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*J Immunol* 2011; 186:2800-2808; Prepublished online 26 January 2011;
doi: 10.4049/jimmunol.1003401
http://www.jimmunol.org/content/186/5/2800

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CXCR4 Expression on Activated B Cells Is Downregulated by CD63 and IL-21

Nobuya Yoshida,*‡ Daisuke Kitayama,* Masafumi Arima,* Akemi Sakamoto,* Ayako Inamine,* Haruko Watanabe-Takano,* Masahiko Hatano,‡ Takao Koike,‡ and Takeshi Tokuhisa*†

CXCR4 expression is critical for localization of centroblasts in the dark zone of germinal centers (GCs), and centrocytes downregulate CXCR4 and thus leave the dark zone to reside in the light zone. However, mechanisms governing CXCR4 downregulation on centrocytes are not known. In this study, we show that the amount of intracellular CXCR4 in centroblasts was similar to that in centrocytes, suggesting differential control of CXCR4 protein expression in these GC B cells. Restimulation of activated B cells with IL-21, which is a major cytokine produced by T follicular helper cells, accelerated CXCR4 internalization by inducing endocytosis-related GRK6 expression. Although CXCR4 expression was downregulated on GC B cells by IL-21 stimulation, CXCR4low centrocytes developed in the spleens of IL-21R–deficient mice, suggesting other mechanisms for downregulation. The level of CD63 (which recruits CXCR4 to late endosome in CD4 T cells) in centrocytes was more than that in centroblasts and was strikingly elevated in activated Bcl6-deficient B cells. Bcl6, a transcriptional repressor, was detected on the chromatin of the CD63 gene in resting B cells, therefore CD63 is a molecular target of Bcl6. Downregulation of CD63 mRNA in activated Bcl6-deficient B cells by small interfering RNA upregulated CXCR4 expression on the B cells. Furthermore, addition of Bcl6 inhibitor to activated B cell cultures increased CD63 mRNA expression in (and downregulated CXCR4 expression on) those activated B cells. Thus, CXCR4 can be downregulated on activated B cells by IL-21–induced endocytosis and CD63-mediated endosomal recruitment, and these mechanisms may contribute to downregulation of CXCR4 on centrocytes. The Journal of Immunology, 2011, 186: 2800–2808.

Germinal centers (GCs) are the site for development of high-affinity memory B cells and long-lived plasma cells (1, 2). After Ag-activated B cells collaborate with activated T follicular helper (Tfh) cells on the follicular border, some of the activated B cells rapidly proliferate in the follicle to generate GCs. These proliferating B cells, centroblasts, undergo somatic hypermutation and form the dark zone of GCs. Then, the centroblasts turn to differentiate to centrocytes with Ig class-switching to IgG. The centrocytes migrate to an area adjacent to the dark zone of GCs. In the area called the light zone of GCs, centrocytes express high-affinity IgG Abs that competitively bind to Ags on follicular dendritic cells and also collaborate with GC–Tfh cells, which produce IL-21 and IL-4 (3). These activated centrocytes scarcely proliferate and differentiate to memory B cells or long-lived plasma cells. The level of a chemokine receptor, CXCR4, on centroblasts is significantly more than that on centrocytes (4, 5), and CXCR4 plays an important role in segregation of dark and light zones in GCs (4). Thus, CXCR4 expression on centrocytes has to be downregulated to leave the dark zone to migrate to the light zone. However, the mechanism of its downregulation on centrocytes is not known.

Because CXCR4 is known as a coreceptor for HIV infection and as a key molecule for cancer metastasis, mechanisms of CXCR4 expression are mainly studied on CD4 T cells and cancer cell lines (6–8). In addition to transcriptional control of the CXCR4 gene (9–12), CXCR4 expression on the cell surface is regulated by its endocytosis and exocytosis. The various endocytosis-related molecules including GRK6, β-arrestin2 (Arrb2), and AIP4 are inducible in CD4 T cells for the clathrin-dependent internalization of CXCR4 (13–16). Of the exocytosis-related molecules, CD63, a ubiquitously expressed tetraspanin, has been identified as a negative regulator of CXCR4 exocytosis in CD4 T cells (17, 18). CD63 interacts with CXCR4 directly through the N-linked glycosylation of CD63. The CD63–CXCR4 complex induces directional CXCR4 trafficking to the endosomes/lysosomes, rather than to the plasma membrane. Thus, activation of the endocytosis-related proteins such as GRK6, Arrb2, and AIP4 and/or that of CD63 may be related to the downregulation of CXCR4 on centrocytes in the light zone of GCs.

We generated GC-like B cells in vitro by sequentially stimulating splenic B cells with anti-IgM Abs and anti-CD40 Abs plus IL-4 and then with IL-21 or IL-4 after a 2-d interval (19). Using the in vitro stimulation system, we have reported that restimulation of activated B cells with IL-21 or IL-4 induced proliferation and differentiation of CXCR4low or CXCR4high B cells, respectively. Thus, IL-21 from Tfh cells may play a role in downregulation of CXCR4 on centrocytes in the light zone of GCs. In this study, we show that restimulation of activated B cells with IL-21 induces GRK6 expression to activate endocytosis of CXCR4.

