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Syk Is Required for Monocyte/Macrophage Chemotaxis to CX3CL1 (Fractalkine)\textsuperscript{1}

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CX3CL1 (fractalkine), the only member of the \(\delta\)/CX3C subclass of chemokines, is a known chemotactic factor for monocytes/macrophages as well as NK cells and T lymphocytes. In several pathologies, excessive production of CX3CL1 at specific sites leads primarily to monocyte/macrophage recruitment, which causes tissue and vascular damage. Despite their clinical relevance, the mechanisms underlying monocyte/macrophage chemotaxis to CX3CL1 remain poorly documented. The present report addresses this issue and identifies cell signaling crucial for this process. Using the murine monocyte/macrophage RAW cell line, we show that CX3CL1 treatment elicits a rapid and transient increase in F-actin and the formation of F-actin-enriched cell protrusions. CX3CL1 also triggers tyrosine phosphorylation of proteins localized in those protrusions. The protein tyrosine kinase Syk is activated upon CX3CL1 treatment, and reduction of Syk expression using RNA-mediated interference results in a specific and massive impairment of RAW cell migration to CX3CL1. Similar results are obtained using the Syk inhibitor, piceatannol. Cells with reduced Syk expression also exhibit a major defect in CX3CL1-induced cytoskeletal remodeling. These data suggest that in monocytes/macrophages, Syk is essential for proper reorganization of the actin cytoskeleton in response to CX3CL1 and is therefore required for cell chemotaxis to CX3CL1. \textit{The Journal of Immunology}, 2005, 175: 3737–3745.

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using RNA-mediated interference to reduce endogenous Syk levels, we show that Syk is required for proper formation of membrane protrusions and cell chemotaxis in response to CX3CL1. Our data demonstrate a new function of Syk downstream of a chemokine receptor besides its well-documented roles in signal transduction from immunoreceptors and integrins in hemopoietic cells.

Materials and Methods

Cells, Abs, and reagents

RAW/LR5 cells (RAW), derived from the murine monocyte/macrophage RAW 264.7 cell line, have been described previously (18) and were cultured in RPMI 1640 medium (Mediatech) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich) and antibiotics (100 IU/ml penicillin/100 μg/ml streptomycin). Murine bone marrow-derived macrophages were isolated and prepared as previously described (19) and were cultured in α-MEM supplemented with 15% FBS, 360 ng/ml recombinant human CSF-1 (Chiron), and antibiotics. All cells were maintained at 37°C in a 5% CO2 atmosphere. Recombinant mouse CX3CL1 (aa 25–105) and mouse CSF-1 (Chiron) were purchased from R&D Systems. Rabbit anti-CX3CR1 Ab (TP501) was obtained from Torrey Pines Biologs; mouse anti-phosphotyrosine (PY99), rabbit anti-Syk (LR and N19), rabbit anti-ERK1/2 (C14), and mouse anti-JNK (F3) Abs were purchased from Santa Cruz Biotechnology; rabbit anti-phospho-Syk (Y519/520 in mouse, Y525/526 in human), mouse anti-phospho-ERK1/2 Abs (T202/Y204), rabbit anti-phospho-p38MAPK (T180/Y182), rabbit anti-phospho-p38 MAPK, and mouse anti-phospho-JNK (T183/Y185) were obtained from Cell Signaling Technology; anti-β-actin (AC-15) Ab was purchased from Sigma-Aldrich. Alexa Fluor 568-phallolidin and all secondary Abs conjugated to Alexa Fluor 488 or 568 used for immunostaining were obtained from Molecular Probes. All secondary Abs conjugated to HRP (used for Western blotting) were purchased from Jackson ImmunoResearch Laboratories. Pertussis toxin from Bordetella pertussis was obtained from Sigma-Aldrich. The Syk inhibitor, piceatannol, and the MEK inhibitor, PD98059, were obtained from EMD Biosciences.

