CXCL9 Causes Heterologous Desensitization of CXCL12-Mediated Memory T Lymphocyte Activation

Oliver Giegold, Nadine Ogrissek, Cornelia Richter, Matthias Schröder, Martina Herrero San Juan, Josef M. Pfeilschifter and Heinfried H. Radeke

J Immunol 2013; 190:3696-3705; Prepublished online 27 February 2013; doi: 10.4049/jimmunol.1101293
http://www.jimmunol.org/content/190/7/3696

References
This article cites 50 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/190/7/3696.full#ref-list-1

Why The JI? Submit online.
- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

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
CXCL9 Causes Heterologous Desensitization of CXCL12-Mediated Memory T Lymphocyte Activation

Oliver Giegold,* Nadine Ogrissek,* Cornelia Richter,† Matthias Schröder,‡
Martina Herrero San Juan,* Josef M. Pfeilschifter,* and Heinfried H. Radeke*

The chemokine receptors CXCR3 and CXCR4 are primarily involved in memory Th1 cell–driven autoimmune diseases. Although recent studies in chronic inflammatory disease showed therapeutic success using combined blockade, details of CXCR3 and CXCR4 synergism are not understood. In this investigation, we intended to unravel the interaction of these chemokine receptors in static and dynamic cell-migration assays at both the cellular and molecular levels. Effects of combined stimulation by murine CXCL9 and CXCL12, ligands of CXCR3 and CXCR4, respectively, were analyzed using a murine central memory Th1 cell clone. Costimulation with CXCL9 desensitized the chemotaxis of Th1 cells toward CXCL12 by up to 54%. This effect was found in murine EL-4 cells, as well as in primary human T cells. Furthermore, under dynamic flow conditions CXCL12-induced crawling and endothelial transmigration of Th1 cells was desensitized by CXCL9. Subsequent experiments uncovered several molecular mechanisms underlying the heterologous cross-regulation of CXCR4 signaling by the CXCR3 ligand. CXCR4 surface expression was reduced, whereas CXCL12-induced Akt phosphorylation and intracellular Ca²⁺ signals were modulated. Moreover, blockade of Rac by NSC23766 revealed differential effects on CXCL12 and CXCL9 chemotaxis and abolished the desensitizing effect of CXCL9. The desensitization of CXCR4 via CXCR3 in memory Th1 cells suggests that their in vivo homeostasis, widely regulated by CXCL12, was far less effective (7). In this study, the question was raised as to what extent CXCR3 and CXCR4 may cooperate to influence leukocyte trafficking. Indeed, in another in vitro system it was shown that combined stimulation of CXCR3 and CXCR4 in plasmacytoid dendritic cells resulted in synergistic effects on chemotaxis (8). Although these findings clearly point to a specific interaction of inflammatory CXCR3 and homeostatic CXCR4 ligands, no detailed knowledge about the molecular interaction of CXCR3 and CXCR4 exists. In our investigation, we focused on their cooperative function specifically in memory Th1 cells, which play a central role in promoting autoimmune disease and are well known to express both chemokine receptors (5, 9). Surprisingly, our experiments revealed that costimulation with CXCL9 led to heterologous desensitization of various CXCL12-mediated effects (crawling, transmigration, and chemotaxis). Until now, desensitization of the CXCR4/CXCL12 axis by stimulation of other chemokine receptors has been described for CCR5 but not for CXCR3 (10–12). In essence, our data suggest that the coordinated interaction of CXCL9 and the homing chemokine CXCL12 enhance egress and inflammatory activity of memory Th1 cells during chronic inflammation.

Materials and Methods

Cells

Experiments were performed with an OVA-specific central memory Th1 cell clone (IF12) generated following s.c. CFA/OVA immunization of BALB/c mice, extraction from regional lymph nodes, and subsequent cloning as described by Radeke et al. (13). This Th1 clone has been characterized extensively by FACS analysis and Ag-presentation assays...
with regard to its OVA-specific cytokine profile, and it was proven to enhance in vivo pathology of glomerulonephritis in an Ag-specific fashion (13, 14). Th1 cells were cultured in RPMI 1640 supplemented with 10% FCS, 100 μM nonessential amino acids, and 5 ng/ml human IL-2. A murine brain endothelial cell line, bEnd3 (kindly provided by D. Vestweber, Max-Planck Institute, Muenster, Germany), was cultured in DMEM with 10% FCS. The murine lymphoma cell line EL-4 and human Jurkat T cells were cultured in RPMI 1640 supplemented with 10% FCS and 100 μM nonessential amino acids. Primary human CD4 memory T cells were isolated from peripheral blood buffy coats of three healthy donors (DRK-Blutspendedienst, Frankfurt am Main, Germany). In detail, PBMCs were separated from buffy coats by Ficoll-Paque PLUS (GE Healthcare, Fairfield, CT) density-gradient centrifugation. An EasySep human memory T cell enrichment Kit (STEMCELL Technologies, Grenoble, France) was used according to the manufacturer’s protocol. We obtained >95% CD4+ T cells (Becton Dickinson, Franklin Lakes, NJ) CD45RO+ (eBioscience, San Diego, CA) and >95% CD3+ T cells (Becton Dickinson), as measured by FACS. Human memory T cells were cultured for 2 d in RPMI 1640 supplemented with 10% FCS, 100 μM nonessential amino acids, and 30 U/ml human IL-2 before use in chemotaxis experiments.

