

A novel Rac-dependent checkpoint in B cell development controls entry into the splenic white pulp and cell survival

Robert B. Henderson,¹ Katarzyna Gryś,¹ Anne Vehlow,² Carine de Bettignies,¹ Agnieszka Zachacz,¹ Tom Henley,³ Martin Turner,³ Facundo Batista,² and Victor L.J. Tybulewicz¹

¹Division of Immune Cell Biology, Medical Research Council National Institute for Medical Research, London NW7 1AA, England, UK

²Cancer Research UK London Research Institute, London WC2A 3PX, England, UK

³The Babraham Institute, Cambridge CB2 4AT, England, UK

Rac1 and Rac2 GTPases transduce signals from multiple receptors leading to cell migration, adhesion, proliferation, and survival. In the absence of Rac1 and Rac2, B cell development is arrested at an IgD⁻ transitional B cell stage that we term transitional type 0 (T0). We show that T0 cells cannot enter the white pulp of the spleen until they mature into the T1 and T2 stages, and that this entry into the white pulp requires integrin and chemokine receptor signaling and is required for cell survival. In the absence of Rac1 and Rac2, transitional B cells are unable to migrate in response to chemokines and cannot enter the splenic white pulp. We propose that loss of Rac1 and Rac2 causes arrest at the T0 stage at least in part because transitional B cells need to migrate into the white pulp to receive survival signals. Finally, we show that in the absence of Syk, a kinase that transduces B cell antigen receptor signals required for positive selection, development is arrested at the same T0 stage, with transitional B cells excluded from the white pulp. Thus, these studies identify a novel developmental checkpoint that coincides with B cell positive selection.

CORRESPONDENCE

Victor L.J. Tybulewicz:
vtybule@nimr.mrc.ac.uk

Abbreviations used: GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; MRF, mature recirculating follicular; MZ, marginal zone; T0, transitional type 0; VavTKO, Vav triple KO.

In mammals, the early phase of B cell development occurs in the bone marrow (Hardy and Hayakawa, 2001). Hematopoietic progenitors located in the marrow differentiate into pro-B cells, which initiate rearrangement of Ig heavy chain genes. Successful rearrangement leads to the production of Ig μ heavy chain, its assembly into the pre-BCR, and signaling from this receptor, resulting in proliferative expansion and differentiation into pre-B cells. Pre-B cells rearrange Ig light chain genes, and if successful, light chains associate with the μ heavy chain, resulting in expression of BCR in the form of IgM on the surface of an immature B cell. Signaling from the BCR in immature B cells allows the cells to move into the late phase of B cell development, B cell positive selection, which occurs in part in the spleen.

Immature B cells migrate from the bone marrow to the spleen, where they are now termed

transitional B cells. Arriving from the blood, transitional B cells first enter the marginal sinus and the red pulp of the spleen, and then migrate across the marginal sinus lining cells into the white pulp (Mebius and Kraal, 2005). Here they acquire expression of IgD, an alternative form of the BCR, and complete their maturation into mature recirculating follicular (MRF) B cells or marginal zone (MZ) B cells. Although MZ B cells reside mainly in the MZ of the spleen, MRF B cells are found primarily in follicles of both the spleen and other lymphoid organs, such as lymph nodes and Peyer's patches, and recirculate between them, exiting lymphoid organs through the lymphatics and returning via the blood vasculature. Thus, cell migration is intimately involved in both B cell development and in the function of mature B cells.

A. Vehlow's present address is Randall Division of Cell and Molecular Biophysics, King's College London, Guy's Campus, London SE1 1UL, England, UK.

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The recirculation of MRF B cells between the splenic and lymph node follicles, and the lymphatic and blood systems, has been shown to require coordinated signaling through chemokine, integrin, and sphingosine-1-phosphate receptors (Cyster, 2005). In contrast, relatively little is known about the movement of immature/transitional B cells from bone marrow to the spleen.

In mice engineered to lack expression of both Rac1 and Rac2, B cell development is blocked at a transitional B cell stage in the spleen (Walmsley et al., 2003). Rac1 and Rac2 are members of the Rho family of GTPases, proteins that transduce signals from antigen receptors such as the BCR, as well as chemokine and integrin receptors (Walmsley et al., 2003; Jaffe and Hall, 2005; Cancelas et al., 2006). Signals from Rac GTPases in turn activate a diverse set of cellular responses, including regulation of the actin cytoskeleton, proliferation, survival, migration, and adhesion. In view of the requirement for signals from the BCR and the BAFF receptor BAFF-R during this late phase of B cell development, the block in B cell development at a transitional B cell stage in the absence of Rac1 and Rac2 could be caused by defects in signaling from either receptor. Indeed in an earlier study, we showed that immature B cells deficient in Rac1 and Rac2 are defective in BAFF-induced survival (Walmsley et al., 2003). However, it is also possible that Rac1 and Rac2 transduce chemokine or integrin receptor signals in immature or transitional B cells that control their exit from the bone marrow, and migration through blood to the red pulp of the spleen and then into the white pulp, finally arriving in the follicles.

Thus, in this study we addressed the question of whether the developmental block seen in the absence of Rac1 and Rac2 was caused at least in part by roles for the GTPases in migration or adhesion of transitional B cells. We show that deficiency of these GTPases leads to developmental arrest at an IgD⁻ transitional B cell stage that we term transitional type 0 (T0), consisting of the most immature splenic transitional B cell immigrants. We show that T0 transitional B cells can freely migrate into the red pulp of the spleen but are unable to enter the white pulp and the follicles until they mature into the T1 and T2 stages. Furthermore, we demonstrate that entry of T1 and T2 transitional B cells into the white pulp requires the integrins LFA-1 and VLA-4, as well as signaling from pertussis toxin-sensitive receptors, most likely chemokine receptors, and we show that migration into the white pulp is required for cell survival. Moreover, we show that although transitional B cells can enter the red pulp in the absence of Rac1 and Rac2, they are unable to enter the white pulp because of defective chemokine receptor signaling, leading to cell death and hence a developmental block. This novel Rac-dependent checkpoint in B cell development, defined by migration of transitional B cells into the white pulp of the spleen, coincides with the positive selection of B cells.

RESULTS

Deficiency of Rac1 and Rac2 leads to identification of an IgD⁻ transitional subset of B cells

In earlier studies, we showed that in the absence of Rac1 and Rac2, B cell development proceeds normally within the bone marrow but is blocked in the spleen at an early T1 stage

(Walmsley et al., 2003). Specifically, the number of T1 B cells was reduced by ~80%, and all subsequent stages of development (T2 and mature subsets) were almost completely absent. The T1 subset of B cells contains the most recently arrived immature B cells, which have migrated from the bone marrow, through the blood to the spleen, where they complete their maturation. In view of the documented role of Rac GTPases in cell mobility and adhesion, we considered whether the developmental defect in B cells deficient in Rac1 and Rac2 may be caused by inefficient migration. To study these processes, we used mice bearing a conditional loxP-flanked allele of *Rac1* (*Rac1^{fllox/flox}*) crossed to *CD19^{Cre/+}* mice to give B lineage-specific deletion of *Rac1* (*Rac1^B*), mice constitutively deficient in *Rac2* (*Rac2^{-/-}*), and mice deficient in both GTPases (*Rac1^BRac2^{-/-}*; Walmsley et al., 2003).

Previously, we analyzed transitional splenic B cell subsets according to expression of CD21, CD23, and CD24 using the system of Loder et al. (1999). We now used an alternative system devised by Allman et al. (2001) in which transitional subsets are identified based on expression of CD93, IgM, and CD23. In this separation, we noted that the T1 subset (CD93⁺IgM^{high}CD23⁻) was heterogeneous with respect to IgD expression, containing both IgD⁻ and IgD⁺ cells (Fig. 1 A). Because immature B cells first express IgM and subsequently IgD, it was likely that the IgD⁻ cells developmentally precede the IgD⁺ cells, and hence, for simplicity, we refer to the former as T0 cells and to the latter as T1 IgD⁺ cells (Fig. 1 A). This assumption about the developmental order of the T0 and T1 IgD⁺ subsets is supported by evidence presented at the end of this section. Enumeration of these subsets showed that deficiency for both Rac1 and Rac2 did not affect the numbers of T0 cells but led to a large decrease in both T1 IgD⁺ and T2 transitional subsets (Fig. 1 B). As previously seen using the Loder et al. (1999) staining protocol (Walmsley et al., 2003), the number of MRF and MZ B cells was also greatly reduced (Fig. 1 B).

We also measured the numbers of both immature and mature B cells in the blood. In the absence of both Rac1 and Rac2 there was a >10-fold increase in the number of immature B cells in the blood (Fig. 1, C and D). More specifically, this increase was in the T0-like immature B cells (Fig. S1 A). These cells are presumably in transit between the bone marrow and spleen. At the same time, there was a decrease in the number of mature B cells in the blood (Fig. 1, C and D), in accordance with large decreases seen in the lymph nodes and spleen. Previous studies have shown that transitional B cell maturation can occur in locations other than the spleen, such as the bone marrow (Cariappa et al., 2007; Lindsley et al., 2007). Examination of bone marrow in mice deficient in both Rac1 and Rac2 showed that although the number of pro-B, pre-B, and T0-like immature B cells was not altered, there was a significant decrease in T1 IgD⁺-like and T2-like immature B cells (Fig. S1 B). Collectively, these results show that in the absence of Rac GTPases, T0-like immature B cells accumulate in the blood, and B cell development is strongly blocked between T0 and T1 IgD⁺ B cells in both the spleen and bone marrow.

To gain further understanding of why *Rac1* and *Rac2* are required for the maturation of T0 B cells into T1 IgD⁺ B cells, we measured the turnover of these subsets by administration of BrdU in the drinking water of the mice either continuously or using a pulse-chase protocol. BrdU is incorporated by cycling pre-B cells in the bone marrow, and because all subsequent subsets are noncycling, the rate at which subsets become BrdU⁺ reflects their intrinsic rate of turnover (Fulcher and Basten, 1997). These studies showed no significant difference in the turnover of *Rac1*^B*Rac2*^{-/-} immature B cells in the

bone marrow and blood, and in splenic T0 or T2 B cell subsets compared with their WT counterparts (Fig. 2). However, the pulse-chase study showed that *Rac*-deficient T1 IgD⁺ splenic B cells turned over more rapidly than WT cells. This mild increase in turnover suggests that the reduced size of the splenic T1 IgD⁺ subset in *Rac1*^B*Rac2*^{-/-} mice is in part caused by more rapid exit of cells from this compartment, perhaps because of increased death. In addition, although the normal size and turnover of the *Rac1*^B*Rac2*^{-/-} T0 subset shows that its output is normal, if these cells are the direct precursors of T1 IgD⁺ cells, we estimate that at most $14 \pm 2\%$ of them mature into the T1 IgD⁺ subset, in contrast to $52 \pm 4\%$ of WT T0 cells. Thus, most *Rac1*^B*Rac2*^{-/-} T0 cells are not becoming T1 IgD⁺ B cells but have other developmental fates. These could include migration to another location or cell death.

Comparison of rates of labeling and turnover of different B lineage subsets shows that immature (T0-like) B cells in the bone marrow label more rapidly than those in the blood and spleen. Splenic T0 and T0-like immature B cells in the blood label with identical kinetics, suggesting that they form one recirculating compartment. Furthermore, splenic T0 B cells acquire BrdU more rapidly than do T1 IgD⁺ B cells, which in turn are more rapid than T2 B cells (Fig. 2). Collectively, these results are consistent with a developmental progression from immature bone marrow B cells to blood/spleen T0 cells to T1 IgD⁺ cells to T2 cells.

