Induction of Eye-Derived Tolerance Does Not Depend on Naturally Occurring CD4\(^+\)CD25\(^+\) T Regulatory Cells

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**PURPOSE.** Regulatory CD4\(^+\) T cells (T regs) arise in the spleens of mice with anterior chamber–associated immune deviation (ACAID), an eye-derived tolerance evoked by injection of antigen into the ocular anterior chamber (AC). The current study was conducted to investigate the possibility that these T regs express CD25 and are derived from natural CD4\(^+\)CD25\(^+\) T cells.

**METHODS.** Naïve T cells from DO11.10 mice were activated in vitro by ovalbumin (OVA)-pulsed, TGF\(/\)H9252-induced ACAID in mice depleted of CD25\(^+\) T regs. CD4\(^+\)CD25\(^+\) T regs before they were injected into OVA-immunized mice or examined for mRNA expression of the regulatory T-cell transcription factor Foxp3. In addition, before AC injection of OVA, systemic depletion of CD25\(^+\) T cells was performed with injections of anti-IL-2 receptor antibody into the mice.

**RESULTS.** OVA-specific T cells from DO11.10 mice expressed CD25 when exposed to OVA-pulsed, TGF\(/\)H-treated APCs, even when the DO11.10 T cells were depleted of CD25\(^+\) cells before their in vitro stimulation. In addition, DH was suppressed in naïve mice that were injected with CD4\(^+\)CD25\(^+\) or CD4\(^+\)CD25\(^-\) ACAID T cells. The CD4\(^+\)CD25\(^-\), but not the CD4\(^+\)CD25\(^+\), ACAID T reg expressed Foxp3. Finally, OVA induced ACAID in mice depleted of CD25\(^+\) T cells.

**CONCLUSIONS.** Some of the CD4\(^+\) T regs of ACAID arise from CD25\(^+\) precursors, and the induction of ACAID is not dependent on the presence of natural CD4\(^+\)CD25\(^+\) T regs. (Invest Ophtalmol Vis Sci. 2006;47:1047–1055) DOI:10.1167/iovs.05-0110

A deviant form of systemic immunity emerges when antigens are introduced into the anterior chamber (AC) of the eye.\(^{1,2}\) This form of eye-derived tolerance termed anterior chamber–associated immune deviation (ACAID) is mediated by at least two populations of antigen-specific T regulator cells (T regs): a CD4\(^+\) population that suppresses the induction of delayed hypersensitivity (an effector regulator), and a CD8\(^+\) population that inhibits the expression of delayed hypersensitivity (an effector regulator).\(^{3-5}\) Pretreatment of genetically susceptible mice with an AC injection of a retinal autoantigen (interphotoreceptor retinol binding protein, IRBP) protects against the acquisition of experimental autoimmune uveitis when the mice subsequently receive a uveitogenic regimen of IRBP and adjuvant.\(^{6}\) Splenic T cells harvested from mice that have received, 7 days previously, an AC injection of IRBP suppress EAU if the adoptive transfer recipients are immunized with IRBP plus adjuvant.\(^{6}\) Thus, T regs generated by AC exposure to a retinal autoantigen can suppress the development of autoimmune directed at antigens in the uveal tract of the eye.

Normal mice possess a population of native T regs that express the surface markers CD4 and CD25.\(^{7-9}\) These cells develop spontaneously in the thymus and suppress T- and B-cell activation in vitro by a cell-contact–dependent mechanism.\(^{7-11}\) More important, mice depleted of CD4\(^+\)CD25\(^+\) T cells are vulnerable to the development of a variety of autoimmune diseases, including insulin independent diabetes mellitus, ophoritis, and thyroiditis.\(^{12-16}\) Mice that are thymectomized on the third postnatal day (a maneuver that depletes the CD4\(^+\)CD25\(^+\) T-cell population) exhibit immune-mediated inflammation in a variety of organs, including the uveal tract of the eye.\(^{17,18}\) Thus, a population of natural T regs exists that has the capacity to protect against a variety of autoimmune diseases. Because the T regs of ACAID suppress EAU, we wondered whether the T regs of ACAID were related to, or perhaps even derived from, this natural CD4\(^+\)CD25\(^+\) T cell population.

**MATERIALS AND METHODS**

**Mice**

Female BALB/c and DO11.10 TCR transgenic mice (BALB/c background), between 5 and 8 weeks of age, were purchased from Jackson Laboratories (Bar Harbor, ME). DO11.10 mice express the transgenic DO11.10 TCR that is specific for the peptide fragment of ovalbumin (OVA), 323-329, in the context of IA\(^\alpha\) and performed under protocols approved by the Schepens Animal Care and Use Committee in accordance with NIH guidelines. All mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Antigens, Adjuvants, and Antibodies**

Bovine serum albumin (BSA) and OVA were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in Hanks’ balanced salt solution (HBSS) at a concentration of 25 mg/mL. Complete Freund’s adjuvant (CFA) containing the heat-killed Mycobacterium tuberculosis strain H37 Ra was obtained from Difco Laboratories (Detroit, MI). The mAbs used for flow cytometry (anti-CD16/CD32, FITC, phycoerythrin (PE), or biotin-conjugated anti-CD25 mAb, FITC or PE-conjugated anti-CD4 mAb, FITC-conjugated anti-CD8 mAb, and mouse IgG [isotype control]) were purchased from BD-PharMingen (San Diego, CA).

