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Immunoregulatory Role of Ocular Macrophages: The Macrophages Produce RANTES to Suppress Experimental Autoimmune Uveitis

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Murine experimental autoimmune uveitis (EAU) is a model of human uveitis. Ocular-infiltrating macrophages play a crucial role in the generation of tissue damage in EAU. In fact, several chemokines are actually produced in the inflamed eye. The aim of this study was to elucidate the role of ocular macrophage-derived chemokines in EAU. C57BL/6 mice were immunized with human interphotoreceptor retinoid binding protein peptide 1–20, and the EAU severity was scored at multiple time points based on microscopic fundus observations (retinal vascular dilatation and exudates) and histological examinations. The peak inflammatory response was observed 1 wk (day 16) after the beginning of macrophage infiltration to the eye (day 9). Ocular-infiltrating cells were enriched or depleted of macrophages by magnetic beads and analyzed by real-time RT-PCR for chemokine mRNA production. We found that the macrophage-enriched cells from the eye produced RANTES, and thus proposed that macrophage-derived RANTES facilitated the ocular inflammations. In contrast to our postulate, neutralization of RANTES by specific Ab in vivo on days 9 and 13 exacerbated EAU. We also found that the ratio of ocular CD4/CD8 T cells was markedly increased after treatment. As a result, RANTES neutralization might exacerbate EAU by modulating the type of T cell subsets recruited to the eye. In conclusion, our data provide insight into the immunoregulatory role of macrophages and RANTES in the pathogenesis of ocular inflammation. Not all macrophage-derived chemokines cause local inflammation, since RANTES produced by ocular macrophages appears to suppress EAU. The Journal of Immunology, 2003, 171: 2652–2659.

Experiential autoimmune uveoretinitis (EAU) is an organ-specific, T cell-mediated, autoimmune disease that can be induced in several animal species by immunization with retinal Ag (interphotoreceptor retinoid-binding protein [IRBP] or S-Ags) emulsified with CFA. The immunization of animals results in a disease that resembles human uveitis. During EAU, the blood-retina barrier is broken, and monocytes/macrophages and Ag-specific T lymphocytes move into the retina and cause tissue damage.

Macrophages are known to play an important role in causing tissue damage in several other experimental autoimmune diseases, including collagen-induced arthritis (4), nephritis (5), thyroiditis (6), and encephalomyelitis (7). In uveoretinitis, macrophages are involved in the phagocytosis of rod outer segments (8–10). Depletion of macrophages in the uveitis model in rats reduced the disease score; therefore, activated macrophages are major effectors of tissue damage in uveitis (11–13). Macrophages also are considered to be potent APCs during the induction of inflammatory autoimmune diseases (14, 15).

The recruitment of leukocytes is a crucial feature of ocular inflammation in EAU. Increased chemokine production is correlated to leukocyte recruitment in the eye. In a recent study of patients, IL-8, inflammatory protein-10 (IP-10), monocyte chemotactant protein-1 (MCP-1), RANTES, and macrophage inflammatory protein-1α (MIP-1α) were significantly increased in the aqueous humor during the active stages of anterior uveitis (16). Chemokines are also associated with the neuroinflammatory processes in experimental allergic encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS) (7). In this model, increases in MCP-1 and MIP-1 mRNA correlated with disease severity and occurred along with or before disease onset (17, 18).

Importantly, a histochemical analysis of MS brain or spinal cord showed that the expression of the CC chemokines, MCP-1, MIP-1, IP-10, and RANTES, correlated with the local infiltration of macrophages (19–21). These chemokines appear to play a role in the macrophage infiltration associated with various forms of MS. To date, little has been known about the role of macrophage-derived chemokines in uveitis. This report tests the hypothesis that increases in macrophage-derived chemokines are responsible for leukocyte infiltration into the eye during uveitis.

RANTES (CCL5) belongs to the CC chemokine family and induces leukocyte migration by binding to specific receptors in the G protein-coupled receptor family (22, 23). It mediates the trafficking...
and homing of classical lymphoid cells such as T cells and monocytes, but also acts on a range of other cells, including basophils, eosinophils, NK cells, dendritic cells, and mast cells (22). RANTES production, which is generated predominantly by CD8+ T cells, epithelial cells, fibroblasts, and platelets, is a particular feature of inflammation. Increased RANTES expression is associated with a wide range of inflammatory disorders and is thought to promote leukocyte infiltration to the sites of inflammation (24). Also, RANTES may be able to activate leukocyte through its G protein-coupled transmembrane receptor (22).

