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Review

CX_3CL1/fractalkine is a novel regulator of normal and malignant human B cell function

Anna Corcione,[1] *Elisa Ferretti and Vito Pistoia*

Laboratory of Oncology, Istituto Di Ricovero e Cura a Carattere Scientifico G. Gaslini, Genova, Italy

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ABSTRACT

CX_3CL1, or fractalkine, the unique member of the CX_3C chemokine family, exists as a transmembrane glycoprotein, as well as in soluble form, each mediating different biological activities, and is constitutively expressed in many hematopoietic and nonhematopoietic tissues. CX_3CR1, the CX_3CL1 exclusive receptor, is a classical GPCR, expressed on NK cells, $CD14^+$ monocytes, and some subpopulation of T cells, B cells, and mast cells. A recent paper by our group has demonstrated for the first time that highly purified human B cells from tonsil and peripheral blood expressed CX_3CR1 at mRNA and protein levels. In particular, tonsil naïve, GC, and memory B cells expressed CX_3CR1, but only GC centrocytes were attracted by soluble CX_3CL1, which with its receptor, are also involved in the pathogenesis of several inflammatory disorders, as well as of cancer. Previous studies have shown that CX_3CR1 is up-regulated in different types of B cell lymphoma, as well as in B-CLL. Recently, we have demonstrated that the CX_3CL1/CX_3CR1 axis is involved in the interaction of B-CLL cells with their microenvironment. Taken together, our data delineate a novel role for the CX_3CL1/CX_3CR1 complex in the biology of normal B cells and B-CLL cells. These topics are the subject of this review article. *J. Leukoc. Biol.* 92: 51–58; 2012.

Introduction

Chemokines are small, proinflammatory cytokines, which coordinate the migration and activation of leukocytes and other types of cells in health and disease [1–3]. Chemokines are characterized by a conserved protein structure, named "chemokine scaffold", strictly dependent on two conserved disulfide bonds connecting cysteine residues, whose relative position determines the identification of four subfamilies: the CC chemokines, which have the first two cysteine residues in adjacent position; the CXC chemokines, which have cysteine residues separated by a single amino acid; the C chemokines, which have a single cysteine residue in the amino terminus; and the CX_3C chemokines, whose exclusive member is CX_3CL1/fractalkine, with three residues separating the cysteine tandem [1–3].

The nomenclature of chemokine ligands is reflected in that of chemokine receptors, which often show ligand promiscuity among members of a defined subfamily.

Chemokine receptors are seven transmembrane domain receptors, whose signaling activity is mediated by heterotrimeric GPCRs and leads to directional cell migration along the ligand gradient [1, 2]. Recently, a group of atypical chemokine receptors, which do not elicit conventional signaling responses, have been described [4, 5]. In inflammatory conditions, these atypical receptors modulate immune responses by acting as decoy/scavenger receptors [4, 5].

Different chemokine receptors coordinate the recruitment of different cell populations [1, 2]. Thus, for example, PMNs are attracted by CXC chemokines binding to CXCR1 and CXCR2; monocytes are mainly recruited by CC chemokines through CCR1, CCR2, and CCR5; Th1 and NK cells are attracted through CXCR3 and CX_3CR1; Th2 and eosinophils are attracted by CC chemokines binding to CCR3 and CCR4.

According to their production, chemokines can be distinguished in "homeostatic" molecules, which control leukocyte homing and lymphocyte recirculation in normal conditions, and "inflammatory" chemokines, produced on demand in response to inflammatory and immune stimuli [6].

Chemokines and chemokine receptors are involved in inflammatory disorders and in cancer and therefore, represent potential targets for the development of innovative therapeutic strategies.

CX_3CL1 AND CX_3CR1 IN HEALTH AND DISEASE STATES

CX_3CL1 is the unique member of the CX_3C family, consisting of a chemokine domain linked to a transmembrane domain via an extended mucin-rich stalk and of an intracellular domain [7, 8]. CX_3CL1 exists in two forms, a membrane-bound and a soluble form, each mediating distinct biological actions.

