Bone marrow stromal cells and the upregulation of interleukin-8 production in human T-cell acute lymphoblastic leukemia through the CXCL12/CXCR4 axis and the NF-κB and JNK/AP-1 pathways

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ABSTRACT

Background

Cytokines released in the bone marrow and thymic microenvironments play a key role in the growth of T-cell acute lymphoblastic leukemia. Among such cytokines, interleukin-8 is highly expressed in T-cell acute lymphoblastic leukemia cells refractory to chemotherapy. In this study we explored whether bone marrow stromal cells can regulate IL-8 expression in T-cell acute lymphoblastic leukemia and investigated the role of the stromal CXCL12 chemokine in this event. We also investigated the roles of the nuclear factor-κB and Jun-N-terminal kinase (JNK)/activating protein (AP)-1 signaling pathways, which contribute to regulate interleukin-8 production in some cells.

Design and Methods

We analyzed the expression of interleukin-8 in primary cells from ten adult patients with T-cell acute lymphoblastic leukemia when these cells were cultured with bone marrow stromal cells or stimulated with exogenous CXCL12. Interleukin-8 mRNA was analyzed by a colorimetric assay. Cytokine production was assayed by cytometric antibody array and flow cytometry. Nuclear factor-κB and JNK/AP-1 activation was investigated by using specific inhibitors of these pathways, immunoblotting, electrophoretic mobility-shift assay and cell transfection assays.

Results

Bone marrow stromal cells upregulated interleukin-8 mRNA in T-cell acute lymphoblastic leukemia cells through the activity of CXCR4, the CXCL12 receptor, as assessed by the use of neutralizing antibodies. Exogenous CXCL12 induced a significant increase in the production of IL-8 mRNA and protein in all T-cell acute lymphoblastic leukemia cases. We showed that CXCL12 activates the nuclear factor-κB and JNK/AP-1 pathways, and that these events are required for increased expression of interleukin-8. Furthermore, the nuclear factor-κB and AP-1 elements of the interleukin-8 promoter are necessary for both constitutive and CXCL12-induced interleukin-8 expression.

Conclusions

Interleukin-8 is physiologically regulated by the CXCL12/CXCR4 axis and the nuclear factor-κB and JNK/AP-1 pathways are required for interleukin-8 expression in T-cell acute lymphoblastic leukemia. We propose that, by upregulating interleukin-8, the bone marrow microenvironment and the CXCL12/CXCR4 axis may play a role in the pathogenesis of T-cell acute lymphoblastic leukemia.

Key words: acute lymphoid leukemias, chemokines, tumor microenvironment.

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Introduction

T-cell acute lymphoblastic leukemia (T-ALL) results from the clonal proliferation of T lymphoid precursors and originates in the thymus. Although in a minority of cases T-ALL cells remain confined to the thymus, in most cases leukemic cells migrate to the bone marrow.[1] Within the microenvironments where T-ALL develops, the establishment of adhesive contacts and the release of cytokines have crucial roles in regulating growth and survival of leukemic cells.[2-6] The gene coding for interleukin-8 (IL-8) has recently been found to be highly expressed in T-ALL cells refractory to chemotherapy and has been proposed to play a role in T-ALL.[7] Originally characterized as a leukocyte chemoattractant, IL-8, also known as CXCL8, has recently been shown to induce angiogenesis and to contribute to cancer progression in humans.[8-12] In hematologic malignancies, increased IL-8 mRNA expression has been associated with prolonged survival of chronic lymphocytic leukemia[13-14] and higher plasma levels of IL-8 are predictive of poor survival in B-lineage ALL.[15] CXCL12, formerly known as stromal-derived factor-1, is constitutively expressed in many types of cells, including bone marrow stromal cells and thymic epithelial cells.[16-17] CXCL12 has a major role in controlling steady-state homeostatic processes of leukocytes and regulates homing and development of leukemic cells.[18-22] Interactions of CXCL12 with CXCR4[23] trigger complex signaling pathways, including phosphorylation of mitogen-activated protein kinase/extracellular signal regulated kinase (MEK/ERK),[24-25] and an increase of nuclear factor (NF)-κB binding activity.[26-27] Several findings suggest that CXCL12 can also affect the expression of genes. Treatment with CXCL12 enhances the production of matrix metalloproteinase 9 in various types of cells, including chronic lymphocytic leukemia cells, myeloma cells and osteoclasts.[28-30] CXCL12 also induces the expression of vascular endothelial growth factor in T-ALL cell lines[31] and regulates IL-8 production in human mast cells, endothelial cells and fibroblast-like synoviocytes.[32-34] Nonetheless, there is very little information on the molecular mechanisms leading to the regulation of gene expression by CXCL12.

In this study, we explored whether bone marrow stromal cells can control IL-8 production in T-ALL cells and investigated the role of CXCL12 in this event. We also examined the contribution of NF-κB and of the Jun-N-terminal kinase (JNK)/activating protein (AP)-1 signaling pathways in CXCL12-induced IL-8 production.

