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Human B Cells Become Highly Responsive to Macrophage-Inflammatory Protein-3α/CC Chemokine Ligand-20 After Cellular Activation Without Changes in CCR6 Expression or Ligand Binding

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CCR6 is the only known receptor for the chemokine macrophage-inflammatory protein (MIP)-3α/CC chemokine ligand (CCL)20. We have shown previously that CCR6 is expressed on peripheral blood B cells, but CCR6 activity on these cells is low in in vitro assays. We report that MIP-3α/CCL20-induced calcium flux and chemotaxis can be enhanced significantly on peripheral blood and tonsillar B cells after activation by cross-linking surface Ag receptors. Of particular interest is the fact that the enhanced activity on B cells was not associated with an increase in CCR6 expression as assessed by levels of receptor mRNA, surface staining, or MIP-3α/CCL20 binding sites, or by a change in the affinity of the receptor for ligand. These data convincingly demonstrate that responses to a chemokine can be regulated solely by changes in the downstream pathways for signal transduction resulting from Ag receptor activation, and establish CCR6 as an efficacious receptor on human B cells. ***The Journal of Immunology,*** **2002, 168: 4871–4880.**

The chemokine family of chemotactic cytokines and their seven-transmembrane domain G protein-coupled receptors are increasingly recognized to play critical roles in lymphocyte trafficking during development, in maintaining homeostasis, and in immune responses (see Ref. 1 for review). Understanding the specific functions of individual receptors and their ligands in lymphocyte physiology has been complicated by the large number of chemokine receptors expressed on lymphocytes and the heterogeneity of lymphocyte populations. Initial steps toward investigating the functions of particular ligand/receptor pairs (or groups, in the case of promiscuous ligands and receptors) include the characterization of the expression and activities of the proteins in lymphocyte subsets and tissues. Most such studies to date have focused on T cells.

Although many chemokine receptors expressed on T cells can also be found on human B cells, such as CXCR4 (2, 3), CXCR5 (4), CXCR3 (5), CCR7 (6), CCR1 (7), and CCR2 (8), in general less is known about the functions of these receptors and their ligands on B cells. Nonetheless, important insights have been gained from analyses of knockout mice. These experiments have shown that CXCR5 is necessary for B cell homeostatic trafficking into follicles (9), that stromal cell-derived factor (SDF)[4]-1/CXC chemokine ligand (CXCL)12 is necessary for B cell lymphopoiesis (10), and that CCR7 is important for retaining B cells in the splenic periarteriolar lymphatic sheath (11).

We and others (12, 13) discovered a gene that encodes a human seven-transmembrane domain G protein-coupled receptor now called CCR6, and demonstrated that it is the receptor for the chemokine macrophage-inflammatory protein (MIP)-3α/CC chemokine ligand (CCL)20 (14–17). CCR6 can also function as a receptor for human β-defensins (18), members of a family of antimicrobial peptides. The MIP-3α/CCL20 gene is expressed in lymphoid tissue, in intestinal epithelium (particularly over Peyer's patches), and in activated monocytes, endothelial cells, dendritic cells, and fibroblasts (19–22). MIP-3α/CCL20 has been described as chemotactic for immature dendritic cells (16, 23) and freshly isolated T cells (21). We have shown that MIP-3α/CCL20 acts selectively on memory T cells (24), and others have reported that MIP-3α/CCL20 is able to trigger the adhesion of memory T cells to ICAM-1-coated glass (25).

Consistent with the activities of MIP-3α/CCL20, the CCR6 mRNA has been found in lymphoid tissue, T and B cells, and selected populations of dendritic cells (12–14, 16, 17, 23, 26). We have shown expression of human CCR6 protein on dendritic cells and freshly isolated memory T cells, including T cells expressing the cutaneous lymphocyte Ag, which directs T cells to the skin, and the gut-associated integrin $\alpha_4\beta_7$, and we demonstrated that CCR6 is fully functional on resting T cells (24). We also found that CCR6 was expressed on peripheral blood B cells (24) but that, unlike for T cells, responses of B cells to MIP-3α/CCL20 were difficult to detect, particularly in assays for chemokine-induced calcium fluxes. In mice, recent data have shown that CCR6 is expressed on various populations of freshly isolated, mature, peripheral B cells, and MIP-3α/CCL20 was shown to have modest activity as a chemotactic factor for these cells (27). Significantly, gene-targeted mice lacking CCR6 showed diminished numbers of cells in lamina propria and Peyer's patch that produced Ab against

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[4] Abbreviations used in this paper: SDF, stromal cell-derived factor; AGS, activator of G protein signaling; BCR, B cell Ag receptor; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; GAP, GTPase-activating protein; MIP, macrophage-inflammatory protein; F-actin, filamentous actin; PLC, phospholipase C; RGS, regulator of G protein signaling; MFI, mean fluorescence intensity.

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orally administered Ags (28). Mice lacking CCR6 have also been reported to have abnormalities in T cell-dependent inflammation (29).

Investigations of chemokine receptor expression and activity on lymphocytes have revealed profound effects for lymphocyte activators such as IL-2 (30). Generally, these studies have reported a correlation between receptor expression and function on a given cell type. In the studies described below we have found that CCR6 preferentially attracts memory B cells, that CCR6 becomes a particularly efficacious receptor on human B cells following cellular activation through Ag receptors, and that this enhancement in CCR6 function is due to changes in the responsiveness of downstream signaling pathways. Our results demonstrate the existence of mechanisms for amplifying chemokine receptor signals and suggest that CCR6 is likely to play an important role in B cell trafficking in humans.

Materials and Methods

Preparation of lymphocytes

Blood products were obtained from normal donors by the Department of Transfusion Medicine, Clinical Center, National Institutes of Health (Bethesda, MD) under a protocol approved by the Institutional Review Board. For analysis of peripheral blood T cells, PBMC were prepared from buffy coats by banding on Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) and cultured in RPMI 1640/10% FBS. Activation of T cells was done using 5 μg/ml OKT3 (Ortho Biotech, Raritan, NJ) for 3 days.

Peripheral blood B cells were isolated by negative selection using two different procedures. For some experiments, cells collected by leukophoresis were isolated by banding on Ficoll-Hypaque and processed as described (31), using L-leucine methyl ester (Sigma-Aldrich, St. Louis, MO) to remove monocytes and NK cells and neuraminidase-treated sheep RBCs to remove T cells. This protocol gave preparations with ~85% B cells, which were used in studies on the flow cytometer where $CD19^+$ cells could be analyzed specifically. To isolate B cells for some experiments, Ficoll-Hypaque-banded cells were reacted with a B cell-enrichment Ab mixture and anti-human CD41 tetramer for 15 min at room temperature, and then incubated with magnetic colloid for an additional 15 min before negative selection over a magnetized column according to the supplier's protocol (StemCell Technologies, Vancouver, Canada). This protocol yielded preparations with >95% B cells. For most experiments, B cells were activated by culturing in RPMI 1640/10% FBS supplemented with 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids, 55 μM 2-ME (all from Life Technologies, Gaithersburg, MD), and 10 μg/ml anti-human IgM $F(ab')_2$ (ICN Pharmaceuticals, Aurora, OH) for 3 days. For some experiments, B cells were also activated using 5 μg/ml anti-human IgM coupled to beads (Irvine Scientific, Santa Ana, CA) plus 20 ng/ml IL-4 (BD PharMingen, San Diego, CA) for 2 days.

Tonsils were obtained as discarded tissue from the Department of Pathology, Children's Hospital (Washington, D.C.), in accord with institutional policy. Tonsil mononuclear cells were isolated as described (32), after which preparations were depleted of T cells using sheep RBCs as above. When indicated, germinal center B cells were removed by using anti-CD38 Ab (BD PharMingen) followed by anti-mouse IgG Dynal beads (Dynal Biotech, Lake Success, NY). This protocol yielded preparations with >95% B cells. To activate naive B cells preferentially, $CD38^-$ cells were cultured in the presence of 10 μg/ml anti-human IgM $F(ab')_2$ (ICN Pharmaceuticals) for 3 days. To activate tonsil memory cells, $CD38^-$ B cells were cultured in the presence of 10 μg/ml anti-IgG $F(ab')_2$ plus 10 μg/ml anti-IgA $F(ab')_2$ (ICN Pharmaceuticals) for 3 days. For all experiments using activated B cells, dead cells were removed by Ficoll-Hypaque banding before additional manipulations.

Flow cytometry

The commercial Abs used and their suppliers were as follows: FITC-conjugated anti-human IgD or CD5, PE-conjugated anti-human CCR6 (clone 11A9), CD19, CD38, or IgM, Cy5PE-conjugated anti-human CD38, biotin-conjugated anti-human IgD, and unconjugated anti-human FcγRII from BD PharMingen; Cy5PE-conjugated anti-human CD19 from Life Technologies; and FITC- or PE-conjugated affinity-purified $F(ab')_2$ goat anti-rabbit IgG from Caltag Laboratories (Burlingame, CA).

For staining human B cells using rabbit Abs against CCR6, one million cells were resuspended in 100 μl of PBS containing 1% FBS and 10 mM HEPES (FACS buffer), and were first incubated with the either anti-FcγRII (BD PharMingen) or Fab of anti-FcγRII mAb IV.3 (Medarex, Princeton, NJ), followed by an incubation for 1 h at 4°C with either 10 μl anti-CCR6 rabbit serum 5146 (24) or preimmune serum, or with 100 μg rabbit IgG that had been purified from preimmune or from anti-human CCR6 serum 5146 over a protein A column (Pierce, Rockford, IL). Finally, the cells were stained with FITC- or PE-conjugated $F(ab')_2$ goat anti-rabbit IgG plus various Abs to B cell markers (and Cy5PE-conjugated streptavidin (BD PharMingen) if appropriate), and analyzed for immunofluorescence using either a FACScan or FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Assaying calcium flux

Calcium flux in lymphocytes was detected using indo-1-loaded cells and a FACSVantage dual-laser flow cytometer (BD Biosciences) as described (33). For some experiments, dye-loaded cells were stained with anti-CD19-PE or anti-CD5-FITC before analysis. Human MIP-3α/CCL20 (Ckβ-4) was kindly provided by Human Genome Sciences (Rockville, MD) or purchased from PeproTech (Rocky Hill, NJ). If required for a positive control for calcium flux, B cells were treated with biotin-conjugated $F(ab')_2$ goat anti-human IgM (Caltag Laboratories) followed by NeutrAvidin (Pierce).

