Transendothelial chemotaxis of human α/β and γ/δ T lymphocytes to chemokines

Stephen J. Roth[1, 2, 3], Thomas G. Diacovo[1, 2, 4], Michael B. Brenner[5, 3], Jean-Pierre Rosat[5, 3], Janet Buccola[2], Craig T. Morita[5, 3] and Timothy A. Springer[2, 3]

[1] Department of Cardiology, Children's Hospital, Boston, USA
[2] The Center for Blood Research, Boston, USA
[3] Harvard Medical School, Boston, USA
[4] Division of Newborn Medicine, Department of Pediatrics, Tufts University School of Medicine, Boston, USA
[5] Lymphocyte Biology Section, Department of Rheumatology and Immunology, Brigham and Women's Hospital, Boston, USA

Two subpopulations of human T lymphocytes expressing different antigen receptors, α/β and γ/δ, emigrate into inflamed tissues in distinctive patterns. We compared the transmigration of α/β and γ/δ T cells to C-C and C-X-C chemokines using an *in vitro* transendothelial chemotaxis assay. The C-C chemokines monocyte chemoattractant protein (MCP)-1, RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β stimulated similar, dose-dependent chemotaxis of purified γ/δ T cells, whereas MCP-1, RANTES, and MIP-1α produced greater chemotaxis of purified α/β T cells than MIP-1β. In contrast, the C-X-C chemokines interleukin (IL)-8 and interferon-γ inducible protein-10 (IP-10) did not promote chemotaxis of either α/β or γ/δ T cells. Three γ/δ T cell clones with differing CD4 and CD8 phenotypes also migrated exclusively to C-C chemokines. Phenotypic analysis of mononuclear cells that transmigrated from an input population of unfractionated peripheral blood mononuclear cells confirmed the results with purified γ/δ T cells. Our data demonstrate that human peripheral blood α/β and γ/δ T cells can transmigrate to MCP-1, RANTES, MIP-1α, and MIP-1β, and suggest that both T lymphocyte subpopulations share the capacity to emigrate in response to C-C chemokines during inflammation.

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1 Introduction

Two subpopulations of human T lymphocytes express distinct antigen receptors, TCRα/β and TCRγ/δ [1, 2]. The majority of T cells in adults express TCRα/β [3, 4]. α/β T cells contribute to immunological host defenses directly through their cytolytic activities and indirectly by producing cytokines that regulate multiple cell types, including other immune cells [5]. Antigen recognition by TCRα/β is associated with expression of the accessory molecules CD4 and CD8 and restricted by expression of MHC molecules [6]. γ/δ T cells have been demonstrated to play a role in host defense against certain bacteria, mycobacteria and parasites [7]. While γ/δ T cells are also capable of cytolysis and cytokine production, most are not MHC restricted. Human γ/δ T cells also bind more effectively than α/β T cells to immobilized P-selectin [8], an adhesion molecule that is up-regulated on platelets and endothelium immediately following the onset of injury or inflammation [9, 10].

α/β and γ/δ T lymphocytes appear to localize to infected or inflamed tissues in distinct migratory patterns. In the synovial fluid of patients with Lyme disease arthritis [11] and specific skin lesions of patients with leprosy or leishmaniasis [12], the frequency of γ/δ T cells is increased three- to fourfold and five- to eightfold relative to peripheral blood, respectively. In affected synovial tissue in rheumatoid arthritis, intestinal epithelium in inflammatory bowel disorders, and selected plaque lesions in multiple sclerosis, there also is an increased proportion of infiltrated γ/δ T cells [13–15]. While the precise role of γ/δ T cells at sites of infection and inflammation is unknown, there is increasing evidence that γ/δ T cells regulate α/β T cell function, making co-localization of these T lymphocyte subpopulations important in the immune response [11, 16–18]. The preferential accumulation of γ/δ T cells at these sites could be due to preferential

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Abbreviations: MCP: Monocyte chemoattractant protein **MIP:** Macrophage inflammatory protein **IP-10:** Interferon-γ-inducible protein-10 **HUVEC:** Human umbilical vein endothelial cells

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migration, or to enhanced *in situ* cell proliferation, retention, or survival. Preferential migration could occur if T cell subpopulations respond selectively to cytokines produced at the inflammatory locus such as chemoattractants, molecules which activate leukocytes and direct their transendothelial migration [19].