Here we show that ocular-infiltrating macrophages produce RANTES during EAU and explore its role in the pathogenesis of local inflammation. Our data provide insight into the immunoregulatory role of ocular macrophages through RANTES, and thus these data support the idea that RANTES contributes in part to the immune privilege of the eye.

Materials and Methods

Mice

Female mice, 8–10 wk old, were used in all experiments. C57BL/6 (B6) mice were obtained from SLC Japan (Shizuoka, Japan). The animals were maintained on food and water ad libitum until they reached the desired weight (20–24 g). All animals were treated humanely and were housed in specific pathogen-free conditions at Kyushu University. All treatment of the animals conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

EAU induction and evaluation

C57BL/6 mice were immunized with human IRBP peptide 1–20 sequence (GPTHLFQPSLVLDMAKVLLD), which has been shown to induce EAU (25, 26). The mice were first immunized s.c. in one footpad and the base of tail with the peptide in 0.2 ml of emulsion in CFA (1/1, v/v), supplemented with Mycobacterium tuberculosis strain H37Ra to 2.5 mg/ml, and then inoculated i.p. with 1.5 g of pertussis toxin.

Funduscopy examinations of mice were performed 9 days following inoculation. Tropicamide (0.5%) was applied to the eyes to dilate the pupil. After confirming mydriasis, the fundus of the eye was examined with a Bonnscope and Super Field NC Lens (Volk Optical, Mentor, OH). Two ophthalmologists performed the clinical assessments in a masked fashion. The presence of vessel dilatation, vessel white focal lesions, vessel white opacifications, and optic disc hyperemia was scored on a scale of 0–3. In brief, the tissue around the eyeball was removed, and eyeball was dissected to remove the cornea and lens. The remaining portion of the eye (including the iris, ciliary body, retina, choroids, etc.) was minced with scissors and shaken in medium supplemented with 0.5 mg/ml of collagenase type D (Roche, Mannheim, Germany) at 37°C for 40 min. As a basic medium, we used RPMI 1640 (Life Technologies) with 10% FBS (Life Technologies/H9262). Recombinant mouse IFN-γ (BD PharMingen) was used to construct a standard curve, and biotinylated rat anti-mouse IFN-γ mAb (XMG1.2; BD PharMingen) was used as the detecting Ab. The plates were treated with alkaline phosphatase-conjugated ExtrAvidin (Sigma-Aldrich, St. Louis, MO), colored substrate by p-nitrophenyl phosphosphate (Sigma-Aldrich), and OD was measured at 405 nm with an MRX Microplate reader (Systeinate Laboratories, Chantilly, VA). The values represent the arithmetic mean of triplicate cultures ± SD.

Abs and reagents

The Abs used for flow cytometric analysis and magnetic bead selection were as follows: Fc Block (anti-mouse FcγRI/IIA/mAb, 2.4G2), biotin-conjugated anti-CD3 (GL3), biotin- or FITC-conjugated anti-NK1.1 mAb (PK136), CyChrome 5-conjugated anti-TCRβ mAb (H57-597), FITC-conjugated CD3 (17A2), FITC-conjugated CD8a (53-6-7), FITC-conjugated anti-ID25 (7D4), FITC-conjugated Gr-1 (RB6-8C5), and PE-conjugated CD4 (RM4-5) were all purchased from BD PharMingen (San Diego, CA). FITC-conjugated anti-CD45 mAb (YW62.3) was purchased from IQ Products (Groningen, The Netherlands). Biotin-conjugated anti-F4/80 mAb (A3-1) was purchased from Caltag (Burlingame, CA). Streptavidin/PE-PE was purchased from Molecular Probes (Eugene, OR).

The Abs used for in vivo treatment were as follows: rat anti-mouse RANTES mAb (53405.111) was purchased from R&D Systems (Minneapolis, MN). Purified rat IgG was purchased from Sigma-Aldrich for use as a control for anti-RANTES mAb. Each Ab was inoculated i.p. (100 μg/mouse) on the days 9 and 13 after IRBP immunization.

