Sustained Activation of Cell Adhesion Is a Differentially Regulated Process in B Lymphopoiesis

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Abstract

It is largely unknown how hematopoietic progenitors are positioned within specialized niches of the bone marrow microenvironment during development. Chemokines such as CXCL12, previously called stromal cell–derived factor 1, are known to activate cell integrins of circulating leukocytes resulting in transient adhesion before extravasation into tissues. However, this short-term effect does not explain the mechanism by which progenitor cells are retained for prolonged periods in the bone marrow. Here we show that in human bone marrow CXCL12 triggers a sustained adhesion response specifically in progenitor (pro- and pre-) B cells. This sustained adhesion diminishes during B cell maturation in the bone marrow and, strikingly, is absent in circulating mature B cells, which exhibit only transient CXCL12-induced adhesion. The duration of adhesion is tightly correlated with CXCL12-induced activation of focal adhesion kinase (FAK), a known molecule involved in integrin-mediated signaling. Sustained adhesion of progenitor B cells is associated with prolonged FAK activation, whereas transient adhesion in circulating B cells is associated with short-lived FAK activation. Moreover, sustained and transient adhesion responses are differentially affected by pharmacological inhibitors of protein kinase C and phosphatidylinositol 3-kinase. These results provide a developmental cell stage–specific mechanism by which chemokines orchestrate hematopoiesis through sustained rather than transient activation of adhesion and cell survival pathways.

Key words: chemokines • adhesion • B cell development • microenvironment • bone marrow

Introduction

Chemokines were initially characterized as cytokines with chemoattractant properties involved in the recruitment of leukocytes to sites of inflammation (1). However, it is now clear that chemokines also participate in many physiological and pathological processes (2, 3). Several chemokines are known to trigger biological responses of B cells (4–7). The chemokine CXCL12, previously called stromal cell–derived factor 1, causes potent calcium mobilization and chemotaxis responses in progenitor B cells in bone marrow (8–10). Moreover, mice lacking either the gene for CXCL12 or its receptor CXCR4 have impaired B lymphopoiesis (11, 12). The mechanisms underlying this defect are not clear. CXCL12 could exert a direct proliferative effect on B cell progenitors (13) and/or have a positioning effect by retaining B cell progenitors in appropriate supportive niches of the bone marrow (14). This positioning effect could be the result of chemotaxis of developing B lineage cells to their distinct bone marrow locations and/or adhesion to bone marrow matrix molecules such as vascular cell adhesion molecule (VCAM)*-1 via the integrin very late antigen (VLA)-4 (15). With respect to the latter, the VCAM-1–VLA-4 interaction also appears necessary for B lymphopoiesis (16, 17).

Integrins are expressed by lymphocytes in low ligand binding activity form, in which they are unable to exhibit firm adhesion (18). The augmentation of integrin-dependent adhesion requires cellular signaling by chemokines or cytokines, which up-regulate integrin affinity and/or avidity (19–21). Certain chemokines, including CXCL12, induce firm arrest of rolling lymphocytes by activating integrins under flow conditions (22). It is speculated that

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**Abbreviations used in this paper:* BIM, bisindolylmaleimide; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; HRP, horseradish peroxidase; MCF, mean channel fluorescence; MEK, mitogen-activated protein kinase kinase; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PTX, pertussis toxin; VCAM, vascular cell adhesion molecule; VLA, very late antigen.

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chemokine-stimulated lymphocytes remain arrested on endothelium for a short time (1–3 min) and either transmigrate to tissues or return to the circulation if signals for transmigration are lacking (23). However, this transient type of chemokine-induced adhesion does not accurately model the bone marrow environment where B cell progenitors are retained for long periods of time in supportive niches for growth and maturation signals (24–26). Moreover, in contrast to the peripheral vasculature, CXCL12 and VCAM-1 are constitutively present at high concentrations in the extravascular bone marrow compartment (5, 27, 28). To simulate the continuous exposure of progenitor B cells to CXCL12 and VCAM-1 in the bone marrow, we have developed an adhesion assay by which cells are first exposed to CXCL12 before incubation in VCAM-1–coated wells. In this assay ("long-term" adhesion assay), the cells are continuously exposed to CXCL12 for 30–60 min. By contrast, in the conventional adhesion assay used to demonstrate transient adhesion to VCAM-1 ("short-term" adhesion assay), cells are first placed in VCAM-1–coated wells and then exposed to CXCL12 for 1–5 min (22).

Using the long-term adhesion assay we demonstrate that bone marrow B cells show a sustained, VLA-4–dependent adhesion response. This sustained CXCL12-mediated adhesion response of developing bone marrow B cells decreases with maturation and is surprisingly absent in mature, peripheral blood B cells. By contrast, using the short-term adhesion assay we observe conventional transient CXCL12-mediated adhesion responses in both bone marrow and peripheral blood B cells. Interestingly, the type of adhesion response closely correlates with the duration of focal adhesion kinase (FAK) activation. Moreover, sustained and transient adhesion responses are differentially affected by pharmacological inhibitors of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3-K). Thus, sustained and transient CXCL12-induced adhesions are distinct physiological processes that are regulated during B lymphopoiesis.

Materials and Methods

Antibodies and Reagents. The following antibodies were used for cell staining: FITC-labeled anti-κ light chain, FITC-labeled anti-λ light chain, PE-labeled anti-IgD, PECy5-labeled anti-CD34 (BD Biosciences), and APC-labeled anti-CD19 (Caltag Laboratories). For integrin surface staining and adhesion blocking, mouse anti–human integrin α_4 monoclonal antibody was used (clone P1H4; Chemicon). The secondary antibody was goat anti–mouse IgG-Cy5–labeled antibody (Jackson ImmunoResearch Laboratories). Antibodies used for immunoblotting were antiphosphotyrosine monoclonal antibody (4G10; provided by T. Roberts, Dana Farber Cancer Institute, Boston, MA) and rabbit anti–FAK polyclonal antibody (Santa Cruz Biotechnology, Inc.). The secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti–mouse IgG-2b antibody (Caltag Laboratories) and HRP-conjugated goat anti–rabbit antibody (Bio-Rad Laboratories). PMA, DMSO, and pertussis toxin (PTX) were from Sigma-Aldrich. Bisindolylmaleimide (BIM)-I, a PKC inhibitor, BIM-V, an inactive analogue of BIM-I (29), wortmannin, a PI3-K inhibitor, and PD98059, a mitogen-activated protein kinase kinase (MEK) inhibitor, were purchased from Calbiochem-Novabiochem. All of the cell culture reagents were from Life Technologies.

Cell Culture and Isolation. REH (pro-B cell line; CRL8286) and HS-Sultan (plasmacytoid cell line; CRL1484) were from American Type Culture Collection. 697 (pre-B) cell line was provided by M.D. Cooper (Howard Hughes Medical Institute, Birmingham, CA). Cells were maintained in RPMI 1640 supplemented with 10% FBS. Heparinized human bone marrow cells were obtained by iliac crest aspiration from healthy adult volunteers after informed consent and in accordance with the guidelines approved by the Institutional Review Committee of the Dana Farber Cancer Institute. Peripheral blood cells were isolated from the buffy coats of healthy donors. Bone marrow or peripheral blood samples were diluted with PBS (Life Technologies) and low density mononuclear cells were isolated by Ficoll-Hypaque (Amersham Biosciences) separation. Cells were then washed twice with PBS, pH 7.4, and resuspended in ice-cold PBS with 2% FBS. For adhesion experiments, B cells were isolated by negative selection using a B cell isolation kit and MACS separation columns (Miltenyi Biotec) according to the manufacturer's protocol. For immunoprecipitation and Western blotting, B cells were isolated by positive selection using CD19 microbeads and MACS separation columns (Miltenyi Biotec) according to the manufacturer's protocol. The purity of B cells isolated from bone marrow as well as peripheral blood was >95%, as estimated by flow cytometry analysis (MoFlo®; DakoCytomation). Isolated B cells were stored at 37°C in StemSpan™ H2000 serum-free medium (StemCell Technologies Inc.) for 12 h before experiments.

