Characterization of marginal zone B cell precursors

Bhaskar Srivastava,[1] William J. Quinn III,[1] Kristin Hazard,[2] Jan Erikson,[2] and David Allman[1]

[1]Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine and [2]The Wistar Institute, Philadelphia, PA 19104

Selection of recently formed B cells into the follicular or marginal zone (MZ) compartments is proposed to occur by way of proliferative intermediates expressing high levels of CD21/35 and CD23. However, we show that CD21/35^{high} CD23^{+} splenocytes are not enriched for proliferative cells, and do not contribute substantially to the generation of follicular B cells. Instead, ontogenic relationships, steady-state labeling kinetics, and adoptive transfer experiments suggest that CD21/35^{high} CD23^{+} splenocytes serve primarily as precursors for MZ B cells, although their developmental potential seems to be broader and is influenced by environmental cues that are associated with lymphopenia. Furthermore, CD21/35^{high} CD23^{+} splenocytes share several key functional characteristics with MZ B cells, including their capacity to trap T-independent antigen and a heightened proliferative response to LPS. These observations challenge previous models of peripheral B cell maturation, and suggest that MZ B cells develop by way of CD21/35^{high} CD23^{+} intermediates.

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CORRESPONDENCE
David Allman:
dallman@mail.med.upenn.edu

Abbreviations used: BCR, B cell receptor; BI, biotin; CFSE, carboxyl fluorescein succinimidyl ester; Fr., fraction; MZ, marginal zone; SA, streptavidin; VCAM, vascular cell adhesion molecule.

In adults, B cells are generated from B-lineage committed precursors in the BM. Newly formed sIgM^{+} B cells in the BM are subjected to multiple selective pressures that purge autoreactive B cells and guide differentiation of the remaining cells into functionally distinct peripheral B cell compartments. Ultimately, immature B cells yield follicular or marginal zone (MZ) B cells, mature subsets that differ in their surface phenotype, anatomic localization, and immunologic function (1).

Newly formed B cells are exported to peripheral lymphoid tissues as functionally immature, or transitional, intermediates (2–7). However, the classification of immature splenic B cells and the resulting models of peripheral B cell maturation are controversial. One classification scheme put forth by Loder et al. (5) used variable surface levels of CD21/35, CD23, and IgD to divide CD24/HSA^{high} splenic B cells into two subsets, termed T1 and T2. The T2 subset was postulated to contain precursors for follicular and MZ B cells, and seemed to be enriched for proliferative cells in vivo. An alternative classification from Allman et al. (6) used the developmental marker AA4 and variable surface levels of IgM and CD23 to delineate three transitional populations, T1 through T3. Allman et al. (6) found that the transitional subsets were nonproliferative in vivo and unresponsive to B cell receptor (BCR) cross-linking ex vivo. Thus, the T2 stage described by Loder et al. (5) may be distinct from the transitional subsets described by Allman et al. (6). For clarity, we refer to the T2 subset described by Loder et al. as CD21/35^{high} CD23^{+} splenocytes.

Several studies question the requirement of CD21/35^{high} CD23^{+} splenocytes as intermediates for follicular B cell development. Mice deficient for the transcription factor Aiolos lack MZ B cells and splenic B cells expressing high levels of IgM, CD21/35, and IgD (likely equivalent to CD21/35^{high} CD23^{+} splenocytes), but have an enlarged pool of hyperactivated follicular B cells (8, 9). Similarly, mice with a conditional deletion of the transmembrane receptor Notch2 (10), or mice that are unable to signal through the Notch pathway (11), lack MZ B cells and CD21/35^{high} CD23^{+} splenocytes, whereas their follicular pool appears normal in size and function. This genetic evidence argues against classification of CD21/35^{high} CD23^{+} splenocytes as intermediates in the development of follicular B cells, and instead, suggests a potential relationship with MZ B cells.