Design and Methods

Patients and cells

Patients were enrolled in the *Italian Gruppo Italiano Malattie EMatologiche dell'Adulto* (GIMEMA) multicenter clinical trials 0496 and 2000 for adult patients with newly diagnosed ALL (protocol registration numbers NCT00439920 and NCT00537550). Written informed consent was obtained from all patients prior to therapy according to the Declaration of Helsinki. The study was approved by an internal review board of the co-ordinating Center. Peripheral blood and/or bone marrow samples with high leukemia involvement (85-100%) were collected from ten patients. Mononuclear cells were isolated by Lymphoprep density gradient centrifugation (Nicomed, Oslo, Norway), subjected to immunophenotypic analysis by flow cytometry as described previously[4] and classified according to their maturation stage using the criteria defined by the European Group for Immunological Characterization of Leukemias (EGIL)[35] (Table 1). Before use, the viability of the cells in each sample, assessed by propidium-iodide dye exclusion, consistently exceeded 90%. Bone marrow stromal cells were obtained from four adult healthy donors, after informed consent, as previously described.[6]

Table 1. Immunophenotype and classification of T-cell acute lymphoblastic leukemia patients.

	Immunophenotype[a]							
T-ALL N.	CD1a	CD2	CD3	CD4	CD5	CD7	CD8	Maturation stage[b]
1	+	+	–	+	+	+	+	III
2	+	+	–	+	+	+	+	III
3	–	–	–	–	+	+	–	II
4	+	+	–	+	+	+	–	III
5	+	+	–	+	+	+	+	III
6	–	–	–	–	+	+	–	II
7	–	+	+	+	+	+	+	IV
8	–	–	+	–	+	+	+	IV
9	+	–	–	–	+	+	–	III
10	–	+	–	+	+	+	+	II

[a]In all cases, greater than 90% of blasts were positive for TdT, and cytoplasmic CD3. [b]T-ALL maturation stages of primary samples were defined as described by Bené et al.[35] Stage II, pre-T-ALL; stage III, cortical T-ALL; stage IV, mature T-ALL.

Co-cultures

Primary T-ALL cells were labeled with the membrane dye PKH26-GL (Sigma, Milan, Italy), added to confluent bone marrow stromal layers at a 10:1 leukemic:stromal-cell ratio, and cultured at 37°C, 5% CO_2, in RPMI 1640 medium (GIBCO), with 10% fetal bovine serum, L-glutamine and antibiotics. Control T-ALL cells were cultured in the same conditions but in the absence of bone marrow stromal cells. At the end of the period of co-culture, cells were detached with a trypsin/EDTA solution and PKH26-GL-fluorescent T-ALL cells were separated by a fluorescence activated cell sorter (FACS Vantage, Becton-Dickinson, Palo Alto, CA, USA). The percentage of cells recovered ranged from 70% to 80%. The purity of recovered cells was greater than 98%. For blocking experiments, neutralizing concentrations (10 μg/mL) of monoclonal antibodies recognizing functional epitopes of CXCR4 (BD Bioscience-Pharmingen), IL-1RI, or

tumor necrosis factor (R&D Systems, Minneapolis, MN, USA), were added to the T-ALL/bone marrow co-cultures at the start of the assay.

IL-8 mRNA analysis

Total RNA was isolated by the guanidinum isothiocyanate/cesium chloride method. IL-8 mRNA levels were quantified using a commercially available colorimetric assay kit (Quantikine mRNA, R&D Systems). IL-8 mRNA values were normalized using β-actin transcript expression as the control house keeping gene. No differences in IL-8 mRNA levels were detected before and after cell sorting (*data not shown*). Differences between treatments were evaluated using the two-tailed Student's t-test and were considered statistically significant when p values were <0.05.

Treatment of T-ALL cells

T-ALL cells ($1.5x10^7$ cells/mL) were cultured for various periods at 37°C, 5% CO_2 with RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, with or without 1 μg/mL CXCL12 (Peprotech, London, UK), 10 ng/mL tumor necrosis factor (Sigma), or 10 ng/mL IL-1β (R&D Systems). In some cases, before CXCL12 stimulation, T-ALL cells were treated with proteasome- (MG-132), JNK- (SP600125) specific inhibitors (Alexis Biochemicals, Lausen, Switzerland), or vehicle alone.

Cytokine production analysis

The production of IL-1, IL-6, IL-8, and tumor necrosis factor was simultaneously measured by a cytometric bead array kit (CBA, BD Bioscience-Pharmingen), according to the manufacturer's instructions. Fluorescence signals were detected using a FACSCalibur flow cytometer (Becton-Dickinson) and data elaborated with built-in dedicated software. The concentration of CXCL12 was determined by an enzyme-linked immunosorbant assay (R&D Systems). Differences were evaluated using the two-tailed Student's t-test and considered statistically significant when p values were <0.05.