Adhesion Assay. Adhesion assays were performed in 96-well plates (Nalge Nunc International) coated with 50 ng of recombinant human VCAM-1 (R&D Systems) per well. At this concentration of VCAM-1, there was no adherence of unstimulated cells. Wells were then washed three times with HBSS containing Hepes (Life Technologies) and blocked with 20% FBS in PBS for 1 h at 37°C.

Short-Term Adhesion Assay. 10^5 cells in 46 μl of adhesion medium (HBSS buffered with Hepes and supplemented with 0.1% BSA) were added to the wells and allowed to settle at 37°C. At the various time intervals, 4 μl of recombinant human CXCL12 (R&D Systems) up to a concentration of 1.0 μM was added to the wells. As a negative control, adhesion medium was added instead of CXCL12 or the assay was performed in BSA-coated wells (Table I).

Table I. *Short-Term Adhesion Assay*

Incubation time in VCAM-1–coated wells	Time of exposure to CXCL12	Total time of assay
min	*min*	*min*
29	1	30
28	2	30
27	3	30
26	4	30
25	5	30

Long-Term Adhesion Assay. Cells were first stimulated with 1.0 μM CXCL12 in suspension at 37°C for defined amounts of time and then placed into VCAM-1–coated wells for 30 min at 37°C. CXCL12 was continuously present during the entire experiment (Table II).

In some studies, cells were incubated with 100 ng/ml PMA for 10 min, 100 ng/ml PTX for 2 h, or 5 μg/ml anti-α_4 integrin monoclonal antibody for 30 min before the adhesion assay. In experiments using pharmacological inhibitors, cells were incubated with different concentrations of BIM-I/BIM-V for 2 h, wortmannin for 45 min, or PD98059 for 2 h at 37°C before the adhesion assay. The optimal concentration of anti-α_4 antibody was determined in preliminary experiments.

Analysis of Adhered Cells. After the adhesion assay, wells were washed manually four times with adhesion medium to remove nonadhered cells. The number of adhered REH cells was determined using the CyQUANT cell proliferation kit (Molecular Probes) and fluorescence of the samples was measured by a microtiter plate fluorometer (DYNEX Technologies). Adhered bone marrow and peripheral blood cells were detached from the bottom of the wells by treatment with 0.01% EDTA (Life Technologies) in PBS. Detached bone marrow B cells were stained to define four stages of human B cell differentiation as previously described (9): the earliest B cell population, designated as pro-B cells, identified as $CD19^+$ $CD34^+$; κ^-/λ^-, pre-B cells, identified as $CD19^+$ $CD34^-$; κ^-/λ^-, followed by immature B cells identified as $CD19^+$ κ^+/λ^+ IgD^-; and mature B cells identified as $CD19^+$ κ^+/λ^+ IgD^+. Detached peripheral blood B cells were defined as $CD19^+$ cells. Incubation and washing was performed at 4°C. After washing with PBS, cells were fixed in 1% paraformaldehyde PBS and analyzed using a MoFlo® cytometer (DakoCytomation). Data are shown as the percent of adhesion calculated from the number of cells determined in the adhered population and the number of cells determined in starting population. For clarity, in experiments using pharmacological inhibitors the results are presented as a percentage of specific adhesion (background adhesion observed in the absence of adhesion stimulus was subtracted from CXCL12- or PMA-stimulated adhesion).

Cell Stimulation, Immunoprecipitation, and Western Blotting. Cells were starved in serum-free RPMI 1640 for 1 h at 37°C and then either left unstimulated or stimulated with CXCL12. Alternatively, cells were first incubated with PTX, BIM-I, or BIM-V for 1 h at 37°C before CXCL12 stimulation. The reactions were stopped by adding 1 ml ice-cold PBS and cells were lysed with lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.6, with protease and phosphatase inhibitors) followed by centrifugation at 13,000 *g*. For immunoprecipitations, the cell lysates were incubated with anti-FAK antibody followed by incubation with protein A Sepharose beads. Immunoprecipitates were washed three times with washing buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.6, and 0.1% Triton X-100) and then eluted by boiling in SDS sample buffer. Protein samples were separated in SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories) for Western blotting. Blots were blocked with 5% BSA in TBS-T buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 0.1% Tween 20) for 1 h and incubated overnight with antiphosphotyrosine monoclonal antibody (4G10). Bound antibody was revealed with HRP-conjugated anti–mouse antibody using enhanced chemiluminescence (Amersham Biosciences) and visualized by autoradiography. The band intensities were determined by densitometry using ImageQuant Version 1.1 (Molecular Dynamics Inc.) and are expressed for each lane as multiples of the control (assigned as a value of 1). The membranes were then stripped and reblotted with anti-FAK antibody followed by enhanced chemiluminescence.

Statistical Analysis. Student's *t* test was used for statistical analysis. The level of significance is indicated by the P value. Data are presented as mean ± SD, unless otherwise indicated.

Table II. *Long-Term Adhesion Assay*

Preincubation time with CXCL12	Incubation time in VCAM-1–coated wells	Total time of assay
min[a]	*min*[a]	*min*
1	30	31
5	30	35
15	30	45
20	30	50
30	30	60

[a]Time of exposure to CXCL12.

Results

Short-Term Stimulation with CXCL12 Induces Transient Adhesion to VCAM-1 in Both Bone Marrow and Peripheral Blood B Cells. As previously reported for circulating human T lymphocytes (22), we found that short-term stimulation with CXCL12 induced rapid but transient adhesion to VCAM-1 of early lineage pro-B cells (REH cell line) as well as of circulating, mature B cells. Adhesion was detected at the concentration of 50 nM, reached a maximum at 1.0 μM, and did not increase at higher concentrations (Fig. 1 A). Adhesion was transient, reaching its peak after 1–2 min of stimulation and decreasing to baseline after 5 min (Fig. 1, B and C). Next, we compared transient CXCL12-mediated adhesion of primary bone marrow and peripheral blood B cells. Transient CXCL12-mediated adhesion was comparable for total bone marrow (containing early and late lineage B cells) and peripheral blood B cells: 21.5% ± 9.0% (mean ± SD) of total bone marrow B cells (Fig. 1 D) and 22.14 ± 7.14% of peripheral blood B cells (Fig. 1 E) adhered to VCAM-1 after 2 min of stimulation with CXCL12.

Long-Term, Continuous Exposure to CXCL12 Induces Differential Adhesion Responses to VCAM-1 in Bone Marrow and Peripheral Blood B Cells. As demonstrated in Fig. 1, short-term stimulation with CXCL12 triggered a robust adhesion of developing bone marrow B cells to VCAM-1 but the transient character of this adhesion response does not reflect the hypothetical role of this chemokine as a bone marrow B cell retention factor (14, 30). High levels of CXCL12 (5, 27, 31, 32), as well as the VLA-4 integrin ligand VCAM-1 (28, 33, 34), are constitutively expressed in the bone marrow microenvironment. To simulate the continuous exposure of progenitor B cells to CXCL12, we performed the long-term adhesion assay, in which cells were first exposed

