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γδ T Cells Promote Anterior Chamber-Associated Immune Deviation and Immune Privilege through Their Production of IL-10

Hossam M. Ashour* and Jerry Y. Niederkorn²†

Anterior chamber-associated immune deviation (ACAID) is a form of peripheral tolerance that is induced by introducing Ags into the anterior chamber (AC) of the eye and is maintained by Ag-specific regulatory T cells (Tregs). ACAID regulates harmful immune responses that can lead to irreparable injury to innocent bystander cells that are incapable of regeneration. This form of immune privilege in the eye is mediated through Tregs and is a product of complex cellular interactions. These involve F4/80⁺ ocular APCs, B cells, NKT cells, CD4⁺CD25⁺ Tregs, and CD8⁺ Tregs. γδ T cells are crucial for the generation of ACAID and for corneal allograft survival. However, the functions of γδ T cells in ACAID are unknown. Several hypotheses were proposed for determining the functions of γδ T cells in ACAID. The results indicate that γδ T cells do not cause direct suppression of delayed-type hypersensitivity nor do they act as tolerogenic APCs. In contrast, γδ T cells were shown to secrete IL-10 and facilitate the generation of ACAID Tregs. Moreover, the contribution of γδ T cells ACAID generation could be replaced by adding exogenous recombinant mouse IL-10 to ACAID spleen cell cultures lacking γδ T cells. The Journal of Immunology, 2006, 177: 8331–8337.

Materials and Methods

Animals

C57BL/6 (H-2b) mice; B6.129P2-Blm1TG1/J (β2-microglobulin knockout (KO) or class I-deficient) mice; δ-chain KO mice (TcrδKO) (C57BL/6J-Tcrδtm1Mom), IL-4 KO mice (B6.129P2-H4tm1Cry1J), IFNg KO mice (B6-IFN-γtm1J/J), and IL-10 KO mice (B6.129P2-IL-10tm1Kor/J) were purchased from The Jackson Laboratory. B6.129-H2-Ab1tm1Gru N12 (MHC-II-deficient) mice were purchased from Taconic Farms. All animals were housed and cared for in accordance with the guidelines of the University Committee for the Humane Care of Laboratory Animals, the National Institutes of Health Guidelines on Laboratory Animal Welfare, and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

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GL3 Ab was produced from hybridoma cells and purified by protein A columns and was graciously provided by Dr. L. Lefrancois (University of Connecticut, Farmington, CT). This Ab inhibits the function of γδ T cells by blocking the TCR (BD Biosciences). PE-conjugated anti-mouse isotype controls used were hamster IgG3 (BD Biosciences) plus complement (Cedarlane Laboratories). The Ab isotype depleted in vitro using purified rat anti-mouse CD8a (Ly-2; BD Biosciences) plus complement (Cedarlane Laboratories). CD8 T cells were depleted in vitro using purified anti-mouse CD8a (or isotype control Ab) plus complement. A LAT assay was performed using OVA plus OVA-immune spleen cells. *, p < 0.01, compared with all other groups except positive control.

Subcutaneous immunization
Mice were immunized by s.c. injection of 250 μg of OVA (Sigma-Aldrich) in PBS and emulsified 1/1 in CFA (Sigma-Aldrich). Each mouse received a 200-μl total volume.

AC injection
A Hamilton automatic dispensing apparatus was used to inject 100 μg (in 5 μl) of OVA into the AC as described previously (7, 14).

DTH assay
An ear swelling assay was used to measure DTH to OVA as described previously (6, 14). Results were expressed as: specific ear swelling = (24-h measurement − 0-h measurement) for experimental ear − (24-h measurement − 0-h measurement) for negative control ear.

Generation of ACAID-like APCs
ACAID-like APCs were generated in vitro using a previously described protocol that has been used extensively for analyzing Tregs in ACAID (5, 8, 28–32). Peritoneal exudate cells were collected from C57BL/6 mice and cultured overnight (2 × 10^6 cells per ml) in complete RPMI 1640 medium supplemented with 10 mg/ml OVA and 2 ng/ml human TGF-β2 (R&D Systems). These ACAID-like APCs induce peripheral tolerance that is identical with ACAID (4, 5, 7).

Generation of ACAID B cells
In an in vitro culture system was used to generate B cells that are capable of inducing the generation of Tregs that express the same phenotype as those induced by AC injection of the Ag (4, 28, 33). OVA-pulsed ACAID-like APCs generated in vitro as described above were cocultured for 48 h with B cells isolated from the spleens of normal C57BL/6 mice using CD45R (B220) microbeads (Miltenyi Biotec). These ACAID-inducing B cells were then adoptively transferred into either normal C57BL/6 mice or γδ T cell KO mice (4 × 10^6 B cells per mouse). The viability of B cells was determined by trypan blue exclusion immediately before the adoptive transfer and was always >95%.