The chemoattractants that are likely to activate lymphocytes *in vivo* are the chemoattractive cytokines or chemokines. Chemokines are a family of small (7–15 kDa), inducible proteins. The C-C chemokines, including monocyte chemoattractant protein (MCP)-1, MCP-2, MCP-3, RANTES, macrophage inflammatory protein (MIP)-1α, and MIP-1β, primarily activate and attract monocytes and T lymphocytes [20–28]. Some also activate eosinophils and basophils, but none attract neutrophils. Most C-X-C chemokines, including IL-8, neutrophil activating protein-2, GRO, and ENA-78, primarily activate and attract neutrophils [29]. None of the C-X-C chemokines attract monocytes except interferon-γ inducible protein-10 (IP-10), which is inactive on neutrophils [30]. IL-8 and IP-10 have been reported by some groups to attract T lymphocytes [30–35], but not by others [22, 27, 28].

To determine if α/β and γ/δ T cells differ in their migratory responses to chemokines, we directly compared purified α/β and γ/δ T cells in transendothelial chemotaxis assays with MCP-1, RANTES, MIP-α, MIP-1β, IL-8 and IP-10. We find that while α/β and γ/δ T cells have distinct response profiles, both subpopulations share the capacity to transmigrate to C-C chemokines but not to IL-8 or IP-10.

2 Results

2.1 Kinetics of α/β and γ/δ T lymphocyte transendothelial chemotaxis

In preliminary transendothelial migration experiments, we established that α/β and γ/δ T cells purified by immunomagnetic positive selection could transmigrate to MCP-1. To determine the kinetics of transmigration to MCP-1, we directly compared the two subpopulations from single blood donors in the transendothelial assay (Fig. 1). Migration of both cell types to MCP-1 at 50 ng/ml increased between 1–5 h and then appeared to plateau by 6 h. In contrast, migration to control media between 1–6 h was consistently less than migration to MCP-1, and did not increase above ≈ 1 %. Based upon these kinetics data, a standard incubation time of 4 h was chosen for subsequent transmigration experiments. Transendothelial migration of purified T cells to C-C chemokines occurs primarily by chemotaxis (directional

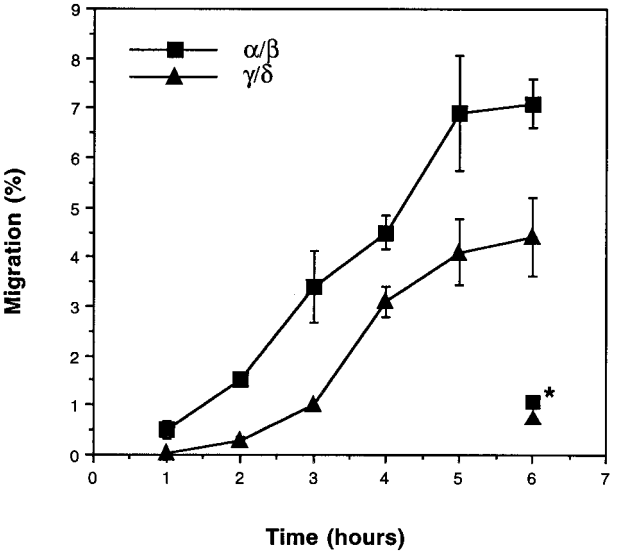

Figure 1. Kinetics of α/β and γ/δ T cell transendothelial chemotaxis to MCP-1. For each time point, 5×10^5 purified α/β or γ/δ T cells were added to duplicate HUVEC-covered Transwell inserts. The percentage of input cells migrated to MCP-1 (50 ng/ml) at 37 °C was determined every hour for 6 h. The two values at 6 h marked with an asterisk (*) represent migration to assay media alone (α/β = 1.1 %; γ/δ = 0.8 %). Data represent the mean α/β or γ/δ cell migration from one of three independent experiments with similar results. Bars represent the range.

locomotion), not by increased random locomotion [28, 36]. To determine if γ/δ T cells migrated by chemotaxis, we performed a checkerboard analysis [37] using the γ/δ T cell clone, JN23, and RANTES. JN23 cells transmigrated by chemotaxis, since significant migration occurred only when a gradient of RANTES existed between bottom and top compartments, with a higher concentration of RANTES in the bottom (Table 1).

