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Bruton's Tyrosine Kinase and Phospholipase Cγ2 Mediate Chemokine-Controlled B Cell Migration and Homing

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SUMMARY

Control of integrin-mediated adhesion and migration by chemokines plays a critical role in B cell development, differentiation, and function; however, the underlying signaling mechanisms are poorly defined. Here we show that the chemokine SDF-1 induced activation of Bruton's tyrosine kinase (Btk) and that integrin-mediated adhesion and migration in response to SDF-1 or CXCL13, as well as in vivo homing to lymphoid organs, was impaired in Btk-deficient (pre-)B cells. Furthermore, SDF-1 induced tyrosine phosphorylation of Phospholipase Cγ2 (PLCγ2), which, unlike activation of the migration regulatory GTPases Rac or Rap1, was mediated by Btk. PLCγ2-deficient B cells also exhibited impaired SDF-1-controlled migration. These results reveal that Btk and PLCγ2 mediate chemokine-controlled migration, thereby providing insights into the control of B cell homeostasis, trafficking, and function, as well as into the pathogenesis of the immunodeficiency disease X-linked agammaglobulinemia (XLA).

INTRODUCTION

Integrin-mediated cell adhesion and migration play a critical role in a wide variety of processes underlying proper organization and function of the immune system, including B cell development and differentiation. During early B cell development, the consecutive generation of pro-, pre-, and immature B cells requires their retention in defined microenvironments in the bone marrow (BM), which is controlled by interactions of integrin $\alpha4\beta1$ with fibronectin (FN) in the extracellular matrix (ECM) and with vascular cell adhesion molecule-1 (VCAM-1)-expressing BM stromal cells (Arroyo et al., 1999; Leuker et al., 2001; Rose et al., 2002; Tokoyoda et al., 2004). In mature B cells, integrins $\alpha4\beta1$ and leukocyte function-associated antigen-1 (LFA-1) mediate high endothelial venule (HEV) attachment and transendothelial migration required for recirculation and homing, and they control cell compartmentalization in peripheral lymphoid tissue (Cyster, 2003, 2005; Koni et al., 2001; Miyasaka and Tanaka, 2004; Rose et al., 2002). Integrins $\alpha4\beta1$ and LFA-1 also play a key role in the T cell-dependent humoral immune response, being involved in migration of naive B cells into B cell follicles and in the interaction of germinal center (GC) B cells with antigen-presenting follicular dendritic cells (FDCs) during antigen-specific B cell differentiation (Brakebusch et al., 2002; Koopman et al., 1991, 1994; Leuker et al., 2001; Rose et al., 2002; Spaargaren et al., 2003).

Chemokines play a prominent role in controlling integrin-mediated adhesion and migration (Kinashi, 2005; Laudanna et al., 2002). As such, the so-called homeostatic chemokines SDF-1 (CXCL12) and CXCL13 (BLC or BCA-1) and their respective G protein-coupled receptors CXCR4 and CXCR5 play a major role in B cell homeostasis, trafficking, and function (Campbell et al., 2003; Cyster, 2003, 2005; Miyasaka and Tanaka, 2004). CXCR4 is expressed by all B cell subsets (Bowman et al., 2000), and SDF-1 is highly expressed by stromal cells in the BM and GCs, by reticulum cells aligning the GCs, and in the splenic red pulp and lymph node medullary cords, and it is present on HEV in lymph nodes and Peyer's patches (Allen et al., 2004; Cyster, 2003, 2005; Okada et al., 2002). SDF-1-CXCR4 signaling plays a critical role in a variety of processes underlying proper B cell development and function, including development and retention of precursor B cells in the BM, homing of mature B cells to secondary lymphoid organs, trafficking and homing of plasma cells to BM, GC organization, and T-independent humoral immune responses (Allen et al., 2004; Cyster, 2003, 2005; Ma et al., 1999; Nagasawa et al., 1996; Nie et al., 2004; Okada et al., 2002; Tokoyoda et al., 2004). CXCR5 is mainly expressed by mature B cells (Bowman et al.,

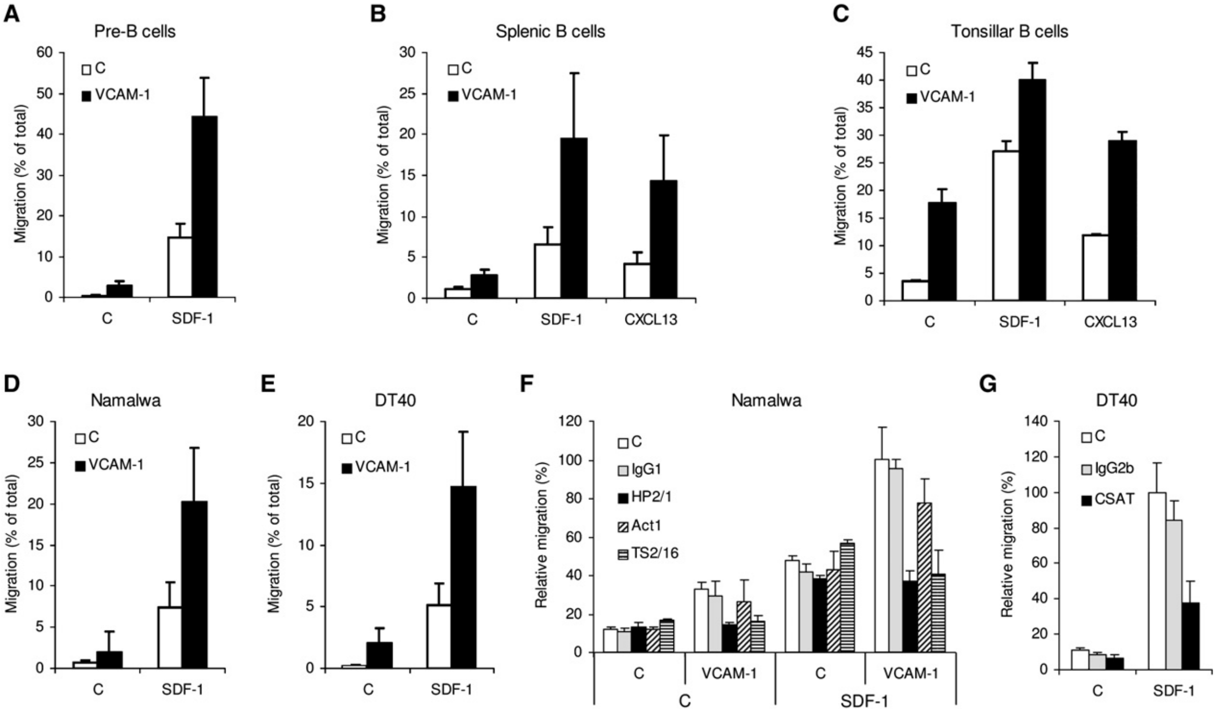

Figure 1. SDF-1 and CXCL13 Induce Integrin-Mediated B Cell Migration on VCAM-1

(A–E) Mouse pre-B cells (A), mouse splenic B cells (B), human tonsillar B cells (C), Namalwa cells (D), and DT40 cells (E) were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) or 500 ng/ml CXCL13 in transwells that were either uncoated or coated with 1 μg/ml VCAM-1, as indicated. The bars represent the means ± SD of a representative experiment (C), or of 6 (A, B, and D) or 14 (E) independent experiments, each assayed in triplicate, and presented as percent migration of total cells.

