Implication for the CD94/NKG2A-Qa-1 system in the generation and function of ocular-induced splenic CD8⁺ regulatory T cells

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Abstract

The injection of antigen into the anterior chamber (AC) induces the production of antigen-specific splenic CD8⁺ regulatory T cells (Tregs) /suppressor T cells that perform the local suppression of delayed-type hypersensitivity (DTH) responses. Because CD94/NKG2A-Qa-1-dependent interactions have been implicated in CD8⁺ Treg-mediated immune suppression and DBA/2J mice are deficient in CD94/NKG2A, we have utilized these mice to test the hypothesis that the CD94/NKG2A-Qa-1 system is essential to the induction and immunosuppressive function of CD8⁺ Tregs in anterior chamber-associated immune deviation (ACAID). We show that: (i) neither ACAID-mediated suppression of DTH to ovalbumin nor splenic Tregs/suppressor T cells was induced in DBA/2J mice that received an injection of antigen into the AC; (ii) splenic CD8⁺ Tregs from ACAID-induced DBA/2Ncr mice suppressed the initiation of DTH when transferred to DBA/2J mice; (iii) following injection of antigen into the AC, intravenous administration of splenocytes or Peripheral Blood Mononuclear Cells (PBMC) isolated from DBA/2Ncr but not from DBA/2J mice transferred suppression of DTH to DBA/2Ncr mice; (iv) antibodies to CD94/NKG2A reduced the ACAID CD8⁺ T cell-mediated suppression of DTH and (v) The deficiency of such immune regulation in DBA/2J mice also correlated with a decreased number of Qa-1⁺ B cells, F4/80⁺ cells, a deficient number of CD94/NKG2A and Qa-1 tetramer binding by CD8⁺ T cells. These results demonstrate that defective ACAID in DBA/2J mice involves multiple regulatory lesions resulting in a lack of induction of a CD8⁺ Treg response and possibly defective CD94/NKG2A-dependent suppression of peripheral cell-mediated immunity.

Introduction

The injection of antigen into the anterior chamber (AC) of an eye induces the systemic impairment of a cell-mediated immune response (1, 2). Antigens introduced into the AC are likely processed by F4/80⁺ antigen-presenting cells (APC) derived from the iris and ciliary body (1–5). It has been proposed that these APC migrate to the thymus (3, 4, 6, 7) and spleen (1, 2, 8, 9) and/or influence circulating APC that migrate to the thymus and spleen (4). After arriving in the spleen, the F4/80⁺ APC initiate a complex series of cellular interactions involving B cells (9, 10–12), recent thymic emigrants (3), γδ T cells (12–14), CD4⁺/CD4⁺ NKT cells (9, 11, 15) and CD8⁺ T cells (16). Recently, it has been reported that one of the transforming growth factor (TGF)-β-responsive T cell-integrin molecules CD103 (αEβ7) is also involved in the generation of CD8⁺ suppressor T cells in anterior chamber-associated immune deviation (ACAID) because CD103⁻/⁻ mice are resistant to the induction of ACAID (17).

The splenic regulatory T cells (Tregs) induced in ACAID are CD4⁺-afferent immunoregulatory T cells (11) which induce the antigen-driven induction of an immune response and antigen-specific CD8⁺-efferent Tregs (1, 16), which regulate the initiation of a delayed-type hypersensitivty (DTH) reaction. Although B cells require both MHC class I and II molecules for the activation of ACAID-inducing Treg (18), antigen presentation by Qa-1, a non-classical MHC I molecule on the surface of activated B cells, stimulates CD8⁺ T cell-suppressive activity (10, 19). The apparent Qa-1 restriction for the presentation of antigen by B cells to CD8⁺ Treg in ACAID is consistent with reports that the induction of the suppression of DTH mediated by CD8⁺ Treg is Qa-1.
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restricted (10). In this regard, it is likely that in ACAID, Qa-1 on APC interacts with CD94/NKG2AR on T cells (20, 21). However, the cellular expression and functional importance of the expression of Qa-1 and CD94/NKG2AR for Qa-1 in ACAID is not known. Accordingly, we have tested the hypothesis that the CD94/NKG2A-Qa-1 system is essential to the induction and immunosuppressive function of CD8⁺ Tregs.