2.2 α/β and γ/δ T lymphocytes undergo transendothelial chemotaxis to MCP-1, RANTES, MCP-1α, and MIP-1β but not IL-8 and IP-10

To compare how α/β and γ/δ T cells migrate to the C-C chemokines MIP-1, RANTES, MIP-1α, and MIP-1β and the C-X-C chemokines IL-8 and IP-10, we purified each subpopulation from single blood donors and tested them over a range of chemokine concentrations. α/β T cells transmigrated to MCP-1, RANTES, MIP-1α, and MIP-1β significantly above control (Fig. 2A). MCP-1, RANTES, and MIP-1α were significantly more potent than MIP-1β for α/β T cells at 100 ng/ml ($p < 0.001$). At 500 ng/ml

Table 1. Checkerboard analysis of γ/δ T lymphocyte transmigration to RANTES[a)]

RANTES (ng/ml) in bottom	Migration (%) RANTES (ng/ml) in top 0	50	100	500
0	0.8 ± 0.36	0.8 ± 0.02	0.7 ± 0.24	1.0 ± 0
50	5.6 ± 0.2	1.1 ± 0.07	0.8 ± 0.14	0.6 ± 0.1
100	6.6 ± 0.41	1.6 ± 0.34	1.3 ± 0.3	1.6 ± 0.04
500	8.6 ± 0.4	3.2 ± 0.6	2.0 ± 0.5	1.6 ± 0.25

a) RANTES was placed into duplicate plate wells (bottom) and HUVEC-covered inserts (top) at the indicated concentrations (0–500 ng/ml). JN23 γ/δ T cell clone cells were added to inserts at 5.0×10^5 cells/insert and incubated for 4 h at 37 °C. Values represent the mean percentage ± half the range of JN23 cells migrated.

MCP-1 attracted significantly more α/β T cells than RANTES or MIP-1α, and RANTES or MIP-1α attracted significantly more T cells than MIP-1β ($p = 0.002$). No response to IL-8 or IP-10 at 1, 10, or 100 ng/ml occurred. These results are nearly identical to the chemotactic responses which we observed with purified $CD3^+$ T cells and these same six chemokines [28].

γ/δ T cells also demonstrated significant, dose-dependent transendothelial chemotaxis only to the four C-C chemokines tested, not to IL-8 or IP-10 (Fig. 2B). In contrast to α/β T cells, γ/δ T cells migrated equally well to all four C-C chemokines at 100 and 500 ng/ml. In further experiments, neither subpopulation transmigrated significantly to IL-8 or IP-10 at 500 ng/ml or to 10^{-7}–10^{-9} M N-formyl-Met-Leu-Phe (fMLP) (data not shown), a formylated peptide that is a potent chemoattractant for neutrophils and monocytes, but not lymphocytes [28, 38]. The function of the IL-8, IP-10, and fMLP used in these assays (and assays described in section 2.3) was confirmed in separate chemotaxis experiments with freshly isolated human neutrophils (IL-8 and fMLP) (data not shown) or cultured, PHA-stimulated PBMC (IP-10) (data not shown).