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Abbreviations used in this article: Ac-H3, acetylation of histone H3 lysine (K) 9 and K14; Arrb2, β-arrestin2; ChIP, chromatin immunoprecipitation; Ct, threshold cycle; GC, germinal center; siRNA, small interfering RNA; Tfh, T follicular helper; WT, wild-type.

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Furthermore, CXCR4 expression was continuously downregulated in activated B cells from Bcl6-deficient (Bcl6<sup>−/−</sup>) mice with overexpression of CD63, and the CD63 gene is a molecular target of Bcl6. We discuss roles of IL-21 and CD63 in maintaining the downregulation of CXCR4 on centrocytes in the light zone of GCs.

Materials and Methods

**Mice**

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan), B6.C<sup>−/−</sup> (20) and IL-21R<sup>−/−</sup> (IL-21R<sup>−/−</sup>) (21) mice were described. All mice were maintained under specific pathogen-free conditions in the animal center of the Graduate School of Medicine, Chiba University. The care of all animals used in the current study was in accordance with Chiba University Animal Care Guidelines.

**Immunization and purification of GC B cells**

Mice were immunized intraperitoneally with 50 μg alumn-precipitated (4-hydroxy-3-nitrophenyl)acetyl<sub>2</sub>-chicken gammaglobulin (Biosearch Technologies) and 1.0 × 10<sup>9</sup> PFU Bordetella pertussis (Nacionala Tesque, Kyoto, Japan). GC B cells were isolated from the spleens of these mice 10 d after immunization. Briefly, spleen cells were first blocked with unconjugated anti-CD32/16 mAbs (2.4G2; BD Pharmingen), followed by incubation with allopregocyanin Cy7-anti-B220 mAbs (BD Pharmingen), FITC-anti-GL7 mAbs (BD Pharmingen), PE-anti-Fas mAbs (BD Pharmingen), and allopregocyanin-anti-CXCR4 mAbs (BD Pharmingen). GC B cells (B220<sup>+</sup>GL7<sup>+</sup>Fas<sup>+</sup>), centroblasts (B220<sup>+</sup>GL7<sup>+</sup>Fas<sup>+</sup>CXCR4<sup>high</sup>), and non-GC B cells (B220<sup>+</sup>GL7<sup>+</sup>Fas<sup>+</sup>) were sorted by a FACSArray II (Becton Dickinson). Purity of each FACS-sorted population was >99%.

**Splenic B cell culture**

Spleen cell suspensions were treated with Trypan blue (Invitrogen) and counted under a microscope after staining with trypan blue (Invitrogen). The care of all animals used in the current study was in accordance with Chiba University Animal Care Guidelines.

**Flow cytometry analysis**

Cells were blocked with unconjugated anti-CD32/16 mAbs followed by incubation with mAbs as indicated: allopregocyanin–anti-IgG1 (BD Pharmingen), FITC–anti-IgG1 (BD Pharmingen), PE–anti-IgG1 (BD Pharmingen), and allopregocyanin–anti-CXCR4 (BD Pharmingen), allopregocyanin–anti-CXCR4 (BD Pharmingen), FITC–anti-CXCR4 (BD Pharmingen), FITC–rat IgG2b for an isotype control (BD Pharmingen), FITC–anti-GL7 (BD Pharmingen), and PE–anti-Fas (BD Pharmingen). Biotinylated Abs were detected by allopregocyanin–streptavidin (BD Pharmingen). Flow cytometric analysis was performed with a FACS Calibur (Becton Dickinson) or a FACS Canto II (Becton Dickinson) using CellQuest software (Becton Dickinson) or FlowJo software (TOMY Digital Biology), respectively. For intracellular staining, cells were stained with FACS Permeabilizing Solution 2 (Becton Dickinson) according to the manufacturer’s instructions. For CFSE staining, purified B cells were labeled with CFSE (Molecular Probes) as described previously (24).

**Real-time quantitative RT-PCR**

RT-PCR was performed as described elsewhere (25). Total RNA was extracted from B cells with the Trizol reagent (Life Technologies). Total RNA was reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen) and Oligo (dT) (Pharmacia), and the cDNAs were used for PCR. After an initial 5-min incubation at 94°C, 50 cycles of PCR were carried out using the following conditions: denaturation at 94°C for 60 s, annealing at 60°C for 60 s, and polymerization at 72°C for 60 s. RT-PCR primers for the cDNA amplification were the following: Bcl6, 5′-CCG-GCT-GAT-CGA-TGG-TGA-A-3′ and 5′-GTC-GAT-CAT-GAG-GT-TG-GA-3′; GRK6, 5′-CCA-TCCCA-TAACCCAGCAACAGA-3′ and 5′-GTT-CGAGATGAGATGA-3′; CXCR4, 5′-ACGGCCGTGTTGAGACT-3′ and 5′-AGGGTCCTGTTGGAGAC-3′; CXCR4<sup>mRNA</sup>, 5′-ATGTTGTTTCCTTACTG-3′ and 5′-GCCGTTCTCTGACCACT-3′; CXCR4<sup>cytoplasmic</sup>, 5′-ACGGCCGTGTTGAGACT-3′ and 5′-AGGGTCCTGTTGGAGAC-3′; CXCR4<sup>intracellular</sup>, 5′-ATGTTGTTTCCTTACTG-3′ and 5′-GCCGTTCTCTGACCACT-3′; Bcl6, 5′-CCAGCTTCTTCTTCTTCTT-3′ and 5′-TGGCATAAGGGT-3′.

