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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.

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A
fier 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) 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 Iggs 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,
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 show that, in contrast to CD77, CXCR4 was able to discriminate two functionally distinct BGC 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-CD19-Pacific Blue (eBioscience) mAbs. Sorts were performed on a FACSAria cell sorter (BD Biosciences). When indicated, CD38-PE-Cy5 anti-CD19-Pacific Blue (eBioscience) mAbs. Sorts were performed on a FACSAria cell sorter (BD Biosciences), anti-CXCR4-PE (BD Biosciences). When indicated, CD38-PE-Cy5 and anti-CD19-Pacific Blue (eBioscience) mAbs. Sorts were performed on a FACSAria cell sorter (BD Biosciences). When indicated, CD38-PE-Cy5 (Beckman Coulter), CD77-FITC, or IgD-FITC (BD Biosciences) were also used to sort B cell subpopulations. Abs used along this study are summarized in Table I.

Cell cycle analysis

Purified human tonsillar B cells (2 x 10⁵) were stained with CD10-energy-coupled dye, CD44-PE-Cy7, CXCR4-PE, and CD77-FITC, 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 (Biostatus). Cells were acquired on a FACSAria 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 x 10⁵ cells/ml and stimulated for 3 days with 50 ng/ml recombinant human CD40L associated with 5 μg/ml cross-linking Ab, 50 U/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 RNeasy 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, PAX5, 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 ∆∆Ct 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⁻ BGC. 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). ANOVA was performed on log transformed data.

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 BGC subsets

Using multicolor flow cytometry, we sought to determine CXCR4 and CXCR5 surface expression on BGC. To address this question we used purified human tonsillar B cells labeled with CD19, CD10, CD44, and CXCR4 (or CXCR5). In the CD19⁺CD44low CD10⁻ B cell 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 CD38lowhigh, CD27high, CD27⁺, and IgD⁺ (Fig. 1B), corresponding to the phenotypic definition of a GC B cell (15). However, the CD44lowCD10⁻CXCR4⁻ subset was shown not to be a pure cell population but rather included B cells with a bright CD38 expression corresponding probably to recently differentiated plasma-blasts (2.71 ± 2.07%, n = 12). In contrast, non-BGC presented a homogeneous and intermediate CXCR4⁺ staining. Concerning the CXCR5 expression, a unique peak was detected on both BGC and non-BGC (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 BGC subsets by immunostaining for CXCR4 on human tonsillar sections (data not shown). In culture and in the absence of CXCL12, CXCR4⁺ BGC 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 BGC subpopulations (9).

Table I. List of Abs used for flow cytometry analysis and cell sorting

<table>
<thead>
<tr>
<th>Anti-Human Abs</th>
<th>Clone</th>
<th>Isotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>IgD-FITC</td>
<td>IA4-2</td>
<td>IgG2a</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>IgG-FITC</td>
<td>G18-145</td>
<td>IgG1</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD77-FITC</td>
<td>5B5</td>
<td>IgM</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CXCR4-PE</td>
<td>12G5</td>
<td>IgG2a</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CXCR5-PE</td>
<td>51505</td>
<td>IgG2b</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>CD10-energy-coupled</td>
<td>ALB1</td>
<td>IgG1</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>IgD-coupled</td>
<td>CD27-PE Cy5</td>
<td>IgG1</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>CD38-PE Cy5</td>
<td>LS198-4-3</td>
<td>IgG1</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>CXCR4-PE Cy7</td>
<td>IM7</td>
<td>IgG2b (rat)</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD19-Pacific Blue</td>
<td>HIB19</td>
<td>IgG1</td>
<td>eBioscience</td>
</tr>
</tbody>
</table>

CXCR4 AND HUMAN GC B CELL SUBSETS
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\(^-\) CD44\(^{low}\) 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\(\%\) 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\(^-\) CD44\(^{low}\) cells and analyzed for forward light scatter (FSC) vs CXCR4 expression. B. Phenotypic characterization of CD19\(^+\) CD10\(^-\) CD44\(^{low}\) 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\(\%\) 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 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 analysis are expressed relative to gene expression in total tonsillar B cells. Cell cycle analysis was performed using the ModFit LT software. Bars represent mean values \(\pm\) SEM (\(\star, p < 0.05; \star\star, p < 0.01\)).