Electrophoretic mobility-shift assay

DNA binding activity of nuclear proteins from primary T-ALL cells was determined using [γ-^{32}P]ATP end-labeled double-strand oligonucleotide encompassing the NF-κB binding site of the IL-8 promoter as the probe (5'-GTGGAATTTCC-3'). A radiolabeled probe (0.04 pmol; specific activity ~$3x10^6$ cpm/pmol) was added to nuclear extracts (5 μg) in binding buffer. In some cases, polyclonal antibodies directed against the RelA/p65 or NFKB1/p50 subunits of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were incubated with nuclear extracts before addition of the probe. Samples were then loaded on a 5% (30:1.2) polyacrylamide gel in 0.05M TBE, and run at 170 V. The gels were dried and exposed to a Kodak phosphor screen. Acquisition and densitometry of the shifted band were performed on a Phosphorimager instrument (Molecular Dynamics, Sunnyvale, CA, USA).

Western blotting

Nuclear proteins (5 μg) from primary T-ALL cells were fractionated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA), and probed with anti-p-c-Jun (phospho-Ser63 and -Ser73) or anti-c-Jun polyclonal antibodies (Santa Cruz Biotechnology). The secondary antibody (anti-rabbit IgG) conjugated with horseradish peroxidase was from Upstate Biotechnology (Lake Placid, NY, USA). Immunodetection was carried out using an enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK).

Cell transfection

Transcriptional activity in Jurkat cells was measured in reporter gene assays after transient transfection of cells with the –162/+44 human IL-8 promoter-luciferase plasmids (wild type, AP-1-, and NF-kB-site mutants,[36] generous gifts from Dr. A. Brasier, University of Texas Medical Branch, Galveston, TX, USA). Logarithmically growing cells were transiently transfected in triplicate with the IL-8 promoter/luciferase plasmids and green fluorescent protein reporter plasmids by electroporation (Electroporator II, Invitrogen, Carlsbad, CA, USA), at 330 V/1000 microfarad. The transfection efficiency of the Jurkat cells for each construct was: wild type=45.6%±2.7, mutant NF-κB=49.8%±7.7, mutant AP-1=42.7%±4.9. Electroporated cells were allowed to grow for 20 hours before CXCL12 treatment. At 5 hours after CXCL12 stimulation, cells were lysed and luciferase activity was measured with the Luciferase Assay System (Promega Corporation, Madison, WI, USA), according to the manufacturer's instruction. Luciferase activity values were normalized with respect to the green fluorescent protein activity, independently measured with a FACSCalibur flow cytometer (BD).

Results

Bone marrow-derived stromal cells increase IL-8-mRNA levels in T-ALL cells through CXCR4

We analyzed IL-8-mRNA expression in primary T-ALL cells cultured with or without human bone marrow stromal cells. T-ALL cells plated onto bone marrow stromal cells showed a significant increase of IL-8 mRNA expression with respect to controls (mean increase±SD=2.8-fold ±1; range 1.8-3.8-fold, n=3; Figure 1A). To determine the molecules of stromal origin responsible for IL-8 up-regulation, we quantified IL-8

production in T-ALL cells treated with bone marrow-stroma conditioned medium. As shown in Figure 1A, this medium induced a significant increase of IL-8 mRNA in T-ALL cells. We, therefore, searched for soluble factors produced by bone marrow stromal cells. Analysis of CXCL12, tumor necrosis factor, and IL-1β in supernatants collected from four independent bone marrow-stroma cultures showed that stromal cells secreted considerable levels of CXCL12 but undetectable amounts of tumor necrosis factor or IL-1β (*data not shown*). CXCL12 production was 1328±501 and 2986±1132 pg/mL ± SD at 4 and 24 hours, respectively. To establish the role of the CXCL12/CXCR4 axis in the bone marrow-mediated increased expression of IL-8, we first analyzed the expression of CXCR4 on T-ALL cells used in this study by flow cytometry. All T-ALL cell samples expressed CXCR4, thus confirming previous results[37] (*Online Supplementary Figure S1*). We, therefore co-cultured T-ALL/bone marrow stomal cells in the presence of anti-CXCR4, anti-tumor necrosis factor, or anti-IL-1RI neutralizing antibodies. As shown in Figure 1B, blockage of CXCR4 molecules significantly inhibited bone marrow-induced IL-8 mRNA expression in T-ALL cells. In contrast, no significant changes in IL-8 mRNA were observed in the presence of antibodies neutralizing tumor necrosis factor or IL-1RI, thus ruling out that undetectable levels of tumor necrosis factor and/or IL-1 can mediate IL-8 up-regulation.