Immature *Rac*-deficient B cells accumulate in the blood because of defective CXCR4-mediated migration to the bone marrow

To investigate further why immature T0-like *Rac1*^B*Rac2*^{-/-} B cells accumulate in the blood, we adoptively transferred splenic T0 cells from *Rac*-deficient mice into WT recipients. The transferred cells were mixed with WT T0 cells to act as a reference population, and the location of the transferred cells was assessed 4 h after transfer. These studies showed that although T0 cells deficient in either *Rac1* or *Rac2* alone localized normally, there was a significant accumulation of *Rac1*^B*Rac2*^{-/-} T0 cells in the blood and a decrease of these cells in the bone marrow (Fig. 3 A). In contrast, the number of *Rac1*^B*Rac2*^{-/-} T0 cells in the spleen was unaltered. These results suggest that the accumulation of immature B cells in the blood of *Rac1*^B*Rac2*^{-/-} mice may be caused by a reduced ability of splenic T0 cells to traffic to the bone marrow.

It is known that the chemokine CXCL12, acting through the CXCR4 receptor, is critical for the retention of developing B lineage cells in the bone marrow and for the homing of mature B cells to the marrow (Ma et al., 1999; Nie et al., 2004). Furthermore, *Rac* GTPases have been implicated in CXCR4 signaling (Cancelas et al., 2006). Thus, we hypothesized that the accumulation of T0 *Rac*-deficient B cells in the blood and their decreased migration back into the bone marrow may be caused by defective CXCR4 function. In support of this, transfer of WT T0 B cells in the presence of an inhibitor of CXCR4 replicated the phenotype of *Rac*-deficient T0 cells, resulting in an accumulation of cells in the

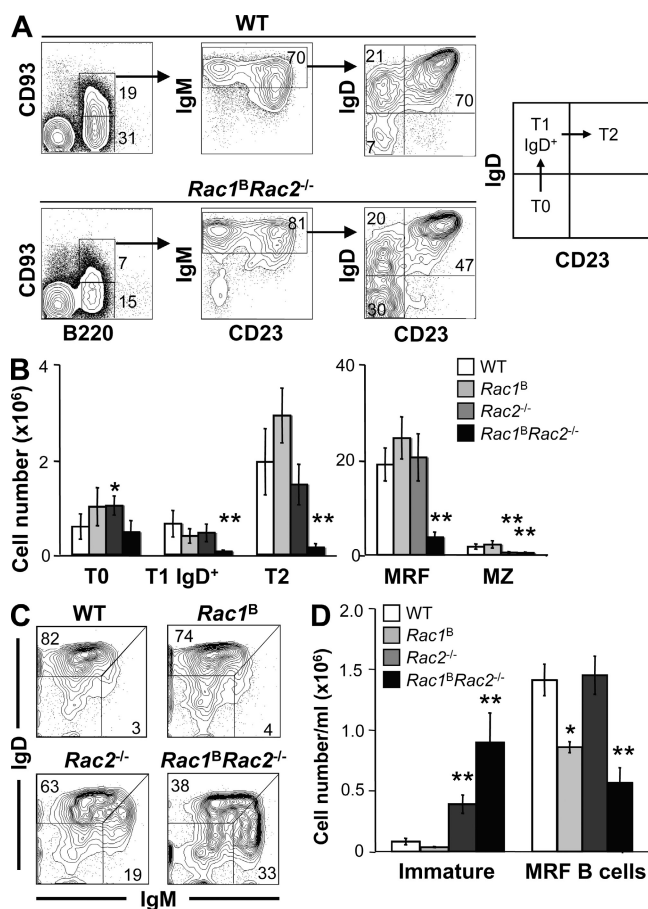


Figure 1. Immature B cells are blocked in splenic development and accumulate in the blood in the absence of *Rac1* and *Rac2*. (A) Contour plots of splenocytes from either WT or *Rac1*^B*Rac2*^{-/-} mice showing separation of B220⁺ cells into immature (CD93⁺) and mature (CD93⁻) B cells. The immature cells were then further gated on IgM⁺ cells and separated according to the expression of IgD and CD23 into T0 (IgD⁻CD23⁻), T1 IgD⁺ (IgD⁺CD23⁻), and T2 (IgD⁺CD23⁺) subsets. Mature (CD93⁻) cells were separated according to expression of IgM and CD23 into MRF (IgM⁺CD23⁺) and MZ (IgM⁺CD23⁻) subsets (not depicted). Numbers show the percentage of cells falling into gates or quadrants. (B) Mean (±SEM) numbers of T0, T1 IgD⁺, T2, MRF, and MZ splenic B cells in mice of the indicated genotypes (*n* = 6). (C) Contour plots showing expression of IgM and IgD on B220⁺ blood lymphocytes. Gates indicate immature (IgM⁺IgD⁻) and MRF (IgD⁺) B cells. Numbers show the percentage of cells falling into gates. (D) Mean (±SEM) numbers of immature and MRF B cells in the blood of mice of the indicated genotypes (*n* = 5). *, *P* < 0.05; **, *P* < 0.01.

blood, a decrease in the bone marrow, and no effect on homing to the spleen (Fig. 3, compare B with A). Furthermore, we found that in the absence of both Rac1 and Rac2, immature bone marrow B cells and splenic T0 B cells are unable to chemotax toward CXCL12, unlike WT cells (Fig. 3 C). This was true even when the assay was performed in the presence of VCAM-1, the ligand for integrin VLA-4 ($\alpha 4\beta 1$), which is activated by CXCL12 and also plays a role in the retention of developing B cells in the bone marrow (Koni et al., 2001). We conclude that the accumulation of Rac1- and Rac2-deficient immature B cells in the blood may result from defective CXCR4-mediated migration of the cells to the bone marrow. We note that in contrast to CXCR4-deficient mice (Nie et al., 2004), we do not see premature release of pro-B and pre-B cells from the bone marrow of *Rac1^BRac2^{-/-}* mice. This is most probably because deletion of *Rac1* in these early subsets is incomplete, only becoming efficient in immature B cells (Walmsley et al., 2003).

Rac-deficient T0 B cells are unable to enter the white pulp of the spleen

The normal homing of Rac-deficient T0 B cells to the spleen (Fig. 3 A) did not explain why development of the cells is arrested. Thus, we examined the maturation and localization of the transferred cells 4 and 24 h after transfer. Flow cytometry showed that at 4 h the transferred cells were still largely T0 in phenotype, whereas by 24 h many of the WT cells had acquired expression of IgD and/or CD23 and thus resembled T1 IgD⁺ and T2 B cells (Fig. 4, A and B). The *Rac1^BRac2^{-/-}* T0 B cells also matured into T1 IgD⁺ and T2 B cells, albeit

less efficiently. Histological analysis demonstrated that at 4 h both WT and Rac-deficient transferred T0 cells were located largely in the red pulp of the spleen. Strikingly, however, by 24 h many of the transferred WT cells had entered the white pulp, whereas the *Rac1^BRac2^{-/-}* cells were largely excluded (Fig. 4 C). Quantitation of these results showed that although Rac1-deficient transitional B cells entered the white pulp normally, Rac2-deficient cells were partially defective and cells missing both GTPases were almost completely arrested in the red pulp (Fig. 4 D). The inability of T0 cells to enter the white pulp 4 h after transfer is most likely caused by their immaturity, as MRF B cells readily home into the white pulp even at this early time point (Fig. 4, C and D). Thus, we conclude that there appears to be no barrier to entry of WT T0 B cells from the blood to the splenic red pulp; however, their subsequent entry into the white pulp requires more time, most likely because they need to mature into T1 IgD⁺ and T2 B cells. In contrast, in the absence of Rac1 and Rac2, transitional B cells are unable to gain entry into the white pulp despite at least some maturation into T1 IgD⁺ and T2 cells.

If some of the transferred *Rac1^BRac2^{-/-}* T0 cells can mature into T1 IgD⁺ and T2 cells, why do these cells not accumulate in the intact mice? One possibility is that the cells die at this developmental stage. We examined this by measuring cell recovery 24 h after transferring WT or Rac-deficient T0 B cells into WT hosts. This clearly showed that although recovery was normal 4 h after transfer (Fig. 3 A), many fewer *Rac1^BRac2^{-/-}* transitional cells were recovered compared with their WT counterparts at 24 h (Fig. 4 E). Strikingly, this reduction was caused by large decreases in the number of T1

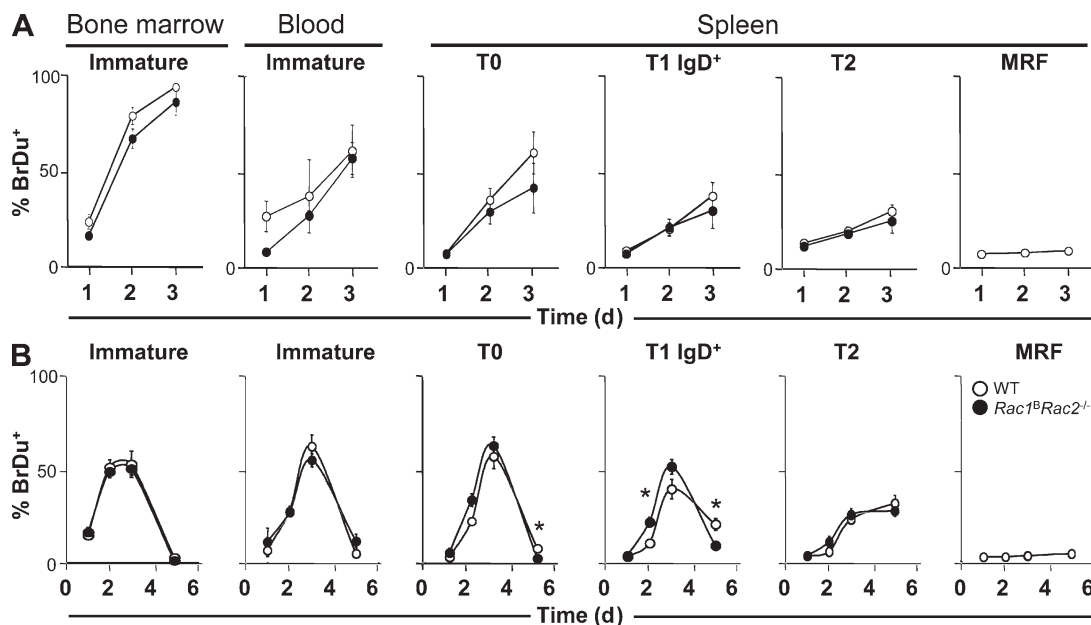


Figure 2. Analysis of population turnover by BrdU incorporation. (A and B) Turnover of immature T0-like B cells in the bone marrow and blood (CD93⁺IgM⁺IgD⁻CD23⁻) and of splenic T0, T1 IgD⁺, T2, and MRF B cells from mice of the indicated genotypes monitored by (A) continuous ($n = 5-6$) or (B) pulse-chase analysis of BrdU incorporation ($n = 4-5$) as a function of time after start of BrdU treatment. BrdU was administered to the mice starting at time 0. For pulse-chase analysis, BrdU was administered for the first 16 h only. Means \pm SEM are shown. *, $P < 0.05$.

IgD⁺ and T2 cells, whereas the number of T0 cells was unaffected. These results imply that *Rac1^BRac2^{-/-}* T0 cells are able to mature into T1 IgD⁺ and T2 cells, but these latter subsets then fail to survive, thus leading to the large reduction in T1 IgD⁺ and T2 cells seen in the mutant mice.

Entry of transitional B cells into the white pulp requires integrins and signaling from pertussis toxin-sensitive receptors

Entry of mature B cells into the white pulp of the spleen has been shown to require the integrins LFA-1 and VLA-4, as well as the function of pertussis toxin-sensitive G protein-coupled receptors (GPCRs), most likely the CXCR5 and CCR7 chemokine receptors (Cyster and Goodnow, 1995; Lo et al., 2003; Ohl et al., 2003). To ascertain if the same is true for the migration of transitional B cells into the white pulp, we transferred WT T0 B cells in the presence of anti-LFA-1

and anti-VLA-4 blocking antibodies, or after treatment with pertussis toxin. Although the antiintegrin antibodies partially reduced the movement of transitional B cells into the white pulp, pertussis toxin almost completely blocked the movement, to a level similar to that seen with *Rac1^BRac2^{-/-}* transitional B cells (Fig. 5 A). Thus, we conclude that, similar to mature B cells, the entry of transitional B cells into the white pulp requires the integrins LFA-1 and VLA-4 as well as pertussis toxin-sensitive receptors.