We sought to resolve controversies regarding the classification of immature B cells and to explore lineage relationships between immature and mature B cell subsets. Accordingly, we incorporated lineage makers from diverse pheno-

typing schemes, and applied kinetic studies to probe the cellular dynamics and developmental relationships among each relevant B cell subset. We find that CD21/35^{high} CD23^{+} splenocytes are a nondividing cell population whose developmental kinetics and functional attributes are most consistent with a direct precursor–product relationship between CD21/35^{high} CD23^{+} splenocytes and MZ B cells.

RESULTS

CD21/35^{high} CD23^{+} splenocytes are distinguishable from most transitional B cells

Several approaches have been used to identify subsets of immature and mature splenic B cells. Whereas most studies agree on the phenotype of follicular (sIgM$^{int/low}$ sIgD^{high} CD23^{+} CD21/35^{int} AA4^{-}) and MZ (sIgM^{high} sIgD^{low} CD23^{low} CD21/35^{high} AA4^{-}) B cells, the surface phenotype of immature B cells is more controversial. Loder et al. (5) defined two sets of transitional B cells, T1 and T2. In this scheme, T1 splenocytes are sIgM^{high} sIgD^{low} CD23^{low} CD21/35^{low}, whereas T2 splenocytes are sIgM^{high} sIgD^{high} CD23^{+} CD21/35$^{int/high}$. An alternative scheme that was proposed by Allman et al. (6) uses the differential surface expression of AA4 to delineate three AA4^{+} transitional pools, termed T1 through T3. T1 splenocytes are sIgM^{high} CD23^{-}, T2 splenocytes are sIgM^{high} CD23^{+}, and T3 splenocytes are sIgM^{low} CD23^{+}. Other studies on peripheral B cell subsets have used combinations of sIgM and sIgD (9) or CD21/35, sIgM, and CD23 (12) to identify immature and mature B cell subsets.

We used seven-color flow cytometry to analyze simultaneously the surface markers that were used in each of these previous studies. Fig. 1 A shows sIgM and CD21/35 levels on B220^{+} splenocytes from an 8-wk-old C57BL/6 mouse. Using initial gates similar to those of Martin and Kearney (12), we defined three fractions (Frs. I–III). These three populations were subdivided based on differential AA4 and CD23 levels, and were given the designations shown in Fig. 1 A. Most cells in Fr. I are AA4^{+} and correspond to the bulk of immature B cells in the adult spleen. The AA4^{+} CD23^{-} population in Fr. I is T1 in the nomenclature of Allman et al. (6) and Loder et al (5), and the AA4^{+} CD23^{+} population is T2 in the nomenclature of Allman et al. Notably, the T2 population defined in this manner includes the CD21/35^{int} fraction of the T2 population as originally defined by Loder et al. (5). The AA4^{-} CD23^{+} sIgM^{high} population of Fr. I corresponds to a recently characterized subset of recirculating cells whose development is independent of the Tec-family tyrosine kinase, Btk (13). Fr. II includes a minor population of AA4^{+} CD23^{+} cells—T3 in the nomenclature of Allman et al.—whereas the bulk of Fr. II cells are AA4^{-} fol-