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Induction and Assay of ACAID and Delayed Hypersensitivity

To induce ACAID, mice received an injection of BSA or OVA (50 μg/2 μL HBSS) into the AC of the right eye, as described previously. For induction of delayed hypersensitivity (DH), 100 μL of an emulsion produced from a 1:1 mixture of OVA (2 mg/mL) and CFA was injected subcutaneously into the nape of the neck. To assess DH, mice received, 7 days after subcutaneous immunization, an intradural inoculation of 200 μg OVA/10 μL HBSS into the right eye pinna. After 24 hours, the ear-swelling response was measured with an engineer’s micrometer (Mitsutoyo, Paramus, NJ). Ear swelling was expressed as follows: specific ear swelling = (24-hour measurement of right ear – 0 hour measurement of right ear) – (24-hour measurement of left ear – 0 hour measurement of left ear) μm. Ear-swelling responses of groups of mice are presented as the mean ± SE.

In Vivo Depletion of CD25⁺⁻ Cells

To create mice deficient in CD25⁺ cells, we administered an intraperitoneal injection of 1 mg rat anti-mouse IL-2R mAb (PC61; BD Pharmingen, San Diego, CA) to normal 5-week-old BALB/c mice three times a week for 2 weeks. Two weeks after the last injection of the mAb, the mice were considered to be depleted of CD25⁺ cells and were used for appropriate experiments. At this time, to quantify the extent of CD25⁺ cell depletion, T cells in the spleen removed from mice treated in this manner were stained with anti-CD4 PE (GK1.5) and anti-CD25 FITC (7D4; BD Pharmingen), and analyzed by flow cytometry.

Preparation of CD25⁺⁻ T Regs for Adoptive Transfer Experiments

Spleens were removed from mice that had received an AC injection of OVA or BSA 7 days earlier. T cells from spleenocyte suspensions were enriched on T-cell separation columns (Immunop; Biotectk laboratory, Houston, TX). In some experiments, purified T cells were further refined by immunomagnetic depletion with anti-CD8a, anti-CD11b, anti-CD4, and anti-Ter-119 (Miltenyi Biotec, Auburn, CA). The negatively selected cells were collected as CD4⁺ T cells. As assayed by flow cytometry, the purity of CD4⁺ cells was >95%. In other experiments, CD4⁺ T cells were depleted of CD25⁺⁺ cells by incubation (20 minutes in a 37°C water bath) with biotin anti-CD25 (7D4) followed by microbeads (15 minutes) and purified by double column−positive selection in a magnet field (Miltenyi Biotec). For adoptive transfer experiments, 5 × 10⁶ CD4⁺ CD25⁻ T cells, or 5 × 10⁵ CD4⁺ CD25⁺⁻ T cells (each number is equivalent to the number to T cells of each type that is contained in 50 ± 10⁶ unselected spleen cells from one mouse) were injected intravenously (IV) into syngeneic recipients. Titration of the CD4⁺ CD25⁻ T cells to less than one mouse equivalent removed the suppressive activity of this population. Of the selected cells suspensions injected, after flow cytometry evaluation, cells were designated CD4⁺ CD25⁻ (>95%) or CD4⁺ CD25⁺⁻ (85%–90%).

Preparation of Peritoneal Exudate Cells

Peritoneal exudate cells (PECs) were obtained from naive BALB/c mice 3 days after they received an intraperitoneal injection of 2 mL of a 3% thioglycollate solution. The cells were washed and resuspended, before they were placed in medium in 24-well culture plates (10⁵/well). PECs were incubated (37°C; CO₂ and air) overnight in the presence or absence of OVA (100 μg/mL) and porcine TGFβ2 (5 ng/mL; R&D Systems, Minneapolis, MI) in serum-free medium composed of RPMI-1640, 10 mM HEPES, 0.1 mM nonessential amino acid, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin (all from BioWhitaker, Walkersville, MD), 1 × 10⁻⁴ M 2-mercaptoethanol (2-ME; Sigma-Aldrich), supplemented with 0.1% BSA (Sigma-Aldrich), ITS⁺ culture supplement (1 mg/mL iron-free transferrin, 10 ng/mL linoleic acid, 0.3 ng/mL Na₂Se, and 0.2 mg/mL Fe(NO₃)₃), and Collaborative-Biochemical Products, Bedford, MA). After overnight culture, plates were washed three times with culture medium to remove TGFβ2 and non-adherent cells. Adherent cells were retained in the wells for use in all subsequent experiments. More than 90% of these cells were F4/80 positive and 97% to 99% of these cells were CD11b positive. CD4⁺ CD25⁺⁻ T cells were not included in these cells.

