Activation of the Small GTPase Rac2 via the B Cell Receptor Regulates B Cell Adhesion and Immunological-Synapse Formation

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SUMMARY

The integrin leukocyte function-associated antigen-1 (LFA-1) is important in the promotion of B cell adhesion, thereby facilitating immunological synapase (IS) formation and B cell activation. Despite this significance, the associated signaling mechanisms regulating LFA-1 activation remain elusive. Here, we show that both isoforms of the small GTPase Rac expressed by primary B cells, Rac1 and Rac2, were activated rapidly downstream of Src-family kinases, guanine-nucleotide exchange factors Vav1 and Vav2, and phosphoinositide-3-kinase (PI3K) after BCR engagement. We identify Rac2, but not Rac1, as critical for B cell adhesion to intercellular adhesion molecule-1 (ICAM-1) and IS formation. Furthermore, B cells expressing constitutively active Rac2 are highly adhesive. We observe that Rac2-deficient B cells exhibit lower amounts of Rap1-GTP and severe actin polymerization defects, identifying a potential mechanism underlying their behavior. We postulate that this critical role for Rac2 in mediating B cell adhesion and IS formation might apply in all lymphocytes.

INTRODUCTION

Recognition of specific antigen by the B cell receptor (BCR) triggers intracellular signaling cascades, leading ultimately to B cell activation, proliferation, and differentiation (Reth and Wienands, 1997). Although soluble antigens can activate B cells, membrane-bound antigens are substantially more efficient in promoting B cell activation. Because B cells will most likely encounter antigen in vivo in the form of immune complexes tethered to cell surfaces by fragment crystallizable (Fc) or complement receptors (Carrasco and Batista, 2006), the mechanisms of membrane-bound-antigen recognition are of particular interest. Indeed, recognition of specific antigen presented by dendritic cells (DCs) (Qi et al., 2006; Wykes et al., 1998), macrophages (Carrasco and Batista, 2007; Phan et al., 2007), or follicular dendritic cells (FDCs) (Szakal et al., 1988) leads to B cell activation. Thus, recognition of membrane-bound antigens is important in the determination of B cell fate in vivo.

The interaction of B cells with membrane-bound ligand is a complex process involving multiple receptors. Leukocyte function-associated antigen-1 (LFA-1) is of particular significance because it has been reported to promote B cell survival in the germinal center (Koopman et al., 1994), where it can bind its main ligand, intracellular adhesion molecule-1 (ICAM-1) on the DC or FDC membrane (Koopman et al., 1991; Kushnir et al., 1998). Importantly, the interaction of LFA-1 with ICAM-1 supports antigen aggregation at limited antigen densities and therefore facilitates B cell activation (Carrasco et al., 2004). We recently established that membrane-bound-antigen recognition by B cells in the presence of ICAM-1 leads to the formation of an immunological synapse (IS) (Carrasco et al., 2004), similar to that described for T cells (Grakoui et al., 1999; Krummel et al., 2000; Monks et al., 1998). IS formation is preceded by a two-phase cellular response, involving spreading and contraction, that facilitates antigen gathering by B cells (Fleire et al., 2006). The IS is organized such that during the cellular response, BCR-antigen complexes are accumulated in the central supramolecular activation cluster (cSMAC), whereas LFA-1 is segregated into the peripheral SMAC (pSMAC). However, signaling downstream of the BCR, which regulates the large-scale cytoskeletal rearrangements involved in IS formation, has not yet been elucidated.

On resting lymphocytes, LFA-1 is maintained in an inactive state. Engagement of cell-surface receptors, such as the BCR or T cell receptor (TCR), generates intracellular signals, leading to LFA-1-mediated cell adhesion (Alon and Dustin, 2007; Dustin and Springer, 1988). This process is termed inside-out activation and results in changes in LFA-1 conformation (affinity) and its distribution on the cell surface (avidity). The signaling mechanisms underlying the inside-out activation of LFA-1 and other integrins have been intensively studied primarily in T cells (Kini- shi, 2005). However, very few studies have addressed this issue in B cells. For example, B cell adhesion mediated by very late antigen-4 (VLA-4) requires the consecutive activation of the...
tyrosine kinases Lyn and Syk, phosphoinositide-3 kinase (PI3K), Bruton’s tyrosine kinase, phospholipase Cγ2, protein kinase C, and inositol 1,4,5-trisphosphate (IP3)-receptor-mediated calcium release (Spaargaren et al., 2003). Furthermore, B cell adhesion to ICAM-1 and fibronectin is substantially compromised in the absence of the SKAP-55 homolog (SKAP-HOM) (Togni et al., 2005). Finally, activation of Rap GTPases downstream of the BCR promotes LFA-1- and VLA-4-mediated B cell adhesion (McLeod et al., 2004).