DBA/2J mice have altered features of immune regulation (22–24) including deficiencies in ocular immune privilege featuring an age-related form of ocular pigment dispersion syndrome, glaucoma and marginal or undetectable ACAID (25, 26). DBA/2J mice also have a deficiency in CD94/NKG2AR (27), which have been implicated in cell-mediated immune regulation (28, 29). Therefore, we have investigated the induction of ACAID in MHC haplotype (H-2b)-matched DBA/2NCr mice with DBA/2J mice to define possible cellular alterations that impact on an ocular influence on the peripheral immune system. We found that both the splenocytes and Peripheral Blood Mononuclear Cells (PBMC) from AC-injected DBA/2J mice did not mount and/or transfer the suppression to antigen-induced DTH as compared with DBA/2NCr mice. Splenocytes containing CD8⁺ T cells from ACAID-induced DBA/2NCr mice were able to suppress the initiation of DTH when transferred to DBA/2J mice in a CD94/NKG2A-dependent manner. Those functional CD8⁺ Tregs from DBA/2NCr mice show higher Qa-1 tetramer binding as compared with DBA/2J mice. Additionally, DBA/2J mice receiving intracameral antigen showed a reduced frequency of Qa-1⁺ on F4/80⁺ cells and CD19⁺ B cells. The number of CD94⁺CD103⁺CD8⁺ and CD94/NKG2A⁺CD8⁺ T cells was also reduced in the AC-injected DBA/2J mice as compared with DBA/2NCr mice. In aggregate, these results demonstrate that the defective immune regulation in DBA/2J mice involves multiple regulatory lesions that may result in a defective CD8⁺ Tregs/suppressor T cell response in ACAID and supports our hypothesis that the Qa-1-CD94/NKG2A system is central to both the afferent and efferent aspects of ACAID-induced CD8⁺ Treg-mediated immune regulation.

Methods

Mice

Female 6- to 10-week, BALB/c, C57BL/6 and DBA/2NCr mice were purchased from the National Cancer Institute (Frederick, MD, USA) and DBA/2J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). DBA/2NHsd were purchased from the National Cancer Institute (Frederick, MD, USA) and DBA/2J mice were purchased from the National Cancer Institute (Frederick, MD, USA). Mice were maintained in the Center for Laboratory Animal Care of the University of Connecticut Health Center. All animals were treated according to an approved University of Connecticut Health Center Animal Care Protocol 2004-092 and the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research.

Reagents

Ovalbumin (OVA) was purchased from Sigma (St Louis, MO, USA). Ficoll-Paque PLUS was purchased from GE Healthcare (Björkagatan 30, 751 84 Uppsala, Sweden).

Allophycocyanin-conjugated CD8 mAb (Ly-2)(53-6.7), biotinylated and purified Qa-1β mAb (6A8.6F10.1A6), PE-conjugated CD19 mAb (1D3), purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block™) (2.4G2) and streptavidin FITC conjugate were purchased from BD Biosciences (San Jose, CA, USA). PE-conjugated F4/80 mAb (CI:A3-1), allophycocyanin-conjugated F4/80 mAb (Cl:A3-1) and PE-conjugated and purified CD94 mAb (18d3) were purchased from Sero-tech (Raleigh, NC, USA). FITC-conjugated CD103 mAb (2E7) was a gift from Beckman Coulter, Inc. (Miami, FL, USA). Fluorochrome-conjugated and purified mAbs for NKG2A/C/E (20d5), NKG2AB6 (16a11) and the negative control (Rat IgG2a) were purchased from eBioscience (San Diego, CA, USA). Qa-1β tetramer was prepared by the NIAID MHC Tetramer Core Facility (Atlanta, GA, USA) using the peptide sequence AMAPRTLLL (30, 31).

