CD1-reactive Natural Killer T Cells Are Required for Development of Systemic Tolerance through an Immune-Privileged Site

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Summary

Systemic tolerance can be elicited by introducing antigen into an immune-privileged site, such as the eye, or directly into the blood. Both routes of immunization result in a selective deficiency of systemic delayed type hypersensitivity. Although the experimental animal model of anterior chamber–associated immune deviation (ACAID) occurs in most mouse strains, ACAID cannot be induced in several mutant mouse strains that are coincidentally deficient in natural killer T (NKT) cells. Therefore, this model for immune-privileged site–mediated tolerance provided us with an excellent format for studying the role of NKT cells in the development of tolerance. The following data show that CD1-reactive NKT cells are required for the development of systemic tolerance induced via the eye as follows: (a) CD1 knockout mice were unable to develop ACAID unless they were reconstituted with NKT cells together with CD1+ antigen-presenting cells; (b) specific antibody depletion of NKT cells in vivo abrogated the development of ACAID; and (c) anti-CD1 monoclonal antibody treatment of wild-type mice prevented ACAID development. Significantly, CD1-reactive NKT cells were not required for intravenously induced systemic tolerance, thereby establishing that different mechanisms mediate development of tolerance to antigens inoculated by these routes. A critical role for NKT cells in the development of systemic tolerance associated with an immune-privileged site suggests a mechanism involving NKT cells in self-tolerance and their defects in autoimmunity.

Key words: innate immunity • immune deviation • anterior chamber–associated immune deviation • autoimmunity • i.v. tolerance

Immune privilege in the eye is attributed to various local factors including the lack of lymphatic drainage (1), Fas ligand expression (2), and multiple immunosuppressive factors in aqueous humor (3–6). In addition to local immunosuppression, ocular immune privilege is associated with the development of an antigen-specific systemic immune deviation. The mechanisms of immune deviation of the ocular type are well studied in an experimental animal model called anterior chamber–associated immune deviation (ACAID)[1] (7–9). ACAID is characterized by a selective deficiency in delayed type hypersensitivity (DTH) and Ig isotypes that fix complement (10, 11). ACAID is fashioned by indigenous, intraocular bone marrow–derived APCs that capture antigen within the anterior chamber (ac) and carry an antigen-specific ACAID-inducing signal via the blood directly to the spleen (10, 12).

The immune deviation induced via the ac is mediated by unique negative regulatory T cells and T helper (Th) cells that are generated in the spleen within 7 d after ac inoculation. The antigen-specific regulatory T cells suppress both the induction (CD4+ afferent–regulatory T cells) and the expression (CD8+ efferent–regulatory T cells) of DTH (13, 14). The afferent regulators interfere with proliferation of CD4 T cells that will be responsible for the DTH response. Alternatively, the efferent-regulatory T cells inhibit the ca-

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[1]Abbreviations used in this paper: ac, anterior chamber; ACAID, anterior chamber–associated immune deviation; B6, C57BL/6; DN, double negative; DTH, delayed type hypersensitivity; ES, embryonic stem; KO, knockout; LAT, local adoptive transfer; PEC, peritoneal exudate cell; RαAsGM1, rabbit antiasialo GM1; WT, wild-type.

pacity of CD4$^+$ T cells to mediate DTH at the elicitation site. In addition, there is a preponderance of CD4$^+$ Th cells with similarities to Th2-type cells that can be associated with the transition to high titers of antigen-specific IgG1 antibodies observed in ACAID (15, 16).

Although ACAID occurs in most mouse strains, several mutant mouse strains, including β2-microglobulin knockout (KO) mice (17), *lpr/lpr* mice (18), and SJL mice (Sonoda, K.-H., and J. Stein-Streilein, unpublished data) fail to develop ACAID and also display a deficit or dysfunction of NKT cells (19–21). NKT cells belong to a specialized population of T lymphocytes that coexpress the TCR α/β chain and NK markers (22). About 85% of the mouse NKT cell population express a restricted TCR repertoire consisting of an invariant TCR α chain (Vα14Jα281 [23–25]). Similarly, NKT cells exist in humans and express the invariant Vα24JαQ TCR α chain (26–29). NKT cells are restricted by MHC class I–like CD1 molecules (29–31), and because the CD1 molecule is also required for the development of NKT cells, CD1 KO mice selectively lack NKT cells (32–34).

In this report, we show that NKT cells are absolutely required for the induction of immune deviation via the ocular but not the intravenous route of inoculation. Moreover, the NKT cells must bind to the CD1 molecule to be able to induce the development of the antigen-specific efferent-regulatory T cells that participate in the immune deviation mechanism.

Materials and Methods

Mice. Female, 8–10-wk-old mice were used in all experiments. C57BL/6 (B6) mice were obtained from Taconic Farms. (B6 × 129/Sv) F1 (F1) mice were obtained from The Jackson Laboratory. CD1 KO mice were generated in the Transgenic Facility, Harvard Medical School, Boston, MA (Exley, M., manuscript submitted for publication). In brief, the CD1 (both CD1.1 and CD1.2) mutation was created in strain 129/Sv-derived embryonic stem (ES) cells. Mutant ES cell clones were injected into B6 blastocysts to obtain chimeric mice. Heterozygous mutant animals were intracrossed in brother–sister mating to obtain (B6 × 129/Sv)F2 (F2) homozygous mutants. In most cases, control wild-type (WT) mice were F2 mice (The Jackson Laboratory), but F1 cells were used as WT in reconstitution experiments. A confirmatory experiment was performed in B6 CD1 KO mice. During the time the experiments were performed, the CD1 mutation was being backcrossed to the B6 parent for five generations (N5). Progeny that lacked the CD1 gene as determined by DNA analyses were chosen for breeders. The animals were maintained on food and water ad libitum until they reached the desired weight (20–24 g). All animals were treated humanely and in accordance with the Schepens Eye Research Institute–Boston Biomedical Research Institute Animal Care and Use Committee and National Institutes of Health guidelines.

Induction of ACAID and Assay for DTH. ACAID was induced in mice by inoculating OVA (50 μg/2 μl in HBSS; Sigma Chemical Co.) into the ac (10) 7 d before sensitizing subcutaneously for DTH. Intravenously induced immune deviation was induced by inoculation of the antigen (OVA, 50 μg/100 μl in HBSS) into the tail vein with a 30-gauge needle 7 d before immunizing for DTH. To induce DTH, mice received a subcutaneous inoculation with OVA (100 μg/ml in HBSS, 50 μl) emul-

sified in CFA (50 μl) and 1 wk later were tested for the development of DTH by an intradermal inoculation of OVA-pulsed peritoneal exudate cells (PECs; 2 × 10^5/10 μl HBSS) into the right ear pinnae. Ear swelling was measured 24 and 48 h later with an engineer's micrometer (Mitutoyo/MTI).

Local Adoptive Transfer. To test for the efferent-regulatory cell of ACAID, a modified local adoptive transfer (LAT) assay was performed as described elsewhere (14). In brief, T (effector) cells were generated in B6 mice or F1 (3–5) by immunizing subcutaneously with OVA in HBSS and CFA. 7 d later the primed T cells were enriched from dissociated spleen cells by removing B cells and macrophages using IMMULAN™ columns (no. BL7020; Biotecx Laboratories, Inc.). Regulator cells were similarly enriched on IMMUNLAN™ columns from spleen cells of ACAID mice 7 d after ac inoculation of OVA. Stimulator cells were OVA-pulsed PECs as described below. Effector (5 × 10^5), stimulator (5 × 10^5), and regulator (5 × 10^5) cells were mixed and resuspended in 10 μl HBSS for intradermal inoculation into the right ear pinnae of naive mice. Ear swelling was measured with an engineer's micrometer at 24 and 48 h. As a negative control, naive T cells from nonmanipulated mice were used as effector cells and regulator cells. Primed T cells were used as effector cells, and naive T cells from nonmanipulated mice were used as regulator cells for positive control.

