Interaction of interleukin-7 and interleukin-3 with the CXCL12-induced proliferation of B-cell progenitor acute lymphoblastic leukemia

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Acknowledgments: we would like to acknowledge Dr. Karen Byth for her assistance with statistical analysis of the data and Dr. Luciano Dalla-Pozza and Dr. Peter Shaw from The Westmead Children's Hospital for assistance with obtaining leukemic bone marrow samples.

Funding: this work was supported by a Faculty of Medicine/Medical Foundation Postgraduate Research Scholarship from the University of Sydney and the NH MRC (JJ), a Dora Lush Postgraduate Research Scholarship from the NH&MRC (SG), the Anthony Rothe Memorial Trust (LB and RB), and the Cancer Institute of NSW (SG and LB).

Manuscript received August 7, 2006. Manuscript accepted February 14, 2007.

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ABSTRACT

Background and Objectives

The chemokine stroma-derived factor 1α (SDF-1α or CXCL12) is essential for proliferation of B lineage acute lymphoblastic leukemia (ALL) cells in their physiological microenvironment, bone marrow stroma. CXCL12 synergizes with cytokines that stimulate myeloid cells, but its interaction with cytokines affecting lymphoid cells has not been examined. We investigated whether interleukin (IL)-7 and IL-3 interact with CXCL12 to regulate ALL proliferation.

Design and Methods

The survival of ALL cells in serum-free cultures, with or without stromal support and cytokines, was assessed by flow cytometry, and proliferation by ^{3}H-thymidine incorporation. Signaling mechanisms were assessed by western blotting of phosphorylated forms of signaling molecules and by the use of specific inhibitors.

Results

CXCL12, IL-3, and IL-7 had only marginal effects on ALL cell survival under serum-free conditions. However, these molecules individually induced significant proliferative responses in stromal cultures of 11 cases of ALL. The combination of CXCL12 with IL-7 or IL-3 produced a variety of responses, with clear synergistic or additive interactions observed in four cases. Synergistic proliferation in response to CXCL12 plus IL-7 was associated with enhanced phosphorylation of the mitogen-activated protein kinases, ERK-1/2 and p38, and AKT, and was partially inhibited by pretreatment of cells with inhibitors for p38 MAPK and phosphatidylinositol 3–kinase, implicating these pathways in the proliferation in response to IL-7 plus CXCL12.

Interpretation and Conclusions

These findings indicate a complex interaction between signaling from the CXCR4 receptor on ALL cells with those initiated by the cytokines IL-7 and IL-3, suggesting that CXCL12 may facilitate ALL proliferation by enhancing cytokine-signaling pathways in responsive cases.

Key words: interleukin-7, interleukin-3, CXCL12, proliferation, B lineage acute lymphoblastic leukemia.

Haematologica 2007; 92:450-459

B-cell progenitor acute lymphoblastic leukemia (ALL) results from the clonal expansion of immature B lineage lymphoid cells. Knowledge of factors contributing to this expansion is central to understanding the pathogenesis of this disease and for the development of new therapeutic strategies. Despite the deregulated expansion of these cells *in vivo*, the leukemic cells from the majority of patients remain highly reliant on the bone marrow microenvironment for their survival and proliferation *in vitro*.[1,2] Cytokines including interleukin (IL)-7, IL-3, stem cell factor and Flt-3 ligand have been examined for their contribution to the proliferation and survival of pre-B ALL cells in culture.[3-6] However, none can substitute for bone marrow stromal support and it is likely that combinations of these and other factors are required for optimal *in vitro* growth and survival. CXCL12 (also known as stromal-derived factor-1 or SDF-1) is a chemokine expressed by a large range of tissues including lymph nodes, lung, brain, kidney, pancreas, spleen, and, within the bone marrow, by stromal cells, osteoblasts and endothelial cells.[7-10] CXCL12 is an essential pre-B-cell growth factor, which is important for the growth and survival of normal and malignant B lineage hematopoietic cells.[11-13] CXCL12 can also enhance the proliferation of normal hematopoietic stem cells and myeloid progenitors *in vitro*, particularly when used in combination with cytokines such as granulocyte-macrophage colony-stimulating factor, thrombopoietin and stem cell factor.[14-18] Interestingly, CXCL12 inhibits the proliferation of normal hematopoietic stem cells in the presence of bone marrow stroma *in vitro* and *in vivo*.[19] We have previously demonstrated, using highly potent and specific antagonists, that CXCL12 makes a significant contribution to the stromal-dependent proliferation of ALL cells *in vitro* without significantly influencing cell survival.[13] Others have reported that CXCL12 in stroma-conditioned medium promotes ALL cell survival in the absence of direct stromal contact.[20] Whether CXCL12 can induce ALL cells to undergo enhanced proliferation in response to lymphoid cytokines, such as IL-7, is not known. Synergistic proliferative effects observed between CXCL12 and myeloid cytokines in the absence of stroma is associated with enhanced phosphorylation of extracellular stress-regulated kinase (ERK1/2), ribosomal S6 kinase (p90RSK) and AKT.[21] IL-3 and IL-7 can also signal through ERK1/2 and AKT, suggesting that synergistic interactions may occur.[22] In this study we examined the potential of CXCL12 in combination with the cytokines IL-7 and IL-3 to promote the survival and proliferation of ALL cells in the absence of serum and evaluated the signal transduction pathways involved.