**CXCR4\(^+\) and CXCR4\(^-\) BGC display, respectively, centroblast and centrocyte features**

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\(^-\) CD44\(^{low}\) 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\(\%\) 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).
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 qRT-PCR 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 qRT-PCR. Moreover, naive (CD38−/H11569, BACH2, PRDM1, IRF4, XBP1) in CXCR4+ cells. Bars represent mean values ± SEM from four independent experiments (*, p < 0.05).

FIGURE 3. Expression of transcriptional factors in CXCR4+ and CXCR4− GC subsets. Differentially regulated transcripts (BCL6, PAX5, BACH2, PRDM1, IRF4, XBP1) in CXCR4+ and CXCR4− BGC in comparison with naive and memory (Mem) B cells are shown. Results of qRT-PCR analysis are expressed relative to gene expression in total tonsillar B cells. Bars represent mean values ± SEM from four independent experiments (*, p < 0.05).

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 S1). Overall, CXCR4+ and CXCR4− BGC 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

FIGURE 4. CXCR4+ cells secrete a higher amount of IgG than does the CXCR4− BGC subset. A, AID and BIC transcripts expression were measured by qRT-PCR on sorted CXCR4+ and CXCR4− BGC. Results are expressed relative to gene expression in total tonsillar B cells and are representative of three independent experiments. B, Left, Purified tonsillar B cells were gated on CD10+ CD44low BGC and analyzed for membrane IgG vs CXCR4 expression. Right, CXCR4+ and CXCR4− BGC were cultured for 3 days in the presence of recombinant CD40L, IL-2, IL-4, and IL-10. Supernatants were analyzed for secreted Ig via capture ELISA. Bars represent mean values ± SEM from six independent experiments (*, p < 0.05).

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 S1). Overall, CXCR4+ and CXCR4− BGC 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

5 The online version of this article contains supplemental material.
marker to discriminate the centroblastic subpopulation, we further
question of whether CXCR4 or CD77 is the most specific
(Fig. 5). While CD77 transcription factor expression in the four different purified subsets
down-regulated compared with CXCR4 and DNA replication (RAD50, BRCA1, BRCA2) were overex-
members), proliferation (MKI67, CCNB1), repair, recombination, and DNA replication (RAD50, BRCA1, BRCA2) were overex-
expression was a much better centroblastic discriminator
results indicated that 745 genes are significantly involved in the
cell subpopulation likely less prone to cell death. Of interest, these
erones genes (HSP90B1, FKBP11, STCH), and trafficking genes
ment showed an increased expression of survival signals (BCL2, E2F1, CCNB1, BRCA1).
so far, the CD77 marker was proposed to discriminate centroblasts
among BGC (3, 4). To address
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+ CXCR4+ and CD77- CXCR4+ belonged certainly to the same cell subpopulation (Fig. 5C). Taken together, our 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

### Table II. Overview of transcriptome results in the comparison of CXCR4+ and CXCR4- BGC

<table>
<thead>
<tr>
<th>Repartition of top molecular and cellular functions</th>
<th>CXCR4+</th>
<th>CXCR4-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle (n = 129)</td>
<td>80</td>
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<tr>
<td>Cellular assembly and organization (n = 49)</td>
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<tr>
<td>DNA replication, recombination, and repair (n = 89)</td>
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<td>Cellular movement (n = 94)</td>
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<tr>
<td>Cytokinesis (n = 19)</td>
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<td>Trafficking (n = 5)</td>
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<td>Infiltration (n = 20)</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>Cellular growth and proliferation (n = 183)</td>
<td>58</td>
<td>42</td>
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<table>
<thead>
<tr>
<th>Top 10 up-regulated genes</th>
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<tr>
<td>WASF3</td>
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<td>SGOL2</td>
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<td>PEG10</td>
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<table>
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<tr>
<th>Ten selected up-regulated genes of interest in the context of BGC</th>
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<tbody>
<tr>
<td>AICDA</td>
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<tr>
<td>BACH2</td>
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<td>ALDH7A1</td>
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<tr>
<td>PLK1</td>
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<tr>
<td>E2F1</td>
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<tr>
<td>CCNB1</td>
<td>4.2</td>
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<tr>
<td>BRC1A1</td>
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<tr>
<td>p53</td>
<td>4.03</td>
</tr>
<tr>
<td>BIRC5</td>
<td>4.5</td>
</tr>
<tr>
<td>MKI67</td>
<td>15.9</td>
</tr>
</tbody>
</table>