Assaying chemotaxis

Chemotaxis assays were performed using the 96-well ChemoTx no. 106-5 microplate (NeuroProbe, Gaithersburg, MD) as previously described (24) or using Transwell membranes (Costar, Cambridge, MA). For the ChemoTx no. 106-5 microplates, purified B cells were resuspended in prewarmed RPMI 1640 containing 1% FBS and 10 mM HEPES at 2×10^6 cells/ml. Chemokines were prewarmed in the RPMI/FBS/HEPES at a concentration of 1 μg/ml. Following a 3.5-h incubation, cells on top of the filter were removed and cold 0.5 mM EDTA in Dulbecco's PBS was added for 20 min at 4°C before centrifugation to dislodge any cells on the filter's underside. The migrated cells in the bottom wells were pooled and counted. For the Transwell assays, purified B cells were resuspended at 10^7 cells/ml in RPMI 1640 containing 0.5% BSA and 10 mM HEPES (pH 8). One hundred microliters of cell suspension was placed in inserts with filters with 5-μm pores and preincubated in wells containing 600 μl of RPMI/BSA/HEPES at 37°C for 30 min before the inserts were placed in wells containing either no chemokine or chemokine at various concentrations, and cells migrating to the lower well after 2 h were collected, counted, and stained for flow cytometry as appropriate.

For inhibitor studies, B cells were incubated in RPMI/BSA/HEPES with or without the following: pertussis toxin (200 ng/ml), genistein (100 μM), herbimycin A (10 μM), or wortmannin (1 μM or 100 nM) at 37°C for 30 min to 2 h before the migration assay. Cell viability was not adversely affected by treatment with any of the compounds or their vehicles (distilled water; DMSO). Pertussis toxin, 4′,5,7-trihydroxyisoflavone (genistein), herbimycin A (*Streptomyces* sp.), and wortmannin (KY 12420) were all purchased from Calbiochem-Novabiochem (San Diego, CA).

Assaying polymerization of F-actin

One-half million B cells were preincubated in 400 μl HBSS buffer containing 10 mM HEPES plus 1% FBS for 20 min before adding 2.5 μg/ml MIP-3α/CCL20 or SDF-1α/CXCL12 and incubating for 5, 15, 30, or 60 s. Cells were then fixed with 4% paraformaldehyde containing 0.5% saponin, 1.7 μg/ml phalloidin, and 132 nM Alexa Fluor 488 phalloidin (Molecular Probes, Eugene, OR) for 10 min on ice, and washed three times with PBS containing 0.1% saponin, 1% BSA, and 0.05% azide. Cells were resuspended with PBS and analyzed on a FACScan, with mean fluorescence intensity (MFI) determined for each sample. The percentage of modulation of MFI was calculated for each sample at each time point as follows: [1 − (MFI before addition of chemokine/MFI after addition of chemokine)] × 100.

Preparation and analysis of RNA

Cells were lysed and RNA was prepared using the TRIzol reagent as specified by the manufacturer (Life Technologies). Agarose/formaldehyde gel electrophoresis, transfer of RNA, and hybridizations and washing were done as described (34). ^{32}P-labeled probes were prepared from fragments using random nonamers and the Megaprime kit according to the manufacturer's protocol (Amersham, Arlington Heights, IL). The CCR6 probe was from a 1.2-kb cDNA fragment (13). cDNAs for regulator of G protein signaling (RGS)1, RGS2, and RGS3 were kind gifts from J. Kehrl (National Institute of Allergy and Infectious Diseases, National Institutes of Health), and their fragments were 1.4, 0.7, and 1.7 kb, respectively. The

RGS14 cDNA was I.M.A.G.E. Consortium clone ID 25842, which contained an insert of 1.7 kb and was obtained from the American Type Culture Collection (Manassas, VA). Hybridization using an end-labeled oligonucleotide probe for the 18S rRNA was done as described (35). Autoradiography/fluorography was done using an intensifying screen.

Assaying MIP-3α/CCL20 binding

Binding sites for MIP-3α/CCL20 were analyzed by displacement of radiolabeled ligand using B cells freshly isolated from peripheral blood or activated with anti-human IgM F(ab′)$_2$ for 3 days. One million B cells were incubated with 0.1 nM ^{125}I-labeled MIP-3α/CCL20 (2200 Ci/mmol; NEN Life Science Products, Boston, MA) and varying concentrations of unlabeled MIP-3α/CCL20 in a total volume of 200 μl of HBSS containing 1% BSA, 25 mM HEPES, and 0.02% NaN_3 for 45 min at room temperature before being spun through sucrose/PBS. The supernatants were aspirated, the bottoms of the microfuge tubes were cut, and cell-bound radioactivity was measured using a Cobra II series Autogamma counting system (Packard Instrument, Meriden, CT). Measurements in each condition were done in triplicate and averaged. Background binding was determined from samples containing 0.1 nM ^{125}I-labeled MIP-3α/CCL20 in the absence of unlabeled ligand and cells, and was subtracted from each experimental value. Data analysis was performed using LIGAND (P. Munson, Analytical Biostatistics, National Institutes of Health).

Results

MIP-3α/CCL20 produces a calcium flux in activated but not resting human peripheral blood B cells

The expression of CCR6 that we had detected on peripheral blood B cells led us to test B cells for responses to MIP-3α/CCL20. As shown in Fig. 1, in contrast with freshly isolated T cells, resting peripheral blood B cells failed to flux calcium after treatment with MIP-3α/CCL20. Nonetheless, these cells showed an appropriate increase in $[Ca]_i$ after the addition of anti-human IgM as a positive control (data not shown). In contrast with the freshly isolated cells, following 3 days of activation by cross-linking surface IgM in the presence of IL-2, B cells were now able to signal in response to MIP-3α/CCL20. Subsequent experiments, some of which are shown below, demonstrated that activation through Ag receptors without the addition of IL-2 was sufficient to confer responsiveness to MIP-3α/CCL20. A response to MIP-3α/CCL20 was not always detectable 1 day after cross-linking surface IgM, with donor-to-donor variability, but was uniformly seen at 2 days (data not shown) and was higher on day 3.

CCR6 expression is not enhanced by cellular activation

Using the anti-CCR6-specific serum 5146, which we have characterized previously (24), we investigated further the expression of CCR6 on peripheral blood B cells and on defined populations of B cells obtained from human tonsils. As shown in Fig. 2, and as was true in multiple experiments, despite the enhanced signaling of MIP-3α/CCL20 after in vitro activation of peripheral blood B cells, activation through surface Ag receptors did not lead to a noticeable increase in the levels of CCR6. Fig. 3*A* shows that both memory and naive tonsillar B cells, like B cells from peripheral blood, stain homogeneously for CCR6, but that CCR6 was not detected on germinal center B cells. As shown in Fig. 3*B*, following treatment with anti-IgM, or with anti-IgG and anti-IgA to activate preferentially naive or memory cells, respectively, surface expression of CCR6 on the tonsillar B cells did not change. The failure of activation to up-regulate CCR6 expression on B cells was a consistent finding whether we stained cells with antiserum 5146, as in Figs. 2 and 3, or with a commercially available anti-CCR6 mAb (clone 11A9; BD PharMingen; data not shown; see Fig. 5). Just as for the cells from peripheral blood, attempts to demonstrate calcium responses to MIP-3α/CCL20 on resting tonsil B cells were unsuccessful (data not shown). However, as shown in Fig. 3*B*, following treatment with anti-IgM or with anti-IgG and anti-IgA, the cells now responded to MIP-3α/CCL20 by fluxing calcium.

Cross-linking Ag receptors enhances CCR6-mediated chemotaxis

Chemotaxis experiments using peripheral blood B cells reproducibly showed small but detectable specific migration of freshly isolated cells in response to MIP-3α/CCL20, expressed as the percentage of input cells migrating to chemokine minus the percentage migrating in the absence of chemokine. However, in 17 of 22 experiments, similar to our findings with the calcium flux assay, cellular activation led to increases in B cell chemotaxis to MIP-3α/CCL20 with a range of ~2- to 20-fold more migration for

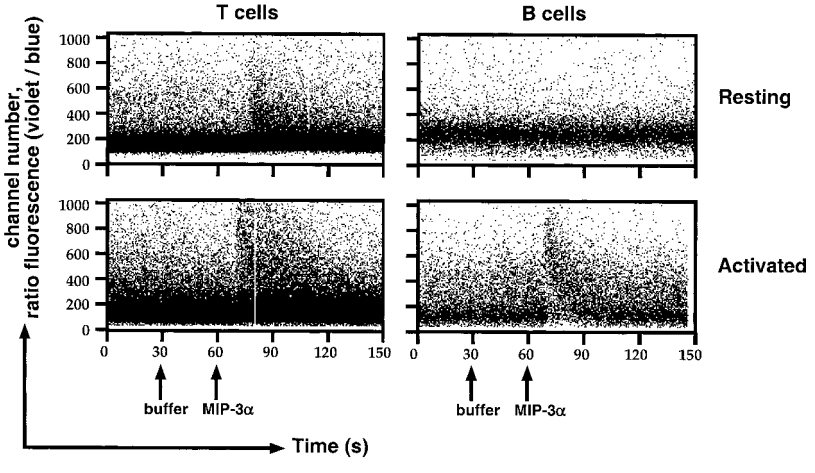

FIGURE 1. MIP-3α/CCL20 induces calcium flux in both resting and activated T cells, but in B cells only after activation. To detect signals in resting T cells, PBMC were isolated, loaded with indo-1, and stained with anti-CD5, and responses to MIP-3α/CCL20 were analyzed on the FACSVantage. To activate T cells, PBMC were cultured in the presence of 5 μg/ml OKT3 for 3 days before being analyzed like the resting cells. Ratio fluorescence was measured over time and is displayed for CD5$^+$ cells. Purified, resting peripheral blood B cells were loaded with indo-1 and stained with anti-CD19. To activate B cells, the purified cells were cultured in the presence of 10 μg/ml anti-IgM F(ab′)$_2$ plus 2 ng/ml rIL-2 for 3 days before being analyzed like the resting cells. Ratio fluorescence was measured over time and is displayed for CD19$^+$ cells. Additions of buffer alone or 1 μg/ml MIP-3α/CCL20 are indicated by arrows. T and B cells were obtained from different donors. Multiple additional donors also showed differences in calcium signaling between resting and activated B cells.