Design and Methods

Antibodies and reagents

The following monoclonal antibodies were purchased: anti-CXCR4-PE (12G5), anti-CD19-APC (SJ25C1), anti-CD19-PE (4G7), anti-IL-7 receptor (hIL-7R-M21), anti-IL-3 receptor-PE (7G3) and anti-Ki67 (BD Pharmingen, Sydney, Australia); anti-Bcl-2 (BD Biosciences, San José, CA, USA); anti-phospho-ERK, anti-ERK, anti-phospho-AKT, anti-AKT, (Cell Signaling Technologies, Beverly, MA, USA); horse radish peroxidase (HRP)-conjugated swine anti-rabbit immunoglobulins (DAKO, Denmark) and HRP-conjugated goat anti-mouse immunoglobulins (Sigma, St Louis, MO, USA). All antibodies were used as recommended by the manufacturer. CXCL12· was purchased from Peprotech (Rocky Hill, NJ, USA) or from Mr. Philip Owen (University of British Columbia, Canada); and IL-7 from Chemicon International (Temecula, CA, USA) and annexin V-fluoroscein isothiocyanate (FITC) from Becton Dickinson (Sydney Australia). TC14012 was synthesized by Mimotopes (Clayton, VIC, Australia). Inhibitors of the following signaling molecules were purchased from Calbiochem-Merck KgaA, Darmstadt, Germany: the MEK inhibitor (PD98059), the PI-3K inhibitor (LY294002), the p38 MAPK inhibitor (SB203580) and its control (SB202474).

Cells

Bone marrow or peripheral blood samples were collected from 17 patients with ALL at the time of diagnosis with informed consent under institutional ethics committee guidelines. Details of the patients' samples are provided in Table 1. Mononuclear cells were separated by density gradient centrifugation and cryopreserved as previously described.[23] Bone marrow mesenchymal (BMM) cells were grown from normal bone marrow mononuclear cells as previously described.[23] A bone marrow stromal cell line transformed with hTERT, here termed hTERT.BMS, was a kind gift of Dr D. Campana (Memphis, TN, USA)[24] and was cultured in RPMI containing 10% fetal calf serum.

Cell Culture

ALL cells were cultured in AIM-V serum free medium and were plated at 10^5-10^6 cells/mL. Confluent stromal layers consisting of hTERT.BMS or BMM cells, which had been irradiated with 30 Gy from a cesium source 7 days prior to plating ALL cells, were present where indicated. hTERT. BMS and BMM cells were washed with AIM-V medium prior to plating ALL cells and cultures containing hTERT.BMS or BMM were harvested using trypsin/EDTA as previously described.[25] Cytokines, CXCL12 and the CXCR4 antagonist TC14012 were added at the following concentrations where indicated: IL-7 (50 U/mL), IL-3 (20 U/mL), CXCL12 (200 ng/mL) and TC14012 (50 μM). Cultures were maintained for 2 to 4 days as specified in a humidified atmosphere containing 5% CO_2 at 37°C. Where indicated cells were cultured with inhibitors of signaling molecules: PD98059 (40 μM), LY294002 (6.65 μM), SB203580 (10 μM) and its control SB202474 (10 μM) for 1 h at 4°C prior to the addition of cytokines and/or CXCL12.

Table 1. Patients' information.

Patient ID	Age/Sex	% Blasts	BM/PB	Immunophenotype	Cytogenetics	%Viability
1786	12/F	90	BM	$CD34^-CD19^+CD10^+CD20^+$	No metaphases	87±4
1809	12/M	94	BM	$CD34^-CD19^+CD10^+CD20^+$	46XY,del(4)(q21q25),-9,add(13) (q14,+add(22)(p13)[9]46XY[11]	90±5
1793	16/M	91	BM	$CD34^-CD19^+CD10^+CD20^-$	46XY	98
1778	10/M	98	PB	$CD19^+CD10^+CD20^-$	46XY,del(13)(q12q14)[5] /46XY[13]	77±4
1848	3/F	96	BM	$CD34^-CD19^+CD10^+CD20^+$	46XX	90±2
1797	2/M	93	BM	$CD34^-CD19^+CD10^+CD20^+$	Hyperdiploid	96±1
0502	4/F	96	BM	$CD34^-CD19^+CD10^+$	Hyperdiploid	87
0426	1/F	90	BM	$CD34^-CD19^+CD10^-$	46XX,t(9,11)(p21;q23)	87
1877	7/M	82	BM	$CD34^-CD19^+CD10^+CD20^+$	46XY	97
0557	4/M	97	BM	$CD34^-CD19^+CD10+CD20^-$	Hyperdiploid	90±3
1817	15/M	96	BM	$CD34^-CD19^+CD10^+CD20^-$	N/A	70±12
1784	6/M	67	BM	$CD34^-CD19^+CD10^+CD20^-$	Hyperdiploid	88±8
1688	5/M	90	BM	$CD34^-CD19^+CD10^+CD20^+$	46XY	86±6
1802	4/M	92	BM	$CD34^-CD19^+CD10^+CD20$-	46XY	79
1870	12/M	96	BM	$CD34^-CD19^+CD10^+CD20^+$	46XY	90
1883	2/F	98	BM	$CD34^-CD19^+CD10^+CD20^+$	47XX,t(1;19)(q23;p13),+8,der (13)t(1;13)(q23;q14)[7]46XX[8]	89
1901	4m/F	100	PB	$CD34^-CD19^+CD10^+CD20^-$	46XX,del(12)(p11.2p13) [6] 46XX[14]	91
0407	N/A	99	BM	$CD34^-CD19^+CD10^+CD20^-$	N/A	94

N/A: not available; BM: bone marrow; PB: peripheral blood. Viability given is that obtained on thawing cryopreserved samples. When this was done on more than one occasion the mean and standard deviation of repeat samples are shown.

