

The B Cell Antigen Receptor Controls Integrin Activity through Btk and PLC γ 2

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Abstract

Integrin-mediated adhesion and B cell antigen receptor (BCR) signaling play a critical role in B cell development and function, including antigen-specific B cell differentiation. Here we show that the BCR controls integrin α 4 β 1 (VLA-4)-mediated adhesion of B cells to vascular cell adhesion molecule-1 and fibronectin. Molecular dissection of the underlying signaling mechanism by a combined biochemical, pharmacological, and genetic approach demonstrates that this BCR-controlled integrin-mediated adhesion requires the (consecutive) activation of Lyn, Syk, phosphatidylinositol 3-kinase, Bruton's tyrosine kinase (Btk), phospholipase C (PLC) γ 2, IP3R-mediated Ca²⁺ release, and PKC. In contrast, activation of mitogen-activated protein kinase kinase (MEK) or extracellular signal-regulated kinase (ERK) is not required, and simultaneous activation of MEK, ERK, and PKB is not sufficient either. Furthermore, Btk is also involved in the control of integrin-mediated adhesion of preB cells. The control of integrin α 4 β 1-mediated B cell adhesion by the BCR involves cytoskeletal reorganization and integrin clustering. These results reveal a novel function for the BCR and Btk, i.e., regulation of integrin α 4 β 1 activity, thereby providing new insights into the control of B cell development and differentiation, as well as into the pathogenesis of the immunodeficiency disease X-linked agammaglobulinemia (XLA).

Key words: lymphocyte adhesion • B-cell development • X-linked agammaglobulinaemia • germinal center • VCAM-1

Introduction

Integrin-mediated cell-cell and cell-matrix adhesion, as well as migration, play an important role in a wide variety of processes controlling B-lymphocyte development and function. During early B cell development in the BM, hematopoietic progenitor cells successively develop into pro-, pre-, and immature B cells, which will emigrate into the bloodstream. This development occurs in defined microenvironments and is controlled by integrin α 4 β 1-mediated interactions with fibronectin (FN) in the ECM and with vascular cell adhesion molecule (VCAM)-1 expressing BM stromal cells (1-4). Furthermore, integrins mediate the transendothelial migration of mature B cells required for their recirculation and homing (5-8), and play a key role in antigen-specific B cell differentiation.

Antigen-specific B cell differentiation takes place within germinal centers (GCs), specialized microenvironments in the B cell area of secondary lymphoid organs (9-11). Here, T cell-dependent humoral immune responses are initiated by the activation of naive B cells in the T cell (paracortical) area, which leads to their migration into B cell follicles where they initiate the formation of GCs. In the GC, the B cells undergo rapid clonal expansion and somatic hypermutation of their immunoglobulin (Ig) genes. The mutated B cells reencounter antigen, presented by follicular dendritic

Abbreviations used in this paper: BCR, B cell antigen receptor; Btk, Bruton's tyrosine kinase; CD, cytochalasin D; CP, Calpeptin; DG, diacylglycerol; ERK, extracellular signal-regulated kinase; FDC, follicular dendritic cell; FN, fibronectin; GC, germinal center; IP3, inositol-1,4,5-trisphosphate; JP, jaspalakinolide; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MFI, mean fluorescence intensity; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PLL, poly-L-lysine; VCAM, vascular cell adhesion molecule; Xid, X-linked immunodeficiency; XLA, X-linked agammaglobulinemia.

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cells (FDCs), and undergo affinity maturation: B cells expressing high affinity B cell antigen receptor (BCR) mutants will be rescued from apoptosis by their interaction with the FDC (11, 12), expand, undergo Ig-isotype switching, and mature into memory B cells or plasma cells. The interaction with FDCs is mediated by the integrins $\alpha 4\beta 1$ and $\alpha L\beta 2$ (LFA-1) expressed on the GC B cell, which engage VCAM-1 and ICAM-1 on the FDC, respectively (13–15). Interestingly, apart from establishing physical contact, outside-in signaling by $\alpha 4\beta 1$ and LFA-1 has been shown to suppress apoptosis of GC B cells (16, 17). Hence, integrin-mediated interactions of GC B cells with FDCs play a key role in antigen-specific B cell differentiation (11). This notion is further supported by the observations that the T cell-dependent humoral immune response is impaired in mice lacking expression of VCAM-1 on FDCs (2) or of integrin $\beta 1$ in the hematopoietic system (18).

Despite the important role of integrins in B cell development and antigen-specific B cell differentiation, the stimuli and underlying signal transduction mechanisms that control integrin activity of B cells are as yet poorly defined. Given the critical role of the (pre)BCR in B cell development and differentiation (19–22), we asked whether the BCR may control integrin-mediated adhesion of B cells and, if so, what is the signal transduction mechanism involved. BCR signal transduction involves a wide variety of signaling molecules (20, 23, 24), some of which, e.g., phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC), protein kinase C (PKC), extracellular signal-regulated kinase (ERK) and several members of the Ras family, have been implicated in the control of integrin activity by other receptors (25, 26). Here, we demonstrate that stimulation of the BCR induces integrin $\alpha 4\beta 1$ -mediated adhesion of B cells to VCAM-1 and FN. Furthermore, our data reveal a critical role for the consecutive activation of Lyn, Syk, PI3K, Bruton's tyrosine kinase (Btk), PLC $\gamma 2$, IP3R-mediated Ca^{2+} release, and PKC in the BCR-controlled inside-out signaling mechanism underlying $\alpha 4\beta 1$ activation, which involves cytoskeletal reorganization and integrin clustering. These results explain how the concerted action of both BCR signaling and integrin activation, as well as outside-in signaling, is controlled and contributes to antigen-specific B cell differentiation. In addition, our study reveals a novel function for Btk, which suggests that a loss of control of integrin activity contributes to the developmental and functional B cell defects observed in the immunodeficiency disease X-linked agammaglobulinemia (XLA).

Materials and Methods

Materials. The following reagents were used in this study: the phosphorylation state-specific antibodies phospho-p44/42 MAP kinase [T202/Y204] against ERK1 and 2 and phospho-Akt [Ser473] against PKB/Akt (New England Biolabs); anti-ERK2 (C-14) and anti-Akt1/2 (H-136; Santa Cruz Biotechnology); the mouse monoclonal IgG1 antibodies HP2/1 against integrin subunit $\alpha 4$ (Immunotech); Act-1 against integrin subunit $\beta 7$ (kindly provided by Dr. A. Lazarovits); TS2/16 against integrin subunit

$\beta 1$ (kindly provided by Dr. F. Sanchez-Madrid); TS1/22 against integrin subunit $\alpha 1$ (ATCC); CSAT IgG2b against the chicken integrin subunit $\beta 1$ (DSHB, University of Iowa); and the rat monoclonal antibodies OXM718-FITC and PS/2-biotin against mouse integrin $\alpha 4$ and $\beta 1$, respectively (Cymbus Biotechnology). Mouse anti-T7-tag (Novagen); anti-CD45R (B220) and anti-mouse IgM microbeads (Miltenyi Biotec); mouse anti-human IgM (MH15; kindly provided by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands). Streptavidin-PE, Streptavidin-FITC, goat-anti-mouse-Biotin, and (Fab) $_2$ -fragments of goat anti-human IgM (DAKO); goat anti-mouse IgM and goat anti-mouse-PE (Southern Biotechnology Associates), (Fab) $_2$ -fragments of goat anti-mouse IgM and goat anti-human Fc-FITC (Jackson Immunoresearch Laboratories), goat anti-chicken IgM (Bethyl Laboratories), and rabbit anti-goat-Biotin (Vector). The pharmacological inhibitors PD-98059, LY-294002, Wortmannin, U-73122, Chelerythrine, cytochalasin D, Jasplakinolide, and Calpeptin (Biomol). Recombinant human sVCAM-1 (R&D Systems), human plasma Fibronectin, BSA (fraction V) and poly-L-Lysine (PLL) (Sigma-Aldrich).

