

Cutting Edge: Hierarchy of Chemokine Receptor and TCR Signals Regulating T Cell Migration and Proliferation¹

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Chemokines play an important role in establishing the distribution of lymphocyte subpopulations in primary and secondary lymphoid tissues and in the recruitment of leukocytes to sites of inflammation. However, the potential of chemokines to down-regulate immune responses has not been demonstrated. We now show that certain chemokine gradients have the potential to suppress T cell activation by preventing formation of the immunological synapse, the specialized cell-cell junction that forms before a T cell can be fully activated. Our data reveals an immunosuppressive potential of chemokines engaging the CXCR3 and CCR7 receptors, but not the CXCR4, CCR2, CCR4, or CCR5 receptors. These results suggest a novel mechanism for T cell ignorance of agonist MHC-peptide complexes based on dominant chemokine gradients. *The Journal of Immunology*, 2000, 165: 15–19.

T cell activation is an essential process for elimination of many microbial infections and for chronic inflammatory processes. T cell activation is initiated by formation of a specialized junction between T cells and APCs, aptly described as an immunological synapse (IS)³ (1). The first manifestation of IS formation in response to agonist MHC-peptide complexes is that the T cell stops migrating (2–4). The ability of agonist MHC-peptide complexes to deliver a stop signal through the TCR may play an important role in selective retention of Ag-specific T cells in tissues and in T cell proliferation. Chemokines have also been shown to arrest T cell rolling under flow (5). However, in tissues, chemokine gradients are thought to motivate and direct T cell mi-

gration (6). For example, chemokine gradients attract activated T cells to APCs (7). In contrast, a gradient leading past the APC would have the potential to suppress the T cell response by preventing T cell stopping and IS formation. The ability of chemokine receptor signaling to compete with TCR signaling for control of T cell migration has not been systematically examined. Therefore, to understand the basic issues in the competition between TCR and chemokine signals, we tested the ability of a panel of chemokines to compete with TCR signals for T cell migration and proliferation. Our results demonstrate that chemokines can be divided into two groups with respect to TCR signals, dominant and subordinate. Dominant chemokine gradients override the TCR-mediated stop signal, while subordinate chemokine gradients do not prevent or reverse the TCR-mediated stop signal. Dominant chemokines have the potential to suppress T cell responses as demonstrated here by inhibition of T cell proliferation. The cells that have bypassed MHC-peptide complexes under the influence of a dominant chemokine gradient still proliferate to subsequent MHC-peptide exposure. This suggests that dominant chemokine gradients render T cells ignorant of agonist MHC-peptide complexes, as opposed to anergic.

Materials and Methods

T cells

Splenocytes from 3A9 TCR transgenic mice (8), provided by E. R. Unanue (St. Louis, MO), were stimulated for 3 days with 1 μ M hen eggwhite lysozyme (HEL). Cells were then expanded with EL-4 supernatant containing 50 U/ml IL-2 activity and used on day 7. T cells from 3A9 TCR transgenic mice interact specifically with IA^k HEL_{48–62}. The 3A9 T cells prepared in this manner make IFN- γ and no IL-4 and are therefore Th1 like.

Transmigration assays

A total of 10⁵ T cells in 100 μ l were added to 5- μ m pore-size, polycarbonate 24-well tissue culture inserts (Costar, Cambridge, MA), with 600 μ l media (or chemokine dilution) in the lower well. After the indicated time the cells in the lower well were counted with the aid of a hemocytometer. All points were determined in triplicate. Murine EBI1 ligand chemokine was a kind gift from J. Cyster (San Francisco, CA), and secondary lymphoid tissue chemokine (SLC) was provided by M. D. Gunn. All other chemokines were obtained from R&D Systems (Minneapolis, MN). IA^k HEL_{48–62} was provided by E. R. Unanue. ICAM-1 expressed in Chinese hamster ovary cells was solubilized with buffered Triton X-100 and captured on YN1/1 Sepharose. ICAM-1 was eluted at low pH in 1% octylglucoside. Molecular densities were determined by immunoradiometric assay using iodinated AW3.18.152 and YN1/1 for IA^k and ICAM-1, respectively. Inserts were coated with 50 μ l of 25 μ g/ml fibronectin for 1 h at 37°C. Then, liquid was aspirated from the inserts, and the inserts were dried at 37°C for 2 h. Alternatively, filters were coated with ICAM-1, IA^k,