Immunofluorescence microscopy

RAW cells or bone marrow-derived macrophages were plated on 12-mm glass cover slips and serum-starved for at least 3 h before stimulation. For stimulation, cells were treated with BWD buffer (20 mM HEPES, 125 mM NaCl, 5 mM KCl, 5 mM dextrose, 10 mM NaHCO3, 1 mM KH2PO4, 1 mM CaCl2, and 1 mM MgCl2; pH 7.4) in the presence or the absence of CX3CL1 at 37°C for the indicated times. Cells were then fixed for 7 min using 3.7% formaldehyde in BWD and were permeabilized for 5 min using 0.2% Triton X-100 in BWD before blocking and incubation with Abs and/or phallolidin. Coverslips were mounted on slides using a mounting medium containing 100% glycerol. For extracellular staining of CX3CR1, live cells were preincubated on ice in BWD containing 1% BSA and a 1/100 dilution of mouse FcBlock (2.4G2 Ab; BD Pharmingen) before adding the primary Ab, followed by the secondary Ab solution, both in cold 1% BSA/BWD. Cells were kept on ice for <1 h, then fixed and mounted as described above. Images shown in Figs. 1, 2, and 8 were taken using the ×60/1.4 oil/1.40 immersion objective of an Olympus IX71 microscope coupled to a Seniscam-x-cooled CCD camera. Images shown in Fig. 3 were taken using a ×60 immersion objective of a confocal laser-scanning microscope (model radiance 2000; Bio-Rad).

Chemotaxis assay

Cell chemotaxis was measured using a transmigration chamber assay with 8-μm pore size inserts (Falcon; BD Biosciences) according to the manufacturer’s instructions. Briefly, the inserts were placed into 24-well plates containing RPMI 1640 in the presence or the absence of CX3CL1. Serum-starved cells (n = 500,000) were then loaded onto the inserts and incubated at 37°C for 4 h. Cell migration was quantified by counting the number of cells that migrated through the insert (at least 10 different randomly selected fields in each well were counted under a phase contrast microscope) and was expressed as the percentage of migrated cells in the absence of any stimulant.

Cellular F-actin measurement

Measurement of total F-actin content was mainly performed as described previously (20). Briefly, 50,000 cells were plated into wells of a 96-well plate (in quadruplicate for each condition) and serum-starved for at least 3 h. Cells were then incubated with BWD in the presence or the absence of CX3CL1 for various times at room temperature, followed by prompt fixation and permeabilization as described in Immunofluorescence microscopy above. Fixed cells were incubated with saturating concentrations of rhodamine-phallolidin and YO-PRO-1 (both from Molecular Probes) to stain F-actin and nucleic acids, respectively. Fluorescence intensities of rhodamine (excitation wavelength, 545 nm; emission wavelength, 590 nm) and YO-PRO-1 (excitation wavelength, 485 nm; emission wavelength, 520 nm) were measured using a plate reader (Polarstar Optima), and the normalized F-actin cellular content (calculated as the ratio of rhodamine to YO-PRO-1 fluorescence) was expressed as the percent increase in response to CX3CL1 compared with the unstimulated condition.

Immunoprecipitation and Western blotting

Cells were lysed in ice-cold buffer A containing 25 mM Tris, 137 mM NaCl, 1% Triton X-100, 1% SDS, 2 mM EDTA, 1 mM orthovanadate, 1 mM benzamidine, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, pH 7.4. For immunoprecipitation, the SDS concentration of the total cell lysate was lowered to <0.2% by dilution in buffer A without SDS before incubation with Abs prebound to Protein A/G Plusagarose beads (Santa Cruz Biotechnology) overnight at 4°C. Beads were then pelleted, washed three times, resuspended in Laemmli buffer, and boiled for 5 min. Total cell lysates and/or immunoprecipitates were resolved by SDS-PAGE, and proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P). Millipor filters that were subsequently blocked using 3% BSA/1% OVA in TBS containing 0.1% Tween 20 before incubation with primary Abs overnight at 4°C. Membranes were then washed and incubated with secondary Abs conjugated to HRP. Signals were visualized using the SuperSignal West Pico chemiluminescent substrate (Pierce), and images were acquired using a Kodak Image Station 440.

