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Differential Expression and Cross-Regulatory Function of RANTES During Mycobacterial (Type 1) and Schistosomal (Type 2) Antigen-Elicited Granulomatous Inflammation

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The role of RANTES in Th1 and Th2 cell-mediated immune responses has been enigmatic. To approach this question, we analyzed RANTES expression and function in murine models of types 1 and 2 cell-mediated pulmonary granulomas elicited with Mycobacterium bovis or Schistosoma mansoni egg Ag-coated beads, respectively. Compared with type 2, type 1 lesions had up to 4-fold greater RANTES protein and mRNA production. Type 1 draining lymph nodes also produced up to 7-fold higher levels of RANTES. Anti-RANTES Ab treatments had opposite effects, decreasing type 1 lesion area by 25% and augmenting type 2 lesions by 50%. The latter was associated with increased IL-4, IL-5, IL-10, and IL-13 production by lymph nodes. Infusion of rRANTES (1 mg/kg/day) did not affect type 1 lesions, but reduced type 2 lesion area by 27% and eosinophils by 40%. Lymph node cultures from RANTES-treated mice had augmented type 1 and impaired type 2 responses. In vitro, RANTES caused selective, dose-related inhibition of IL-4 that was largely dependent on CCR1 receptors. In conclusion, RANTES plays different roles in types 1 and 2 granuloma formation, promoting the former and mediating cross-regulatory inhibition of the latter. Moreover, RANTES may have therapeutic potential in the treatment of established type 2 hypersensitivity. The Journal of Immunology, 1999, 163: 165–173.

The C-C chemokine, RANTES, is produced by a wide variety of cell types, including lymphocytes, macrophages, fibroblasts, platelets, smooth muscle, endothelial, and epithelial cells (1–8). With respect to target receptor specificity, RANTES shows promiscuous binding to multiple chemokine receptors, including CCR1, CCR3, CCR4, and CCR5 (9). In addition to its chemotactic properties, RANTES has been shown to have T cell-stimulatory activity (10, 11). Since it is chemotactic for eosinophils, mononuclear phagocytes, basophils and mast cells, it has been postulated to be important in inflammatory reactions, especially of the allergy-associated type (12, 13). Indeed, in the decade since its discovery, numerous reports have provided evidence of associations with asthma and allergic rhinitis (12–16). In addition, there are reported associations with arthritis (17), sarcoidosis (18), and inflammatory bowel disease (19). The above studies provided largely circumstantial evidence for RANTES participation in inflammation, and there is little direct evidence demonstrating the in vivo function of this molecule. Moreover, there is confusion regarding the particular role of RANTES in Th1 and Th2 cell-mediated responses. As mentioned, many studies have demonstrated associations with allergy-related type 2 responses, yet recent reports show preferential expression in type 1 immune responses (20).

To help clarify these issues, we performed a detailed analysis of the expression and function of RANTES in defined models of polarized type 1 (Th1) and type 2 (Th2) cell-mediated pulmonary granuloma formation elicited with soluble protein Ags of Mycobacterium bovis or Schistosoma mansoni, respectively (21). The type 1 granuloma is composed mostly of mononuclear cells and is largely dependent on IFN-.gamma and TNF-alpha, whereas the type 2 granuloma is eosinophil-rich and is largely dependent on IL-4. Our findings clearly show a differential expression in the two models with RANTES predominating in the type 1 response at both the site of granuloma formation and draining lymphoid tissue. In vivo depletion analysis revealed differing roles for RANTES in the two types of responses. Specifically, RANTES contributed to type 1 inflammation and limited the type 2 response analogous to the cross-regulatory effect of IFN-gamma (22, 23). Thus, our findings suggest that RANTES is an endogenous regulatory molecule that participates differently in type 1 and 2 inflammatory responses.

Materials and Methods

Animals

Female CBA/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6 × 129 mice with knockout of the CCR1 receptor gene were prepared as previously described (24). Wild-type control B6 × 129 mice were obtained from The Jackson Laboratory. All mice were maintained under specific pathogen-free conditions and provided with food and water ad libitum.

Sensitization and granuloma induction

Type 1 and 2 granulomas were generated as follows. Briefly, mice were sensitized by s.c. injection with 20 μg purified protein derivative (PPD)³ (Department of Agriculture, Veterinary Division, Ames, IA) of M. bovis purified protein derivative; SEA, schistosome egg Ags; CON, control; h, human.

