CXCL12 SIGNALING IS INDEPENDENT OF JAK2 AND JAK3

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SUMMARY
Janus kinases (Jaks) are a small family of cytoplasmic tyrosine kinases, critical for signaling by Type I and II cytokine receptors. The importance of Jaks in signaling by these receptors has been firmly established by analysis of mutant cell lines, the generation of Jak knockout mice and the identification of patients with Jak3 mutations. While a number of other ligands that do not bind Type I and II cytokine receptors have also been reported to activate Jaks, the requirement for Jaks in signaling by these receptors is less clear. Chemokines for example, which bind seven transmembrane receptors, have been reported to activate Jaks and principally through the use of pharmacological inhibitors, it has been argued that Jaks are essential for chemokine signaling. In the present study, we focused on CXCR4, which binds the chemokine CXCL12 or stromal cell-derived factor-1, a cytokine that has been reported to activate Jak2 and Jak3. We found that the lack of Jak3 had no effect on CXCL12 signaling or chemotaxis nor did overexpression of wild-versions of the kinase. Similarly, overexpression of wild-type or catalytically-inactive Jak2 or “knocking-down” Jak2 expression using siRNA also had no effect.

We also found that in primary lymphocytes, CXCL12 did not induce appreciable phosphorylation of any of the Jaks compared to cytokines for which these kinases are required. Additionally, little or no Stat (signal transducer and activator of transcription) phosphorylation was detected. Thus, we conclude that in contrast to previous reports, Jaks, especially Jak3, are unlikely to play an essential role in chemokine signaling.

INTRODUCTION
The discovery of Janus kinases (Jaks) and Signal Transducers and Activators of Transcription (Stats) and elucidation of their role in cytokine signaling provided a new paradigm by which rapid membrane to nucleus signaling could be accomplished (1-4). Type I and II cytokine receptors bind Janus kinases, a small family of protein tyrosine kinases consisting of four members, Jak1, Jak2, Jak3 and Tyk2 (2-4) by the membrane proximal regions of the receptor cytosolic domains. The Jaks bind via their amino-terminal FERM (band 4.1, ezrin, radixin, moesin) domains and upon cytokine binding become activated by transphosphorylation (5,6). The Jaks then phosphorylate cytokine receptors on cytosolic tyrosine residues, forming docking sites for...
proteins with phosphotyrosine binding- or src homology 2 (SH2)-domains. Stats are one key family of proteins with SH2 domains, which are recruited in this manner and are themselves phosphorylated by Jaks. This allows the Stats to dimerize, resulting in their nuclear accumulation (7). The essential function of Jaks and Stats in signaling via Type I and II cytokine receptors has been clearly established by studies of mutant cell lines, knockout mice and humans with severe combined immunodeficiency due to Jak3 mutations (1-4,8). In addition though, a number of studies have reported that Jaks and Stats are activated by ligands that do not bind Type I and II cytokine receptors. Importantly, some of these studies have also concluded that Jaks are essential for signaling by such ligands.

Chemokines have a broad range of critical functions in the immune response ranging from recruitment and trafficking of inflammatory cells to lymphopoiesis, angiogenesis, and lymphoid organ development. As their name implies, a major action is to induce a chemotactic response in cells; as such, they play important roles in various inflammatory and infectious diseases (9-12). Chemokine receptors belong to the well-known seven-transmembrane domain receptor family known to signal via trimeric G-proteins. Chemokines in particular signal via the pertussis toxin-sensitive Gi subfamily, inducing phosphorylation of multiple substrates including Pyk2, AKT and ERK1/2 (13,14). Chemokines also activate phospholipase Cβ2 and β3, which hydrolyze phosphatidylinositol bisphosphate to form diacylglycerol and inositol trisphosphate thereby activating protein kinase C and inducing mobilization of intracellular calcium stores respectively.