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³ Abbreviations used in this paper: IS, immunological synapse; HEL, hen eggwhite lysozyme; SLC, secondary lymphoid tissue chemokine; SDF, stromal cell-derived factor; MIP, macrophage inflammatory protein; IP-10, IFN-inducible protein-10; MDC, macrophage-derived chemokine; MCP, macrophage chemoattractant protein.

or IA^k HEL₄₈₋₆₂ by dilution of the octylglucoside to 0.1% in 50 μ l in the filter insert. In this case, the inserts were not dried. Rather, they were then blocked with 5% nonfat dried milk for 1 h and then washed with media. Only the upper surface of the filter is coated with protein, and thus cells are not retained on the bottom of the filters. In addition, cells were stained with 0.1% crystal violet both before and after exposure to each chemokine and examined microscopically for cell aggregation. Each condition was performed in triplicate for the calculation of mean and SD values.

Results and Discussion

Prior experiments with time-lapse microscopy demonstrated that effector T cells from TCR transgenic mice migrate rapidly on ICAM-1-coated surfaces and stop migrating when they encounter Ag in the form of a specific MHC-peptide complex (3). We established a system in which T cells could be simultaneously exposed to Ag and chemokine gradients. This was based on coating adhesion molecules and MHC-peptide complexes on the top surface of a 5- μ m pore-size polycarbonate filter. The filter separated two chambers, a top chamber into which T cells are placed and a bottom chamber into which the cells that cross the filter are collected. In vitro-activated T cells were used for these experiments, because they adhere to and migrate through the ICAM-1-coated filters. Furthermore, these effector cells express a broad repertoire of chemokine receptors, allowing comparison of the response to multiple chemokines. Spontaneous migration of T cells on filters coated with ICAM-1 alone or ICAM-1 and irrelevant MHC-peptide complexes resulted in rapid migration across the filter and collection of \sim 50% of the cells in the lower chamber after 1 h (Fig. 1A). In contrast, the Ag-specific T cells did not migrate through filters coated with ICAM-1 and the activating MHC-peptide complex (Fig. 1A). This inhibition of spontaneous migration was dose dependent (Fig. 1B). Migration of $>$ 98% of the T cells was stopped for 2–4 h, after which the T cells began to migrate to the lower chamber and to proliferate with peak induction at 12 h (Fig. 1C). Thus, the stop signal is sustained long enough to ensure full T cell activation, at which time the T cells release themselves to migrate and proliferate. This is reminiscent of *in vivo* experiments in which Ag-activated T cells are retained in the lymph node until they are released as proliferating effector cells (9). These experiments demonstrate a 2- to 4-h time period in which the results of competition between the chemokine go signal and Ag stop signal can be determined.

The optimal forward gradient of each chemokine required to enhance T cell migration was determined and then used in subsequent experiments (Fig. 2A) (10). A forward gradient is generated by placing the chemokine only in the lower chamber of the transwell on the opposite side of the filter from the T cells. The optimal concentrations we determined are consistent with earlier studies for each chemokine (7, 10–14). Next, Ag-specific T cells were loaded onto filters coated with ICAM-1 and the activating MHC-peptide complex and incubated for 1 h. The filters were then washed to remove nonadherent cells such that any migrating cells in the subsequent hour would represent cells that were released early from the Ag stop signal. The adherent cells were subjected to optimal forward chemokine gradients of stromal cell-derived factor (SDF)-1 α , macrophage inflammatory protein (MIP)-1 α , SLC, MIP-3 β , IFN-inducible protein-10 (IP-10), RANTES, macrophage-derived chemokine (MDC), and macrophage chemoattractant protein (MCP)-1. SDF-1 α , MIP-1 α , RANTES, MDC, and MCP-1 did not overcome the Ag stop signal, with $<$ 1% of cells migrating to the bottom chamber. Filters were examined microscopically both before and after exposure of the cells to these chemokines; failure of the cells to transmigrate in response to these chemokines did not result from cell aggregation. Strikingly, SLC, MIP-3 β , and IP-10 all attracted at least 10% of effector T cells