³Abbreviations used in this paper: PPD, purified protein derivative; SEA, schistosome egg Ags; CON, control; h, human.
incorporated in 0.25 ml CFA (Sigma, St. Louis, MO; product no. F-5881) or 3000 S. mansoni eggs suspended in 0.5 ml PBS. Then, 14–16 days later, PPD and egg-sensitized mice were respectively challenged i.v. with 6000 Sepharose 4B beads (in 0.5 ml PBS) covalently coupled with PPD or to soluble schistosome egg Ags (SEA) obtained from the World Health Organization (Geneva, Switzerland). Nonimmune control (CON) granulomas were generated by injection of Ag-free beads.

Cytokines, Abs, and cytokine assays

All cytokines and chemokines used in this study were obtained as purified carrier-free recombinant proteins from PeproTech (Rocky Hill, NJ) and R&D Systems (Minneapolis, MN). Human rRANTES, kindly provided by Dr. T. Wells (Glaxo Wellcome Research and Development, Research Triangle Park, NC), was used for in vivo osmotic pump studies that required large doses of the chemokine. Murine RANTES and IL-2, -4, -5, -13, and IFN-γ were measured by standard ELISA using commercially available reagents (R&D Systems and Pharmingen (San Diego, CA)). Sensitivities ranged from 15 to 50 pg/ml. IL-2 was measured by sandwich ELISA, as previously described, with sensitivity to 50 pg/ml (25).

Goat anti-murine RANTES was prepared on contract with Lampire Biological Laboratories (Pipersville, PA) using a 14-aa carboxyl-terminal peptide of murine RANTES kindly provided by Dr. B. Daugherty (Merck, Whitehouse Station, NJ). Titer was determined to be >1:10^4 by direct ELISA, and neutralization was confirmed by inhibition of RANTES-elicted eosinophil chemotaxis. Specifically, a 1:1000 dilution of antisera completely neutralized 10 ng of recombinant murine RANTES with no effect on eotaxin-induced chemotaxis.

In vivo cytokine depletion protocol

At the time of bead challenge, mice were given an i.p. injection of 5 mg of protein A-purified rabbit anti-murine RANTES IgG. All polyclonal Ab was depleted of any intrinsic anti-PPD activity by prior passage over a solid-phase PPD immunosorbent column. Nonimmune goat IgG served as a control.

In vivo cytokine treatment by osmotic pump implantation

Miniosmotic pumps were implanted as previously described (26). Briefly, 2 days before bead challenge, mice were subjected to ketamine anesthesia, then the abdomen was shaved and cleaned with 70% alcohol. An 8-mm incision was made in the skin and peritoneum through which an osmotic pump (Alza, Palo Alto, CA) was implanted, containing 100 µl of carrier-free human RANTES (hRANTES) solution. The concentration was adjusted to achieve a dosage of 1 mg/kg/day given as constant infusion (830 ng/mouse/h). Control pumps contained the vehicle, PBS. After 2 days, mice were challenged i.v. with Ag-coated beads. Then, 3 days after bead challenge, lungs and lymph nodes were excised and cultures prepared as described below. In all experiments, samples of lungs were fixed in 10% buffered formalin for morphometric analysis, while the remaining lung was used for granuloma isolation, dispersal, and cell differential analysis.

Morphometry

At 1, 2, 4, or 8 days after challenge, lungs and lymph nodes were excised and prepared as described below. In some experiments, lungs were inflated and fixed with 10% buffered formalin for morphometric analysis. Granuloma area was measured blindly in hematoxylin- and eosin-stained sections of paraffin-embedded lungs using a morphometer and software program (The Morphometer, Woodshole, MA). A minimum of 20 lesions was measured per lung. Only granulomas with full cross-sections of the bead nidus were measured.