Preparation of OVA-pulsed PECs. PECs were obtained from peritoneal washes of mice 3 d after they received an intraperitoneal inoculation of 2.5 ml of 3% aged thioglycolate solution (Sigma Chemical Co.). After counting, PECs were cultured with OVA (5 mg/ml) in a 24-well culture plate in serum-free medium (RPMI 1640 medium, 10 mM Hepes, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (BioWhittaker), and supplemented with 0.1% bovine serum albumin (Sigma Chemical Co.), ITS$^+$ culture supplement (1 μg/ml iron-free transferrin, 10 ng/ml linoleic acid, 0.3 ng/ml Na$_2$Se, and 0.2 μg/ml Fe(NO$_3$)$_3$; Collaborative Biomedical Products). Nonadherent cells were removed from the cultures after 18 h by three washes, and the remaining adherent cells were collected by vigorous pipetting with cold medium (4°C) before washing (three times in HBSS) to remove free OVA.

Abs. The Abs used for flow cytometry analysis were as follows: Fc Block™ (anti–mouse FcRγ II/III mAb, 2.4G2), biotin or FITC-conjugated anti-NK1.1 mAb (PK136), biotin-conjugated anti-Ly49C (5E6), biotin-conjugated anti-CD1 mAb (1B1), FITC-conjugated anti-CD3 mAb (145-2C11), Cy-Chrome 5–conjugated anti–TCR-β mAb (H57-597) were all purchased from PharMingen. PE-conjugated anti-B220 mAb (RA3-6B2) and PE-conjugated Mac-1 (M1/70.15) were purchased from CALTAG Laboratories. Rabbit antiasialo GM1 Ab (RαAsGM1) was purchased from Wako Chemicals USA, Inc.; streptavidin-PE was purchased from Jackson ImmunoResearch Labs, Inc. and FITC-conjugated goat anti–rabbit IgG was purchased from Sigma Chemical Co.

The Abs used for in vivo treatment were as follows: anti-NK1.1 mAb (PK136, mouse IgG2a) and anti-Ly49C (5E6, mouse IgG2a) were purified from mouse ascites using protein A column chromatography (GIBCO BRL) in our laboratory. Purified mouse IgG was purchased from Sigma Chemical Co. for use as control for anti-NK1.1 mAb and anti-Ly49C. RαAsGM1 was purchased from Wako Chemicals USA, Inc., and purified rabbit IgG was purchased from Sigma Chemical Co. Anti-CD1 mAb (3C11, rat IgM) was also purified from mouse ascites using protein A columns. Purified rat IgM isotype control (R4-22) was purchased from PharMingen and used as control for anti-CD1 mAb.

Flow Cytometry. Splenic NK and NKT cells were analyzed by flow cytometry. RBCs were lysed by adding Tris-buffered ammonium chloride to a cell pellet of spleen cells. Staining was performed in the presence of saturating concentration of Fc Block™ (blocks FcRγ II/IIIs). Cells were stained with the following three reagents and colors (using concentrations recommended by the manufacturer): biotin-conjugated anti-NK1.1 mAb counterstained with streptavidin-PE; CyChrome 5–conjugated anti–TCR-β chain mAb; and FITC-conjugated anti-CD3 mAb. In some experiments the cells were stained with RαAsGM1 Ab and counterstained with FITC-conjugated goat anti–rabbit IgG and with CyChrome 5–conjugated anti–TCR β chain mAb. Stained cells were analyzed on an EPICS XL flow cytometer (Coulter). The absolute number of splenic NKT cells detected in flow cytometry was calculated from the percent of NKT cells in the number of viable cells. The total number of viable cells harvested from the spleens before staining was determined by the trypan blue exclusion method.

Depletion of NK and NKT Cells In Vivo. To deplete NK cells in vivo, 100 μl of PBS containing one of the following RαAsGM1 (250 μg), mouse anti-NK1.1 Ab (50 μg), rabbit IgG (250 μg), or mouse IgG (50 μg) was injected into the tail vein of B6 mice. To deplete both NK and NKT cells in vivo, a mixture of anti-NK1.1 mAb and anti-Ly49C (50 μg + 50 μg) or mouse IgG (100 μg) was injected into the tail vein of B6 mice. 24 h later, spleen cells from Ab-treated animals were monitored for the presence of NK or NKT cells by flow cytometry using Abs that detected an NK marker that was different from the target of the Ab used in the depletion treatment. 24 h after the cell depletion treatments, B6 mice were inoculated ac with OVA (50 μg/2 μl). 7 d after ac inoculation, the ability to suppress a primed DTH response was tested in a LAT assay. Before enriching for the regulator T cells from spleens from the ac-inoculated mice, the NK and NKT cells were monitored again to confirm their absence.

Blocking of NKT–CD1 Cell Interaction In Vivo. Purified anti-CD1 mAb (3C11) or control rat IgM mAb (50 μg in 100 μl PBS) was injected into the tail vein of B6 mice to block the interaction of NKT cells with CD1. It is reported that 3C11 blocks the NKT cell–CD1 interaction in vitro (31). Flow cytometry studies of spleen cells from the 3C11-treated mice confirmed that the CD1+ cells (biotin-conjugated anti-CD1 mAb [1B1] counterstained by streptavidin-PE) were neither depleted nor showed changes in the populations of T cells (FITC-conjugated anti-CD3 mAb), B cells (PE-conjugated anti-B22 mAb), NK and NKT cells (triple staining: FITC-conjugated anti-CD3 mAb, CyChrome 5–conjugated anti–TCR β chain mAb, and biotin-conjugated anti-NK1.1 mAb counterstained by streptavidin-PE), and macrophages (PE-conjugated Mac-1) (data not shown).

Depletion of NK1.1+ or CD1+ Cells In Vitro. After RBC lysis, spleen cells were treated with FITC-conjugated anti-NK1.1 mAb, biotin-conjugated anti-Ly49C, and MicroBeads-conjugated anti–mouse pan-NK cells (DX5) (Miltenyi Biotec), and washed twice in PBS (pH 7.2) containing 0.5% BSA and 2 mM EDTA. Ab-labeled cells were treated with anti-FITC MicroBeads and streptavidin MicroBeads (Miltenyi Biotec) for 15 min, and washed twice. To harvest NK and NKT cell–enriched and depleted populations, cells were applied to Type MS+ positive selection column with MiniMACS (Miltenyi Biotec). Cells were stained with Cy-Chrome 5–conjugated anti–TCR β chain mAb, and depletion or enrichment was confirmed by flow cytometry. Cell numbers of depleted populations were adjusted to approximate the number used in the control studies.

For reconstitution experiments, CD1+ cells were depleted from the spleen cells. Following RBC lysis, column-enriched splenic T cells were incubated with biotin-conjugated anti-CD1 (1B1) and then treated with streptavidin MicroBeads before they were applied to Type MS+ positive selection column with Mini-MACS. The CD1+ cell–depleted population in the effluent wash was counterstained by streptavidin-PE (Jackson ImmunoResearch Labs, Inc.) and analyzed by flow cytometry to confirm the quality of the depletion technique.