Flow cytometry

The numbers of ocular-infiltrating cells were adjusted to the appropriate concentrations and then were stained by two colors with FITC anti-CD45 mAb and biotin-conjugated anti-F4/80 mAb, followed by streptavidin/R-PE. These macrophages are positive for CD45 and F4/80 surface molecules. As a result, the CD45 and F4/80 double-stained cells were analyzed as macrophages. Flow cytometry was performed with EPICS XL (Beckman Coulter, Mannheim, Germany). The number of intraocular-infiltrating macrophages was calculated using the percentage of each population within the gate and the precounted total number of viable cells using trypan blue dye exclusion. Gates were set for viable cells by forward and side scatter analysis.

Macrophage enrichment

For macrophage enrichment, the ocular-infiltrating cells were treated with biotin-conjugated anti-F4/80 mAb before magnetic bead selection. Ab-bead-bound cells were treated with streptavidin MicroBeads (Miltenyi Biotec, Gladbach, Germany) for 15 min and washed twice in PBS (pH 7.2) containing 0.5% BSA and 2 μM EDTA. To harvest macrophage-enriched cells, cells were applied to type MS+ positive selection column with MiniMACS (Miltenyi Biotec). Selected cells were stained by FITC-conjugated anti-CD45 mAb and biotin-conjugated anti-F4/80 mAb.
CD45 (RM4-5) and streptavidin-R-PE for counterstaining with biotin-conjugated anti-F4/80 mAb (A3-1), and then the degree of enrichment was confirmed by flow cytometry.

We performed not only the positive selection, but also the negative selection of ocular-infiltrating macrophages. Ocular-infiltrating cells were treated with a mixture of FITC-conjugated anti-CD3 mAb, FITC-conjugated anti-RT20 mAb, FITC-conjugated anti-Gr-1 mAb, and biotin-conjugated anti-NK1.1 mAb before magnetic bead selection. Thereafter, the cells were treated with a mixture of anti-FITC MicroBeads and streptavidin MicroBeads (Miltenyi Biotec) for 15 min and then washed twice. To harvest the population depleted with T cells, B cells, NK cells, and the neutrophils, cells were applied to a type M5+ positive selection column with MiniMACS (Miltenyi Biotec). All Ab-labeled cells were removed by magnetic field, and negatively selected macrophages were collected as eluted cells. These cells were stained by streptavidin-R-PE for counterstaining with biotin-conjugated Abs, and then the enrichment was confirmed by flow cytometry. The cell number of the enriched populations was adjusted to approximate the number used in the control studies.

**Conventional and real-time RT-PCR**

Total mRNA were extracted from column-enriched macrophages and other remaining populations (macrophage-eliminated) in ocular-infiltrating cells 16 days after immunization using TRZol (Life Technologies). Total mRNA was reverse transcribed using GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT). First-strand cDNA (20 μl final volume) was synthesized using AMV reverse transcriptase (Roche, Indianapolis, IN) according to the manufacturer’s guidelines. cDNA was subjected to PCR with a 20-μl volume containing 2 μl of LightCycler DNA Master SYBER Green 1 (containing Taq DNA polymerase, reaction buffer, dNTP mix, SYBER Green I dye, and 10 mM MgCl2) (×10 concentration; Roche), 2.4 μl of MgCl2 stock solution (25 mM), 11.6 μl of H2O, 1 μl of sense primer (10 pmol), 1 μl of antisense primer (10 pmol), and 2 μl of sample cDNA. PCR was performed by LightCycler instrument (Roche). The PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. The intensity of ethidium bromide was measured using image sensor (Densitograph, ATTO, Tokyo, Japan) with a computer-controlled display. The amount of RNA in each sample was standardized by the preliminary amplification for GAPDH and readjusting the sample concentration according to the densitometry reading of GAPDH bands, as described above. The adjusting systems were repeated until the GAPDH bands were equalized in serially diluted samples. The primers were listed below. For amplification: GAPDH: sense, 5′-GGC CGC TCT AGG CAC CAA-3′; and antisense, 5′-CTC TTT GAT GTC ACA CTG GTC AC-3′; β-actin: sense, 5′-ACA GGT CAA ACT ACA ACT CCA-3′; and antisense, 5′-TCA GCT TTT GAT GTC ACA CTG GTC AC-3′; RANTES: sense, 5′-AGA TTC GCA GAG CAA AGG TTG CAA GAG CAA-3′; and antisense, 5′-TTA CTG GTC ACA CTG GTC AC-3′ (product size, 252 bp).