Intact granuloma and lymph node cell culture

Intact and dispersed granulomas were prepared as previously described (21). A total of 2 ml of intact granuloma suspension (1500 lesions/ml) was cultured in sterile 100-mm plastic culture dishes in the presence or absence of 5 µg/ml PPD or SEA. Supernatants were collected at 24 h and assayed for RANTES production. A 200 cell differential analysis was performed on duplicate Wright-stained cytospin preparations of dispersed granulomas. Mediastinal lymph nodes were collected aseptically at the time of lung harvest and teased into a single cell suspension. After washing, the cells were cultured in RPMI-FBS at 5 x 10^5 cells/ml in 100-mm dishes with 5 µg/ml PPD or SEA for 24 h at 37°C in a humidified 5% CO2 atmosphere. In some studies, the cultures were stimulated with 0, 1, 10, or 100 ng/ml murine RANTES and cultured as above. Supernatants were collected by centrifugation and stored at −45°C before performing cytokine assays.

RNA extraction

Perfused lung lobes, excluding major bronchi, were snap frozen with liquid N2, and total cellular RNA was extracted by a modified method of Chirgwin et al. (27) and Jonas et al. (28). The frozen tissues were suspended in extraction buffer (250 mM Tris (pH 8.0), 0.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-ME), homogenized, then added to an equal volume of extraction buffer (100 mM Tris (pH 8.0), 10 mM EDTA, and 1% SDS). The mixture was then serially extracted with chloroform-pheno/ol and chloroform-isooamyl alcohol. The RNA is then precipitated at −70°C in ethyl alcohol, washed, and precipitated. The pellet was finally dissolved in diethyl pyrocarbonate water, and RNA concentrations were determined spectrophotometrically before storage at −70°C. Yields are routinely >100 µg.

Primers and probes

Primers and probes were designed based upon mRNA nucleotide sequences downloaded from the National Center for Biotechnology Information (NCBI) database and using primer designing software (Premier Biosoft International, Palo Alto, CA). Designed primer and probe sequences (5′ to 3′) for murine RANTES (NCBI accession no. M77747) and endoplasmic reticulum-specific cyclophilin (NCBI accession no. M60456) mRNAs were as follows: murine RANTES sense primer, GTG-CCC-AGC-CTA-AGG-AGT-ATT-T; antisense primer, A-AGA-CTA-GTG-AGG-GAA-AGC-TTG-TTC; murine cyclophilin sense primer, GTG-GCC-TTC-GTC-GTC-CTT-TT; antisense primer, C-CTT-CTT-CCT-GTG-CCA-CTT-CTT-C; probe sequence, CAT-CTG-GTC-ATC-AAG-GAC-TTC-A (spanning bases 296-318). Primers and probes were prepared by Genosys Biotechnologies (The Woodlands, TX). Probes were biotinylated with biotin-UTP using a standard 3′ end-labeling kit (Boehringer Mannheim, Indianapolis, IN). Unincorporated biotin was removed with QuickSpin columns (Boehringer Mannheim). Incorporation was confirmed by nitrilcellulose blotting followed by streptavidin-alkaline phosphatase detection.

RT-PCR ELISA detection of cytokine mRNA

The isolated RNA was reverse transcribed to DNA as follows. To 20 µg of RNA (in 25 µl of diethyl pyrocarbonate water) was added 3.6 µl of RNA-sin (Boehringer Mannheim) and 10 µl of random hexamer solution (500 ng/ml, Promega, Madison, WI), followed by heating to 70°C for 5 min in a thermocycler (Perkin-Elmer 9600; Perkin-Elmer, Norwalk, CT). The temperature was then reduced to 43°C, then 69 µl of a first strand buffer (Life Technologies, Grand Island, NY) containing dTT, dNTP, and 1000 U Moloney murine leukemia virus RT was added (29). The mixture was incubated for 2 h, and then the reaction was stopped by heating to 70°C. The DNA was then subjected to PCR (30). Briefly, 5 µl of DNA was added to a PCR buffer containing unlabeled dNTPs (0.2 mM of each) plus digoxigenin-labeled dUTP, 1 µg sense primer, 1 µg antisense primer, and 5 U Taq polymerase (all from Boehringer Mannheim) in a thin-walled PCR tube. Amplification was then performed in a thermocycler as follows: 4 min at 95°C followed by 25 cycles of 1 min at 95°C, 2 min at 57°C, and 1 min at 72°C. After cycling, there was a DNA extension period of 2 min at 72°C, then samples were stored at −20°C before analysis.

