Cutting Edge: C-C Chemokine Receptor 6 Is Essential for Arrest of a Subset of Memory T Cells on Activated Dermal Microvascular Endothelial Cells Under Physiologic Flow Conditions In Vitro

David J. Fitzhugh, Shubhada Naik, S. Wright Caughman and Sam T. Hwang

*J Immunol* 2000; 165:6677-6681; doi: 10.4049/jimmunol.165.12.6677

http://www.jimmunol.org/content/165/12/6677

---

**References**

This article cites 28 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/165/12/6677.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
Memory T cells (mTC) express multiple chemokine receptors (including CCR4 and CCR6) that may potentially be involved in their arrest on inflamed endothelium. Herein, we specifically addressed whether CCR6 is required for mTC to arrest on TNF-α-activated human dermal microvascular endothelial cells (HDMEC) in vitro under shear stress conditions. Recombinant liver and activation-regulated chemokine (LARC)/CCL20 (a CCR6 ligand) induced firm arrest of cutaneous lymphocyte Ag+ mTC in a flow chamber system using purified substrates. Strikingly, desensitization of CCR6 with LARC, but not thymus and activation-regulated chemokine/CCL17 or secondary lymphoid tissue chemokine/CCL21, caused a 50–75% decrease ($p < 0.001$) in arrest of mTC on HDMEC, which was indistinguishable from the reduction observed when total mTC were treated with pertussis toxin ($p > 0.5$). CCR6-depleted mTC also had a markedly reduced ability to arrest on HDMEC. Our results suggest that LARC production by activated endothelial cells and CCR6 expression by mTC may be critical components in the pertussis toxin-sensitive arrest of mTC on activated HDMEC. The Journal of Immunology, 2000, 165: 6677–6681.

Chemokines acting through seven transmembrane-spanning G-protein-coupled receptors are believed to be critical in the migration of lymphocytes out of blood vessels into peripheral tissue and secondary lymphoid organs. In the multistep model of immune cell recruitment, various adhesion molecules including the selectins mediate transient (rolling) interactions with endothelial cells, whereas chemokines have been shown to induce firm arrest via integrins in vitro (1) as well as in vivo (2).

Naive (CD45RA+ and memory (CD45RO+) T cells migrate to different sites of the body, in part because of their differential expression of specific adhesion molecules (i.e., selectins) and chemokine receptors (CKRs) (3). Memory T cells (mTC) efficiently interact with E- and P-selectin and VCAM-1 (4). Some mTC express a carbohydrate modification of P-selectin glycoprotein ligand-1 termed cutaneous lymphocyte Ag (CLA) that acts as a ligand for E-selectin (5, 6). Interestingly, CLA+ mTC preferentially express CCR4, and infarmed dermal blood vessels express thymus and activation-regulated chemokine (TARC/CCL17), a CCR4 ligand (7). Together, CLA and CCR4 may contribute to specific mTC trafficking to inflamed skin.

Liver and activation-regulated chemokine (LARC)/macrophage-inflammatory protein (MIP)-3α/Exodus-1/CCL20 (8–10) is one of a small number of chemokines including stromal-derived factor (SDF)-1/CXCL12, secondary lymphoid tissue chemokine (SLC)/CCL21, EBI1-ligand chemokine/CCL19, and TARC that have been demonstrated to induce arrest of lymphocytes under physiologic flow conditions (1, 7, 11). TNF-α has been shown to up-regulate LARC by HUVEC (10). The receptor of LARC, CCR6 (12, 13), is expressed by 40–50% of peripheral blood memory, but not naive, T cells (14). Because our own preliminary results (S.T.H., unpublished data) and those of others (15) have suggested that CCR6 may be up-regulated by skin T cells in psoriasis, we hypothesized that the expression of LARC by microvascular endothelial cells might contribute to the arrest of circulating CCR6+ mTC on acutely inflamed vascular endothelium.

We demonstrate striking up-regulation of LARC by cytokine-activated human dermal microvascular endothelial cells (HDMEC). Two separate lines of evidence reveal a predominant role for CCR6 in the arrest of mTC on activated endothelial cells under physiologic flow conditions. Thus, our data suggest that LARC and its ligand, CCR6, may play critical roles in the early entry of mTC into peripheral sites of inflammation in vivo.