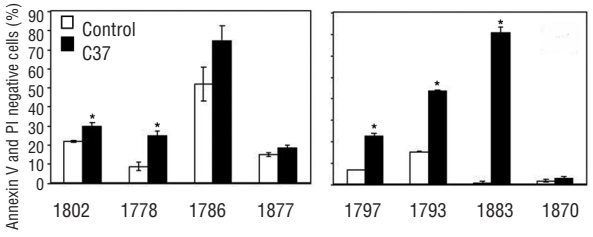

Figure 1. Effect of CXCL12, IL-7 and IL-3 on ALL cell viability. ALL cell viability after 4 days of culture in the absence of stroma with media alone (Control) or with CXCL12, IL-3 and IL-7 (C37) as determined by the absence of annexin V and PI fluorescence. Bars indicate the standard deviation of duplicate determinations. *$p<0.05$.

Flow cytometry

ALL cells were labeled with directly conjugated monoclonal antibodies or unlabeled primary antibodies followed by sheep anti-mouse FITC as previously described.[26] For intracellular staining, cells were fixed first in IntraPrep fixation reagent (Beckman Coulter, Sydney, Australia) for 15 minutes, washed with phosphate-buffered saline, and then treated with the IntraPrep permeabilization reagent (Beckman Coulter) for 10 minutes. Both steps were performed at room temperature. Intracellular, antigen-specific antibodies were then used for labeling. Cells were analyzed on a FACSCalibur flow cytometer.

Viability and proliferation assays

The cell recovery of viable ALL cells was assessed by flow cytometry after 4 days of culture in serum-free conditions in the presence or absence of stromal support. Recovered cells were labeled with CD19-APC, annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions. Viable ALL cells were identified by forward and side scatter properties, CD19 expression and the absence of annexin V and PI staining. In some experiments viable cell recovery was enumerated using the above labeling strategy with the addition of True Count beads (BD Pharmingen). Proliferation was assessed by ^{3}H-thymidine incorporation as previously described.[13] Briefly, ALL cells were plated in quadruplicate in a 96-well format and 1 μCu of ^{3}H-thymidine (Pharmacia Amersham, Sunnyvale, CA, USA) was added per well on day 4 and incubated overnight at 37°C. Cells were harvested onto glass fiber filters (Packard, Meriden, CT, USA) and analyzed on a TopCount plate reader.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was extracted from 5-10×10^6 cells using 1 mL of Trizol reagent (Invitrogen, Grand Island, NY, USA) and cDNA synthesized using oligo dT primers and the reverse transcriptase AMV (Promega, Madison, WI, USA). PCR amplification was performed on a Hybaid thermal cycler PCR machine (Hybaid Research, Australia) using 2.5U of Taq polymerase (Promega) in a 50 μL reaction and the following specific primers: CXCL12;[27] IL-7,[28] Flt-3L forward[29] and Flt-3L reverse,[30] and GAPDH forward (acgcatttggtcgtattggg) and reverse (tgattttggagggatctcgc).

Western blotting

Western blotting was performed as previously described.[31] Briefly, between 15 and 40×10^6 ALL cells, in RPMI containing 0.5% bovine serum albumin, were stimulated with CXCL12 (100 ng/mL) and/or IL-7 (50 U/mL) for the specified time periods at 37°C. Cell pellets were lysed in 10 mM Tris, 150 mM NaCl, pH 7.5 containing 1 mM EDTA, 2 mM Na_3VO_4, 2 mM Na_3VO_4, 10 mM NaF, 1% Triton X-100 and protease inhibitors and lysates clarified by centrifugation. Equal amounts of protein were loaded in each lane of a 7.5% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel and

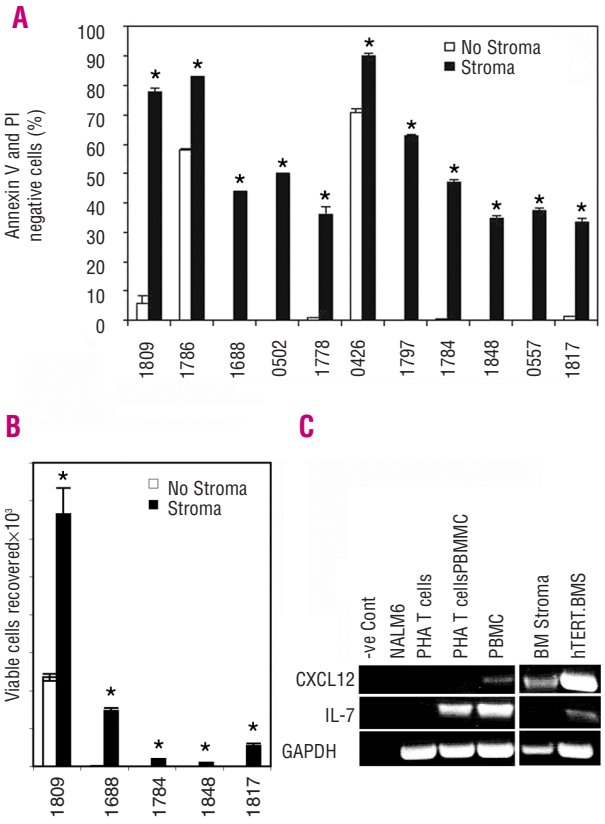

Figure 2. Stromal support of ALL cell viability. (A). Viability of ALL cells was determined after 4 days in culture in the presence or absence of stromal support. Viable ALL cells were identified by CD19 expression and the absence of annexin V fluorescence and the absence of PI staining. Patients' ID numbers are indicated. Bars indicate the standard deviation of duplicate determinations. *$p<0.05$. (B). Numbers of viable cells recovered from culture of five of the samples shown in (A). (C). Expression of cytokines and chemokines by bone marrow (BM) stroma and hTERT.BMS. RT-PCR for CXCL12 and IL-7 was performed on RNA isolated from the pre-B ALL cell line NALM6, phytohemagglutinin (PHA)-stimulated T cells, peripheral blood mononuclear cells (PBMC), BM stroma and hTERT.BMS. GAPDH expression was assessed as a positive control for each cDNA. Results obtained in the absence of cDNA are shown in the negative control (–ve Cont) lane.

transferred onto nitrocellulose. Phosphorylated and total proteins were detected sequentially on the same membrane using specific primary antibodies, secondary antibodies conjugated to horseradish peroxidase and enhanced chemiluminescence (Perkin Elmer, Boston, MA, USA). Bands were quantitated by densitometry.

