

# Corruption of Human Follicular B-Lymphocyte Trafficking by a B-Cell Superantigen

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Protein A (SpA) of *Staphylococcus aureus* is known to target the paratope of immunoglobulins expressing V<sub>H</sub>3 genes, and to delete marginal zone B cells and B-1a *in vivo*. We have discovered that SpA endows *S. aureus* with the potential to subvert B-cell trafficking in the host. We found that SpA, whose Fc-binding site has been inactivated, binds essentially to naïve B cells and induces a long-lasting decrease in CXCR4 expression and in B-cell chemotaxis to CXCL12. Competition experiments indicated that SpA does not interfere with binding of CXCR4 ligands and does not directly bind to CXCR4. This conclusion is strongly supported by the inability of SpA to modulate clathrin-mediated CXCR4 internalization, which contrasts with the potent effect of anti-immunoglobulin M (IgM) antibodies. Microscopy and biochemical experiments confirmed that SpA binds to the surface IgM/IgD complex and induces its clathrin-dependent internalization. Concomitantly, the SpA-induced signaling leads to protein kinase C-dependent CXCR4 downmodulation, suggesting that SpA impairs the recycling of CXCR4, a postclathrin process that leads to either degradation into lysosomes or *de novo* expression at the cell surface. In addition to providing novel insight into disruption of B-cell trafficking by an infectious agent, our findings may have therapeutic implications. Because CXCR4 has been associated with cancer metastasis and with certain autoimmune diseases, SpA behaves as an evolutionary tailored highly specific, chemokine receptor inhibitor that may have value in addition to conventional cytotoxic therapy in patients with various malignancies and immune-mediated diseases.

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## INTRODUCTION

Expression of tailored evasion proteins is a common strategy used by several pathogens, such as *Staphylococcus aureus*. This bacterium is a leading cause of human infections worldwide in healthy and immune-compromised individuals, and it has developed an exceptional ability to exploit host immune functions. Several of the clinically important inter-

actions of *S. aureus* are mediated by protein A (SpA), a surface virulence factor that is highly conserved between strains (1). First, through its Xr repeated sequences, SpA was found to induce interleukin-6 (IL-6) and interferon- $\beta$  (IFN $\beta$ ) secretion in airway epithelial cells as well as in lymphocytes (2). It is of note that a point had to be ruled out. IFN $\beta$  is a major immune actor that modulates the

antibody response and the chemotactic response of B cells to sphingosine-1 phosphate (3,4). Second, SpA can activate epithelial cells through Toll-like receptor 2 and tumor necrosis factor R1, with potential pathological implications (5,6). Third, SpA binding to the Fc fragment of circulating Ig activates the classical complement pathway and elicits tissue inflammation mediated by conventional antigen-antibody complexes (7). By contrast, the *S. aureus* extracellular fibrinogen-binding protein inhibits C3d fragment interaction with complement receptor 2 (CR2), thus preventing CR2-mediated B-cell activation (8). Fourth, SpA targets B cells that express Ig V<sub>H</sub>3 genes, and acts as a superantigen through its binding to the Ig paratope (9-11).

Through such unconventional binding, SpA interacts with 30%-50% of circulating human B cells and induces cell prolifer-

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eration or apoptosis, according to the B-cell target. Recent experiments revealed that administration of soluble SpA to transgenic mice expressing fully human Ig reduces B-1a lymphocyte numbers in the peritoneal cavity and marginal zone (MGZ) B cells in the spleen. This depletion impaired the type 2 T-cell-independent response and decreased immunoglobulin M (IgM)-expressing B cells more strongly than IgG-expressing  $V_H3^+$  B cells (12). Although IgMs are also expressed by all naïve B cells (surface [S]IgD<sup>high</sup>SigM<sup>low</sup> CD27<sup>+</sup>) and by a small proportion of mutated memory B cells (SIgM<sup>+</sup>SIgD<sup>-</sup>CD27<sup>+</sup>), no significant loss in follicular B cells was observed in these SpA-treated mice (12). The preferential depletion of MGZ B cells probably depends on their increased sensitivity to B-cell receptor (BCR)-mediated apoptosis (13) and their exposure to the bloodstream as a first line of innate-like B-cell effectors (12). Besides the strong and long-lasting MGZ B-cell depletion, a more limited and transient loss in follicular B cells was described in SpA-treated mice (14), which might suggest that B-cell trafficking is also transiently impaired.

Lymphocyte recirculation, which is critical for effective immunity, is tightly regulated by the expression of adhesion molecules and chemokine receptors on lymphocytes combined with the spatial and temporal expression of their corresponding ligands in a variety of tissues (15). In the bone marrow, the CXCL12/CXCR4 pair is important for the retention of precursor B cells, and also for that of long-lived plasma cells in particular niches. CXCL12- or CXCR4-deficient mice have impaired B-cell lymphopoiesis and abnormal numbers of circulating immature B cells (16,17). Superimposed on the role of the CXCL12/CXCR4 pair, the balance between CXCR5/CXCL13 and CCR7/CCL21 pairs controls the organization of B-cell and T-cell areas in lymphoid tissues and the appropriate relocation of mature follicular B cells during immune responses (18,19). Both naïve and memory follicular B cells express CXCR4, CXCR5 and CCR7 and migrate in response to their ligands: CXCL12,

CXCL13, and CCL21 or CCL19, respectively. However, antigen (Ag), inflammatory cytokines, and interactions with T cells can strongly modulate B-cell chemotaxis by impairing chemokine receptor expression or signaling (20–22). BCR ligation decreases the response to CXCL12 through protein kinase C (PKC)-dependent CXCR4 internalization (23), whereas type I IFN enhances B-cell chemotaxis to CXCL12 by accelerating CXCR4 internalization and impairing signaling (24).

To gain further insight into the pathogenic impact of *S. aureus* on humoral immunity, we investigated whether SpA devoid of its Fc binding capacity (Mod-SpA) can directly alter human B-cell chemotaxis. We show that SpA binds essentially to naïve B cells and induces a long-lasting decrease in CXCR4 expression and chemotactic response to CXCL12. Mod-SpA does not directly bind CXCR4, but it induces CXCR4 downmodulation from the B-cell surface through BCR-dependent PKC activation.

## MATERIALS AND METHODS

### B Cells and Culture

The BJAB cell line was obtained from ECACC (Salisbury, UK). We isolated resting B cells from palatine tonsils using a three-step purification as previously described (25). The resulting B-cell population, comprising naïve and memory B cells, is herein called “B cells.” It was >98% CD19<sup>+</sup> or CD20<sup>+</sup>, >93% CD44<sup>+</sup>, <1% CD3<sup>+</sup> (T cells), CD14<sup>+</sup> (monocytes) and CD38<sup>+</sup> (germinal center B cells). The viability of these cells was consistently higher than 98%.

