CD8⁺ T Regulatory Cells Use a Novel Genetic Program that Includes CD103 to Suppress Th1 Immunity in Eye-Derived Tolerance

Hiroshi Keino,¹,² Sharmila Masli,¹ Shuji Sasaki,¹ J. Wayne Streilein,¹,⁵ and Joan Stein-Streilein¹

PURPOSE. The peripheral tolerance that arises after injection of antigen into the anterior chamber (anterior chamber–associated immune deviation; ACAID) is associated in part with CD8⁺ T cells that suppress the expression of Th1 and Th2 immunity. The purpose of these studies was to determine the genes and molecules that are critical for CD8⁺ T regulatory cell (T reg) functions in ACAID.

METHODS. Ovalbumin (OVA)-specific CD8⁺ T cells from T-cell receptor (TCR) transgenic OT-1 mice acquire effector regulatory properties similar to in vivo–generated CD8⁺ T regs after stimulation with OVA-pulsed TGF-β²-treated APCs. Changes in the genetic program associated with acquisition of effector regulatory function in OT-1 CD8⁺ T cells in vitro were determined by DNA microarray analyses and confirmed by RT-PCR analyses and biological assays.

RESULTS. T regulatory OT-1 T cells acquired a novel transcriptional pattern indicative of their function. Genes for molecules associated with TGF-β function, resistance to TCR-triggered apoptosis, and localization of cells to antigen deposition in peripheral tissues were upregulated, and genes related to cytolysis were downregulated. Further study showed that CD103, a cell-adhesion molecule that binds E-cadherin, was highly upregulated in in vivo–generated ACAID T regs and was necessary for their suppression of T-cell activation in vitro.

CONCLUSIONS. OT-1 CD8 T cells modulated in vitro by exposure to antigen-pulsed, TGF-β²-treated APCs expressed genes related to immune suppression. Thus, the necessity for CD103 emerges in the effector CD8⁺ T-cell regulatory mechanisms in eye-derived tolerance. (Invest Ophthalmol Vis Sci. 2006;47: 1533–1542) DOI:10.1167/iovs.04-1454

Injection of antigen into the anterior chamber (AC) of the eye dramatically alters the systemic immune response to that specific antigen.¹ This response, a type of tolerance derived via the eye and termed anterior chamber–associated immune deviation (ACAID), is inducible with a wide variety of antigens, including soluble proteins, viral-encoded proteins, and minor histocompatibility antigens.¹,² In ACAID, intraocular injection of antigen prevents the development and expression of delayed-type hypersensitivity (DH) responses in experimental animals, even when they are appropriately immunized. A considerable body of experimental evidence indicates that ACAID is triggered by indigenous, intraocular bone marrow–derived antigen-presenting cells (APCs) that capture antigen within the AC and deliver an antigen-specific, ACAID-inducing signal via the blood directly to the spleen.³,⁴ ACAID mice express two functionally distinct populations of regulatory T cells that suppress DH.⁵–¹⁰ One population of regulatory T cells express CD4 and prevent the induction of immunity that leads to the generation of DH effectors (i.e., effector regulators). Another population expresses CD8 and inhibits the expression of DH in vivo (i.e., effector regulators). The molecular mechanisms by which these cells accomplish their regulatory functions are largely unknown. Unfortunately, the frequency of these putative regulator T cells in lymphoid organs of mice with ACAID has been too low for their collection and a detailed molecular analysis. To overcome this technical barrier, we turned to T-cell receptor (TCR) transgenic mice, which have lymphoid organs that contain a high frequency of T cells expressing T-cell receptors specific for defined peptides of ovalbumin (OVA) in the context of major histocompatibility complex (MHC) class I (K¹, OT-I) and class II (I-A¹, DO11.10). We recently reported that OVA-specific CD8⁺ OT-I T cells that are exposed in vitro to OVA-pulsed, TGF-β²-pretreated APCs acquire the ability to suppress expression of DH when adoptively transferred into OVA-immune C57BL/6 mice.¹¹ Because this is a key property of CD8⁺ effector T regulatory cells (T regs) in ACAID, study of in vitro-generated CD8⁺ OT-I T regs offered us the possibility of identifying relevant molecules and mechanisms of suppression.

Although TGF-β is known to be involved in the DH-inhibitory activities of regulatory T cells in ACAID,¹¹–¹³ there is little additional information that implicates other molecules that are involved in the generation of these T regulators or in enabling the regulators to mediate suppression. In this study, we used DNA microarray analysis to compare patterns of gene expression in regulatory OT-I CD8⁺ T cells generated in vitro with patterns from nonregulatory OT-I CD8⁺ T cells. Based on our results, we identified expected and, more importantly, unexpected genes with products that may influence the capacities of these T cells to perform their regulatory function in eye-derived tolerance. Several categories of differentially expressed genes were found to promote regulatory function, including genes that enable these T regs (1) to synthesize and secrete active TGF-β, (2) to express signaling receptors for active TGF-β, and (3) to resist antigen-triggered apoptosis. Several other categories of differentially expressed genes were found that prevent the generation of effector cells, including genes that rob the cells of their native capacity to lyse target cells and inhibit their secretion of proinflammatory Th1-type cytokines. The most strongly upregulated gene in CD8⁺ T regs generated in vitro encodes the cell surface integrin CD103. Using CD103 knockout (KO) mice, we found that expression of CD103 molecules by CD8⁺ T regs was absolutely essential to the capacity of these T regs to inhibit bystander OVA-primed T-cell

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activation in vitro and that expression of CD103 molecules by mice that received AC injections of OVA was essential in the induction of ACD. Hence, the generation of effector molecules and T regulators. Thus, CD103 expression is essential in both the inductive and effector stages of ACD.