Cell Lines and Culture. The Burkitt's lymphoma cell line Namalwa clone V3M (27) and the human preB cell line Nalm6 (28) were cultured as described. The chicken bursal lymphoma B cell line DT40 and DT40 cells deficient for both Lyn and Syk (29), Btk (29), PLC $\gamma 2$ (30), and for all three types of IP3R (31), obtained from Riken Cell Bank (Tsukuba Science City, Japan) with permission from Dr. T. Kurosaki (Kansai Medical University, Moriguchi, Japan), were cultured at 39.5°C as described (32). All DT40 cells, i.e., WT, gene-deficient and reconstituted (see below), showed similar expression of surface IgM and integrin $\beta 1$, as determined by FACS analysis, using goat anti-chicken IgM (10 μ g/ml) and rabbit anti-goat-Biotin/Streptavidin-PE, or CSAT (3 μ g/ml) and goat anti-mouse-PE, respectively, analyzed with a FACSCaliburTM (Beckton Dickinson) using CELLQuest software.

Isolation of Primary Tonsillar and Splenic B Cells. Human tonsillar B cells were isolated essentially as previously described (16). Typically, the obtained cell population contains >97% B cells as determined by FACS analysis. Isolated B cells were maintained in RPMI containing 10% FCS and were used immediately or after overnight storage at 4°C.

Mouse splenic B cells from C57BL/6 mice, which were bred and maintained at the animal care facility of the Academic Medical Center, were obtained using the MACS system (Miltenyi Biotec) by positive selection with anti-CD45R (B220) microbeads, essentially according to manufacturer's instruction.

Mice and BM Cultures. Btk⁻/LacZ mice were crossed onto a C57BL/6 background for over 6 generations, and Btk genotyped as described (33). 3–83 μ δ Ig transgenic mice which bear 3–83 Ig heavy and κ -light chain transgenes (anti-H-2Kkb) on a B10.D2 background (34), were crossed with WT or Btk-deficient mice. These mice were bred and maintained at the animal care facility of the Erasmus MC (Rotterdam, the Netherlands).

To obtain mouse preB cells or B cells, IL-7-driven primary BM cultures of WT and Btk-deficient mice, or of 3–83 transgenic WT and Btk-deficient mice, respectively, were performed essentially as described (35, 36). In brief, BM cells were erythrocyte depleted and cultured at 10⁷ cells/4 ml/well in 6-well plates in IMDM supplemented with 10% FCS and 100 U/ml murine IL-7 (R&D) for 5 d. Naive B cells from IL-7-driven BM cultures of 3–83 μ δ Ig transgenic WT and Btk-deficient mice were used since mature B cells from Btk-deficient mice have an aberrant immature IgM^{high} phenotype. The IL-7 driven BM cultures of the nontransgenic

WT or Btk-deficient mice typically resulted in >98% B220⁺ cells and ~80% and 95% preB cells (B220⁺/IgM⁻), respectively. Cultures from 3–83 transgenic mice contained >95% B220⁺/IgM⁺ B cells. Expression levels of integrin $\alpha 4$ and $\beta 1$ on mice preB cells were determined by FACS analysis using OXM718-FITC (1:20) and PS/2-biotin (1:80) antibodies, respectively.

Generation of Reconstituted DT40 Cells. The reconstitution of the Btk- or PLC $\gamma 2$ -deficient DT40 cell lines was performed by electroporation, using pApuro expression vectors containing the T7-tagged cDNAs for human Btk (29) and rat PLC $\gamma 2$ (30), which were provided by Dr. T. Kurosaki (Kansai Medical University, Moriguchi, Japan). Briefly, 25 μ g linearized plasmid DNA (pApuro-T7-Btk or T7-PLC $\gamma 2$) was added to 10^7 cells in 0.5 ml culture medium in a 0.4 cm electrode gap Gene Pulser Cuvette (BioRad) and electroporated using a Gene Pulser Apparatus with Capacitance Extender (BioRad) at 250 V, 960 μ F. After 24 h recovery at 39.5°C in DT40 medium, cells were selected in DT40 medium containing 0.5 μ g/ml Puromycin (Sigma). Puromycin-resistant clones were screened for expression of the transgene by immunoblotting with anti-T7. At least three clones of each reconstituted stable cell line were tested and found to be functionally restored in BCR-induced adhesion.

Immunoblotting. After stimulation of 10^7 cells/ml RPMI with 10 μ g/ml anti-IgM antibody, cells were directly lysed in SDS-PAGE sample buffer. 2×10^5 cells were applied on a 10% SDS-PAGE gel and blotted with anti-phospho-mitogen-activated protein kinase (MAPK)1/2 and anti-phospho-PKB, followed by HRP-conjugated goat anti-rabbit and developed by enhanced chemiluminescence (Amersham Pharmacia). To confirm equal expression (WT versus deficient DT40 cells) and loading, the blots were stripped or occasionally blots were generated in a parallel fashion, and incubated with the antibodies anti-ERK2 and anti-PKB.

Cell Adhesion Assay. Adhesion assays were done in triplicate on round-bottom 96-well plates (Costar) coated overnight at 4°C with PBS containing, unless otherwise indicated, 1 μ g/ml sVCAM-1, 10 μ g/ml Fibronectin, 4% BSA, or for 15 min at 37°C with 1 mg/ml poly-L-lysine (PLL), and blocked for 2 h at 37°C with 4% BSA in RPMI 1640. Cells were pretreated with the pharmacological inhibitors at 37°C or anti-integrin antibodies TS1/22 (1:1000), Act-1 (1:100), TS2/16 (1:5), and HP2/1 (1:250) at 4°C for 30 min in RPMI with 1% BSA. Subsequently, cells were stimulated with either 10 μ g/ml mouse anti-human IgM MH15, goat anti-chicken IgM, (Fab)₂ fragments of anti-human or anti-mouse IgM, anti-mouse IgM microbeads (1:300) or 50 ng/ml PMA, and 1.5×10^5 Namalwa or DT40 cells, or 3×10^5 primary B cells, were plated in 100 μ l/well and incubated at 37°C (39.5°C for DT40) for 30 min, unless otherwise indicated. After extensive washing of the plate with RPMI containing 1% BSA to remove nonadhering cells, the adherent cells were fixed for 10 min with 10% glutaraldehyde in PBS and stained for 45 min with 0.5% crystal violet in 20% methanol. After extensive washing with water, the dye was eluted in methanol and absorbance was measured after 5 to 40 min at 570 nm on a spectrophotometer (Microplate Reader 450, Biorad). Background absorbance (no cells added) was subtracted. Absorbance due to nonspecific adhesion, as determined in wells coated with 4% BSA, was always less than 10% of the absorbance of anti-IgM-stimulated cells. Maximal (100%) adhesion was determined by applying the PMA-stimulated cells to wells coated with the positively charged PLL, resulting in nonspecific adhesion of all cells, without washing the wells before fixation (see also Fig. 1, A–D). Unless otherwise indicated, adhesion of the nonpretreated anti-

IgM-stimulated cells was normalized to 100% and the bars represent the means \pm SD of a triplicate experiment representative of at least three independent experiments.

Soluble VCAM-1 Binding Assay. DT40 cells were incubated with or without 10 μ g/ml goat anti-chicken IgM in RPMI + 1% BSA for 10 min at 39.5°C. Subsequently 5×10^4 cells were incubated in a 96-well V-shaped bottom plate without or with 2 or 10 μ g/ml soluble VCAM-1-Fc (kindly provided by Y. van Kooyk) in the absence or presence of 3 mM MnCl₂ in a final volume of 25 μ l for 30 min at 4°C (similar results were obtained when anti-IgM or MnCl₂ and VCAM-1-Fc were added simultaneously and cells were incubated for 30 min at 39.5°C). After washing the cells twice, the cells were incubated with goat anti-human Fc-FITC (1:200) in RPMI + 1% BSA for 30 min at 4°C, washed twice, and analyzed by FACS. Mean fluorescence intensity (MFI) values are depicted after subtraction of the MFI values obtained in the absence of soluble VCAM-1-Fc.

