

$\gamma\delta$ T Cells from Tolerized $\alpha\beta$ T Cell Receptor (TCR)-deficient Mice Inhibit Contact Sensitivity-Effector T Cells In Vivo, and Their Interferon- γ Production In Vitro

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Summary

Contact sensitivity (CS) responses to reactive hapten Ag, such as picryl chloride (PCI) or oxazolone (OX), are classical examples of T cell-mediated immune responses in vivo that are clearly subject to multifaceted regulation. There is abundant evidence that downregulation of CS may be mediated by T cells exposed to high doses of Ag. This is termed high dose Ag tolerance. To clarify the T cell types that effect CS responses and mediate their downregulation, we have undertaken studies of CS in mice congenitally deficient in specific subsets of lymphocytes. The first such studies, using $\alpha\beta$ T cell-deficient (TCR $\alpha^{-/-}$) mice, are presented here. The results clearly show that TCR $\alpha^{-/-}$ mice cannot mount CS, implicating $\alpha\beta$ T cells as the critical CS-effector cells. However, TCR $\alpha^{-/-}$ mice can, after high dose tolerance, downregulate $\alpha^{+/+}$ CS-effector T cells adoptively transferred into them. By mixing ex vivo and then adoptive cell transfers in vivo, the active downregulatory cells in tolerized $\alpha^{-/-}$ mice are shown to include $\gamma\delta$ TCR $^{+}$ cells that also can downregulate interferon- γ production by the targeted CS-effector cells in vitro. Downregulation by $\gamma\delta$ cells showed specificity for hapten, but was not restricted by the MHC. Together, these findings establish that $\gamma\delta$ T cells cannot fulfill CS-effector functions performed by $\alpha\beta$ T cells, but may fulfill an Ag-specific downregulatory role that may be directly comparable to reports of Ag-specific downregulation of IgE antibody responses by $\gamma\delta$ T cells. Comparisons are likewise considered with downregulation by $\gamma\delta$ T cells occurring in immune responses to pathogens, tumors, and allografts, and in systemic autoimmunity.

Cutaneous contact sensitivity (CS)¹ responses to contact sensitizing hapten Ag such as picryl chloride (PCI) and oxazolone (OX) are classical manifestations of T cell-mediated immunity in vivo. In previously sensitized hosts, CS and the related delayed-type hypersensitivity (DTH) reaction, are manifest as macroscopically measurable inflammation (skin swelling) that peaks at 24–48 h after topical cutaneous Ag challenge (1).

It appears that early after challenge with hapten, local tissue mast cells (2, 3) and blood platelets (4) are induced to

release the vasoactive amine serotonin (2–6) that facilitates local extravasation and subsequent migration and activation of Ag-specific CS-effector lymphocytes. Available data strongly suggest, but do not prove, that the responding late-acting lymphocytes are exclusively MHC class II-restricted, $\alpha\beta$ TCR $^{+}$, CS-effector T cells (7, 8), which customarily make Th1-type effector cytokines, such as IFN- γ (9–11).

By contrast to the conventional CS-effector response, which is induced by immunization via skin painting with reactive hapten Ag, mice respond to high intravenous doses of soluble hapten Ag by developing tolerance. This high dose Ag tolerance is of particular significance with regard to the capacity of individuals that become tolerant toward contact hapten allergens, and may also underlie aspects of tolerance to self Ag. Numerous studies have demonstrated

¹Abbreviations used in this paper: DN, double negative T cells (CD4 $^{-}$, CD8 $^{-}$); CS, contact sensitivity; DTH, delayed-type hypersensitivity; MRBC, mouse red blood cells; OX, oxazolone; PCI, picryl chloride; TMB, tetramethylbenzidine; TNBSA, Trinitro-benzene sulfonic acid.

that high dose tolerance can be associated with the development of regulatory T cells that limit the response of CS-effector T cells (12–17). In this regard, it is unclear whether or not such CS-downregulatory T cells are exclusively $\alpha\beta$ T cells, or whether they may include $\gamma\delta$ T cells.

An assay commonly used to demonstrate high dose downregulation of CS, is the mixing together in vitro of spleen cells from mice tolerized intravenously with high doses of Ag, together with CS-effector cells from contact sensitized mice. Thereafter, the mixed regulatory and CS-effector cells are adoptively transferred to naive recipients, and a subsequent measurement is made of the transferred CS-effector response in the recipients. The studies reported in this manuscript use this mixing assay and other established assays, along with use of TCR-deficient $\alpha^{-/-}$ mice, to assess the cells responsible for CS elicitation and for CS downregulation, respectively. The studies show that $\gamma\delta$ T cells cannot substitute for $\alpha\beta$ T cells as effectors of CS elicitation, but that $\gamma\delta$ T cells can mediate downregulation of both $\alpha\beta$ CS effectors in vivo and IFN- γ production by these CS effectors in vitro. The studies are particularly pertinent given the reported capacity of $\gamma\delta$ T cells to negatively regulate $\alpha\beta$ T cell-driven responses in allergic (18, 19) and other immune responses.

Materials and Methods

Mice. 5–7-wk-old 129/J ($H2^b$), BDF₁ ($H2^b \times ^d$), C57Bl/6 ($H2^b$), BALB/c ($H2^d$), and CBA/J ($H2^k$) mice were obtained from Jackson Labs (Bar Harbor, ME). TCR $\alpha^{-/-}$ and TCR $\alpha^{+/-}$ mice (20–22) with different but defined MHC backgrounds that included $H2^b$, $H2^d$, and $H2^{b+d}$, were supplied locally. All mice were maintained in microisolator cages, and changed in a laminar flow hood. MHC haplotypes were determined by FACS[®] with anti-MHC class I^b (PE-conjugated), and class I^d (FITC-conjugated) mAb (PharMingen, San Diego, CA).

Reagents. Picryl chloride TNP chloride (PCI) obtained from Chemica Alta (Edmonton, Alberta, Canada) was recrystallized from methanol/H₂O, and protected from light and humidity. Trinitro-benzene sulfonic acid (TNBSA) (Eastman Fine Chemicals, Rochester, NY); oxazolone (4-ethoxymethylene-2-phenyloxazolone) (OX) (British Drug Houses via Gallard-Schlesinger Inds. Inc., Carle Place, NY); and anti-hamster IgG antibody-coated magnetic beads (1 μ m iron magnetic particles; Advanced Magnetics, Inc., Cambridge, MA) were obtained from the manufacturers. Anti-TCR $\gamma\delta$ mAb (clone UC7-135D5, hamster IgG), was obtained from Dr. J. Bluestone (University of Chicago, Chicago, IL; 23). Low-tox rabbit complement C was obtained from Pel-Freez Biologicals (Brown Deer, WI).

Active Immunization. Mice were actively sensitized by topical application of 0.15 ml of 5% PCI or 3% OX in 1:3 acetone-ethanol mixture, to the shaved abdomen, chest, and hind feet on day 0. 4 d later, zero time ear thickness was measured with an engineer's micrometer (Mitutoyo Mfg. Co., Tokyo, Japan) before local ear skin challenge via topical application to both sides of both ears of one drop of 0.8% PCI or OX solution in olive oil (for 129/J, BALB/c, TCR $\alpha^{+/-}$ and $\alpha^{-/-}$), or 1% PCI to less reactive BDF₁ or C57Bl/6. The subsequent optimal increase in ear thickness was measured at 24 h, and occasionally 48 h, after challenge as re-

ported (1–8), and expressed in units of 0.01 mm \pm SE. In all experiments, a group of nonimmune control animals was also challenged on the ears with 0.8% PCI or OX, and the resulting background increase in ear thickness (\sim 2 U at 24 h) was subtracted from responses of experimental groups to yield net ear swelling responses, shown in the figures.

Induction of High Ag Dose Tolerance. For induction of TNP-specific high dose tolerance, mice received two intravenous injections of 3 mg TNBSA (1% TNBSA in distilled H₂O, readjusted to pH 7.2 with 1 M NaOH) in 0.3 ml on days 0 and 3. On day 7, spleens from these TNBSA-injected mice were harvested as a source of putative regulatory cells. Alternatively, mice were tolerized by intravenous injection of TNP-conjugated syngeneic spleen cells.