**Materials and Methods**

**Mice**

C57BL/6 (B6) mice, between 6 and 8 weeks of age, were purchased from Taconic Farms (Germantown, NY). Mice were used as a source of peripheral exudate cells (PECs). BALB/c mice at 6 to 8 weeks of age were purchased from Taconic Farms. The Jackson Laboratory (Bar Harbor, ME), OT-I TCR transgenic mice (C57BL/6 background, obtained originally from the Jackson Laboratory) were maintained in our facility. T cells from OT-I mice recognize a peptide (residues 257-264) derived from OVA in the context of Kb. OT-I mice were used as the source of T cells. OVA-specific TCR transgenic T cells in OT-I mice were identified by surface flow cytometry for the expression of CD8 and Vβ. In general, 30% to 40% of CD3⁺ T cells in OT-I lymphoid cell suspensions expressed CD8 and TCR Vβ. CD103 (Igκ) gene knockout mice 14 (backcrossed 10 generations to BALB/c) were provided by Gregg Hadley (University of Maryland Medical School, Baltimore, MD) and maintained in our animal facility. Thrombospondin (TSP)-1 KO mice (C57BL/6 background)15 were provided by Jack Lawler (Beth Israel Deaconess Hospital, Boston, MA). All animals were treated according to NIH guidelines with approval from the Schepens Animal Care and Use Committee (ACUC) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Culture Medium**

Serum-free medium was used for all cell cultures and was composed of RPMI 1640, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin, all from BioWhittaker, Walkersville, MD, 1 × 10⁻⁵ M 2-mercaptoethanol (ME; Sigma-Aldrich, St. Louis, MO), supplemented with 0.1% bovine serum albumin (Sigma-Aldrich), ITS⁺ culture supplement (1 μg/mL iron-free transferrin, 0.3 μg/mL Na₂Se, and 0.2 μg/mL Fe(NO₃)₃, Collaborative Biochemical Products, Bedford, MA).

**Regents: Antigens, Adjuvants, and Antibodies**

OVA was purchased from Sigma-Aldrich and dissolved in Hanks’ balanced salt solution (HBSS) at a concentration of 25 mg/mL. Complete Freund’s adjuvant (CFA) containing heat-killed Mycobacterium tuberculosis strain H37 Ra was purchased from Difco Laboratories (Detroit, MI). Porcine TGF-β2 and anti-pan TGF-β neutralizing antibody were purchased from R&D Systems (Minneapolis, MN). The Abs used for flow cytometry analysis were: Fc block (anti-mouse Fcγ II/III mAb, clone 2G12), phycoerythrin (PE)-conjugated anti-CD4 mAb (clone GK1.5), FITC- or PE-conjugated anti-CD8 mAb (clone 53-6.7), FITC-conjugated anti-TCR Vβ1, 5.2 mAb (clone MR9-4), FITC-conjugated or purified anti-CD103 mAb (clone NM290), and purified or FITC- or PE-conjugated isotype controls (all purchased from BD-PharMingen; San Diego, CA). Purified anti-E-cadherin mAb (clone ECD-2) was obtained from Zymed (South San Francisco, CA). FITC-conjugated anti-rat Ig polyclonal Ab was purchased from BD-PharMingen.

**Preparation of Pure OT-I T Cells**

Spleens were removed from OT-I mice and pressed through nylon mesh to produce a single cell suspension. Red blood cells were lysed with Tris-NH₄Cl. The remaining cells were washed three times with RPMI 1640 and passed through T-cell columns (Immunal; Biotec Laboratory, Houston TX). After enrichment, the percentages of T cells were monitored by CD4 and CD8 staining and flow cytometric analyses. Together, CD4⁺ and CD8⁺ cells accounted for >90% of the cells in enriched suspensions.

**Preparation of PECs Pretreated with TGF-β2**

PECs were harvested from normal C57BL/6 mice that received 2.5 mL of thioglycollate (Sigma-Aldrich) intraperitoneally 3 days earlier. In some experiments, PECs were harvested from TSP-1 KO mice. As described previously,16,17 the recovered cells were washed and resuspended, placed in 24-well culture plates (1 × 10⁶/well) and treated with or without 5 ng/mL porcine TGF-β2 in serum-free medium at 37°C in an atmosphere of 5% CO₂. After overnight culture, plates were washed three times with culture medium to remove TGF-β2 and nonadherent cells. Adherent cells were retained in the wells for use in all subsequent experiments. More than 92% of the adherent cells were F4/80⁺ and 99% of the cells were CD11b⁺.

