Selective Diapedesis of Th1 Cells Induced by Endothelial Cell RANTES

Toshihisa Kawai, Makoto Seki, Kenji Hiromatsu, Jean W. Eastcott, Gerald F. M. Watts, Motoyuki Sugai, Daniel J. Smith, Steven A. Porcelli and Martin A. Taubman

*J Immunol* 1999; 163:3269-3278;
http://www.jimmunol.org/content/163/6/3269

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**  This article **cites 46 articles**, 24 of which you can access for free at: http://www.jimmunol.org/content/163/6/3269.full#ref-list-1

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

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

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Selective Diapedesis of Th1 Cells Induced by Endothelial Cell RANTES

Toshihisa Kawai,* Makoto Seki, Keiichi Hiromatsu, Jean W. Eastcott,* Gerald F. M. Watts, Motoyuki Sugai, Daniel J. Smith,* Steven A. Porcelli, and Martin A. Taubman

Dissociated CD4 T cells can be divided into Th1 and Th2 types based on the cytokines they produce. Differential expression of chemokine receptors on either the Th1-type or the Th2-type cell suggests that Th1-type and Th2-type cells differ not only in cytokine production but also in their migratory capacity. Stimulation of endothelial cells with IFN-γ selectively enhanced transmigration of Th1-type cells, but not Th2-type cells, in a transendothelial migration assay. Enhanced transmigration of Th1-type cells was dependent on the chemokine RANTES produced by endothelial cells, as indicated by the findings that Ab neutralizing RANTES, or Ab to its receptor CCR5, inhibited transmigration. Neutralizing Ab to chemokines macrophage-inflammatory protein-1α or monocyte chemotactic protein-1 did not inhibit Th1 selective migration. Whereas anti-CD18 and anti-CD54 blocked basal levels of Th1-type cell adherence to endothelial cells and also inhibited transmigration, anti-RANTES blocked only transmigration, indicating that RANTES appeared to induce transmigration of adherent T cells. RANTES seemed to promote diapedesis of adherent Th1-type cells by augmenting pseudopod formation in conjunction with actin rearrangement by a pathway that was sensitive to the phosphoinositol 3-kinase inhibitor wortmannin and to the Rho GTP-binding protein inhibitor, epidermal cell differentiation inhibitor. Thus, enhancement of Th1-type selective migration appeared to be responsible for the diapedesis induced by interaction between CCR5 on Th1-type cells and RANTES produced by endothelial cells. Further evidence that CCR5 and RANTES play a modulatory role in Th1-type selective migration derives from the abrogation of this migration by anti-RANTES and anti-CCR5 Abs. The Journal of Immunology, 1999, 163: 3269–3278.

1 This work was supported by National Institute of Dental and Craniofacial Research Grant DE-03420. 2 Current address: Mitsubishi Chemical Corp., Yokohama Research Center, Yokohama, Japan. 3 Address correspondence and reprint requests to Dr. Martin A. Taubman, Department of Immunology, The Forsyth Institute, 140 Fenway, Boston, MA 02115. E-mail address: mtaubman@forsyth.org

Abbreviations used in this paper: CXCR3, CXC-chemokine receptor-3; ECC, endothelial clone cells; MIP, macrophage-inflammatory protein; MCP, monocyte chemotactic protein; EDIN, epidermal cell differentiation inhibitor; PI-3 kinase, phosphoinositide 3-kinase; IP-10, IFN-inducible protein 10.

Copyright © 1999 by The American Association of Immunologists
augmenting pseudopod formation beneath the endothelial cell layer. Our findings point to the new concept that discrimination of a Th1-type or Th2-type polarized trafficking pattern is regulated at the diapedesis step.

Materials and Methods

Rat CD4⁺ T clone cells

T clone cells were isolated from the cervical lymph nodes of Rowett rats that were immunized with the Gram-negative periodontal disease pathogen Actinobacillus actinomycetemcomitans ATCC 43718 (strain Y4) and maintained as previously described (17, 18). Th1-type CD4⁺ clone cells (G21 and G23) specific for A. actinomycetemcomitans 29-kDa outer membrane protein (Omp29) (19), Th2-type CD4⁺ clone reactive to an A. actinomycetemcomitans Ag different from Omp29 (G26) and Th2-type CD4⁺ clone cells specific for Omp29 (F10 and F13) were activated by incubation with formalin-fixed A. actinomycetemcomitans and irradiated (3300 rad) syngeneic rat spleen cells. Rat recombinant IL-2 (1 U/ml; Serotec, Bicester, U.K.) or conditioned medium from Con A-stimulated spleen culture was added to the Th2-type cell culture.

