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CXCR4 Expression Functionally Discriminates Centroblasts versus Centrocytes within Human Germinal Center B Cells

Gersende Caron,2*† Simon Le Gallou,2* Thierry Lamy,*‡ Karin Tarte,*† and Thierry Fest3*†

The human germinal center is a highly dynamic structure where B cells conduct their terminal differentiation and traffic following chemokine gradients. The rapidly dividing centroblasts and the nondividing centrocytes represent the two major B cell subsets present in germinal center and also the most common normal counterparts for a majority of lymphomas. CD77 expression was previously associated to proliferating centroblasts undergoing somatic hypermutation, but data from transcriptional studies demonstrate that CD77 is not a reliable marker to discriminate human centroblasts from centrocytes. Herein we were able for the first time to separate these two subpopulations based on the expression of the chemokine receptor CXCR4 allowing their characterization. Phenotypic and functional features were especially explored, giving an accurate definition of CXCR4+ centroblasts compared with CXCR4− centrocytes. We show that CXCR4+ and CXCR4− germinal center B cells present a clear dichotomy in terms of proliferation, transcription factor expression, Ig production, and somatic hypermutation regulation. Microarray analysis identified an extensive gene list segregating these B cells, including highly relevant genes according to previous knowledge. By gene set enrichment analysis we demonstrated that the centroblastic gene expression signature was significantly enriched in Burkitt’s lymphomas. Collectively, our findings show that CXCR4 expression can properly separate human centroblasts from centrocytes and offer now the possibility to have purified normal counterparts of mature B cell-derived malignancies. The Journal of Immunology, 2009, 182: 7595–7602.

After Ag encounter in the edge of the T cell zone, Ag-specific B cells, in the company of Ag-primed T cells, will migrate into the B follicle of peripheral lymphoid tissues to generate a germinal center (GC)4 reaction, the functional niche required for the production of high-affinity Abs during adaptive immune response. Around days 7–10 after initial Ag priming, the GC follicle is polarized into a so-called “dark zone” of rapidly dividing centroblasts processing Ab maturation and a “light zone” of small nondividing centrocytes that undergo selection based on the affinity of their surface Igs for the inducing Ag. Finally, cells proceed to the terminal differentiation into Ab-producing plasma cells or memory B cells (1).

GC B cells (BGC) are thought to be the normal counterpart of some human B cell malignancies, including follicular lymphoma, diffuse large B cell lymphoma, and Burkitt’s lymphoma (BL) (2). Thus, a better knowledge of centroblast and centrocyte biology, through the comprehensive molecular and functional study of highly pure cell populations, is a prerequisite to fully understand lymphomagenesis. No relevant surface marker is available to functionally separate human centroblasts from centrocytes. So far, only CD77 expression was used to characterize proliferating centroblasts engaged in the somatic hypermutation (SHM) process, whereas CD77 is no longer expressed on centrocytes involved in the class switch recombination (CSR) process (3, 4). However, recent gene expression studies show that only limited differentially expressed genes were detected when BGC were segregated according to the CD77 marker (5–7). Remarkably, CD77+ cells represent a heterogeneous subset, which share the CD77+ cell proliferation program, DNA damage, and replication checkpoint activation and polycomb group protein expression (8), suggesting that centroblasts comprise cells lacking CD77. These data reinforced the need to have a better marker to discriminate human BGC subsets.

In mice, CXCR4 expression on BGC clusters the proliferating centroblasts in a unique site of the GC, thereby creating the dark zone rich in CXCL12, whereas the CXCR5-CXCL13 pair attracts BGC to the light zone (9). This compartmentalization is highly dynamic, and data recently confirmed by two-photon intravital microscopic studies described a B cell trafficking within each zone and interzonal migration of infrequent cells that may repeat one or several rounds of Ag-driven mutations (10–13). Moreover, a mathematical modeling of BGC migration suggests that the spatiotemporal GC organization is associated to a transient sensitivity of centroblasts and centrocytes to CXCL12 and to CXCL13, respectively (14). Thus, chemokine receptor expression levels by B cells and their regulation appear to be critical for GC organization. To gain insight into the human BGC compartmentalization, we analyzed the expression of both CXCR4 and CXCR5 on BGC. We observed that CXCR4 membrane expression segregated nicely the cells into a positive and
a negative subpopulation, with negative B cells presenting a CXCL12-induced CXCR4 internalization. We have further analyzed the proliferation status and the transcriptional profile of these two subpopulations and showed that, in contrast to CD77, CXCR4 was able to discriminate two functionally distinct BCG subsets. Importantly, we demonstrated for the first time that one marker, that is, CXCR4, was able to delineate a homogeneous B cell subpopulation that corresponds to human centroblasts, giving us the opportunity to characterize and compare them as well to their malignant counterpart.