(F) Namalwa cells were preincubated for 1 hr at 4°C with medium alone (C), 10 μg/ml of an IgG1 isotype control (IgG1), or antibody Act-1 blocking α4β7 (Act-1), HP2/1 blocking α4β1 (HP2/1), or antibody TS2/16 activating integrin α4β1 (TS2/16). Subsequently, cells were allowed to migrate for 2 hr in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in transwells that were either uncoated or coated with 1 μg/ml VCAM-1, as indicated.

(G) DT40 cells were preincubated for 1 hr at 4°C with medium alone (C), or 10 μg/ml of an IgG2b isotype control (IgG2b) or antibody CSAT against integrin subunit β1 (CSAT). Subsequently, cells were allowed to migrate for 2 hr in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in transwells that were either uncoated or coated with 1 μg/ml VCAM-1, as indicated.

(F and G) The migration was normalized to 100% for the cells not pretreated with antibody allowed to migrate in the presence of SDF-1 in transwells coated with 1 μg/ml VCAM-1. The bars represent the means ± SD of an experiment representative of three independent experiments, each performed in triplicate.

2000), and CXCL13 is produced in follicles and is present on HEV of lymph nodes and Peyer's patches (Miyasaka and Tanaka, 2004). CXCL13-CXCR5 signaling is required for migration of naive B cells into follicles and for GC organization (Allen et al., 2004; Ansel et al., 2000; Campbell et al., 2003; Cyster, 2005; Forster et al., 1996; Okada et al., 2002).

Despite the important role of chemokine-controlled integrin-mediated migration in B cell development and function as well as in the pathogenesis of B cell malignancies and chronic inflammatory or autoimmune diseases, the underlying signal transduction mechanisms are as yet poorly defined (Kinashi, 2005; Laudanna et al., 2002). Interestingly, many of the B cell defects in mice deficient in SDF-1-CXCR4, CXCL13-CXCR5, VCAM-1, or α4 integrin, such as impaired development and retention of B cell precursors in the BM and impaired B cell differentiation and immune responses (Allen et al., 2004; Ansel et al., 2000; Arroyo et al., 1999; Forster et al., 1996; Leuker et al., 2001; Ma et al., 1999; Nagasawa et al., 1996; Nie et al., 2004; Okada et al., 2002), are similar to the defects observed in the immunodeficiency diseases X-linked immunodeficiency (Xid) in mice and XLA in men, caused by loss-of-function germline mutations in the cytoplasmic tyrosine kinase Btk (Cariappa et al., 1999; Conley et al., 2005; Ellmeier et al., 2000; Hendriks et al., 1996; Khan et al., 1995; Middendorp et al., 2002; Nomura et al., 2000). Combining this notion with our recent finding that Btk is required for the control of integrin-mediated adhesion by the B cell antigen receptor (BCR) (Spaargaren et al., 2003), we hypothesized that Btk may be involved in the signaling mechanism underlying chemokine-controlled integrin-mediated migration. Here, we demonstrate that SDF-1- or CXCL13-controlled integrin-mediated adhesion and migration, as well as in vivo homing, of pre-B and B cells was indeed mediated by Btk. Furthermore, Btk mediated SDF-1-induced phosphorylation of PLCγ2, and PLCγ2 mediated SDF-1-controlled migration as well.

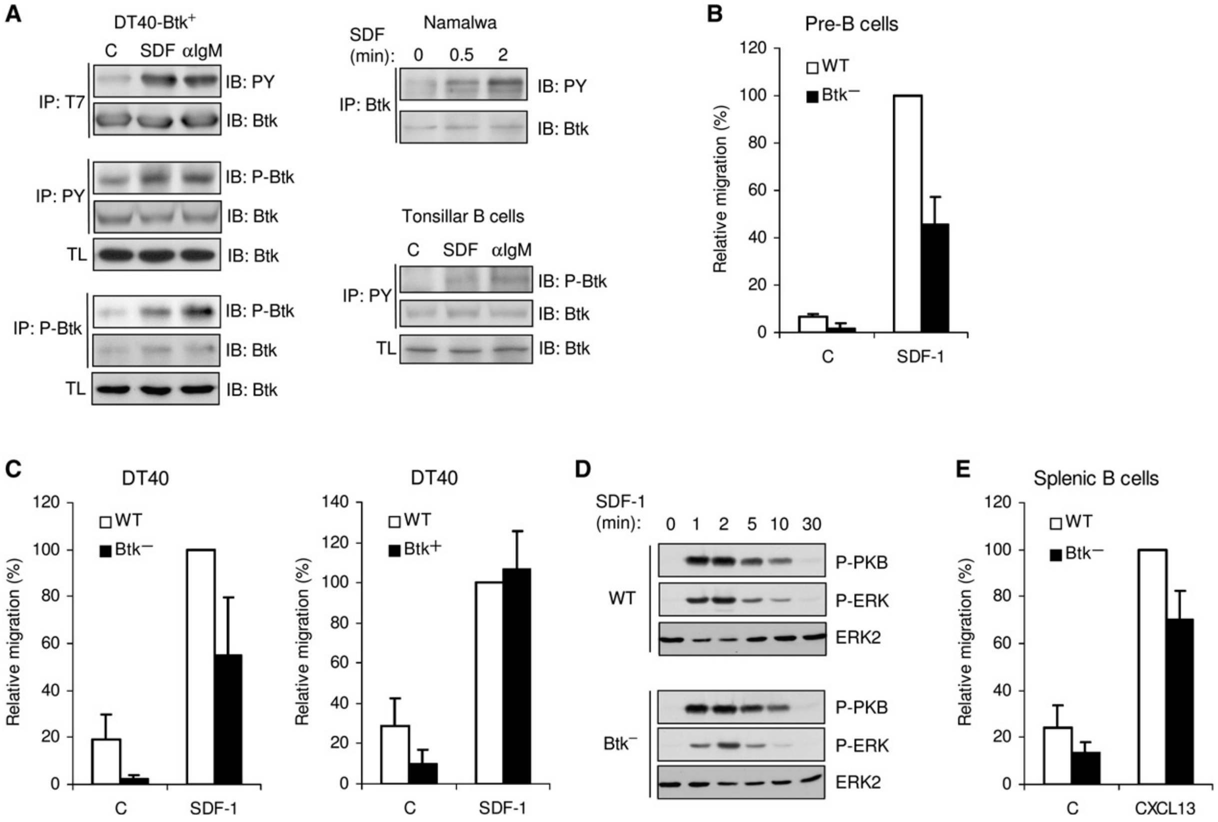

Figure 2. Btk Mediates Chemokine-Controlled Migration

(A) Btk-deficient DT40 cells reconstituted with T7-tagged Btk (Btk$^+$), Namalwa cells, and human tonsillar B cells were stimulated with SDF-1 or anti-IgM for 2 min. Lysates were immunoprecipitated (IP) by means of anti-Btk (Btk), anti-T7 (T7), anti-phospho-Btk (P-Btk), or anti-phosphotyrosine (PY), and the IPs were immunoblotted (IB) with anti-phospo-Btk or anti-phosphotyrosine, as indicated. As a control, the immunoprecipitates and total lysates (TL) were (re-)probed with anti-Btk.

(B) Wild-type (WT) or Btk-deficient (Btk$^-$) murine pre-B cells were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in transwells coated with 1 μg/ml VCAM-1 (n = 6). The bars represent the means ± SD of six independent experiments, each performed in triplicate.