The protooncogenic Vav family of hematopoietic-specific proteins (Vav1, Vav2, and Vav3) is rapidly tyrosine phosphorylated upon stimulation of lymphocyte receptors (Johmura et al., 2003; Kurosaki, 2002; Movilla and Bustelo, 1999) and is essential for B cell development (Doody et al., 2001; Tarakhovsky et al., 1995). One of the main functions of the Vav proteins is as guanine-nucleotide exchange factors (GEFs) for Rho GTPases such as Rac1 and Rac2, which are key regulators of rearrangements of the cytoskeleton. Whereas Rac1 is universally expressed, the expression of Rac2 is restricted to the hematopoietic system (Jaffe and Hall, 2005; Walmsley et al., 2003). Thus, given the role of Rho GTPases, Vav proteins might act as key players in linking receptor signaling to rearrangements of the actin cytoskeleton.

In this study, we have investigated the BCR-mediated signaling pathways that contribute to LFA-1-mediated adhesion and pSMAC formation at the IS. Engagement of the BCR led to activation of LFA-1-mediated B cell adhesion through a signaling pathway involving Src-family tyrosine kinases (SFKs), Vav1, Vav2, PI3K, and Rac2. This pathway for activation of LFA-1-mediated B cell adhesion and the associated cytoskeleton rearrangements necessary for IS formation are likely to be important for integrin activation in all lymphocytes.

RESULTS

Src-Family Tyrosine Kinases Are Required for B Cell Adhesion and IS Formation

To examine whether SFKs are required for LFA-1-mediated B cell adhesion, we stimulated naive C57BL/6 (WT) B cells with anti-IgM and settled onto planar lipid bilayers containing glycosylphosphatidylinositol (GPI)-linked ICAM-1. As shown in Figure 1A, BCR stimulation resulted in the formation of close contacts between B cells and the bilayers, visualized as dark areas by interference-reflection microscopy (IRM). IRM provided an effective means of measuring LFA-1-mediated B cell adhesion because binding to soluble ICAM-1 or beads coated with ICAM-1 could not be assessed after BCR stimulation (data not shown). Quantification of B cell adhesion frequency revealed a 75% increase after BCR crosslinking relative to unstimulated B cells (Figure 1B). Pretreatment of wild-type (WT) B cells with the SFK inhibitors PP1 and PP2 resulted in a dose-dependent decrease in B cell adhesion, with a complete loss at 100 μM PP1 or PP2. Because ICAM-1 is the primary ligand for LFA-1, these results indicate that SFKs play a crucial role in mediating LFA-1-dependent B cell adhesion after BCR stimulation.

We next determined whether SFK inhibition affects formation of the pSMAC or the gathering of antigen into the cSMAC of the IS. To explore this in detail, we used MD4 transgenic (MD4-Tg) mice, whose B cells bear a BCR with high affinity for hen egg lysozyme (HEL) (Goodnow et al., 1988). MD4 B cells were treated with 100 μM PP1, and their interaction with lipid bilayers containing HEL as antigen and ICAM-1 was visualized by confocal microscopy (Carrasco et al., 2004). As shown in Figures 1C and 1D, the capacity of PP1-treated B cells to form a pSMAC was severely impaired when compared to untreated B cells. The absence of the pSMAC also correlated with a significant reduction in the B cell contact area with the bilayer, as assessed by IRM (Figure 1E; p ≤ 0.01). Furthermore, PP1 treatment abrogated cSMAC formation in 60% of B cells and significantly reduced the number of accumulated antigen molecules in comparison to control B cells even at a high density of antigen (Figures 1C, 1F, and 1G; p ≤ 0.01). Treatment of MD4 B cells with 100 μM PP2 produced similar results (data not shown). Thus, our data reveal that antigen engagement by BCR triggers SFK-dependent signals that are essential for LFA-1-mediated adhesion, pSMAC formation, and antigen accumulation.

Vav1 and Vav2 Regulate B Cell Adhesion and IS Formation

We observed a significant impairment of BCR-induced adhesion to ICAM-1 in B cells derived from Vav1−/−, Vav2−/−, and Vav1−/−Vav2−/− mice compared to WT B cells (Figure 2A; p ≤ 0.05). This defect was further pronounced in Vav1−/−Vav2−/− B cells, indicating that both isoforms can function to promote LFA-1-mediated adhesion. Importantly, when treated with phorbol ester (PDBu) and ionomycin, these deficient cells were able to adhere equally as well as WT B cells, indicating that the intrinsic capacity of Vav1−/−Vav2−/− cells to adhere to ICAM-1 was not itself impaired (Figure S1 available online). In contrast, Vav2−/− B cells did not exhibit any defect in adhesion, suggesting a mechanism for LFA-1-mediated B cell adhesion independent of Vav3 (data not shown).

