the age range of 30 to 60 and >15% of the population over 65 years of age⁶ and is one of the leading reasons patients seek ophthalmic care. The clinical response in humans with dry eye include ocular discomfort, blurred and fluctuating vision, altered corneal barrier function, corneal ulceration, and increased proinflammatory cytokines and proteases in the tear film.⁷ Cellular infiltration of CD4⁺ T cells into the conjunctiva and CD4⁺ T cell cytokines in the tear film can be detected in patients with dry eye and in animal models of dry eye.^{8–17}

Previously, we reported that CD4⁺ T cells are the main effector cells in experimental dry eye disease.⁸ For these studies, an adoptive transfer model was used in which euthymic mice were placed in a dry, desiccating stress environment (DS) induced by low humidity (<40%), injections of scopolamine, and constant air flow produced by a fan.¹⁸ This animal model of dry eye is characterized by decreased tear production, decreased conjunctival goblet cells, increased cellular infiltration into the lacrimal functional unit, and a T_H1 phenotype.^{16–18} Adoptive transfer of pathogenic CD4⁺ T cells from the superficial cervical lymph node of euthymic mice subjected to DS results in decreased tear production, loss of goblet cells, secretion of proinflammatory cytokines on the ocular surface, and severe inflammatory cellular infiltration of the lacrimal functional unit.⁸ These experiments emphasize the importance of CD4⁺ T cells in dry eye and offer therapeutic targets.

Adoptive transfer of pathogenic CD4⁺ T cells into euthymic (wild-type) mice does not result in significant disease suggesting that CD4⁺CD25⁺ regulatory T cells play a key modulatory role in dry eye disease. Elimination of CD25⁺ T cells in euthymic mice before their receiving adoptively transferred pathogenic CD4⁺ T cells results in dry eye disease.⁸ By contrast, reconstitution of T cell-deficient nude mice with pathogenic CD4⁺ T cells and CD4⁺CD25⁺Foxp3⁺ regulatory T cells results in ablation of disease transfer.⁸ These results demonstrate the importance of regulatory T cells in maintaining a homeostatic environment at the ocular surface.

We previously reported greater severity of keratoconjunctivitis in C57BL/6 mice. It was possible to induce dry eye, albeit milder, on the BALB/c background. It has been reported that BALB/c mice have a larger repertoire of regulatory T cells and that these regulatory T cells are more efficient at suppressing CD4⁺CD25⁺ effector T cells than are C57BL/6 mice.¹⁹ It was our goal in this study to determine whether there were any significant differences in the ability of freshly isolated or in

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vitro-expanded regulatory T cells to mitigate dry eye disease between these two strains of mice.

Two criteria must be met for investigating ex vivo expansion of regulatory T cells. First, it must be determined whether regulatory T cells can be expanded in vitro while maintaining the regulatory T-cell phenotype. If these cells can be expanded and maintain a normal phenotype, then the second requirement is to determine whether these expanded cells can abrogate disease in a nude mouse recipient. If regulatory T cells from this model can be expanded and still ameliorate disease on adoptive transfer, this in vitro model would provide a unique tool for evaluating ways to induce regulatory T-cell activity in vivo to prevent chronic ocular surface inflammation.

MATERIALS AND METHODS

Mice

Female euthymic BALB/c (BALB/cAnNCrI) and athymic BALB/c nude mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). C57BL/6 (C57BL/6NTac) and B6.Cg/NTac-Foxn1^{nu}NE9 were purchased from Taconic, Inc. (Germantown, NY). Mice were used at 6 to 10 weeks of age. Animal studies approval was obtained from the Allergan Animal Care and Use Committee. All studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Desiccating Stress (DS) in Mice

Dry eye was induced by treating mice with SC injections of scopolamine hydrobromide (0.5 mg/0.2 mL; Sigma-Aldrich, St. Louis, MO) three times a day alternating between the left and right flanks. Up to four mice were placed in a cage containing perforated plastic screens on two sides of the cage to permit airflow from fans (one fan on each side of the cage) for 16 h/d in a hood (AirClean Systems, Raleigh, NC). Room humidity was kept below 40%. DS was administered for 5 consecutive days.^{8,18}

In Vitro Expansion of Regulatory T Cells

Superficial cervical lymph node cells and spleen cells from DS-treated C57BL/6 or BALB/c mice were obtained by gently processing between the ends of two sterile frosted slides. CD4⁺CD25⁺ T cells were isolated by using a mouse regulatory T cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol.