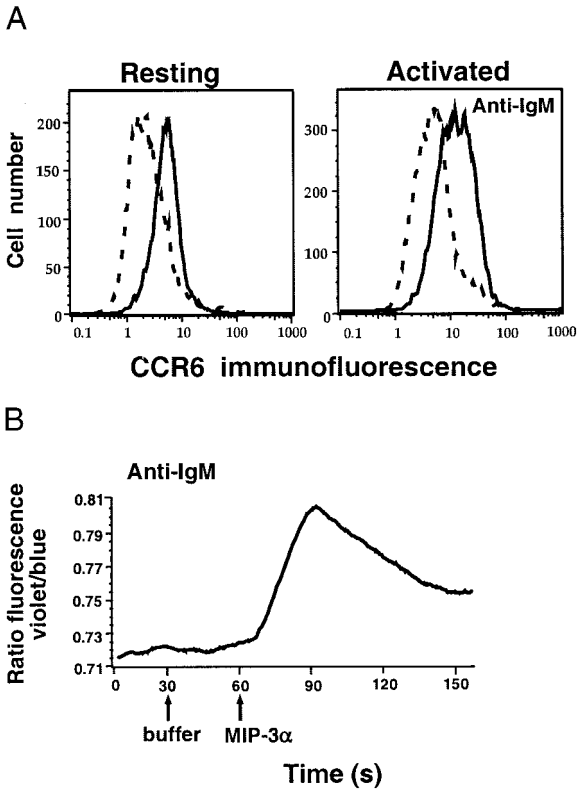

FIGURE 2. Cellular activation potentiates responses to MIP-3α/CCL20 on human peripheral blood B cells without up-regulating receptor expression. *A*, Peripheral blood B cells, either freshly isolated or after activation with anti-IgM $F(ab')_2$ for 3 days, were stained with IgG purified from preimmune serum (dashed lines) or from anti-CCR6 serum (solid lines) followed by PE-conjugated $F(ab')_2$ goat anti-rabbit IgG and Cy5PE-conjugated anti-CD19. Staining with the rabbit Abs is shown for the $CD19^+$ cells. *B*, The activated B cells shown in *A* were loaded with indo-1 and analyzed for changes in intracellular calcium on a FACSVantage flow cytometer. Averages of the ratios of fluorescence of the cells are plotted vs time. Additions of buffer alone or 1 μg/ml MIP-3α/CCL20 are indicated by arrows. Similar results were found with cells from multiple donors.

the activated as compared with resting cells. Applying a paired *t* test to the data from all 22 experiments revealed that the increase in chemotaxis after B cell activation was highly significant, with a *p* value of < 0.0001. Fig. 4*A* shows results with one donor where the difference was 12-fold. Fig. 4*B* shows migratory responses of resting and 3-day-activated peripheral blood B cells from a second donor, demonstrating that as compared with resting cells the activated cells responded at lower doses of MIP-3α/CCL20 and also responded better at optimal concentrations of ligand. The numbers of cells showing MIP-3α/CCL20-specific migration were significantly greater for the activated than for the resting cells at 100, 1000, and 1500 ng/ml MIP-3α/CCL20 ($p < 0.01$). As shown in Fig. 4*B*, the percentage of input cells migrating spontaneously in the absence of chemokine was also higher for the activated vs the freshly isolated cells ($p < 0.02$ for the data in Fig. 4*B*), and the possible significance of this observation is discussed below. Surface staining was done together with each chemotaxis experiment and demonstrated no increase in CCR6 expression on activated as compared with resting cells (data not shown). Analyzing chemotaxis to MIP-3α/CCL20 among human tonsillar B cells also revealed enhanced migration in activated as compared with freshly isolated cells, and this enhancement occurred whether activation was through cross-linking surface IgM or through IgG plus IgA (data not shown). Using peripheral blood B cells, we compared the migration to MIP-3α/CCL20 with migration to other B cell-attracting chemokines at a single dose of chemokine on both resting and activated cells. As shown in Fig. 4*C*, the activity of MIP-3α/CCL20 was comparable to that seen with the other chemokines, which also showed significantly enhanced activities on activated as compared with resting cells ($p < 0.01$ for all resting/activated pairs except for SDF-1α/CXCL12).

While this manuscript was under review, Krzysiek et al. (36) reported that cross-linking B cell Ag receptor (BCR) on human cells led to down-regulation of both CCR6 expression and function as measured by flow cytometry and induction of filamentous actin (F-actin) polymerization. Migratory responses to MIP-3α/CCL20 were not shown. Their activation protocol differed from ours in using anti-IgM-coated beads instead of soluble anti-IgM $F(ab')_2$, and their most dramatic effects were seen when IL-4 was also added. Fig. 5 shows an experiment where peripheral blood B cells, either freshly isolated or after activation with soluble anti-IgM $F(ab')_2$ or with anti-IgM-coated beads plus IL-4, were analyzed for CCR6 expression, migration to MIP-3α/CCL20, and MIP-3α/CCL20-induced F-actin polymerization. As expected, although soluble anti-IgM $F(ab')_2$ did not alter CCR6 expression, the migratory response was increased as compared with resting cells ($p < 0.01$). As compared with resting cells, these cells showed no change in the F-actin polymerization assay. Using the alternative activation protocol, we found, as reported, that there was a fall both in CCR6 expression and in the rapid MIP-3α/CCL20-induced F-actin polymerization. Despite this, specific migration to MIP-3α/CCL20 was higher than it was for resting cells ($p < 0.01$). In three additional experiments using anti-IgM-coated beads plus IL-4 where CCR6 expression reproducibly fell, specific migratory responses to MIP-3α/CCL20 either increased or stayed the same. In no case did chemotactic responses decrease as compared with those for freshly isolated cells. These data that receptor function is either maintained or increased in the face of lower receptor levels reinforce our finding that responses to MIP-3α/CCL20 are enhanced on BCR-activated cells on a per-receptor basis. These data also demonstrate that assays for chemokine-mediated F-actin polymerization do not necessarily vary concordantly with those for chemotaxis.

Because the efficacy of chemokines can differ between naive and memory lymphocytes (37), we analyzed the phenotypes of the peripheral blood B cells migrating to MIP-3α/CCL20. As shown in Fig. 6, although MIP-3α/CCL20 attracted both naive and memory B cells, memory cells responded disproportionately.

Resting and activated B cells express similar levels of CCR6 mRNA and binding sites for MIP-3α/CCL20

To complement the flow cytometry data on CCR6 expression, we analyzed the levels of CCR6 mRNA in resting and activated tonsillar B cells. As shown in Fig. 7 and supporting the results shown above, Northern analysis showed no significant change in CCR6 mRNA levels in tonsillar B cells after 3 days of activation with anti-IgM, anti-IgG, and anti-IgA. Finally, we performed competitive binding assays for MIP-3α/CCL20 with peripheral blood B cells, both freshly isolated and at 3 days after cross-linking surface IgM. Fig. 8 displays the binding data for the same cells used for the chemotaxis experiment shown in Fig. 4*A*. Both the number of binding sites per cell (~6500) and the affinity of these sites for MIP-3α/CCL20 (with a K_d of ~0.5–1 nM) were unchanged after cellular activation. These data rule out the possibility that ligand-receptor interactions could have been altered, for example through posttranslational modifications of receptors such as glycosylation or sulfation, or that enhanced responses to MIP-3α/CCL20 on the