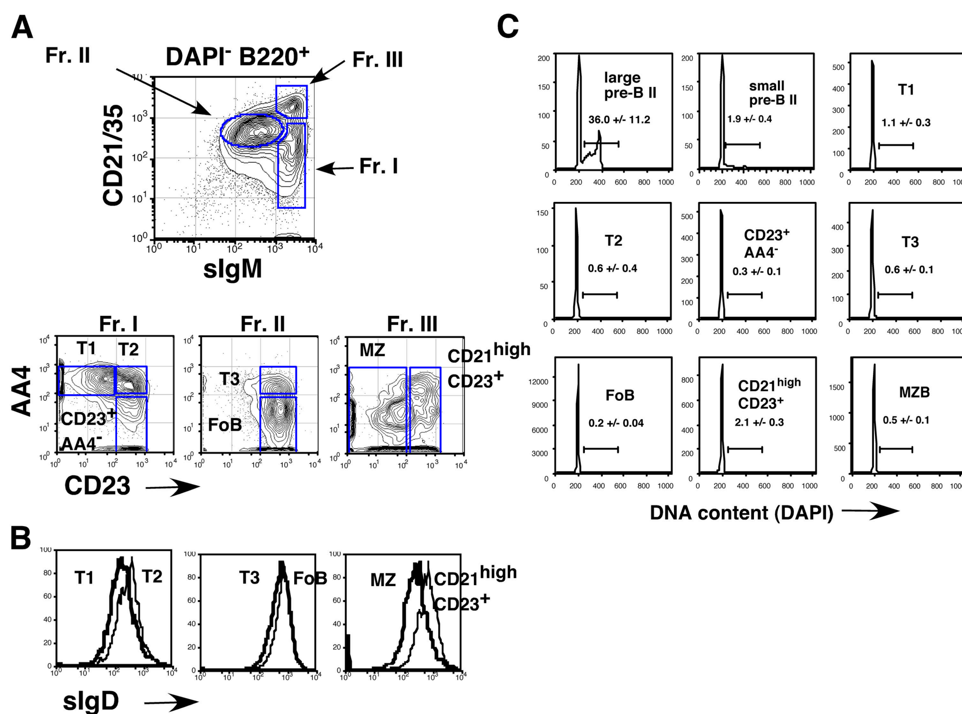


Figure 1. Resolution of splenic B cell subsets. (A) Splenocytes from an 8-wk-old C57BL/6 mouse were harvested and stained with sIgM-FITC, CD23-PE, CD21/35-PECy5.5, B220-APCCy7, AA4-APC, and sIgD-biotin revealed with SA-PECy7. (Top row) sIgM and CD21/35 levels on FSC- and SSC-gated DAPI^{-} B220^{+} lymphocytes. (Bottom row) CD23 and AA4 levels on the three fractions defined above. (B) sIgD levels on six populations defined in (A). (C) Cells from spleen or BM were stained for surface markers as in 1, A and B, fixed, permeabilized, and stained with DAPI. Line graphs show DAPI profiles on the seven splenic populations and two BM populations (gated as described in the text) for positive and negative controls. Gates show the fraction of cells in G_2/M, with average percentages and standard deviations derived from three separate mice shown above the gate.

licular B cells. Approximately one third of the cells within Fr. III are CD23$^+$ cells that correspond to the CD21/35^{high} fraction of the Loder et al. (5) T2 subset; the remainder of Fr. III contains CD23^{low} MZ B cells. Many CD21/35^{high} CD23$^+$ splenocytes are AA4^{low}, which suggests that these cells include recent emigrants from the BM, but constitute a more mature subset than the uniformly AA4$^+$ T1 and T2 subsets. Relative levels of sIgD are illustrated in Fig. 1 B. As reported previously (6), sIgD levels increase as cells mature from T1 to T2 and are high on T3 and follicular B cells. As reported by Loder et al. (5), sIgD levels are high on CD21/35^{high} CD23$^+$ splenocytes but are lower on MZ B cells. Thus, this flow cytometric scheme allows for clear resolution of seven subpopulations of adult splenic B cells, and directly shows that AA4$^+$ CD23$^+$ CD21/35^{low} "T2" transitional B cells are distinct from the CD21/35^{high} CD23$^+$ splenic B cell subset that was described by Loder et al.

AA4^{low} CD21/35^{high} CD23$^+$ splenocytes are nonproliferative in vivo

Previous analyses reported that ≤15% of CD21/35^{high} CD23$^+$ splenocytes are contained within the G_2/M phase of the cell cycle (5). This observation, combined with other studies that suggested that these splenocytes proliferate, upregulate survival pathways, and differentiate into follicular B cells upon BCR cross-linking (14, 15), supports the existence of a BCR-mediated proliferative burst that is associated with the selection and expansion of useful clones