**Immunoblot**

Cultured B cells were washed with PBS and lysed with a lysis buffer (1% Nonidet P-40, 5% glycerol, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 μg/ml leupeptin, 0.1 mM PMSE, 1 mM DTT, 1 μg/ml pepstatin A, 10 mM Na<sub>2</sub>VO<sub>4</sub>, and 10 mM NaF). Cell lysate was subjected to SDS-PAGE, and immunoblot was carried out as described previously (26). Primary Abs used were goat polyclonal IgG anti-CD63 Abs (R-13, sc-31213; Santa Cruz), 5′-CCAGCTTCTTCTTCTTCTT-3′ and 5′-TGGCATAAGGGT-3′.

**Nucleofection of small interfering RNA**

Predesigned CD63 small interfering RNA (siRNA) (s63677, s63678, and s63679; Silencer Select Predesigned siRNA; Ambion) and scramble siRNA (Silencer Select Negative Control #1 siRNA; Ambion) were purchased, and 500 pM each of siRNA was transfected to purified B cells previously stimulated with anti-IgM Abs and anti-CD40 mAbs plus rIL-4 (2 U/ml), using the nucleofector I device (Amaxa). Nucleofection of each siRNA was performed according to the manufacturer’s recommendations.

**Flow cytometry analysis**

Cells were blocked with unconjugated anti-CD32/16 mAbs followed by incubation with mAbs as indicated: allopregocyanin–anti-IgG1 (BD Pharmingen), FITC–anti-IgG1 (BD Pharmingen), PE–anti-IgG1 (BD Pharmingen), and allopregocyanin Cy7–anti-B220 (BD Pharmingen), biotinylated anti-CXCR4 (BD Pharmingen), allopregocyanin–anti-CXCR4 (BD Pharmingen), allopregocyanin–anti-CXCR4 (BD Pharmingen), FITC–anti-CXCR4 (BD Pharmingen), FITC–rat IgG2b for an isotype control (BD Pharmingen), FITC–anti-GL7 (BD Pharmingen), and PE–anti-Fas (BD Pharmingen). Biotinylated Abs were detected by allopregocyanin–streptavidin (BD Pharmingen). Flow cytometric analysis was performed with a FACS Calibur (Becton Dickinson) or a FACS Canto II (Becton Dickinson) using CellQuest software (Becton Dickinson) or FlowJo software (TOMY Digital Biology), respectively. For intracellular staining, cells were stained with FACS Permeabilizing Solution 2 (Becton Dickinson) according to the manufacturer’s instructions. For CFSE staining, purified B cells were labeled with CFSE (Molecular Probes) as described previously (24).

**FIGURE 1.** Restimulation of activated B cells with IL-21 and IL-4 inversely regulates CXCR4 expression on those B cells in vitro. Naïve B cells were cultured with anti-IgM Abs and anti-CD40 mAbs plus a low dose of IL-4 for 7 d. IL-21 or IL-4 was added at day 2 of culture. A, IgG1 and CXCR4 expression on activated B220<sup>+</sup> B cells at day 4 of culture were analyzed by flow cytometry. The numbers in the plot indicate the percentage of each gate. Data are presented as a representative of five independent experiments. B, Numbers of CXCR4<sup>high</sup> and CXCR4<sup>low</sup> B cells in the culture were calculated by the flow cytometry profiles. Open and filled circles indicate IL-4 and IL-21 restimulated B cells, respectively.
Cruz Biotechnology) and goat polyclonal IgG anti-actin Abs (C-11, sc-1615; Santa Cruz Biotechnology).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using a ChIP assay kit (Upstate Biotechnology) and conducted according to the manufacturer’s recommendations as described (27). Briefly, protein and DNA in B cells (3 × 10^7/ml) were cross-linked by adding formaldehyde solution (37%; Fisher Scientific) directly, and then these cells were lysed by SDS lysis buffer containing protease inhibitors. The lysates were subjected to sonication to reduce DNA length to less than 1 kb. Chromatin immunoprecipitation was performed using a specific Ab to the protein of interest overnight at 4°C. The amount of the objective DNA region in the immunoprecipitated chromatin was analyzed by quantitative PCR analysis. Real-time PCR was performed in 20-μl reaction volumes containing iQ SYBR-Green Supermix (Bio-Rad), 200 nM of each primer, and 1 μl each ChIP DNA fraction using the CFX96 real-time PCR detection system (Bio-Rad). PCR cycle parameters were 3 min at 95°C, 45 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, followed by melting curve analysis. The threshold cycle (Ct) (i.e., the cycle number at which the amount of amplified DNA of interest reached a fixed threshold) was determined subsequently. Relative quantification of ChIP DNA was calculated by the comparative Ct method described elsewhere (28). Each ChIP DNA fractions’ Ct value was normalized to the input DNA fraction Ct value for the same quantitative PCR assay (ΔCt [normalized ChIP] = Ct [ChIP] − Ct [Input]). The normalized ChIP fraction Ct value was adjusted for the normalized background [rabbit IgG] fraction Ct value (ΔΔCt [ChIP/IgG] = ΔCt [normalized ChIP] − ΔCt [normalized IgG]). ChIP fold enrichment above the sample specific background was calculated as 2 ^ − ΔΔCt [ChIP/IgG]. The following three sets of primers were used in the ChIP assays: CD63-1, 5′-TGTCCTAACCCTAGTTGTAAG-3′; CD63-2, 5′-GGGTGACCCCGGATAAGAG-3′; and CD63-3, 5′-TAACACGGAGTTCTCCATCCC-3′.