Abbreviations: ADAM=a disintegrin and metalloprotease domain, B-CLL=B-chronic lymphocytic leukemia, FDC=follicular DC, FL=follicular lymphoma, GC=germinal center, MCL=mantle cell lymphoma, MSC=mesenchymal stromal cell, NLC=nurse-like cell, T_{FH}=T follicular helper, TGB=tingible body macrophage, V=variable

1. Correspondence: Laboratory of Oncology, G. Gaslini Institute, Largo G. Gaslini 5, 16148 Genova, Italy. E-mail: annacorcione@ospedale-gaslini.ge.it

The soluble CX_3CL1 form acts as a conventional chemokine, which attracts monocytes, NK cells, T cells, and B cells [9, 10]. Constitutive shedding of CX_3CL1 is operated by ADAM10, whereas shedding under inflammatory conditions is mediated by ADAM17 [11, 12]. The membrane-bound CX_3CL1 form serves as an adhesion protein that promotes firm adhesion of leukocytes under static and flow conditions without activating integrins [13]. CX_3CL1 is expressed on endothelial and epithelial cells, lymphocytes, neurons, microglial cells, and osteoblasts [14–20].

CX_3CL1-dependent chemotaxis and adhesion are mediated by the GPCR CX_3CR1, which is expressed on human NK cells, monocytes, Th1 $CD4^+$ cells, $CD8^+$ T cells, mast cells, and B cells [9, 10].

CX_3CL1 and its receptor are implicated in several inflammatory processes, including allergic asthma, rheumatoid arthritis, Crohn's disease, and atheroslerosis. In patients with Crohn's disease, there was a significant increase in CX_3CL1 mRNA expression in inflamed lesions compared with noninflamed colonic mucosa, suggesting an important role of this chemokine in intestinal inflammation [21]. Patients with allergic asthma and rhinitis show increased concentrations of circulating soluble CX_3CL1 and increased expression of CX_3CR1 on naïve and memory $CD4^+$ T cells. Therefore, the CX_3CL1/CX_3CR1 axis contributes to the recruitment of circulating $CD4^+$ T cells in the airways following allergen contact [22].

Up-regulation of CX_3CL1 in synovial fibroblasts, macrophages, DCs, and T cells from rheumatoid arthritis has also been observed, indicating a proinflammatory role for the chemokine and its receptor in the pathogenesis of the disease [23, 24].

In atherosclerotic lesions, CX_3CR1 has been shown to be expressed by monocytes accumulated in response to chronic inflammatory injury and by smooth muscle cells. In contrast, CX_3CL1 is released from vascular endothelium and binds to CX_3CR1, expressed on endothelial cells, indicating that this chemokine is involved in leukocyte accumulation and smooth muscle cell migration in the atherosclerotic lesions [25, 26].

CX_3CL1 and CX_3CR1 play a major role in the neuron/microglia cross-talk and contribute to the maintenance of homeostasis in the brain under conditions of inflammation or injury. Neurons and astrocytes are major producers of the ligand [27], whereas microglial cells express the receptor [28]. High CX_3CR1 expression has also been detected in neuroblastoma cell lines [29] and primary tumors [30] and found to mediate increased invasiveness of neuroblastoma cells in response to CX_3CL1.

Up-regulation of CX_3CR1 has been reported in glomerulonephritis, where CX_3CL1 acts as a chemoattractant and adhesion molecule for the majority of leukocytes that infiltrates the kidney [31]. The CX_3CL1/CX_3CR1 axis also possesses anti- and protumoral activity. In colorectal cancer patients, CX_3CL1 is thought to have a crucial role in the recruitment of tumor-infiltrating leukocytes into and out of the lamina propria via its receptor, resulting in high survival rate and better patient prognosis [32]. Patients with hepatocellular carcinoma have high expression of CX_3CL1/CX_3CR1, and this expression correlates with better prognosis and fewer local and distant metastases [33].

In other solid malignancies, CX_3CL1 has been shown to promote tumor growth and metastasis. Thus, bone marrow endothelial cells and differentiated osteoblasts expressed CX_3CL1, which was cleaved from the cell surface as a soluble form and attracted prostate cancer cells. Therefore, the CX_3CR1/CX_3CL1 system plays a role in prostate cancer cell adhesion and extravasation steps [34, 35] and enhances their metastatic potential.

Tumor cells from human pancreatic ductal adenocarcinoma patients strongly expressed CX_3CR1 and infiltrated the local peripheral nerves expressing CX_3CL1, suggesting that CX_3CR1 has an important role in tumor neurotropism and invasion [36].

