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Antigen-presenting cells are targets of regulatory T cells similar to those that mediate anterior chamber-associated immune deviation

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Abstract *Purpose:* To identify the means by which in-vitro-generated regulatory T cells, similar to those in anterior chamber-associated immune deviation (ACAID), suppress antigen-specific T-cell responses. *Methods:* T regulators (T regs), generated by stimulating ovalbumin (OVA)-specific Tcr transgenic DO11.10 T cells with OVA-pulsed, TGF- β 2-treated peritoneal exudates cells (PEC), or their supernatants were added to OVA-pulsed PEC that were used to activate DO11.10 T cells in vitro or to suppress OVA-specific delayed hypersensitivity (DH) induction in vivo. *Results:* OVA-pulsed PECs exposed in vitro to TGF- β -producing T regs or their supernatants failed to activate DO11.10 T cells in vitro, and suppressed DH in mice immunized with OVA plus adjuvant. *Conclusion:* T cells exposed to TGF- β 2-pretreated, antigen-pulsed PECs secrete soluble factors, including active TGF- β that regulate OVA-specific responses by forcing antigen-presenting cells to promote deviant T-cell activation in vitro and in vivo.

Key words T lymphocytes; cytokines; tolerance; suppression; eye

Introduction Intraocular injection of exogenous antigens induces a stereotypic deviant systemic immune response termed anterior chamber-associated immune deviation (ACAID).^{1–4} The key antigen-specific characteristics of this response are failed induction and expression of delayed hypersensitivity (DH),⁵ combined with intact, and often enhanced, production of antibodies,⁶ and primed and clonally expanded cytotoxic T-cell precursors.⁷ Evidence indicates that indigenous intraocular antigen-presenting cells (APCs) capture injected antigen, migrate across the trabecular meshwork to escape from the eye, and traffic via the blood to the spleen.^{8–11} In this secondary lym-

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phoid organ, the regulatory T cells that dictate the unique features of ACAID are activated. It is important to understand the mechanism by which regulatory T cells suppress the antigen-specific immune response of naive T cells, because the deviant immune response of ACAID plays a central role in the phenomenon of ocular immune privilege and serves as a model for regulation of immunopathogenic responses at other sites in the body.

Conventional APCs (harvested from peritoneal exudate, or from peripheral blood) that are pulsed with antigen in the presence of transforming growth factor (TGF)- β and then injected intravenously into naive mice induce a deviant systemic immune response similar to ACAID.¹² Recently, we reported that peritoneal macrophages treated in vitro with TGF- β 2 secrete reduced amounts of interleukin-12 (IL-12), express low levels of CD40, and produce enhanced amounts of mature TGF- β compared with untreated APCs.^{13,14} When pulsed with ovalbumin (OVA), TGF- β 2-treated APCs resemble untreated APCs in their ability to induce proliferation of naive T cells harvested from Tcr transgenic DO11.10 mice.¹⁵ However, OVA-specific transgenic T cells stimulated with OVA-pulsed, TGF- β 2-treated APCs in this manner suppress DH when injected intradermally with OVA-primed T cells and OVA-pulsed APCs.¹⁵ Thus, TGF- β 2 alters the functional properties of APCs in a manner that enables them to induce regulator T cells that suppress the immunogenic inflammation of DH.

In the present study, we examined the mechanisms by which regulatory T cells of this type suppress responses of naive DO11.10 T cells. Our results indicate that transgenic T cells activated in this manner to become regulators acted directly on APCs to prevent them from activating naive T cells. Moreover, these in vitro-generated regulatory T cell-produced soluble factor(s), including active TGF- β 2, were at least partly responsible for this direct inhibitory effect on APCs.

Materials and methods

MICE Female BALB/c mice (6–8 weeks of age) were purchased from Taconic farms (Germantown, NY, USA). These mice were used as a source of peritoneal exudate cells (PECs). DO11.10 Tcr transgenic mice were maintained in our colony (original parents were a kind gift of Dr. Dennis Loh) on an H-2^d background. These mice were used as a source of T cells in the present study. DO11.10 mice express the DO11.10 Tcr that is specific for the peptide fragment of OVA, 323–339, in the context of I-A^d.^{16,17}

CULTURE MEDIUM Serum-free medium was used for all macrophage cultures. This medium was composed of RPMI 1640, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin (all from Biowhitaker, Walkersville, MD, USA), 1×10^{-5} M 2-ME (Sigma Chemical, St. Louis, MO, USA), supplemented with 0.1% bovine serum albumin (BSA; Sigma Chemical Inc.), and ITS⁺ culture supplement (1 μ g/ml iron-free transferrin, 10 ng/ml linoleic acid, 0.3 ng/ml Na₂Se, and 0.2 μ g/ml Fe(NO₃)₃; Collaborative Biochemical Products, Bedford, MA, USA). Complete medium

was used for all T-cell cultures. This medium was supplemented with 5% fetal bovine serum (FBS) and 2 mM L-glutamine, instead of BSA and ITS⁺.

REAGENTS Porcine TGF- β 2 and anti-TGF- β 2 neutralizing antibody were purchased from R&D systems (Minneapolis, MN, USA). Anti-Thy-1 antibody was purchased from PharMingen (San Diego, CA, USA). OVA was obtained from Sigma Chemical Inc.

PREPARATION OF PURE DO11.10 T CELLS Spleens were removed from DO11.10 mice and pressed through nylon mesh to produce a single-cell suspension. Red blood cells were lysed with Tris-NH₄Cl. The remaining nucleated cells were washed three times with RPMI 1640 and passed through T-cell enrichment columns (Biotex Laboratories Houston, TX, USA). The resultant cell suspension contained >98% CD3⁺ cells.