In this study, we focused on the ligand-receptor pair CXCL12 and CXCR4. CXCR4 is widely expressed, but unlike many other chemokine receptors that share multiple ligands, CXCL12 is its only ligand, allowing one to know with certainty what receptor was being analyzed (26). In addition, a previous study reported that Jak2 and Jak3 were transiently phosphorylated in response to CXCL12 (15), although another reported that Jak1, Jak2 and Tyk2 but not Jak3 were activated (18). Similarly, CXCL12 has been variably reported to activate Stats 1, 2, 3, and 5 (15). Importantly, it has also been concluded that Jaks are an essential aspect of chemokine signalling. Jak2 and Jak3 were reported to
associate with and phosphorylate CXCR4 and putatively selective Jak inhibitors block CXCL12 signalling, as measured by inhibition of substrate phosphorylation, calcium mobilization and cell migration (18,27). Unfortunately, these inhibitors are not selective for Jaks (28). We therefore sought to reassess the importance of the Jaks in CXCL12 signaling.

MATERIALS AND METHODS

Reagents, antibodies and plasmids.

The following reagents were purchased from the indicated suppliers: monoclonal antiphosphotyrosine antibody (4G10) and polyclonal antibodies against Jak2, Tyk2, phospho-Stat1 (Y701) (Upstate, Waltham, MA); monoclonal antiphospho-Stat3 and polyclonal anti-Stat1, -Stat2, -Stat4, -Stat6, -phosphoERK1/2 and -ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal anti-phosphorylated (Y693)-STAT4 and monoclonal anti-phospho Stat5 (Zymed Laboratories, South San Francisco, CA); polyclonal anti-Stat3 (Biosource International, Camarillo, CA); PE-conjugated anti-human CXCR4 monoclonal antibody (12G5) (BD Biosciences Pharmingen, San Diego, CA). Polyclonal antibodies against human Jak3 and Stat5 were raised in our laboratory as previously described (29,30). Human IFNγ IL-4 and IL-12 were purchased from R&D Systems (Minneapolis, MN). Human CXCL12 (SDF-1γ) was obtained from PeproTech (Rocky Hill, NJ). Human IL-2 was provided by Dr. Craig Reynolds (National Cancer Institute, Frederick, MD) and human IFNγ was provided by Dr. Gerald M. Feldman (FDA, Bethesda, MD). Phytohemaglutinin (PHA) was purchased from Sigma-Aldrich (St. Louis, MO). Fura-2 was purchased from Molecular Probes (Eugene, OR).

The cDNA constructs encoding wild type Jak3 and catalytically inactive Jak3, pME18SJak3 and pME18SJak3K855A respectively were previously described (31-33). The constructs encoding wild-type Jak2 and catalytically inactive Jak2 were kindly provided by Dr. James N. Ihle (St. Jude Children’s Research Hospital, Memphis, TN) and Christin Carter-Su (University of Michigan, Ann Arbor, MI). The CXCR4 cDNA was cloned into pCDNA3 as described (34,35).

Cell culture, stimulation, and transfection.

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by apheresis and sedimented over Ficoll-Paque™ PLUS (Amersham Pharmacia Biotech AB, Uppsala Sweden). Cells were stimulated with 1 μg/ml PHA for 3 days in RPMI 1640 medium (Biosource International) supplemented with 10% fetal bovine serum (Biosource International), 2 mM L-glutamine (Invitrogen Corporation, Carlsbad, CA), 10 mM HEPES (Biosource International) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Biosource International). This was followed by an additional 48 hrs of stimulation with IL-2 (100 U/ml) to maximize IL-2R and Jak3 expression (2, 12). Cells were washed with acidified medium to remove bound IL-2, incubated in serum-free medium and re-stimulated with the cytokines as indicated. EBV transformed human B cell line from healthy donor and a Jak3 deficient patient (36), were cultured with complete RPMI 1640 medium containing 10% fetal bovine serum. The human embryonic kidney cell line, HEK293, was cultured with DMEM containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and was transfected using Lipofectamine2000 (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s protocol.
For siRNA experiments, Jurkat cells were cultured in complete RPMI-1640, and were split daily to ensure log phase growth at the time of transfection. At the time of transfection, 1 \times 10^7 cells in RPMI-1640 in a volume of 0.4 ml were mixed with 200 pmol of Jak2 SmartPool siRNA or 200 pmol of a scrambled, non-specific siRNA (Dharmacon, Lafayette, CO), and transferred to a 0.4 cm gap cuvette (Bio-Rad, Hercules, CA). The cells were electroporated at 262V, 1040 \mu F, 720 Ohms using a BTX ECM 630 (Genetronics Inc., San Diego, CA). The transfected cells were transferred to 6 well plates and cultured in RPMI. After 24 h incubation, RNA was isolated using a Qiagen Rneasy kit (Qiagen, Valencia, CA) and reverse transcribed using a TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA). Jak2 mRNA expression in transfected cells was determined by real-time PCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The primer/probe sequences for Jak2 were follows: Primers: 5’-TGGAGCTTTGGAGTGGTTCTG-3 and 5’TGCCAATCATACGTTAATATC-3’. Probe: 5’-6-FAMAACTTTTCACATACATTGAGAAGAGAAGTCCAC-TAMRA-3’. Analysis of GAPDH mRNA levels was performed using a commercially available primer/probe set (Applied Biosystems). Jak2 mRNA levels were normalized against GAPDH levels, and compared to the levels in mock-transfected control cells for an individual experiment, which were arbitrarily designated a value of 1.0.