Statistical analysis

Statistical analysis was done using unpaired Student t test, and p values <0.05 were considered to be significant.

Results

Downregulation of CXCR4 on activated B cells restimulated with IL-21 is due to acceleration of CXCR4 endocytosis

When splenic B cells were sequentially stimulated with anti-IgM Abs and anti-CD40 mAbs plus a low dose of IL-4 (which did not induce Ig class-switching) and with IL-21 or IL-4 after a 2-d

**FIGURE 2.** Restimulation of activated B cells with IL-21 downregulates CXCR4 expression on those B cells. Naive B cells were cultured with anti-IgM Abs and anti-CD40 mAbs plus a low dose of IL-4 for 4 d. A. CFSE-labeled naive B cells were cultured. IL-21 (filled histograms), IL-4 (open histograms), or nothing (open histogram with a broken line) was sequentially added at day 2 of culture. B220+ cells in the culture were analyzed by flow cytometry. Data are presented as a representative of three independent experiments. B. CXCR4low and CXCR4high B cells at day 2 of culture were sorted by FACS and restimulated with IL-21 or IL-4 for another 2 d. CXCR4 expression on these B cells was analyzed by flow cytometry. The number in each histogram indicates the mean fluorescence intensity. Data are presented as a representative of four independent experiments.

**FIGURE 3.** Restimulation of activated B cells with IL-21 accelerates CXCR4 endocytosis. Naive B cells were cultured with anti-IgM Abs and anti-CD40 mAbs plus a low dose of IL-4 for 4 d. IL-21 or IL-4 was sequentially added at day 2 of culture. A. Cell surface and intracellular CXCR4 expression of these B cells was analyzed by flow cytometry. Filled and open histograms indicate IL-21 and IL-4 restimulated B cells, respectively. The isotype control of each CXCR4 staining is represented as an open histogram with a thin broken line. The numbers in each histogram indicate the mean fluorescence intensity (isotype controls). The isotype control of each CXCR4 staining is represented as an open histogram with a thin broken line. The numbers in each histogram indicate the mean fluorescence intensity (isotype controls). Data are presented as a representative of five independent experiments. B. Dynasore (Dyn) was added in the culture at day 2 of culture. CXCR4 expression on these B cells was analyzed by flow cytometry. Filled and open histograms indicate activated B cells cultured with and without Dyn, respectively. The numbers in each histogram indicate the mean fluorescence intensity (isotype controls). Data are presented as a representative of four independent experiments. C. Expression of GRK6 and AIP4 mRNA in these activated B cells was analyzed by real-time quantitative RT-PCR. Results represent means ± SD of three independent experiments. Bars in figures represent mean values ± SD. *p < 0.05. N.S., not significant.
interval, these restimulations induced two distinct subsets of IgG1 B cells, CXCR4\textsuperscript{low} or CXCR4\textsuperscript{high} IgG1 B cells, respectively (Fig. 1A). Although the number of the activated B cells restimulated with IL-21 decreased after day 5 of culture because of the differentiation to plasma cells, kinetic studies of activated B cells clearly demonstrated that restimulation of activated B cells with IL-21 or IL-4 induced proliferation of CXCR4\textsuperscript{low} or CXCR4\textsuperscript{high} activated B cells including IgG1 B cells, respectively (Fig. 1B).