Abbreviations used in this paper: CKR, chemokine receptor; HDMEC, human dermal microvascular endothelial cell; mTC, memory T cell(s); CCL, C-C chemokine ligand; CLA, cutaneous lymphocyte Ag; LARC, liver and activation-regulated chemokine; MIP, macrophage-inflammatory protein; TARC, thymus and activation-regulated chemokine; SDF, stromal-derived factor; SLC, secondary lymphoid tissue chemokine; FKN, fractalkine; PTX, pertussis toxin.
Materials and Methods

Reagents and cell culture

Recombinant human chemokines were purchased from PeproTech (Rocky Hill, NJ). Human E-selectin/ Ig chimera (16) was provided by Dr. S. Rosen (University of California, San Francisco, CA). Soluble human ICAM-1 and a non-function-blocking mouse anti-human ICAM-1 mAb (P79, mouse IgGl) were gifts of Dr. T. Kishimoto (Boehringer-Ingelheim, Ridgefield, CT). PE-conjugated anti-β2, α5, β1 integrin and FITC-labeled anti-CLA were purchased from PharMingen (San Diego, CA).

HDMEC were isolated from neonatal foreskins, as described (17, 18), and used for these studies in passages 3–6.

Quantitative RT-PCR

Confluent monolayers of HDMEC were stimulated by addition of 10 ng/ml recombinant human TNF-α (R&D Systems, Minneapolis, MN) for 6–8 h at 37°C; control cells were left unstimulated. After total RNA and first-strand cDNA synthesis, real-time quantitative PCR was performed with an ABI PRISM 7700 using SYBR Green (PE Biosystems, Foster City, CA). Threshold cycle (Ct) values were assigned according to the cycle number at which a fixed fluorescent intensity was achieved. A ΔΔCt value was calculated by Ct (target) – Ct (G3PDH). Fold induction of genes in the TNF-α-treated samples relative to the unstimulated samples was then calculated according to the following expression, where ΔΔCt is the difference between ΔCt TNF-α and ΔCt unstimulated: 2-ΔΔCt. The primer sequences were used in the real-time PCR amplifications, all of which generated amplions 65–88 bp long: G3PDH: forward (F) 5'-ACCACACTCCTCCACCTTTGA-3'; reverse (R) 5'-CATACGGATAATGAGCTTGACAA-3'; LARC (F) 5'-TCTGGCCTTGTGATGCTCA-3'; RARC (F) 5'-AGGATGCGCAGTTTTTTGTAA-3'; fractalkine (FKN) (F) 5'-CTCCCTGCGCTTCCTCTCCT-3'; R-5'-ATCCTCTCGGATCTTCTGAGA-3'; IL-8 (F) 5'-GTCGACTTGGCCTCCTCTTCT-3'; CAT (F) 5'-TGGCCGATTGCTGACAA-3'; TARC (F) 5'-CAAAGTTGCTTGCTGCTTCTGA-3'; CX3C chemokine (19). LARC expression was up-regulated by 170-fold (Fig. 1A). Protein expression paralleled mRNA expression with E-selectin and FKN being expressed on up to 30% of HDMEC between 5 and 10 h after stimulation (Fig. 1B). Interestingly, although stimulated TARC expression was up-regulated by ~200-fold in the presence of TNF-α, this enhanced level of expression was ~250-fold lower than the expression of LARC mRNA concentrations in the presence of TNF-α, a result also observed by conventional PCR with gel analysis.

Isolation of mTC populations

Human PBMC from volunteer-derived buffy coats were enriched by centrifugation over Histopaque 1077 (Sigma, St. Louis, MO). After overnight culture of PBMC in complete RPMI 1640 with 10% FCS (cRPMI), mTC were depleted of CD14+/CD19- cells with specific mAb-conjugated Dy- nal magnetic beads. CD45RA+ mTC were removed by labeling with anti-CD45RA mAb (PharMingen) and depleting with anti-mouse IgG-conjugated magnetic beads. The resultant population was consistently >95% CD45RO- mTC as measured by FACs analysis. CCR6- mTC were obtained by labeling the total mTC population with anti-CCR6 (mAb 195, mouse IgG2b, R&D Systems) followed by a FITC-conjugated secondary Ab. After washing, CCR6+ and CCR6- mTC populations were purified using the MACs anti-FITC micromagnetic bead system (Miltenyi Biotec, Auburn, CA). After selection, we obtained <5% and >85% CCR6+ mTC in the CCR6+ and CCR6- fractions, respectively.