Reagents

The following reagents were used in this study: recombinant murine or human CXCL9 and CXCL12 (PeproTech, Rocky Hill, NJ), recombinant Fc-Chimera of murine E-selectin and ICAM-1 (R&D Systems, Minneapolis, MN), human IL-2 (Strathmann, Hamburg, Germany), AMD3100 (Sigma-Aldrich, St. Louis, MO), NIBR2130 (kindly provided by Novartis), FURA 2-AM (Sigma-Aldrich), CFSE (eBioscience), and diginton (Sigma-Aldrich). Edfedosine, PP2, and Piceatannol were purchased from Tocris Bioscience (Bristol, U.K.). NCS23766, G60976, LY294002, Thapsigargin, and Ro 318220 were from Calbiochem/Merck Biosciences (Schwalbach, Germany).

Flow cytometry

Following stimulation as outlined below, T lymphocytes were washed twice, incubated (5 min/4°C) with Mouse Fc block (Becton Dickinson), and stained (30 min/4°C) with the following Abs (coupled to respective fluorochromes): human (h)CXCR3-FITC, hCXCR4-allophycocyanin, murine (m)CXCR3-allophycocyanin, mCXCR4-FITC, rat IgG2a-allophycocyanin, and rat IgG2b-FITC (R&D Systems). Cells were washed twice and fixed with fixing buffer (PBS with 1% paraformaldehyde [PFA], 1% FCS, and 0.1% sodium azide). For intracellular staining, cells were fixed and permeabilized with PFA/Saponin (4% PFA and 0.1% saponin in PBS). Further wash steps and staining were performed in the presence of 0.1% saponin in PBS, as described before. Data were acquired with a FACS-Calibur for Th1 cells (IF12) or with a FACScanto II for primary human T cells (Becton Dickinson) and analyzed using FlowJo software (TreeStar, Ashland, OR).

Chemotaxis assays

Cell-migration assays were performed in 96-well Transwell plates (Corning, Lowell, MA) with 3.0-μm-pore polycarbonate filters coated with bovine fibronectin (Sigma-Aldrich), essentially as described previously (15, 16). Pretreated or nontreated T cells were diluted in RPMI 1640 medium with 0.5% BSA. First, the wells of the lower plate were filled with 235 μl solution (with or without reagents). Next, the insert filter plate was placed on top of the lower wells, and 75 μl T cell suspension (2.0 × 105 cells with or without reagents) was pipetted into the insert (≈ upper well). After 2 h of incubation (5% CO2/37°C), the transmigrated cells in the lower wells were counted. To this end, cells were transferred into a V-bottom 96-well plate and centrifuged. Pellets were washed in PBS and resuspended in 175 μl fixing buffer (PBS with 1% PFA, 1% FCS, and 0.1% sodium azide) containing 10 × 103 CFSE-labeled Th1 cells as internal standard. To increase the precision of cell counting, cell numbers were automatically calculated by Fl Solutions (Hitachi), according to Grynkiewicz et al. (18). The shift in the basal Ca2+ level in measurements of 40 min stimulation with CXCL9, due to continuous efflux of FURA 2-AM into the medium, was corrected by including values of a control probe.

Statistics

Statistical significance for multiple comparisons was assessed using one-way ANOVA and the Tukey posttest or using two-way ANOVA with the Bonferroni posttest. Statistical significance was two-tailed and set at 5%. All error bars are single SD. The Prism software package (Graph Pad Prism 5.01 for Windows) was used for data collection and presentation.