To assess the influence of these genetic losses on the formation of the IS in an antigen-specific system, we generated Vav1−/−, Vav2−/−, and Vav1−/−Vav2−/− mice carrying HEL-specific B cells by backcrossing the Vav-deficient mice with MD4-Tg mice. As depicted in Figures 2B–2E, we observed a marked reduction in B cell contact area with the bilayer compared to MD4-Tg WT B cells, likely as a result of their inability to attach to ICAM-1. This was accompanied by a significant reduction in both the frequency of pSMAC formation and antigen accumulation at limited HEL density (p ≤ 0.05). The observed behavior of MD4-Tg Vav1−/−Vav2−/− B cells was not as a consequence of general abrogation of BCR signaling because we observed high levels of phosphotyrosine at the site of antigen accumulation (Figure S2). Moreover, their behavior was not an artifact of the MD4-Tg background because similar deficiencies were observed in Vav1−/−, Vav2−/−, and Vav1−/−Vav2−/− B cells in response to bilayers loaded with anti-κ chain as a surrogate antigen (Figure S3). Furthermore, in agreement with our previous observations (Carrasco et al., 2004), the contact area of WT B cells was dependent on the presence of ICAM-1 (Figure S1). In contrast, Vav1−/−, Vav2−/−, and Vav1−/−Vav2−/− B cells did not exhibit this dependency, confirming that this shortcoming in adhesion does indeed occur as a result of their inability to bind to ICAM-1 through LFA-1 (Figure S3). Taken together, these results demonstrate that Vav1 and Vav2 play key roles in coupling the initial BCR signaling events to LFA-1-dependent
B cell adhesion, concomitant pSMAC formation, and efficient antigen accumulation.

Rac1 and Rac2 Play Distinct Roles Downstream of the BCR in Primary B Cells

To further outline the pathway linking BCR signaling with LFA-1-mediated B cell adhesion, we investigated the role of Rac1 and Rac2. We assessed the capacity for adhesion to ICAM-1 of naive B cells derived from Cd19^{cre/+} (WT), Cd19^{cre/+}Rac1^{fx/fx} (Rac1^{−/−}), and Cd19^{cre/+}Rac2^{fx/fx} (Rac2^{−/−}) mice, where floxed alleles are efficiently deleted in mature recirculating follicular B cells (Walmsley et al., 2003). As shown in Figure 3A, adhesion of Rac2^{−/−} B cells to ICAM-1 was significantly reduced compared to WT B cells (p ≤ 0.05). In contrast, Rac1^{−/−} B cells did not show any significant defect in B cell adhesion (p > 0.05). In line with these results, the contact area with the bilayer was significantly decreased in Rac2^{−/−} B cells but unaffected in B cells lacking Rac1 (Figures 3B–3D; p < 0.01 compared with p > 0.05). B cells deficient in Rac2 also showed a marked impairment in their ability to aggregate ICAM-1 in a pSMAC. In contrast, we detected no defect in pSMAC formation by Rac1^{−/−} B cells. Consequently, at limited antigen density, Rac2^{−/−} B cells accumulated fewer antigen molecules than did Rac1^{−/−} or WT B cells (Figure 3E). Thus, these findings confirm an essential role for Rac2, but not for Rac1, in the activation of LFA-1-mediated B cell adhesion upon antigen recognition.
PI3K Is Required for BCR-Mediated Rac Activation, B Cell Adhesion, and IS Formation

We then examined the role of PI3K by using the inhibitor wortmannin (WM) on the BCR-mediated activation of Rac1 and Rac2 with a p21-activated kinase (PAK) binding assay. As shown in Figure 4A, both GTPases are activated within 1 min of BCR crosslinking. However, inhibition of PI3K activity with WM prior to BCR stimulation reduced dramatically the activation of Rac1 and abolished the activation of Rac2, thus demonstrating PI3K-dependent regulation of Rac1 and Rac2 activation. Rac1 and Rac2 activation also required SFKs and was impaired in the absence of Vav1 and Vav2 (Figure S4). We would therefore expect that inhibition of PI3K would result in a defect in B cell adhesion and pSMAC formation. Indeed, treatment of WT B cells with the PI3K inhibitors WM or LY294002 (LY) prior to BCR stimulation resulted in a concomitant dose-dependent decrease of B cell adhesion to ICAM-1 (Figure 4B). Furthermore, WM treatment considerably reduced the contact area of MD4-Tg control cells with bilayers containing HEL and ICAM-1 (Figure S5A; p ≤ 0.01). Furthermore, as expected because of their adhesive defect, a low percentage of MD4 p1100-deficient B cells formed a pSMAC, and their contact area with the bilayer was strongly reduced (Figures S5B–S5D). Therefore, our results indicate that the p1100 catalytic subunit of PI3K is required for Rac2 activation, subsequent enhanced LFA-1-mediated B cell adhesion, and pSMAC formation.