In addition, pertussis toxin treatment partially blocked maturation of the T0 cells into T1 IgD⁺ and T2 cells and reduced cell recovery at 24 h (Fig. 5, B and C). Notably, pertussis toxin treatment caused a drop in recovery of T1 IgD⁺ and T2 cells (Fig. 5 C). We note the similarity of this result to that seen in cells deficient in Rac GTPases (compare with Fig. 4 E). Collectively, these results suggest that both Rac GTPases and pertussis toxin-sensitive receptors are required for the entry of transitional B cells into the white pulp and for the survival of T1 IgD⁺ and T2 B cells.

Rac1 and Rac2 are required for chemokine responsiveness of transitional B cells

In view of the pertussis toxin sensitivity of transitional B cell migration into the splenic white pulp, we investigated the ability of transitional B cells to respond to CCL21, CXCL12, and CXCL13. In Transwell migration assays, we found that as cells matured from T0 to T1 IgD⁺ to T2, they became more responsive to chemokines, especially to CCL21 and CXCL13, which are known to direct migration of lymphocytes into the T and B cell areas of the white pulp, respectively (Fig. 6 A; Ohl et al., 2003). This maturation was accompanied by an increase in surface levels of LFA-1 (both α L and β 2 subunits) and of CXCR5, the receptor for CXCL13, whereas the levels of VLA-4 (both α 4 and β 1 subunits) and of CXCR4, the receptor for CXCL12, remained unchanged (Fig. 6 B). The migration of transitional B cells was enhanced in the presence of ICAM-1, a ligand for LFA-1, or in the presence of MadCAM-1 and VCAM-1, ligands for VLA-4 (Fig. 6 C). In all cases, however, the responsiveness of the cells again increased as they matured from T0 to T1 IgD⁺ to T2. These results are in agreement with our observation that transitional B cells need to mature from the T0 stage to the T1 IgD⁺ or T2 stages before they can enter the white pulp, and that this migration is dependent on both integrins and pertussis toxin-sensitive receptors. We note that MadCAM-1 is expressed in the marginal sinus lining cells and that ICAM-1 and VCAM-1 are expressed in the white pulp, and thus, these integrin ligands are located in the right place to mediate entry of transitional B cells into the white pulp or their subsequent retention (Lo et al., 2003).

Next, we examined the role of Rac1 and Rac2 in the chemokine responsiveness of transitional B cells. Using the Transwell assay system, we found that in the absence of both GTPases, T0, T1 IgD⁺, and T2 B cells were almost completely unresponsive to CCL21, CXCL12, and CXCL13 (Fig. 6 D). This was not caused by defective receptor expression,

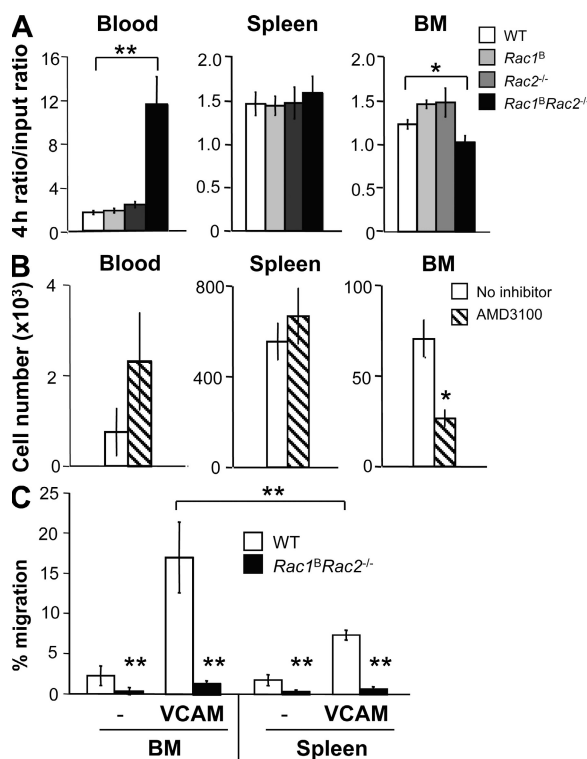


Figure 3. Accumulation of Rac-deficient immature B cells in the blood may be caused by defective CXCR4 signaling. (A) Splenic T0 B cells from mice of the indicated genotypes (IgM^bLy5.2⁺) mixed with splenic T0 B cells from 129S8 (IgM^bLy5.2⁺) mice were injected into B6.SJL (IgM^bLy5.1⁺) mice. Graph shows the mean (\pm SEM) IgM^bLy5.2⁺ to IgM^bLy5.1⁺ ratio of B cells in the blood, spleen, and bone marrow 4 h after transfer, normalized to the input ratio ($n = 5-17$). (B) Mean (\pm SEM) number of IgM^b cells recovered from the blood, spleen, and bone marrow of B6.SJL mice into which WT splenic T0 B cells had been transferred 4 h earlier in the absence or presence of the CXCR4 inhibitor AMD3100 ($n = 4$). (C) Mean (\pm SEM) migration of immature bone marrow or splenic T0 cells from mice of the indicated genotypes in a Transwell assay in response to CXCL12. Wells were coated or not (-) with VCAM-1 ($n = 6$). *, $P < 0.05$; **, $P < 0.01$. BM, bone marrow.

as the surface levels of both CXCR5 and CXCR4 were unchanged in the mutant cells (unpublished data). This result, together with the requirement for a pertussis toxin-sensitive receptor for entry into the white pulp, suggests that *Rac1^BRac2^{-/-}* transitional B cells are unable to enter the white pulp because

of defective chemokine receptor function. Furthermore, this failure to enter the white pulp may account in part for the defective survival of *Rac*-deficient transitional B cells. This conclusion implies that the reduced survival of *Rac1^BRac2^{-/-}* T1 IgD⁺ and T2 B cells in the adoptive transfer

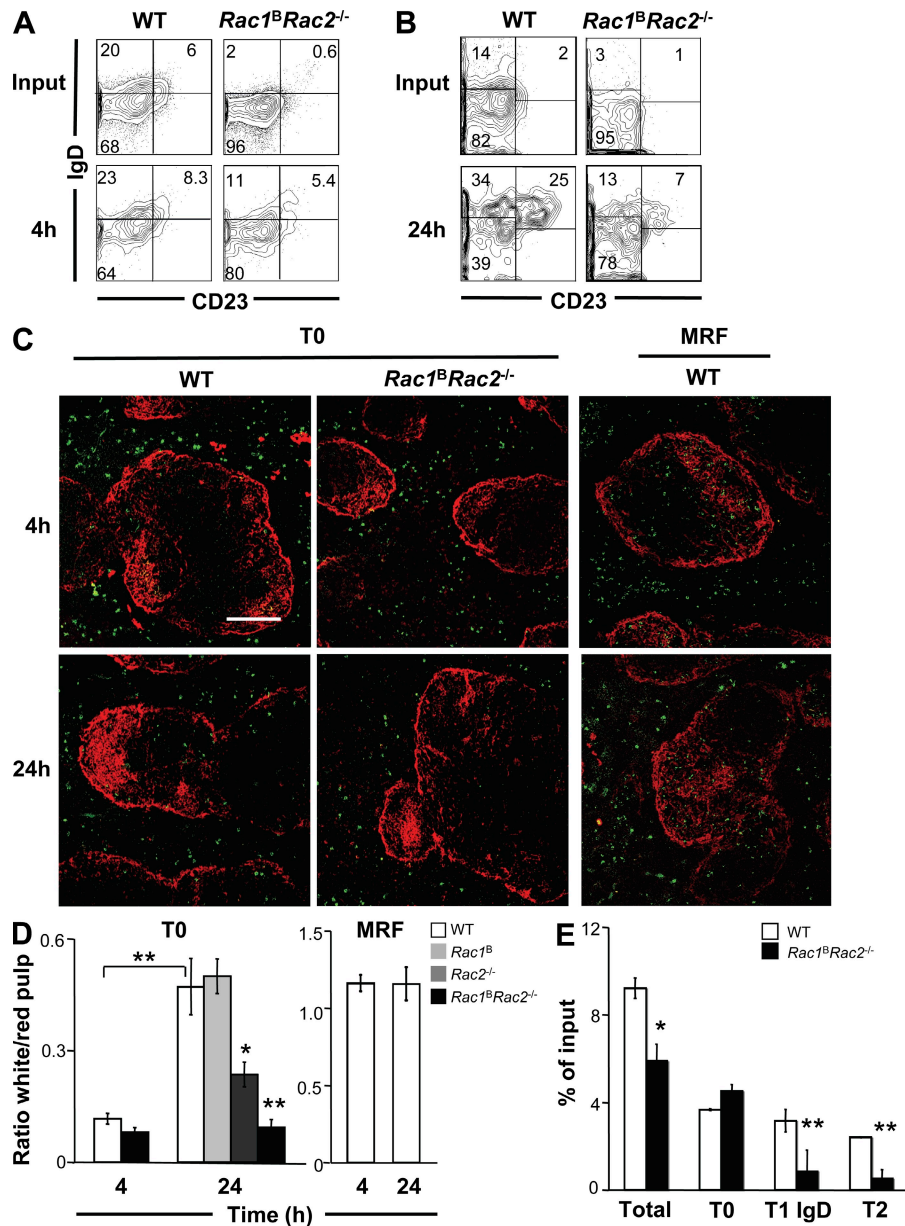


Figure 4. Transitional B cells deficient in both *Rac1* and *Rac2* fail to enter the white pulp of the spleen. (A and B) Contour plots show IgD and CD23 expression on B220⁺IgM^b cells from 129S8 (IgM^b) mice into which splenic T0 B cells from WT or *Rac1^BRac2^{-/-}* (IgM^b) mice had been transferred (A) 4 h or (B) 24 h earlier. The input cells before transfer are shown for comparison. Numbers indicate percentages of cells falling into quadrants. (C) Images showing immunofluorescence staining of sections from spleens of mice into which WT or *Rac1^BRac2^{-/-}* (IgM^b) T0 B cells had been transferred 4 or 24 h earlier. Staining for IgM^b (green) identifies transferred T0 B cells, and MadCAM-1 (red) defines the edges of the white pulp. (right) Transferred WT MRF B cells for comparison. Bar, 150 μm. (D) Mean (±SEM) ratio of transferred IgM^b T0 or MRF B cells ending up in white relative to red splenic pulp at 4 or 24 h after transfer, quantitated using sections such as those shown in C (n = 4–6). (E) Splenic T0 B cells from mice of the indicated genotypes (IgM^bLy5.2⁺) were injected into 129S8 (IgM^bLy5.2⁺) mice. Graph shows the mean (±SEM) percent recovery of transitional B cells in the spleen 24 h after transfer of T0 cells of the indicated genotype. Recovery of total transitional cells is shown, as well as subdivision of these into T0, T1 IgD⁺, and T2 cells (n = 5–8). *, P < 0.05; **, P < 0.01.

studies (Fig. 4 E) would not be further impaired by treatment with pertussis toxin, and this is indeed what we find (Fig. 5, D and E).