Detection of PCR products was performed as follows. Initially, a series of amplification reactions using unlabeled dNTPs was performed, and the products were analyzed by standard agarose gel electrophoresis to confirm that primers yielded predicted products. Once confirmed, labeled products were generated and detected by PCR-ELISA (31). Briefly, 30 µl of amplified RANTES or 5 µl of cyclophilin product was added to a sterile microtiter plate containing 200 µl of denaturing buffer (30°C, plus 4 mg/ml of denaturing agent (Boehringer Mannheim)) and incubated 10 min. Due to the high degree of assay sensitivity and the relative greater incidence of cyclophilin mRNA species, less cyclophilin product is used to keep the final absorbance signal within readable range. Next, 500 µl of hybridization buffer containing 4 ng/ml of appropriate (target gene) biotinylated probe was added. Negative controls included tubes with no DNA or DNA with inappropriate probe. The solution was mixed and 200-µl portions were distributed into duplicate wells of a microtiter plate. Amplification was then performed in a thermocycler as follows: 4 min at 95°C followed by 25 cycles of 1 min at 95°C, 2 min at 57°C, and 1 min at 72°C. After cycling, there was a DNA extension period of 6 min at 72°C, then samples were stored at −20°C before analysis.

The amount of color is directly related to the amount of amplification product added. A 96-well plate ELISA reader is used to measure OD at 405 nm at 15 and 30 min. Relative mRNA is then calculated as follows: Relative mRNA ratio = [(OD target gene)/(OD cyclophilin, challenged lung)]/[(OD target gene)/(OD cyclophilin, unchallenged lung)].
Unlike simple gel detection, the PCR-ELISA method employs a hybridization step that specifically binds target amplicons. Consequently, it is highly specific and has proven to be 10- to 100-fold more sensitive than gel detection and allows amplification cycles to be kept to a minimum.

Statistics
The paired Student’s t test was used to compare paired groups. ANOVA was used for multigroup analysis. Values of $p \geq 0.05$ were considered to indicate lack of significance.

Results
RANTES is differentially expressed during type 1 and 2 granuloma formation
To determine the dynamics of RANTES expression during synchronized type 1 and 2 granuloma formation, we measured levels of spontaneous and Ag-elicited RANTES in the culture supernatants of intact lesions isolated at 1, 2, 4, and 8 days of development. This was temporally related to granuloma size, local RANTES protein secretion, and mRNA expression. Fig. 1 shows the results of this multiparameter analysis. Compared with type 2 SEA and non-Ag CON lesions, the type 1 PPD response was clearly associated with the greatest overall spontaneous and Ag-elicited RANTES production by granulomas ($F > 1.0$ and $p < 0.05$ for days 1 and 2, as compared with other groups). Levels of RANTES mRNA were likewise greater for the type 1 response and paralleled the protein expression. Among the type 1 granuloma cultures, the greatest production occurred on days 1 and 2 corresponding to the rapid growth period, 0–4 days. Interestingly, foreign-body lesions elicited by non-Ag CON beads showed modestly increased levels of RANTES in response to Ag stimulus (i.e., combined PPD and SEA Ags). These lesions, which resemble a primordial type 1 response, nonspecifically recruit mononuclear cells that could produce RANTES as an innate response to Ag.

We next measured levels of RANTES in cultures of corresponding draining lymph nodes. As shown in Fig. 2, levels of Ag-elicited RANTES were notably higher during the type 1 PPD response. Even spontaneous production was significantly higher on days 2 and 4 ($F = 11, p = 0.007$ and $F = 43, p = 0.0001$, respectively). Interestingly, the kinetics of RANTES production differed from the type 1 granulomas, increasing to a maximum on day 4 ($F > 1$ and $p < 0.05$, as compared with days 1, 2, and 8). These kinetics paralleled lymphoproliferation as determined by assessment of lymph node cell yields (data not shown).

In vivo RANTES depletion has opposite effects on type 1 and 2 granuloma formation
To test the in vivo function of RANTES, types 1 and 2 granulomas were induced in mice treated with a neutralizing preparation of goat anti-murine RANTES IgG or a corresponding dose of non-immune goat IgG. On day 4 of granuloma formation, mice were sacrificed, and lesion sizes were determined. As shown in Fig. 3, anti-RANTES treatment decreased type 1 granuloma area by 25% and augmented type 2 granuloma area by 50%. This finding indicated that RANTES functioned differently in the two systems, supporting type 1 and inhibiting type 2 inflammation.