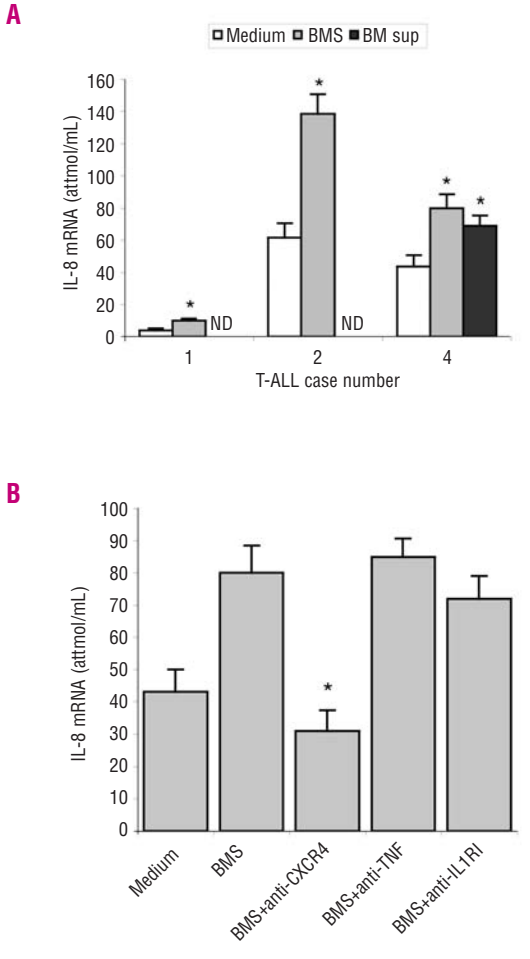

Figure 1. Bone marrow stromal cells require CXCR4 to stimulate IL-8 mRNA production in T-ALL. (A) Primary T-ALL cells were stained with the fluorescent dye PKH26-GL and cultured in medium alone (Medium), with bone marrow stromal cells (BMS), or in the presence of bone marrow-stroma conditioned medium (BM sup) for 4 hours. After co-culture, T-ALL cells were sorted by FACS and analyzed for IL-8-specific mRNA using a colorimetric assay kit. Cells from T-ALL case numbers 1, 2, and 4 were used in this experiment. An asterisk indicates that the differences between the effects of BMS or BM-sup treatments vs. the medium alone are statistically significant ($p<0.05$). (B) Primary T-ALL cells were stained with the fluorescent dye PKH26-GL and cultured with bone marrow stromal cells (BMS) in the presence of monoclonal antibodies (10μg/mL) neutralizing CXCR4, tumor necrosis factor (TNF), or IL-1RI . After co-culture, T-ALL cells were sorted by FACS and analyzed for IL-8-specific mRNA using a colorimetric assay kit. Values represent the average of two independent experiments. Error bars indicate standard deviations. An asterisk indicates that the differences between the effects of BMS+antiCXCR4 vs. BMS treatments are statistically significant ($p<0.05$).

CXCL12 increases IL-8 production in T-ALL cells

CXCL12 induced a significant and sustained decrease of CXCR4 expression in T-ALL (*data not shown*), thus indicating its stimulation.[38] To establish whether CXCL12 could modulate IL-8 production, primary T-ALL cells were incubated for 5 and 24 hours with and without exogenous CXCL12 and the culture supernatants analyzed for the presence of IL-8. Maximal differences in IL-8 levels between medium- and CXCL12-stimulated cultures were observed at 5 hours (*data not shown*). Dose-response curves indicated that 1 μg/mL CXCL12 induced the maximal response (*data not shown*). Therefore, all experiments were performed with 1μg/mL CXCL12 and the cytokine production assayed at 5 hours. As shown in Figure 2A, all T-ALL cell samples constitutively secreted IL-8 in the culture medium. CXCL12 treatment significantly increased IL-8 production (2.5±1.0 fold-increase ± SD; range 1.4-4.4 fold, n=10, Figure 2A) at levels comparable to those induced by tumor necrosis factor, but significantly lower than those induced by IL-1 (Figure 2B). Treatment of T-ALL cells with CXCL12 also up-regulated IL-6 production, although the levels of IL-6 production were 20-fold lower than those of IL-8 (Figure 2C). In contrast, CXCL12 did not influence the production of IL-1β or tumor necrosis factor, as shown by cytometric array assay (Figure 2C). We then confirmed the CXCL12-mediated production of IL-8 at the mRNA level. Colorimetric assay showed that CXCL12 induced IL-8 mRNA expression that was evident 1.5 hours after stimulation and maximal at 4 hours after stimulation (Figure 2D). Dose-response experiments performed at 4 hours indicated that an increase of IL-8 mRNA occurred when CXCL12 was present at a concentration of 10 ng/mL; maximal induction of IL-8 mRNA was achieved at a CXCL12 concentration of 1000 ng/mL. The levels of IL-8 mRNA induced by bone marrow stromal cells were comparable to those achieved with 10 ng/mL CXCL12 (Figure 2E).