**Immunoprecipitation, SDS-PAGE and Western blot analysis**

Cytokine- or CXCL12-stimulated PBMC (50 X 10^5) were lysed in buffer containing 0.5% Triton X-100, 50 mM Tris pH7.5, 300 mM NaCl, 2 mM EDTA, 0.4 mM sodium orthovanadate, 10 \mu g/ml aprotinin, 10 \mu g/ml leupeptin, and 0.25 \mu g/ml NPGB and clarified lysates were immunoprecipitated as described previously (2). Immunoprecipitates and lysates were separated in SDS-PAGE (8%-12.5%) and transferred to Immobilon™-P PVDF membrane (Millipore Corporation, Bedford, MA) and immunoblotted with the indicated antibody. Blots were stripped in with 2% SDS, 62.5 mM Tris pH 6.8, 100 mM 2-mercaptoethanol and then rebotted to document equivalent protein loading.

**Calcium Flux**

Cells (2 x10^6) were incubated in 1ml PBS containing 1.5 \mu M Fura-2/AM at 37°C for 30 min, washed twice in HBSS (Invitrogen Corporation, Carlsbad, CA) and suspended in 2 ml HBSS. Cells were stimulated with CXCL12 in a continuously stirred cuvette at 37°C and changes in intracellular calcium levels were detected fluorimetrically using a Ratio Fluorescence Spectrometer (model MS-III; Photon Technology Inc., South Brunswick, NJ) (37). Data were recorded every 20 ms as the ratio of fluorescence emitted at 510 nM following sequential excitation at 340 and 380 nM. Alternatively, Jurkat cells were incubated with 16 \mu M Fura-red and 16 \mu M Fluo-4AM (Molecular Probes, Eugene, OR) for 45 min at 37°C, washed twice in cold RPMI, and resuspended in cold Tyrode’s buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5.6 mM glucose, 20 mM HEPES). The cells were stimulated with CXCL12 at 37°C, and changes in dye fluorescence over time were determined by flow cytometry. Calcium mobilization was expressed as the ratio of Flou-4AM to Fura-red fluorescence intensity over time.

**Chemotaxis Assay**

Cells (1.5 x 10^5) in 25 \mu l of medium were applied to the upper wells of a 96 well chemotaxis plate (5-\mu m pore size membrane, Neuro Probe, Inc., Gaithersburg, MD) with or without CXCL12 (1-
100 nM final concentration) in RPMI in the lower wells, and incubated at 37°C for 2 hr. Cells adhered to the filter were dislodged by incubating with PBS containing 3% EDTA for 30 min at 4°C and centrifuging (500 x g) for 10 min. The number of cells migrating to the lower well was enumerated by MTT staining.

RESULTS AND DISCUSSION

CXCR4 signaling is not affected by Jak2 and Jak3 in the HEK293 cells. Using an inhibitor that blocks both Jaks as well as other kinases, previous studies have concluded that Jaks are essential for chemokine signaling (18,20,22,27,28). However, the use of pharmacologic inhibitors may lead to erroneous conclusions and therefore need to be verified by genetic approaches. To begin study this question in more detail, we first employed HEK 293 cells. These cells do not express Jak3 (figure 1A, lower panel, lane 1) and express extremely low levels of Jak2 (upper panel, lane 1); as assessed by real-time PCR, Jak2 levels were approximately 2.5% of the level found in peripheral blood lymphocytes (data not shown). After transfection, wild-type and mutant versions of these kinases were expressed at high levels (panel B, lanes 2-5). In addition, the levels of CXCR4 were comparable among the transfected cells (data not shown).