Granuloma composition analysis showed typical patterns for type 1 and 2 lesions, with lymphocytes and large mononuclear cells dominating the former and the addition of a significant component of eosinophils in the latter (Table I). Ab treatment did not cause changes in these major cell populations, suggesting that RANTES was not selectively recruiting mononuclear cells or eosinophils. However, alterations in specific lymphocyte subpopulations could not be ruled out.

In vivo RANTES depletion modulates cytokine production by draining lymphoid tissues
Since RANTES was produced in regional lymphoid tissues, it was of interest to determine whether its depletion influenced draining

FIGURE 1. Expression of RANTES protein and mRNA during synchronized PPD (type 1), SEA (type 2), and non-Ag control bead-elicited pulmonary granuloma formation. Upper panel, Kinetics of granuloma development over the 8-day study period. Bars are mean lesion cross-sectional areas ± SE. Middle panel, Production of RANTES protein from cultured granulomas (1500 lesions/ml). Bars show means ± SE of spontaneous and Ag-elicited levels of RANTES. Control lesions were stimulated with both PPD and SEA. Lower panel, RANTES mRNA expression. Bars show the mean ratios ± SE of cyclophilin-normalized signal of granulomatous to unchallenged lungs. Baseline signal is valued at 1 (dashed line). Bars for all panels were derived from a total of six to nine mice per group. Group values were compared by ANOVA.
lymph node cytokine-producing capacity. As shown in Fig. 4, typ-ical type 1 and 2 cytokine profiles were obtained in the two mod-els. The type 1 (PPD) response, dominated by IFN-γ and IL-2, was generally resistant to RANTES depletion. In contrast, the type 2 cytokine profile was broadly augmented with significant increases in IL-4, IL-5, IL-10, and IL-13. In summary, endogenous RAN-TES appeared to temper both type 2 granuloma formation and its corresponding regional cytokine response. However, RANTES was not critical to supporting the Th1 profile during the PPD response.

Parenteral RANTES administration reduces type 2, but not type 1, inflammation

To test the direct effect of RANTES on type 1 and 2 inflammation, granulomas were induced in mice implanted with miniosmotic pumps providing a constant i.p. infusion (20 μg per mouse per day) of recombinant hRANTES, which is 85% homologous to mu-rine RANTES at the amino acid level (32). Because of the high degree of homology, hRANTES is poorly immunogenic in mice, therefore vehicle could serve as an appropriate control substance. Fig. 5 shows the effect of the treatment on granuloma sizes on day 3. Type 1, PPD lesions were statistically unaffected, although there was a trend to larger lesions, and if values are extrapolated to volumes, then a significant augmentation could be detected (data not shown). In contrast, the average type 2, SEA bead granuloma area was reduced by 27% (40% after subtracting the area occupied by the bead nidus). Composition analysis of granulomas showed no change in the type 1 lesions, but there was a significant decrease (~40%) in the percentage of eosinophils in type 2 granuloma, resulting in a corresponding increase in the proportion of lympho-cytes (Table II). Further analysis of cytokine profiles in draining lymph node cultures likewise revealed opposing effects. Fig. 6 shows Ag-elicited levels of cytokines in lymph node cultures. Compared with day 4, type 1 lymph node cultures on day 3 tend to show less IFN-γ and increased IL-12 (compare Figs. 4 and 6), yet the type 1 profile is clearly present. In these cultures, IFN-γ and IL-2 were augmented by RANTES infusion with no change in IL-12. Small increases in IL-5 and IL-13 were also noted, indicating that like Th1 clones, type 2 cytokines are reduced but not totally eliminated from the type 1 response. In contrast, during the type 2 response, there was broad impairment of IL-2, IL-4, IL-5, IL-10, and IL-13. Thus, RANTES had opposite effects, augmenting the type 1 and inhibiting the type 2 response. Because the effects were broad, the bulk of the cytokines generally maintained the same relative proportions.