Results

Effects of CXCL9 on CXCL12-induced chemotactic responses of Th1 cells

In a first series of experiments, we analyzed the chemotactic response of the memory Th1 cell clone (IF12) induced directly by CXCL9 and CXCL12. As expected, Th1 cells exhibited a strong chemotactic response toward gradients of CXCL9 (10 nM) and CXCL12 (10 nM) equivalent to 122 and 80 ng/ml, respectively (Fig. 1A). Moreover, an additive chemotactic response was determined for stimulation with both chemokines given simultaneously (Fig. 1B). CXCL9 might influence subsequent chemotaxis mediated by
CXCL9 affects the chemotactic response of Th1 cells toward CXCL12. (A) Th1 cells were exposed to gradients of CXCL9 (10 nM), CXCL12 (10 nM), or a combination of both (each 10 nM). Data are mean ± SD of n = 6 in triplicates. (B) Th1 cells were preincubated with CXCL9 (10 nM) and washed after 30 min or left untreated as control. Afterward, cells were exposed to gradients of CXCL9 (10 nM) or CXCL12 (10 nM). Data are mean ± SD of n = 12 in triplicates. ***p < 0.001, versus medium control or as indicated. ns, Not significant.

CXCL12 differentially. Therefore, Th1 cells were pretreated with CXCL9 (10 nM) for 30 min and washed twice after this incubation period. Subsequently, cells were allowed to migrate toward CXCL9 (10 nM) or CXCL12 (10 nM). Surprisingly, in addition to the expected homologous desensitization of CXCL9-mediated effects, we observed a heterologous desensitization of CXCL12 effects: CXCL9 treatment significantly reduced chemotaxis toward CXCL9 by 60.4% (p < 0.001) and CXCL12 by 28.0% (p < 0.001, Fig. 1B).

CXCL9/CXCR3 axis desensitized the chemotactic effects of CXCL12/CXCR4 but not vice versa

In the following experiments we tested whether CXCL12-mediated chemotaxis of Th1 cells is also modulated by a continuous presence of CXCL9 or vice versa. Hence, we loaded CXCL12 or CXCL9 (10 nM) into the lower wells of a chemotaxis chamber to establish a gradient, and we added CXCL9 or CXCL12 into the upper and lower wells, respectively, in equimolar concentrations (Fig. 2A–D). Interestingly, CXCL12 had no significant influence on CXCL9-mediated chemotaxis of Th1 cells; rather, it showed a tendency to sensitize chemotaxis toward CXCL9 (Fig. 2A). In contrast to this observation and in accordance with previous findings, chemotaxis toward CXCL12 was significantly reduced by the presence of CXCL9 for both concentrations used, up to 53.8% at 10 nM CXCL9 (p < 0.001, Fig. 2C). To determine the time dependency of the observed desensitization, Th1 cells were treated with CXCL9 (10 nM) for 2 or 1 h or immediately before the experiments started. CXCL9 was added to the upper and lower wells in equimolar concentrations. The resulting data revealed no difference in the extent of CXCL9’s desensitizing effect with respect to the duration of CXCL9 treatment (Fig. 2D). Analogously, stimulation with CXCL12 (10 nM) did not show any significant time-dependent effects (Fig. 2B). To determine the relevance of these results, we determined whether previous findings could be reproduced in murine (EL-4) and human (Jurkat) T cell lines. Indeed, by choosing the same setup for the chemotaxis assay with graded CXCL12 (40 nM) and costimulation with CXCL9 (40 nM), compared with noncostimulated cells EL-4 and Jurkat T cells exhibited a reduced directed migration toward CXCL12 by 55.5 and 42.8%, respectively (Fig. 2E, 2F). Finally, we tested primary human memory CD4⁺ T cells, isolated from PBMCs with the same experimental setting. After costimulation with CXCL9 (40 nM), human memory CD4⁺ T cells showed an impaired chemotaxis toward graded CXCL12 (40 nM) compared with noncostimulated cells. Although this reduced migration (by 22.7%) was not as strong as observed in the previous experiments with other cells, it was highly reproducible and significant (Fig. 2G). Therefore, we were interested in studying the number of CXCR3⁺ CXCR4⁺ T cells compared with CXCR3⁻ CXCR4⁺ T cells of human memory CD4⁺ T cells. We found that 26.6 ± 6.9% of all CXCR4⁺ T cells were CXCR3⁺, and 24.6 ± 6.2% of all measured T cells were CXCR3⁺ CXCR4⁺ T cells (mean ± SD, n = 3 donors, Fig. 2H). Of note, experiments performed with CXCL10 (10 nM) and CXCL11 (10 nM) instead of CXCL9 for costimulation of Th1 cells revealed that only CXCL11 was able to mediate a significant reduction (by 43.3 ± 12.7%) in CXCL12-driven chemotaxis (10 nM) (p < 0.01; mean ± SD, n = 5 independent experiments; data not shown).