Materials and Methods
Purification of human B cell subpopulations
Tonsils were obtained from routine tonsillectomies performed at Children’s Clinique La Sagesse at Rennes in accordance with ethical recommendations. After mincing, tonsillar mononuclear cells were isolated by Ficoll density centrifugation (Sigma-Aldrich). B cells were purified by negative selection using magnetic cell separation (B Cell Isolation Kit II; Miltenyi Biotec). The purity of the B cell fraction was routinely >99%. The CD10+CD44−/−CXCR4+ and CXCR4− B cell subpopulations were separated by FACS sorting using anti-CXCR4-PE (BD Biosciences), anti-CD10-energy-coupled dye (Beckman Coulter), anti-CD44-PE-Cy7, and anti-CXCR5-PE (R&D Systems). Abs used along this study are summarized in Table I.

Cell cycle analysis
Purified human tonsillar B cells (2 × 10⁶) were stained with CD10-energy-coupled dye, CD44-PE-Cy7, CXCR4-PE, and CD77-IFT, fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences), washed and resuspended in Perm/Wash buffer (BD Biosciences), and stained with 5 μM DRAQ5 DNA probe (Biotium). Cells were acquired on a FACSArria flow cytometer, and B cell subpopulations were analyzed for cell cycle phases using ModFit LT 3.1 software (Verity Software House).

Ig secretion assay
Human B cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FCS (Biowest) and antibiotics (Invitrogen) at 7.5 × 10⁶ cells/ml and stimulated for 3 days with 50 ng/ml recombinant human CD40L associated with 5 μg/ml cross-linking Ab, 50 UI/ml IL-2, 10 ng/ml IL-10, and 10 ng/ml IL-4 (R&D Systems), IgG, IgA, and IgM secretion was assessed by ELISA using a goat anti-human Ig for coating and secondary HRP-coupled Abs specific for γ-, α-, or μ-chains, respectively (all from Jackson ImmunoResearch Laboratories).

Quantitative real-time PCR
Total RNA was isolated from cells using an RNaseasy Mini Kit or Micro Kit (Qiagen), and cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time RT-PCR was performed using the TaqMan Universal PCR Master Mix, No AmpErase UNG, and Assays on Demand (Applied Biosystems). TaqMan Gene Expression assays for BCL6, PRDM1, PAIX, BACH2, IRF4, XBP1, CXCR4, CCNB1, BIC, and AID were purchased from Applied Biosystems. Gene expression levels were quantified using B2M as endogenous control. Results are expressed relative to total B cell fractions using the ΔΔCt comparative method.

Gene expression profile analysis
Total RNA from each purified population was extracted using an RNeasy kit (Qiagen), and RNA integrity was assessed using a bioanalyzer (Agilent Technologies). The hybridization was done onto Human Genome U133 Plus 2.0 GeneChips (Affymetrix) (data are available at www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc, accession no. GSE15271) following a standardized protocol develop by the Core Facility Platform of the Centre Hospitalier Universitaire de Montpellier. A total of four human tonsils have been sorted for CXCR4+ and CXCR4− BCG. The normalized hybridization intensity for each probe set was calculated using the GeneChip robust multiarray analysis method; background noise was decreased by eliminating probe set with a CV <0.8. Both analyses were done with an ArrayAssist software package (Agilent Technologies). An asymptotic paired t test was conducted selecting probe sets with a p value of <0.05 and fold change of >3.0 (ArrayAssist Software). Clustering analysis was done by using Partek Genomics Suite software with Pearson’s dissimilarity and average linkage methods, and gene functional analysis was assed with Ingenuity Pathways Analysis software (Ingenuity Systems). BL data were analyzed by principal components analysis, and 12 out of 18 cases (available on http://llmpp.nih.gov/BLI), which presented a homogeneous repartition, were selected for gene set enrichment analyses (ArrayAssist Software).

