Mechanisms of Peripheral Tolerance following Intracameral Inoculation Are Independent of IL-13 or STAT6

Takahiko Nakamura, Ania Terajewicz and Joan Stein-Streilein

*J Immunol* 2005; 175:2643-2646; doi: 10.4049/jimmunol.175.4.2643
http://www.jimmunol.org/content/175/4/2643

**References**
This article cites 38 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/175/4/2643.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Mechanisms of Peripheral Tolerance following Intracameral Inoculation Are Independent of IL-13 or STAT6

Takahiko Nakamura, Ania Terajewicz, and Joan Stein-Streilein

The peripheral tolerance that is elicited by the anterior chamber-associated immune deviation (ACAID) protocol is characterized by impairment of Th1 responses such as delayed-type hypersensitivity. It has been proposed that suppression of Th1 responses is mediated by a deviation toward Th2 responses. Because NKT cells have a prominent role in ACAID and NKT cell-derived IL-13 is required in a tumor model of tolerance, we postulated that NKT cell-derived Th2 cytokines might have a role in ACAID. However, contrary to the tumor model, in this study we show that NKT cells from IL-13-deficient mice or IL-4/IL-13 double deficient mice were able to reconstitute the capability of Jα18-deficient mice (lacking invariant NKT) to develop peripheral tolerance postintracameral inoculation of Ag. Also, we were able to induce peripheral tolerance directly in IL-13-deficient, IL-4/IL-13-double deficient, and STAT6-deficient mice by inoculation of Ag into their eye. We conclude that neither IL-4 nor IL-13 cytokines are required for the generation of efferent CD8+ T regulatory cells during eye-induced peripheral tolerance. We propose that Ags inoculated into the anterior chamber of the eye induce the immunoresponse to deviate from producing immune T effector cells to producing efferent T regulatory cells, rather than deviating from Th1- to Th2-type effector cells. The Journal of Immunology, 2005, 175: 2643–2646.

Once Ag is introduced in the eye, bone marrow-derived F4/80+ APCs capture the Ag in the eye and travel via the blood vessels to the marginal zone (MZ) of the spleen (1, 2). The eye-derived APCs secrete MIP-2 chemokine that attracts invariant NKT (iNKT) cells along the way to the MZ (2). Following ligation of their TCR with CD1d expressed by the F4/80+ APC and MZ B cells, iNKT cells produce IL-10 and secrete RANTES (3). RANTES, in turn, recruits more F4/80+ APCs and T cells to the MZ, and the cells assemble in clusters (4). The appearance of Ag-specific, CD8+ T regulatory (Tr) cells depends on the presence of CD1d-restricted iNKT cells (4, 5). In contrast to a recent report that anterior chamber-associated immune deviation (ACAID) induction suppresses Th2 responses (6), earlier reports suggested that ACAID deviated T cell responses from a Th1- to a Th2-type response to effect the suppression of delayed-type hypersensitivity (DTH). In keeping with this notion is the observation that ACAID mice are unable to make complement-fixing Abs but adequately produce IgE and IgG1 (7). Contrary to the fact that IL-4/IL-13 mice are able to generate CD8+ Tr cells that suppress DTH, spleen cells from wild-type (WT) mice 7 days post-anterior chamber (a.c.) inoculation produce significant amounts of IL-4 when restimulated in vitro with Ag (8), indicating a role for IL-4 in a yet undetermined aspect of ACAID.

TGF-β is an essential cytokine needed in the generation of Tr cells in ACAID (9, 10). The need for Ag inoculation into the eye for tolerance induction can be bypassed, by i.v. injection of TGF-β-treated, Ag-pulsed APC (tolerogenic APC) into naive syngeneic recipients and the subsequent generation of CD8+ Tr cells capable of suppressing DTH responses (7). Addition of anti-TGF-β mAb to peritoneal exudate cell (PEC) cultured in aqueous humor abolishes the ability of aqueous humor-cultured PEC to induce ACAID or to generate CD8+ Tr in recipients (9, 10). In fact, TGF-β treatment and Ag exposure of a variety of APC populations produce a semi-mature APC that induces most, if not all, aspects of peripheral tolerance induced by Ag inoculation into the eye.

