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NKT Cell-Derived RANTES Recruits APCs and CD8+ T Cells to the Spleen During the Generation of Regulatory T Cells in Tolerance

Douglas E. Faunce*† and Joan Stein-Streilein2*‡

The induction of peripheral tolerance via immune privileged sites such as the eye requires splenic colocalization of NKT cells and CD1d+ tolerogenic F4/80+ APCs, both of which are needed for the generation of CD8+–regulatory T (Tr) cells. Whereas tolerogenic APCs secrete the chemokine macrophage-inflammatory protein-2 for the purpose of recruiting NKT cells, the signals responsible for recruiting potential Tr cells and additional APCs to the spleen are not known. Here we examined the ability of CD1d-stimulated NKT cells to produce chemokines that can recruit other cells needed for tolerance. Our results show that NKT cells stimulated by either CD1d-transfected fibroblasts in vitro or CD1d+ tolerogenic APCs both in vivo and ex vivo produced RANTES in a CD1d-dependent manner. The requirement for RANTES in tolerance was demonstrated by studies in which RANTES blockade in vivo prevented not only APC accumulation in the spleen but also the generation of CD8+ Tr cells that suppress Th1 immunity. Thus, CD1d-restricted NKT cells provide critical signals for orchestrating the accumulation of cells needed for tolerance induction. These data expand our current knowledge of RANTES beyond its role in Th1 immune responses to show its importance in tolerance induction and add a novel aspect to our understanding of the role of NKT cells in tolerance. Understanding the precise mechanisms involved in tolerance induction may lead to more effective therapeutic strategies for autoimmunity and graft rejection. The Journal of Immunology, 2002, 169: 31–38.

In recent years, NKT cells have received considerable attention for their ability to promote Th1-like immunity (1–3). However, work done by our laboratory and others has also demonstrated their central importance in tolerance to both foreign and self-Ags (4–7). It is believed that the ability of the NKT cells to shape the T cell response results partly from their ability to rapidly release large quantities of immunomodulatory cytokines. Whether NKT cells provide a source of chemokines for controlling cell migration during tolerance remains unknown. There are many reports of chemokine production by conventional lymphocytes, monocytes, macrophages, and dendritic cells, yet there is little or no information on chemokine production by NKT cells. NKT cells are unconventional lymphocytes that express most NK cell markers and an invariant, CD1d-restricted TCR. Once their TCR is stimulated by CD1d, NKT cells can release cytokines, including IFN-γ, IL-4, and IL-10, very quickly (8–10).

Chemokines are well known for their roles in coordinating leukocyte migration during conventional immunity and inflammation; however, their roles in regulating immune cell movement during tolerance induction are not well understood. Chemokines belong to a family of low molecular mass cytokines (8–10 kDa) that direct the migration of leukocytes during immune and inflammatory responses (11–14). Categorized according to the positions of cysteines in their N termini, the chemokine families are subdivided into four groups including CC, CXC, CX3C, and C. Among the best studied chemokines are RANTES, a member of the CC family, and IL-8, the prototypic chemokine belonging to the CXC family (15). During the past decade, chemokines were found not only to control cellular migration but also to modulate the expression of cytoskeletal associated genes, activate intracellular signal transduction events such as Ca2+ flux, and induce cytokine production (11, 12, 16).

The chemokines responsible for directing leukocyte migration during immune homeostasis and inflammation are well characterized (11, 12, 17, 18), but there is a general lack of information regarding the role of chemokines and cell trafficking during the induction of peripheral tolerance. Previously, we showed that during the induction of tolerance to Ags injected into the eye, eye-derived APCs selectively increased expression of macrophage-inflammatory protein (MIP)1,2 during their preferential migration to the splenic marginal zones (MZ) and that MIP-2 was responsible for recruiting NKT cells to the spleen (19). Additionally, we observed that the number of F4/80+ APCs increased in the MZ where they formed discrete clusters with the NKT cells and CD8+ T cells in the splenic MZ where they remained at least 7 days (19). This is in contrast to cellular interactions during immune inflammatory responses in which the interacting cells migrate from the MZ to the T cell areas within 4–6 h of entrance into the spleen.
(20). These data also suggest that there must be migration and retention signals involved in the cell cluster formation in the MZ.