To induce OX-specific tolerance, mice were injected intravenously once with 0.3 ml of 10% OX-conjugated mouse red blood cells (MRBC), or with OX-conjugated spleen cells (5×10^7) on days 0 and 3. The MRBC or spleen cells were labeled with OX as described previously (12). Briefly, 1 ml of packed MRBC was resuspended in 10 ml of DPBS (calcium- and magnesium-free PBS) and mixed for 30 min with freshly prepared 20 ml aqueous OX solution (containing 20 mg OX). The OX-MRBC were then washed twice with PBS containing 5% FCS, and before use, the pelleted OX-MRBC were resuspended to 10% in PBS. Spleens from mice injected intravenously with OX-MRBC, or with OX spleen cells, were harvested on d 7 as a source of OX-specific regulatory cells.

Adoptive Cell Transfer of CS Responses and Cell Mixing Assay. Donors of CS-immune effector cells were contact sensitized with 5% PCI or 3% OX on days 0 and 3. Immune lymph node and spleen cells were harvested on day 7 and 7×10^7 were injected intravenously into normal syngeneic recipients. Immediately after transfer, recipients were challenged on the ears with 0.8% PCI or OX in olive oil. The increase in ear swelling was determined 24 h, and sometimes 48 h later. It was compared to actively sensitized positive controls and to negative controls that were simply challenged on the ears with 0.8% PCI or OX.

For the cell mixing assay, $5\text{--}7 \times 10^7$ CS-effector immune lymph node and spleen cells from PCI or OX contact sensitized $\alpha^{+/+}$ donors were incubated for 30 min at 37°C with 5×10^7 regulatory spleen cells from mice intravenously tolerized with TNBSA or with OX-MRBC. Regulatory spleen cells were from tolerized $\alpha^{-/-}$ mice, or control $\alpha^{+/+}$ 129/J, C57Bl/6, BALB/c, or BDF₁ mice. Positive controls were immune CS-effector cells incubated without regulatory cells. After incubation, washed mixed cells from each group were transferred intravenously, and recipients were tested for CS responses by ear challenge with topical application of 0.8% PCI or OX, within 30 min immediately after transfer.

Transfer into Tolerized Mice to Confirm Active Tolerance and Ag-specificity. To test tolerance and Ag specificity of downregulatory cells in another system, $\alpha^{-/-}$ mice were tolerized with a high dose of TNBSA or with OX-MRBC intravenously on days 0 and 3 before serving as cell transfer recipients. Then on day 7, nontolerized control or TNP- or OX- tolerized $\alpha^{-/-}$ mice received 7×10^7 CS-effector cells from normal TCR $\alpha^{+/+}$ mice (BALB/c) that were Ag homologous, or were of unrelated Ag specificity (TNP versus OX). $\alpha^{-/-}$ recipient mice were then challenged immediately on the ears with homologous hapten (TNP or OX), and ear tested for 24 h ear swelling.

Immuno-magnetic Bead Cell Fractionation. Spleen cells (1.5×10^8) from four TNBSA-tolerized $\alpha^{+/+}$ 129/J, or $\alpha^{-/-}$ donor

mice were incubated in 20 ml UC7 hamster anti-TCR $\gamma\delta$ mAb (12 $\mu\text{g/ml}$) hybridoma supernatant for 60 min on ice. After mAb coating, cells were washed three times with PBS + 2% FCS, and resuspended in 40 ml PBS + 2% FCS containing goat anti-hamster Ig-coated paramagnetic beads at 5–10 beads per target cell. The cells were then incubated in a flat vertical 50-ml flask for 30 min on ice, after which a magnet (Advanced Magnetics Inc., Cambridge, MA) was applied to one side of the flask. 10 min later, magnetic bead nonadherent cells were recovered, and then the magnetic bead adherent cells were recovered. Nonseparated regulatory cells were used in experiments shown in Figs. 3, 5, and 6 b.

Recoveries of $\gamma\delta$ cells were as follows. For 129/J TNBSA-tolerized donors, we harvested 15×10^7 separated spleen cells from three donors, obtaining $\sim 10^6$ anti- $\gamma\delta$ magnetic bead positive cells that were 60% $\gamma\delta^+$ by FACS[®]. Thus, there were $\sim 1.6 \times 10^5$ $\gamma\delta$ cells transferred to each of four recipients, while there were 10×10^5 $\gamma\delta$ cells in the starting spleen of one donor mouse. For $\alpha^{-/-}$ TNBSA-tolerized donors, we harvested 20×10^7 spleen cells from three donors, obtaining 3.4×10^6 anti- $\gamma\delta$ bead positive total cells that were on average 80% $\gamma\delta^+$ by FACS[®], thus allowing for 7×10^5 $\gamma\delta$ -enriched cells transferred to each of four recipients. FACS[®] analysis of the anti- $\gamma\delta$ immunobead separated subpopulations was presented elsewhere (24), in which we reported that the percentage of $\gamma\delta$ cells adhering to the anti- $\gamma\delta$ beads can vary between 55–95% of the total adherent cells; the rest are not specifically bound, and on assay did not have biologic activity. Also, we noted that unavoidable splenic macrophage phagocytosis of some mAb-coated $\gamma\delta$ cells that were attached to beads, probably contributed to variability in $\gamma\delta$ T cell recoveries.

Mixing of CS-effector Cells and Magnetic Bead-enriched Regulatory Cells. Immune CS-effector cells (7×10^7) were incubated with 5×10^7 unseparated regulatory T cells, with an equivalent number of separated magnetic bead positive $\gamma\delta$ cells, or with an equivalent number of magnetic bead nonadherent $\gamma\delta^-$ cells (i.e., $\alpha\beta$ TCR⁺-remaining cells) originating from either tolerized 129/J $\alpha^{+/+}$ mice or TCR $\alpha^{-/-}$ mice for 30 min at 37°C. The washed effector and regulatory cell mixture was then injected intraperitoneally into naive 129/J recipients that were tested for CS responses by challenging ears with 0.8% PCl or OX in olive oil the next day. Recipients were then measured for ear swelling 24 h after Ag challenge to the ears.

In Vitro Culture of CS-effector Cells and Hapten-conjugated APC for Elaboration of IFN- γ , and Regulation by Tolerized Cells. A single cell suspension of just CS-effector lymph node cells was obtained aseptically from PCl contact sensitized mice. Suspensions of regulatory cells were from spleen cells of mice that were injected with TNBSA or OX-MRBC intravenously on days 0 and 3, or from control nonimmune mice. In some experiments, spleen cells from TNBSA-tolerized mice, or nonimmune controls, were incubated with 1 μg biotinylated anti- $\gamma\delta$ TCR (GL3; Pharmingen) per 10^6 cells at 2×10^7 cells/ml for 20 min on ice. Then, 10 μl of streptavidin-conjugated magnetic microbeads (Miltenyi, Sunnyvale, CA and BioTek Instrs. Inc., Sunnyvale, CA) per 10^7 cells were added and incubated for 15 min on ice. After washing, this cell and bead suspension was applied to a MiniMACS magnetic separation column (Miltenyi). After recovering of nonadherent $\gamma\delta$ -depleted cells and washing the column, $\gamma\delta$ -enriched adherent cells were eluted. Equivalent numbers of $\gamma\delta$ -enriched, or $\gamma\delta$ -depleted cells were added in vitro as regulatory cells, to the CS-effector cells (1:1; 2×10^5 /well), and then were added together for 72 h to mitomycin C-treated TNP- or OX-conjugated normal spleen cells as APC (2×10^5) in 0.2 ml of RPMI 1640 containing 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 2 mM L-glu-

tamine, 25 mM Hepes, 5×10^{-5} M/2-mercaptoethanol, and 10% fetal calf serum. At 48 h, culture supernatants were collected for IFN- γ quantitation.