In vitro flow adhesion assay

Human mTC were either unlabeled or labeled with calcine-AM (Molecular Probes, Eugene, OR) per manufacturer’s instructions and were exposed to pertussis toxin (PTX, 100 ng/ml, Sigma) in some cases for 2–3 h at 37°C. mTC were allowed to equilibrate at 37°C for 30 min before the flow assay and then suspended at 5×106 cells/ml in cRPMI in a 12-mI syringe, which was fixed to a precision syringe pump (Harvard Apparatus, Holliston, MA).

For flow assays using recombinant proteins, droplets of E-selectin/human Ig (2 μg/ml) and an anti-human ICAM-1 mAb (P79, 1 μg/ml) in 100 μl Tris-buffered saline (pH 9.0), were applied overnight to 35-mm non-treated plastic culture dishes at 4°C as described (11). After a brief rinse with PBS, chemokines (50 μl, 10 μg/ml) in PBS were applied for 1.5 h at room temperature. After two PBS rinses, soluble ICAM-1 (100 μl, 10 μg/ml) in PBS/0.1% BSA was applied to the plate for 1 h at room temperature. A parallel plate flow chamber apparatus (Glycotech, Rockville, MD) was affixed to the tissue culture plate and flow adjusted to achieve a shear stress of 1.5 dynes/cm2. Unlabeled mTC were observed by phase microscopy. Five minutes after initiation of flow (when large numbers of arrested and/or rolling cells could be observed), randomly selected fields were videotaped for 20–30 s and subjected to digital video analysis of 10-s segments using NIH Image 1.62 and cell-tracking macros designed by K. Tangemann. Adherent cells were defined as cells that did not move during the 10-s interval. All other cells interacting with the plate were defined as rolling cells. Results were expressed as the adherent:rolling cell mean ratio (10 fields were analyzed per condition). On plates coated with ICAM-1 only, capture/rolling/arrest was not observed.

For attachment experiments using calcine-labeled mTC, HDMEC were grown to confluence in 35-mm tissue culture dishes and stimulated with 10 ng/ml recombinant human TNF-α for 6–8 h at 37°C. Interactions were allowed to establish for the first 5 min of flow at a shear stress of 1.5 dynes/cm2 at RT. While maintaining this shear stress, images were then captured under fluorescent illumination with an exposure time of 2 s, such that only cells that had not moved within this time frame on the endothelial monolayer would generate a single-cell image. Rolling cells appeared as streaks because of movement during the long exposure time while arrested cells appeared single points. Arrested cells were resistant to detachment at shear stresses >10 dynes/cm2 (data not shown). Images obtained using a ×4 objective (1.7 mm²/image in surface area) were typically captured during each run by a CCD camera, systematically sampling fields from eight different sectors of the exposed monolayer within a span of 2 min. The numbers of firmly arrested cells in each image were then quantified manually and expressed as the mean number of adherent cells per field ± SEM. Statistical significance (Student’s t test) was calculated using Microsoft Excel. All flow experiments were repeated at least three times with similar results.

Results and Discussion

Chemokine expression by HDMEC was examined by real-time, quantitative RT-PCR. LARC and TARC were analyzed along with two genes known to be up-regulated by endothelial cells with TNF-α stimulation: E-selectin and FKN, a membrane-tethered CX3C chemokine (19). LARC expression was up-regulated by 170-fold (Fig. 1A). Protein expression paralleled mRNA expression with E-selectin and FKN being expressed on up to 30% of HDMEC between 5 and 10 h after stimulation (Fig. 1B). Interestingly, although stimulated TARC expression was up-regulated by ~200-fold in the presence of TNF-α, this enhanced level of expression was ~250-fold lower than the expression of LARC mRNA concentrations in the presence of TNF-α, a result also observed by conventional PCR with gel analysis.

CCR6 is expressed by both CLA+ and α4β7+ mTC (14). By quantitative FACs analysis (Fig. 2A), we found that CLA was expressed on both CCR6+ and CCR6- mTC in roughly equal proportions (~15%). Isolated populations of CCR6-negative and–positive mTC showed similar distributions of β2, β1, and α4 integrins (Fig. 2, B–D). Thus, CCR6 is found on both CLA+ and CLA- mTC.

