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Regulation of CXCR3 and CXCR4 expression during terminal differentiation of memory B cells into plasma cells

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C-X-C motif chemokine receptor 3 (CXCR3) and CXCR4 expressed on immunoglobulin G (IgG)–plasma-cell precursors formed in memory immune responses are crucial modulators of the homing of these cells. Here, we studied the regulation of the expression of these chemokine receptors during the differentiation of human memory B cells into plasma cells. We show that CXCR3 is absent on CD27⁻ naive B cells but is expressed on a fraction of memory B cells, preferentially on those coexpress-ing IgG1. On differentiation into plasma-cell precursors, CXCR3⁺ memory B cells maintain the expression of this chemokine receptor. CXCR3⁻ memory B cells up-regulate CXCR3 and migrate toward concentration gradients of its ligands only when costimulated with interferon γ (IFN-γ), but not interleukin 4 (IL-4), IL-1β, IL-6, IFN-α, IFN-β, or tumor necrosis factor α (TNF-α). In contrast, the differentiation of CXCR4⁻ B cells into plasma cells is gener-ally accompanied by the induction of CXCR4 expression. These results show that lack of CXCR4 expression on plasma-cell precursors is not a limiting factor for plasma-cell homing and that the expression of CXCR3 on memory B cells and plasma-cell precursors is induced by IFN-γ, provided in human T helper type 1 (Th1)–biased immune responses. Once induced in memory B cells, CXCR3 expression remains part of the individual cellular memory. (Blood. 2005;105: 3965-3971)

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Introduction

Antibodies secreted by terminal differentiated B cells are important in immune defense,[1] but also can contribute to the pathogenesis of autoimmune diseases.[2] On stimulation by antigen and CD40 signaling by T cells, naive B cells can form germinal centers yielding memory B cells expressing antibodies of higher affinities.[3] On a second contact with antigen, memory B cells can differentiate into proliferating antibody-secreting plasmablasts found in the extrafollicular areas of secondary lymphoid tissues.[4] These cells further differentiate into mature nondividing plasma cells either remaining within the tissues of their origin or accumulating in mucosa-associated tissues, bone marrow, and sites of inflammation.[5,6] Plasma-cell homing to different tissues is a crucial regulator of the strength and duration of humoral immune responses. Maintained antibody responses are associated with plasma-cell homing to the bone marrow.[7] Under pathologic conditions, long-term survival of plasma cells is also supported in chronically inflamed tissues.[8,9] The local production of antibodies by plasma cells resident at such sites is likely to be important in clearing pathogens. In individuals suffering from systemic inflammatory autoimmune diseases, disturbed plasma-cell localization is associated with the production of autoantibody titers refractory to conventional therapy.[10]

During differentiation into plasma cells, B cells change their chemokine receptor expression profile.[5,6,11] Expression of C-C chemokine receptor type 9 (CCR9) and CCR10 allows homing of immunoglobulin A (IgA) plasma cells to mucosal tissues.[12,13] IgG⁺ plasma-cell precursors formed in a secondary immune response against systemic antigenic challenge migrate toward ligands for C-X-C motif chemokine receptor 3 (CXCR3) and CXCR4, likely allowing them to migrate to inflamed tissue and bone marrow, respectively.[14,15] Additionally, both chemokine receptors and their cognate ligands likely also play a role for the localization of activated B cells, plasmablasts, and plasma cells within secondary lymphoid tissues. Plasma cells surrounding the germinal centers are colocalized with CXCL12, the ligand for CXCR4.[16,17] In the draining lymph nodes, CXCL9 is produced by a subset of high endothelial venules (HEVs) surrounding the B-cell follicles.[18]

Here, we show that in vivo, CXCR3 is expressed only on a fraction of human memory B cells, preferentially those expressing IgG1. Once induced, the expression of this chemokine receptor is maintained during plasma-cell differentiation. Thus, the information whether this chemokine receptor is used during an immune response seems to be part of the B-cell memory. On CXCR3⁻ B cells, interferon γ (IFN-γ), but not interleukin 4 (IL-4), IL-1β, IL-6, IFN-α, IFN-β, or tumor necrosis factor α (TNF-α), induces the expression of this receptor. For that, IFN-γ must be present before day 3 following activation of the B cells. In vivo, during this period, activated B cells are located within the follicular areas, colocalized with T cells,

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suggesting that CXCR3 expression is induced by human T helper type 1 (Th1) cells.

In contrast, the majority of memory B cells express CXCR4. On B cells lacking this receptor, on activation with various stimuli, plasma-cell differentiation is generally accompanied by induction of CXCR4 expression. Thus, lack of CXCR4 expression on plasma-cell precursors is not a limiting factor for plasma-cell homing to the bone marrow.