The transfected cells were loaded with Fura2 and intracellular calcium concentration was monitored in a spectrofluorimeter following stimulation with CXCL12 as a readout of CXCR4 signaling. As shown in figure 1B, CXCL12 (100 nM) induced mobilization of intracellular calcium within seconds of its application to cells. However, expression of wild-type Jak2 or Jak3 did not enhance the calcium signal nor did catalytically inactive kinases interfere with signaling; this was apparent other doses of CXCL12 used (data shown). We interpret these results to show that in HEK 293 cells, Jak2 and Jak3 play no appreciable role in CXCR4 signaling as measured calcium mobilization. The lack of effect of the catalytically inactive Jaks, which were massively overexpressed and presumably would interfere with other Jaks, might also suggest that Jaks, in general, are not important for CXCR4 signaling.

CXCL12 signaling is not impaired in the Jak3 deficient lymphocytes. While the data presented above argue against a role of Jaks in CXCR4 signaling, it was possible that Jaks have cell-specific functions. In other words, it remained possible that Jaks might be selectively important in chemokine signaling in lymphocytes. While we did not favor this hypothesis, we thought it essential to directly test it.

Mutation of Jak3 in humans results in severe combined immunodeficiency (36,38). Typically, these patients lack T and NK cells, however B cells develop in these patients and it is possible to generate EBV-transformed cell lines for analysis of signaling. We therefore selected one line from a patient that we have previously studied in detail, which had comparable expression of CXCR4 with control cells (data not shown). The patient is homozygous for a mutation that results in a missense mutation of Jak3 in the FERM domain (Y100C). This mutant Jak is poorly expressed, but in addition, does not bind its cognate cytokine receptor, γc, and is catalytically inactive; consequently this mutant is completely unable to support cytokine signaling (5,36,39). We therefore compared CXCL12 signaling in normal B cells that expressed Jak3 with the Jak3 deficient cell line. Of note, calcium mobilization was not diminished in the Jak3 deficient line (Fig. 2A) after stimulation with either 10 nM or 100 nM CXCL12, even though CXCR4 expression was comparable. In addition, CXCR4-mediated calcium flux was not impaired in other Jak3-
deficient cell lines, although receptor expression was not necessarily comparable (data not shown). Perhaps the most relevant functional consequence of chemokine signaling is chemotaxis. We therefore next examined CXCL12-induced chemotaxis in the EBV-B cells from a normal individual and the Jak3-SCID patient, but found no impairment in the chemotactic response to CXCL12 in the Jak3-deficient cells (Fig. 2B). Thus, we conclude that the presence or absence of Jak3 has no effect on CXCR4 signaling in lymphocytes, similar to what was found in the HEK 293 cells.

CXCR4-dependent migration of murine Jak3−/− lymphocytes has recently been reported to be reduced (27,40-42). However, we found that Jak3 deficient cells have severely impaired expression of CXCR4. This confounds the interpretation of functional experiments and limits the utility of these cells in studying the effects of CXCL12 (supplementary data, discussed further below).