In Vitro Preparation of DO11.10 T Regs

DO11.10 T cells from spleens of DO11.10 mice were enriched on T-cell separation columns (Immunop; Biotectk). After enrichment, the percentage of T cells was monitored by CD4 and CD8 staining and flow cytometric analyses. Together, CD4⁺ and CD8⁺ cells account for ~87% cells. To create suspensions enriched for CD25⁺ T cells, separation-enriched splenic T cells were treated with FITC-conjugated anti-CD25 mAbs (BD-PharMingen, San Diego, CA) for 20 minutes, and washed twice. CD25⁺ T cells were harvested with a fluorescence-activated cell sorter (EPICS Cell Sorter; Beckman Coulter, Fremont, CA). The percentage of CD4⁺ CD25⁺ T cells within the total cell suspension was less than 0.25%. Purified whole DO11.10 T cells (3 × 10⁷/well) or CD25⁺ DO11.10 T cells (3 × 10⁷/well) were added to 24-well plates containing TGFβ2 pretreated, OVA-pulsed PECs. After 72 hours of culturing in serum-free medium, nonadherent live cells were separated by centrifugation over a linear density gradient (Ficoll-Isoaque; GE Healthcare, Piscataway, NJ) and collected as regulatory T cells. DO11.10 T cells similarly cultured with (TGFβ2 untreated) OVA-pulsed PECs were collected as nonregulatory T cells. Both regulatory and nonregulatory T cells were exposed to γ-irradiation (2000R) for use in subsequent experiments.

Proliferation Assay

Along with RBC-lysed, γ-irradiated (3000 rads) BALB/c mice spleen cells as APCs, spleen cells (5 × 10⁵), purified by cell sorter, were cultured for 3 days in 96-well, round-bottomed plates in RPMI supplemented with 10% FCS (10 mM HEPES, 0.1 mM nonessential amino acid, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin; all from BioWhitaker), 1 × 10⁻⁴ M 2-mercaptoethanol (2-ME; Sigma-Aldrich). Anti-CD3 mAbs (145-2C11; BD-PharMingen) at a final concentration of 1.0 μg/mL was added to the culture for stimulation. The cultures were sustained for 72 hours, pulsed with 0.5 μCi [³H]-thymidine 8 hours before termination, and then harvested onto glass filters with an automated cell harvester (Tomtec, Orange, CT). Radioactivity was assessed by liquid scintillation spectrometry, and the amount expressed as counts per minutes.

Interferon-γ Assay

To assay culture supernatant for content of interferon (IFN-γ), we established cultures in which γ-irradiated (2000 rads) DO11.10 T cells (1 × 10⁷/well, T reg) that were first cultured with OVA-pulsed, TGFβ2-pretreated (or not) PECs were added to 96-well culture plates containing naive DO11.10 T cells (4 × 10⁵/well) and OVA-pulsed PECs (1 × 10⁵/well). In some experiments, γ-irradiated (2000 rads) DO11.10 T cells that were first cultured with OVA-pulsed, TGFβ2-pretreated (or not) PECs were added to 96-well culture plates containing OVA-pulsed PECs in the absence of naive DO11.10 T cells. After 96 hours of culture, supernatants were collected and analyzed by quantitative capture enzyme-linked immunosorbent assay (ELISA), according to the manufacturer’s instruction (BD-PharMingen). Rat mAb to mouse cytokine IFN-γ (R4-6A2) was used as the coating Ab. A biotinylated rat mAb to mouse cytokine IFN-γ (XMG1.2) was used as the detecting Ab.

RT-PCR for Foxp3

Total RNA was extracted from CD4⁺ CD25⁺⁻ and CD4⁺ CD25⁺ T cells freshly isolated from naive mice or from mice that received AC injection of OVA using the RNA stat-60 kit (TelTest, Friendswood, TX). Of the total RNA, 1.0 μg was reversed transcribed for single-stranded cDNA, using an oligo dT primer with reverse transcriptase (Promega, Stratagene, CA). The cDNA was then used for PCR amplification, and the PCR products were sequenced.
Madison, WI). For PCR amplification, cDNAs were amplified using primers as follows: GAPDH, 5'-GGTGAAGGTGGTGAGACGGGA-3' and 5'-TGTATGGGTGCTGCTGTCGGT-3', giving an amplification product of 245 bp, and Foxp3, 5'-CACGTCGTACATGGCCTCT-3' and 5'-CATTGGCAAGAGTGTTGTA-3', giving an amplification product of 382 bp.26 PCR was performed in a 50-μL amplification mixture containing 1× polymerase buffer, 2.5 mM MgCl₂, 0.2 μM each dNTP, 1 μM of forward and reverse primers, and 1.25 U Taq polymerase (Perkin Elmer, Wellesley, MA). PCR cycling conditions were 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. After 28 cycles of amplification, the PCR products were separated by 1.0% agarose gel containing 0.5 μg/mL ethidium bromide electrophoresis. Densitometric measurement of the bands was used to calculate a ratio to the gene of interest, GAPDH.