PREPARATION OF PECS PRETREATED WITH TGF- β 2 PECs were harvested from normal BALB/c mice that had received 2 ml thioglycolate (Sigma Chemical Inc.) intraperitoneally three days earlier. The cells were washed, placed in 24-(1×10^6 /well) or 96-well (1×10^5 /well) culture plates, pulsed with various concentrations of OVA, and treated with or without 5 ng/ml porcine TGF- β 2 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- β 2 and non-adherent cells. Adherent cells were retained in the wells for use in all subsequent experiments. More than 90% of these adherent cells were F4/80⁺ macrophages.

PREPARATION OF 'REGULATORY' T CELLS Purified DO11.10 T cells (2×10^5 /well) were added to 24-well plates containing TGF- β 2 (5 ng/ml)-pretreated, OVA (50 μ g/ml)-pulsed PECs (1×10^6 /well), and cultured in complete medium for 48 hours at 37°C in an atmosphere of 5% CO₂. Nonadherent cells were collected as 'regulatory' T cells. Flow cytometric analysis of the recovered nonadherent cells for expression of CD3 and F4/80 revealed that >99% of the cells in suspension were CD3⁺, F4/80⁻ (data not shown). DO11.10 T cells similarly cultured with TGF- β 2-untreated, OVA (50 μ g/ml)-pulsed PECs were collected as 'activated' T cells. 'Regulatory' and 'activated' T cells were either injected i.v. into naive BALB/c mice (in-vivo experiments) or co-cultured with TGF- β 2-untreated, OVA-pulsed PECs or naive DO11.10 T cells in vitro.

CYTOKINE ASSAYS To assay culture supernatants for content of interferon (IFN)- γ , interleukin (IL)-2, and IL-4, DO11.10 T cells (2×10^4 /well) were added to 96-well plates containing TGF- β 2-pretreated (or not), OVA-pulsed PECs (1×10^5 /well) and cultured in complete medium for 24 or 48 hours. In some experiments, DO11.10 T cells were added to 96-well plates containing either TGF- β 2-untreated, OVA-pulsed PECs that had been previously exposed to 'regulatory' or 'activated' T cells, or OVA-pulsed PECs that had been previously treated

with supernatants from 'regulatory' or 'activated' T cells. At each time point, supernatants were collected and analyzed by quantitative capture enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (PharMingen). Rat monoclonal antibodies (mAbs) to mouse cytokine IFN- γ (R4-6A2), IL-2 (JES6-1A12), and IL-4 (BVD4-1D11) were purchased from PharMingen and used as coating Abs. Biotinylated rat mAbs to mouse cytokines IFN- γ (XMG1.2), IL-2 (JES6-5H4), and IL-4 (BVD6-24G2; PharMingen) were used as detecting Abs.

TGF- β ASSAYS To assay culture supernatants for TGF- β content, DO11.10 T cells (2×10^5 /well), that had been previously stimulated by TGF- β 2-treated (or not), OVA-pulsed PECs, were added to 24-well plates containing TGF- β 2-untreated, OVA-pulsed PECs (1×10^6 /well) for re-stimulation and cultured in serum-free medium for 24 hours. Supernatants were collected at 24 hours and analyzed for total and biologically active (mature) TGF- β using Mv1Lu cells (ATCC, Rockville, MD, USA). To detect mature TGF- β , the supernatants were diluted 1:4 with EMEM (Eagle's minimum essential medium; Biowhitaker), which consisted of 2 mM L-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.5% FBS. Diluted supernatant (100 μ l) was added to 96-well flat-bottom plates. To measure total TGF- β , the supernatants were pretreated with 1 N HCl (1:10) for one hour (activate any latent TGF- β present), then neutralized with a mixture of 1 N NaOH:1 M HEPES (1:5). These acidified supernatants were diluted 1:10 with complete EMEM with 0.5% FBS, and 100 μ l diluted supernatant was added to a 96-well flat-bottom plate. Mv1Lu cells (1×10^5 cells/100 μ l) were added into each well of the 96-well flat-bottom plate, and cultured for 24 hours at 37°C in an atmosphere of 5% CO₂. Cultures were pulsed with 1 μ Ci [³H]-thymidine 4 hours before termination, then harvested onto glass filters using an automated cell harvester (Tomtec, Orange, CT, USA). Radioactivity was assessed by liquid scintillation spectrometry. Half-maximal inhibition was determined by polynomial regression on log-log transformation of standard curves and experimental samples. The results were expressed as pg/ml. By subtracting the amount of mature TGF- β from the amount of total TGF- β , the amount of latent TGF- β can be determined.

PROLIFERATION ASSAY OF T CELLS ACTIVATED IN VITRO DO11.10 T cells (2×10^4 /well) were added to 96-well plates containing TGF- β 2-pretreated (or not), OVA-pulsed PECs (1×10^5 /well) and cultured in complete medium for 24–96 hours at 37°C in an atmosphere of 5% CO₂. In some experiments, X-irradiated (2000 R) DO11.10 T cells (2×10^4 /well), first cultured with OVA-pulsed, TGF- β 2-pretreated (or not) PECs, were then co-cultured in 96-well plates with or without OVA-pulsed PECs and naive DO11.10 T cells. The cultures were sustained for 24, 48, 72, or 96 hours, pulsed with 0.5 μ Ci [³H]-thymidine eight hours before termination, and then harvested onto glass filters using an automated cell harvester. Radioactivity was assessed by liquid scintillation spectrometry, and the amount expressed as counts per minute.