A modified protocol adapted from Tang et al.²⁰ was used to expand regulatory T cells in vitro. Isolated CD4⁺CD25⁺ regulatory T cells were placed in culture at 2×10^6 cells/2 mL/well (24 well plate) in RPMI 1640 medium with 7% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, 2 mM glutamine, 50 μ M 2-ME, 1 mM sodium pyruvate, 0.01 mM nonessential amino acids, and 10 mM HEPES. Anti-mouse CD3- and anti-mouse CD28-coated 4 μ m polystyrene magnetic beads (Dyna; Invitrogen, Carlsbad, CA) were added at a 1:1 ratio. Recombinant mouse IL-2 (20 ng/mL; R&D Systems, Minneapolis, MN) was added to each well. The cultures were monitored daily and maintained at 0.7 to 1×10^6 cells/mL. At the end of the seventh day in culture, a magnet was used to remove the beads. The cells were analyzed for CD4⁺, CD25⁺, and intracellular Foxp3⁺ expression by flow cytometry. Supernatants from in vitro regulatory T cells were analyzed with a bioassay system (Luminex Corp., Austin, TX) for TGF- β and IL-10 cytokine levels.

Flow Cytometry

To determine surface expression of CD4 and CD25, 5×10^5 cells/100 μ L FACS buffer (PBS, 0.02% sodium azide [Sigma-Aldrich] and 2% bovine serum albumin) were incubated, first with 1 μ g/tube of purified anti-mouse CD16/32 (BD-Pharmingen, San Diego, CA) and placed on ice for 10 minutes to eliminate potential Fc binding of the primary antibody. The cells were then incubated with biotin rat anti-mouse

CD4 (cloneGK1.5) and PE labeled anti-mouse CD25 (IL-2 receptor α chain, p55, clone PC61 at concentrations of 1μ g/ 5×10^5 cells for 30 minutes at 4°C). The isotype control antibodies used were biotin rat IgG2b, κ and PE rat IgG1, λ . The cells were washed two times in FACS buffer and resuspended at 5×10^5 cells/100 μ L buffer. The tubes containing biotin-labeled antibody received 1.5 μ L of an accessory staining pigment (Streptavidin PerCP; BD-Pharmingen) and were placed on ice for 20 minutes in the dark. For intracellular staining of Foxp3, the cells were resuspended in 350 μ L of 2% formaldehyde (methanol free) and incubated 15 minutes at 4°C in the dark. They were washed in FACS buffer and resuspended in 0.5% saponin (Sigma-Aldrich) in FACS buffer. One microgram per tube of anti CD16/32 was added for 10 minutes on ice in the dark; 1 μ g/tube of FITC anti-mouse Foxp3 (clone PCH101, eBiosciences, San Diego, CA) or 1 μ g/tube of FITC rat IgG2a isotype (eBiosciences) was added to the appropriate cell groups and put on ice for 30 minutes in the dark. The cells were washed with 1 mL 0.5% saponin buffer and, resuspended in 500 μ L of flow cytometry buffer. Expression was analyzed (FACSCalibur with CellQuest software; BD Biosciences, Mountain View, CA).

Adoptive Transfer of Dry Eye

Spleen or superficial cervical lymph node cells were collected after 5 days of exposure to DS and enriched for CD4⁺ T cells or CD4⁺CD25⁺ T regulatory cells by using the appropriate magnetic microbeads (MACS System; Miltenyi Biotec) according to the manufacturer's instructions. The cells were analyzed by flow cytometry to determine T-cell purity (87% purity) and intraperitoneally (IP) injected into nude mouse recipients at 5×10^6 CD4⁺ T cells and in some cases in separate IP injections of 5×10^6 CD4⁺CD25⁺ T regulatory cells. Tissues from recipient athymic nude mice were collected 60 hours after adoptive transfer of cells.

Bioassay

For tear collection, 1.5 μ L of PBS was placed on each eye, and then 1 μ L of tear was collected from both eyes and placed in 8 μ L of cytokine assay buffer (Beadlyte; Millipore, Billerica, MA). Buffer and tear fluid were collected by capillary action using a 1- μ L volume glass capillary tube (Drummond Scientific, Broomhall, PA) that was placed in the tear meniscus of the lateral canthus. Samples were frozen at -80°C until the time of assay. Cytokine levels in tears (IL-12, TNF- α , and IFN γ) and supernatants of in vitro expanded regulatory T cells (TGF- β , IL-10) were analyzed by using the corresponding cytokine pairs (Beadmate; Millipore). For the bioassay (Luminex Corp.), a 96-well filter plate (Millipore) was prewetted with 25 μ L of cytokine assay buffer (Beadlyte; Millipore). A vacuum manifold (Millipore) was used to aspirate the buffer from the wells. For tears, 10 μ L of diluted (2 μ L tears to 8 μ L Beadlyte buffer) tear sample was placed in each well. The beads (25 μ L) were pipetted into the wells. Standard curves for each cytokine were generated in duplicate by placing 10 μ L of the appropriate dilution of standards purchased from Upstate (Lake Placid, NY). The plate was incubated overnight with gentle shaking in the dark at 4°C. The plate was washed with cytokine assay buffer and wash buffer was eliminated by using a vacuum manifold (Millipore). Twenty-five microliters of the appropriate biotin-conjugated secondary antibody (Upstate) was added to each well for 90 minutes at room temperature with gentle shaking. The beads were incubated with streptavidin-phycoerythrin (1:25 dilution in Beadlyte assay buffer) for 30 minutes at room temperature with gentle shaking. The beads were washed, resuspended in 125 μ L of cytokine assay buffer, and analyzed (system 100; Luminex Corp.). The mean fluorescence intensities obtained from 50 beads per cytokine minimum were analyzed (Beadview software; Upstate). Standard curves were generated (eight data points including a zero standard run in duplicate) using a four- or five-parametric logistic curve. R^2 was between 0.99 and 1. Data are expressed in picograms or nanograms per milliliter.