Materials and methods

Media and cytokines

Cell culture medium RPMI 1640 (Life Technologies, Paisley, United Kingdom) was used, supplemented with 10% fetal calf serum (FCS), 20 μM β_2-mercaptoethanol, 10 mM L-glutamate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 μg/L gentamicin, all purchased from Invitrogen (Carlsbad, CA). In various culture conditions the following cytokines were added: IL-2, IL-4, IL-10 (AMS Biotechnology, Wiesbaden, Germany); IL-1β, IL-6, IFN-γ (R&D Systems, Minneapolis, MN); TNF-α (Sigma-Aldrich, Steinheim, Germany); and IFN-αA, and IFN-β1a (PBL Biomedical Laboratories, Piscataway, NJ).

Cell culture

B cells were purified from human blood of healthy donors. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by a standard Ficoll-Hypaque PLUS (Amersham Biosciences, Uppsala, Sweden) gradient method. B cells were enriched from PBMCs to more than 98% purity (data not shown) by magnetic-associated cell sorting (MACS). PBMCs were incubated 15 minutes at 4°C with antihuman CD19 microbeads as indicated by the manufacturer (Miltenyi Biotec; Bergisch Gladbach, Germany), washed, and loaded on 2 succeeding light-sensitive (LS) columns (Milteny Biotec). For some cultures, CXCR3$^-$ or CXCR4$^-$ cells were isolated by fluorescence-activated cell sorting (FACS DIVA, Becton Dickinson, Heidelberg, Germany). The isolated cells were mainly cultured in a 2-step culture system. B cells were initially cocultured with the murine thymoma cell line EL4-B5 (50 Gy irradiated)[19], constitutively expressing CD40L with the addition of recombinant IL-2 (50 U/mL) and IL-10 (50 U/mL) for a B cell/T cell ratio of 1:10. After 3 days of culture, B cells were isolated by MACS by using antihuman CD19 beads as described. Subsequently, they were cultured for another 5 days in the presence of recombinant IL-2 (50 U/mL) and IL-10 (50 U/mL). In some cultures IL-4 (100 U/mL), IL-1β (50 U/mL), IL-6 (50 U/mL), IFN-αA (20 U/mL, 200 U/mL, 6600 U/mL), IFN-β1a (20 U/mL, 200 U/mL, 6600 U/mL), IFN-γ (200 U/mL, 2000 U/mL), or TNF-α (10 ng/mL) was added to the culture. Alternatively, B cells were stimulated with IL-2 (50 U/mL) and IL-10 (50 U/mL) and cytosine phosphate guanine (CpG) 2006 (0.3 nmol/mL; Tibmolbiol, Berlin, Germany) or *Staphylococcus aureus* Cowan I (50 ng/mL; Pansorbin cells, Calbiochem, San Diego, CA). To achieve optimal stimulation of naive B cells, CpG 2006 (0.3 nmol/mL, Tibmolbiol), B-cell receptor cross-linking with F(ab$'$)$_2$ fragment of goat anti–human immunoglobulin (2 μg/mL; Jackson ImmunoResearch, West Grove, PA), IL-2 (50 U/mL), and IL10 (50 U/mL) were used for stimulation. Approval was obtained from the Ethik Comission of the Charite, University of Berlin institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki.

Staining and flow cytometry

Isolated PBMCs were stained with antihuman CXCR3-phycoerythrin (PE), CXCR4-allophycocyanin (APC), CD19-biotinylated, IgM-fluorescein isothiocyanate (FITC) purchased from BD Pharmingen (Le Pont de Claix, France), and IgA-FITC (Cymbus Biotechnology, Hampshire, United Kingdom). The following antibodies were produced and coupled in the institute to fluorochromes or biotin: CD19-FITC (clone BU 12), CD27-PE (clone 2 E 4), IgG1-FITC (clone IDC-1), IgG2-biotinylated (clone G 18-21), and IgG3-biotinylated (clone G 18-3). CD20-FITC, CD19-biotinylated, CD27-

FITC, CD38-APC, CXCR3-PE, and CXCR4-PE were purchased from BD Pharmingen; streptavidin-peridinin chlorophyll protein (PerCP; BD Pharmingen) was used as a secondary reagent. Dead cells were excluded with DAPI dilactate (4$'$,6-diamino-2-phenylindole, dilactate; 0.2 μg/mL; Molecular Probes, Eugene, OR). Cells were incubated with staining reagents in phosphate-buffered saline/bovine serum albumin (PBS/BSA, 0.5%) for 10 minutes on ice. Analysis was performed by flow cytometry (LSR I, Becton Dickinson). Cell cycles were measured by flow cytometry using 5- and 6-carboxyfluorescein diacetate–succinimidyl ester (CFDA-SE) as described (Molecular Probes).[20]