(C) Wild-type (WT), Btk-deficient (Btk$^-$), or Btk-deficient DT40 cells reconstituted with Btk (Btk$^+$) were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in transwells coated with 1 μg/ml VCAM-1 (n = 8). The bars represent the means ± SD of eight independent experiments, each performed in triplicate.

(D) Wild-type (WT) or Btk-deficient (Btk$^-$) DT40 cells were stimulated with SDF-1 for the indicated period of time (min) and immunoblots are probed with anti-phospho-PKB (P-PKB) and anti-phospho-MAPK (P-ERK) and reprobed with anti-ERK2, as indicated.

(E) Wild-type (WT) or Btk-deficient (Btk$^-$) murine splenic B cells were allowed to migrate in the absence (C) or presence of 500 ng/ml CXCL13 in transwells coated with 1 μg/ml VCAM-1 (n = 3). The bars represent the means ± SD of three independent experiments, each performed in triplicate.

This function for Btk implies that impaired chemokine-controlled migration may contribute to the developmental and functional B cell defects observed in XLA and Xid.

RESULTS

SDF-1 and CXCL13 Induce Integrin-Mediated Migration of B Cells

To study the control of integrin-mediated B cell migration by the chemokines SDF-1 and CXCL13, murine BM-derived pre-B cells, murine splenic B cells, and human tonsillar B cells were assayed for migration toward these chemokines in a transwell system of which the membrane was either uncoated or coated with VCAM-1 or FN. Although SDF-1 strongly induced the migration of these primary B cells on uncoated membranes, migration toward SDF-1 was more pronounced ($\sim$3-fold increase) on membranes coated with VCAM-1 (Figures 1A–1C). Similar results were obtained for migration on FN (not shown) and for migration of murine splenic B cells and tonsillar B cells toward CXCL13 (Figures 1B and 1C).

VCAM-1 and FN (not shown) also enhanced migration toward SDF-1 of the human GC B cell-like cell line Namalwa (Figure 1D) and the chicken DT40 B cells (Figure 1E) by approximately 3-fold. As shown in Figure 1F, the integrin $\alpha4\beta1$-blocking antibody HP2/1 and the $\alpha4\beta1$-activating antibody TS2/16 completely abolished VCAM-1-mediated migration of Namalwa cells toward SDF-1, whereas no effect was observed with either an isotype control IgG1 or the integrin $\alpha4\beta7$-blocking antibody Act-1. Furthermore, antibody CSAT, directed against the chicken integrin $\beta1$ subunit, strongly suppressed SDF-1-induced migration of DT40 cells on VCAM-1 (Figure 1G). Taken together, our data demonstrate that SDF-1 and CXCL13 control integrin (α4β1)-mediated migration of primary B cells and B cell lines.

Btk Mediates Chemokine-Controlled Migration

Because many of the defects observed in mice deficient in SDF-1-CXCR4, CXCL13-CXCR5, VCAM-1, or α4 integrin are also observed in Xid or Btk-deficient mice and XLA patients (as elaborated in the Discussion), and because we have recently established that Btk is required for the control of integrin-mediated adhesion by the BCR (Spaargaren et al., 2003), we hypothesized that Btk might be involved in the signaling mechanism underlying chemokine-controlled integrin-mediated migration.

In support of a possible role for Btk in chemokine-controlled migration, we observed SDF-1-induced tyrosine phosphorylation of Btk in DT40 cells, Namalwa cells, and human tonsillar B cells (Figure 2A). Phosphorylation of Btk could be detected with either a general phosphotyrosine antibody or a phospho-specific antibody for the autophosphorylation site Y223, reflecting Btk activation. Typically, SDF-1 induced Btk phosphorylation in a fast and transient fashion, with optimal phosphorylation being observed between 30 s and 2 min. A similar degree of Btk phosphorylation was observed after suboptimal duration of BCR stimulation (i.e., 2 min) with anti-IgM (Figure 2A); however, optimal 5 min BCR stimulation resulted in stronger Btk phosphorylation (not shown).

To directly examine a possible role for Btk in SDF-1-controlled integrin-mediated migration, we used pre-B cells derived from Btk-deficient mice. Interestingly, as shown in Figure 2B, Btk-deficient pre-B cells exhibited ~55% reduced SDF-1-induced migration on VCAM-1 in comparison to WT pre-B cells. Notably, surface expression of CXCR4, integrin α4 and β1 was similar on WT and Btk-deficient pre-B cells (see Figure S1 in the Supplemental Data available online). In DT40 cells, Btk deficiency also resulted in a reduction (by ~45%) of SDF-1-controlled migration (Figure 2C). This defect was not due to clonal variation because it could be completely restored by stable transfection of a Btk expression construct (Figure 2C). Furthermore, as shown in Figure 2D, whereas SDF-1-induced activation of the MAP kinase ERK2 (MAPK1) was slightly reduced, activation of PKB (Akt) was not affected in the Btk-deficient cells, demonstrating that the impaired migration is not due to a general signaling defect. To examine the role of Btk in the control of B cell migration by CXCL13, we used splenic B cells, because pre-B cells and DT40 cells do not respond to CXCL13 (not shown). As shown in Figure 2E, Btk-deficient B cells also exhibited impaired migration on VCAM-1 toward CXCL13 (by ~30%). Notably, surface expression of CXCR5 and integrin β1 was similar on WT and Btk-deficient splenic B cells (Figure S2).

In conclusion, these data identify Btk as a component of the SDF-1-CXCR4 signaling cascade and reveal a regulatory function for Btk in chemokine-controlled integrin-mediated migration of (pre-)B cells.

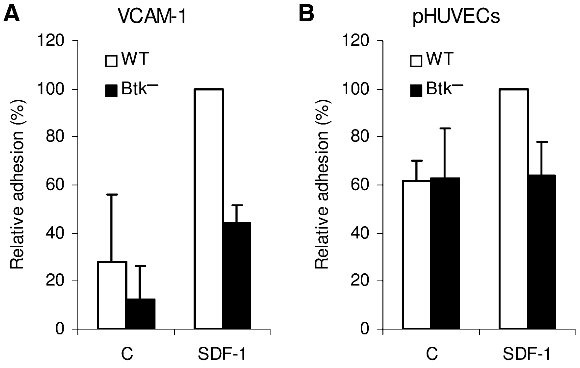

Figure 3. SDF-1-Controlled Adhesion Is Impaired in Btk-Deficient B Cells

(A) Wild-type (WT) or Btk-deficient DT40 cells (Btk$^-$) were allowed to adhere to wells coated with 1 µg/ml VCAM-1 coimmobilized without (C) or with 150 ng/ml SDF-1 (SDF-1) (n = 4; each in triplicate).

(B) Wild-type (WT) or Btk-deficient DT40 cells (Btk$^-$) were perfused at a shear stress of 0.8 dyn/cm^2 over primary HUVECs overlaid without (C) or with 10 ng/ml SDF-1 (SDF-1) (n = 3; each in duplicate). The bars in (A) and (B) represent the means ± SD of the indicated number of experiments.