In our efforts to understand cell trafficking and interactions during tolerance induction, we proposed that CD1d/TCR ligation of NKT cells stimulated them to release chemokines that orchestrate the colocalization of additional cells needed for the generation of Tr cells and tolerogenic immune responses. Furthermore, disruption of colocalization of crucial cell types is implied if interference with chemokine signaling prevents generation of Tr cells and tolerance. A useful model for elucidating mechanisms involved in tolerance induction is anterior chamber-associated immune deviation (ACAID). ACAID is demonstrated experimentally by the inability of anterior chamber (a.c.)-inoculated mice to display Ag-specific delayed-type hypersensitivity (DTH) responses in the periphery (21). ACAID requires both the eye and spleen to remain intact for the first 3 days after a.c. injection, after which only the spleen is required (22). Once eye-derived APCs arrive in the spleen, they engage newly recruited NKT cells and stimulate their production of the immunosuppressive cytokine IL-10 (3) that contributes to the generation of Ag-specific CD8<sup>+</sup> T-regulatory (Tr) cells that actively suppress the expression phase of DTH (21, 23).

Here we present data to show that stimulation of NKT cells by CD1d-bearing, tolerogenic APCs (both in vitro and in vivo) leads to their production of RANTES and the subsequent recruitment of the additional APCs needed for tolerance. Although many cell types are reported to make RANTES during inflammatory conditions, we report that the NKT cell-derived RANTES is critical for the generation of CD8<sup>+</sup> Tr cells during tolerance induction.

Materials and Methods

**Mice**

Female C57BL/6 mice used in these experiments were obtained from the Schepens Eye Research Institute Vivarium (Boston, MA) or Taconic Farms (Taconic, NY). 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 Schepens Eye Research Institute Animal Care and Use Committee and the National Institute of Health guidelines.

**Cell culture and cell enrichment**

DN32.D3 NKT cells were maintained in RPMI, 10% FCS (Life Technologies, Gaithersburg, MD). Parental L cells and L cells transfected with murine CD1d (1) were maintained in DMEM, 10% FCS (Life Technologies). For all experiments described here, cells were cultured in serum-free medium (SMF) consisting of RPMI 1640, 10 mM HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Life Technologies), supplemented with 0.1% BSA and ITS<sup>+</sup> culture supplement (1 µg/ml iron-free transferrin, 10 ng/ml linoleic acid, 0.3 ng/ml Na<sub>2</sub>Se, and 0.2 µg/ml Fe(NO<sub>3</sub>)<sub>3</sub>; Collaborative Biomedical, Medford, MA). The peritoneal exudate cells (PECs) used in the in vitro coculture systems were cultured overnight in SMF ± OVA (5 ng/ml) ± TGF-β2 (5 ng/ml). PECs were then washed with HBSS to remove excess OVA and TGF-β. Enriched T and NKT cells were isolated using IMMULAN goat anti-mouse IgG-concoated beads (BiotecX Laboratories, Houston, TX). Enriched T and NKT cells (2.0 × 10<sup>6</sup> cells/ml) were cocultured with the PECs in six-well plates for either 30 min or 1 h. NKT cells were depleted from IMMULAN-enriched cells by incubation with anti-NK1.1 mAb (clone PK136), followed by incubation with baby rabbit complement (Pel-Freez, Brown Deer, WI).

**Isolation and analysis of chemokine mRNA**

Total cellular RNA was isolated from DN32.D3 NKT cells, IMMULAN-enriched T cells, and FACS-sorted NKT cells using TriZol reagent (Life Technologies). RNA isolation was performed according to the manufacturer’s protocol. Chemokine mRNA was analyzed with the RiboQuant multiprobe RNase protection assay (RPA) and the mK<sup>+</sup>-mouse chemokine (chemokine) DNA immobilization set (BD Pharmingen, San Diego, CA) encoding lymphotixin (Lm), RANTES, eotaxin, MIP-1α, and -1B, MIP-2, inflammatory protein-10 (IP-10), monocyte chemotactant protein-1 (MCP-1), T cell activation (TCAS3), L32, and GAPDH. Total RNA (5 µg) was used for the RPA, followed by electrophoresis on polyacrylamide sequencing gels. The gels were dried at 80°C for 1 h, and the mRNA bands were detected by phosphorimaging with a Bio-Rad Molecular FX Imaging system (Bio-Rad, Hercules, CA). Chemokine mRNA quantification was completed with QuantiOne Molecular Imaging Software (Bio-Rad). To compensate for loading imperfections and for quantitative purposes, chemokine mRNA bands were normalized to GAPDH bands. Scanning densitometry results are expressed as relative OD units (OD/OD<sub>GAPDH</sub> where “x” is the OD of the chemokine band of interest).

**RT-PCR**

Total cellular RNA was isolated from FACS-sorted NKT cells using TRIzol, and 100 ng of total RNA were reverse transcribed and amplified using the Access RT-PCR system (Promega, Madison, WI) according to the manufacturer’s specifications. RT-PCR products were resolved by electrophoresis in a 1.5% agarose gel containing GelStar nucleic acid stain (FMC BioProducts, Rockland, ME). The bands were visualized and gels were photographed using a Bio-Rad Molecular FX Imaging station and GelDoc (Bio-Rad Laboratories). The primers used were: murine RANTES, sense 5′-GGT CCC ACC TCA AGG AGT AT-3′, antisense 5′-GGG AAC CTT AT A TA CAG GGT CA-3′; murine β-actin, sense 5′-GGT GCC CGC TCT AGG CAC CAA-3′, antisense 5′-CTC TTT GAT TGC AGC CAG GAT TTC-3′.