B cells ( $2 \times 10^6$ /mL) were cultured in RPMI 1640-glutamax medium containing 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 IU/mL penicillin, 100 µg/mL streptomycin, 1 mmol/L sodium pyruvate (all from Invitrogen SARL, Cergy-Pontoise, France) and 10% heat-inactivated fetal calf serum (FCS) (PAA Laboratories GmbH, Les Mureaux, France), subse-

quently termed complete medium (CM). The endotoxin concentration in the culture medium and reagents used was below 1 ng/mL as indicated by the manufacturers.

### Recombinant Protein A

Purified recombinant protein A (SpA), produced in *Escherichia coli*, was obtained from Repligen (Waltham, MA, USA). Selective blockade of the Fc binding site of purified SpA was performed by treatment with iodine monochloride. This process neither affects its  $V_H3$  binding activity nor introduces endotoxin contamination (12,26). This modified protein A and its biotinylated form used in flow cytometry are herein called “Mod-SpA” and “biotinylated-Mod-SpA,” respectively.

### Flow Cytometric Analysis

BJAB cells or primary B cells were stained with 5 µg/mL biotinylated-Mod-SpA for 30 min at 4°C followed by Alexa fluor 488-conjugated streptavidin (AF488-Streptavidin, Invitrogen SARL) for 30 min at 4°C. In some experiments, B cells were preincubated for 30 min at 4°C with 0.5 µg/mL purified mouse anti-human CD32 monoclonal antibody (mAb) (BD Biosciences, Le Pont de Claix, France) and 10 ng/mL tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) or 100 ng/mL CXCL12 (both from R&D Systems, Abingdon, UK) before staining with biotinylated-Mod-SpA. The phenotype of SpA<sup>+</sup> B cells was determined by polychromatic staining with CD20-PECy7, CD44-APC, CD27-PE, IgD-PE or IgD-FITC mAb or matched isotype controls conjugated to PECy7/APC/PE/FITC (all from BD Biosciences). We collected data using LSRI Flow cytometer and analyzed it using Cell quest software (both from BD Biosciences). We gated on viable cells and analyzed 10,000 cells/sample.

### Calcium Mobilization

BJAB cells ( $2 \times 10^6$ /test) were loaded for 30 min at 37°C with 1 µmol/L Indo1/AM (Molecular Probes, Invitrogen SARL) in Hanks balanced salt solution (HBSS) buffer containing 1 mmol/L

CaCl<sub>2</sub>, 1 mmol/L MgCl<sub>2</sub> and 1% FCS. Cells were washed and then resuspended in HBSS buffer and incubated at 37°C for 5 min before stimulation with F(ab')<sub>2</sub> goat anti-human IgM antibody (Ab) (10 µg/mL; Beckman Coulter, Villepinte, France) or with biotinylated-Mod-SpA (5 µg/mL) and streptavidin (Beckman Coulter). Indo-1 fluorescence was collected on an LSRII flow cytometer with the following configuration: ultraviolet excitation at 325 nm, and emission at 405/20 nm and 500/11 nm for bound and free probe, respectively.

### Proliferation Assays

B cells (2 × 10<sup>5</sup> cells/well/200 µL) were activated by incubation in CM for 3 d with one or more of the following: F(ab')<sub>2</sub> goat anti-human IgM Ab (1 to 10 µg/mL; Beckman Coulter), formalinized cell-wall extracts from *S. aureus* Cowan I strain (SAC, 10<sup>-5</sup> to 10<sup>-4</sup> vol/vol; Calbiochem, La Jolla, CA), Mod-SpA (0.01 to 10 µg/mL) and IL-2 (R&D systems, 20 ng/mL). Proliferation was measured by supplying the cultures with a pulse of 1 µCi/well of [*methyl*-<sup>3</sup>H] thymidine (Amersham, Les Ulis, France) for the last 16 h of the third day of incubation. Cells were collected by filtration through a glass-fiber filter, and [<sup>3</sup>H] thymidine incorporation was measured in a β-scintillation counter (Betaplate 1205, EGG Wallac, Turku, Finland). Results are expressed as counts per minute (mean of triplicates ± SEM).

### Cytokine Production

B cells (2 × 10<sup>5</sup> cells/well/200 µL) were cultured in triplicates for 2 d in CM with one or more of the following: F(ab')<sub>2</sub> goat anti-human IgM Ab (10 µg/mL), Mod-SpA (10 µg/mL), CD40L (R&D Systems, 50 ng/mL) and IL-4 (R&D Systems, 20 ng/mL). IL-6 and MIP-1β concentrations were measured in cell-free supernatants with specific ELISA kits purchased from Beckman Coulter and R&D Systems, respectively. Results are expressed as the mean concentration (pg/mL) ± standard error of the mean (SEM).

### Receptor Internalization

B cells (2 × 10<sup>6</sup>/mL) were cultured for various periods of time in medium with 10 µg/mL Mod-SpA, 10 µg/mL F(ab')<sub>2</sub> goat anti-human IgM Ab or 100 ng/mL CXCL12 (R&D systems). Cells were washed in ice-cold medium and stained with CD20-PECy7 and either CD19-PE, IgM-FITC, IgD-PE (all from BD Biosciences), CCR7-APC, CXCR5-PE or CXCR4-PE (all from R&D Systems) for 30 min at 4°C. Two-color immunofluorescence analysis was performed on 10,000 viable cells after gating on CD20<sup>+</sup> cells. Data are expressed as the mean percentage ± SEM mean channel fluorescence intensity (MFI) values for residual surface expression as previously described (24).

In some experiments, B cells were preincubated for 10 min at 37°C with 20 µmol/L PP2 (*src* kinase inhibitor), 5 µmol/L Herbimycin (Herb; a tyrosine kinase inhibitor), 1 µmol/L wortmannin (WMN; a phosphoinositide 3-kinase [PI3K] inhibitor), 2 µmol/L U73122 (a phospholipase C [PLC] inhibitor) and 5 µmol/L chelerythrine chloride (CC; a PKC inhibitors) before treatment with Mod-SpA, goat anti-IgM Ab or CXCL12. Inhibitors were from Biomol International (Enzo Life Sciences, Villeurbanne, France) or Calbiochem. B cells were also treated with 2 mmol/L methyl β-cyclodextrin (MβCD; Sigma, St Louis, MO) or 0.4 mol/L sucrose for 1 h at 37°C before adding Mod-SpA, goat anti-IgM Ab or CXCL12.

### In Vitro Chemotaxis Assay

A chemotaxis assay was carried out as described (24). In brief, 5 × 10<sup>5</sup> B cells in 100 µL of prewarmed RPMI 1640 containing 10 mmol/L HEPES and 1% FCS were transmigrated through 5-µm pore size bare filter Transwell inserts (Costar, Cambridge, MA) for 3 h at 37°C in response to CXCL12 (250 ng/mL). After exclusion of cell debris by forward- and side-scatter gating, the migrated cells were counted with an LSRII cytometer for 60 s. Results are shown as the percentage of specific migration, from

which background migration to control medium was subtracted.