Entry of transitional B cells into the splenic white pulp coincides with B cell positive selection

Migration of immature B cells from the bone marrow to the splenic white pulp and other secondary lymphoid organs is a

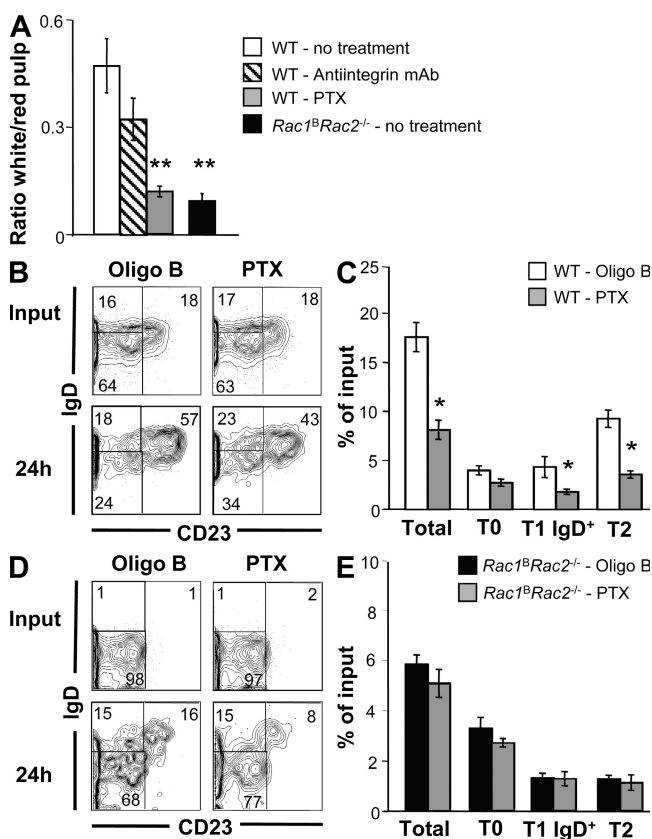


Figure 5. Pertussis toxin blocks entry of T0 splenic B cells into the white pulp, as well as their survival. (A) Mean (\pm SEM) ratio of transferred IgM^{hi} T0 B cells ending up in white relative to red splenic pulp at 24 h after transfers, as described in Fig. 4 (A–C). Transferred T0 cells were from WT or *Rac1^BRac2^{-/-}* mice. In some transfers of WT T0 B cells, the mice were pretreated with anti-LFA-1 and anti- α 4 blocking antibodies (antiintegrin mAb), or the cells were treated with pertussis toxin ($n = 4–6$). (B and D) Contour plots show expression of IgD and CD23 on B220⁺IgM^{hi} splenocytes from 129S8 (IgM^{hi}) mice into which (B) WT or (D) *Rac1^BRac2^{-/-}* (T0 B cells (both IgM^{hi})) had been transferred 24 h earlier and had been either pretreated with pertussis toxin or an oligomer of the B subunit of pertussis toxin (Oligo B). Oligo B controlled for any effects of pertussis toxin independent of the ADP-ribosylation activity of the A subunit, which inactivates G α i-coupled GPCRs. Staining of input cells before transfer is shown for comparison. Numbers indicate percentages of cells falling into quadrants. (C and E) Graphs show the mean (\pm SEM) percent recovery of transitional B cells in the spleens of 129S8 mice 24 h after transfer of WT (C; $n = 4–5$) or *Rac1^BRac2^{-/-}* (E; $n = 6–7$) T0 B cells pretreated with Oligo B or pertussis toxin. Recovery of total transitional cells is shown as well as subdivision of these into T0, T1 IgD⁺, and T2 cells. *, $P < 0.05$; **, $P < 0.01$. PTX, pertussis toxin.

consequence of positive selection for cells that have successfully rearranged light chain genes, resulting in expression of cell-surface IgM. The identification of Rac GTPase-dependent entry of transitional B cells into the splenic white pulp suggested that this process might represent a checkpoint during B cell positive selection. To evaluate this possibility, we examined development of B lineage cells in the absence of Syk, a tyrosine kinase critical for signaling from the BCR (Turner et al., 2000). As reported previously (Cheng et al., 1995; Turner et al., 1995, 1997), in the absence of Syk we found reduced numbers of pre-B and immature B cells in the bone marrow and no splenic mature B cells at all, reflecting a partial block at the pre-BCR checkpoint and a complete block at the BCR checkpoint, B cell positive selection (Fig. 7 B). However, we were able to find a small number of transitional B cells in the spleen, almost all of which were T0 cells, with only $18 \pm 3\%$ falling into T1 IgD⁺ or T2 subsets (Fig. 7, A and B). Furthermore, the large majority of these Syk-deficient transitional B cells were located in the red pulp, with only $11 \pm 5\%$ in the white pulp (Fig. 7 C). These studies show that like the Rac GTPases, the Syk kinase is required for transitional B cells to enter the white pulp, and we propose that this process is a critical feature of BCR-driven positive selection.

Ectopic expression of Bcl-xL rescues the developmental defect in Rac-deficient transitional B cells

The similarity in the developmental arrest and in the survival and migratory defects between *Rac1^BRac2^{-/-}* and pertussis toxin-treated transitional B cells leads us to propose that although Rac-deficient transitional T0 B cells are able to differentiate into the T1 IgD⁺ and T2 stages, they are unable to migrate into the splenic white pulp and hence die because they cannot receive an essential survival signal that is only available in the white pulp. However, an alternative interpretation, based on the similarity of the developmental block in *Rac1^BRac2^{-/-}* and *Syk^{-/-}* transitional B cells, is that the Rac GTPases, like Syk, are required to transduce a BCR signal leading to differentiation of the cells into mature B cells. To distinguish these possibilities, we used retroviral gene transduction to ectopically express the prosurvival Bcl-xL protein in *Rac1^BRac2^{-/-}* transitional B cells. If the requirement for Rac GTPases is to transduce chemokine receptor signals, allowing transitional B cells to migrate into the white pulp and hence receive a survival signal, expression of Bcl-xL should rescue cell survival and hence reverse the developmental block without affecting the migratory defect. If, on the other hand, Rac proteins transduce BCR positive selection signals required for cell maturation, then ectopic expression of Bcl-xL will increase cell survival but will not rescue the developmental block. Exactly this outcome was seen using the related prosurvival Bcl2 protein, which was unable to rescue the developmental defect in *Syk^{-/-}* transitional B cells (Turner et al., 1997).

Bcl-xL expression was forced by using the MIGR1-Bcl-xL retroviral vector to transduce bone marrow cells from WT or *Rac1^BRac2^{-/-}* mice, which were then used to reconstitute

irradiated Rag1-deficient mice. The MIGR1–Bcl-xL vector clearly gave rise to increased Bcl-xL expression in both WT and Rac-deficient transitional B cells (Fig. 8 A). Although increased Bcl-xL expression had no effect on the development of WT transitional cells, it resulted in a decrease in the percentage of Rac-deficient transitional B cells at the T0 stage and a comparable increase in the percentage of T1 IgD⁺ cells (Fig. 8, B and C). As expected, Bcl-xL overexpression did not rescue the defective chemotaxis of Rac-deficient transitional B cells (Fig. 8 D). This result shows that the developmental block in *Rac1^BRac2^{-/-}* mice is caused by the failure of transitional B cells to survive, and supports the hypothesis that Rac GTPases are essential for the survival of transi-

tional B cells because they transduce chemokine signals, allowing the cells to migrate to the white pulp where they receive survival signals.

The Vav exchange factors are not required for entry into the white pulp

The Vav family of guanine nucleotide exchange factors (GEFs) transduce antigen receptor signals to the activation of Rac proteins in both B and T cells (Turner and Billadeau, 2002; Tybulewicz, 2005). They have also been shown to play critical roles in lymphocyte development, most likely because they transduce antigen receptor signals controlling lymphocyte selection. In the absence of all three family members,

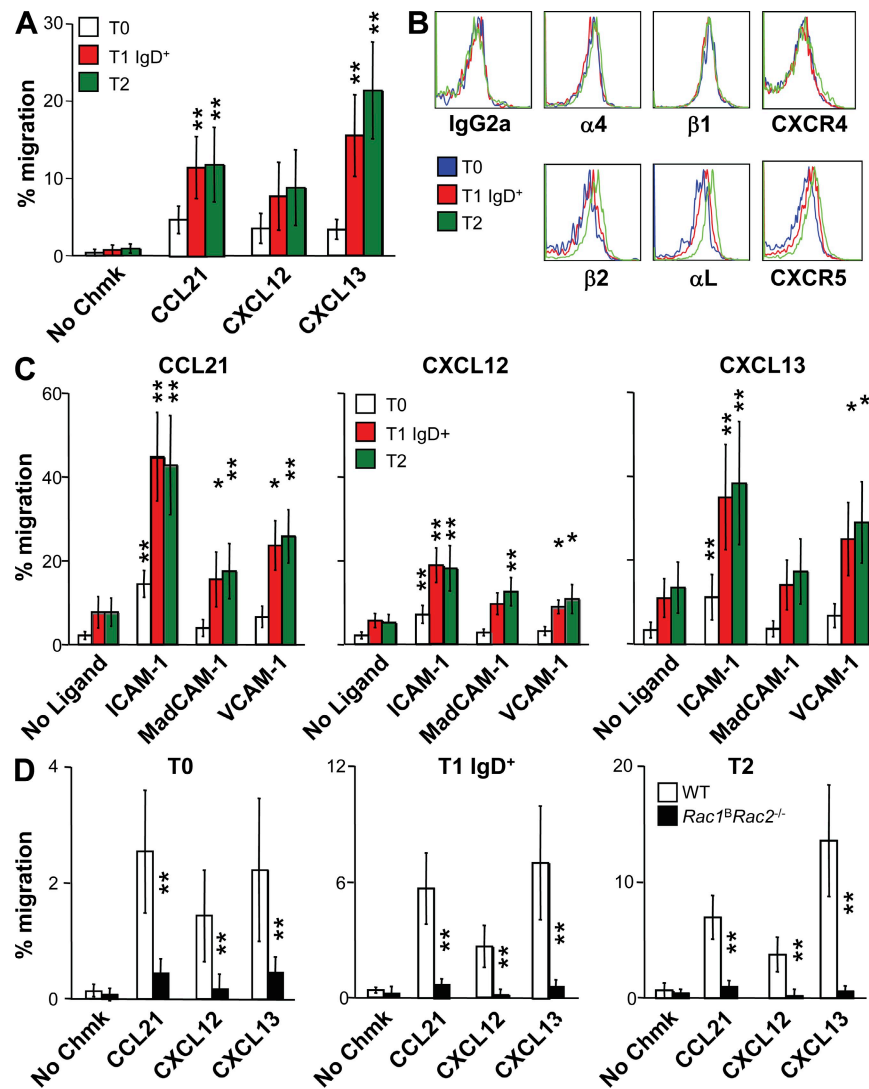


Figure 6. Migration in response to lymphoid chemokines is defective in transitional B cells deficient in Rac1 and Rac2. (A) Mean (±SEM) migration in a Transwell assay of T0, T1 IgD⁺, and T2 splenic B cells from a WT mouse in response to CCL21, CXCL12, CXCL13, or no chemokine (n = 6). (B) Histograms showing cell-surface levels of α4, αL, β1, and β2 integrins, and CXCR4 and CXCR5 on T0, T1 IgD⁺, and T2 B cell subsets. (C) Graph as in A, with migration in response to different chemokines assayed in the presence of the integrin ligands ICAM-1, MadCAM-1, or VCAM-1, or with no ligand present (n = 6). (D) Graph as in A, comparing chemokine-induced migration of transitional B cells from WT and *Rac1^BRac2^{-/-}* mice (n = 6). *, P < 0.05; **, P < 0.01. No Chmk, no chemokine.

Vav1, Vav2, and Vav3, B cell development is almost completely blocked at a transitional B cell stage (Fujikawa et al., 2003; Vigorito et al., 2005). In view of the apparent similarity of this developmental phenotype to that in Rac-deficient mice, we considered whether there may be a Vav–Rac signaling pathway controlling B cell development at the point of entry into the splenic white pulp. Thus, we compared B cell development and the migration of transitional B cells in mice missing either the Vav or Rac proteins.

Enumeration of splenic B cell subsets demonstrated that in mice missing all three Vav proteins (Vav triple KO [VavTKO]), the number of T0 cells was elevated and the number of T1 IgD⁺ cells was unchanged, whereas the numbers of T2, MZ, and MRF B cells were reduced (Fig. 9, A and B), in agreement with previous reports showing a block in development between the T1 and T2 stages (Fujikawa et al., 2003; Vigorito et al., 2005). Analysis of chemokine responsiveness showed that lack of Vav proteins partially reduces chemotaxis induced by CCL21 and CXCL13 in T0, T1 IgD⁺, and T2 B cells, and has no effect on CXCL12 responses; however, these reductions are not as large as those seen in *Rac1^BRac2^{-/-}* B lineage cells (Fig. 9 C). In agreement with this, migration of transitional B cells into the white pulp was not affected by the absence of Vav proteins (Fig. 9 D). Collectively, these results show that the absence of Vav proteins results in a very different B cell phenotype compared with Rac-deficient mice. The developmental block in VavTKO mice is at a later stage than that in *Rac1^BRac2^{-/-}* mice, and the B cells respond reasonably well to chemokines

and are able to traffic normally to the splenic white pulp. Hence, the defects in chemokine responses and migration to the white pulp seen in the absence of Rac proteins must reflect pathways using Rac GEFs other than the Vav family.