Although LARC has been reported to stimulate the arrest of α4β7+ mTC under flow conditions (1), the ability of LARC to stimulate arrest of CLA+ T cells under flow conditions has not been addressed. This issue is important because skin (CLA+) vs intestinal (CLA-) homing T cells may have differences in CKR and adhesion molecule expression that alter their trafficking patterns (20). We analyzed the ability of chemokines to induce arrest in mTC on purified substrates (E-selectin/Ig and sICAM-1) by calculating the proportion of arrested to rolling cells as described in Materials and Methods. The chemokine SDF-1 was used as positive control for arrest because its receptor (CXCR4) is found on virtually 100% of mTC and because SDF-1 is highly effective in inducing firm adherence of lymphocytes under flow conditions (1). In the absence of chemokine, individual mTC typically rolled but did not arrest (Fig. 3A). In the presence of SDF-1, however, mTC arrested on the order of seconds yielding a plateau in the distance vs time plot (Fig. 3B) and a high adherent:rolling cell ratio (Fig. 3E). LARC also induced arrest in a substantial proportion of mTC although its efficacy was not as high as that of SDF-1 (Fig. 3D). LARC also required a longer period of time to induce arrest (Fig. 3C). PTX treatment of mTC blocked firm arrest but did not influence mTC rolling (Fig. 3, D and E). CCR6-depleted mTC lost much of their ability to arrest on LARC-coated plates (Fig. 3D) but arrested well in the presence of SDF-1 (Fig. 3E), showing that depletion of CCR6+ mTC did not remove the ability of the remaining mTC to either roll on E-selectin or arrest on ICAM-1.

Downloaded from http://www.jimmunol.org/ by guest on April 15, 2017
SDF-1 was three to four times more effective in inducing arrest than LARC after adjusting for the higher proportion of mTC that expressed CXCR4 compared with CCR6. Thus, LARC induced the arrest of CLA$^{+}$ mTC in a PTX-sensitive fashion under physiologic flow conditions.

When flowing mTC over activated HDMEC, interactions in the absence of TNF-$\alpha$ treatment were negligible, and arrested T cells were rarely observed. After exposing endothelial monolayers to TNF-$\alpha$ for 6–8 h, many T cells were observed to transiently bind, roll, and arrest. In this system, mTC adhesion was strongly dependent on the expression of CLA by mTC and E-selectin by the activated endothelial cells. Depletion of the CLA$^{-}$ subpopulation of mTC using immunomagnetic bead methods (>95% effective) led to a 75% decrease in the number of arrested cells compared with total mTC ($p < 0.001$). Furthermore, treatment of activated endothelial cells with an anti-E-selectin mAb (Endogen, Cambridge, MA) led to an 80% ($p < 0.001$ compared with total mTC) decrease in arrested cells at the end of the flow period. Although the majority of T cell arrest could be blocked by PTX (Figs. 4 and 5), other molecules such as FKN (21) and VLA-4 (22) may have contributed to PTX-independent arrest of mTC via direct ligand-receptor interactions.

Two independent experimental methods demonstrated that CCR6 was critical for firm arrest of mTC to activated HDMEC. First, mTC, CCR6-depleted T cells, and PTX-treated mTC were separately applied to the flow chamber containing activated HDMEC. At the end of the loading period, CCR6$^{+}$ mTC had dramatically fewer accumulated T cells than the total mTC population, which was approximately the same as the numbers seen with PTX treatment of the entire mTC fraction (Fig. 4). Because surface adhesion molecule receptor expression was similar between CCR6$^{+}$ and CCR6$^{-}$ mTC (Fig. 2), differential expression of CLA or critical integrins could not account for the dramatic reduction in arrest of the CCR6-depleted mTC on TNF-$\alpha$ activated HDMEC.