Healthy and malignant ovarian epithelial cells also expressed CX_3CL1, and its production in epithelial ovarian cancer is correlated to cell proliferation and tumor growth [37].

Recent studies in the CNS tumors have demonstrated that CX_3CR1 is overexpressed at mRNA and protein levels in solid human astrocytomas of different grades and in glioblastomas [38–40].

EXPRESSION AND FUNCTION OF THE CX_3CL1/CX_3CR1 AXIS ON NORMAL B LYMPHOCYTES

Naive B cells encounter specific antigen in the T cell area of secondary lymphoid organs, where they are activated and subsequently colonize primary lymphoid follicles to initiate the GC reaction. GC B lymphocytes undergo proliferation, class-switch recombination, antibody gene diversification, and affinity maturation. Based on histological appearance, two distinct areas, called dark and light zones, respectively, are identified in the GC. According to the classical model, B cells in the dark zone, called centroblasts, proliferate and somatically hypermutate Ig V genes and then move to the light zone, where they are denominated centrocytes and are selected based on the affinity of the BCR for antigen[41]. Following selection, centrocytes differentiate extrafollicularly into memory cells or plasmablasts [41] (**Fig. 1**).

Recent studies based on mouse experiments have delineated two additional models for B cell trafficking in the GC: the cyclic re-entry model, whereby B cells, which have migrated from the dark to the light zone and have been selected here, subsequently return to the dark zone for further proliferation; and the intrazonal recirculation model, whereby most B cells reside in the dark or light zone, and selection may operate independently in both areas [42] (Fig. 1). A quite recent study provided further evidence in support of the classical model discussed above by showing that mouse GC B cells have a preference to migrate from the dark to the light zone [43].

Mouse and human studies agree on the fact that GC B cells must be rescued from spontaneous apoptosis to migrate along chemokine gradients [44, 45]. In vitro human GC B cells migrate to anti-Ig antibodies or CXCL12 only, following CD40 mAb and IL-4-mediated rescue from apoptosis [45, 46]. Murine GC B cells overexpressing Bcl-2 under control of the Ig μ enhancer and therefore, resistant to spontaneous apoptosis show different homing properties regulated by chemokine gradients in vivo [44]. In particular, centroblasts express high levels of CXCR4 and home to the dark zone, where the CXCR4

