CD4⁺ NKT Cells, But Not Conventional CD4⁺ T Cells, Are Required to Generate Efferent CD8⁺ T Regulatory Cells Following Antigen Inoculation in an Immune-Privileged Site

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CD4\(^+\) NKT Cells, But Not Conventional CD4\(^+\) T Cells, Are Required to Generate Efferent CD8\(^+\) T Regulatory Cells Following Antigen Inoculation in an Immune-Privileged Site

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Following inoculation of Ag into the anterior chamber (a.c.), systemic tolerance develops that is mediated in part by Ag-specific efferent CD8\(^+\) T regulatory (Tr) cells. This model of tolerance is called a.c.-associated immune deviation. The generation of the efferent CD8\(^+\) Tr cell in a.c.-associated immune deviation is dependent on IL-10-producing, CD1d-restricted, invariant V\alpha14\(^+\) NKT (iNKT) cells. The iNKT cell subpopulations are either CD4\(^+\) or CD4\(^-\)CD8\(^-\) double negative. This report identifies the subpopulation of iNKT cells that is important for induction of the efferent Tr cell. Because MHC class II\(^-/-\) (class II\(^-/-\)) mice generate efferent Tr cells following a.c. inoculation, we conclude that conventional CD4\(^+\) T cells are not needed for the development of efferent CD8\(^+\) T cells. Furthermore, Ab depletion of CD4\(^+\) cells in both wild-type mice (remove both conventional and CD4\(^+\) NKT cells) and class II\(^-/-\) mice (remove CD4\(^+\) NKT cells) abrogated the generation of Tr cells. We conclude that CD4\(^+\) NKT cells, but not the class II molecule or conventional CD4\(^+\) T cells, are required for generation of efferent CD8\(^+\) Tr cells following Ag introduction into the eye. Understanding the mechanisms that lead to the generation of efferent CD8\(^+\) Tr cells may lead to novel immunotherapy for immune inflammatory diseases.

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entral tolerance is induced in the thymus during development, whereas peripheral tolerance is an active process in the adult, and both are primarily concerned with controlling self-reactive lymphocytes. Moreover, peripheral tolerance may be attained through apoptosis, anergy, or T cell regulation (1). Here we are studying the mechanisms involved in the generation of efferent T regulatory (Tr\(^+\)) cells.

The idea of T cell regulation or T suppressor cells arose in the late 1960s and seemed to peak in the 1970s and early 1980s. In both mice and men, suppression was shown in vitro to require interactions between CD8\(^+\) cells and CD4\(^+\) cells (1–3). Moreover, it appeared during this time that a CD4\(^+\) T cell subset was required for induction of CD8\(^+\) Tr suppressor as well as being a subset of target for the CD8\(^+\) T suppressor (4–6). Currently, most reports about Tr cells focus on CD4\(^+\) Tr cells, and only a few studies explore questions about efferent CD8\(^+\) Tr cells (6, 7). Here we address the mechanisms that give rise to the CD8\(^+\) Tr cells in an immune privilege model of tolerance induced in the eye, called anterior chamber-associated immune deviation (ACAIT) (8).

CD8\(^+\) Tr cells that suppress effector Th1 and Th2 cell function are induced following inoculation of Ag into the anterior chamber (a.c.) of the eye (7–10). Once Ag is introduced in the eye, bone marrow-derived F4/80\(^+\) APCs, indigenous to the eye, capture the Ag and travel via the blood to the marginal zone (MZ) of the spleen (11). The eye-derived APCs secrete macrophage inflammatory protein-2 chemokine that attracts NKT cells along the way to the MZ (12). In turn, after stimulation by CD1d expressed by the F4/80\(^+\) APCs, NKT cells secrete RANTES that, in turn, recruits more F4/80\(^+\) APCs and T cells to the MZ where clusters of cells accumulate (13). CD8\(^+\) Tr cells that suppress Th1 and Th2 effector cells appear in the spleen within 7 days of a.c. inoculation and are dependent on the presence of CD1d-restricted invariant NKT (iNKT) cells (14).