**Analysis of RANTES protein**

Supernatants from L-CD1d/DN32.D3 cocultures that had been stored at −20°C were thawed at room temperature and assayed for RANTES protein using a murine RANTES Quantikine-M sandwich ELISA (R&D Systems, Minneapolis, MN). All samples were assayed undiluted and were measured in duplicate. The ELISA was performed according to the manufacturer’s instructions.

**Antibodies**

The Abs used for flow cytometry were as follows: FITC-conjugated NK1.1 (PK136), FITC-conjugated CD3ε (BD Pharmingen); and PE-conjugated F4/80 (Caltag Laboratories, Burlingame, CA). The Abs used for in vivo assays were: rat anti-mouse RANTES (R&D Systems); rat anti-mouse CD1d (purified from hybridoma clone 3C11; gift of Dr. S. Balk, Beth Israel Deaconess Medical Center, Boston, MA); rat IgM (BD Pharmingen), and rat IgG (Sigma-Aldrich, St. Louis, MO). Abs delivered in vivo were suspended in 100 µl sterile PBS and injected i.v.

**Intracellular cytokine immunostaining for detection of RANTES**

Erythrocyte-free, total splenocyte suspensions were immunostained for cell surface markers Cy5-TCR-β chain and FITC-NK-1.1 as previously described (19). The cells were then fixed and permeabilized with PermeaFix (Ortho Diagnostics, Raritan, NJ) according to the manufacturer’s protocol and incubated with biotinylated anti-RANTES mAb (R&D Systems) followed by R-PE-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA). To prevent background staining due to endogeneous biotin, the cells were treated with the DAKO Biotin Blocking System (DAKO, Carpinteria, CA) before incubation with the biotinylated anti-RANTES mAb. The percent of RANTES-positive NKT cells was determined by flow cytometric analyses of PE-positive cells within the NKT (FITC<sup>+</sup> Cy5<sup>+</sup>) population.

**Anterior chamber inoculation of Ag**

Ags were administered into the anterior chambers of mice anesthetized with ketamine-xylazine as previously described (19). Briefly, the cornea was punctured with a 30-gauge needle, and the aqueous humor was drained (~2 µl). The anterior segment of the eye was reinflated with ~2 µl of air. Using finely drawn glass needles, 50 µg of OVA (2 µl of a 25-µg/ml solution in HBSS) were instilled into the anterior chamber, displacing the air. The OVA solutions used in all studies were passed through DetoxiGel AfinityPak polymyxin B columns (Pierce, Rockford, IL) to remove contaminating endotoxin-LPS (98% efficiency of endotoxin removal of concentrations up to 2 ng/ml).

**Local adoptive transfer assay (LAT)**

LAT was used to test for the presence or absence of regulatory CD8<sup>+</sup> T cells as previously described (3, 19). Briefly, OVA-primed effector T cells were generated by immunizing C57BL/6 mice with OVA in CFA (Sigma-Aldrich). Seven days later, the spleens were collected and enriched for Tr cells by passage over IMMULAN columns (Biotecx). Tr cells were enriched from the spleens of mice that received anti-RANTES mAb or control IgG (i.p.) 7 days post-OVA or HBSS (a.c.). Stimulator cells were
obtained by culturing thioglycolate-elicited PECs with OVA (5 ng/ml) overnight. Stimulator, effector, and regulatory cells (5 × 10^5 each) were resuspended in 10 μl HBSS and injected intradermally into the ear pinnae of naive mice. The change in ear thickness was measured at 24 and 48 h after ear challenge using an engineer’s micrometer (Mitutoyo; MTI, Paramus, NJ).

Results

CD1d stimulates DN32.D3 NKT cells to express RANTES

CD1d stimulation of the invariant TCR on NKT cells induces their expression of immunomodulatory cytokines including IL-4, IL-10, and IFN-γ (1–3). To determine whether CD1d stimulation induced the expression of chemokines in NKT cells, we cocultured CD1d-transfected fibroblasts (or untransfected control cells) (24) with NKT hybridoma (DN32.D3) cells. After 30 min or 1, 2, 4, or 8 h of coculture, total cellular RNA was isolated from the DN32.D3 NKT cells and examined for chemokine mRNA expression with a MultiProbe RPA. NKT cells that were cocultured with untransfected fibroblasts constitutively expressed low levels of RANTES mRNA throughout the time course (Fig. 1A). In contrast, NKT cells cocultured with CD1d transfectants increased their expression of RANTES mRNA over time, beginning with 30 min, peaking at 4 h, and returning to baseline after 8 h (Fig. 1, A and B).