Chemotaxis assay

A chemotaxis assay recently described[14] was modified to analyze human cells. Chemotaxis assays were performed in 5-μm pore Transwell inserts (Costar, Corning, NY) coated with 50 μL human fibronectin (10 μg/mL; Sigma-Aldrich). Cells harvested from culture were washed and diluted in prewarmed (37°C) assay medium (RPMI 1640 supplemented with 0.5% BSA, low endotoxin; Sigma-Aldrich) at a concentration of 2.5 to 5 × 10^6 cells/mL. The lower Transwell chamber was filled with 600 μL assay medium supplemented with the chemokine CXCL9 (100 nM; R&D Systems) and 100 μL cells was added to upper chamber and incubated for 90 minutes at 37°C in a atmosphere of 5% CO$_2$. Subsequently, IgG-secreting cell numbers before and after migration were quantified by solid-phase enzyme-linked immunospot (ELISPOT) assay. Migration toward medium alone was less than 2% (basal migration).

ELISPOT

IgA-, IgG-, or IgM-secreting cells were quantified by ELISPOT.[21] Antibodies used for ELISPOT were goat anti–human isotype antibodies (Sigma-Aldrich; for coating used 5 μg/mL) and biotin labeled goat anti–human isotype antibodies (Sigma-Aldrich; for detection 1 μg/mL). Streptavidin-alkaline phosphatase (Roche, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Sigma-Aldrich) were used for development as described.[14]

Results

The expression of CXCR3 on memory B cells is correlated with coexpression of IgG1

In mice, the chemokine receptors CXCR3 and CXCR4 are important in the regulation of the localization of IgG-plasma cells formed within memory immune responses.[14] In the present study, the expression of these chemokine receptors was determined on human memory B cells from peripheral blood of healthy donors and correlated to the expression of particular antibody isotypes by FACS. B cells were identified by the expression of CD19; naive and memory B cells were distinguished by the absence and presence of CD27 expression, respectively.[22] About 6.4% ± 4.3% of naive B cells expressed CXCR3 and 87.6% ± 8.8% expressed CXCR4. Among the population of memory B cells, 32.1% ± 9.5% expressed CXCR3 and 68.1% ± 2.9% expressed CXCR4 (data not shown). No correlation was found between CXCR4 expression and the coexpression of the antibody isotypes IgG1, IgG2, IgG3, IgA, or IgM on individual cells (Figure 1). In contrast, CXCR3 expression was associated with the coexpression of IgG1. About 34.7% ± 3.9% of memory cells expressing IgG1, but only 10% ± 6.3% expressing IgG2 ($P < .001$), did coexpress CXCR3 (Figure 1). The differences in CXCR3 expression between memory B cells expressing IgG1 and IgG3 ($P < .030$) or IgM ($P < .043$) were also significant, but less pronounced.
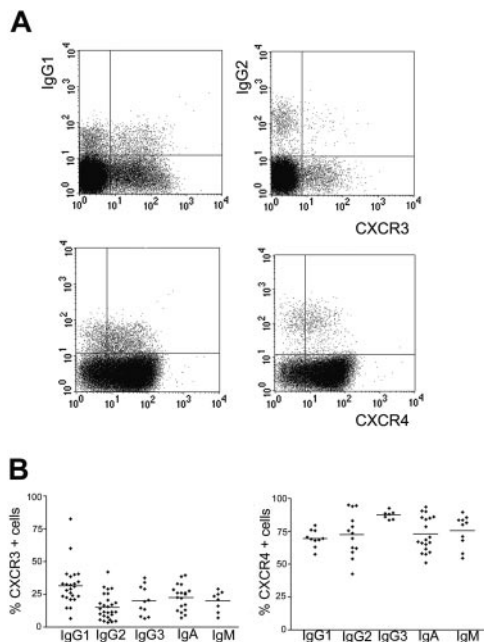
Figure 1. CXCR3 is preferentially expressed on memory B cells coexpressing IgG1. PBMCs were stained for CD19, antibody isotypes, CXCR3, and CXCR4 and analyzed by FACS. To identify IgM memory cells, cells were additionally stained for CD27. Dead cells were excluded by DAPI staining. (A) Representative FACS analysis. Dot plots were gated on living CD19$^+$ B cells. IgM$^+$ cells were additionally gated for CD27$^+$ cells, a marker for memory B cells. (B) Frequencies of CXCR3$^+$ and CXCR4$^+$ memory B cells expressing the antibody isotypes indicated. Cells were analyzed by FACS as described. Each dot resembles cell frequencies of one individual donor. Differences between CXCR3$^+$/IgG1$^+$ and CXCR3$^+$/IgG2$^+$ B cells were significant ($P < .05$). The horizontal bars show the mean value.