Injection of antigen into the AC

For the induction of ACAID, antigen was injected into an AC of the mice as described previously (6). Briefly, mice were anesthetized by intraperitoneal injection of ketamine (75 mg kg⁻¹)/xylazine (15 mg kg⁻¹). Under a dissecting microscope, a transcorneal paracentesis was performed on an eye using an 18-gauge needle; 4–5 μl PBS (pH 7.2) containing 50 μg OVA was then injected into the AC by manually controlled microinjection using a 33-gauge needle on tubing attached to a Hamilton syringe (Stoelting Co., Wood Dale, IL, USA). Mice were immunized 7 days after the injection of antigen into the AC.

Immunization

Mice received a subcutaneous (s.c.) injection of 200 μg OVA emulsified 1:1 in CFA (Sigma) in a total volume of 50 μl 7 days after AC injection.

Cell preparation

Spleens were removed from mice that received an injection of antigen into the AC and disrupted through a 70-μm nylon cell strainer (BD Falcon, Bedford, MA, USA) with a 1-ml syringe plunger in PBS (pH 7.2). The cell suspension was washed with PBS and centrifuged at 200 × g, 6–8 min. BD Pharm Lyse (BD Biosciences) was used for lysing the erythrocytes according to the manufacturer's protocol. The cells (AC-SPL cells) were then washed twice with PBS. The cell pellets were re-suspended in PBS. CD8⁺ AC-SPL T cells were prepared by washing splenocytes two times with PBS and suspended in BD™ IMag separation buffer (BD Biosciences, Rockville, MD, USA). The AC-SPL cells were separated by immunomagnetic beads into a CD8⁺ and CD8⁻ population with a BD™ (BD Biosciences) CD8⁺ T lymphocyte enrichment set according to the manufacturer's protocol. Enrichment of the cells was assessed by FACS and found to be 92% CD8⁺ T cells and CD8⁻ fractions were <3% CD8⁺ T cells (data not shown). PBMC were collected by tail vein bleeding and enriched by Ficoll-Hypaque (GE Healthcare, Orlando, FL, USA) density gradient-cut according the manufacturer's protocol and as described elsewhere (3).
Delayed-type hypersensitivity

The DTH response to OVA was measured using a footpad swelling assay (10). Mice were challenged by intradermal (i.d.) injection of OVA (200 μg) in 40 μl PBS into the left footpad 7 days after s.c. immunization with OVA. Footpad swelling was measured before and after the antigenic challenge with an engineer’s digital micrometer (Mitutoyo, Tokyo, Japan). Micrometers of swelling were determined by computing the difference in thickness between the challenged footpad and the unchallenged footpad before and after challenge. Each measurement was then corrected by subtracting the 24–48 h difference in the footpads of challenged and unchallenged footpads of naive mice.

Adoptive transfer of cells: local transfer of suppression

Suppressor splenocytes in the AC-SPL cells were assayed by the local transfer of suppression (LTS) assay (4). AC-SPL cells donor mice, that received an AC injection of OVA (50 μg per 5 μl) into an AC on day 0 and immunized on day 7, were euthanized on day 14 by cervical dislocation, spleens were removed, diced and expressed through a 70 μm nylon mesh (BD Biosciences) into PBS. The cells were washed twice with PBS and re-suspended in PBS. AC-SPL cells (25 × 10^3 cells per 40 μl PBS) from the donors of suppressor T cells were injected i.c. into the footpad during antigenic challenge with 200 μg OVA. In addition, in some experiments, antibodies to CD94/NKG2A and the control antibody (10 μg per 100 μl) were pre-incubated at 4°C for 60 min with the AC-SPL or AC-SPL CD8+ T cells and OVA (200 μg) at the time of footpad challenge. The antibodies were found to block the respective receptors and were found to be non-cytolytic (data not shown) (32).

Adoptive transfers of AC-PBMC and AC-SPL cells intravenously

PBMC from AC-injected donors (AC-PBMC) and AC-SPL cells (2 × 10^6 and 5 × 10^6 cells, respectively, in 100 μl PBS) were injected intravenously (i.v.) to naive mice and the mice were immunized 1 day later. DTH was determined in the recipients 7 days after the immunization.