ELISA Quantitation of IFN- γ in Culture Supernatants. Quantitative sandwich ELISA for IFN- γ was performed with two different mAb specific for mouse IFN- γ (clones R4-6A2 and XMG1.2; Pharmingen). 1 $\mu\text{g/ml}$ of capture mAb to IFN- γ in 0.1 M Na_2CO_3 , pH 8.3, was briefly coated onto 96-well microtrays (Corning Glass Incorporated, Corning, NY) overnight at 4°C. After blocking with 3% dry milk in PBS, samples and standard recombinant mouse IFN- γ (Genzyme Corp., Cambridge, MA) were applied to the wells and incubated overnight at 4°C. Then, 0.5 $\mu\text{g/ml}$ of biotinylated separate mAb to IFN- γ was applied for 45 min at 25°C, followed by incubation with 1:3,000 dilution of horseradish peroxidase-conjugated streptavidin (Vector Labs., Inc., Burlingame, CA), for 30 min at 25°C. TMB (tetramethylbenzidine) microwell peroxidase substrate, and TMB one component stop solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were used for reaction development and were read at OD 405.

Statistics. Double-tailed Student's *t* test was used to assess the significance of differences between groups, with $P < 0.05$ taken as a minimum level of significance.

Results

TCR $\alpha^{-/-}$ Mice Have No 24 h CS Responses. CS responses in contact sensitized TCR $\alpha^{-/-}$ mice and control 129/J mice were compared by sensitizing (via abdominal skin painting) with concentrated PCl (5%), followed 4 d later by topical challenge with dilute PCl (0.8%) applied to the ears, and subsequent measurement of ear swelling 24 h later. It was

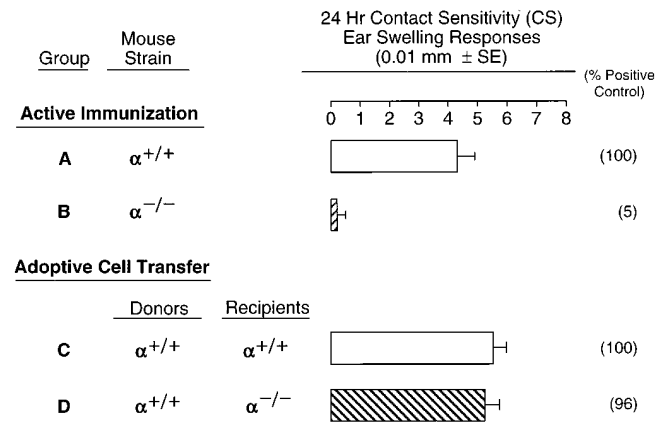


Figure 1. Actively sensitized TCR α knock out mice ($\alpha^{-/-}$) have no classical late 24 h cutaneous CS responses, but can serve as adoptive cell transfer recipients. TCR $\alpha^{+/+}$ 129/J (group A) or TCR $\alpha^{-/-}$ (group B) mice, were contact sensitized with 0.15 ml of 5% PCl skin painting. 4 d later, animals were challenged on the ears with 0.8% PCl, and CS responses were determined 24 h later by measuring increases in ear thickness with an engineer's micrometer. Results were expressed as U \times 0.01 mm \pm SE. Background ear swelling in PCl challenged controls that were not sensitized, were subtracted from experimental groups to give net ear swelling, as is shown. BDF₁ (group C) $\alpha^{+/+}$ immune cells from PCl contact sensitized donors were transferred intravenously to BDF₁ recipients, or to TCR $\alpha^{-/-}$ H2^b recipients (group D) that were ear challenged similarly, and then, 24 h ear swelling responses were determined.

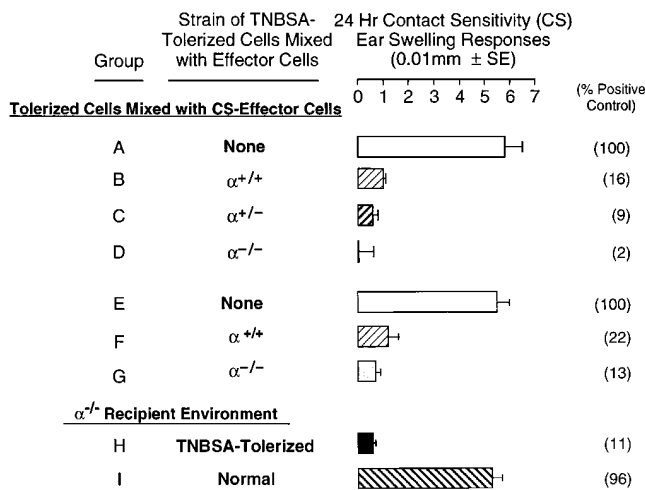


Figure 2. High dose Ag tolerance of $\text{TCR}\alpha^{-/-}$ mice induces cells that downregulate CS and generate an inhibitory environment. As an assay for generation of downregulatory cells, CS-effector cells (7×10^7) from PCI contact sensitized 129/J mice (groups A–D) were incubated for 30 min at 37°C , alone (group A, positive controls), or with 5×10^7 putative regulatory cells induced by two intravenous injections of TNBSA in $\text{TCR}\alpha^{+/+}$ 129/J (group B), $\text{TCR}\alpha^{+/-}$ (group C), or $\text{TCR}\alpha^{-/-}$ (group D) mice. The cell mixture was then washed and transferred intravenously into naive syngeneic 129/J recipients that immediately were challenged on the ears with 0.8% PCI. Groups B–D were significantly different ($P < 0.001$) from control group A. In groups E–I, from a pooled separate experiment, $\text{TCR}\alpha^{+/+}$ BDF₁ mice or their $\text{TCR}\alpha^{-/-}$ H2^{b,d} counterparts were used. BDF₁ are poorly CS-reactive and thus were skin sensitized with PCI twice (days 0 and 3). On d 7, immune CS-effector lymph node and spleen cells (7×10^7), from these mice were injected intravenously into normal BDF₁ mice (groups E–G), or into $\text{TCR}\alpha^{-/-}$ recipients (groups H and I). Tolerized downregulatory cells were induced in BDF₁ or $\text{TCR}\alpha^{-/-}$ mice by two intravenous injections of 5×10^7 TNP-conjugated $\text{TCR}\alpha^{-/-}$ spleen cells on days 0 and 3. Spleens from these tolerized mice served as a source of downregulatory cells respectively in groups F and G, in which 3.5×10^7 tolerized cells were mixed and incubated with BDF₁ PCI-immune cells (7×10^7), before being injected intravenously together into the recipients. In group H, recipient $\text{TCR}\alpha^{-/-}$ animals were tolerized via prior intravenous injection of TNP-conjugated spleen cells, i.e., they were tolerized by a procedure similar to inducing regulatory cells used in the mixing and adoptive transfer experiments (groups E–G). Statistical significance: group E vs F, I and H, $P < 0.005$. Each group consisted of three animals. In group I, BDF₁ CS-immune cells were transferred successfully into untreated $\text{TCR}\alpha^{-/-}$ mice.

found that $\alpha\beta$ T cell-bearing 129/J mice developed a classical 24 h ear swelling response (Fig. 1, group A), whereas $\text{TCR}\alpha^{-/-}$ mice did not (Fig. 1, group B).

When $\alpha^{-/-}$ mice were used as recipients of $\alpha\beta^+$ cells from MHC-matched, immunized $\alpha\beta$ T cell-sufficient BDF₁ mice, they could mount CS upon ear challenge with hapten (Fig. 1, group D), similar to normal mice (Fig. 1, group C). This demonstrated that the vascular and cellular environment for eliciting CS was normal in $\text{TCR}\alpha^{-/-}$ knock-out mice. The finding that the 24 h component of CS cannot be elicited in actively sensitized $\text{TCR}\alpha^{-/-}$ mice is consistent with other studies indicating that the active CS-effector cell type is $\text{TCR}\alpha\beta^+$ (7, 8, 24–26).