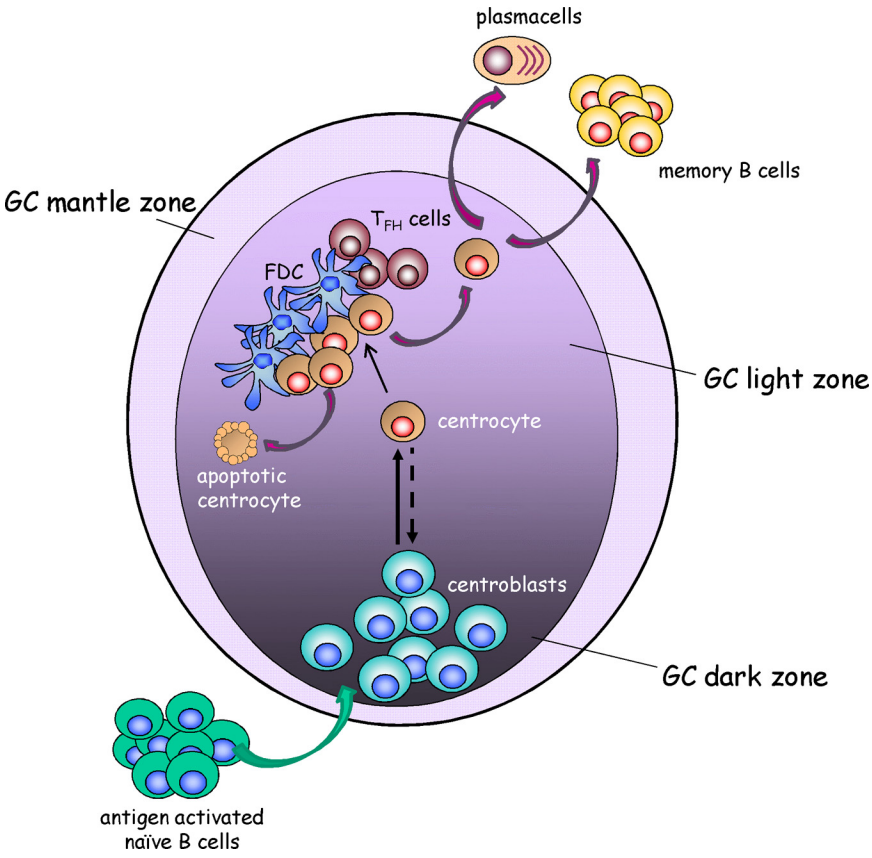

Figure 1. Models for B cell trafficking in the GC. In the classical model, antigen-activated, naïve B cells populate the dark zone of the GC. Here, these cells transform into centroblasts, which proliferate, mutate IgV genes, and migrate to the light zone (continuous black line), where they differentiate into centrocytes. In the light zone, centrocytes undergo selection by interacting with FDCs and T_{FH} cells and finally, differentiate into plasma cells or memory B cells or else die by apoptosis. In the cyclic reentry model, B cells migrate from the dark to the light zone (continuous black line) and may subsequently return to the dark zone (dashed black line) for further proliferation. An additional model (not shown) postulates intrazonal migration, whereby most B cells are confined in the dark or light zone, and selection may operate independently in both areas.

ligand CXCL12 is abundantly expressed. Centrocytes expressing high levels of CXCR5 accumulate in the light zone of the GC, where the CXCR5 ligand CXCL13 is expressed at elevated concentrations [44]. Migration of centroblasts from the dark to the light zone of the GC is promoted by down-regulation of CXCR4 and up-regulation of CXCR5 [44].

We have recently reinvestigated with different approaches the expression of CX_3CR1 in the major B cell subsets from tonsil and peripheral blood and demonstrated unambiguously that human B cells expressed CX_3CR1 at mRNA and protein levels [10].

Approximately one-half of human naïve (IgD^+, $CD27^-$), GC ($CD10^+$), and memory ($CD27^+$) $CD19^+$ B cells expressed CX_3CR1, but soluble CX_3CL1 attracted only freshly isolated GC B cells. Signal transduction induced by CX_3CL1 in GC B cells was found to be similar to that reported previously for other cell types [47, 48] and involved phosphorylation of

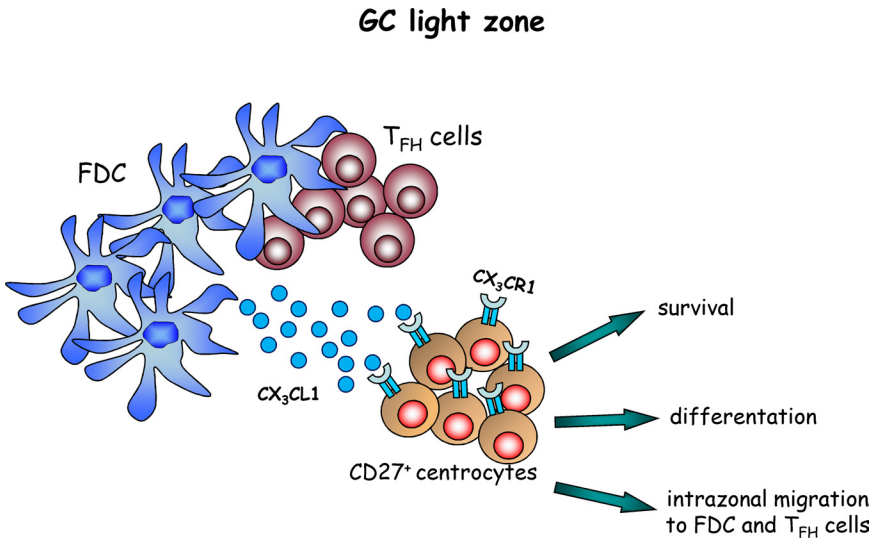

Figure 2. Model for interaction among FDC, T_{FH}, and CX_3CR1^+ $CD27^+$ centrocytes. In the light zone of human GC, FDC and T_{FH} cells release CX_3CL1 (blue dots), which attracts a subset of $CD27^+$ centrocytes expressing CX_3CR1 on the cell surface. This interaction promotes survival, differentiation, and intrazonal migration of $CD27^+$ centrocytes to FDCs and T_{FH} cells.