Reconstitution of CD1 KO Mice. CD1 KO mice were γ-irradiated (cesium, 500 rad, Mark 1 irradiator; J.L. Shepherd and Associates) 1 d before receiving 2×10^7/mouse whole spleen cells derived from F1 mice or spleen cells depleted of their NK1.1+ cells by magnetic beads. 7 d later, reconstituted CD1 KO mice were inoculated (ac) with OVA (50 μg/2 μl in HBSS). Spleens were removed 1 wk after the ac inoculation, dissociated cells were pooled, and splenic T cells were enriched as described above. Enriched splenic T cells were transferred to naive F1 mice as regulator cells with effector (derived from F1 mice) and stimulator cells (derived from F1 mice) prepared as described above and tested in a LAT assay. Any host versus graft disease that might have occurred was undetectable and did not interfere with the experimental outcome 15 d after reconstitution.

Statistics. Data were subjected to analysis by analysis of variance and Scheffe's test. A value of $P \leq 0.05$ was considered significant.

Results

ACAID Induction Correlates with an Increase in Splenic NKT Cells. ACAID is a well-established experimental animal model for the study of immune deviation mediated through an immune-privileged site. Modulation of the model and analysis of the subsequent effects on the im-
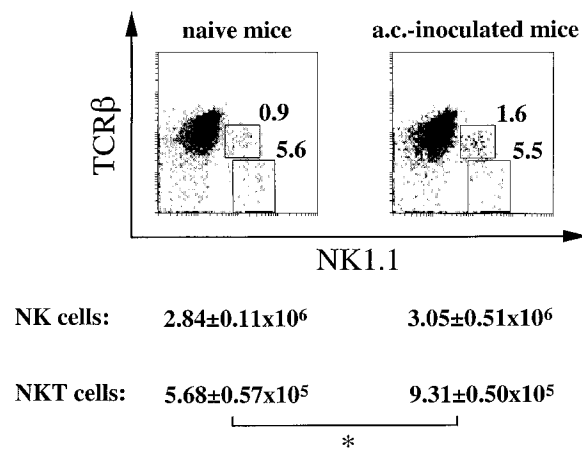

	naive mice	a.c.-inoculated mice
NK cells:	2.84±0.11x10⁶	3.05±0.51x10⁶
NKT cells:	5.68±0.57x10⁵	9.31±0.50x10⁵

Figure 1. Generation of ACAID correlates with increased number of NKT cells in the spleen. ACAID was induced in mice as described previously. In brief, B6 mice were killed 7 d after ac inoculation of OVA. Column-enriched splenic T cells were harvested from ac-inoculated and uninoculated mice by application to IMMULAN™ columns, and stained for analysis by flow cytometry to determine the ratio of NK and NKT cells present within the lymphocyte gate for individual animals. Fluorescence for the TCR β chain (CyChrome 5) and the NK1.1 marker (PE) are shown on the ordinate and abscissa, respectively. The percentage of cells within the NK cell quadrant (rectangle) and the NKT cell quadrant (square) are indicated for the representative experiment shown. Absolute number (mean ± SEM) of NK and NKT cells was calculated from the precounted viable cell numbers in individual animals ($n = 5$), and is shown below the abscissa. Significant differences ($P \leq 0.05$) are indicated by an asterisk.

mune deviation–associated systemic tolerance can be assessed directly in the whole mouse, or in a LAT assay (14) for detection of antigen-specific regulatory T cells associated with ACAID. The postulate that NKT cells are important in ACAID was first assessed by looking for an ACAID-associated increase of NKT cells in the spleen.

B6 mice were inoculated (ac) with OVA, and 7 d later the spleens were extirpated, cells dissociated, and the numbers of NK and NKT cells were analyzed by flow cytometry after staining for the TCR β chain and the NK1.1 molecule. Analysis was performed on five individual mice per group. The flow cytometry data showed that the ratio of NKT cells to total gated lymphocytes from spleens (de-

pleted of B cells and macrophages) was increased in all ACAID mice compared with naive mice (Fig. 1), subcutaneously or intravenously inoculated mice (data not shown) at 7 d after ac inoculation. In contrast to NKT cells, the number of NK cells did not change in the spleen during ACAID induction. Both the percent and the absolute number of NKT cells in the spleen began to increase as early as day 3 (data not shown), and peaked at day 7 (Fig. 1). These data show an association of splenic accumulation of NKT cells, not NK cells, with the induction of ACAID.

CD1 KO Mice Failed to Induce ACAID. To directly test the involvement of the NKT cells in the development of ACAID, we examined the potential of CD1-deficient mice
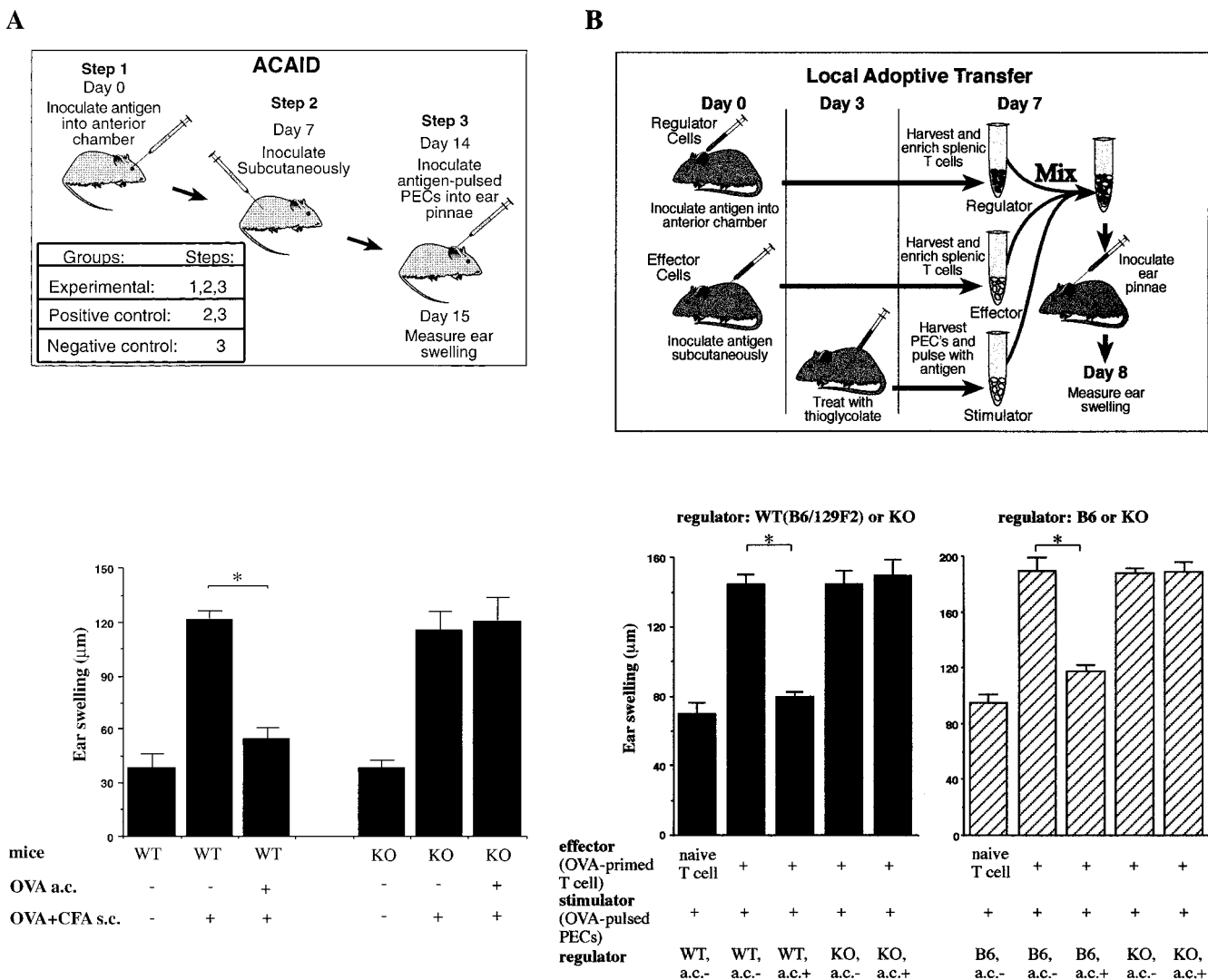