Polarization of Th1 and Th2 cells

Naïve CD4⁺ T lymphocytes were isolated by passing suspensions of axillary and lateral axillary lymph node cells through nylon wool and glass wool columns. The CD8⁺ population was excluded by panning with mAb anti-rat CD8 (OX8; Serotec)-coated plates. At least 95% of these cells were CD4⁺ when tested with anti-CD4 mAb (W3/25; Sera Lab, Crawley Down, U.K.). For the establishment of polarized line T cells, CD4⁺ cells were treated in 24-well plates with immobilized anti-rat TCR-αβ mAb (R73, 1 μg/ml; gift of Dr. Th. Hüning, Institut für Virologie und Immunobiologie, Würzburg, Germany) and soluble anti-CD28 mAb (JJ319, 200 ng/ml; PharMingen, San Diego, CA). For the establishment of polarized Th1 lines, mouse recombinant IL-12 (2 ng/ml; gift from Genetic Institute, Cambridge, MA) was added. To develop Th2 lines, rat recombinant IL-4 (10 ng/ml, PepTec, Rocky Hill, NJ), mAb hamster anti-mouse IL-12 p35 (1 μg/ml; gift from Genzyme, Cambridge, MA), and recombinant rat IL-2 (1 U/ml) were added. Purified CD4⁺ T cells (10⁶/well) were cultured for 5 days under the conditions described. To assess functional capabilities, the T cell lines were restimulated as described above. To verify the nature of the T cell lines, the cells were restimulated with immobilized anti-TCR-αβ and soluble anti-CD28 alone for 24 h to produce culture supernatant for IL-4 and IFN-γ ELISA.

Rat IL-4 and IFN-γ ELISA

The concentration of IL-4 in the culture supernatant was detected with a rat IL-4 ELISA kit with a lower detection limit of 15 pm (BioSource International, Camarillo, CA). The amount of IFN-γ in the culture supernatant was measured by sandwich ELISA. Affinity-purified goat anti-rat IFN-γ (2 μg/ml) was applied to the ELISA plate. The samples and standard rat recombinant IFN-γ (PepTec) were diluted in the diluent buffer of the mouse IFN-γ ELISA kit (BioSource International). The ELISA reaction was developed with the reagents, anti-rat IFN-γ mAb-biotin conjugate (2 μg/ml; DB-1, BioSource International) and avidin-conjugated peroxidase (×4000 dilution, Boehringer Mannheim, Indianapolis, IN). PBS containing 0.02% Tween 20 was used as the diluent and washing solution. The ELISA color was developed for 5 min by reaction of substrate o-phenylenediamine dihydrochloride (Sigma, St. Louis, MO), 2.5 mg/ml in 0.1 M phosphate citrate buffer (pH 5.5) with 0.03% hydrogen peroxide, and terminated by 2 N H₂SO₄. The lower detection limit of rat IFN-γ ELISA was 10 pg/ml.

Endothelial clone cells (ECC)

The establishment and the characteristics of a Rowett rat aorta endothelial clone cell (MAT-1) have been reported previously (18). ECC were maintained in 10% FBS-RPMI medium supplemented with 2.5% rat conditioned medium. Rat brain conditioned medium was prepared from a 2-day culture supernatant of minced rat whole brain tissue (4–6 mo old) in 15 ml of RPMI 1640 complete medium. The morphology and phenotype of MAT-1 were stable after >100 passages, showing no change in the expression pattern of ICAM-1, VCAM-1, HMC class I, HMC class II, very late Ags-1, inducible nitric oxide synthase, cyclooxygenase-2, RANTES, and von Willebrand factor.

Rabbit polyclonal anti-rat chemokine Abs and rat cross-reactive anti-human CCR5

Rabbit polyclonal anti-rat RANTES (PepTec), anti-rat MIP-1α (PepTec) and anti-rat monocytie chemotactic protein-1 (MCP-1; PepTec) reacted in direct ELISA with recombinant rat RANTES, MIP-1α, and MCP-1 (PepTec), respectively. No cross-reactivity to these other recombinant chemokines was observed in direct ELISA when these sera were tested against wells coated with 0.1 μg/ml of the respective recombinant chemokines (RANTES, MIP-1α, and MCP-1). Transiently, no interaction with the chemokines exhibited by G23 (stimulated with APC and A. actinomycetemcomitans for RANTES and MIP-1α, or with IL-2 alone for MCP-1) to each of the chemokines above was inhibited only by the respective corresponding Abs. RANTES-induced transendothelial chemotaxis of G23 was also inhibited by CCR5 mAb (anti-human CCR5 mAb, which cross-reacts with rat CCR5, clone 45502.111; IgG2b, R&D Systems, Minneapolis, MN). According to the manufacturer’s report (R&D Systems), this mAb (#45502.111) reacts with the amino-terminal domain of human CCR5 (20) which has considerable homology with rat CCR5 (87% in the first 73 amino acids). This mAb also blocks human recombinant CCR5-induced calcium flux on human CCR5-transfected Swiss 3T3 cells, and it reacts with CCR5 on fixed human PBMC in flow cytometry.