Vav1, Vav2, Rac2, and PI3K Regulate BCR-Dependent Rap1 Activation

The GTPase Rap1 has been implicated as a regulator of LFA-1-mediated B cell adhesion, and pSMAC formation. Indeed, treatment of WT B cells with the PI3K inhibitors WM or LY294002 (LY) prior to BCR stimulation resulted in a concomitant dose-dependent decrease of B cell adhesion to ICAM-1 (Figure 4B). Furthermore, WM treatment considerably reduced the contact area of MD4-Tg control cells with bilayers containing HEL and ICAM-1 and completely abolished pSMAC formation (Figures 4C–4E). As a result, the number of HEL molecules aggregated was significantly decreased in WM-treated B cells when compared to the MD4-Tg control (Figure 4F; p ≤ 0.01). Thus, our data demonstrate a vital role for PI3K in regulating B cell adhesion and IS formation.
Rac2 Controls B Cell Adhesion and IS Formation

Constitutively Active Rac2 Restores Adhesion of Signaling-Deficient B Cells

Given the crucial role of Rac2 in promoting B cell adhesion and IS formation in response to membrane-antigen recognition, we examined the biological consequences of the overexpression of constitutively active Rac2 (Rac2V12) in primary antigen-specific Tg (3.83-Tg) B lymphocytes. As shown in Figure 6A, B cells overexpressing Rac2V12 were able to accumulate antigen and segregate ICAM-1 in a manner similar to control B cells in a classical mature IS. Indeed, it was clear that the area of antigen accumulation in these cells was greatly enhanced, and we presume that this results from a stimulation of B cell adhesion. This difference in enhancement was not observed in vector-only and uninfected control cells (data not shown).

We were prevented from further characterization of the role of Rac2 in these primary B cells because the retroviral transduction process involves stimulation — and thus the associated activation of adhesion — of lipopolysaccharide (LPS)-induced blasts. As an alternative, we have assessed the overexpression of Rac2V12 in A20 B cells. As shown in Figure 6B, overexpression of Rac2V12 stimulated high levels of LFA-1-mediated B cell adhesion. In addition, similar stimulation of adhesion was observed after the overexpression of constitutively active Vav1 (Vav1 CA), PI3K (PI3K CA), Rac1 (Rac1Q61L), and Rap1 (Rap1V12), further reinforcing our proposed signaling pathway. However, it is worth noting that the capacity for adhesion observed on overexpression of Rac1Q61L was partial compared with that observed for Rac2V12. We assume that in this case, Rac1, which is highly homologous to Rac2, can adopt the role usually performed by Rac2 in B cell adhesion when it is expressed at elevated amounts in an activated form.

In order to assess the consequences of this Rac2-mediated increase in B cell adhesion on IS formation, we have overexpressed Rac2V12 in signaling-incompetent A20 (A20-IgM-H2) B cells (Williams et al., 1994). The A20-IgM-H2 B cells express a chimeric BCR capable of binding with high affinity to HEL antigen but unable to associate with the Igαβ sheath and initiate B cell signaling. We found that A20-IgM-H2 B cells were unable to adhere to bilayers containing HEL and ICAM-1, unlike A20 B cells expressing a WT version of the BCR (Figures 6C and 6D). It can be seen in Figure 6E that this severely compromised adhesion of A20-IgM-H2 B cells resulted from their inability to activate LFA-1-mediated B cell adhesion. Overexpression of Rac2V12 in A20-IgM-H2 B cells was sufficient to restore LFA-1-mediated adhesion (Figures 6C–6E). This effect was Rac2V12 specific because A20-IgM-H2 clones transfected with empty vector did not show a reconstitution of their contact area relative to control cells. However, as shown in Figure 6F, it is clear that the stimulation of ICAM-1 aggregation resulting from Rac2V12 overexpression was not accompanied by the formation of a structured pSMAC, as observed in the classical IS arrangement displayed in A20 B cells expressing a WT version of the BCR. In line with these observations, activation of LFA-1 through Mn2+ was not sufficient to permit the formation of the structured pSMAC in the absence of Vav, Rac, and PI3K activity (Figure S5E). These observations taken together suggest that, in concert with integrin activation, additional processes mediated by BCR threshold required for triggering a cellular response. In contrast, Rac1-deficient B cells displayed no significant difference in amounts of Rap1-GTP compared with WT B cells (p > 0.1). Thus, these data indicate that SFKs, Vav1, Vav2, PI3K, and Rac2, but not Rac1, are involved in the regulation of Rap1 activity in response to BCR engagement.

