Chemokine receptor expression in EBV-associated lymphoproliferation in hu/SCID mice: implications for CXCL12/CXCR4 axis in lymphoma generation

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The mechanisms by which intraperitoneal injection of peripheral blood mononuclear cells (PBMCs) from Epstein-Barr virus (EBV)–seropositive donors into severe combined immunodeficient (SCID) mice gives rise to lymphomas (hu/SCID tumors) are far from clear. This study addressed whether chemokine receptors and their ligands could be implicated in this experimental model. CXCR4 was found to be highly expressed in hu/SCID tumors; surface expression of CXCR4 was prevalently limited to a tumor cell subset poorly expressing CD23, whereas the CXCR4 ligand, CXCL12, was predominantly expressed by the tumor subpopulation expressing CD23. In vitro inhibition of this autocrine/paracrine CXCL12/CXCR4 axis significantly inhibited lymphoma proliferation and survival. Furthermore, CXCL12 was expressed in cells recovered from the mouse peritoneal cavity early after PBMC transfer as well as by EBV-transformed B cells but not by resting or activated B lymphocytes; also, lymphoma development was associated with a dramatic increase in the levels of murine CXCL12 present in the peritoneal cavity. Finally, antagonizing the CXCL12/CXCR4 axis in vivo strongly counteracted lymphoma development. These studies demonstrate that CXCL12 expression may be associated with EBV infection and suggest that the CXCR4/CXCL12 axis may participate in the EBV-associated lymphomagenesis process in immunodeficient hosts. (Blood. 2005;105:931-939)

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Introduction

Epstein-Barr virus (EBV) is implicated in the pathogenesis of at least 3 malignant disorders of B cells: Burkitt lymphoma, Hodgkin disease, and B-cell lymphoproliferative disease (BLPD) seen in immunosuppressed allograft recipients.1 The severe combined immunodeficient (SCID) mouse, which lacks functional B and T cells, is a suitable animal model for the study of EBV-associated lymphoproliferation.3,4 SCID mice injected intraperitoneally with peripheral blood mononuclear cells (PBMCs) from EBV-positive donors develop EBV-positive human B-cell lymphomas (hu/SCID tumors), which display a surface phenotype closely resembling that seen in BLPD with low expression of EBV latent proteins and presenting a mixture of lymphoblastoid and plasmacytoid cells.5 The events occurring in the peritoneal cavity of PBMC-injected mice are very complex and entail T-cell activation and massive cytokine production, both of which favor the expansion of B lymphocytes, including EBV-positive B-cell precursors. In this regard, we showed that the presence of T lymphocytes within the cellular inoculum is necessary for lymphoma development, and treatment of PBMC-injected mice with cyclosporin A paradoxically counteracts tumor generation.7,8 Recently, we also demonstrated that no EBV reactivation occurs during the lymphomagenesis process, and tumor masses originate from the expansion of the very few latently infected B lymphocytes present in the inoculum.9

Hu/SCID tumors consist of multiple masses at the hepatic hilus, lower splanchnic region, and within the mesenteric tissue, the privileged sites of primary and/or metastatic localization being peritoneum, liver, and lymph nodes. Interestingly, all these organs have been reported to produce high levels of the CXCR4 ligand (CXCL12, previously called stromal cell–derived factor-1 [SDF-1])10,11; indeed, no tumor development occurs when PBMCs from EBV-positive donors are injected by other routes, suggesting a critical role of the peritoneal environment for lymphomagenesis. In this regard, it has recently been shown that CXCL12, produced by mesothelial cells lining the peritoneum, is involved in the persistence of peritoneal B lymphocytes in mice11; indeed, CXCR4 is expressed on mature B cells.12