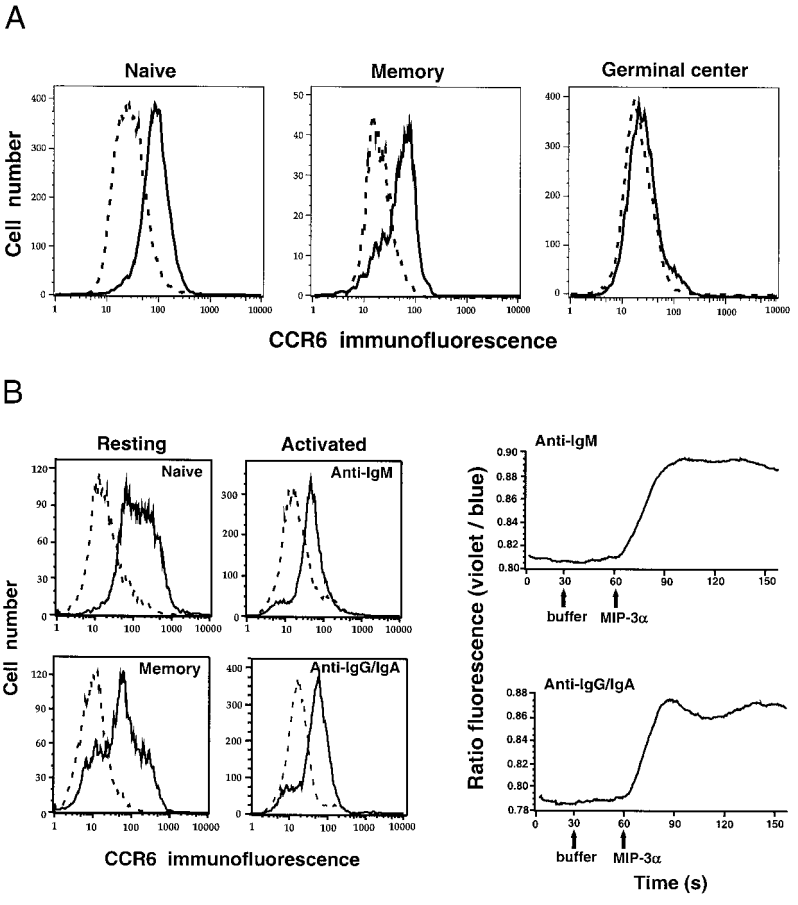

FIGURE 3. CCR6 is expressed on both memory and naive tonsillar B cells, and cellular activation potentiates responses to MIP-3α/CCL20 on these cells without up-regulating CCR6 expression. *A*, Expression of CCR6 on tonsillar B cells. Purified tonsillar B cells, all of which were $CD19^+$ (data not shown), were stained with preimmune serum (dashed lines) or anti-CCR6 serum (solid lines) plus biotin-conjugated anti-human IgD followed by FITC-conjugated $F(ab')_2$ goat anti-rabbit IgG, Cy5PE-conjugated streptavidin, and PE-conjugated anti-human CD38. CCR6 immunofluorescence is shown for naive (IgD^+CD38^-), memory (IgD^-CD38^-), and germinal center (IgD^-CD38^+) cells. *B*, Expression and signaling through CCR6 on naive and memory B cells after activation. *Left panel*, Tonsillar B cells were depleted of germinal center cells and stained with Ab purified from preimmune serum (dashed lines) or anti-CCR6 serum (solid lines) followed by FITC-conjugated anti-IgD and PE-conjugated $F(ab')_2$ goat anti-rabbit IgG. Naive cells and memory cells were identified based on their expression of IgD. To activate preferentially tonsillar naive or memory B cells, the germinal center-depleted tonsillar B cells were cultured in the presence of anti-IgM $F(ab')_2$, or in the presence of anti-IgG $F(ab')_2$ plus anti-IgA $F(ab')_2$, respectively, for 3 days before staining with the rabbit Abs as described for the fresh cells. *Right panel*, The activated B cells shown in *left panel* were loaded with indo-1 and analyzed for changes in intracellular calcium as described for Fig. 2. Data for staining and calcium flux of differentially activated tonsillar cells are shown for cells from one of three donors that gave similar results.

activated cells could have been due to induction of an as yet unidentified MIP-3α/CCL20 receptor.

More than one change in downstream signaling pathways is likely responsible for enhanced CCR6-mediated responses in activated B cells

Our observations that the activated B cells showed enhanced responses to MIP-3α/CCL20 in both calcium flux and chemotaxis assays without changes in CCR6 expression or ligand binding, and that responses mediated by multiple chemokine receptors were potentiated on the activated B cells, suggested that an early signaling event and one shared by the chemokine receptors could be affected in the activated cells. Activation of G proteins is the immediate consequence of chemokine receptor stimulation, and changing G protein signaling would affect both calcium flux and chemotaxis and be shared among receptors. G protein function can be altered by changes in the levels and/or activities of RGS proteins, which are GTPase-activating proteins (GAPs) for the α subunits of heterotrimeric G proteins (reviewed in Refs. 38 and 39), and which can function as inhibitors of chemokine receptor signaling (40) (see *Discussion*). We analyzed levels of mRNAs for a number of RGS proteins whose expression has been reported in B cells, including RGS1, RGS2, RGS3, and RGS14 (41–43). Northern blot analysis detected minimal signal in freshly isolated peripheral blood B cells for RGS3 and RGS14, with no significant change after BCR cross-linking, although samples from B cells stimulated for 24 h with PMA and *Staphylococcus aureus* Cowan I bacteria as positive controls showed bands of the expected mobilities (data not shown). In contrast, probes for mRNAs for RGS1 and RGS2 revealed expression in the resting cells that was down-regulated after 2 days of stimulation with anti-IgM $F(ab')_2$, as shown in Fig. 9.

The down-regulation of RGS proteins was consistent with the enhanced responses to MIP-3α/CCL20 in the activated B cells. However, careful consideration of the data suggested that changes in G protein signaling alone might not account for the chemotaxis component of the enhanced response. Even though significantly higher numbers of activated vs resting B cells migrated to MIP-3α/CCL20, as noted above, the numbers of activated cells migrating without chemokine also rose, so that the chemotactic index (numbers of cells migrating with chemokine divided by the numbers of cells migrating without chemokine) was not increased in the activated cells (data not shown). This suggested that in the activated B cells the signals from CCR6 (and other receptors) might be amplified by shared pathways that were also responsible for the increased numbers of cells migrating without chemokine. We investigated this possibility by testing the effects of inhibitors of signaling molecules on both chemokine-independent and chemokine-mediated migration of the activated cells. As shown in Fig. 10, we found that pertussis toxin inhibited chemokine-mediated migration but not chemokine-independent migration, while inhibitors of protein tyrosine kinases (genistein and herbimycin) and phosphoinositide 3-kinases (wortmannin) inhibited both types of migration on the activated B cells. These data were consistent with the presumption based on the chemotactic indices that the activated B cells had up-regulated the activity of common pathways responsible for cell migration, and that the enhanced migration to MIP-3α/CCL20 might be due to amplification of CCR6/G_i protein-mediated signals using these same pathways. As discussed below, together with the findings for calcium signaling and the RGS genes, these data suggest that multiple pathways affecting chemokine receptor signaling were likely to have been altered in the activated B cells.

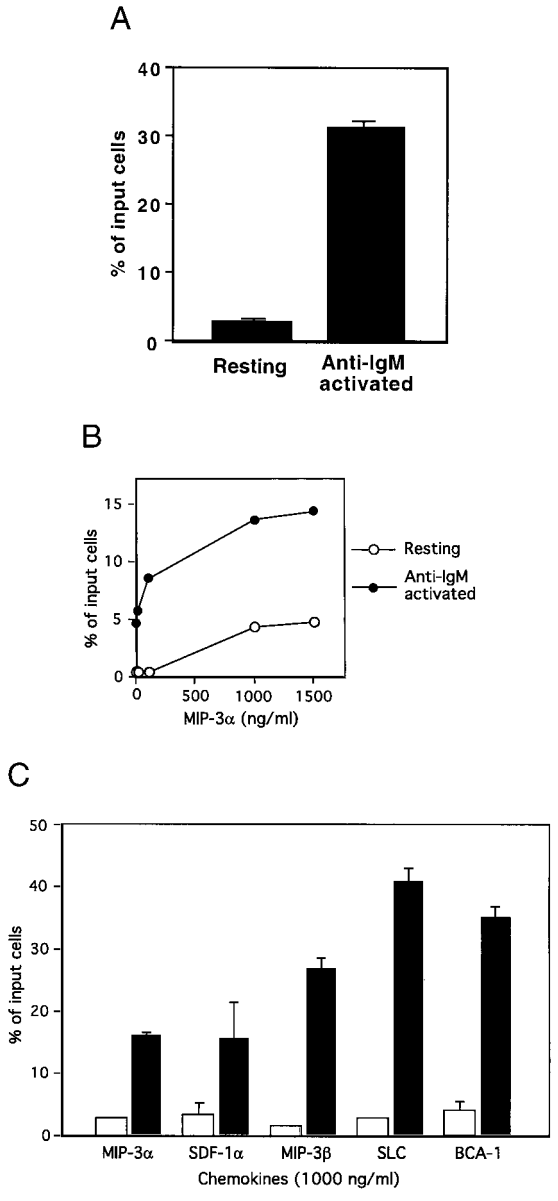

FIGURE 4. MIP-3α/CCL20 preferentially induces chemotaxis of activated, as compared with resting, B cells. *A*, Chemotaxis of peripheral blood B cells. One million B cells, either freshly isolated or after 3 days of activation with anti-IgM F(ab′)$_2$, were placed in a Transwell insert with a membrane having 5-μm pores, and cells migrating into the lower chamber containing 1 μg/ml MIP-3α/CCL20 were counted following a 2-h incubation. The number of cells migrating in the absence of MIP-3α/CCL20 (0.25% for freshly isolated cells and 7.4% for activated cells) was subtracted to yield chemokine-dependent migration, and the values shown are the means from duplicate wells along with SDs. The difference between resting and activated cells was significant, with a value of $p = 0.0006$. Data are for cells from 1 of 22 experiments. See text for details. *B*, Chemotaxis of resting and activated B cells to varying doses of MIP-3α/CCL20. Peripheral blood B cells from a single donor, either freshly isolated or 3 days after activation with anti-IgM F(ab′)$_2$, were assayed for chemotaxis as in *A* with 0, 10, 100, 1000, and 1500 ng/ml MIP-3α/CCL20 in the bottom wells. Values are displayed without subtraction of the numbers of cells migrating in the absence of chemokine. Error bars are too small to be seen. The numbers of cells showing specific migration to MIP-3α/CCL20 (calculated by subtracting the numbers of cells migrating without chemokine) were significantly greater for the activated than for the resting cells at 100, 1000, and 1500 ng/ml MIP-3α/CCL20 ($p < 0.01$). The numbers of cells migrating in the absence of chemokine was also greater for the activated vs resting cells ($p < 0.02$). Cells from a second donor gave similar results. *C*, Chemotaxis of resting and activated B cells to B cell active chemokines. Peripheral blood B cells from a single donor, either freshly isolated or 3 days after activation with anti-IgM F(ab′)$_2$, were assayed for chemotaxis as in *A* with 1 μg/ml MIP-3α/CCL20, SDF-1α/CXCL12, MIP-3β/CCL19, secondary lymphoid tissue chemoattractant/CCL21, B cell-attracting chemokine-1/CXCL13, or no chemokine in the bottom wells. The number of cells migrating in the absence of chemokine (0.14% for freshly isolated cells and 4.7% for activated cells) was subtracted to yield chemokine-dependent migration, and the values shown are the means from duplicate wells along with SDs. Open bars represent values for freshly isolated cells; filled bars represent values for activated cells. Differences between freshly isolated and activated cells were significant for all chemokines ($p < 0.01$) except for SDF-1α/CXCL12. Similar results were found in another complete experiment and in several additional experiments where subsets of these chemokines were tested.