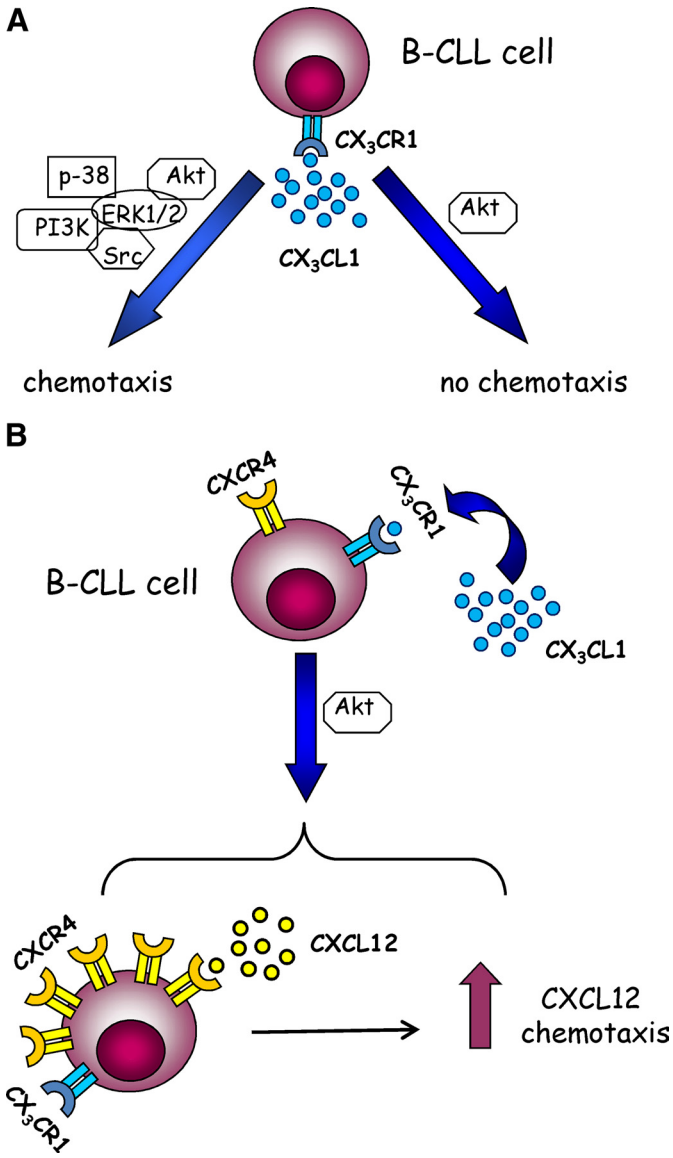

Figure 3. (A) CX_3CR1-driven B-CLL signaling pathways. Upon incubation with CX_3CL1 (blue dots), migrating B-CLL cells show phosphorylation of PI3K, Erk1/2, Akt, Src, and p38. In contrast, nonmigrating B-CLL samples display Akt phosphorylation only. (B) Up-regulation of CXCR4 expression and function in CX_3CL1-treated CLL cells. B-CLL cell fractions, attracted or not by CX_3CL1, undergo Akt phosphorylation-dependent up-regulation of CXCR4 and increased chemotaxis to CXCL12 (yellow dots) upon incubation with CX_3CL1 (blue dots).

PI3K, Erk1/2, p38, Akt, and Src. In contrast, naïve and memory B cells (non-GC B cells), which were not attracted by CX_3CL1, showed a limited signaling pattern involving phosphorylation of Akt and Src, but not PI3K, p38, and ERK1/2.

CX_3CR1^+ GC B cells were almost devoid of $CD77^+$ centroblasts and contained two mutually exclusive subsets expressing CD23 or CD27, respectively. The $CD23^+$ subset displayed an immunophenotype consistent with that of antigen-activated, naïve B cells, which have recently accessed the GC, and was not attracted by CX_3CL1 in chemotaxis assays [49]. The $CD27^+$ subset had an immunophenotypic profile consistent with that of centrocytes and migrated to CX_3CL1.

The rate of IgV gene somatic mutations was similar in CX_3CR1^+ and CX_3CR1^- GC B cells, consistent with the similar expression of CD27, a memory B cell marker associated with IgV gene mutations [50], in the two cell fractions.