**Preparation of Regulatory T Cells and RNA Isolation**

OT-I T cells (4 × 10⁵) were cultured in 24-well plates containing TGF-β2-treated or untreated PECs, and 400 μg/mL OVA. In some experiments, OT-I T cells were cultured in the presence of pan-TGF-β antibody for neutralization (1 μg/mL). After 72 hours, nonadherent OT-I T cells were harvested and purified by immunomagnetic depletion with anti-CD4, anti-CD11b, anti-B220, anti-DX5, and anti-TER-119 (Miltenyi Biotech, Auburn, CA). The negatively selected cells were collected as CD8⁺ T cells. Cell purity, as examined by flow cytometry, showed that >95% were CD8⁺. Total RNA was extracted from these cells (RNA Stat-60 Kit; Tel-Test, Friendswood, TX and purified (RNeasy columns; Qiagen, Valencia, CA), according to the manufacturers’ instructions.

**DNA Microarray Hybridization and Analysis**

Preparation of the biotinylated RNA probe was performed as described (GeneChip Expression Analysis Manual; Affymetrix, Santa Clara, CA). In brief, 10 μg of total RNA was converted to double-stranded cDNA, by using oligo-dT primers. Biotinylated RNA was generated with high-yield reagents (BioArray; Enzo Diagnostics, Farmingdale, NY), according to the manufacturers’ protocol. Samples were hybridized to murine genome U74Av2 chips (Affymetrix) and read, using a microarray chip scanner (GeneChip scanner; Affymetrix) and accompanying gene expression software, at the Bauer Center for Genomics Research at Harvard University.

**Semiquantitative Reverse Transcription–Polymerase Chain Reaction**

**Real-Time PCR Assay**

A real-time PCR assay (SYBR Green; Applied Biosystems, Inc. [ABI] Foster City, CA) was used to determine the relative quantitative expression of selected genes. Sequences of the primers used for these genes are as follows: IL-12Rb1, forward (F), 5'-CAG TTC TGG GAA CAG GAC GAT ACT, reverse (R), 5'-GGG GTC GTC TTG GTC CAG TTG TA; granzyme B, F, 5'-GAG ACC TGG TGC GAT CTT AAT AAA, R, 5'-AGG AAT ATG TCA GTT GGG TTG TCA; CD103, F, 5'-GCC GTC GTC ACT ACT TTT GAT; MMP9, F, 5'-AAG GTG CTA GCC GGC GAA CAG R, 5'-GAA AGG AAT GGG GAT CCG TGT TTA; and MMP12, F, 5'-GTT GTT TGG GAC CAG TAT GGA TAA, R, 5'-GAT GCC GAC TGG GAC CAG TAT AAT AAA. Amplification reactions were set up with mastermix (SurePRIME&Q-GBio; QBiogene, Carlsbad, CA). Briefly, each reaction contained 1× mastermix, 100 μM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.02 U/μL DNA polymerase (SurePRIME&Qbiogene), 200 nM forward and 200 nM reverse primers; 1:2000 nucleic acid gel stain (SYBR green 1 dye; Molecular Probes Inc., Eugene, OR), and a 1:50 dilution of cDNA. The reactions were performed in 96-well plates (MicroAmp; ABI) capped with optical caps (MicroAmp; ABI) and amplified for 40 cycles (Prism 7900HT; ABI) with the standard PCR parameters (thermal profile: 50°C for 2 minutes; 1 cycle, 95°C for 15 minutes; 1 cycle, 52 to 55°C for 1 minute; 40 cycles, 72°C for 30 seconds). Dissociation curves were analyzed to ensure product speci-
ficity and amplicon identification based on Tm (melting temperature) values. The data generated from reactions was analyzed by plotting ΔRn (normalized) fluorescence signal versus the cycle number. An arbitrary threshold was set at the midpoint of the log ΔRn versus cycle number plot. The Ct values (thresholds) calculated from this plot were used to determine relative quantitation of gene expression by applying comparative Ct method (ΔΔCt). ΔΔCt was calculated by subtracting the Ct of the reference gene from the Ct (target gene). The reference gene was GAPDH. Comparative expression level was then calculated by converting ΔΔCt from a logarithmic to a linear value. Fold change, 2^−ΔΔCt.

Flow Cytometry
Cells in staining buffer (PBS, 1% fetal calf serum, 0.1% sodium azide) were incubated with blocking reagent (Fc blocker, 2 μg/10^6 cells). All cells were incubated on ice for 20 to 30 minutes and washed twice before the next reagents were added. In some experiments with PECs, aliquots of 2 × 10^7 PECs were incubated with ECCD-2 which recognizes the extracellular domains of E-cadherin in a buffer containing PBS, 1 mM CaCl2, and 1% bovine serum albumin (PBS-Ca-BSA) for 30 minutes at 4°C. Cells were washed three times in PBS followed by incubation with FITC-conjugated goat anti-rat Ig antibody for E-cadherin in PBS-Ca-BSA for 30 minutes at 4°C. Flow cytometry analysis of E-cadherin was performed in the absence of the blocking agent of Fc receptors on PECs, because FITC-conjugated anti-Ig antibody yielded high-background fluorescence in cells treated with antibodies for Fc blocking. Negative controls included direct staining with secondary antibody in the absence of the first antibody. Stained cells were analyzed on a flow cytometer (EPICS XL; Beckman Coulter, Miami, FL).