NKT cells are a unique subset of T cells that exist in both mouse and humans (15–17). A major subset of NKT cell subsets is restricted by the MHC class I-like molecule CD1d (18–20), which is known to be expressed on cells of haemopoietic origin (dendritic cells, B cells, T cells, macrophages) and liver (21). CD1d-restricted NKT cells include CD4\(^+\) and CD4\(^-\)CD8\(^-\) double-negative (DN) subsets and express a heavily biased TCR repertoire, with the majority expressing an invariant V\alpha14J\alpha281 TCR-\(\alpha\)-chain and V\beta8.2, V\beta2, or V\beta7 TCR-\(\beta\)-chains in mice. A similar subpopulation of NKT cells exists in the human and is defined by its invariant V\alpha24J\alphaQ TCR chain (18, 22–24). NKT cells are abundant in the bone marrow, thymus, and liver and are also found in spleen and other peripheral lymphoid organs (25–27).

NKT cells in mice and men are able to produce large amounts of cytokines within minutes of a signal (28). The phenotype of human NKT cell subsets correlates with the production of a unique set of cytokines: CD4\(^+\) NKT cells produced both Th1- and Th2-
type cytokines and IL-10; DN NKT cells produced only Th1-type cytokines (29, 30). During ACAID induction in the mouse iNKT cells produce IL-10, but not IL-4 (31) and thus may represent a different activation pathway. Here we explore the phenotype of the iNKT cell that is required and the role of conventional CD4+ T cells and MHC class II (class II) in the development of CD8+ Tr cells in ACAID.

Materials and Methods
Mice
Eight- to 20-wk-old mice were used in all experiments. The female C57BL/6 (B6) mice used in these experiments were obtained from the Schepens Eye Research Institute vivarium (Boston, MA) or Taconic Farms (Germantown, NY). Female MHC class II-deficient (class II−/−) mice on a B6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). Jax281−/− breeders were generated at Chiba University (Chiba, Japan) and were backcrossed nine generations to B6 mice (N9); they were a gift from M. Taniguchi (Chiba University Graduate School of Medicine, Chiba, Japan). Mice were housed on a 12-h light, 12-h dark cycle and were provided food and water ad libitum. All animals were treated humanely and in accordance with the guidelines set forth by the Schepens Eye Research Institute Animal Care and Use Committee and National Institute of Health guidelines.

Abs and flow cytometry
Anti-CD4 mAb (GK1.5) was used for depletion of CD4+ cells in vivo. The following Abs were used for flow cytometric analysis: CyChrome 5 (Cy5)-conjugated anti-TCRβ mAb (H57-597) and FITC- or PE-conjugated anti-CD4 mAb (RM4-4), FITC-conjugated anti-CD8 mAb (Ly-2, clone 53-6.7), and biotin-conjugated anti-NK1.1 mAb (PK136). They were purchased from BD PharMingen (San Diego, CA). PE-conjugated streptavidin was and biotin-conjugated anti-NK1.1 mAb (PK136). They were purchased from BD PharMingen (San Diego, CA). PE-conjugated streptavidin was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Rat anti-mouse FcγR II/III mAb (2.4G2 ascites) was used as the Fc block. Splenic NKT cells were analyzed by flow cytometry as previously described on an EPICS XL flow cytometer (Beckman Coulter, Miami, FL).

Preparation of the α-galactosylceramide (α-GalCer)-loaded CD1d tetrameric complex.

The lyophilized powder of α-GalCer was dissolved in 0.5% Tween 20 (J. T. Baker, Sanford, MI) at a concentration of 220 μg/ml by incubation for 24 h at 37°C with agitation, and stored at −20°C. Before use, the Ag was thawed at room temperature, then sonicated for at least 10 min at 37°C. CD1d tetramers were generated using a dimeric fusion protein of murine CD1d-Fc (32). Fluorescent tetramers were prepared from complexes of Alexa 488 dye-labeled protein A (Molecular Probes, Eugene, OR) and the CD1d-Fc fusion protein as previously described (29). CD1d tetramers were loaded with Ag by incubation for 72 h at 37°C with a 4/1 molar ratio of α-GalCer dissolved in 0.5% Tween 20 or were mock-treated with an equivalent volume of 0.5% Tween alone as a negative control. The Ag-loaded or mock-treated CD1d tetramers were used for flow cytometric staining as described previously (29).