ASSESSMENT OF DELAYED HYPERSENSITIVITY (DH) DH was accomplished as described^{18,19} previously with minor modification. Seven days following s.c. immunization with OVA (200 µg/ml)-pulsed PEC (1×10^6 /mouse), BALB/c mice received intradermal (i.d.) inoculation of OVA (200 µg/10 µl) into the right ear pinnae. The ear-swelling response was then assessed 24 and 48 hours later using an engineer's micrometer (Mitutoyo 227-101, MTI Corporation, Paramus, NJ, USA). Experimental, and positive and negative control panels contained five mice each. Each experiment was repeated at least twice with similar results. Representative experimental results of individual experiments are presented in the figures.

STATISTICAL ANALYSES Results of experiments were analyzed by ANOVA and Scheffe test. Mean values were considered to be significantly different when $p < 0.05$.

Results

IN-VITRO AND IN-VIVO REGULATORY PROPERTIES OF DO11.10 T CELLS STIMULATED IN VITRO BY OVA-PULSED, TGF- β 2-TREATED PECs We have previously reported that DO11.10 T cells exposed to OVA-pulsed, TGF- β 2-treated PECs are activated in a deviant manner. While these cells remain capable of proliferating in response to antigen stimulation, they fail to secrete IFN γ , whereas DO11.10 T cells exposed to OVA-pulsed, untreated PECs both proliferate and secrete large amounts of IFN γ .¹³ Suppression of T-cell activation by TGF- β -treated PECs is dependent upon TGF- β elaborated by the PECs, since neutralization of this growth factor restores the capacity of T cells to respond to OVA stimulation.¹⁴ Moreover, T cells stimulated with OVA-pulsed, TGF- β 2-treated PECs differentiate into 'regulators'. That is, we have previously reported that T cells harvested after incubation with OVA-pulsed, TGF- β 2-treated PECs display the capacity to suppress bystander T-cell proliferation in vivo and to suppress induction of OVA-specific DH in vivo.¹⁵ Our first goal in the present experiments was to determine whether DO11.10 T cells stimulated in vitro by OVA-pulsed, TGF- β 2-treated PECs were able to alter the responses of bystander naive DO11.10 T cells stimulated in vitro with OVA-pulsed APCs. Achieving this goal was important in order to analyze mechanisms of suppression and to identify the cellular targets of the suppression.

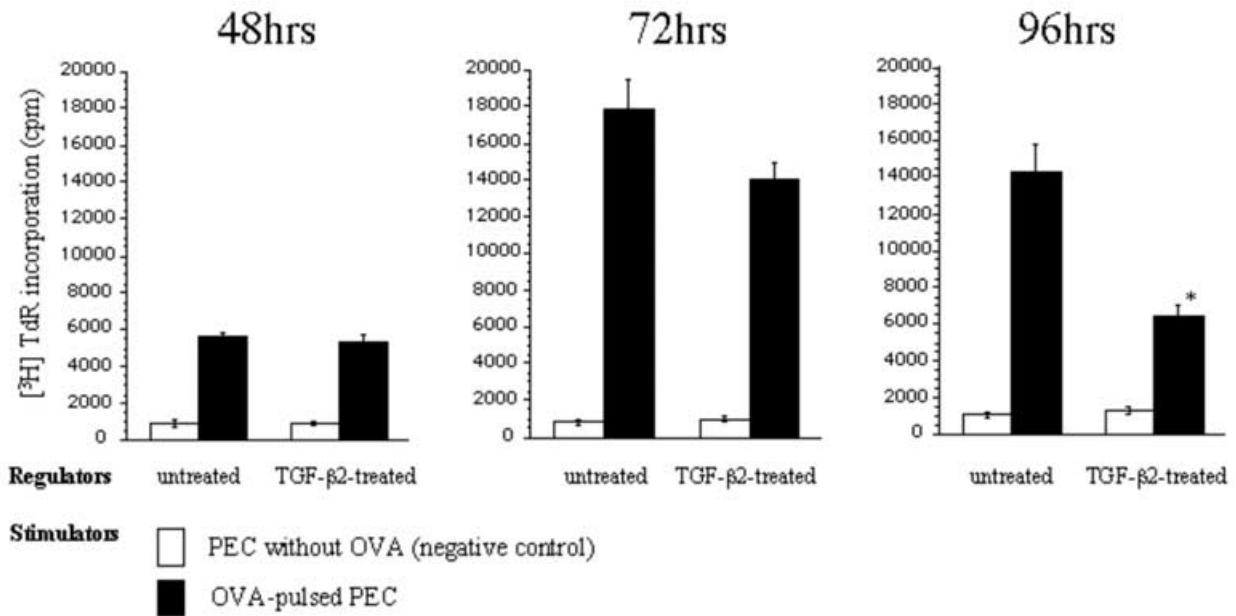
Purified DO11.10 T cells were stimulated by OVA-pulsed, TGF- β 2-pretreated PECs for 48 hours, washed, exposed to X-irradiation (2000 R), and used as 'TGF- β -treated regulators'. In separate culture dishes, freshly prepared PECs were cultured overnight with OVA, then washed and plated as 'stimulators' in a 96-well culture plate. Naive DO11.10 T cells were added to these cultures as 'responders', and then 'TGF- β -treated regulators' previously exposed to TGF- β -treated PECs were added. In positive control cultures, X-irradiated DO11.10 T cells that had been stimulated by TGF- β 2-untreated PECs were added as 'untreated regulators'. Proliferation responses were measured at 48, 72, and 96 hours, and supernatants were collected after 96 hours to measure IFN- γ secretion. The results of a representative experiment

are displayed in Figure 1. After 48 and 72 hours, the presence of regulatory T cells had no discernible effect on responder T-cell proliferation. However, at 96 hours, responder T cells cultured in the presence of 'TGF- β -treated regulators' proliferated poorly compared to responder T cells cultured in the presence of 'untreated regulators' (Fig. 1A). In addition, IFN- γ secretion was significantly suppressed in cultures containing 'TGF- β -treated regulators' (Fig. 1B). These results suggest that 'TGF- β -treated' regulators have the capacity to interfere with the activation signal that enables bystander T cells to proliferate fully in response to stimulation with OVA-pulsed APCs.