Flow cytometry

T cells were stained with mouse mAb to rat CD18 (IgG2a, Endogen, Boston, MA), anti-CCR5 (clone 45502.111) or isotype control mouse mAb (clone 45531.111, IgG2b (R&D Systems)); clone PA20, IgG1; and clone PF18, IgG2a (21)) followed by FITC-labeled Fab(‘), anti-mouse IgG (Jackson ImmunoResearch Laboratory, West Grove, PA). All viable T cells were isolated by Isolymph (Gallard-Schlesinger, Carle Place, NY) gradient centrifugation and fixed with 2% paraformaldehyde before mAb staining. Endothelial cells were removed from culture flasks by washing with 0.05% EDTA-PBS and single-cell suspensions were stained with anti-rat ICAM-1 (IgG1, Serotec) followed by FITC-labeled Fab(‘), anti-mouse IgG. Fluorescence data were collected by using logarithmic amplification on 20,000 T cells or 5,000 endothelial cells as determined by forward light scatter intensity on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

CCR5 and RANTES detection by RT-PCR

Total RNA was extracted from the cells using RNAzolB as described in the protocol of the manufacturer (Tel-Test, Friendswood, TX). First-strand cDNA was prepared by Superscript II (Life Technologies, Gaithersburg, MD), hexanucleotide mixture (Boehringer Mannheim) and 100 ng of sample RNA. PCR was performed on the resulting cDNA from each sample with specific primers for rat RANTES and β-actin which were previously described elsewhere (22, 23). The PCR primer set specific to rat CCR5 was designed based on the following sequences: 5’ primer, ATCTATGACATCGATGAG; 3’ primer, ATTTGAGCACTTCTTTTTGAGAT. The specificity of this primer set was searched by basic local alignment search tool and showed no cross-reactivity to any rat gene. cDNA was amplified 20 or 30 cycles (94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and final elongation time of 10 min at 72°C) for CCR5 or RANTES with β-actin as an internal control. PCR products were separated in 1.7% agarose gels and stained with ethidium bromide.

RANTES detection by immunoprecipitation

After stimulation of ECC with IFN-γ for 18 h, cells were incubated in methionine-free RPMI containing 2% dialyzed FBS for 30 min and were labeled with 100 μCi of [l-35S]methionine (DuPont, Wilmington, DE) for 3 h. The harvested supernatant was mixed with 1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM PMSE, and protease inhibitor mixture (Boehringer Mannheim) and precleared by control rabbit IgG. The supernatant was immunoprecipitated using rabbit anti-RANTES or anti-MIP-1α or anti-MCP-1 antisera and GammaBind Plus Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ). The samples were electrophoresed on a 10–20% polyacrylamide gradient gel and exposed to x-ray film.

T lymphocyte adhesion to ECC

ECC were cultured in 96-well tissue culture plates and allowed to reach confluency. T clone cells or T line cells were labeled with [3H]thymidine (2 μCi/ml) for 6 h and were incubated with the Ag and spleen APC or with anti-TCR-αβ mAb and anti-CD28 mAb. [3H]thymidine-labeled T lymphocytes (2 × 10⁷/100 μl/well) were applied to the confluent layer of ECC with saturating concentration of inhibiting Abs (10 μg/ml) in RPMI 1640 with 10% FBS. After incubation for various times in the tissue...
culture incubator, the wells were washed three times with RPMI 1640 to remove nonadherent lymphocytes. NaOH, 2 N, 100 μl, was added to lyse the cells. Adherence was quantitated by analyzing the radioactivity in the well compared with the radioactivity from whole lymphocytes applied to a well. Incubation for 3 min was determined to be optimal for adhesion assessment, because a maximal 45–55% of T cells bound to the ECC. By 6 min, the T cells were beginning to extend pseudopods beneath the ECC.

T lymphocyte transmigration across an ECC confluent layer

The T lymphocyte transmigration system was modified from the method of Carr et al. (24) and performed as reported previously (18). Briefly, a single-cell suspension of ECC (3–5 × 10^5 cells/ml in RPMI with 10% FBS) was applied onto 0.2% gelatin-coated cell culture inserts (polyethylene terephthalate filter, 3-μm pore size, 9.0-mm diameter, 24-well format; Becton Dickinson) and cultured for 1 or 2 days. Confluent ECC on a filter membrane were either cultured for 24 h in medium alone or stimulated with recombinant rat IFN-γ (1000 U/ml, Life Technologies). T cells (2 × 10^5 cells/filter) in RPMI with 10% FBS were overlaid on the ECC confluent layer with or without blocking Abs (10 μg/ml), and incubated in a 37°C, 5% CO_2 atmosphere. The lymphocytes transmigrated across the ECC (transendothelial migration) in 3–5 h into the lower wells of the plate and were harvested, resuspended in 40 μl, and counted in a hemocytometer. Ethidium bromide and acridine orange were routinely used for staining cells before counting.

Fluorescence labeling of cytoplasm and F-actin of adherent T cells on ECC

T cells were labeled with the thiol-reactive, live cell labeling reagent, Cell Tracker Green 5-chloromethylfluorescein diacetate (Molecular Probes, Eugene, OR). T cells (2 × 10^5/200 μl, in RPMI with 10% FBS) were pre-incubated in the presence or absence of Wortmannin (100 nM, Biomol Research Laboratories, Plymouth Meeting, PA), epidermal cell differentiation inhibitor (EDIN, 100 ng/ml (25)), cytochalasin D (100 ng/ml, Sigma), or anti-CCR5 (10 μg/ml) for 30 min and washed twice. T cells were applied onto confluent IFN-γ-stimulated or unstimulated ECC on a coverglass and incubated in a 37°C, 5% CO_2 atmosphere. In some wells, anti-RANTES was applied to the ECC together with T cells. After incubation for 3 to 6 min, nonadherent T cells on ECC were washed off and fixed with 2% paraformaldehyde in PBS for 10 min, and cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min. F-actin was stained by Texas Red-conjugated phalloidin (Molecular Probes). Immunofluorescence was analyzed by fluorescence microscopy at ×400 or ×1000 magnification.