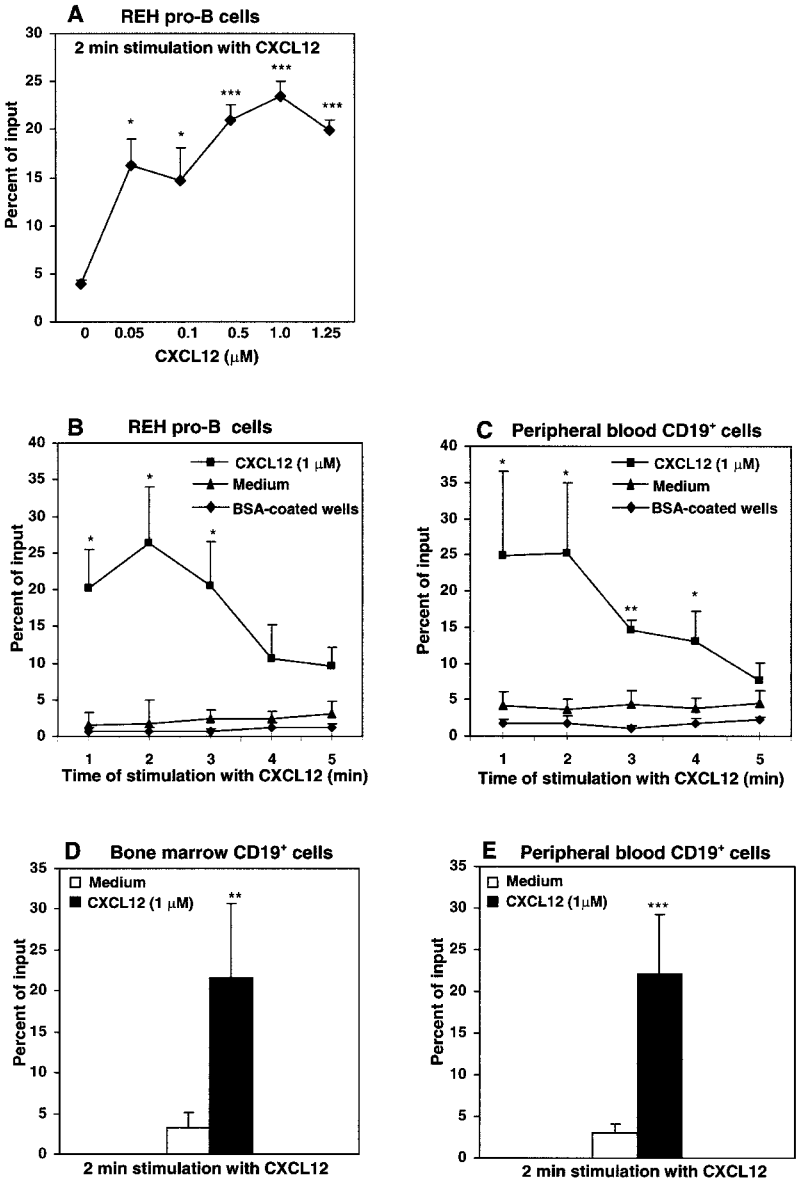

Figure 1. CXCL12 induces rapid and transient adhesion of peripheral blood as well as bone marrow B cells to VCAM-1 using the short-term assay conditions (refer to Materials and Methods). (A) REH cells were incubated in VCAM-1–coated wells for 28 min and then stimulated with different concentrations of CXCL12 for 2 min followed by the removal of nonadherent cells and quantitation of adhesion as described in Materials and Methods. (B and C) Kinetics of CXCL12-induced transient adhesion of REH pro-B and peripheral blood B cells to VCAM-1 is shown. Cells were incubated in VCAM-1– or BSA-coated wells for 25–29 min and after that time 1.0 μM CXCL12 was added for 5 to 1 min followed by the removal of nonadherent cells and quantitation of adhesion as described in Materials and Methods. Data represent the mean ± SD of three separate experiments, each performed in triplicate. (D and E) Comparison of transient CXCL12-induced adhesion of bone marrow and peripheral blood B cells to VCAM-1. The adhesion assay was conducted using 1 μM CXCL12 as described in A. Data represent the mean ± SD of five (bone marrow) or six (peripheral blood) separate experiments, each performed in triplicate. *, **, and ***, statistical significance as compared with negative control and assessed as $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively.

to CXCL12 for up to 30 min and then incubated in VCAM-1–coated wells for another 30 min. We found that long-term stimulation with CXCL12 induced strong and sustained adhesion of REH pro-B cells to VCAM-1, which reached a maximum at 1 μM of CXCL12 (Fig. 2 A) and persisted for at least 60 min (Fig. 2 B). Similarly, primary total bone marrow $CD19^+$ B cells, which were exposed to CXCL12 for 1 or 30 min before 30 min of incubation in VCAM-1–coated wells, demonstrated strong, sustained adhesion after long-term stimulation (Fig. 2 C, middle). In sharp contrast, peripheral blood B cells showed no adhesion responses after prolonged CXCL12 stimulation (Fig. 2 C, right). However, peripheral blood B cells responded with strong up-regulation of adhesion after PMA stimulation (not depicted), suggesting that peripheral blood B cells were still able to up-regulate their adhesion to integrin ligands through chemokine receptor–independent pathways (35).

CXCL12-induced Adhesion of Bone Marrow B Cells to VCAM-1 Decreases with Maturation. Next, sustained (Fig. 3, A and B) and transient (Fig. 3 C) adhesion responses were determined for individual bone marrow B cell subsets using long-term or short-term adhesion assay conditions, respectively (refer to Materials and Methods). We observed a gradual decrease in the CXCL12-induced adhesion response during B cell maturation. The sustained adhesion response was stronger for early lineage (pro- and pre-) B cells than for mature B cells from bone marrow ($P < 0.0005$). Similarly, the transient adhesion response was stronger for early lineage B cells than for mature bone marrow B cells ($P < 0.05$).

Peripheral blood B cells exhibited a transient adhesion response, which was not statistically different from late lineage, mature B cells in bone marrow (Fig. 3 C). The overall responsiveness of bone marrow B cells to integrin acti-