### Immunofluorescence Microscopy

After staining in suspension, B cells (1 × 10<sup>5</sup>/slide) were spun for 5 min at 900g with a Shandon Cytospin<sup>®</sup> Cytocentrifuge (Thermo Scientific, St-Herblain, France) and fixed with paraformaldehyde 4%. Slides were mounted in 4,6-diamidino-2-phenylindole (DAPI)-Fluoromount-G<sup>™</sup> medium (Clinisciences, Montrouge, France) and were analyzed with a Zeiss Axioplan 2 microscope. Data acquisition was performed with Mercator 4.42 software (ExplorNova, La Rochelle, France).

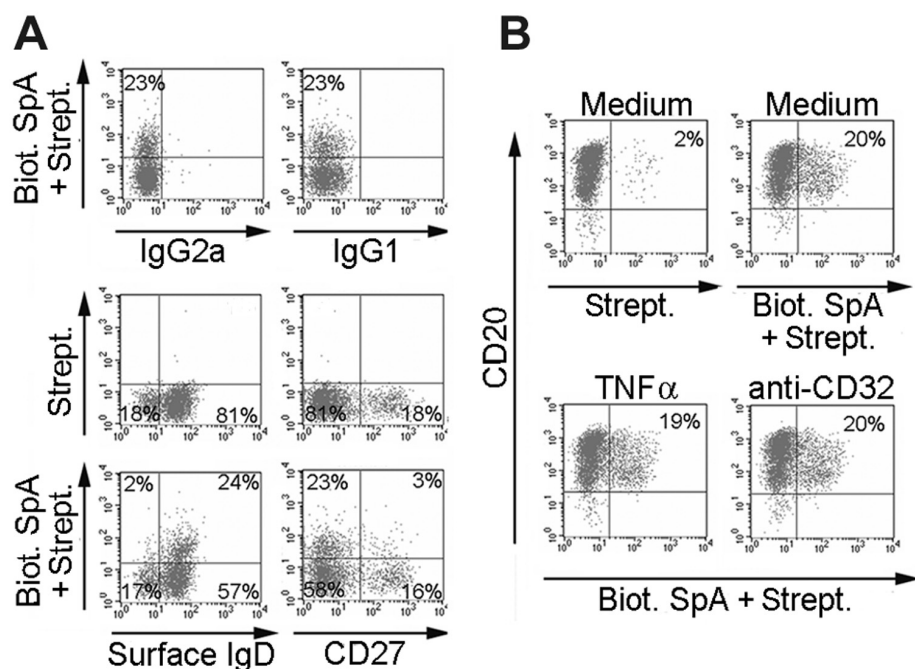
### Statistical Analysis

Data were analyzed with GraphPad Prism software (GraphPad, San Diego CA). *p* Values <0.05 were considered significant.

## RESULTS

### Protein A of *S. aureus* Binds More Naive than Memory B Cells

First, we assessed the binding capacity of biotinylated-Mod-SpA to resting tonsillar B cells (CD20<sup>+</sup>CD44<sup>high</sup>). Staining of B cells from one representative donor with anti-CD20 mAb and either anti-SIgD mAb (Figure 1A, left panel) or anti-CD27 mAb (Figure 1A, right panel) showed that Mod-SpA binds 23 (upper panel) to 26% (lower panels) B cells and is coexpressed with 24% SIgD or 3% CD27 of 26% B cells, respectively. Similar results obtained in seven independent donors showed that 26% ± 2.0% (mean ± SEM) B cells were Mod-SpA<sup>+</sup>, with 21% ± 2.1% and 6% ± 0.6% B cells coexpressing SIgD or CD27, respectively. Therefore, Mod-SpA<sup>+</sup> B cells contained 83% ± 3.6% naïve B cells and 23% ± 4.1% memory B cells. Preincubation with anti-CD32 blocking mAb or with TNFα did not prevent the Mod-SpA binding to B cells (Figure 1B), indicating that neither FcγRII (CD32) nor TNF-RI participates in Mod-SpA binding to B cells.



**Figure 1.** Flow cytometric analysis of SpA binding to primary B cells. (A) B cells were stained with CD20-PEcy7 mAb, biot-Mod-SpA plus AF488-Streptavidin and IgG-PE mAb (left panels) or CD27-PE mAb (right panels). Data from one representative experiment of seven are shown. The percentages of positive cells gated on CD20<sup>+</sup> cells are indicated in each quadrant. (B) B cells were incubated with medium, 10  $\mu$ g/mL CD32 mAb or 10 ng/mL TNF $\alpha$  for 30 min at 4°C before staining with CD20-PEcy7 mAb and biot-Mod-SpA plus AF488-Streptavidin. Data from one representative experiment of 7 are shown. The percentages of Mod-SpA positive cells are indicated in the upper right quadrants.

### Protein A Induces IL-6 Production, but Not B-Cell Proliferation

Because Mod-SpA was previously shown to preferentially bind to V<sub>H</sub>3<sup>+</sup> SIgM B cells and because *S. aureus* particles (SAC) promote polyclonal B-cell proliferation, we tested whether Mod-SpA triggers B-cell proliferation. Unlike SAC and anti-IgM Ab, Mod-SpA did not induce B-cell proliferation, even in the presence of IL2 (Figure 2A). To determine whether SpA triggers progression into the cell cycle and intracellular Ca<sup>++</sup> mobilization, we analyzed the ability of SpA to induce Ca<sup>++</sup> release in a V<sub>H</sub>3<sup>+</sup> SIgM<sup>+</sup> B-cell line, BJAB. In contrast to anti-IgM cross-linking, biotinylated-Mod-SpA did not trigger Ca<sup>++</sup> release in these cells, even after cross-linking with streptavidin (Figure 2B). However, Mod-SpA increased IL-6 production in IL-4- and CD40L-treated B cells by 1.8  $\pm$

0.6-fold (range 1.2–2.8-fold) (Figure 2C, but not that of MIP-1 $\beta$ ) (Figure 2D). The production of both cytokines was strongly enhanced by anti-IgM Ab (Figures 2C, D). Thus, compared with BCR stimulation, Mod-SpA only partially activates B cells.