In view of these observations, we addressed whether chemokine receptors and their ligands could be implicated in EBV-mediated lymphomagenesis in this experimental model. Aims of our study were (1) to define the chemokine receptor expression profile of lymphoblastoid cell lines (LCLs) and hu/SCID tumors; (2) to assess whether chemokines were differentially expressed following cellular transformation by EBV; and (3) to evaluate whether any of the latter could contribute to the outgrowth of EBV-transformed B cells in the immunocompromised host.
We report here that lymphoma development in PBMC-injected SCID mice is associated with down-regulated expression by EBV-transformed B cells of the chemokine receptors CCR6, CCR7, and CXCR5, while CXCR3 and CXCR4 are expressed at moderately high levels. We show that most hu/SCID tumors express functional CXCR4 receptors and that CXCL12 affects several biologic responses, including migration, adhesion, proliferation, and invasion. In adjunct, we demonstrate that CXCL12 expression may be related to immortalization of B cells by EBV. We also demonstrate that the hu/SCID tumor subset poorly expressing CD23 expresses functional CXCR4; meanwhile, the tumor subset expressing CD23 is the main producer of CXCL12, suggesting a potential chemotactic loop between these 2 cell populations. Finally, antagonizing the CXCR4/CXCL12 axis through the use of a neutralizing anti-CXCL12 antibody (Ab) or a CXCR4 antagonist was able to counteract lymphoma growth. Because CXCR4 is expressed in the human counterpart of hu/SCID tumors,13,14 these observations may be relevant to the human setting and open new avenues to therapeutic approaches in B-cell non-Hodgkin lymphomas.

Materials and methods

Isolation of PBMCs and B-cell purification

PBMCs were isolated by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation as reported elsewhere,15 washed 4 times with RPMI medium, counted, and used as such for establishment of LCLs and inoculation of SCID mice or separated further. LCLs were generated and maintained as previously reported.16 Purified B cells were obtained from PBMCs by positive selection using magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer’s recommendations.17 Recovered B cells were of 95% to 98% purity, as determined by flow cytometric analysis. B-cell cultures were set up as previously described.18

Generation of human B-cell tumors in SCID mice

SCID mice were purchased from Charles River (Wilmington, MA) and maintained in our animal facilities under pathogen-free conditions. Procedures involving animals and their care conformed with institutional guidelines that comply with national and international laws and policies (European Community EEC Council Directive 86/609, OJ L 358, December 12, 1987). Groups of 7- to 9-week-old SCID mice were injected intraperitoneally with 70 to 100 × 10⁶ unfractionated PBMCs, observed daily for signs of illness, and killed by cervical dislocation when necessary for experimentation. The animals were treated with intraperitoneal injections of a polyclonal goat anti-human CXCL2 Ab (kindly provided by Prof Robert M. Strieter, UCLA, Los Angeles, CA; 50 μL per mouse per injection); 500 ng of either human or murine CXCL12 in PBS. The animals were treated with intraperitoneal injections of a polyclonal goat anti-human CXCL2 Ab (kindly provided by Prof Robert M. Strieter, UCLA, Los Angeles, CA; 50 μL per mouse per injection); 500 ng of either human or murine CXCL12 in PBS.

In vitro adhesion assays

Adhesion of hu/SCID tumor cells to human umbilical vein endothelial cells (HUVECs) and fibronectin was assayed as previously described,20 with minor modifications. Briefly, hu/SCID tumor cells (1 × 10⁶) were labeled with the fluorescent dye calcein acetoxymethyl ester (calcine-AM; Molecular Probes) and were subsequently added to 30 to 60 minutes to 96-well culture plates covered by HUVECs, bovine serum albumin (BSA; 40 μg/mL; Sigma, St Louis, MO), or fibronectin (10 μg/mL). After the incubation period, the nonadherent cells were removed by vigorously washing the wells with RPMI 1640 medium (Life Technologies, Grand Island, NY) 3 times. Adherent cells were collected and counted by flow cytometry. A gate was set using the calcein labeling of the lymphoma cells to exclude HUVECs from the counts. Results were expressed as percentage of adherent cells (ie, number of adherent cells per number of input cells).

For inhibition studies, hu/SCID tumor cells were preincubated with a neutralizing anti-CXCL12 Ab (1:100) or 1 μM TC14012, a CXCR4-specific inhibitor,24 for 30 minutes at 37°C before being plated in HUVEC- or fibronectin-coated wells.