Statistical Analysis

The results of the experiments were analyzed with ANOVA and the Scheffé test. Mean results were considered to be significantly different at \( P < 0.05 \).

RESULTS

Expression of CD25 in ACAID-Type CD4⁺ T Regs

We have demonstrated that OVA-specific T cells (from Tcr transgenic DO11.10 donors) that are exposed in vitro to OVA-pulsed, TGFβ2-treated APCs proliferate, albeit less than do similar T cells exposed to OVA-pulsed, TGFβ-untreated APCs.21 Moreover, T cells stimulated by antigen-pulsed, TGFβ2-treated APCs acquire the capacity to suppress T-cell activation in an antigen-specific manner.22,23 Because T-cell proliferation depends on IL-2 secretion and signaling through the IL-2 receptor, we wondered whether T cells that proliferate in response to OVA-pulsed, TGFβ2-treated APCs express CD25 (the IL-2 receptor α chain). Splenic T cells (greater than 75% CD4⁺), obtained from DO11.10 mice, were exposed to OVA-pulsed, TGFβ2-treated PECs. In positive control cultures, similar T cells exposed to OVA-pulsed, TGFβ-untreated PECs. Negative control experiments consisted of DO11.10 T cells exposed to PECs alone. After 72 hours, the nonadherent T cells were harvested, stained with anti-CD25 and anti-CD4 antibodies, and analyzed by flow cytometry. CD25 expression was significantly upregulated on CD4⁺ T cells exposed to OVA-pulsed PECs, treated with TGFβ or not (Fig. 1). The upregulation of CD25 expression on cells was consistent with their proliferation. Because the transgenic T cells resemble the CD4⁺ Tregs of ACAID in their capacity to regulate bystander T effector cells, and because at least some of these in vitro-activated T cells may have been CD25⁺ at the time the culture was initiated, we conclude that naïve CD4⁺ CD25⁺ DO11.10 T cells have the potential to differentiate into ACAID type T regs.

In Vitro Generation of T Regs of the ACAID Type

As mentioned, OVA-specific DO11.10 T cells exposed in vitro to OVA-pulsed, TGFβ2-treated APCs acquire regulatory properties (i.e., in secondary cocultures, they suppress IFNγ production by fresh bystander DO11.10 T cells exposed to OVA-pulsed APCs).22,23 Using this model system, we tested whether the in vitro generated Tregs arose from CD25⁺ and/or CD25⁻ Do11.10 T cells. Column-purified splenic T cells (typically containing approximately 87% CD4⁺ and CD8⁺ cells) were obtained from Do11.10 donors. After fluorescence-activated cell sorting the percentage of the total cells expressing CD4⁺ and CD25⁺ was reduced from 2.87% to 0.21% (Fig. 2A). Although we considered this sorted population to be depleted of CD25⁺ cells, the small number of contaminating CD4⁺ CD25⁻ cells in the sorted cell suspension required us to determine whether the putative CD25-depleted cell suspension still displayed regulatory capacity. One way to determine whether natural CD4⁺ CD25⁻ Tregs are present is to examine whether a suspension of T cells containing this population displays the capacity to suppress anti-CD3 activation of CD4⁺ CD25⁻ depleted T cells.11 Therefore, we generated sorted populations of CD4⁺ T cells that were depleted of or enriched for, CD25⁻ cells. A portion of each of these cell suspensions was removed and used as "regulators" in T-cell proliferation assays in which CD4⁺ CD25⁻ depleted cells were stimulated with anti-CD3 mAb (1.0 μg/mL) for 72 hours, alone or in the presence of CD25-depleted or CD25-enriched regulators (Fig. 2B). We observed that CD25-enriched regulators inhibited the proliferation of anti-CD3-stimulated CD4⁺ CD25⁻ T cells. By contrast, CD25-depleted Tregs failed to suppress anti-CD3-induced T-cell proliferation. We conclude that naïve CD4⁺ Do11.10 T cells contain a population of natural CD25⁺ T regulators and that naïve CD4⁺ Do11.10 T cells depleted of CD25⁺ cells by cell sorting lose their capacity to suppress proliferation of naïve CD4⁺ CD25⁻ T cells in vitro.