TGF-β, IL-10, IL-4, and IL-13 are all capable of interacting to down-regulate immune responses. TGF-β is produced by various types of cells and usually secreted in latent form (11). Both IL-10 and IL-13 are reported to induce secretion and activation of TGF-β. IL-10 induces TGF-β secretion by Tr cells in a mouse model of experimental colitis (12). IL-12 and IFN-γ inhibit TGF-β production of CD4+ T cells, whereas IL-10 enhances TGF-β production by indirectly affecting IL-12 secretion (and production) (13). Activated immune cells show reduced TGF-β2 receptor expression, but IL-10 reverses the down-regulation (14). IL-13 is reported to stimulate production of active TGF-β in a pulmonary fibrosis model (15). Furthermore, IL-4 and IL-13 down-regulate IFN-γ production and thus prevent the interference with intracellular TGF-β signaling by IFN-γ-dependent SMAD-7 production (16). More recently, it was reported that tumor effector cells could be generated in STAT6-deficient mice but not in IL-4α receptor-deficient mice, implicating a role for IL-13-dependent immunosuppression in tumor growth (17).

These conflicting reports suggest a need to clarify the role of Th2 cytokines in ACAID. The focus of this investigation was to evaluate the contribution of the Th2 cytokine, IL-13, in generating CD8+ Tr cells in ACAID. Moreover, in another model of tumor immunosuppression, IL-13-producing NKT cells regulate anti-tumor immunity.
Materials and Methods

Mice

Female BALB/c mice were purchased from Taconic Farms; IL-13−/− and IL-4−/−/IL-13−/− mouse breeders were generous gifts from A. McKenzie. University of Oxford (Oxford, U.K.). IL-13−/− mice were generated from 129 × C57BL/6 mice, with the disruption in exon 1 (21), and were backcrossed for seven generations onto the BALB/c strain. IL-4−/−/IL-13−/− mice were generated from 129 × C57BL/6 mice (22) and backcrossed for seven generations onto the BALB/c strain. STAT6−/− mice on C129.S2 background and WT controls were obtained from The Jackson Laboratory. Jα18−/− (BALB/c background) mice were bred in the Schepps vivarium from breeders that were a generous gift from M. Taniguchi (Chiba University Graduate School of Medicine, Chiba, Japan). Mice were housed on a 12:12-h light-dark cycle and provided food and water ad libitum. All animals were treated humanely and in accordance with the guidelines set forth by the Schepps Eye Research Institute Animal Care and Use Committee and National Institutes of Health guidelines.

Peripheral tolerance induction via the eye and assay for DTH

Peripheral tolerance was induced as previously described (23). In brief, 2 μl of OVA (50 μg) in PBS were inoculated into the a.c. of the eye using a specially prepared glass needle. One week later, mice were immunized with OVA (100 μg/50 μl in HBSS) emulsified in CFA (50 μl). To test for DTH, OVA (200 μg in 10 μl PBS) was inoculated into the ear pinnae 1 wk after immunization, and ear swelling was measured 24 h later with an engineer’s micrometer (Mitsutoyo).

Reconstitution of Jα18−/− mice and ACAID induction

To reconstitute the iNKT cells deficiency in Jα18−/− mice, Jα281−/− mice were gamma-irradiated (cesium, 200 rad, Mark 1 irradiator; J. L. Shepherd) 24 h before they received (i.v.) pan-T cell IMMULAN column-enriched spleen cells (10^7) from either WT, IL-13−/−, or IL-4−/−/IL-13−/− mice. Twenty-four hours after reconstitution, Jα281−/− mice were inoculated (a.c.) with OVA (50 μg/2 μl in PBS) to generate Ag-specific CD8+ Tr cells.