Statistics

The ^{3}H-thymidine proliferation data were analyzed using the statistics software package SPSS for Windows, Version 11. A significance level of 5% was used throughout. In order to stabilize the variance, the count per minute data were square root transformed. A two-way analysis of variance was used to investigate the effects of the chemokine CXCL12 and the cytokine IL-7 in the co-culture experiments with stroma.

Results

CXCL12 and cytokine combinations do not maintain the viability of ALL cells *in vitro*. ALL cells undergo rapid apoptosis in the absence of stromal support[1] and CXCL12 has been reported to be an important component of stromal cell conditioned medium that inhibits ALL cell death.[20] We have previously reported that the inhibition of CXCL12 in stromal cultures does not significantly affect ALL cell survival.[13] However, it is possible that in these cultures other factors, particularly those involving direct ALL cell/stromal cell contact, compensate for the absence of CXCL12. Nishii *et al*. reported that purified CXCL12 enhances ALL cell survival,[20] so we examined the effect of CXCL12 when used alone in the absence of stroma and assessed viability by trypan blue exclusion. We did not detect any significant improvement in viable cell recovery in the presence of CXCL12 when used as a single agent, or in combination with the cytokines IL-7 and IL-3 (mean viability of 18.8±20.5 for cells in AIM-V, 20.7±18.9 for cells in CXCL12 alone, 21.2±24.3 for cells in CXCL12 and IL-3, and 18.1±24.1 for cells in CXCL12 and IL-7, n=9). This suggests that CXCL12 alone or the combination of CXCL12 and the cytokines tested does not enhance ALL cell survival in culture. In order to investigate this more closely, we examined eight cases for their survival in the presence of CXCL12, IL-3 and IL-7 using PI and annexin V staining to identify dead and dying cells. Although five of the eight cases (1802, 1778, 1797, 1793 and 1883) demonstrated a statistically significant increase in viability in the presence of CXCL12, IL-3 and IL-7 (Figure 1), in three of these five cases less than 10% of cells survived. Overall it appears that CXCL12 does little to enhance the survival of ALL cells in culture, even when used in combination with IL-7 and IL-3.

ALL cells proliferate in response to CXCL12 and IL-7 or IL-3 when supported by stroma

To examine the role of CXCL12 in ALL cell proliferation under conditions supportive of ALL cell viability, 11 ALL cases were cultured on a human stromal cell line, hTERT-BMS, and one on BMM, in the absence of serum for 4 days. Stromal support enhanced the viability of ALL cells from 12.4±25.9% (range, 0.0%-70.7%) to 54.1±20.8% (range, 33.2-90.1%, $p<0.001$, n=11) (Figure 2A). The change in the percentage of viable cells recovered was not due to the removal of dead cells by stromal cells, as the total viable cells recovered from stroma-free cultures was reduced by more than 65% as compared to the mumber recovered from stroma-supported cultures (Figure 2B). This demonstrates that stromal support results in a genuine increase in the number of viable cells recovered from the cultures, a finding consistent with previous reports.[1] Human bone marrow stroma and hTERT.BMS produce significant amounts of CXCL12

(Figure 2C), and therefore TC14012, a potent and highly specific CXCL12 antagonist, was used to determine the contribution of CXCL12 to the proliferation of ALL cells in the presence of stromal support. All cases examined displayed CXCL12-dependent proliferation on stroma, with TC14012 inhibiting proliferation by an average of 44.7±20.4% (range, 13.5-79.0%, p=0.03 compared to control) (Figure 3). This is consistent with our previous findings[13] confirming the important role of CXCL12 in ALL cell proliferation. Baseline proliferation was considered to be that observed in the presence of TC14012, where the effects of stromal-derived CXCL12 are inhibited. Using this system, all cases of ALL examined showed increased proliferation in response to CXCL12, 58% (cases 1786, 1809, 0502, 1784, 1778, 0426 and 1901) in response to the addition of IL-7, and 25% (cases 1784, 1778 and 0426) in response to additional IL-3. When combinations of CXCL12 and cytokines were tested, 75% of samples (cases 1817, 1778, 0502, 1797, 1688, 0426, 1809, 1786, and 1901) responded to CXCL12 and IL-7, and 58% (cases 1817, 0502, 1797, 0426, 1778, 1809 and 1901) to CXCL12 and IL-3 (Figure 3). In contrast, in cases 1784, 0557 and 1848 the addition of IL-7 or IL-3 antagonized the proliferative effects of CXCL12, and in case 1786 only IL-3 had this effect. In cases in which no response to IL-7 or IL-3 was observed, this was not due to the absence of IL-7 (IL-7R) and IL-3 (IL-3R) receptors as their presence was confirmed by flow cytometry (Figure 4A and *data not shown*). When the actions of CXCL12 were blocked by TC14012 there was no correlation between IL-7R or IL-3R expression and the response of the ALL samples to IL-7 or IL-3 respectively (Figure 4B). Similarly when no TC14012 was added, there was no correlation between CXCR4 or IL-3R expression and proliferative responses to their respective agonists. However, in the presence of CXCL12 (no added TC14012) there was a significant correlation between IL-7R expression and the proliferative response of ALL cells to IL-7 (p=0.001) (Figure 4C). This suggests that CXCL12 is required for optimal responses to IL-7 by the IL-7 receptor.