Depletion of CD4+ cells
To deplete CD4+ cells, 200 μl of anti-CD4 mAb (GK1.5 ascites, diluted 1/4 in PBS) was injected i.p. into mice. The depletion of CD4+ cells was monitored in spleen cells by flow cytometry. Cells were stained with Cy5-conjugated anti-TCRβ mAb, and FITC-conjugated anti-CD4 mAb (RM4-4). The percentages of the CD4+ population in the total lymphocyte population before and after GK1.5 treatment were 14.1 and 0.18% in wild-type (WT) mice, and 2.16 and 0.05% in class II−/− mice, respectively.

ACAID induction and assay for delayed-type hypersensitivity (DTH)
ACAID was induced as previously described (33). In brief, 2 μl of OVA in PBS (25 mg/ml) was inoculated into the a.c. of the eye using a glass needle. One week later mice were immunized s.c. with OVA (100 μg/50 μl in HBSS) emulsified in CFA (50 μl). To test for DTH, OVA-pulsed, thioglycolate-induced peritoneal exudate cells (PECs; 2 × 107/10 μl of HBSS) were inoculated intradermally (i.d.) into the ear pinnae, and ear swelling was measured 24 h later with an engineer’s micrometer (Mitutoyo, Paramus, NJ).

Local adoptive transfer (LAT) assay
Tr cell function was tested in a LAT assay (9). In brief, effector cells were generated in B6 mice immunized s.c. with OVA (100 μg/50 μl of HBSS) and CFA (50 μl). Ten days later, effector T cells from harvested spleen cells were enriched on pan-T cell IMMULAN columns (Biotex Laboratories, Watford, U.K.). Naive T cells from unmanipulated mice were used as effector cells for the negative control group. Regulator cells were similarly enriched on pan-T cell IMMULAN columns from spleen cells of ACAID mice 7 days after a.c. inoculation of OVA. Stimulator cells were prepared by pulsing thioglycolate-induced PECs (1 × 108/ml) with OVA (5 mg/ml). Effector cells (5 × 104), stimulator cells (2 × 104), and Tr cells (5 × 104) were resuspended in 10 μl of HBSS and inoculated i.d. into the ear pinnae of naive B6 mice. Ear thickness was measured with an engineer’s micrometer at 24 h. Splenic T cells from unmanipulated mice were

FIGURE 1. Flow cytometric dot plot of immunostained NKT cells. Spleen cells were collected from WT mice 7 days post a.c. inoculation of OVA, stained with biotin-conjugated anti-NK1.1 mAb, and counterstained with streptavidin-PE and either FITC-conjugated anti-CD4 mAb or anti-CD8 mAb, and with Cy5-conjugated anti-TCRβ. A. Flow cytometric analyses of NK1.1+ T subpopulations in the spleens of one naive and one ACAID mouse. The NK1.1+ TCRβ+ lymphocytes within the circular gate (left panels) were analyzed for the presence of CD4 or CD8 surface expression. Left panels, Percentage of NK1.1+ TCRβ+ cells in the whole lymphocyte-gated population (30,000 events collected); center and right panels, CD4+ and CD8+ subpopulations, within the NK1.1+ TCRβ+ lymphocytes indicated by the circular gate in the left panels. The data shown represent an individual mouse within an experimental group of three mice. B, Bar graph of the number of NK1.1+ TCRβ+ cells from a.c.-inoculated mice. n, number of mice per group. The experiment was repeated twice with similar results.
used as regulatory cells for the positive control. In some experiments OVA-sensitized whole spleen cells were used as effector and stimulator cells. In that case, whole spleen cells (10^6) and Tr cells (10^6) were resuspended in 10 µl of HBSS in the presence of OVA (10 mg/ml) and inoculated i.d. into the ear pinnae of naive B6 mice.

Reconstitution of Jα281−/− mice

To reconstitute Jα281−/− mice with CD4+ NKT cells, pan-T cell IMMULAN column-enriched spleen T cells from class II−/− mice were stained with FITC-conjugated anti-CD4 mAb, counterstained with anti-FITC MicroBeads (Miltenyi Biotec, Auburn, CA), and applied to a type MS positive selection column with MiniMACS (Miltenyi Biotec). Enriched CD4+ cells (2 × 10^6/mouse) were injected i.v. into Jα281−/− mice. Twenty-four hours after reconstitution, Jα281−/− mice were inoculated (a.c.) with OVA (50 µg/2 µl of PBS) to test ACAID induction.