Analysis of Integrin $\alpha 4\beta 1$ Clustering by Confocal Microscopy. DT40 cells (1.5×10^5) were incubated with or without 10 μ g/ml goat anti-chicken IgM in RPMI for different time periods at 39.5°C. Subsequently, cells were fixed with 4% paraformaldehyde in PBS for 15 min at RT, incubated with 7.5 μ g/ml CSAT for 30 min at 4°C, washed, incubated with biotinylated goat-anti-mouse (1:400) for 30 min at 4°C, washed, incubated with Streptavidin-FITC (1:200) for 15 min at 4°C, and washed again. Finally, the cell suspension was spotted onto 0.1% PLL-coated slides, and after 5 min the slides were rinsed and cells were mounted in Vectashield for confocal laser scanning microscopy, using a Leica TCS SP2 with a krypton/argon laser at 488 nm and a 63 \times lens. To evaluate integrin clustering, horizontal 0.6 μ m optical serial sections were taken throughout the cell. Clustering was inspected at midheight optical sections and by whole cell projections along the z-axis using maximum fluorescence values.

Statistical Analysis. The unpaired two-tailed Student's *t* test was used to determine the significance of differences between means. All relevant comparisons (e.g., control versus inhibitors or WT versus deficient DT40) were significantly different ($P < 0.01$).

Results

BCR Activation Induces Integrin $\alpha 4\beta 1$ -mediated B Cell Adhesion to VCAM-1 and FN. To investigate whether the BCR is able to control B cell adhesion, freshly isolated human tonsillar and murine splenic B cells were stimulated with anti-IgM antibody to ligate and activate the BCR, or with the phorbol ester PMA to activate PKC, and plated on a surface coated with either VCAM-1 or FN. As shown in Fig. 1, A and B, BCR activation, as well as PMA treatment, resulted in a strong stimulation of adhesion to both VCAM-1 and FN. Anti-IgM stimulation, as well as PMA-treatment of the human B cell line Namalwa, which resembles GC B cells, also resulted in strongly enhanced adhesion to both VCAM-1 and FN (Fig. 1 C). Similar results were obtained using chicken DT40 B cells (Fig. 1 D).

As shown in Fig. 1 E, antibody HP2/1, known to block $\alpha 4\beta 1$ function, specifically and completely blocked both basal as well as BCR-induced adhesion of Namalwa cells to VCAM-1, whereas no effect was observed with either an isotype control IgG1, or the specific antibodies TS1/22, blocking LFA-1, or Act-1, blocking integrin $\alpha 4\beta 7$. On the