The supernatants were tested on a 50- μ L volume of sample. All beads, biotinylated secondary antibodies, and streptavidin were added

in 25- μ L volumes, as described earlier. The IL-10 and TGF- β beadmates were tested in separate assays. TGF- β is present in a latent dimer form associated with a binding protein. Acid activation induces release of TGF- β in a biologically active form. Supernatants being tested for TGF- β levels were brought to between pH 1 and 2 with 1 N HCl and incubated at 4°C for 1 hour. The sample was neutralized with 1 N NaOH to reach pH 7. The media were processed through the activation step and run as a control due to the reported levels of TGF- β found in serum.

Immunofluorescence

Freshly isolated or 7-day in vitro regulatory T cells were prepared by cytopsin for immunofluorescence staining using specific rat anti-mouse CD4 (L3T4) monoclonal antibody (clone H129.19; 2 μ g/mL; BD-Pharmingen), rat anti-mouse CD25 (clone 7D4; 5 μ g/mL; BD-Pharmingen), and anti-mouse/rat Foxp3 (clone FJK-16s; 5 μ g/mL; eBioscience). FITC donkey anti-rat (Invitrogen-Molecular Probe, Eugene, OR) was used as the secondary for CD4 and Foxp3 staining. Freshly isolated cells were labeled with PE anti-CD25 as outlined earlier for the isolation procedure. Sections for Foxp3 were fixed in cold acetone for 10 minutes. Sections for CD4 and CD25 were fixed in 4% paraformaldehyde for 10 minutes. Slides were washed in PBS (for Foxp3, 0.1% Triton-S 100 in PBS) and blocked with 2% donkey serum for 15 minutes at room temperature, and the primary antibody was added (anti-CD4, 2 μ g/mL, anti-CD25, 5 μ g/mL, and Foxp3, 5 μ g/mL). The slides were washed, blocked again with 2% donkey serum, and incubated with conjugated secondary antibody (2 μ g/mL), washed, and mounted.

Statistical Analysis

Values for cell counts in tissue specimens and cytokine levels in tears were evaluated by either Student's *t*-test or ANOVA.

RESULTS

In Vitro Regulatory T Cell Proliferation

Because of the limited number of regulatory T cells in vivo and the inherent loss of cells through the isolation procedure, it became necessary to expand regulatory T cells in vitro to begin to determine the mechanism of action used by these cells to inhibit ocular inflammation. A modified protocol adapted from Tang et al.²⁰ was used to expand regulatory T cells in vitro. Spleen and superficial cervical lymph node regulatory T cells were isolated using the magnetic isolation system (Miltenyi). Both C57BL/6 and BALB/c CD4⁺CD25⁺ T cells expanded approximately 10-fold over 7 days in culture with stimulation by anti-CD3 and anti-CD28 coated beads and IL-2 (20 ng/mL) (Fig. 1).

Flow Cytometric and Immunohistological Comparison of Freshly Isolated and In Vitro-Expanded Regulatory T Cells

Regulatory T cells were analyzed by flow cytometry before and after 7 days in vitro. In vitro regulatory T cells from both C57BL/6 and BALB/c mice, when compared with freshly isolated regulatory T cells, had a similar percentage of cells expressing CD4 (Fig. 2A). CD25 expression was higher on day 7 compared with the freshly isolated regulatory T cells for both C57BL/6 (78% freshly isolated, 98% in vitro) and BALB/c (85% freshly isolated, 98% in vitro). The intensity of CD25 per cell increased in both mouse strains. Polarization of the regulatory T cells due to the growth conditions at the 7-day time point may account for the higher expression of CD25 seen in the regulatory T cells cultured in vitro. The percentage of Foxp3 cells was comparable between freshly isolated and in vitro regulatory T cells. However, a small decrease in Foxp3 intensity per cell was detected. The detection of comparable levels of CD4⁺, CD25⁺, and Foxp3⁺ cells in freshly isolated and in