Flow cytometry analysis

Cells were incubated in staining buffer (PBS, 1% fetal calf serum, 0.1% sodium azide) with 0.5 μg ml⁻¹ anti-CD16/CD32 antibodies as a blocking reagent (BD Biosciences) in PBS for 10 min and then incubated with fluorochrome-labeled antibodies to cell-surface markers and control antibodies for 30 min. After washing, the labeled cells were detected by flow cytometry using a FACSCalibur and analyzed by CellQuest software (BD Biosciences). A total of >10 000 cells were collected for each sample. Splenocytes were further gated for respective live cell populations.

Statistics

For each experiment, experimental and control groups contained three animals per group. Differences between groups were analyzed by Student’s t test. P values <0.05 were considered significant.

CD94/NKG2A in CD8+ Treg-mediated suppression

Results

DTH is not suppressed in DBA/2J mice by the injection of antigen into the AC

Because DBA/2J mice show defective immune regulation (22–24), including a deficiency of ocular immune privilege (25, 26), we compared the induction of the suppression of DTH by ACAID in three different DBA/2 mouse strains: DBA/2J, DBA/2Ncr and DBA/2Nhsd and also with C57BL/6 (H-2b) and BALB/c (H-2d) mice. OVA was injected into the AC and 1 week later the mice were immunized to OVA. One week after immunizing, footpads were challenged with OVA. We found that the DTH response to OVA was significantly reduced in all the mouse strains (that received an injection of OVA into the AC (P < 0.05) except DBA/2J mice (Table 1). This result demonstrates that the suppression of DTH induced by the injection of antigen into the AC is not induced in DBA/2J mice.

DBA/2J AC-SPL cells do not transfer the local suppression of DTH

To determine whether the AC-SPL suppressor cells from H-2d haplotype-matched mice (i.e. BALB/c, DBA/2Ncr and DBA/2J) can transfer the suppression of DTH to immunized DBA/2J mice, 25 000 AC-SPL cells (provide maximum suppression in the LTS, R.E.C. unpublished observation) from BALB/c, DBA/2Ncr and DBA/2J mice were injected into the footpads of OVA-immunized DBA/2J mice at the time the footpads were challenged with OVA. BALB/c and DBA/2Ncr AC-SPL cells were found to be effective in suppressing the DTH response in immunized DBA/2J mice but DBA/2J AC-SPL cells did not suppress the DTH reaction (P > 0.05) when transferred to immunized DBA/2J mice (Fig. 1A). AC-SPL cells from BALB/c and DBA/2Ncr mice also suppressed DTH to OVA in BALB/c and DBA/2Ncr immunized mice (data not shown). AC-SPL cells from DBA/2Ncr mice enriched for CD8+ cells to 92% transferred the suppression of DTH. However, CD8+ cells from the same AC-SPL cell

Table 1. DTH is not suppressed in DBA/2J mice by the injection of antigen into the AC

<table>
<thead>
<tr>
<th>Mice strain</th>
<th>Naive (μm)</th>
<th>Imm (μm)</th>
<th>AC + Imm (μm)</th>
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<tbody>
<tr>
<td>BALB/c</td>
<td>14 ± 15.63</td>
<td>290 ± 71.18</td>
<td>116 ± 21.82 (*)</td>
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<tr>
<td>C57BL/6</td>
<td>247 ± 9</td>
<td>44 ± 30 (*)</td>
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<tr>
<td>DBA/2Ncr</td>
<td>19.5 ± 33.18</td>
<td>260.5 ± 6.65</td>
<td>114 ± 26 (*)</td>
</tr>
<tr>
<td>DBA/2Nhsd</td>
<td>27.33 ± 25.79</td>
<td>326 ± 49.51</td>
<td>100 ± 31.09 (*)</td>
</tr>
<tr>
<td>DBA/2J</td>
<td>8 ± 14.4</td>
<td>305 ± 60.7</td>
<td>269 ± 85.65 (NS)</td>
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One week after receiving an AC injection of OVA, mice were immunized to OVA and a footpad of the immunized mice was challenged with OVA by intradermal injection 1 week after immunization. DTH was measured by micrometer of footpad swelling 24 h after the footpad was challenged with i.d. OVA. Data are representative of three similar experiments with similar results [values expressed as mean swelling ± SD; *P < 0.05 as compared with immunized (Imm) group; AC + Imm, AC-injected and immunized mice; NS, non-significant as compared with immunized (Imm) group; ND, not detectable swelling]. All experimental and control groups contained three mice per group to measure DTH.
population (<3% CD8+ cells) did not transfer suppression in the LTS assay (Fig. 1B). These results show that DBA/2J mice do not generate functional suppressor AC-SPL cells but the DTH response can be suppressed in DBA/2J mice by transferring functional AC-SPL cells containing CD8+ T suppressor cells from DBA/2NCr mice.