Figure 2. ACAID in CD1 KO mice. (A) ACAID induction in CD1 KO mice. For clarity, the inset shows the ACAID protocol. Five CD1 KO mice and five WT mice were inoculated (ac) with OVA 7 d before subcutaneous sensitization with OVA and CFA. Mice were challenged with OVA-pulsed PECs derived from F1 mice into the ear pinnae 7 d after subcutaneous sensitization. Ear swelling measurements (24 h after ear challenge) are shown on the ordinate. Treatment of the mice in each group represented by the bars is shown below the abscissa. Significant differences ($P \le 0.05$) are indicated by an asterisk. (B) Regulatory cell induction in CD1 KO mice. A LAT assay (inset shows the LAT protocol) was performed to assess the development of efferent regulatory T cells. The black bar represents results using (B6 × 129)F2 CD1 KO mice. Column-enriched splenic T cells harvested from ac-inoculated WT mice or CD1 KO mice (five per group) 7 d earlier were used as regulator cells and cotransferred into F1 mice (five per group) with effector and stimulator cells from F1 mice. The hatched bars represent results from similar studies using B6 KO and B6 mice. Ear swelling measurements (24 h after ear challenge) are shown on the ordinate, and the mixture of cells inoculated into the ear pinnae is indicated below the abscissa. Significant differences ($P \le 0.05$) are indicated by an asterisk.

to develop immune deviation after ac inoculation of antigen. Since the CD1 molecule is essential for NKT cell development (30, 31), CD1 KO mice do not have NKT cells, but do have NK cells and other lymphocyte subpopulations (32–34). CD1 KO mice and control WT mice were inoculated (ac) with OVA 7 d before subcutaneous sensitization with OVA and CFA, and challenged into the ear pinnae with OVA-pulsed PECs 7 d after subcutaneous sensitization (Fig. 2 A, protocol inset). When ear swelling was measured 24 and 48 h later, it was observed that ac-inoculated CD1 KO mice developed a positive DTH (ear swelling) response, but ac-inoculated WT mice exhibited a suppressed response (Fig. 2 A). While the level of the DTH response in CD1 KO mice was similar to that in WT mice, CD1 KO mice did not develop ACAID.

To further assess the NKT cell-dependent ACAID mechanism, spleen cells from ac-inoculated CD1 KO and WT mice were tested in a LAT assay (Fig. 2 B, black bars, protocol inset). In brief, CD1 KO mice were inoculated (ac) with

OVA 7 d before harvesting, dissociating, and enriching T cells from the spleens for use as regulator cells. Regulator T cells were then cotransferred with OVA-primed T cells (effector cells from F1 mice) and OVA-pulsed PECs (stimulator cells from F1 mice) into the ear of F1 mice. In contrast to regulator cells from WT mice, regulator T cells from CD1 KO mice that received OVA were unable to suppress the DTH response (Fig. 2 B). An experiment was performed in B6 CD1 KO mice (hatched bars) to confirm the role of NKT cells in ACAID in a genetically homogeneous background. In this case, effector and stimulator cells were prepared from B6 mice, and then cells were transferred to the B6 recipient. These results show that NKT cells or other CD1-dependent populations were needed for the development of ACAID in general, and for the generation of the antigen-specific efferent regulatory T cells, in particular.

Adoptive Transfer of NKT Cells and CD1$^+$ APCs Reconstitutes the ACAID-inducing Ability in the CD1 KO Mice. To confirm whether the defect in the CD1 KO mice that led
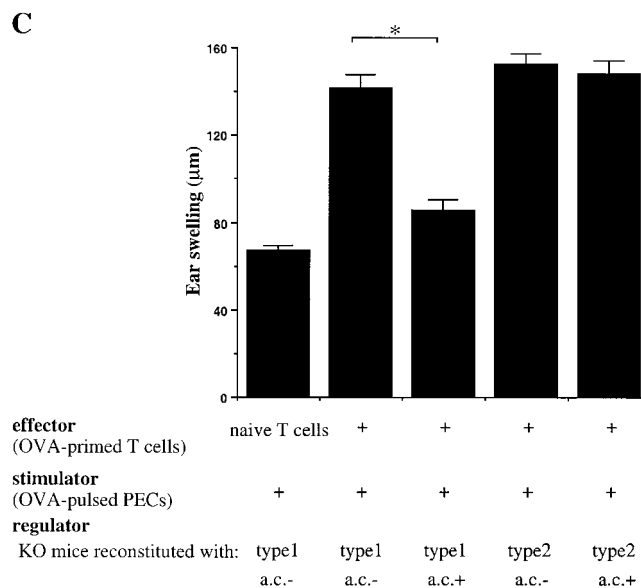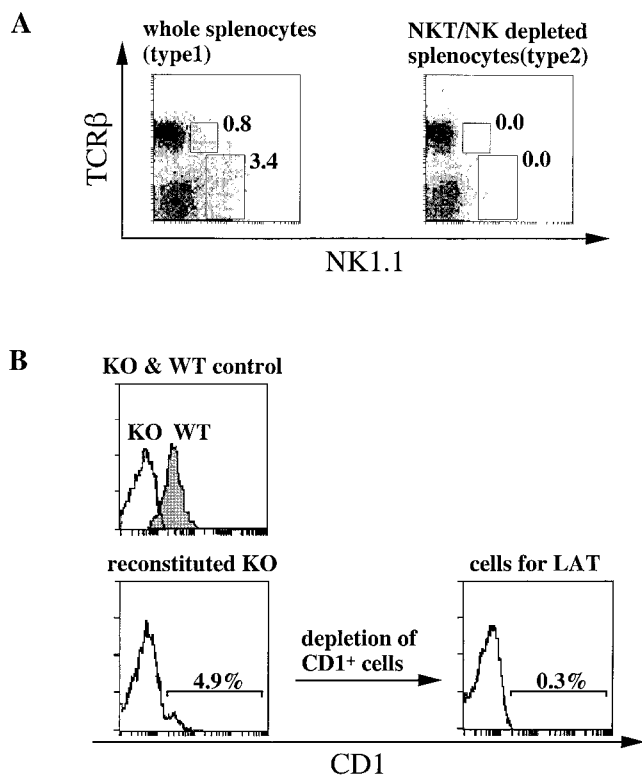