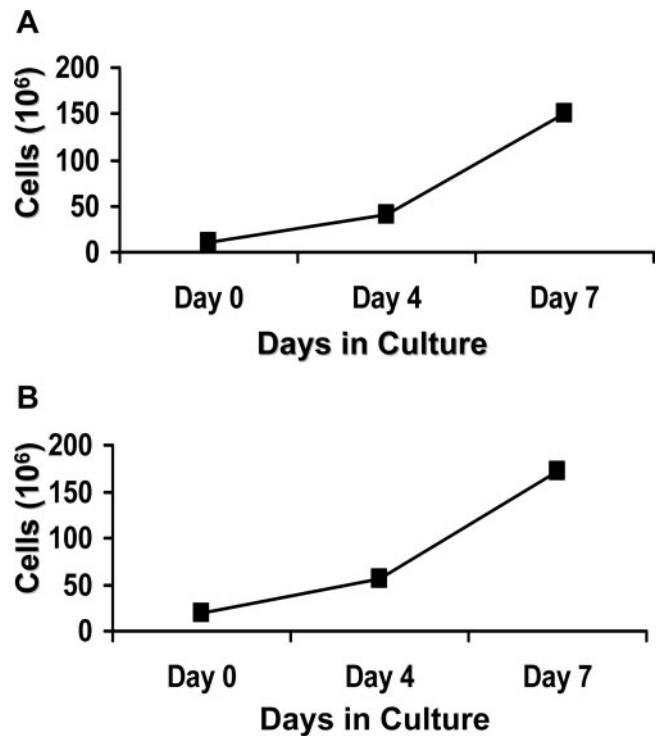


FIGURE 1. C57BL/6 and BALB/c regulatory T cells can be expanded in vitro. C57BL/6 (A) or BALB/c WT (B) superficial cervical lymph node and spleen cells were run over a CD4⁺CD25⁺ separation column (MACS Miltenyi) to isolate regulatory T cells. The CD4⁺CD25⁺ cells were plated at 1×10^6 cells/mL in a 2-mL volume and incubated with equal numbers of anti-CD3 and anti-CD28 coated beads and 20 ng/mL of recombinant mouse IL-2. Both C57BL/6 and BALB/c CD4⁺CD25⁺ T cells expanded approximately 10-fold after 7 days in culture.

vitro-expanded populations of regulatory T cells was further supported by immunofluorescence studies. In both the C57BL/6 and BALB/c backgrounds, the two groups had comparable levels of fluorescence for the three markers tested (Figs. 2B, 2C).

In Vitro Regulatory T Cells Secrete TGF- β and IL-10

The ability of expanded regulatory T cells to secrete anti-inflammatory cytokines was examined by the bioassay system (Luminex Corp.). Cell supernatants from C57BL/6 or BALB/c in vitro regulatory T cells were collected on day 7 and analyzed for the presence of TGF- β and IL-10, two immunosuppressive cytokines secreted by regulatory T cells. TGF- β was present in the supernatants at significantly increased levels compared with the media TGF- β levels (Fig. 3). Expanded regulatory T cells also secreted the immunosuppressive cytokine IL-10 at high levels (Fig. 3). These results suggest that cultured regulatory T cells maintain a normal regulatory T-cell phenotype for at least 7 days.

Reconstitution of T-Cell-Deficient Nude Mice with CD4⁺ T Cells and In Vitro Expanded CD4⁺CD25⁺Foxp3⁺ Regulatory T Cells

The ability of in vitro-expanded regulatory T cells to suppress dry eye in athymic mice after in vivo reconstitution of T-cell-deficient nude mice with pathogenic donor CD4⁺ T cells from mice exposed to DS was evaluated. The ratios of in vitro regulatory T cells to CD4⁺ pathogenic T cells tested were 1:2 and 1:1 for C57BL/6 mice and 1:4, 1:2, and 1:1 for the BALB/c mice. The ratios of