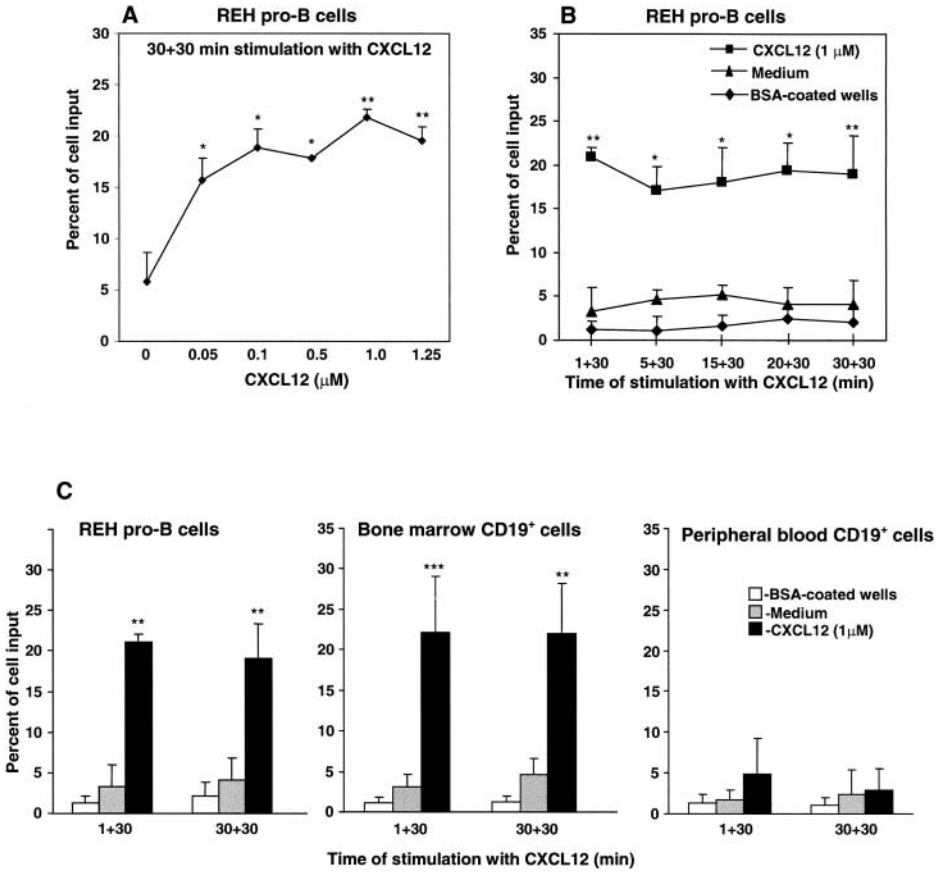

Figure 2. CXCL12 induces sustained adhesion to VCAM-1 of bone marrow but not peripheral blood B cells using long-term assay conditions (refer to Materials and Methods). (A) REH cells were pretreated with different concentrations of CXCL12 for 30 min and then incubated in VCAM-1–coated wells for 30 min followed by the removal of nonadherent cells and quantitation of adhesion as described in Materials and Methods. (B and C) Cells were pretreated with 1.0 μM CXCL12 for 1–30 min (time shown as a first number on x axis) and then incubated in VCAM-1– or BSA-coated wells for 30 min (time shown as a second number on x axis) followed by the removal of nonadherent cells and quantitation of adhesion as described in Materials and Methods. CXCL12 was present throughout the incubation period. Kinetics of CXCL12-induced sustained adhesion of REH pro-B cells to VCAM-1 is shown in B and the comparison of sustained CXCL12-induced adhesion of REH, bone marrow, and peripheral blood $CD19^+$ cells to VCAM-1 is shown in C. Data represent the mean ± SD of three (REH cells), four (bone marrow), and five (peripheral blood) independent experiments, each performed in triplicate. *, **, and ***, statistical significance as compared with negative control and are assessed as $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively.

vation was unchanged throughout development as measured by PMA-induced adhesion (not depicted).

CXCL12-induced Adhesion to VCAM-1 Is VLA-4 Integrin Dependent. To examine if CXCL12-induced B cell adhesion is dependent on VLA-4 integrin activation, REH or peripheral blood B cells were incubated with 5 μg/ml anti–VLA-4 monoclonal antibody before the adhesion assay. We found that both sustained (Fig. 4 A) and transient (Fig. 4, B and C) adhesion responses were completely blocked. In contrast, cells treated with isotype-matched nonblocking antibody exhibited strong CXCL12-induced adhesion to VCAM-1.

Expression of VLA-4 Integrin Does Not Change During Human B Cell Development. We examined surface expression of α_4 integrin chain on developing bone marrow B cells to determine if the decrease in adhesion response of bone marrow B cells to CXCL12 during maturation could be explained by a reduction in VLA-4 expression. Flow cytometric analysis revealed high levels of α_4 chain on every analyzed subset of bone marrow or peripheral blood B cells: 93% of pro-B, 92% of pre-B, 98% of immature, 76% of mature bone marrow B cells (Fig. 4 D), and 78% of peripheral blood B cells expressed α_4 (Fig. 4 E). Moreover, the calculated mean channel fluorescence (MCF) was similar for each B cell population. There was no statistical difference in the mean percentage of $VLA\text{-}4^+$ cells as well as MFC values between cell populations. This finding suggests that changes in integrin adhesiveness rather than an increase in the number of molecules are relevant to this process.

We also questioned whether prolonged exposure to CXCL12 could affect α_4 expression levels. Consequently, we evaluated the expression of α_4 on bone marrow and peripheral blood B cells after 5, 15, and 30 min of incubation with 1.0 μM CXCL12 (not depicted). The expression of α_4 was unchanged suggesting that CXCL12 does not downmodulate α_4 integrin expression on bone marrow or peripheral blood B cells.

CXCL12-induced Adhesion to VCAM-1 Is $G\alpha_i$ Protein-coupled Receptor and PKC Dependent. To explore the role of $G\alpha_i$ protein–dependent signaling in the activation of B cell adhesion by CXCL12, we pretreated the cells with PTX at a concentration of 100 ng/ml. We found that sustained CXCL12-induced adhesion responses of early pro-B cells (Fig. 5 A) as well as transient responses of both early and mature B cells (Fig. 5, B and C) were completely blocked by PTX. In contrast, PMA-induced adhesion, which activates integrins independently on G protein–coupled receptor (36), was not altered by PTX (Fig. 5). Next, we evaluated the role of PKC, a key molecule involved in chemokine- and integrin-triggered signaling pathways (37, 38). We used the highly selective metabolic inhibitor of PKC, BIM-I, and as a negative control its inactive analogue BIM-V (29). We found that BIM-I blocked both

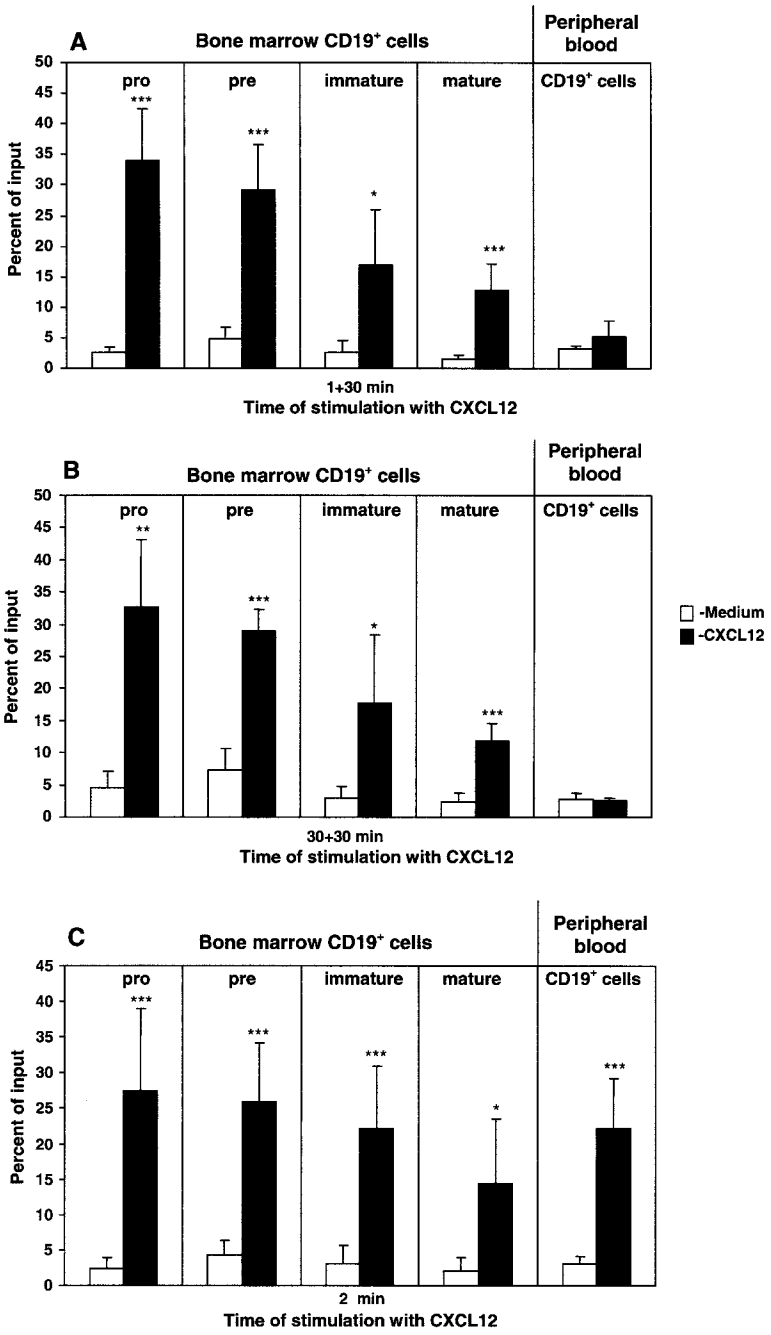

Figure 3. Decreasing response of B cells to CXCL12-induced adhesion to VCAM-1 during maturation. Sustained adhesion responses by developmental B cell subsets are shown in A and B. Transient adhesion responses by B cell subsets are shown in C. Long-term adhesion assay (A and B) was conducted as described in Fig. 2 and short-term adhesion assay (C) was conducted as described in Fig. 1. Data represent the mean ± SD of four (bone marrow) and five (peripheral blood) experiments, each performed in triplicate. *, **, and ***, statistical significance between adhesion of CXCL12- and medium-stimulated cells (negative control) and are assessed as $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively. The difference in CXCL12-induced adhesion response between pro-/pre-B cells and mature bone marrow B cells was statistically significant for sustained adhesion ($P < 0.0005$) as well as for transient adhesion ($P < 0.05$).

sustained (REH cells) and transient adhesion (peripheral blood and REH cells). The inhibition by BIM-I occurred in a dose-dependent manner but required a concentration of 1,000 nM for statistically significant inhibition compared with the negative control, BIM-V. As an example, data are shown for the inhibition of transient adhesion of peripheral blood B cells (Fig. 5 D). However, as shown in Fig. 5, E and F, we consistently found that BIM-I more effectively blocked sustained adhesion (by ± 90%) than transient adhesion (by ± 45%) responses. This difference in BIM-I inhibition between sustained and transient adhesion is highly statistically significant ($P = 0.01$), suggesting that the signaling pathways involved in sustained versus transient adhesion are qualitatively different.