### Protein A Significantly Reduces CXCR4 Expression and CXCL12-Mediated B-Cell Chemotaxis

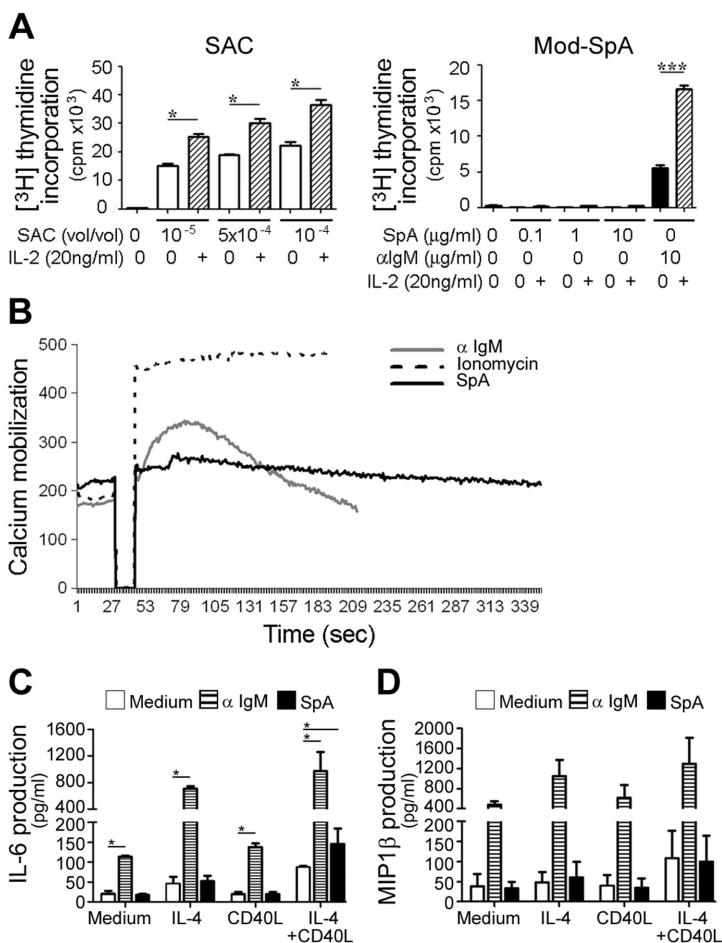
Because BCR ligation was previously shown to downmodulate surface CXCR4 expression (22), we assessed whether Mod-SpA also impairs CXCR4 expression on B cells. Our data showed that preincubation of B cells with Mod-SpA significantly decreased CXCR4 expression (MFI from 167 to 49) at 37°C (Figure 3A), but not at 4°C (data not shown). Kinetic studies of B cells from two independent donors showed that Mod-SpA

rapidly decreased the intensity of CXCR4 expression by 37%  $\pm$  2.8% after 30 min, and by 50%  $\pm$  0.2% after 1 h. A steady decrease in CXCR4 expression was reached after 4 h with 71%  $\pm$  11.3% at 4 h, 64%  $\pm$  2.7% at 6 h, and 62%  $\pm$  1.7% at 24 h, whereas the basal CXCR4 expression (in the presence of medium) increased after 6 h (Figure 3B).

In preliminary experiments, we found that the CXCR4 downmodulation induced by CXCL12 or by polyclonal anti-IgM Ab was maximal after 2 h or 16 h, respectively. Therefore, we first compared the intensity of CXCR4 downmodulation after culturing B cells for 2 h in medium alone or in the presence of 10  $\mu$ g/mL Mod-SpA or 100 ng/mL CXCL12. We observed that Mod-SpA and CXCL12 similarly decreased CXCR4 expression by 60%  $\pm$  14% and 62%  $\pm$  13%, respectively (Figure 3C). After culturing B cells for 16 h in medium alone or in the presence of 10  $\mu$ g/mL Mod-SpA or 10  $\mu$ g/mL anti-IgM Ab, we found that Mod-SpA was more efficient than anti-IgM Ab in downmodulating CXCR4 with a reduction of the MFI values by 67%  $\pm$  5.6% and 48%  $\pm$  5.8%, respectively (Figure 3D). This SpA-induced decrease in CXCR4 expression was concomitant with a reduced chemotaxis to CXCL12: 24%  $\pm$  4%, 18%  $\pm$  7.5% and 42%  $\pm$  8% B cells migrated in response to CXCL12 after treatment with Mod-SpA, anti-IgM Ab or medium, respectively (Figure 3E).

### Protein A Decreases the Expression of SIgM and SIgD, but Not of CD19

In addition to decreased CXCR4 expression, BCR ligation was shown to differently modulate CXCR5 and CCR7 expression (22). Accordingly, we compared the effect of Mod-SpA on the surface expression of CXCR4, CXCR5 and CCR7 on B cells. Our data showed that Mod-SpA did not modify the expression of CXCR5 and CCR7, whereas it decreased that of CXCR4 by 50% (MFI from 114 to 57) on the same cells (Figure 4). Similarly, Mod-SpA decreased SIgD and SIgM expression by 19% (MFI from 316 to 255) and 23% (MFI from 56 to 43), respectively.



**Figure 2.** Functional consequences of SpA binding to B cells. (A) B cells from two donors were cultured for 3 d in medium with or without graded doses of SAC (left panel), Mod-SpA or goat anti-human IgM Ab (right panel), with or without IL-2. Proliferative response was measured by incorporation of <sup>3</sup>H-thymidine during the last 16 h of the culture. Triplicates of one donor are showed and expressed as mean cpm ± SEM. Data have been analyzed with the paired *t* test (\**p* < 0.05; \*\*\**p* < 0.001). (B) Calcium mobilization assay was performed on BJAB cells (2 × 10<sup>6</sup>/mL) loaded with Indo/AM and stimulated at 37°C with ionomycin (discontinued line), anti-IgM Ab (gray line) or biotinylated Mod-SpA plus streptavidin (black line). Indo-1 fluorescence was monitored during 350 s and the ratio (emission at 405/20 nm/emission at 500/11) was depicted. One representative experiment of two is shown. (C, D) B cells were cultured for 2 d in medium, IL-4 and/or CD40L in the presence of medium alone (open bars), 10 μg/mL goat anti-human IgM Ab (hatched bars) or 10 μg/mL Mod-SpA (black bars). Production of IL-6 (C, *n* = 3) and MIP1β (D, *n* = 2) was assessed by ELISA in cell-free supernatants. Data are expressed as mean ± SEM pg/mL. One-tailed Mann-Whitney test was used to determine statistical significance and *p* values are indicated as \**p* < 0.05.

Decreased SIgD expression in the whole B-cell population was associated with the detection of a B-cell population expressing intermediary levels of SIgD (24% to 32% of the whole SIgD<sup>+</sup> B cells). These data suggests that SpA preferentially af-

fects a subset of naïve B cells. By contrast, the expression of CD19, a member of the BCR signaling complex, remained unchanged (Figure 4). In three independent experiments, Mod-SpA decreased the expression of SIgD, SIgM, CD19 and

CXCR4 by 29% ± 7.4%, 27% ± 5.7%, 1% ± 4% and 43% ± 13.9% in whole B cells, respectively. Anti-IgM Ab decreased the expression of SIgD, CD19 and CXCR4 by 33.7% ± 19.5%, 19.3% ± 5.1% and 37.7% ± 4.7%, respectively. These distinct effects on CD19 and SIgD suggest that Mod-SpA dissociates CD19 from BCR expressing IgM and IgD.

