

# T cell sensitivity to TGF- $\beta$ is required for the effector function but not the generation of splenic CD8<sup>+</sup> regulatory T cells induced via the injection of antigen into the anterior chamber

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## Abstract

The introduction of antigen into the anterior chamber (AC) of the eye induces the production of antigen-specific splenic CD8<sup>+</sup> regulatory T cells (AC-SPL cells) that suppress a delayed-type hypersensitivity (DTH) reaction in immunized mice. Because the generation of these regulatory T cells is also induced by exposure to transforming growth factor (TGF)- $\beta$  and antigen or F4/80<sup>+</sup> cells exposed to TGF- $\beta$  and antigen *in vitro*, we investigated (i) whether these cells are produced in dominant negative receptor for transforming growth factor  $\beta$  receptor type II (dnTGF $\beta$ RII) or Cbl-b<sup>-/-</sup> mice whose T cells are resistant to TGF- $\beta$ , (ii) whether DTH is suppressed by wild type (WT) CD8<sup>+</sup> AC-SPL cells in Cbl-b<sup>-/-</sup> and dnTGF $\beta$ RII mice and (iii) the effect of antibodies to TGF- $\beta$  on the suppression of DTH by CD8<sup>+</sup> AC-SPL cells. DnTGF $\beta$ RII immunized and Cbl-b<sup>-/-</sup> mice produced splenic CD8<sup>+</sup> regulatory cells after the intracameral injection of antigen and immunization. The suppression of a DTH reaction by CD8<sup>+</sup> AC-SPL cells in WT mice was blocked by the local inclusion of antibodies to TGF- $\beta$  when WT splenic CD8<sup>+</sup> AC-SPL cells were injected into the DTH reaction site. Moreover, the DTH reaction in immunized dnTGF $\beta$ RII and Cbl-b<sup>-/-</sup> mice was not suppressed by the transfer of WT CD8<sup>+</sup> AC-SPL cells to the site challenged with antigen. In aggregate, these observations suggest that T cell sensitivity to TGF- $\beta$  is not an obligate requirement for the *in vivo* induction of CD8<sup>+</sup> AC-SPL T cells but the suppression of an *in vivo* DTH reaction by CD8<sup>+</sup> AC-SPL cells is dependent on TGF- $\beta$ .

## Introduction

The antigen-specific suppression of T cells to self and non-self antigens in T<sub>h</sub>1 and T<sub>h</sub>2 immune responses is effected by CD8<sup>+</sup> regulatory T cells that are distinct from CD8<sup>+</sup> cytotoxic T cells (1–6). In addition to induction, CD8<sup>+</sup> regulatory T cells, unlike CD4<sup>+</sup>, CD25<sup>+</sup>, Foxp3<sup>+</sup> regulatory T cells, suppress immunized T cells (7). Moreover, the immunosuppressive activity of CD8<sup>+</sup> regulatory T cells is detected after an immune response to the antigen (1, 4), suggesting that antigen-specific CD8<sup>+</sup> regulatory T cells are induced and/or amplified during

an immune response but the requirements and/or mechanisms of the activation of these cells *in vivo* are not clear.

CD8<sup>+</sup> regulatory T cells are induced by the injection of antigen into the anterior chamber (AC) of an eye [AC-SPL cells (6, 8–12)] or *in vitro* by the ligation of 4-1BB (13, 14), transforming growth factor (TGF)- $\beta$  (15) or TGF- $\beta$ , antigen-pulsed F4/80<sup>+</sup> cells (16). The immunosuppressive properties of TGF- $\beta$  (17) suggest that this cytokine could be a suppressive mechanism used *in vivo* by CD8<sup>+</sup> regulatory T cells. Accordingly,

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TGF- $\beta$  potentially could have an afferent and/or efferent role in the immunosuppression mediated by CD8<sup>+</sup> regulatory T cells.

In the current studies, we investigated requirements for T cell sensitivity to TGF- $\beta$  for the generation *in vivo* of splenic CD8<sup>+</sup> regulatory T cells induced by the injection of antigen into the AC by using two genetically engineered strains of mice, dominant negative receptor for transforming growth factor  $\beta$  receptor type II (dnTGF $\beta$ RII) or Cbl-b<sup>-/-</sup>, resistant to TGF- $\beta$ . In the second strain, the E3 ubiquitin ligase Cbl-b has been specifically deleted [Cbl-b<sup>-/-</sup> mice (18, 19)]. Cbl-b, a member of the Cbl/Sli family of molecular adaptors and a ubiquitin ligase, has been shown to negatively regulate CD28-dependent T cell activation. Recently, we reported that Cbl-b<sup>-/-</sup> T cells, though not constitutively activated, are resistant to the inhibitory effects of TGF- $\beta$  (20, 21). Cbl-b<sup>-/-</sup> T cells display increased proliferation after TCR stimulation and produce increased amounts of IL-2, but not IFN- $\beta$  or tumor necrosis factor- $\alpha$ . Cbl-b<sup>-/-</sup> mice have T cells that are independent of CD28 co-stimulation, in that they do not require CD28 engagement for IL-2 production and proliferation (18, 19). Moreover, Cbl-b<sup>-/-</sup> mice develop spontaneous autoimmunity characterized by auto-antibody production, infiltration of activated T and B lymphocytes into multiple organs, and parenchymal damage and are highly susceptible to experimental autoimmune encephalomyelitis (18, 19). In the second TGF- $\beta$ -resistant strain, mice transgenically express a dnTGF $\beta$ RII in CD4<sup>+</sup> and CD8<sup>+</sup> T cells (22). This dominant negative TGF- $\beta$  receptor prevents TGF- $\beta$  from signaling normally in T cells and T cells from dnTGF $\beta$ RII mice respond well to antigen but do not respond to TGF- $\beta$ . A significant percentage of the T cells from dnTGF $\beta$ RII mice show evidence of prior activation in terms of CD44 expression (22).