Figure 3. Rac2, but Not Rac1, Regulates B Cell Adhesion to ICAM-1 and IS Formation

(A) WT, Rac1−/−, and Rac2−/− B cells stimulated by 5 μg/ml anti-IgM (Fab′)2 or unstimulated were settled onto bilayers containing ICAM-1. The percentage of cells adhering was evaluated in four independent fields, each containing 30–40 B cells. The mean percentage (± SE) is shown. Data are representative of three different experiments. (B–E) Naive WT, Rac1−/−, and Rac2−/− B cells were settled onto bilayers containing anti-κ (κ+κ, green) and ICAM-1 (red), DIC, fluorescence, and IRM images are shown (B). The scale bar represents 3 μm. Quantification of pSMAC frequency (C), the B cell-bilayer contact area (D), and the number of anti-κ molecules aggregated (E) is shown. For each condition, four independent fields, each containing 10–20 B cells, were analyzed. The mean values (± SE) are shown. Data are representative of three different experiments (t test: * indicates p ≤ 0.05, and ** indicates p ≤ 0.01).
signaling, such as underlying cytoskeletal rearrangements, are required for formation of the organized IS.

Overall, our results show that increased Rac2 activity, either by overexpression of Rac2V12 or other activators of its function, is sufficient to restore LFA-1-mediated adhesion in B cells; however, alone it cannot induce pSMAC formation. Our data suggest that a localized, coordinated signal triggered by antigen engagement of the BCR is necessary for organized LFA-1 segregation at the IS.

**Vav1, Vav2, Rac2, and PI3K Regulate Cytoskeletal Rearrangements in B Cells**

Because induction of B cell adhesion is not sufficient for pSMAC formation, we ascertained whether abnormal underlying cytoskeletal changes could account for the inability of the different deficient B cells to form a mature IS. To test this, we visualized F-actin polarization in WM-treated, Vav1−/−, Vav2−/−, Vav1−/− Vav2−/−, Rac1−/−, and Rac2−/− B cells by phalloidin staining. In WT B cells, we found that F-actin was polarized toward the IS and localized predominantly at the pSMAC (Figure 7A). In contrast, the amount of polarized F-actin was markedly reduced in WM-treated, Vav1−/−, Vav2−/−, Vav1−/− Vav2−/−, and Rac2−/− B cells (Figures 7A–7C). The observed reduction in F-actin polarization in the Vav-deficient cells was similar in the absence of ICAM-1 (Figure S7). Consistent with the observation that pSMAC formation in Rac1−/− B cells was normal, F-actin polarization in Rac1-deficient cells was also unaffected (Figures 7A–7C). Furthermore, overexpression of constitutively active Rac2 in signaling-incompetent A20-IgM-H2 B cells induced the formation of F-actin-rich lamellipodia at the IS (Figure 7D).

These data demonstrate that antigen recognition by the BCR triggers cytoskeletal rearrangements, such as F-actin polarization, through a signaling cascade involving the sequential activation of Vav1, Vav2, PI3K, and Rac2 (Figure S8). Rearrangement of the actin cytoskeleton is, in turn, crucial for B cell polarization, B cell adhesion, and pSMAC formation at the IS.