CXCL9 treatment desensitized CXCL12-mediated activation of Th1 cells under flow conditions

To prove our concept that CXCL9 desensitizes the CXCL12/CXCR4 axis in an in vivo–like situation, we performed adhesion and migration assays under flow conditions. Therefore, flow chambers were coated with E-selectin and ICAM-1 Fc-chimeric proteins, which enable determination of cell interactions limited to these molecules. Additionally, CXCL12 (10 nM, 1 h preincubation) was immobilized onto the flow chamber slide. The pretreatment of Th1 cells with CXCL9 revealed no difference in the total number of interacting cells, but it resulted in a decrease in cellular activity and motility. Thus, an increase in the number of adherent cells is only dependent on the reduction in crawling and not on a induction of adhesion (Fig. 3A–C). The decreased ratio of crawling/adherent cells significantly clarifies the shift to less cellular activity and motility triggered by CXCL9 pretreatment (p < 0.05; Fig. 3D).

CXCL9 treatment desensitized CXCL12-induced crawling and endothelial transmigration of Th1 cells in flow chamber assays

To get detailed information about differential processes of Th1 cell extravasation (adhesion, crawling, and transmigration), we performed flow chamber experiments in slides with a confluent endothelial monolayer (b.End3). In these assays, exogenous rCXCL12 (100 nM) was allowed to bind to the endothelium for 30 min. In parallel, the Th1 memory clones were preincubated with CXCL9 (10 nM) for 20 min or were left untreated (Fig. 4). Experimental data were collected during the subsequent flow of Th1 cells over pretreated endothelial cells. High numbers of interacting Th1 cells were observed in all settings. Most of these cells showed enhanced motility and crawled on top of the endothelium (Fig. 4B, 4D, 4E). In the entire series of experiments we demonstrated an increased rate of crawling cells after endothelial cell pretreatment with CXCL12 (p < 0.05). Moreover, stimulation with CXCL12 significantly enhanced transendothelial migration (p < 0.05; Fig.
According to the results from the previous static chemotaxis assay, treatment of Th1 cells with CXCL9 (10 nM) resulted in a significant heterologous desensitization of CXCL12-induced effects. CXCL9 treatment provoked a reduction in crawling and transmigrated Th1 cells by 61.0 and 80.7%, respectively (*p*, 0.05; Fig. 4B). Furthermore, the ratio of crawling/adherent cells was clearly decreased by CXCL9 treatment (**p*, 0.01; Fig. 4E). In conclusion, these data revealed diminished cellular activity and motility after CXCL9 preincubation compared with cells stimulated by CXCL12 alone.

Cross-regulation of CXCR4 receptor by the CXCR3 agonist CXCL9

The results from functional assays raised the question of whether CXCL9 treatment modulates the cell surface expression of CXCR4. Thus, Th1 cells were stimulated either with CXCL9 (10 nM) or CXCL12 (10 nM) for 90 min and analyzed for surface expression of CXCR3 and CXCR4 by flow cytometry at various time points (Fig. 5A, 5B). The vast majority of Th1 cells showed a clear CXCR3 surface expression. Internalization of CXCR3 was only detectable by stimulation with its specific ligand CXCL9 and not with CXCL12 (Fig. 5A). Surface CXCR4 expression of Th1 clone cells was low and only detectable in up to 10% of all cells. However, a tendency toward CXCR4 receptor internalization mediated by both CXCL9 and CXCL12 was detectable (Fig. 5B). Because of the discrepancy between low surface expression of CXCR4 and the pronounced chemotactic response of Th1 cells to CXCL12, we reasoned that CXCR4 receptors might be retained intracellularly (19). Therefore, we stained Th1 cells for intracellular CXCR4 and CXCR3. For CXCR3 expression, no evidence for additional intracellular stores could be found (Fig. 5C, 5D). However, most of the Th1 cells were now CXCR4+ (Fig. 5E, 5F), which confirms the reports of other investigators regarding intracellular storage of CXCL12 receptors (19, 20). In one of our previous studies (14), we characterized the IF12 Th1 cell clone as having a central memory Th1 phenotype. This was demonstrated...
clearly by an mRNA expression profile that included the proinflammatory chemokine receptors CCR2, CCR5, and CXCR3, as well as the homing chemokine receptors CCR7 and CXCR4 (9).