Statistical analysis
Statistical analyses were performed using GraphPad Prism software (GraphPad Software), and p values were calculated by two-tailed Student’s t test.

Results
CXCR4 membrane expression defines two human BCG subsets
Using multicolor flow cytometry, we sought to determine CXCR4 and CXCR5 surface expression on BCG. To address this question we used purified human tonsillar B cells labeled with CD19, CD10, CD44, and CXCR5 (or CXCR8). In the CD19+CD44−/−CD10− BCG population, we identified by flow cytometry and quantitative RT-PCR (qRT-PCR) (Fig. 1, A and E, respectively) two cell subsets based on CXCR4 expression. Both of them were CD38high, CD20high, CD27+; both were identified as CXCR4 surface expression, the CXCR4 molecule was detected in a homogeneous and intermediate CXCR4− B cell subpopulation but rather included B cells with a bright CD38 expression corresponding probably to recently differentiated plasma cells (2.71 ± 2.07%, n = 12). In contrast, non-BCG presented a homogeneous and intermediate CXCR4− staining. Concerning the CXCR5 expression, a unique peak was detected on both BCG and non-BCG (Fig. 1C). Interestingly, despite its undetectable surface expression, the CXCR4 molecule was detected in the cytoplasm of permeabilized CXCR4+ cells (Fig. 1D, left panel), suggesting a posttranslational regulation of the molecule. Due to the presence of high levels of intracytoplasmic CXCR4, we were unable to discriminate the two BCG subsets by immunostaining for CXCR4 on human tonsillar sections (data not shown). In culture and in the absence of CXCL12, CXCR4− BCG gained progressively CXCR4 on their surface (Fig. 1D, right panel) and all cells became CXCR4+ after 24 h (data not shown). This membrane recovery may be blocked when cells were kept at 4°C (data not shown) or when CXCL12 was added in the culture (Fig. 1D, right panel). Since all of these data were obtained on tonsillar B cells, we decided to test cells issued from reactive lymph nodes and found identical results emphasizing the interest of the CXCR4 chemokine receptor in the characterization of GC-derived B cells (data not shown). At this point we could conclude that in humans, as previously described in mice, the CXCR4 membrane receptor was able to segregate two BCG subpopulations (9).
We next compared CXCR4⁺ and CXCR4⁻ BGC and sought to determine whether CXCR4 expression correlates with the centroblast stage of BGC maturation. We first assessed cell proliferation on purified tonsillar B cells gated on CD10⁺CD44low BGC and analyzed for forward light scatter vs CXCR4 expression (Fig. 1A, right panel). In contrast to CXCR4⁺ cells, CXCR4⁻ BGC contained large cells (24.94 ± 3.87% of CXCR4⁺ BGC, n = 25), and the DNA content analysis revealed that 30% of CXCR4⁺ cells were cycling compared with 10% for the CXCR4⁻ subset (Fig. 2A).

**FIGURE 1.** CXCR4 membrane expression defines two BGC subsets in human tonsil. Surface expression of CXCR4 and CXCR5 was performed by immunofluorescence analysis on purified tonsillar B cells using a combination of anti-CD10, anti-CD44, and anti-CXCR4 or anti-CXCR5 Abs. A, BGC were gated as CD10⁺CD44low cells and analyzed for forward light scatter (FSC) vs CXCR4 expression. B, Phenotypic characterization of CD19⁺CD10⁺CD44low BGC and analyzed for forward light scatter vs CXCR4 expression. Data shown are representative of five independent experiments. C, Histogram plots show CXCR4 (upper panels) or CXCR5 (lower panels) expression on GC (left) and non-GC B cells (right). Data shown are representative of 10 tonsillar preparations. D, Left, Intracytoplasmic expression of CXCR4 on sorted CXCR4⁺ BGC using mAb 12G5 (black line) or an isotype control mAb (gray histogram). Right, Surface expression of CXCR4 on sorted CXCR4⁺ BGC 4 h after culture in complete medium, in the absence (black line) or presence (dotted line) of 500 ng/ml recombinant CXCL12. Data are representative of four independent experiments. E, CXCR4 transcript expression was measured by qRT-PCR on sorted CXCR4⁺ and CXCR4⁻ BGC in comparison with naive and memory (Mem) B cells. Results of qRT-PCR analysis are expressed relative to gene expression in total tonsillar B cells. Bars represent mean values ± SEM from three independent experiments (*, p < 0.05; **, p < 0.01).