To perform a quantitative analysis, real-time RT-PCR assay was conducted to detect RANTES mRNA expression by these cells. The cDNA was diluted 1/2 (40 μl final volume), and aliquots of 2 μl were analyzed by real-time PCR with a Light Cycler instrument. The total cDNA from the cells was added to the 18 μl of master mixture (FastStart DNA Master Hybridization Probes; Roche) according to the manufacturer’s instructions. To determine the amount of RANTES mRNA RT-PCR amplification, target (RANTES) and control (β-actin) hybridization probes were mixed with target and control PCR primers, respectively. This mixture was transferred to a set of thermal cycler tubes and transcribed at 42°C for 30 min, followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min. RANTES mRNA expression was estimated from the ratio of fluorescence intensity to β-actin. PCR primers used for these analyses are as follows: RANTES: sense, 5′-AGATTCGCCCTGACTCATC-3′; and antisense, 5′-CTCCTGGTTGCTGACACACTT-3′; β-actin: sense, 5′-CTCTGTAATGCCTCTGCTGTA-3′; and antisense, 5′-CCAGAGCAATTTCTCTTCTCAGC-3′ (3′ label, fluorescein); and antisense, 5′-CCAGAGCAATTTCTCTTCTCAGC-3′ (3′ label, fluorescein); and antisense, 5′-CCAGAGCAATTTCTCTTCTCAGC-3′ (3′ label, fluorescein); and antisense, 5′-AGATTCGCCCTGACTCATC-3′ (3′ label, fluorescein). The probes used for these analyses are as follows: RANTES: sense, 5′-GGATATTTCTCTTCTCAGCAGCAGAATGT-3′ (3′ label, fluorescein); and antisense, 5′-TCACTCTCCTGCTGCAAGTCT-3′. The probes used for these analyses are as follows: RANTES: sense, 5′-GGATATTTCTCTTCTCAGCAGCAGAATGT-3′ (3′ label, fluorescein); and antisense, 5′-TCACTCTCCTGCTGCAAGTCT-3′. The probes used for these analyses are as follows: RANTES: sense, 5′-GGATATTTCTCTTCTCAGCAGCAGAATGT-3′ (3′ label, fluorescein); and antisense, 5′-TCACTCTCCTGCTGCAAGTCT-3′. The probes used for these analyses are as follows: RANTES: sense, 5′-GGATATTTCTCTTCTCAGCAGCAGAATGT-3′ (3′ label, fluorescein); and antisense, 5′-TCACTCTCCTGCTGCAAGTCT-3′ (3′ label, fluorescein). The data were analyzed to determine any significant differences among the experimental groups using Student’s t test. A value of p ≤ 0.05 was considered significant.

**Results**

**Kinetics of ocular-infiltrating macrophage in EAU**

To examine the kinetics of ocular-infiltrating macrophages in EAU, we isolated the ocular-infiltrating cells using collagenase treatment and analyzed them by flow cytometry. While few F4/80+ macrophages were detected in the cells from eyes of naive animals, F4/80+ macrophages were clearly detected (~40% of ocular-infiltrating cells) in the gated live cells harvested from EAU eyes (Fig. 1A). Ocular macrophage infiltration was first observed 9 days after immunization, peaked 1 wk later (day 16), and thereafter gradually decreased (Fig. 1B).

**Ocular-infiltrating macrophages are the major cellular source of RANTES**

It is well established that macrophage-derived cytokines/chemokines contribute to tissue damage during severe experimental autoimmune diseases (11, 12). Since cytokines and chemokines appear to be crucial for ocular inflammation (16), we postulated that ocular-infiltrating macrophages actually produced the cytokines and chemokines that mediated EAU. To investigate the macrophage-producing cytokine and chemokine profile, ocular-infiltrating cells derived from day 16 EAU mice were divided into a macrophage-enriched population and a macrophage-eliminated population by magnetic beads. The purity of each population was confirmed by flow cytometry. The ratios of the F4/80+ CD45+ macrophages in the macrophage-enriched and macrophage-eliminated population were 97.8 and 4.6%, respectively (Fig. 2A). Thereafter, total RNA was extracted from each sample.