High Dose Ag Tolerance of $\text{TCR}\alpha^{-/-}$ mice Induces Regulatory Spleen Cells that Downregulate CS. Because $\text{TCR}\alpha^{-/-}$ mice do not mount CS responses, it is impossible to determine

directly whether high dose Ag tolerization of such mice induces downregulatory cells. Instead, CS-effector cells (7×10^7) from contact-sensitized 129/J mice were mixed and incubated for 30 min at 37°C with 5×10^7 spleen cells from $\alpha^{-/-}$ mice, that were harvested on d 7 after two intravenous injections of tolerogenic TNBSA. Analogous TNBSA-tolerized cells were derived from TNBSA-treated MHC haplotype-matched $\text{TCR}\alpha^{+/-}$ mice, or $\text{TCR}\alpha^{+/+}$ (129/J) mice. The cells were then transferred back to haplotype-matched recipient mice that were challenged with hapten.

Fig. 2 shows that upon transfer back to recipient mice, CS-effector cells elicit a 24 h response that can be significantly reduced by preincubation of the effector cells with cells from tolerized $\text{TCR}\alpha^{+/+}$ mice (Fig. 2, group B), $\text{TCR}\alpha^{+/-}$ mice (Fig. 2, group C), or $\text{TCR}\alpha^{-/-}$ mice (Fig. 2, group D). Essentially the same results were obtained when effector cells from sensitized BDF₁ mice were mixed with cells from either TNBSA-tolerized BDF₁ mice or TNBSA-tolerized $\text{TCR}\alpha^{-/-}$ mice (Fig. 2, groups E–G).

A prediction that follows from the data shown in Fig. 2 (groups A–G) is that $\text{TCR}\alpha^{-/-}$ mice will not support the actions of adoptively transferred $\text{TCR}\alpha^{+/+}$ CS-effector cells if the recipient $\alpha^{-/-}$ mice are subject to high dose Ag tolerance beforehand. The data in Fig. 2, groups H and I confirm this prediction. This result is important in that it rules out that the detection of $\text{TCR}\alpha^{-/-}$ downregulatory cells in adoptive transfer experiments (Fig. 2, groups D and G) was artefactual, due to manipulation of the cells in vitro and their subsequent transfer. Furthermore, induction of high dose tolerance by TNBSA in $\text{TCR}\alpha^{-/-}$ mice was reproducibly demonstrated using transfers from individual $\text{TCR}\alpha^{-}$ mice (data not shown), indicating the generality of the findings.

Expression of $\gamma\delta$ TCR on CS-regulatory Cells from Tolerized $\text{TCR}\alpha^{-/-}$ Mice. To determine the phenotype of CS-regulatory cells induced by high Ag dose tolerance, splenocytes from TNBSA-tolerized mice were fractionated by use of anti-TCR $\gamma\delta$ mAb-coated magnetic beads. $\gamma\delta^+$, $\gamma\delta^-$, or unfractionated tolerized cells were then mixed for 30 min at 37°C with lymph node and spleen CS-effector cells (7×10^7) from contact immunized, haplotype-matched mice, and the mixture transferred to recipients that were subsequently assayed for CS responses by ear challenge. In these experiments, anti- $\gamma\delta$ bead adherent cells ranged from 55 to 95% $\gamma\delta^+$ when reanalyzed by FACS[®] (see Materials and Methods). Thus, we estimate that $\geq 10^6$ $\gamma\delta$ cells were adoptively transferred into each recipient when unseparated $\text{TCR}\alpha^{-/-}$ cells were used, and about $\geq 7 \times 10^5$ $\gamma\delta$ cells were transferred when magnetic bead $\gamma\delta$ -enriched cells were used.

The data in experiments 1 and 2 of Fig. 3 show that while the cells from TNBSA-treated $\alpha^{+/+}$ 129/J mice that are responsible for downregulating CS effectors are exclusively TCR $\gamma\delta^-$ (group D), the most potent regulatory cell fraction from tolerized $\text{TCR}\alpha^{-/-}$ mice scored as TCR $\gamma\delta^+$ (Fig. 3, group F). Similar results were obtained in experiment three, in which cells from H2^d $\text{TCR}\alpha^{-/-}$ mice were

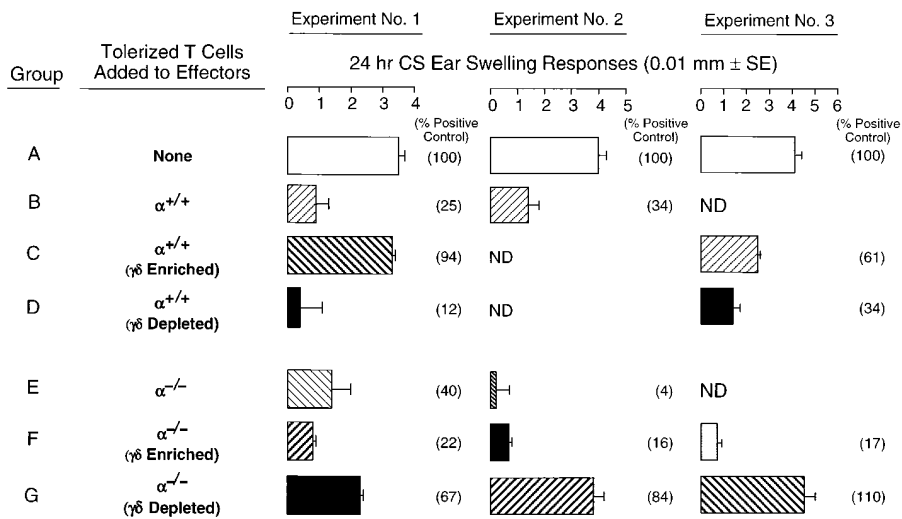


Figure 3. $\gamma\delta$ T cells are responsible for CS downregulatory activity of high dose Ag tolerized $\text{TCR}\alpha^{-/-}$ mice. In experiments 1 and 2, spleen cells (1.5×10^8) from TNBSA intravenously tolerized 129/J (group B), or $\text{TCR}\alpha^{-/-}$ animals (group E), were separated into $\gamma\delta \text{TCR}^+$ (groups C and F), and $\gamma\delta \text{TCR}^-$ (groups D and G) subpopulations by anti- $\gamma\delta$ TCR mAb treatment in solution, and then incubation with anti-hamster Ig-coated para magnetic beads, and subsequently separated in vitro by exposure to a magnet. In experiment 3, similar cell fractionation used $\alpha^{+/+}$ BALB/c and their $\alpha^{-/-}$ H2^d counterparts. Immune CS-effector cells (7×10^7) were incubated for 30 min at 37°C with downregulatory, separated, magnetic bead positive cells ($\gamma\delta \text{TCR}^+$) (groups C and F), or magnetic bead negative ($\gamma\delta \text{TCR}^-$) cells (groups D and G), that were from either TNBSA-tolerized 129/J or BALB/c mice (groups C, D), or TNBSA-

tolerized $\text{TCR}\alpha^{-/-}$ animals (groups F, G). Cell mixtures were then washed and injected intraperitoneally into naive recipients. As a positive control, CS-effector cells incubated without regulatory cells were transferred intraperitoneally (group A). The next day, animals were challenged on the ears with 0.8% PCl in olive oil and the increment in ear swelling was determined 24 h later. Statistical significance: group A vs B and D, $P < 0.01$; group A vs group E, $P < 0.05$; and group A vs group F, $P < 0.001$.

compared with cells from H2^d BALB/c mice for their response to high dose Ag tolerance. As before, when measuring the capacity of tolerized cells to downregulate a CS-effector response to PCl, the TCR $\gamma\delta^+$ fraction from $\text{TCR}\alpha^{-/-}$ mice, but not from $\alpha^{+/+}$ BALB/c mice, was again active (experiment 3, group C). Conversely, the active fraction from normal $\alpha^{-/-}$ mice was TCR $\alpha\delta^+$ (group D), and the TCR $\gamma\delta^-$ fraction from $\text{TCR}\alpha^{-/-}$ mice (group E) contained negligible residual activity.