Chemotaxis and chemoinvasion assay

Migration and invasion were assayed in modified Boyden chambers (Neuro Probe, Gaithersburg, MD) using 13-mm polycarbonate filters of 8-μm pore size as previously described.25 Membranes were uncoated for chemotaxis or coated with 25 μg Matrigel (Becton Dickinson) for invasion assays. The lower compartments were filled with RPMI supplemented with 0.1% BSA with or without CXCL12 (100 to 200 ng/mL). Hu/SCID tumor cells were preincubated for 30 minutes at 37°C in the presence of 1 μM TC14012, anti-CXCL12 Ab, or in control conditions (RPMI-0.1% BSA) and subsequently loaded (3 × 10⁵) onto the upper compartments of the modified
for 48 hours with 1/H9262 in vivo–generated hu/SCID tumors. Data are summarized in Figure 1. Circulating B cells expressed CCR6, CCR7, CXCR3, CXCR4, and CXCR5 (Figure 1, circles); very rarely, CCR1, CCR4, CCR5, and CCR2 were expressed at low levels (data not shown). In contrast, EBV-immortalized LCL cells expressed CCR7, CXCR3, and CXCR4 at a lower level (P < .05), with CCR6 and CXCR5 being frequently undetectable (Figure 1, squares); rarely, CCR1, CCR3, and CCR4 were positive at low levels (data not shown). Hu/SCID tumors presented a mixed chemokine receptor expression profile (Figure 1, triangles), with CXCR3 and CXCR4 being substantially expressed, while CCR6, CCR7, and CXCR5 were significantly more weakly expressed compared with freshly isolated B lymphocytes (P < .05); rarely, CCR1, CCR2, and CCR4 were positive at low levels (data not shown). In general, a lower percent expression of chemokine receptors was also paralleled by a sizable reduction in mean fluorescence intensity (MFI) of the cells (data not shown). Altogether, these data demonstrate that EBV transformation in vitro is associated with a significant down-regulation of the expression of all chemokine receptors studied (CCR6, CCR7, CXCR3, CXCR4, and CXCR5); on the contrary, EBV-positive hu/SCID tumors generated after PBMC transfer into SCID mice continue to express substantial levels of CXCR3 and CXCR4, comparable to those present in freshly isolated B cells, though down-modulating CCR6, CCR7, and CXCR5.

**CXCR4 is functional and is predominantly expressed by a CD23low hu/SCID tumor cell population**

The functionality of the chemokine receptors expressed on hu/SCID tumor cells was evaluated by a chemotaxis assay. In most cases CXCL12 gave the greatest chemotactic response (data not shown), and because tumors in our experimental model consist of multiple peritoneal masses with frequent involvement of liver, spleen, and abdominal lymph nodes, sites known to produce high levels of CXCL12, subsequent experiments focused on the CXCL12/CXCR4 axis. As shown in Figure 2A, chemotaxis to murine CXCL12 ELISA

Confocal microscopic analysis was executed as previously described. Cells were stained with the following primary Abs: mouse anti-CXCR4 mAb (R&D Systems) and rabbit anti-human CXCL12 Ab (PeproTech), while the secondary Abs used were Alexa 594–conjugated anti–mouse Ab (Molecular Probes; red signal) and an Alexa 488–conjugated antirabbit Ab (Molecular Probes; green signal).

Peritoneal washings were executed as previously described. For each time point considered, 3 to 5 mice were killed and the liquid obtained from the peritoneal washings of each time point was pooled together. These pools were concentrated 50 times with Centriplus Centrifugal Filter Devices (YM-3; Millipore, Billerica, MA) to obtain a final volume of 300 to 500 μL. The quantity of murine CXCL12 present in the peritoneal washings was then determined using an antibody sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems) with a sensibility of more than 44 pg/mL.

**Statistical analysis**

Results were expressed as mean value ± SD. Statistical analyses of the in vitro data were performed using the nonparametric Mann-Whitney test; in vivo experiments, Kaplan-Meier survival curves were analyzed with the Mantel-Haenszel test.