RANTES directly modulates IL-4 production in vitro

The above studies indicated that RANTES plays a differential reg-ulatory role in cytokine production during type 1 and 2 granuloma formation. To begin to explore potential mechanisms, PPD- or SEA-sensitized lymphoid cultures from mice at day 4 of granuloma formation were exposed to specific Ags and graded doses of exogenous murine RANTES in vitro. Representative type 1 and 2 cytokines (IL-2, IFN-γ, IL-4, and IL-5) were then measured after 24 h of culture. As shown in Fig. 7, type 1 (PPD) and type 2 (SEA)
responses had the expected Th1- and Th2-related cytokine profiles with IL-2 and IFN-γ dominating the former and IL-4 and IL-5 dominating in the latter. In response to RANTES treatment, the SEA-sensitive cultures displayed a specific dose-dependent inhibition of IL-4, dropping to nearly 50% of control levels at 100 ng/ml (ANOVA, 0 ng/ml vs 1 ng/ml, \( p < 0.05 \); 1 ng/ml vs 10 ng/ml, \( p < 0.008 \)). Interestingly, IL-5 was unaffected. In the type 1 (PPD) cultures, RANTES had no consistent effect. Thus, a selective inhibitory effect of mRANTES on IL-4 could be demonstrated in vitro. However, this was unlike the broad effects on type 2 cytokines that we observed when RANTES was administered in vivo over several days, suggesting the presence of intermediary events leading to in vivo changes.

RANTES mediates its effect on IL-4 via the CCR1 receptor

RANTES is known to interact with a number of known chemokine receptors including CCR1, -3, -4, and -5 (9). As an initial effort to determine which of these receptors was involved in IL-4 regulation, we compared the effects of RANTES treatment on day 4, type 2-responsive draining lymph node cultures from wild-type and CCR1 receptor knockout mice. This was likewise compared with the in vitro effect of eotaxin, a chemokine thought to selectively bind to the CCR3 receptor. Fig. 8 shows the results of these studies. The first observation that became apparent upon inspecting the baseline response was that CCR1 knockout mice produced higher levels of IL-2 and IL-4 than wild-type mice. This effect did not extend to IFN-γ, which tended to be lower than controls. Ag-elicited IL-5 levels were comparable. However, we did note that spontaneous levels of IL-5 and -13 were greater in CCR1 knockout cultures (data not shown). With regard to RANTES treatment, the B6 × 129 wild-type mice responded similarly to the CBA strain, showing a selective dose-dependent inhibition of IL-4 production. In CCR1 knockout cultures, RANTES failed to demonstrate statistically significant effects. The CCR3 agonist, eotaxin, did not

Table II. Effect of in vivo RANTES infusion on day 3 type 1 and 2 granuloma cell compositiona

<table>
<thead>
<tr>
<th>Lesion and Treatment</th>
<th>Lymphocytes</th>
<th>Large Mononuclear</th>
<th>Eosinophils</th>
<th>Neutrophils</th>
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<td>PPD-bead</td>
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<td></td>
</tr>
<tr>
<td>Vehicle (PBS)</td>
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<td>33 ± 13</td>
<td>2 ± 3</td>
<td>9 ± 2</td>
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<tr>
<td>RANTES</td>
<td>54 ± 4</td>
<td>35 ± 6</td>
<td>5 ± 1</td>
<td>7 ± 1</td>
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<tr>
<td>SEA-bead</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (PBS)</td>
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<td>39 ± 1</td>
<td>18 ± 3</td>
<td>4 ± 2</td>
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<td>50 ± 8*</td>
<td>42 ± 1</td>
<td>10 ± 3*</td>
<td>5 ± 1</td>
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</tbody>
</table>

* Numbers represent mean percentages ± SD. Groups of PPD- or SEA-sensitized mice were each implanted with miniosmotic pumps releasing a constant infusion of recombinant hRANTES (20 μg/day) or vehicle (PBS). Two days later, the groups were respectively challenged i.v. with PPD or SEA Ag-beads to induce granulomas. On day 3, lesions were collected and dispersed, then stained cytospin smears were prepared for differential analysis. Four mice per group.

* \( p < .05 \).
effect IL-4 in either wild-type or knockout strains. However, as we previously reported (33), 100 ng/ml eotaxin augmented IFN-\(\gamma\) levels (ANOVA, \(p<0.001\) and \(p<0.0001\) for control and knockout mice, as compared with 0 ng/ml). Surprisingly, eotaxin at 100 ng/ml inhibited IL-5 in CCR1 knockout, but not in wild-type, cultures, suggesting that CCR1 deficiency enhanced sensitivity to the CCR3 agonist.