In vitro ACAID model of Treg cell generation
We used an in vitro spleen cell culture system that generates Tregs that express the same properties and surface markers as Tregs produced by AC injection (4–7, 9, 34). These in vitro-generated Tregs are Ag-specific CD8^- T cells that can directly inhibit DTH (7).
A

FIGURE 3. γδ T cells do not play an Ag presentation role in ACAID.

A, γδ T cell KO mice were reconstituted with 5 × 10^6 γδ T cells from MHC-I KO mice, MHC-II KO mice, or wild-type mice. OVA was injected into the AC 7 days after γδ T cell reconstitution. Mice were immunized s.c. with OVA plus CFA 7 days after the AC injections with OVA. DTH responses to OVA were assessed 7 days after the s.c. immunization using ACAID-like APCs (5 × 10^6) were added to a large petri dish (Falcon 3003; BD Biosciences) containing 5 × 10^5 spleen cells harvested from either normal C57BL/6 mice or γδ T cell KO mice. Spleen cell cultures were incubated for 5–7 days at 37°C before being tested for the presence of Tregs. Viability of the in vitro-generated Tregs was always >95% as assessed by trypan blue exclusion.

In some experiments, two spleen equivalents of γδ T cells (sorted from spleen cells of C57BL/6 mice, MHC-I KO mice, or MHC-II KO) were used to reconstitute spleen cell cultures from γδ T cell KO mice. In our hands, the yield of γδ T cells from two spleens ranged from 5 × 10^3 to 10^6 γδ T cells. One group of the reconstituting γδ T cells was treated with chloroquine (80 μM/2 × 10^5 cells) (Sigma-Aldrich), checked for viability by trypan blue exclusion, and then added to reconstitute the in vitro spleen cell cultures. In other groups, 10 ng/ml of either recombinant mouse (rm) IL-10 (R&D Systems) or rmIL-4 (R&D Systems) was added to the culture medium.

Local adoptive transfer (LAT) assay

This assay was used to test for Tregs in ACAID (7, 9). Putative Tregs were injected (1 × 10^6 cells in 10 μl) with spleen cells (1 × 10^6 cells in 10 μl) collected from s.c. immunized donors and 10 mg/ml OVA into the left ear pinna of a naive mouse. The presence of Tregs was demonstrated by the suppression of the ear swelling responses mediated by immune spleen cells.

Statistics

Statistical significance of DTH was determined using Student’s t test.

Results

γδ T cells are necessary for the induction of ACAID

ACAID is a sequential process that is contingent on the presence of multiple cell populations in the spleen (3, 35). Among these, the Ag-presenting B cell population and γδ T cell population are both crucial for ACAID generation (7, 14). To determine the role of γδ T cells in the induction of ACAID, it was important to determine whether they acted upstream or downstream of B cells following AC injection of the Ag. The initial steps in the induction of ACAID can be recapitulated in vitro by coculturing Ag-pulsed, ACAID-like APCs with spleen cells from normal mice. After 5–7 days of in vitro culture, CD8+ Tregs are generated that have the same properties of Tregs induced by AC injection of the Ag. To determine whether γδ T cells acted downstream from B cells in the induction of ACAID, we tested the capacity of ACAID-inducing B cells to generate ACAID in mice deficient in γδ T cells. ACAID-inducing B cells were generated in vitro by coculturing OVA-pulsed, ACAID-like APCs with purified B cells suspensions for 2 days. B cells generated in such cultures will induce ACAID when adoptively transferred to naive mice (7, 8, 34). Accordingly, ACAID B cells were adoptively transferred into either γδ T cell KO mice or wild-type C57BL/6 mice depleted of γδ T cells using anti-γδ T cell Ab. The recipients of adoptively transferred B cells were immunized s.c. with OVA plus CFA and subsequently tested for OVA-specific DTH. The results indicated that, as expected,