**Results**

**EBV transformation of B cells in vitro (LCLs) and in vivo (hu/SCID tumors) is associated with modulation of chemokine receptor expression**

The chemokine receptor expression profile of EBV-transformed B cells has not been as yet extensively addressed. Using a panel of mAbs directed against CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CXCR1, CXCR2, CXCR3, CXCR4, and CXCR5, we compared the chemokine receptor expression profile in freshly isolated B lymphocytes, in vitro EBV-immortalized LCL cells, and in vivo–generated hu/SCID tumors. Data are summarized in Figure 2B. The chemokine receptor expression profile (Figure 1, triangles), with CXCR3 and CXCR4 being substantially expressed, while CCR6, CCR7, and CXCR5 were significantly more weakly expressed compared with freshly isolated B lymphocytes (P < .05); rarely, CCR1, CCR2, and CCR4 were positive at low levels (data not shown). In general, a lower percent expression of chemokine receptors was also paralleled by a sizable reduction in mean fluorescence intensity (MFI) of the cells (data not shown). Altogether, these data demonstrate that EBV transformation in vitro is associated with a significant down-regulation of the expression of all chemokine receptors studied (CCR6, CCR7, CXCR3, CXCR4, and CXCR5); on the contrary, EBV-positive hu/SCID tumors generated after PBMC transfer into SCID mice continue to express substantial levels of CXCR3 and CXCR4, comparable to those present in freshly isolated B cells, though down-modulating CCR6, CCR7, and CXCR5.

**Conical polymerization**

To evaluate the effect of CXCL12 on cytoskeleton rearrangement of different tumor subsets, freshly isolated hu/SCID lymphoma cells were labeled with PE-conjugated anti-CD23 mAb at 4°C for 30 minutes. Cells were washed and incubated at 37°C in RPMI medium before assessing cytoskeleton rearrangement. Actin polymerization experiments were performed as previously described.11

**Proliferation and survival assays**

For proliferation and survival studies evaluating the role of endogenous CXCL12, freshly isolated hu/SCID tumor cell suspensions treated with or without anti-CXCL12 Abs (1:20) or TC14012 (50 to 100 μM) were pulsed for 48 hours with 1 μCi [37 Bq] [methyl-3H]thymidine (Amersham, Arlington Heights, IL) or labeled with FITC-conjugated annexin V (PharMingen, BD Biosciences, San Diego, CA) plus propidium iodide (PI; Molecular Probes).27 Subsequently, thymidine incorporation was evaluated on a scintillation β-plate counter, or the percentage of cells undergoing apoptosis was determined by flow cytometry. Annexin V–positive PI–negative and annexin V–positive PI–positive cells correspond to apoptotic and necrotic cells, respectively.

**Confocal microscopy**

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**CXCR4 is functional and is predominantly expressed by a CD23low hu/SCID tumor cell population**

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**Figure 1. Modulation of chemokine receptor expression in EBV-transformed B cells in vitro (LCLs) and in vivo (hu/SCID tumors).** Flow cytometric analysis for CCR6, CCR7, CXCR3, CXCR4, and CXCR5 expression on B lymphocytes (○), LCL cells (■), and freshly isolated hu/SCID tumor cells (▲). Each point shows the percentage of cells expressing the indicated chemokine receptor. Horizontal bars represent the mean value of each group; the asterisks denote a significant difference compared with B cells (P < .05).
CXCL12 could be significantly blocked by pretreatment of cells with neutralizing Abs to CXCL12 and the CXCR4 antagonist TC14012. Further, no significant increase of migration was observed with a uniform distribution of CXCL12, indicating that this is a gradient-dependent chemotactic response. It has been reported that hu/SCID tumors are composed of 2 phenotypically distinct B-cell subsets: CD23low (and CD38high [CD38hi]) and CD23intermediate (CD23int) and CD38low, demonstrated to harbor a different EBV gene expression profile; on the other hand, LCL cells have been reported to be homogenously CD23low/CD38int/low and only show a reduced CD23 expression when transferred into SCID mice. We thus investigated whether CXCR4 expression on hu/SCID tumor cells segregated with the B-cell phenotype. To analyze the coexpression of membrane CXCR4 and CD23 on hu/SCID tumors, the tumor cells were examined cytometrically after staining with FITC anti-CD23 and PE anti-CXCR4 mAbs. As shown in Figure 2B (upper panel), in agreement with previously published data, within hu/SCID tumor cells 2 cell subpopulations were discernable on the basis of CD23 expression, one CD23low and one CD23int; this was not the case for LCLs, where virtually all cells showed high CD23 expression (Figure 2B, lower panel). Further, a clear segregation in CXCR4 expression was observed in the 2 hu/SCID tumor cell subpopulations (Figure 2C), with CXCR4 surface expression being predominantly confined to the CD23low subset (Figure 2C, top left panel). On the other hand, both tumor cell subsets expressed consistent levels of internal CXCR4 (Figure 2C). To determine the functional relevance of the generation of the 2 cell subsets, we investigated F-actin modulation following incubation with increasing concentrations of CXCL12. As shown in Figure 2D, a significant increase in F-actin polymerization could be observed only in the CD23low tumor subset, suggesting that this subset is the main responder to CXCL12.