Our next goal was to determine whether the ACAID type Tregs generated in vitro by stimulating naïve Do11.10 T cells with OVA-pulsed, TGFβ2-treated APCs22,23 were derived from CD25⁺ or CD25⁻ precursors. T cells were purified from spleens of naïve Do11.10 mice as described earlier, and a portion of the suspension was depleted of CD25⁺ cells by cell sorting. Whole and depleted populations were then exposed in vitro to TGFβ2-treated, OVA-pulsed PECs. In companion cultures, similar T-cell suspensions were exposed to OVA-pulsed PECs (no TGFβ2). After 72 hours, the nonadherent T cells were collected, mitomycin C-treated, and added as regulators to secondary cultures in which fresh Do11.10 T cells were mixed with OVA-pulsed PECs and cultured for 4 days before being analyzed by ELISA for IFNγ content. The results of a representative experiment are presented in Figures 2C and 2D (top). Replete T cells (containing CD25⁺ cells), exposed first to untreated PECs, induced responder T cells to produce copious amounts...
The role of natural CD4+ Tregs in ACAID was assessed. (A) Flow cytometry analyses of extent of depletion of sorted CD25+ cells. Numbers indicate the percentage of cells within quadrants. (B) The capacity of CD25-depleted cell suspensions to suppress naive T-cell proliferation. The ordinate shows the phenotype of cells added to cultures containing naive CD25-depleted CD4+ T cells plus anti-CD3 mAb (1.0 µg/mL) plus γ-irradiated naive splenocytes as APCs for 72 hours. [3H]thymidine was added for the terminal 8 hours and radioisotope incorporation was measured (abscissa). Bars, mean ± SEM. The experiment was repeated twice with similar results. (C, D) Analyses of DO11.10 Tregs on IFN-γ production. (C) Unselected or (D) CD25+-depleted DO11.10 T cells after stimulated with PECs treated as indicated stimulated with OVA-pulsed, TGFβ2-treated PECs for 72 hours, stimulation with OVA-pulsed PEC in the absence of TGFβ2. In vitro-stimulated γ-irradiated cells were added as regulators to these cells in the presence of responder T cells (naive DO11.10 T cells; top). γ-Irradiated DO11.10 T cells (Tregs) that were first cultured with OVA-pulsed, TGFβ2-pretreated (or not) PECs were added to 96-well culture plates containing OVA-pulsed PEC in the absence of naive DO11.10 T cells (bottom). Culture supernatants were harvested after 96 hours, and assayed for IFN-γ by ELISA. Each data point represents the mean ± SEM of triplicate cultures. *Significant difference (P < 0.05), ‡Not detected. The experiment was repeated twice with similar results.

**Effect of CD25+ and CD25− ACAID-Type CD4+ T Regs on DH**

Because in vitro-generated cells are imperfect models of the Tregs that are generated in vivo and because the presence of the transgene may have altered the thymic-dependent generation of natural CD4+ CD25+ Tregs in DO11.10 mice, we tested the role of natural CD4+ CD25+ Tregs in vivo ACAID. We tested whether the CD4+ Tregs that are typically generated in the spleens of normal BALB/c mice with ACAID are derived from natural CD25+ Tregs or from CD25− precursors. OVA were injected into the AC of naive BALB/c mice. Seven days later, spleens were removed from these mice, and fractionated into an enriched CD4+ T cell population. The CD4+ T cells used for adoptive transfer of ACAID T regulators were either depleted of CD25+ cells before intravenous injection into naive recipients, or were positively selected for CD25+ cells before intravenous injection. In these experiments, cell sorting was performed using magnetic beads. The efficiency of depletion and enrichment in these experiments is presented in Figure 3A. CD25-depleted cell suspensions contained 0.68% CD4+ CD25+ cells, and CD25-enriched cell suspensions contained 86.7% CD4+ CD25+ cells. Positive sensitization controls received an intravenous injection of HBSS. Two hours after adoptive transfer, recipients were immunized subcutaneously with OVA plus CFA. One week later, OVA were injected intradermally into the ear pinnae of all groups of mice, plus the negative controls, and ear swelling was assessed 24 hours later. Recipients of either CD4+ CD25+ T cells or CD25− T cells displayed suppressed ear-swelling responses (Fig. 3B). As anticipated, mice that re-
received an AC injection of OVA exhibited less ear swelling after sensitization and ear challenge with OVA. These results indicate that both CD4⁺ CD25⁺ and CD4⁺ CD25⁻ T cells are capable of functioning as T regs in ACAID. Whereas the CD4⁺ T regs of ACAID are reported to be antigen-specific,⁴,²⁴ natural CD4⁺ CD25⁺ T regs are generally regarded as antigen nonspecific,²⁵,²⁶ although they can be expanded by an antigen-specific signal.²⁷,²⁸ Thus, our next experiments examined the antigen specificity of suppression mediated by these two types of T reg populations. First, we tested whether the CD4⁺ T regs found in the spleens of mice with ACAID induced by an AC injection of BSA were capable of suppressing DH to an unrelated antigen (OVA). Splenic CD4⁺ T cells were harvested from these mice and separated into CD25⁺-enriched and CD25⁻-depopulated populations. Enriched cells were adoptively transferred into naïve BALB/c mice. Within 2 hours, the recipients were immunized subcutaneously with OVA. As ACAID controls, mice that received only an AC injection of BSA or OVA were immunized subcutaneously with OVA and CFA 1 week later. All groups of mice, plus negative controls, were challenged intradermally in the ear with OVA. Mice that received an AC injection of OVA displayed impaired ear swelling, whereas mice that received an AC injection of BSA, displayed intense ear swelling—indicating that the impaired DH of ACAID is antigen specific (Fig. 3C). Moreover, adoptive transfer recipients of CD4⁺ T cells from donors that received an AC injection of BSA displayed OVA-specific DH of comparable intensity to positive controls. This was true whether the adoptively transferred cells were first depleted of CD25⁺ cells or enriched for these cells. CD4⁺ or CD25⁻ T regs in ACAID suppress DH only in an antigen-specific way.