SDF-1-Controlled Adhesion Is Impaired in Btk-Deficient B Cells

Chemokine-controlled cell migration is a complex process that involves proper coordination of cell polarity, cytoskeletal reorganization, and control of integrin localization and activity. We have previously demonstrated that Btk mediates BCR-controlled integrin-mediated adhesion of B cells (Spaargaren et al., 2003), so we examined whether Btk is also involved in SDF-1-controlled integrin-mediated adhesion. A static adhesion assay showed adhesion to VCAM-1 in the presence of SDF-1 to be reduced by ~55% in the Btk-deficient DT40 cells in comparison to the WT DT40 cells (Figure 3A). In a more physiological assay, i.e., under flow adhesion to SDF-1-presenting primary HUVECs (at a shear stress of 0.8 dyn/cm^2), the stimulation of adhesion by SDF-1 was completely abolished in Btk-deficient cells (Figure 3B). Taken together, these results demonstrate that Btk also mediates SDF-1-controlled integrin-mediated adhesion, which may underlie the impaired chemokine-controlled migration of Btk-deficient B cells.

In Vivo Homing Is Impaired in Btk-Deficient Pre-B and Immature B Cells

B cell homing is critically dependent upon the differential expression and coordinated action of B cell integrins, endothelial cell adhesion molecules, and specific chemokines and their cognate receptors, including SDF-1-CXCR4 and CXCL13-CXCR5 (Campbell et al., 2003; Cyster, 2003, 2005; Miyasaka and Tanaka, 2004). Therefore, to study the in vivo consequence of impaired chemokine-controlled adhesion and migration resulting from Btk deficiency, we investigated the homing capacity of pre-B and immature B cells from Btk-deficient mice versus WT mice. Given the B cell developmental arrest in

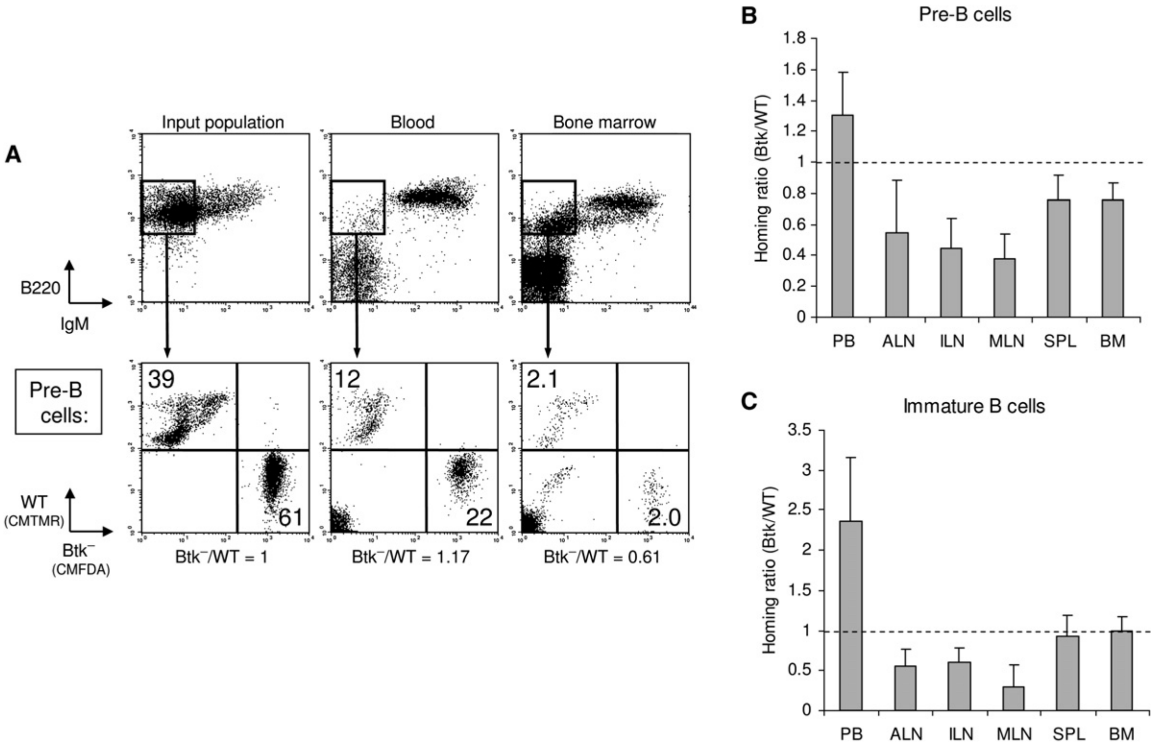

Figure 4. In Vivo Homing Is Impaired in Btk-Deficient Pre-B and Immature B Cells

Cells from IL-7-driven cultures of BM from wild-type (WT) or Btk-deficient (Btk$^-$) mice were labeled with CMFDA or CMTMR, mixed, and injected into C57Bl/6 mice. After 3 hr, peripheral blood (PB), inguinal lymph nodes (ILN), axillary lymph nodes (ALN), mesenteric lymph nodes (MLN), spleen (SPL), and bone marrow (BM) were collected and FACS analyzed.

(A) An example of the FACS analysis of the input population, peripheral blood, and BM of a representative mouse. The B220$^+$IgM$^-$ pre-B cell fractions were gated and analyzed for the two dye-labeled populations. The percentages of labeled cells are given within the quadrants of the dot plots. The homing ratio of Btk-deficient (Btk$^-$) versus WT B cells was corrected for the input ratio (normalized to 1).

(B) The homing ratio of Btk$^-$ versus WT B220$^+$IgM$^-$ pre-B cells in the indicated tissues (n = 5; i.e., 5 Btk/WT-combinations analyzed in 10 recipient mice, including a dye swap). p values were <0.05 for ALN, spleen, and BM, and <0.005 for ILN and MLN. The bars represent the means ± SD of the indicated number of combinations.

(C) The homing ratio of Btk$^-$ versus WT B220$^+$IgM$^+$ immature B cells in the indicated tissues (n = 7; i.e., 14 recipient mice). p values were <0.01 for blood, ALN, ILN, and MLN. The bars represent the means ± SD of the indicated number of combinations.

Btk-deficient mice, we used an IL-7-driven BM culture system to obtain better developmentally matched WT and Btk-deficient pre-B and immature B cell populations. 3 hr after adoptive transfer, lymphoid organs were collected to determine the homing ratio of the Btk-deficient B cells compared to WT cells (Figure 4A). Interestingly, the accumulation of the Btk-deficient pre-B cells was reduced in axillary (by $\sim$45%), inguinal ($\sim$55%), and mesenteric ($\sim$60%) lymph nodes, the spleen ($\sim$25%), and in BM ($\sim$25%) (Figure 4B). Btk-deficient IgM$^+$ immature B cells also exhibited impaired homing to the peripheral (by $\sim$40%–45%) and mesenteric ($\sim$70%) lymph nodes, but not to the spleen or BM (Figure 4C). Consistent with a homing defect, and opposing a possible survival disadvantage, elevated numbers of Btk-deficient B cells were recovered from the peripheral blood (Figure 4). These results clearly demonstrate that Btk-deficient pre-B and immature B cells suffer from an intrinsic defect in the in vivo homing to (secondary) lymphoid organs, characteristic of impaired chemokine-controlled migration.

The Role of Lyn and Syk Tyrosine Kinases and PI3K in SDF-1-Controlled Migration

Recent studies have revealed an important role for cytoplasmic tyrosine kinases in signaling by G protein-coupled receptors, and the cytoplasmic tyrosine kinases Lyn and Syk play a prominent role in BCR-controlled activation of Btk (Kurosaki, 2002). Consistent with a putative similar role for these kinases in SDF-1-induced activation of Btk, SDF-1-controlled migration on VCAM-1 was reduced by $\sim$50% in DT40 cells deficient in both Lyn and Syk (Figure S3A and Supplemental Results).