To examine the relationship between proliferation and CXCR4 expression on activated B cells, naive B cells labeled with CFSE were sequentially stimulated for 4 d. As shown in Fig. 2A, being associated with cell cycle progression, restimulation of activated B cells with IL-21 or IL-4 decreased or increased the amount of CXCR4 on those B cells, respectively. To confirm the regulation of CXCR4 expression on activated B cells by IL-21 or IL-4, we isolated CXCR4\textsuperscript{high} and CXCR4\textsuperscript{low} activated B cells at day 2 of culture before restimulation by FACS. These isolated B cells were restimulated with IL-21 or IL-4 for 2 d. The IL-21 or IL-4 restimulation clearly decreased or increased the amount of CXCR4 on activated B cells, respectively (Fig. 2B).

When we examined the amount of intracellular CXCR4 protein in sequentially stimulated B cells and compared it with that of surface CXCR4 on these cells at day 4 of culture, the difference in amounts of intracellular CXCR4 between IgG1 B cells restimulated with IL-21 or IL-4 was much less than that for amounts of cell surface CXCR4 (Fig. 3A). These results suggested that restimulation of activated B cells with IL-21 downregulates CXCR4 expression on IgG1 B cells by endocytosis. To examine CXCR4 endocytosis in activated B cells restimulated with IL-21, Dynasore, a dynamin inhibitor, was added at day 2 of the culture. Dynasore increased the amount of CXCR4 on activated B cells restimulated with IL-21 but not with IL-4, indicating an acceleration of CXCR4 endocytosis in activated B cells restimulated with IL-21 (Fig. 3B). Then, we measured expression of GRK6, AIP4, and Arrb2 mRNA in sequentially activated B cells at day 4 of culture by real-time quantitative RT-PCR. The amount of GRK6 mRNA but not that of AIP4 or Arrb2 (data not shown) increased in activated B cells restimulated with IL-21 but not with IL-4 (Fig. 3C).

The amount of CD63 mRNA in centrocytes is more than that in centroblasts

In GCs, upregulation and downregulation of CXCR4 allows GC B cells to localize in dark and light zones, respectively (4). To examine the effect of IL-21 on CXCR4 downregulation in GC B cells, wild-type (WT) and IL-21R\textsuperscript{-/-} GC B cells were sorted from spleen cells 10 d after immunization and cultured with IL-21 for 48 h. CXCR4 expression on WT GC B cells but not on IL-21R\textsuperscript{-/-} GC B cells was downregulated with IL-21 stimulation (Fig. 4A). However, when we analyzed CXCR4 expression on GC B cells from immunized IL-21R\textsuperscript{-/-} mice, CXCR4\textsuperscript{low} GC B cells

![Image](https://image-url.com)
were detected in the spleens of IL-21R−/− mice, and the amount of CXCR4 expression on CXCR4low IL-21R−/− GC B cells was almost equivalent to that on CXCR4low WT GC B cells (Fig. 4B). These results suggested that the CXCR4 downregulation on centrocytes in the light zone of GCs could not be explained only by the acceleration of CXCR4 endocytosis by IL-21.

To elucidate the mechanism of CXCR4 downregulation on centrocytes, we isolated CXCR4high GC B cells (centroblasts) and CXCR4low GC B cells (centrocytes) from immunized WT and IL-21R−/− mice and analyzed expression of Bcl6, IRF-4, and CXCR4 mRNA in those GC B cells by real-time quantitative RT-PCR. The amount of Bcl6 mRNA in centroblasts was more than that in centrocytes from both WT and IL-21R−/− mice, and the amount of IRF-4 mRNA was inversely correlated with that of Bcl6 (Fig. 4C). Surprisingly, the amount of both CXCR4 mRNA and intracellular CXCR4 protein (Fig. 4B) in the centroblasts was similar to that in the centrocytes from both WT and IL-21R−/− mice, suggesting acceleration of endocytosis and/or deceleration of exocytosis of CXCR4 in centrocytes. However, amounts of GRK6 mRNA in centrocytes from WT and IL-21R−/− mice were not more than those in centroblasts. Because CD63 is known to traffic CXCR4 protein to late endosome in CD4 T cells (17), we examined CD63 mRNA expression in these GC B cells. Indeed, CD63 mRNA expression was upregulated in activated B cells restimulated with anti-IgM Abs and anti-CD40 mAbs plus a low dose of IL-4 for 2 d were restimulated with CXCL12, and the amount of CD63 on their cell surfaces was analyzed by flow cytometry. Though the amount of CXCR4 on activated B cells was downregulated 6 h after CXCL12 restimulation, expression completely recovered 24 h after restimulation (Fig. 5A). Moreover, CD63 mRNA expression in these B cells was not induced after CXCL12 restimulation.

Then, we focused on Bcl6, a transcriptional repressor, to elucidate the mechanism of CXCR4 downregulation. We noted that expression of Bcl6 and CD63 mRNA was inversely correlated in centroblasts and centrocytes, suggesting downregulation of CD63 mRNA in centroblasts by Bcl6. Thus, we examined expression of CD63 mRNA and protein in activated B cells restimulated with IL-21 or IL-4. Amounts of CD63 mRNA and protein were strikingly elevated in activated B cells−/− B cells restimulated with IL-21 or IL-4 (Fig. 5B, 5C). The amounts of CD63 mRNA and protein in naive Bcl6−/− B cells were also more than those in naive WT B cells, suggesting CD63 as a molecular target of Bcl6.