**CXCR4 Internalizes Concomitantly with Protein A**

To verify that protein A does not mask the CXCR4 epitope recognized by the mAb, we incubated B cells at 4°C with medium alone (Figure 5A, left panel) or biotinylated-Mod-SpA (Figure 5A, right panel) before staining with the CXCR4 mAb. Regardless of the presence of Mod-SpA during the incubation period, CXCR4 mAb recognized more than 80% B cells with a similar staining intensity (Figure 5A). On the other hand, preincubation of B cells with CXCL12 did not downmodulate the binding of biotinylated-Mod-SpA (Figure 5B). Thus, the protein A-induced decrease in CXCR4 expression is not due to competition with its ligands.

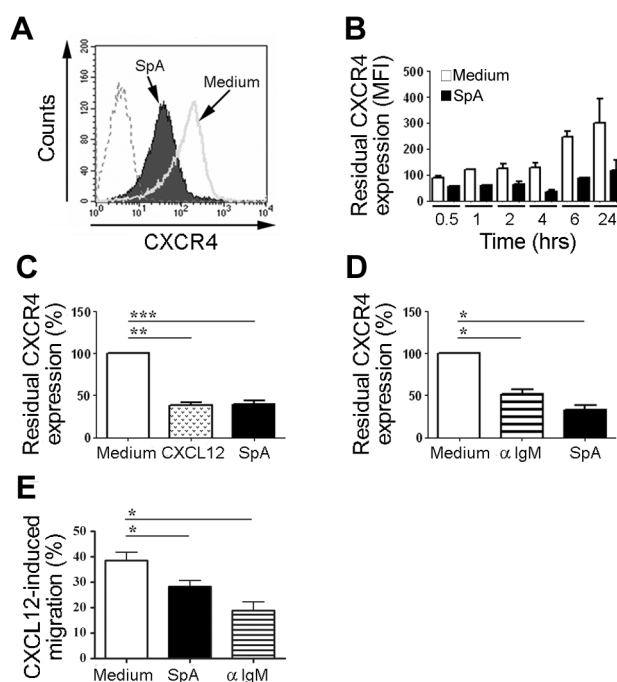
We then asked whether the observed CXCR4 modulation is associated with receptor internalization. To better distinguish between membrane and cytoplasmic CXCR4 staining, we used the V<sub>H</sub>3<sup>+</sup>, CXCR4<sup>+</sup> B-cell line BJAB. Accordingly, BJAB cells were stained at 4°C with CXCR4 mAb and incubated for 2 h with medium alone or 10 μg/mL Mod-SpA at 37°C before we assessed the surface and cytoplasmic expression of CXCR4 with AF488-conjugated goat anti-mouse Ab (Figure 5C). After incubation in medium alone, most CXCR4 expression remained at the cell surface, whereas increased amounts of CXCR4 were present in the cytoplasm of B cells incubated with Mod-SpA. These experiments suggest that protein A contributes to CXCR4 internalization into B cells.

To determine whether protein A penetrates into primary B cells after its membrane binding, we compared its residual surface staining after incubation for 2 h

at 4°C or at 37°C. Whereas 21% of B cells were Mod-SpA<sup>+</sup> after incubation at 4°C, only 13% of B cells were Mod-SpA<sup>+</sup> after incubation at 37°C. The intensity of Mod-SpA staining was also 2-fold lower after incubation at 37°C than at 4°C (MFI = 321 versus 664, Figure 5D). At 4°C, Mod-SpA staining was homogeneously distributed at the B-cell surface, whereas surface patches and intracellular Mod-SpA staining were observed after incubation at 37°C (Figure 5E). This finding strongly suggests that Mod-SpA is internalized after its binding to the B-cell surface and that CXCR4 downmodulation occurs concomitantly.

### Protein A Decreases CXCR4 Expression after Its Internalization by the BCR

It has been well established that BCR internalization is strictly dependent on clathrin whereas both clathrin- and caveolin-dependent mechanisms have been previously reported for CXCR4 internalization. Consistent with our previous data suggesting that CXCR4 internalization in B cells occurs essentially through caveolin-independent pathways (27), here we found that depletion of cholesterol by M $\beta$ CD marginally decreased CXCL12-induced CXCR4 internalization. In contrast, pretreatment of B cells with hypertonic sucrose strongly inhibited CXCL12-induced CXCR4 internalization (Figure 6A). In three independent experiments, CXCL12-induced internalization of CXCR4 was 57.3%  $\pm$  3.2% and 41.3%  $\pm$  5.7% after incubation with medium or M $\beta$ CD, respectively, but reduced to 18.3%  $\pm$  12.7% after incubation with sucrose. In contrast, Mod-SpA-induced CXCR4 downregulation was 47%  $\pm$  2.6%, 52.7%  $\pm$  14.5% and 40.3%  $\pm$  5.1% after incubation with medium, M $\beta$ CD and sucrose, respectively. Pretreatment of B cells with hypertonic sucrose, but not with M $\beta$ CD, totally prevented the Mod-SpA-induced internalization of SIgD (Figure 6B). Therefore, internalization of protein A probably occurs through its binding to the BCR by a clathrin-dependent mechanism, but not directly through its binding to CXCR4.



**Figure 3.** SpA impairs CXCR4 expression and CXCL12-induced B-cell chemotaxis. (A) B cells were incubated with medium alone or 10  $\mu$ g/mL Mod-SpA for 2 h at 37°C before staining with CXCR4-PE mAb. One representative experiment of 13 is shown. (B) B cells were incubated with medium alone (open bars) or 10  $\mu$ g/mL Mod-SpA (black bars) for 0.5 to 24 h at 37°C before staining with CXCR4-PE mAb. Data are expressed as the mean  $\pm$  SEM percentage of MFI values for residual surface CXCR4 expression obtained from two donors. (C) B cells were incubated with medium, 100 ng/mL CXCL12 or 10  $\mu$ g/mL Mod-SpA for 2 h at 37°C before staining with CXCR4-PE mAb. Results are expressed as the mean  $\pm$  SEM percentage of MFI values for residual surface CXCR4 expression obtained from 13 different donors. Two-tailed Wilcoxon test was used to determine statistical significance, and *p* values are indicated as \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001. (D) B cells were incubated with medium alone, 10  $\mu$ g/mL anti-IgM Ab or Mod-SpA for 16 h at 37°C before staining with CXCR4-PE mAb. Results are expressed as the mean  $\pm$  SEM percentage of MFI values for residual surface CXCR4 expression obtained from seven different donors. Two-tailed Wilcoxon test was used to determine statistical significance and *p* values are indicated as \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001. (E) B cells were incubated with medium alone, 10  $\mu$ g/mL anti-IgM Ab or Mod-SpA for 16 h at 37°C. Medium, anti-IgM Ab and Mod-SpA-treated B cells were analyzed for migration to 100 ng/mL CXCL12. Results are expressed as the mean  $\pm$  SEM percentage of specifically migrating cells obtained from 11 different donors. Two-tailed Wilcoxon test was used to determine statistical significance, and *p* values are indicated as \**p* < 0.05.