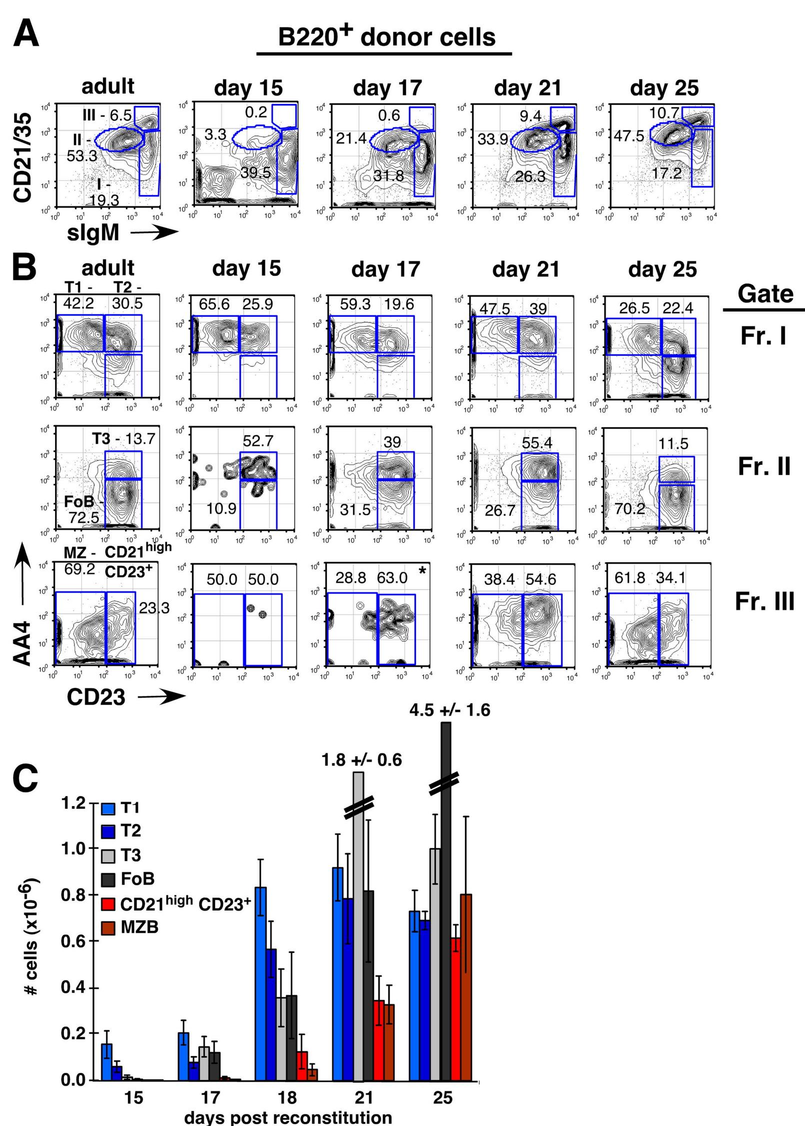


Figure 2. Reconstitution of splenic B cell subsets. Chimeras were generated by reconstituting irradiated C57BL/6 (Ly5^{B6}) hosts with congenic B6.Ly5.2 (Ly5^{SJL}) B220-depleted BM. The composition of the peripheral B cell pool was assessed daily starting on day 12 using the markers in Figure 1 (except IgD) and incorporating congenic markers. (A) CD21/35 and sIgM levels on donor-gated DAPI$^-$ B220$^+$ cells on the indicated days. (B) CD23 and AA4 levels on the three gated fractions shown in A. (C) Absolute cells numbers for each population at the indicated time points.

among late transitional B cells. However, several studies that evaluated in vivo BrdU incorporation rates failed to observe rapid accumulation of $BrdU^+$ mature B cells as predicted (3, 6, 7). Accordingly, we directly examined cell cycle profiles for the splenic B cell subsets that are illustrated in Fig. 1 A. For these analyses, cellular doublets were excluded from postdata collection analyses (see Materials and methods). As shown in Fig. 1 C, as expected, ≤36% and ≤3% of BM large pre–B II cells ($B220^+$ $CD25^+$ FSC^{high}) and small pre–B II cells ($B220^+$ $CD25^+$ FSC^{low}), respectively, were contained within the G_2/M phase of the cell cycle (16). However, none of the splenic B cell subsets that were defined in Fig. 1 A, including the $CD21/35^{high}$ $CD23^+$ subpopulation, was enriched for cells in the S or G_2/M phases of the cell cycle (Fig. 1 C). We conclude that $CD21/35^{high}$ $CD23^+$ splenocytes are not enriched for proliferative cells in vivo.