We show that (i) dnTGF $\beta$ RII and Cbl-b<sup>-/-</sup> mice that received an intracameral injection of antigen produce CD8<sup>+</sup> AC-SPL cells that suppress a DTH reaction when transferred to wild-type (WT) recipients; (ii) The DTH reaction cannot be suppressed in immunized dnTGF $\beta$ RII and Cbl-b<sup>-/-</sup> recipients by transferred WT CD8<sup>+</sup> AC-SPL cells. (iii) Antibodies to TGF- $\beta$  inhibit the local *in vivo* suppression of a DTH reaction by CD8<sup>+</sup> regulatory spleen cells induced by the injection of antigen into the AC. These results suggest that sensitivity to TGF- $\beta$  by T cells is not obligate for the *in vivo* induction of CD8<sup>+</sup> AC-SPL cells induced via the AC but TGF- $\beta$  is likely a suppressive mechanism utilized by CD8<sup>+</sup> AC-SPL cells.

## Methods

### Mice

Female or male C57BL/6 mice 8–10 weeks old were purchased from Charles River/NCI Laboratories (Wilmington, MA, USA). Cbl-b-deficient mice (18, 19) on a C57BL/6 background, a gift from Dr H. Gu (National Institutes of Health, Bethesda, MD, USA) are bred in our facilities under specific, pathogen-free conditions. DnTGF $\beta$ RII mice [CD4-dnTGF $\beta$ RII (22)] derived from Dr Flavell's colonies at Yale University are bred in our facilities under specific, pathogen-free conditions. All mice are maintained in the Center for Laboratory Animal Care of the University of Connecticut Health Center. All work with animals was approved previously by the University of Connecticut Health Center Animal Care Committee

(ACC2007-380, ACC2007-379). All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Antigens/immunization

Trinitrophenol-BSA 2,4,6, Trinitrobenzene sulphonic acid (TNP), BSA and ovalbumin (OVA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). TNP-BSA was prepared as described by Li *et al.* (10). Picryl chloride (PCI), 2-chloro-1,3,5-trinitrobenzene [the antigenic equivalent of TNP used to elicit contact sensitivity (CS)] was purchased as 2-chloro-5-tryptthane from Chemical Alta Ltd (Edmonton, Alberta, Canada). Mice were immunized by a single subcutaneous injection of 200  $\mu$ g TNP-BSA in 50  $\mu$ l CFA (Sigma-Aldrich, St Louis, MO, USA). In general, the mice were challenged to induce CS to TNP 7–9 days after the mice were immunized.

OVA Mice received s.c. 200  $\mu$ g OVA in 50  $\mu$ l CFA to a flank.

### Induction of DTH reaction

A CS reaction to TNP in TNP-BSA-sensitized or naive mice was induced by the epicutaneous application of 15  $\mu$ l 1% PCI in acetone:olive oil (4:1) to a footpad (10). A DTH reaction (we define the DTH reaction as the swelling induced by the application of antigen to an immunized recipient to distinguish the DTH reaction from the initiation of a T<sub>H</sub>1 response) to OVA was induced by an intra-dermal injection of 50  $\mu$ g OVA in 30  $\mu$ l PBS (pH 7.2). The DTH response was usually measured approximately 24–48 h after the application of PCI or injection of OVA by measuring footpad thickness with an engineer's digital micrometer (Mitutoyo, Tokyo, Japan). Before challenge, mice were anesthetized with ketamine (75 mg kg<sup>-1</sup>)/xylazine (15 mg kg<sup>-1</sup>) and the thickness of each footpad was measured. One footpad was then challenged with antigen and the other with vehicle only. Twenty-four hours later the mice were anesthetized with ketamine/xylazine and the thickness of each footpad was measured. The thickness of each footpad before the application of PCI was subtracted from that of the thickness 24 h after the application of PCI. The increase in the thickness of the footpad defined as swelling was computed as the difference in the increase in micrometers between the 24 h and 0 h measurement of the thickness of the challenged footpad minus that of the unchallenged footpad.

### Injection of antigen into the AC (intracameral injection)

Mice were anesthetized with ketamine/xylazine (75 mg kg<sup>-1</sup>)/xylazine (15 mg kg<sup>-1</sup>) and, under a dissecting microscope, ~3  $\mu$ l PBS containing 4  $\mu$ g TNP-BSA or 50  $\mu$ g OVA was injected into the AC. A 32g needle attached to a cannula attached to a manually controlled Hamilton syringe (Stoelting Co., Wood Dale, IL, USA) was inserted into the AC. The mice recovered ~30 min after the injection; they exhibited no distress and began eating and drinking normally. Seven days after the intracameral injection of antigen, mice were immunized with TNP-BSA as described (9, 10).