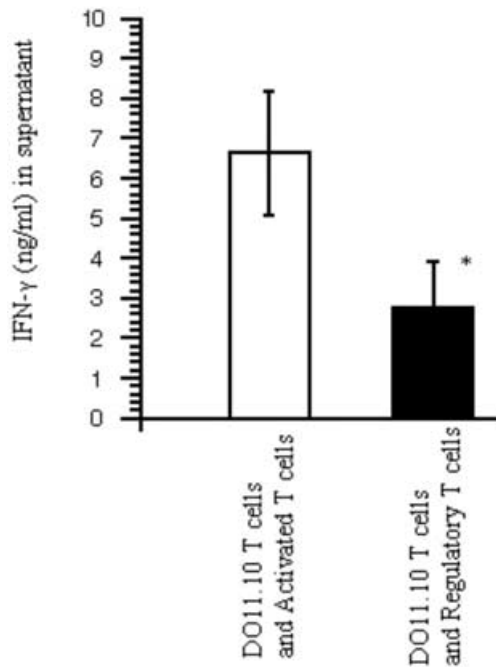
CAPACITY OF PECS EXPOSED TO REGULATOR DO11.10 T CELLS TO STIMULATE NAIVE DO11.10 T CELLS IN VITRO The ability of regulator T cells first exposed in vitro to TGF- β 2-treated PECs to suppress bystander T-cell activation in subsequent cultures offered an opportunity to inquire into the cellular target of the suppression. Two possibilities exist: (a) regulator T cells influence the antigen-presentation properties of APCs; and (b) regulator T cells directly suppress bystander T cells. We chose to examine the former possibility first, hypothesizing that APCs are the primary targets of regulatory T cells. A flow diagram of this type of experiment is presented in Figure 2. To test this idea, we generated in vitro regulatory DO11.10 T cells and prepared fresh PECs that were cultured overnight with OVA. Regulator T cells were then added to 96-well plates containing freshly prepared OVA-pulsed PECs. After 24 hours co-culture, the plates were washed to remove regulatory T cells and putative soluble factors. In addition, the plates were treated with anti-Thy 1 antibodies plus complement in order to remove any remaining vestiges of regulator T cells. Naive DO11.10 T cells were then added to these plates containing OVA-PECs exposed overnight to regulator T cells. After 24 hours, supernatants were collected and assayed for cytokine production (IFN- γ , IL-2, and IL-4) using a quantitative capture ELISA. As indicated in Figure 3, IFN- γ secretion by DO11.10 T cells stimulated with OVA-PECs exposed to regulatory T cells was significantly less than that secreted by DO11.10 T cells stimulated with OVA-PECs previously exposed to activated T cells (positive control). Responding DO11.10 T cells secreted equivalent amounts of IL-2 and IL-4 whether their APCs had previously encountered regulator or activated T cells (data not shown). This result suggests that regulator T cells, generated upon exposure to TGF- β 2-treated PECs, alter the functional properties of APCs such that the latter are inefficient at stimulating naive DO11.10 T cells to secrete IFN- γ .

CAPACITY OF PECS EXPOSED TO REGULATOR DO11.10 T CELLS TO SUPPRESS DH RESPONSES IN VIVO Regulator T cells, created by exposing naive DO11.10 T cells in vitro to OVA-pulsed, TGF- β 2-treated PECs, were next tested for their potential to alter the ability of naive mice to develop DH when appropriately immunized. PECs were cultured overnight in the presence of regulator T cells (or in-vitro activated T cells). The T cells were then discarded and the adherent PECs were collected and injected i.v. (1×10^6) into naive BALB/c mice. Positive control mice received PECs exposed either to T cells stimulated with

A.



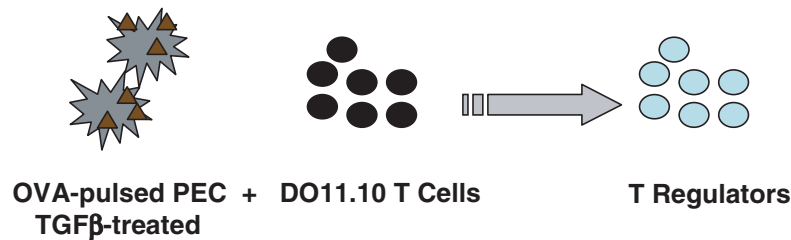
B.