CXCL12 induces NF-κB binding activity and c-Jun phosphorylation in T-ALL cells

NF-κB and JNK/AP-1 pathways have been implicated in IL-8 transcriptional activation in various types of cells.[39] To determine whether these pathways are activated by CXCL12 in primary T-ALL cells, we first explored whether CXCL12 stimulation increased NF-κB binding activity and found that it did indeed cause a marked increase of constitutive NF-κB binding activity. The increase peaked at 30 minutes, and returned to basal levels at 60 minutes after stimulation (Figure 3A). The delayed complex contained RelA/p65 NF-κB but not the NFKB1/p50 subunit, as shown by supershift analysis (Figure 3B). Next we evaluated whether CXCL12 could activate AP-1 proteins. Electrophoretic mobility-shift assay revealed high constitutive binding activity of nuclear extracts to an AP-1 probe and no differences between binding activities of untreated or CXCL12-stimulated cells (*data not shown*). These findings are in agreement with previous studies showing that, in many cell types, AP-1 proteins are constitutively bound to their DNA elements and transcriptional activity is regulated by phosphorylation of transactivation domains.[39] We, therefore, analyzed whether CXCL12 could phosphorylate the transactivation domain of c-Jun, a member of the AP-1 family directly phosphorylated by JNK. Primary T-ALL cells were stimulated with CXCL12 and c-Jun phosphorylation was analyzed by immunoblotting. Figure 3C illustrates that CXCL12 induced a significant increase of c-Jun phosphorylation, which was obvious at 15 minutes after stimulation and still present at 60 minutes after stimulation.

NF-κB and JNK pathways are required for CXCL12-mediated IL-8 production in T-ALL cells

We explored the functional role of NF-κB and JNK activation by using specific inhibition of these pathway: MG-132 is a potent proteasome inhibitor that blocks NF-κB activation and SP600125 is an

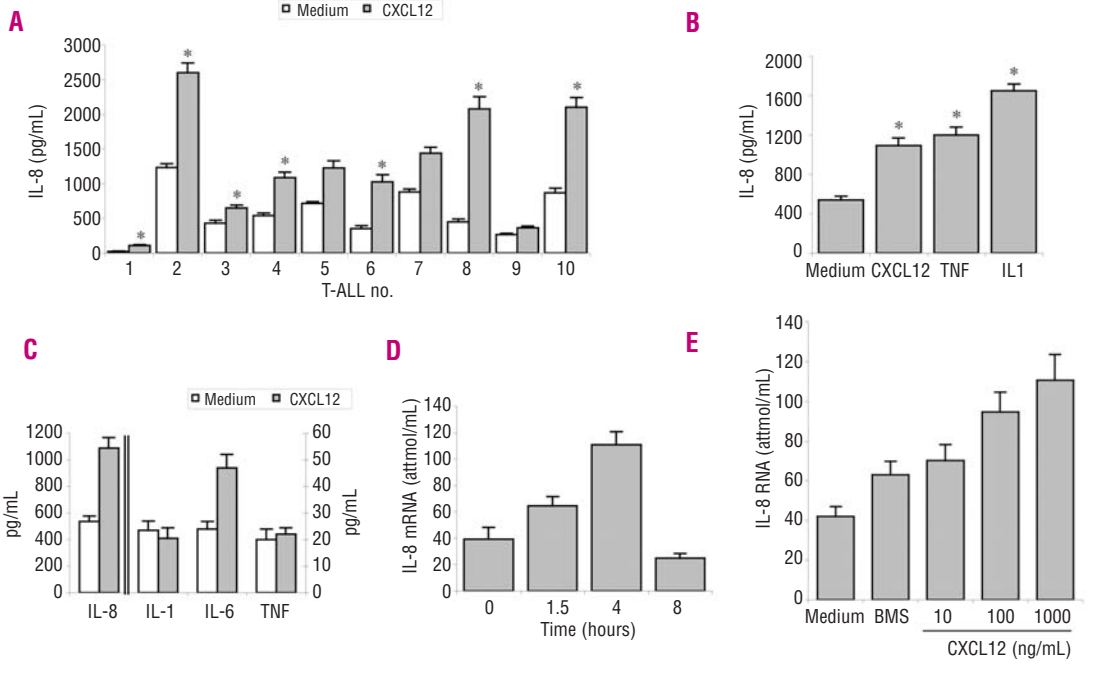

Figure 2. CXCL12 induces IL-8 production in T-ALL. (A) Primary T-ALL cells were stimulated with CXCL12 (1 μg/mL) for 5 hours in RPMI medium with 10% fetal calf serum. After incubation, the concentration of IL-8 in the cell-free supernatants was quantified by cytometric bead array assay. Values (in pg/mL) represent the average of triplicate experiments. Error bars indicate standard deviations. An asterisk indicates that the difference between the effects of CXCL12 vs. the medium alone is statistically significant ($p<0.05$). (B) The levels of IL-8 produced by CXCL12-stimulated (1μg/mL) T-ALL cells were compared with those produced after incubation with tumor necrosis factor (TNF) (10 ng/mL) or IL-1, (10 ng/mL). Cells from T-ALL cases number 1, 3, 4, and 5 were used in this experiment. Values (in pg/mL) represent the average of three independent experiments from the representative T-ALL case number 4. Similar data were obtained from the other T-ALL cells. Error bars indicate standard deviations. An asterisk indicates that the difference between the effects of cytokines vs. the medium alone is statistically significant ($p<0.05$). (C) IL-1, IL-6, IL-8, and TNF concentration in the cell-free supernatant of primary T-ALL cells stimulated with CXCL12 (1 μg/mL) for 5 hours was determined by cytometric bead array assay. Values (in pg/mL) represent the average of three independent experiments from the representative T-ALL case number 4. Similar data were obtained from the other T-ALL cells. Error bars indicate standard deviations. (D) Total RNA was extracted from primary T-ALL cells stimulated or not with CXCL12 for the indicated time. IL-8 and β-actin, used as the control house-keeping gene, mRNA was quantified using a colorimetric assay kit. Cells from T-ALL cases number 1, 2, 5, 6, and 8 were used in this experiment. Values (in attmol/mL) represent the average of three independent experiments from the representative T-ALL case number 5. Similar data were obtained from the other T-ALL cells. Error bars indicate standard deviations. (E) T-ALL cells were stimulated with different doses of CXCL12 or cultured in the presence of bone marrow stromal cells (BMS) for 4 hours. After co-culture, T-ALL cells were sorted by FACS. Total RNA was extracted from stimulated or unstimulated primary T-ALL cells (case number 5). IL-8 and β-actin, used as the control house-keeping gene, mRNA was quantified using a colorimetric assay kit.