**DISCUSSION**

By using a combination of biochemical, genetic, and imaging tools, we have outlined key molecular requirements coupling BCR-antigen engagement with cytoskeletal rearrangements that lead to IS formation. The results presented here reveal a signaling cascade that is likely to be applicable in all lymphocytes. Downstream of antigen recognition by the BCR, this pathway involves SFKs, the family of Vav proteins, and the small GTPases Rac2 and Rap1, linking them as crucial regulators of LFA-1-mediated B cell adhesion to ICAM-1. We have also found that Rac1 and Vav3 are dispensable for BCR-induced LFA-1-mediated adhesion.
The observation that SFKs have a critical function in the induction of LFA-1-mediated adhesion to ICAM-1 in primary naive B cells is in agreement with recent studies reporting a role for the tyrosine kinases Lyn and Syk for B cell adhesion to VCAM-1 and fibronectin (Spaargaren et al., 2003). We found that B cells deficient in Vav1 and Vav2 displayed a substantial defect in adhesion and IS formation. This role for the Vav-family proteins has not been identified previously in B cells, though these observations are supported by earlier studies in T cells. Wulfing and Davis found that the accumulation of TCR-MHC at the T cell/antigen-presenting cell (APC) interface and actin cytoskeleton rearrangements were defective in Vav1-deficient CD4+ T cells (Wulfing et al., 2000). However, whereas Vav1 appears to be the main player in TCR signal transduction, our data support the notion that signal transduction through highly homologous Vav2 is as important in B cells (Doody et al., 2001; Tedford et al., 2001), and both can function in the control of B cell adhesion, cytoskeleton rearrangement, and IS formation. Activation of LFA-1-mediated B cell adhesion was also strongly dependent on PI3K activity, in a manner similar to that observed for VLA-4-mediated B cell adhesion (Spaargaren et al., 2003). Together, these data identify PI3K as a key player in mediating integrin activation in B cells. Interestingly, we found that B cell adhesion to ICAM-1 and IS formation are substantially reduced in the absence of the p110δ catalytic subunit of PI3K, in contrast to LFA-1-mediated T cell adhesion, which is independent of the p110δ subunit (Okkenhaug et al., 2002).

Previous work has investigated the regulation of Rho GTPases by Vav proteins and PI3K (Crespo et al., 1997; Inabe et al., 2002; Kurosaki, 2002). We show here that in B cells, downstream of BCR engagement, SFKs, Vav1, Vav2, and PI3K regulate activation of the small GTPases Rac1 and Rac2. However, LFA-1-mediated adhesion requires only Rac2. Overexpression of constitutively active Rac2 rendered B cells highly adhesive and enhanced their capacity to aggregate antigen. Furthermore, adhesion of A20 B cells expressing a signaling-deficient BCR to ICAM-1 could be restored simply by overexpression of constitutively active Rac2. Although there is evidence implicating Rac proteins in the regulation of VLA-4 in T cells (D’Souza-Schorey et al., 1998), here we assign a unique role to Rac2 in the activation of integrin-mediated adhesion in lymphocytes. Interestingly, Rac2, but not Rac1, contributed to the activation of the small GTPase Rap1, previously reported to regulate B cell adhesion to ICAM-1 and VCAM-1 (McLeod et al., 2004). At this stage, we have not elucidated a mechanism by which activation of Rap1 occurs, though we postulate that it might occur through Rac2-mediated regulation of cytoskeleton rearrangements to create an environment most favorable for the activation of Rap1. Indeed, it has been shown previously that the spatial localization of Rap1 is involved in the regulation of its activity in facilitating integrin activation in T cells (Kliche et al., 2006). It seems likely, therefore, that the organized cellular segregation of Vav, Rac2, and Rap1 allows crosstalk and thus cooperation between different intracellular signaling pathways (Bos, 2005).

Rac1 and Rac2 are both expressed in mature B cells, but they appear to have different functions, even though they have a high degree of sequence identity. Although at this stage we cannot identify the role of Rac2 in B cell adhesion and IS formation, suggesting a unique role for Rac2 in B cell signaling.
exclude that these observations are due to differences in expression, such differential functioning is not without precedence. For instance, Rac1 and Rac2 have been shown to have distinct functions in both primitive hematopoietic cells and differentiated neutrophils (Glogauer et al., 2003; Gu et al., 2003). The most elegant demonstration of this differential functioning has been reported by the Williams lab. This study showed that C-terminal motifs within Rac2 were sufficient to determine its specific subcellular localization and associated function (Filippi et al., 2004). We postulate that a similar mechanism might apply in lymphocytes, where activated Rac2 is recruited specifically to the site of B cell adhesion. This is supported by our biochemical observation that prior to stimulation, B cells have detectable amounts of active Rac2 but do not adhere. Furthermore, the amounts of GTP-bound Rac2 increase no more than three times upon BCR crosslinking. Thus, redistribution of the spatial pool of active Rac2 to the site of adhesion might be a critical step in the promotion of B cell attachment.