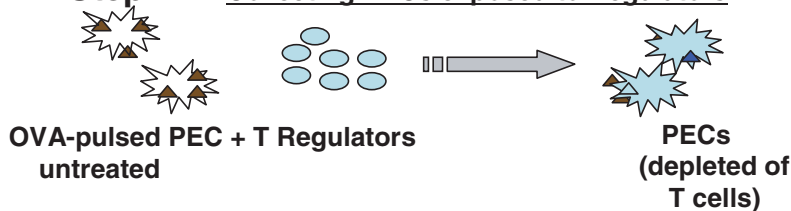
OVA-pulsed PECs, or to naive BALB/c T cells. Experimental and positive control mice were immunized s.c. with OVA emulsified in CFA one week later. Negative control mice received neither PECs nor OVA immunization. After one week, the ear pinnae of all mice were challenged i.d. with OVA, and ear-swelling responses were measured 24 and 48 hours later. Mice that had received OVA-pulsed PECs exposed to

Fig. 1. Impairment of proliferation response and IFN- γ secretion of naive DO11.10 T cells co-cultured with X-irradiated DO11.10 T cells stimulated by OVA-pulsed, TGF- β 2-treated PECs. DO11.10 T cells were stimulated with OVA-pulsed, TGF- β 2-treated PECs for 48 hours, then washed and exposed to X-irradiation (2000 R). For positive controls, DO11.10 T cells were X-irradiated after stimulation with OVA-pulsed, TGF- β 2-untreated PECs. PECs (1×10^5 /well) were cultured overnight with 50 μ g/ml OVA, then washed and prepared as 'stimulators' in a 96-well culture plate. In-vitro-activated, X-irradiated T cells (1×10^4 /well) were added as 'regulators' to cell mixtures comprised of naive DO11.10 T cells as 'responders'. (A) T-cell proliferation response was measured at 48, 72, and 96 hours. $[^3\text{H}]$ -thymidine was added 8 hours prior to termination of culture. (B) After 96 hours, the culture supernatants were harvested and assayed for IFN- γ content using ELISA. Each data point represents mean $\pm$ SD of triplicate cultures. *Indicates responses significantly lower than untreated controls (p < 0.05).

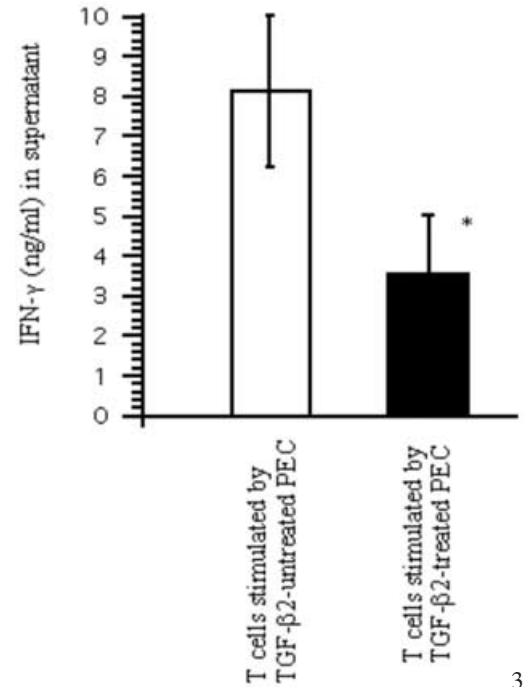
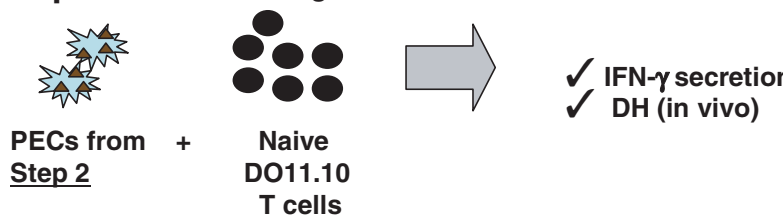
Step 1. Generation of Regulators :



Step 2. Collecting APCs exposed to Regulators :



Step 3. Assessing APC function



3

Fig. 2. Flow diagram of culture experiments to test effect of regulatory T cells on APCs.

Fig. 3. Secretion of IFN-γ by naive DO11.10 T cells stimulated with OVA-pulsed PECs that had been exposed to regulatory DO11.10 T cells activated in vitro with OVA-pulsed, TGF-β₂-treated PECs. Regulatory DO11.10 T cells were induced in vitro by a 48-hour culture with OVA-pulsed, TGF-β₂-treated PECs (black bars) or by non-TGF-β₂-treated PECs (open bars). Freshly prepared PECs (1×10^5 /well) were cultured overnight with 50 μg/ml OVA in a 96-well plate, then washed and co-cultured with regulatory DO11.10 T cells (1×10^5 /well). After a 24-hour co-culture, regulatory T cells were completely removed and naive DO11.10 T cells (2×10^4 /well) were added to the culture plate. After 24 hours, the culture supernatants were harvested and assayed for IFN-γ content by ELISA. Each data point represents mean $\pm$ SD of triplicate cultures.

*Indicates response significantly lower than untreated control ($p < 0.05$).

activated T cells or naive BALB/c T cells displayed vigorous DH responses following immunization with OVA in CFA (Fig. 4, groups B and C). In contrast, mice that received OVA-PECs exposed to regulator T cells displayed weak ear-swelling responses, indistinguishable from negative controls (Fig. 4, group D). These results indicate that OVA-pulsed PEC exposed in vitro to regulator T cells induced immune deviation similar to ACAID when injected into naive mice. We conclude that APCs are an important target of the regulatory properties of DO11.10 T cells first activated in vitro by OVA-pulsed, TGF-β₂-treated PECs.