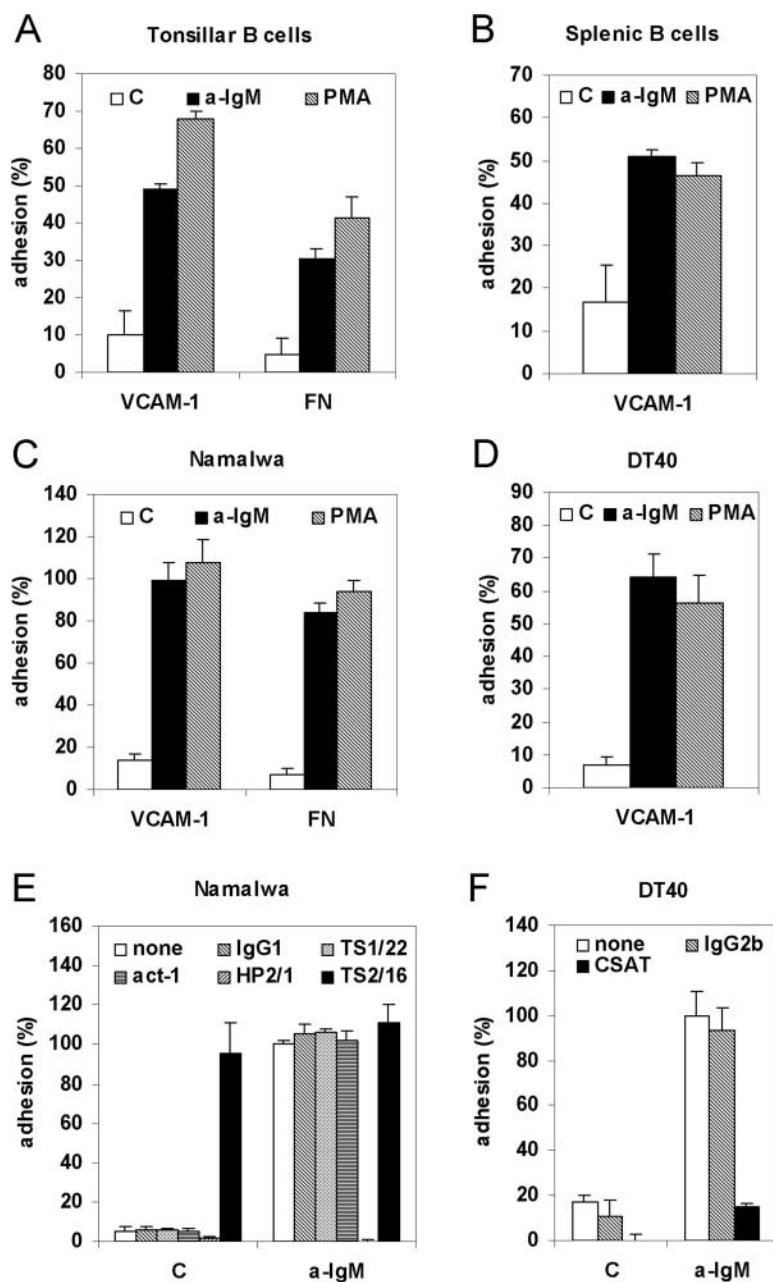


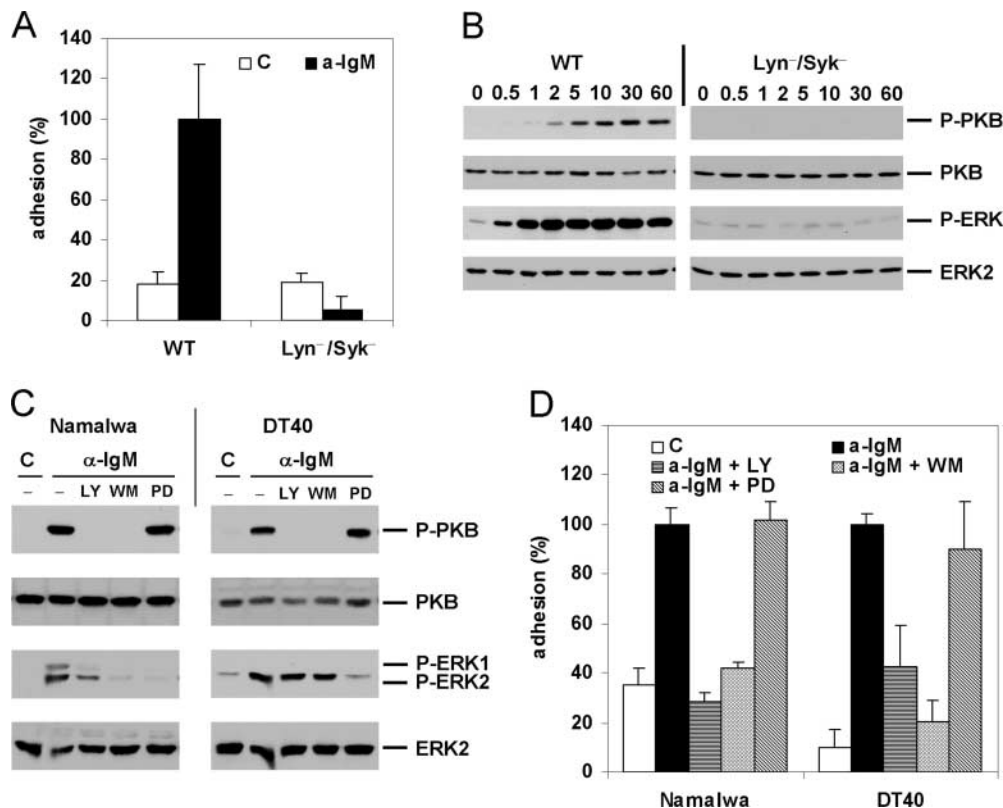
Figure 1. BCR activation induces integrin $\alpha 4\beta 1$ -mediated B cell adhesion to VCAM-1 and FN. (A) Primary tonsillar B cells were not stimulated (C) or stimulated with (Fab)₂-fragments of anti-IgM (a-IgM) or PMA and plated for 20 min on a surface coated with either VCAM-1 or FN, as indicated. (B) Mouse splenic B cells were not stimulated (C) or stimulated with (Fab)₂-fragments of anti-IgM (a-IgM) or PMA and plated on a surface coated with 0.3 μ g/ml VCAM-1. (C) Namalwa cells were not stimulated (C) or stimulated with anti-IgM (a-IgM) or PMA and plated on a surface coated with either VCAM-1 or FN, as indicated. (D) DT40 cells were not stimulated (C) or stimulated with anti-IgM (a-IgM) or PMA and plated on a surface coated with VCAM-1. (E) Namalwa cells were preincubated for 1 h at 4°C with medium alone (none), 10 μ g/ml of an IgG1 isotype control (IgG1), or antibody TS1/22 blocking LFA-1 (TS1/22), Act-1 blocking $\alpha 4\beta 7$ (Act-1), HP2/1 blocking $\alpha 4\beta 1$ (HP2/1), or antibody TS2/16 activating integrin $\alpha 4\beta 1$ (TS2/16). Subsequently, cells were not stimulated (C) or stimulated with anti-IgM (a-IgM), as indicated, and plated on a surface coated with VCAM-1. (F) DT40 cells were preincubated for 1 h at 4°C with medium alone (none), or 10 μ g/ml of an IgG2b isotype control (IgG2b) or antibody CSAT against integrin subunit $\beta 1$ (CSAT). Subsequently, cells were stimulated with anti-IgM and plated on a surface coated with VCAM-1. (A–D) The adhesion is presented as absolute adhesion, with input being determined by adhesion of all cells to PLL (= 100% adhesion). (E and F) The adhesion was normalized to 100% for the anti-IgM-stimulated cells.

other hand, antibody TS2/16, known to activate integrin $\alpha 4\beta 1$, strongly promoted adhesion to VCAM-1. Furthermore, the antibody CSAT, directed against the chicken integrin $\beta 1$ subunit, blocked the BCR-controlled adhesion of DT40 cells to VCAM-1 (Fig. 1 F). Taken together, our data demonstrate that stimulation of the BCR of either primary B cells or B cell lines results in their enhanced adhesion to VCAM-1, which is mediated by integrin $\alpha 4\beta 1$.

BCR-controlled Adhesion Requires Activation of Lyn, Syk, and PI3K, but Not MAPK Kinase or ERK. Given the proximal and prominent role in BCR signaling for Lyn, a Src-family tyrosine kinase, and Syk, a member of the ZAP70/Syk family of tyrosine kinases, we investigated their possible role in the regulation of integrin-mediated

adhesion by the BCR. As shown in Fig. 2 A, in Lyn/Syk double-deficient DT40 cells the BCR-induced adhesion to VCAM-1 was completely abolished.

As shown in Fig. 2, B and C, BCR stimulation of DT40 and Namalwa cells results in a strong activation of PKB/Akt-1 and the MAP kinase ERK2/MAPK1 and, in Namalwa cells, also of ERK1. In agreement with previous studies (37–41), and confirming the prominent role of Lyn and Syk in BCR signaling, the BCR-induced activation of PKB and ERK2 was completely abolished in the Lyn/Syk-deficient cells (Fig. 2 B), whereas their activation by PMA was not affected (data not depicted). Furthermore, the activation of ERK1 and 2 is completely abolished by pretreatment of the cells with the MAPK kinase (MEK) inhibitor



PD-98059 (PD), but not in DT40, and only partially in Namalwa, by treatment with the PI3K inhibitors LY-294002 (LY) or Wortmannin (WM) (Fig. 2 C). By contrast, PKB activation was completely abrogated by pretreatment with the PI3K inhibitors LY and WM, but was not affected by the MEK inhibitor PD (Fig. 2 C). Thus, BCR stimulation results in activation of PI3K, which is required for the activation of PKB and, in Namalwa, is also partially involved in the activation of ERK.

PI3K, MEK, and ERK have previously been implicated in the regulation of integrin activity by other stimuli (25). As shown in Fig. 2 D, the BCR-induced integrin-mediated adhesion to VCAM-1 of both Namalwa and DT40 cells was completely abolished upon treatment of the cells with the PI3K inhibitors LY or WM, whereas treatment with the MEK inhibitor PD had no effect. Taken together, these data demonstrate that Lyn- and Syk-dependent activation of PI3K is required for BCR-induced $\alpha 4\beta 1$ activation, whereas activation of MEK or ERK is not involved.

Btk Is Required for BCR-controlled Adhesion. PI3K activation results in the formation of phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 mediates the plasma membrane translocation and activation of several signaling molecules, including the cytoplasmic tyrosine kinase Btk, the serine/threonine kinase PKB, or the exchange factor Vav, as a

consequence of the interaction of their PH domains with PIP3. Interestingly, using Btk-deficient DT40 cells, we observed a complete loss of BCR-, but not PMA-, induced adhesion to VCAM-1 (Fig. 3 A). This defect is not due to clonal variation since it could be completely restored by stable transfection of an expression construct encoding Btk (Fig. 3 A). In agreement with previous data, we observed that, whereas ERK2 activation was reduced in the Btk-deficient DT40 cells (40), PKB activation remained unaffected (37, 41; however see 39) (Fig. 3 B). Thus, whereas (PI3K-mediated) activation of PKB is not sufficient, Btk is required for activation of integrin $\alpha 4\beta 1$ by the BCR.

These observations are particularly interesting since Btk-deficiency causes the B cell immunodeficiency disease XLA in humans and X-linked immunodeficiency (*Xid*) in mice. Therefore, we further studied the role of Btk in BCR-induced adhesion using B cells derived from Btk-deficient mice. As shown in Fig. 3 C, Btk-deficiency did not affect BCR-controlled integrin-mediated adhesion of mouse B cells. This difference with the chicken DT40 cells may be due to species-specific functional redundancy of Btk for other tec-family kinases in mouse B cells (see Discussion).

Pre-B cell differentiation, which is controlled by the presumably constitutively active ligand-independent pre-

Figure 2. BCR-controlled adhesion is dependent on Lyn and Syk and requires activation of PI3K, but not of MEK or ERK. (A) WT or Lyn/Syk double-deficient DT40 cells (Lyn⁻¹/Syk⁻¹) were not stimulated (C) or stimulated with anti-IgM (a-IgM) and plated on a surface coated with VCAM-1. (B) Activation of PKB and ERK in WT or Lyn/Syk double-deficient DT40 cells (Lyn⁻¹/Syk⁻¹) after stimulation with anti-IgM for the indicated period of time (min). The immunoblots are probed with anti-phospho-PKB (P-PKB) and anti-phospho-MAPK (P-ERK) and reprobbed with anti-ERK2 and anti-PKB, as indicated. (C) Activation of PKB and ERK in Namalwa cells (left) or DT40 cells (right) which were pretreated with 20 μ M LY294002 (LY), 100 nM Wortmannin (WM), 50 μ M PD98059 (PD), or left untreated (-) for 30 min, and stimulated with anti-IgM (a-IgM) or not (C) for 5 min, as indicated. The blots are probed as in B. (D) Namalwa cells (left) or DT40 cells (right) were pretreated with 50 μ M LY294002 (LY), 100 nM Wortmannin (WM), 50 μ M PD98059 (PD), or left untreated for 30 min, and not stimulated (C) or stimulated with anti-IgM (a-IgM) and plated on a surface coated with VCAM-1.