Previous studies have also implicated PI3K in chemokine-controlled migration (Kinashi, 2005; Laudanna et al., 2002) and in activation of Btk (Kurosaki, 2002). However, in comparison to the reduced migration in Btk-deficient DT40 cells ($\sim$45%) (Figure 2C), pretreatment of DT40 or Namalwa cells with the unrelated PI3K inhibitors Wortmannin (WM) and LY294002 (LY) caused only a minor reduction of SDF-1-induced migration ($\sim$20%) (Figure S4B and Supplemental Results). Yet, the residual migration of

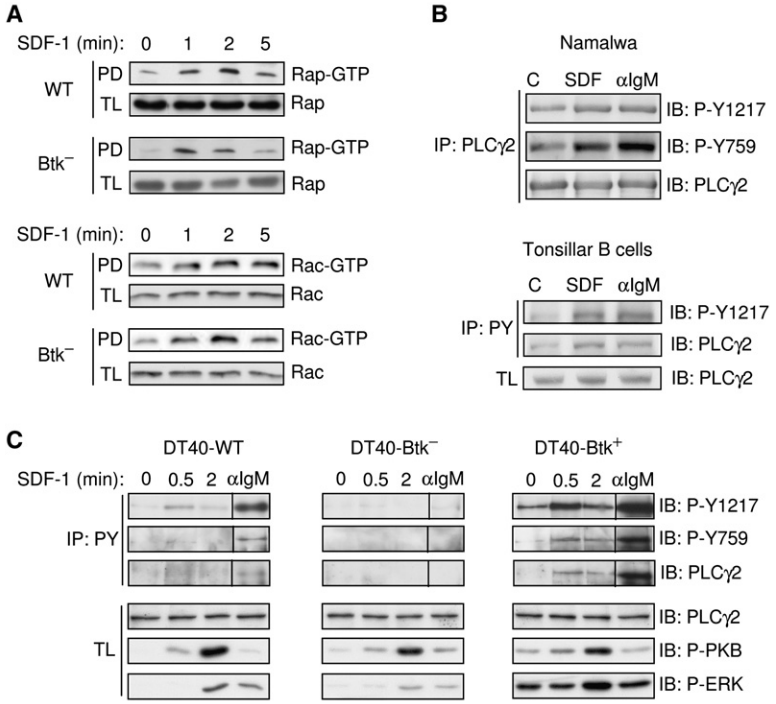

Figure 5. Btk Mediates SDF-1-Induced Phosphorylation of PLCγ2 but Not Activation of Rap1 or Rac

(A) WT DT40 cells (WT) or Btk-deficient DT40 cells (Btk^{-}) were stimulated with SDF-1 for 1, 2, or 5 min, and activation of the GTPases was determined by GTPase pull down and immunoblotting with anti-Rap1 (Rap-GTP) or anti-Rac (Rac-GTP). In parallel, total lysates were immunoblotted with anti-Rap1 (Rap) or anti-Rac1 (Rac).

(B and C) Namalwa cells or tonsillar B cells (B), and WT DT40 cells (WT), Btk-deficient DT40 cells (Btk^{-}), or Btk-deficient DT40 cells reconstituted with Btk (Btk^{+}) (C) were stimulated with SDF-1 or anti-IgM for 2 min. The lysates were immunoprecipitated (IP) by means of anti-PLCγ2 (PLCγ2) or anti-phosphotyrosine (PY), and the IPs were immunoblotted (IB) with anti-phospo-PLCγ2 (P-Y1217 or P-Y759), as indicated. As a control, the immunoprecipitates and total lysates (TL) were (re-)probed with anti-PLCγ2. Phosphorylation of PKB and ERK2 in the cell lysates was analyzed as in Figure 2D.

the Btk-deficient cells toward SDF-1 could be further reduced by ∼60% upon PI3K inhibition (Figure S4B). Taken together, these results suggest that PI3K and Btk mediate SDF-1-controlled migration in a parallel fashion, independent of each other.

Btk Mediates SDF-1-Induced Phosphorylation of PLCγ2 but Not Activation of Rap1 or Rac

Next, we wished to explore which signaling molecules may control chemokine-controlled migration downstream of Btk. The small GTPases Rap1 and Rac have both been implicated in SDF-1-controlled migration of B lymphocytes (Kinashi, 2005; McLeod et al., 2002). Indeed, GTPase pull-down assays revealed that SDF-1 stimulation of DT40 cells resulted in enhanced amounts of GTP-bound Rap1 and Rac; however, similar Rap1 and Rac activation was observed upon SDF-1 stimulation of Btk-deficient DT40 cells (Figure 5A). Thus, SDF-1-induced activation of neither Rap1 nor Rac is mediated by Btk.

Another candidate is PLCγ2, which has been identified as a direct substrate for Btk (Kurosaki, 2002). Furthermore, we have recently established an important role for PLCγ2 in BCR-controlled integrin-mediated adhesion (Spaargaren et al., 2003). Interestingly, SDF-1 stimulation resulted in enhanced tyrosine phosphorylation of PLCγ2 in Namalwa cells, human tonsillar B cells, and DT40 cells (Figures 5B and 5C). SDF-1-induced phosphorylation of PLCγ2 could be detected with a general phosphotyrosine antibody as well as by phospho-specific PLCγ2 antibodies directed against Y1217 and the Btk substrate site Y759. Importantly, SDF-1-induced tyrosine phosphorylation of PLCγ2 was severely reduced in the Btk-deficient DT40 cells and could be completely restored by stable transfection of a Btk expression construct (Figure 5C). Most likely because of higher Btk expression, PLCγ2 phosphorylation was even more pronounced in these reconstituted cells. Furthermore, SDF-1-induced phosphorylation of PKB (analyzed in the same cell lysates) was not affected by the absence of Btk (Figure 5C), thereby emphasizing the specificity of the defect in PLCγ2 phosphorylation. Thus, these data demonstrate that Btk mediates SDF-1-induced phosphorylation of PLCγ2.

PLCγ2 Mediates SDF-1-Controlled Migration

To examine a possible role for PLCγ2 in SDF-1-controlled migration, we used PLCγ2-deficient DT40 cells. As shown in Figure 6A, PLCγ2-deficient DT40 cells showed a reduction of SDF-1-controlled migration on VCAM-1 by ∼55%. This defect could be completely restored by gene complementation with a PLCγ2 expression construct (Figure 6A), demonstrating that the impaired migration is not due to clonal variation. Similar to Btk-deficient DT40 cells (Figure 5C), SDF-1-induced phosphorylation of PKB was not affected, but phosphorylation of ERK2 was reduced in PLCγ2-deficient DT40 cells (Figure 6B). Interestingly, treatment of Namalwa or DT40 cells with the PLC inhibitor U73122 almost completely abolished SDF-1-induced migration on VCAM-1 (Figure 6C). These data indicate that PLCγ2, together with other isoforms of PLC, plays an important role in SDF-1-CXCR4 signaling and SDF-1-controlled B cell migration.