Effect of PI3-K and Extracellular Signal-regulated Kinase (ERK) Inhibition on CXCL12-induced B Cell Adhesion to VCAM-1. Recent studies using activated T cells indicate that CXCL12 has the unique ability to cause sustained activation of both PI3-K and ERK-2, in contrast to the transient signaling response typical for other chemokines (39). We pretreated REH cells with different concentrations of wortmannin, a PI3-K inhibitor, or with PD98059, a MEK inhibitor that acts directly upstream of ERK, before the adhesion experiments. We observed that similar to BIM-I, as

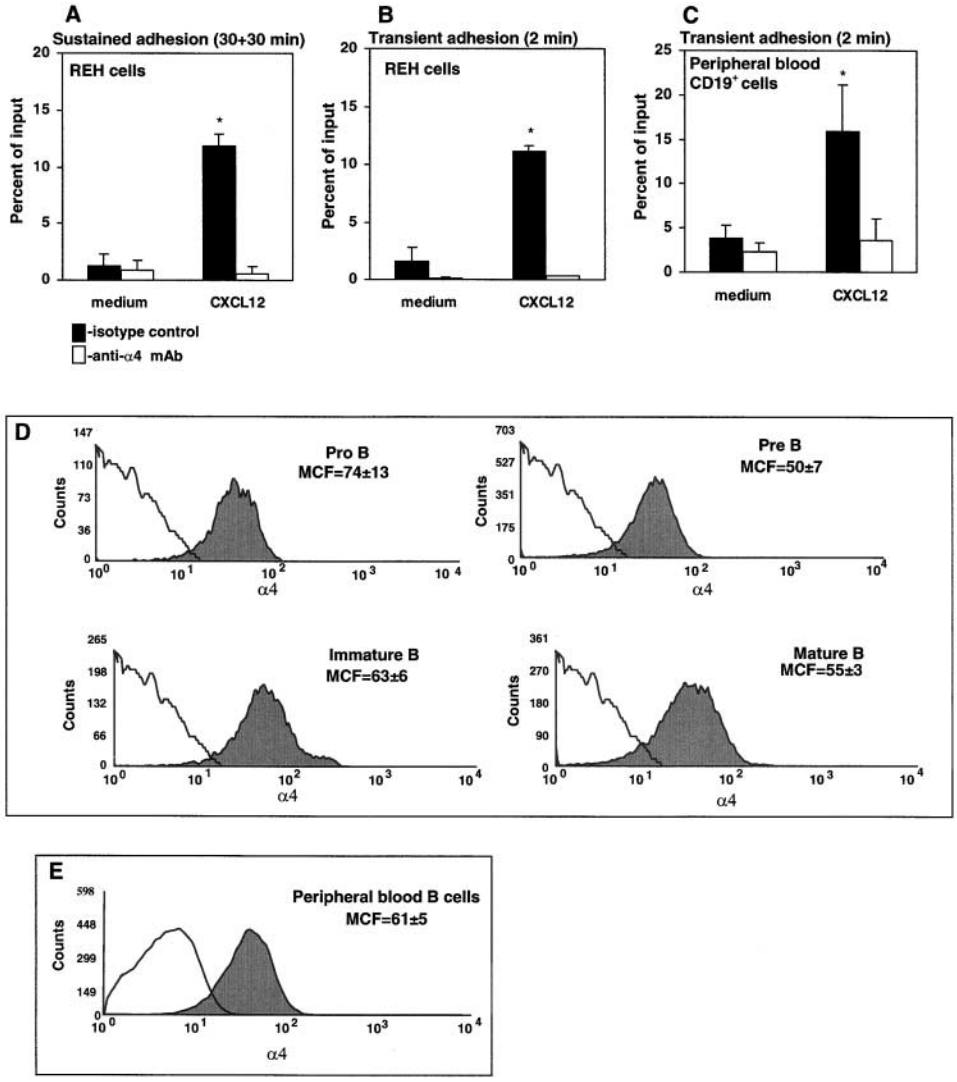

Figure 4. CXCL12-mediated adhesion of B cells to VCAM-1 is integrin VLA-4 dependent. (A–C) CXCL12-mediated adhesion to VCAM-1 is blocked by anti-α4 monoclonal antibody. Before the adhesion assay, REH pro-B cells (A and B) or peripheral blood $CD19^+$ cells (C) were incubated with 5 μg/ml anti–VLA-4 monoclonal antibody or with matching isotype control antibody for 30 min. Cells were then subjected to either long-term (A) or short-term (B and C) adhesion assays as described in Materials and Methods. Data represent the mean ± SD of three independent experiments, each performed in triplicate. *, statistical significance and is assessed as $P < 0.01$. (D and E) Expression of α_4 integrin chain does not change during B cell development. Expression of α_4 integrin chain on bone marrow B cell subpopulations (D) and total peripheral blood B cells (E) is shown. The black line represents cells stained with the isotype control and the shaded part of the histogram represents cells stained with anti-α_4 antibody. The values of MCF represent the average of three independent experiments ± SD. The differences between MCF values of different subsets are not statistically significant.

little as 10 nM of wortmannin completely inhibited the sustained adhesion response but only partially inhibited the transient adhesion response of REH pro-B cells (Fig. 6, A and B). The difference in inhibition of sustained and transient adhesion by wortmannin at 10 nM was highly significant ($P = 0.001$). Thus, this finding also supports the concept that the CXCL12-induced signaling pathways in sustained and transient adhesion differ at some level. In contrast to PKC and PI3-K inhibitors, the MEK inhibitor PD98059 used at a broad range of concentrations did not influence CXCL12-mediated adhesion of REH cells to VCAM-1 (Fig. 6, C and D).