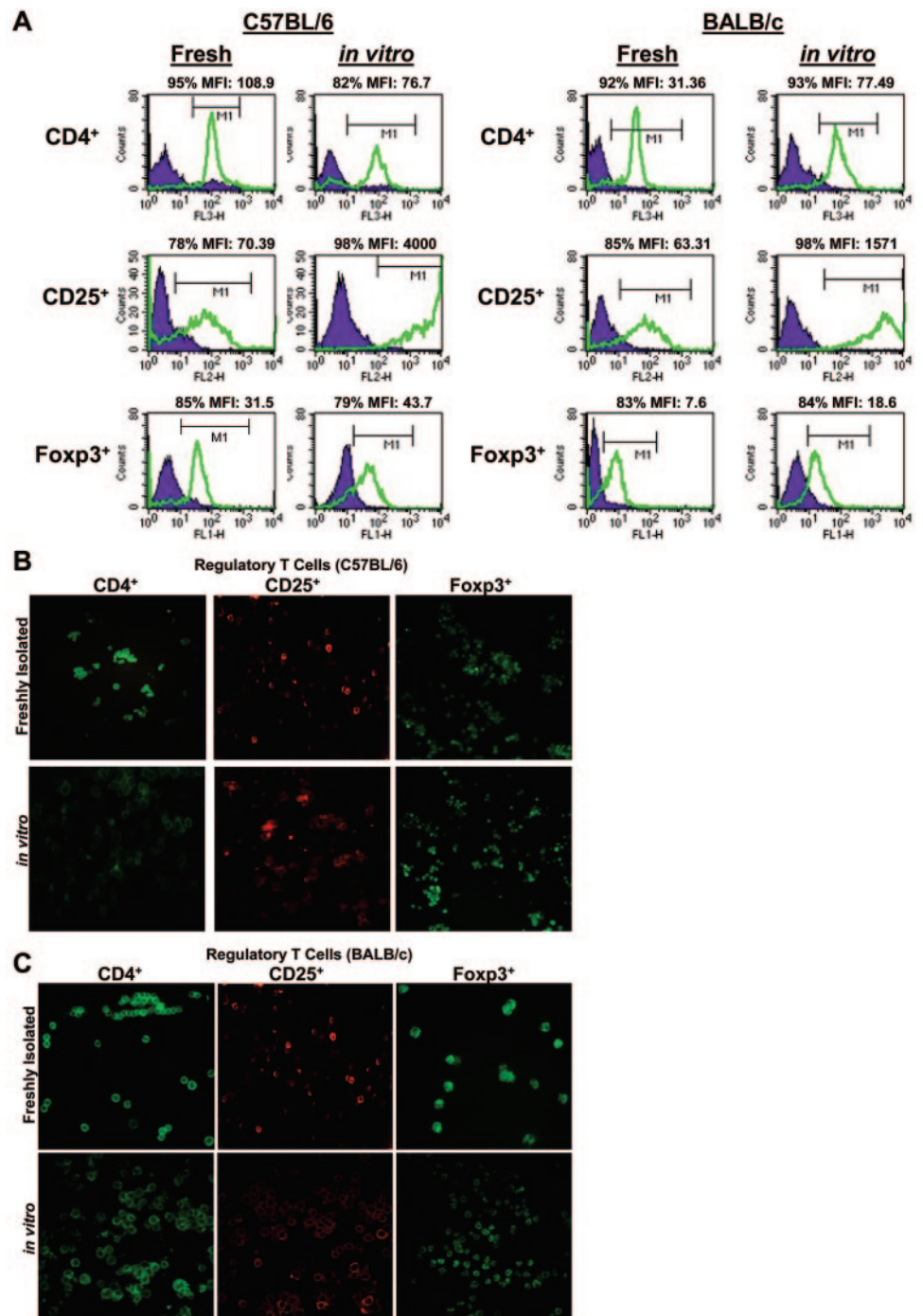


FIGURE 2. (A) Freshly isolated regulatory T cells and in vitro regulatory T cells (7 days in culture) were stained for cell membrane expression of CD4 and CD25, or stained intracellularly for Foxp3. The cells were analyzed by flow cytometry. In vitro regulatory T cells maintained a comparable or higher expression of all three molecules analyzed, providing evidence that regulatory T cells cultured in vitro for 7 days keep the phenotype expressed by freshly isolated regulatory T cells. Cells were positive for expression as represented by the shift to the right (green line). Purple: the appropriate isotype control. The percentage of positive cells and geo mean fluorescence intensity are shown. (B, C) Immunofluorescent staining of freshly isolated and in vitro (7 days) regulatory T cells from both C57BL/6 and BALB/c mice for CD4⁺, CD25⁺, and Foxp3⁺ revealed similar levels of expression in all groups. Freshly isolated regulatory T cells were stained with a PE-anti-CD25 antibody as part of the isolation procedure. FITC-conjugated antibodies were used for CD4⁺ and Foxp3⁺ and PE-conjugated CD25⁺ antibody, to determine expression levels on the 7-day in vitro cells.

freshly isolated regulatory T cells to CD4⁺ pathogenic T cells tested were 1:4 and 1:1 for both strains of mice.

C57BL/6 WT mice were exposed to DS for 5 days. CD4⁺ T cells from the spleens and superficial cervical lymph nodes of these mice were collected and adoptively transferred (5×10^6 cells/mouse) in the absence or presence of either 2.5×10^6 (1:2) or 5×10^6 (1:1) in vitro regulatory T cells into C57BL/6 nude mice. Histopathologic analysis of nude mouse recipients of both CD4⁺ pathogenic T cells and in vitro regulatory T cells at a 1:1 ratio revealed a significant decrease in conjunctival cellular infiltration compared with the heavy cellular infiltration seen in the conjunctiva of mice receiving only pathogenic DS-treated CD4⁺ T cells (Fig. 4). However, there were no differences in mononuclear cell counts in the conjunctivae of

nude mice receiving a ratio of 1:2 (T Regs:CD4⁺Path) compared to mice receiving pathogenic CD4⁺ T cells alone (Fig. 4). Athymic mice reconstituted with CD4⁺ pathogenic T cells and freshly isolated regulatory T cells (1:4 and 1:1) had a significant decrease in conjunctival cellular infiltration.