Generation of cells with stable reduced Syk expression using RNA-mediated interference

Reduction of Syk expression was achieved through the retroviral infection of RAW cells with short hairpin RNAs directed against the Syk mRNA, using pSUPER.retro.puro (pSUPER) plasmids (Oligoengene). Two different 19-nt target sequences were selected in the mouse Syk cDNA coding sequence as follows: sh#1, 5′-GGCACACACTACAGTACC-3′ (177–203; SH2 domain); and sh#2, 5′-GGGAAATGTAGTTGCATCC-3′ (627–645, kinase domain); each one was used to design a 60-nt oligo, which was subcloned between the BglII and HindIII restriction sites of the pSUPER vector according to the manufacturer’s instructions. The resulting pSUPER/Sysk#1 and pSUPER/Sysk#2 plasmids were transfected into a 293T-based packaging cell line. The corresponding culture supernatants were then used to retrovirally infect RAW cells. Infected cells were selected on the basis of their resistance to puromycin (brought by the pSUPER plasmids), and their level of Syk protein expression was monitored by Western blotting. Puromycin-resistant heterogeneous cell populations as well as single cell-derived populations were obtained with significant (>70%) and stable (for a period of at least 3 wk) reduction of Syk. As a control, the same set of experiments was performed using a mock short hairpin RNA (shRNA) sequence corresponding to sh#1 in a scrambled order (5′-TCCACACACC TACACGGC-3′), referred to as scrambled later in the text.

Phagocytosis assay

The ability of cells to perform FcγR-mediated phagocytosis was assessed as described previously (18). Briefly, cells were incubated with a suspension of sheep erythrocytes opsonized with rabbit IgG (IgG-RBC) for 30 min at 37°C in BWD. Noninternalized IgG-RBCs were then removed by washes, followed by hypotonic lysis. At least 100 cells were observed by phase contrast microscopy, and the number of erythrocytes in each cell was counted and expressed as the average number of particles ingested per cell (phagocytic index).

Data analysis

All results were calculated as the mean ± SEM. Data were analyzed using Student’s t test, and differences between two means with a value of p < 0.05 were regarded as significant.

\footnote{Abbreviations used in this paper: pSUPER, pSUPER.retro.puro; scr, scrambled-treated; shRNA, short hairpin RNA; WASp, Wiskott-Aldrich syndrome protein; NI, noninfected.}
Results

RAW cells as a model to study CX3CL1 chemotaxis

To study monocyte/macrophage chemotaxis in response to CX3CL1 (fractalkine), we first selected a cell model that was appropriate for this purpose. RAW 264.7 cells have been extensively used as a bona fide replica for moderately differentiated macrophages and have been shown to respond to physiological stimuli regulating proliferation (21), production of NO (22, 23) and cytokines (22, 24), phagocytosis (18, 25, 26), or chemotaxis (27). To determine whether RAW cells could also represent a valid model to study CX3CL1-induced chemotaxis, we first verified that they expressed CX3CR1 at their surface by performing immunostaining on live cells at 4°C, a temperature known to prevent internalization (18). A bright staining was observed when cells were incubated with CX3CR1 Ab, but no significant staining was detected using corresponding matching isotype IgG (Fig. 1A). The ability of RAW cells to chemotax to soluble CX3CL1 was then assessed using a transmigration chamber assay as described in Materials and Methods. Increasing the dose of CX3CL1 in the lower compartment of the chamber resulted in a significant increase in cell migration starting at the dose of 1 ng/ml (Fig. 1B). Both 50 and 150 ng/ml CX3CL1 appeared to trigger a comparable maximal cell response. To determine whether CX3CL1 stimulated RAW cell chemotaxis (i.e., directional cell movement dependent on a concentration gradient) and/or chemokinesis (i.e., random cell motility stimulated in a gradient-independent manner), we added 50 ng/ml CX3CL1 only in the top compartment of the chamber. In this case, cell migration remained similar to the basal level (Fig. 1C), clearly indicating that a concentration gradient was required for efficient RAW cell migration in response to CX3CL1. Preincubation of RAW cells with either CX3CR1 neutralizing Ab (the same as used in Fig. 1A) or pertussis toxin both resulted in a massive inhibition of CX3CL1-induced cell migration, indicating a specific involvement of CX3CR1 as well as one or more pertussis toxin-sensitive G proteins in this process (Fig. 1, D and E). These data indicated that RAW cells expressed a functional receptor for CX3CL1 and exhibited genuine chemotaxis toward this molecule, in accordance with the behavior of monocytes/macrophages in vivo.