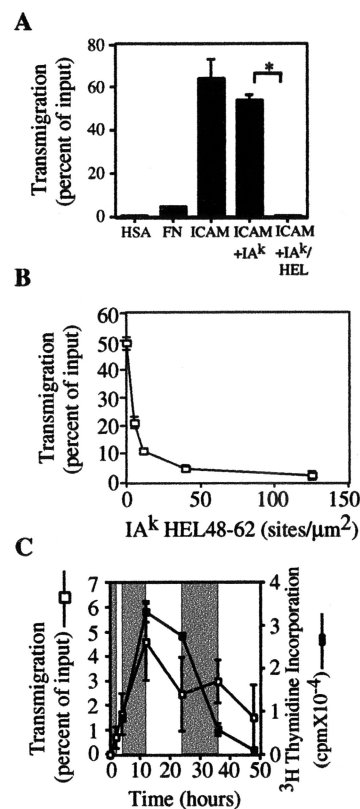


FIGURE 1. Effect of agonist MHC-peptide complexes adsorbed to filters on T cell migration and proliferation. *A*, One-hour transmigration of 3A9 T cells through protein-coated filters. T cells were allowed to transmigrate through filters coated with 1% human serum albumin (HSA), 25 μ g/ml fibronectin (FN), ICAM-1 (1000 sites/ μ m²), or ICAM-1 and an irrelevant MHC-peptide complex, IA^k with endogenously loaded peptide (100 sites/ μ m²) or with IA^k HEL₄₈₋₆₂ (100 sites/ μ m²). Note that no chemokine is required for this spontaneous migration. *, $p < 0.0003$. *B*, Density of MHC-peptide complex required to stop 3A9 T cells in a 1-h transmigration assay with ICAM-1-coated filter. *C*, Time course of spontaneous T cell migration and proliferation. A total of 10^5 3A9 T cell blasts were added to transwell inserts coated with ICAM-1 and IA^k HEL₄₈₋₆₂ and allowed to migrate for the indicated time spans (shading to emphasize time periods). At the indicated time points, transmigrated cells from the preceding time span were collected, and three wells were pooled, counted, and transferred to an untreated well of a 96-well plate. At 48 h, the cells were pulsed with 0.4 μ Ci [³H]thymidine per well for 12 h. Then, the cells were harvested onto filters for scintillation counting. Results are representative of three experiments.

across the Ag-coated filters (Fig. 2B). Furthermore, cells exposed simultaneously to the Ag and chemokine signals migrated in response to SLC, MIP-3 β , and IP-10. However, the agonist MHC-peptide-coated filters stopped 3A9 T cells simultaneously exposed to gradients of SDF-1 α or MIP-1 α (data not shown). Thus, migration signals produced by receptors CXCR4 (SDF-1 α), CCR2 (MCP-1), CCR4 (MDC), and CCR5 (RANTES and MIP-1 α) are subordinate to TCR signals, while signals produced by CCR7 (SLC and MIP-3 β) and CXCR3 (IP-10) are dominant over TCR signals. Dominant chemokine gradients block or disrupt IS formation.

It was important to determine the form of chemokine presentation that leads to reversal of IS formation. When the chemokine SLC was included in the chamber with the T cells (reverse gradient), or in both the chambers (no gradient), there was no increase

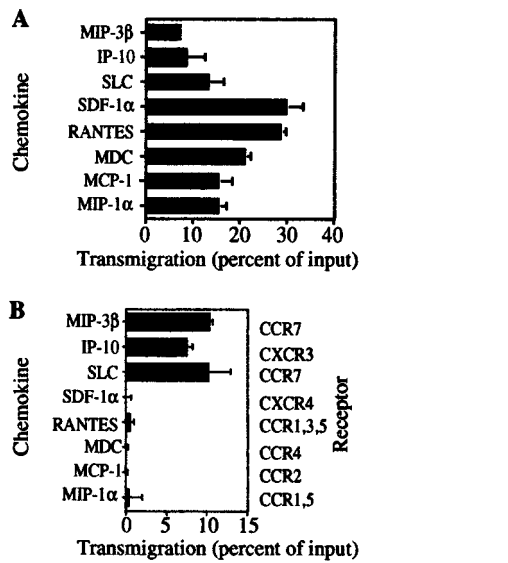


FIGURE 2. Effect of chemokine gradients on T cell migration through agonist MHC-peptide complex-coated filters. *A*, Efficient migration of T cells through fibronectin-coated inserts in response to optimal chemokine gradients. Optimal concentrations of chemokines were determined in preliminary experiments: MIP-3 β (0.5 μ g/ml), IP-10 (0.5 μ g/ml), SLC (0.5 μ g/ml), RANTES (100 ng/ml), MCP-1 (100 ng/ml), MDC (1 μ g/ml), and MIP-1 α (1 ng/ml). Transmigration through fibronectin-coated inserts in the absence of chemokine is 6%. Results are representative of three experiments. *B*, Assay for competition of MHC-peptide stop signal and chemokine signals. Inserts were coated with ICAM-1 and IA^k HEL_{48–62}. A total of 10⁵ 3A9 T cells were added to the upper chamber and allowed to interact with the protein-coated insert. After 1 h, the upper chambers were gently washed to remove unbound cells. The filters and remaining bound cells were then transferred to wells containing individual chemokines (same concentration as *A*). Spontaneous transmigration (1.9%) through the ICAM-1- and IA^k HEL_{48–62}-coated filters was subtracted from each condition as background migration. Results are representative of seven experiments.