Second, as previously demonstrated using CCR7$^{+}$ naive T cells (2), a CKR can be specifically inhibited by exposing it to high concentrations of its ligand. Treatment of mTC with LARC before and during the flow adhesion assay led to a 60% decrease ($p < 0.001$) in number of arrested mTC compared with the untreated control (Fig. 5). This degree of inhibition was comparable with the inhibition of arrest demonstrated by PTX. By contrast, the arrest of mTC treated with TARC and SLC was not significantly different from control. Therefore, CCR6 (but not CCR4 or CCR7) is required for arrest of mTC in this system.

mTC express a number of CKRs (23), and endothelial cells express multiple chemokines including FKN, monocyte chemoattractant protein-1, IL-8, RANTES, and MIP-1$\beta$ (reviewed in Ref. 24). Our data, however, suggest a surprisingly dominant role for
CCR6 and LARC in the acute arrest of mTC to activated dermal endothelium. Although ours is an in vitro study, the proteins acutely expressed by TNF-α-stimulated HDMEC in vitro closely parallel those expressed in vivo when human subjects were injected with TNF-α intradermally (25). Moreover, a recent report demonstrated that protein expression of CCR6 and chemotactic sensitivity to LARC are up-regulated in the T cells of psoriatic patients (15), which lends support to the possible importance of CCR6 in vivo. Although TARC and CCR4 may potentially be important for homing of mTC to skin (7), we found that HDMEC expression of TARC was relatively low (compared with LARC) under our activation conditions and, accordingly, desensitization of CCR4 with TARC (Fig. 5) did not diminish the adhesion of mTC to activated HDMEC. Protein expression by dermal endothelial cells changes as the inflammatory process continues over time. For instance,

FIGURE 4. CCR6− mTC arrest poorly on stimulated HDMEC. Total mTC, PTX-treated, and CCR6− cells from a single donor were allowed to flow over TNF-α-stimulated HDMEC as described in Materials and Methods. Results are expressed as the mean arrested cells/field ± SEM.

FIGURE 5. Pretreatment of mTC with LARC inhibits firm arrest on HDMEC under physiologic flow conditions. Labeled mTC were treated with the indicated chemokines at 1 μg/ml for 30 min at 37°C in cRPMI. Other cells were left untreated or treated with PTX. At the end of pretreatment, mTC groups were maintained at 5 × 10^5 cells/ml in cRPMI containing chemokines at 1 μg/ml (if previously added) and introduced into the flow chamber containing activated HDMEC. Adherent cells were quantified and displayed as mean adherent cells/field ± SEM (n = 8; *, p < 0.001). For TARC and SLC treated mTC, no significant difference was observed compared with untreated mTC (n = 8; **, p > 0.5).
L-selectin ligands are not detected on ovine dermal blood vessels until 3 days after the onset of inflammation and are not maximally expressed until day 6 (26). Thus, it is possible that TARC is expressed in vivo at a later time point or that TARC and LARC are coinduced in vivo early in inflammation.

Our results clearly show that CCR6 is required for nearly all PTX-sensitive arrest on HDMEC under flow conditions in vitro. In vivo, however, the chemokine environment at the endothelial interface is likely to be complex. For example, it has been shown that endothelial cells can transcytose chemokines synthesized by other cells (27). By itself, CCR6 is unlikely to confer skin-homing properties because it is expressed by both \( \alpha_\text{b} \beta_\text{a} \) and \( \text{CLA} \text{mTC} \). Circulating CCR6+ mTC, however, may constitute a pool of T cells poised to enter acutely inflamed dermal- or intestinal-associated tissue depending on their coexpression of appropriate rolling/tethering receptors. Thus, in acute inflammatory conditions such as contact dermatitis, inhibition of CCR6 on mTC may be an effective means of delaying or inhibiting T cell homing to skin. Interestingly, LARC has been detected in the epidermis and in some dermal blood vessels in noninflamed skin (28). Conceivably, low level expression of LARC may enhance T cell recruitment to the skin and epidermis for immune surveillance under noninflammatory conditions. Future experiments will focus on the expression patterns of LARC and TARC under chronic as well as acute inflammatory conditions in vivo and on clarifying the precise roles of their receptors in selective mTC homing.

Acknowledgments

We thank Drs. Mark Udey, Joshua Farber, and Stephen Katz for helpful discussions; Dr. Kirsten Tangemann for help with the flow chamber and data analysis; and Erik Gonzalez for technical assistance.

References

cytoe capture, firm adhesion, and activation under physiologic flow. J. Exp. Med. 188:141.