**FIGURE 2.** Cell cycle analysis in CXCR4⁺ and CXCR4⁻ GC subsets. A, Cell cycle status was determined using DRAQ5 dye on GC CXCR4⁺ and CXCR4⁻ cells. Dot and histogram plots are representative of three independent experiments. B, Frequency of cells in each stage of the cell cycle (left panel, n = 4) and cyclin B1 transcript expression (right panel, n = 3) among BGC. Results of qRT-PCR expression are relative to gene expression in total tonsillar B cells. Cell cycle analysis was performed using the ModFit LT software. Bars represent mean values ± SEM (**, p < 0.01).
Remarkably, large CXCR4+ cells were highly proliferating, as 74.27% (±4.91%; n = 3) of them were in S and G2/M phases of the cell cycle (data not shown). Altogether, according to the presence or not of CXCR4 expression, two different BGC compartments may be defined in terms of cell proliferation (Fig. 2B, left panel) sustained by a differential cyclin B1 expression (Fig. 2B, right panel).

To account for stage-specific transcription factors expression in Ag-activated B cells, we assessed by qRTPCR the expression of six factors (BCL6, BACH2, PAX5, IRF4, PRDM1, and XBP1), which may present a mutually exclusive expression in the context of specific B cell subpopulations (Fig. 3) (16). To rule out the possibility of having contaminating plasmablasts in the CXCR4+ cell compartment, the CD38bright cells were discarded by cell sorting before qRTPCR. Moreover, naive (CD38−/IgD−) and memory (CD38+/IgD+) B cells were tested in parallel. Whereas the two subsets expressed transcriptional factors relative to GC origin, results showed a statistically significant down-regulation of BCL6, PAX5, and BACH2 and an up-regulation of PRDM1 and IRF4 in the CXCR4+ subset compared with CXCR4− cells (Fig. 3).

We next wanted to explore further the functionality of CXCR4+ and CXCR4− subpopulations to verify recent knowledge about GC biology. Classically, SHM occurs in the dark zone and CSR in the light zone of the GC, and recently microRNA-155 has been described as a major regulator of GC reaction by down-modulating activation-induced cytidine deaminase (AID) in B cells undergoing CSR (17). Taking into account that microRNA-155 is a functional end product of the BIC gene and that both expressions are constitutive of the GC reaction by down-modulating the light zone of the GC, and recently microRNA-155 has been described as a major regulator of GC reaction by down-modulating the light zone of the GC. Classically, SHM occurs in the dark zone and CSR in the light zone of the GC. Altogether, according to the presence or not of CXCR4 expression, two different BGC compartments may be defined in terms of cell proliferation (Fig. 2B, left panel) sustained by a differential cyclin B1 expression (Fig. 2B, right panel).

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Taken together, results from proliferation, transcription factor expression analysis, and Ig exploration suggest that CXCR4+ and CXCR4− BGC correspond respectively to the centroblast and the centrocyte stage of B cell differentiation.

Transcriptional analysis of the two major B cell compartments constitutive of the GC reaction

To define further the CXCR4+ centroblasts and CXCR4− centrocytes, a comparison of transcripts expressed by these two BGC subsets was conducted by microarray analysis. We identified 1039 probe sets corresponding to 745 specific genes differentially expressed between these two cell subsets within a range from 3 to 67.47 (probe set list in supplemental Table SI). Overall, CXCR4+ and CXCR4− compartments displayed 464 (among 640 probe sets) vs 281 (among 399 probe sets) specific up-regulated genes, respectively. Table II shows the top molecular and cellular functions as well as specific gene lists highlighted through this comparison. Briefly, genes implicated in alignment, orientation, and segregation of chromosomes (CENPE, CENPF, kinesin family...
CXCR4 expression is a much better centroblastic discriminator than CD77.