CXCL9 modulated CXCL12-induced Akt phosphorylation and [Ca\textsuperscript{2+}] mobilization

After analyzing the effects of CXCL9 on CXCR4 expression, we investigated signaling molecules downstream of CXCR4, which are known to be affected by a heterologous desensitization of CXCR4 (10–12). By Western blotting, we determined phosphorylation of Akt, a prominent mediator of the CXCR4-signaling cascade. In several studies, Akt was affected by CCR5-dependent desensitization of CXCR4 signaling (11, 12). Akt is a major downstream effector of PI3K, and PI3K can mediate chemotaxis in T lymphocytes upon CXCL12 stimulation (21). Phosphorylation of Akt was analyzed after Th1 cell pretreatment with CXCL9 (10 nM) for 30 min or with control medium and additional stimulation with CXCL12 (10 nM) for 5, 10, or 15 min (Fig. 5G). Akt was phosphorylated after CXCL12 stimulation and peaked after 5 min. Interestingly, prestimulation with CXCL9 significantly reduced the CXCL12-mediated phosphorylation of Akt when stimulation with CXCL12 lasted 5 min. For samples with longer incubation times, sustained Akt phosphorylation was detected when cells were pretreated with CXCL9.

In further experiments, we measured [Ca\textsuperscript{2+}] mobilization as an important second messenger upon subsequent CXCL9 or CXCL12 stimulation and the influence of a preceding CXCL9 treatment (Fig. 5H). Both chemokines, when applied as a single stimulus, induced a rapid increase in [Ca\textsuperscript{2+}], within a few seconds. CXCL9 stimulation yielded a much higher peak in the average Ca\textsuperscript{2+} concentration (570 nM) compared with CXCL12 (220 nM). The pretreatment of Th1 cells with CXCL9 for 40 min caused a delay in the CXCL12-induced Ca\textsuperscript{2+} signal by \(\sim 70\) s but, remarkably, enhanced its magnitude up to 780 nM compared with untreated CXCL9 cells. In contrast, additional CXCL9-mediated Ca\textsuperscript{2+} signals were virtually abrogated.

Inhibition of Rac efficiently blocked CXCL9-induced reduction of CXCL12-mediated T cell chemotaxis

To characterize the signaling that mediates the functional desensitization of CXCL12-induced chemotaxis by CXCL9, we used

FIGURE 3. Pretreatment of Th1 cells with CXCL9 blocked CXCL12-mediated effects under dynamic flow conditions. (A–D) Th1 cells were preincubated with CXCL9 (10 nM) for 20 or 40 min and perfused with a laminar shear stress of 0.4 dyn/cm\(^2\) over E-selectin and ICAM-1 Fc-Chimera–coated slides. Additionally, CXCL12 (10 nM) was immobilized on the slides in indicated probes. Interactions of cells (adhesion and crawling) were counted after 4.5 min. Data are mean \(\pm\) SD of \(n = 5\). (D) To point out the change in migratory cell activity, the ratio of [number of crawling cells]/[number of adherent cells] was calculated. Data are mean of ratios \(\pm\) SD. *\(p < 0.05\), Mann–Whitney U test.