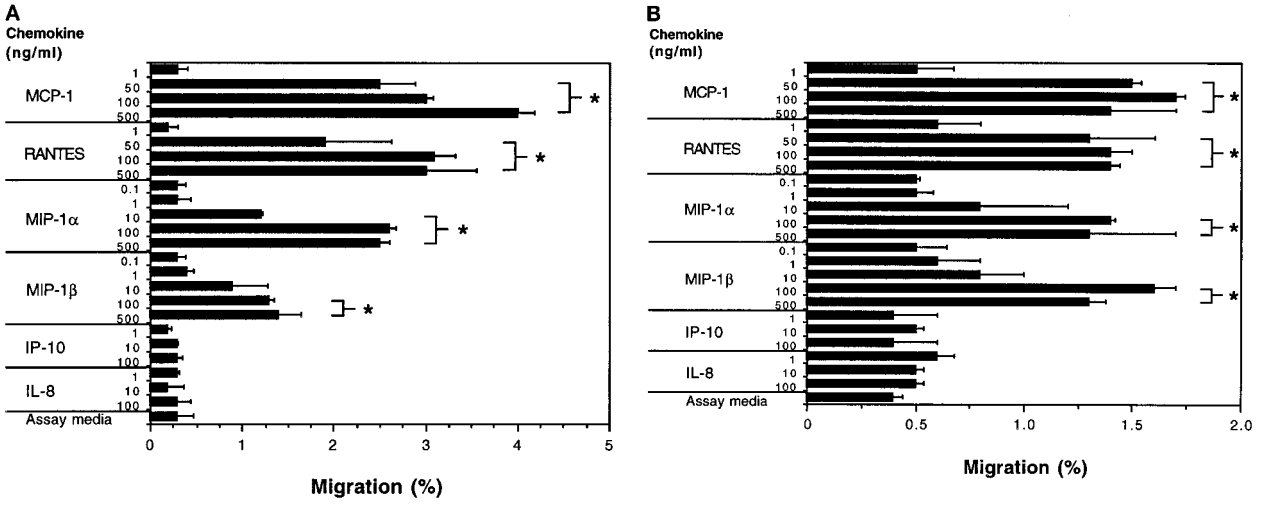

Figure 2. α/β and γ/δ T cell transendothelial chemotaxis to C-C and C-X-C chemokines. Purified α/β (A) or γ/δ (B) T cells were added to duplicate HUVEC-covered Transwell inserts and allowed to migrate to the indicated concentrations of MCP-1, RANTES, MIP-1α, MIP-1β, IP-10, IL-8, or assay media alone for 4 h at 37 °C. Data represent the mean percentage migration from one of three independent experiments with similar results. Bars represent the range. * : $p < 0.05$, compared to assay media control.