Rac2 is required for LFA-1–mediated adhesion

Chemokine receptor signaling activates integrin-mediated adhesion, a process believed to be critical in migration of lymphocytes between the blood and the peripheral lymphoid organs. Furthermore, Rac proteins have been implicated in both inside-out signaling leading to the activation of integrins and outside-in signaling from integrins resulting in firm adhesion (Rose et al., 2007). Thus, the inability of Rac-deficient transitional B cells to migrate into the splenic white pulp may be caused by defects in signaling from chemokine receptors, integrins, or both. To investigate this further, we studied integrin-mediated adhesion in Rac-deficient B cells. Because we were unable to detect such adhesion using transitional cells, we studied the role of Rac proteins in chemokine and integrin function using mature B cells. In addition, because mature B cells deficient in both GTPases do not develop in *Rac1^BRac2^{-/-}* mice (Walmsley et al., 2003), we were instead limited to B cells missing either Rac1 or Rac2 alone.

Transfer experiments showed that although WT and Rac1-deficient mature B cells efficiently enter the splenic white pulp, the absence of Rac2 causes a marked reduction in this migration (Fig. 10 A). To measure integrin-mediated adhesion of B cells, we made use of a system in which the

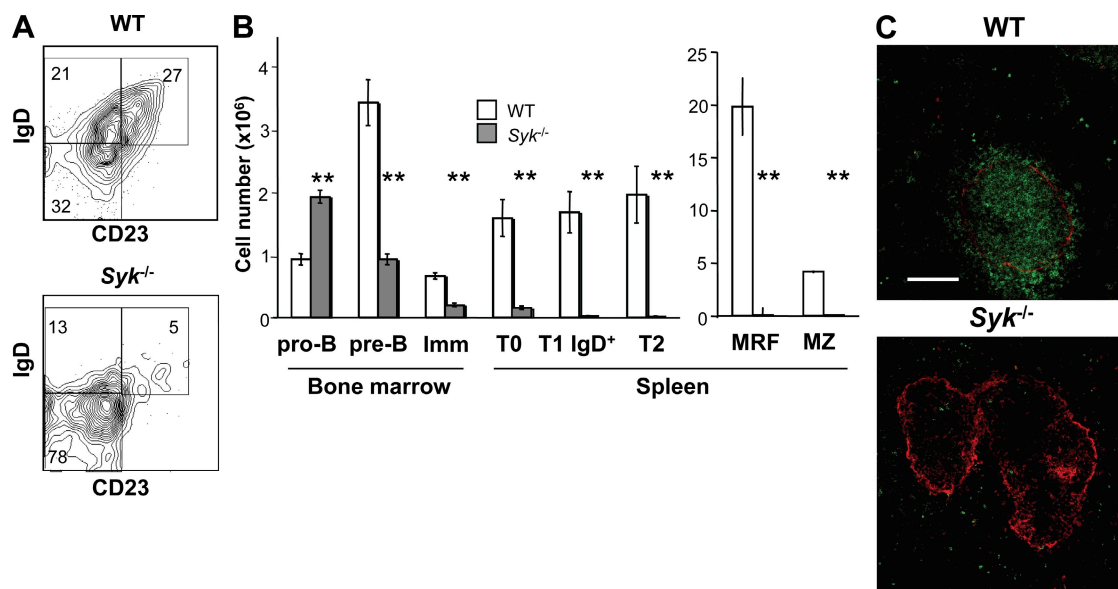


Figure 7. Loss of Syk causes developmental arrest at the T0 transitional B cell stage. (A) Contour plots show IgD and CD23 expression on B220⁺CD93⁺IgM^{hi} splenocytes from irradiated B6.SJL-*Rag2*^{-/-} mice reconstituted 8 wk earlier with *Syk*^{-/-} or WT fetal liver. Numbers indicate percentages of cells falling into quadrants. (B) Mean (\pm SEM) numbers of pro-B (B220⁺IgM⁻IgD⁻CD2⁻), pre-B (B220⁺IgM⁻IgD⁻CD2⁺), and immature T0-like (Imm; B220⁺CD93⁺IgM⁺IgD⁻CD23⁻) cells in the bone marrow and splenic subsets (defined as in Fig. 1 B) of radiation chimeras described in A ($n = 6$). (C) Images showing immunofluorescence staining of sections from spleens of irradiated 129S8 (IgM^h) mice reconstituted 8 wk earlier with *Syk*^{-/-} or WT fetal liver. Staining for IgM^h (green) identifies donor B cells, and MadCAM-1 (red) defines the edges of the white pulp. Bar, 150 μ m. **, $P < 0.01$.

integrin ligand ICAM-1 was tethered to a planar lipid bilayer (Carrasco et al., 2004). We found that although WT and Rac1-deficient B cells adhered strongly to ICAM-1 in response to CXCL12 and CXCL13, *Rac2*^{-/-} B cells showed a large reduction in adhesion (Fig. 10 B). A similar reduced adhesion was also seen after treatment of *Rac2*^{-/-} B cells with Mn²⁺, which activates integrins directly. In contrast, treatment of B cells with phorbol ester and ionomycin resulted in efficient adhesion, irrespective of genotype, demonstrating that Rac2-deficient B cells are able to adhere to ICAM-1 if signaling pathways are bypassed pharmacologically. Collectively, these results show that Rac2 plays an important role in outside-in signaling from LFA-1 that leads to firm adhesion, and may also participate in transducing

inside-out signals from chemokine receptors leading to LFA-1 activation. In contrast, no role for Rac1 could be seen in these processes.

We previously reported that Rac2 was also required for BCR-induced adhesion to ICAM-1, and suggested that this might be caused by a role for Rac2 in the activation of Rap1, a GTPase implicated as a regulator of LFA-1, because BCR-induced Rap1 activation was partially reduced in *Rac2*^{-/-} B cells (Arana et al., 2008). To investigate whether the same may be true for chemokine receptor signaling, we examined Rap1 activation in response to CXCL13. In contrast to our earlier studies with BCR stimulation, we found that both Rac1- and Rac2-deficient B cells activated Rap1 normally (Fig. 10 C). Thus Rac2 appears to play a selective

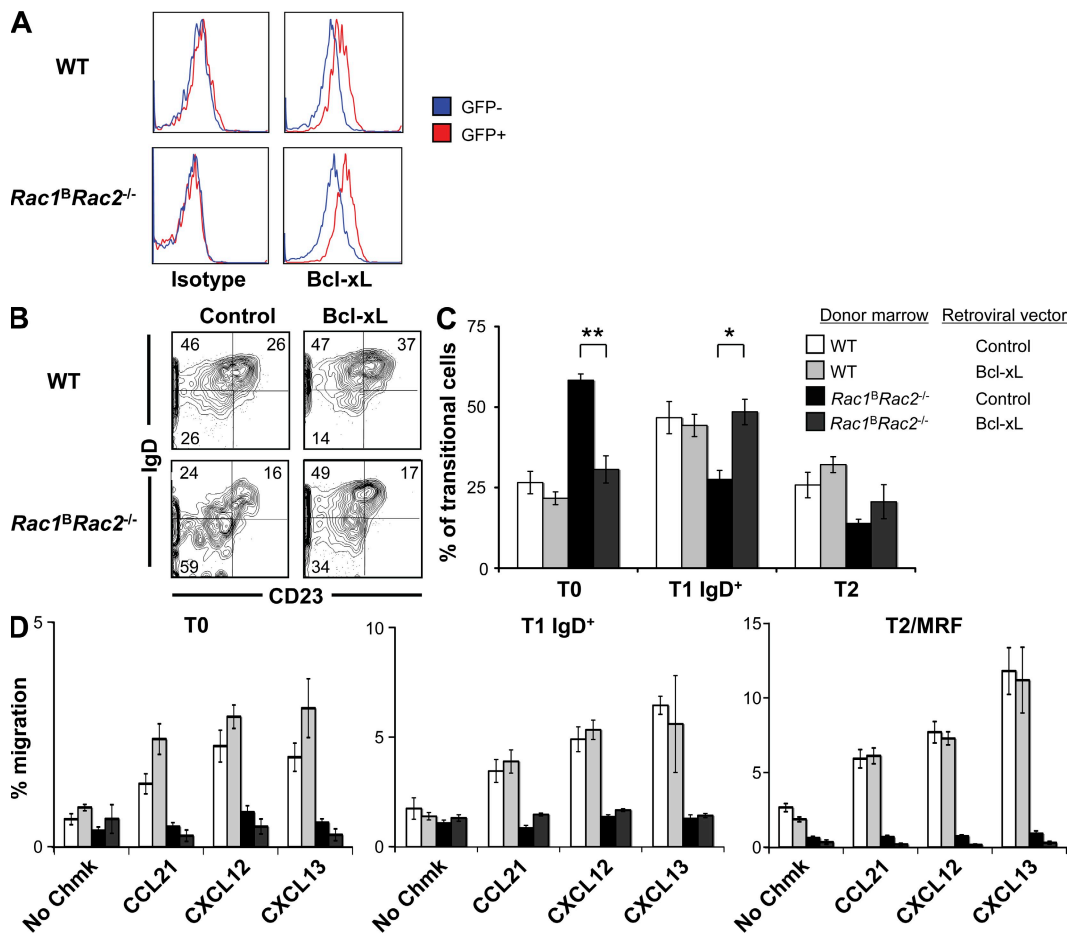


Figure 8. Expression of Bcl-xL in transitional B cells deficient in Rac1 and Rac2 rescues survival and development but not migration. Irradiated Rag1-deficient mice were reconstituted with bone marrow cells from WT or *Rac1*^β*Rac2*^{-/-} mice that had been infected with MIGR1-Bcl-xL, a retroviral vector expressing GFP and Bcl-xL, or with a control vector (MIGR1) expressing only GFP. (A) Histograms show fluorescence of staining with an anti-Bcl-xL or isotype control antibody of GFP⁺ or GFP⁻ splenic T2 B cells from chimeras reconstituted with WT or *Rac1*^β*Rac2*^{-/-} bone marrow cells infected with MIGR1-Bcl-xL. No increase in Bcl-xL expression was seen in T2 cells from chimeras reconstituted with cells infected with the MIGR1 vector control (not depicted; *n* = 3). (B) Contour plots show IgD and CD23 expression on B220⁺CD93⁺IgM^{hi}GFP⁺ splenocytes from chimeras reconstituted with WT or *Rac1*^β*Rac2*^{-/-} bone marrow cells infected with MIGR1 (Control) or MIGR1-Bcl-xL (Bcl-xL) vectors. Numbers indicate percentages of cells falling into quadrants. (C) Mean (±SEM) percentage of B220⁺CD93⁺IgM^{hi}GFP⁺ transitional splenocytes from chimeras described in B that were T0 (IgD⁻CD23⁻), T1 IgD⁺ (IgD⁺CD23⁻), and T2 (IgD⁺CD23⁺), gated as in B (*n* > 4). (D) Mean (±SEM) percent migration in a Transwell assay of B220⁺IgM^{hi}GFP⁺ T0, T1 IgD⁺, and T2/MRF splenocytes from chimeras described in B. Migration was measured in response to no chemokine or the indicated chemokines. Colors of bars are as in C (*n* > 8). *, *P* < 0.05; **, *P* < 0.01. No Chmk, no chemokine.

role in transducing BCR but not chemokine receptor signals leading to Rap1 activation. Finally, we examined chemokine-induced actin polymerization, a process known to be regu-

lated by chemokine receptor signaling. Absence of Rac2 resulted in reduced basal and chemokine-induced actin polymerization (Fig. 10 D). Collectively, these results show