FIGURE 4. CXCL9 reduced CXCL12-mediated crawling and endothelial transmigration of Th1 cells. (A–D) Th1 cells were either prestimulated with CXCL9 (10 nM) for 20 min or left untreated and then were perfused with a laminar shear stress of 0.5 dyn/cm\(^2\) over slides with endothelial monolayers (b.End3). Endothelium was either treated with CXCL12 (100 nM) or left untreated. Interactions of cells (adhesion, crawling, and transmigration) were counted after 15 min flow. Data are mean \(\pm\) SD of \(n = 5\) in triplicates, one-way ANOVA, and Tukey posttest. (E) To elucidate the change in migratory cell activity, the ratio of [number of crawling cells]/[number of adherent cells] was calculated. Data are mean of ratios \(\pm\) SD. *\(p < 0.05\), **\(p < 0.01\), versus medium control or as indicated, Mann–Whitney U test.
inhibitors of relevant signaling molecules potentially activated by the CXCR3 receptor (Fig. 6A–I). The chemotaxis of Th1 cells toward graded CXCL9 and CXCL12 was blocked by the inhibitors of ZAP70 (Piceatannol; Fig. 6B), Lck (PP2; Fig. 6C), PI3K (LY294002; Fig. 6E), and protein kinase C (PKC; α and β isoforms; Gö6976; Fig. 6F). The blockade upon costimulation with inhibitors of PKCs (all isozymes; Ro 31-8220; data not shown) and Rac (NNSC23766; Fig. 6H) only reduced CXCL12-mediated chemotaxis upon NSC23766 treatment by 58.8% (p < 0.01). Rac belongs to the Rho GTPases and is mainly involved in regulating actin polymerization at the leading edge during directed cell migration. Surprisingly, costimulation with NSC23766 increased CXCL9 directed migration by 76.0% (p < 0.001; Fig. 6H). The inhibition of phospholipase C (Edelfosine; data not shown) and sarco/endoplasmic reticulum Ca²⁺-ATPase (Thapsigargin; Fig. 6D) had no significant impact on chemokine-mediated chemotaxis. Focusing on the inhibitory effect of CXCL9 on CXCL12-mediated chemotaxis, we found that Piceatannol and PP2 caused only a marginal reduction (Fig. 6A). Thapsigargin, which is known to increase the [Ca²⁺]i by permanent depletion of cellular Ca²⁺ stores in the endoplasmic reticulum, tended to intensify the blocking effect of CXCL9 (Fig. 6A). NIBR2130, a highly specific antagonist for CXCR3 receptors, was used as positive control, and it efficiently inhibited the desensitizing effects of CXCL9 mediated via the CXCR3 receptor (Fig. 6A, 6G). Interestingly, during a sequential treatment of Th1 cells, first with NSC23766 and then with CXCL9, NSC23766 significantly abrogated the CXCL9-dependent inhibition of CXCL12-driven chemotaxis (Fig. 6A, 6I). Together with the finding that costimulation with NSC23766 differentially affected CXCL9 and CXCL12-dependent chemotaxis, the data suggest that Rac most likely is involved in this heterologous interaction between CXCR3 and CXCR4 (22, 23).

Discussion

The results of our study revealed a desensitization of major CXCL12-induced effects in central memory Th1 cells by sequential or simultaneous treatment with CXCL9. These interactions of the CXCL9- and CXCL12-signaling apparatus affected gradient-dependent chemotaxis, as well as crawling and transmigration in a dynamic in vivo–like flow situation.

Under static conditions, two general forms of cellular responses initiated by chemotacticants were distinguished: chemokinesis, the reaction by which the multidirectional locomotion of cells was determined by uniform, nongraded concentrations of chemotacticants, and chemotaxis, the reaction by which a directional locomotion was performed along a chemotactic gradient (24). Our results revealed that stimulation with CXCL9 desensitized chemotaxis of the Th1 cells toward subsequent CXCL12. This heterologous effect of CXCR3 ligands was observed irrespective of sequential or simultaneous treatment with CXCL9 (Figs. 1B, 2C, 2D). Moreover, the effect was reproducible in murine EL-4 and human Jurkat T cell lines, as well as in primary human memory CD4⁺ T cells, pointing out the effect as physiologically relevant in two different species (Fig. 2E–G). Other studies also reported cross-desensitization of the CXCL12/CXCR4-mediated chemotaxis of T lymphocytes by the chemokine receptor CCR5 (11), the α-opioid receptor (25), and the TCR complex (26). Consistent with our data, the results of these investigations showed a partial
desensitization of CXCL12-induced chemotaxis by ∼50%. Of note, CXCL9 (40 nM) enhanced chemokinesis slightly (Fig. 2C). These observations led us to suggest that CXCL9 might disturb Th1 cell orientation or directional movement within a CXCL12 gradient. Results of our subsequent experiments with a combination of rectified gradients of CXCL9 and CXCL12 supported this hypothesis, yielding an additive effect compared with stimulation with a single chemokine (Fig. 1A). Under these conditions, T lymphocytes were geared to the gradient of CXCL9, and the chemokinetic effect of CXCL12, which does not seem to be influenced by CXCL9, contributed an additive effect to chemotaxis. In an analogous setup, synergistic effects were observed for human CD3+ CD45RA- cells from peripheral blood (8). A recent publication described that CXCL9 and CXCL12 can form hetero-complexes, which showed an enhanced CXCR4-mediated chemotaxis in CD8+ T cells compared with CXCL12 alone (27). In our experiments, the prestimulation with CXCL9 was immediately followed by washing of the cells, indicating that the desensitizing mechanism is independent of a chemokine hetero-complex formation (Fig. 1B).