### Preparation of splenic AC-induced regulatory cells (AC-SPL cells)

Fourteen days after receiving an injection of TNP-BSA into the AC and 7 days after the AC-injected mice were

immunized, spleens were removed from the mice, pooled, diced and expressed through a 40-mm nylon mesh (BD Falcon, Bedford, MA, USA) with a 1-ml syringe plunger into PBS. The cells were washed 2 $\times$  and suspended in PBS at  $1 \times 10^8$  ml<sup>-1</sup>. The cell suspension was washed with PBS and centrifuged at  $200 \times g$  for 6–8 min. BD Pharm Lyse (BD Biosciences, San Jose, CA, USA) was used for lysing the erythrocytes according to the manufacturer's protocol. The cells were then washed twice with PBS and re-suspended in PBS. CD8<sup>+</sup> and CD8<sup>-</sup> spleen cells were prepared by washing splenocytes two times with PBS and re-suspending the cells in BD<sup>TM</sup> IMag separation buffer (BD Biosciences, Rockville, MD, USA). The spleen cells were separated by immunomagnetic beads into CD8<sup>+</sup> and CD8<sup>-</sup> populations with a BD<sup>TM</sup> CD8 T lymphocyte enrichment set (BD Biosciences) according to the manufacturer's protocol. Enrichment of the cells was assessed by flow cytometry and found to be >87–92% pure CD8<sup>+</sup> T cells. The CD8<sup>-</sup> fractions showed <3% CD8<sup>+</sup> T cells (data not shown).

#### Local transfer of suppression (LTS)

AC-SPL cells were assayed for the suppression of the DTH reaction by the LTS assay (10). Spleen cells were counted with a Coulter counter and unless otherwise specified 10 000 or 25 000 cells in 40  $\mu$ l PBS were injected subcutaneously into the footpad of immunized mice immediately following epicutaneous challenge with PCI. Swelling was measured 24 h later.

The use of anti-TGF- $\beta$  antibodies in the LTS was performed by admixing AC-SPL cells from donors that received an intracameral injection of antigen ( $1 \times 10^6$  ml<sup>-1</sup> PBS) with monoclonal anti-TGF- $\beta$  (TGF- $\beta$ 1,2,3) IgG1 antibody (MAB #1835, R&D Systems, Minneapolis, MN, USA) or isotype control MOPC 21 IgG (Sigma, St Louis, MO, USA) so that 40  $\mu$ l contained 15  $\mu$ g antibody or MOPC 21 isotype control. The cells were held at 4°C for 30 min and then injected intradermally into the footpad of sensitized recipients immediately after the footpad was challenged with antigen. Some immunized mice received an injection of anti-TGF- $\beta$  antibody only after challenge.

#### Statistics

Statistical significance was calculated by one-way analysis of variance. *P*-values were determined by the Student–Neuman–Keuls test.

## Results

#### Antigen-specific local suppression of the DTH reaction by CD8<sup>+</sup> AC-SPL cells

To confirm and demonstrate the phenotype of AC-SPL cells that suppress locally the DTH reaction in the LTS, graded numbers of CD8<sup>+</sup>, CD8<sup>-</sup> or unseparated AC-SPL cells from donors that received an intracameral injection of TNP–BSA were injected into the footpads of TNP–BSA-immunized mice immediately after the footpads were challenged with epicutaneous PCI. The swelling reaction in the footpads was eliminated or reduced significantly in the recipients of unseparated or CD8<sup>+</sup> but not CD8<sup>-</sup> AC-SPL cells (Fig. 1). The enrichment

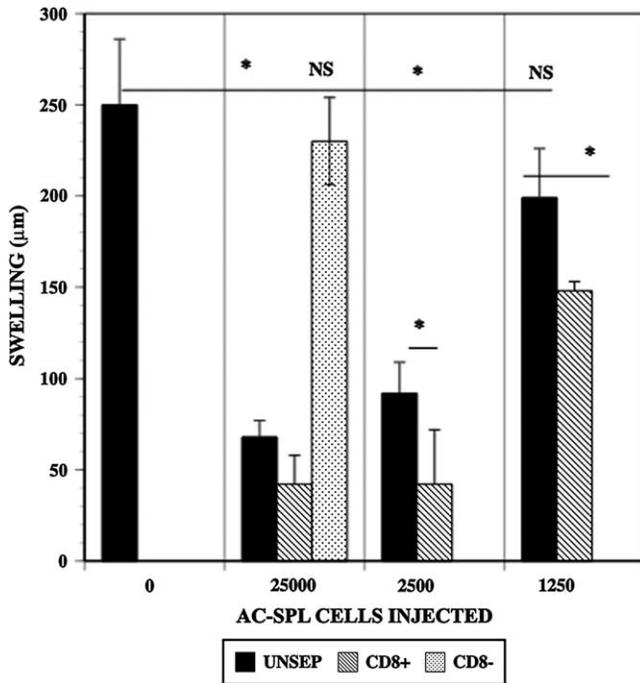
for CD8<sup>+</sup> regulatory spleen cells is shown by the suppression of PCI-induced swelling by limiting numbers (1, 250) of CD8<sup>+</sup> AC-SPL cells while that number of unseparated AC-SPL cells did not suppress swelling significantly. CD8<sup>+</sup> AC-SPL cells recovered from donors that received intracameral injections of TNP–BSA or OVA were assayed for the ability to suppress the DTH reaction when injected into the footpads of mice immunized to TNP–BSA or OVA after the footpad was challenged with PCI or OVA. As shown in Fig. 2, CD8<sup>+</sup> spleen cells recovered from donors receiving intracameral TNP–BSA suppressed the swelling reaction of TNP–BSA-immunized mice challenged with PCI but not that of OVA-sensitized mice challenged with OVA. Conversely, AC-SPL cells from mice receiving intracameral OVA suppress the OVA-induced swelling in OVA-immunized mice but not PCI-induced swelling in TNP–BSA-immunized mice (data not shown). Therefore, the results in Figs 1 and 2 document further that the local, antigen-specific suppression of the DTH reaction in immunized recipients by transferred AC-SPL cells in the LTS is due to CD8<sup>+</sup> regulatory T cells.