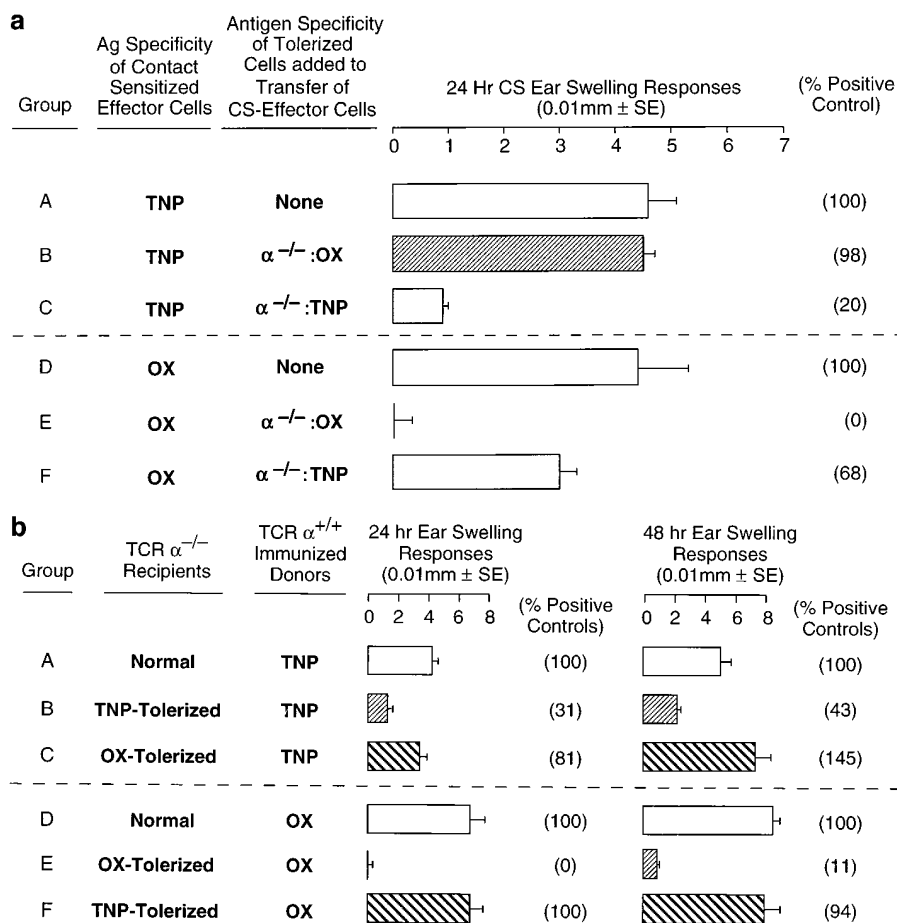
$\gamma\delta$ T Cell-mediated Regulatory Activity Shows Antigen Specificity. The discovery that $\gamma\delta$ T cells in $\text{TCR}\alpha^{-/-}$ mice can mediate downregulation of CS effector cells prompted an investigation of Ag specificity, since $\gamma\delta$ T cells and $\alpha\beta$ cells appear to have a different set of Ag specificities. To test this, cells from $\text{TCR}\alpha^{-/-}$ mice tolerized with high doses of OX or TNP, were mixed with CS-effector cells from $\text{TCR}\alpha^{+/+}$ mice sensitized with PCl or OX, and compared in their capacities to downregulate the 24 h CS response after transfer back to recipient mice. Data presented in Fig. 4 a show no capacity of cells from OX-tolerized $\text{TCR}\alpha^{-/-}$ mice to influence the CS response to TNP (group B), whereas TNP responses were inhibited significantly after mixing effector cells with cells from TNP-tolerized $\text{TCR}\alpha^{-/-}$ mice (Fig. 4 a, group C). Data in Fig. 4 a, groups D–F demonstrate that reciprocal Ag specificity was likewise apparent (i.e., cells from OX-tolerized $\text{TCR}\alpha^{-/-}$ mice downregulated effector cells from OX-sensitized mice, but not $\alpha^{+/+}$ TNP-immune cells) (group E versus F).

To confirm the above findings we tested Ag-specificity in tolerized $\alpha^{-/-}$ mice by attempting to transfer $\alpha^{+/+}$ CS-effector cells into these recipients. $\alpha^{-/-}$ mice were tolerized with a high dose of TNBSA or OX-MRBC intravenously before cell transfer. Then, on day 7, control (non-tolerized) or TNP or OX-tolerized $\alpha^{-/-}$ mice received 7×10^7 TNP or OX immune cells from normal $\alpha^{+/+}$ (BALB/c)

mice. The data presented in Fig. 4 b show there was no ability to adoptively transfer immunity into TNP tolerized $\alpha^{-/-}$ mice with TNP-specific CS-effector cells (group B), while TNP-immune CS-effector cells transferred normal CS responses into OX-tolerized $\alpha^{-/-}$ recipients (group C). Similar data were obtained when OX-specific immune cells were transferred (group D versus F). Thus, total inhibition of adoptive cell transfer was obtained when OX-specific immune CS-effector cells were transferred to OX-tolerized $\alpha^{-/-}$ mice (Fig. 4 b, group E), whereas OX CS was successfully transferred to TNP-tolerized $\alpha^{-/-}$ mice (Fig. 4 b, group F). Thus, this system that did not involve cell harvest, cell mixing, nor cell transfer, confirmed the presence of active Ag-specific downregulation in high dose-tolerized $\alpha^{-/-}$ mice.

$\gamma\delta$ T Cell-mediated Regulatory Activity is MHC Unrestricted. To determine possible MHC restriction of $\gamma\delta$ downregulatory T cells, magnetic bead-enriched $\gamma\delta \text{TCR}^+$ cells from TNBSA-tolerized $\alpha^{-/-}$ mice were incubated with 7×10^7 PCl-immune CS-effector cells from MHC-compatible (129/J, H2^b), or -incompatible (CBA/J, H2^k) mice, for 30 min at 37°C, followed by subsequent transfer to recipients syngeneic with the CS-effector cells. The recipient mice were challenged immediately (before any significant host response to the allogeneic $\gamma\delta$ graft could develop), and ear swelling responses determined. The results in Fig. 5 show that $\gamma\delta^+$ cells from TNBSA-tolerized $\text{TCR}\alpha^{-/-}$ mice downregulated CS-effector responses of allogeneic CBA/J H2^k CS-effector cells (group D), as effectively as the regulation of H2-matched CS-effector cells (group B).

Phenotype and dose-response Activity of CS-regulatory $\gamma\delta$ T Cells. To establish the phenotype of $\gamma\delta$ T cells capable of inhibiting CS-effector responses, spleen cells from TNBSA-tolerized $\alpha^{-/-}$ mice were treated with anti-CD4 or anti-CD8 mAb or PBS, and then rabbit complement 1:75.



not involve transfers and manipulation of the putative regulatory cells. $\alpha^{-/-}$ mice were tolerized by intravenously injection of TNBSA or OX-MRBC before serving as recipients for transfer of CS-effector cells from normal BALB/c mice that were of homologous or unrelated Ag (TNP or OX) specificity. The experiments included groups of positive and negative controls. Mice were then challenged immediately on the ears with homologous hapten (TNP or OX), and then tested for CS ear swelling 24 and 48 h later. Statistical analysis at 24 h using the two tailed Student's *t* test showed: group B vs A, $P < 0.01$; group B vs C, $P < 0.02$; group E vs D, $P < 0.01$; group E vs F, $P < 0.01$. For 48 h: group B vs A, $P < 0.02$; group B vs C, $P < 0.01$; group E vs D, $P < 0.001$; and group E vs F, $P < 0.01$.