Local adoptive transfer (LAT) assay

To assess the development of effector regulatory cell function, a modified LAT assay was performed (23). In brief, effector cells were generated in WT BALB/c mice immunized (s.c.) with OVA (100 μg, 1:1 HBSS:CFA). Ten days later, effector T cells were enriched using pan-T cell IMMULAN columns (Biotecx Laboratories). 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 mice 7 days after a.c. inoculation of OVA. Stimulator cells were prepared by pulsing thioglycolate-induced PEC (10^7/ml) with OVA (5 mg/ml). Effector cells (5 × 10^5), stimulator cells (2 × 10^5), and Tr cells (5 × 10^5) were admixed and resuspended in 10 μl of HBSS and inoculated 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 used as regulatory cells for positive control.

Statistics

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

Results

NKT cells do not need to make IL-13 to reconstitute ACAID in Jα18-deficient mice

Based on the role of NKT cell-derived IL-13 in a tumor model of suppression, we tested whether NKT cell-derived IL-13 played a role in ACAID (18). It is known that Jα18−/− mice are unable to develop peripheral tolerance post-eye inoculation of Ag, unless they are reconstituted with NKT cells from WT mice (4). To test the hypotheses that IL-13 derived from NKT cells was required for peripheral tolerance induction, Jα18−/− mice were reconstituted with T cell-enriched spleen cells (pan-T cell Immunol column passed) from WT, IL-13−/−, or IL-4−/−/IL-13−/− mice, and their eff erent Tr cell function was assessed in a LAT assay (Fig. 1). T cells from nonreconstituted Jα18−/− mice were unable to suppress DTH ear swelling, but T cells harvested from Jα18−/− mice that were reconstituted with IMMULAN-enriched T cells from WT, IL-13−/−, or IL-4−/−/IL-13−/− mice suppressed the adoptively transferred DTH response. Because the Jα18−/− mice are only missing the iNKT cell population, we felt that a mixed T cell population was better to use for reconstitution than an enriched NKT cell population, which might be altered by Ab binding and the sorting process.

IL-13−/−/IL-4−/−/IL-13−/− mice developed ACAID

Because Jα18−/− mice do have cells that are capable of producing IL-13, we tested whether IL-13 derived from non-NKT cells were important for tolerance development in IL-13−/− mice. We reasoned that if IL-13 were important for eye-induced peripheral tolerance, we would not be able to induce ACAID in IL-13−/− mice. In brief, IL-13−/− mice were inoculated (a.c.) with OVA 7 days before sensitization (s.c.) with OVA and CFA and 14 days before ear challenge with the Ag. The DTH response was suppressed (evidence of peripheral tolerance) in the IL-13-deficient mice, indicating that there is no requirement for IL-13 in the generation of the eff erent Tr cell responsible for diminishing ear swelling (Fig. 2).

Although it has been reported that IL-4−/− mice get ACAID, we further explored the role of IL-4 in ACAID induction in IL-4−/−/
IL-13−/− double deficient mice. Again, ACAID induced by intracameral inoculation of Ag suppressed DTH responses; thus, we concluded that neither IL-4 nor IL-13 is needed for generation of the efferent CD8+ Tr cell in ACAID.

**STAT6−/− mice developed peripheral tolerance via the eye**

To further confirm the independence of eye-induced peripheral tolerance from either IL-4 or IL-13 signals, STAT6−/− mice were tested for their susceptibility to peripheral tolerance induction by a.c. inoculation of Ag (Fig. 3). Similar to results with IL-4−/− on IL-13−/− mice, a.c. inoculation of STAT6−/− mice induced tolerance to the Ag, and ear challenge of the mice with Ag showed a significant suppression of Ag-induced DTH compared with non-a.c.-inoculated mice. This result confirms our postulate that induction of peripheral tolerance via the eye is not dependent on signals derived through the IL-4α or the IL-13β receptors because both of these receptors use STAT6 in their pathway.