#### AC-SPL CD8<sup>+</sup> regulatory cells are produced by dnTGF $\beta$ R11 mice or *Cbl-b*<sup>-/-</sup> mice

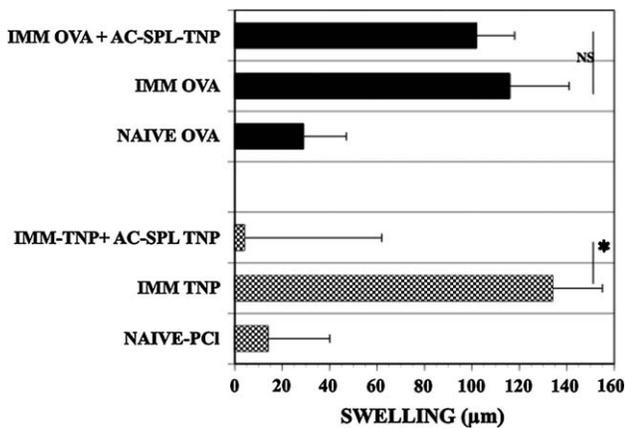
To determine whether sensitivity to TGF- $\beta$  by T cells is required by the T cells *in vivo* to generate splenic CD8<sup>+</sup> regulatory cells after the intracameral injection of antigen, we investigated whether CD8<sup>+</sup> regulatory T cells are generated in dnTGF $\beta$ R11 and *Cbl-b*<sup>-/-</sup> mice after these mice received an injection of TNP–BSA into the AC. WT, dnTGF $\beta$ R11 or *Cbl-b*<sup>-/-</sup> mice received an intracameral injection of TNP–BSA. One week later, the mice were immunized with TNP–BSA and 1 week after immunizing, spleen cells were recovered from these mice and injected into the footpads of TNP–BSA-sensitized WT mice immediately after the footpads were challenged with epicutaneous PCI. Fig. 3(A and B) demonstrate that the injection of unseparated WT but not unseparated *Cbl-b*<sup>-/-</sup> or dnTGF $\beta$ R11-derived AC-SPL cells into the footpad at a challenge site at the time of challenge resulted in a significant reduction in footpad swelling in the immunized recipient. However, swelling was suppressed in the footpads of the recipients of *Cbl-b*<sup>-/-</sup> or dnTGF $\beta$ R11-derived CD8<sup>+</sup> AC-SPL cells despite the fact that unseparated *Cbl-b*<sup>-/-</sup> or dnTGF $\beta$ R11-derived CD8<sup>+</sup> AC-SPL did not suppress the DTH reaction. Moreover, the intracameral injection of TNP–BSA into *Cbl-b*<sup>-/-</sup> or dnTGF $\beta$ R11 mice induced the production of peripheral blood mononuclear cells or thymocytes that induce splenic CD8<sup>+</sup> AC-SPL cells (data not shown).

#### WT CD8<sup>+</sup> spleen cells from mice receiving intracameral antigen do not suppress DTH in TGF- $\beta$ -resistant *Cbl-b*<sup>-/-</sup> or dnTGF $\beta$ R11 mice

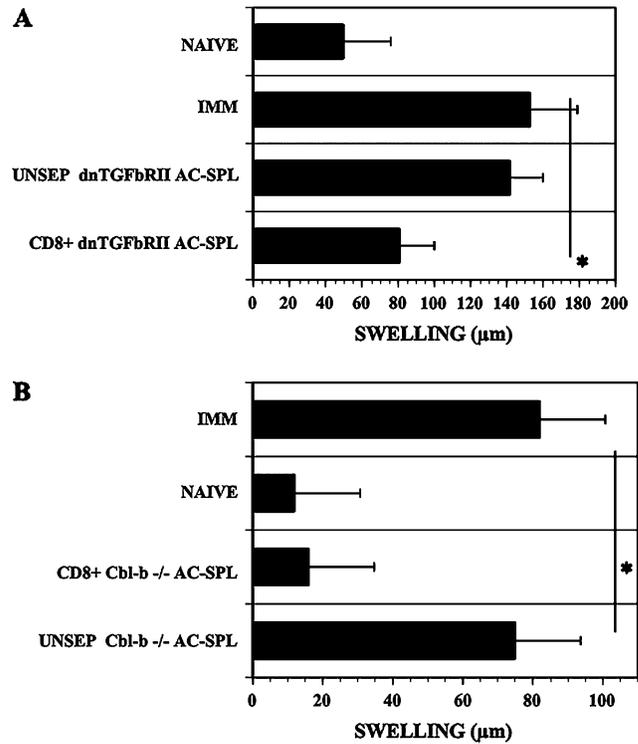
Because unseparated AC-SPL cells from TGF- $\beta$ R11 or *Cbl-b*<sup>-/-</sup> mice did not suppress the DTH reaction but CD8<sup>+</sup> AC-SPL cells did suppress the DTH reaction when transferred to immunized recipients, we reasoned that the AC-SPL cells from TGF- $\beta$ R11 or *Cbl-b*<sup>-/-</sup> mice might contain T cells resistant to suppression effected by TGF- $\beta$ . These cells could transfer a hypersensitivity reaction to the recipient mice.