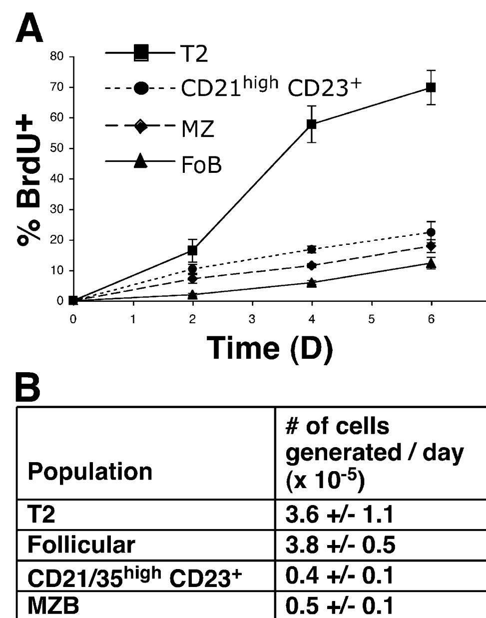
Developmental kinetics of $CD21/35^{high}$ $CD23^+$ splenocytes

Given the genetic data that implicate $CD21/35^{high}$ $CD23^+$ splenocytes in the generation of MZ B cells (9, 10), we sought to determine developmental relationships for these populations using two complementary approaches. First, we conducted reconstitution experiments in which congenic, B220-depleted $B6.Ly5^{SJL}$ BM was transferred into irradiated C57BL/6 ($Ly5^{B6}$) hosts, and the composition of the donor-derived peripheral B cell pool was assessed each day following reconstitution, beginning on day 15. Second, we used continuous in vivo BrdU labeling to assess steady-state turnover and production rates in 8-wk-old C57BL/6 mice.

Fig. 2 illustrates donor-derived B cells ($B220^+$ $Ly5^{SJL+}$) in host spleens on the indicated days after reconstitution. As expected, at early time points, reconstitution was restricted to Fr. I, with the T1 and T2 $AA4^+$ populations evident at this time. In addition, the very few cells detected in Fr. II on day 15 were restricted to the $AA4^+$ T3 pool. Initial reconstitution of the follicular pool was evidenced first on day 17. By analyzing 200,000 cells per sample, we were able to detect small numbers of $AA4^{low}$ $CD21/35^{high}$ $CD23^+$ cells and MZ B cells at this time (Fig. 2 B, day 17). These data are consistent with the reported delayed emergence of MZ B cells (17). Furthermore, reconstitution of the $CD21/35^{high}$ $CD23^+$ pool slightly preceded reconstitution of the MZ B pool, as judged by the early predominance of $CD21/35^{high}$ $CD23^+$ cells when compared with MZ B cells (Fig. 2 B, panel with asterisk). Thus, $CD21/35^{high}$ $CD23^+$ cells emerged well after $AA4^+$ transitional populations T1 through T3, and slightly before MZ B cells. Fig. 2 C illustrates the absolute number of cells in each population as a function of time. Significant numbers of $AA4^+$ transitional and follicular B cells were noted readily before detection of the $CD21/35^{high}$ $CD23^+$ subset, which shows that most $CD21/35^{high}$ $CD23^+$ splenocytes arose well after initial reconstitution of follicular B cells. These data argue against classification of $CD21/35^{high}$ $CD23^+$ splenocytes as precursors in follicular B cell development, and instead, suggest a close developmental relationship between $AA4^{low}$ $sIgM^{high}$ $CD21/35^{high}$ $CD23^+$ splenocytes and MZ B cells.