DISCUSSION

Our results reveal that Btk and PLCγ2 mediate chemokine-controlled integrin-mediated migration. Besides

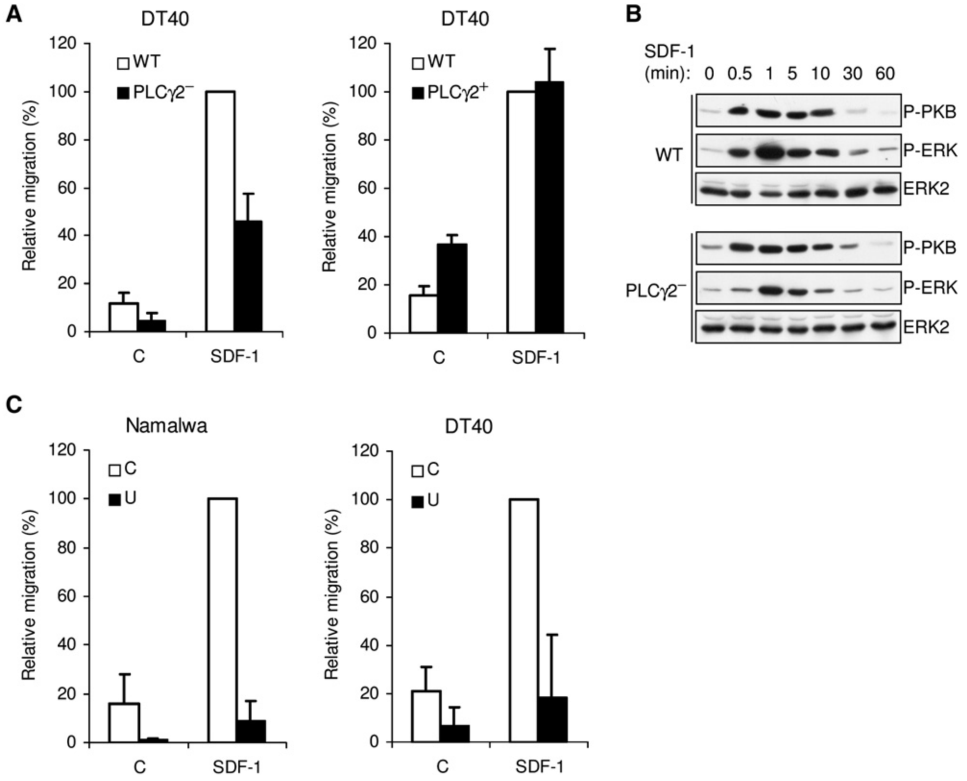

Figure 6. PLCγ2 Mediates SDF-1-Controlled Migration

(A) Wild-type (WT), PLCγ2-deficient (PLCγ2$^-$), or PLCγ2-deficient DT40 cells reconstituted with PLCγ2 (PLCγ2$^+$) were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in transwells coated with 1 μg/ml VCAM-1, as indicated (n = 6). The bars represent the means ± SD of six independent experiments, each performed in triplicate.

(B) Activation of PKB and ERK in wild-type (WT) or PLCγ2 (PLCγ2$^-$)-deficient DT40 cells after stimulation with SDF-1 for the indicated period of time (min). The blots are probed as in Figure 2D.

(C) Namalwa cells (left) or DT40 cells (right) were pretreated with 2.5 μM U73122 (U) or left untreated for 30 min, and subsequently cells were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in transwells coated with 1 μg/ml VCAM-1 (n = 5). The bars represent the means ± SD of five independent experiments, each performed in triplicate.

providing several answers and new insights, this study also raises some new questions and challenges for future studies.

How do chemokines control Btk activity? In BCR signaling, the PI3K product PIP3 is implicated in membrane recruitment of Btk, enabling activation of Btk by Lyn, whereas Syk, through phosphorylation of the adaptor BLNK (SLP-65 or BASH), facilitates activation of PLCγ2 by Btk (Kurosaki, 2002). Consistent with a putative role for these tyrosine kinases in activation of Btk and/or PLCγ2 by SDF-1, SDF-1-controlled migration was impaired in B cells deficient in Lyn and Syk. In contrast, our observations suggest that PI3K does not mediate activation of Btk by SDF-1. Yet, SDF-1-induced membrane recruitment of a Btk-GFP fusion protein in Hela cells was reported to be sensitive to PI3K inhibition (Nore et al., 2000). However, supporting our results, and opposing the general perception that activation of Btk is strictly dependent upon its PI3K-mediated membrane recruitment, BCR-controlled phosphorylation and activation of Btk (and PLCγ2) is not affected in B cells deficient in the regulatory PI3K subunit p85α or the catalytic subunit p110δ, nor in primary B cells treated with LY or WM (Jou et al., 2002; Suzuki et al., 2003). Likewise, in accordance with the minor effect of PI3K inhibition on SDF-1-induced B cell migration, several recent studies have challenged the general concept that PI3K activation is indispensable for chemokine-controlled cell migration (Nombela-Arrieta et al., 2004; Smit et al., 2003; Ward, 2004). Nevertheless, the residual migration of Btk-deficient cells was largely PI3K dependent. Most likely, this involves the catalytic p110δ and adaptor p85α subunits (Figure 7), which, in contrast to the catalytic subunit p110γ, have been implicated in chemokine-controlled B cell migration (Nombela-Arrieta et al., 2004; Reif et al., 2004). Recently, B cells were shown to require the scaffolding protein DOCK2 for efficient SDF-1- and CXCL13-controlled migration and integrin-mediated adhesion to ICAM-1 and VCAM-1, respectively (Kinashi, 2005; Nombela-Arrieta et al., 2004). DOCK2, like Btk, mediates chemokine-controlled migration in a largely PI3K-independent fashion (Nombela-Arrieta et al., 2004), so it is tempting to speculate that DOCK2 may be involved in chemokine-induced Btk or PLCγ2 activation (Figure 7). Alternatively, by analogy to

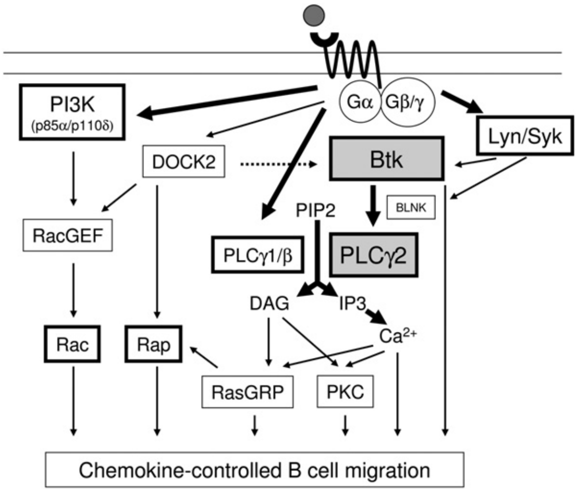

Figure 7. The Role of Btk and PLCγ2 in the Signaling Cascades Underlying Chemokine-Controlled Integrin-Mediated Migration

A schematic representation of the (putative) signaling pathways underlying chemokine-controlled integrin (α4β1)-mediated migration is shown. The proteins studied and connections established in this study are represented by the bold boxes and bold arrows, respectively. See the Discussion section for further detail.

BCR signaling (Kurosaki, 2002), the adaptor protein BLNK may be involved. Finally, as previously established for Gα (αq and α12) and Gβ/γ subunits (Bence et al., 1997; Lowry and Huang, 2002), the membrane recruitment and activation of Btk may be mediated by the direct interaction of chemokine receptor-activated G protein subunits (Figure 7).