In order to determine the nature of the interaction between CXCL12 and IL-7 or IL-3, we performed a two-way analysis of variance. True synergy between CXCL12 and IL-7 was observed in cases 1809 and 1817 and 1901 and between CXCL12 and IL-3 in case 1809. An additive response between CXCL12 and IL-7 was also detected in case 1778 and between CXCL12 and IL-3 in cases 1817 and 1778 (Figure 3). These results highlight the essential role of CXCL12 in the proliferation of ALL on stroma. They also demonstrate a complex pattern of interaction between the signaling pathways initiated by CXCL12 and IL-7 and IL-3, with additive or synergistic interactions between CXCL12 and IL-7 or IL-3 in promoting the proliferation of ALL cells in some cases, but antagonistic effects observed in other cases.

CXCL12 enhances signaling through PI-3K, p38MAPK and AKT by IL-7

Since we had limited numbers of cells for performing western blots, and as IL-7 more frequently produced stimulatory interactions with CXCL12, we limited our study of signaling events to the combination of CXCL12 and IL-

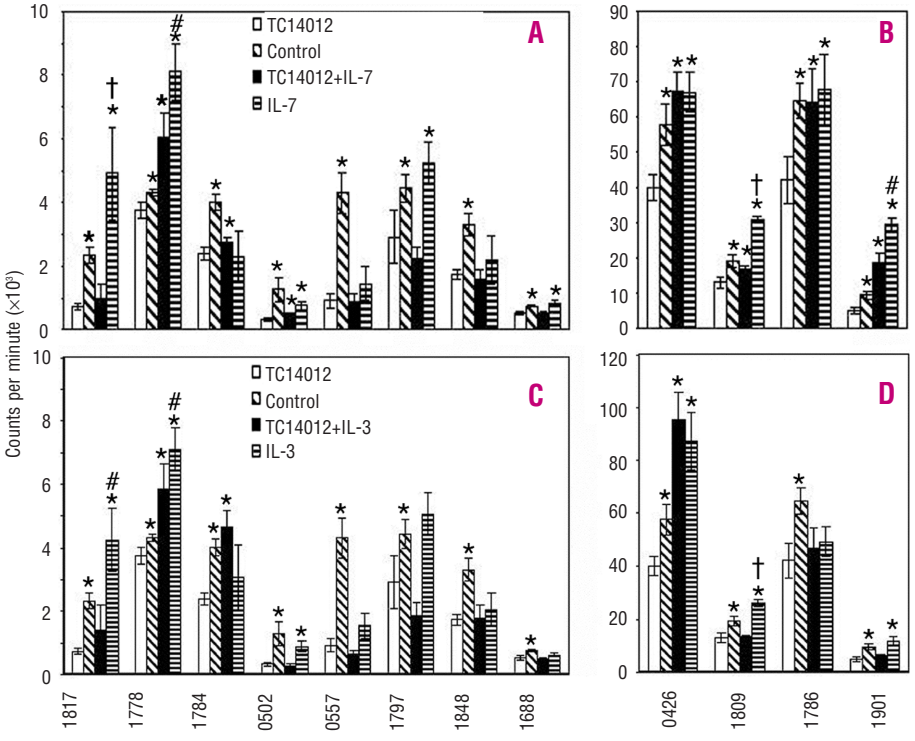

Figure 3. Proliferative responses of ALL cells to CXCL12 and/or IL-7 in the presence of stromal support. ALL cells were cultured on stroma in the absence or presence of CXCL12, IL-3 and IL-7. Exogenous CXCL12 was not added to the cultures but the presence of CXCL12 was regulated by the addition of the specific CXCR4 antagonist TC14012. The proliferation was assessed after 4 days of culture by ^{3}H-thymidine incorporation. Data for IL-7 are shown in A and B and data for IL-3 in C and D. Data are shown on separate graphs for clarity due to the variability of counts per minute (cpm) observed between individual cases. Bars represent the standard deviation of quadruplicate determinations. *p<0.05 indicating an increase in proliferation, # indicating an additive effect of CXCL12 and IL-7 or IL-3 on proliferation and † indicating a synergistic effect of CXCL12 and IL-7 or IL-3 on proliferation.

7 in cases in which the combination produced additive or synergistic effects. CXCL12 activates signaling through the PI-3K/AKT, p38MAPK and MEK/ERKs pathways in ALL.[31] IL-7 is also known to activate the PI-3K pathway in normal human B-cell precursors.[22] We examined whether there was evidence of synergistic or additive phosphorylation of AKT, p38MAPK or ERK when ALL cells were treated with CXCL12 and IL-7 in combination. These experiments were performed on three cases for which sufficient cells for western blotting were available. In case 1901 we had obtained an exceptionally large sample from the patient at diagnosis, while cells from cases 1786 and 1809 were successfully expanded *in vitro* in the presence of bone marrow stromal support. As we have previously reported, AKT, p38MAPK and ERK were all phosphorylated following CXCL12 treatment, with maximum effect occurring after 2 to 5 mins in all three cases examined for AKT and ERK, and between 2 and 10 minutes for p38 MAPK. IL-7 also induced phosphorylation of AKT in two of the three cases, p38 MAPK in all three cases and ERK in one of the three cases (Figure 5 and *data not shown*). The combination of IL-7 and CXCL12 resulted in clear synergistic effects on the phosphorylation of ERK, p38 MAPK and AKT in case 1901. The combination also produced at least additive effects on ERK and p38MAPK (1809) or AKT (1786) phosphorylation in the remaining two cases. These data suggest that CXCL12 and IL-7 synergize to enhance proliferation by cooperative signaling events in responsive cases.