Tear collection for bioassay was taken on the morning of day 3 from the nude mice recipients of different ratios of regulatory T cells and CD4⁺Path and analyzed for the proinflammatory cytokines IL-12, IFN- γ , and TNF- α . Mice receiving adoptive transfer of CD4⁺Path T cells had elevated tear levels of IL-12, IFN- γ , and TNF- α (Fig. 4). Reconstitution of nude mice with ratios of in vitro regulatory T cells:CD4⁺Path at 1:1 resulted in a significant decrease in tear levels of IL-12, IFN- γ , and TNF- α (Fig. 4). IFN- γ and TNF- α tear levels were significantly

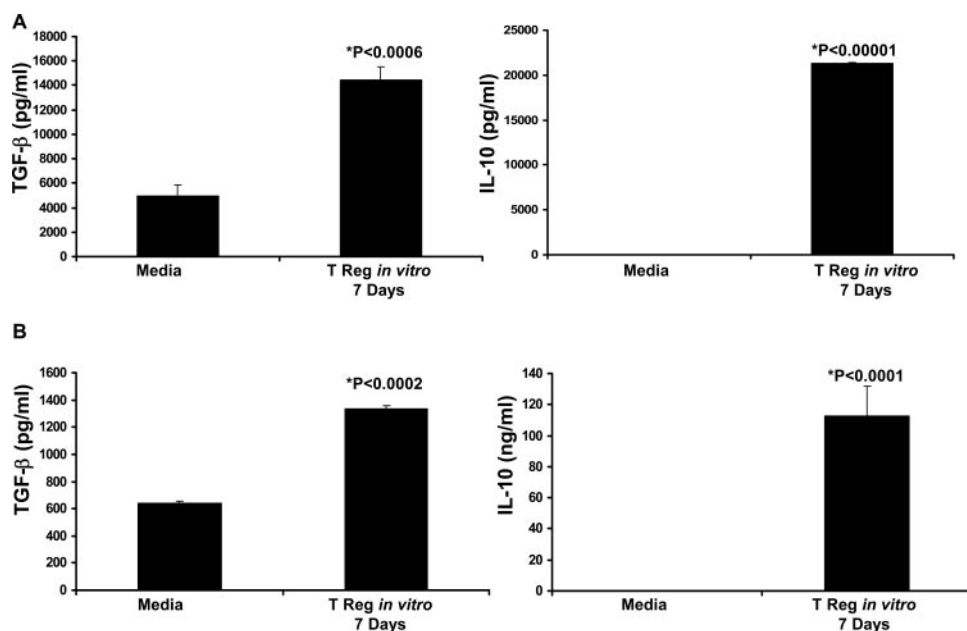


FIGURE 3. In vitro regulatory T cells (7 days in culture) secreted the immunosuppressive cytokines TGF- β and IL-10 into the supernatant (A) C57BL/6 background and (B) BALB/c background. Cell supernatants were analyzed by a bioassay system (model 100; Luminex).

decreased in recipients of 1:4 and 1:1 ratios of freshly isolated regulatory T cell:CD4⁺Path (Fig. 4).

The dose titration of freshly isolated and in vitro regulatory T cells was also tested on the nude BALB/c recipient mouse tears for the proinflammatory cytokines IL-12, IFN- γ , and TNF- α . Mice receiving adoptively transferred CD4⁺Path had elevated levels of tear IL-12, IFN- γ , and TNF- α (Fig. 5). Reconstitution of nude mice with ratios of in vitro regulatory T

cells:CD4⁺Path at 1:4, 1:2, and 1:1 resulted in significant reduction in the tear levels of IL-12, and TNF- α (Fig. 5).

DISCUSSION

The ocular surface is constantly challenged by everyday environmental stress such as low humidity, allergy, wind, and