**Discussion**

During the 1970s and later, ACAID was thought to promote suppression by inducing a deviation in the immune response from a Th1- to a Th2-type response (8, 24, 25). However, Kosiewicz et al. (8) reported that, although IL-4 was generally up-regulated after a.c. injection of Ag in naive mice, ACAID could be induced in IL-4−/− mice. Although these authors did not consider a role for IL-4 in the generation of the efferent CD8+ Tr cell, they did show that the CD8+ Tr cell function as suppressor cell did not depend on Th2 cytokines. The possibility remains that IL-4 is needed for the humoral response during the ACAID process (26). It is known that there is both an afferent Tr cell and an efferent Tr cell generated following the injection of Ag into the eye (20, 27). However, most assessments of peripheral tolerance in this model are by measurement of the efferent arm of a DTH response, i.e., suppression of ear swelling (28). Such suppression is necessarily induced by an efferent Tr cell shown to be Ag specific and CD8+ (6, 27, 29). However, this choice of assessment further contributed to the idea that the Th1 response is suppressed by a dominant Th2 response. The fact that there is also a switch in types of Abs during tolerance induction compared with induction of inflammatory immune responses further supported the idea that introduction of Ag into the a.c. deviated immune responses toward the Th2 phenotype (7). Moreover, recall responses of spleen cells from ACAID mice produced IL-4 in vitro (8). Because Th2 responses are capable of suppressing Th1 responses, the idea that ACAID regulated DTH responses by releasing Th2 cytokines is more secure.

However, conflicting data was also reported, because peripheral tolerance was induced in IL-4−/− mice post-a.c. inoculation of Ag (7), and, later, Katagiri et al. (6) showed that both ACAID induction and ACAID-like tolerogenic APC (generated in vitro by exposure of PEC to TGF-β and Ag) were able to down-regulate Th2 responses in a lung model of airway hyperreactivity and inflammation in the mouse. Also contradictory to Th2 cells being involved in ACAID is our report that conventional CD4+ T cells are not needed for the generation of CD8+ Tr cells (30). In that study, we showed that intracameral inoculation of Ag induces peripheral tolerance and CD8+ Tr cells into the eye of class II-deficient mice (which lack conventional CD4+ T cells) (30). Thus CD4+ Th cells are not needed for the generation of the efferent CD8+ Tr cell in ACAID. Alternatively, although IL-4 is predominately made by CD4+ T cells, IL-13 could still be produced in class II-deficient mice by many cell types, thus allowing for the possibility that select Th2 cytokines contribute to ACAID. A report by Terabe et al. stimulated our interest when it showed that, in a mouse model of tumor recurrence, NKT cell-derived IL-13 was a major contributor to the tolerance (18) of the tumor. However, we show in this study that IL-13 has no role in the development of efferent Tr cells and peripheral tolerance in ACAID.

IL-4 and IL-13 operate through the IL-4 and IL-13 receptors, respectively. Both receptors share the α-chain of the IL-4 receptor (31, 32) and promote the STAT6 factor for activation (33). Contact hypersensitivity to various allergens is reduced in STAT6−/− mice, whereas DTH reaction to sheep RBC is maintained (34). IL-13 is associated with the stimulation of TGF-β production in the development of fibrosis (15). Because neither IL-13 nor STAT6 is required for generation of CD8+ Tr cells in peripheral tolerance induced in the eye, the other mechanisms known for stimulating and activating TGF-β, including thrombospondin (35) and IL-10 (15), may be sufficient.
There are mechanistic differences between the tumor tolerance model and peripheral tolerance related to a.c. inoculation. Unlike peripheral tolerance induced in the eye, iNKT cell-derived IL-13 activates Grit1^+ C11b^+ myeloid-derived suppressor cells, which in turn produce immunosuppressive TGF-β (19). The role of iNKT cells in eye-induced peripheral tolerance is that they appear to facilitate cellular interactions within the MZ of the spleen where CD8^+ Tr cells are generated. The effector CD8^+ Tr cell suppresses immune effector cells (24) in an Ag-specific fashion. There is some evidence that the iNKT cells promote the recruitment of cells to the MZ of the spleen (36), as well as create a tolerance-inducing environment by secreting immunosuppressive factors (37, 38).

In conclusion, our data suggest that IL-4, IL-13, and STAT6 are not needed for the generation of Ag-specific effector CD8^+ Tr cells. Together with the fact that conventional CD4^+ T cells are also not needed (30), these data strongly contradict the notion that inoculation of Ag into the eye induces a deviation from Th1- to Th2-type cytokines. In fact, we show here that Th2 cytokines and Th2-type cytokines. In fact, we show here that Th2 cytokines and Th2-type cytokines.

References