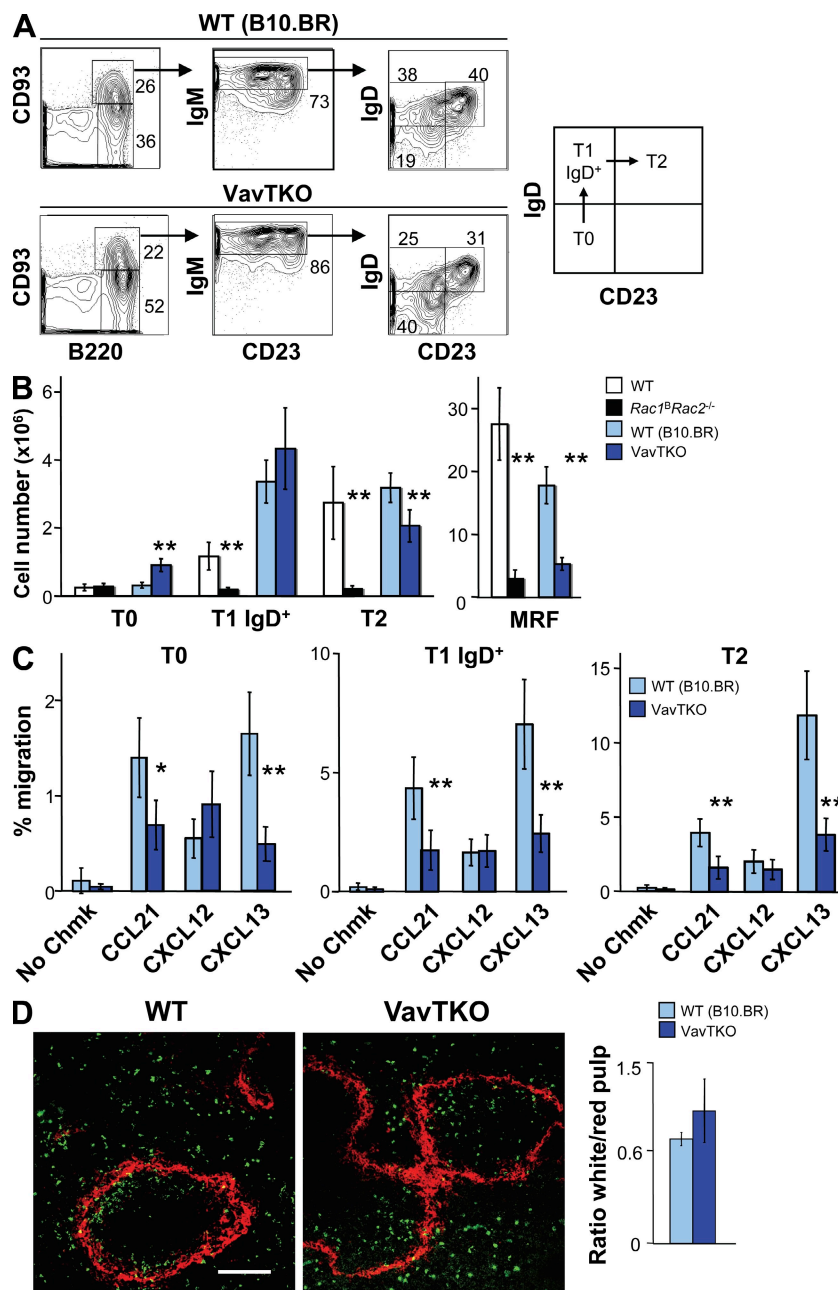


Figure 9. Vav family proteins are not required for entry into splenic white pulp. (A) Contour plots of splenocytes from either WT (B10.BR) or *Vav1*^{-/-}*Vav2*^{-/-}*Vav3*^{-/-} (VavTKO) mice showing separation of B220⁺ cells into immature (CD93⁺) and mature (CD93⁻) B cells. Note that the VavTKO mice are on a B10.BR background and are therefore compared with WT B10.BR mice. The immature cells were further gated on IgM⁺ cells and separated according to the expression of IgD and CD23 into T0 (IgD⁻CD23⁻), T1 IgD⁺ (IgD⁺CD23⁻), and T2 (IgD⁺CD23⁺) subsets. Mature (CD93⁻) cells were separated according to expression of IgM and CD23 into MRF (IgM⁺CD23⁺) and MZ (IgM⁺CD23⁻) subsets (not depicted). Numbers show percentages of cells falling into gates or quadrants. (B) Mean (±SEM) number of splenic T0, T1 IgD⁺, T2, and MRF B cells in WT (*n* = 5), *Rac1*^B*Rac2*^{-/-} (*n* = 7), B10.BR, and VavTKO mice (*n* = 7). (C) Mean (±SEM) migration in a Transwell assay of T0, T1 IgD⁺, and T2 splenic B cells from WT (B10.BR) or VavTKO mice in response to the indicated chemokines (*n* = 6). (D) Images showing immunofluorescence staining of sections from spleens of mice into which WT (B10.BR) or VavTKO (IgM^b) T0 B cells had been transferred 24 h earlier. Staining for IgM^b (green) identifies transferred T0 B cells, and MadCAM-1 (red) defines the edges of the white pulp. Bar, 150 μm. The graph shows the mean (±SEM) ratio of transferred IgM^b T0 B cells ending up in white relative to red splenic pulp 24 h after transfer (*n* = 4). *, *P* < 0.05; **, *P* < 0.01. No Chmk, no chemokine.

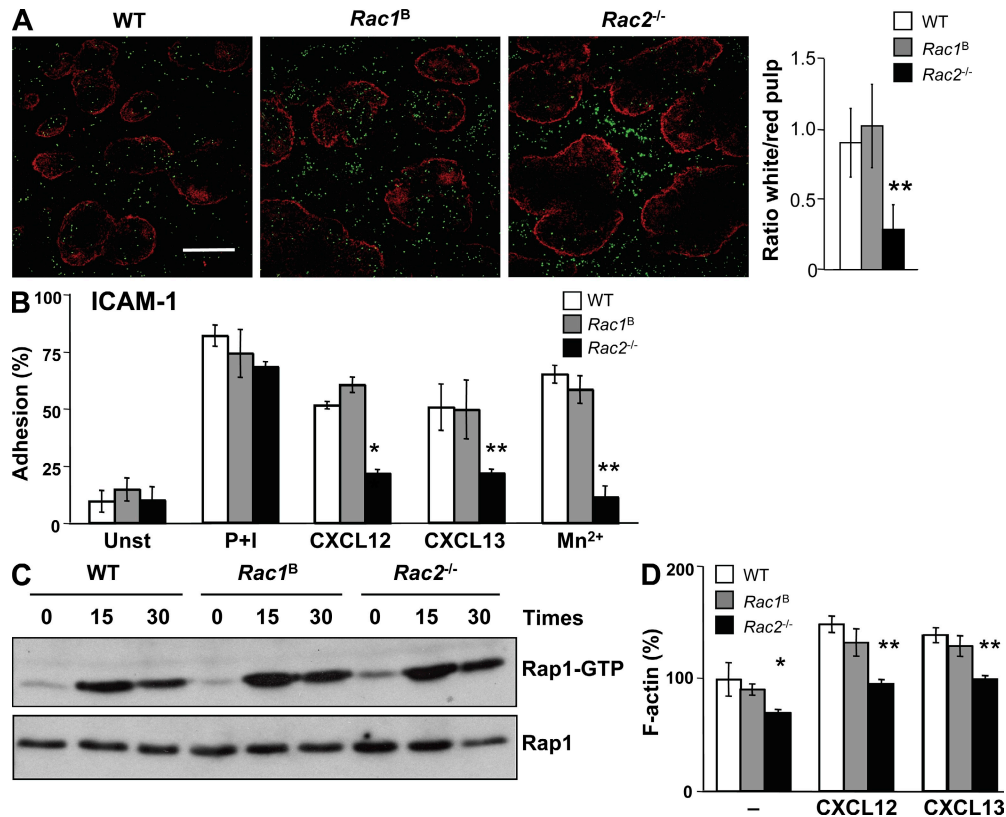


Figure 10. Rac1 and Rac2 are required for chemokine-induced integrin adhesion. (A) Images showing immunofluorescence staining of sections from spleens of mice into which WT, *Rac1^B*, or *Rac2^{-/-}* CFSE-labeled MRF B cells had been transferred 4 h earlier. CFSE (green) identifies transferred B cells, and staining for MadCAM-1 (red) defines the edges of the white pulp. Bar, 300 μ m. The graph shows the mean (\pm SEM) ratio of transferred MRF B cells ending up in white relative to red splenic pulp 4 h after transfer ($n = 5$). (B) Mean (\pm SEM) adhesion of splenic MRF B cells from mice of the indicated genotypes to either ICAM-1 or VCAM-1 incorporated into lipid bilayers. B cells were either unstimulated (Unst) or treated with phorbol 12,13-dibutyrate and ionomycin (P+I), CXCL12, CXCL13, or Mn²⁺. (C) Immunoblots showing levels of Rap1-GTP pulled down using a GST-RBD-RalGDS fusion protein, and of total Rap1 in cell lysates of splenic MRF B cells from mice of the indicated genotypes stimulated with CXCL13 for the indicated times. (D) Graph showing mean (\pm SEM) F-actin content in MRF B cells either unstimulated (–) or stimulated with the indicated chemokines, normalized to the F-actin content of WT unstimulated cells (100%; $n = 5$). Statistical significance in B and D was determined using an unpaired *t* test. *, $P < 0.05$; **, $P < 0.01$.

that Rac2 participates in chemokine receptor signals leading to actin polymerization but not in those required for Rap1 activation.

DISCUSSION

Analysis of splenic B cell development in mice deficient in Rac1 and Rac2 has allowed us to identify a transitional subset that we term T0. Allman et al. (2001) previously described the T1 subset as the earliest B cell immigrants into the spleen. We have now subdivided these cells on the basis of IgD expression into T0 (IgD⁻) cells and T1 IgD⁺ cells. Our studies support the hypothesis that T0 cells are direct precursors of T1 IgD⁺ and T2 cells. BrdU labeling experiments show that splenic T0 cells acquire BrdU more slowly than immature B cells in the bone marrow but do so more rapidly than splenic T1 IgD⁺ and T2 cells. Furthermore, transferred T0 cells mature into T1 IgD⁺ and T2 cells within 24 h. Recent studies have shown that immature B cells can mature into T2 cells already in the marrow, before migrating to the spleen and other peripheral lymphoid organs (Shahaf et al., 2004; Cariappa et al., 2007;

Lindsley et al., 2007). Our BrdU incorporation data support this conclusion, as we find that the output from the splenic T1 IgD⁺ compartment is insufficient to account for the observed cellular input into the T2 compartment, indicating that there must be input from other sources. Indeed, although most immature B cells in the bone marrow are like T0, some express IgD and hence are more like T1 IgD⁺ or T2 cells. Thus, it appears that the transitional B cells migrating from the bone marrow to the spleen are likely to be a mixture of T0, T1 IgD⁺, and T2 cells, as was seen in the blood (Fig. S1 A).

This subdivision into T0 and T1 IgD⁺ subsets appears to be of functional significance, as we show that T0 B cells are unable to enter the splenic white pulp until they mature into the T1 IgD⁺ or T2 subsets. The homing and retention of developing B cells into different lymphoid organs is under the control of chemokine receptors. Although early B cell progenitors (pro-B and pre-B cells) require CXCR4 for retention in the bone marrow, homing and retention of mature B cells in the spleen require CXCR5 and CCR7 (Ma et al., 1999; Ohl et al., 2003; Nie et al., 2004). This suggests that

changing responsiveness to chemokines may determine the localization of developing B cells. In keeping with this, responsiveness of B cell progenitors in the marrow to CXCL12, the ligand for CXCR4, decreases as the cells mature from pro-B through pre-B to immature B cells, reaching a low point in mature B cells (Glodek et al., 2003). In contrast, mature B cells acquire the ability to respond to the CCR7 ligands CCL19 and CCL21 and to CXCL13, the ligand for CXCR5 (Bowman et al., 2000). Our analysis of the chemokine responsiveness of transitional B cells has shown that although T0 B cells respond poorly to CXCL12, CXCL13, and CCL21, T1 IgD⁺ and T2 B cells show increasing responses as maturation proceeds. Collectively, these data suggest that during early B cell development in the bone marrow, B lineage cells gradually lose responsiveness to CXCL12, reaching a low in the immature/T0 stage, thereby allowing them to exit the bone marrow. Upon arrival in the spleen, T0 cells mature into the T1 IgD⁺ and T2 stages, acquire responsiveness to CCL21 and CXCL13, and are thus able to enter the splenic white pulp and eventually the follicles.