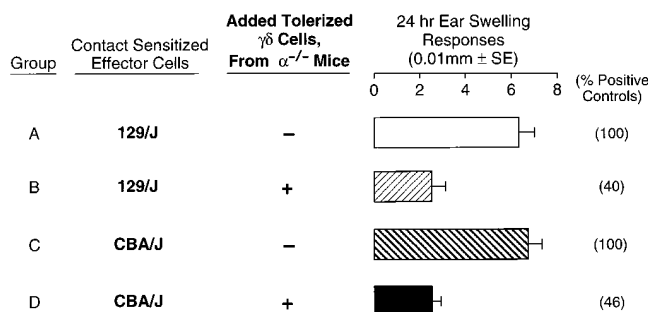


Figure 5. Lack of MHC restriction of $\gamma\delta$ downregulatory cells from TNBSA-tolerized TCR $\alpha^{-/-}$ mice. Magnetic bead positive ($\gamma\delta$ TCR $^{+}$) cells from TNBSA-tolerized $\alpha^{-/-}$ mice were incubated with 7×10^7 cells from MHC compatible 129/J H2^{b,d}, or MHC incompatible (CBA/J, H2^k) mice for 30 min at 37°C (groups B and D). As a positive control, we used immune cells from 129/J or CBA/J mice that were incubated without regulatory cells in medium alone (groups A and C). Then cells were washed and injected intraperitoneally into recipients that were syngeneic with the immune cells, and were skin tested for CS responsiveness. Statistical significance: group A vs B, and group C vs D, $P < 0.001$.

Figure 4. (a) Reciprocal Ag specificity of splenic $\gamma\delta^{+}$ regulatory cells from TNP or OX-tolerized $\alpha^{-/-}$ mice that act on TNP versus OX-immune CS-effector T cells. BDF₁ mice were used as donors and recipients of PCI or OX immune cells, while their H2^{b,d} TCR $\alpha^{-/-}$ counterparts were used as donors of tolerized regulatory cells. To tolerize spleens of TCR $\alpha^{-/-}$ mice, normal $\alpha^{-/-}$ spleen cells were conjugated in vitro with TNP or OX, and 5×10^7 hapten-conjugated self cells were injected intravenously into $\alpha^{-/-}$ mice on days 0 and 3, and then spleen cell suspensions from these tolerized recipients were prepared on day 7 as putative regulatory cells. BDF₁ CS-effector cell donors were contact skin-sensitized with TNP (via PCI painting) (groups A–C), or with OX (groups D–F), on days 0 and 3, and then spleen and lymph node cells were transferred into BDF₁ recipients on day 7, either alone (7×10^7 cells) (groups A and D), or mixed with Ag homologous downregulatory $\alpha^{-/-}$ cells (groups C and E), or Ag heterologous downregulatory cells (5×10^7) from TCR $\alpha^{-/-}$ mice (groups B and F). Adoptive cell recipients that were MHC homologous to CS-effector cells that were cotransferred, were challenged on the ears with TNP or OX hapten, and ear swelling measurements were made 24 h later. Each group consisted of three mice. Statistical significance: group A vs C, $P < 0.001$; and group D vs E, $P < 0.002$; group D vs F NS. (b) Antigen-specificity of tolerance in $\alpha^{-/-}$ mice tested as recipients of $\alpha^{+/+}$ CS-effector cells. To confirm Ag-specificity of tolerized $\alpha^{-/-}$ mice we used an assay that did

The three resultant populations of TCR $\alpha^{-/-}$ cells were then individually incubated with 129/J immune CS-effector cells for 30 min at 37°C, and each mixture of cells was subsequently assayed for CS-effector function after transfer to naive recipients. The results in Fig. 6 a demonstrate once more that TNBSA-treated TCR $\alpha^{-/-}$ mice are a reliable source of downregulation of CS-effectors (group B), and that this activity is unaffected by treatment with either anti-CD4 (group C), or anti-CD8 (group D). Results are consistent with the active regulators being double negative (DN) $\gamma\delta$ T cells.

DN $\gamma\delta$ T cells have been reported in the lymphoid system of essentially all vertebrates examined, but they occur in mice in very low numbers relative to $\alpha\beta$ T cells. Therefore, to assess the potency of DN $\gamma\delta$ T cells to downregulate CS, a dose-response experiment was performed. 10-fold dilutions (2.5×10^5 , 2.5×10^4 , or 2.5×10^3) of 60% $\gamma\delta$ TCR $^{+}$ cells (purified from TNBSA-tolerized $\alpha^{-/-}$ mice by use of magnetic beads) were incubated, each with

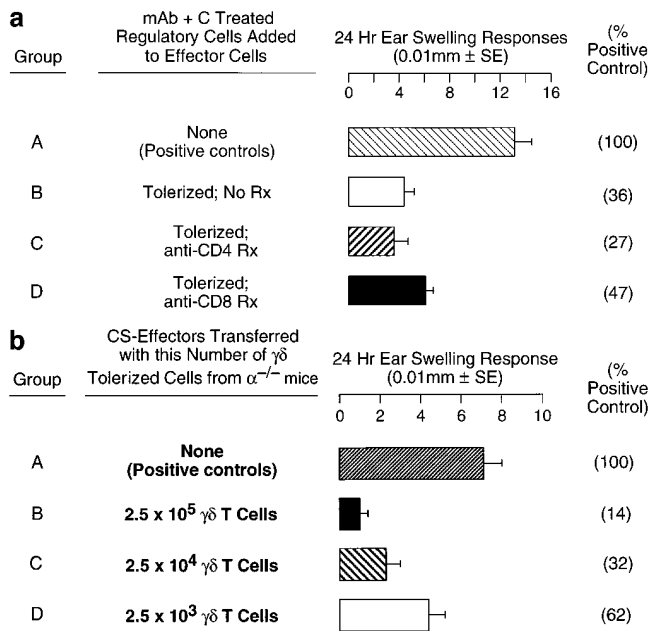


Figure 6. Phenotype (a) and dose response of $\gamma\delta$ downregulatory cells derived from TNBSA-tolerized $\alpha^{-/-}$ mice (b). (a) Phenotype: To determine phenotype of downregulatory cells, spleen cells from TNBSA-tolerized $\alpha^{-/-}$ mice were incubated with medium alone, or with mAb (anti-CD4 or anti-CD8) for 40 min on ice. Cells were washed and incubated with rabbit complement for 45 min at 37°C, and were washed and resuspended in PBS. 7×10^7 CS-effector cells were incubated with medium alone (group A, positive control), with 5×10^7 untreated tolerized cells (group B), or with 5×10^7 anti-CD4 + C treated tolerized cells, (group C), or with 5×10^7 anti-CD8 + C treated tolerized cells, (group D). Statistical significance: group A vs B, and group A vs C $P < 0.001$, and group A vs D $P < 0.01$. (b)-Dose response: 7×10^7 immune CS effector cells were incubated with medium alone (group A, positive controls), or with 10-fold decreasing dilutions (2.5×10^5 , 2.5×10^4 , or 2.5×10^3 cells) of magnetic bead positive ($\gamma\delta$ TCR⁺) downregulatory cells from $\alpha^{-/-}$ TNBSA-tolerized mice, for 30 min at 37°C (groups B–D). After incubation, the cell mixtures were washed and injected into recipients that were challenged on the ears, and measured for CS responses at 24 h. Statistical significance: group A vs B and C, $P < 0.001$, group A vs D, $P < 0.01$.

7×10^7 PCI-immune CS-effector 129/J cells for 30 min at 37°C, and then were harvested, washed, and transferred. Recipient ear swelling results (Fig. 6 b) showed a strong dose response of the assay to the inocula (groups B–D). Even the dose of 2.5×10^3 $\gamma\delta$ T cells had activity in the assay (group D), albeit weaker than the other doses. The capacity of very small numbers of downregulatory $\gamma\delta$ T cells to score in this bio-assay is discussed below.

$\gamma\delta$ Cells From TNBSA-tolerized TCR $\alpha^{-/-}$ Mice Inhibit In Vitro Production of IFN- γ by TNP-specific CS-effector T Cells. Since IFN- γ is a central cytokine in Th-1-mediated responses, such as DTH and CS, we determined whether IFN- γ production by CS-effector TNP-immune cells was affected by regulatory cells induced by high dose intravenous TNBSA or OX tolerogenesis. Using two different anti-IFN- γ antibodies in an ELISA assay, it was first established that 2×10^5 lymph node cells from contact immunized mice, stimulated in vitro by TNP-conjugated APC, secreted >3 ng/ml IFN- γ over a 48 h period. Com-

pared to production of IFN- γ by CS-effector cells mixed with nonimmune spleen cells (Fig. 7 a, groups A and D), IFN- γ production by CS-effector cells and TNP-APC was substantially inhibited by mixing with spleen cells from intravenous TNBSA tolerized normal $\alpha^{+/+}$ mice (Fig. 7 a, group B) and also with spleen cells from intravenous TNBSA tolerized $\alpha^{-/-}$ mice (Fig. 7 a, group E). Also, as controls, spleen cells from nonimmune and nontolerized animals incubated alone or in the presence of TNP or OX-APC did not secrete detectable levels (0.1 ng) of IFN- γ (data not shown).