The CXCR4 ligand, CXCL12, is expressed by LCLs and a CD23-expressing hu/SCID tumor cell population but not resting or activated B cells

EBV-transformed B lymphocytes have a propensity to produce autocrine factors for their own growth and survival. We evaluated by both RT-PCR and fluorescence-activated cell sorter (FACS) analysis whether LCLs and hu/SCID tumors expressed the CXCR4 ligand, CXCL12, thus possibly activating an autocrine loop; indeed, this has not been investigated so far. As shown in Figure 3A, all LCLs (lanes 1 to 5) and hu/SCID tumor cells (lanes 6 to 10) tested expressed mRNA for SDF-1/H9251/CXCL12 and most also expressed SDF-1/H9252/CXCL12 (data not shown), with the α isoform being the predominant transcript in most cases. To investigate whether the expression of CXCL12 by LCLs and hu/SCID tumor cells was simply associated to the activation state of these cells or more strictly linked to EBV transformation, B cells were purified and stimulated in vitro with anti-CD40 mAb and interleukin-4 (IL-4) for 3 to 5 days. RT-PCR analysis of these samples disclosed that no specific transcripts for SDF-1α/CXCL12 or SDF-1β/CXCL12 could be evidenced in both freshly isolated (Figure 3A, lanes 1 to 5) and activated (lanes 6 to 10) samples, suggesting that the observed expression is not driven by activation signals.
The expression of CXCL12 and β-actin in LCL cells and hu/SCID tumor cells was evaluated by RT-PCR. Representative results from 5 LCLs and 5 hu/SCID tumors are shown. Lanes 12 and 13 correspond to a representative case of purified resting B cells and to the same B cells after 48 hours of in vitro stimulation with anti-CD40/IL-4, respectively. (B-C) The intracellular expression of CXCL12 in LCL cells and hu/SCID tumor cells was evaluated by cytofluorimetric analysis. (B) The fluorograms (from top to bottom) show normal B cells not expressing CXCL12, LCL cells, and hu/SCID tumor cells. (C) The expression of CXCL12 in the CD23lo (lower right) and CD23hi (upper right) tumor cell subsets is shown. A representative experiment of 3 consecutive experiments is shown. (D) Confocal microscopic analysis evaluating the coexpression of surface CXCR4 and intracellular CXCL12 in hu/SCID tumors. Cells were fixed, stained with anti-CXCR4 Ab, and subsequently permeabilized before incubation with anti-CXCL12 Ab. The signal for CXCR4 is shown in red, while the CXCL12 signal is in green. Areas of colocalization are shown in yellow.

In vitro CXCL12 neutralization inhibits hu/SCID lymphoma cell proliferation and survival

We showed thus far that hu/SCID tumors express CXCL12 and that they can efficiently respond to exogenous CXCL12. We thus set out to investigate the possible functional role of endogenous CXCL12 on hu/SCID cell biology. We first investigated whether CXCL12 neutralization by the use of anti-CXCL12 neutralizing Abs or the use of a recently described specific blocking agent for CXCR4, such as TC14012, affected lymphoma growth in vitro. Indeed, as shown in Figure 4A, treatment of ex vivo–obtained lymphoma cells for 2 days with neutralizing anti-CXCL12 Abs (1:20 dilution) or TC14012 (50 to 100 μM) significantly reduced lymphoma cell proliferation (P < .05). On the other hand, the goat preimmune serum used as control at the same dilution did not significantly alter lymphoma proliferation (Figure 4A). We next tested the effect of
blocking the endogenous CXCL12 on apoptosis of hu/SCID lymphoma cells. To this end, ex vivo–obtained lymphoma cells were treated for 2 days with neutralizing anti-CXCL12 Abs (1:20 dilution) or TC14012 (50 to 100 μM) and then analyzed by flow cytometry after staining with FITC-conjugated annexin V and PI. Treatment of cells with either anti-CXCL12 Abs or TC14012 determined a significant increase in annexin V–positive cells (data not shown). However, a difference seemed to emerge in their mode of action, because the neutralizing anti-CXCL12 Abs determined mainly an increase in early apoptosis (annexin V–positive PI-negative), while the CXCR4 antagonist acted mainly on late apoptosis (annexin V–positive PI-positive) (Figure 4C). Notably, the concentrations of the neutralizing anti-CXCL12 Abs (1:100 dilution) or TC14012 (1 to 5 μM) used in functional assays such as chemotaxis, adhesion, or chemoinvasion did not affect either the proliferation or survival of lymphoma cells (data not shown). On the whole, these data suggest that survival and proliferation of hu/SCID tumors are in part mediated by CXCL12/CXCR4 interactions and autocrine secretion of CXCL12.