### Signaling Pathways Involved in Protein A-Induced CXCR4 Modulation

Considering that activation of cytoplasmic effectors downstream the BCR can participate in SpA-induced CXCR4 internalization, we treated B cells with various inhibitors or medium before incubating them with Mod-SpA or soluble anti-IgM Ab for 16 h. Decrease in CXCR4 expression induced by anti-IgM Ab was

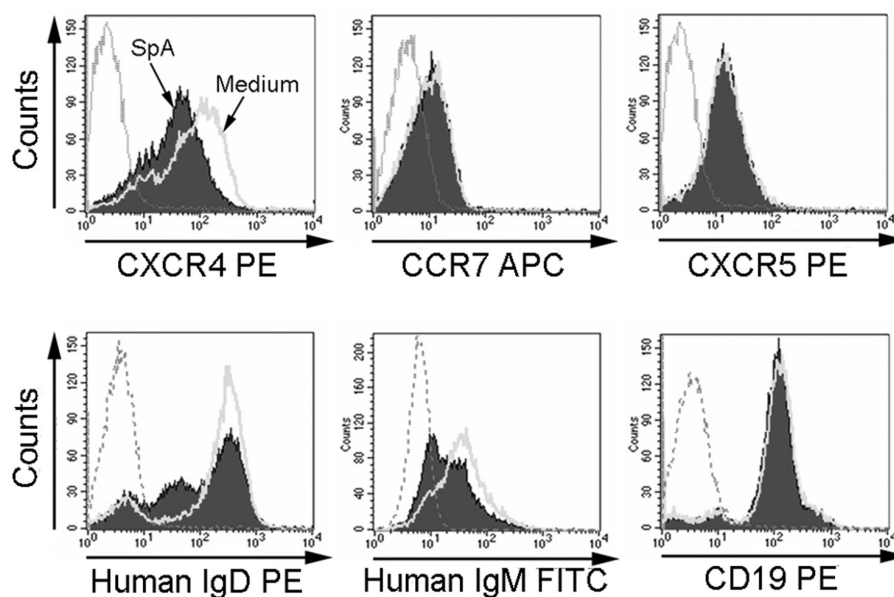
partially prevented by inhibition of src kinases (PP2 and Herb), PLC (U73122), PI3K (WMN) and PKC (CC) activation (Figure 6B). In contrast, Mod-SpA-induced CXCR4 downregulation was only inhibited by CC, an inhibitor of all PKC (Figure 6C). Incubation with BAPTA, a Ca<sup>++</sup> chelator, also prevented the anti-IgM Ab-induced CXCR4 decrease, but not that induced by Mod-SpA

(data not shown). Because of the strong autofluorescence of piceatannol, it was not possible to evaluate whether SYK participated in BCR- or SpA-induced CXCR4 internalization. Consistent with previous observations (24), CXCL12-dependent internalization was independent of Src (PP2) and PI3K (WMN) activation, whereas it was partially inhibited by CC (Figure 6D). Our present findings indicate that PKC-induced phosphorylation of CXCR4 may contribute to protein A-induced CXCR4 downmodulation.

## DISCUSSION

To evade immune surveillance, a common strategy for infectious agents is to produce a variety of molecules that subvert immune responses and cell trafficking. For example, gram-negative bacteria, including *Bordetella pertussis*, produce toxins that either block their signal transduction (28) or downregulate chemokine receptor expression, thereby impairing immunomodulatory functions of human neutrophils and monocytes (29). Staphylococcal superantigenic enterotoxins, such as staphylococcal enterotoxin A (SEA) and SEB, induce a rapid, agonist-independent mechanism of downmodulating chemokine receptors and responsiveness of monocytes to chemokine ligands (30). As a result, stimulation of monocytes with either SEA or SEB led to inhibition of CC chemokine-directed migration and  $Ca^{2+}$  mobilization, thus demonstrating that gram-positive bacteria-derived SAgs can suppress functional responsiveness of monocytes to chemokines (30). In other bacteria, several species attain chemoattractant inhibition by secreting proteases that disable chemoattractants. For example, proteases from *Pseudomonas aeruginosa*, *Serratia marcescens*, *Porphyromonas gingivalis*, *Streptococcus pyogenes* and streptococci groups A, B and G degrade anaphylatoxins and chemokines (31).

Here, we have discovered that *S. aureus* has developed another strategy to downregulate a chemokine receptor in the host. We found that, along with its *in*