Figure 3. Effect of NKT cell reconstitution on ability of CD1 KO mice to develop ACAID. (A) Flow cytometry confirmation of NK and NKT cell depletion in vitro. Spleen cells from F1 mice were treated with FITC-conjugated anti-NK1.1 mAb, biotin-conjugated Ly49C mAbs, and MicroBead-conjugated anti–mouse pan-NK cell Ab before treatment with anti-FITC MicroBeads and streptavidin MicroBeads, and exposure to a magnetic field to remove NK and NKT cells. The negatively selected cells, and the similarly treated whole spleen cell population not exposed to the magnetic field, were stained with CyChrome 5–conjugated anti–TCR β chain mAb and analyzed by flow cytometry. Fluorescence for CyChrome 5–TCR β chain and FITC-NK1.1 are shown on the ordinate and abscissa, respectively. The percentage of cells within the NKT cell (square) and the NK cell (rectangle) quadrants are listed in the blocks before (whole splenocytes, type 1) and after (NK and NKT depleted, type 2) depletion. (B) Flow cytometry confirmation of CD1$^+$ cell depletion. 7 d after reconstitution with whole spleen cells (type 1) or NK and NKT cell–depleted spleen cells (type 2, Fig. 3 A), five mice from each reconstituted CD1 KO mouse group were inoculated (ac) with OVA. Column-enriched splenic T cells were harvested 1 wk after ac inoculation, and CD1$^+$ cells were removed. Cells were stained with biotin-conjugated anti-CD1 (1B1), then treated with streptavidin MicroBeads and applied to MiniMACS columns to deplete CD1$^+$ cells. Flow cytometry–generated graphs show the relative number of cells (ordinate) versus the increasing fluorescence channels (abscissa) that identify PE-streptavidin-biotin–conjugated anti-CD1 mAb–labeled cells. "KO & WT control" shows the fluorescence pattern of splenic T cells from unreconstituted CD1 KO (open) or WT (shaded) mice; "reconstituted KO" shows the pattern of CD1$^+$ cells after reconstitution; "cells for LAT" shows the pattern of CD1$^+$ cells after negative selection. The percentage of CD1$^+$ cells is indicated in each of the lower blocks. (C) LAT assay for CD1$^-$ T cell regulator function. CD1$^-$ T cells from reconstituted CD1 KO mice (regulator) were cotransferred with effector and stimulator cells from F1 mice into the ear pinnae of naive F1 mice. Adoptively transferred regulator cells from reconstituted mice that did not receive ac inoculation were used as a control. Ear swelling measurements (24 h after ear challenge) are shown on the ordinate, and the identity of the cell mixture inoculated into the ear pinnae for each group (five per group) is indicated below the abscissa. Significant differences ($P \leq 0.05$) are indicated by an asterisk.

to the failure of ACAID was actually the NKT cell deficiency, we reconstituted CD1 KO mice with whole spleen cells from WT mice (F1) (containing both NKT cells and CD1$^+$ APCs), or spleen cells immunomagnetically depleted of NK1.1$^+$ lymphocytes (that still contained CD1$^+$ APCs). The successful depletion of NKT and NK cells was confirmed by flow cytometry analysis (Fig. 3 A). 7 d after reconstitution, the mice were inoculated (ac) with OVA, and after 8 d spleen cells were harvested for T cell enrichment. A typical profile of enriched splenic T cells from reconstituted CD1 KO mice (Fig. 3 B, bottom left panel) shows that ~4.9% of T cells were donor derived (CD1$^+$). (Total of CD1$^+$ cells in the non-T splenic cells was 25%; data not shown.) To analyze the regulatory potential of the host CD1$^-$ T cells, the spleen cells were further negatively selected against CD1 expression to yield populations that were CD1$^-$ (Fig. 3 B, bottom right panel). These cells were then assessed as regulator cells in the LAT assay.

We observed that whole spleen cells were able to reconstitute the ACAID-inducing ability of the CD1 KO mice. In contrast, spleen cells depleted of NKT cells did not restore ACAID-inducing ability (Fig. 3 C). Because CD1 is expressed on all WT bone marrow–derived cells that were transferred, these results also show that antigen-specific-efferent regulatory cells that were generated did not express CD1, indicating that the transferred CD1$^+$ NKT cells were not the effector cell in the regulation of the expression of DTH. The data support the postulate that NKT cells are essential in the induction of ACAID, and function by supporting the generation of antigen-specific efferent-regulatory T cells.

NKT Cells Are Not Efferent DTH–regulatory Cells. To confirm that NKT cells do not have a direct role in efferent regulation of DTH in another way, we depleted cells expressing NK1.1 antigens from the regulator cell population before testing in a LAT assay using specific Ab treatment and magnetic beads selection (Fig. 4 A). Treated cells were then assessed by flow cytometry before cotransferring with primed T cells and OVA-pulsed PECs to the ear pinnae. The results of the LAT assay showed that the NK and NKT cell–depleted populations retained their DTH-regulatory capability. Thus, the efferent-regulatory cell is a conventional NK1.1$^-$ T cell. It is known that activated NKT cells can downregulate their NK1.1 molecules in vitro (35). Therefore, we were aware that the in vivo–activated NKT cell may not express NK1.1, and the Ab depletion of NK1.1$^+$ cells treatment may not work. However, as also shown in Fig. 1, after OVA was inoculated in the ac NK1.1$^+$ T cells were clearly present in the spleen. Therefore, together with our observations that CD1$^-$ T cells (from CD1$^+$ NKT cell–reconstituted CD1 KO mice) can function as regulators of DTH (Fig. 3 C), these data confirm that NKT cells are not direct efferent regulators of DTH in ACAID.

NKT Cells, Not NK Cells, Are Required for the Induction of the Regulatory T Cells in ACAID. Another approach to assess the importance of NKT cells in induction of systemic tolerance used Abs specific for NK markers to create CD1$^+$

mice with selective depletion of the NK1.1$^+$ cell populations. B6 mice were depleted of NKT cells and NK cells, or NK cells only, and their ability to generate the efferent regulatory T cell in ACAID was compared to syngeneic mice treated with isotype control Ab. We reported previously that it is difficult to remove the NKT cell population in vivo with Abs to typical NK cell markers (36). Specifically, the inoculation of mice with anti-NK1.1 mAb or RαAsGM1 Ab depletes NK cells, but not NKT cells, although they both express the target antigens (36; and Fig. 5 A and B). Because the NKT cells that remained in the spleens after in vivo anti-NK1.1 mAb treatment expressed Ly49C (36), we mixed the anti-NK1.1 and anti-Ly49C Abs and effectively depleted NKT cells in the mice for these studies (Fig. 5 B). 24 h after the mixed Ab treatment,
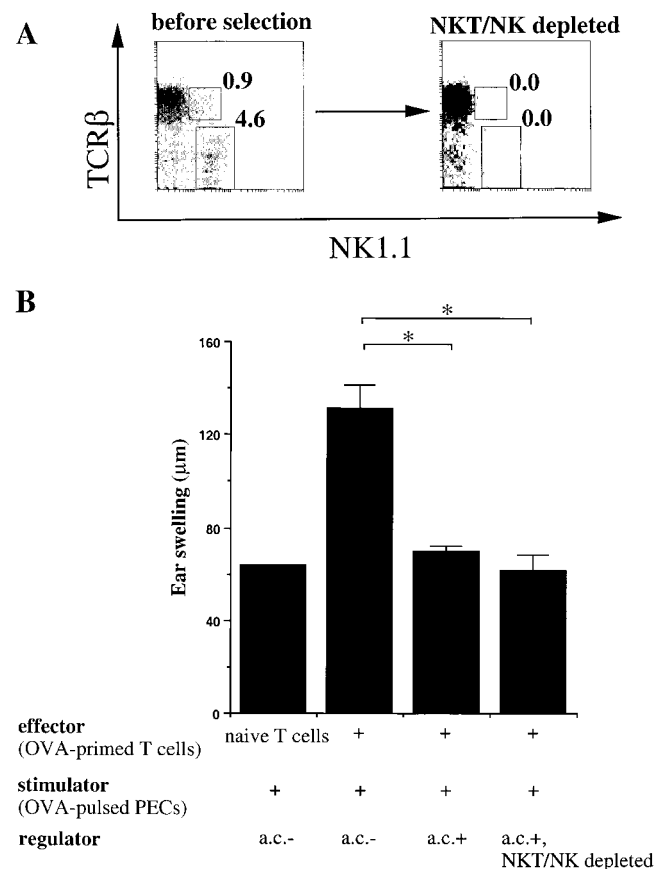