CXCL12 signaling is not impaired when Jak2 levels are reduced. Gene targeting of Jak2 results in embryonic lethality due to impairment of erythropoietin signaling, making it difficult to study the contribution of Jak2 to chemokine signaling. Embryonic fibroblast cell lines can be obtained from these mice, but in preliminary studies we found that CXCL12 signaling and CXCR4 expression was inconsistent in the cells (data not shown). We therefore turned to an alternative approach. The human T cell line Jurkat already has relatively low levels of Jak2 compared to normal peripheral blood lymphocytes (about one-eighth the level of mRNA as determined by real-time PCR) and also expresses CXCR4. Nonetheless, Jurkat cells have equivalent dose-response to CXCL12 as measured by calcium mobilization to other lymphoid lines, which express higher levels of Jak2 and Jak3 (data not shown). We next used siRNA to further knock down Jak2 levels to determine if this influenced CXCL12-induced calcium flux. Jurkat cells were transfected with a Jak2-specific siRNA, or a control siRNA, and Jak2 mRNA levels were assessed by real-time PCR. As shown in figure 3A, expression of Jak2 mRNA was reduced approximately 70% after siRNA transfection compared to non-transfected cells, while transfection with a control siRNA had no effect on Jak2 mRNA levels. Despite the reduction in Jak2 levels however, there was no reduction in CXCL12-induced calcium flux (Figure 3B). Taken together with the data provide in figures 1 and 2, we believe it is unlikely that Jak2 or Jak3 are critical for CXCR4 signaling as was suggested by inhibitor experiments.

**CXCL12 fails to induce Jak or Stat phosphorylation in primary human lymphocytes.** Because our data argued that Jaks are are required for CXCL12 signalling, we next sought to determine if this stimulus was a potent inducer of Jak and Stat activation. Previous studies have reported activation of both Jaks and Stats, but chemokines were either not compared to cytokines or were not studied in primary lymphocytes, so the magnitude and relevance of activation has not been entirely clear. We therefore set out to directly compare Jak and Stat activation by CXCL12 in primary lymphocytes with the appropriate cytokine known to be a potent activator. First, we demonstrated CXCR4 was highly expressed on human PBMC stimulated with PHA and IL-2 (data not shown). In response to CXCL12 (100 nM), ERK1/2 was rapidly phosphorylated (Fig. 4A). Using the same cells, we stimulated PBMC with CXCL12 comparing this stimulus to the appropriate cytokine known to activate a given Jak. However, as shown in figure 4, we failed to detect significant Jak1 (panel B), Jak2 (panel C), Jak3 (panel D) or Tyk2 (panel E) phosphorylation or in response to CXCL12,
whereas the appropriate control cytokine readily induced phosphorylation.

In parallel experiments, we also analyzed Stat phosphorylation. As is evident in figure 5, we failed to detect substantial phosphorylation of Stat1 (panel A), Stat2 (panel B), Stat3 (panel C), Stat4 (panel D), Stat5 (panel E) or Stat6 (panel F) in response to CXCL12, whereas the appropriate control cytokine elicited phosphorylation in all cases.

Several studies have asserted that Jaks are key signalling elements for chemokine receptors. In fact, it has been proposed that chemokine receptors dimerize and that Jak activation is an essential first step; subsequent activation of trimeric G-proteins and their downstream effectors are Jak-dependent. However, this remains a controversial model, as discussed in detail by Thelen and Baggiolini, who make a strong case against the need for dimerization (43). In this study, we present data that argue against a critical requirement for Jaks in chemokine signalling.

We would argue that studies that make a case for a critical role of Jaks in chemokine signalling, have a number of limitations. In several studies, the requirement for Jaks was inferred through the use of pharmacologic inhibitors, which may have unexpected effects and do not reliably establish biochemical pathways. These drugs are not potent Jak inhibitors and have relatively little selectivity amongst the Jaks. More importantly, they also inhibit other protein tyrosine kinases at similar or even lower concentrations than what is need to inhibit Jaks (28). Instead, we employed more stringent genetic approaches and have found no evidence for a requirement of Jaks in chemokine signalling focusing on one particular chemokine/chemokine receptor pair, CXCL12/CXCR4. We did this by overexpressing wild-type and mutant versions of Jak2 and Jak3 and found no alteration in chemokine-dependent calcium mobilization. Using RNA interference, we found that knocking down Jak2 levels also did not affect CXCR4 signalling.

Cells from Jak3-SCID patients also had no impairment in CXCL12 calcium mobilization or chemotaxis. A recent study that examined CXCL12 signalling in murine Jak3 deficient thymocytes concluded that chemotaxis is impaired (27). The authors also concluded that CXCR4 expression was normal at the level of mRNA. However, using flow cytometry we have not found this to be the case; as is evident in supplementary figure 1, CXCR4 expression is markedly reduced in Jak3−/− thymocytes and splenocytes. In addition, the level of CXCR4 expression on thymocytes is quite low compared to peripheral lymphocytes. Thus the impaired responsiveness to CXCL12 can be readily explained alterations in receptor expression. Thus, these cells are not useful in assessing if Jak3 is required for CXCL12. Taken together with our data in human cells, we conclude that these data do not indicate a requirement for Jak3 in CXCL12/CCR4 function.