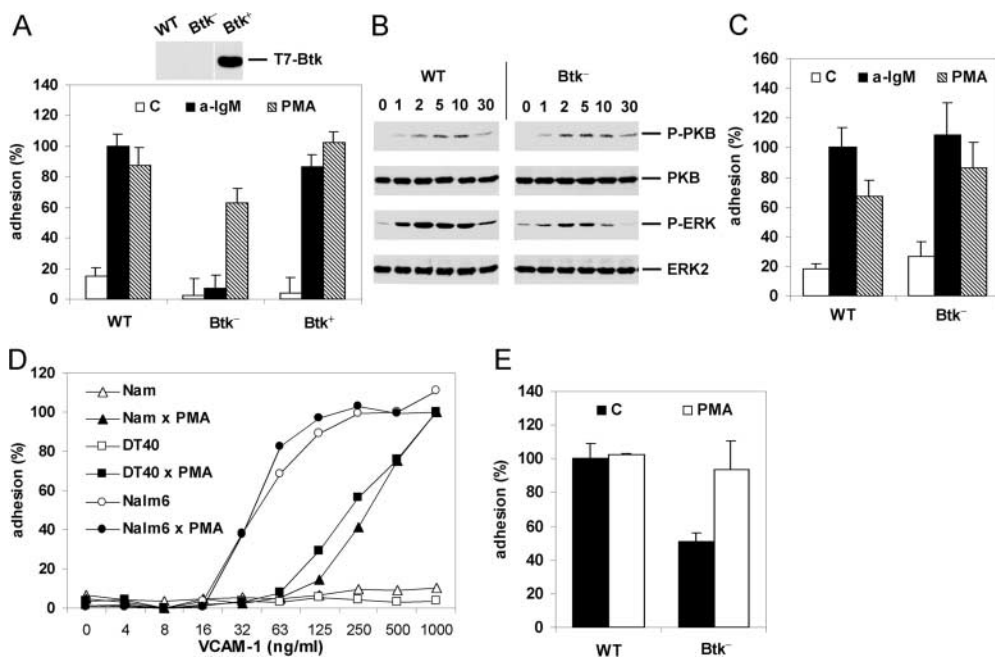


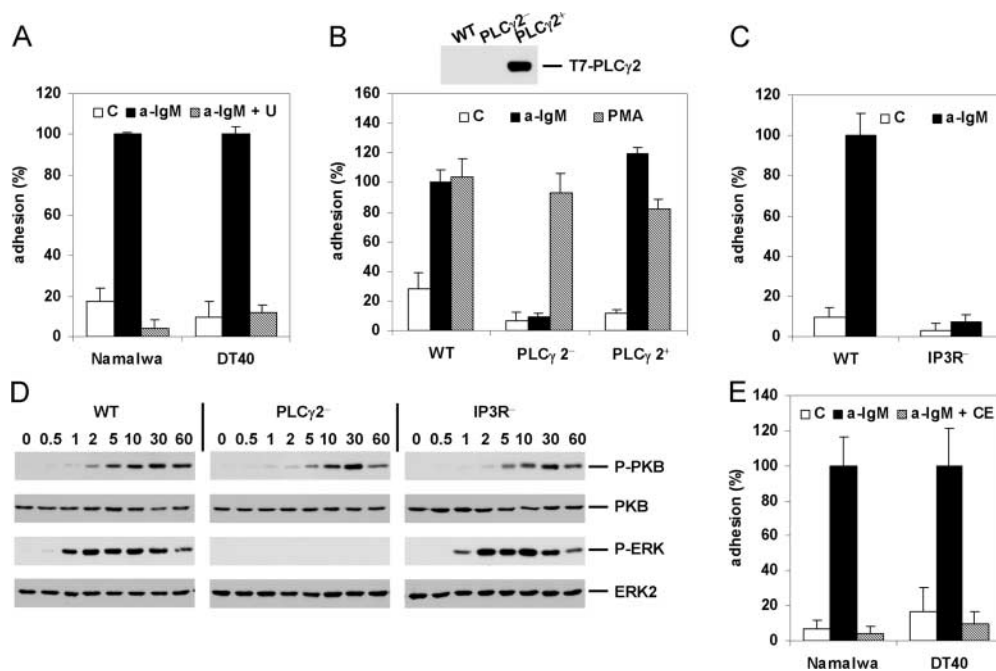
Figure 3. Btk is required for BCR-controlled adhesion. (A) WT, Btk-deficient (Btk^{-}), or Btk-deficient DT40 cells reconstituted with Btk (Btk^{+}) were not stimulated (C) or stimulated with anti-IgM (a-IgM) or PMA and plated on a surface coated with VCAM-1. The inset shows expression of Btk in the Btk-reconstituted DT40 cell-line as detected by immunoblotting with anti-T7. (B) Activation of PKB and ERK in WT or Btk-deficient (Btk^{-}) DT40 cells after stimulation with anti-IgM for the indicated period of time (min). The blots are probed as in Fig. 2 B. (C) B cells from WT or Btk-deficient (Btk^{-}) mice were not stimulated (C) or stimulated with anti-IgM (a-IgM) or PMA and plated for 15 min on a surface coated with 0.3 $\mu\text{g/ml}$ VCAM-1. The experiment shown is representative for two independent experiments. (D) Nalm6 (circles), DT40 (squares), or Namalwa (triangles) cells were not stimulated (open symbols) or stimulated with PMA (closed symbols) and plated for 20 min on a surface coated with different concentrations of VCAM-1. The adhesion was normalized to 100% for the PMA-stimulated cells plated on 1 $\mu\text{g/ml}$ VCAM-1. The experiment shown is representative for two independent experiments. (E) Pre-B cells from WT or Btk-deficient (Btk^{-}) mice were not stimulated (C) or stimulated with PMA and plated for 15 min on a surface coated with 0.3 $\mu\text{g/ml}$ VCAM-1. The adhesion was normalized to 100% for the unstimulated WT preB cells. The bars represent the means \pm SD of 2 WT and 2 Btk-deficient mice, each assayed in triplicate. In a total of three independent experiments (6 WT and 6 Btk-deficient mice), the basal adhesion of the Btk-deficient mice was $54\% \pm 10\%$ in comparison to WT preB cells.

BCR (19, 21, 22, 42), is impaired in Btk-deficient mice (33, 43) and almost completely arrested in XLA patients (44, 45). Interestingly, using mouse preB cells and the human preB cell line Nalm6, in which Btk is constitutively phosphorylated (46, 47), we observed high basal integrin-mediated adhesion which, in contrast to DT40 or Namalwa cells, could not be further enhanced by PMA (Fig. 3, D and E). Noteworthy, the PI3K inhibitor Wortmannin reduced the high basal adhesion of the Nalm6 cells (on 0.125 $\mu\text{g/ml}$ VCAM-1; data not depicted). Moreover, preB cells derived from Btk-deficient mice showed an $\sim 50\%$ lower basal adhesion in comparison to WT preB cells, which could be enhanced by PMA up to the level of adhesion of the WT preB cells (Fig. 3 E). The WT and Btk-deficient preB cells display similar expression levels of integrin $\alpha 4$ and $\beta 1$, as determined by FACS analysis (data not depicted). Since the preBCR signals in a presumably constitutively active ligand-independent fashion involving Btk (42, 46, 48, 49), these results suggest that Btk mediates integrin activation by the preBCR. In conclusion, our data reveal a critical regulatory function for Btk in the (pre)BCR-controlled integrin-mediated adhesion.