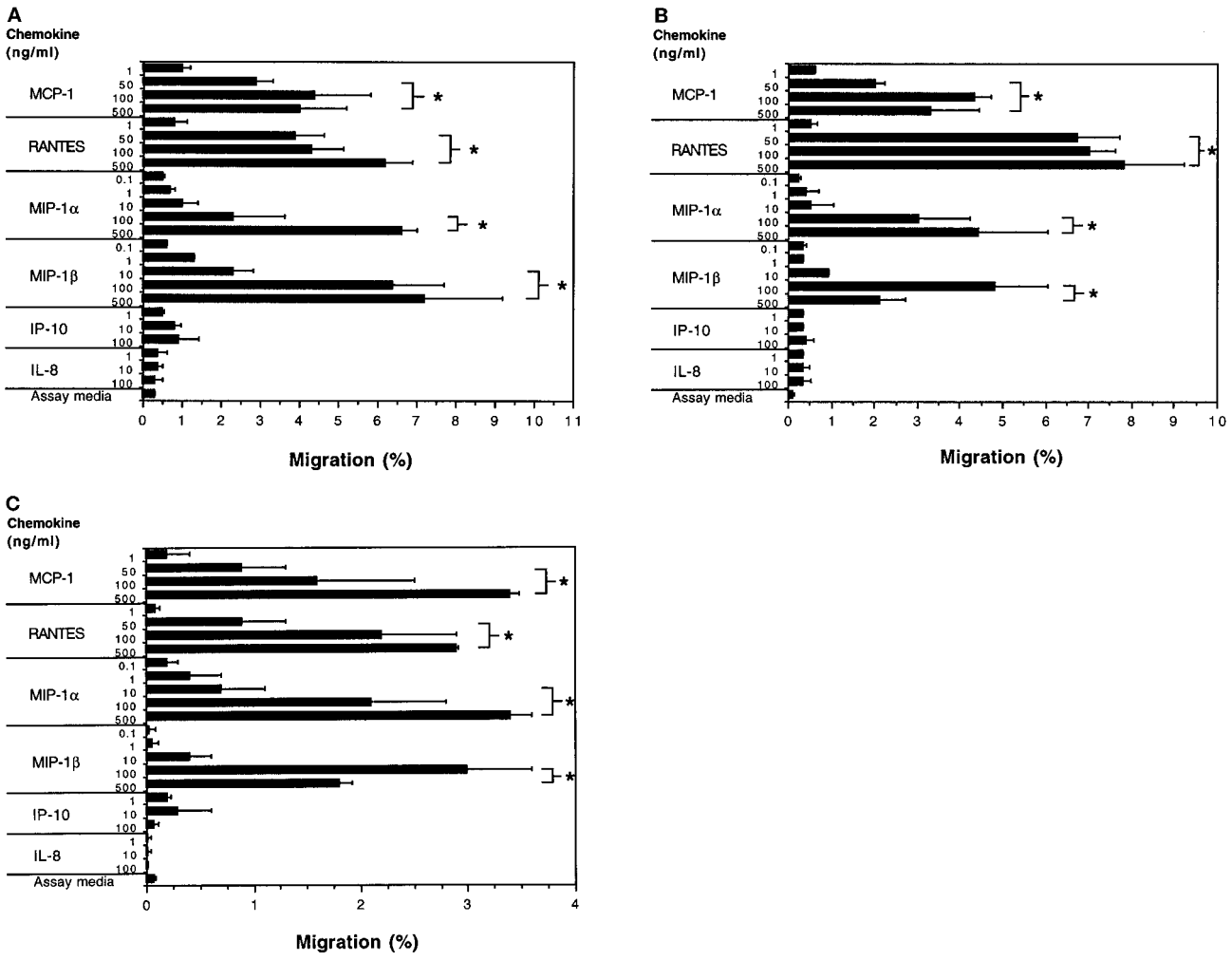

Figure 3. Transendothelial chemotaxis of the γ/δ T cell clones JN23, CP.1.5, and CP.1.15 to C-C and C-X-C chemokines. JN23 (A), CP.1.5 (B), or CP.1.15 (C) cells were added to duplicate HUVEC-covered Transwell inserts and allowed to migrate to the indicated concentrations of MCP-1, RANTES, MIP-1α, MIP-1β, IP-10, IL-8, or assay media alone for 4 h at 37 °C. Data represent the mean percentage migration from one of three independent experiments with similar results. Bars represent the range. *: $p < 0.05$, compared to assay media control.

2.3 Chemotactic response of functional subsets of γ/δ T lymphocytes

Expression of the accessory molecules CD4 and CD8 divides α/β T cells into two phenotypically distinct subsets [5]. In contrast, the majority of γ/δ T cells are $CD4^-$ and $CD8^-$, although clones that are $CD4^+$ or $CD8^+$ have been derived and characterized [39]. To determine if γ/δ T cells that vary in CD4 and CD8 expression demonstrate functional differences in chemotaxis, we tested three γ/δ clones, JN23 ($CD4^+/CD8^-$), CP.1.5 ($CD4^-/CD8^+$), and CP.1.15 ($CD4^-/CD8^-$), in the transendothelial assay (Fig. 3A–C). All three clones migrated significantly only to the C-C chemokines. As noted with purified γ/δ T cells, the γ/δ clones responded equally well to all four C-C chemokines except for clone CP.1.5, which showed greater migration to RANTES (Fig. 3B).

2.4 γ/δ T lymphocytes from an input population of PBMC transmigrate to C-C chemokines

The γ/δ T cells used in the chemotaxis experiments of Figs. 1 and 2 were purified by positive immunomagnetic selection using mAb to the α/β or γ/δTCR. Since mAb binding of the TCR-associated CD3 complex has been shown to alter lymphocyte responsiveness to chemokines in chemotaxis assays [22, 40], we wished to confirm that γ/δ T cells without mAb bound to their TCR:CD3 complex transmigrated similarly. Therefore,

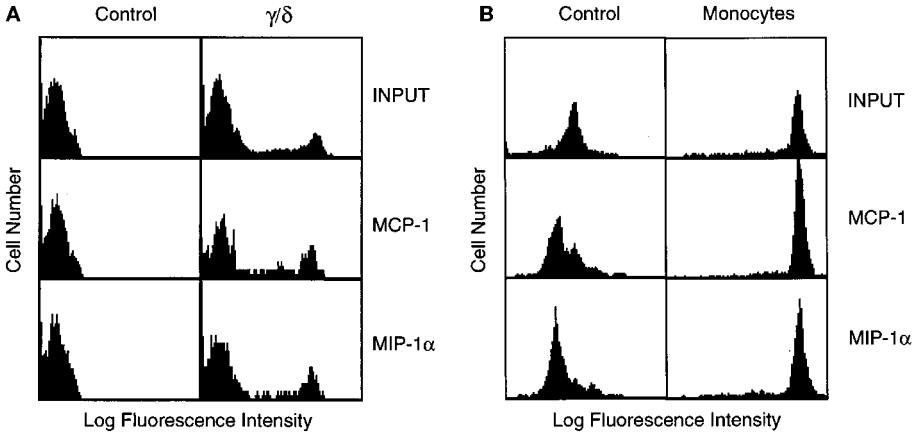

Figure 4. γ/δ T cells in unfractionated PBMC undergo transendothelial chemotaxis to C-C chemokines. Mononuclear cells which transmigrated to MCP-1 or MIP-1α at 100 ng/ml for 4 h at 37 °C were harvested, and along with input PBMC were washed, stained with mAb X63 (control), anti-TCRδ1 mAb to γ/δ on T cells, MY4 mAb to CD14 on monocytes, or BMA 031 mAb to α/β on T cells and subjected to flow cytometry. Forward and 90° scatter gates were set to exclude endothelial cell debris, and lymphocytes and monocytes were resolved by scatter-gating. (A) Lymphocytes; (B) Monocytes. Of the lymphocyte subpopulation, γ/δ T cells constituted 10 % of input PBMC, 15 % of cells migrated to MCP-1, and 13 % of cells migrated to MIP-1α. Of the total scatter-gated mononuclear cells (lymphocytes + monocytes), monocytes constituted 18 % of input PBMC, 39 % of cells migrated to MCP-1, and 28 % of cells migrated to MIP-1α. Histograms are from one of two experiments with similar results.