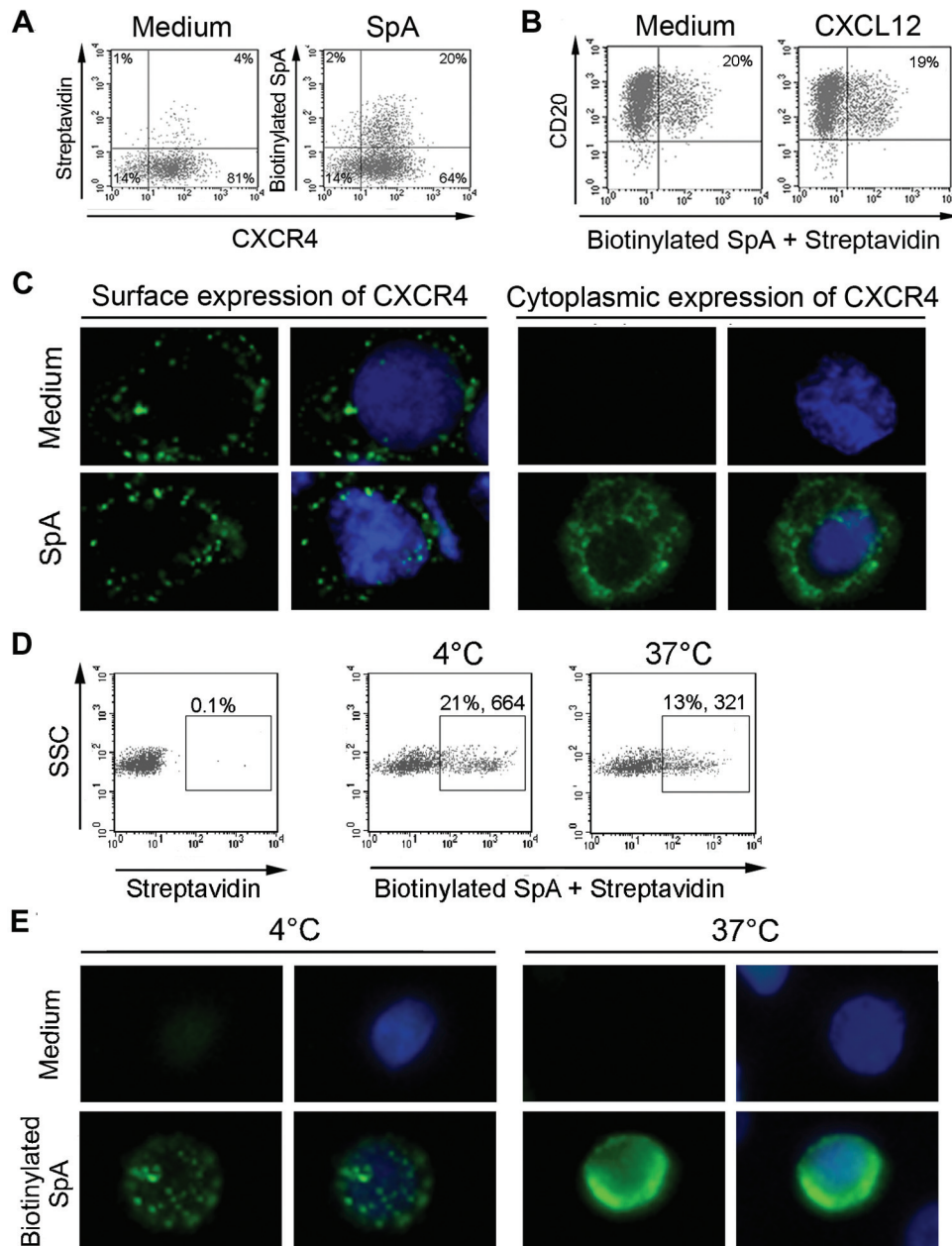
**Figure 4.** Phenotypic consequences of SpA binding on primary B cells. B cells were incubated with medium (bold line, empty histogram) or 10  $\mu$ g/mL Mod-SpA (gray histogram) for 2 h at 37°C before staining with CXCR4-PE, CXCR5-PE, CCR7-APC, IgD-PE, IgM-FITC or CD19-PE mAb. One representative experiment of three is shown.

*in vivo* deleting effects on MGZ and B1 B cells (12), *S. aureus* protein A inhibits B-cell chemotaxis to CXCL12 through downmodulation of surface CXCR4 expression. The effect was temperature dependent and long lasting, with an average decrease of 62% after 24 h of SpA treatment. Consistent with a preferential protein A binding to naïve B cells, SpA decreased the expression of SIgM and SIgD as efficiently as anti-IgM Ab, but left unchanged that of CD19. The uncoupling between SIgM/D and CD19 probably explains the lack of calcium release in B cells treated with SpA and the lack of cell proliferation, because CD19 is required for maximal calcium mobilization and proliferation (32). Whereas Mod-SpA did not achieve full BCR activation, primary B cells exposed to Mod-SpA produced significant amounts of IL-6, but not of MIP1 $\beta$ . Through its ability to stimulate IL-6 production in the absence of B-cell proliferation, Mod-SpA might preferentially engage IgM-expressing B cells into plasma cell differentiation. This hypothesis is consistent with the increase in specific IgM, but not IgG, serum levels

observed in SpA-pretreated mice immunized with tetanus toxoid (12).

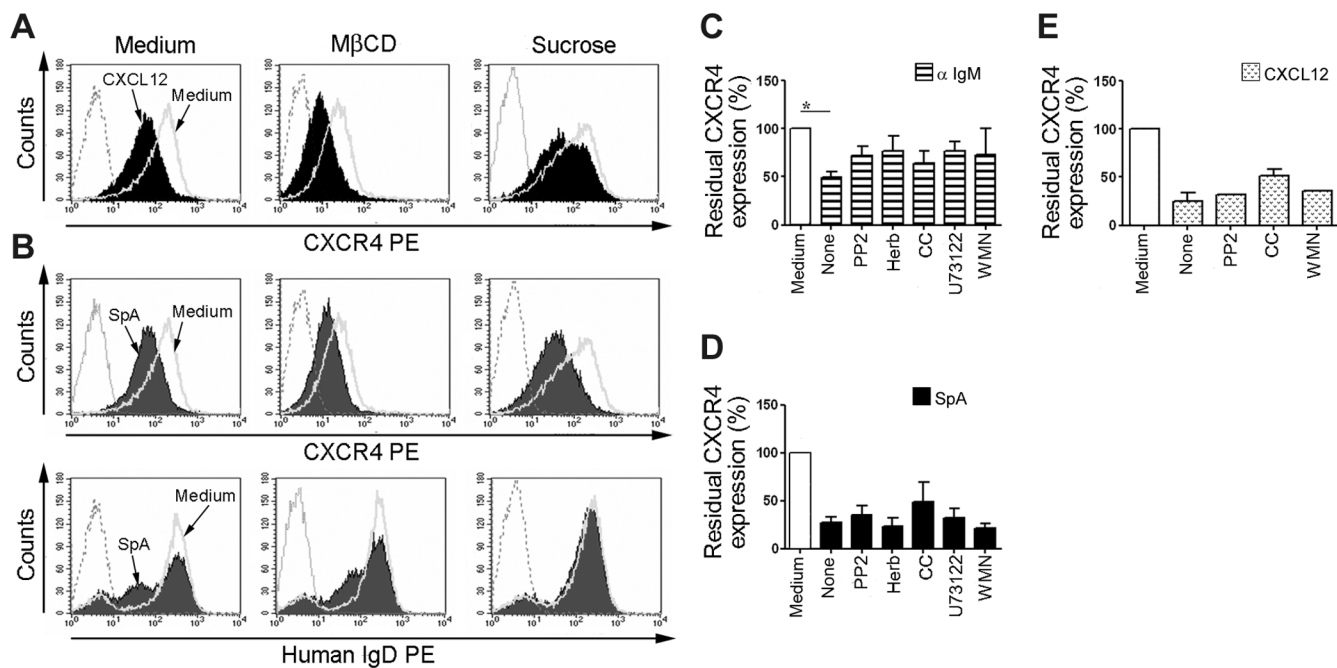
The observation that SpA preferentially binds naïve B cells relative to memory B cells suggests that somatic mutations may decrease the affinity of SpA for  $V_H3$ -expressing BCR, thereby accounting for its preferential binding to naïve B cells. In previous work, we showed that 77% of  $V_H3^+$  human Ig bind SpA, that positive Igs were derived from eight  $V_H3$  germline genes (3-7, 3-11, 3-21, 3-23, 3-30, 3-30.3, 3-33 and 3-73), and that the 3-23 and 3-30 genes were the most frequently encountered (33). Consistent with the observations made herein, we also found that the proportion of  $V_H3^+$  IgM that exhibited SpA binding was higher than that of  $V_H3^+$  IgG.