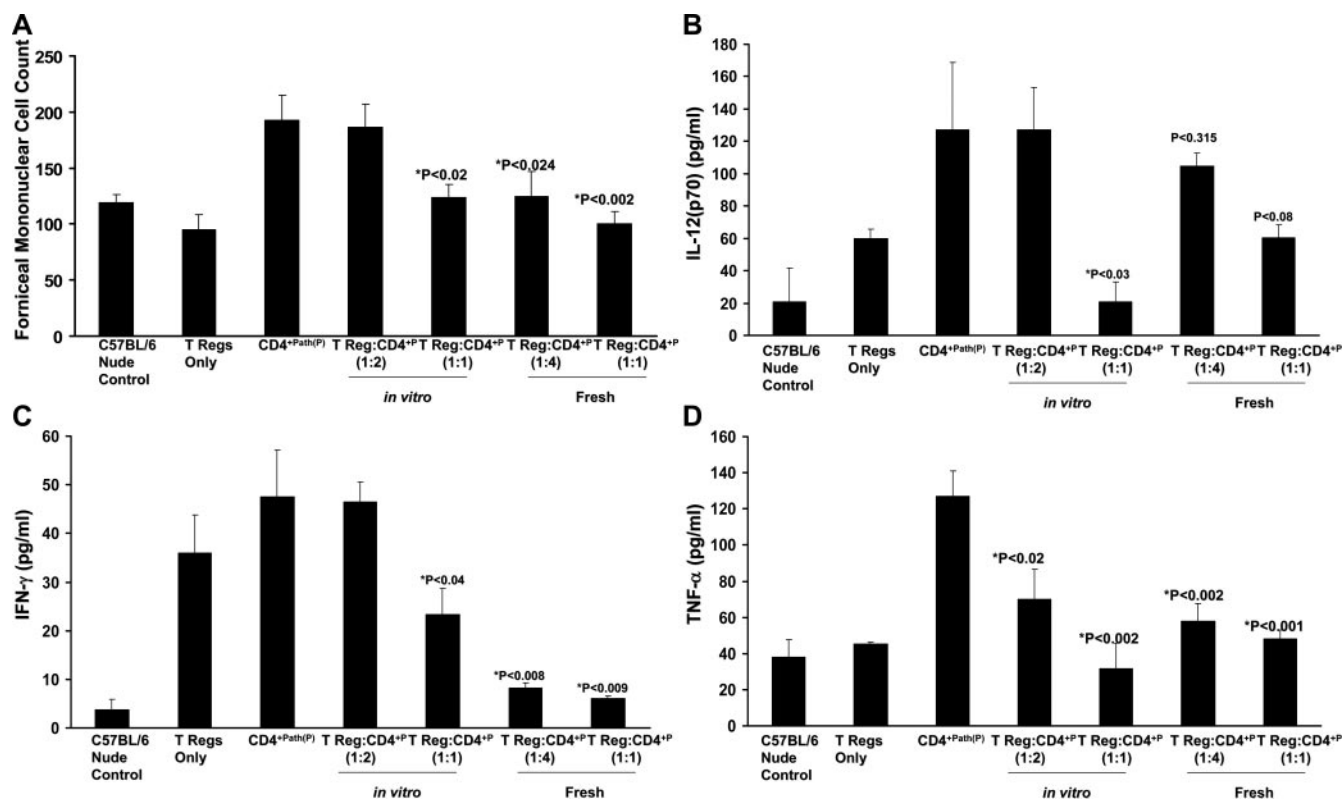


FIGURE 4. Conjunctival mononuclear cell counts and tear levels of inflammatory cytokines from nude mouse recipients (C57BL/6 background) of adoptive transfer of pathogenic CD4⁺ T cells in the absence or presence of in vitro or freshly isolated regulatory T cells ($n = 5$). Probabilities are in comparison with the CD4⁺Path-only recipients.