CX3CL1 induces reorganization of the actin cytoskeleton

The ability of cells to move implies a dynamic remodeling of the actin cytoskeleton that governs the extension of cell protrusions and the retraction of the cell body (reviewed in Ref. 28). To document the cell response to CX3CL1 in terms of morphology and actin dynamics, we stimulated RAW cells for various times with 50 ng/ml CX3CL1 before fixation and F-actin staining using phalloidin. We typically observed a robust formation of thin and undulating F-actin-enriched membrane protrusions (also termed ruffles) on the dorsal and lateral sides of the cells, which appeared to be maximal after 1-min stimulation (Fig. 2A). Quantitative measurement of the total F-actin cellular content showed a rapid increase detectable within 10 s after addition of CX3CL1 and peaking at 1 min to represent an ∼30% increase of F-actin compared with nonstimulated cells (Fig. 2B). The amount of F-actin then decreased to reach levels close to the basal after 3 min. Therefore, RAW cells massively reorganized their cytoskeleton upon CX3CL1 treatment, resulting in a rapid and transient increase in

FIGURE 1. RAW cells chemotax toward CX3CL1 (fractalkine). A, Live cells were incubated with rabbit anti-CX3CR1 Ab or control IgG at 4°C before fixation and incubation with anti-rabbit IgG Ab conjugated to Alexa Fluor 488. Immunofluorescence images are shown as well as the corresponding phase contrast images (see insets). Scale bar = 10 µm. B, Cell migration in response to increasing doses of CX3CL1 was measured as described in Materials and Methods. C, To distinguish between chemotaxis and chemokinesis, cell migration was assessed in response to 50 ng/ml CX3CL1 added in either the bottom chamber or the top chamber of the transmigration apparatus. D, The specificity of CX3CL1-induced chemotaxis was verified by preincubating the cells with 10 µg/ml CX3CR1 neutralizing Ab or control IgG for 1 h before subjecting the cells to the transmigration assay. E, Cells were preincubated for 5 h with or without 250 ng/ml pertussis toxin (PTX) before measuring cell migration as described above. n = 3, *p < 0.05 compared with the corresponding controls.
the total F-actin amount and the formation of dorso-lateral membrane ruffles, suggesting the acquisition of a motile phenotype.

**CX3CL1 stimulates discrete tyrosine phosphorylation**

After binding to G protein-coupled receptors, several chemokines have been shown to stimulate tyrosine kinase activity, which appears to be required for accurate signaling and cell chemotaxis (29). We therefore asked whether CX3CL1 could trigger tyrosine phosphorylation in monocytes/macrophages. Total lysates were prepared from RAW cells treated with 50 ng/ml CX3CL1 for various times and subjected to Western blotting using an Ab recognizing phosphotyrosine (PY) residues (Fig. 3A). Multiple bands corresponding to phosphotyrosine-containing proteins were clearly induced after 30 s and 1 min of CX3CL1 treatment before returning to levels comparable to unstimulated cells after 5 min (Fig. 3A). To determine the intracellular localization of these tyrosine-phosphorylated proteins, we performed immunofluorescence experiments using the phosphotyrosine-specific Ab in costaining experiments together with F-actin (Fig. 3B). Confocal microscopy indicated that tyrosine-phosphorylated proteins were significantly colocalized with the typical F-actin-rich protrusions induced in RAW cells upon CX3CL1 treatment, especially at the dorsal surface (see Fig. 3B). CX3CL1 stimulation of primary macrophages derived from progenitors isolated from mouse bone marrow led to a similar cell response with the formation of membrane ruffles enriched in both F-actin and tyrosine-phosphorylated proteins (Fig. 3C).