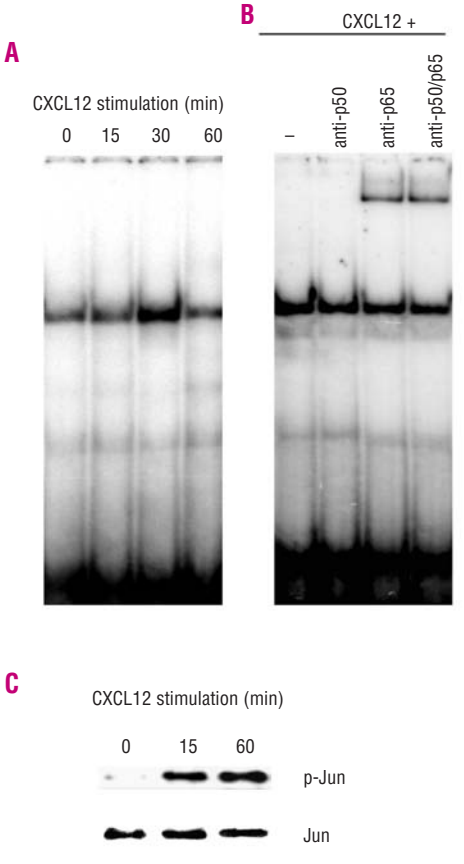

Figure 3. CXCL12 induces NF-κB binding activity and c-Jun phosphorylation in T-ALL cells. (A) NF-κB binding activity of nuclear proteins from T-ALL cells stimulated with CXCL12 for the indicated time was examined by electrophoretic mobility shift assay. Cells from T-ALL cases number 1, 4, 5, 8, and 9 were used in this experiment. Results from T-ALL case number 1 are shown. Similar data were obtained from the other T-ALL cells. (B) Nuclear proteins from T-ALL cells stimulated with CXCL12 for 30 minutes were subjected to the electrophoretic mobility shift assay with a NF-κB probe in the presence of anti-p50 and/or anti-p65 antibodies. Cells from T-ALL cases number 1 and 5 were used in this experiment. Results from the representative T-ALL case number 1 are shown. (C) The amount of phospho-c-Jun was determined on nuclear proteins by western blot analysis; as a loading control, the amount of c-Jun protein is shown at the bottom. Cells from T-ALL cases number 1, 4, and 5 were used in this experiment. Results from T-ALL case number 1 are shown. Similar data were obtained from the other cases.