Electron microscopic analysis

The methods were modified from a previous report of Parton (26). The clone G23 cells were incubated with unstimulated or IFN-γ-stimulated ECC on the polyethylene terephthalate filter for 8 min. This was followed by the removal of all media and gently rinsing once with PBS to remove nonadherent cells and proteins. The membranes were fixed in 2.5% glutaraldehyde/1% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 20 min at room temperature before being cut into small strips (2 × 4 mm), followed by 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4), and 1% uranyl acetate in 0.1 M maleate buffer (pH 5.2). After dehydration with sequential concentration of ethanol from 50 to 100%, the membranes were immersed in 1 ml 100% propylene for 20 min. Subsequently, the samples were incubated with Spurr’s resin (Electron Microscopy Science, Fort Washington, PA): propylene oxide, 1:3, 1:1, 3:1 for 2 h each; 100% Spurr’s resin for 12 h; followed by fresh 100% Spurr’s resin for 1 h at 60°C for polymerization. Finally, thin sections were stained with 3% uranyl acetate in 50% methanol for 10 min and Reynolds lead citrate for 30 s. Electron micrographs were obtained with a JEOL 100 CX transmission electron microscope operated at an accelerating voltage of 80 kV.

Table I. Cytokine production by Th1- or Th2-type cell lines and clones

<table>
<thead>
<tr>
<th></th>
<th>Th1 line</th>
<th>G21</th>
<th>G23</th>
<th>G26</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ (ng/ml)</td>
<td>48.3</td>
<td>9.8</td>
<td>7.9</td>
<td>7.2</td>
</tr>
<tr>
<td>IL-4 (pg/ml)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

—, Nondetectable by ELISA methods at the lower detection level of 10 pg/ml for IFN-γ, 15 pg/ml for IL-4.

Results

Stimulation of endothelial cells with IFN-γ selectively enhances transmigration of Th1-type cells

To examine Th1 or Th2 subset specific transendothelial-migration, we developed polarized Th1 or Th2 lines by stimulation with immobilized anti-TCR-αβ and soluble anti-CD28 mAb in the presence of IL-12 or IL-4, respectively. The characteristic of Th1- or Th2-type cells was ascertained by the cytokine production pattern of IFN-γ and IL-4 (Table I). The Th1 polarized line and clones G21, G23, and G26 produced only detectable IFN-γ, whereas the Th2 polarized line and clones F10 and F13 produced only detectable IL-4.

To determine the effects of stimulation of endothelial cells with the Th1 cytokine IFN-γ, we tested transendothelial migration of Th1-type clone cells and the polarized Th1 line or Th2-type clone cells and the Th2 line, respectively (Fig. 1). Stimulation of ECC with IFN-γ enhanced transendothelial migration of the Th1 clone cells (G21, G23, and G26) and polarized Th1 line cells. In contrast, IFN-γ treatment of ECC enhanced the transendothelial migration of neither the Th2-type line cells nor Th2-type clone cells (F10 and F13). These Th2 lines or clones demonstrated a modest level of basal transmigration as opposed to naive CD4⁺ T cells, which showed little basal migration and also no response to IFN-γ-simulated ECC. Although naive CD4⁺ T cells did not demonstrate
IFN-γ-mediated enhancement of transmigration, such enhancement was observed when these T cells were polarized to a Th1 phenotype by stimulation with immobilized anti-TCR-αβ and soluble anti-CD28 mAb for 3 days in the presence of IL-12 (Fig. 1).

CCR5 and LFA-1 expression by Ag-activated T clone cells

Because it has been shown that CCR5 is preferentially expressed on Th1 cells by Northern blotting (4) and flow cytometry analysis (7), we hypothesized that CCR5 might be responsible for Th1-specific migration induced by IFN-γ-stimulated ECC. Expression of CCR5 mRNA by Th1 cells was first tested by RT-PCR (Fig. 2A). Expression of CCR5 mRNA by stimulated Th1 clone or Th1 line cells was detected. Little or no CCR5 mRNA production was observed by unstimulated Th1 clone, naive CD4+ T, stimulated Th2 clone, or Th2 line cells. We further tested surface expression of CCR5 by Th1 or Th2 clone cells by means of flow cytometric analyses (Fig. 2B). CCR5 was expressed only on Th1-type clone cells, but not on Th2-type cells or naive CD4+ T cells. T cells need to receive special signals from adhesion molecules or chemokine receptors for transmigration after loose adhesion to endothelial cells. Thus, we also examined adhesion molecule expression on the same battery of T cells (Fig. 2B). Previous studies (27) have demonstrated the importance of adhesion interaction between ICAM-1 (CD54) and LFA-1 (CD11a/CD18) for T cell transmigration across endothelium, and adhesion molecules may be associated with Th1-specific transmigration. However, the expression of CD18 was similarly increased on both Th1 and Th2 clone cells (Fig. 2B) and on polarized line cells (data not shown) when compared with naive T cells, suggesting that levels of this integrin did not determine the observed transmigration difference in Th1 as opposed to Th2 cells but could affect differences observed between activated opposed to naive cells.