To determine the phenotype of regulatory cells responsible for the in vitro suppression of IFN- γ production, $\gamma\delta$ TCR⁺ or $\gamma\delta$ TCR⁻ cells in the spleens were separated, using anti- $\gamma\delta$ antibody coated magnetic beads that were used to separate spleen cells from nontolerized, TNBSA-tolerized, or OX-tolerized TCR $\alpha^{+/+}$ or TCR $\alpha^{-/-}$ mice, and then equivalent numbers of each cell subpopulation were added to TNP-immune CS-effector cells in vitro, and cultured together with TNP-hapten-conjugated APC. There was a striking suppression of IFN- γ production by $\gamma\delta^+$ cells from TNBSA-tolerized $\alpha^{-/-}$ mice (Fig. 7 b, group E, left) that did not occur with $\gamma\delta^+$ cells from TNBSA-tolerized $\alpha^{+/+}$ mice (Fig. 7 b, group B, left). In contrast, the inhibitory activity of TNBSA-tolerized $\alpha^{+/+}$ mice was shown to be due to $\gamma\delta^-$ cells (Fig. 7 b, group B, right), and thus was probably due to $\alpha\beta$ T cells. In contrast to the downregulatory activity of $\gamma\delta^+$ cells from TNBSA-tolerized $\alpha^{-/-}$ mice, the $\gamma\delta^-$ cells of these mice were totally without inhibitory activity (Fig. 7 b, group E, right), as were OX-tolerized $\gamma\delta^+$ and $\gamma\delta^-$ cells (Fig. 7 b, group C and F, left), again verifying Ag-specificity of $\gamma\delta^+$ cells mediating tolerance.

These in vitro findings were consistent with the in vivo findings. Thus, we concluded that suppressive cells in Ag-tolerized $\alpha^{-/-}$ mice were potent Ag-specific $\gamma\delta$ downregulatory T cells, both in vivo with contact sensitivity, and in vitro with IFN- γ production, while T cells that inhibited CS in vivo and IFN- γ in vitro that were obtained from normal $\alpha^{+/+}$ mice, were not $\gamma\delta$ T cells, and probably were $\alpha\beta$ T cells. The failure of most cell mixtures tested in Fig. 7 b to reduce IFN- γ production demonstrated that selected reductions in IFN- γ production were not readily attributable to nonspecific effects of extra cells in the assay, such as binding of IFN- γ to IFN- γ receptors, or to degradation of secreted IFN- γ .

Discussion

$\alpha\beta$ T cell-deficient mice do not elicit the classical 24 h component of CS after active contact sensitization and subsequent skin challenge. This result extends previous findings implicating $\alpha\beta$ T cells as principal effectors in CS responses (7, 8, 25, 26): i.e., $\alpha\beta$ T cells and $\gamma\delta$ T cells are not redundant in their functional capacity as effector cells in CS. By contrast, TCR $\alpha^{-/-}$ mice can respond to high dose Ag tolerance by the induction of cells that can downregulate CS-effector activity from $\alpha^{+/+}$ immunized donors.

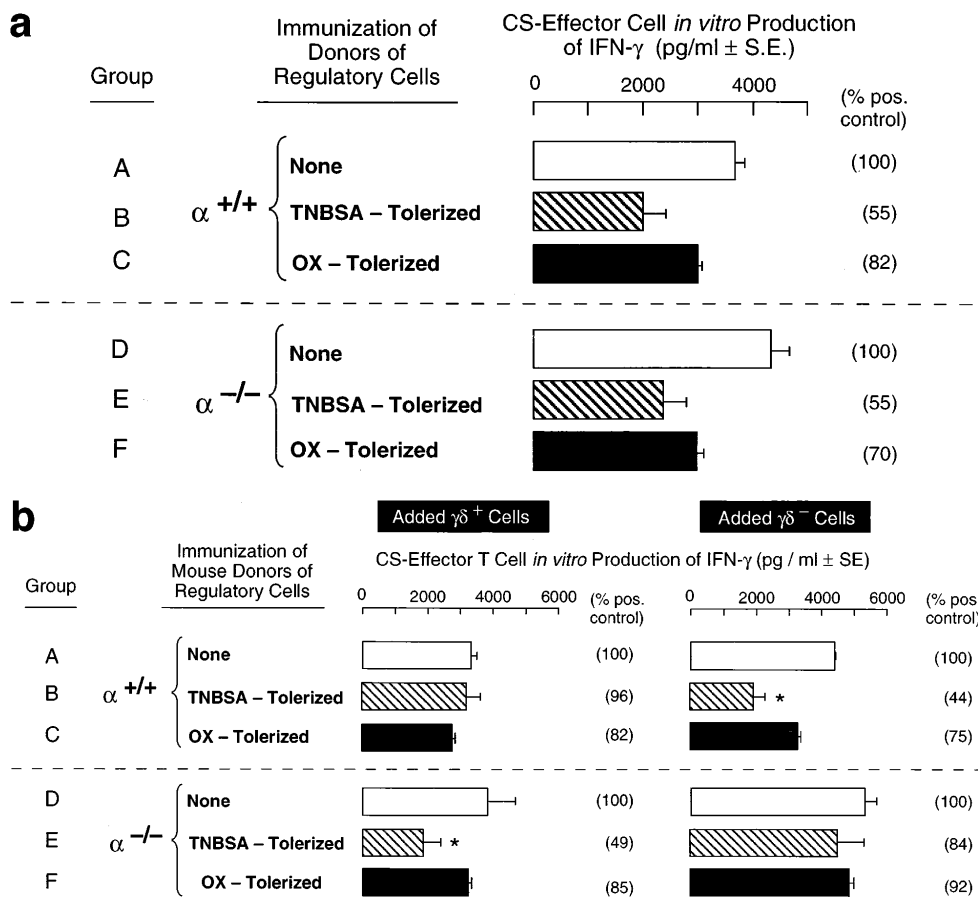


Figure 7. (a) $\gamma\delta$ T cells in spleens of TNBSA intravenously tolerized $\alpha^{-/-}$ mice Ag-specifically downregulate IFN- γ production by CS-effector T cells. 2×10^5 TNP-immune CS-effector cells from $\alpha^{+/+}$ mice were cultured *in vitro* in the presence of TNP-conjugated normal spleen cells as APC, either with or without added regulatory cells (group A), or with regulatory cells of two different specificities: either TNBSA-tolerized or OX-tolerized. These regulatory cells were either from $\alpha^{+/+}$ normal mice (groups B and C), or were harvested from TNBSA-tolerized or OX-tolerized $\alpha^{-/-}$ mice (groups E and F, respectively). At the end of 48 h, supernatants from the individual microwell cultures were harvested and assayed subsequently for IFN- γ content with a double mAb capture ELISA immunoassay. Statistics: group A vs B, $P < 0.004$; group C vs A, $P < 0.01$; group E vs D, $P < 0.004$; and group F vs D, $P < 0.02$. (b) Phenotype of *in vitro* $\gamma\delta$ down regulatory cells: 2×10^5 lymph node CS-effector cells from PCI-sensitized donors were cultured in individual wells of flat-bottomed wells of microtrays in quadruplicate with TNP- or OX-conjugated syngeneic spleen cells as APC, either alone (groups A and D), or with regulatory cell subpopulations from TNBSA-tolerized or OX-tolerized donors (group B and groups C, E, and F). These regulatory cells came from either normal $\alpha^{+/+}$ mice (groups B and C), or from matched TCR α knockout $\alpha^{-/-}$ mice. Prior to addition to microcultures, these regulatory cells were each separated by immunomagnetic bead techniques, using anti- $\gamma\delta$ TCR mAb, into $\gamma\delta$ -enriched cells ($\gamma\delta^+$, left), or $\gamma\delta$ -depleted cells ($\gamma\delta^-$, right). After 48 h culture of the various cell mixtures, supernatants were harvested from individual wells and assayed subsequently using a two mAb capture ELISA immunoassay for IFN- γ content. Statistics: group A vs B (left), NS; group A vs B (right), $P < 0.001$; group A vs C (right), $P < 0.001$; group D vs E (left), $P < 0.002$; group D vs F (left), NS; group D vs E (right), NS.