The maturation of B cells through the transitional stages corresponds to the point at which the processes of positive and negative B cell selection are occurring (Hardy and Hayakawa, 2001). Immature B cells that have successfully generated a functional BCR in the form of IgM are positively selected, allowing them to continue maturation; cells that fail to make such a BCR are destined to die. In contrast, immature B cells that generate a BCR with high affinity for a self-antigen are eliminated by apoptosis, anergy, or receptor editing in a process termed negative selection. The outcome of these selection events is dependent on BCR signaling. Weak BCR signaling is thought to drive positive selection, whereas strong BCR signaling leads to negative selection. In this study, we have shown that in the absence of Syk, a critical signal transducing molecule downstream of the BCR required for positive selection (Turner et al., 1997), B cell maturation is blocked at the splenic T0 stage, with cells located primarily in the red pulp. Thus, we propose that a critical outcome of the BCR signals driving positive selection is a change in the ability of immature and transitional B cells to respond to different chemokines. Such BCR signals would cause immature B cells to become less responsive to CXCL12 and more responsive to CCL21 and CXCL13, thereby causing the cells to exit the marrow and home to the white pulp of the spleen. Furthermore, we propose that the maturation of T0 B cells into T1 IgD⁺ B cells, which can occur in either the bone marrow or spleen, represents the point at which B cell positive selection is acting to change cellular homing.

The strong developmental block between T0 and T1 IgD⁺ B cells in Rac-deficient mice is most likely caused by a critical role for the GTPases in survival of T1 IgD⁺ and T2 cells. We found no evidence that the absence of Rac GTPases causes maturational arrest, because there is no accumulation of T0 cells in Rac-deficient mice. In contrast, mutant T0 cells were able to mature into T1 IgD⁺ and T2 cells after transfer into WT hosts, but these latter populations survived

poorly. In support of this, expression of Bcl-xL in *Rac1^BRac2^{-/-}* transitional B cells reversed the developmental block, resulting in increased numbers of T1 IgD⁺ cells. In contrast, earlier experiments had shown that ectopic expression of Bcl-2 did not rescue the developmental block in *Syk^{-/-}* transitional B cells (Turner et al., 1997). Collectively, these studies suggest that although Syk is required for the maturation of transitional B cells, presumably because it transduces BCR signals that drive this differentiation process, the Rac GTPases regulate survival of post-T0 stages but not the maturational process itself.

The similarity of the *Rac1^BRac2^{-/-}* phenotype to that seen after treatment with pertussis toxin and the inability of Rac-deficient transitional cells to respond to CCL21 and CXCL13 suggest that in both cases the impaired survival of T1 IgD⁺ and T2 cells may be caused by a failure to migrate to the splenic white pulp in response to chemokines. This migration may be required for transitional B cells to access niches providing survival factors. A key survival factor for transitional B cells is BAFF. Because BAFF is a soluble protein, it is presumably present in the red pulp. However, the observed failure of pertussis toxin-treated T1 IgD⁺ and T2 cells to survive in the red pulp suggests that either BAFF must be presented to the cells within the white pulp, or that BAFF is not sufficient for survival and another survival signal is required that can only be delivered in the white pulp. Although our results focus on early transitional B cell development, further maturational steps within late transitional B cells have been well defined in terms of response to BAFF and selection into the MRF and MZ pools (Saito et al., 2003; Pillai et al., 2004; Srivastava et al., 2005; Meyer-Bahlburg et al., 2008). Here, once again, signals from the BCR and environmental cues are critically involved.

We note that in the absence of Rac GTPases we also see a developmental block between T0- and T1 IgD⁺-like immature B cells in the bone marrow. By analogy with our results in the spleen, this block may indicate that survival of T1 IgD⁺- and T2-like immature bone marrow B cells also requires migration into an appropriate niche, such as the sinusoidal niche shown to be the location of MRF B cells and some immature B cells in the bone marrow (Cariappa et al., 2005; Pereira et al., 2009). We speculate that the developmental block in the bone marrow of Rac-deficient mice may be caused by an essential role for Rac GTPases in transducing signals from cannabinoid receptor 2, a GPCR required for the retention of immature B cells in bone marrow sinusoids (Pereira et al., 2009). More generally, this suggests that GPCRs other than chemokine receptors may also be important in directing B cell migration and hence development.

In earlier work we showed that Rac-deficient immature bone marrow B cells respond poorly to BAFF in vitro and proposed that this may contribute to the developmental block in the mutant mice (Walmsley et al., 2003). The current study suggests that the loss of Rac1 and Rac2 has also affected another key pathway required for survival of transitional B cells involving chemokine-induced migration into the white pulp. We note that the reduction in T1 cells in mice with a mutation

in the BAFF receptor BAFF-R is less severe than that in *Rac1^BRac2^{-/-}* mice (Lentz et al., 1998; Thompson et al., 2001; Yan et al., 2001; Amanna et al., 2003), and hence we propose that the developmental block seen in the absence of Rac1 and Rac2 is likely to be caused by the combination of defects in both BAFF responsiveness and migration into the white pulp.

The precise biochemical role for Rac GTPases in responses to chemokines remains unclear. An important event downstream of chemokine signaling is the activation of integrins by inside-out signaling, binding of integrins to their ligands, outside-in signaling from the integrin, and subsequent adhesion. We have shown that chemokine-induced adhesion through the integrin LFA-1 is dependent on Rac2 in mature B cells. The defective adhesion to ICAM-1 in *Rac2^{-/-}* B cells treated with Mn²⁺ suggests that Rac2 is required to transduce outside-in signals from LFA-1. These might potentially involve regulation of the actin cytoskeleton, because this is a critical event in cell adhesion, and we and others have shown that Rac2-deficient B cells have decreased levels of polymerized actin (Crocker et al., 2002). From the results presented in this study it is not possible to deduce if Rac GTPases also play a role in inside-out activation of integrins. That they may do so is suggested by our recently reported study showing that Rac2 deficiency resulted in a small decrease in BCR-induced activation of the Rap1 GTPase, which is a key transducer of inside-out signals leading to integrin activation (Arana et al., 2008). However, we now find that chemokine-induced Rap1 activation is unaffected by the absence of Rac2, suggesting that Rac2 participates differentially in BCR and chemokine receptor signaling pathways.

Earlier reports have suggested that in T cells, Vav proteins may have important roles in transducing CXCR4 signals to the activation of integrins and that they may also transduce outside-in signals from both β 1 and β 2 integrins, presumably via Rac GTPases (del Pozo et al., 2003; Sánchez-Martín et al., 2004; García-Bernal et al., 2005; Vicente-Manzanares et al., 2005). However, our studies using VavTKO mice show that a deficiency of all three Vav proteins causes a B cell developmental block, which is distinct from that seen in the absence of Rac1 and Rac2, has no effect on the ability of transitional B cells to enter the splenic white pulp, and only partially affects chemokine-induced migration. These results demonstrate that the Vav proteins do not play an important role downstream of chemokine or integrin receptors, at least in transitional B cells. Instead, a more likely candidate for a GEF that transduces chemokine receptor signals to the activation of Rac GTPases is DOCK2, because DOCK2-deficient B cells show greatly reduced CXCR4- and CXCR5-induced migration, and decreased Rac1 and integrin activation (Fukui et al., 2001; Nombela-Arrieta et al., 2004).

In conclusion, we have identified an IgD⁻ transitional subset of B cells that we term T0, which corresponds to the most recently arrived developing B cells in the spleen. We have shown that these cells are unable to enter the splenic white pulp until they mature into T1 IgD⁺ and T2 B cells and that this migration is essential for survival. Furthermore, in

the absence of Rac1 and Rac2, B cell development is arrested at the T0 stage potentially because the later transitional subsets are unable to respond to chemokines and, thus, to migrate into the white pulp and survive. The same developmental block at the T0 stage is seen in the absence of Syk, suggesting that the maturation of T0 cells into T1 and T2 cells and their subsequent migration into the white pulp may represent a key step in the positive selection of developing B cells.

MATERIALS AND METHODS

Mice

Mice bearing a conditional loxP-flanked allele of *Rac1* (*Rac1^{fllox/fllox}*; Walmsley et al., 2003) and *Rac2*-deficient mice (*Rac2^{-/-}*; Roberts et al., 1999) were crossed with *CD19^{Cre/+}* mice in which the Cre recombinase had been knocked into the *CD19* gene (Rickert et al., 1997) to generate mice deficient in Rac1 (*Rac1^{fllox/fllox}CD19^{Cre/+}*; *Rac1^B*), *Rac2* (*Rac2^{-/-}CD19^{Cre/+}*; *Rac2^{-/-}*), or both (*Rac1^{fllox/fllox}Rac2^{-/-}CD19^{Cre/+}*; *Rac1^BRac2^{-/-}*). *CD19^{Cre/+}* mice were used as control WT mice. Mice deficient in *Vav1*, *Vav2*, and *Vav3* (*Vav1^{-/-}Vav2^{-/-}Vav3^{-/-}*) on a B10.BR background (Vigorito et al., 2005) were compared with WT B10.BR mice. Mice heterozygous for a null mutation in *Syk* (*Syk^{+/-}*) were maintained on a B6 background (Turner et al., 1995). Inbred strains used in this study included 129S8, C57BL6/J, B6.SJL, B10.BR, and C3H/HeJ. *Rac2^{-/-}* mice were obtained from D. Williams (Children's Hospital, Boston, MA) and *CD19^{Cre/+}* mice were obtained from J. Roes (University College London, London, England, UK). All mice were bred, maintained, and used under the control of a project license issued by the UK Home Office.

Flow cytometric analysis and cell sorting

Bone marrow cells were obtained by flushing out the femurs and tibias of both hind legs. Splenocytes were isolated by pushing the spleen through a 70- μ m sieve, and blood, taken by cardiac puncture, was collected into 50 μ l of 0.5 M EDTA. Erythrocytes were lysed by incubating cell suspensions for 2 min in 150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.2–7.4. Cells were incubated with Fc receptor blocking antibody 2.4G2 (anti-CD16/CD32) before staining at 4°C in PBS, 0.5% BSA, 0.01% Na₂S₂O₈ containing the specific pretitered antibodies. Biotin- and fluorophore-conjugated antibodies against the following cell-surface proteins were used: IgM, IgM^b, IgM^b, IgD, CD2, CD23, B220, CD93, CD45.1, CD45.2, CXCR4, CXCR5, CD11a (α L integrin), CD18 (β 2 integrin), CD29 (β 1 integrin), and CD49d (α 4 integrin), as well as streptavidin conjugates (obtained from BD, eBioscience, SouthernBiotech, Jackson Immuno-Research Laboratories, Inc., and Invitrogen). To measure intracellular levels of Bcl-xL, cells were stained with antibodies to cell-surface proteins as described, fixed in 3% paraformaldehyde at room temperature for 25 min, permeabilized in 0.1% NP-40 for 2 min, blocked with 1% BSA for 20 min, and stained with PE-conjugated anti-Bcl-xL (7B2.5; Abcam) or IgG3 as an isotype control. Five- or six-color analysis was performed on cytometers (LSR II and FACSCanto II; BD), and data were analyzed using FlowJo software (Tree Star, Inc.). Cell numbers were calculated for a whole spleen or for the marrow from two femurs and two tibias.

To measure turnover of cell populations, mice were injected i.p. with 1 mg BrdU in PBS (Sigma-Aldrich). Thereafter, the mice were given 1 mg/ml BrdU in their drinking water either continuously or, for a pulse-chase study, for a period of only 16 h before being returned to normal drinking water. Splenocytes were harvested at different times and stained with antibodies to cell-surface proteins, and BrdU incorporation into DNA was measured using a BrdU with DNase kit (BD) according to the manufacturer's instructions.