Although the RPA template contained probes for several chemokines (Ltn, eotaxin, RANTES, MIP-1α and -1β, MCP-1, IP-10, and TCA3), only RANTES was expressed after CD1d stimulation of DN32.D3 NKT cells. A very minor level of eotaxin expression was also noted among CD1d-stimulated NKT cells (band located directly beneath the RANTES band in Fig. 1); however, the level of expression does not likely represent a biologically relevant change in mRNA.

To show that RANTES protein secretion followed mRNA expression, we performed ELISA on the supernatants from CD1d fibroblast/DN32.D3 NKT cocultures (Fig. 1C). As expected, CD1d-stimulated NKT cells began to secrete RANTES protein after 1 h of stimulation. RANTES protein production was greatest in samples collected after 4 h of coculture. Moreover, the RANTES protein production was CD1d dependent because DN32.D3 NKT cells cocultured with untransfected fibroblasts secreted only baseline levels of RANTES. Because the transfected fibroblasts do not express activation receptors other than CD1d, we concluded that stimulation by CD1d appears to be sufficient for the production of RANTES by NKT cells.

Stimulation of ex vivo NKT cells by tolerogenic APCs leads to the rapid production of chemokines

Because DN32.D3 NKT cells hybridoma cells do not necessarily express all of the characteristics of freshly isolated NKT cells, we examined the production of chemokines by ex vivo NKT cells after culturing with resting, tolerogenic, or immunogenic APCs in vitro. In brief, IMMULAN-enriched T and NKT cells were prepared from the spleens of B6 mice as previously described (7) and

![FIGURE 1. Chemokine mRNA expression in DN32.D3 NKT cells after coculture with CD1d-transfected fibroblasts. Confluent monolayers of L cells (L-DAP-3 fibroblasts) either untransfected (L cells) or transfected with a murine CD1d construct (L-CD1d) were established in 25-mm tissue culture dishes. DN32.D3 NKT cells (2.0 × 10⁶/ml) were added to L cell or L-CD1 cultures for 30 min or 1, 2, 4, or 8 h, after which the NKT cells were isolated and processed to obtain total cellular RNA. Five micrograms of RNA were examined using the chemokine MultiProbe RPA system. A, The hybridization products were resolved on a polyacrylamide sequencing gel and detected by phosphorimaging. Although several chemokines (Ltn, RANTES, eotaxin, MIP-1β and -1α, MCP-1, IP-10, and TCA-3) are encoded on the RPA template, only RANTES was expressed by the stimulated DN32 cells. B, Results of scanning densitometry. Data are representative of three experiments in which similar results were obtained. Arbitrary OD units were calculated as a ratio of RANTES to GAPDH expression. C, RANTES protein secretion in supernatants from CD1d/DN32.D3 NKT cocultures was confirmed by sandwich ELISA.](http://www.jimmunol.org/)

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cocultured (30 min or 1 h) with unstimulated, OVA-pulsed immunogenic, or OVA + TGF-β-treated tolerogenic APCs. At the end of the coculture period, nonadherent T and NKT cells were aspirated and examined for chemokine mRNA expression by RPA. Compared with IMMULAN-enriched cells cocultured with either resting or immunogenic APCs, cells cocultured with tolerogenic APCs increased their expression of RANTES mRNA 3- to 4-fold after 30 min of coculture (Fig. 2). In similar experiments that examined the kinetics of chemokine expression by IMMULAN-enriched cells stimulated by tolerogenic APCs, we observed that RANTES expression peaked at 30 min and gradually decreased over the course of 8 h (data not shown). Additionally, we observed that RANTES expression was unique to cocultures of IMMULAN-enriched cells and tolerogenic (OVA plus TGF-β-treated) APCs and did not occur when cells were cocultured with APCs treated with either OVA or TGF-β alone. To determine the contribution of NKT cells to the chemokine profile, parallel groups of IMMULAN-enriched cells were depleted of NKT cells by pretreatment with anti-NK1.1 mAb and baby rabbit complement. IMMULAN-enriched cells that were depleted of NKT cells by Ab and complement before coculture with tolerogenic APCs did not have increased expression of RANTES mRNA at this time (Fig. 2). Thus, we concluded that tolerogenic APCs were able to induce the rapid expression of RANTES by NKT cells. In addition to RANTES, we also observed that tolerogenic APCs induced a modest increase in the expression of MIP-1α and MIP-1β that disappeared when IMMULAN fractions were depleted of NKT cells. The potential roles of MIP-1α and MIP-1β were not pursued in this study.