we performed a phenotypic analysis of cells that transmigrated in large-scale transendothelial chemotaxis assays using an unfractionated PBMC input population. Since inadequate numbers of cells transmigrated to IL-8 and IP-10 for flow cytometric analysis, only data from experiments with C-C chemokines were obtained. PBMC were allowed to transmigrate to MCP-1 or MIP-1α at 100 ng/ml for 4 h, and the input and migrated cells were stained with mAb and subjected to flow cytometry. Lymphocytes and monocytes were resolved by scatter-gating. The lymphocytes were ~ 70 % TCRα/β$^+$ and 10 % TCRγ/δ$^+$. γ/δ T cells were present among the transmigrated cells, confirming that unfractionated γ/δ cells can undergo chemotaxis to these two C-C chemokines (Fig. 4A). The number of γ/δ T cells which transmigrated to MCP-1 and MIP-1α was similar, consistent with the chemotactic response observed with purified γ/δ cells as the input population (Fig. 2B). In contrast, while monocytes migrated both to MCP-1 and MIP-1α, approximately 1.4-fold more monocytes migrated to MCP-1, demonstrating a differential response to these chemokines (Fig. 4B). In additional phenotyping experiments, no significant difference was observed in the number of γ/δ T cells that migrated to RANTES or MIP-1β versus MCP-1 and MIP-1α (data not shown).

3 Discussion

We have directly compared the chemotactic response of human α/β and γ/δ T lymphocytes to six C-C and C-X-C chemokines in transendothelial chemotaxis assays. All six chemokines have been detected in affected tissue at inflammatory sites and reported to promote T lymphocyte chemotaxis in *in vitro* chemotaxis assays. We find that purified α/β and γ/δ T cells undergo transendothelial chemotaxis only to the C-C chemokines MCP-1, RANTES, MIP-1α, and MIP-1β, and not to the C-X-C chemokines IL-8 and IP-10. Our results demonstrate that despite clear differences between α/β and γ/δ T cells in cell surface phenotype, antigen recognition mechanisms, and migratory behavior, these two subpopulations share the ability to transmigrate to C-C chemokines. However, the chemotactic responses of α/β and γ/δ T cells to C-C chemokines are not identical. Differences in responsiveness to C-C chemokines between α/β and γ/δ T cells may contribute to their distinctive patterns of tissue migration during inflammation.

To our knowledge, *in vitro* experiments evaluating human γ/δ T cell chemotaxis have not been published. However, peripheral blood γ/δ T cells have been reported to exhibit a greater capacity than CD8$^+$ or CD4$^+$ α/β T cells to transmigrate spontaneously across human umbilical vein endothelial cells (HUVEC) cultured on collagen gels

[41]. In the transendothelial chemotaxis assay, spontaneous transmigration is measured as background migration in control wells containing assay media. We found no significant difference between α/β and γ/δ T cells in the low percentage of spontaneous transmigration (consistently $< 0.5\,\%$ at 4 h incubation; see Figs. 1 and 2). Unstimulated HUVEC were used in our assays because cytokine activation of HUVEC (*e.g.* with IL-1 or TNF-α) significantly increases background T cell migration [36] and could promote HUVEC chemokine production, which would complicate the interpretation of migration results.

We previously found MCP-1 to be a more efficacious chemoattractant than RANTES, MIP-1α, or MIP-1β for purified $CD3^+$ T lymphocytes [28]. MCP-1 also promoted significant γ/δ T cell chemotaxis, but the response was more comparable to the other C-C chemokines. The chemotaxis of purified γ/δ T cells to MCP-1 varied between approximately 1.5–3.0 % at 4 h, a range similar to that obtained here with purified α/β T cells and previously with $CD3^+$, $CD4^+$, and $CD8^+$ T cell subsets [28]. The kinetics of transmigration, with insignificant migration at < 2 h and a plateau by 6 h, are also similar for α/β and γ/δ T cells.