CAPACITY OF PECS EXPOSED TO SUPERNATANTS DERIVED FROM REGULATOR DO11.10 T CELLS TO STIMULATE NAIVE DO11.10 T CELLS IN VITRO Our next goal was to gain insight into the mechanism(s) by which regulator T cells modify the functional properties of APCs. We considered two non-mutually exclusive possibilities: (a)

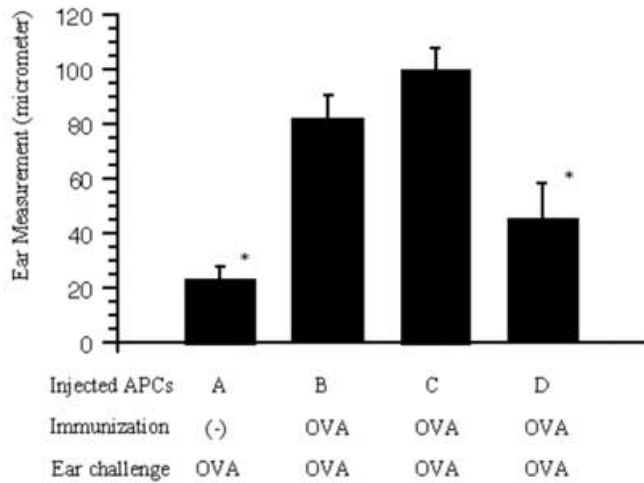


Fig. 4. Regulatory CD4⁺ T cells endow PECs with ACAID-inducing capabilities. Regulatory DO11.10 T cells were induced in vitro by a 48-hour culture with OVA-pulsed, TGF- β 2-treated PECs. Freshly prepared PECs (1×10^6 /well) were cultured overnight with 50 μ g/ml OVA in a 24-well plate, then washed and co-cultured with regulatory DO11.10 T cells (1×10^6 /well). After a 24-hour co-culture, regulatory T cells were completely removed and adherent PECs were harvested and injected i.v. into the lateral tail vein of naive BALB/c mice (1×10^5 /mouse). Seven days later, experimental and positive control mice were immunized with OVA emulsified in CFA (100 μ g). One week later, all groups received ear challenges with OVA (200 μ g/10 μ l). Bars represent the mean ear-swelling measurements of mice 24 hours post challenge with OVA. Group A: no APC. Group B: APCs cultured with naive BALB/c T cells. Group C: APC cultured with DO11.10 T cells stimulated by TGF- β 2-untreated PEC. Group D: APC cultured with DO11.10 T cells stimulated by TGF- β 2-treated PEC. *Indicates responses significantly lower than positive control (B) ($p < 0.05$).

regulator T cells secrete factors that modify APCs; and (b) regulator T cells through direct cell-to-cell contact modify APCs. To approach (a), regulator T cells were prepared as before by exposing DO11.10 T cells to OVA-pulsed, TGF- β 2-treated PECs. The T cells were then re-stimulated with freshly prepared OVA-pulsed PECs for 24 hours. Supernatants were removed from these cultures and added to another culture plate containing freshly prepared OVA-pulsed PECs for treatment. At the same time, a portion of the supernatants was assayed for cytokine content (IFN- γ and TGF- β). After treating PECs for 24 hours with supernatants, the plates were washed to remove soluble factors. Naive DO11.10 T cells were added to these plates and cultured with adherent PECs that had been pretreated with soluble factor from regulatory T cells. Supernatants were collected and assayed for cytokine production (IFN- γ) at 24 hours. The results of a representative experiment are displayed in Figures 5 and 6. At first, supernatants from cultures containing regulator T cells re-stimulated by OVA-pulsed, TGF- β 2-untreated PECs contained significantly less IFN- γ compared with supernatants from cultures containing untreated regulator T cells (Fig. 5A). At the same time, supernatants from cultures containing regulator T cells re-stimulated by OVA-pulsed, TGF- β 2-untreated PECs contained much larger amounts of mature TGF- β (Fig. 5B) than controls. We presume that enhanced levels of TGF- β in supernatants from cultures containing regulator T cells arise primarily from these T cells, implying that regulator T cells retain their capacity to secrete TGF- β even after re-stimulation by TGF- β 2-untreated PEC. Moreover, the supernatant from cultures containing regulator T cells and TGF- β 2-untreated PECs altered the capacity of fresh OVA-pulsed PEC to stimulate naive DO11.10 T cells, and the latter cells secreted lesser amounts of IFN- γ (Fig. 6) than controls. We conclude that a soluble factor is released into supernatants of cultures containing regulator T cells and TGF- β 2-untreated antigen-presenting cells, and this factor plays an important role in suppressing responses of naive T cells.

Discussion OVA-specific DO11.10 T cells that are activated by exposure in vitro to OVA-pulsed, TGF- β -pretreated APCs possess a