Figure 4. Analysis of NKT and NK cells as efferent regulatory DTH cells in the LAT assay. (A) Flow cytometry confirmation of NKT and NK cell depletion in vitro. Column-enriched splenic T cells were harvested from B6 mice 7 d after OVA (ac) inoculation. All NK1.1$^+$ cells were removed from the T cell–enriched populations with a magnetic field after treatment with a mixture of FITC–anti-NK1.1, biotin–anti-Ly49C mAbs, and anti–pan-NK cell conjugated Microbeads, and anti-FITC and streptavidin MicroBeads. CyChrome 5–conjugated anti–TCR β chain and FITC-conjugated anti-NK1.1 cells are shown in the dot plots for cells before and after treatment. The percentage of labeled cells is shown next to the square and rectangle for NKT and NK cells, respectively. (B) The effect of NKT and NK cell depletion on efferent regulation of DTH. Ear swelling was measured 24 h after cell transfer of the various cell mixtures (indicated below the abscissa for each bar) into the ear pinnae of naive syngeneic mice (five per group), and is shown on the ordinate. Significant differences ($P \leq 0.05$) are indicated by an asterisk.
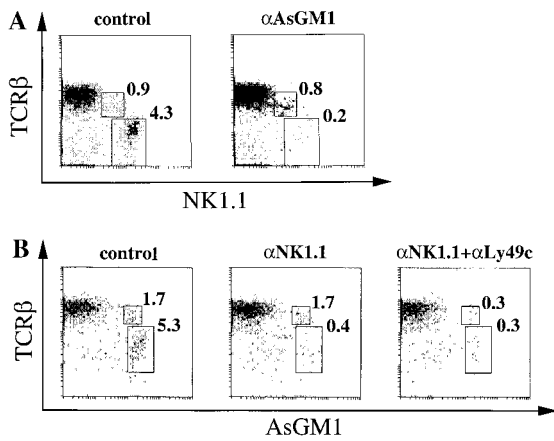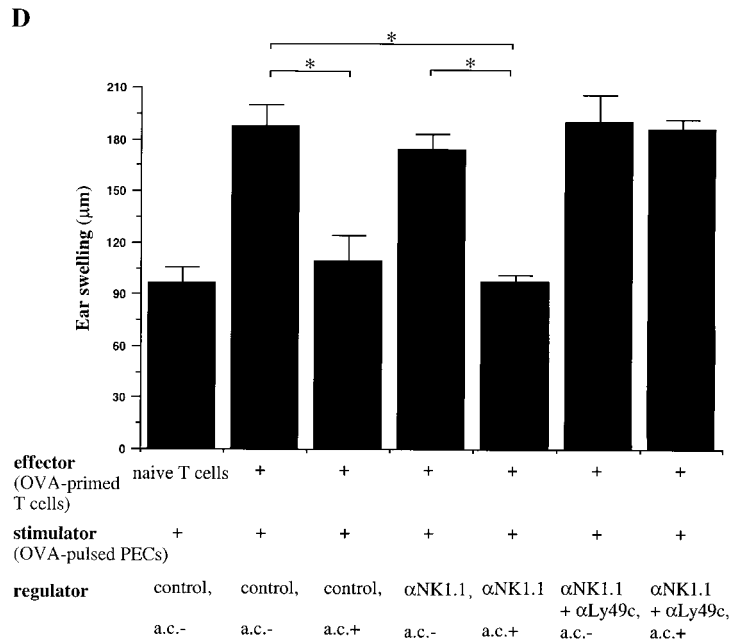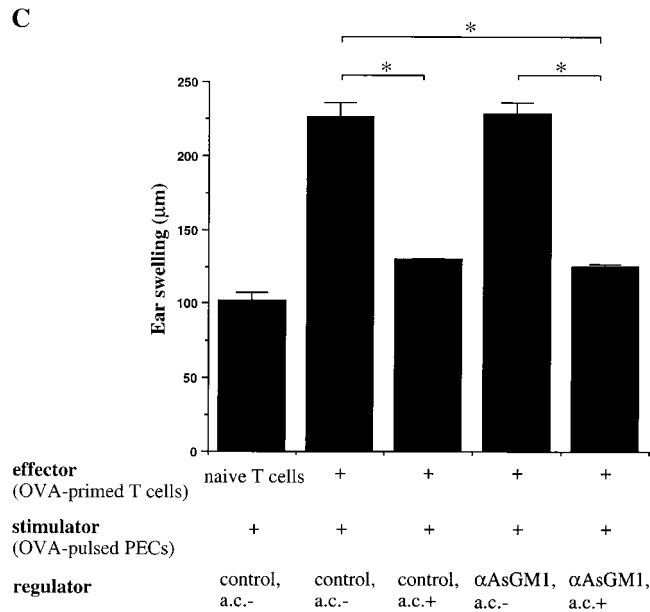
A

TCRβ | NK1.1

control: 0.9 / 4.3
αAsGM1: 0.8 / 0.2

B

TCRβ | AsGM1

control: 1.7 / 5.3
αNK1.1: 1.7 / 0.4
αNK1.1+αLy49c: 0.3 / 0.3

C

Ear swelling (μm)

effector (OVA-primed T cells)	naive T cells	+	+	+	+
stimulator (OVA-pulsed PECs)	+	+	+	+	+
regulator		control, a.c.-	control, a.c.+	αAsGM1, a.c.-	αAsGM1, a.c.+
		control, a.c.-			

D

Ear swelling (μm)

effector (OVA-primed T cells)	naive T cells	+	+	+	+	+	+
stimulator (OVA-pulsed PECs)	+	+	+	+	+	+	+
regulator		control, a.c.-	control, a.c.-	control, a.c.+	αNK1.1, a.c.-	αNK1.1, a.c.+	αNK1.1 + αLy49c, a.c.-	αNK1.1 + αLy49c, a.c.+