In vivo maturation, migration, and survival

To isolate T0 B cells for transfer assays, splenocytes from 2-wk-old mice were depleted using biotinylated anti-IgD and anti-CD23 antibodies and streptavidin M-280 Dynabeads (Invitrogen). In the resulting cell preparation, typically >70% were T0 (IgD⁻CD23⁻) B cells and >95% were CD23⁻

B cells. To isolate MRF B cells, splenocytes from 6–8-wk-old mice were depleted using biotinylated antibodies to CD43, Gr1, and Mac1, resulting in a preparation containing >90% B cells.

Maturation and survival assay. Transitional B cells from the *CD19^{Cre/+}* strains (IgM^{b+}) were injected i.v. into 129S8 (IgM^{a+}) recipients (1–3 × 10⁷ cells/mouse). Input samples, as well as splenocytes recovered 4 and 24 h after transfer were stained with antibodies to IgD, IgM^a, IgM^b, CD23, CD93, and B220.

Competitive migration assays. Transitional B cells from the *CD19^{Cre/+}* strains (IgM^{b+}Ly5.2⁺) were mixed with an equal number of transitional B cells from 129S8 mice (IgM^{a+}Ly5.2⁺) and were injected i.v. into B6.SJL (IgM^{b+}Ly5.1⁺) recipients (1–3 × 10⁷ cells of each population/mouse). The input cell mixture and cells recovered from the blood, spleen, and bone marrow 4 h after transfer were analyzed by staining with antibodies to IgM^a, IgM^b, Ly5.1, and Ly5.2. Efficiencies of migration and survival were calculated by normalizing the IgM^b/IgM^a ratio of recovered Ly5.2⁺ cells to the IgM^b/IgM^a ratio of injected cells.

Migration into splenic white pulp. Transitional or MRF B cells from the *CD19^{Cre/+}* strains (IgM^{b+}) were injected i.v. into 129S8 (IgM^{a+}) recipients. Transitional B cells from the *Vav1^{-/-}Vav2^{-/-}Vav3^{-/-}* or B10.BR strains (IgM^{b+}) were injected i.v. into C3H/HeJ (IgM^{a+}) recipients. In all cases, 1–3 × 10⁷ cells were injected per mouse. MRF B cells were labeled with 2 μM CFSE (Invitrogen) in Dulbecco's-PBS (Invitrogen) for 10 min at 37°C and washed before injection. Spleens were harvested 4 and 24 h after transfer and snap frozen in liquid nitrogen.

To inhibit CXCR4 function, recipient mice were injected s.c. with 5 mg/kg AMD3100 (Sigma-Aldrich) 30 min before transfer of cells. To block LFA-1 and VLA-4, mice were injected i.p. with blocking antibodies anti-CD11a (H68) and anti-CD49d (PS/2; 100 μg/mouse; a gift from N. Hogg, Cancer Research UK London Research Institute, London, England, UK) 20 min before transfer of cells. In some experiments, transitional B cells were pretreated with pertussis toxin or an oligomer of the B subunit of pertussis toxin (both at 100 ng/ml; Sigma-Aldrich) for 2 h at 37°C before injection.

Immunohistochemistry

5–10-μm cryostat sections from snap-frozen spleens were dried for 1 h at room temperature, fixed in 90% acetone for 20 min, air dried for 10 min, incubated for 5 min in Tris, pH 7.6, and stained with mouse anti-IgM^b-FITC (AF6-78) and rat anti-MadCAM-1 (MECA-367; AbD Serotec) for 1–1.5 h. Where MRF B cells had been transferred, the sections were also stained with mouse anti-IgD^b-FITC (217-170). After washing, slides were incubated in a moist chamber with the secondary reagents anti-FITC labeled with Alexa Fluor 488 and anti-rat IgG labeled with Alexa Fluor 647 (Invitrogen), which had been preabsorbed to 10% normal mouse serum and centrifuged to remove aggregates. Slides were washed in 0.5 M Tris, pH 7.6, washed in water, and mounted in fluorescent mounting medium (Dako). Images of five distinct regions were captured per spleen using an HCPL APO dry objective (20×, NA 0.7) on a confocal microscope (TSC SP2 AOBIS; Leica). Red and white pulp areas, delineated by MadCAM-1 expression, were identified using Image J software (available at <http://rsbweb.nih.gov/ij/>), the number of B cells in each area was counted, and cell density was established by dividing the cell number by area. Finally, the ratio of cell density in white to red pulp areas was calculated. All cell counts were performed by an operator blind to genotype.

In vitro migration

Transwell plates (5-μm pore size; Costar) were either uncoated or precoated with 0.5 μg/ml of recombinant mICAM-1Fc, 3 μg/ml mMadCAM-1Fc, or 3 μg/ml mVCAM-1Fc (R&D Systems), washed three times with PBS, and preincubated with chemotaxis buffer RPMI 1640, 2 mM glutamine, 0.5% BSA at 37°C for 1 h. 1 μg/ml CCL21, 0.2 μg/ml CXCL12, or 1 μg/ml CXCL13 (R&D Systems) were added to the lower chamber, whereas splenocytes prepared from 4-wk-old mice were added to the upper chamber

(10⁶ cells/well). Cells were allowed to migrate for 3 h at 37°C through the Transwell insert. Cells that had migrated into the lower chamber were collected, a set number of PerCP CaliBRITE beads (BD) were added to allow quantification of cell number, and cells were stained with antibodies to B220, CD93, CD23, IgM, and IgD. Flow cytometric analysis established percentages of cells falling into T0, T1 IgD⁺, and T2 subsets, and recovery of CaliBRITE beads was used to calculate absolute numbers of cells that had transmigrated, which was then expressed as the percent migration compared with the number of cells in each of these subsets in the input population of cells. Data shown in the figures are representative of three independent experiments.

Ectopic expression of Bcl-xL in radiation chimeras

Plat-E packaging cells (Morita et al., 2000) were transfected with either the control GFP-expressing MIGR1 retroviral vector (Pear et al., 1998) or the MIGR1-Bcl-xL vector using Genejuice (EMD) according to the manufacturer's protocol. MIGR1-Bcl-xL consists of MIGR1 with a human Bcl-xL cDNA inserted into the EcoRI site such that it expresses both Bcl-xL and GFP (provided by D. Allman and W. Pear, University of Pennsylvania, Philadelphia, PA). Supernatant containing retrovirus was harvested from the cells 48–72 h after transfection, passed through a 0.45-μm filter, and concentrated by centrifugation at 15,000 g for 2 h at 4°C.

Bone marrow cells were harvested from WT or *Rac1^BRac2^{-/-}* mice treated 5 d earlier with 100 mg/kg 5-fluorouracil i.p. (Autogen Bioclear) and cultured overnight in DMEM, 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1× nonessential amino acids (Invitrogen), 2 mM glutamine, 50 μM 2-mercaptoethanol, 6 ng/ml IL-3, 10 ng/ml IL-6, and 100 ng/ml stem cell factor (all growth factors were obtained from PeproTech), and were then infected by culturing in plates coated with 25 μg/ml of recombinant human fibronectin fragment (Retronectin; Lonza) in the presence of concentrated retroviral supernatant for 24 h, and were infected a second time with fresh retroviral supernatant for a further 24 h. Infected bone marrow cells were injected i.v. into B6-*Rag1^{tm1Mom/tm1Mom}* mice that had received two doses of 5 Gy of total body irradiation from a ¹³⁷Cs source administered 3 h apart. Bone marrow cells from one donor mouse were transferred into two recipient mice. Mice were treated with Baytril (Bayer HealthCare) in their drinking water and analyzed 4–5 wk after reconstitution.

Radiation chimeras with Syk-deficient hematopoietic cells

To examine B cell development in the absence of Syk, E15.5 fetal liver cells from *Syk^{-/-}* or *Syk^{+/+}* embryos generated by intercrossing of *Syk^{+/-}* mice were used to reconstitute the hematopoietic system of either B6.SJL-*Rag2^{-/-}* (Ly5.1⁺) mice given 4.5 Gy of irradiation from a ¹³⁷Cs source or 129S8 mice given two doses of 4.5 Gy of irradiation 3 h apart to minimize gastrointestinal tract damage. The B6.SJL-*Rag2^{-/-}* chimeras were used to calculate numbers of cells in B cell subsets; the 129S8 chimeras were used to generate splenic sections. Mice were treated with Baytril in their drinking water for at least 4 wk after transfer and were analyzed 6–8 wk after the transfer.

Adhesion assay

Planar lipid bilayers containing glycosylphosphatidylinositol-linked Alexa Fluor 532-conjugated ICAM-1 (100 molecules/μm²) were prepared in FCS2 chambers (Bioprocess) as described previously (Carrasco et al., 2004). Bilayers were preincubated with 250 ng/ml CXCL12 or CXCL13, and 10⁷ MRF B cells were incubated with bilayers for 20 min at 37°C in PBS, 0.5% FCS, 2 mM MgCl₂, 0.5 mM CaCl₂, 5.5 mM glucose. Alternatively, B cells were pretreated with 10 ng/ml phorbol 12,13-dibutyrate, 1 μg/ml ionomycin for 20 min at 37°C before placing on bilayers. To switch integrins into their active conformation, B cells were incubated with bilayers for 20 min at 37°C in 10 mM MnCl₂, 20 mM Hepes, 140 mM NaCl, 2 mg/ml glucose. Several images were taken of independent areas on bilayers with interference reflection microscopy (IRM) and analyzed as described previously to establish the percentage of adherent cells (Arana et al., 2008). B cells with a minimum 2 μm² of IRM dark area were defined as being adherent. Data shown in the figures are representative of three independent experiments. Statistical analysis was performed using an unpaired *t* test.

Rap1 activation assay

Purified splenic MRF B cells were resuspended in HBSS ($1-2 \times 10^7$ cells per stimulation) and stimulated with $0.5 \mu\text{g/ml}$ CXCL13 at 37°C . Reactions were stopped by addition of an equal volume of cold $2\times$ Rap lysis buffer (50 mM Tris HCl, pH 7.5, 300 mM NaCl, 10 mM MgCl_2 , 2% IGEPAL, 2 mM dithiothreitol, 10% glycerol, 1:50 vol/vol protease inhibitor cocktail [P8340; Sigma-Aldrich]). Active Rap1-GTP was pulled down and analyzed by SDS-PAGE and immunoblotting according to the EZ-Detect Rap1 activation kit protocol (Thermo Fisher Scientific). Signal on the immunoblots was revealed using anti-rabbit Ig-horseradish peroxidase, and blots were washed six times for 10 min in PBS, 0.05% Tween 20 and developed using the West Femto Maximum Sensitivity Substrate (SuperSignal; Thermo Fisher Scientific). Data shown in the figures are representative of three independent experiments.

F-Actin measurement

2×10^6 purified splenic MRF B cells were resuspended in 0.5 ml HBSS and stimulated with $0.5 \mu\text{g/ml}$ CXCL12 or 200 ng/ml CXCL13 at 37°C for 15 s. Reactions were stopped by addition of paraformaldehyde to 3.7%, washed with 50 mM NH_4Cl , permeabilized in 0.1% NP-40 for 2 min, blocked with 1% BSA for 20 min, stained for 30 min with 0.25 U Alexa Fluor 647-conjugated phalloidin (Invitrogen), washed, and analyzed by flow cytometry. Within each experiment, the mean of triplicate F-actin content measurements was normalized to that in unstimulated WT cells, which was set to 100%. Data from five independent experiments were combined and statistical analysis was performed using an unpaired *t* test.

Statistical analysis

All statistical comparisons were performed using the nonparametric two-tailed Mann-Whitney *U* test unless otherwise stated. Unless otherwise indicated, asterisks in figures indicate significant differences compared with WT cells.

Online supplemental material

Fig. S1 shows the separation of immature B cells according to expression of IgD and CD23 in the blood and bone marrow of WT and *Rac1^{fl}Rac2^{-/-}* mice. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20091489/DC1>.

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