$CD3^+$ T cells and $CD4^+$, $CD8^+$, and $CD45R0^+$ T cell subsets undergo transendothelial chemotaxis to MCP-1, MCP-2, MCP-3, RANTES, MIP-1α, and MIP-1β, but not to IL-8 or IP-10 [28]. Since the majority of peripheral blood $CD3^+$ T cells are $TCR\alpha/\beta^+$ [3], we expected that $CD3^+$ and α/β T cells would migrate to the same chemokines. Purified α/β T cells migrated significantly better to MCP-1, RANTES, and MIP-1α than to MIP-1β. Purified γ/δ T cells migrated to the same chemokines. However, in contrast to α/β T cells, γ/δ T cells migrated to MIP-1β equally well as to MCP-1, RANTES, and MIP-1α. Phenotyping experiments with unfractionated PBMC as the input population also showed equivalent chemotactic efficacy at 100 ng/ml for γ/δ T cells using these four C-C chemokines. Therefore, while both subpopulations are capable of migrating to each of these four C-C chemokines, differences in responsiveness are detectable *in vitro.*

Absence of chemotaxis to IP-10 of α/β T cells purified from unstimulated PBMC is consistent with our published results with resting $CD3^+$ T cells [28] and the results of others [30, 42]. Only T cells activated *in vitro,* not resting T cells from peripheral blood, have been shown to undergo chemotaxis to IP-10. Unstimulated γ/δ T cells are similar to unactivated α/β T cells in that they also do not migrate to IP-10. However, because the γ/δ T cell clones used in these experiments are stimulated during *in vitro* preparation [39], they might be expected to migrate to IP-10. Activated human T cells, but not resting T cells, B cells, monocytes, or granulocytes have been shown to express a chemokine receptor, CXCR3, which is specific for IP-10 and the related C-X-C chemokine Mig [42, 43]. The absence of chemotaxis to IP-10 by γ/δ T cell clones could be due either to lack of expression or to low expression of an IP-10 receptor (*i.e.* CXCR3) on clone cells. γ/δ T cells have not been assessed for CXCR3 expression. Although we have not tested α/β or γ/δ T cells for chemotaxis to Mig, we would not expect resting cells of either T cell subpopulation to transmigrate based on what is known about CXCR3 expression [42].

Our results are relevant to the current understanding of the migratory behavior of α/β and γ/δ T cells *in vivo.* Our data suggest that α/β and γ/δ T lymphocyte subpopulations can transmigrate from peripheral blood in response to the C-C chemokines MCP-1, RANTES, MIP-1α, and MIP-1β during an inflammatory response. Both increased messenger RNA and protein expression of these C-C chemokines have been reported in inflammatory lesions, including tuberculoid and sarcoid granulomas [44] and rheumatoid synovial tissue [15]. Although the function of γ/δ T cells in local immune responses is uncertain, there is increasing evidence for antigen-specific immunoregulation by γ/δ T cells on α/β T cells in both infectious (*e.g.* Lyme arthritis) [11] and autoimmune (*e.g.* diabetes mellitus and systemic lupus erythematosus) [17, 18] inflammatory responses. The shared responsiveness of α/β and γ/δ T cells to four different C-C chemokines as shown here may provide a mechanism for attracting both T lymphocyte subpopulations into inflamed tissues at the same time. Furthermore, differences in preferences for these four C-C chemokines as shown here may contribute to the distinctive distribution of T lymphocyte subsets at inflammatory loci in specific diseases.

C-C chemokines thus may function to attract γ/δ T cells to local sites of infection. T cell activation, both through antigen binding to the TCR and chemokine stimulation, increases the adhesiveness of integrin molecules, greatly enhancing their binding to counterreceptors on endothelium and extracellular matrix proteins [45, 46]. Chemokines are likely to function in concert with antigen-TCR binding to promote migration specificity and subsequent lymphocyte expansion and/or retention of γ/δ T cells at inflammatory sites.

4 Materials and methods

4.1 Chemokines

Purified recombinant human chemokines were obtained from the following sources: MCP-1, RANTES, MIP-1α, MIP-1β and IL-8 from both Genzyme (Cambridge, MA) and Peprotech (Rocky Hill, NJ); IP-10 from both Peprotech and as a gift of A. Luster (Harvard Medical School, Boston, MA).

4.2 Antibodies

MY4 (anti-CD14, IgG2b [47] was from Coulter Immunology (Hialeah, FL); BMA 031 (pan-TCRα/β, IgG1) and anti-TCRδ1 (pan-TCRγ/δ, IgG1) were either unmodified or biotinylated [48]. X63 (myeloma IgG1) was used as a negative control.