Once induced in B cells, CXCR3 expression is part of the cellular memory

To induce differentiation into plasma cells, B cells were stimulated by cytokines plus CD40 ligation or CpG as described in "Materials and methods." With both methods, following 8 days in culture, 10% to 40% of cells showed a CD20$^-$/CD38^{++} plasma-cell phenotype (Figure 2A). As evaluated by ELISPOT, similar frequen-

cies of cultured cells secreted antibodies (data not shown). In accordance with earlier results,[23,24] under these stimulation conditions preferentially CD27$^+$ memory B cells were activated to differentiate into plasma cells (data not shown).

To analyze possible changes in the expression of CXCR3 during their differentiation into plasma cells, before culture, CD19$^+$ B cells were sorted into CXCR3$^-$ and CXCR3$^+$ fractions by FACS. When cultures were started with CXCR3$^-$ or CXCR3$^+$ B cells, between 13.7% ± 1.5% or 81.0% ± 4.8% of B cells and plasma cells, respectively, expressed this chemokine receptor at day 3 and day 8 (Figure 2B). This result shows that once induced in memory B cells, CXCR3 expression remains stable during their differentiation into plasma cells and is part of the individual cellular memory.

CXCR3 expression is induced by IFN-γ

The question how the expression of CXCR3 is induced in B cells was addressed next. Ligands for CXCR3 attract lymphocytes into inflamed tissues,[25,26] favoring inflammatory cytokines like IFN-α, IFN-β, IL-1β, IL-6, or TNF-α as candidates potentially inducing CXCR3 on B cells. Other candidates were IFN-γ and IL-4, characteristic for Th1 and Th2 responses, respectively.[27] The latter options are based on the finding that the expression of CXCR3 on B cells is correlated to the coexpression of IgG1, an antibody isotype induced by T cell–derived cytokines.

To identify the factors regulating CXCR3 expression, CXCR3$^-$ B cells were activated by CD40 ligand together with IL-2 and IL-10 as described and cultures were supplemented with the candidate cytokines listed. When no additional cytokines were added, the frequency of CXCR3$^+$ cells among the CD20$^+$/CD38$^{+/-}$ B cells and CD20$^-$/CD38^{++} plasma cells were 19.5% ± 3.8% at day 8 of culture. Addition of IL-1β, IL-4, IL-6, or TNF-α did not alter the frequency of CXCR3$^+$ cells (Figure 3; Table 1). In contrast, addition of 200 U/mL IFN-γ led to a significant increase in the frequency of CXCR3$^+$ B cells and plasma cells to 50% ± 6.5%, measured at day 8 (Figure 3). Addition of a 10-fold higher concentration of IFN-γ resulted in
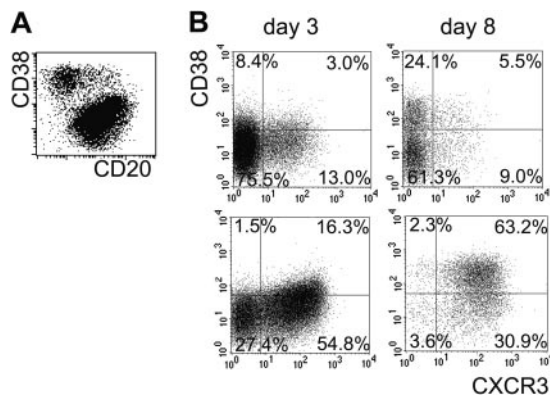
Figure 2. CXCR3$^+$ B cells maintain the expression of this receptor during plasma-cell differentiation. (A) Plasma-cell differentiation was induced on isolated B cells as described. Following 8 days of stimulation, between 10% and 40% of B cells had undergone differentiation into CD38^{++}/CD20$^-$ plasma cells. (B) Prior to stimulation, cells underwent FACS into CXCR3$^-$ and CXCR3$^+$ B cells. At day 3 and day 8 of culture, CD19$^+$ B cells and plasma cells were analyzed for the expression of CXCR3 and CXCR4. Upper plots show cultures started with CXCR3$^-$ B cells; lower plots show cultures started with CXCR3$^+$ B cells. Shown are results from one representative experiment of five. Percentages indicate the percentage of CD38 and CXCR3 expression in each quadrant gated an CD19$^+$ lymphocytes.
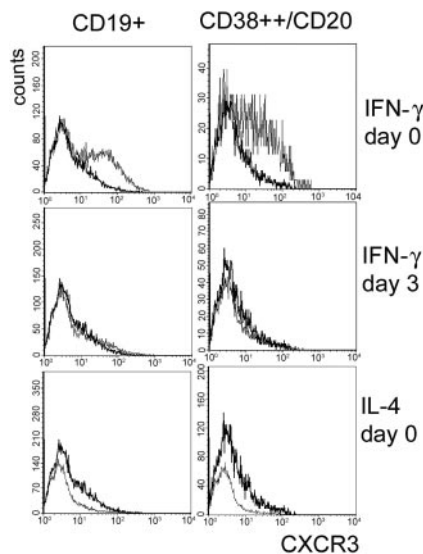
Figure 3. Early costimulation with IFN-γ led to the up-regulation of CXCR3 expression. Sorted CXCR3$^-$ peripheral blood B cells were activated with CD40L, IL-2, and IL-10 as described (bold lines). To some cultures, IFN-γ or IL-4 was added at day 0 or day 3 as indicated (thin lines). CD19$^+$ B cells (left column) and CD38^{++}/CD20$^-$/CD19$^+$ plasma cells (right column) were analyzed at day 8 of culture. Data shown are representative for one of more than four experiments (Table 1).