Our results reveal a signaling cascade, involving SFKs, Vav proteins, and Rac2, for the regulation of LFA-1 inside-out activation and subsequent B cell adhesion to ICAM-1. We envisage two divergent pathways downstream of Vav1 and Vav2, one of which involves Rac1 and is dispensable for integrin-mediated adhesion. The other connects BCR-proximal signaling with activation of LFA-1-mediated adhesion and pSMAC formation, through the activation of Rac2 and Rap1. Thus far, the investigation of LFA-1-mediated B cell adhesion has not identified any effectors as being involved in differential clustering of BCR and LFA-1 at the IS. We expect that such pathways within B cells, similar to those found in T cells involving Wiskott-Aldrich syndrome protein (WASP) and the Drosophila melanogaster myoblast city (CDM)-family protein, dedicator of cytokinesis 2 (DOCK2) (Krawczyk et al., 2002), might exist and be elucidated in the future. However, it is worth noting at this stage that different signaling pathways controlling LFA-1 activation in lymphocytes have been identified previously, as illustrated by the differences in requirements for adaptor proteins (Griffiths et al., 2001; Peterson et al., 2001). For example, the ADAP-SKAP55 signaling module has been shown to be mandatory for integrin activation in T cells (Kliche et al., 2006), and although B cell adhesion is
severely impaired in the absence of SKAP-HOM, it seems dispensable for T cell adhesion (Togni et al., 2005). Because we have identified a unique role for Rac2 in activating LFA-1-mediated B cell adhesion downstream of the Vav proteins, we postulate that the pathway that we have identified is likely to be generally applicable for the activation of integrins in all lymphocytes.

Samelson and colleagues have suggested that TCR signaling occurs in microclusters where receptors and adaptor molecules are recruited (Bunnell et al., 2002). Furthermore, the groups of Dustin and Saito demonstrated that the formation of signaling clusters is a continuous process taking place in the periphery of the IS to sustain T cell signaling (Varma et al., 2006; Yokosuka et al., 2005). Recently, we have identified a similar situation in B cells, with the pSMAC forming the site for continuous receptor engagement and prolonged signaling (Depoil et al., 2008). In the future, it would be interesting to investigate the spatial and temporal regulation of integrin activity with high-resolution total-internal-reflection fluorescence microscopy (TIRFM). Such studies would offer insight into the dynamic and differential recruitment of signaling molecules, such as Vav1, Vav2, and Rac2, into microclusters and whether this temporary relocalization constitutes the foundation for pSMAC formation. Finally, the determination of the role of other small GTPases, such as Cdc42 and Rho, and their downstream effectors, in the formation of the B cell IS will be crucial in understanding how the interplay between intracellular signaling and cytoskeleton rearrangements drives the segregation of lymphocyte receptors and the formation of an IS.

EXPERIMENTAL PROCEDURES

Mice and Splenic B Cell Purification

MD4 transgenic mice [B cells expressing HEL-specific BCR (Goodnow et al., 1988)] were crossed with Vav1−/−, Vav2−/−, Vav1−/−Vav2−/−, and Pik3ca−/− (p110δ-deficient) mice (Clayton et al., 2002; Doody et al., 2001; Turner et al., 1997) so that MD4 Vav1−/−, MD4 Vav2−/−, MD4 Vav1−/−Vav2−/−, and MD4 Pik3ca−/− (p110δ-deficient) mice could be obtained. 3.83 transgenic mice [B cells expressing H-2Kk-specific BCR that also recognize p31 (Carrasco et al., 2004)] were used for retroviral transfection with Rac2V12. Cd19cre/− WT, Cd19cre/−Rac1f/fc, and Cd19cre/−Rac2f/fc were used where floxed alleles

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**Figure 7. F-Actin Polarization at the IS Depends on PI3K, Vav1, Vav2, and Rac2**

(A–C) WT, WM-treated, Vav1−/−, Vav2−/−, Vav1−/−Vav2−/−, Rac1−/−, and Rac2−/− B cells were settled onto bilayers containing antigen (HEL, except for Rac1−/− and Rac2−/−, where anti-κ was used as antigen; shown in green) and ICAM-1, permeabilized, and fixed after 20 min, and F-actin (gray) was stained with phalloidin. DIC images and fluorescence images of the xz plane (middle panels) and the xy plane (bottom panels) of representative cells are shown (A). Quantification of the amount of F-actin polarized (B) and the area of F-actin at the contact interface (C) is shown. Three independent fields, each containing 30–40 cells, were analyzed. The mean percentage (± SE) is shown. Data are representative of two different experiments.

(D) A20-IgM-H2 B cells untransfected (control), vector only, or Rac2V12 transfected were settled onto bilayers containing HEL (green) and ICAM-1, permeabilized, and fixed after 20 min, and F-actin (gray) was stained with phalloidin. DIC images and fluorescence images of the xz plane (middle panels) and the xy plane (bottom panels) of representative cells are shown.
were efficiently deleted from mature circulating follicular B cells (Rickert et al., 1997; Roberts et al., 1999; Walmsley et al., 2003). Splenic naive B cells were purified by negative selection as described previously (Carrasco et al., 2004). This method resulted in an enrichment of naive B cells up to 95–98%. All experiments were approved by the Cancer Research UK Animal Ethics Committee and the United Kingdom Home Office.