Administration of Antigens
For intracocular injection, mice were injected a range of OVA (50 μg/2 μL HBSS) into the AC of the right eye, as described previously.7,18 For conventional sensitization, 100 μL of an emulsion produced from a 1:1 mixture of OVA (2 mg/mL), and CFA was injected subcutaneously (SC) into the nape of the neck.

DH Assay
Induction and assessment of DH were performed as previously described.7,18 Seven days after immunization, mice received an intradermal injection of 200 μg/10 μL HBSS of OVA into the right ear pinna. In some experiments, OVA-pulsed PECs (prepared as described earlier, 5 × 10^7/10 μL HBSS) were used as the antigen,19 even though background is higher when OVA-pulsed PECs were used than when antigen alone was used. There was swelling in both cases when primed T cells were added. Ear-swelling was expressed as follows: specific ear-swelling = (24 hours measurement of right ear − 0 hour measurement of right ear) − (24 hours measurement of left ear − 0 hour measurement of left ear) in micrometers. Ear-swelling responses of groups of mice are presented as the mean ± SEM. At 24 and 48 hours, ear-swelling was measured with a micrometer (Mitsutoyo, Paramus, NJ).

Preparation of T-Cell-Reconstituted Mice
CD103 KO mice were reconstituted with CD103-expressing T cells by a modification of an original method previously described.12 In brief, unfractonated, purified T cells and purified CD8+ T cells were prepared from spleens and lymph nodes of normal BALB/c mice, as described earlier. Recipient CD103 KO mice received naïve unfractonated (3–4 × 10^7/mouse) or purified CD8+ (5–8 × 10^7/mouse) BALB/c T cells, intravenously (IV) 24 hours before use.

Adoptive Transfer of Regulatory T Cells
T cells were enriched from spleens of mice that had received an AC injection of OVA 7 days earlier. Some of the T cells were depleted of CD103+ cells by incubation with biotin anti-CD103 mAb (M290) for 20 minutes, washed and incubated with streptavidin microbeads for 15 minutes, and purified by column-negative selection in a magnetic field (Miltenyi Biotec). CD103-depleted T cells (enrichment was 99.9% for both CD4+CD103+ and CD8+CD103+ T cells) or undepleted T cells from mice with ACAID (10^7) and T cells from naïve BALB/c mice (10^7) were inoculated IV into BALB/c recipients that had been immunized 7 days earlier with OVA plus CFA. Immediately after IV infusion, recipients received an ear pinna challenge with OVA (200 μg/10 μL) or OVA-pulsed PECs (5 × 10^7/10 μL HBSS). Positive control mice received only OVA/CFA immunization 7 days earlier. Negative controls were injected into the right ear pinna only, with OVA or OVA-pulsed PECs. Ear-swelling responses were measured 24 hours after intradermal injections.

Local Adoptive Transfer of Regulatory T Cells
To test for the effector regulator cells of ACAID, a modified local adoptive transfer (LAT) assay was performed, as described elsewhere.7,19,20 OVA-primed T cells (responder cells) were generated in BALB/c mice by immunization with OVA and CFA, and 7 days later the primed T cells were dissociated from the draining lymph nodes by removing B cells and macrophages (Immunul; Biotecx Laboratory) column. Regulator cells were enriched on the column from spleen cells of mice that had received an AC injection with OVA 7 days earlier. Some of the regulator T cells were depleted of their CD103+ cells by incubation with biotin anti-CD103 mAb, washed and incubated with streptavidin microbeads for 15 minutes, and purified by column-negative selection in a magnetic field (Miltenyi Biotec). Stimulator cells were OVA-pulsed PECs as described earlier. Responder cells (5 × 10^7), stimulator cells (5 × 10^5), and regulator cells (5 × 10^7) were mixed and resuspended in 10 μL HBSS for inoculation into the right ear pinna of naïve mice. Ear-swelling was measured at 24 and 48 hours. As a negative control, naïve T cells from unmanipulated mice were used as responder cells and regulator cells. Primed T cells were used as responder cells, and naïve T cells from unmanipulated mice were used as regulator cells for a positive control.

Statistical Analysis
Data were subjected to analysis by ANOVA and the Scheffe test. Means were considered to be significantly different when P ≤ 0.05.

RESULTS
Comparison of Gene Expression Profiles in Regulatory and Nonregulatory CD8+ T Cells by DNA Microarray Analysis
To determine the molecules that might be involved in the generation of as well as the regulatory properties of the regulatory CD8+ T cell of ACAID that suppress expression of Th1 and Th2 effector cells, we compared RNA isolated from CD8+ OT-I T cells exposed to OVA-pulsed, TGFB2-treated APCs (T reg) or those exposed to OVA-pulsed, untreated APCs T effector cells (non-T reg). We used DNA microarrays that analyze 12,000 murine genes and expressed sequence tags (ESTs). Summaries of genes differentially expressed between the two cell populations are shown in Table 1. The signal ratio (T reg/non-T reg) of each of the 12,000 genes was calculated. Genes with a threefold increase in this ratio were defined arbitrarily as upregulated in T reg, whereas those with a threefold decrease in this ratio were defined as downregulated. Using these criteria, 45 genes were found to be upregulated, and 16 genes were found to be downregulated in T reg compared with non-T reg.