Splenic NKT cells from a.c.-inoculated mice express RANTES in a CD1d-dependent manner

To test the postulate that NKT cells that accumulate in the spleen during tolerance induction increase their expression of RANTES mRNA, total cellular RNA was isolated from splenic NKT cells (collected by FACS sorting) 3 and 7 days after a.c. inoculation of B6 mice with OVA (Fig. 3A). RANTES mRNA was detected in the samples by RT-PCR with mouse RANTES-specific primers. NKT cells isolated 7 days after inoculation of OVA (a.c.) expressed 2- to 3-fold higher levels of RANTES mRNA than NKT cells from naive mice or mice that received OVA 3 days before (Fig. 3, B and C). Production of RANTES protein by NKT cells was confirmed by intracellular cytokine staining and flow cytometry of spleen cells that were collected 7 days after intraocular inoculation of OVA (or HBSS as a control). Inoculation of OVA led to a 2-fold increase in the number of splenic RANTES-positive NKT cells (Fig. 4). RANTES production was exclusive to the NKT cell population, because neither conventional T cells nor NK cells increased their production of the RANTES protein, regardless of whether the mice were given OVA (a.c.) or vehicle (data not shown).

To determine whether RANTES expression by NKT cells during tolerance in vivo was CD1d dependent, B6 mice were given anti-CD1d mAb (clone 3C11) or control rat IgM (i.v.) on the same day they received inoculations of OVA (a.c.). In a previous publication, we confirmed that i.v. administration of anti-CD1d mAb did not eliminate CD1d+ APCs (7). Four days after the initial mAb treatment, the mice received a second injection of Ab; after another 3 days, splenic NKT cells were collected by FACS sorting (Fig. 5A) and RANTES mRNA expression was assessed by RT-PCR. NKT cells from a.c.-inoculated mice that received control IgM expressed higher levels of RANTES mRNA than NKT cells from naive mice (Fig. 5B). In contrast, RANTES mRNA expression was not increased in NKT cells harvested from a.c.-inoculated mice that received anti-CD1d mAb (i.v.) (Fig. 5B). Thus, the increased expression of RANTES mRNA in NKT cells that accumulate in the spleen during tolerance must be dependent on interaction with CD1d, presumably expressed on the APC.

NKT-derived RANTES recruits additional F4/80+ APCs and CD8+ T cells

Our previous reports showed cell clusters in the spleen that contained F4/80+ APCs (dendritic cells) and NKT cells (19). Confocal microscopy of immunostained spleen sections from a.c.-inoculated mice showed a substantial increase in the number of F4/80+ cells localizing in the marginal zones, indeed far more than could have migrated from the iris and ciliary body of one mouse eye. Because RANTES is a potent macrophage and DC chemoattractant, we wondered whether the NKT-derived RANTES recruited the additional F4/80+ APCs to the spleen during tolerance induction.
B6 mice were given inoculations of OVA or HBSS (a.c.) in conjunction with rat IgG or a neutralizing anti-RANTES mAb (i.v.). Four days later, all mice received a second injection of Abs; 3 days after that, the number of F4/80$^+$ cells in the spleen was calculated by flow cytometry analyses of cells immunostained with PE-conjugated anti-F4/80 mAb. Naive B6 mice had an average of $4.9 \times 10^6$ F4/80$^+$ cells per spleen, whereas after anterior chamber inoculation with OVA the number of F4/80$^+$ cells increased to an average of $8.7 \times 10^6$ per spleen (Fig. 6). The differences between naive and a.c.-inoculated were statistically significant ($p < 0.05$).

In contrast, when mice received OVA (a.c.) in conjunction with systemic anti-RANTES neutralizing mAb, the number of splenic F4/80$^+$ APCs did not increase (Fig. 6).

Because RANTES is also a lymphocyte chemoattractant and CD8$^+$ T cells are integral members of the tolerogenic clusters observed by our laboratory previously, we tested whether NKT-derived RANTES could also recruit naive CD8$^+$ T cells. Supernatants from CD1d-stimulated NKT cells (DN32.D3) were tested in Boyden microchemotaxis assays for their ability to induce active migration of FACS-sorted naive CD8$^+$ T cells. We observed that supernatants from NKT cells (DN32.D3) that were stimulated by CD1d-transfected fibroblasts induced chemotaxis of naive CD8$^+$ T cells, whereas supernatants from DN32.D3 NKT cells cocultured with untransfected fibroblasts did not (Fig. 7). Moreover, if the supernatants from CD1d-stimulated NKT cells were treated with anti-RANTES mAb for 30 min before being used in Boyden chemotaxis assays, the supernatants failed to induce CD8$^+$ chemotaxis. Thus, through their production of RANTES, NKT cells can recruit additional F4/80$^+$ APCs and CD8$^+$ T cells that are needed for tolerance.