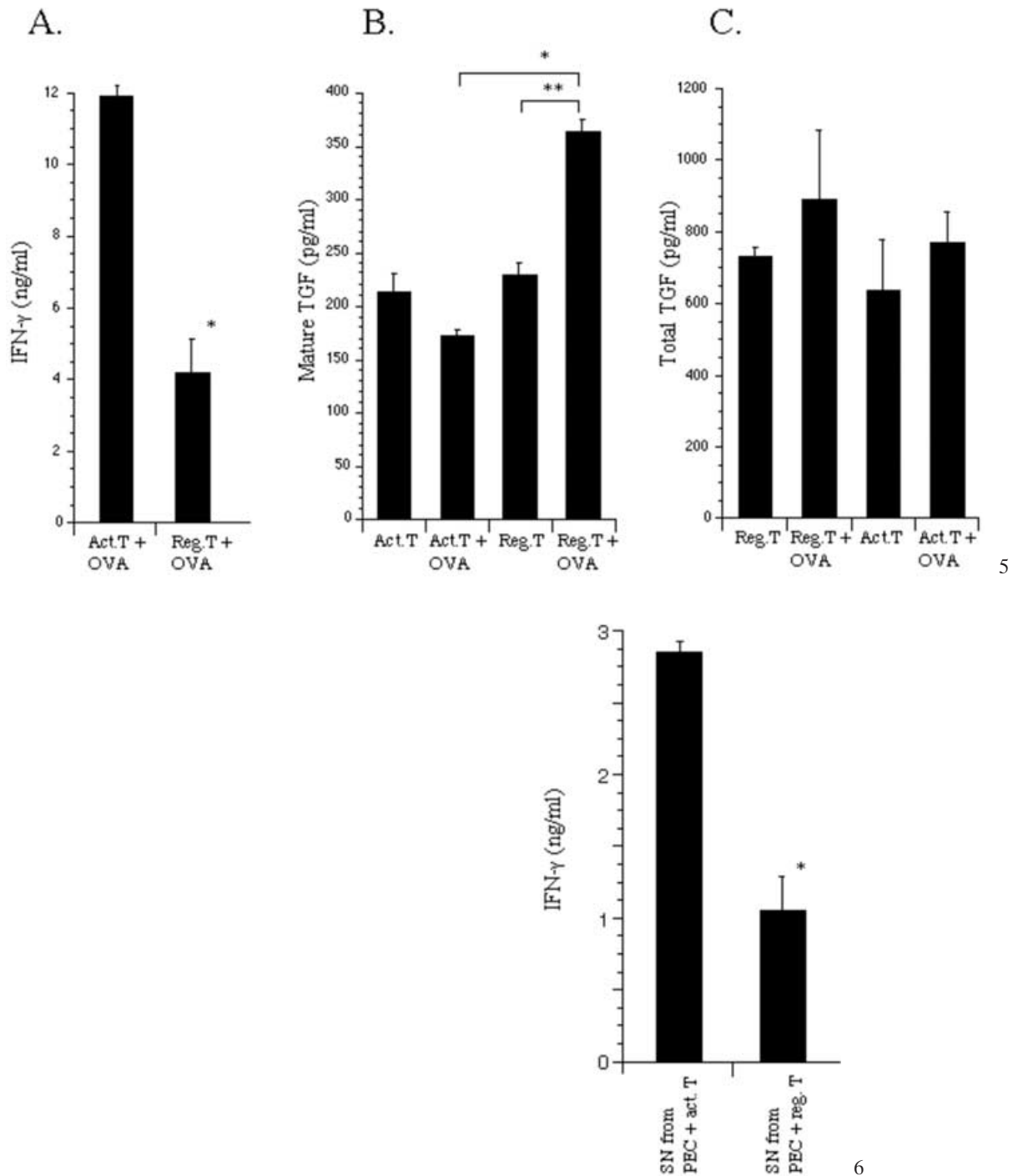


Fig. 5. Secretion of IFN- γ and TGF- β by regulatory DO11.10 T cells following re-activation with OVA-pulsed PECs. Regulatory DO11.10 T cells, induced in vitro by a 48-hour culture with OVA-pulsed, TGF- β 2-treated PECs, were harvested, then washed and added (2×10^4 /well) to a 96-well plate containing freshly prepared OVA-PECs (1×10^5 /well) that had been cultured overnight with 50 μ g/ml OVA. After 24 hours, the culture supernatants were harvested and assayed for (A) IFN- γ , (B) mature TGF- β , and (C) total TGF- β content using ELISA (IFN- γ) or bioassay (TGF- β). Each data point represents mean $\pm$ SD of triplicate cultures. *Difference between Act T + OVA and Reg T + OVA is significant ($p < 0.05$). **Difference between Reg T + OVA and Reg T without OVA is significant ($p < 0.05$).

Fig. 6. Secretion of IFN- γ by regulatory DO11.10 T cells following re-activation with OVA-pulsed PECs. Regulatory DO11.10 T cells, induced in vitro by a 48-hour culture with OVA-pulsed, TGF- β 2-treated PECs, were harvested, then washed and re-stimulated (2×10^5 /well) with OVA-pulsed, TGF- β 2-untreated PECs (1×10^6 /well) in a 24-well plate. After 24 hours, the culture supernatants were harvested and added to a 96-well plate containing freshly prepared OVA-PECs (1×10^5 /well) that had been cultured overnight with 50 μ g/ml OVA. After a 24-hour treatment of PECs, soluble factors were completely removed by washing and naive DO11.10 T cells (2×10^4 /well) were added to the culture plate. After 24 hours, the culture supernatants were harvested and assayed for IFN- γ content by ELISA. Each data point represents mean $\pm$ SD of triplicate cultures. *Indicates value significantly lower than control ($p < 0.05$).

novel set of characteristics that may be pertinent to ACAID. We have previously reported that T cells activated in this manner acquire the capacity to suppress the induction and then the expression of delayed hypersensitivity *in vivo*.¹⁵ In the current communication, we present evidence that APCs are important targets of the regulatory properties of DO11.10 T cells first activated *in vitro* by OVA-pulsed, TGF- β 2-treated APCs. On the one hand, OVA-pulsed APCs, exposed overnight to regulatory DO11.10 T cells, poorly stimulated naive DO11.10 T cells to secrete IFN- γ , even after treatment with anti-Thy-1 antibodies in order to remove any remaining vestiges of regulator T cells. On the other hand, BALB/c mice that received OVA-PECs exposed to regulatory DO11.10 T cells prior to immunization with OVA plus adjuvant failed to display DH responses, whereas mice that received OVA-PECs exposed to activated DO11.10 T cells (or naive BALB/c T cells) prior to OVA immunization displayed vigorous DH responses. Together, these results indicate that regulatory DO11.10 T cells, generated upon exposure to TGF- β 2-treated PECs, alter the functional properties of APCs that they encounter subsequently such that the latter are inefficient at activating and converting naive CD4⁺ T cells into DH-effector cells.