**Involvement of Syk downstream of CX3CR1**

Two lines of evidence prompted us to investigate the putative involvement of the Syk tyrosine kinase in the CX3CL1-induced signaling pathways. Firstly, Syk has been shown to be phosphorylated...
in the MonoMac6 monocyte cell line even though the functional relevance of this modification in the CX3CL1 signal transduction had not been explored (11), and secondly, CX3CL1 treatment of RAW cells resulted in the prominent increase in the phosphotyrosine content of immunoreactive species with an electrophoresis mobility slightly below 75 kDa, consistent with the molecular mass of Syk (see Fig. 3A). We first sought to establish whether Syk was activated downstream of CX3CR1 in RAW cells by performing immunoprecipitation experiments. Cells were incubated with or without 50 ng/ml CX3CL1 for 1 min before lysis under denaturing conditions and immunoprecipitation (IP) of Syk. Immunoprecipitants were then subjected to anti-phosphotyrosine Western blotting (WB PY). The amount of Syk immunoprecipitated in each condition was determined by probing the same samples with the Syk Ab used for the immunoprecipitation. CX3CL1 treatment of RAW cells resulted in an increase in the tyrosine phosphorylation level of Syk (Fig. 4A, upper panel). We also performed the reverse experiment where phosphotyrosine-containing proteins were immunoprecipitated (IP PY) from lysates of cells treated or not with CX3CL1. Immunoprecipitates were then probed for the presence of Syk specifically phosphorylated on tyrosine residues 519/520 (WB P-Syk). These residues lie within the Syk activation loop, and their phosphorylation has been shown to be required for Syk activity (30). CX3CL1 treatment resulted in an increased amount of activated Syk in RAW cells (Fig. 4A, lower panel). To determine whether Syk activity could play a role in cell chemotaxis toward CX3CL1, we used the Syk-selective inhibitor piceatannol. RAW cells were pretreated with 50 μM piceatannol or with DMSO as a control (vehicle) for 1 h before being subjected to a transmigration assay in response to CX3CL1 in the presence or the absence of the inhibitor. Piceatannol treatment of cells led to the abolishment of their ability to chemotax to CX3CL1 (Fig. 4B). No difference in cell viability was observed between DMSO- and piceatannol-pre-treated cells, as assessed by the trypan blue exclusion method. CX3CL1 treatment of RAW cells increased the level of tyrosine phosphorylation of Syk, in particular on key residues required for its activity and pharmacological inhibition of Syk activity dramatically inhibited cell chemotaxis in response to CX3CL1.

Reduction of Syk expression in RAW cells results in a severely impaired ability of cells to chemotax to CX3CL1

To demonstrate the specific involvement of Syk in CX3CL1-induced cell chemotaxis, we chose to inhibit Syk expression using an RNA-mediated interference approach. RAW cells were retroviral infected with pSUP plasmids containing either two different sequences coding for shRNA targeting the Syk transcript or a control scrambled sequence coding for a mock shRNA, as described in Materials and Methods. After puromycin selection, infected cells were screened for Syk expression by Western blotting. As shown in Fig. 5A, 70–80% of Syk protein levels were reduced in Syk shRNA-treated cells compared with scrambled-treated (scr) or noninfected (NI) cells, without affecting the expression of CX3CR1, β-actin, and piceatannol or with DMSO as a control (vehicle) for 1 h before being subjected to a transmigration assay in response to CX3CL1 in the presence or the absence of the inhibitor. Piceatannol treatment of cells led to the abolishment of their ability to chemotax to CX3CL1 (Fig. 4B). No difference in cell viability was observed between DMSO- and piceatannol-pre-treated cells, as assessed by the trypan blue exclusion method. CX3CL1 treatment of RAW cells increased the level of tyrosine phosphorylation of Syk, in particular on key residues required for its activity and pharmacological inhibition of Syk activity dramatically inhibited cell chemotaxis in response to CX3CL1.

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CX3CR1 and β-actin. No significant difference in the level of Syk reduction was observed using the two different target sequences, sh#1 and sh#2. Significant Syk protein reduction was detectable in puromycin-resistant heterogeneous cell populations (see Fig. 5A) as well as in single cell-derived populations generated by limit dilution cloning (not shown). The cell viability of Syk or mock shRNA-treated cells was assessed with the trypan blue exclusion method, and >95% of all cells were found to be viable. Unless otherwise noted, all subsequent experiments were conducted using at least two independent batches of shRNA-treated cells showing at least 70% Syk protein reduction. As a control, we also tested the functional repercussion of Syk reduction in RAW cells on FcγR-mediated phagocytosis, a process known to be Syk dependent (31). As expected, the ability to internalize IgG-coated erythrocytes was markedly reduced in Syk shRNA-treated cells compared with scr or NI cells (Fig. 5B). This observation is in agreement with a disrupted signaling downstream of the FcγR, as previously reported in Syk−/− macrophages (31).