**CXCL12 stimulates the adhesive and invasive properties of hu/SCID tumor cells**

CXCL12 has been reported to regulate the function of several integrins on normal human hematopoietic cells; we thus investigated whether CXCL12 also regulated integrin activity in hu/SCID tumor cells. As shown in Figure 5A, CXCL12 consistently increased the adhesion of hu/SCID tumor cells to both fibronectin (Figure 5A, middle panel) and HUVECs (Figure 5A, right panel), whereas it did not affect their adhesion to BSA (Figure 5A, left panel).

Because tumor cell invasion is one of the major characteristics of malignant cells, we evaluated this feature using a chemoinvasion Matrigel assay. As shown in Figure 5B, the invasive capability of hu/SCID tumors was increased in the presence of a CXCL12 gradient (Figure 5B, open columns). In addition, TC14012 significantly inhibited both the adhesive and invasive properties of these cells (Figure 5, gray columns), thus indicating that the invasion-promoting effect of CXCL12 was a specific phenomenon; similar results were obtained when neutralizing anti-CXCL12 Abs were used (data not shown).

**CXCL12 is expressed in vivo during the early phases of EBV-mediated lymphomagenesis, and its neutralization inhibits tumor growth in vivo**

We finally directly addressed the possible relevance of the CXCL12/CXCR4 axis in the lymphomagenesis process of this experimental model. To this end, we first wondered whether CXCL12 and CXCR4 were indeed expressed in the mouse peritoneal microenvironment. Thus, cells were recovered from the peritoneal cavity of SCID mice at 4, 8, and 16 days following intraperitoneal transfer of PBMCs from EBV-positive donors, and the expression of human and murine CXCL12 and human CXCR4 was determined by RT-PCR. In addition, the peritoneal washings were evaluated for the presence of murine CXCL12 by ELISA. We used primers specific for murine and human CXCL12 (Figure 6A), because under our experimental conditions a band corresponding to human CXCL12 could only be evidenced on human cells but not on murine tissues (Figure 6A). At the same time, primers specific for murine CXCL12 gave a band only when mouse tissues were used (Figure 6A). As shown in Figure 6B, both human and mouse CXCL12 transcripts were undetectable in PBMCs immediately prior to injection; meanwhile, they were both readily evidenced in the cell populations recovered at all the time points considered. On the other hand, the CXCR4 transcript was always consistently detected in all cell populations studied (Figure 6B).

RT-PCR results seemed to indicate that murine CXCL12 is the main isoform present in the early stages of lymphomagenesis. We thus determined whether murine CXCL12 protein was effectively present in the peritoneal cavity and how it varied during the lymphomagenesis process. As shown in Figure 6C, low levels of murine CXCL12 were present in the peritoneal washings prior to PBMC injection; however, during the course of the lymphomagenesis process there was a considerable increase in the protein levels of CXCL12, reaching a peak at the moment of killing.