Expression of Foxp3 in ACAID-type CD4⁺ T Regs

The transcription factor Foxp3 has been circumstantially linked to the regulatory functions of CD4⁺ CD25⁺ Tregs.²⁹,⁳⁰ In fact, natural Tregs are missing from mice with the Foxp3 gene disrupted. We anticipated that ACAID Tregs that arise from CD25⁺ precursors would express Foxp3, but we wondered whether expression of this gene would be upregulated in ACAID T regs derived from CD25⁻ precursors. To assess whether Foxp3 expression is associated with the induction of CD4⁺ ACAID T regs, we compared the expression of the Foxp3 transcript in CD4⁺ CD25⁺ or CD4⁺ CD25⁻ T cells derived from naïve mice and from mice that had received an AC injection of OVA 7 days previously. Purified CD4⁺ CD25⁺ or CD4⁺ CD25⁻ T cells, but not CD4⁺ CD25⁻ T cells, in naïve mice expressed Foxp3 (Fig. 4A). Similarly, CD4⁺ CD25⁺ T cells isolated from mice with ACAID expressed Foxp3 (Fig. 4B). In contrast, the anti-CD25 mAbs (CD4⁺ CD25⁺ T cells). Numbers indicate percentages of cells within quadrants. (B) Systemic adoptive transfer. CD25⁺-enriched (⁵ × 10⁵) and CD25⁻-depleted cell (⁵ × 10⁵) suspensions were enriched from OVA AC-inoculated mice and transferred (intravenously) to naïve BALB/c mice. Two hours later, the recipient mice were immunized (subcutaneously) with OVA plus CFA, and 1 week later DH was analyzed. The treatment of recipient mice is indicated under the bars along the abscissa. Data are expressed as mean ear-swelling responses ± SEM (n = 5). *Mean results significantly lower than the positive control (P ≤ 0.05). The experiment was repeated twice with similar results. (C) Antigen specificity of T reg cells. BSA was injected into the AC of one eye of naïve BALB/c mice. Seven days later, CD4⁺ splenic T cells were harvested, separated into CD25⁺-enriched or CD25⁻-depleted cell suspensions by magnetic bead cell sorting, and injected (intravenously) into normal BALB/c mice. As ACAID control, OVA or BSA was injected into the AC of normal BALB/c mice. Data are expressed as the mean ear swelling responses ± SEM (n = 5). *Mean results significantly lower than those of the positive control (P ≤ 0.05).
expression of Foxp3 was detected only at low levels in CD4^+CD25^+ T cells obtained from the spleens of mice with ACAID. Moreover, the level of Foxp3 mRNA expression was virtually identical in CD25^- T cells harvested from naive mice and mice that received an AC injection of OVA 1 week previously. These results indicate that the CD4^+CD25^- ACAID T regs harvested from spleens of mice injected AC with OVA do not need to enhance expression of Foxp3 to enable them to mediate suppression of induction of OVA-specific DH.

**Induction of ACAID in Mice Depleted of CD25^+ T Cells**

To this point, ACAID has routinely been induced by injection of antigen into the AC of the eyes of normal mice whose lymphoid compartments contain natural CD4^+CD25^+ T regs. Because the latter cells have been shown to be important natural regulators of certain autoimmune diseases and can be used to suppress tissue allograft rejection, it is reasonable to inquire whether the T regs of ACAID arise from CD4^+CD25^- precursor T cells, and whether ACAID can be induced in mice in which CD4^+CD25^- T cells have been depleted. To determine whether cells of this type give rise to the CD4^+ T regs of ACAID, naive BALB/c mice were treated intraperitoneally with anti-CD25 antibodies for 2 weeks, using a regimen that profoundly depleted the number of CD4^+CD25^- T cells from the periphery. To document the extent of CD4^+CD25^- T cell depletion by this regimen, spleen cell suspensions harvested at the time of inoculation with OVA (day 0) were analyzed by flow cytometry (Fig. 5A). To document the extent of CD4^+CD25^- T-cell depletion by this regimen, spleen cell suspensions harvested at the time of AC injection with OVA were analyzed by flow cytometry. Splenic CD4^+CD25^- T cells were reduced from approximately 7.7% (in untreated mice) to 0.7% in mice treated with anti-CD25 antibodies (Fig. 5B).