Table 1. Influence of cytokines on the expression of CXCR3

Culture conditions	CXCR3+ cell frequencies	Change in CXCR3+ cells	No. of experiments
CD40L, IL-2, IL-10 (basal condition)	19.5% ± 3.8%	0	8
+IFN-γ (200 U/mL) d 0	50.0% ± 6.5%	30.5% ± 10.3%	8
+IFN-γ (2000 U/mL) d 0	65.8% ± 4.7%	34.7% ± 8.5%	3
+IFN-γ (200 U/mL) d 3	12.8% ± 1.0%	4.5% ± 4.8%	3
+IFN-α/β (20 U/mL) d 0	23.4% ± 1.8%	3.9% ± 5.6%	4
+IFN-α/β (200 U/mL) d 0	18.4% ± 1.6%	−1.1% ± 5.4%	4
+IFN-α/β (6600 U/mL) d 0	24.1% ± 1.8%	4.6% ± 5.6%	4
+IL-4 (100 U/mL) d 0	22.4% ± 5.5%	3.7% ± 9.3%	5
+IL-6 (50 U/mL) d 0	22.9% ± 3.7%	−8.2% ± 7.5%	3
+IL-1β (50 U/mL) d 0	33.6% ± 0.7%	0.6% ± 4.5%	2
+TNF-α (200 U/mL) d 0	33.1% ± 0.7%	−2.2% ± 4.5%	2
CpG 2006, IL-2, IL-10	25.8% ± 2.6%	0	5
+IFN-γ (200 U/mL) d 0	43.4% ± 2.3%	30.5% ± 10.3%	5

Sorted CXCR3− peripheral blood B cells were stimulated with either CD40L-bearing EL4-B5 cells, IL-2, and IL-10, or with CpG 2006, IL-2, and IL-10 to induce differentiation into CD38++/CD20− plasma cells (basal stimulation condition). Additional cytokines were added at day 0 or at day 3 as indicated. This table shows the frequencies of CXCR3+ cells in individual cultures and the changes induced by the added cytokines compared to cells cultured under basal culture conditions in the same experiment. Results are mean values from repeated experiments.

lower frequencies of plasma cells, but did not further increase in the frequencies of CXCR3+ cells. Also, cultures supplemented with IFN-α, IFN-β, IL-1β, or IL-4 showed in some, but not all, experiments an increase in the frequencies of CD38++/CD20− plasma cells at day 8. Addition of IFN-γ later than day 3 showed no effect on the expression of CXCR3 on the activated B cells (Figure 3), showing that the expression of this chemokine receptor is induced within the first 3 days of plasma-cell differentiation.

In these experiments, as a source of CD40L, irradiated thymoma cells were used. To exclude the possibility that the expression of CXCR3 on B cells was not directly induced by IFN-γ, but by another factor expressed by the thymoma cells on stimulation with IFN-γ, in other experiments purified B cells were stimulated by CpG, in the absence of any other cell type. Again, addition of IFN-γ increased the frequency of CXCR3+ B cells and plasma cells up to 43.4% ± 2.% (Table 1).