CXCL12 Induces Prolonged FAK Activation in Early Lineage but Not Mature B Cells. FAK is thought to play an important role in cell adhesion and migration (40). Therefore, we assessed FAK activation after CXCL12 stimulation in developing bone marrow and circulating peripheral blood B cells. We first determined that CXCL12 induces FAK tyrosine phosphorylation in REH pro-B cells in a dose-dependent manner. The phosphorylation was evident at a concentration of 50 nM, reached a maximum at 100 nM, and decreased at 500 nM but was still detectable at 1–2 μM CXCL12 (Fig. 7 A). Next, we stimulated primary bone marrow and peripheral blood B cells as well as cell lines representing different stages of B cell development, i.e., REH pro-B cells, 697 pre-B cells, and HS Sultan postgerminal center B cells, with 100 nM CXCL12 for different periods of time. We detected remarkable differences in the kinetics of CXCL12-induced FAK phosphorylation between early lineage and mature B cells. In early lineage B cells, CXCL12 induced a markedly prolonged FAK phosphorylation that was detectable for up to 20 min in REH cells and up to 30 min in 697 cells (Fig. 7 B, top and middle). Stimulation of cells with higher concentrations (1,000 nM) of CXCL12 did not additionally prolong FAK phosphorylation (not depicted). In contrast, in mature peripheral blood B cells (Fig. 7 C, bottom) and HS Sultan cells (Fig. 7 B, bottom) FAK phosphorylation peaked at 1 min and reversed to baseline level 3 min after CXCL12 stimulation. In primary total bone marrow B

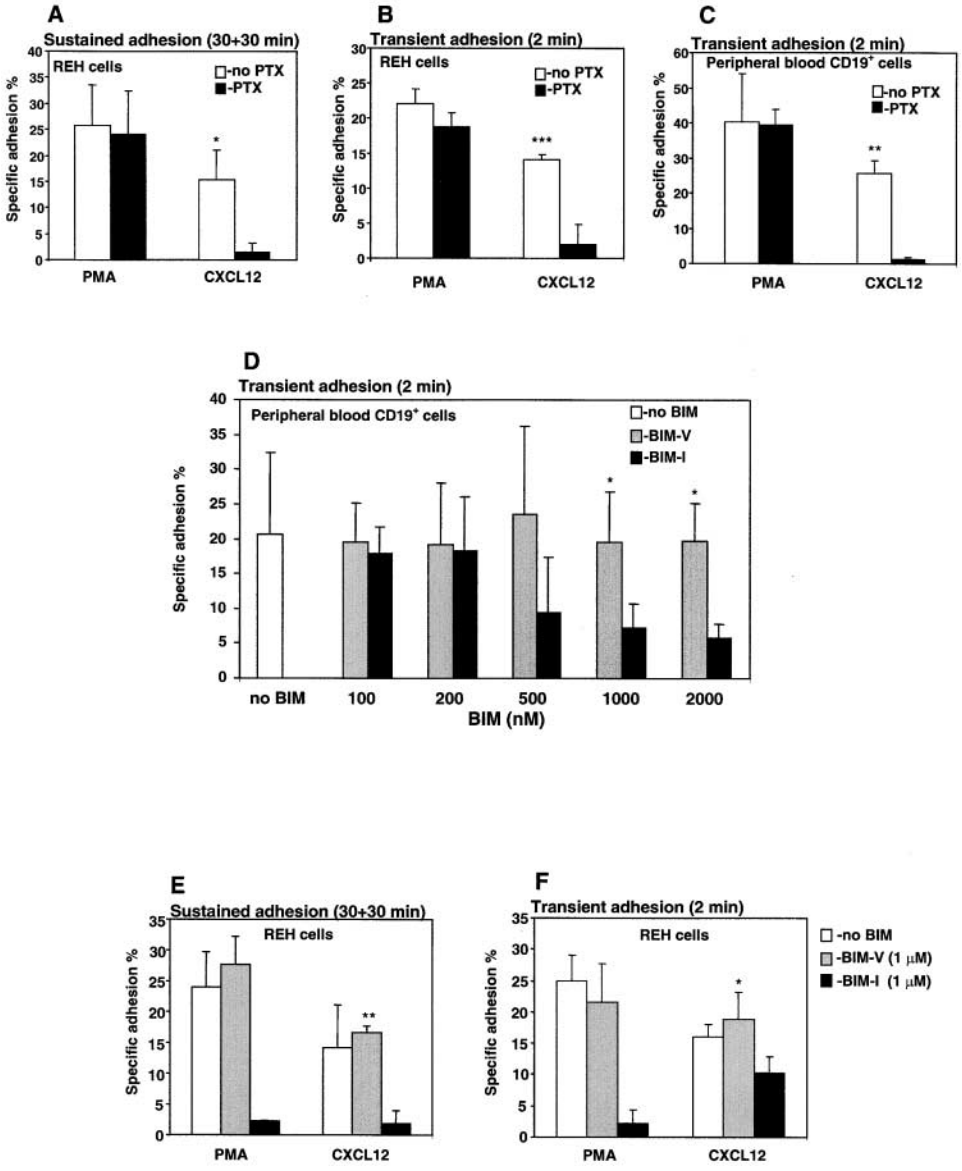

Figure 5. CXCL12-mediated adhesion of B cells to VCAM-1 is $G\alpha_i$ protein–coupled receptor and PKC dependent. (A and B) REH pro-B cells or (C) peripheral blood $CD19^+$ cells were incubated with or without 100 ng/ml PTX for 2 h at 37°C. Cells were then subjected to either long-term (A) or short-term (B and C) adhesion assays as described in Materials and Methods. Otherwise, after incubation with PTX, separate cell aliquots were stimulated with 100 ng/ml PMA for 10 min and then placed into VCAM-1–coated wells for 30 min. (D) Peripheral blood $CD19^+$ cells were either left untreated or were incubated with different concentrations of BIM-I, PKC inhibitor, or its negative control BIM-V for 2 h at 37°C. Cells were then subjected to short-term adhesion assay as described in Materials and Methods. (E and F) REH cells were either left untreated or were incubated with 1 μM BIM-I or BIM-V for 2 h at 37°C followed by short-term (F) or long-term (E) adhesion assay as described in Materials and Methods. Separate cell aliquots were stimulated with 100 ng/ml PMA for 10 min after incubation with BIM-I/BIM-V and before being placed onto VCAM-1–coated wells for 30 min. Results are shown as specific adhesion (background adhesion in the absence of a stimulus was subtracted from CXCL12- or PMA-induced adhesion). Data represent the mean ± SD of three independent experiments, each performed in triplicate. *, **, and ***, statistical significance between adhesion of PTX-treated or untreated cells (A–C) or between BIM-V– and BIM-I–treated cells (D–F) and are assessed as $P < 0.05$, $P < 0.02$, and $P < 0.01$, respectively.

cells FAK phosphorylation was also protracted, detectable for 10 min, and then declined to baseline (Fig. 7 C, top). The shorter duration of FAK phosphorylation in total bone marrow cells compared with the pro-/pre-B cell lines was ascribed to the fact that total bone marrow B cells included both early and late lineage B cell subsets. Total bone marrow B cell fraction contains a significant number of mature B cells (40–60% of total B cells), in which FAK activation is of much shorter duration than in the early lineage B cells. CXCL12-induced FAK phosphorylation was mediated by $G\alpha_i$ protein–coupled receptor signaling, as cell incubation with PTX completely blocked FAK activation (Fig. 7 D).

Taken together, we demonstrate that CXCL12 induces prolonged FAK phosphorylation in bone marrow B cells, in contrast to short-lived FAK phosphorylation in peripheral blood B cells. Importantly, prolonged FAK phosphorylation in bone marrow B cells correlates with sustained adhesion of these cells to VCAM-1.

CXCL12-induced FAK Phosphorylation in B Cells Is PKC Dependent. To investigate whether CXCL12-induced FAK phosphorylation is PKC dependent, we incubated REH cells with different concentrations of BIM-I or its negative control, BIM-V. FAK phosphorylation induced after long-term (20 min) CXCL12 stimulation was completely inhibited by BIM-I treatment (Fig. 8 A). In contrast, FAK activation after short-term (3 min) CXCL12 stimulation (Fig. 8 C) was only partially blocked by BIM-1, even at high concentrations (2.0 μM). The incubation of cells with BIM-V, the inactive analogue of BIM-I, did not affect CXCL12-induced FAK phosphorylation (Fig. 8, B and D). The difference in BIM-I inhibition of FAK phosphorylation after long-term and short-term CXCL12 stimulation of REH cells closely correlates with the differential