BrdU-labeling kinetics

To evaluate further the lineage relationships suggested by Fig. 2, we examined the steady-state cellular dynamics of each subset illustrated in Fig. 1 by way of continuous in vivo BrdU labeling. Cohorts of 8-wk-old C57BL/6 adults were inoculated i.p. with BrdU every 12 h, and BrdU incorporation was assessed at 2, 4, or 6 days. As reported previously (6), the $AA4^+$ transitional population T2 labeled rapidly, with ∼60% labeling by day 4. In contrast, follicular B cells, $CD21/35^{high}$ $CD23^+$ splenocytes, and MZ B cells all labeled relatively slowly, with 5%, 10%, and 15% labeling on day 4, respectively (Fig. 3 A). We further estimated production rates for each pool. The number of $CD21/35^{high}$ $CD23^+$ B cells produced per day was markedly insufficient to sustain the follicular B cell pool. Whereas 3.8×10^5 follicular B cells were produced per day, only 0.4×10^5 $CD21/35^{high}$ $CD23^+$ B cells were generated per day (Fig. 3 B). Significantly, $CD21/35^{high}$ $CD23^+$ splenocytes exhibited slightly accelerated labeling kinetics relative to MZ B cells (Fig. 3 A), and production rates for $CD21/35^{high}$ $CD23^+$ B cells mirrored those for MZ B cells (Fig. 3 B). These data, together with the reconstitution data in Fig. 2, suggest a direct pre-



Population	# of cells generated / day (x 10^{-5})
T2	3.6 +/- 1.1
Follicular	3.8 +/- 0.5
$CD21/35^{high}$ $CD23^+$	0.4 +/- 0.1
MZB	0.5 +/- 0.1

Figure 3. BrdU labeling of peripheral B cell pools. 8-wk-old C57BL/6 mice (three mice per time point) were inoculated i.p. with BrdU every 12 h for 2, 4, or 6 d. Mice were killed, and splenocytes were stained with the surface markers in Fig. 1 (excluding IgD), and then stained intracellularly for BrdU incorporation as described in Materials and methods. (A) Percent labeling within the T2, follicular B cell (FoB), $CD21/35^{high}$ $CD23^+$, and marginal zone B (MZB) cell pools as a function of time. (B) Number of cells generated in those pools per day estimated by calculating the average number of $BrdU^+$ cells generated each day between days 2 and 4.