Together, these experiments identified IFN-γ as a direct inducer of CXCR3 expression during terminal B-cell differentiation. They also demonstrate that the induction of CXCR3 expression occurs before day 3 following B cells are initially activated. That is before cells with a plasma-cell phenotype appear in culture as well as in the course of a specific immune response in vivo.[4,28,29]

Stimulation with IFN-γ increases the frequency of IgG-secreting cells that migrate toward concentration gradients of CXCL9

The expression of a chemokine receptor on a particular cell does not necessarily correlate with its capability to migrate toward the concentration gradients of the corresponding chemokines.[30,31] Therefore, we tested whether the addition of IFN-γ to the B-cell culture system not only leads to the induction of CXCR3 expression, but also enhances the frequency of IgG-secreting plasma cells migrating toward CXCL9, one of the ligands for CXCR3. For this, CXCR3− B cells were stimulated in the presence or absence of recombinant IFN-γ. This cytokine was added in concentrations not influencing the numbers of IgG-secreting plasma cells induced. The percentage of those cells migrating toward CXCL9 was tested in chemotaxis assays (Figure 4). In cultures not supplemented with

IFN-γ, the mean frequency of IgG-secreting cells migrating toward CXCL9 was 9.5% ± 3.8%. Addition of IFN-γ enhanced this frequency to 37.2% ± 15.2%. This increase in IgG-secreting cells migrating toward CXCL9 is comparable with the increase in the frequency of CXCR3-expressing plasma cells observed in that cultures, indicating that IFN-γ–induced CXCR3 expression allows activated B cells to migrate toward the cognate ligands of this receptor.

CXCR4 is constitutively up-regulated during T-dependent and T-independent plasma-cell differentiation

It had been shown in mice that plasmablasts formed in the course of a T-dependent immune response express CXCR4 and remain positive for this receptor during their differentiation into plasma cells.[11,14] CXCR4-deficient plasma cells accumulate only in severely reduced numbers in the bone marrow.[17]

About 68.1% ± 2.9% of human peripheral blood CD27+ memory B cells express CXCR4 (Figure 1 and data not shown). Following stimulation, the frequency of CXCR4+ cells among the activated CD20−/CD38+/− B cells and CD20−/CD38++ plasma cells increased slightly to 90% and 95%, respectively (data not shown). To test whether this was due to up-regulation of this chemokine receptor or due to a selective recruitment of CXCR4+ B cells into the plasma-cell differentiation pathway, CXCR4− B cells were enriched to more than 98% purity. These cells, roughly 72% of them memory B cells, were activated by IL-2 and IL-10 and either CD40L, S aureus Cowan I (SAC), or CpG. These culture methods resemble T-dependent and T-independent B-cell stimulations, respectively. After 3 days, in all cultures, 50.1% ± 9.4% of B cells already expressed CXCR4. At day 8, 75.2% ± 4.8% of plasma cells expressed CXCR4 (Figure 5). About 87.6% ± 8.8% of isolated CD27− naive B cells expressed this chemokine receptor. Following stimulation under T-independent activation conditions by B-cell receptor cross-linking, these cells also maintained CXCR4 expression (data not shown). Together, these results indicate that differentiation of naive and memory B cells into plasma cells is generally accompanied by induction of the expression of this chemokine receptor.

Up-regulation of the expression of CXCR3 and CXCR4 does not depend on cell-cycle progression

Recent data revealed that the frequency of CXCR3+ T cells following activation increases with the number of cell divisions. In
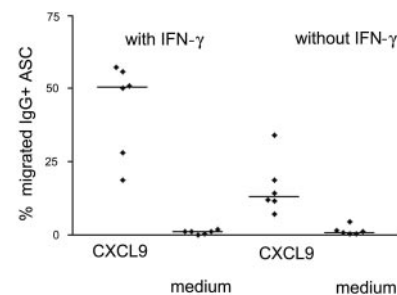
Figure 4. IgG-secreting cells formed in cultures supplemented with IFN-γ showed enhanced migration toward CXCL9. Plasma-cell differentiation was induced on sorted CXCR3+ or CXCR3− peripheral blood B cells in the presence or absence of IFN-γ. After 8 days of culture, migration of IgG+ antibody-secreting cells (ASC) toward 100 nM CXCL9 was analyzed by Transwell chemotaxis assays. IgG-secreting cells were quantified by ELISPOT. Each dot represents the percentage of migrating IgG-secreting cells of one experiment. Differences between frequencies of migrating cells from cultures with and without IFN-γ are statistically significant (P < .05). Horizontal bars indicate the median value.
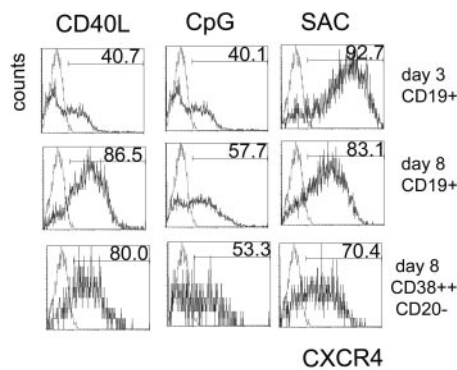
Figure 5. Plasma-cell differentiation is generally accompanied by the up-regulation of CXCR4 expression. Sorted CXCR4⁻ peripheral blood B cells were stimulated with IL-2, IL-10, and CD40L (left), CpG 2006 (middle), or *S aureus* Cowan I (SAC, right). Histogram plots show CXCR4 expression of CXCR4⁻ sorted B cells before culture (day 0, thin line) and CXCR4 expression of CD19⁺ B cells and CD38⁺⁺/CD20⁻ plasma cells at day 3 and day 8 (bold line). Shown is a representative result of 3. Values at top right indicate the percentage of CXCR3-expressing CD19⁺ and CD38⁺⁺/CD20⁻ cells, respectively, compared with CXCR4⁻-sorted cells.