How do Btk and PLCγ2 control chemokine-induced migration? Our results strongly indicate that Btk mediates chemokine-controlled migration through PLCγ2, which is highly expressed in all B cell subsets (Figure 7). To our knowledge, the only previous evidence for a possible role for PLC-γ in chemokine signaling is the observation that SDF-1 induces tyrosine phosphorylation of PLC-γ (isotype undefined) in hematopoietic progenitor cells (Wang et al., 2000). In addition, since SDF-1-induced migration could be completely abolished with the general PLC inhibitor U-73122, other PLC isotypes, such as PLCγ1, which is highly expressed in pro- and pre-B cells, or PLCβ, may be involved as well (Figure 7). Similar to our observations, CXCR3-mediated migration of T lymphocytes is relatively insensitive to PI3K inhibition, but can be completely abolished by PLC inhibition (Smit et al., 2003). The observed critical role of PLC, including PLCγ2, in chemokine-controlled migration points toward an important downstream role for calcium- and/or DAG-dependent signaling molecules. Likely candidates are the classical and novel isoforms of PKC and the RasGRP (CalDAG-GEF) family of exchange factors, which act on different Ras family GTPases, including Ras and Rap1 (Kinashi, 2005). Indeed, Rap1 was shown to be involved in SDF-1-induced migration of B lymphocytes (McLeod et al., 2002; Shimonaka et al., 2003). However, SDF-1-induced activation of Rap1 was not impaired in Btk- or PLCγ2-deficient B cells. Similarly, activation of Rap1 by SDF-1 is not impaired in Jurkat T cells deficient in PLCγ1, the major PLC-γ isotype in T cells (Katagiri et al., 2004). The activation of Rac, another GTPase implicated in chemokine-controlled migration, can occur through PI3K-dependent and PI3K-independent mechanisms (Kinashi, 2005; Ward, 2004). Noteworthy, DOCK2 also mediates activation of Rac (and Rap) (Nombela-Arrieta et al., 2004; Sanui et al., 2003). However, SDF-1-induced activation of Rac was not impaired in Btk- or PLCγ2-deficient B cells. Taken together, our data indicate that Btk, Rap1, and Rac act in parallel signaling pathways in chemokine-controlled B cell migration (Figure 7).

What about the role of other Tec family kinases in chemokine-controlled migration? Several members of the Tec family of tyrosine kinases, consisting of Tec, Btk, Itk, Rlk, and Bmx, have previously been implicated in cytoskeletal reorganization, integrin-mediated adhesion, or migration (Berg et al., 2005). In B cells, we have previously shown that Btk mediates BCR-controlled α4β1-mediated adhesion, which involves cytoskeletal reorganization (Spaargaren et al., 2003). Similarly, in T cells, Itk mediates TCR-controlled actin polymerization and activation of β1 integrins (Berg et al., 2005). Moreover, two recent studies revealed a critical role for the Tec family kinases Itk and Rlk in chemokine-controlled migration of T cells (Fischer et al., 2004; Takesono et al., 2004). Furthermore, in neutrophils, the chemotactic peptide fMLP induces the membrane recruitment and activation of Tec, Btk, and Bmx (Lachance et al., 2002), which, like the chemotactic response, can be suppressed by the pharmacological Tec family kinase inhibitor LFM-A13 (Gilbert et al., 2003). Recently, LFM-A13 was also reported to inhibit SDF-1-induced B cell migration and homing, and solely based upon the use of this inhibitor, the authors propose a role for Btk in these processes (Ortolano et al., 2006). We would like to emphasize, however, that LFM-A13 is not a specific Btk inhibitor: it is a potent inhibitor of the other Tec family kinases as well (Chau et al., 2005; Fernandes et al., 2005; Gilbert et al., 2003), and LFM-A13 is an equally efficient inhibitor of the nonrelated kinase JAK2 (van den Akker et al., 2004), which has also been implicated in SDF-1-induced lymphocyte migration (Soriano et al., 2003; Zhang et al., 2001). Yet, combining all of the above with our current findings, Tec family kinases appear to play an important role in chemokine-controlled adhesion and migration, at least in lymphocytes. Given the observed partial redundancy of Btk with Tec in murine B cells (Ellmeier et al., 2000), it would be interesting to determine whether Tec (or another Tec family member) is responsible for the residual migration observed in Btk-deficient (pre-)B cells. Furthermore, by analogy to the role of Btk and PLCγ2 in chemokine-controlled B cell migration, it would be interesting to determine whether chemokine-controlled migration of T lymphocytes involves Itk- or Rlk-mediated phosphorylation and activation of PLCγ1, the major PLC-γ isotype in T cells.

What are the implications for XLA and Xid? Our study demonstrates a direct role for Btk in signaling by CXCR4 and in chemokine-controlled adhesion, migration, and homing. Interestingly, loss-of-function germline mutations in the *BTK* gene give rise to the B cell immunodeficiency disease XLA in humans and Xid in mice. XLA patients show a severe reduction in mature B cell numbers (>99%) (Conley et al., 2005; Nomura et al., 2000), and in Xid- and the phenotypically identical Btk-deficient mice, mature B cell numbers are ~50% reduced (Khan et al., 1995). The earliest role for Btk occurs during B cell development at the progression from pre-B to immature B cells, which is severely impaired in XLA patients and to a lesser extent in Xid and Btk-deficient mice (Conley et al., 2005; Hendriks et al., 1996; Middendorp et al., 2002). In XLA, this involves a proliferation defect of the μH-chain-positive pre-B cells (Nomura et al., 2000), and in Xid mice this involves a defect in cellular maturation of pre-B cells (Middendorp et al., 2002). Furthermore, Xid mice have been reported to display a defect in the retention of immature B cells in the BM (Cariappa et al., 1999). Interestingly, α4 integrin-deficient pre-B cells also display a proliferation defect (Arroyo et al., 1999), and SDF-1 was originally identified as a pre-B cell growth-stimulating factor. Moreover, our results as well as previous studies (Bowman et al., 2000; Tokoyoda et al., 2004) show that SDF-1 controls α4β1-mediated adhesion and migration of pre-B and immature B cells, and mice deficient in SDF-1-CXCR4, VCAM-1, or α4 integrin display defects in development and retention of precursor (pro-, pre-, and immature) B cells in the BM (Arroyo et al., 1999; Leuker et al., 2001; Ma et al., 1999; Nagasawa et al., 1996; Nie et al., 2004). In the mature B cell population, Btk-deficient mice reveal a defect in follicular entry of long-lived recirculating follicular cells, in follicle and GC formation, in T-independent immune response, and a strongly reduced primary and variably affected secondary T-dependent immune response, whereas both responses are absent in Btk/Tec double-deficient mice (Ellmeier et al., 2000; Hendriks et al., 1996; Khan et al., 1995; Ridderstad et al., 1996; Vinuesa et al., 2001). Similarly, mice deficient in either SDF-1 or CXCR4 and mice deficient in either CXCL13 or CXCR5 display defects in migration of naive B cells into follicles, GC B cell migration, and GC organization (Allen et al., 2004; Ansel et al., 2000; Forster et al., 1996; Okada et al., 2002), CXCR4-deficient mice show a loss of T-independent immune response (Nie et al., 2004), and mice lacking either VCAM-1 expression on FDCs or expression of integrin β1 in the hematopoietic system exhibit an impaired T-dependent immune response (Brakebusch et al., 2002; Koni et al., 2001; Leuker et al., 2001). Thus, based upon our current findings, impaired chemokine (SDF-1 or CXCL13)-controlled adhesion and migration, required for localization of pre-B and immature B cells in the appropriate BM niches and for emigration of immature B cells from the BM into the blood and to secondary lymphoid organs, may very well contribute to the defects in early B cell development in XLA and Xid and to the partial defects in localization, differentiation, and responses of mature B cells in Xid mice.