CX_3CL1 was found to be abundantly expressed in the GC, as well as in the tonsil subepithelial area and follicular mantle. CX_3CL1 was released constitutively by T_{FH} cells and FDC lines. CX_3CL1 production by both cell types, which home in the light zone of the GC, was increased upon stimulation with TNF or IFN-γ. Notably, in this respect, GC B cells undergoing apoptosis were found to express CX_3CL1, which recruited CX_3CR1^+ TGBs to the GC. Here, TGBs are responsible for the clearance of apoptotic cells [51]. Accordingly, CX_3CR1-deficient mice showed reduced recruitment of TGB to the GC of lymphoid follicles [52]. These data are in contrast with those reported by other investigators, whereby CX_3CL1 expression was induced in human GC B cells via the BCR and CD40 triggering [16], which prevents spontaneous apoptosis of the latter cells [53].

It has been suggested that T_{FH} cells may collaborate with FDC in centrocyte rescue from apoptosis [42] (see Fig. 1). On this ground, we propose a model, whereby human FDC and T_{FH} cells release in the light zone of GC-soluble CX_3CL1, which attracts a subset of $CD27^+$ human centrocytes, potentially impacting on their survival and differentiation (**Fig. 2**).

No morphological or immunophenotypic abnormalities were detected in splenic GC from $CX_3CR1^{-/-}$ and $CX_3CL1^{-/-}$ versus WT mice, which had been immunized with OVA. However, significant impairment of OVA-specific IgG production was detected in $CX_3CR1^{-/-}$ or $CX_3CL1^{-/-}$ versus WT mice, indicating that the CX_3CL1/CX_3CR1 system is involved in the control of unknown step(s) of B cell differentiation to antibody-producing cells.

THE CROSS-TALK BETWEEN THE CX_3CR1/CX_3CL1 SYSTEM AND TUMOR MICROENVIRONMENT IN MATURE B-CELL MALIGNANCIES: THE CASE OF B-CLL

The microenvironment of mature B cell tumors mimicks the physiological microenvironment of the tissue where the tumor develops. The composition of such tumor microenvironment may include various subsets of T cells [54]; endothelial cells lining neoangiogenetic microvessels [55]; and different types of stromal cells, such as monocyte-derived NLCs [56], MSCs [57], or FDCs [58], all of which promote the survival of malignant cells.

Chemokine receptors and their ligands control cell trafficking in the microenvironment of B cell tumors. Malignant cells from FL and MCL express chemokine receptors, in particular, CXCR4 and CXCR5, which allow homing and retention of the tumor elements within stromal niches [45, 59–62].

The homing to and within the bone marrow of malignant B cells, such as those from multiple myeloma [63] and B-CLL