BCR-controlled Adhesion Requires PLC $\gamma 2$ Activation, IP3R-mediated Ca $^{2+}$ Release, and PKC Activation. Given the intriguing critical role of Btk in BCR-induced integrin activation, we wished to further extend and strengthen our observations by exploring which signaling molecules may control this process downstream from Btk. A major candidate is PLC $\gamma 2$, since it has been implicated in BCR signal-

ing, and has been identified as a substrate for both Btk and Syk (29). Indeed, as shown in Fig. 4 A, treatment of Namalwa or DT40 cells with the PLC inhibitor U73122 abolished BCR-induced VCAM-1 adhesion. Noteworthy, U73122 also strongly reduced the high basal adhesion of the Nalm6 cells (on 0.125 $\mu\text{g/ml}$ VCAM-1) (data not depicted). Moreover, PLC $\gamma 2$ -deficient DT40 cells showed a complete loss of BCR-, but not PMA-, induced VCAM-1 adhesion (Fig. 4 B), which could be completely restored by gene complementation with a PLC $\gamma 2$ expression construct (Fig. 4 B).

PLC activation results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP $_2$) into inositol-1,4,5-trisphosphate (IP $_3$) and diacylglycerol (DG). IP $_3$ binds to IP $_3$ Rs on the endoplasmic reticulum resulting in the release of Ca $^{2+}$, whereas DG activates PKC. Using IP $_3$ R-triple-deficient DT40 cells, in which the genes encoding all three types of IP $_3$ Rs expressed in these cells have been inactivated resulting in a loss of BCR-mediated Ca $^{2+}$ release (31), we observed a complete loss of BCR-induced VCAM-1 adhesion (Fig. 4 C). Furthermore, in agreement with previous reports (32, 37, 39, 41), PKB activation does not require any PLC $\gamma 2$ - or IP $_3$ R-mediated signals, whereas ERK activation involves DG-dependent activation of PKC but not IP $_3$ R-mediated Ca $^{2+}$ -release (Fig. 4 D). Thus, the IP $_3$ R-deficient B cells reveal that simultaneous activation of both PKB and ERK is not sufficient for integrin $\alpha 4\beta 1$ activation (Fig. 4, C and D). Finally, treatment of Namalwa or DT40 cells with the PKC inhibitor Chelerythrine



(D) Activation of PKB and ERK in WT, PLC γ 2(PLC γ 2⁻), or IP3R(IP3R⁻)-deficient DT40 cells after stimulation with anti-IgM for the indicated period of time (min). The blots are probed as in Fig. 2 B. (E) Namalwa cells (left) or DT40 cells (right) were pretreated with 5 or 10 μ M Chelerythrine (CE), respectively, or left untreated for 30 min, and subsequently not stimulated (C) or stimulated with anti-IgM (a-IgM) and plated on a surface coated with VCAM-1.

abolished PMA- (data not depicted) and BCR-induced VCAM-1 adhesion (Fig. 4 E), whereas treatment with the Ca²⁺-ionophore Ionomycin did not result in enhanced integrin-mediated adhesion (data not depicted). Combined, these data indicate that activation of PLC γ 2 and subsequently the concomitant IP3R-mediated release of calcium and DG-mediated activation of PKC are required for BCR-controlled integrin α 4 β 1-mediated adhesion.

BCR-controlled Integrin α 4 β 1-mediated Adhesion Involves Cytoskeletal Reorganization and Integrin Clustering. Integrin activation can be accomplished by a conformational change resulting in enhanced ligand affinity, by an increase in lateral mobility and clustering resulting in enhanced avidity, or both (25, 26). As shown in Fig. 5 A, whereas MnCl₂ treatment resulted in enhanced binding of soluble VCAM-1 to DT40 cells, no change of α 4 β 1 affinity for VCAM-1 was observed after stimulation with anti-IgM. In contrast, confocal microscopy analysis revealed enhanced α 4 β 1 clustering after anti-IgM stimulation of DT40 cells. Stimulation with anti-IgM for 15 min results in the formation of many small clusters, whereas after 30 min rather large clusters of α 4 β 1 are observed (Fig. 5 B). PMA stimulation results in the formation of larger and more diffuse α 4 β 1 clusters in WT and Btk-deficient DT40 cells (Fig. 5 B). In Btk-deficient DT40 cells, however, consistent with their disability to adhere to VCAM-1 upon BCR activation (Fig. 3), no anti-IgM-induced α 4 β 1 clustering is observed (Fig. 5 B).

The actin cytoskeleton has been implicated in the control of clustering of integrins, including of LFA-1 and α 4 β 1, and in integrin-mediated adhesion (26, 50–54). To investigate whether (re)organization of the actin cytoskele-

ton contributes to the BCR-induced α 4 β 1-mediated adhesion, and to provide further support for the role of α 4 β 1 clustering in this process, we examined the effect of Cytochalasin D (CD), which prevents actin filament polymerization and organization, and Jaspilkinolide (JP), which prevents actin depolymerization and stabilizes preexisting actin filaments. Since the Ca²⁺-dependent protease Calpain has previously been implicated in TCR-, chemokine-, and PMA-induced LFA-1 clustering and LFA-1-mediated T cell adhesion (53, 55), and given the observed critical role of IP3R-mediated Ca²⁺-release in BCR-controlled α 4 β 1 activation (Fig. 4 C), we also studied the effect of an inhibitor of Calpain, Calpeptin (CP). As shown in Fig. 5 C, pretreatment of DT40 and Namalwa cells with CD, JP, or CP completely abolished the BCR-induced α 4 β 1-mediated adhesion to VCAM-1. Taken together, these data indicate that the control of integrin α 4 β 1-mediated B cell adhesion by the BCR involves cytoskeletal (re)organization and stimulation of integrin avidity.

Discussion

The Inside-out Signaling Mechanism Underlying BCR-controlled Integrin α 4 β 1 Activation. In this study we demonstrate that stimulation of the BCR results in inside-out activation of integrin α 4 β 1 and consequently in strongly enhanced adhesion of B cells to FN and VCAM-1 (Fig. 1). Using a combined biochemical, pharmacological, and genetic approach, we also show that the tyrosine kinases Lyn and Syk, as well as PI3K, are required for BCR-controlled α 4 β 1-mediated adhesion (Fig. 2). By contrast, activation of

Figure 4. BCR-controlled adhesion requires activation of PLC γ 2, IP3R-mediated Ca²⁺ release, and PKC. (A) Namalwa cells (left) or DT40 cells (right) were pretreated with 1 or 2.5 μ M U73122 (U), respectively, or left untreated for 30 min, and subsequently not stimulated (C) or stimulated with anti-IgM (a-IgM) and plated on a surface coated with VCAM-1. (B) WT, PLC γ 2-deficient (PLC γ 2⁻), or PLC γ 2-deficient DT40 cells reconstituted with PLC γ 2 (PLC γ 2⁺) were not stimulated (C) or stimulated with anti-IgM (a-IgM) or PMA and plated on a surface coated with VCAM-1. The inset shows expression of PLC γ 2 in the PLC γ 2-reconstituted DT40 cell-line as detected by immunoblotting with anti-T7. (C) WT or IP3R-deficient DT40 cells (IP3R⁻) were not stimulated (C) or stimulated with anti-IgM (a-IgM) and plated on a surface coated with VCAM-1.