RANTES is required for the generation of CD8$^+$ Tr cells in an in vivo model of NKT cell-dependent peripheral tolerance

Because colocalization of the APCs, NKT cells, and CD8$^+$ T cells is presumably required for tolerance, we postulated that in the absence of RANTES, CD8$^+$ Tr cell would not be generated, presumably because the required cells would associate with one another. To block the effects of RANTES during tolerance in vivo,
mice were inoculated (a.c.) with OVA and given either anti-RANTES mAb or control rat IgG (i.v. Days 0 and 4). Seven days after a.c. inoculation, splenic T cells were collected and examined for their ability to suppress DTH responses in a local adoptive transfer (LAT) assay. While T cells from a.c.-inoculated mice that received control rat IgG suppressed the subsequent DTH response, T cells from mice that received a.c. inoculation of OVA in conjunction with systemic anti-RANTES mAb did not (Fig. 8). T cells

![Figure 6](image-url)

**FIGURE 6.** Involvement of RANTES in the accumulation of F4/80<sup>+</sup> APCs in the spleen after a.c. inoculation of Ag. Total splenocytes from either naive B6 mice or those that received OVA (a.c.) in conjunction with control rat IgG or anti-RANTES mAb (i.v.) 7 days earlier were immunostained with PE-conjugated anti-F4/80 mAb and analyzed by flow cytometry to determine the number of F4/80<sup>+</sup> cells. Total number of F4/80<sup>+</sup> cells per spleen ± SEM. n = 4 mice per group. Data are representative of two experiments in which similar results were obtained. Statistical differences were determined by Student’s t test. * p < 0.05.

![Figure 7](image-url)

**FIGURE 7.** Chemoattraction of CD8<sup>+</sup> T cells by supernatants from CD1d-stimulated NKT cells. CD8<sup>+</sup> and CD4<sup>+</sup> T cells were enriched from the spleens of naive B6 mice by FACS sorting and tested for their ability to migrate across Boyden chamber chemotaxis membranes in response to supernatants obtained from NKT cells (DN32.D3) that had been stimulated with CD1d-transfected fibroblasts for 2 h. SFM alone was used as a control for unstimulated NKT cells and baseline chemotaxis. The contribution of RANTES to the T cell chemotactic response was tested by pretreating the supernatants with either control rat IgG or anti-RANTES mAb (clone 3C11) i.v. on days 0 and 4 after a.c. injection.

**FIGURE 5.** RANTES mRNA expression in a.c.-inoculated mice cotreated with anti-(α)-CD1d mAb. A. Flow cytometric dot blots show the degree of enrichment of the FACS-sorted NKT cells from each group. Areas labeled M, K, and O indicate gates for T cells, NKT cells, and NK cells, respectively. B. Representative gel shows the products of RT-PCR for murine RANTES and β-actin done on NKT cells collected from the spleens of mice 7 days after they were inoculated with OVA (a.c.). Mice were also given 100 μg (total dose) of either rat control IgM or anti-CD1 mAb (clone 3C11) i.v. on days 0 and 4 after a.c. injection.
of NKT cells not only influences the cytokine milieu that supports differentiation of Tr cells but also initiates production of chemokines that can recruit lymphocytes and additional APCs into the tolerogenic microenvironment. We further propose that the initial eye-derived APCs travel to the marginal zone to recruit NKT cells, where together, the two cell types initiate an amplification cascade that recruits additional F4/80+ APCs needed for maintenance of the tolerogenic microenvironment that supports differentiation of CD8+ T lymphocytes into regulatory cells. Because F4/80 is a marker for multiple populations of macrophages and dendritic cells, it remains to be shown whether the F4/80+ cells recruited by RANTES are dendritic cells or macrophages. Confocal microscopic evaluation of the recruited F4/80+ cells shows that they exhibit a typical dendritic morphology (19).

In our vitro coculture experiments, we observed a lack of increased RANTES mRNA expression when NK1.1+ cells were depleted with specific Ab. Although anti-NK1.1 mAb depleted both NK and NKT cells, we observed in our ex vivo intracellular cytokine staining experiments that during tolerance induction, RANTES protein was produced only by NKT and not conventional T or NK cells. Moreover, RANTES expression among splenic NKT cells was CD1d dependent, and if the chemokine was neutralized, the generation of CD8+ Tr cells failed. Together, with our previous reports showing CD1d dependency for Tr cell generation, these findings support the concept of a direct effect of NKT cell-derived RANTES in the generation of CD8+ Tr cells.