We looked for Bcl6-binding sequences in the CD63 gene. Within the 4.8-kb sequence spanning −1.0 kb upstream to +1.0 kb downstream from the CD63 locus (MGI: 99529), four putative Bcl6-binding sequences (BS1: −846 to −838, 5′-TTCTGGTAAGA-3′; BS2: 1492 to 1500, 5′-TTCTGGAGGAG-3′; BS3: +2288 to +2296, 5′-TTCTCCAGAA-3′, and +2409 to +2417, 5′-TTCTAGGAA-3′ from the CD63 transcription start site) were identified by using match module of gene regulation (http://www.gene-regulation.com) (Fig. 5D). Then, we investigated Bcl6 binding and acetylation of histone H3 lysine (K) 9 and K14 (Ac-H3) at each BS site of naive Bcl6−/− and WT B cells by ChIP assay. Bcl6 binding was detected at BS2 and BS3 of WT B cells. Levels of Ac-H3 at BS2 and BS3 of Bcl6−/− B cells were more than those of WT B cells although those at BS1 were similar between them. When we examined Bcl6

**FIGURE 5.** Bcl6 negatively regulates CD63 expression in activated B cells. A, Naive WT B cells were cultured with anti-IgM Abs and anti-CD40 mAbs plus a low dose of IL-4 for 4 d. CXCL12 was added at day 2 of culture. CXCR4 expression on and CD63 mRNA expression in these activated B cells 6, 24, and 48 h after addition were analyzed by flow cytometry and by real-time quantitative RT-PCR, respectively. The number in each histogram indicates the mean fluorescence intensity. Data are presented as a representative of three independent experiments. Results represent means ± SD of triplicate culture. B and C, Naive B cells from WT (open bars) and Bcl6−/− (KO, filled bars) mice were cultured with anti-IgM Abs and anti-CD40 mAbs plus a low dose of IL-4 for 4 d. IL-21 or IL-4 was sequentially added at day 2 of culture. B, CD63 mRNA expression in naive and these activated B cells was measured by real-time quantitative RT-PCR. Results represent means ± SD of triplicate culture. Bars in figures represent mean values ± SD. *p < 0.05, **p < 0.01. C, CD63 protein in these B cells was detected by Western blot analysis. Data are presented as a representative of three independent experiments. D, Naive B cells from WT (open bars) and Bcl6−/− (KO, filled bars) mice were cultured with anti-IgM Abs and anti-CD40 mAbs plus a low dose of IL-4 for 4 d. The top figure indicates the CD63 gene map (boxes; exons) and putative Bcl6/STAT (BS)-binding sites (triangles). Relative amounts of Ac-H3 at and Bcl6 binding to the BS sites were measured by ChIP assay. Data are indicated by mean values ± SD of triplicate real-time quantitative RT-PCR. Results are presented as representative of three independent experiments. *p < 0.05. N.S., not significant.
binding and Ac-H3 at the CD63 locus of activated Bcl6−/− and WT B cells. Bcl6 binding was diminished in activated WT B cells, and the level of Ac-H3 increased at the locus in activated WT B cells but not in activated Bcl6−/− B cells. These results strongly suggested that the CD63 gene is a molecular target of Bcl6.

### Bcl6 upregulates CXCR4 expression on activated B cells by silencing the CD63 gene

We examined CXCR4 expression on activated B cells from Bcl6−/− mice. Naive Bcl6−/− B cells were sequentially stimulated with anti-IgM Abs and anti-CD40 mAbs plus IL-4 and with IL-21 or IL-4 after a 2-d interval. CXCR4 expression was downregulated on activated Bcl6−/− B cells after restimulation not only with IL-21 but also with IL-4 (Fig. 6A). Although the number of the IL-21–restimulated Bcl6−/− B cells decreased after day 4 of culture because of the differentiation to plasma cells and the apoptosis, kinetic studies of activated Bcl6−/− B cells clearly demonstrated that restimulation with IL-21 or IL-4 induced demonstration of CXCR4low B cells including IgG1 B cells (Fig. 6B). When we analyzed the amount of intracellular CXCR4 protein in activated Bcl6−/− B cells restimulated with IL-21 or IL-4, these activated Bcl6−/− B cells produced similar amounts of CXCR4 mRNA (Fig. 6C) and protein (Fig. 6D) compared with those of activated WT B cells. Furthermore, we added Dynasore to the culture to determine whether or not Bcl6 deficiency affects CXCR4 internalization. Although CXCR4 expression on activated Bcl6−/− B cells restimulated with IL-21 was slightly increased by the addition of Dynasore, CXCR4 on activated Bcl6−/− B cells restimulated with IL-4 was not increased at all (Fig. 6E).