in T cell migration through Ag-coated filters. Only when the SLC gradient originated from the lower chamber, opposite the T cells, would they migrate through the agonist MHC-peptide-coated filters (Fig. 3A). Similar results were obtained for migration in re-

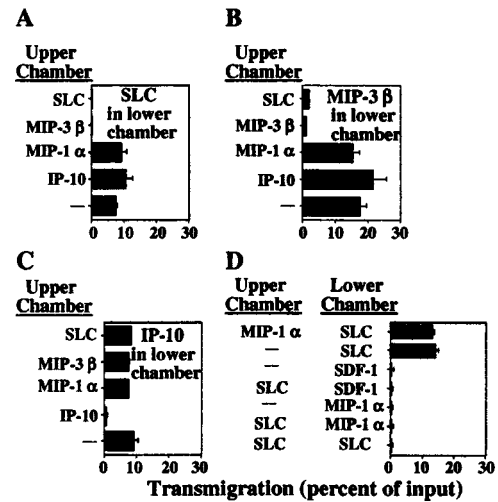


FIGURE 3. Requirement of chemokine gradient for reversal of Ag-induced T cell stop signal. 3A9 T cell blasts were allowed to interact with inserts coated with ICAM-1 and IA^k HEL_{48–62} as in Fig. 2*B*. After removal of unbound cells, 100 μ l of media alone or containing the indicated chemokine was added to each insert containing the remaining bound cells. The inserts were then immediately transferred into wells containing 600 μ l media plus (*A*) SLC, (*B*) MIP-3 β , (*C*) IP-10, or (*D*) the indicated chemokines (concentrations as in Fig. 2). After 1 h, transmigrated cells were counted. Results are representative of three experiments.

sponse to MIP-3 β (Fig. 3*B*) and IP-10 (Fig. 3*C*). As expected, reverse gradients of chemokines that interact with the same receptor as the chemokine in the forward gradient (SLC and MIP-3 β) restored IS formation (Fig. 3, *A* and *B*). Furthermore, treatment of T cells with a nongradient form of SLC did not allow the T cells to migrate across the agonist MHC-peptide-coated filter in response to a gradient of SDF-1 α or MIP-1 α (Fig. 3*D*). Thus, the dominant effect of SLC, MIP-3 β , or IP-10 over the Ag stop signal requires that the T cells migrate from low to high chemokine concentration.

T cells require sustained interaction with agonist MHC-peptide complexes to proliferate and initiate an immune response (15). In previous studies the separation of T cells from surfaces bearing agonist MHC-peptide complexes was forced mechanically (15).

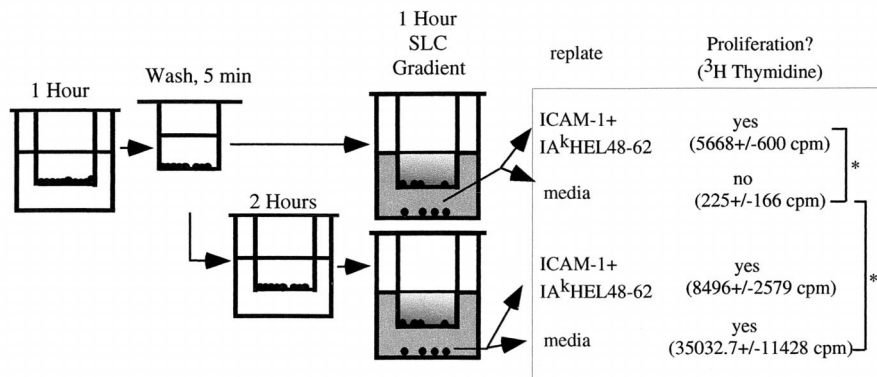


FIGURE 4. Dominant chemokine can prevent T cell proliferation in response to agonist MHC-peptide complexes. Cells were allowed to interact with ICAM-1- and IA^k HEL_{48–62}-coated inserts for 1 h. Unbound cells were removed. Then, inserts with remaining bound cells were immediately transferred to wells containing chemokine or were allowed to incubate for another 2 h without a chemokine gradient, after which they were placed in a chemokine gradient. After 1 h, transmigrated cells were counted and transferred to an uncoated or ICAM-1- and IA^k HEL_{48–62}-coated 96-well plate. After 48 h, cells were pulsed with 0.4 μ Ci [³H]thymidine for 12 h, harvested, and counted. *, *p* < 0.02. Results are representative of two experiments.