AC-PBMC and AC-SPL cells from AC-injected DBA/2J mice do not induce ACAID

Intravenous injection of PBMC from mice that received an injection of antigen into the AC (AC-PBMC) and AC-SPL cells induce the suppression of DTH to the antigen but do not suppress the local DTH response in the LTS (3, 16, 33). To determine if AC-PBMC or AC-SPL cells from DBA/2J mice induce the suppression of DTH to the antigen, we injected i.v. AC-PBMC or AC-SPL cells from DBA/2NCr mice to DBA/2J mice. One day after the cell transfer, the recipient mice were immunized and assessed for DTH 7 days later. Intravenous injection of AC-PBMC and AC-SPL cells recovered from DBA/2NCr mice induced the suppression of DTH in DBA/2NCr mice but not DBA/2J mice. In contrast, AC-PBMC and AC-SPL cells from DBA/2J mice did not transfer the induction of suppression of DTH to either DBA/2J or DBA/2NCr mice (Fig. 2). This result demonstrates that afferent (inducer) AC-PBMC and AC-SPL cells from DBA/2NCr mice are functional in DBA/2NCr mice but not in DBA/2J mice. Conversely, neither AC-PBMC nor AC-SPL cells from DBA/2J mice can induce the suppression of DTH to DBA/2NCr mice.

The expression of the CD94-Qa-1 axis is deficient in DBA/2J lymphoid, monocytic and iris cells

The above results show that DBA/2J mice behave differentially as compared with DBA/2NCr mice with respect to both the afferent and efferent aspects of ACAID. DBA/2J mice are deficient in the expression of CD94/NKG2A(27) and most of the tissue in the AC express Qa-1, implicated in ocular immune privilege (34). Moreover, CD94/NKG2A and Qa-1 have been implicated in CD8+ Treg function (20, 21, 28, 29) and B cell-driven, Qa-1-restricted induction of ACAID (10). Therefore, we investigated the expression of Qa-1 and the binding of Qa-1 tetramers by DBA/2J monocytic, lymphoid and iris cells and the expression of these markers in both DBA/2NCr and DBA/2J mice.

The irides of DBA/2NCr mice have a greater number of iris cells expressing Qa-1 than DBA/2J mice (Fig. 3A). DBA/2J mice have less CD94+NKG2A+CD8+ T cells (Fig. 3Bi) as compared with DBA/2NCr AC-SPL cells. Moreover, DBA/2J mice have less splenic CD103, a TGF-β-dependent T cell integrin (17, 35), expressing CD8+CD94+ T cells than DBA/2NCr mice (Fig. 3C). Additionally, there is a reduced binding of Qa-15 tetramer by DBA/2J AC-SPL CD8+ T cells (Fig. 3Bi). Similarly, Qa-15, the ligand for CD94/NKG2AR, was also found to be reduced on F4/80+ and CD19+ cells, AC-SPL cells (Fig. 3D and E) and iris cells (Fig. 3A) from DBA/2J mice as compared with DBA/2NCr mice. Higher CD8+CD94+ T cells, Qa-15+F4/80+ and Qa-15+CD19+ cells were also found in AC-SPL cells from BALB/c mice as compared with DBA/2J mice (data not shown). Together these results demonstrate a reduced