- | Number of genes differentially expressed.
- | Percentage of the total gene expressed for each considering cellular function.
- | Results confirmed by qRT-PCR.
CXCR4 surface expression discriminates the two B cell compartments that constitute the GC, that is, centroblasts and centrocytes. Results issued from cell cycle distribution, transcription factor expression, and IgG production, reinforced by data obtained from gene expression profiling, allowed us to discriminate the proliferating CXCR4+ centroblasts undergoing SHM from CXCR4- centrocytes, which correspond to a nondividing cell population in the way of terminal differentiation, expressing Ig and chaperone molecules. Taken together, our findings indicate that in human chronically inflamed tissue, the proliferating B compartment seems to be restricted to centroblasts, CXCR4+ cells, unlike recently described in mouse acute GC, where proliferating cells were found in both CXCR4+ and CXCR4- subsets (23). In mice, the chemokine receptors CXCR4 and CXCR5 are essential for GC polarization (9). CXCR4 membrane expression is needed for positioning BGC in the dark zone rich in CXCL12, whereas the CXCL13/CXCR5 pair is required to attract the former B cells in the light zone. Accordingly, our results demonstrated that CXCR4 surface expression is also restricted to the human centroblasts and segregated nicely these cells from centrocytes. Moreover, we observed that the CXCR4 molecule was still present at the cytoplasmic level in CXCR4+ centroblasts and segregated nicely these cells from centrocytes. We compared the expression of transcription factors between BGC and between the CXCR4+ and CXCR4- subpopulation as well (data not shown). A data set of 7110 probe sets, presenting a SD/mean of >0.8, was kept for further analyses. Unsupervised clustering yielded a clear segregation between malignant and normal BGC and between the CXCR4+ and CXCR4- subpopulation as well (data not shown). We next used a gene set enrichment analysis and sought to identify whether the 640 probe sets up-regulated in CXCR4+ correlated with the BL when the analysis was done with CXCR4 as reference (22). Of the 640 probe sets, 268 were in the data set and presented a distribution that formerly indicated that CXCR4+ signature expression was significantly enriched in BL (p = 0.003, false discovery rate of 0.033). In contrast, among 399 probe sets up-regulated in CXCR4− cells, 329 were present in the data set but their distribution demonstrated the absence of correlation between centrocytes and BL. Interestingly, by restricting to the 1039 probe sets that distinguished centroblasts from centrocytes, we were able to cluster the CXCR4+ cells with the Burkitt cases using an unsupervised clustering (Fig. 6). These results showed that despite a clear segregation between malignant and nonmalignant B cells, BL cells maintain a centroblastic expression profile.

Discussion

In this study, using purified B cells from human tonsil and reactive lymph nodes, we demonstrated for the first time in humans that CXCR4 surface expression discriminates the two B cell compartments that constitute the GC, that is, centroblasts and centrocytes. Results issued from cell cycle distribution, transcription factor expression, and IgG production, reinforced by data obtained from gene expression profiling, allowed us to discriminate the proliferating CXCR4+ centroblasts undergoing SHM from CXCR4- centrocytes, which correspond to a nondividing cell population in the way of terminal differentiation, expressing Ig and chaperone molecules. Taken together, our findings indicate that in human chronically inflamed tissue, the proliferating B compartment seems to be restricted to centroblasts, CXCR4+ cells, unlike recently described in mouse acute GC, where proliferating cells were found in both CXCR4+ and CXCR4- subsets (23). In mice, the chemokine receptors CXCR4 and CXCR5 are essential for GC polarization (9). CXCR4 membrane expression is needed for positioning BGC in the dark zone rich in CXCL12, whereas the CXCL13/CXCR5 pair is required to attract the former B cells in the light zone. Accordingly, our results demonstrated that CXCR4 surface expression is also restricted to the human centroblasts and segregated nicely these cells from centrocytes. Moreover, we observed that the CXCR4 molecule was still present at the cytoplasmic level in CXCR4+ cells, and the presence of CXCL12 in culture blocked the acquisition of CXCR4 on the surface of these cells. Thes data suggest that CXCR4− BGC in vivo have internalized CXCR4 after being in the presence of a large amount of CXCL12 in the dark zone, concomitant to their migration toward the light zone (24). Additionally, a transcriptional regulation of CXCR4 seems to exist in the GC, which is in agreement with our microarray and qRT-PCR data; CXCR4 mRNA expression increased when B cells started the GC reaction, then dropped dramatically at the centrocytic stage and finally re-increased once cells exited the GC (Fig. 1E). Mechanisms involved in this process are still under investigation. Recent data from two-photon microscopic studies demonstrated the highly dynamic structure of the GC, with an interzonal circulation and a cyclic reentry of B cells in both the dark and light zones, correlated with specific chemokine receptor expression levels (14, 23). This cell trafficking between dark and light zones might explain why some of CXCR4+ cells were IgG+ whereas the CXCR4− cells were IgG−.