Competition experiments indicated that SpA neither interferes with the binding of CXCR4 ligands nor binds to CXCR4. The inability of SpA to modulate early events of clathrin-mediated CXCR4 internalization strongly supports this conclusion. Microscopy and biochemical experiments confirmed that SpA binds to the SIgM/D complex and



**Figure 5.** After binding to B cells, SpA is internalized and induces CXCR4 internalization. (A) B cells were stained with biotinylated Mod-SpA plus AF488-Streptavidin and CXCR4-PE at 4°C for 30 min. One representative staining of three is shown. The percentage of Mod-SpA-positive cells is indicated in the upper right quadrant. (B) B cells were incubated with medium or 100 ng/mL CXCL12 for 30 min at 4°C before staining with CD20-PEcy7 and biotinylated Mod-SpA plus AF488-Streptavidin. Data from one representative experiment of three are shown. The percentage of Mod-SpA-positive cells is indicated in the upper right quadrant. (C) BJAB cells were stained with CXCR4 mAb alone, and then incubated for 2 h with medium alone or 10  $\mu$ g/mL Mod-SpA at 37°C. Addition of AF488-conjugated goat anti-mouse Ab (green) before or after permeabilization of BJAB cells was used to detect residual surface CXCR4 expression (left panel) or internalized CXCR4 (right panel) by fluorescent microscopy. Nuclei were visualized with DAPI (blue). One representative experiment of two is shown. (D) B cells were stained in medium with AF488-Streptavidin alone or biot-Mod-SpA plus AF488-Streptavidin at 4°C for 30 min then incubated for 2 h at 4°C or 37°C. The residual surface Mod-SpA expression was analyzed by flow cytometry. The percentages and MFI of SpA-positive cells are shown in the upper right quadrant. One representative experiment of three is shown. (E) Location of Mod-SpA (green) was assessed by fluorescent microscopy after incubation at 4°C (left panel) or 37°C (right panel). Nuclei were visualized with DAPI (blue). One representative experiment of three is shown.





**Figure 6.** SpA-induced CXCR4 downregulation is concomitant with BCR internalization and depends on PKC. (A) B cells were incubated with medium, 2 mmol/L MβCD or 0.4 mol/L sucrose for 1 h at 37°C before treatment with medium (empty histogram) or 100 ng/mL CXCL12 (black histogram) for 2 h at 37°C. Then, expression of CXCR4 on B cells was assessed by flow cytometry. (B) B cells were incubated with medium, 2 mmol/L MβCD or 0.4 mol/L sucrose for 1 h at 37°C before treatment with medium (empty histogram) or 10 μg/mL Mod-SpA (gray histogram) for 2 h at 37°C. Then expression of CXCR4 (upper panel) and SIgD (lower panel) on B cells was assessed by flow cytometry. (C, E) B cells from three to six independent donors were first cultured for 30 min at 37°C with medium alone (white bar), 20 μmol/L PP2, 5 μmol/L Herb, 5 μmol/L CC, 2 μmol/L U73122 or 5 μmol/L WMN. Cells were then incubated with 10 μg/mL anti-IgM Ab (C, hatched bars), 10 μg/mL Mod-SpA (D, black bars) or 100 nmol/L CXCL12 (E, dotted bars) for 16 h (C, D) or 2 h (E) at 37°C. After staining with CD20-PEcy7 and CXCR4-PE mAbs or IgG1-PE control, surface expression of CXCR4 was assessed by flow cytometry. Results are expressed as the mean ± SEM percentage of MFI values for residual surface CXCR4 expression. Two-tailed Wilcoxon test was used to determine statistical significance, and *p* values are indicated as \**p* < 0.05.

induces its clathrin-dependent internalization. Concomitantly, SpA-induced BCR signaling leads to PKC-dependent CXCR4 downmodulation. Whereas *src* kinases, PLC, PI3K and PKC contribute to anti-IgM-induced CXCR4 internalization, only PKC was involved in the SpA-mediated effect. The lack of PI3K involvement is consistent with CD19 being uncoupled from the BCR complex after SpA stimulation. In the absence of *src* activation, PKC activation might be induced downstream of SYK, a crucial kinase for BCR-mediated signaling. Consistently, SYK and ZAP70 were previously shown to interact with CXCR4 after its physical association with the T-cell receptor (34), and SYK was recently found to be involved in BCR-

induced CXCR4 downmodulation in B cells from patients with chronic lymphocytic leukemia (35). Whereas PKC-mediated phosphorylation of the cytoplasmic tail of chemokine receptors generally favors the recruitment of β-arrestin and the formation of clathrin vesicles (36), SpA-induced CXCR4 downmodulation is not prevented by treatment with hypertonic sucrose. Therefore, we infer that SpA-induced PKC phosphorylation impairs late events in CXCR4 internalization. Reminiscent of BCR-induced CXCR4 downmodulation in patients with chronic lymphocytic leukemia (35), SpA might increase proteasome-mediated CXCR4 degradation. A prolonged sequestration in cytoplasmic structures is another po-

tential mechanism, although it has been more frequently observed with recycling chemokine receptors, that is, CCR5. Other observations indicate that ligand-induced phosphorylation of different serine/threonine residues on the cytoplasmic tail of CXCR4 is associated with distinct kinetics of CXCR4 internalization and recruitment of specific GRK (37). As previously shown for CCR5, the recycling of which is modified by synthetic agonists (38), SpA may induce a prolonged sequestration of CXCR4 in endosomes or in the trans-Golgi.

Our findings may have therapeutic implications. CXCR4 is expressed in a variety of cell types. It is the most widely expressed chemokine receptor in many different hematological and solid cancers

and has been associated with cancer dissemination and poor prognosis, including breast cancer, prostate cancer to the bone marrow, colon cancer to the liver, and undifferentiated thyroid cancer (39). In addition to metastasis, CXCR4 has been associated with some autoimmune diseases, such as lupus (40). Because CXCR4 antagonists may have value in addition to that of conventional cytotoxic therapy in patients with various malignancies and immune diseases, several CXCR4 antagonists have been developed. At least one of them, the small-molecule heterocyclin bicyclam AMD3100 (41), is under evaluation in phase 3 clinical trials for mobilization of hematopoietic stem cells (ClinicalTrials.gov; <http://clinicaltrials.gov/ct2/show/nct0075335>).

## CONCLUSION

Our studies revealed that *S. aureus* produces an evolutionary tailored, highly specific, chemokine receptor inhibitor. Modified protein A could thus serve as a supplement to direct cytotoxic therapy to suppress metastasis of B-cell lymphomas, thus adding a possible treatment option for this poor prognosis group. In autoimmune diseases, in which B cells play a key role (42), antagonizing CXCR4 was found to have beneficial effects on disease progression in experimental animals (40), which lends further support to the potential therapeutic use of protein A as a CXCR4 antagonist.

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## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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