We then assessed the ability of cells with reduced Syk expression to chemotax toward CX3CL1. Using the transmigration assay with 50 ng/ml CX3CL1 in the lower chamber, we compared the migration of cells with reduced Syk expression obtained from infection with two different shRNA (sh#1 and sh#2) to NI cells and scr cells (Fig. 6A). Cells with reduced Syk expression showed an almost total inhibition of chemotaxis toward CX3CL1 compared with control cells. Interestingly, the ability of Syk shRNA-treated cells to migrate toward CSF-1, a well-known monocyte/macrophage chemoattractant that binds the receptor tyrosine kinase CSF-1R (32), was not significantly altered compared with that of mock shRNA-treated cells (Fig. 6B).

We also compared RAW cells with normal and reduced Syk expression in terms of MAPK activation, because others have

![FIGURE 6.](http://www.jimmunol.org/)

**FIGURE 6.** Cells with reduced Syk expression show impaired migration toward CX3CL1. A, Syk shRNA-treated cell migration in response to CX3CL1 was determined using a Transwell assay as described previously and compared with NI and scr cells. n ≥ 4. * p < 0.05 compared with CX3CL1-induced migration in NI cells. B, The ability of Syk shRNA and scr cells to migrate in response to 20 ng/ml CSF-1 was also evaluated. n = 3.

![FIGURE 7.](http://www.jimmunol.org/)

**FIGURE 7.** CX3CL1 activation of ERK1/2 is not required for RAW cell chemotaxis. A, RAW cells were stimulated with CX3CL1 for the indicated times before Western blotting of the corresponding Triton-soluble whole cell lysates. The activation statuses of ERK1/2 (p42/44MAPK), p38MAPK, and JNK1 were assessed using phosphospecific Abs, and the corresponding total (phosphorylation-independent) levels of MAPK expression are shown below as proof of equal protein loading. B, CX3CL1-induced ERK1/2 activation levels were compared between scrambled and Syk shRNA-treated cells. Results shown were obtained with a clone exhibiting 80% Syk protein reduction. C, RAW cells were preincubated for 1 h with 30 μM PD98059 (MEK inhibitor) or with DMSO (vehicle) before being subjected to a transmigration assay. PD98059 efficacy was confirmed through its ability to abolish ERK1/2 phosphorylation (see inset). For all experiments, n = 3.
reported a piceatannol-sensitive activation of members of the MAPK family in chemokine-stimulated monocytes (11, 33). As shown in Fig. 7A, CX3CL1 treatment of RAW cells activated ERK1/2 (p42/44MAPK) within 3 min, and both proteins remained activated for at least 15 min. However, the phosphorylation levels of p38MAPK and JNK1 were not significantly increased in response to CX3CL1, even after a 3-h treatment (not shown). Consistent with the results reported by Cambien et al. (11) using piceatannol, CX3CL1-induced ERK1/2 activation was abolished in Syk shRNA-treated cells, suggesting that Syk might be upstream of ERK (Fig. 7B). Nevertheless, inhibition of the MEK/ERK pathway with PD98059 did not result in any reduction of the ability of RAW cells to chemotax toward CX3CL1 (Fig. 7C). These observations suggested that Syk might not be a key mediator of the ERK function required for RAW cell migration in response to CX3CL1. Overall, these data suggested a MAPK-independent requirement for Syk in RAW cell chemotaxis in response to CX3CL1, but not to CSF-1.

Syk is required for the CX3CL1-induced reorganization of the actin cytoskeleton

Finally, we analyzed the phenotype of RAW cells with reduced Syk expression with respect to F-actin rearrangement in response to CX3CL1. Strikingly, the increase in F-actin cellular content induced upon CX3CL1 treatment (as shown previously in Fig. 2B) was dramatically inhibited in Syk shRNA-treated cells compared with scr cells or NI cells (Fig. 8A). Accordingly, the ability of cells with reduced Syk expression to extend membrane protrusions in response to CX3CL1 was also severely compromised (Fig. 8B). Quantitative analysis of the extent of protrusions in response to CX3CL1 was performed as described previously (18) and is shown below the representative images. Instead of exhibiting typical F-actin-rich ruffles upon CX3CL1 treatment, Syk shRNA-treated cells appeared to be rounder, devoid of specific cytoskeletal structures, and occasionally displayed small F-actin aggregates that remained identical in the presence or the absence of CX3CL1. Overall, we concluded that reduction of Syk expression in RAW cells resulted in a major defect in their cytoskeletal reorganization in response to CX3CL1, which itself is presumably the cause of the inability of cells to properly chemotax to CX3CL1.