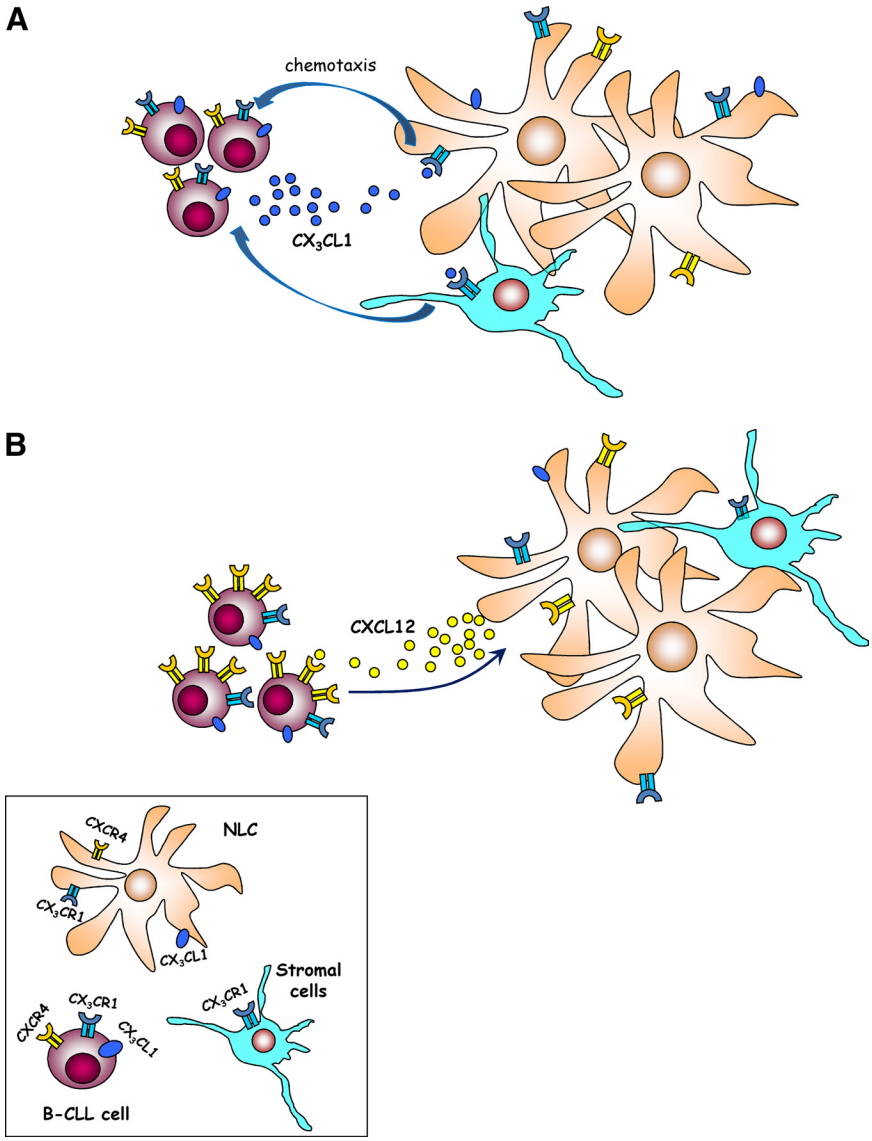

Figure 4. Interaction between B-CLL cells and their microenvironment (A). B-CLL cells express CX_3CR1 and CX_3CL1 on the cell surface and constitutively release the soluble form of the chemokine (blue dots). NLCs coexpress CX_3CL1 and CX_3CR1, but only a fraction of them is attracted by B-CLL-derived CX_3CL1 (upper arrow). Stromal cells, which express CX_3CR1 [74], may also be attracted by B-CLL-derived CX_3CL1 (lower arrow). (B) Up-regulation of CXCR4 on B-CLL cells induced by CX_3CL1 enhances the attraction of leukemic cells (arrow) toward NLCs secreting CXCL12 (yellow dots).

[64], is mediated by the CXCR4 receptor binding to CXCL12, expressed by MSCs [65]. In addition, CXCR4 and CXCR5 triggering stimulates growth and survival of B-CLL cells [54].

Previous studies have shown that the CX_3CR1 receptor is up-regulated in different types of B cell lymphoma, as well as in B-CLL [66, 67]. CX_3CL1 has been found to be released by apoptotic Burkitt lymphoma cells (which originate from the GC) and to attract TGB to the tumor site [52].

We have recently delineated a novel role for the CX_3CL1/CX_3CR1 axis in the B-CLL microenvironment [68]. CX_3CR1 was found to be expressed by B-CLL cells, but only a fraction of them was attracted in vitro by its ligand CX_3CL1. Signal transduction experiments with migrating and nonmigrating malignant cell fractions demonstrated that upon incubation with CX_3CL1, the former cells showed increased phosphorylation of PI3K, Erk1/2, Akt, and Src and de novo induction of phosphorylation of p38 [47], similarly to that observed with normal GC B cells [10]. In contrast, B-CLL cells, which did not migrate to CX_3CL1, displayed only augmented Akt phosphorylation, similarly to that observed with normal non-GC B cells [10].

All leukemic B cell samples up-regulated CXCR4 expression upon incubation with CX_3CL1, and this was paralleled by increased chemotaxis to CXCL12. CXCR4 up-regulation was counteracted by the Akt inhibitor, irrespective of the fact that B-CLL cell fractions were attracted or not by CX_3CL1. Therefore, leukemic cells that migrated to CX_3CL1 used two different signaling pathways for CX_3CL1-induced chemotaxis and CXCR4 up-regulation, respectively, sharing Akt phosphorylation only [68] (**Fig. 3**).