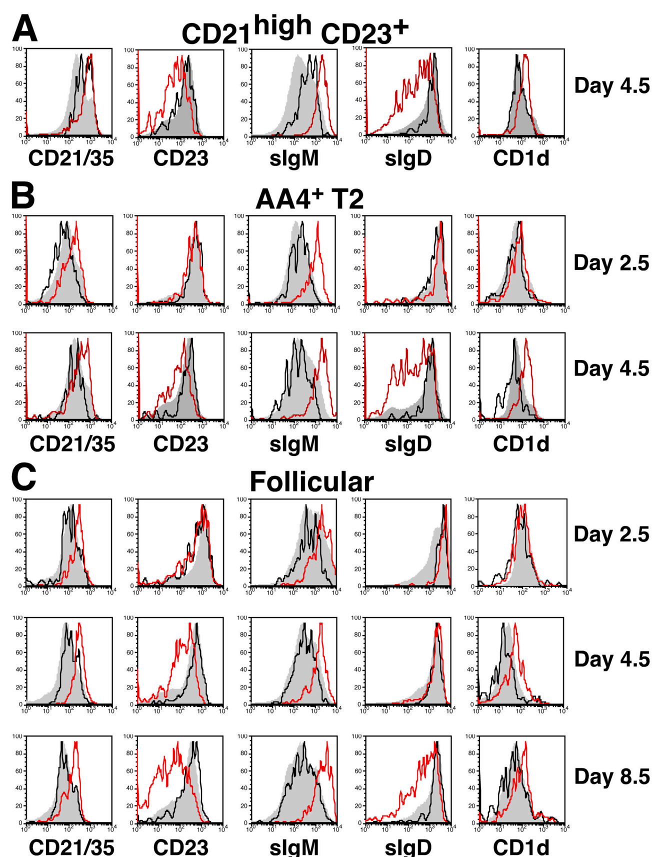


Figure 4. Adoptive transfer of peripheral B cell subsets. Cells were sorted from splenocytes of B6.Ly5^{SJL} mice using the surface markers in Fig. 1 (excluding IgD), and then transferred into C57BL/6 or C57BL/6-backcrossed RAG2$^{-/-}$ (both Ly5^{B6+}) hosts. Host splenocytes were harvested at the indicated time points, and were stained with the indicated antibodies, including anti-Ly5^{B6} antibodies. Line graphs show overlays of surface markers on donor B cells (identified as Ly5^{B6-} B220^{+}): red, donor cells in RAG2$^{-/-}$ hosts; black, donor cells in C57BL/6 hosts; gray, host splenic B cells (Ly5^{B6+} B220^{+}). (A) Phenotype of CD21/35^{high} CD23^{+} splenocytes at day 4.5 after transfer. (B) Phenotype of AA4^{+} T2 splenocytes at days 2.5 and 4 after transfer. (C) Phenotype of follicular B cells at days 2.5, 4.5, and 8.5 after transfer.

cursor–product relationship for CD21/35^{high} CD23^{+} and MZ B cells.

CD21/35^{high} CD23^{+} splenocytes are intermediates in the development of MZ B cells

Next, we conducted several adoptive transfer experiments to assess directly whether CD21/35^{high} CD23^{+} splenocytes are MZ B cell precursors. We sorted and transferred 250,000 CD21/35^{high} CD23^{+} splenocytes from B6.Ly5^{SJL} donors into C57BL/6 or RAG2$^{-/-}$ (both Ly5^{B6+}) hosts. 4.5 d after transfer, we assessed the phenotype of donor B cells by gating on B220^{+} Ly5^{B6-} splenocytes. As seen in Fig. 4 A, CD21/35^{high} CD23^{+} splenocytes efficiently gave rise to CD21/35^{high} IgM^{high} CD23^{low} CD1d^{high} MZ B cells in RAG2$^{-/-}$ hosts, but seemed to lose some CD21/35 and sIgM expression while maintaining CD23 and sIgD expression in C57BL/6 hosts. Consistent with previous data (18), recoveries in both host types typically were 1–2% (unpublished data). We conclude that CD21/35^{high} CD23^{+} splenocytes can give rise to MZ B cells in lymphopenic hosts but seem to give rise to follicular B cells in replete hosts; this suggests that competitive pressures may influence peripheral B cell fate.

Because AA4^{+} T2 B cells are considered late-stage immature B cells, we reasoned that these cells should give rise to MZ B cells and follicular B cells upon adoptive transfer. Furthermore, if the CD21/35^{high} CD23^{+} population is an intermediate in MZ B cell development, the AA4^{+} T2 splenocytes might pass through this stage as they differentiate into MZ B cells. Accordingly, we sorted and transferred 250,000 AA4^{+} T2 cells from B6.Ly5^{SJL} donors into C57BL/6 or RAG2$^{-/-}$ hosts. The donor inoculum for AA4^{+} T2 splenocytes contained only 0.1–0.2% contamination by MZ B cells (unpublished data). Given a 1–2% homing efficiency, this implies that virtually no MZ B cells would be transferred to the host. As shown in Fig. 4 B, AA4^{+} T2 splenocytes efficiently gave rise to MZ B cells in RAG2$^{-/-}$ hosts at day 4, but gave rise to CD21/35^{int} IgM$^{int/lo}$ CD23^{+} CD1d^{low} follicular B cells in C57BL/6 hosts. Importantly, at day 2.5 after transfer, the AA4^{+} T2 splenocytes in RAG2$^{-/-}$ hosts expressed high levels of CD21/35 and sIgM, and maintained high expression of CD23 and sIgD (Fig. 2 B, first row). Thus, AA4^{+} T2 splenocytes transit through a CD21/35^{high} CD23^{+} stage en route to the MZ B stage.