Signaling through p38 MAPK and PI-3K provides the principal proliferative signals to pre-B ALL cells in response to IL-7 and CXCL12

Our results demonstrating evidence of enhanced proliferation in response to IL-7 and CXCL12 and enhanced phosphorylation of AKT, ERK and p38 MAPK suggested the possibility of cross-talk between IL-7 and CXCL12. In order to investigate pathways involved in synergistic proliferation to IL-7 and CXCL12 we evaluated the proliferative responses of four cases (1786, 0407, 1901 and 1809), with case 1786 being expanded *in vitro* on stroma to generate sufficient cells. As can be seen in Figure 6B this case demonstrated synergistic proliferation in response to IL-7 and CXCL12 following *in vitro* expansion. Cells were pretreated with inhibitors selective for MEK (PD98059), PI-3K (LY294002) or p38 MAPK (SB203580) for 1 hour prior to assessment of proliferation by ^{3}H-thymidine incorporation. The specific control compound for SB203580, SB202474, was also included when appropriate. DMSO controls were used for PD98059 and LY294002. LY294002 (n=4), SB203580 (n=3) and PD98059 (n=4) inhibited proliferation in response to CXCL12 alone in all cases tested (Figure 6A and B; control). LY294002 (n=4), SB203580 (n=3) and PD98059 (n=4) also inhibited proliferation in response to IL-7 alone, (i.e. when CXCL12 was blocked by TC14012) (Figure 6A and B; TC14012 + IL-7). Similarly

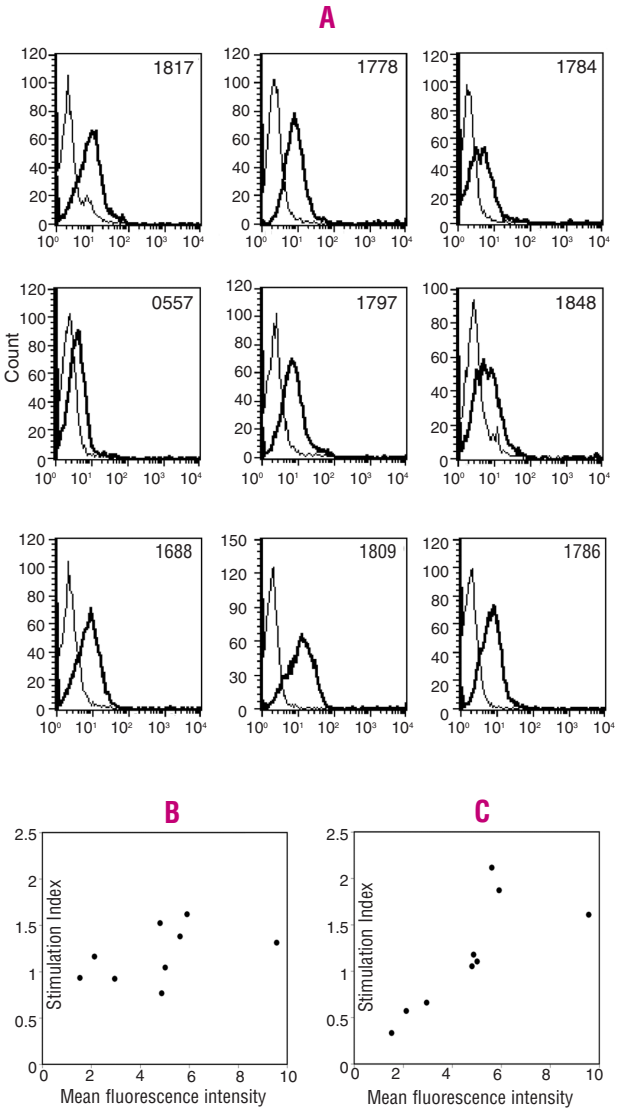

Figure 4. Correlation between IL-7R expression and the response of ALL to IL-7 requires the presence of CXCL12. (A) Expression of IL-7R by ALL cells as determined by flow cytometry. Heavy lines represent staining for IL-7R and thin lines that for the isotype control. (B) The correlation between IL-7R expression and the response to IL-7 in the presence of TC14012 to block CXCL12 binding to CXCR4. (C) The correlation between IL-7R expression and the response to IL-7 in the presence of CXCL12 (i.e. no addition of TC14012). The proliferative responses are given as a stimulation index relative to cells cultured in identical conditions but in the absence of IL-7.

the synergistic proliferation observed when CXCL12 and IL-7 were both present was inhibited by LY294002 (n=4) and SB203580 (n=3), but PD98059 inhibited this proliferation in only three of the four cases (Figure 6A and B; IL-7). We also examined the effect of these inhibitors on the combination of IL-7 and CXCL12 in the absence of stromal support in case 1809, which demonstrated a strong proliferative response to IL-7 and CXCL12 in the absence of stroma (8494±390 cpm in control cultures compared to 26723±1991 cpm in cultures treated with IL-7 and CXCL12). The proliferative response to the combination of CXCL12 and IL-7 was inhibited by LY294002 (100%