Discussion

Our data provide evidence for two major points: 1) that CCR6 is an efficacious chemokine receptor on human peripheral blood and tonsillar B cells, particularly after cellular activation, and 2) that responses to MIP-3α/CCL20 can be enhanced by cellular activation without changes in CCR6 expression or ligand binding.

Using B cells from peripheral blood and tonsils we showed that on resting cells CCR6 preferentially attracts the memory subset and that CCR6-mediated calcium flux and chemotaxis can be up-regulated by activating naive cells through surface IgM, or memory cells through surface IgG and IgA. In our previous work, we described that CCR6 is expressed and active on resting memory T cells expressing both skin-homing and gut-homing surface markers (24). A number of laboratories have described the expression of CCR6 on human dendritic cells (16, 17, 23, 24, 44), particularly on immature cells (23, 44). These dendritic cells are able to process Ag and activate memory T cells at peripheral sites (44).

Our finding that B cell activation through BCR led to enhanced responses to MIP-3α/CCL20 raises the possibility that CCR6 may have a role particularly on Ag-activated B cells. Although germinal center cells from tonsils did not stain for the receptor, it is possible that CCR6 functions on other populations of activated B cells in vivo. These would include naive follicular cells or marginal zone B cells in the initial stages after contact with Ag, and other noncentroblast B lymphoblasts, including memory B cells that may be activated in situ. High levels of expression of MIP-3α/CCL20 have been found in the epithelium overlying human tonsils (44), and the tissue adjacent to the epithelium is rich in memory/activated B cells (45, 46). Taken together, the data suggest that MIP-3α/CCL20 and CCR6 may be important in humans for initiating anamnestic responses by recruiting and/or positioning memory T cells, memory/activated B cells, and immature dendritic cells in tissue, particularly at mucosal sites.

Our data on the effects of B cell activation on CCR6-mediated responses demonstrate changes in postreceptor signaling pathways, and thereby convincingly establish a receptor-independent mechanism for up-regulating responses to chemokines in activated lymphocytes. A priori, a parsimonious explanation for our findings that both chemotaxis and calcium responses were enhanced would be increased activity of heterotrimeric G proteins, the proximal components shared between the signaling pathways (see Ref. 47 for review).

RGS proteins are factors that could modify the outcome of G protein activation through their ability to function as GTPase activators (GAPs) for the α subunits of the G_i, G_q, and $G_{12/13}$ subfamilies (reviewed in Refs. 38, 39, and 48). In their function as GAPs, the RGS proteins are able to shorten the $t_{1/2}$ of the activated, GTP-bound G_α subunit. Although genes for RGS proteins

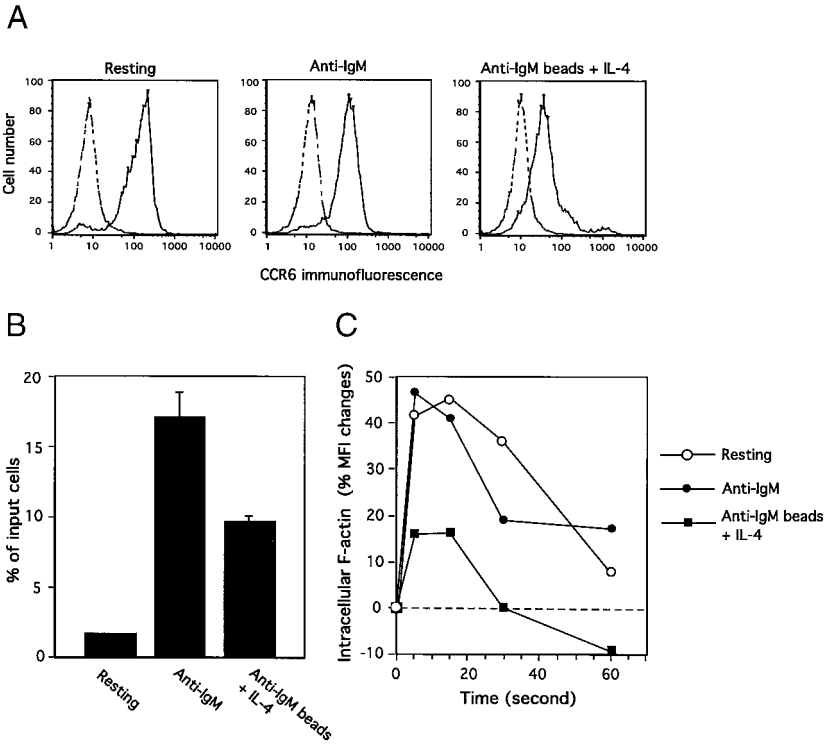

FIGURE 5. Chemotaxis to MIP-3α/CCL20 can be enhanced in B cells activated by a protocol that decreases expression of CCR6. Peripheral blood B cells from a single donor were analyzed at the time of isolation or 2 days after being activated with anti-IgM $F(ab')_2$ alone or with anti-IgM-coated beads plus IL-4. *A*, Cells were stained with PE-conjugated isotype control (dashed lines) or PE-conjugated anti-CCR6 Ab, clone 11A9 (BD PharMingen) (solid lines). *B*, Cells were assayed for chemotaxis as described for Fig. 4*A*. The number of cells migrating in the absence of MIP-3α/CCL20 (0.1% for freshly isolated cells, 6.2% for cells activated with anti-IgM alone, and 4.1% for cells activated with anti-IgM-coated beads plus IL-4) was subtracted to yield chemokine-specific migration, and the values shown are the means from duplicate wells along with SDs. The values for resting cells vs cells after activation with anti-IgM alone or with anti-IgM-coated beads plus IL-4 were significantly different ($p < 0.01$ in each case). *C*, Cells were treated with or without 2.5 μg/ml MIP-3α/CCL20 and assayed for changes in polymerization of F-actin using Alexa Fluor phalloidin at the times shown. Changes in intracellular polymerized F-actin were calculated as described in *Materials and Methods*.

have generally been found to be induced after lymphocyte activation (40, 42, 43, 49), we found that mRNAs for RGS1 and RGS2 were in fact down-regulated at 2 days after BCR cross-linking, similar to what has been reported for RGS3 in mouse B cells (43). Diminished expression of RGS proteins would be expected to up-regulate signals from chemokine receptors. RGS1 is a GAP for $G_{i\alpha}$ and $G_{q\alpha}$ and has been shown to inhibit calcium and chemotactic responses mediated by a number of chemoattractant receptors (40, 42, 43, 50). RGS2 has been reported to serve as a GAP for $G_{i\alpha}$ and $G_{q\alpha}$, although preferential activity for $G_{q\alpha}$ has been reported (51). $G_{q\alpha}$ is an efficient activator of phospholipase C (PLC)-β, which produces inositol 1,4,5-trisphosphate and triggers a rise in intracellular calcium, but a role for G_q proteins in cell migration, at least as assayed in chemotaxis assays in vitro, is less clear. Consistent with a preference for $G_{q\alpha}$ over $G_{i\alpha}$, transfection of RGS2 into cell lines had either no or modest activity in inhibiting chemokine-mediated chemotaxis (43, 50).

Despite the appeal of attributing our findings to RGS-mediated changes in G protein signaling, the chemotaxis data suggest that this is unlikely to be the sole mechanism for the enhanced responses in the activated cells. In addition to the increase in the percentage of the activated B cells migrating to chemokine, the number of these cells migrating across the filters in the absence of chemokine was also elevated, so that the ratio of chemokine-directed vs spontaneously migrating cells (chemotactic index) was not increased (see Fig. 4*B*). This does not mean that increased

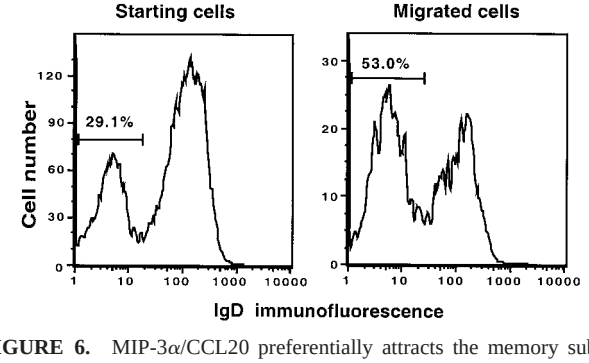

FIGURE 6. MIP-3α/CCL20 preferentially attracts the memory subset of resting peripheral blood B cells. The freshly isolated peripheral blood B cells from the chemotaxis experiment shown in Fig. 4*A* were used to determine migration of naive and memory subsets. The starting population and cells migrating to four lower wells containing MIP-3α/CCL20 were stained with Cy5PE-conjugated anti-human CD19 and FITC-conjugated anti-human IgD and analyzed by flow cytometry. Histograms show intensities of staining for IgD on $CD19^+$ cells to identify naive (IgD^+) vs memory (IgD^-) B cells. Two additional experiments gave similar results.