The differentially expressed genes are arranged in Table 1 according to four functional categories that were chosen based on known functions of these genes: (1) synthesis, secretion, activation, and receptor binding of TGF-β, (2) inhibition and loss of NK and CD8+ T effector function, (3) preferential localization to peripheral sites (nonlymphoid) of antigen deposition, and (4) capacity to resist apoptosis triggered by TCR ligation.
Table 1. Differential Gene Expression in OT-I T Cells*

<table>
<thead>
<tr>
<th>Common Name (GenBank Accession No.)</th>
<th>Gene Product Description</th>
<th>Change (x-Fold)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL9 (M30136)</td>
<td>Stimulates TGF-β production by LPS-stimulated monocytes</td>
<td>+23.48</td>
<td>21</td>
</tr>
<tr>
<td>MMP9 (X72795)</td>
<td>Converts latent to active TGF-β</td>
<td>+21.32</td>
<td>22 23</td>
</tr>
<tr>
<td>Retinoic acid receptor-α (RAR-α) (M60969)</td>
<td>Enhances TGF-β expression</td>
<td>+14.05</td>
<td>24 25</td>
</tr>
<tr>
<td>Thyroglobulin (AF070186)</td>
<td>Inhibits TGF-β receptor binding</td>
<td>−28.08</td>
<td>26</td>
</tr>
<tr>
<td>Serine protease inhibitor 4 (X70296)</td>
<td>Prevents TGF-β activation</td>
<td>−4.79</td>
<td>27</td>
</tr>
<tr>
<td>Serine protease inhibitor 6 (U96700)</td>
<td>Prevents TGF-β activation</td>
<td>+3.81</td>
<td>27</td>
</tr>
<tr>
<td>II. Inhibition and loss of NK and CD8 T effectors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD94 (AF030311)</td>
<td>Binds class I b→ inactivation</td>
<td>+3.53</td>
<td>28 29</td>
</tr>
<tr>
<td>IFN-γ (K00083)</td>
<td>Proinflammatory function</td>
<td>−4.78</td>
<td>30</td>
</tr>
<tr>
<td>IL-12 receptor β1 (U23922)</td>
<td>IFN-γ production</td>
<td>−3.38</td>
<td>31</td>
</tr>
<tr>
<td>Granzyyme B (M12302)</td>
<td>Lytic activation</td>
<td>−3.24</td>
<td>32</td>
</tr>
<tr>
<td>III. Localization to site of antigen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD103 (AV268095)</td>
<td>Promotes adhesion during migration</td>
<td>+85.12</td>
<td>36 55 33</td>
</tr>
<tr>
<td>CCR8 (AF001277)</td>
<td>Promotes chemotaxis toward TCA-3</td>
<td>+20.29</td>
<td>34</td>
</tr>
<tr>
<td>IV. Antiapoptotic function</td>
<td>Prevents apoptosis</td>
<td>+17.68</td>
<td>35</td>
</tr>
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</table>

* Cells were exposed to TGF-β2-untreated OVA-pulsed PECs (non-T regs) versus OT-I T cells exposed to TGF-β2-treated OVA-pulsed PECs (T regs).

**Confirmation of Differentially Expressed Genes**

To confirm that the observed differences in DNA microarray analysis correlated with differences in steady state levels of the corresponding mRNAs, the expression patterns of selected genes were determined by real-time RT-PCR, using primers specific for the genes shown in the Materials and Methods section. Levels of selected genes relative to that of a housekeeping gene (GAPDH) were compared between T reg- and non-T reg-derived RNA. This method confirmed the differential expression of each of the selected genes in the expected manner (Fig. 1). mRNA expression of CD103, MMP-9, CCR8, and RAR-α was upregulated in T regs, whereas expression of granzyme B and IL-12 receptor β1 was downregulated. Furthermore, CD94 protein expression on T regs and non-T regs was examined by flow cytometry. Increased cell surface levels of CD94 were detectable on T regs compared with non-T regs (data not shown).

**CD103 Expression on CD8⁺ Regulatory T Cells**

Because the discovery that CD8⁺ T cells in ACAID are capable of inhibiting the expression of DH in vivo and in vitro,5-10 we focused our efforts on identifying other marker molecules for ACAID T regulatory cells and analyzed their mode of action. In this study, we found that expression of the CD103 gene, an integrin known to be expressed by intraepithelial lymphocytes (IELs),14,36 was increased (85-fold) in our regulatory CD8⁺ T cells generated in vitro. Thus, subsequent experiments were designed to determine whether increased expression of the CD103 gene was essential for the regulatory function of the CD8⁺ T cells and for ACAID. We first confirmed that the CD103 protein was expressed on the surface of T regs, by flow cytometry. The percentage of CD103-expressing OT-I T cells increased when these cells were exposed to OVA-pulsed, TGF-β2-treated PECs for 72 hours (82.8%; Fig. 2). By contrast, fewer OT-I T cells expressed CD103 after exposure to OVA-pulsed, untreated PECs (0.53%). Thus, nearly all OT-I T cells acquired the capacity to express surface CD103 during their conversion from cytotoxic (effector) to regulatory T cells by exposure to OVA-pulsed, TGF-β2-treated APCs.11