Statistics

Data were analyzed by ANOVA and Scheffé’s test. A value of p ≤ 0.05 was considered significant.

Results

Number of CD4+ NKT cells in the spleen increases following a.c. inoculation

Previously, we showed that CD1d-restricted iNKT cells were necessary for the establishment of ACAID (14). Also, published data established that the Tr cell that suppresses the effector arm of a Th1 response in ACAID is CD8+ (8). It is therefore well established that iNKT cells are required for the production of efferent CD8+ Tr cells. However, it was never established whether the iNKT cells that are responsible for generating CD8+ Tr cells were CD4+ or DN. Whole splenocytes from naive mice or from a.c.-inoculated mice (day 7) were harvested and immunostained with Cy5-conjugated DN. Whole splenocytes from naive mice or from a.c.-inoculated mice (day 7) were harvested and immunostained with Cy5-conjugated anti-TCR mAb, biotin-conjugated NK1.1 mAb counterstained with streptavidin-PE and either FITC-conjugated anti-CD4 mAb or anti-CD8 mAb (Fig. 1A). Among NK1.1+ TCRβ+ cells that were increased in the spleens of ACAID mice (14), only the CD4+ population increased significantly (Fig. 1B). Of the NK1.1+ TCRβ+ cells, ~85% expressed the invariant chain for their TCR. Since we showed previously that the NKT cell required for ACAID expressed the invariant receptor (iNKT), we wanted to confirm that the CD4+ NKT cell that was increased was an iNKT cell (34). Splenocytes were stained with anti-TCRβ mAb, anti-CD4 mAb, and α-GalCer-loaded CD1d tetramer (Fig. 2A). Anti-TCRβ intermediate α-GalCer-loaded CD1d tetramer+ cells (Fig. 2A, circle in left panels) were determined as iNKT cells. The number of total iNKT cells increased after a.c. inoculation with OVA. Among the iNKT cell population (Fig. 2A, left panels) there was a significant increase in the number of CD4+ cells, while the CD4− population showed no significant change (Fig. 2B). Thus, we conclude that it is the CD4+ iNKT cells that increase in the spleen following a.c. inoculation.

Class II−/− mice generate Tr after a.c. inoculation

Since class II−/− mice lack conventional CD4+ T cells but still have CD4+ NKT cells (35), we tested whether conventional CD4+ T cells were required for the development of Tr cells. Class II−/− or WT mice were inoculated (a.c.) with OVA, but since conventional CD4+ T cells are needed for the expression of a DTH response, the Tr function of enriched T cells from spleens of a.c.-inoculated class II−/− mice was tested in a LAT assay (Fig. 3). The enriched T cells from OVA a.c.-inoculated class II−/− mice suppressed the adoptively transferred DTH response induced in the recipient’s ear (Fig. 3). Thus, even in the absence of both the class II molecule and conventional CD4+ T cells, efferent Tr cells are generated in ACAID.

Ab depletion of CD4+ cells prevents the generations of Tr cells

To further address the issue of a role for a CD4+ NKT cell population in ACAID development, class II−/− and WT mice were treated with CD4-specific depleting Ab (GK1.5) 1 day before inoculation (a.c.) of OVA. Seven days later, T cells were enriched