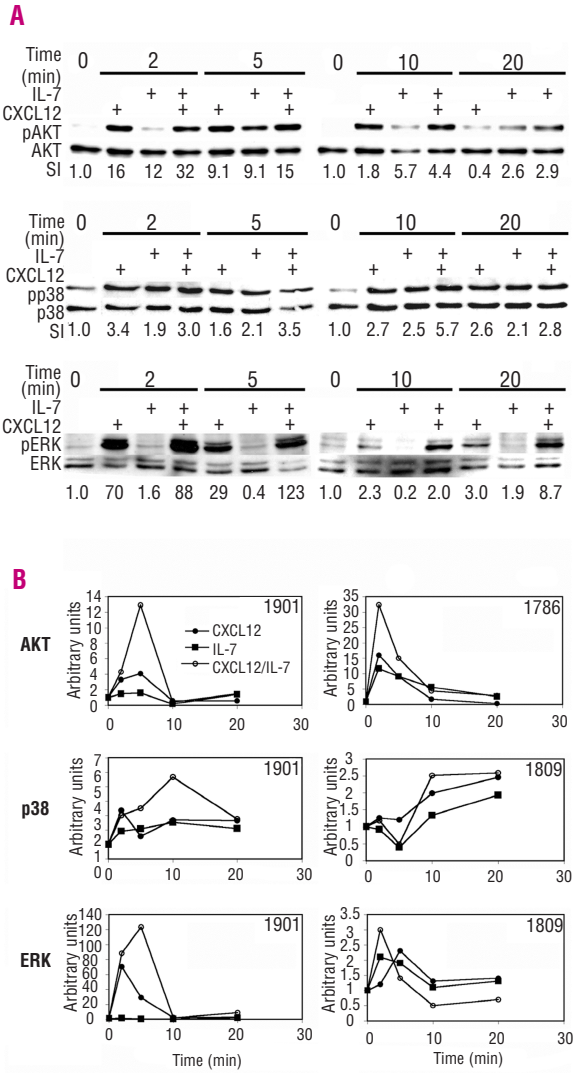

Figure 5. Phosphorylation of p38, AKT and ERK in ALL cells treated with CXCL12, IL-7 or the combination of both agents. Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with antibodies to phosphorylated ERK, p38 MAPK or AKT and subsequently stripped and reprobed with antibodies to total proteins. The 2 and 5 min time points and 10 and 20 minute time points were run on separate gels. The 0 time point was included on both gels for normalization. (A) Western blots of phosphorylated and total AKT from patient 1786 and phosphorylated and total p38 MAPK and ERK from patient 1901 are shown. SI indicates the stimulation index, defined as the intensity of the band from the phosphorylated protein relative to that of the total protein and normalized to a value of 1 in unstimulated samples. (B) Quantitation of the phosphorylation of ERK, p38 MAPK or AKT in stimulated ALL cells. The signal from the phosphorylated form was corrected for loading. Patients' ID numbers are given on each graph.

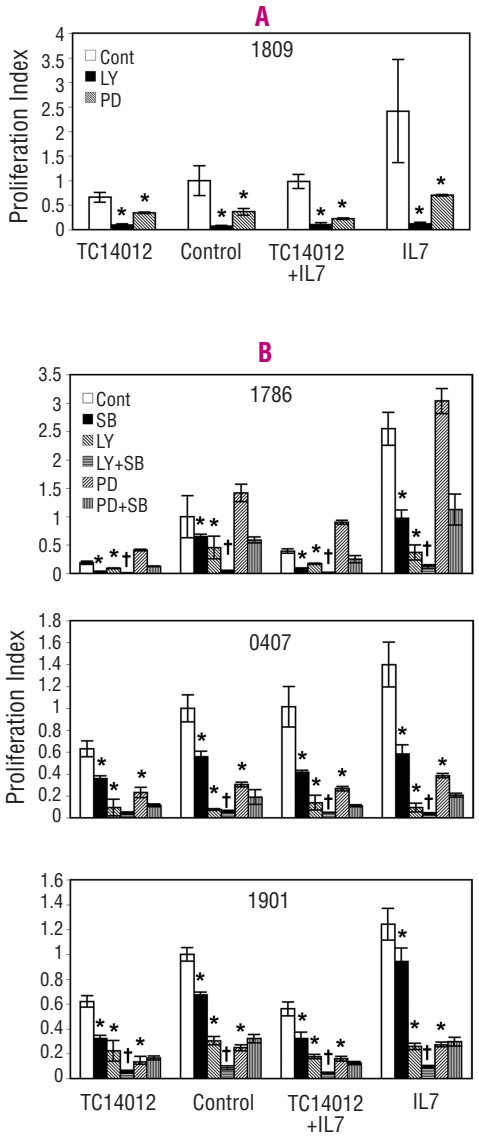

Figure 6. PI-3K and p38 MAPK provide the major proliferative signals from CXCL12 and IL-7. Cells from indicated cases were cultured on stroma in the presence or absence of IL-7 and/or CXCL12. Exogenous CXCL12 was not added to the cultures but the presence of CXCL12 was regulated by the addition of the specific CXCR4 antagonist TC14012. Cells were treated with the indicated inhibitors for 1 h prior to plating in proliferation assays. Proliferation was assessed by ^{3}H-thymidine incorporation on day 4 of culture. (A) inhibition of MEK and PI-3K, and (B) inhibition of MEK and PI-3K in combination with inhibition of p38 MAPK. The bars indicate the mean and standard deviation of quadruplicate determinations. The data shown are representative of at least two repeat experiments. *$p<0.05$ compared to cells cultured on stroma in the absence of inhibitors of signaling molecules. *$p<0.05$ compared to cells treated with SB203580 (SB), LY294002 (LY) and PD98059 (PD).

inhibition) and SB203580 (92% inhibition) while PD98059 had no inhibitory effect. The effect of combining inhibitors of cell signaling was also investigated in cases 1786, 0407 and 1901. The combination of PD98059 and SB203580 had no effect above that observed for SB203580 or PD98059 alone in two of the three cases, but was more effective in the remaining case (0407). However, the combination of LY294002 and SB203580 almost completely eliminated proliferative responses in all conditions in all cases examined (Figure 6B). The small residual responses suggest that other mechanisms may be involved. These data show that signaling through each of the activated pathways can play a role in the proliferative responses of ALL cells to CXCL12, IL-7 or their combination, but that signaling through PI-3K appears to be the most important, since inhibiting this kinase produced major reductions in proliferative responses in all cases.