We determined whether the premature separation of T cells from the agonist MHC-peptide complex could be accomplished in a more natural manner with a dominant chemokine gradient. We found that an SLC gradient did inhibit the proliferation of T cells by pulling them through the agonist MHC-peptide-coated substrate before commitment of the T cell to a program of proliferation (Fig. 4). T cells that migrated through the agonist MHC-peptide-coated filter in response to SLC gradients within 1 h did not proliferate. These cells did proliferate when replated in wells with agonist MHC-peptide complexes and ICAM-1, demonstrating that they were competent to proliferate. Additionally, if cells were allowed to interact with the agonist MHC-peptide-coated filter for 3 h before exposure to the SLC gradient, then a significant level of proliferation was detected in the migrating cells when they were replated without Ag. The reduced proliferative activity of cells replated on ICAM-1- and MHC-peptide-coated wells in comparison to uncoated wells may be caused by Ag-induced cell death (15). Thus, 3–4 h of IS formation is sufficient to commit an effector T cell to a program of proliferation, and exposure to an SLC gradient does not reverse this programming. Dominant chemokines have the ability to induce T cell ignorance of Ag when they act early in the IS formation process. This process does not result in T cell anergy or nonresponsiveness, because the T cells would proliferate in response to a subsequent Ag challenge.

Our data indicate an order of command between chemokine receptors and the TCR where some chemokines are dominant (SLC, MIP-3 β , and IP-10) and others are subordinate (SDF-1 α , MIP-1 α , RANTES, MDC, and MCP-1) to agonist MHC-peptide complexes. The chemokine receptors are members of the serpentine receptor family linked to heterotrimeric G proteins, while the TCR signals through initiation of a tyrosine kinase cascade. The biochemical mechanism for the communication between serpentine and Ag receptors may involve protein kinase C isoforms that are implicated in down-regulation of CXCR4 responses (16, 17) and may play a role in IS formation (18). While arrest of B cell migration subsequent to Ag receptor engagement has not been demonstrated, SLC enhances chemotaxis of Ag-stimulated B cells, but SDF-1 α has no effect (19). This suggests that in B cells also, Ag receptor engagement inactivates CXCR4, but not CCR7.

The dominant chemokine receptor CCR7 has been shown to play an important role in T cell homeostasis in vivo. CCR7 mutant mice and mice lacking SLC expression show deficits in naive T cell and dendritic cell migration to peripheral lymph nodes (20–22). On activated human T cells, CCR7 has been implicated as a marker for a population of central memory T cells that recirculate through lymph nodes (23). In polarized mouse T cells, CCR7 and CXCR3 are expressed preferentially on the Th1 population and CCR7 expression targets these cells to the T cell areas of the spleen (24). We can speculate that dominant chemokines may orchestrate exit of Th1 cells from lymph nodes in the continued presence of Ag-positive dendritic cells and may help the Th1 cells navigate in Ag-rich tissue sites without becoming locked into the first encounter with an Ag-positive cell. The role of dominant chemokines and their receptors in controlling recognition of agonist MHC-peptide complexes in vivo needs to be addressed given the established importance of these receptors in T cell migration in vivo.

Our results suggest that pairs of dominant chemokines may play a role in coordinating inflammation and Ag responses. We have identified one chemokine pair with this potential, SLC and MIP-3 β , which both bind the dominant CCR7 receptor. SLC is expressed by lymphatic endothelial cells (12) and may guide T cells

to draining lymphatics to exit tissues (25). Thus, in the absence of inflammation, a T cell may follow an SLC gradient and exit tissues, even in the presence of cognate Ag. However, under inflammatory conditions, local production of MIP-3 β by activated macrophages and differentiated dendritic cells (26, 27) could functionally saturate CCR7, preventing T cells from sensing the constitutive SLC gradient and allowing IS formation in response to Ag. We have demonstrated that SLC gradients can induce T cell ignorance of Ag. Thus, chemokines not only guide T cells to the APC, but may dictate the actions of the T cell in response to agonist MHC-peptide complexes.

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