To identify the specific cytokines or chemokines that derived from ocular-infiltrating macrophages, we first identified the different cytokines and chemokines found in macrophage-positive and -negative populations by RT-PCR (including IL-1β, IL-6, IL-12, GM-CSF, TNF-α, IFN-γ, MCP-1, RANTES, IP-10, MIP-1αβ, and MIF). As a result, RANTES was found to be the only chemokine that was highly associated with macrophages (Fig. 2B). To achieve a quantitative assessment, real-time PCR was performed. As shown in Fig. 2C, most of the RANTES was derived from a macrophage-enriched population on day 16 of EAU. We further confirmed this fact in the negatively selected macrophages (T cells, B cells, NK cells, and neutrophils were removed by magnetic beads) to eliminate the possibility that the RANTES production was not due to activation through anti-F4/80 Ab binding. Importantly, the negatively selected macrophages produced equivalent amount of RANTES in EAU as the positively selected macrophages (Fig. 2C). These data show that the ocular-infiltrating macrophages are the major cellular source of RANTES during EAU.

**Neutralization of RANTES exacerbated EAU**

RANTES is associated with inflammation in various autoimmune diseases (30), but the precise role of RANTES in EAU is not known. To investigate the role of macrophage-derived RANTES in EAU generation, we inactivated neutralizing anti-RANTES Ab into the mice on day 9, which is the beginning of the recruitment of infiltrating macrophages to the eye (Fig. 1B). We expected that blocking an inflammatory chemokine such as RANTES would suppress EAU. However, anti-RANTES Ab treatment of the mice exacerbated the clinical symptoms of EAU throughout the time course (Fig. 3A). The typical pictures of the ocular fundus and anterior segment
Representative prepared (see immunization the mice were euthanized, and ocular-infiltrating cells were
investigated with human IRBP peptide 1 similarly treated cells derived from the eyes of naive animals (number of.
In the control Ig-treated and another group of splenocytes between control and anti-RANTES Ig-treated animals (data not shown). We observed that cells from control Ig- and anti-RANTES Ig-treated groups produced IFN-γ predominantly (Fig. 4), while IL-4 production was not detectable (<5 pg/ml). Thus, Th1 cells were induced in the RANTES-neutralized animals. Although the IFN-γ-producing ability of the RANTES-neutralizing animals seemed to be impaired compared with that of the control Ig-treated animals, the anti-RANTES-treated animals showed more severe EAU than the control Ig-treated animals.

**RANTES neutralization changed the subsets of ocular-infiltrating T cells**

Since RANTES is primarily a chemoattractant for cells, we reasoned that if it were neutralized, there would be a difference in the cells that were recruited to the eye. The phenotypes of ocular-infiltrating cells were examined by flow cytometry. The number of ocular-infiltrating macrophages was not significantly different on days 13 and 16 post-IRBP immunization (Fig. 5A). While the total number of T cells slightly increased in the eye of anti-RANTES-treated mice, there was not a significant difference compared with the number of T cells in the eye of the control Ig-treated mice on the day 16 (Fig. 5B). RANTES neutralization did not affect either macrophage or T cell accumulation in EAU. We then hypothesized that the neutralization of macrophage-derived RANTES might influence the T cell subpopulations recruited to the eye during EAU. The ratio of CD4/CD8 ocular-infiltrating T cells in the eyes of anti-RANTES Ig-treated mice was significantly increased (Fig. 5C). CD4+ Th1 cells are well established to be the main effector cells in the murine EAU model. RANTES neutralization, therefore, results in the predominant accumulation of CD4+ effector T cells to the eye.

We also examined both the ratio and the number of CD4+ T cell, CD8+ T cell, and other cell populations in the draining lymph nodes (inguinal lymph nodes) and spleen after anti-RANTES Ig inoculation by flow cytometry. No difference was seen between the control Ig-treated and anti-RANTES Ig-treated animals regarding the ratio and number of T cell subsets, B cells, macrophages, and neutrophils (data not shown). Thus, compared with the dramatic changes in the eye, anti-RANTES treatment induced minimal changes in the peripheral lymphoid organs.

We also checked the in vivo blocking ability of anti-RANTES mAb by peritoneal cell recruitment assay. Mice were i.p. inoculated with recombinant RANTES to recruit the cells to peritoneal cavity. One hour later either anti-RANTES (100 μg/100 μl of PBS/mouse; n = 5) or control Ig (100 μg/100 μl of PBS/mouse; n = 5) was i.p. inoculated. At 18 h from recombinant RANTES inoculation, total lavage was pooled for individual mice, and total cells collected were counted. The anti-RANTES mAb significantly blocked the RANTES-dependent cell recruitment into the peritoneal cavity (control Ig, 3.57 ± 1.20 × 10⁶/mouse; anti-RANTES mAb, 0.75 ± 0.23 × 10⁶/mouse; p = 0.009).