FIGURE 2. Preferential expression of CCR5 mRNA and selective surface expression of CCR5 on Th1 clone cells or Th1 line cells. A, RT-PCR specific for rat CCR5 (477 bp) or β-actin (607 bp) was performed on the total RNA isolated from unstimulated Th1 clone G23, Ag-stimulated G23 or Th2 clone F13, naive CD4+ T cells, stimulated Th1 line, or stimulated Th2 line cells. T clones and polarized T lines were stimulated for 3 d as described in Materials and Methods. Naive CD4+ T cells were freshly isolated from rat axillary lymph nodes. B, Surface expression of CCR5 and CD18 was analyzed on Th1 clone cells (G21, G23, G26) and Th2 clone cells (F10, F13) after 3 days of stimulation. Cells were stained with control mAb (open histograms) or mAb to CD18 or CCR5 (shaded histograms) followed by FITC-conjugated rat anti-mouse IgG. The data scales are logarithmic. One representative experiment of three is shown.

FIGURE 3. ICAM-1 expression and soluble RANTES production identified from IFN-γ-stimulated ECC. A, Enhancement of ICAM-1 expression on ECC after treatment with IFN-γ. Confluent ECC were incubated with IFN-γ for 24 h. ICAM-1 expression (shaded histograms) was monitored by flow cytometry with mAb (1A29) and compared with an isotype matched control mAb (open histograms). Data are shown as log mean fluorescence channel on the x-axis, and cell number on the y-axis. B, RANTES mRNA induction on ECC by IFN-γ stimulation. ECC were stimulated with IFN-γ for the times (hours) shown, and RT-PCR specific to rat RANTES mRNA (221 bp) and β-actin mRNA (607 bp) was performed on the total RNA isolated from ECC. C, Identification of soluble RANTES production by IFN-γ-stimulated ECC. Confluent ECC treated with or without IFN-γ (1000 U/ml) for 18 h, freshly isolated CD4+ T cells, or Ag-activated (with APC for 3 days) G23 Th1-type clone cells (2 × 10^6 cells) stimulated with APC and formalin-killed A. actinomycescomitans for 3 days, were incubated with [35S]methionine for 3 h. The culture supernatants were immunoprecipitated with rabbit polyclonal anti-rat RANTES Abs. The arrow indicates the position of RANTES corresponding to 7.9 kDa. D, Selective production of RANTES and MCP-1, but not MIP-1α, by IFN-γ-stimulated ECC. Confluent ECC treated with or without IFN-γ were labeled with [35S]methionine for 3 h. The culture supernatants were immunoprecipitated with anti-RANTES, anti-MIP-1α, or anti-MCP-1 polyclonal Abs. The expected band size of RANTES (7.9 kDa) or MCP-1 (14.1 kDa) is indicated by a solid arrow or an open arrow, respectively.
ICAM-1 and RANTES expression by IFN-γ-stimulated ECC

Stimulation of ECC with IFN-γ induced enhanced ICAM-1 expression (a counterligand of LFA-1 (Fig. 3A)). IFN-γ also induced RANTES mRNA detected by RT-PCR (Fig. 3B) and RANTES protein expression observed by immunoprecipitation (Fig. 3C). Although T clone cells (G23) were not able to produce RANTES after 3 days of activation with APC and Ag, this was the time period (3 days) when T clone cells exhibited maximal transmigration across endothelial cells, implying that T cell production of RANTES was not a factor in T cell transmigration. Importantly, no RANTES is produced by ECC in the absence of IFN-γ. Of the three chemokines that can bind to CCR5, RANTES, but not MIP-1α or MIP-1β, can be induced on endothelial cells by IFN-γ stimulation (28, 29). We also observed that ECC stimulation with IFN-γ induced the protein expression of RANTES and MCP-1, but not MIP-1α (Fig. 3D).

To assess the role of CCR5 in the selective transmigration of Th1 cells, we examined T cell transendothelial chemotaxis to RANTES directly. One of the CC-chemokines, RANTES, MIP-1α, or MCP-1, was applied to the lower compartment of the unstimulated ECC confluent layer. Then, Th1-type T clone cells (G23) or Th2-type T clone cells (F13) were applied on top of the ECC layer (Fig. 4). Ag-stimulated G23 cells responded to RANTES (G23) or Th2-type T clone cells (F13) were applied on top of the stimulated ECC confluent layer. Then, Th1-type T clone cells were capable of enhanced transmigration.