**Fig. 1.** CD8<sup>+</sup> AC-SPL cells suppress DTH in WT mice in the LTS. WT (C57BL/6) mice received an injection of TNP-BSA into the AC. One week later, these mice were immunized with TNP-BSA and CFA. Seven days later, the spleens were recovered from the AC-injected mice, pooled and a spleen cell suspension was prepared (AC-SPL cells). Spleen cells were separated into CD8<sup>+</sup> and CD8<sup>-</sup> cells by immunomagnetic beads;  $1.25 \times 10^3$  to  $2.5 \times 10^4$  unseparated (UNSEP), CD8<sup>+</sup> or CD8<sup>-</sup> AC-SPL cells were injected into the footpads of TNP-BSA-immunized (IMM) or naive C57BL/6 mice immediately after the footpads were challenged with epicutaneous PCI. The thickness of the footpads before and 24 h after challenge was measured to compute the swelling of the footpad. Data represents one of three experiments with similar results and is the mean  $\pm$  SEM swelling of five mice per group. \**P* < 0.01.



**Fig. 2.** Antigen-specific suppression of DTH in the LTS by CD8<sup>+</sup> AC-SPL cells. CD8<sup>+</sup> AC-SPL cells recovered from mice that received an intracameral injection of TNP-BSA were then immunized with TNP-BSA and injected into the footpads of TNP-BSA-immunized or OVA-immunized mice immediately after the footpad of the immunized mice was challenged with epicutaneous PCI or intra-dermal OVA, respectively. Swelling was measured 24 h after challenge. Data are the mean  $\pm$  SEM for the results obtained in two experiments with eight mice per group. \**P* < 0.01.

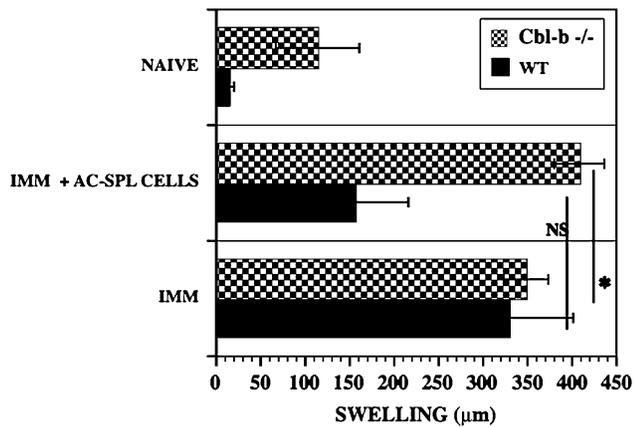


**Fig. 3.** Intracameral injection of antigen induces CD8<sup>+</sup> regulatory T cells in dnTGFβRII mice and Cbl-b<sup>-/-</sup> mice. (A) One week after dnTGFβRII mice received an intracameral injection of TNP-BSA, the mice were immunized with TNP-BSA and CFA. One week later, spleens were recovered, spleen cell suspensions prepared and separated into CD8<sup>+</sup> and CD8<sup>-</sup> populations by immunomagnetic beads. IMM, immunized WT recipients received 25 000 unseparated AC-SPL cells (UNSEP) or separated AC-SPL cells. NAIVE, non-immune mice. Swelling is the mean  $\pm$  SEM of data obtained in three experiments of 9–10 mice per group. \**P* < 0.01. (B) One week later, Cbl-b<sup>-/-</sup> mice received an intracameral injection of TNP-BSA, the mice were immunized with TNP-BSA and CFA. One week after immunization, spleens were recovered and spleen cell suspensions prepared and separated with immunomagnetic beads into CD8<sup>+</sup> and CD8<sup>-</sup> populations. TNP-BSA-immunized recipient WT mice received an intradermal injection of 10 000 unseparated (UNSEP), CD8<sup>+</sup> or CD8<sup>-</sup> AC-SPL cells into the challenged footpad immediately after challenge. Swelling was measured 24 h after challenge with epicutaneous PCI. Data are the mean and SEM of two experiments, six to eight mice per group. \**P* < 0.05.

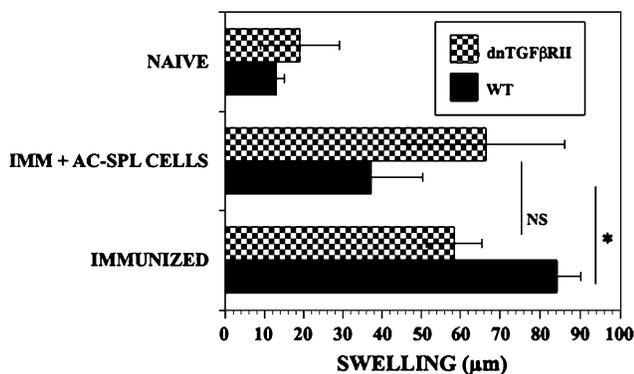
To investigate this hypothesis, we determined whether the DTH reaction was suppressed by AC-SPL cells in mice resistant to TGF- $\beta$ . Because Cbl-b<sup>-/-</sup> mice lacking the ubiquitin ligase Cbl-b are resistant to the immunosuppressive effects of TGF- $\beta$  (20, 21), spleen cells from WT donors that received intracameral TNP-BSA were injected into a footpad of TNP-BSA-immunized Cbl-b<sup>-/-</sup> or WT mice immediately after the mice were challenged with an epicutaneous application of PCI. Swelling was measured 24 h later. Immunized Cbl-b<sup>-/-</sup> and WT mice both showed robust a DTH reaction when challenged with PCI. WT spleen cells suppressed the DTH response of WT mice to naive levels but, in contrast, the DTH response of Cbl-b<sup>-/-</sup> mice receiving WT spleen cells was not suppressed (Fig. 4).