Figure 5. ACAID in NK and NKT cell–depleted mice. (A) Flow cytometry confirmation of NK cell depletion. Data in the dot plots confirm the presence (control) and absence of NK cells (RαAsGM1 treatment). B6 mice were inoculated intravenously with RαAsGM1 Ab or purified rabbit IgG. Column-enriched splenic T cells (includes NK and NKT cells) were harvested 24 h later and stained with CyChrome 5–conjugated anti–TCR β chain and PE-streptavidin-biotin–conjugated anti-NK1.1 mAbs, and analyzed by flow cytometry. The cell population that is analyzed is indicated above the block, and the percentage of NKT cells and NK cells is indicated by the square and rectangle, respectively, within the dot plot shown. (B) Flow cytometry confirmation that mixed antibody treatment removed NKT as well as NK cells. B6 mice were inoculated with mouse IgG, anti-NK1.1 mAb, or a mixture of anti-NK1.1 and anti-Ly49C mAbs. Column-enriched splenic T cells harvested 24 h later were stained by CyChrome 5–conjugated TCR β chain mAb and FITC-conjugated goat anti–rabbit and RαAsGM1, and assessed by flow cytometry. (C) LAT assay for role of NK cells in generation of T-regulatory cell in ACAID. Mice (five per group) were inoculated (ac) with OVA 24 h after treatment with purified rabbit IgG, or RαAsGM1 Ab. 7 d later, column-enriched splenic T cells were harvested from the ac-inoculated mice (regulator), and cotransferred with effector and stimulator cells (from B6 mice) into ear pinnae of five syngeneic naive mice. Ear swelling measurements (24 h after ear challenge) are shown on the ordinate. The phenotype of the cell mixtures that were injected into the ear pinnae is indicated below the abscissa for each bar. Significant differences ($P \leq 0.05$) are indicated by an asterisk. (D) LAT assay to test the role of NKT cells in generation of T-regulatory cells. Each of five mice was treated with mouse IgG, anti-NK1.1 mAb, or a mixture of anti-NK1.1 and anti-Ly49C mAb treatment 1 d before inoculation (ac) with OVA. 7 d later, column-enriched splenic T cells were harvested from the ac-inoculated mice (regulator), and cotransferred with effector and stimulator cells (from B6 mice) into ear pinnae of five syngeneic naive mice. Ear swelling measurements (24 h after ear challenge) are shown on the ordinate, and the phenotype of the cell mixture inoculated into the ears is indicated below the abscissa for each group (five per group) indicated by the bar. Significant differences ($P \leq 0.05$) are indicated by an asterisk.

mice were inoculated (ac) with OVA, and the differently treated groups of mice were tested 1 wk later for their ability to generate efferent-regulatory T cells in a LAT assay. As expected, the NK-only depleted mice, previously treated with either RαAsGM$_1$ or anti-NK1.1 mAb, developed antigen-specific efferent-regulatory T cells (Fig. 5, C and D), but the NKT and NK cell–depleted mice did not (Fig. 5 D). Therefore, together with studies in the CD1 KO mice (Fig. 3), these data show that the CD1-dependent cell responsible for the development of systemic toler-

ance and the generation of regulatory T cells in ACAID is the CD1-dependent NKT cell.

NKT Cell–CD1 Interactions Are Required for Induction of Efferent-regulatory T Cells in ACAID. Clearly CD1 is needed for the development of NKT cells, but it is unknown if CD1 is required for NKT cell function in the generation of regulatory T cells in ACAID. We reasoned that if NKT cell interactions with CD1 were required for ACAID induction, we might be able to block ACAID by blocking the NKT cell interaction with CD1. Previously,

anti-CD1 mAbs (3C11) were successfully used in vitro to block NKT cell–CD1 interactions (31). Therefore, mice were treated with anti-CD1 mAb 1 d before being inoculated (ac) with OVA and 8 d before harvesting the spleens, dissociating the cells, and column-enriching the splenic T cells for use as regulator cells in a LAT assay. 24 h after anti-CD1 treatment (3C11), dissociated spleen cells were stained with a different Ab for CD1 (1B1) to assess the presence of CD1$^+$ cells in the spleen. Flow cytometry analyses showed that the Ab treatment did not alter the ratio or absolute number of CD1$^+$ cells. In addition, anti-CD1 mAb treatment of mice did not alter the ratio or absolute number of NKT and NK cells (stained by anti–TCR-β and anti-NK1.1), T cells (stained by anti-CD3), B cells (stained by anti-B220), and macrophages (anti–Mac-1) in their spleens (data not shown). Groups of mice treated with control Ab developed antigen-specific efferent-regulatory T cells, but mice treated with anti-CD1 mAb did not (Fig. 6). Because the Ab treatment did not eliminate CD1$^+$ cells, the most likely explanation is that an interaction between NKT cells and CD1 was blocked by the anti-CD1 mAb. Thus, we postulate that an interaction between the NKT cell and the CD1 molecule is required for the NKT cell to function in ACAID.

Intravenously Induced Systemic Tolerance Is Not Defective in CD1 KO Mouse. Since antigen inoculated into the ac is carried to the spleen, an argument could be made that there are few differences between the induction mechanisms of ACAID and the mechanisms involved in intravenously induced systemic tolerance. In fact, it could be argued that the leakage of antigen from the venules in the eye into the blood during ACAID induces intravenous tolerance. However, differences between the mechanisms involved in immune deviation via the ac or intravenous route have been
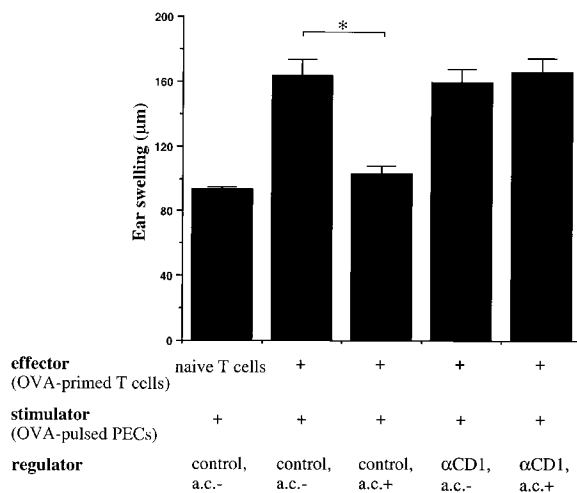
reported (13). It has also been reported that intravenously induced tolerance generates CD8$^+$ afferent-regulatory T cells in contrast to ACAID, which generates both CD4$^+$ afferent-regulatory T cells and CD8$^+$ efferent-regulatory T cells (14).

CD1 KO mice and WT mice were inoculated ac or intravenously with OVA 7 d before subcutaneous sensitization with OVA and CFA and testing for DTH by challenging 14 d later with OVA-pulsed PECs into the ear. As before, ear swelling was measured 24 and 48 h later. As expected, WT mice developed immune deviation regardless of the route of inoculation (Fig. 7). Importantly, in contrast to the inability of CD1 KO mice to develop immune deviation when inoculated ac, intravenously treated CD1 KO mice were fully capable of developing immune deviation, and showed reduced ear-swelling responses (Fig. 7). Therefore, NKT cells do not participate in intravenously induced immune deviation, and ACAID is indeed a separate entity, with unique and locally maintained mechanisms of regulation.

Discussion

This report shows that NKT cells (a) are required for the development of ACAID; (b) are specifically required for the generation of an efferent-regulatory T cell; (c) may interact with a CD1-expressing cell to mediate regulatory mechanisms for suppressing DTH responses in ACAID; and (d) are not required for intravenous tolerance. In addition, a relationship between ACAID and self-tolerance is suggested by reports that induction of ACAID in mice both prevented the onset of and also suppressed ongoing experimental autoimmune uveitis (37). Furthermore, while several previously published reports imply a role for NKT cells in preventing certain autoimmune disease in humans
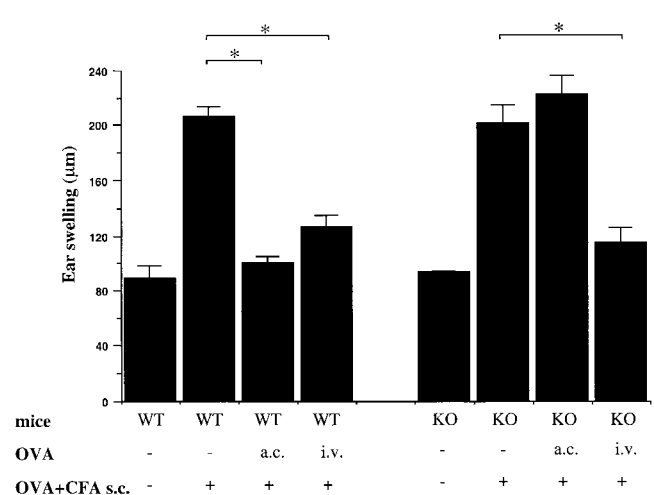