an ear swelling assay. *, p < 0.01, compared with each of the three groups of γδ T cell reconstituted mice. B, Absence of γδ T cells precludes the generation of Tregs in vitro. ACAID-like APCs from B6 mice were generated in vitro, incubated (5 × 10^6) with spleen cells (5 × 10^6) from either B6 mice or γδ T cell KO mice. After 5–7 days, the spleen cell suspensions were tested in a LAT assay for their capacity to suppress DTH responses to OVA. C, ACAID-like APCs were generated in vitro, incubated (5 × 10^6) with spleen cells (5 × 10^6) from γδ T cell KO mice without or with γδ T cells from B6 mice, MHC-II KO mice, or MHC-I KO mice. Another group was incubated with chloroquine-treated γδ T cells from B6 mice. After 5–7 days, the spleen cell suspensions were tested in a LAT assay for their capacity to suppress DTH responses to OVA. *, p < 0.01, compared with positive control.
The splenic phase of ACAID involves the interactions between ocular APCs, B cells, NKT cells, CD4+ T cells, CD8+ T cells, and γδ T cells. Because γδ T cells act downstream from ACAID B cells, it is possible that γδ T cells are, in fact, the end-stage Tregs that suppress the expression of DTH. To test this hypothesis, ACAID Tregs were generated in vitro and spleen cell cultures were depleted of γδ T cells immediately before testing for Treg cell activity in a LAT assay. Previous studies have demonstrated that ACAID Tregs are CD8+ (36). Therefore, as a control, the in vitro generated Tregs were treated with anti-CD8 Ab plus complement to remove Treg activity. The results indicated that, as expected, removal of CD8+ T cells abolished Treg activity and allowed full expression of OVA-specific DTH (Fig. 2). By contrast, depletion of γδ T cells did not remove Treg activity and indicated that γδ T cells do not act as the end-stage Tregs in ACAID. The use of isotype controls for each of the depleting Abs did not interfere with the generation of Tregs as expected.

γδ T cells do not act as ACAID Tregs

It has been demonstrated recently that γδ T cells can act as APCs (37, 38). ACAID culminates in the generation of MHC-II-restricted CD4+ Tregs and MHC-I-restricted CD8+ Tregs and thus requires APCs that present Ag on MHC-I and MHC-II molecules (8). If γδ T cells act as APCs for the induction of ACAID, then reconstituting γδ T cell KO mice with γδ T cells from either MHC-I- or MHC-II-deficient donors should not restore ACAID in γδ T cell KO recipients. This hypothesis was tested by reconstituting γδ T cell KO mice with 5 × 10^5 γδ T cells from wild-type C57BL/6 mice, MHC-I-deficient mice, or MHC-II-deficient mice. One week after reconstitution, OVA was injected into the AC of reconstituted γδ T cell KO mice and control mice. Seven days later, mice were immunized s.c. with OVA emulsified in CFA. OVA-specific DTH was assessed 7 days after the s.c. immunization. As anticipated, nonreconstituted γδ T cell KO mice failed to develop ACAID (Fig. 3A). However, reconstitution with γδ T cells restored the capacity of γδ T cell KO mice to develop ACAID. Importantly, reconstitution with γδ T cells from either MHC-I- or MHC-II-deficient donors successfully restored ACAID, indicating that γδ T cells did not act as APCs for the induction of ACAID.

Additional experiments using spleen cell cultures confirmed that γδ T cells were needed for the generation of ACAID Tregs in vitro.
γδ T cells produce IL-10 to induce ACAID

Studies have shown that γδ T cells have the capacity to produce a variety of cytokines, including IL-10, IL-4, and IFN-γ (40–44). Other reports have suggested that the immunosuppressive function of γδ T cells is mediated mainly by cytokines (42, 45). Because ACAID is a Th2-like phenomenon with an immunosuppressive consequence, we hypothesized that γδ T cells need to secrete Th2 cytokines (such as IL-10 and IL-4) for the generation of efferent Tregs and down-regulation of DTH. Although IFN-γ is a signature cytokine for Th1 cells, it is also necessary for the generation of Tregs in some models and is known to mitigate some Th1-immune-mediated diseases (46). The possibility that production of IL-4, IL-10, or IFN-γ by γδ T cells was involved in the induction of ACAID was explored.

γδ T cell KO mice were reconstituted with $5 \times 10^5$ γδ T cells from IFN-γ KO mice, IL-4 KO mice, or IL-10 KO mice. One week after reconstitution, OVA was injected into the AC of reconstituted γδ T cell KO mice and control mice. Seven days later, mice were immunized s.c. with OVA emulsified in CFA. OVA-specific DTH was assessed 7 days after the s.c. immunization. The lack of either IFN-γ or IL-4 did not affect the ability of γδ T cells to reconstitute the generation of ACAID (Fig. 4). However, γδ T cells from IL-10 KO donors were incapable of restoring ACAID in γδ T cell KO mice and indicated that production of this cytokine was crucial for the γδ T cell’s contribution to the induction of ACAID (Fig. 4).

The aforementioned ACAID spleen cell culture system was used to confirm the role of γδ T cell-derived IL-10 in the induction of ACAID. OVA-pulsed ACAID-like APCs were added to spleen cell cultures from γδ T cell KO mice. Spleen cell cultures were then supplemented with rmIL-10, rmIL-4, or γδ T cells from wild-type mice. The generation of ACAID Tregs was determined 5 days later using the aforementioned LAT assay. The results indicated that ACAID was restored in spleen cell cultures from γδ T cell KO mice by the addition of rmIL-10 or reconstitution with γδ T cells from wild-type mice (Fig. 5).