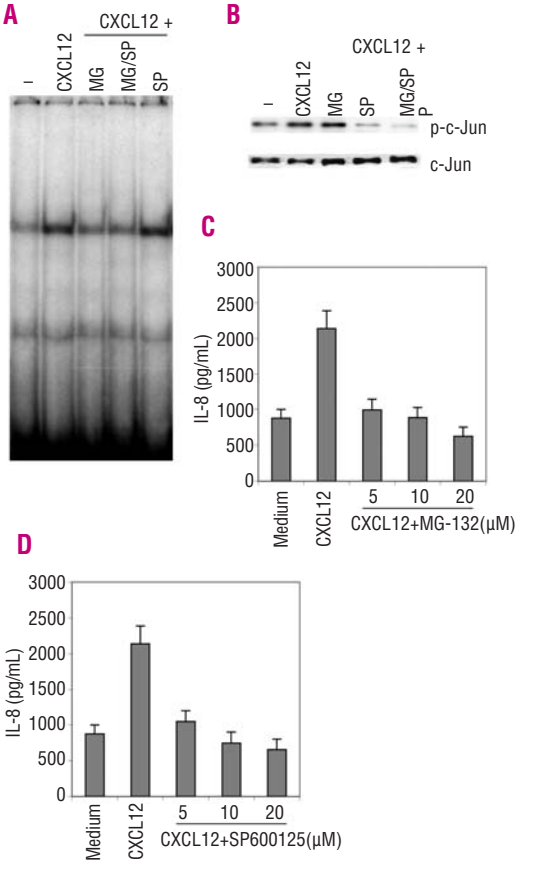

Figure 4. NF-κB and JNK activation are required for CXCL12-mediated IL-8 production in T-ALL. (A-B) T-ALL cells were pre-treated with 10 μM MG-132 and/or 10 μM SP600125 for 1 hour, and then stimulated with 1 μg/mL CXCL12 for 30 minutes. Electrophoretic mobility-shift assay with a NF-κB probe (A) and western blot analysis with anti-p-c-Jun antibody (B) were performed to confirm that MG-132 (MG) and SP-600125 (SP) specifically inhibit NF-κB activity and the JNK pathway, respectively. As the loading control of the western blot, the amount of c-Jun protein is shown at the bottom of panel B. Cells from T-ALL cases number 5 and 10 were used in these experiments. Results from the representative T-ALL case number 5 are shown. (C-D) T-ALL cells were pre-treated with the indicated concentrations of MG-132 (C), or SP600125 (D) for 1 hour, and then stimulated with 1 μg/mL CXCL12 for 5 hours in duplicate wells. In control samples, i.e. Medium or CXCL12 in the absence of inhibitors, vehicle alone was added to the medium. After incubation, the concentration of IL-8 was quantified by a cytometric bead array system in the cell-free supernatant. Cells from T-ALL cases number 1, 5, and 10 were used in these experiments. Results from T-ALL case number 10 are shown. Values represent the mean ± SD of three independent experiments. Similar data were obtained from the other cases.

anthrapyrazolone compound that selectively inhibits JNK activity. These inhibitors did not affect T-ALL cell viability at the time point (5 hours) chosen to determine cytokine production (*data not shown*). CXCL12-mediated NF-κB binding activity was inhibited when T-ALL cells were pretreated with MG-132, but not with SP600125 (Figure 4A). By contrast, c-Jun phosphorylation was specifically abrogated by SP600125 but unaffected by MG-132 (Figure 4B). NF-κB binding activity inhibition correlated with a clear-cut reduction of IL-8 production in CXCL12-stimulated T-ALL cells pretreated with different concentrations of MG-132 (Figure 4C). Similarly, IL-8 production was reduced in CXCL12-stimulated T-ALL cells in the presence of different amounts of SP600125 (Figure 4D).

Effects of NF-κB and AP-1 site mutations on IL-8 gene activation by CXCL12 stimulation

To establish the role of NF-κB and AP-1 *cis* elements of the IL-8 promoter in the responsiveness to CXCL12 stimulation, the human T-ALL cell line Jurkat was transiently transfected with luciferase reporter gene containing the wild type, NF-κB- mutated or AP-1-mutated human IL-8 promoter. In preliminary assays, we evalu-

ated whether Jurkat cells could be representative of primary T-ALL cells. Jurkat cells expressed CXCR4 molecules, as demonstrated by flow cytometry analysis (*data not shown*) and showed IL-8 up-regulation following CXCL12 stimulation (25±2 to 49±4 pg/mL±SD, 1.8 fold-increase at 5 hours, *data not shown*). The Jurkat cells were transfected with IL-8 promoter/luciferase plasmids and luciferase activity was measured at 5 hours after CXCL12 stimulation. As shown in Figure 5, CXCL12 treatment induced a significant increase of promoter activity, consistent with the observed increased IL-8 production. Mutations at either the NF-κB or AP-1 binding sites affected both basal and CXCL12-induced activities of the promoter.

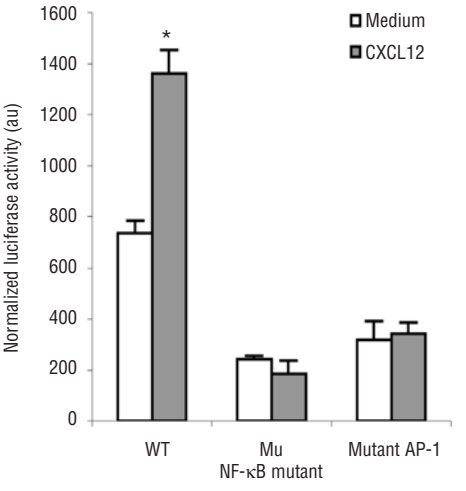

Figure 5. Effects of NF-κB and AP-1 site mutation in the IL-8 promoter sequence on promoter activity. Jurkat cells were transiently transfected with wild type (WT), NF-κB site mutant or AP-1-site mutant plasmids of the –162 IL-8 gene promoter and then stimulated with recombinant CXCL12 (1 μg/mL) for 5 hours. Untreated cells served as controls. For each sample, luciferase activity values were normalized to the green fluorescent protein expression. Data are expressed as mean ± SD of normalized luciferase activity of four independent experiments performed in triplicate. The asterisk indicates that the difference between the effects of CXCL12 vs. the medium alone is statistically significant ($p<0.05$).

Discussion

In this study, we explored the ability of the bone marrow microenvironment to regulate the expression of IL-8 in T-ALL. We show for the first time that: (i) bone marrow stromal cells induce IL-8 mRNA upregulation in human primary T-ALL cells; (ii) the CXCL12/CXCR4 axis is required for the stroma-mediated IL-8 induction; (iii) IL-8 is upregulated in response to exogenous CXCL12; (iv) CXCL12 activates NF-κB and JNK/AP-1 pathways, which are required for IL-8 upregulation; and (v) NF-κB and AP-1 *cis*-elements within the IL-8 promoter are necessary for the constitutive and CXCL12-induced IL-8 expression.