neic spleen cells as APC, either alone (groups A and D), or with regulatory cell subpopulations from TNBSA-tolerized or OX-tolerized donors (group B and groups C, E, and F). These regulatory cells came from either normal $\alpha^{+/+}$ mice (groups B and C), or from matched TCR α knockout $\alpha^{-/-}$ mice. Prior to addition to microcultures, these regulatory cells were each separated by immunomagnetic bead techniques, using anti- $\gamma\delta$ TCR mAb, into $\gamma\delta$ -enriched cells ($\gamma\delta^+$, left), or $\gamma\delta$ -depleted cells ($\gamma\delta^-$, right). After 48 h culture of the various cell mixtures, supernatants were harvested from individual wells and assayed subsequently using a two mAb capture ELISA immunoassay for IFN- γ content. Statistics: group A vs B (left), NS; group A vs B (right), $P < 0.001$; group A vs C (right), $P < 0.001$; group D vs E (left), $P < 0.002$; group D vs F (left), NS; group D vs E (right), NS.

This was shown in two ways: splenocytes from tolerized TCR $\alpha^{-/-}$ mice downregulate CS-effector cells with which they are mixed *in vitro*, before cotransfer and elicitation of CS, and high dose Ag-treated tolerized TCR $\alpha^{-/-}$ mice inhibit the transfer of CS-effectors that function normally in untreated TCR $\alpha^{-/-}$ mice.

The experiments presented here demonstrate that $\gamma\delta$ cell-enriched fractions from TCR $\alpha^{-/-}$ mice are better mediators of high dose Ag tolerance than are $\gamma\delta$ cell-depleted fractions, suggesting that $\gamma\delta$ T cells are the active regulatory cells, as opposed to TCR $\beta^+\alpha^-$ cells that are a potential feature of TCR $\alpha^{-/-}$ mice (27–28). The lack of conventional MHC restriction shown by the TCR $\alpha^{-/-}$ regulatory cells, and their CD4 $^-$ CD8 $^-$ phenotype are both consistent with most assays of $\gamma\delta$ function (29). The apparent hapten specificity of the regulatory $\gamma\delta$ cells demonstrated herein for the first time may be considered consistent with the hypothesis that the potential diversity (30) of the $\gamma\delta$ TCR is greater than that of any other Ag receptor (31). Thus, overall, downregulation can be mediated by Ag-specific $\gamma\delta$

T cells, and need not be solely mediated by specific $\alpha\beta$ T cells. However, in this study we have provided no evidence that the $\gamma\delta$ cells directly recognized either PCI/TNP or OX, nor whether the $\gamma\delta$ cells recognized either conventional APCs, or the effector T cells with which they were mixed, or some other cells. Conceivably all of these cells may have been haptenated.

Commonly, it has been argued that TCR $\alpha^{-/-}$ mice are deficient in immune effector responses because $\alpha\beta$ cells are uniquely capable of specifically responding to the challenging antigen. However, the apparent capacity of $\gamma\delta$ cells in TCR $\alpha^{-/-}$ mice to distinguish between CS-effector cells that are responsive to different hapten Ag raises questions about the completeness and/or validity of this explanation. Possibly, potential $\gamma\delta$ effector cells responsive to challenge by specific Ag may be unable to mount strong immune effector responses because they cannot interact efficiently with the professional antigen presenting cell system. This may be for physiologic reasons; e.g., $\gamma\delta$ cells may occur in an inappropriate anatomical location, in insufficient num-

bers, or produce inappropriate effector molecules. However, equally attractive is the idea that $\gamma\delta$ cells, like B cells, only rarely recognize molecular complexes of antigenic peptides and MHC. In such a case, $\gamma\delta$ cells would not "learn" that the body was infected from APCs and could not be expected to mount a conventional immune response of the kind that we associate with APC- $\alpha\beta$ T cell interactions (31).

Provocatively, the data presented here bear striking similarities with the role of $\gamma\delta$ cells reported in another system, namely the IgE allergic responsiveness of mice to protein antigen aerosols (18, 19). Thus $\gamma\delta$ T cells reduced ovalbumin-specific IgE responses in rats and mice that were tolerized with ovalbumin administered via repetitive respiratory aerosol administration (18, 19). Not only were $\gamma\delta$ T cells shown to be active in both cases, but strikingly low numbers of $\gamma\delta$ T cells per animal ($\sim 2 \times 10^3$) were effective, as also was the case here (Fig. 6 *b*). Likewise, systemic oral tolerance to ovalbumin was suggested to be due to $\gamma\delta$ T cells (32). Recently it was also shown that mice deficient in $\gamma\delta$ T cells, and responding to infection with a natural intestinal pathogen, *Eimeria*, had exaggerated gut immunopathology attributable to unrestrained $\alpha\beta$ T cell function, or the effects thereof, suggesting a downregulatory function of $\gamma\delta$ T cells (33). In other studies, $\gamma\delta$ T cells from *T. cruzi* infected mice were shown to be suppressive in vivo and in vitro, correlating with autoimmunity (34). Finally, hepatic $\gamma\delta$ T cells could adoptively transfer tolerance to allogeneic skin grafts, and also reduced lymphokine production (35). $\gamma\delta$ T cells in tumor bearing mice were reported to decrease anti-tumor cytotoxic T cell activity (36). Thus, the data presented here, in the classical CS system, provide a clear demonstration of a fundamental functional phenotype of $\gamma\delta$ cells that is operative in numerous systems, including allergy to aerosolized antigen, oral tolerance, autoimmunity, transplantation, tumor immunity, and pathogenesis of infectious diseases. As such, the downregulation of $\alpha\beta$ T cells

by the effects of $\gamma\delta$ T cells is emerging as a major and probably general functional capacity.

The downregulatory effect of $\gamma\delta$ T cells that we describe may in part be effected by a direct or indirect inhibition of IFN γ production by the CS-effector cells, as our results suggest. The mechanism for this inhibition has yet to be resolved. An obvious possibility is the production by $\gamma\delta$ T cells of cytokines that inhibit T cell production of IFN- γ , such as TGF β , IL-4, or IL-10. The production by $\gamma\delta$ T cells of IL-4 would be consistent with the capacity of $\gamma\delta$ T cells from TCR $\alpha^{-/-}$ mice to support copious production of antibodies (22). Thus, regulation of CS by $\gamma\delta$ T cells may be another instance of Th2 regulation or diversion of Th1 cells (37). Alternatively, $\gamma\delta$ T cells may regulate CS-effector $\alpha\beta$ T cells by their being eliminated via injury, apoptosis, or cytotoxic lysis. There are many reports that $\gamma\delta$ T cells possess cytotoxic activity (38, 39). However, it is noteworthy that we have recent evidence that in vitro tolerance for IFN- γ production by splenocytes of TNBSA-tolerized mice can be reversed by IL-12 (Ushio, H., and P.W. Askenase, unpublished results), suggesting that cytotoxicity may not be the mechanism for $\gamma\delta$ T cell-mediated TNBSA tolerance.

In summary, DTH and CS are examples of important in vivo Th1 responses that can play a crucial role in defense against diverse microbial pathogens and tumor cells, and as effectors of some allergic and autoimmune diseases. This may be the reason for such tight regulation of these responses. It is possible that changes in immunoregulation could be a cause for decreased immune resistance in serious infections, or in cancers, or perhaps for the abnormally increased immune responses that occur in allergies and autoimmunity. The findings of the current study clearly establish that $\gamma\delta$ T cells can function as a newly recognized component of immune downregulation, and may be able to do so in an Ag-specific fashion, the underlying mechanism of which needs now to be defined.

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