A distinctive feature of the B-CLL microenvironment is the formation of proliferation centers of B-CLL cells (pseudofollicles) not found in other lymphoproliferative disorders [69]. Pseudofollicles contain stromal cells, $CD68^+$ myeloid cells related to NLCs [70], activated Th cells, and FDCs, all of which provide antiapoptotic and proliferative stimuli to tumor cells [70]. We detected expression of CX_3CL1 and CX_3CR1 in proliferation centers within LN and bone marrow from B-CLL

patients. CX_3CL1 was expressed predominantly by the neoplastic elements, whereas CX_3CR1 was detected in the latter cells and stromal cells. In particular, $CD68^+$ myeloid cells were found to express CX_3CR1 but not CX_3CL1 [68].

NLCs differentiate in vitro into large, adherent cells from blood monocytes cocultured with B-CLL cells [56, 58]. NLCs secrete CXCL12 [56] and CXCL13 [71], which attract B-CLL cells via CXCR4 and CXCR5, respectively, and protect them from spontaneous or drug-induced apoptosis [56, 72].

NLCs express additional molecules, providing survival signals to B-CLL cells via the respective receptors, such as B cell-activating factor and a proliferation-inducing ligand [72], and CD31, together with plexin-B1 [73].

In our study, NLCs generated from B-CLL patients expressed CX_3CL1 and CX_3CR1 on the cell surface (**Fig. 4A**). However, no soluble CX_3CL1 was released in NLC culture supernatants, suggesting that CX_3CL1 may serve as an adhesion molecule [9]. B-CLL cells expressed CX_3CL1 on the cell surface and constitutively released the soluble chemokine, which attracted NLCs from a subgroup of B-CLL patients (Fig. 4A). Stromal cells, which express CX_3CR1 [74], may also be attracted by CLL cell-derived CX_3CL1 (Fig. 4A).

The phenotypic and functional up-regulation of CXCR4 on B-CLL cells, induced by binding of soluble CX_3CL1 to CX_3CR1, may enhance the attraction of leukemic cells toward NLCs secreting CXCL12, which acts as a prosurvival factor for B-CLL cells [56] (Fig. 4B). According to the model proposed here, all of the above CX_3CL1 cell interactions in the tumor microenvironment may translate into modulation of B-CLL cell survival.

Preliminary data by our group show that the CX_3CR1 receptor is also expressed on FL, MCL, and marginal zone lymphoma cells, considered the malignant counterparts of GC, naïve, and memory B lymphocytes, respectively. Experiments are in progress to clarify the functional activity of this receptor in such B cell lymphoproliferative disorders (unpublished results).

CONCLUDING REMARKS

CX_3CL1 and its receptor CX_3CR1 are implicated in several inflammatory and neoplastic disorders. We have shown for the first time that human naïve, GC, and memory B cells express CX_3CR1, but only a subset of GC B cells is attracted by soluble CX_3CL1. This cell fraction has a CX_3CR1^+ $CD27^+$ immunophenotype, consistent with that of centrocytes, and CX_3CL1 is produced by T_{FH} cells and FDCs, suggesting that the CX_3CL1/CX_3CR1 axis acts as a regulator of human centrocyte trafficking in the light zone of the GC of secondary lymphoid follicles.

We have also discussed recent results obtained by our group, delineating a novel role for the CX_3CL1/CX_3CR1 complex in B-CLL. Leukemic cell fractions expressed CX_3CR1 and CX_3CL1 and constitutively released the soluble form of the chemokine, but only a part of them migrated to CX_3CL1. NLCs generated in vitro from B-CLL patients coexpressed CX_3CR1 and CX_3CL1 but did not secrete the soluble form of the chemokine. CX_3CL1 attracted some but not all NLC suspensions. Up-regulation of CXCR4 on B-CLL cells induced by interaction of soluble CX_3CL1 with surface CX_3CR1 enhanced the attraction of leukemic cells toward NLCs secreting CXCL12. Thus, the CX_3CL1/CX_3CR1 axis appears to represent a novel addition to the complex molecular network regulating the interactions of B-CLL cells with their microenvironment.

AUTHORSHIP

A.C. designed and supervised in vitro and in vivo studies, analyzed data, and wrote the paper. E.F. performed in vitro and in vivo studies. V.P. reviewed the paper.

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