The ability of TGF-β to convert PECs into ACAID-promoting cells has been shown to be dependent on the ability of the APCs to secrete their own active TGF-β.16,52 and TGF-β has been reported to control CD103 expression on CD8⁺ T cells.35 Therefore, we determined whether TGF-β had a role in the upregulation of CD103 in OT-I T regs. Two types of experiments were performed. First, PECs were pulsed with OVA without TGF-β2 (risen to remove unbound TGF-β) and were used to stimulate OT-I T cells in the presence or absence of a pan anti-TGF-β mAb. The T cells were then harvested and analyzed by flow cytometry for expression of CD103. Treatment with anti-TGF-β antibody partially inhibited the expression of CD103 on OT-I T cells responding to OVA-pulsed, TGF-β2-treated PECs (from 73% without Ab to 42% in the presence of Ab). These results support the notion that TGF-β produced by the TGF-β-treated PECs is necessary for the expression of CD103. To that end, we reconfirmed the original demonstration by Hadley et al.36 that CD8⁺ T cells upregulate CD103 expression when exposed in vitro to TGF-β.

**Development of Eye-Derived Tolerance in CD103 KO Mice**

The lack of T reg activity in AC-inoculated CD103 KO mice suggests that eye-derived tolerance (ACAID) may not develop in CD103 KO mice. To examine this possibility, we treated groups of CD103 KO and WT mice with an AC injection of OVA. Seven days later, these mice were immunized SC with 200 μg OVA, and ear-pinning responses were measured 24 hours after the challenge. As expected, groups of AC-injected WT mice displayed a reduced ear-swelling response compared with a positive control group, indicating suppressed OVA-specific DH (Fig. 3). By contrast, a similarly treated group of CD103 KO mice displayed ear-swelling responses comparable to that in the positive control group. This lack of suppression of OVA-specific DH indicates absence of eye-derived tolerance. These results are consistent with our observations in in vitro experiments that indicate the absence of regulatory activity in splenic T cells of CD103 KO mice receiving an AC inoculation of OVA. Thus, both results suggest that expression of CD103 protein is necessary for the development of eye-derived tolerance (ACAID).

**Role of CD103 Expressed on T Cells in the Development of Eye-Derived Tolerance**

Microarray analysis detected increased CD103 expressed in OT-I T reg- and non-T reg-derived RNA. This method confirmed the differential expression of each of the selected genes in the expected manner (Fig. 1). mRNA expression of CD103, MMP-9, CCR8, and RAR-α was upregulated in T regs, whereas expression of granzyme B and IL-12 receptor β1 was downregulated. Furthermore, CD94 protein expression on T regs and non-T regs was examined by flow cytometry. Increased cell surface levels of CD94 were detectable on T regs compared with non-T regs (data not shown). Therefore, we determined whether TGF-β had a role in the upregulation of CD103 in OT-I T regs. Two types of experiments were performed. First, PECs were pulsed with OVA without TGF-β2 (risen to remove unbound TGF-β) and were used to stimulate OT-I T cells in the presence or absence of a pan anti-TGF-β mAb. The T cells were then harvested and analyzed by flow cytometry for expression of CD103. Treatment with anti-TGF-β antibody partially inhibited the expression of CD103 on OT-I T cells responding to OVA-pulsed, TGF-β2-treated PECs (from 73% without Ab to 42% in the presence of Ab). These results support the notion that TGF-β produced by the TGF-β-treated PECs is necessary for the expression of CD103. To that end, we reconfirmed the original demonstration by Hadley et al.36 that CD8⁺ T cells upregulate CD103 expression when exposed in vitro to TGF-β.
mice did not develop eye-derived tolerance after AC injection of OVA addresses the question of whether CD103 expression in T cells is relevant. Therefore, to examine whether CD103 expression by T cells is essential for eye-derived tolerance, we reconstituted CD103 KO mice with wild-type (WT) T cells expressing CD103. One mouse equivalent each of purified T cells from WT (3–4 × 10⁷/mouse) or CD8⁺ (5–8 × 10⁶/mouse) T cells were transferred into individuals within the groups of CD103 KO mice. Twenty-four hours later, each recipient received an AC injection of OVA. Seven days later, the mice were immunized (SC) with OVA/CFA and 1 week later were injected in the ear pinna with an OVA challenge, to determine their ability to express OVA-specific DH. Positive control CD103 KO mice were immunized (SC) with OVA/CFA and 1 week later were injected in the ear pinna with an OVA challenge, to determine their ability to express OVA-specific DH.