contrast, the frequency of CXCR4⁺ T cells decreases with cell-cycle progression.[32,33]

Here, we tested whether up-regulation of CXCR3 and CXCR4 on B cells is associated with the number of cell divisions. B cells negative for the respective chemokine receptor were stained with CFDA-SE and stimulated with CpG, IL-2, and IL-10. To induce the expression of CXCR3 on CXCR3⁻ B cells, IFN-γ was added. At day 3 of the cultures, expression of CXCR3, CXCR4, and content of CFDA-SE was analyzed (Figure 6). A fraction of B cells that had not undergone a complete cell division had already up-regulated CXCR3 and CXCR4. The frequencies of B cells that had up-regulated these receptors did not significantly differ between those that had proliferated and those that had not. These results indicate that the expression of CXCR3 and CXCR4 on activated B cells does not depend on proliferation. This is in accordance with the observation that the induction of CXCR3 expression occurs early following activation.

Discussion

CXCR3, CXCR4, and their cognate chemokines are important regulators for the homing of murine IgG-secreting plasma cells formed in memory immune responses.[14] The presence of CXCR4 on human bone marrow plasma cells had been reported.[34,35] The importance of CXCR4 for plasma-cell homing to the bone marrow had been clearly demonstrated in CXCR4-deficient fetal liver chimeras. In these mice, normal numbers of plasma cells are formed but compared to wild type, the numbers of plasma cells accumulating in the bone marrow are reduced to about 30%.[17] Plasma-cell homing to bone marrow is a specific feature of T-dependent immune responses.[36] These findings prompted us to question whether up-regulation of CXCR4 expression on plasma-cell precursors determines their capabilities to home to the bone marrow and whether induction of the expression of this receptor depends on T cell–derived signals. As shown in the present study, in peripheral blood, the great majority of all mature naive and memory B cells express CXCR4 and remain positive for this receptor following activation and differentiation into plasma cells. On activation of B cells by all stimuli tested, including T-independent signals, plasma-cell differentiation generally seems to be accompanied by the induction of CXCR4 expression. These

results suggest that CXCR4 expression is not limited to plasma-cell precursors formed in T-dependent responses and that during plasma-cell differentiation, the decision for bone marrow homing is not made by the induction to express CXCR4. Recent results showed that all human bone marrow plasma cells also express CXCR6 and CCR10.[34] It remains to be elucidated whether plasma-cell homing to the bone marrow is regulated by the modulation of CXCR4 function or by other chemokine receptors.

Plasma cells can accumulate within chronically inflamed tissues due to in situ formation[37] and due to efficient immigration from secondary lymphoid tissues.[8] Ligands for CXCR3 likely attract plasma-cell precursors to sites of inflammation.[14,38] There, the CXCR3 ligands CXCL9, CXCL10, and CXCL11 are expressed in high quantities, attracting activated CXCR3-bearing leukocytes.[25] The production of CXCL9 in high endothelial venules surrounding the B-cell follicles[18] may indicate that the expression of CXCR3 on activated B cells also modulates their recirculation through, or release from, the follicles, hence influencing their differentiation. This idea is supported by the observation that the expression of CXCR3 on B cells is induced early following activation, even before these cells had divided.

The data presented here identified IFN-γ as a potent inducer of CXCR3 expression during terminal B-cell differentiation. In cultures started with CXCR3⁻ B cells not supplemented with IFN-γ, only a small fraction of activated B cells and plasma cells expressed this chemokine receptor at the end of the culture period. It could not be excluded that another factor, like IFN-γ able to induce CXCR3 expression on B cells, was present in these cultures. Other possibilities include the presence of low levels of IFN-γ in the FCS supplementing the cultures or the autocrine secretion of this cytokine by the activated B cells. It is also possible that B cells up-regulating CXCR3 in the cultures had been stimulated by IFN-γ in vivo, prior to their isolation.