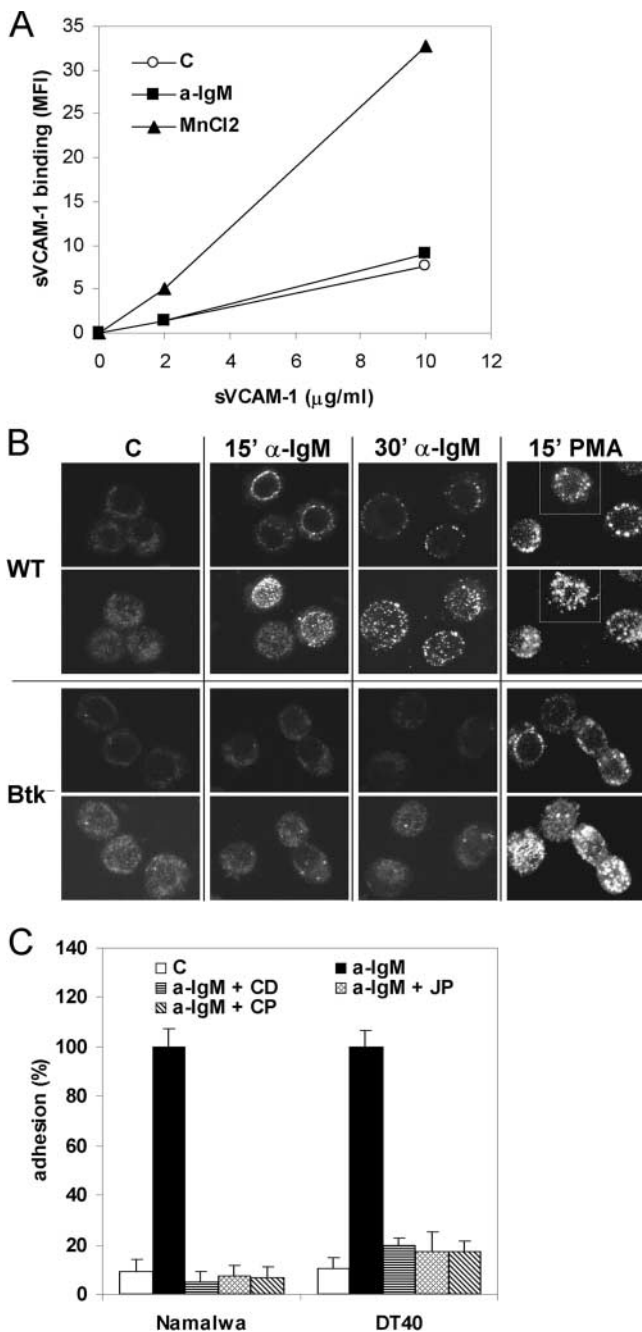


Figure 5. BCR-controlled integrin $\alpha 4\beta 1$ -mediated adhesion involves integrin clustering and cytoskeletal reorganization. (A) DT40 cells were incubated without (open circles) or with 10 $\mu\text{g}/\text{ml}$ anti-IgM (closed squares) or 3 mM MnCl_2 (closed triangles) and 0, 2, or 10 $\mu\text{g}/\text{ml}$ soluble VCAM-1-Fc, as indicated. Binding of soluble VCAM-1-Fc was measured by FACS analysis. Mean fluorescence intensity (MFI) values of a representative experiment are depicted. (B) DT40 cells, either WT or Btk-deficient (Btk^-), were not stimulated (C) or stimulated for 15 or 30 min with anti-IgM (a-IgM) or PMA, as indicated. Clustering of integrin $\alpha 4\beta 1$ was analyzed by means of confocal microscopy. For each condition, representative images of optical midheight sections (1st and 3rd row), and corresponding whole cell projections along the z-axis using maximum fluorescence values (2nd and 4th row), are shown. (C) Namalwa cells (left) or DT40 cells (right) were pretreated for 30 min with 1 $\mu\text{g}/\text{ml}$ Cytochalasin D (CD), 1 μM Jaspalakinolide (JP), 100 $\mu\text{g}/\text{ml}$ Calpeptin (CP), or left untreated, and subsequently not stimulated (C) or stimulated with anti-IgM (a-IgM) and plated on a surface coated with VCAM-1.

MEK or the MAP kinases ERK1 or 2 is not involved (Fig. 2 D). In concordance with the observed critical role of Lyn, Syk, and PI3K in $\alpha 4\beta 1$ activation, activation of PI3K by the BCR can be mediated by Lyn (56, 57), and, via the adaptor protein BCAP, by Syk (24, 38, 39). The use of Btk-, PLC $\gamma 2$ -, and IP3R-deficient B cells revealed that PI3K-controlled activation of PKB, either alone or in combination with ERK2, is not sufficient for $\alpha 4\beta 1$ activation (Figs. 3 and 4). Interestingly, we found that the PI3K-controlled tyrosine kinase Btk is a critical mediator of BCR-induced adhesion (Fig. 3). Btk activation requires its translocation to the plasma membrane by the interaction of its PH domain with the PI3K product PIP₃, and phosphorylation of Y551 in the kinase domain activation loop (58). Indeed, reflecting their role in PI3K activation or Y551 phosphorylation, BCR-controlled Btk activation is mediated by Lyn and Syk (23, 24). We also found that the Btk substrate PLC $\gamma 2$ mediates the BCR-controlled adhesion (Fig. 4, A and B). PLC $\gamma 2$ activation by the BCR requires phosphorylation of the adaptor BLNK by Syk, thus creating docking sites for the SH2 domains of Btk and PLC $\gamma 2$. This brings PLC $\gamma 2$ into close proximity of Btk, resulting in its phosphorylation and activation by Btk (24). The apparent bifurcation of the signaling cascade underlying $\alpha 4\beta 1$ activation downstream of PLC $\gamma 2$, i.e., concomitant IP₃-mediated Ca^{2+} release and DG-mediated activation of PKC (Fig. 4, C and E), either indicates the involvement of a Ca^{2+} -dependent DG-activated PKC isotype (i.e., PKC α or β), or the requirement for PKC activation and Ca^{2+} release in a parallel fashion. Taken together, we have identified a (linear) cascade of essential signaling components underlying BCR-controlled $\alpha 4\beta 1$ activation, i.e., the (consecutive) activation of Lyn, Syk, PI3K, Btk, PLC $\gamma 2$, IP₃R-mediated Ca^{2+} release and PKC (Fig. 6), and although any alternative or branching pathways may also play a role, activation of those pathways alone will not be sufficient for $\alpha 4\beta 1$ activation by the BCR.

$\alpha 4\beta 1$ -mediated adhesion can be controlled by integrin clustering (avidity) (50, 51, 54), or by active regulation of $\alpha 4\beta 1$ affinity (59, 60). Our data revealed that the stimulation of the BCR results in enhanced $\alpha 4\beta 1$ avidity, whereas no change in affinity was observed (Fig. 5). The role for $\alpha 4\beta 1$ avidity regulation in the control of adhesion is further supported by our observation that BCR-induced $\alpha 4\beta 1$ -mediated adhesion is controlled by Ca^{2+} -release, activation of Calpain and (re)organization of the actin cytoskeleton (Figs. 4 and 5), since these processes have previously been implicated in clustering of integrins, in particular of LFA-1, underlying TCR- and chemokine-induced LFA-1-mediated adhesion (26, 51–55). In addition, recent studies also implicated membrane lipid rafts in the positive control of clustering and activation of integrins, indicating that the role of the cytoskeleton may involve stabilization of raft structures as well (61, 62). Combining our data with these studies, we propose that upon BCR stimulation $\alpha 4\beta 1$ may initially be released from a cytoskeletal constraint by Ca^{2+} -mediated Calpain activation (CP-sensitive) or actin depolymerization (JP-sensitive), followed by