So far, the CD77 marker was proposed to discriminate centroblasts (CD77+) from centrocytes (CD77-) among BGC (3, 4). To address the question of whether CXCR4 or CD77 is the most specific marker to discriminate the centroblastic subpopulation, we further cell sorted the CXCR4+ and CXCR4- BGC based on the presence or not of CD77. Subsequently, we evaluated the cell cycle and transcription factor expression in the four different purified subsets (Fig. 5). While CD77+ cells were mainly CXCR4+ (78.5 ± 5.2%, n = 14), CD77- cells also expressed for the most part CXCR4 on their surface (62 ± 7.3%, n = 14) (Fig. 5A), meaning that CD77- cells were in the majority centroblastic cells. Our cell cycle analysis showed that both CD77+ CXCR4+ and CD77- CXCR4+ cells were similarly actively cycling (Fig. 5B, left panel). Conversely, CD77- cells were split into a proliferating and a nondividing subset according to CXCR4 expression, that is, CXCR4+ vs CXCR4-, respectively. Cyclin B1 expression assessment confirmed these later results (Fig. 5B, right panel). Expression of transcription factors by qRT-PCR clearly showed that CD77+ cells were like indeed to the same cell subpopulation (Fig. 5C). Taken together, these results illustrated that CXCR4 membrane expression discriminates more precisely centroblast vs centrocyte stages than does CD77.

Transcriptional analysis of BL compared with CXCR4+ and CXCR4− BGC subsets

Our findings permit us to now dispose of normal centroblasts for further explorations, offering the possibility for new insights about B cell development in human GC. Moreover, we can also explore new ways concerning lymphomagenesis, and one obvious approach would be to compare normal centroblasts with their malignant counterparts. Centroblastic non-Hodgkin’s B cell lymphomas represented a heterogeneous entity in which was isolated a specific proliferation that harbored a characteristic genetic signature and was classified as BL (20, 21). We therefore decided to compare CXCR4+ and CXCR4− Affymetrix data to gene expression profiling on 18 BL available from the Staudt and coworkers

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<th>Top 10 up-regulated genes</th>
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a Number of genes differentially expressed.
b Percentage of the total gene expressed for each considering cellular function.
c Results confirmed by qRT-PCR.
FIGURE 5. CXCR4 discriminates more precisely centroblast vs centrocyte stages than does CD77. A, Left, BGC were gated as CD10+CD44low cells and analyzed for CD77 vs CXCR4 expression. Right, Histograms represent percentage of CXCR4+ B cells in CD77+ and CD77− subsets. Bars represent mean values ± SEM (n = 14) (**, p < 0.01). B, Left, Cell cycle status was determined using DRAQ5 dye on BGC based on their CXCR4 and CD77 expressions. Histogram plots are representative of three independent experiments. Right, Cyclin B1 transcript expression was measured by qRT-PCR. Results are expressed relative to gene expression in total tonsillar B cells. Data shown are representative of experiments performed with three different tonsil samples.
CXCR4 expression. Our analysis demonstrates that transcription factors implicated in the GC formation, such as BCL6 and BACH2, and in the B cell identity, such as PAX5, are up-regulated at the mRNA level in both GC subsets. We distinguished statistically significant differences between the CXCR4+ and CXCR4− compartments for BACH2, PRDM1, IRF4, and BCL6, showing as expected from mice models an inverse regulation of IRF4 and BCL6 within the GC. More precisely, these molecules modified their expression following a rocking motion associated with two specific transient zones of the GC, that is, when B cells enter and exit the CXCR4+ stage of differentiation (25). Thus, starting from naive B cells, a striking increase of BCL6 and BACH2 expression associated with a repression of IRF4 was detected in CXCR4+ BGC, whereas CXCR4− cells down-regulated both BCL6 and BACH2 and reexpressed IRF4 associated to the emergence of PRDM1 expression. These data definitively place the CXCR4+ BGC on the final track of BGC maturation before occurrence of memory B cell or plasma cell commitment. Indeed, compared with CD38bright plasmablasts, CXCR4− BGC expressed 5.5- and 3.8-fold lower XBP1 and PRDM1, respectively (data not shown). Taken together, such assessment of transcription factor regulation in primary normal human BGC has never been done previously. Our results are in accordance with a published paper that characterized at the protein levels by immunohistochemistry the expression pattern of transcription factors within the GC on embedded tissues (25). Thus, the segregation of the BGC subsets based on the 1039 probe sets, which differentiate centroblasts from centrocytes. Red, high expression; blue, low expression.