Like conventional T lymphocytes, NKT cells need to be stimulated to increase their RANTES production. Unlike conventional lymphocytes that require 3–5 days after stimulation by Ag and MHC to begin to express RANTES (25, 26), NKT cells produce RANTES mRNA in minutes and RANTES protein within hours of CD1d stimulation of their invariant TCR. The kinetics and magnitude of RANTES production by NKT cells are similar to their production of other cytokines.

The mechanisms used by tolerogenic but apparently not immunogenic APCs to induce RANTES in NKT cells require further study. All APCs express CD1d; however, APCs treated simultaneously with Ag and TGF-β express significantly higher levels of CD1d on their cell surface than APCs treated with either Ag alone or TGF-β alone (Ref. 27 and our unpublished observations). Although the magnitude of CD1d expression clearly differs between subsets of APCs and CD1d is sufficient for stimulation, it is unlikely that levels of CD1d alone are responsible for the induction of RANTES in NKT cells associated with tolerance. Besides expressing high levels of CD1d, tolerogenic APCs exhibit low B7.1 and B7.2, low CD40, and low MHC II (28). Moreover, the tolerogenic APCs secrete TGF-β and IL-10, two cytokines known to influence lymphocyte function and phenotype. Thus, the ability of tolerogenic (but not immunogenic) APCs to induce rapid RANTES production by NKT cells may result from a combination of CD1 stimulation of the NKT cells and signals within the tolerogenic microenvironment, although this remains to be proved.

Several cell types including T cells, monocytes, and dendritic cells produce RANTES; however, our studies show that during tolerance only NKT cells produce the chemokine, implying the importance of NKT-derived RANTES in the promotion of tolerogenic cell clusters. Although our experiments here did not directly address the impact of RANTES on colocalization and clustering of cells in the splenic MZ, we show that disruption of either CD1d stimulation of NKT cells or RANTES signaling prevents the end result of the tolerogenic cluster formation, implying their disruption as well.

**Discussion**

This report presents the unique findings that CD1d-restricted NKT cells that encounter tolerogenic APCs (both in vitro and in vivo) rapidly increase their expression of the lymphocyte and dendritic cell-macrophage chemoattractant, RANTES. Using a model of peripheral tolerance induction in mice, we demonstrated that NKT cells that accumulated in the spleen increased their expression of RANTES in a CD1d-dependent manner and that a function of the chemokine was to recruit additional F4/80+ APCs to the spleen. The requirement for RANTES protein production in vivo was also demonstrated by experiments in which the generation of CD8+ Tr cells failed when neutralizing anti-RANTES mAb was given systemically.

Previous reports from our laboratory showed that during tolerance induction, CD1d stimulation of NKT cells was required for CD8+ Tr cell formation and that CD8+ Tr differentiation was linked directly to the ability of NKT cells to produce IL-10 (3, 7). The data presented here support the concept that CD1d stimulation from naive mice treated with rat IgG did not interfere with the generation of the local DTH response (Fig. 7). In conclusion, RANTES contributes to the process of tolerance induction by recruiting F4/80+ APCs and potential CD8+ Tr cells to the microenvironment that nurtures the differentiation of CD8+ Tr cells.

**FIGURE 8.** LAT assay using regulator cells from a.c.-inoculated, anti-RANTES-treated mice. Mice were given OVA (a.c.) or HBSS in conjunction with either systemic anti-RANTES mAb or control IgG at days 0 and 4 after a.c. injection (n = 5 per group; 100 μg total dose). Splenic T cells were collected 7 days after a.c. injection and enriched by IMMULAN-negative selection columns and used as regulatory cells for LAT assay using regulator cells from a.c.-inoculated, anti-RANTES-treated mice. Mice were given OVA (a.c.) or HBSS in conjunction with either systemic anti-RANTES mAb or control IgG at days 0 and 4 after a.c. injection (n = 5 per group). Ear thickness was determined before ear pinna injection and at 24 and 48 h postinjection. Data are represented as mean change in ear thickness (microns, ± SEM). Ear thickness was determined before ear pinna injection and at 24 and 48 h postinjection. Data are represented as mean change in ear thickness at 24 h after injection ± SEM. The composition of cells injected into the ear for the LAT assay is indicated under each bar. *, p < 0.05. Statistical differences were determined by ANOVA and Neuman-Keuls post hoc analysis. Data are representative of two experiments in which similar results were obtained.