Jaks have also been implicated in chemokine signalling based on their ligand-induced phosphorylation. It might be argued that although the genetic approaches used in the present study excluded Jak2 and Jak3 as being essential elements in chemokine signalling, but Jaks in general might still play an important role. Importantly though, in previous studies the level of Jak phosphorylation induced by chemokines was modest and was not directly compared to stimulation with the appropriate cytokines known to evoke Jak activation. In addition, many of these studies employed cell lines not primary cells. We would argue that the lack of appreciable phosphorylation demonstrated in the present study argues against the contention that Jaks are redundantly involved in chemokine signalling.
Nonetheless, since a cell devoid of all Jaks has not been created, we cannot formally exclude this possibility. We also did not observe appreciable Stat activation by CXCL12 and the lack of Stat activation by chemokines has also been reported by others (44,45). Thus it is tempting to speculate that Stats may also not be involved in chemokine signaling. However, it should be pointed out that Stat-deficient cells were not examined. In addition, though the one finds the expression “Jak/Stat pathway” commonly used, it is very clear that there are Jak-independent means of activating Stats. Therefore it is more difficult to conclusively exclude a role of Stats in chemokine signaling at this point.

Based on our results however, we would argue that the evidence implicating Jaks in signaling by CXCR4 and perhaps other chemokine receptors is not compelling. The findings based on a pharmacologic inhibitor are simply not borne out when using genetic approaches. We interpret our data to argue for a much more restricted function of Janus kinases; their dedicated function in cytokine receptor signaling is well-established but the data supporting the function in other receptors is not. In view of our data, the pharmacological studies may be interpreted to suggest that tyrosine kinases other than Jaks are involved in chemokine signaling.
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FIGURE LEGENDS

Figure 1 CXCL12 signaling is independent of Jak2 and Jak3 in the HEK293 cells. HEK293 cells were transfected with the indicated plasmids encoding CXCR4, wild type (WT) and catalytically-inactive or dominant negative (DN) versions of Jak2 and Jak3, after which CXCR4 expression was analyzed by flow cytometry. Cell lysates were separated by 8% SDS-PAGE transferred to PVDF membranes and immunoblotted with polyclonal rabbit anti-Jak2 or Jak3 antibody (A). After 24-hrs in serum free medium, the transfected HEK293 cells were loaded with Fura-2 and changes in intracellular calcium concentrations were monitored in a spectrofluorimeter before and after CXCL12 stimulation (100 nM) (B).

Figure 2. CXCL12 signaling and chemotaxis are not impaired in Jak3 deficient lymphocytes. EBV transformed cells from a patient homozygous for a missense mutation resulting in the replacement of tyrosine 100 with a cysteine residue (Y100C) were compared to a cells from a normal individual. The Y100C mutation not only decreases the expression of Jak3 protein but also disrupts its kinase activity and binding to the common $\gamma$ chain (5,32,46). The cells were stained by PE-conjugated mouse anti-human monoclonal CXCR4 antibody and found to have equivalent receptor expression. The cells were then loaded with Fura-2 to measure intracellular calcium concentration before and after CXCL12 stimulation (A). To assess chemotaxis in response to CXCL12, varying concentrations of ligand (0 -100 nM) were added to the lower wells of chemotaxis plate. Cells from a normal individual and the Jak3-SCID patient (1.5 X 10^5 cells in 25 $\mu$l) were placed in the upper wells. The plate was incubated at 37 °C for 2 hrs and the cells migrating to the lower chamber were enumerated by MTT assay (B).