4.3 Preparation of leukocyte populations

Purified α/β and γ/δ T lymphocytes were isolated from single donor human platelet pheresis residues by positive selection using magnetic cell sorting according to the manufacturer's protocol (Miltenyi Biotec, Sunnyvale, CA), as described [8]. Briefly, PBMC were isolated by gradient centrifugation through Ficoll-Hypaque (Sigma, St. Louis, MO), washed to remove platelets, resuspended at 2×10^7 cells/ml in PBS with 1 % BSA and 5 mM EDTA at pH 7.4 (PBE buffer), and then incubated for 30 min on ice with 5–10 μg/ml of either biotinylated mAb BMA 031 or anti-TCRδ1. Following two washes with cold PBE buffer, a 1:100 dilution of FITC-conjugated streptavidin (CALTAG Laboratories, San Francisco, CA) was added and the cells incubated for 30 min on ice. Cells were then washed with cold PBS, incubated 10 min on ice with biotin-conjugated magnetic microbeads (MACS, Miltenyi Biotec), and passed over an AS1 magnetic column (MACS). Purity was assessed by immunofluorescence flow cytometry and was >95 % for both α/β and γ/δ preparations. Cell viability was >95 % as determined by trypan blue exclusion.

Purified α/β, γ/δ, and γ/δ clone cells were labeled with 0.5 μg/ml 2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF) (Molecular Probes, Eugene, OR) for 30 min at 37 °C in assay media, a 1:1 mixture of medium 199 (BioWhittaker, Walkersville, MD) and RPMI 1640 (Sigma) plus 0.5 % human serum albumin, to facilitate counting of migrated cells [36].

4.4 Derivation and maintenance of γ/δ T lymphocyte clones

The γ/δ T lymphocyte clones CP.1.5, CP.1.15, and JN23 were derived from purified γ/δ T lymphocytes by limiting dilution with PHA stimulation as described [39]. Periodic restimulation with allogenic PBL, Epstein-Barr virus-transformed B cells, and PHA-P was performed to maintain clones. Gradient centrifugation through Ficoll-Hypaque was performed on previously frozen clones to separate viable from nonviable cells before use in chemotaxis assays.

4.5 Transendothelial chemotaxis assay

HUVEC were harvested, cultured, and passaged as previously described [49]. Lymphocyte transendothelial chemotaxis assays were performed as described [36]. Briefly, HUVEC at passage 2 or 3 were grown to confluence on type I collagen-coated, 6.5-mm diameter Transwell (Costar, Cambridge, MA) tissue culture inserts of 8 μm pore size. Purified lymphocytes at 5×10^5/insert in assay media were added to duplicate inserts, chemokines or control media placed into the wells of 24-well cluster plates, and the assembled plates incubated at 37 °C, 5 % CO_2 for the period of chemotaxis. Lymphocytes which migrated into the bottom wells were resuspended, allowed to settle, and then counted in four separate locations/well bottom on a fluorescent microscope (Diaphot-TMD, Nikon, Garden City, NJ). Duplicate wells without inserts containing a 1:20 dilution of input cells served to establish input cell counts. Migration, expressed as the percentage of input cells migrated, was calculated by dividing the average number of migrated cells/well bottom by the average number of input cells/well bottom.

4.6 Flow cytometry of migrated mononuclear cells

The transendothelial chemotaxis assay described above was performed in larger scale using HUVEC-covered 24.5-mm Transwell inserts, 6-well cluster plates, and 5×10^6 unlabeled PBMC in 1 ml/insert. PBMC were allowed to transmigrate to MCP-1 or MIP-1α at 100 ng/ml in four to six wells/chemokine for 4 h. Following incubation, the migrated cells were harvested, and input and migrated cells washed and resuspended in L-15 medium (GibcoBRL, Grand Island, NY) with 2.5 % (vol/vol) FCS at 4 °C. Cells were incubated for 30 min on ice with a saturating concentration of mAb MY4, BMA 031, anti-TCRδ1, or X63, washed, and then incubated with a 1/20 dilution of FITC-conjugated second antibody [goat anti-mouse Ig(G + M)] (Zymed Laboratories, So. San Francisco, CA). Flow cytometry was performed using a FACScan (Becton Dickinson, San José, CA) with forward versus 90° scatter gates set to exclude endothelial debris as described [25].

4.7 Statistical analysis

A square root transformation of cell count data and nested analysis of variance (ANOVA) were used to compare mean counts of cells migrated to chemokines or media controls. If ANOVA detected a statistically significant difference in mean counts, the Tukey method of multiple comparisons was applied. A p value of < 0.05 was considered to be statistically significant for all analyses.

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Correspondence: Timothy A. Springer, The Center for Blood Research, 200 Longwood Avenue, Boston, MA 02115, USA
Fax: +1-617-2 78 32 32