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**Discussion**

This report presents the unique findings that CD1d-restricted NKT cells that encounter tolerogenic APCs (both in vitro and in vivo) rapidly increase their expression of the lymphocyte and dendritic cell-macrophage chemoattractant, RANTES. Using a model of peripheral tolerance induction in mice, we demonstrated that NKT cells that accumulated in the spleen increased their expression of RANTES in a CD1d-dependent manner and that a function of the chemokine was to recruit additional F4/80+ APCs to the spleen. The requirement for RANTES protein production in vivo was also demonstrated by experiments in which the generation of CD8+ Tr cells failed when neutralizing anti-RANTES mAb was given systemically.

Previous reports from our laboratory showed that during tolerance induction, CD1d stimulation of NKT cells was required for CD8+ Tr cell formation and that CD8+ Tr differentiation was linked directly to the ability of NKT cells to produce IL-10 (3, 7). The data presented here support the concept that CD1d stimulation of NKT cells not only influences the cytokine milieu that supports differentiation of Tr cells but also initiates production of chemokines that can recruit lymphocytes and additional APCs into the tolerogenic microenvironment. We further propose that the initial eye-derived APCs travel to the marginal zone to recruit NKT cells, where together, the two cell types initiate an amplification cascade that recruits additional F4/80+ APCs needed for maintenance of the tolerogenic microenvironment that supports differentiation of CD8+ T lymphocytes into regulatory cells. Because F4/80 is a marker for multiple populations of macrophages and dendritic cells, it remains to be shown whether the F4/80+ cells recruited by RANTES are dendritic cells or macrophages. Confocal microscopic evaluation of the recruited F4/80+ cells shows that they exhibit a typical dendritic morphology (19).

In our vitro coculture experiments, we observed a lack of increased RANTES mRNA expression when NK1.1+ cells were depleted with specific Ab. Although anti-NK1.1 mAb depleted both NK and NKT cells, we observed in our ex vivo intracellular cytokine staining experiments that during tolerance induction, RANTES protein was produced only by NKT and not conventional T or NK cells. Moreover, RANTES expression among splenic NKT cells was CD1d dependent, and if the chemokine was neutralized, the generation of CD8+ Tr cells failed. Together, with our previous reports showing CD1d dependency for Tr cell generation, these findings support the concept of a direct effect of NKT cell-derived RANTES in the generation of CD8+ Tr cells.

Like conventional T lymphocytes, NKT cells need to be stimulated to increase their RANTES production. Unlike conventional lymphocytes that require 3–5 days after stimulation by Ag and MHC to begin to express RANTES (25, 26), NKT cells produce RANTES mRNA in minutes and RANTES protein within hours of CD1d stimulation of their invariant TCR. The kinetics and magnitude of RANTES production by NKT cells are similar to their production of other cytokines.

The mechanisms used by tolerogenic but apparently not immunogenic APCs to induce RANTES in NKT cells require further study. All APCs express CD1d; however, APCs treated simultaneously with Ag and TGF-β express significantly higher levels of CD1d on their cell surface than APCs treated with either Ag alone or TGF-β alone (Ref. 27 and our unpublished observations). Although the magnitude of CD1d expression clearly differs between subsets of APCs and CD1d is sufficient for stimulation, it is unlikely that levels of CD1d alone are responsible for the induction of RANTES in NKT cells associated with tolerance. Besides expressing high levels of CD1d, tolerogenic APCs exhibit low B7.1 and B7.2, low CD40, and low MHC II (28). Moreover, the tolerogenic APCs secrete TGF-β and IL-10, two cytokines known to influence lymphocyte function and phenotype. Thus, the ability of tolerogenic (but not immunogenic) APCs to induce rapid RANTES production by NKT cells may result from a combination of CD1 stimulation of the NKT cells and signals within the tolerogenic microenvironment, although this remains to be proved.

Several cell types including T cells, monocytes, and dendritic cells produce RANTES; however, our studies show that during tolerance only NKT cells produce the chemokine, implying the importance of NKT-derived RANTES in the promotion of tolerogenic cell clusters. Although our experiments here did not directly address the impact of RANTES on colocalization and clustering of cells in the splenic MZ, we show that disruption of either CD1d stimulation of NKT cells or RANTES signaling prevents the end result of the tolerogenic cluster formation, implying their disruption as well.
The data reported here expand the current knowledge of RANTES beyond its role in Th1-like immune responses to include tolerance and the generation of T cells. Our data raise the possibility that defective chemokine production may contribute to flaws in cell trafficking events that are needed for the generation of self-tolerance, thus allowing the clinical appearance of certain autoimmune diseases. Additionally, our findings suggest that therapeutic targeting of RANTES signaling pathways, as is being done for treatment of HIV infection, may interfere with the generation or maintenance of tolerance to self-Ags. Understanding the precise mechanisms involved in tolerance induction will enable the development of more effective treatment strategies for circumstances where tolerance to self (for prevention of autoimmune disease) or foreign Ags (prevention of organ transplant rejection) is preferred.

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