In view of these data, which suggested the possibility that a CXCL12/CXCR4 autocrine/paracrine loop could be at work in lymphoma development and progression, we wondered whether interfering with the CXCL12/CXCR4 axis could affect lymphoma development in SCID mice. To this end, we chose to use freshly obtained hu/SCID tumor cell suspensions to generate tumors in naive SCID mice. Hu/SCID lymphoma cells were used instead of PBMCs from EBV-positive donors because of the well-known problem of the very high heterogeneity in the generation of lymphomas in SCID mice among different donors, which would have complicated the interpretation of the effects of

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**Figure 5. Effect of CXCL12 on the adhesive and invasive properties of hu/SCID tumor cells.** (A) Effect of CXCL12 on adhesion of hu/SCID tumor cells to fibronectin and endothelial cells. The panels from left to right represent adhesion of hu/SCID tumor cells to bovine serum albumin (BSA), fibronectin, and HUVECs. Unstimulated cells are shown as black bars; open and gray bars represent hu/SCID tumor cells stimulated with CXCL12/SDF-1α (200 ng/mL) in the absence and in the presence, respectively, of the CXCR4 antagonist TC14012. Data are shown as mean ± SEM of 3 experiments performed in triplicate. Asterisks denote a significant difference compared with untreated cells (*P < 0.05); 2 asterisks denote a significant inhibition by TC14012 of the adhesive properties of CXCL12-stimulated cells (*P < 0.005). (B) Cells invading the Matrigel were collected from the lower compartments and counted as detailed in “Materials and methods”; results are expressed as number of cells per high power field (HPF) and represent mean values ± SEM of 3 replicate determinations for each tumor. Hu/SCID lymphoma cells showed significant invasion through Matrigel toward a CXCL12 gradient (open columns); this phenomenon was inhibited by treating the cells with TC14012 (gray columns). The single asterisk denotes a significant difference (*P < 0.05) compared with unstimulated cells; 2 asterisks denote a significant inhibition by TC14012 of the invasive properties of CXCL12-stimulated cells (*P < 0.005). Results obtained in 3 different hu/SCID tumor masses representative of 4 consecutive experiments are shown.
neutralization of the CXCR4/CXCL12 axis on lymphoma development. Hu/SCID tumor-injected mice were then treated with intraperitoneal injections of a neutralizing anti-CXCL12 Ab or adequate controls. For CXCR4 neutralization, 4F-benzoyl-TN14003, a recently reported T140 derivative shown to have increased biostability with respect to TC14012,18 was administered by subcutaneous injection using Alzet pumps beginning from the day preceding transplantation of hu/SCID lymphoma cells for 4 consecutive weeks. As shown in Figure 6D, in experiments evaluating the effect of CXCL12 neutralization, all control animals succumbed within 50 days after tumor cell transfer; meanwhile, mouse treatment with the neutralizing anti-CXCL12 Ab significantly delayed lymphoma development (Figure 6D; P < .0002), with 50% of anti-CXCL12–treated animals being tumor free after 180 days. A similar effect on animal survival was observed when 4F-benzoyl-TN14003 was used (Figure 6E; P < .05).

Discussion

In this article we addressed the contribution of the chemokine system to the outgrowth of EBV-positive B-cell lymphomas in SCID mice injected intraperitoneally with PBMCs from EBV-positive donors. We showed that most in vitro–transformed LCLs strongly down-regulated the expression of most chemokine receptors, compared with normal B lymphocytes; meanwhile, most hu/SCID tumors analyzed expressed CXCR3 and CXCR4 at moderately high levels and CCR6, CCR7, and CXCR5 at intermediate/low levels. That in vivo and in vitro immortalization of circulating B cells by EBV is associated with down-regulation of CCR6, CCR7, and CXCR5 expression may be partially linked to the activation state of these cells.34 Our data concerning LCLs partially confirm those recently reported by other workers,35 in that we also found a down-regulation of CXCR4 and CXCR5; however, we were not able to document any significant up-regulation of CCR6 surface expression in LCL cells. Notably, only a few LCL samples were considered by these authors;35 in addition, the modulation of chemokine receptor expression (in comparison with a single peripheral blood B-cell sample examined) was solely based on a semiquantitative RT-PCR analysis,35 while our conclusions are based on protein expression by cytofluorimetry on a much larger number of samples. On the other hand, this apparent discrepancy might depend on the very high heterogeneity between LCLs, as also encountered in the present study (Figure 1), and the modulation of some chemokine receptors associated with in vitro culture (data not shown). The differential profile of chemokine receptor expression between LCLs and hu/SCID tumor cells, with these latter expressing moderately high CXCR3 and CXCR4 levels, may also partially reflect a different stage of maturation of these 2 cell populations.35 It is well known that plasma cells express CXCR4 while down-modulating CCR7 and CXCR5, whereas activated B cells down-modulate CCR6 (Brandes et al34 and data not shown). Thus, the down-regulation of CCR6, CCR7, and CXCR5 seen in hu/SCID tumors may partially reflect their stage of differentiation and their activation state.