Having confirmed that a 14-day treatment regimen with anti-CD25 antibody depleted mice of the function of natural CD25^- T regs at the time of AC injection of OVA, we next determined whether mice treated in this manner could acquire ACAID. OVA were injected into the AC of mice that had been treated with antibody for 14 days and rested for an additional 14 days. For the antibody treatment control, naive mice that had not received OVA into the AC were also treated with anti-CD25 antibody for the same time interval. ACAID control mice received an AC injection of OVA, but no anti-CD25 antibody treatment.

Seven days after AC injection of OVA, all mice plus the positive control were immunized with OVA plus CFA. When ears were challenged with OVA 1 week later, ear-swelling responses of both non-anti CD25-treated mice and mice that received the anti-CD25 antibody were impaired (Fig. 5C). Thus, ACAID was induced in mice in which the complement of natural CD4^+CD25^- T cells was greatly reduced at the time of antigen injected into the AC. This implies that ACAID induction is independent of, and perhaps even irrelevant to, the presence of natural CD4^+CD25^- T regs.

**DISCUSSION**

Two generic types of regulatory T cells are described in the recent literature. One type, of which natural CD4^+CD25^- T cells are the prototype, develops spontaneously within the thymus of normal mice. These T regs are considered to be nonspecific, in part because they express a heterogeneous spectrum of T-cell receptors for antigen, and because they have been demonstrated to inhibit bystander T cells in vitro without regard to antigen specificity. Of importance, CD4^+CD25^- T regs have been demonstrated to play a major role in suppressing the development of certain spontaneous and induced
autoimmune disease. The other generic type of regulatory T cells arises as a consequence of diverse experimental manipulations, and the list of such manipulations is quite long. On this list are the regulatory T cells that are found in the spleens of mice in which ACAID develops after introduction of antigen into the AC of the eye. In fact, at least two functionally distinct types of ACAID Tregs have been defined, one of which is CD8+ and suppresses the expression of Th1- and Th2-type immunity. These cells have been referred to as effector suppressors. The other type of ACAID Tregs is CD4+ and suppresses the induction of Th1- and Th2-type immune responses. These cells have been referred to as afferent suppressors. Afferent ACAID Tregs bear a certain resemblance to natural CD4+CD25+ Tregs, because they both express CD4. Moreover, both ACAID Tregs and natural CD4+CD25+ Tregs have been reported to suppress autoimmune disease. They differ to the extent that the suppression by ACAID Tregs has been shown repeatedly to be antigen specific, whereas that is not usually the case with natural Tregs.

The results in this study make the case that some ACAID Tregs, especially the CD4+ afferent regulators, can arise from T-cell precursors that are CD25 negative. Previously Kezuka and Streilein reported that DO11.10 T cells treated with TGFβ in vitro are capable of suppressing DH in vivo. We present the first evidence in support of this case: CD25-depleted CD4+ DO11.10 T cells differentiated readily into regulatory T cells when stimulated in vitro with OVA-pulsed, TGFβ2-treated APCs—just as do CD4+ DO11.10 T cells that include CD25+ cells. This is of interest because DO11.10 T cells are actually activated when they are exposed in vitro to OVA-pulsed, TGFβ2-treated APCs. This stimulation induces the cells to undergo several rounds of division, which correlates with their production of IL-2 and expression of IL-2 receptors. Thus, these activated cells become CD25+ because CD25 represents the α chain of the mature IL-2 receptor. We suspect that CD25 expression on IL-2 receptor-positive T cells activated in vitro in this manner has a different biological meaning from the expression of CD25 alone on natural Tregs. We have also examined in vitro-stimulated DO11.10 T cells for expression of the glucocorticoid-induced TNF receptor family-related gene (GITR), considered to be a marker for natural Tregs. We found that GITR expression was equally upregulated in DO11.10 T cells stimulated in vitro with OVA-pulsed, TGFβ2-treated or OVA-pulsed, TGFβ2-unstimulated APCs (data not shown). Thus, neither upregulation of CD25 nor upregulation of GITR enables us to distinguish between DO11.10 T cells with regulatory properties (stimulated with TGFβ2-treated APCs) and DO11.10 T cells with a Th1 effector function (stimulated by non-TGFβ-treated APCs). These findings raise the notion that expression of CD25 on T cells with regulatory capacity need not reflect a direct functional or lineage relationship with natural CD4+CD25+ Treg.