### Planar Lipid Bilayers and Confocal Microscopy
Planar lipid bilayers containing GPI-linked Alexa-532-conjugated ICAM-1 were prepared in FCS2 chambers (Bioptechs), as described previously (Carrasco et al., 2004). Antigens, monobiotinylated HEL, rat anti-mouse κ, or p31 (Carrasco et al., 2004), were loaded in the bilayers with Alexa 633-streptavidin (Molecular Probes). Images were acquired with a Zeiss Axiovert LSM 510-META inverted microscope with a 63× oil-immersion objective (Zeiss, Germany). Differential interference contrast (DIC) and confocal fluorescence images were obtained simultaneously. IRM images were analyzed with Velocity software (Improvision, UK).

### Inhibitor Treatment
Naïve B cells were treated with various concentrations of inhibitors PP1, PP2, LY294002 (all Calbiochem), or wortmannin (Sigma) in adhesions assays, or 100 μM PP1 and 100 nM Wortmannin in IS formation assays, for 30 min at 37°C in IS buffer.

### Adhesion Assay
So that the adhesion of naive B cells to ICAM-1 could be measured, 10 × 10^6 cells were stimulated with 5 μg/ml goat anti-mouse IgM (Fab′)2 polyclonal antibody (Jackson) for 30 min at 37°C in IS buffer and then incubated on bilayers containing 80 molecules/μm² Alexa532-labeled GPI-linked ICAM-1. Several images were taken of independent areas on bilayers with IRM. The B cell-bilayer adhesion area was quantified by measurement of the dark IRM area and B cell adhesion was determined by counting of the dark IRM signals as a percentage of total B cells, with a minimum area of 2 μm² defined as adhesion.

### Immunological Synapse Formation Assay
Naïve B cells were settled onto planar lipid bilayers containing 80 molecules/μm² Alexa532-labeled GPI-linked ICAM-1 and antigen at a density of 35 (for antigen accumulation) or 150 (pSMAc formation and contact area) molecules/μm². In the case of SFK-inhibitor treatment, a higher antigen density (150 molecules/μm²) was used for antigen accumulation because an effect was observed even at higher antigen density. DIC, confocal fluorescence, and IRM images were taken after 15–20 min of several independent areas, pSMAc frequency was determined by counting of B cells aggregating antigen and forming a pSMAc as a percentage of total B cells. The B cell contact area and the total amount of accumulated antigen were quantified as described previously (Fleire et al., 2006).

### Rac1, Rac2, and Rap1 Activation Assay
Ten million (for Rac1 and Rap1 activation assays) and twenty million (for Rac2 activation assays) naïve B cells were resuspended and stimulated with 10 μg/ml goat anti-mouse IgM (Fab′)2. B cell supernatants, after lysis and centrifugation (Amaxa Biosystems), were efficiently deleted from mature circulating follicular B cells (Rickert et al., 1997; Roberts et al., 1999; Walmsley et al., 2003). Splenic naive B cells were purified by negative selection as described previously (Carrasco et al., 2004). This method resulted in an enrichment of naive B cells up to 95–98%. All experiments were approved by the Cancer Research UK Animal Ethics Committee and the United Kingdom Home Office.

### Rac2 Transfection and B Cell Activation Assays
Constitutively active versions of the proteins were either point mutations (Rac1, Rac2, and Rap1) or fusions to CD2 domain (Vav and PI3K). Constitutively active Rap1 was a kind gift from Doreen Cantrell’s lab, and constitutively active Rac1 was a kind gift from Salvador Benitah Aznar’s lab. Former constructions were transiently transfected in A20 cells. For stable transfections, Rac2V12 was cotransfected with a plasmid encoding Hygromycin B resistance into A20-IgM-H2 B cells (Williams et al., 1994) with the electroporation (Amazyx Biosystems). Hygromycin B-resistant stable A20-IgM-H2 clones expressing Rac2V12 were selected by their expression of GFP.

### pTyr and F-Actin Staining on Lipid Bilayers
B cells in contact with bilayers containing 80 molecules/μm² of ICAM-1 and 150 molecules/μm² of Ag were fixed with 4% paraformaldehyde (PFA), permeabilized, and stained with Alexa543-phalloidin (Molecular Probes, Invitrogen) or IgG2/P17 phosphotyrosine antibodies (Cancer Research UK) as described (Fleire et al., 2006).

### Supplemental Data
Eight figures are available at http://www.immunity.com/cgi/content/full/28/1/88/DC1/.

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### REFERENCES