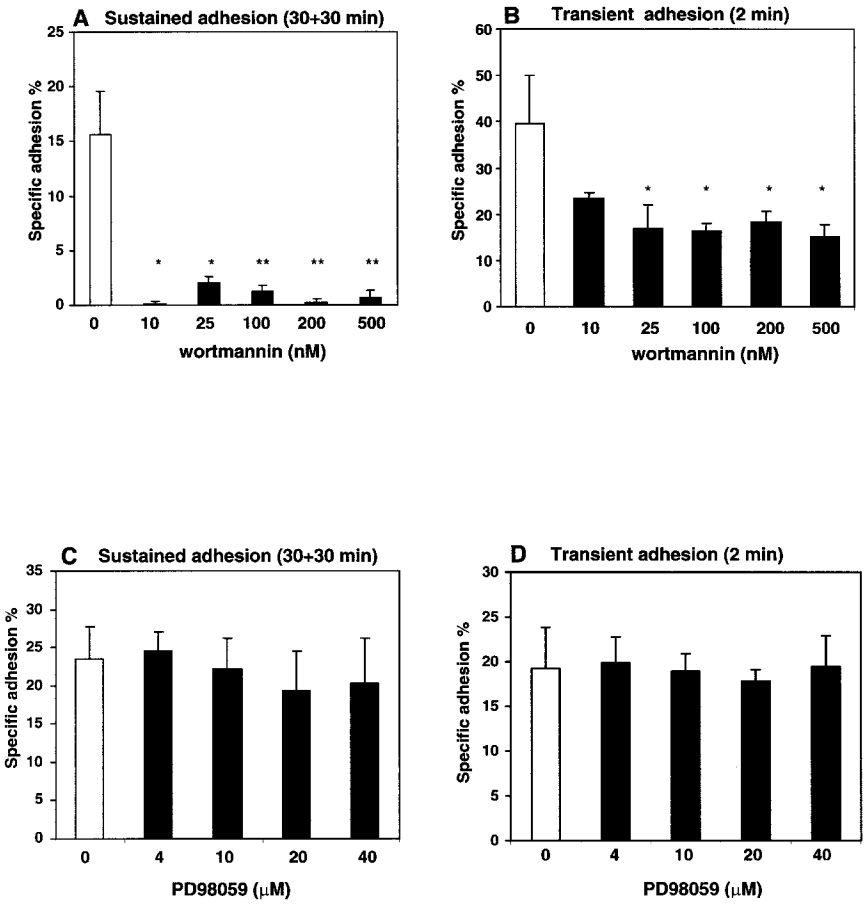

Figure 6. CXCL12-induced adhesion of B cells to VCAM-1 is PI3-K but not MEK dependent. REH cells were pretreated with the carrier (DMSO) or with different concentrations of wortmannin for 45 min (A and B) or PD98059 for 2 h (C and D). Cells were then subjected to either long-term (A and C) or short-term (B and D) adhesion assays as described in Materials and Methods. Results are shown as specific adhesion (background adhesion in the absence of a stimulus was subtracted from CXCL12-induced adhesion). Data represent the mean ± SD of three independent experiments, each performed in triplicate. * and **, statistical significance between adhesion of wortmannin- and carrier-treated cells and are assessed as $P < 0.05$ and $P < 0.02$, respectively.

inhibitory effect of BIM-I on sustained and transient CXCL12-induced adhesion (Fig. 5, E and F).

Discussion

In the bone marrow, developing B cells must remain in close contact with stromal cells to receive signals necessary for growth and maturation (26, 41, 42). Within the extravascular hematopoietic compartment, the earliest progenitor B cells (pro-B cells) are located within or near the endosteum (43). As bone marrow B cells differentiate, they move toward the sinusoids along stromal cell processes and after expressing sIgM enter the peripheral circulation (44). The mechanisms by which developing B cells are appropriately positioned in the bone marrow are not known. Independent gene targeting experiments in mice have suggested that both the CXCL12/CXCR4 and VLA-4/VCAM-1 axes are critical for B lymphopoiesis (11, 12, 16, 45, 46). We speculate that these are related phenomena because CXCL12 and other chemokines can transiently activate cell surface integrins of all known leukocyte types including T cells, neutrophils, monocytes and eosinophils (21, 22, 47–49), and most importantly, $CD34^+$ bone marrow progenitor cells (50). In this study we show that as for T cells, CXCL12 also triggers rapid, transient VLA-4–dependent adhesion of bone marrow and peripheral blood B cells. However, the kinetics of CXCL12-mediated adhesion is markedly different when bone marrow B cells are first exposed to CXCL12 before being placed in VCAM-coated wells. The continuous exposure to CXCL12 and VCAM-1, which we believe models the bone marrow environment (27, 28), causes sustained rather than transient adhesion to VCAM-1. This sustained CXCL12-mediated adhesion response gradually diminishes as B cells mature in the bone marrow and is absent in circulating peripheral blood B cells (Figs. 2 and 3). Therefore, we propose that sustained CXCL12-mediated adhesion is an important process in the retention of developing progenitor cells within the bone marrow microenvironments for prolonged periods of time (24, 25). Because under short-term assay conditions progenitor B cells (refer to Fig. 1) exhibit transient CXCL12-mediated adhesion, it is plausible that transient CXCL12-induced adhesion plays a role in the homing of early B lineage cells (e.g., during fetal development and perhaps later in ontogeny) to bone marrow, where VCAM-1 is constitutively expressed on endothelial cells (28). Moreover, transient CXCL12-mediated adhesion likely plays a role in the trafficking of circulating B cells to sites of tissue inflammation, where VCAM-1 expression is up-regulated (51). The decrease in CXCL12-induced adhesion responses (sustained and transient) during B cell maturation (Fig. 3) is in agreement with an