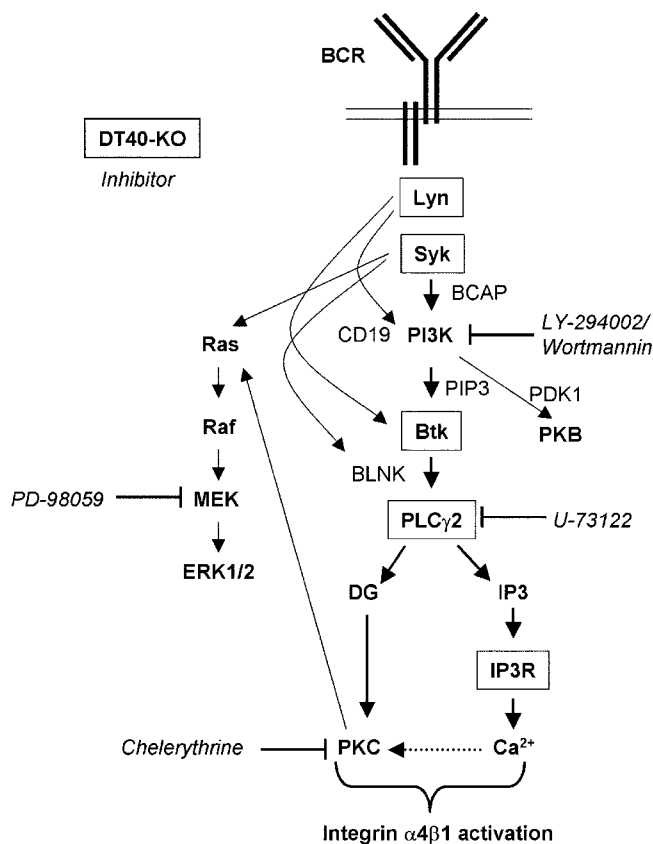


Figure 6. Molecular dissection of the signaling cascade underlying BCR-controlled integrin $\alpha 4\beta 1$ -mediated adhesion. A schematic representation of the signaling pathway underlying the inside-out activation mechanism of integrin $\alpha 4\beta 1$ upon stimulation of the BCR is shown. The proteins encoded for by the inactivated genes in the DT40 cells and the pharmacological inhibitors used in this study are boxed and italicized, respectively. For sake of clarity, the role of actin cytoskeleton (re)organization is not included. See the Discussion section for further detail.

mobilization to lipid rafts which are stabilized by the cytoskeleton (JP- and CD-sensitive), and the subsequent formation of $\alpha 4\beta 1$ clusters which may become tethered to the (repolymerized) actin cytoskeleton again (JP- and CD-sensitive), resulting in enhanced $\alpha 4\beta 1$ avidity and adhesion.

Integrin $\alpha 4\beta 1$ -mediated Adhesion in B Cell Development and Differentiation. During B cell development both the pre-BCR (19, 21, 22), and integrin-mediated adhesion to VCAM-1-expressing BM stromal cells and ECM (1, 3, 4), play a critical role in the survival of preB cells and their differentiation into immature B cells. The preBCR, which presumably signals in a ligand-independent fashion, controls signaling molecules similar to the BCR (42, 48). Therefore, it is tempting to speculate that expression of the preBCR controls integrin activity (see Fig. 3, D and E), which may be required for retention of the preB cells in the proper BM microenvironment and their transition into immature B cells.

Previously, we and others have shown that the interaction of GC B cells with FDCs, which plays a major role in antigen-specific B cell differentiation, is mediated by $\alpha 4\beta 1$ (2,

13, 14), and that adhesion through $\alpha 4\beta 1$ suppresses apoptosis of GC B cells (16, 17). Combined with our present findings, we propose that antigen-specific B cell differentiation is controlled by stimulation of the BCR by the FDC-presented antigen, generating signals that result in inside-out integrin activation. This will “lock” the interaction of the GC B cell with the FDC. The enhanced $\alpha 4\beta 1$ -mediated adhesion to FDCs may counteract GC B cell migration along a chemokine gradient, e.g., of BLC. After the initial migration arrest, the integrin-mediated formation of clusters of B cells and FDCs will give rise to the structural microenvironment of the GC. The retention of the GC B cells in these GCs and their contact with the FDC plays an important role in affinity maturation: without the proper signals provided by the GC microenvironment in general, and by the FDCs in particular, GC B cells will not proliferate and undergo apoptosis (12, 15). Moreover, the interaction with the FDC also generates integrin-mediated anti-apoptotic outside-in signals which may directly contribute to the clonal selection of the GC B cells (16, 17). Thus, by competing for antigen and integrin-mediated survival signals, B cells expressing a high affinity BCR will prevent or terminate the BCR signal in a B cell of lower affinity, which will consequently “unlock” from the FDC, resulting in apoptosis.

A Critical Role for Btk in the Control of Integrin Activity: Implications for XLA and Xid. This study is the first to demonstrate a critical role for Btk (and PLC $\gamma 2$ and IP3Rs) in the control of integrin activity (Fig. 3). 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%) and consequently in Ig serum levels (44, 45). In *Xid* and the phenotypically identical Btk-deficient mice, however, mature B cell numbers are only 50% reduced (63). This may be due to functional redundancy of Btk with other Tec family members in murine B cells, as illustrated by the ability to overcome signaling defects in Btk-deficient DT40 cells and XLA patient B cells by expression of other Tec family members (64, 65), and the observation that, in contrast to the Btk- or Tec-deficient mice, mature B-cells are almost absent in Btk/Tec double-deficient mice (66). In particular since, in contrast to Btk-deficient chicken DT40 cells (29) and XLA patient B cells (65), *Xid* mice mature B cells also show (near) normal BCR-induced activation of PLC $\gamma 2$ (67), PI hydrolysis (68), and Ca $^{2+}$ release (67–69), this species-specific redundancy may explain why Btk-deficient murine naive B cells exhibit normal BCR-induced adhesion as well (Fig. 3 C).

The earliest role for Btk in B cell development occurs at the switch from preB cells to immature B cells, which is severely impaired in XLA patients and, to a lesser extent, in *Xid* and *Btk* $^{-/-}$ mice (33, 43, 44). In XLA this is due to a proliferation defect of the μ H-chain positive preB cells (45). Supported by the observation that the proliferation of preB cells from $\alpha 4$ integrin-deficient mice is also reduced (4), and because the preBCR signals through Btk (46, 48, 49), our results suggest that the loss of control of integrin-mediated adhesion by the preBCR may contribute to this

proliferation defect in XLA preB cells (Fig. 3, D and E; see also discussion above). In addition, both Btk and integrins are involved in the retention (2, 70) and positive selection (63, 71) of immature B cells in the BM, as well as in the development, survival and follicular entry of long-lived recirculating follicular cells (33, 63, 72). Furthermore, besides a loss of T-independent antigen responses, *Xid* and Btk-deficient mice show a strongly reduced primary and a variably affected secondary T-dependent antigen response, whereas both responses are absent in Btk/*Tec* double-deficient, which is accompanied by smaller follicles and less GCs (63, 66, 73, 74). Gain-of-function E41K-Btk transgenic mice also revealed a loss of the T-dependent antigen response, smaller follicles and the absence of marginal zones and GCs (72). This may reflect a defect in (BCR-controlled) $\alpha 4\beta 1$ activity of GC B cells, which, as discussed above, has an important role in GC formation and antigen-specific B cell differentiation. Finally, it is noteworthy that mice deficient in the p85 subunit of PI3K, BLNK, PLC $\gamma 2$, or PKC β , which like Btk play a critical role in the signaling cascade underlying BCR-controlled integrin activity (Figs. 2, 4, and 6), reveal a *Xid*-like phenotype as well (75).

In conclusion, whereas the control of integrin activity by the (pre)BCR plays an important role in B cell development and antigen-specific B cell differentiation, the loss of control of integrin activity, due to loss-of-function germline mutations of *BTK*, may contribute to the developmental and functional B cell defects as observed in *Xid* mice and XLA patients.

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