**Th1 cells were induced in the RANTES-neutralized animals**

It is clearly established that autoreactive Th1 cells mediate EAU (31), and EAU induction is correlated with T cell-derived IFN-γ production (32). Thus, one possibility to explain our observation is that anti-RANTES Ab treatment modulated the autoreactive Th1 cell induction and changed the Th1/Th2 balance. We compared IFN-γ and IL-4 production from a mixture of immunized lymph node cells and splenocytes between the control Ig-treated and anti-RANTES Ig-treated mice on day 16. There was no difference in the total mixture of splenocytes and regional lymph node cell numbers harvested between control and anti-RANTES Ig-treated animals (data not shown). We observed that cells from control Ig- and anti-RANTES Ig-treated groups produced IFN-γ predominantly (Fig. 4), while IL-4 production was not detectable (<5 pg/ml). Thus, Th1 cells were induced in the RANTES-neutralized animals. Although the IFN-γ-producing ability of the RANTES-neutralizing animals seemed to be impaired compared with that of the control Ig-treated animals, the anti-RANTES-treated animals showed more severe EAU than the control Ig-treated animals.

**FIGURE 1.** Kinetics of ocular-infiltrating macrophages in EAU. A. Representative fluorescence pattern of ocular macrophages based on flow cytometry findings. C57BL/6 mice (n = 3, total of six eyes) were immunized with human IRBP peptide 1–20 that induces EAU. On day 16 after immunization the mice were euthanized, and ocular-infiltrating cells were prepared (see Materials and Methods). As a negative control we used similarly treated cells derived from the eyes of naive animals (number of mice = 3, total of six eyes). Cells were counted (trypan blue dye exclusion), stained with biotin-conjugated anti-F4/80 mAb (A3-1), counterstained by streptavidin/R-PE, and analyzed by flow cytometry. Leukocyte gates were set according to the forward and side scatters for live cells. The fluorescence pattern of cells from naive animals (shaded) and EAU animals (open) are indicated with the percentage of F4/80+ macrophages in the live cells in the panel. The gate was set according to the data using isotype control Ab in the EAU sample. The experiment was repeated a minimum of three times. B. The kinetics regarding the number of ocular macrophage in EAU. At multiple time points after IRBP immunization, the number of ocular-infiltrating macrophages per eye was calculated based on the percentage of each population in the gate of the precounted total number of viable cells using trypan blue dye exclusion (n = 5/time point). The average number of right and left eyes represented the number of macrophages for each individual animal. The experiment was repeated twice.

in each group are shown in Fig. 3B. In the control Ig-treated animals, the optic disc was slightly vague compared with naive mice, and there was no sign of synchia in the iris. The animals in which RANTES was neutralized presented a completely obscured margin of the optic disc, severe retinal exudate with retinal vein dilatation, and posterior synchia in iris. In addition, histological examination on day 16 confirmed that ocular inflammation was exacerbated when RANTES was neutralized (Fig. 3C). There was only mild cellular infiltration in the eyes of animals treated with control Ig, while the eyes of mice treated with anti-RANTES Ig showed massive infiltration of inflammatory cells at multiple parts of vitreous cavity and retina and edema in the retina (Fig. 3C, upper pictures).

To exclude the possibility that immune complex might be formed in the anti-RANTES Ig-treated EAU mice, we stained and analyzed paraffin-fixed sections immunohistologically. As a result, no immune complex was observed in either the section derived from the anti-RANTES Ig-treated mice or that from the control Ig-treated mice (Fig. 3D).
To investigate the contribution of potential regulatory cells against ocular macrophage-derived RANTES in EAU, we also analyzed the ratio of CD25+ T cells, NK T cells, and γδ T cells in ocular-infiltrating cells as potential immunoregulatory T cells on day 16 after immunization. CD25+ T cells in the TCRβ-chain-positive gate were 1.8 ± 0.52 and 1.9 ± 0.88% in the control Ig-treated (n = 5) and anti-RANTES Ig-treated (n = 5) groups, respectively. NK1.1+ T cells in the TCRβ-chain-positive gate (NK T cells) were 0.9 ± 0.43 and 0.8 ± 0.75% in the control Ig-treated (n = 5) and anti-RANTES Ig-treated (n = 5) groups, respectively. The ratios of TCRγδ+ T cells in the CD3-positive gate (γδ T cells) were 5.1 ± 1.3 and 4.8 ± 2.5% in the control I-treated (n = 5) and anti-RANTES Ig-treated (n = 5) groups, respectively. There was no statistical difference between the control Ig and anti-RANTES Ig-treated groups regarding the ratio of these cells.