Fig. 1. DBA/2J spleen cells do not suppress DTH in a LTS mediated by ACAID. (A) AC-SPL cells from H-2d haplotype-matched mice (i.e. BALB/c, DBA/2NCr and DBA/2J) were transferred to measure the suppression of the DTH response to immunized DBA/2J mice. Twenty-five thousand AC-SPL cells from BALB/c, DBA/2NCr and DBA/2J mice that received an injection of OVA into the AC were injected into the footpad of OVA-immunized DBA/2J mice at the time of footpad challenge with OVA. The DTH response was measured by micrometer of footpad swelling 24 h after challenge. The open and closed bar graphs show the comparative data from BALB/c; DBA/2J and DBA/2NCr; DBA/2J mice, respectively, for the LTS by AC-SPL cells to the OVA-immunized DBA/2J mice. (B) Five thousand purified CD8+ AC-SPL cells and the CD8− cells from DBA/2NCr mice that received an injection of OVA into the AC were transferred to the immunized DBA/2J mice to measure the suppression of the DTH response to DBA/2J mice. Purified CD8+ AC-SPL cells from DBA/2NCr mice were injected into the footpad of OVA-immunized DBA/2J mice at the time of footpad challenge with OVA. The DTH response was measured by micrometer of footpad swelling. Data are representative of two similar experiments with similar results [values expressed as mean ± SD, *P < 0.05 and NS = non-significant as compared with immunized (IMM) group, IMM + AC-SPL = LTS of AC-SPL cells in the immunized mice, D/2J = DBA/2J mice and D/2N = DBA/2NCr mice]. All experimental and control groups contained three mice per group to measure DTH.
Fig. 2. AC-PBMC and AC-SPL cells from DBA/2J do not transfer the induction of ACAID. (A) PBMC (2 × 10^6) from DBA/2NCr and DBA/2J mice that received an injection of OVA into the AC (AC-PBMC) and also (B) AC-SPL cells (5 × 10^6) from DBA/2J mice were transferred i.v. to both naive DBA/2NCr and DBA/2J mice. One day after the cell transfer, the recipient mice were immunized. A footpad of the immunized mice was challenged with OVA through i.d. injection 1 week after immunization and assessed for DTH 24 h later. [values expressed as mean ± SD, *P < 0.05 and NS = non-significant as compared with immunized (IMM) group, 2N = DBA/2NCr mice, 2J = DBA/2J mice, 2N AC-PBMC + 2N IMM = transfer of 2N AC-PBMC to immunized 2N mice, 2J AC-PBMC + 2N IMM = transfer of 2J AC-PBMC to immunized 2N mice, 2J AC-PBMC + 2J IMM = transfer of 2J AC-PBMC to immunized 2J mice, 2N PBMC + 2N IMM = transfer of 2N AC-PBMC to immunized 2N mice, 2J PBMC + 2J IMM = transfer of 2N AC-PBMC to immunized 2J mice, 2N AC-SPL + 2N IMM = transfer of 2N AC-SPL cells to immunized 2N mice, 2J AC-SPL + 2N IMM = transfer of 2J AC-SPL cells to immunized 2N mice, 2J AC-SPL + 2J IMM = transfer of 2J AC-SPL cells to immunized 2J mice, 2N AC-SPL + 2J IMM = transfer of 2N AC-SPL cells to immunized 2J mice]. All experimental and control groups contained three mice per group to measure DTH.