To further elucidate the functional aspects of this desensitization of CXCL12-mediated effects in detail, we examined the migration of Th1 cells in dynamic flow assays. Th1 cells are exposed to shear stress, which can change the cell response to chemokines and the interactions of chemokines (28). Flow chamber assays are useful in vitro models to study in vivo–like flow-dependent endothelial interactions of T lymphocytes within the extravasation cascade (29). During an initial accumulation phase, cells become firmly adherent to the surface by activation of \( \alpha_b \)-integrins (e.g., very late Ag-4 or lymphocyte function-associated Ag 1 [LFA-1]). Chemokines mainly induce the change from a lower to a higher active conformation of \( \alpha_b \)-integrins (30). Further activation of cells by chemokines results in crawling, migration over respective intravascular surfaces, and transmigration through the endothelial cell layer (31). According to our results and those of Engelhardt (32), the interactions between T cells and the brain endothelial cell line b.End3 are primarily mediated via VCAM-1 and very late Ag-4 or ICAM-1 and LFA-1, whereas rolling is virtually absent. Interestingly, we observed that the Th1 cell clone IF12 has the ability to interact directly with addressins (e.g., ICAM-1 or VCAM-1) and independently of chemokine stimulation, as described for peripheral blood T cells of RA patients, which express LFA-1 at high levels already in an active conformation (33). Therefore, the phase of adhesion was mostly transient in our experiments, even on surfaces coated purely with E-selectin and ICAM-1 (Fig. 3). We detected increased numbers of Th1 cells that exhibited crawling and transmigration behavior when endothelial cells were stimulated with CXCL12, as described by other investigators (Fig. 4).
In our approach, CXCL12 actively supported transendothelial migration, because the rate of transmigration increased several-fold compared with that of crawling cells. Notably, and most likely relevant for chronic inflammation in vivo, along these steps of extravasation, CXCL9 as a proinflammatory chemokine impedes cell polarization and motility induced by the homeostatic chemokine CXCL12 (Figs. 3, 4). As a consequence, fewer cells interacted biophysically with the endothelium. Therefore, the increased number of adherent cells is mainly due to a reduced crawling and transmigration rate. In conclusion, CXCL9 desensitizes CXCL12-induced migration in static assays, as well as in experiments under shear stress conditions.

Unraveling the cellular mechanisms that could drive this desensitization of CXCL12-mediated effects, we investigated changes in the CXCR4 surface expression after CXCL9 stimulation. Indeed, we measured a cross-regulation of CXCR4 receptor expression by flow cytometry. The ∼40% decrease in basal CXCR4 receptor surface expression did not reach the level of the ligand-specific internalization upon CXCL12 stimulation (Fig. 5B). Although up to 10% of all Th1 cells expressed CXCR4 on the plasma membrane, the majority of cells stored CXCR4 intracellularly (Fig. 5E, 5F). The observed reduction in CXCR4 surface expression upon CXCL9 stimulation may also be the result of a modulation in transporting CXCR4 from intercellular vesicles to plasma membrane and was not necessarily a result of receptor desensitization and internalization. Nevertheless, the observed magnitude of CXCR4 downregulation is similar to comparable investigations reporting CXCR4 cross-desensitization by stimulation with ligands for CCR5, the opioid, or the TCR (25, 26, 34). To test whether CXCL12-dependent activation of prominent signaling molecules is modulated by CXCL9, we measured phosphorylation of Akt (Ser473), which was shown to be affected by a heterologous desensitization of CXCR4 upon CCR5 receptor stimulation (10, 11). Interestingly, when cells were pretreated with CXCL9, phosphorylation of Akt was reduced after a 5-min stimulation with CXCL12 (Fig. 5G). In contrast to nontreated cells, this activation signal was sustained and declined much more slowly. Obviously, CXCL9 did not just desensitize CXCR4 signaling; instead, it acted as a modulator of the phosphorylation signal of Akt.

Classically, stimulation of chemokine receptors like CXCR3 and CXCR4 causes a Gs-dependent increase in [Ca2+]i, acting as an important second messenger. In our experiments, pretreatment of Th1 cells with CXCL9 modulated CXCL12-induced [Ca2+]i mobilization in two ways. First, the usually prompt cytosolic influx of Ca2+ was delayed by >1 min (Fig. 5H). This delay in the Ca2+ signal could explain the reduced interactions between Th1 and endothelial cells in flow chamber assays, because it was reported that CXCL12 can activate the adhesion molecule LFA-1 via the Ca2+ and diacylglycerol-regulated guanine nucleotide exchange factor I (35). Second, the maximal measured [Ca2+]i increased by an average of 3.5-fold, up to 818 nM. Several studies reported correlations between a high intracellular calcium concentration and reduced motility of T lymphocytes (23, 36). Hence, it was hypothesized that a high [Ca2+]i mobilization could act as a stop signal in T cell migration. Accordingly, modulation of the CXCL12-induced calcium signal by CXCL9 could explain, at least in part, the functional desensitization of CXCR4 signaling with regard to distinct important steps in T lymphocyte extravasation. Increasing [Ca2+]i by stimulation with Thapsigargin tended to enhance the desensitizing effect of CXCL9 on CXCL12-induced chemotaxis (Fig. 6A, 6D).