The second piece of evidence that supports a distinction between ACAID Tregs and natural CD4+CD25+ Tregs is that impaired capacity to acquire OVA-specific DH (ACAID) was transferred adaptively to naïve mice using CD25-depleted CD4+ T cells harvested from spleens of mice that had previously received an AC injection of OVA. This is in line with a recent report that CD25+ Treg are present within skin transplants and suppress graft rejection. Similarly, CD25+ Treg have recently been demonstrated to participate in nasal and oral tolerance. In aggregate, these findings support our data that indicate that at least one subset of CD4+ T cells that display the capacity to regulate induction of OVA-specific immunity do not need to express CD25 to become Tregs or to perform their regulatory functions.

This analysis of our experimental results is biased in the sense that it emphasizes the capacity of CD4+CD25+ T cells of ACAID to function as regulatory cells. However, our results also indicate that CD4+CD25+ T cells are not capable of differentiating into, and functioning as, afferent ACAID Tregs. Ten times fewer CD4+CD25+ T cells than CD4+CD25+ T cells harvested from ACAID mice were able to prevent recipient mice from acquiring OVA-specific DH. In addition, we confirmed that CD4+CD25+ spleen cells (from ACAID mice) up-regulate the expression of Foxp3. This observation is supported by a recent report that showed that conversion of TGFβ peripheral CD4+CD25+ T cells to CD4+CD25+ regulatory T cells correlated with the expression of Foxp3. Thus both CD4+CD25+ and CD4+CD25- T cells have the capacity to become afferent ACAID Tregs, but we also point out that Foxp3 is not unregulated in ACAID spleen cells depleted of CD25+ cells. This finding raises the possibility that upregulation of Foxp3 is not necessary for CD4+ T cells to acquire the ACAID Treg phenotype and function.

The issue of antigen specificity of Tregs is complicated. Although ACAID Tregs are antigen specific, the antigen specificity requirement is less clear for natural Tregs. Although some reports claim that CD4+CD25+ Tregs are anergic, recent studies have demonstrated that these cells are capable of proliferating. In particular, Yamazaki et al. have recently demonstrated that both steady state and mature antigen-processing dendritic cells induce proliferation of adoptively transferred CD4+CD25+ T cells. The image of these cells as anergic arises from attempts to stimulate their proliferation in vitro, whereas the cells are readily able to proliferate in vivo. They point out, however, that antigen itself was not necessary when dendritic cells promoted proliferation of natural Tregs in vivo. Nonetheless, the proliferating cells continued to display their regulatory functions. Gavin et al. have reported that while CD4+CD25+ Tregs are relatively hyporesponsive to antigenic stimulation in vivo, the same cells proliferate quite well in the presence of lymphopenia. Moreover, their capacity to suppress is augmented. These results suggest the possibility that antigen stimulation of the TcR may not be necessary, or even helpful, when natural Tregs proliferate in vivo. Hori et al. studied the specificity requirements of CD4+CD25+ Tregs in mice bearing TcR transgenic T cells specific for myelin basic protein. Their evidence suggests that natural Tregs with TcR specific for antigen express their effector function (suppression of experimental allergic encephalomyelitis) in an antigen-specific manner, but that their thymic selection or commitment is not dependent on expression of these TcR cells.

The results of our final experiment, in which we attempted to induce ACAID in mice depleted of CD25+ cells, moves the analysis to a more relevant level. In this study, the proportion of CD25+ T cells declined by more than 90% after anti-CD25 mAb treatment, and this level of CD25+ cell depletion was the same as that reported recently by others to have functional consequences (i.e., loss of natural suppressor activity). Injection of OVA into the AC of eyes of mice largely, if not completely, depleted CD25+ cells, induced ACAID and was still able to prevent these mice from acquiring OVA-specific DH when they were immunized subsequently. This result strongly supports the view that CD25+ precursors are not essential in ACAID induction. However, a very recent study suggests otherwise (i.e., CD25+ T cells are required to produce suppressor cells in the in vitro ACAID culture system). This latter study focused on the need for CD25+ T cells in the generation of ACAID suppressor cells in vitro cultures, whereas we have examined the requirement for CD25+ T cells in ACAID induction in vivo. Our results support the view that CD25+ T cells are not required in vivo, and therefore we conclude that induction of ACAID in vivo is independent of the presence of
CD4+CD25+ T cells. Moreover CD4+CD25+ T are also not required for the development of CD8+ T reg cells in ACAID, since AC inoculation of antigen into class II-deficient mice is effective in generating ACAID CD8+ T reggs.48 Although it appears that CD25+ T cells can acquire ACAID-like regulatory function in in vitro experiments and in contrived cell-transfer experiments, we believe that CD25+ natural Tregs are largely irrelevant to ACAID induced in the intact mouse.

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References