Fig. 3. Differential expression of CD94 and Qa-1 by DBA/2NCr and DBA/2J AC-SPL cells. (A) Differential expression of Qa-1 protein on the iris cells of DBA/2NCr and DBA/2J mice. (B)(i) Comparison of CD94^+NKGA^+ cells on AC-SPL cells on CD8^-gated cells from DBA/2NCr and DBA/2J mice representing percent positive value by flow cytometric analysis (values expressed as mean ± SD). B(ii) Qa-1 tetramer binding (%) on splenic CD8^+ T cells. (C) Comparison of CD94^+CD103^+ AC-SPL cells (CD8^-gated cells) from DBA/2NCr and DBA/2J mice representing percent positive value by flow cytometric analysis. (D) Comparison of Qa-1^+F4/80^+ and Qa-1^+CD19^+ AC-SPL cells from DBA/2NCr and DBA/2J mice representing percent positive value by flow cytometric analysis. (E) Graphical representation of % Qa-1^+ AC-SPL cells in DBA/2NCr and DBA/2J mice (values expressed as mean ± SEM, *P < 0.05). Results are representative of three to five experiments.
frequency of CD94/NKG2A-expressing CD8+ T cells, lesser binding of Qa-1 tetramer on CD8+ T cells and also a decrease of Qa-1 molecules on the two potential immunoregulatory APC in DBA/2J mice.

**Suppression of DTH to antigen by AC-SPL-CD8+ T cells requires functional CD94/NKG2AR**

To investigate a role for CD94/NKG2AR in the ability of AC-SPL cells to suppress DTH, we used antibodies to CD94/NKG2A (30, 32, 36, 37) in the DTH suppression assay by transferring an admixture of antibody-pre-treated (10 µg per 100 µl) AC-SPL cells and OVA (200 µg) into the footpad of the immunized DBA/2Ncr mice. We found that the inclusion of CD94/NKG2A antibodies inhibited the suppression of DTH with DBA/2Ncr AC-SPL cells in DBA/2Ncr mice (86.2% suppression of the DTH response was observed with the transfer of AC-SPL cells alone, P < 0.05) (Fig. 4). The suppression of DTH was reduced with the blocking of CD94/NKG2AR. The antibodies do not suppress the DTH response in the immunized mice (data not shown). We have also found that DBA/2Ncr AC-SPL-CD8+ T cells transfer suppression of a DTH response in a CD94/NKG2A-dependent manner (data not shown) similar to AC-SPL cells (Fig. 4). These results suggest that the suppression of DTH to antigen by ACAID-CD8+ T cells requires functional CD94/NKG2AR.

**Discussion**

DBA/2J mice are the only reported mouse strain, which are naturally deficient in CD94/NKG2AR (27). The loss of ocular immune privilege, ocular immunosuppressive microenvironment, a susceptibility to glaucoma and an inability to induce ACAID (24–26) suggest a defect in immune regulation in DBA/2J mice. Because ACAID is due to multiple afferent and efferent steps, we investigated possible cellular lesions in the ACAID model in DBA/2J mice in context of CD94/NKG2A-Qa-1 axis. Recently, it was reported that Qa-1 is ubiquitously expressed in most tissues of the AC and this report proposed a role for Qa-1 in the maintenance of ocular immune privilege (34). Consistent with those observations, we observed that DBA/2Ncr mice have a higher number of Qa-1++ iris cells that were not detected in DBA/2J mice (Fig. 3A).

The ability of CD8+–enriched but not CD8+ AC-SPL cells from DBA/2Ncr AC-SPL cells to transfer the suppression of the DTH response to antigen (Fig. 1B) in the LTS is consistent with previous observations that the AC-SPL cells that suppress directly the initiation of the DTH reaction are CD8+ based on the loss of the ability to transfer suppression when CD8+ AC-SPL cells are removed or the transfer of suppression by purified CD8+ T cells (6, 16). It has been reported that suppression of DTH in ACAID by efferent CD8+ Tregs/suppressor T cells does not require conventional CD4+ Tregs (15) and the naturally occurring CD4+CD25+ FoxP3+ Tregs may not be involved in ACAID (38). However, it has been shown that the inducible CD4+CD25+ Tregs may participate in the induction of the CD8+ Tregs mediated by ACAID (11). Additionally, in Herpes simplex virus-1 infection an association between CD4+FoxP3+ Tregs and CD8+ memory T cells has been reported (37). Moreover, the suppression of DTH mediated by the CD8+ AC-SPL cells is antigen specific (1, 10) and it could be associated with functional CD94/NKG2AR (Fig. 4) and its ligand, Qa-1 (10). In addition, if the TCR of CD8+ Treg is restricted by Qa-1 (29), suppression may require ligation of Qa-1: peptide complexes on the effector cells. Our results with anti-CD94/NKG2A-blocking antibodies (Fig. 4) suggest that the efferent CD8+ Treg cells in AC-SPL cells that mediate the suppression of DTH require CD94/NKG2AR.