Role of CD103 in Efferent Suppression of DH in Eye-Derived Tolerance

It has been reported that, in eye-derived tolerance, two distinct populations of Tregs are involved in the suppression of DH responses after tolerance induction through the eye. Although CD8⁺ Tregs are known to suppress the induction (afferent phase) of the DH response, the suppression (efferent phase) of DH is attributed to the CD8⁺ Treg population.⁴⁻¹⁰ To confirm the necessity of CD103 expression in the efferent suppression of DH followed by AC inoculation of OVA, we performed two types of adoptive transfer experiments: systemic and LAT. Spleen cells were obtained from AC-injected or untreated BALB/c mice and served as a source of regulatory cells. T cells were purified from these spleens, which then were depleted (from 8.15% to 0.02%) of CD103-bearing cells by using magnetic bead sorting. For systemic adoptive transfer experiments, CD103-replete or -depleted T cells (10⁷/mouse) were injected IV into OVA/CFA immunized BALB/c mice. Positive control mice were immunized SC only. OVA-primed recipient mice that received T cells IV were injected in the ear pinna with a challenge of either OVA or OVA-pulsed PECs within 2 hours of receiving the T cells. A suppressed ear-swelling response was detected in the recipients of replete T cells (Fig. 5). Similar results were obtained when the ears of adoptive transfer recipients were challenged with OVA only (data not shown). By contrast, ear-swelling responses were not suppressed in recipients of CD103-depleted T cells. Next, we used an LAT, in which regulatory cells derived from the spleens of AC-inoculated BALB/c mice were mixed with OVA-primed T cells (responders) and OVA-pulsed PECs (stimulators) and injected into the ear pinnae of naïve syngeneic mice. Ear-swelling was measured at 24-hour intervals (Fig. 6). Whereas replete T cells from AC-injected donors suppressed ear-swelling responses, CD103-depleted T cells from similar donors did not (Fig. 7). Together,
the results of our systemic transfer and LAT assays support the postulate that CD103 expression on T regs is necessary for the suppression of the efferent phase of the DH response.

**DISCUSSION**

Over the past decade, there has been a resurgence of interest in the functional properties of regulatory T cells and in the role(s) these cells play in suppressing autoimmune diseases and promoting the acceptance of solid tissue allografts. Research in this area has been stifled in part by the inability to obtain a sufficient number of antigen-specific T regulatory cells to enable molecular analysis. Because of the relative abundance of natural CD4$^{+}$/CD25$^{+}$ T regulatory cells in normal mice and humans, it has been possible to use microchip gene array technology to define genes that are differentially expressed in these cells and are therefore candidates for conferring regulatory properties on these cells. We adopted a similar approach to understanding the molecular mechanisms by which afferent and efferent regulatory T cells in eye-derived tolerance suppress the induction and expression, respectively, of Th1-type immunity to antigens injected into the AC of the eye, TCR transgenic mice such as DO11.10 (TCR recognizes OVA peptides 323-339 in the context of I-Ad) and OT-I (TCR recognizes OVA peptides 257-264 in the context of K$^{b}$). We showed recently that DO11.10 and OT-I T cells stimulated in vitro with OVA-pulsed, TGF-$\beta$-treated APCs differentiate into T regulatory cells, while veering away from their natural inclination to develop into Th1 cells or cytotoxic T cells, respectively.$^{11,12,43}$ The functional properties of DO11.10 and OT-I in vitro–generated T regulatory cells resemble, respectively, the properties previously described for CD4$^{+}$-afferent and CD8$^{+}$-efferent regulatory T cells in ACAIDS.$^{6-10}$

In this communication, we report the results of experiments designed to determine genes that are differentially expressed in OT-I T cells exposed to OVA-pulsed, TGF-$\beta$-treated or untreated APCs. We assigned priority of interest to those genes with the greatest change (threefold or greater) in expression compared with genes in OT-I T cells exposed to OVA-pulsed, TGF-$\beta$-untreated APCs and with the known ability
of the gene products to influence the following parameters of T cell function: (1) capacity to synthesize, activate, secrete, and respond to TGF-β, (2) capacity to perform effector functions such as lysis of target cells and secretion of proinflammatory cytokines, (3) ability to localize via vascular migration to selected peripheral tissues and to adhere to bystander immune and parenchymal cells, and (4) resistance to TCR-triggered apoptosis.

TGF-β is known to be a crucial immunomodulatory factor within the eye, in the induction of ACAID by eye-derived APCs, and in the suppression of DH by ACAID T regulators. It was therefore gratifying to find several strongly up- and downregulated genes (14–23-fold increases) with the potential to influence TGF-β production and responsiveness in OT-I T cells converted into T regs in vitro (Table 1, category I). Although dramatic changes in gene expression profiles by OT-I T cells exposed to OVA-pulsed, TGF-β-treated APCs correlated with gain of function (regulation), there were also changes in gene expression that correlated with loss of function (effector). Downregulated genes that correlated with loss of effector function by OT-I T cells (Table 1, category II) help explain why OT-I T cells that convert into T regulators in vitro no longer display effector functions. Presumably, these functions would be inimical to the inhibition of inflammation, which is the goal of their suppressor activities. Knowing that the efferent CD8+ T regulators of ACAID originate in the spleen and then disseminate peripherally implies that molecular factors governing migration and adhesion are likely to be important to expression of their regulatory function (Table 1, category III).