The observed results show that the induction of CXCR3 occurs within the first 3 days of plasma-cell differentiation, that is, before B cells acquire a plasma-cell phenotype, thus suggesting that up-regulation of CXCR3 expression occurs on activated B cells, but not during later differentiation stages on plasmablasts or plasma cells. In the course of an immune response in vivo, before day 3 of antigenic challenge, B cells are found in the follicular areas
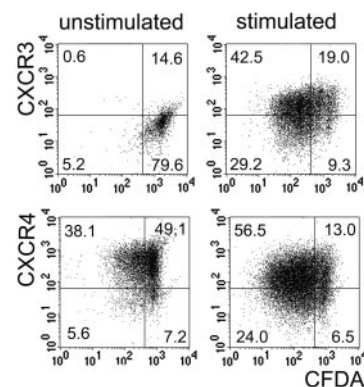
Figure 6. Up-regulation of CXCR3 and CXCR4 expression on activated B cells does not depend on proliferation. Sorted CXCR3⁻ or CXCR4⁻ peripheral-blood B cells where labeled with CFDA-SE and stimulated with CpG, IL-2, and IL-10. IFN-γ was added to CXCR3⁻ B cells to induce the expression of CXCR3. Following 3 days cultures started with CXCR3⁻ B cells were analyzed for CXCR3 expression, cultures started with CXCR4⁻ B cells for CXCR4 expression, and compared to the loss of CFDA-SE by FACS. Borders for chemokine receptor staining were set according to isotype controls. One representative result of 3 is shown. Values represent the percentage of CDFA content and CXCR3 or CXCR4 expression, respectively, in each quadrant.

adjacent to T-helper cells.[39] These T-effector cells are classified into Th1 and Th2 cells, originally defined by their capacity to secrete either IFN-γ or IL-4, respectively.[27] In contrast to IFN-γ, IL-4 does not induce the expression of CXCR3 on B cells. To our knowledge, no other cell type present in the follicles had been reported to secrete IFN-γ, thus indicating that help by Th1 cells is the prominent mechanism inducing CXCR3 on activated B cells. Noteworthy, it had been shown that the expression of CXCR3 on T cells is correlated to a Th1 phenotype.[40] Interestingly, in the present study, a positive correlation between the expression of IgG1 and CXCR3 on memory B cells was observed. Murine B cells undergo immunoglobulin class switch to IgG1 in response to stimulation by IL-4.[41] In human cells, this cytokine induces class switch to IgG4 and IgE,[42,43] whereas several factors seem to be able to induce class switch of human B cells to IgG1 in vitro.[44,45] During Lyme borreliosis infection, a pathogen inducing a strong IFN-γ response, IgG1, together with IgG3, is the dominant immunoglobulin isotype.[46] The latter report is in line with the data presented here showing that IgG1 expression on human B cells isolated from peripheral blood is positively correlated to the coexpression of CXCR3, a chemokine receptor that is upregulated in response to IFN-γ.

It could not be excluded that other factors than IFN-γ can induce CXCR3 expression on terminally differentiating B cells. However, the results presented in this study suggest that the following scenario is at least one mechanism leading to the accumulation of plasma cells within inflamed tissues. At that site, interferons, mainly IFN-γ, induce the expression of the CXCR3 ligands CXCL9, CXCL10, and CXCL11, allowing the attraction of CXCR3-bearing leucocytes.[25] Antigen-presenting cells (APCs)

immigrating from the site of inflammation induce the formation of IFN-γ–secreting Th1 cells in the draining lymph nodes. These T cells can stimulate activated B cells specific for the same antigen to express CXCR3, thus supporting their accumulation within the adjacent inflamed tissue.

It had been shown by Butcher and colleagues that peripheral antibody-secreting cells induced by vaccination via parenteral or mucosal routes differ in their homing potential resembled by the expression pattern of tissue specific adhesion molecules.[47] The authors proposed that the site of original B-cell activation determines the preferential homing potentials of antibody-secreting cells to mucosal sites or the systemic compartment. The data presented here show that B cells stimulated with IFN-γ are induced to express CXCR3, allowing them to migrate to the site of inflammation. This could happen in inflamed tissues or in secondary lymphoid organs where IFN-γ production is induced by APCs or T-cell immigrants from the inflamed tissue. Thus, similar to the homing of activated B cells to the mucosal or systemic compartment, the factor inducing their homing potential to inflamed tissue originates from the target site. Once induced in memory B cells, CXCR3 expression and function remain stable during reactivation and differentiation into plasma cells, thus indicating that the homing potential of B cells to inflammatory tissues is part of their individual cellular memory.

Acknowledgments

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