Figure 3. Reduction of Jak2 levels does not affect CXCL12-induced calcium flux. Jurkat cells were transfected with Jak2-specific siRNA or a control siRNA (Dharmacon) as described in the Materials and Methods, and incubated 24 hr and Jak2 mRNA levels were determined by real-time PCR. Jak2 levels were normalized to GAPDH expression, and are presented as normalized to the mock-transfected control, which was arbitrarily assigned a value of 1.0. (A) Cells were loaded with Flou-4AM and Fura-red and calcium mobilization was assessed by flow cytometry after stimulation with CXCL12 (100 nM – light arrow). After 420 seconds the cells were stimulated with ionomycin (5 nM – heavy arrow) to document sufficient loading. The results are representative of 3 separate experiments.

Figure 4. CXCL12 induces ERK1/2 but not Jak phosphorylation in primary human mononuclear cells. PBMC were obtained from normal donors, isolated over Ficoll-Hypaque and cultured with PHA (1 $\mu$g/ml) for 3 days, and IL-2 (100 U/ml) for an additional for 2 days to maximize cytokine receptor, Jak3 and Stat expression (29,47). The cells were stained by PE-conjugated mouse anti-human monoclonal CXCR4 antibody to document receptor expression. After culturing in serum free medium, the cells were stimulated for 15 minutes at 37°C with CXCL12 (100 nM) or the following cytokines as indicated: IL-2 (1000 U/ml), IFN-\(\gamma\) (1000 U/ml), IFN-\(\alpha\) (1 $\mu$g/ml), IL-12 (50 ng/ml) or IL-4 (20 ng/ml). Cells were lysed and separated on 12.5% SDS-polyacrylamide gels. The proteins were electrotransferred to membranes and immunoblotted with anti-phospho-
ERK1/2 antibody (A). Lysates were also immunoprecipitated with the indicated anti-Jak antibodies (B-Jak1, C-Jak2, D-Jak3, E-Tyk2). The immunoprecipitates were electrophoresed, transferred to PVDF membranes and blotted with antiphosphotyrosine antibody (pY). Membranes were stripped and reblootted with anti-Jak antibody to assess equivalent loading.

Figure 5. CXCL12 does not induce Stat phosphorylation in primary human mononuclear cells. PBMC were isolated and cultured with PHA and IL-2 as above, and after culture in serum-free medium, the cells were stimulated for 15 minutes at 37°C with CXCL12 (100 nM) or the indicated cytokines, after which cells were lysed in detergent. For Stat1 (A), Stat3 (C), Stat4 (D) and Stat5 (E), lysates were directly separated on 12.5% SDS-polyacrylamide gels, transferred, and blotted with anti-phosphoStat antibody. For Stat2, (B) and Stat6 (F), cell lysates were immunoprecipitated with anti-Stat2 or anti-Stat6, and the immunoprecipitates were electrophoresed, transferred and immunoblotted with antiphosphotyrosine antibody (pY). Membranes were stripped and reblootted with anti-Stat antibody to document equivalent loading of samples.
Supplementary figure 1. Splenocytes and thymocytes from C57Bl/6 Jak3−/− mice and wild-type controls were stained with FITC anti-CXCR4 (12G5, BD Biosciences Pharmingen, San Diego, CA; solid and dashed lines respectively,) or an isotype control (dotted line) and analyzed by flow cytometry. The mean channel of fluorescence for CXCR4 expression was as follows: wild-type splenocytes, 27.3; Jak3−/− splenocytes, 16.4; wild-type thymocytes, 282; Jak3−/− thymocytes, 128.
Figure 1A
Figure 1B

B

control

WT Jak2

DN Jak2

340/380 ratio

WT Jak3

DN Jak3

time (sec)
Figure 2

A

WT

Jak3

SCID

340/380 ratio

Time (sec)

B

%Migrating

CXCL12 (nM)

WT

Y100C
Figure 3
Figure 4

A

CXCL12 (min) 0 1 5

IB: α-pERK1/2
α- ERK2

B

None IL-2 CXCL12

IB: α-pY
α- Jak1

IP: Jak1

C

None IFN-γ CXCL12

IB: α-pY
α- Jak2

IP: Jak2

D

None IL-2 CXCL12

IB: α-pY
α- Jak3

IP: Jak3

E

None IL-12 CXCL12

IB: α-pY
α- Tyk2

IP: Tyk2
Figure 5
CXCL12 signaling is independent of Jak2 and Jak3
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