The prominent role of TGF- β 2 in generating regulatory T cells *in vitro* from naive DO11.10 T cells reflects a similarly prominent role for this cytokine in ACAID. Eye-derived APCs, that capture antigen locally and migrate to the spleen for presentation to T cells, reside in an ocular microenvironment rich in TGF- β 2.¹⁸ Aqueous humor that fills the anterior chamber not only contains TGF- β 2,¹⁹ but this fluid has been demonstrated to confer ACAID-inducing properties on conventional APCs.¹² PECs that are pulsed with antigen *in vitro* in the presence of aqueous humor induce ACAID when injected intravenously into naive mice, and this property of aqueous humor is nullified if neutralizing anti-TGF- β antibodies are present during the *in-vitro* incubation.²⁰ In addition, we have previously reported that TGF- β -pretreated APCs acquire the ability to secrete mature TGF- β in an autocrine fashion,¹³ and that DO11.10 T cells activated *in vitro* by OVA-pulsed, TGF- β -pretreated APCs secrete large amounts of mature TGF- β .¹⁵ Thus, TGF- β not only equips APCs to promote ACAID, but it promotes the ability of TGF- β -treated APCs and regulatory T cells to secrete mature TGF- β as well. Aqueous humor or TGF- β -treated APCs differ from APCs of the type described by Gratchev et al.²¹ These authors demonstrated that macrophages polarized by treatment with IL-4, IL-10 or PGE₂ exhibit the capacity to deviate responding T cells toward the Th2 phenotype. OVA-pulsed PECs treated with aqueous humor or TGF- β induce DO11.10 T cells to secrete IL-4 and TGF- β , but the responding T cells do not secrete either IL-5 or IL-10.^{14,22} Moreover, mice that receive an intravenous injection of OVA-pulsed, TGF- β -treated PECs do not acquire OVA-specific immunity of the Th2 type. In fact, Th2 immunity is suppressed by pre-treatment of mice with OVA injected into the anterior chamber or with an intravenous injection of OVA-pulsed, TGF- β -treated PECs.

The results of our present experiments further suggest that regulatory T cells modify the functional properties of APCs through the secretion of soluble factor(s), a likely candidate being TGF- β . *In-vitro*-activated regulatory T cells and/or APCs secreted large amounts of mature TGF-

β when TGF- β -treated regulators were re-stimulated with freshly prepared OVA-pulsed APCs. Moreover, these supernatants containing large amounts of mature TGF- β modified the functional properties of fresh APCs such that naive DO11.10 T cells stimulated by these cells secreted much less IFN- γ . It is of interest that the total amount of TGF- β secreted during regulatory T cell-APC conjugation was unchanged, whereas the amount of mature TGF- β secreted was enhanced in an antigen-dependent manner. Based on our recent discovery of a key role for thrombospondin in ACAID induction,²³ there is the possibility that regulatory T cells endow APCs with the capacity to secrete soluble factor(s), such as thrombospondin, that in turn convert latent TGF- β to its mature form. It is important to understand the mechanism(s) by which regulatory T cells inhibit immune responses of bystander naive T cells. Our current results suggest that regulator T cells modify the functional properties of APCs, including bystander APCs, through secretion of activated TGF- β . In this manner, the regulatory T cells of ACAID can establish in secondary lymphoid organs novel microenvironments conducive to activation of further regulatory cells.

Inhibitory activities of regulatory T cells are believed to be central to the prevention of autoimmune diseases, allergies, transplant rejection, and immune-deficiency disorders. Recent evidence indicates that multiple types of regulatory T cells may exist. Different subsets of CD4 and CD8 T cells show regulatory activities that are mediated either by immunosuppressive cytokines or by contact-dependent mechanisms.²⁴⁻²⁷ Since we only examined the putative effects of regulatory T cells on APCs in our experiments, the possibility exists that bystander T cells may also serve as direct cellular targets of the regulatory T cells. Furthermore, although we demonstrated that regulatory T cells altered the functional properties of APCs through soluble factors, it remains possible that regulator CD4⁺ T cells can also convert fresh APCs to ACAID-ogenic APCs through cell-to-cell contact. Experiments to test these possibilities are underway.

When foreign antigens are used for immunization leading to DH, a large number of APCs are activated *in vivo*, and these in turn activate naive T cells to become effector T cells that will play a key role in mediating DH. In the ACAID system, nominal antigen is injected into the eye and regulatory CD4⁺ and CD8⁺ T cells are activated by eye-derived APCs in the spleen even before the mice are immunized systemically with the same antigen.²⁸ The presence of regulatory T cells at the time of systemic immunization with the same antigen means that APCs (at least those within the spleen) have already been altered by soluble factors released from regulatory T cells. Consequently, the T cells that are responsible for DH are not efficiently generated. We hypothesize that regulatory T cells have a pervasive effect on large numbers of APCs, inhibiting their capacity to induce effector T cells upon subsequent immunization. Our results suggest that regulatory DO11.10 (presumably CD4⁺) T cells convert fresh APCs to ACAID-inducing APCs through the release of soluble factors, one of which is active TGF- β . We propose that this step is central to the cascade of subsequent cellular events that result in the generation of CD4⁺ and CD8⁺ regulatory T cells and the phenomenon of ACAID.

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