The Rho GTPase Rac, which is regulated by CXCR4 signaling, is an essential key regulator in coordinated directional migration (37, 38). Rac acts as a switch between several signaling pathways and primarily regulates lamellipodia formation by actin polymerization. T lymphocytes express the two isoforms: Rac1 and Rac2. Although these isoforms can mediate differential effects, they still reveal a strong functional redundancy (39, 40). Moreover, CXCL12-induced transendothelial migration of T lymphocytes can be partially blocked by inhibition or depletion of Rac (41). In contrast, the effects of inhibition or depletion of Rac on CXCR3-dependent chemotaxis are unknown. Inhibition of eotaxin-induced responses, acting via the CCR3 receptor and Rac2, was reported only for the CXCR3 ligand CXCL9. No CCR3 expression, typically found on Th2 cells (42), was detectable at the mRNA level for the Th1 cell clone IF12 (14). Additionally, the reduction in CXCL12-induced chemotaxis, observed upon stimulation with CXCL9, was completely blocked by the specific CXCR3 antagonist NIBR2130, which indicates that CXCR3 mediates this effect. Notably, in Transwell assays we measured differential effects on CXCL9- and CXCL12-induced chemotaxis by blocking Rac with a specific inhibitor (NSC23766). Chemotaxis toward CXCL12 was reduced, in accordance with the above-mentioned studies. However, after blocking Rac we measured increased numbers of cells migrating toward CXCL9. Furthermore, sequential treatment of Th1 cells with NSC23766 and CXCL9, compared with a single treatment, revealed no additive effects on desensitization of CXCL12-induced chemotaxis. These findings suggest that chemotaxis of CXCL12 in Th1 cells is mediated primarily by guanine nucleotide exchange factors (GEFs) that interact with the binding site of the T cell lymphoma invasion and metastasis-inducing protein 1, because NSC23766 specifically blocks this binding site on the Rac molecule. Other GEF binding sites are not affected by NSC23766, and it is obvious that CXCL9 regulates the activity of Rac via other GEFs (e.g., Vav-1). Most excitingly, the experiments done using NSC23766 pretreatment of Th1 cell abolished the desensitizing effect of CXCL9. This suggests that the downstream signaling of the CXCR4 receptor competed with the dominant signals of the CXCR3 receptor for regulation of Rac or that stimulation with CXCL9 led to a blockade of a signaling pathway of CXCR4 that activates Rac via the T cell lymphoma invasion and metastasis-inducing protein 1 binding site. A potential interaction between CXCR3 and CXCR4 might occur within the signal transduction pathway via the TCR complex and could modulate the activity or the localization of Rac. Interestingly, Rac1 is activated by stimulation with CXCL12 via cross-regulation of the TCR with CXCR4, whereas ZAP70 is phosphorylated; this was shown to be essential for directional T cell migration in a CXCL12 gradient (43, 44). Similarly, Dar and Knechtle (45) described cross-regulation between the TCR and CXCR3. They showed that CXCR3-dependent chemotaxis is regulated via the TCR and ZAP70. Consequently, the costimulation with CXCL9 particularly could interfere with the signal transduction of CXCR4 via the TCR. In contrast, our experiments revealed that the blockade of ZAP70 and Lck with Piceatannol or PP2, respectively, only tended to block the desensitization of CXCL12-induced chemotaxis upon CXCL9 treatment. Additionally, the long-lasting CXCR4-modulating effects of CXCL9 could be mediated by a Gαq- or ZAP70-dependent activation of PKCs. Orsini et al. (46) demonstrated the effective phosphorylation of CXCR4 by activation of PKCs with PMA, resulting in receptor internalization. The role of PKCs, particularly in the case of heterologous desensitization of CXCR4 (e.g., after TCR stimulation) was verified (26, 47). Chemotaxis experiments performed with two PKC inhibitors completely failed to reduce the CXCL9-induced desensitization (Fig. 6A). Altogether, our data suggest that CXCL9 partially inhibits CXCL12-induced migration by modulating CXCR4 receptor sig-
Disclosures
The authors have no financial conflicts of interest.

References
Lymphocyte crawling and transendothelial migration require chemokine triggering of high-affinity LFA-1 integrin. *Immunity* 30: 384–396.