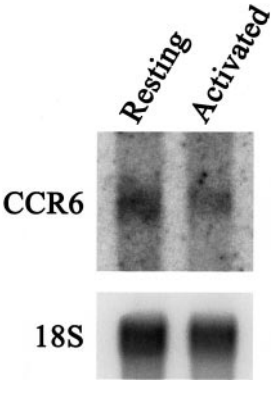

FIGURE 7. Levels of CCR6 mRNA do not differ between resting and activated tonsillar B cells. Total RNA was prepared from tonsillar B cells from a single donor, either freshly isolated or after 3 days of activation with anti-IgM $F(ab')_2$, anti-IgG $F(ab')_2$, and anti-IgA $F(ab')_2$. Seven-microgram samples of RNA were fractionated in a 1.2% agarose-formaldehyde gel, transferred to supported nitrocellulose, and hybridized first to a radiolabeled CCR6 cDNA probe and subsequently, to demonstrate equal loading, to a radiolabeled 18S rRNA oligonucleotide probe. The CCR6 band ran at the predicted position slightly below the 28S rRNA. Analysis of RNA prepared from cells from a second donor also showed no difference in CCR6 mRNA between freshly isolated and activated cells.

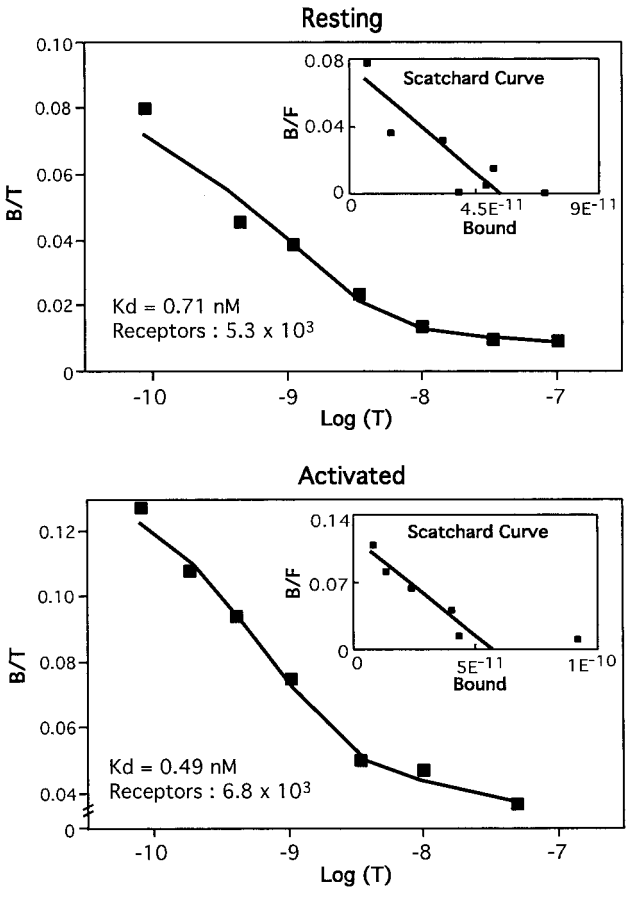

FIGURE 8. Binding sites for MIP-3α/CCL20 do not differ between resting and activated B cells. The freshly isolated and 3-day-activated B cells used for the chemotaxis experiment shown in Fig. 4*A* were analyzed for MIP-3α/CCL20 binding using a radiolabeled ligand displacement assay. Measurements in each condition were done in triplicate and averaged. Data analysis was performed using LIGAND. B, T, and F represent bound, total, and free ligand, respectively. Two other experiments also showed no significant differences in K_d or in binding sites per cell for the freshly isolated vs activated B cells.

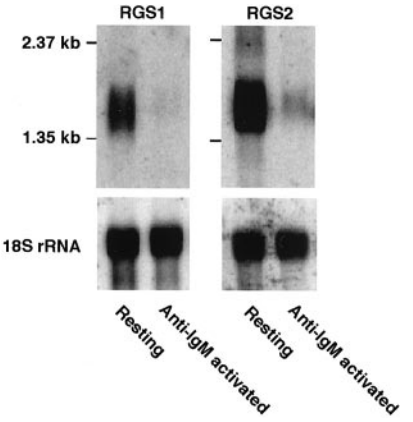

FIGURE 9. mRNAs for RGS1 and RGS2 are down-regulated in preparations of peripheral blood B cells after activation with anti-IgM. Total RNA was prepared from peripheral blood B cells from a single donor, either freshly isolated or after 2 days of activation with anti-IgM F(ab′)$_2$. Fifteen-microgram samples of RNA were fractionated in a 1.2% agarose-formaldehyde gel, transferred to supported nitrocellulose, and hybridized first to radiolabeled RGS1 or RGS2 cDNA probes and subsequently, to demonstrate equal loading, to a radiolabeled 18S rRNA oligonucleotide probe. The RGS1 and RGS2 mRNAs demonstrated their expected mobilities as shown. Analysis of RNA prepared from 2-day-activated cells from a second donor and from 1-day-activated cells from a third donor gave similar results.

migration to MIP-3α/CCL20 was due simply to the increase in the numbers of cells migrating spontaneously, because we showed that chemokine-specific migration, which was calculated by subtracting the numbers of spontaneously migrating cells from those migrating to chemokine, was increased after B cell activation. Rather, our data suggested that while chemokine-independent signals and chemokine-induced signals resulted in much different numbers of cells migrating, these signals were being amplified to similar extents as a consequence of B cell activation. The simplest explanation for these findings is that chemokine-independent and chemokine-dependent migration were being amplified through shared pathways.

We investigated this possibility using genistein, herbimycin, wortmannin, and pertussis toxin, inhibitors of protein tyrosine kinases, phosphoinositide 3-kinases, and receptor-mediated activation of G_i proteins, to determine whether we could separate the enhanced chemokine-independent and chemokine-dependent migration by blocking the former without eliminating the latter. We could not. Inhibitors of the two classes of kinases blocked both chemokine-independent and chemokine-dependent migration, and although pertussis toxin eliminated the latter it had no effect on the former, consistent with the supposition that although chemokine-induced signals required receptor activation of G_i proteins, both sorts of migration were amplified through common pathways. The results with pertussis toxin ruled out the possibility that the increased spontaneous migration of the activated cells was due to G_i protein-coupled receptor activation through autocrine or paracrine factors, although this is a phenomenon for which there is precedence (52, 53).

G_i protein signals are the primary (but not the only (see Ref. 54)) class of G protein signals with a role in cell migration (55, 56); thus, given the evidence for shared pathways for chemokine-independent and chemokine-dependent migration, the failure of pertussis toxin to inhibit chemokine-independent migration would argue against increased activity of G proteins, and by implication a fall in RGS proteins, as responsible for the enhanced migration of the activated B cells. Therefore, these considerations favor the possibility that the enhanced motility of the activated cells was due to effects on any of a number of downstream points in the signaling pathway, such as on one or more of the protein tyrosine kinases that we have shown are necessary, as a class, for chemokine-dependent and chemokine-independent migration. Both the *src* and the *syk* families of protein tyrosine kinases have been shown to be in the pathway of chemokine-induced signals important for migration (57, 58). That said, it is possible, however, for G_i (or other G) proteins to be activated by factors acting independently of receptors, such as by the activators of G protein signaling (AGS) proteins (59), which would not be sensitive to inhibition by pertussis toxin. $G_{i\alpha}$ turned on in the activated cells by a receptor-independent mechanism such as AGS1 (59) could in turn enhance cell motility and could be regulated by RGS1 or other RGS proteins, as has in fact been reported (60). However, there is as yet little information on the biological roles of the AGS proteins.

Regarding the enhanced calcium signals in the activated B cells, although it is possible that downstream pathways such as those we have implicated in the chemotaxis responses could be contributory, the well-established pathway for chemokine receptor-induced increases in intracellular calcium is through direct activation of PLC-β by $G_{q\alpha}$ or the $\beta\gamma$ dimer (61). Taken together, the data