Our laboratory reported a few years ago that DO11.10 T cells stimulated in vitro with OVA-pulsed, TGF-β-treated APCs are spared from the apoptosis that is the fate of...
That suppressed T cell activation in vitro. Finally, CD8+ ACAID, and this resulted in the generation of splenic T cells measured at 24 hours. Mean apoptosis after antigen stimulation 48 of regulatory T cells may contribute to their stability. Thus, because TGF-β1-treated APCs promote ACAID by rescuing antigen-activated T cells from apoptosis, it is not a surprise that anti-apoptotic genes expression by regulatory T cells appears to be absolutely essential for these cells to suppress antigen-specific Th1-type immunity in vivo—that is, eye derived tolerance (ACAID).

Most of the DO11.10 T cells stimulated in vitro with OVA-pulsed, TGF-β1-untreated APCs. We suspect that prevention of apoptosis after antigen stimulation of regulatory T cells may contribute to their stability. Thus, because TGF-β1-treated APCs promote ACAID by rescuing antigen-activated T cells from apoptosis, it is not a surprise that anti-apoptotic genes such as IGF-1 (Table 1, category IV) are highly upregulated in OT-I T regs.

Because CD103 (a cell-adhesion molecule that binds E-cadherin) was recently shown to be upregulated in natural CD4+ CD25+ T regulators, and because it was the most highly upregulated in OT-I T cells, we chose to study the role of CD103 in eye-derived tolerance. Our results indicate that expression of CD103 was upregulated on nearly all in vitro-generated OT-I T reg cells and that this expression was dependent, at least in part, on active TGF-β1, presumably produced by the APCs and the T cells themselves. Moreover, we found that CD103 expression was necessary in vivo for induction of ACAID, and this resulted in the generation of splenic T cells that suppressed T cell activation in vitro. Finally, CD8+ T regs from spleens of mice with ACAID proved to be unable to transfer suppression of DH to OVA-primed mice if the splenic cells were first depleted of CD103-bearing cells. Thus, CD103 expression by regulatory T cells appears to be absolutely essential for these cells to suppress antigen-specific Th1-type immunity in vivo—that is, eye derived tolerance (ACAID).

There are at least two ways in which CD103 interactions with E-cadherin could be necessary to achieve this outcome. First, it can be expressed by APCs, and our own results indicate that PECs treated with OVA and TGF-β1 upregulate their expression of E-cadherin (Keino H, unpublished observations, 2004). Thus, the conversion of naïve OT-I T cells into T regs on exposure to OVA-pulsed, TGF-β1-treated APCs may be facilitated by interactions between CD103 on the T cells and E-cadherin on the APC, thus implicating CD103 in the induction of CD8+ T regs in ACAID. Alternatively, local APCs participate in activating primed Th1 cells at the site of antigen deposition in DH reactions. Thus, E-cadherin expression by APCs within the stroma of OVA-injected ear pinnae may be necessary for effector CD103+ CD8+ T regs to effect suppression of the inflammatory response. It is worth pointing out that CD103/E-cadherin-adhesive interactions are enhanced by signaling via the T cell receptor complex, which makes it possible that CD103 stabilizes and retains CD8+ T regulators at the site of antigen deposition. We are attracted to this possibility, because CD103-depleted ACAID T regulators failed to suppress DH expression in the LAT assay.

A second way in which CD103 interactions with E-cadherin could be important relates to the expression of E-cadherin on vascular endothelial cells. CD103 binding to E-cadherin on vascular endothelial cells is an attractive possibility, because it has been shown that CD103 functions to promote the entry or retention of CD8+ cytotoxic T cells into the epithelial compartment of allografts. One way to interpret our finding that CD103-depleted T regulators from mice with ACAID failed...
to suppress DH in an adoptive transfer experiment is that CD103 expression is necessary for the cells to localize first to the local vessels then into the stroma at the site of antigen injection. This is supported by Huehn et al.\textsuperscript{52} who showed a correlation of suppressive capacity of CD103\textsuperscript{+} expressing T cells subsides with their preferential migration to inflamed sites. A very recent paper by Schwarz et al.\textsuperscript{30} provides evidence that the reason regulatory T cells generated after ultraviolet B radiation of the skin fail to suppress the expression of hapten-specific contact hypersensitivity is that the regulatory cells lack the requisite adhesion molecules that enable T cells to migrate into the skin. Together, these considerations lead us to propose that upregulation of CD103 creates a novel functional program that promotes migration of effector T regulators to the nonlymphoid sites where antigen is deposited—that is, ear pinnae where OVA is injected to elicit DH.

Our immediate goal is to identify key up- and downregulated genes in OT-I T regulatory cells and to determine whether these genes are essential in the generation of these cells or in their capacity to effect suppression. Our near-term goal is to use molecular strategies to interrupt expression or to induce selective expression of individual genes within these OT-I T cells and then within nontransgenic CD8\textsuperscript{+} T cells obtained from naive or primed donors. Our ultimate goal is to use these strategies to create regulatory T cells at will with the capacity to suppress immunogenic inflammation in experimental animal model systems as a preamble to attempting these strategies in humans with immunopathogenic diseases and to promote allograft acceptance.

**References**


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