To more specifically test the hypothesis that sensitivity to TGF- $\beta$  is required for the suppression of DTH *in vivo* by

AC-SPL cells, spleen cells recovered from WT mice that had received an intracameral injection of TNP-BSA were tested for suppressive ability in the LTS in WT or dnTGF $\beta$ RII mice. T cells in dnTGF $\beta$ RII mice are largely resistant to the effects of TGF- $\beta$  as a result of the T cell-specific expression of a dominant negative TGF- $\beta$  receptor II (22). Although the PCI-induced footpad swelling responses were generally smaller in this series of experiments, the DTH responses were suppressed in the WT mice receiving the WT regulatory spleen cells while the responses were not significantly suppressed in the dnTGF $\beta$ RII mice receiving the WT regulatory spleen cells (Fig. 5).



**Fig. 4.** DTH in Cbl-b<sup>-/-</sup> mice is resistant to suppression by WT-AC-SPL cells. Twenty-five thousand AC-SPL cells from WT (C57BL/6), Cbl-b<sup>+/+</sup> mice that received intracameral TNP-BSA were injected into footpads intradermally immediately after the footpads of TNP-BSA-immunized Cbl-b<sup>-/-</sup> and WT mice were challenged with epicutaneous PCI. Swelling was measured 24 h after challenge. Data represent the pooled results of three experiments and are the mean swelling  $\pm$  SEM of 8–9 mice per group. \* $P$  < 0.03. NAIVE, non-immunized; IMM, immunized.



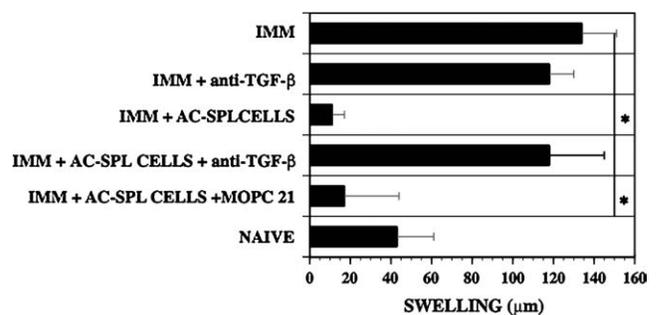
**Fig. 5.** DTH in dnTGF $\beta$ RII mice is resistant to suppression by WT-AC-SPL cells. Twenty-five thousand AC-SPL cells from C57BL/6 mice that received intracameral TNP-BSA were injected into footpads intradermally immediately after the footpads of TNP-BSA-immunized dnTGF $\beta$ RII mice or WT mice were challenged with epicutaneous PCI. Swelling was measured 24 h after challenge. The data represent the pooled mean swelling  $\pm$  SEM of 9–10 mice per group in three experiments. \* $P$  < 0.02. Immunized: immunized, challenged mice; NAIVE: swelling in challenged, non-immunized mice. NAIVE, non-immunized; IMM, immunized.

#### The suppression of the DTH reaction by CD8<sup>+</sup> AC-SPL cells is inhibited by antibodies to TGF- $\beta$

Because CD8<sup>+</sup> T cells in the spleens of mice that received an intracameral injection of antigen produce TGF- $\beta$  (12, 23) and T cells resistant to TGF- $\beta$  are resistant to suppression mediated by CD8<sup>+</sup> AC-SPL cells, we reasoned that the DTH-suppressive mechanism of the CD8<sup>+</sup> AC-SPL cells may be dependent on TGF- $\beta$ . To test this hypothesis, antibodies that recognize TGF- $\beta$  1–3 were included in the inoculum of C57BL/6 AC-SPL cells injected into footpads of TNP-BSA-immunized WT mice immediately after the challenge with epicutaneous PCI. The swelling of the footpads that received AC-SPL cells and control IgG1 antibody (MOPC 21) was suppressed significantly, i.e.  $\sim$ 75% less swelling than that of challenged, immunized mice that did not receive regulatory spleen cells (Fig. 6). However, the swelling of the footpads that received AC-SPL cells and anti-TGF- $\beta$  antibodies was not significantly different from that of challenged immunized mice that received PCI only or PCI and the isotype control antibody MOPC 21. Similar results were obtained when mice received unseparated AC-SPL cells  $\pm$  anti-TGF- $\beta$  antibody (data not shown).

#### Discussion

The injection of antigen into the AC generates circulating F4/80<sup>+</sup> cells that migrate to the spleen and thymus (10, 24–26). Thymic NKT cells, activated by these F4/80<sup>+</sup> cells migrate to the spleen where, in concert with F4/80<sup>+</sup> cells, marginal zone B cells and splenic NKT cells activate regulatory CD8<sup>+</sup> T cells (11). Whether these F4/80<sup>+</sup> cells were circulating cells recruited to the AC and then recirculated or were resident in the iris is not known. However, TGF- $\beta$  in aqueous humor and/or produced by iris F4/80<sup>+</sup> cells induces a suppressive phenotype in F4/80<sup>+</sup> cells (25). Therefore, we investigated whether TGF- $\beta$  is required directly by T cells *in vivo* for the generation of splenic CD8<sup>+</sup> regulatory T cells after the