FIGURE 2. Flow cytometric dot plot of tetramer-stained iNKT cells. Spleen cells were collected from WT mice 7 days post a.c. inoculation of OVA and were stained with Alexa 488 dye-labeled, αGalCer-loaded CD1d tetramer, PE-conjugated anti-CD4 mAb, and Cy5-conjugated anti-TCRβ. A, Flow cytometric analyses of iNKT subpopulations in the spleens of one naive and one ACAID mouse. The iNKT lymphocytes within the circular gate (left panels) were analyzed for the presence of CD4 surface expression. Left panels, Percentage of iNKT cells in the whole lymphocyte-gated population; right panels, CD4+ subpopulation within the iNKT lymphocytes indicated by the circular gate in the left panels. The data shown are one of five individual results from each experimental group. B, Bar graph of iNKT cell subpopulations within experimental groups. Absolute cell numbers are indicated on the ordinate. iNKT cells from naive mice; n, iNKT cells from a.c.-inoculated mice. a, number of mice per group. The experiment was repeated twice with similar results.
Splenic Tr cells were collected from WT or class II−/− mice 7 days after a.c. inoculation with OVA. Splenic T cells from WT mice that were immunized s.c. with OVA (100 μg/50 μl in HBSS) emulsified in CFA (50 μl) were used as effector T cells (Te). Te cells were mixed with Tr cells and stimulator cells (OVA-pulsed PECs) and were injected into the ear pinnae of naive mice. As a positive control, pan-T cell, IMMULAN column-enriched spleen cells from naive mice were used for regulatory T cells, and for the negative control, pan-T cell, IMMULAN column-enriched spleen cells from naive mice were also used for effector cells. The change (Δ) in ear swelling (24 h after ear challenge) is shown on the ordinate, and the identity of the cell mixture inoculated into the ear pinnae for each group is indicated below the abscissa. n, number of mice per group. Significant differences (p ≤ 0.05) are indicated by an asterisk. This is a representative result of two similar experiments.

**FIGURE 4.** LAT assay for Tr function in CD4-depleted WT and class II−/− mice. CD4+ cells were depleted in both WT and class II−/− mice by inoculation of CD4+–specific mAb ( GK1.5) i.p. Mice were inoculated (a.c.) with OVA (50 μg/2 μl of PBS) 24 h after the treatment. Seven days later, pan-T cell, IMMULAN column-enriched splenic T cells were harvested from the a.c.-inoculated mice and used as Tr cells in a LAT assay. Change (Δ) in ear swelling (24 h after ear challenge) are shown on the ordinate. The cell mixtures that were injected into the ear pinnae of each group are indicated below the abscissa for each bar. n, number of mice per group. Significant differences (p ≤ 0.05) are indicated by an asterisk. Data shown are representative result of two similar experiments.

**FIGURE 5.** ACAID in Jα281−/− mice (iNKT deficient) after CD4+ NKT cell reconstitution. Pan-T cell IMMULAN column-passed spleen cells from naïve class II−/− mice were stained with FITC-conjugated anti-CD4 mAb, counterstained with anti-FITC magnet beads, and applied to a type MS+ positive selection column with MiniMACS to enrich CD4+ NKT cells. Jα281−/− mice were either reconstituted or not with CD4+ NKT cells 24 h before they were given an a.c. inoculation with OVA. Seven days after a.c. inoculation mice were immunized s.c. with OVA (100 μg/50 μl in HBSS) emulsified in CFA (50 μl). Seven days after OVA s.c. immunization, OVA-pulsed, thioglycolate-induced PECs (2 × 10^7/10 μl HBSS) were inoculated i.d. into the ear; the change in ear swelling was measured 24 h after the injection and is shown on the ordinate. The treatment of the mice is listed under the abscissa. n, number of mice per group. Significant differences (p ≤ 0.05) are indicated by an asterisk.

**CD4+ NKT cells reconstitute ACAID in Jα281−/− mice**

Another approach to the question was to determine whether CD4+ iNKT cells were sufficient to reconstitute ACAID in Jα281−/− mice. Jα281−/− mice were reconstituted with CD4+ T cells from class II−/− mice 24 h before OVA inoculation (a.c.). One week later, mice were immunized s.c. with OVA (100 μg/50 μl in HBSS) emulsified in CFA (50 μl). OVA-pulsed, thioglycolate-induced PECs (2 × 10^7/10 μl of HBSS) were inoculated i.d. into the ear pinnae of naive mice, and ear swelling was measured 24 h later (Fig. 5). Because Jα281−/− mice only lack iNKT cells, we again concluded that CD4+ iNKT cell are required for the generation of effector Tr cells.

**Discussion**

In this report we show that CD4+ iNKT cells are essential for generating Tr cells in ACAID, but CD4− iNKT cells (or DN, since there are no CD8+ iNKT cells in the mouse (34)) and conventional class II-dependent CD4+ T cells are not. Previously, it was established that the Tr cells that regulate effector cells induced by...
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