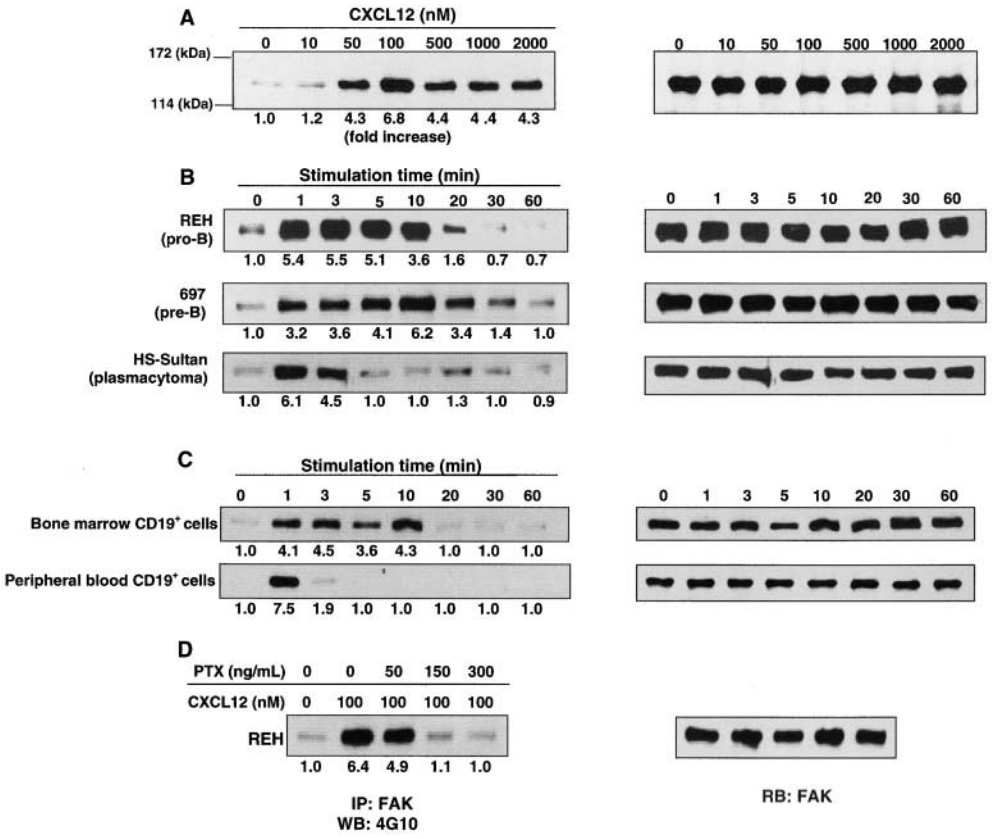

Figure 7. CXCL12-induced FAK phosphorylation is prolonged in early B cells but not in mature B cells. (A) CXCL12-stimulated FAK phosphorylation of REH pro-B cells is concentration dependent. REH cells were stimulated with indicated CXCL12 concentrations for 3 min. Immunoprecipitation (IP) with anti-FAK antibody was then performed followed by Western blot (WB) with antiphosphotyrosine antibody (4G10). The membranes were then stripped and reblotted (RB) with anti-FAK antibody to ensure equal protein loading (as shown in the right panels). Numbers under each lane are based on densitometry values and indicate the fold increase of phosphorylated FAK, expressed as multiples of the control (assigned as a value of 1). (B) REH, 697, and HS Sultan cells or (C) isolated bone marrow or peripheral blood B cells were stimulated with 100 nM CXCL12 for the indicated times and FAK phosphorylation was evaluated as described in A. (D) CXCL12-induced FAK phosphorylation is $G\alpha_i$ protein–coupled receptor dependent. REH cells were incubated for 1 h with the indicated concentrations of PTX and then stimulated with 100 nM CXCL12 for 3 min followed by the evaluation of FAK phosphorylation as described in Fig. 7 A. Each experiment was repeated three times from which the representative blots are shown.

overall decrease in CXCL12-induced responsiveness (as measured by chemotaxis and calcium mobilization) during B cell development in human bone marrow (8–10). The decrease in CXCL12-induced responses is disproportionate to the constant CXCR4 (9) and VLA-4 surface expression levels (Fig. 4). Therefore, we propose that

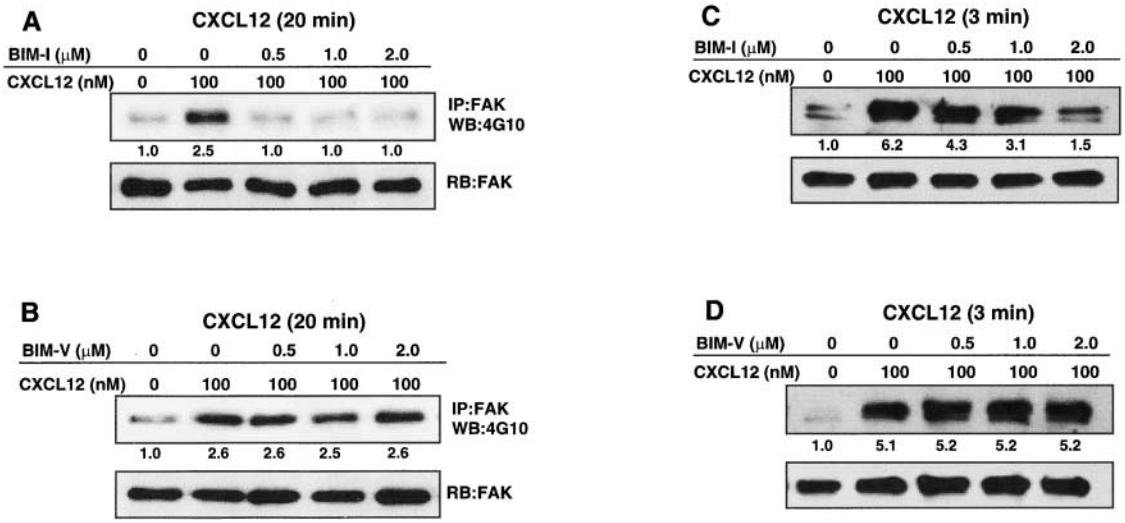

Figure 8. CXCL12-induced FAK phosphorylation of early B cells is PKC dependent. REH cells were exposed to different concentrations of BIM-I or its negative control BIM-V and then stimulated with 100 nM CXCL12 for 20 (A and B) or 3 min (C and D). Immunoprecipitation (IP) with anti-FAK antibody was performed followed by Western blot (WB) with antiphosphotyrosine antibody (4G10). The membranes were then stripped and reblotted (RB) with anti-FAK antibody to verify equal protein loading (as shown in the bottom panels). Numbers under each lane indicate the fold increase of phosphorylated FAK based on densitometry values. Data are representative of three independent experiments.

CXCL12/CXCR4-mediated cell activation is regulated by postreceptor signaling pathways during B cell development.

Both sustained and transient adhesion responses are affected by pharmacological inhibitors of PKC and PI3-K. Interestingly however, although sustained adhesion of bone marrow pro-B cells is completely blocked by the above mentioned inhibitors, transient adhesion of bone marrow pro-B and circulating peripheral blood B cells is only partially blocked (Figs. 5 and 6). This observation suggests that the signaling pathways in sustained and transient CXCL12-induced adhesion are qualitatively different. In contrast, the MEK inhibitor, PD98059, did not influence CXCL12-induced adhesion, which implies that the mitogen-activated protein kinase pathway does not contribute to integrin activation in the REH pro-B cells. A similar observation has been noted in bone marrow–derived leukemia and myeloma cell lines (50, 52).

We also find that FAK activation is differentially regulated during B cell development. Our studies show that CXCL12 can mediate prolonged FAK phosphorylation in bone marrow–derived progenitor B cells correlating with their sustained adhesion response (Fig. 7). In contrast, in circulating mature B cells, CXCL12 induces only brief FAK phosphorylation correlating with their transient adhesion response. The basis for this correlation between CXCL12-induced adhesion and FAK phosphorylation is not straightforward. First, the mechanisms by which FAK activation influences cell adhesion are not well understood. When $FAK^{-/-}$ and $FAK^{+/+}$ murine fibroblast-like cells are placed on a substrate such as fibronectin, the $FAK^{-/-}$ cells exhibit an increased number of focal adhesions and a nonpolar morphology suggesting a role of FAK in focal adhesion disassembly rather than in focal adhesion formation (53). It is not clear how these data relate to the studies presented here. In the experiments using murine ($FAK^{-/-}$ and $FAK^{+/+}$) fibroblast-like cells, FAK activation or the lack thereof is occurring through outside-in signaling, i.e., through ligand-induced integrin activation. In contrast, in our experiments both inside-out (i.e., CXCL12-induced) as well as outside-in (i.e., VLA-4/VCAM-mediated) integrin signaling events contribute to FAK activation (54). The prolonged activation of FAK in primary and cell line–derived bone marrow B cells is remarkable because chemokines characteristically trigger transient activation of signaling molecules (22, 55). However, recently Tilton et al. (39) showed that CXCL12 induced prolonged phosphorylation of protein kinase B and ERK-2 in IL-2–expanded T lymphocytes, thus indicating that sustained activation of signaling can occur in hematopoietic cells. Remarkably, we show that FAK phosphorylation after long-term (i.e., 20 min) CXCL12 stimulation is completely blocked by the PKC inhibitor BIM-I, whereas FAK phosphorylation after short-term (i.e., 3 min) CXCL12 stimulation is only partially blocked (Fig. 8). This result parallels the differential effect of PKC inhibition in sustained versus transient CXCL12-induced adhesion (Fig. 5, E and F) and provides additional evidence for the close relationship between CXCL12-induced FAK phosphorylation and the activation of cell adhesion.

In summary, we demonstrate that sustained adhesion to VCAM-1 is triggered by CXCL12 in progenitor but not mature B cells and thus may play an important role in the retention of developing B cells in their microenvironmental niches in the bone marrow. As progenitor B cells differentiate, CXCL12 responsiveness gradually diminishes possibly enabling $sIgM^+$ B cells to exit and enter the peripheral circulation. The sustained adhesion response is tightly associated with prolonged activation of FAK in progenitor B cells. This association is intriguing because FAK plays an important role in cell adhesion, cell motility, as well as in cell survival (56). We conclude that sustained signaling through CXCR4 might be an essential physiologic process in B lymphopoiesis.

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