**Fig. 6.** Anti-TGF- $\beta$  antibodies inhibit the suppressive activity of AC-SPL cells in the LTS. WT mice were immunized with TNP-BSA 1 week after they received an intracameral injection of TNP-BSA. One week after immunizing, spleen cell suspensions were prepared. The cell suspensions were incubated for 30 min at 4°C. in PBS, PBS + 15  $\mu$ g anti-TGF- $\beta$  1–3 antibodies or control MOPC 21 IgG1 antibody. After incubation, the cells  $\pm$  antibodies or anti-TGF- $\beta$  antibody only were injected into footpads of TNP-BSA-immunized recipients immediately after the footpad received an epicutaneous challenge with PCI. Swelling was measured 24 h later. Data represent the mean swelling  $\pm$  SEM of six mice per group pooled from two experiments. \* $P$  < 0.01. NAIVE, non-immunized; IMM, immunized.

injection of antigen into the AC. Although unseparated dnTGF $\beta$ R11 and Cbl-b<sup>-/-</sup> AC-SPL cells did not suppress the DTH reaction when injected into the footpads of immunized mice immediately after antigenic challenge, CD8<sup>+</sup> AC-SPL cells enriched from the unseparated cell population suppressed the DTH response (Fig. 3A and B). The unseparated cell population could contain CD4<sup>+</sup>-sensitized T cells resistant to TGF- $\beta$ -mediated suppression that would transfer the DTH reaction to the sensitized recipients. Because Cbl-b<sup>-/-</sup> and dnTGF $\beta$ R11 mice produce CD8<sup>+</sup> regulatory AC-SPL cells, the generation of circulating F4/80<sup>+</sup> cells and regulatory thymocytes is also intact (data not shown). We have observed that the spleens of dnTGF $\beta$ R11 mice receiving intracameral antigen and immunization contain different numbers of DTH-inducing T cells and CD8<sup>+</sup> regulatory T cells such that each cell population can be demonstrated when transferring higher or lower numbers of cells (R. E. Cone and Y. Lemire, unpublished observations). TGF- $\beta$  can 'convert' activated CD8<sup>+</sup> T cells to a suppressive phenotype *in vitro* (15). Therefore, our results do not preclude a direct role for TGF- $\beta$  in the *in vivo* generation of CD8<sup>+</sup> regulatory T cells after the intracameral injection of antigen. In particular, if some circulating CD8<sup>+</sup> regulatory T cells migrate to the AC perhaps they will be 'converted' to regulatory T cells in the AC by TGF- $\beta$  in the aqueous humor. However, F4/80<sup>+</sup> cells in the AC likely have a suppressive phenotype due to the TGF- $\beta$  in aqueous humor and are central to the induction of peripheral regulatory T cells.

CD8<sup>+</sup> regulatory T cells, unlike CD4<sup>+</sup>, CD25<sup>+</sup> FoxP3<sup>+</sup> regulatory T cells suppress activated T cells (1–3, 7). Moreover, CD8<sup>+</sup> regulatory T cells are detected after immunization and there is no evidence at present for naturally occurring CD8<sup>+</sup> regulatory T cells. Unlike CD4<sup>+</sup> Treg, the suppression effected by CD8<sup>+</sup> Treg is highly antigen specific (1, 2) and may be directed toward TCR V-region epitopes (1, 5). Like CD4<sup>+</sup> Treg, the suppressive mechanisms of CD8<sup>+</sup> regulatory T cells have not been defined. This issue is complicated by the description of several types of CD8<sup>+</sup> regulatory T cells that may suppress by distinct mechanisms (27–29). CD8<sup>+</sup> regulatory T cells can be induced *in vivo* (1–6) or *in vitro* (15, 16). We chose to investigate a mechanism of suppression effected by CD8<sup>+</sup> regulatory T cells induced *in vivo* because it is well established that the injection of antigen into the AC induces the production of antigen-specific, splenic CD8<sup>+</sup> regulatory T cells that are capable of suppressing a DTH reaction in sensitized recipients (6, 8–12, 26, 30). We confirmed that the suppression of the DTH reaction in the LTS is specific to the antigen injected into the AC. CD8<sup>-</sup> or CD4<sup>+</sup> AC-SPL cells do not suppress the DTH reaction in the LTS or the local adoptive transfer assay (8–10, 31). Therefore, it is unlikely that CD4/CD25<sup>+</sup> T cells suppress the DTH reaction in the LTS. However, intracamerally induced CD4<sup>+</sup> T cells induce CD8<sup>+</sup> regulatory T cells (23, 25, 32). Splenic regulatory CD8<sup>+</sup> T cells are induced *in vitro* by TGF- $\beta$  or TGF- $\beta$ , antigen-pulsed F4/80<sup>+</sup> cells (15, 16). CD8<sup>+</sup> regulatory T cells are induced (converted?) *in vitro* by TGF- $\beta$ , the *in vitro* ligation of 4-1BB by anti-4-1BB antibodies (13, 14) or the injection of antigen into the AC (12, 23) produce TGF- $\beta$ . Therefore, we asked whether TGF- $\beta$  could be a mechanism used by CD8<sup>+</sup> regulatory AC-SPL cells to mediate the sup-