In conclusion, our results demonstrate that Btk and PLCγ2 mediate chemokine-controlled B cell adhesion and migration, which play an important role in B cell development and function as well as in the pathogenesis of B cell malignancies and chronic inflammatory or autoimmune diseases. Furthermore, our results indicate that impaired adhesion and migration, resulting from loss-of-function germline mutations of *BTK*, may contribute to the developmental and functional B cell defects observed in XLA patients and Xid mice.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures online for complete methods.

Materials

The following reagents were used in this study: phosphorylation state-specific antibodies phospho-p44/42 MAP kinase [T202/Y204], phospho-Akt [S473], phospho-Btk [Y223], phospho-PLCγ2 [Y759], and phospho-PLCγ2 [Y1217] (Cell Signaling Technology); phosphotyrosine antibodies PY20 (BD Biosciences) and 4G10 (Upstate Biotechnology); rabbit polyclonal antibodies against ERK2 (C-14), Akt1/2 (H-136), Btk (C20), PLCγ2 (Q20 and H160) (Santa Cruz Biotechnology), and CXCR4 (AB 1846) (Chemicon); mouse monoclonal antibodies against T7 (Novagen), Btk (G149-11), Rap1, and Rac1 (BD Biosciences); mouse monoclonal IgG1 antibodies HP2/1 against integrin subunit α4 (Immunotech), Act-1 against integrin subunit β7 (kindly provided by A. Lazarovits), TS2/16 against integrin subunit β1 (kindly provided by F. Sanchez-Madrid), CSAT IgG2b against the chicken integrin subunit β1 (DSHB, University of Iowa); rat and hamster monoclonal antibodies PS/2-biotin and OXM718-FITC against mouse integrin α4 and β1, respectively (Chemicon), and 2G8-biotin against CXCR5 (BD Biosciences); anti-CD45R(B220) microbeads (Miltenyi Biotec); mouse anti-human IgM (MH15) (Sanquin, Amsterdam, the Netherlands), goat anti-chicken IgM (Bethyl Laboratories); anti-B220-FITC (Leinco Technologies), anti-IgM-biotin (BD Biosciences), Streptavidin-PE, Streptavidin-FITC, goat anti-mouse-Biotin, goat anti-mouse-PE (Southern Biotechnology Associates), Swine anti-Rabbit-FITC (DAKO), and rabbit anti-goat-Biotin (Vector); pharmacological inhibitors PD-98059, LY-294002, Wortmannin, U-73122 (Biomol); recombinant human sVCAM-1, SDF-1α and BCA-1/CXCL13 (R&D Systems), human plasma FN, and BSA (fraction V) (Sigma); CMFDA and CMTMR (Molecular Probes).

Isolation of Tonsillar B Cells, Murine Pre-B Cells, and Splenic B Cells

Human tonsillar B cells, mouse splenic B cells from Btk-deficient mice and WT littermate controls, and mouse pre-B cells (IL-7-driven primary BM cultures of WT and Btk-deficient mice) were obtained essentially as described (Koopman et al., 1994; Middendorp et al., 2002; Spaargaren et al., 2003).

Cell Migration Assay

Migration assays were performed in triplicate with transwells (Costar) coated with 1 μg/ml sVCAM-1 or 10 μg/ml FN. The lower compartment contained 100 ng/ml SDF-1 or 500 ng/ml CXCL13, and the cells were applied to the upper compartment and allowed to migrate for 5 hr (DT40 and Namalwa), 3.5 hr (tonsillar B cells), 2.5 hr (splenic B cells), or 1 hr (pre-B cells) at 37°C (39.5°C for DT40), unless otherwise indicated. The amount of viable migrated cells was determined by FACS and expressed as a percentage of the input. Unless otherwise indicated, the migration of nonpretreated WT cells on VCAM-1-coated transwells in the presence of SDF-1 or CXCL13 was normalized to 100%, and the bars represent the means ± SD of at least three independent experiments, each assayed in triplicate.

Cell Adhesion Assays

The static cell adhesion to VCAM-1 was assayed essentially as described (Spaargaren et al., 2003), except that 150 ng/ml SDF-1 was coimmobilized with 1 μg/ml VCAM-1 on the plates, the plates were spun directly after applying the cells to the plate, and the cells were allowed to adhere for 2 min.

The under flow cell adhesion to HUVECs was assayed essentially as described (da Costa Martins et al., 2006). In brief, primary HUVECs were overlaid with 10 ng/ml SDF-1 prior to perfusion, DT40 cells were perfused at 0.8 dyn/cm^2 for 5 min, and 20 randomized images recorded between 2 and 5 min were analyzed to determine the average amount of adhering cells per field.

In both assays, the adhesion of WT cells in the presence SDF-1 was normalized to 100%.

Homing Assay

Cells from IL-7-driven BM cultures of WT and Btk-deficient mice were labeled with either CMFDA or CMTMR, mixed (1:1), and injected intraveneously in C57Bl/6 mice. Each WT/Btk$^-$ combination was analyzed by adoptive transfer of two recipient mice, which included a dye-swap. After 3 hr, lymphoid organs were collected and FACS analyzed to identify pre-B and immature B cells by their B220/IgM profile and to quantify dye-labeled cells. The homing ratio (Btk-deficient/WT cells) was corrected for the input ratio (which was normalized to 1).

Immunoprecipitation and Immunoblotting

For analysis of Btk and PLCγ2 tyrosine phosphorylation, cells were stimulated with 200 ng/ml SDF-1 or 10 μg/ml anti-IgM and immunoprecipitated with anti-Btk (C20), anti-T7, anti-P-Btk, anti-PLCγ2 (Q20), or anti-phosphotyrosine (PY20 or 4G10). Immunoprecipitates and total lysates were analyzed by SDS-PAGE and immunoblotting with anti-P-Btk, anti-Btk (G149-11), anti-P-PLCγ2, anti-PLCγ2 (H160), or anti-phosphotyrosine (4G10).

For analysis of ERK and PKB phosphorylation, cells were stimulated with 100 ng/ml SDF-1 and immunoblotted with anti-phospho-MAPK and anti-phospho-PKB, as described (Spaargaren et al., 2003).

Rac and Rap1 Activity Pull-Down Assays

Cells were stimulated with SDF-1 (100 ng/ml), and cell lysates were prepared and used immediately for GTPase pull-down assays with GST-RalGDS-RBD or GST-PAK-RBD fusion protein for Rap and Rac, respectively. Bound proteins were eluted with sample buffer, separated by 15% SDS-PAGE, and immunoblotted with anti-Rap1 or anti-Rac1.

Statistical Analysis

The unpaired two-tailed Student's t test was used to determine the significance of differences between means. Unless otherwise indicated, all relevant comparisons (e.g., control versus inhibitors or WT versus gene-deficient cells) were significantly different ($p < 0.05$).

Supplemental Data

Supplemental Data include four figures, Supplemental Results, and Supplemental Experimental Procedures and can be found with this article online at http://www.immunity.com/cgi/content/full/26/1/93/DC1/.

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