Discussion

The dependence of the majority of ALL cells on bone marrrow-derived stromal factors for survival and proliferation has been known for over a decade. However, the precise nature of the factors responsible remains unclear. CXCL12 is a stroma-derived chemokine which, in addition to inducing chemotaxis, mediates the proliferation and survival of various normal and malignant hematopoietic cell types.[11,16,20,21] We have previously shown that CXCL12 causes stroma-dependent proliferation of ALL cells, using the highly potent and specific CXCR4 antagonists, AMD3100 and TC14012.[13] This contrasts with the effect of CXCL12 antagonists on normal hematopoietic stem cells in stromal cultures.[19] In this study we examined the interaction of CXCL12 with the cytokines IL-3 and IL-7, in the presence or absence of stromal support, in maintaining ALL cell viability and promoting proliferation. Although other investigators have reported that IL-3 and IL-7 stimulate ALL cell proliferation and survival, the responsiveness was not, overall, always convincing and varied considerably among patients and between studies.[3,4,32-36] In this study we used these cytokines with the addition of CXCL12 in serum-free cultures. In contrast to the work published by Nishii *et al*. we did not find that CXCL12 reliably enhanced ALL cell survival when used alone or in combination with IL-3 and IL-7.[20] The reason for this discrepancy is unclear, although Nishii *et al*. used serum in their experiments, which may have contained additional undefined factors contributing to ALL cell survival. One recent study reported that stromal layers resulted in decreased survival of ALL cells.[37] However, in agreement with a large number of preceding studies, we found that the presence of a stromal layer greatly enhanced the survival of ALL cells.[1,13,20,38,39] The factors responsible for stromal-dependent proliferation appear to include both contact and currently unidentified soluble factors.[1,39] Nishii *et al.* demonstrated that IL-7 and IL-3 could enhance the survival of ALL cells only when the cells were cultured in the presence of stroma,[20] but we were unable to confirm these results. We also found that IL-7 and IL-3 were capable of inducing ALL cell proliferation in the presence of a supportive stromal layer. The interaction between these cytokines and CXCL12 in influencing the proliferation of primary ALL cases was demonstrated to be complex. In some cases CXCL12 produced additive or synergistic effects when combined with IL-7 or IL-3, while in other cases antagonistic effects were observed. The reasons for the latter are unclear but IL-7 and IL-3 have been reported to induce differentiation and apoptosis in a subpopulation of ALL cells.[35,40] Synergistic interactions between CXCL12 and Flt-3 ligand, granulocyte-macrophage colony-stimulating factor, stem cell factor and thrombopoietin have been observed in normal and leukemic myeloid progenitors cultured in the absence of stroma, resulting in enhanced survival, chemotaxis and proliferation.[21,41] In these studies CXCL12-enhanced responses to cytokines were associated with augmented signaling through ERK/MEK and PI-3K/AKT pathways, although the causative role of this was not always confirmed. In this study we observed similar interactions between CXCL12 and IL-7 on phosphorylation of AKT and ERK proteins, as well as p38 MAPK, in ALL cells, suggesting that these pathways may underpin the effects on proliferation in these cells. A significant role for MEK/ERK signaling in the proliferative responses of ALL to the combination of CXCL12 and IL-7 could not be demonstrated in all cases. The effectiveness of the MEK inhibitor was confirmed by western blotting of phosphorylated ERK in CXCL12-stimulated cells (*data not shown*). This was surprising considering that MEK/ERK signaling is frequently associated with proliferative responses and is activated by CXCL12 in a number of cell types.[21,42,43] In contrast inhibition of signaling through PI-3K and p38 MAPK significantly inhibited the proliferation of ALL cells in response to the combination of IL-7 and CXCL12, suggesting that these pathways are the most important for the transmission of proliferative signals from these factors. The PI-3K pathway is known to be activated by IL-7 in normal B-cell progenitors and has recently been shown to be essential for IL-7-mediated proliferative responses in T-ALL cells, while no role for MEK/ERK could be demonstrated.[22,44] Similarly activation of p38 MAPK plays a significant role in T-cell proliferation in response to IL-7,[45] but conversely p38 MAPK is also activated following IL-7 withdrawal, and has been linked to cell cycle arrest due to degradation of Cdc25A[46] and cell death.[47] This study has confirmed that CXCL12 is indeed a major stromal factor involved in regulating ALL cell biology. However the effects of CXCL12 on ALL cell survival appear to be marginal, and the mechanisms responsible for maintaining ALL viability, particularly in contact with stromal cells, remain largely unknown. Despite this, it is clear that CXCL12 can interact with cytokines in the presence of stromal support to modulate ALL proliferation. It is likely that *in vivo* responses to CXCL12 and cytokines, where cell survival is optimal, will be found to be even greater and more frequent than those observed here, and that disrupting proliferative signals, such as those mediated through p38 MAPK and PI-3K, may provide new therapeutic strategies for B-cell progenitor ALL.

Authors' Contributions

JJ performed most of the in experimental work examining ALL cell survival and proliferation and some of the western blotting; RB performed the majority of the western blotting work; SG performed the proliferation experiments using the inhibitors of cell signaling; KB gave significant intellectual input to the project and assisted with editing the manuscript; LB wrote the paper and supervised the researchers and students undertaking the laboratory work. She also made a significant intellectual input to the project.

Conflict of interest

The authors reported no potential conflicts of interest.

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