**Discussion**

The biologic functions of RANTES have largely been defined in vitro, with significant emphasis on its eosinophil, monocyte, and T cell chemotactic properties (34–36). Consequently, we and others have generated circumstantial evidence suggesting a proinflammatory role for RANTES in allergic conditions (12–16, 37). However, other than local administration (38), direct in vivo testing of RANTES function has been lacking. Moreover, there are reports challenging RANTES role in eosinophil recruitment. For example, a careful temporal analysis of allergen-challenged human subjects failed to show an association of RANTES with eosinophil recruitment (39). Furthermore, RANTES had been demonstrated in conditions that are not traditionally considered allergic-type, such as delayed-type hypersensitivity (2), arthritis (17), sarcoidosis (18), and endotoxemia (40). In addition, Schrum et al. (20) reported that human cells show strong expression of RANTES in the type 1 response to the Gram-negative bacterium, *Yersinia enterocolitica*, but weak expression in the type 2 response to the nematode *Acanthocheilonema viteae*. Others have also presented evidence that IFN-\(\gamma\) and TNF-\(\alpha\) augment RANTES, whereas type 2 cytokines IL-4 and IL-13 reduce expression (7, 41, 42).

To help clarify the function of RANTES in vivo, we performed a detailed multiparameter analysis of RANTES expression and function in defined models of polarized type 1 and 2 pulmonary granuloma formation. Both protein and mRNA measurements revealed strong expression of RANTES during the type 1 (Th1) response to *M. bovis* PPD with weaker expression in the type 2 (Th2) response to the *S. mansoni* egg Ags. This was in complete accord with studies of human cells reported by Schrum et al. (20) and noted above. Our results demonstrated differential production at both the local sites of inflammation and in draining lymphoid tissues. The association with the type 1 response fits well with reports showing that IFN-\(\gamma\) and TNF-\(\alpha\) promote RANTES production (41, 42), since these cytokines characterize the type 1 granuloma (43). This notion is also supported by our previously reported observation that RANTES and TNF-\(\alpha\) mRNA are reduced in IFN-\(\gamma\) knockout mice during the type 1 granulomatous response (44).
Because of the synchronized nature of our models, we were able to define the time course of local and regional RANTES production. Maximal local production during the type 1 response occurred during the cellular recruitment phase, 1–4 days. In addition, RANTES neutralization resulted in decreased granuloma size. These findings are fully consistent with RANTES chemotactic role and concur with studies showing that Th1 cells are preferentially attracted to RANTES (45). However, in view of RANTES reported effects on T cells (10, 11), other interpretations must be considered. The chemotactic function of RANTES may be secondary to immunoregulatory effects. If RANTES is promoting local Th1 cell activation, then neutralization might similarly cause decreased inflammation, independent of chemotactic function. Alternatively, or in addition, if RANTES is inhibiting the cross-regulatory influence of type 2 cytokines, like IL-4 and IL-13 (which are present but at low levels in type 1 lesions), then its neutralization might lead to lesion impairment by enhancing the effect of these opposing cytokines. Further studies will be needed to determine which mechanisms are participating.

In lymph nodes, RANTES production paralleled the lymphoproliferative response maximizing on day 4 and suggesting the expansion of RANTES producing cells, possibly Th1 clones as the studies of Schrum et al. (20) would suggest. Interestingly, Ag-stimulated cultures produced significant levels of RANTES within 24 h. Based on in vitro studies, this seems rapid since Ag-stimulated T cells reportedly produce RANTES primarily as a late transcription (3–5 days) product (46). This would suggest that production occurs more rapidly after in vivo priming than other RANTES-producing cells are present.

Our depletion studies provided compelling and novel findings regarding the role of RANTES in the type 1 and 2 responses. Although RANTES may act as an eosinophil chemotactic agent in acute allergy (37), it did not seem to recruit eosinophils in the type 2 granuloma. In fact, endogenous RANTES supported the type 1 and inhibited the type 2 granuloma. This result was analogous to the cross-regulatory effect reported with IFN-\(\gamma\) depletion (22, 23). Since maximal RANTES production is in part dependent on IFN-\(\gamma\), it would suggest that RANTES is a downstream cross-regulatory agent. Furthermore, it is known that IL-4 and IL-13 down-regulate RANTES (7, 41, 42), thus defining a cross-regulatory circuit analogous to that involving IFN-\(\gamma\) and IL-10 (47). In this scenario, RANTES would be another element in defining the balance of Th1 and Th2 cell expression.