However, addition of IL-4 did not restore the generation of ACAID. The effect shown with rmIL-10 was not merely a nonspecific effect of IL-10 on spleen cells, because no Tregs were detected unless OVA-pulsed APCs were present in IL-10 supplemented culture medium. These results suggest that the major function of γδ T cells in the induction of ACAID is their secretion of IL-10.

Discussion

Peripheral tolerance that is induced when Ag enters an immune privileged site, such as the eye, regulates harmful immune responses that can lead to irreparable injury to innocent bystander cells that are incapable of regeneration. Corneal endothelial cells and cells forming the retina are examples of terminally differentiated ocular cells that cannot undergo mitosis and regenerate. Injury to either of these cell populations can lead to blindness. Whether ACAID prevents the generation or the expression of autoimmune Th1 immune responses in the eye under normal physiological conditions remains to be established. However, it is noteworthy that inducing ACAID by AC injection of either retinal-specific autoantigens or corneal alloantigens results in the mitigation of experimental autoimmune uveitis and the acceptance of corneal allografts, respectively (47–49).

The induction of ACAID involves a complex series of events and the participation of at least four organs (eye, thymus, spleen, and sympathetic nervous system) and at least six different cell populations (ocular APCs (3), B cells (7, 8), γδ T cells (14, 45, 50), NKT cells (32, 51), CD4+ T cells (5, 6), and CD8+ T cells (36)). After capturing Ag in the AC, F4/80+ ocular APCs migrate to the thymus (33) and spleen (52). In the spleen, the F4/80+ ocular APCs interact with NKT cells, CD4+ T cells, and B cells (51, 53). Recent evidence suggests that splenic B cells capture antigenic peptides released by the F4/80+ ocular APCs and present these Ags to both CD4+ and CD8+ T cells, leading to the generation of efferent-acting Tregs (8). Where γδ T cells function in the induction of ACAID remains to be identified.

There are several strategic points in the induction of ACAID where γδ T cells might function. The recent report that γδ T cells can function as APCs (37) led us to test the hypothesis that γδ T cells act as ancillary APCs in the induction of ACAID. However, two findings argue against this role. First, chloroquine treatment inhibits the Ag-presenting function of γδ T cells (37) yet does not affect the capacity of γδ T cells to contribute to the generation of

![Figure 5](http://www.jimmunol.org/Download/8355F5.jpg)
ACAID. The induction of ACAID requires simultaneous presentation of Ags on both MHC-I and MHC-II molecules (8), yet the present findings indicate that γδ T cells from mice deficient in the expression of either MHC-I or MHC-II molecules were still capable of contributing to the induction of ACAID.

The results reported in this study indicate that the γδ T cell acts downstream from the ACAID B cell, as adoptive transfer of ACAID B cells into γδ T cell KO mice fails to induce ACAID. We considered the obvious explanation that γδ T cells act as effector Treg cells that inhibited the expression of DTH, as γδ T cells are known to secrete immunosuppressive and anti-inflammatory molecules (45). Moreover, some γδ T cell populations express the CD8 molecule, which is also found on ACAID efferent Tregs (11). However, our findings demonstrate that removal of IL-10 KO donors cannot. Moreover, the contribution of γδ T cells from ACAID CD8+ Treg suspensions does not abolish CD8+ T cell-mediated suppression of DTH, thereby confirming that γδ T cells do not function as ACAID effector Tregs.

We are attracted to the hypothesis that γδ T cells act as ancillary producers of IL-10, which is known to be crucial for the induction of ACAID (54). This proposition is supported by the finding that γδ T cells from wild-type mice, IL-4 KO mice, or IFN-γ KO mice can restore ACAID in γδ T cell KO mice, while γδ T cells from IL-10 KO donors cannot. Moreover, the contribution of γδ T cells in the induction of ACAID could be replaced by simply adding exogenous rmIL-10 cytokine to ACAID spleen cell cultures lacking γδ T cells. These results fit well with data showing a cytokine secretion function for γδ T cells in other systems (40–45, 55).

Many unanswered questions remain in relation to the role of γδ T cells in ACAID. The segregation of γδ T cells into functionally specialized cell populations in correlation with TCR variable gene expression (57), raises an interesting yet challenging question. This question is determined to which of these subpopulations of γδ T cells is particularly involved in ACAID and what kind of interaction it has with other γδ T cells and other cells. Because γδ T cells were shown to interact with cells of the innate system at many levels (57), unraveling these interactions in the immunoregulatory setting of ACAID is also important. Finally, the details of the Ag recognition process by γδ T cells in ACAID need to be thoroughly investigated.

Disclosures

The authors have no financial interest in this study.

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