To confirm the CD63-mediated CXCR4 downregulation on activated Bcl6−/− B cells, we examined the effect of CD63 siRNA on CXCR4 expression. Naive Bcl6−/− B cells were cultured with anti-IgM Abs and anti-CD40 mAbs plus a low dose of IL-4 for 4 d. Three types of predesigned CD63 siRNA or scramble siRNA were transfected to activated Bcl6−/− B cells at day 2 of culture. CXCR4 expression on Bcl6−/− B cells transfected with each CD63 siRNA increased compared with that on Bcl6−/− B cells transfected with scramble siRNA at day 4 of culture (Fig. 7A). We confirmed the downregulation of CD63 mRNA in CD63 siRNA (s63679)-transfected Bcl6−/− B cells (Fig. 7B).

Furthermore, we examined the effect of Bcl6 inhibitor on cell surface CXCR4 expression and CD63 mRNA expression in activated WT B cells. Naive WT B cells were cultured with anti-IgM Abs and anti-CD40 mAbs plus a low dose of IL-4 for 4 d. Bcl6 inhibitor was added to the WT B cell culture twice a day for 4 d. CXCR4 expression was partially downregulated on activated B cells cultured with the Bcl6 inhibitor (Fig. 7C). CD63 mRNA expression increased in the activated B cells after addition, and the

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**FIGURE 6.** CXCR4 expression is downregulated on activated Bcl6−/− B cells in vitro. Naive Bcl6−/− (A–E) and WT (C, D) B cells were cultured with anti-IgM Abs and anti-CD40 mAbs plus a low dose of IL-4 for 7 d. IL-21 or IL-4 was added at day 2 of culture. A, IgG1 and CXCR4 expression on activated Bcl6−/− B cells at day 4 of culture was analyzed by flow cytometry. The numbers in the flow cytometry profile indicate the percentage of each gate. Data are presented as a representative of five independent experiments. B, Numbers of CXCR4high and CXCR4low Bcl6−/− B cells in the culture were calculated by the flow cytometry profiles. Open and filled circles indicate IL-4 and IL-21 restimulated B cells, respectively. C, CXCR4 mRNA expression in activated B cells was measured by real-time quantitative RT-PCR. Filled and open bars indicate Bcl6−/− and WT B cells, respectively. Results represent means ± SD of triplicate culture. Bars in figures represent mean values ± SD. N.S., not significant. D, Cell surface and intracellular CXCR4 expression of activated B cells was analyzed by flow cytometry. Filled and open histograms indicate Bcl6−/− and WT B cells, respectively. The isotype control of each CXCR4 staining is represented as an open histogram with a thin broken line. The numbers in each histogram indicate the mean fluorescence intensity (isotype controls <12). Data are presented as a representative of five independent experiments. E, Dynasore (Dyn) was added in the culture at day 2 of culture. CXCR4 expression on these activated Bcl6−/− B cells was analyzed by flow cytometry. Filled and open histograms indicate Bcl6−/− B cells cultured with and without Dyn, respectively. The numbers in each histogram indicate the mean fluorescence intensity. Data are presented as a representative of three independent experiments.
Discussion

CXCR4 expression on GC B cells plays an important role in controlling dark and light zone segregation (4). CXCR4 expression is upregulated in centroblasts localized in the dark zone differentiate to centrocytes, downregulate CXCR4, and move to the light zone in GCs. When centrocytes were cultured in vitro without any stimulation, CXCR4 was upregulated on centrocytes within 4 h after culture (5). Thus, CXCR4 is actively downregulated on centrocytes. However, the mechanism of CXCR4 downregulation on centrocytes is not fully understood. Although CXCL12 stimulation transiently downregulates CXCR4 expression on CD4 T cells (29) and activated B cells, expression was completely recovered on activated B cells within 24 h after stimulation. Thus, it is impossible to explain the continuous downregulation of CXCR4 expression on centrocytes by CXCL12 stimulation. A previous article reported that CXCR4 expression on GC B cells was regulated by the level of its transcription (5). However, we have shown here that the amount of CXCR4 mRNA in centroblasts was similar to that in centrocytes of WT mice. Although it has been proved that CXCR4 mRNA expression is mainly controlled by nuclear respiratory factor-1 and yin-yang 1 in CD4 T cells (9–11), neither nuclear respiratory factor-1 mRNA nor yin-yang 1 mRNA was induced in activated B cells or GC B cells (data not shown). These results strongly suggested that CXCR4 expression on GC B cells is regulated at the post-transcriptional level.

We showed two alternative mechanisms that downregulate CXCR4 expression on activated B cells. First, we showed that IL-21 stimulation accelerated CXCR4 endocytosis in activated B cells by increasing GRK6 expression. Because IL-21 produced by Tfh cells (3) is required for GC B cells to maintain the size of GCs (30) and for centrocytes to differentiate to long-lived plasma cells (31, 32), the IL-21–induced CXCR4 downregulation may explain the maintenance of CXCR4 expression on centrocytes in the light zone. Indeed, IL-21 stimulation downregulated CXCR4 expression on FACS-isolated GC B cells. However, the IL-21–mediated CXCR4 downregulation was not clearly detected on GC B cells from WT mice compared with that from IL-21R−/− mice at day 7 (data not shown), day 10, and day 14 (data not shown) after immunization. We tried to detect CXCR4 endocytosis in centrocytes in vitro, but FACS-isolated GC B cells could not be cultured with Dynasore for the period of time long enough to detect CXCR4 upregulation. Although we were not able to show clear results proving IL-21–induced CXCR4 downregulation in centrocytes in vivo, IL-21 has a potential to control CXCR4 downregulation not only on activated B cells but also on GC B cells.