This regulatory role was further revealed by observed effects on lymphoid tissue during the type 2 response, where RANTES depletion broadly augmented and exogenous administration broadly abrogated type 2 cytokines. Although it might be argued that the observed effects on granulomas were due to complex modifications of chemotactic gradients, it is difficult to conceive how this would apply to the changes in regional lymph node cytokine profiles. In view of RANTES role in T cell activation (10, 11), it seems more reasonable to propose that our findings reflect regulation of Th2
lymphocyte function or maturation. Although not providing a detailed mechanism, our in vitro studies would support this explanation. During the in vitro Ag-elicted response, RANTES inhibited IL-4 production, but not IL-5. Superficially, this seems inconsistent with the broad effects on type 2 cytokines observed following in vivo treatments, but based on current paradigms of T cell maturation, there is likely no contradiction. Since IL-4 is an upstream promotor of Th2 development, its abrogation by RANTES would eventually broadly degrade the type 2 profile, as we observed in over 5 days of in vivo RANTES treatment. However, in vitro cultures treated for just 24 h may only show effects on the sensitive target cell population. Downstream changes in other Th2 cytokines would require longer exposure. Although it is possible that RANTES mediates specific IL-4 gene regulation, we would rather offer that RANTES acts on a precursor IL-4-producing stage of Th2 cell development with mature Th2 cells being resistant. This would explain why RANTES treatment achieved only 50% inhibition of IL-4 in vitro. This explanation is purely speculative and many basic mechanistic questions have yet to be resolved, such as characterization of the specific source(s) and target(s) of RANTES action within lymphoid tissues.

Whatever the underlying mechanism, our findings indicate that the action of RANTES on the type 2 lymphoid response involves ligation of the CCR1 receptor. This was unexpected since CCR1 is only one of several potential RANTES receptors (9). The importance of this receptor was suggested by the baseline augmentation of IL-4 production in CCR1 knockout mice, but this was directly demonstrated by their failure to respond to RANTES-mediated IL-4 inhibition. Of course, future studies designed to reintroduce the CCR1 receptor will help to confirm its regulatory role. Interestingly, IL-4 inhibition could not be replicated by the CCR3 agonist, eotaxin. In view of reports showing that CCR3 is selectively expressed on Th2 cells (48), the participation of the CCR1 receptor in regulating the type 2 response suggests an additional level of complexity. If mature Th2 memory cells cannot directly respond to RANTES via CCR1, then indirect effects must be considered, such as intermediary cells or, as proposed above, stage-specific regulation. Recent evidence suggests the existence of a dynamic T cell subset selective expression of chemokine receptors that is under cytokine-mediated control (49, 50). Therefore, lymphocyte regulation via transient, stage-specific chemokine receptor expression is a reasonable hypothesis. It should be noted that a detailed analysis of type 1 and 2 granuloma formation in CCR1 knockout mice is underway and will be reported later.

Although RANTES clearly appears to temper the type 2 response, its role in type 1 lymphoid response is unclear. A seeming inconsistent finding was that during the type 1 (PPD) response, RANTES depletion did not reduce Th1 cytokines, yet parenteral RANTES administration augmented production. A simple explanation is that due to chemokine redundancy, RANTES is not by itself essential in promoting the Th1 response. We have reported that both monocyte chemotactant protein-1/JE and macrophage-inflammatory protein (MIP)-α can promote IFN-γ production in lymphoid cultures, and, like RANTES, MIP-1α inhibits IL-4 production (51). Thus, there is definite potential for redundancy. The administration of exogenous RANTES may simply augment a mixed pool of endogenous agonists that promote the type 1 response, yet elimination of the endogenous RANTES component may not be sufficient to impair the response.

In summary, this is the first comprehensive in vivo investigation of RANTES expression and function in polarized type 1 and 2, T cell-mediated granuloma formation. Our studies clarify a number of confusing observations and provide novel evidence for RANTES as a cross-regulatory molecule that tempers type 2, hypersensitivity inflammation. Finally, we demonstrate the potential use of exogenous RANTES to temper established type 2 hypersensitivity states.

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References