### Discussion

Ocular-infiltrating macrophages are known to contribute to inflammation during EAU (10, 12). However, in this paper we demonstrate that a large number of macrophages were recruited to the eye in murine EAU and produced RANTES, which appears to regulate inflammation, since neutralization of macrophage-derived RANTES by the specific Ab exacerbated ocular inflammation and resulted in the predominant recruitment of pathogenic CD4+ T cells.

This is not the first report to demonstrate the immunoregulatory potential of ocular macrophages in EAU. Laliotou et al. (33) demonstrated the accumulation of IL-10-producing macrophages in animals made tolerant for retinal Ag. In addition, Robertson et al. (34) reported that resident retinal macrophages and those from recovery phase EAU behave in a similar manner as macrophages conditioned by anti-inflammatory cytokines such as TGF-β. However, our studies show for the first time that macrophage-derived RANTES may participate during the peak clinical period in terminating local inflammation. RANTES may be responsible for recruiting CD8+ T cells that regulate the response or encourage the recovery.

We believe that the systemic anti-RANTES Ig inoculation efficiently neutralizes RANTES derived from ocular macrophages in this experiment. In another study i.p. inoculated Ab was reported to actually be delivered to the eye (35, 36), and our data showed that macrophages were the only cells that actively produce RANTES in EAU (Fig. 1). Since macrophages begin to enter the eye on day 9 (Fig. 1B), Ab inoculation was given 9 days post-IRBP immunization. In addition, giving the Ab at 9 days minimized the ability of Ab to interfere with the role of RANTES during the induction phase. Autoreactive Th1 cells were developed in the CD3-positive gate (10-40%) compared with CD4+ T cells (3-10%) (38). Since fewer CD8+ T cells were recruited in the absence of RANTES, we propose that ocular macrophage-derived RT-PCR). The CDNA derived from macrophage-eliminated populations (Mac., positively enriched macrophages (Mac.+, positive selection), and negatively enriched macrophages by elimination of T cells, B cells, NK cells, and neutrophils (Mac., negative selection) was analyzed by real-time PCR with a Light Cycler instrument. The target (RANTES) and control (β-actin) hybridization probes were mixed with target and control PCR primers, respectively. RANTES mRNA expression was estimated from the ratio of fluorescence intensity to β-actin. Each experiment was repeated a minimum of three times.
RANTES recruits CCR5$^+$ CD8$^+$ T cells predominantly to the inflammation site. Although Th1 CD4$^+$ T cells are the major effector T cells for the induction of EAU (31), our data indicate that CD8$^+$ T cells are either the immunoregulatory T cells or at least non-pathogenic T cells in EAU. Perhaps RANTES neutralization resulted in a decrease in the number of nonpathogenic CD8$^+$ T cells, while, in contrast, increasing the number of pathogenic CD4$^+$ T cells instead and exacerbating EAU. Faunce et al. (39) recently demonstrated that NK T cell-derived RANTES recruits CD8$^+$ T cells to the spleen to induce CD8$^+$ regulatory T cells and systemic tolerance induced by Ag inoculation into the eye. This report is compatible with our idea, and thus the possibility arises that CD8$^+$ T regulatory cells were induced in the secondary lymphoid organ, then recruited to the eye by ocular-infiltrating macrophage-derived RANTES during the course of EAU.