Discussion

The CX3CL1 (fractalkine)/CX3CR1 ligand/receptor pair has fostered increasing interest in the past few years due to its emerging involvement in various clinical diseases, such as rheumatoid arthritis (4–6), cardiovascular diseases (7–10), cardiac allograft rejection (34, 35), glomerulonephritis (36), or HIV infection (37, 38). Indeed, overexpression or ectopic expression of CX3CL1 in various pathological tissues has been shown to be associated with significant CX3CR1-expressing leukocyte infiltration, contributing to the progression of the disease (reviewed in Ref. 39). Additional pathologically relevant cell types susceptible to interact with CX3CL1 include human CMV, whose US28 broad-spectrum receptor has been shown to bind CX3CL1 with a high affinity (40), and human cancer cells, which express functional CX3CR1 (41). Together, these observations support the idea of a broad function of the CX3CL1/CX3CR1 system in pathogenesis through the recruitment of various cell types to various sources of CX3CL1. Cells of the monocyte/macrophage lineage represent the main leukocyte subset to accumulate at sites such as the thoracic aorta in atherosclerosis or the synovium in rheumatoid arthritis and have been shown to actively contribute to the progression of these diseases. Their ability to chemotax to CX3CL1 is likely to account for a major part of their recruitment. This report provides the first data documenting intracellular signaling involved in monocyte/macrophage chemotaxis toward CX3CL1.

In this study we have characterized the monocyte/macrophage response to CX3CL1 in terms of actin dynamics with high temporal resolution. Very few studies have examined this aspect, and quantitative measurement of F-actin cellular content in response to CX3CL1 has been performed only on dendritic cells (42) and T lymphocyte subsets (43). These cell types were found to increase their amount of F-actin within 30 s after addition of CX3CL1 even though, in both cases, the time scale used did not have sufficient resolution to determine whether the maximal response occurred at this time point. In addition, a significant cytoskeletal reorganization

FIGURE 8. CX3CL1-induced cytoskeletal reorganization is disrupted in cells with reduced Syk expression. A, The ability of cells to increase their F-actin content after a 1-min CX3CL1 stimulation was compared among NI, scr, and Syk shRNA-treated (sh) cells, as previously described. n = 4 independent experiments using heterogeneous cell populations. *, p < 0.05 compared with NI cells. B, Scrambled and Syk shRNA-treated cells were either untreated or treated with CX3CL1 for 1 min, and their ability to exhibit F-actin-rich cell protrusions (ruffles) was compared using F-actin staining. Representative images of three independent experiments are shown. Scale bar = 10 μm. The extent of CX3CL1-induced ruffles in individual cells was scored as described previously (18); protrusion indexes were calculated as the average of at least 50 cells in three different experiments and expressed as a percentage of scr cells. n = 3. *, p < 0.05 compared with scrambled.
has been observed in microglial cells, which started to round up and display a peripheral band of actin bundles after 10 min of CX3CL1 treatment (44). This suggests that the morphological and temporal nature of the actin response to CX3CL1 depends on the cell type.

Our results demonstrate that the protein tyrosine kinase Syk is activated in response to CX3CL1 and is required for the reorganization of the actin cytoskeleton (i.e., formation of membrane ruffles) and proper migration of RAW cells. Syk is primarily, but not only, expressed in hemopoietic cells and is known to be essential for the function of BCR, TCR, and FcR (reviewed in Ref. 45). Interestingly, signaling through ZAP-70, the second member of the Syk/ZAP-70 family of protein tyrosine kinases, has been shown to be essential to mediate stromal cell-derived factor-1α (CXCL12) chemotaxis in T cells (46, 47). A potential role for Syk itself downstream of chemokine receptors is more elusive even if its activation has been reported in response to MIP-1β/CCL4 in the pre-B lymphoma cell line L1.2 transfected with CCR5 and in primary T lymphocytes (48) as well as in hemopoietic progenitor cells after activation of CXCR3 (49). Phosphorylation of Syk has also been reported in MonoMac6 cells in response to CX3CL1 (11) and CCL2 (MCP-1) (33). However, the functional significance of these events in terms of cell chemotaxis was either apparently negligible (49) or had not been investigated (11, 33, 48). It should also be noted that the activation of Syk in hemopoietic progenitor cells only occurred after 30 min of stimulation, at which time the early events governing cell chemotaxis and motility have already taken place (49). Overall, the heterogeneity of cell models and chemokines used in these different studies render comparisons difficult, because the kinetics and functional role of Syk activation might be significantly different under these conditions.