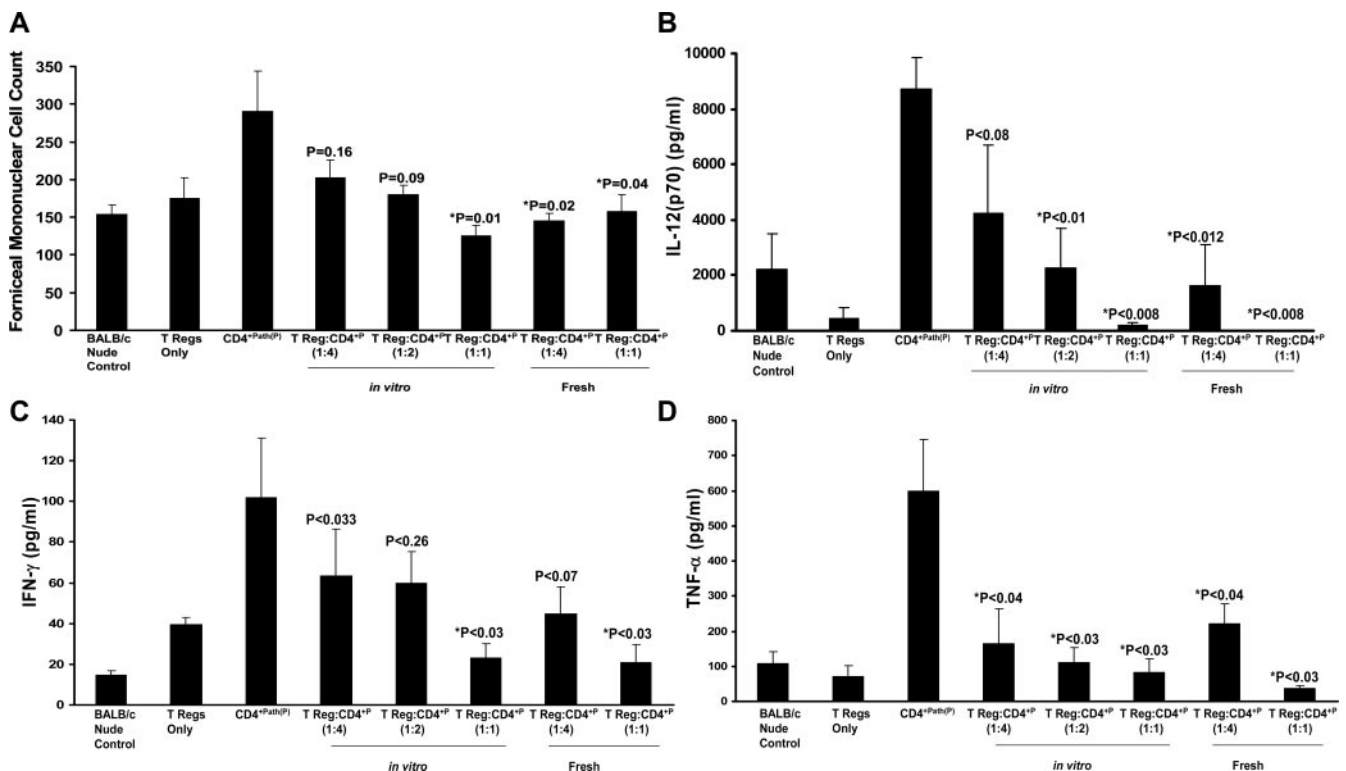


FIGURE 5. Conjunctival mononuclear cell counts and tear levels of inflammatory cytokines from nude mouse recipients (BALB/c background) of adoptive transfer of pathogenic CD4⁺ T cells, in the absence or presence of in vitro or freshly isolated regulatory T cells. Probabilities are in comparison with the CD4⁺Path-only recipients.

contact lenses. Therefore, there must be mechanisms in place to inhibit the initiation of inflammation related diseases such as dry eye. It is well documented that the CD4⁺CD25⁺Foxp3⁺ population of regulatory T cells modulates immune responses. To determine how regulatory T cells mechanistically prevent the development of dry eye, it became necessary to expand these cells in vitro.

Regulatory T cells cultured for 7 days in the presence of rmIL-2 and beads coated with anti-CD28 and anti-CD3 maintained their production of the anti-inflammatory cytokines IL-10 and TGF-β and functioned similarly to freshly isolated regulatory T cells in suppressing dry eye disease. The cultured regulatory T cells produced in vivo immunosuppressive activity that was comparable to freshly isolated regulatory T cells on a per cell basis. In our studies, inflammatory responses were ablated most efficiently at 1:1 ratios of T Regs:CD4⁺Path for both C57BL/6 and BALB/c mice. In BALB/c mice, IL-12(p70) and TNF-α tear levels were significantly inhibited at a 1:2 ratio. Decreases, while not significantly inhibited, were detected in the mononuclear cells counts and IFN-γ levels in the BALB/c mice at a 1:2 ratio. In C57BL/6 mice, only TNF-α tear levels were significantly inhibited at a 1:2 ratio. These results suggest that in our studies, BALB/c regulatory T cells are more efficient at ablating CD4⁺CD25⁺ effector T-cell responses compared with the C57BL/6 strain of regulatory T cells.

The exact mechanisms used by regulatory T cells to suppress inflammation are currently under intense investigation. The mechanism of action used by regulatory T cells to prevent ocular surface inflammation as well as other T-cell-mediated diseases has not been defined. There have been several suggested methods by which regulatory T cells may function to inhibit T effector cell responses. One mechanism is through the secretion of the anti-inflammatory cytokines TGF-β and IL-10. If this were the case, future experiments would be

needed to demonstrate that neutralization of IL-10 or TGF-β in vivo restores tear production of proinflammatory cytokines in recipients of pathogenic T cells and regulatory T cells. Several in vivo models suggest that secretion of these cytokines may be the main mechanism of regulatory T-cell activity.^{21–22}

Animal models for dry have been developed, but not all of these models assume an autoimmune component.²³ Our previously published results⁸ suggest that DS results in the exposure of shared antigenic epitopes in the ocular surface that induce pathogenic CD4⁺ T cells that produce lacrimal keratoconjunctivitis, which, under homeostatic conditions, would be restrained by regulatory T cells. Antigen-specific regulatory T cells have been more effective in animal models of inflammatory disease.^{24–26} The specific antigen responsible for initiating dry eye disease has not been clearly defined. α-Fodrin was identified as a candidate autoantigen in Sjögren's syndrome.²⁷ If the exact antigen(s) responsible for causing dry eye could be identified, then regulatory T cells could be expanded in vitro that are specific for the ocular surface dry eye antigen.

The ability to expand regulatory T cells in vitro is important because of the limited number of regulatory T cells in vivo. It allows for investigation of regulatory mechanisms and provides an experimental platform on which to evaluate candidate therapeutics. Therapeutic manipulation of regulatory T cells is a potential treatment for immune-mediated ocular surface diseases.

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