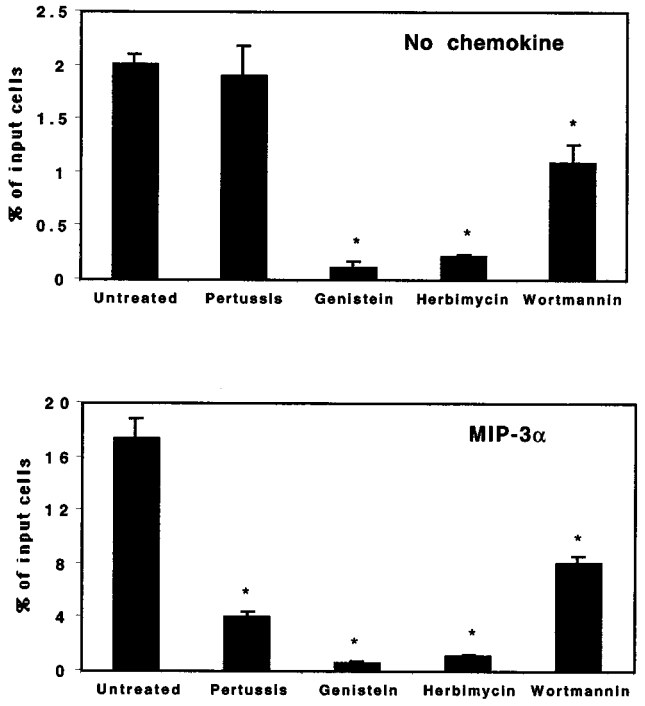

FIGURE 10. Chemokine-dependent chemotaxis of activated B cells is blocked by factors that inhibit chemokine-independent migration. After 3 days of activation with anti-IgM F(ab′)$_2$, human B cells were treated with the following compounds: pertussis toxin (200 ng/ml), genistein (100 μM), herbimycin A (10 μM), and wortmannin (1 μM) at 37°C for 2 h. Untreated cells were used as a control. After treatment, cells were placed in a Transwell insert with a membrane having 5-μm pores, and cells migrating into the lower chamber containing either chemotaxis medium (*upper panel*) or 1 μg/ml MIP-3α/CCL20 (*lower panel*) were counted following a 2-h incubation. The number of cells migrating was expressed as a percentage of the input cells. Numbers for cells migrating to MIP-3α/CCL20 are shown without subtraction of cells migrating in the absence of chemokine. Duplicate wells were used for each condition and error bars represent SEMs for the duplicate values. *, Significant difference ($p < 0.05$) between the numbers of migrating treated vs untreated cells. Data shown are from one representative of five independent experiments, some of which used wortmannin at 100 nM with similar results.

suggest that enhanced responses to chemokines in the activated B cells may be due to changes in multiple signaling components, with RGS effects on G_q/G_i proteins potentially contributing to increases in PLC-β-dependent calcium signals, and changes in downstream pathways that depend on protein tyrosine kinases and phosphoinositide 3-kinases being primarily responsible for increases in cell migration. Additional work will be needed to clarify the contributions of each component to the specific outcomes of receptor activation in these different cellular contexts.

At the time this manuscript was submitted and subsequent to its submission, a number of papers have appeared addressing the roles of CCR6 on human or mouse B cells. Brandes et al. (62) reported no enhancement of migration to MIP-3α/CCL20 after B cell activation, possibly because they activated cells with LPS or anti-CD40 and IL-4 and not through BCR. Krzysiek et al. (36) reported findings similar to ours on the expression pattern of CCR6 on tonsillar B cells and the preferential activity of MIP-3α/CCL20 on memory B cells from peripheral blood. However, they found that both CCR6 expression and function, as assayed by MIP-3α/CCL20-induced F-actin polymerization, were diminished after activation through BCR. When we activated B cells using precisely their protocol, as discussed in *Results* and shown in Fig. 5, we reproduced their findings. However, while following their procedure we measured migration to MIP-3α/CCL20, which they did not report, and here we found that, despite a fall in CCR6 expression, chemotaxis either increased or stayed the same, depending on the donor, on the activated as compared with the freshly isolated cells. In no case did migration to MIP-3α/CCL20 diminish, despite a significant fall in CCR6 expression induced by their activation protocol, demonstrating in every case that per receptor the chemotactic response to MIP-3α/CCL20 was enhanced in the activated B cells. Our data also demonstrate a discordance between changes in the assays for F-actin polymerization and chemotaxis, consistent with the complexity and divergence in the pathways regulating these responses (47). Finally, Bowman et al. (27) reported that cells from germinal centers of Peyer's patches from C57BL/6 (but not BALB/c) mice expressed low levels of CCR6 yet responded to MIP-3α/CCL20 better than the brighter-staining follicular B cells, again consistent with our observations that activated B cells can have enhanced responses to MIP-3α/CCL20 independently of CCR6 expression.

In mice, targeted disruption/deletion of the CCR6 gene has resulted in diminished numbers of Peyer's patch and lamina propria cells producing specific Ab as part of a primary response to orally administered Ags, and in diminished production of secreted IgA against a virus (28), and serum IgG2b against a protein Ag (29). Our data suggest that the deficits in B cell function in these mice might be the direct result of the absence of CCR6 on B cells. Taken together, the available data suggest an important role for CCR6 in trafficking and/or other aspects of the physiology of activated B cells in both mice and humans.

Acknowledgments

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References

1. Zlotnik, A., J. Morales, and J. A. Hedrick. 1999. Recent advances in chemokines and chemokine receptors. *Crit. Rev. Immunol. 19:1.*
2. Federsppiel, B., I. G. Melhado, A. M. Duncan, A. Delaney, K. Schappert, I. Clark-Lewis, and F. R. Jirik. 1993. Molecular cloning of the cDNA and chromosome localization of the gene for a putative seven-transmembrane segment (7-TMS) receptor isolated from human spleen. *Genomics 16:707.*
3. Bleul, C. C., L. Wu, J. A. Hoxie, T. A. Springer, and C. R. Mackay. 1997. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc. Natl. Acad. Sci. USA 94:1925.*
4. Forster, R., T. Emrich, E. Kremmer, and M. Lipp. 1994. Expression of the G-protein-coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells. *Blood 84:830.*
5. Qin, S., J. B. Rottman, P. Myers, N. Kassam, M. Weinblatt, M. Loetscher, A. E. Koch, B. Moser, and C. R. Mackay. 1998. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J. Clin. Invest. 101:746.*
6. Birkenbach, M., K. Josefsen, R. Yalamanchili, G. Lenoir, and E. Kieff. 1993. Epstein-Barr virus-induced genes: first lymphocyte-specific G protein-coupled peptide receptors. *J. Virol. 67:2209.*
7. Gao, J.-L., D. B. Kuhns, H. L. Tiffany, D. McDermott, X. Li, U. Franccke, and P. M. Murphy. 1993. Structure and functional expression of the human macrophage inflammatory protein 1α/RANTES receptor. *J. Exp. Med. 177:1421.*
8. Frade, J. M., M. Mellado, G. del Real, J. C. Gutierrez-Ramos, P. Lind, and A. C. Martinez. 1997. Characterization of the CCR2 chemokine receptor: functional CCR2 receptor expression in B cells. *J. Immunol. 159:5576.*
9. Forster, R., A. E. Mattis, E. Kremmer, E. Wolf, G. Brem, and M. Lipp. 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell 87:1037.*
10. Nagasawa, T., S. Hirota, K. Tachibana, N. Takakura, S. Nishikawa, Y. Kitamura, N. Yoshida, H. Kikutnai, and T. Kishimoto. 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature 382:635.*
11. Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell 99:23.*
12. Zaballos, A., R. Varona, J. Gutierrez, P. Lind, and G. Marquez. 1996. Molecular cloning and RNA expression of two new human chemokine receptor-like genes. *Biochem. Biophys. Res. Commun. 227:846.*