We compared the expression of transcription factors between naive B cells, memory B cells, and the two BGC subsets based on
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-high and CXCR4-low 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-high 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-high BGC, whereas CXCR4-low cells down-regulated both BCL6 and BACH2 and reexpressed IRF4 associated to the emergence of PRDM1 expression. These data definitively place the CXCR4-high BGC on the final track of BGC maturation before occurrence of memory B cell or plasma cell commitment. Indeed, compared with CD38bright plasmablasts, CXCR4-high 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 CXCR4 membrane expression will give the opportunity to generate a GC in vitro model allowing a better understanding of the GC biology.

CD77 expression was previously used to discriminate BGC. Thus, the CD77-high subset was associated to centroblasts, while the CD77-low B cell subset was associated to centrocytes. Our data revealed that the proportion of the CD77-high and CD77-low B cell subsets in GC (39.3% vs 60.7%, respectively; n = 14, data not shown) is clearly at odds with GC biology, where proliferating centroblasts represent the majority of the B cell population. The proportion of CXCR4-high and CXCR4-low B cells in GC (68.6% vs 31.4%; n = 14, data not shown) is more consistent with our knowledge of GC. Moreover, a previous study demonstrated that not only the CD77-high cells, but also the CD77-low cells, are proliferating (5). Here we confirmed that the CD77-low subset was a heterogeneous subpopulation for proliferation program. Flow cytometry analysis revealed that proliferative and nondividing BGC correspond to the CXCR4-high and CXCR4-low subsets, respectively, independently of CD77 expression. Polycomb gene complexes were recently identified as novel regulators of hematopoiesis, and they appear to be expressed differently in a nonoverlapping pattern in follicular B cells (8, 26). Thus, their expression profile reflects distinct B cell differentiation stages in human GC. EED and EZH2, two polycomb genes shown to be expressed in the dark zone centroblasts, were up-regulated at the mRNA level in CXCR4-high CD77-high BGC subsets, while BMI-1, another polycomb gene expressed in light zone centrocytes, was up-regulated in the CXCR4-low CD77-high B cells (data not shown). Interestingly, several studies on gene expression profiling compared CD77-high with CD77-low BGC and detected only a limited differentially expressed gene list with a partial overlap between them, confirming the poor discriminatory power of the CD77 marker for BGC (5–7). When we compared our 745 gene list implicated in the centroblast/centrocyte transition with data obtained by Nakayama et al. (7), among their 51 up-regulated genes in CD77-low cells, 30 genes were also up-regulated in CXCR4-high cells, and 4 genes among the 8 found preferentially expressed by CD77-high cells were up-regulated in the CXCR4-high subset. A comparison with microarray data of Klein et al. (6) revealed that only 2 of 19 genes of this study (IL-8 and FCGR) based on the CD77 expression were found in our microarray analysis. Finally, B3GALT3, the only BGC discriminant marker implicated in CD77 biosynthesis found in the study of Högerkorp and Borrebaeck (5), was not found to be differentially expressed between CXCR4-high and CXCR4-low BGC. The high proportion of CXCR4-high cells in both CD77-high and CD77-low BGC...
terpart of GC-derived B cell neoplasias. We are now able to isolate and to study more precisely the CXCR4
expression profile of this neoplasia, demonstrating
that it is a surrogate marker for separating normal 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 seg-
regation of BGC gives the opportunity to obtain the normal counter-
part of GC-derived B cell neoplasias. We are now able to isolate a highly proliferative human BGC compartment, which corre-
sponds to normal centroblasts. Among the latter, some aggressive forms of GC-derived lymphomas may emerge giving either a hetero-
egeneous group of disease, that is, diffuse large B cell lympho-
mas, or a well-defined centroblastic malignancy, the Burkitt’s lym-
phoma, which is driven and primarily caused by a C-MYC deregulation (20, 21). In this study, the comparison of our mi-
croarray 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-de-
rivced 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 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 lymphoma 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 char-
acterize the two B cell compartments involved in the GC micro-
architecture, that is, the dark zone and light zone, by adding the
architecture, that is, the dark zone and light zone, by adding the
identifier to Ig class switching.

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Disclosures
The authors have no financial conflicts of interest.