In monocytes/macrophages, Syk has previously been shown to play a role in actin-dependent processes, such as FcγR-mediated phagocytosis (31) and integrin-mediated adhesion (50). In this study we show that CX3CL1 induces a rise in F-actin in RAW cells that is dramatically inhibited in cells with reduced Syk expression, suggesting a function for Syk in the control of actin dynamics. How Syk could control such a process is unknown, but, importantly, several studies indicate a link between Syk and the actin cytoskeleton. Syk has been reported to directly phosphorylate at least two actin-binding proteins: the cortactin-related hemopoietic lineage cell-specific protein 1, which binds to the Arp2/3 complex and promotes actin assembly and branching (51, 52), and SH3P7, a protein of unknown function, isolated from the phosphotyrosine immunoreactive fraction induced after lymphocyte activation (53). Notably, Syk-mediated phosphorylation of hemopoietic lineage cell-specific protein 1 was required for its translocation to lipid raft microdomain together with Arp2/3 and the actin regulator Wiskott-Aldrich syndrome protein (WASP) after BCR cross-linking (54). In addition, Syk has been shown to bind WASp through the adapter CrkL in platelets (55), and an interaction between Syk and the WASp-interacting protein has recently been reported upon FcεRII activation in mast cells (56). A similar protein association has also been described in T cells, where the Syk-related protein ZAP-70 was shown to be part of a complex including WASp, WASp-interacting protein, and CrkL (57). Together, these data suggest that in hemopoietic cells, one or more protein complexes involving Syk might be relevant for the control of actin assembly in response to different stimuli. Whether a similar signaling complex is present and/or can be induced after CX3CR1 activation in monocytes/macrophages remains to be determined.

Importantly, the cytoskeletal remodeling defect observed in cells with reduced Syk expression is clearly associated with their inability to efficiently chemotax toward CX3CL1. Using a Transwell assay, the difference in the number of migrated cells between Syk shRNA-treated cells and control cells was significant after a 4-h stimulation (Fig. 6) and was still comparable after a 16-h stimulation (not shown), indicating that cells with reduced Syk expression were not simply delayed in their ability to respond to CX3CL1. In addition, cell migration to CSF-1, a chemotactic factor acting through a tyrosine kinase cell surface receptor, appeared to be normal in cells with reduced Syk expression. These data together with the observation that Syk does not appear to be essential for neutrophil migration in response to the bacterial peptide fMLP or MIP-2 (58) suggest that the role of Syk in cell signaling is receptor selective and/or cell specific. Our results support the idea that Syk is required for the actin-driven cell motility in response to CX3CL1, but we cannot rule out the possibility that reduced migration in cells with reduced Syk expression could also be due to a defect in other steps involved in chemotaxis, i.e., sensing of the chemokine gradient or establishment and maintenance of cell polarity (28). Additional experiments are required to address these issues. Also, very little is known about CX3CL1 signal transduction in monocytes/macrophages, and it is likely that other signaling components participate in the regulation of CX3CL1-induced chemotaxis. In particular, the possible contributions of a PI3K pathway, reported to be activated downstream of CX3CR1 (11), remain to be investigated as well as its potential interaction with Syk, as described in neutrophils upon LPS stimulation (59).

In conclusion, we have identified the protein tyrosine kinase Syk as a key signaling component in CX3CL1-induced chemotaxis of monocytes/macrophages, indicating for the first time a clear positive requirement of Syk in a migratory process and shedding new light upon the as yet understudied cell signaling downstream of CX3CR1.

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Disclosures
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