13. Liao, F., H.-H. Lee, and J. M. Farber. 1997. Cloning of STRL22, a new human gene encoding a G protein-coupled receptor related to chemokine receptors and located on chromosome 6q27. *Genomics 40:175.*
14. Baba, M., T. Imai, M. Nishimura, M. Kakizaki, S. Takagi, K. Hieshima, H. Nomiyama, and O. Yoshie. 1997. Identification of CCR6, the specific receptor for a novel lymphocyte-directed CC chemokine LARC. *J. Biol. Chem. 272:14893.*
15. Liao, F., R. Alderson, J. Su, S. J. Ullrich, B. L. Kreider, and J. M. Farber. 1997. STRL22 is a receptor for the CC chemokine MIP-3α. *Biochim. Biophys. Acta 236:212.*
16. Power, C. A., D. J. Church, A. Meyer, S. Alouani, A. E. I. Proudfoot, I. Clark-Lewis, S. Sozzani, A. Mantovani, and T. N. C. Wells. 1997. Cloning and characterization of a specific receptor for the novel CC chemokine MIP-3α from lung dendritic cells. *J. Exp. Med. 186:825.*
17. Greaves, D. R., W. Wang, D. J. Dairaghi, M. C. Dieu, B. Saint-Vis, K. Franz-Bacon, D. Rossi, C. Caux, T. McClanahan, S. Gordon, et al. 1997. CCR6, a CC chemokine receptor that interacts with macrophage inflammatory protein 3α and is highly expressed in human dendritic cells. *J. Exp. Med. 186: 837.*
18. Yang, D., O. Chertov, S. N. Bykovskaia, Q. Chen, M. J. Buffo, J. Shogan, M. Anderson, J. M. Schroder, J. M. Wang, O. M. Howard, and J. J. Oppenheim. 1999. β-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science 286:525.*
19. Rossi, D. L., A. P. Vicari, K. Franz-Bacon, T. K. McClanahan, and A. Zlotnik. 1997. Identification through bioinformatics of two new macrophage proinflammatory human chemokines: MIP-3α and MIP-3β. *J. Immunol. 158:1033.*
20. Hromas, R., P. W. Gray, D. Chantry, R. Godiska, M. Krathwohl, K. Fife, G. I. Bell, J. Takeda, S. Aronica, M. Gordon, et al. 1997. Cloning and characterization of exodus, a novel β-chemokine. *Blood 89:3315.*
21. Hieshima, K., T. Imai, G. Opdenakker, J. Van Damme, J. Kusuda, H. Tei, Y. Sakaki, K. Takatsuki, R. Miura, O. Yoshie, and H. Nomiyama. 1997. Molecular cloning of a novel human CC chemokine liver and activation-regulated chemokine (LARC) expressed in liver: chemotactic activity for lymphocytes and gene localization on chromosome 2. *J. Biol. Chem. 272:5846.*
22. Tanaka, Y., T. Imai, M. Baba, I. Ishikawa, M. Uehira, H. Nomiyama, and O. Yoshie. 1999. Selective expression of liver and activation-regulated chemokine (LARC) in intestinal epithelium in mice and humans. *Eur. J. Immunol. 29:633.*
23. Yang, D., O. M. Howard, Q. Chen, and J. J. Oppenheim. 1999. Cutting edge: immature dendritic cells generated from monocytes in the presence of TGF-β1 express functional C-C chemokine receptor 6. *J. Immunol. 163:1737.*
24. Liao, F., R. L. Rabin, C. S. Smith, G. Sharma, T. B. Nutman, and J. M. Farber. 1999. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3α. *J. Immunol. 162:186.*
25. Campbell, J. J., J. Hedrick, A. Zlotnik, M. A. Siani, D. A. Thompson, and E. C. Butcher. 1998. Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science 279:381.*
26. Liao, F., G. Alkhatib, K. W. C. Peden, G. Sharma, E. A. Berger, and J. M. Farber. 1997. STRL33, a novel chemokine receptor-like protein, functions as a fusion cofactor for both macrophage-tropic and T cell line-tropic HIV-1. *J. Exp. Med. 185:2015.*
27. Bowman, E. P., J. J. Campbell, D. Soler, Z. Dong, N. Manlongat, D. Picarella, R. R. Hardy, and E. C. Butcher. 2000. Developmental switches in chemokine response profiles during B cell differentiation and maturation. *J. Exp. Med. 191: 1303.*
28. Cook, D. N., D. M. Prosser, R. Forster, J. Zhang, N. A. Kuklin, S. J. Abbondanzo, X.-D. Niu, S.-C. Chen, D. J. Manfra, M. T. Wiekowski, et al. 2000. CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity 12:495.*
29. Varona, R., R. Villares, L. Carramolino, I. Goya, A. Zaballos, J. Gutierrez, M. Torres, A. C. Martinez, and G. Marquez. 2001. CCR6-deficient mice have impaired leukocyte homeostasis and altered contact hypersensitivity and delayed-type hypersensitivity responses. *J. Clin. Invest. 107:R37.*
30. Loetscher, P., M. Seitz, M. Baggiolini, and B. Moser. 1996. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *J. Exp. Med. 184:569.*
31. Splawski, J. B., E. M. Eisenstein, and K. S. Chua. 1999. Isolation of B cell populations. In *Current Protocols in Immunology*, Unit 7.5. J. Coligan, A. Kruisbeek, D. Margulies, E. Shevach, and W. Strober, eds. Wiley, New York.
32. Ryon, J. J. 1999. Isolation of mononuclear cells from tonsillar tissue. In *Current Protocols in Immunology*, Unit 7.8. J. Coligan, A. Kruisbeek, D. Margulies, E. Shevach, and W. Strober, ed. Wiley, New York.
33. Rabin, R. L., M. K. Park, F. Liao, R. Swofford, D. Stephany, and J. M. Farber. 1999. Chemokine receptor responses on T cells are achieved through regulation of both receptor expression and signaling. *J. Immunol. 162:3840.*
34. Vanguri, P., and J. Farber. 1990. Identification of CRG-2: an interferon-inducible mRNA predicted to encode a murine monokine. *J. Biol. Chem. 265:15049.*
35. Amichay, D., R. T. Gazzinelli, G. Karupiah, T. R. Moench, A. Sher, and J. M. Farber. 1996. Genes for chemokines MuMig and Crg-2 are induced in protozoan and viral infections in response to IFN-γ with patterns of tissue expression that suggest nonredundant roles in vivo. *J. Immunol. 157:4511.*
36. Krzysiek, R., E. A. Lefevre, J. Bernard, A. Foussat, P. Galanaud, F. Louache, and Y. Richard. 2000. Regulation of CCR6 chemokine receptor expression and responsiveness to macrophage inflammatory protein-3α/CCL20 in human B cells. *Blood 96:2338.*
37. Schall, T. J., K. Bacon, K. J. Toy, and D. V. Goeddel. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature 347:669.*
38. Kehrl, J. H. 1998. Heterotrimeric G protein signaling: roles in immune function and fine-tuning by RGS proteins. *Immunity 8:1.*
39. De Vries, L., B. Zheng, T. Fischer, E. Elenko, and M. G. Farquhar. 2000. The regulator of G protein signaling family. *Annu. Rev. Pharmacol. Toxicol. 40:235.*
40. Druey, K. M., K. J. Blumer, V. H. Kang, and J. H. Kehrl. 1996. Inhibition of G-protein-mediated MAP kinase activation by a new mammalian gene family. *Nature 379:742.*
41. Cho, H., T. Kozasa, K. Takekoshi, J. De Gunzburg, and J. H. Kehrl. 2000. RGS14, a GTPase-activating protein for $G_{i\alpha}$, attenuates $G_{i\alpha}$- and $G_{13\alpha}$-mediated signaling pathways. *Mol. Pharmacol. 58:569.*
42. Moratz, C., V. H. Kang, K. M. Druey, C. S. Shi, A. Scheschonka, P. M. Murphy, T. Kozasa, and J. H. Kehrl. 2000. Regulator of G protein signaling 1 (RGS1) markedly impairs $G_{i\alpha}$ signaling responses of B lymphocytes. *J. Immunol. 164: 1829.*
43. Reif, K., and J. G. Cyster. 2000. RGS molecule expression in murine B lymphocytes and ability to down-regulate chemotaxis to lymphoid chemokines. *J. Immunol. 164:4720.*
44. Dieu, M. C., B. Vanbervliet, A. Vicari, J. M. Bridon, E. Oldham, S. Ait-Yahia, F. Briere, A. Zlotnik, S. Lebecque, and C. Caux. 1998. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med. 188:373.*
45. Dono, M., V. L. Burgio, C. Tacchetti, A. Favre, A. Augliera, S. Zupo, G. Taborelli, N. Chiorazzi, C. E. Grossi, and M. Ferrarini. 1996. Subepithelial B cells in the human palatine tonsil. I. Morphologic, cytochemical and phenotypic characterization. *Eur. J. Immunol. 26:2035.*
46. Brandtzaeg, P., E. S. Baekkevold, I. N. Farstad, F. L. Jahnsen, F. E. Johansen, E. M. Nilsen, and T. Yamanaka. 1999. Regional specialization in the mucosal immune system: what happens in the microcompartments? *Immunol. Today 20: 141.*
47. Wu, D., C. K. Huang, and H. Jiang. 2000. Roles of phospholipid signaling in chemoattractant-induced responses. *J. Cell Sci. 113:2935.*
48. Ross, E. M., and T. M. Wilkie. 2000. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu. Rev. Biochem. 69:795.*
49. Hong, J. X., G. L. Wilson, C. H. Fox, and J. H. Kehrl. 1993. Isolation and characterization of a novel B cell activation gene. *J. Immunol. 150:3895.*
50. Bowman, E. P., J. J. Campbell, K. M. Druey, A. Scheschonka, J. H. Kehrl, and E. C. Butcher. 1998. Regulation of chemotactic and proadhesive responses to chemoattractant receptors by RGS (regulator of G-protein signaling) family members. *J. Biol. Chem. 273:28040.*
51. Heximer, S. P., N. Watson, M. E. Linder, K. J. Blumer, and J. R. Hepler. 1997. RGS2/G0S8 is a selective inhibitor of $G_{q\alpha}$ function. *Proc. Natl. Acad. Sci. USA 94:14389.*
52. Dunlevy, J. R., and J. R. Couchman. 1995. Interleukin-8 induces motile behavior and loss of focal adhesions in primary fibroblasts. *J. Cell Sci. 108:311.*
53. Hobson, J. P., H. M. Rosenfeldt, L. S. Barak, A. Olivera, S. Poulton, M. G. Caron, S. Milstien, and S. Spiegel. 2001. Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. *Science 291:1800.*
54. Offermanns, S., V. Mancino, J. P. Revel, and M. I. Simon. 1997. Vascular system defects and impaired cell chemokinesis as a result of $G_{\alpha 13}$ deficiency. *Science 275:533.*
55. Arai, H., C. L. Tsou, and I. F. Charo. 1997. Chemotaxis in a lymphocyte cell line transfected with C-C chemokine receptor 2B: evidence that directed migration is mediated by $\beta\gamma$ dimers released by activation of $G_{\alpha i}$-coupled receptors. *Proc. Natl. Acad. Sci. USA 94:14495.*
56. Neptune, E. R., and H. R. Bourne. 1997. Receptors induce chemotaxis by releasing the $\beta\gamma$ subunit of G_i, not by activating G_q or G_s. *Proc. Natl. Acad. Sci. USA 94:14489.*
57. Cambien, B., M. Pomeranz, M. A. Millet, B. Rossi, and A. Schmid-Alliana. 2001. Signal transduction involved in MCP-1-mediated monocytic transendothelial migration. *Blood 97:359.*
58. Ottoson, N. C., J. T. Pribila, A. S. Chan, and Y. Shimizu. 2001. Cutting edge: T cell migration regulated by CXCR4 chemokine receptor signaling to ZAP-70 tyrosine kinase. *J. Immunol. 167:1857.*
59. Cismowski, M. J., C. Ma, C. Ribas, X. Xie, M. Spruyt, J. S. Lizano, S. M. Lanier, and E. Duzic. 2000. Activation of heterotrimeric G-protein signaling by a *ras*-related protein: implications for signal integration. *J. Biol. Chem. 275:23421.*
60. Cismowski, M. J., A. Takesono, C. Ma, J. S. Lizano, X. Xie, H. Fuernkranz, S. M. Lanier, and E. Duzic. 1999. Genetic screens in yeast to identify mammalian nonreceptor modulators of G-protein signaling. *Nat. Biotechnol. 17:878.*
61. Rhee, S. G. 2001. Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem. 70:281.*
62. Brandes, M., D. F. Legler, B. Spoerri, P. Schaerli, and B. Moser. 2000. Activation-dependent modulation of B lymphocyte migration to chemokines. *Int. Immunol. 12:1285.*