Recently, gene expression profile analysis showed that the IL-8 gene is more highly expressed in cells from refractory T-ALL patients than in those who respond to induction chemotherapy.[7] Our experiments suggest that stromal cells can modulate the functions of IL-8 in the bone marrow microenvironment by regulating the production of this cytokine in leukemic cells. IL-8 up-regulation requires the activity of CXCL12. Furthermore, CXCL12 treatment results in a prolonged decrease of CXCR4 surface expression (*data not shown*), which is consistent with its stimulation.[38] CXCL12 stimulation of T-ALL cells upregulates IL-8 mRNA and protein. The increase in IL-8 mRNA is detectable at 1.5 hours following stimulation, thus suggesting a direct role for CXCL12 in inducing IL-8 upregulation. The increase in IL-8 parallels the upregulation of IL-6. This finding is consistent with the fact that the IL-8 and IL-6 genes share key mechanisms of transcriptional regulation (i.e. NF-κB, AP-1, CEBP) and appear to be co-regulated in several types of cells.[39]

CXCL12 stimulation induces the simultaneous activation of F-κ-B and c-Jun in the nucleus of T-ALL cells. Although several studies have reported that CXCL12 can induce NF-κB binding activity,[26-27] our data show for the first time that CXCL12 activates the NF-κB pathway in primary T-ALL cells. It has been described that CXCL12 stimulation can activate the JNK pathway in human sarcoma cells, whereas it is unable to activate this pathway in other types of cells.[25-27] We first report that CXCL12 induces phosphorylation of c-Jun in T-ALL, thus suggesting the activation of JNK.

IL-8 production is regulated primarily at the level of gene transcription and NF-κB is considered to play a central role in this process.[39] Consistently, we show that CXCL12-induced IL-8 production is dependent on NF-κB activation. The CXCL12-induced binding complex contains p65 but not p50 subunits, in accordance with previous findings that p65 homodimers bound preferentially to IL-8 κB sites and had a selective, major involvement in IL-8 gene expression.[40] The core IL-8 promoter also contains the binding site for the AP-1 transcription factor.[39] Transcriptional activity of the AP-1 proteins is regulated by phosphorylation of transactivation domains and JNK is considered to activate IL-8 via c-Jun and the AP-1 *cis*-element.[39] Herein, we describe that the JNK pathway is essential for IL-8 induction mediated by CXCL12. The functional involvement of the NF-κB and JNK/AP-1 pathways was confirmed by transient transfection analysis of the human IL-8 promoter, which showed that the binding sites for NF-κB and AP-1 were necessary for IL-8 promoter activation following CXCL12 stimulation. Mutations at either the NF-κB or AP-1 binding sites also affected basal activity of the promoter, thus indicating the requirement of both binding sites for constitutive activation of the IL-8 promoter in T-ALL cells.

Taken together, our results support the model that CXCL12 activates IL-8 transcription via upregulation of NF-κB and AP-1 activity in the nucleus.

There is increasing evidence suggesting a critical role for the CXCL12/CXCR4 axis in leukemia progression by controlling homing and survival of neoplastic cells.[20–22,24,41] Our study highlights a role for CXCL12 in regulating gene expression of cytokines potentially involved in leukemia, thus suggesting that CXCL12 can amplify their potential biological effects on leukemic and/or microenvironmental cells.

T-ALL cells do not express detectable levels of IL-8 receptors, namely CXCR1 and CXCR2, on the cell surface (*data not shown*). The possible role of IL-8 in T-ALL may, therefore, derive from its potential functions as a chemotactic and angiogenic factor. Increasing evidence indicates that angiogenesis is involved in the pathogenesis of ALL.[42,15] However, the role of IL-8 in T-ALL angiogenesis and pathogenesis remains to be elucidated.

In conclusion, we propose that CXCL12, produced by bone marrow microenvironmental cells, can regulate IL-8 expression in T-ALL. We determined that the activity of NF-κB and AP-1 transcription factors is central to constitutive and induced IL-8 expression. The involvement of NF-κB is of particular interest as this transcription factor is emerging as a key molecule in the establishment of T-ALL and, as a consequence, NF-κB-inhibiting agents are considered attractive candidates for the treatment of T-ALL.[43] IL-8 could be one NF-κB-target gene involved in the progression of T-ALL. The characterization of molecular mechanisms leading to IL-8 upregulation could, therefore, be relevant to elucidate the natural history of T-ALL and design possible alternative therapeutic strategies.

Authorship and Discosures

MTS designed and performed the research, and wrote the manuscript. MD, FC, MR, OP, GM, SC and CA performed the research. FV and MK contributed to writing the manuscript. MP supervised the EMSA experiments. AS and RF contributed to interpretation of data. GP designed the research.

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