Figure 6. In vivo blocking of NKT cell–CD1 interaction abrogated the ACAID. Five B6 mice were inoculated intravenously with rat IgM or anti-CD1 (3C11) Abs 1 d before ac inoculation with OVA. 7 d later, column-enriched splenic T cells harvested from ac-inoculated mice (regulator) were cotransferred with effector and stimulator cells into the ear pinnae of naive syngeneic mice (five per group). Ear swelling measurements from B6 mice (24 h after ear challenge) are shown on the ordinate, and cell mixture inoculated is indicated below each bar on the abscissa. Significant differences ($P \leq 0.05$) are indicated by an asterisk.

Figure 7. Comparison of ACAID versus intravenously-induced tolerance in CD1 KO mice. CD1 KO mice and control WT mice (five mice per group) were inoculated ac or intravenously with OVA 7 d before subcutaneous sensitization with OVA and CFA. OVA-inoculated mice were challenged in their ear pinnae with OVA-pulsed PECs from F1 mice 7 d after sensitization. Ear swelling measurements (24 h after ear challenge) are shown on the ordinate, and treatment of mice is indicated below the abscissa for each group (five per group) indicated by the bar. Significant differences ($P \leq 0.05$) are indicated by an asterisk.

(38, 39) and in mice (20, 40, 41), the mechanisms through which NKT cells might regulate autoimmunity remain unclear. Therefore, these results indicate that the autoimmunity associated with NKT cell defects (such as type 1 diabetes, scleroderma) may be mediated by disruption of organ-specific tolerance mechanisms that are similar to those mediating systemic tolerance to antigens introduced through immune-privileged sites.

The systemic tolerance to antigens introduced into the ac (ACAID) involves several steps. In the initial step, antigen introduced into the ac is carried to the spleen by specialized eye-derived F4/80$^+$ APCs (42). A CD8$^+$ efferent or effector stage regulatory T cell that can suppress a subsequent DTH response to the specific antigen is then generated. This study further shows that CD1-reactive NKT cells are required for the generation of the ACAID regulatory T cell. On the other hand, the LAT studies of CD1 KO mice reconstituted with CD1$^+$ NKT cells show that CD1d expression by ACAID efferent-regulatory cells was not necessary (Fig. 3 B and C). Anti-CD1 blocking experiments nonetheless indicate a role for CD1, presumably functioning as a ligand for NKT cells in ACAID. Therefore, we suggest that the critical CD1-dependent interaction is between the NKT cell and the specialized ac-derived APC, and that NKT cell interactions with particular APCs may similarly be defective in some autoimmune diseases associated with loss of NKT cell function.

The ac contains aqueous humor that is filled with a mixture of immunosuppressive components including TGF-β (3–6, 43). Moreover, we observed that addition of TGF β2 to thioglycolate-induced PECs in vitro induced an increased expression of their CD1d molecules (Sonoda, K.-H., and J. Stein–Streilein, unpublished observations). Thus, the eye-derived APCs may similarly express increased levels of CD1, and thereby stimulate NKT cells in the spleen. Alternative, and not mutually exclusive, hypotheses are that NKT cells are activated by a CD1-presented endogenous lipid antigen, accessory molecule, or cytokine produced by the APC.

A further alternative is that the NKT cells recognize CD1d expressed by another cell type in the spleen. Such a possible CD1$^+$ NKT cell stimulator in the spleen is the marginal zone B cell. Niederkorn and colleagues showed that splenic B cells are needed for ACAID, and suggested that eye-derived APCs "hand over" their antigen and allow the splenic B cells to induce ACAID (44, 45). Consistent with this hypothesis is the observation that the APCs in the spleen that express the highest density of CD1 are the marginal zone B cells (46).

Activated NKT cells may produce large amounts of a variety of cytokines (35) that, in the splenic microenvironment, likely contribute to the development of the efferent-regulatory T cell in ACAID. Cytokines, such as IL-4, may directly modulate activity of the efferent-regulatory cell. While IL-4 is most commonly thought to mediate NKT cell–dependent T cell regulation, we do not propose it to be the critical cytokine in NKT cell–dependent generation of the efferent-regulatory T cell since ACAID occurs in IL-4 KO mice (16, 47, 48). Another potentially important cytokine, already commonly associated with the development of ACAID, is TGF-β (43). A report has been published showing NKT cell production of TGF-β (49), and another shows that TGF-β may modulate APC function (50). Thus, the possibility arises that NKT cells respond to the CD1 or other signals by upregulating TGF-β production or its conversion from latent to active form. Strengthening this possibility is a recent report by Kosiewicz et al. showing that both CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^-$ (double negative, DN) T cells from ACAID spleens produced TGF-β (16). NKT cells are notably either CD4$^+$ or DN (22).

A significant technical observation in this study is that in our hands NKT cells are resistant to in vivo antibody treatments known to remove NK cells. While others report removal of NKT cells by anti-NK1.1 mAb (51, 52), we could only eliminate NK cells and not NKT cells with either anti-NK1.1 mAb or RαAsGM1 Ab alone. However, when we used a mixture of Abs, we effectively eliminated NKT cells as well as NK cells. These results were not surprising, as we previously reported that while activating apoptosis in NK cells, the NK antigen–specific (anti-NK1.1) Ab treatment activated IL-4 synthesis in NKT cells (36). In our laboratory, when the same Ab was used to label cells for flow cytometry that was used for elimination, we could not find the cells because the antigen was either masked or downregulated. Thus, we consistently used different mAbs for depletion studies and the flow cytometric analyses.

In contrast to ACAID, intravenous tolerance could be induced in CD1 KO mice. The relationship between intravenous tolerance and ACAID is unclear, but intravenous tolerance does differ from ACAID in several respects. The intravenous administration of antigen cannot suppress the immune response in previously immunized hosts, whereas presentation of antigen via the ac does downregulate ongoing DTH responses in previously immunized hosts (53). Moreover, ACAID is mediated by both CD4$^+$ afferent regulatory T cells and CD8$^+$ efferent regulatory T cells (13), whereas intravenously induced tolerance only required CD8$^+$ afferent-regulatory cells (14). (In fact, because intravenous tolerance mechanism does not require an efferent-regulatory cell, we could not test the intravenous tolerance capability of CD1 KO mice in a LAT assay.) It is proposed that intravenous tolerance reflects the intrinsic response of T cells to antigen in the absence of costimulatory molecules, and therefore may not require additional cells (54). In contrast, this report clearly shows that ACAID involves interactions between multiple cells, a process that may be necessary in order to suppress established immune responses. A recent paper by Zeng et al. suggests that NKT cells may be responsible for assisting the generation of antigen-specific regulatory T cells in the bone marrow, perhaps to regulate immune responses to self-antigens displayed on other bone marrow–developing cells (55). Thus, it seems likely that CD1-reactive NKT cells may not only be unique regulators of self-reactivity in immune privileged sites (ACAID), but also may contribute to self-tolerance through regional specialization in a variety of organs, tissues, and microenvironments in general.

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