Recently, it has been described that EBV latent genes can also modify chemokine receptor expression.35 Hu/SCID tumors in general exhibit low levels of latent EBV transcripts such as Epstein-Barr nuclear antigen-1 (EBNA-1), EBNA-2, and latent membrane protein-1 (LMP-1) with respect to LCLs.5 Thus, differential expression of EBV latent genes between LCLs and hu/SCID tumors may also help explain the differences in the chemokine receptor profile between LCLs and hu/SCID tumors. In this regard, as outlined previously and reported by others,5,28 hu/SCID tumors are composed of 2 phenotypically distinct B-cell subsets: CD23+/CD38+ and CD23+/CD38+,; meanwhile, LCLs have been
reported to be homogeneously CD23hiCD38int/low.5,28 The CD23low/CD38hi cell population present within hu/SCID tumors expresses lytic cycle EBV transcripts, while the CD23hi/CD38int population only expresses the latent cycle transcripts EBNA-1, EBNA-2, and LMP-1.1 We demonstrated that surface CXCR4 is predominantly expressed by the CD23low tumor cell subset, previously shown to have a low proliferative index and high-level Ig secretion, typical of plasma cells.5 Thus, it is conceivable that differential expression of EBV genes could translate into modulation of chemokine receptor expression, with CXCR4 up-regulation by lytic gene products.

Given these premises, we concentrated our attention on the CXCR4 ligand, CXCL12, to evaluate whether a putative autocrine loop could be at work in the lymphomagenesis process of this experimental model. We showed for the first time that EBV-transformed B cells, both LCLs and hu/SCID tumors, are able to express CXCL12, in contrast to resting and in vitro–activated B lymphocytes. Interestingly, CXCL12 was shown to be produced predominantly by the CD23th tumor cell subset, which expresses low levels of surface CXCR4 (sCXCR4) but high levels of internal CXCR4. It has been previously shown that the transfer of a tumor cell line (generated after in vitro growth of hu/SCID lymphomas) expressing CD23 at intermediate levels to SCID mice entails a substantial shift to low CD23 expression. Because the former expresses low levels of sCXCR4, while the latter expresses good levels of sCXCR4, it could be inferred that the CD23th/CD38hiCXCR4low (lymphoblastoid-like) subset shifts phenotype to CD23low/CD38hiCXCR4int (plasmacytoid-like) after transfer and growth in SCID mice with gain of CXCL12 responsiveness and that the plasmacytoid cells originate from the lymphoblastoid cells.28 We thus expand the current knowledge regarding this experimental model, proposing that the differentiation of plasmacytoid cells from lymphoblastoid cells involves gain of CXCR4 surface expression (and responsiveness) and reduced production of CXCL12 and that the CXCL12/CXCR4 axis may be important in the homeostasis of the 2 tumor subsets that are found within lymphomas. Furthermore, in vitro treatment of hu/SCID lymphoma cells with neutralizing anti-CXCL12 Abs or TC14012 decreased cell survival and proliferation, implying autocrine regulation of cells with neutralizing ant-CXCL12 Abs or TC14012 decreased lymphoma survival and proliferation, implying autocrine regulation of CXCR4-specific antagonists. In any case, these findings strongly suggest that the CXCR4/CXCL12 axis could play a significant role in favoring the outgrowth and dissemination of EBV-transformed cells in PBMC-injected SCID mice. Because CXCR4 expression has been proven in the human tumor counterpart of this experimental model,13,14 the CXCL12/CXCR4 axis could indeed become an attractive target for therapy in lymphoma-bearing patients, possibly through the use of small molecular antagonists of CXCR4.

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Chemokine receptor expression in EBV-associated lymphoproliferation in hu/SCID mice: implications for CXCL12/CXCR4 axis in lymphoma generation

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