To measure the induction of ACAID by the afferent immunoregulatory cells (e.g. function of CD4+ Tregs, tolerogenic B cells, F4/80+ cells and γδ T cell), we transferred i.v. AC-PBMC and AC-SPL cells and then immunized the recipients (16, 33). Our results show that DBA/2J mice also have afferent lesions reflected in defective AC-PBMC and AC-SPL cells (constituting F4/80+ cells, B cells and CD4+ T cells). These afferent AC-SPL cells or AC-PBMC from DBA/2J mice do not induce ACAID when transferred i.v., as compared with DBA/2Ncr mice (Fig. 2). Our results demonstrate that DBA/2J mice do not generate functional CD8+ Tregs, even when they are the recipients of AC-PBMC or afferent AC-SPL cells that transfer ACAID. In this regard, induced DBA/2J CD8+ Tregs may not be functional because of a deficiency of CD94–NKG2A-Qa-1 interactions necessary to mediate suppression.

CD103, a T cell integrin (αEβ7) molecule, has been reported to be a TGF-β-dependent biomarker that is associated with CD8+ Treg functions (35). It has been suggested that CD8+ Tregs may regulate ACAID in a TGF-β-dependent manner by altering the DTH response to antigen (17). We found an increase in the splenic CD8+CD103+ T cell population in DBA/2Ncr mice as compared with DBA/2J
mice after the injection of OVA into the AC. In addition, our preliminary data suggest that suppression of the DTH response by ACAID is TGF-β dependent (S. Chattopadhyay and R. E. Cone, unpublished results). These observations suggest that a deficiency in CD94/NKG2AR and the CD103 co-receptors on CD8+ T cells may be associated with a deficiency in functional CD8+ Treg cells in DBA/2J mice. Taken together with the expression of CD94, our results suggest that the phenotype of CD8+ suppressor T cells/Tregs induced by intracameral antigen is CD8+CD94+NKG2A+CD103+.

The role of CD94R in CD8+ T cell-mediated suppression is not clear. CD94/NKG2AR may facilitate antigen binding if the CD8+ T cell is restricted and/or this receptor may deliver a signal to the CD8+ Treg/suppressor T cell to secrete immunosuppressive cytokines when Qa-1 is bound. Experiments are in progress to clarify these issues. The expression of Qa-1b molecules by ACAID-inducing APC (Fig. 3) may be required to induce both afferent regulatory and effector CD8+ Treg cells. However, other cellular subsets including γδ T cells (12, 39) may also induce CD94/NKG2A-expressing CD8+ Treg in ACAID. Moreover, in addition to the activation of CD8+ Treg cells, the antigen-specific suppression of effector cells is likely restricted by Qa-1 as shown elsewhere (20, 40) and by our preliminary data (R. E. Cone et al., unpublished results). Our results show less binding of Qa-1 tetramer by DBA/2J mice CD8+ T cells (Fig. 3) as compared with ACAID-inducing DBA/2Ncr mice. Therefore, a deficiency in Qa-1 and its inhibitory receptor CD94/NKG2A complex may contribute to the disrupted ocular immune privilege in DBA/2J mice. The association between functional CD94/NKG2AR and ACAID (deficient in DBA/2J mice) indicates that this deficiency is extended to a loss of an ocular influence on the peripheral immune response manifested as ACAID.

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Abbreviations
AC anterior chamber
ACAI D anterior chamber-associated immune deviation
APC antigen-presenting cells
DTH delayed-type hypersensitivity
i.d. intradermal
i.v. intravenous
LTS local transfer of suppression
OVA ovalbumin
PBMC peripheral blood mononuclear cells
s.c. subcutaneous
TGF transforming growth factor
Treg regulatory T cell

References
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