RANTES does not affect T cell adhesion to ECC

Because the T cell chemotactic effect in IFN-γ-stimulated ECC was inhibited by Abs to RANTES, we considered that RANTES could affect T cell adhesion to ECC. No effect on G23 adhesion to unstimulated ECC was observed when various concentrations of recombinant RANTES or IFN-γ-stimulated ECC supernatant were added to the G23, ECC adhesion system (Fig. 5B). Also, no effect was observed when Th2 (F13) or polarized Th1 cells were substituted for G23 in the assay of adhesion to unstimulated ECC (data not shown).

Inhibition of Th1-type selective migration by both anti-RANTES and anti-CCR5 Ab

Further to examine the effect of endothelial-derived RANTES on adhesion, we tested the effects of polyclonal anti-RANTES, monoclonal anti-CCR5, anti-CD18 (LFA-1β), and anti-CD54 (ICAM-1)
Abs on adhesion (3 min; Fig. 6A) and transmigration (3 h; Fig. 6B) of Th1-type clone G23. G23 demonstrated increased adhesion to IFN-γ-stimulated ECC when compared with unstimulated ECC. Anti-CD18 mAb and anti-CD54 mAb significantly inhibited the Th1-type clone G23 adhesion to either unstimulated or IFN-γ-stimulated ECC, whereas anti-CCR-5 or anti-RANTES had no effects on adhesion of G23 to either unstimulated or IFN-γ-stimulated ECC. Transmigration of G23 was significantly enhanced on IFN-γ-stimulated ECC compared with unstimulated ECC, and this enhanced transmigration was abolished by Abs to CD18, CD54, CCR5, or RANTES (Fig. 6B). In contrast, anti-CCR5 or anti-RANTES had no effect on the basal level of transmigration of G23 across unstimulated ECC (mean, 5684 transmigrated cells/well (Fig. 6B)), although this was inhibited by anti-CD18 (mean, 2281 transmigrated cells/well (Fig. 6B)) and anti-CD54 (mean, 3268 transmigrated cells/well (Fig. 6B)). These results suggested that transmigration induced by RANTES binding to CCR5 on Th1 cells was not simply the result of enhanced integrin-mediated adhesion and thus implied chemokine involvement in a subsequent step after adhesion.

We also investigated the effect of RANTES produced by ECC on transendothelial migration of polarized Th1 and Th2 line cells (Fig. 7A, Th2 line; Fig. 7B, Th1 line). Transendothelial migration of Th2 line cells was not enhanced by IFN-γ stimulation of ECC. None of the blocking Abs reactive to CCR5, RANTES, or MCP-1...
inhibited the basal Th2 line cell transendothelial migration. Transmigration of Th1 line cells was enhanced by IFN-γ stimulation of ECC. Only the enhanced transmigration of Th1 line cells by IFN-γ were utilized. Polarized Th2-type cell transmigration was not enhanced by IFN-γ-stimulated ECC (B). Only enhanced transmigration on the IFN-γ-stimulated ECC compared with unstimulated ECC (B). Only enhanced transmigration on the IFN-γ-stimulated ECC was abrogated by anti-CCR5 and anti-RANTES Abs, but not by anti-MIP-1α or anti-MCP-1.

**RANTES-CCR5 interaction activates PI-3 kinase and/or Rho and induces actin rearrangement**

We further explored the mechanism for the enhancement of Th1-type cell transmigration induced by endothelial cell RANTES using pharmacological agents known to interfere in signaling pathways activated by chemokines. Chemokine receptors are linked to the heterotrimeric guanine nucleotide-binding protein (G protein), which transduces signals through activation of PI-3 kinase (31). In addition, the coupling of Rho GTP-binding proteins to G proteins linked to the chemokine receptor triggers integrin-mediated leukocyte adhesion (32). This process is also thought to involve actin rearrangement, which is necessary for cell migration (16). Hence, we tested the hypothesis that RANTES-CCR5 interaction could activate PI-3 kinase and/or induce Rho-related actin rearrangement. Preincubation of G23 cells with recombinant RANTES for 3 min enhanced transendothelial migration even in the absence of a chemotactic factor in the lower compartment of the transmigration system (Fig. 8). This enhancement was abolished in a dose-dependent manner by the PI-3 kinase inhibitor wortmannin, the Staphylococcus aureus toxin EDIN which deactivates Rho (25), and by cytochalasin D which inhibits actin rearrangement. In contrast, the basal transmigration of G23 preincubated in medium alone was not affected by either wortmannin or EDIN but was inhibited by cytochalasin D (Fig. 8). This observation suggested that RANTES enhanced T cell motility through activation of PI-3 kinase and the Rho-related pathway during the 3 min of chemokine pretreatment.