pression of DTH. We determined whether the DTH reaction is suppressed by CD8<sup>+</sup> AC-SPL cells in the presence of antibodies to TGF- $\beta$  or whether the DTH reaction in immunized mice with T cells resistant to TGF- $\beta$  is suppressed by CD8<sup>+</sup> AC-SPL cells in the LTS. Our observation of a complete inhibition *in vivo* of the suppression of DTH to PCI by TNP-specific-BSA-AC-SPL cells by antibodies to TGF- $\beta$  isoforms 1–3 is similar to our observation of the anti-TGF- $\beta$ -induced inhibition of the suppression of OVA-induced DTH by OVA-specific AC-SPL cells (33). The inhibition of CD8<sup>+</sup> AC-SPL cell-mediated suppression of DTH is consistent with a report that the DTH reaction is enhanced by antibodies to anti-TGF- $\beta$  injected into the immunized recipients at the site challenged with antigen to elicit a DTH reaction (34). Moreover, we observed that two strains of mice, Cbl-b<sup>-/-</sup> and dnTGF $\beta$ R11, in which the T cells are resistant to TGF- $\beta$  based on different mechanisms, are also resistant to the suppression of the DTH reaction by CD8<sup>+</sup> AC-SPL cells. In one strain, dnTGF $\beta$ R11, there is a proximal defect in TGF- $\beta$  signaling in T cells resulting from the transgenic expression of a dnTGF $\beta$ R11 (17, 22). In the second strain, Cbl-b<sup>-/-</sup> mice have a normal receptor for TGF- $\beta$  but the absence of the ubiquitin ligase Cbl-b renders the cells incapable of responding to TGF- $\beta$  signaling (20, 21).

Although both strains may have immunological defects, we show that the DTH response to immunization is similar (or sometimes stronger) than that induced in a WT mouse. In these studies, we did not generally use isolated splenic CD8<sup>+</sup> cell populations to suppress a local DTH reaction because we demonstrated that CD8<sup>+</sup> but not CD4<sup>+</sup> splenic regulatory T cells induced by the injection of antigen into the AC selectively suppress the *in vivo* DTH reaction (Fig. 1). Moreover, it is unlikely that the suppressive activity of the CD8<sup>+</sup> regulatory spleen cells induced by intracameral antigen is mediated through cytotoxicity because in the LTS suppression of the DTH reaction by regulatory spleen cells is independent of perforin and FasL (9).

Mice deficient in Cbl-b have T cells that are CD28 independent in their activation and hyperactive in their responses (18–21). In addition, Cbl-b<sup>-/-</sup> mice develop spontaneous autoimmunity and have increased susceptibility to elicited autoimmunity (18, 19). Although Cbl-b<sup>-/-</sup> mice have normal numbers of functional CD4<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells, their effector T cells are resistant to the suppression mediated by both TGF- $\beta$  and CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (20). Cbl-b<sup>-/-</sup> effector T cells have been shown to express normal levels of TGF- $\beta$ R11, though an abnormality in Cbl-b<sup>-/-</sup> T cells in the phosphorylation of Smad2 has been described (20). In the present studies, we extended these findings and demonstrate that Cbl-b<sup>-/-</sup> effector T cells are also resistant *in vivo* to CD8<sup>+</sup> regulatory T cells.

Because the resistance of Cbl-b<sup>-/-</sup> mice to TGF- $\beta$  is likely due to the complexities of signaling dysfunction in these mice, we also investigated TGF- $\beta$  resistance in dnTGF $\beta$ R11 mice in which the resistance to TGF- $\beta$  is a result of a dominant negative TGF- $\beta$  receptor II expressed specifically on T cells (16, 22). As with DTH effector cells in Cbl-b<sup>-/-</sup> mice, DTH effector T cells in dnTGF $\beta$ R11 mice were resistant to suppression by splenic CD8<sup>+</sup> regulatory spleen cells. That both strains, resistant to TGF- $\beta$  by completely different mechanisms, are

resistant to the suppression effected by CD8<sup>+</sup> regulatory T cells suggests that the resistance of the two strains to suppression mediated by CD8<sup>+</sup> regulatory T cells has a common basis.

CD8<sup>+</sup> T (OT-1) cells converted to a suppressive phenotype by culture with TGF- $\beta$  and OVA suppressed the induction of OVA-specific cytotoxic T cells *in vitro* and the rejection of a cardiac allograft in mice transgenic for OVA by a TGF- $\beta$ -independent mechanism (15). Because the CD8<sup>+</sup> regulatory T cells were induced by exposure to exogenous TGF- $\beta$  and antigen *in vitro*, they may differ from some CD8<sup>+</sup> regulatory T cells induced *in vivo*. Different subtypes of CD8<sup>+</sup> regulatory T cells have been described that function by distinct non-lytic mechanisms (27, 28). Our results with antibodies to TGF- $\beta$  and the resistance of TGF- $\beta$ -resistant mice to the suppression of the DTH reaction by CD8<sup>+</sup> AC-SPL cells provide evidence that TGF- $\beta$  is an efferent suppressive mechanism of these cells. However, the LTS assay measures the suppression of DTH by a limited number of cells. It is possible that the use of larger numbers of cells may reveal additional suppressive mechanisms. Additionally, T cell-derived TGF- $\beta$ 1 has pleiotropic effects and may promote T<sub>H</sub>17 cell differentiation while suppressing T<sub>H</sub>1 cell differentiation (35, 36). Our experiments have not identified the TGF- $\beta$  isoforms responsible for immunosuppression by CD8<sup>+</sup> regulatory T cells nor the induction of AC-induced F4/80<sup>+</sup> T cells required to induce immunoregulatory thymocytes or splenic CD8<sup>+</sup> T cells. Accordingly, the afferent and efferent roles for TGF- $\beta$  in the suppression of cell-mediated immunity induced by the intracameral injection of antigen are likely dependent on the complexity of the phenomenon under investigation.

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## Abbreviations

AC	anterior chamber
CS	contact sensitivity
dnTGF $\beta$ RII	dominant negative receptor for transforming growth factor $\beta$ receptor type II
DTH	delayed-type hypersensitivity
LTS	local transfer of suppression
OVA	ovalbumin
PCI	picryl chloride
TGF	transforming growth factor
TNP	trinitrophenol
WT	wild type

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