**FIGURE 6.** Transcriptional analysis of BL compared with CXCR4+ and CXCR4− BGC subsets. Heat map represents mRNA expression of 4 CXCR4+, 4 CXCR4−, and 18 BL. Unsupervised clustering result is based on the 1039 probe sets, which differentiate centroblasts from centrocytes. Red, high expression; blue, low expression.
subsets probably explains why only limited differentially expressed genes were detected when BGC were segregated according to the CD77 marker (5). Finally, CD44 expression level was also proposed as a surrogate marker between centroblasts (CD44+) and centrocytes (CD44−) (27). In our transcriptomic comparison, CD44 was found to be 5-fold more highly expressed in CXCR4+ than in CXCR4− cells. By flow cytometry, relative mean fluorescence intensity of the CD44 labeling was only slightly lower in the CXCR4+ compared with CXCR4− BGC subset (26 ± 2.9 vs 29.8 ± 3.9, respectively; n = 8, p < 0.01; in comparison, non-BGC have a mean fluorescence intensity of 326 ± 61). Even if this finding confirms the interest of CD44 marker for separating human centroblasts from centrocytes, CXCR4 is by far the best discriminator.

 Knowledge of the major steps of normal B cell differentiation is necessary to fully understand lymphoma biology. The good segregation of BGC gives the opportunity to obtain the normal counterpart of GC-derived B cell neoplasias. We are now able to isolate a highly proliferative human BGC compartment, which corresponds to normal centroblasts. Among the latter, some aggressive forms of GC-derived lymphomas may emerge giving either a heterogeneous group of disease, that is, diffuse large B cell lymphomas, or a well-defined centroblastic malignancy, the Burkitt’s lymphoma, which is driven and primarily caused by a C-MYC deregulation (20, 21). In this study, we compared our microarray data with BL gene expression profile revealed that only a CXCR4+ signature was enriched in this neoplasia, demonstrating that the normal counterpart of BL is a centroblast. The proportions of centroblastic and centrocytic cells are important to determine the lymphoma stage. Thus, follicular lymphoma, another GC-derived malignancy, is divided into three grades depending on the number of large cells that appear under the microscope. Large cells tend to behave a bit more aggressively than do small cells. It will therefore be interesting to extend the comparison of BGC subset gene expression profiles with other GC-derived B cell neoplasias, such as follicular lymphoma and diffuse large B cell lymphomas, and to study more precisely the CXCR4+ and CXCR4− compartments in these pathologies. BL and diffuse large B cell lymphomas represent two distinct entities in the World Health Organization classification, but some atypical cases, with boundaries difficult to mark between these two pathologies, have led to define a “Burkitt-like” group. The BGC segregation could offer the possibility to redefine this atypical group of lymphoma.

In summary, for the first time we were able to isolate and characterize the two B cell compartments involved in the GC microarchitecture, that is, the dark zone and light zone, by adding the CXCR4 marker to the rest of the BGC panel. The centroblast stage seems to be a unique stage of the B cell development entirely dedicated to cell proliferation and highly susceptible to cell death. The fact that we can now separate the BGC in functionally distinct centroblasts and centrocytes should provide new insights into the mechanisms that regulate normal BGC biology and that lead to the development of B cell malignancies.

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Disclosures
The authors have no financial conflicts of interest.

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