**Endothelial cell RANTES augments pseudopod formation by adherent T cells in conjunction with actin rearrangement**

The extension of pseudopods in response to migratory stimuli is universally coupled with local actin polymerization (16). To investigate T cell morphology in situ, Th1-type clone G23 T cells were visualized by live cell staining with green fluorescent dye (Figs. 9, a–j). F-actin of both T cells and ECC was stained with Texas Red-conjugated phalloidin (Molecular Probes). In the early 3-min incubation, the adherent G23 cells were round and formed few or no pseudopods on G23 cells on anti-RANTES treated IFN-γ-stimulated ECC. At higher magnification, no pseudopods were observed on G23 cells on anti-RANTES treated IFN-γ-stimulated ECC when the plane of focus was either at the ECC surface (Fig. 9g) or in the cytoplasm of the ECC (Fig. 9h). However, pseudopod elongation...
Red-conjugated phalloidin (Molecular Probes). G23 cells on the IFN-γ incubation, samples were permeabilized, and F-actin was stained by Texas Green in the cytoplasm and applied to the ECC confluent layer on coverslip. G23 cells were labeled with Cell Tracker when focused on ECC surface (Fig. 9g) or on IFN-γ-stimulated ECC for 8 min extended pseudopods beneath the ECC layer (Fig. 9f), in marked contrast to the absence of pseudopod formation by G23 incubated on unstimulated ECC for the same time period (Fig. 9k).

Discussion

Our data indicated that RANTES secreted by IFN-γ-stimulated endothelial cells enhanced the transmigration of Th1-type cells, but not Th2 cells or naive CD4+ T cells because of the selective expression of CCR5 on Th1 cells. Although both RANTES and MIP-1αβ interact with CCR5 (8), only RANTES production by ECC was induced by IFN-γ stimulation in this study. This finding is supported by reports (9, 28) indicating that human endothelial cell production of RANTES in vitro is stimulated by IFN-γ and that stimulation of endothelial cells with IFN-γ mixed with TNF-α and IL-1β induces only RANTES production, and not MIP-1α or -1β (9). In clinical situations characterized by Th1-type inflammation such as DTH granuloma lesions in lymph nodes associated with sarcoidosis or tuberculosis, RANTES was produced in situ by macrophages and by endothelial cells, in particular (29). Also, ~80% of T cells in rheumatoid arthritis synovial fluid expressed CCR5, a significant enrichment over the 15% expression in blood (6). These findings led to suggestions that RANTES might play a role in the selective accumulation of cells characterizing cell-mediated immune reactions (29) and that CCR5 is a marker for T cells associated with Th1-type inflammatory reactions (6). Here we demonstrated functional interaction between CCR5 on Th1 cells and RANTES produced by endothelial cells in the context of T cell transendothelial migration. Both anti-RANTES and anti-CCR5 Ab could abrogate Th1-type selective migration in vitro (Figs. 6B and 7B). Therefore, we have hypothesized that the interaction between CCR5 and RANTES can account for selective transmigration and polarization of Th1-type cells in various types of inflammatory lesions.

Cell migration is an important process in a variety of biological phenomena, embryogenesis, angiogenesis, fibroblast migration in wound healing, metastasis, and lymphocyte immigration (16). This process is distinct from the cell to cell adhesion event, because it is characterized by simultaneous attachment at the leading edge and detachment at the rear end of the motile cell. Furthermore, diapedesis, the lymphocyte transmigration step across an endothelial cell layer, requires proteolytic remodeling of the extracellular matrix of basement membrane (33). A particularly strong signal would be expected to induce diapedesis by the adherent lymphocyte on the luminal surface of the endothelial cell layer. Cytoskeletal reconstruction by actin rearrangement is well studied in cell migration phenomena. The activation of leukocyte adhesion through integrin is induced by chemokine receptor stimulation associated with the GTP-binding protein RhoA (32). The Rho family and PI-3, PI-5 are relevant signal transduction molecules involved in the cell migration processes by activating actin rearrangement on unstimulated ECC (k) demonstrated extended pseudopods, whereas most cells of IFN-γ-stimulated ECC (l) showed extended pseudopods.
In the present study, the signal from RANTES which induced pseudopod formation within 6 min was inhibited by wortmannin and EDIN, suggesting very rapid signal transduction by RANTES receptors. RANTES is a unique chemokine that activates dual T cell signaling pathways (34) by activating an initial transient (20–30 s) G protein-dependent signal, and a second long-lasting (2–3 min) signal mediated by protein tyrosine kinase. RANTES stimulation of T lymphocytes induces the redistribution of ICAM-3 at the uropod in conjunction with the cytoskeletal protein moesin (35). These data indicate that RANTES not only up-regulates actin rearrangement for T cell locomotion, but also induces other required biological functions for transmigration.

In addition to CCR5, CXCR3 has been clinically associated with similar types of Th1 inflammatory lesions (6). CXCR3 may be less significant than CCR5 in Th1 cell accumulation in inflammatory lesions. Although CCR5 and CXCR3 are expressed on human Th1-type cells (4, 6), CCR5 seems to be characteristic of Th1 lymphocytes, because CXCR3 can be expressed on both Th1 and Th2 cells cultured with IL-2 (7). Although CXCR3 (36) is the receptor for IP-10 (IFN-inducible protein 10), MIG (monokine induced by IFN-γ), and I-TAC (IFN-inducible T cell α chemotactant), all inducible by IFN-γ stimulation of endothelial cells (37), it does not appear that these chemokines are significant in transmigration. The CXC chemokines IL-8 or IP-10 are not able to induce transendothelial chemotaxis of memory-type CD3+ lymphocytes, whereas RANTES can induce transendothelial chemotaxis of such CD3+ lymphocytes in the same system (38). Also, chemotactant activity of recombinant RANTES for human peripheral blood T lymphocytes is 2-fold greater than recombinant IP-10 at the optimal concentration of each chemokine (39). Because CXCR3 is also found on Th2 lymphocytes, if it were significant in transmigration, we would expect that IFN-γ stimulation of ECC would induce CXC chemokines which should produce Th2 transmigration. This did not occur (Fig. 1). Therefore, although there appears to be potential for contribution of CXC chemokines and CXCR3 to transmigration, CCR5 which is selectively expressed on Th1 cells, is the key to regulation of Th1 transmigration by RANTES produced by IFN-γ-stimulated endothelial cells.