Second, we showed that CD63, which traffics CXCR4 to late endosome in CD4 T cells (17), downregulates CXCR4 expression on activated B cells. The amount of CD63 mRNA was inversely correlated with that of Bcl6 mRNA in GC B cells, and the amount of CD63 protein was strikingly augmented in activated Bcl6−/− B cells. Bcl6 binding was detected on the CD63 gene locus of naive B cells. Thus, the CD63 gene is a molecular target of Bcl6. It should be noted, however, that CD63 mRNA was detected in centroblasts, which express a large amount of Bcl6. CD63, a ubiquitously expressed tetraspanin, may be required in centroblasts. Because the amount of CD63 mRNA in centrocytes was more than that in centroblasts, the larger amount of CD63 can contribute to the CXCR4 downregulation on centrocytes. Therefore, IL-21–induced and CD63-mediated CXCR4 downregulation may contribute to maintain CXCR4 downregulation on centrocytes in the light zone.

We showed that IL-21 restimulation induced GRK6 mRNA to accelerate endocytosis of CXCR4 in activated B cells. Because IL-21 stimulation does induce Bcl6 in activated B cells (19, 33, 34), CXCR4 expression might be upregulated on activated B cells by Bcl6-mediated CD63 downregulation. However, CD63 was not significantly downregulated in activated B cells restimulated with IL-21 compared with that in activated B cells without restimulation. IL-21 stimulation also induces Blimp-1 in activated B cells, and Blimp-1 mutually represses Bcl6 expression in activated B cells (33). Indeed, the restimulation of activated B cells with IL-21 induced Blimp-1 (19) and protected against the Bcl6-mediated downregulation of CD63. Therefore, the restimulation of activated B cells with IL-21 downregulates CXCR4 expression on activated B cells by acceleration of the GRK6-mediated endocytosis and CD63-induced endosome trafficking. IL-21 acts as the inducer of Bcl6 in early phase of GC B cells such as centroblasts.
and as the inducer of Blimp-1 in the late phase of GC B cells such as centrocytes (34). Thus, these two regulatory mechanisms can cooperate together to maintain CXCR4 downregulation on activated B cells and probably on centrocytes.

Signal transduction pathways of the IL-21−induced and the CD63-mediated CXCR4 downregulation on activated B cells may be new targets for therapy of HIV infection and WHIM syndrome. CXCR4 is a part of the receptor for HIV, and the downregulation of CXCR4 on CD4 T cells protected against HIV infection (35). Because these two regulatory mechanisms may work in CD4 T cells, activation of these two mechanisms in CD4 T cells could protect against HIV infection. In WHIM syndrome, heterozygous truncating mutations in CXCR4 have been proposed to lead to altered lymphocyte trafficking (36). Leukocytes from WHIM patients show impaired association of GRK6 with CXCR4 and delayed recruitment of Arrb2 to CXCR4 leading to slower internalization of the receptor (15). The impaired CXCR4 signaling in WHIM syndrome is thought to result in defective B-cell functions (36). Thus, IL-21 stimulation induces GRK6 expression, which may enhance GRK6-CXCR4 association to weaken the abnormality of B-cell functions in WHIM patients.

It has been shown that coengagement of CD3 and CD63 induces a potent costimulatory signal in T cells (37), and that the engagement of CD63 resulted in rapid translocation of MHC class II molecules to the endocytic pathway in dendritic cells (38), suggesting that CD63 downregulation participates in Ag presentation on Ag-activated B cells. We found that expression of MHC class II on activated Bcl6−/− B cells was lower than that on activated WT mice. Thus, CD63 downregulation by Bcl6 may be important not only for CXCR4 expression on centroblasts but also for Ag presentation on Ag-activated B cells.

In summary, we have found two novel mechanisms of CXCR4 downregulation on activated B cells. These two mechanisms may contribute to the maintenance of CXCR4 downregulation on centrocytes. Further study of these two mechanisms may provide new insights to development of high-affinity memory B cells and long-lived plasma cells in GCs.

Acknowledgments
We thank Dr. M. Osawa (Chiba University), Dr. A. Iwama (Chiba University), and H. Satake for technical assistance, Dr. M. Osawa (Chiba University), Dr. A. Iwama (Chiba University), and H. Satake for technical assistance, Dr. M.J. Grusby (Harvard School of Medicine) for plasmid expression vectors.

Disclosures
The authors have no financial conflicts of interest.

References