Another possible explanation for our data is that RANTES neutralization inhibited the ocular recruitment of so-called natural suppressor cells. CD25$^+$ T cells (40), NK T cells (41, 42), and γδ T cells to the spleen to induce CD8$^+$ regulatory T cells and systemic tolerance induced by Ag inoculation into the eye. This report is compatible with our idea, and thus the possibility arises that CD8$^+$ T regulatory cells were induced in the secondary lymphoid organ, then recruited to the eye by ocular-infiltrating macrophage-derived RANTES during the course of EAU.
cells (43, 44) have all been reported to be suppressor cells in several systems. We thus examined the number of CD25+ T cells, NK T cells, and γδ T cells in ocular infiltrating cells. CD25+ T cells and NK T cells were barely detected (<2.0%) on day 16 after immunization, and no difference was observed between the control Ig and anti-RANTES Ig-treated groups. Although γδ T cells were detected in certain amounts, there was no difference between these groups either. Macrophage-derived RANTES may thus have no effect on other potential regulatory cells in EAU.

Although the IFN-γ-producing ability tends to decrease rather than increase (Fig. 4), anti-RANTES Ig treatment of mice exacerbated clinical symptoms of EAU. However, we still considered that EAU can be a Th1-mediated disease, because IFN-γ sometimes has dual roles. Neutralization of RANTES was performed after the induction of autoreactive Th1 cells; thus, anti-RANTES interfered with the resolution, not the induction, of EAU. Although not studied, IFN-γ may also play a role in the resolution. In fact, IFN-γ-deficient mice showed severe EAU induced by IRBP peptide (45).

Several reports show the local chemokine expression in EAU (46, 47). Adamus et al. (46) recently showed the highest expression of RANTES, MCP-1, and MIP-1α in the eye to be detected at the onset of anterior uveitis associated with rat EAE. Although RANTES expression was observed during the entire course of the disease, anti-RANTES treatment had no effect on the progression of the clinical disease. The discrepancy between these results and ours may be due to a difference in the models. The other reports studied uveitis associated with EAE in rats, which may be mediated by different mechanisms from those involved in EAU in mice. Also, in contrast to our experiment, anti-RANTES Ig was inoculated at the initiation of EAE in their experiments. Therefore, neutralizing any RANTES role affects the induction of the autoimmune responses.

Our data provide a novel insight into a new feature of macrophages during ocular and (perhaps) other local inflammatory dis-

FIGURE 4. Pattern of systemic cytokine production in the RANTES-neutralizing mice in EAU. The spleens and inguinal lymph nodes were removed from four or five mice on day 16 after IRBP immunization in either the control Ig-treated or anti-RANTES Ig-treated group. The cells were incubated with or without IRBP peptide (10 μg/ml) in 96-well culture plates for 48 h. Supernatants were collected, and IFN-γ concentration was measured by quantitative capture ELISA. The values represent the arithmetic mean of triplicate cultures ± SD. A significant difference (p ≤ 0.05) is indicated (+). p values were 0.63 × 10−4 (control Ig-treated mice) and 0.0051 (anti-RANTES Ig-treated mice). The experiment was repeated three times.

FIGURE 5. The phenotype of ocular-infiltrating T cells after neutralizing RANTES. A and B, Comparison of the number of ocular macrophages (A) and T cells (B). Ocular-infiltrating cells were prepared from either control Ig-treated or anti-RANTES Ig-treated EAU mice (n = 4, total of eight eyes) on days 13 and 16 as described in Fig. 1. The cells were stained by biotin-conjugated anti-F4/80 mAb (A3-1) and counterstained by streptavidin/R-PE or CyChrome 5-conjugated anti-TCRβ mAb (H57-597), and flow cytometry was performed as described in Fig. 1. The numbers of ocular-infiltrating macrophages (F4/80+ cells) and T cells (TCRβ+ cells) per eye were calculated based on the percentage of each population in the gate of the precounted total number of viable cells using trypan blue dye exclusion. The average numbers of right and left eyes represented the numbers of macrophages and T cells for each individual animal. The experiment was repeated three times. C, The CD4/CD8 ratio of ocular-infiltrating T cells after treatment with anti-RANTES mAb. Ocular-infiltrating cells were triple-stained with CyChrome 5-conjugated anti-TCRβ mAb (H57-597), PE-conjugated CD4 (RM4-5), and FITC-conjugated CD8a (53-6.7). The ratio of ocular-infiltrating CD4+ and CD8+ T cells in the individual eyes was determined in the gated TCRβ+positive T cells (n = 4, total of eight eyes). The average ratio for the right and left eyes represented the CD4/CD8 ratio for each individual animal. The experiment was repeated three times. A significant difference (p = 0.0022) is indicated (+).

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