RANTES or MIP-1β does not induce rapid adhesion of lymphocytes to ICAM-1 in 3 min compared with stromal cell-derived factor-1α which shows dramatic adhesion to ICAM-1 during the same period of incubation (15). The 3-min incubation reflects more the physiological conditions of blood flow. Our results also showed that RANTES produced by ECC was not responsible for the T cell adhesion to IFN-γ-stimulated ECC in 3 min (Figs. 5, 6, and 9). Despite the lack of responsibility for direct adhesion, RANTES induced diapedesis of T cells within 6 min of incubation (Fig. 9).

It has been demonstrated that T cells can produce RANTES (40, 41). However, in our experiments no detectable RANTES was produced by Th1 clone cells during 3 days of Ag stimulation (Fig. 3C). In contrast to many T cell products that are expressed within hours of activation, such as IL-2, the gene encoding RANTES is up-regulated ~5 days after stimulation of T cells with Ag or mitogen (42). Thus, RANTES produced by T cells is unlikely to have contributed to the induction of Th1 transmigration in our system.

In general, costimulation of T cells with anti-CD28 skewed the T cell response toward Th2 type (43, 44), and in vitro T cell stimulation with both anti-CD3 and anti-CD28 could induce Th2-type response in which CCR4, but not CCR5, would be preferentially expressed (4). In the present study, Th1 polarization with anti-TCR-αβ and anti-CD28 in the presence of IL-12 induced CCR5 expression and chemotactic response to RANTES. In contrast, Th2 polarization with anti-TCR-αβ and anti-CD28 in the presence of IL-4 did not induce CCR5 expression or responsiveness to RANTES. Thus, the various factors known to contribute to Th cell polarization (TCR, CD28, IL-4, and IL-12) also appear to have important roles in the regulation of chemokine receptors.

Naive T cells preferentially migrated into peripheral lymph nodes (13), whereas memory T cells (CD45RO+CD29-) traffic into inflamed tissue (45) or exhibit preferential transmigration across endothelial cell layers in vitro (46) compared with CD45RA+ (naive) T cells. We have demonstrated that the presence of Ag in gingival tissue can attract and retain Ag-specific T clone cells (G23 and F13) locally (17). The Th1- and Th2-type clone cells in this study were memory-type T cells (CD25+, CD45RC-, very late Ag-4+), and those Th1- and Th2-type clone cells demonstrated more transmigration across ECC than naive T cells (Fig. 1). Therefore, we believe our transmigration model reflects memory-type T cell migration into local inflammation.

Endothelial cells regulate neutrophil transmigration by endogenous IL-8 (47). Therefore, it is quite plausible that chemokines produced by endothelial cells are also significant in regulating Th1 cell transmigration from the blood stream. Chemokines appear to target distinct subsets of lymphocytes more selectively than adhesion interactions that are mediated in many cases by molecules, like LFA-1, that were distributed over the entire leukocyte population. Therefore, the study of regulatory mechanisms and the generation of appropriate inhibitors of specific chemokines could be relevant as a therapeutic approach for intervention in many types of immune-mediated diseases. Both chemokines and chemokine receptors comprise large families (8, 48), and it seems feasible that RANTES-CCR5 interaction is not the only chemokine-chemokine receptor interaction that regulates Th1-type cell transmigration. The present study demonstrates the selective activity of RANTES in Th1 transmigration and suggests an important role for the RANTES-CCR5 interaction in the induction of diapedesis by Th1 cells and their recruitment into sites of inflammation.

**Acknowledgments**

We thank Dr. B. Moser (Theodor-Kocher Institute, University of Bern) for his excellent critical comments and discussion, Dr. Thomas Hüning (Institut für Virologie und Immunobiologie) for R73 hybridoma, Dr. Ziedonis Skobe (The Forsyth Institute) for assistance with graphics preparation, and Jan Schafer for manuscript preparation.

**References**


Science 272:60.

25. Campbell, J. J., J. Hedrick, A. Zlotnik, M. A. Siani, D. A. Thompson, and 
E. C. Butcher. 1998. Chemokines and the arrest of lymphocytes rolling under 
flow conditions. Science 279:381.

Cytobiology 248:587.


transmigration. In Mucosal Solutions, Advances in Mucosal Immunology, Vol. I.

32. Laudanna, C., J. J. Campbell, and E. C. Butcher. 1996. Role of Rho in chemoat-