Because the host environment influenced whether recovered AA4^{+} T2 cells exhibited a follicular or MZ B cell surface phenotype (Fig. 4 B), we further reasoned that mature follicular B cells also might yield MZ B cells when transferred into B cell–deficient, but not B cell–sufficient, hosts. Previous studies that employed transfer of unfractionated B cells from the mouse lymph node or rat thoracic duct suggested that mature B cells generate MZ-like B cells upon transfer into lymphopenic hosts (19, 20). However, these MZ-like cells also may derive from late transitional B cells, which are readily detectable in the lymph node and among recirculating B cells in the peripheral blood (unpublished data). Accordingly, we determined whether highly purified splenic follicular B cells, as defined in Fig. 1, yielded MZ B cells when transferred into RAG2$^{-/-}$ hosts, and whether such differentiation was accompanied by the initial acquisition of a sIgM^{high} CD21/35^{high} CD23^{+} surface phenotype. As shown in Fig. 4 C, at day 2.5 after transfer, donor B cells that were recovered from RAG2$^{-/-}$ hosts were CD21/35^{high} CD23^{+} sIgM^{high}. By day 4.5, a subset of the donor B cells had down-regulated CD23, and by day 8.5, donor B cells also were sIgD^{low}, which indicated further differentiation along the MZ B cell pathway (Fig. 4 C). In contrast, at day 2.5 and all subsequent time points, donor B cells that were recovered from C57BL/6 hosts retained a sIgM^{low} CD21/35^{low} CD23^{+} surface phenotype that is typical of follicular B cells. We also conducted immunohistochemical and functional analyses to test whether

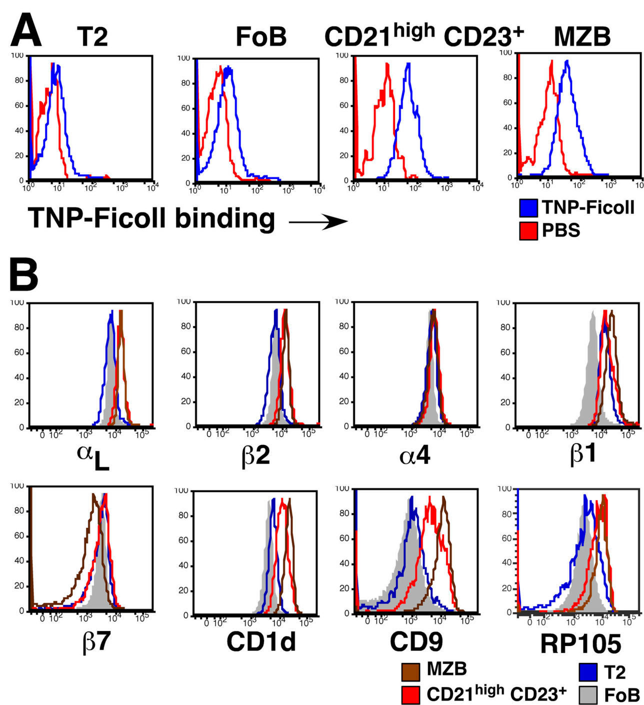


Figure 5. Trapping of T-independent antigen and integrin expression. (A) C57BL/6 mice were injected with 500 μg of TNP-Ficoll in PBS or PBS alone. 30 min later, splenocytes were stained with the surface markers in Fig. 1 (except IgD) and biotinylated anti-TNP-Ficoll (revealed with SA-PECy7). The levels of bound TNP-Ficoll are shown as line graph overlays; red and blue lines show signals from PBS-injected and TNP-Ficoll injected mice, respectively. (B) Expression of MZ-specific surface markers, including integrins, CD1d, CD9, and RP105, on T2, follicular B cell (FoB), CD21/35^{high} CD23^{+}, and MZ B cells. Surface levels are shown as line graph overlays.

transferred follicular B cells localized to the MZ and acquired attributes that are characteristic of MZ B cells (unpublished data). Transferred follicular B cells colocalized with MOMA^{+} metallophillic macrophages in the splenic MZ (21), and acquired the capacity to trap T-independent antigens (22, 23) and stimulate allogeneic CD4^{+} T cells in a mixed lymphocyte reaction (24). Again, cell recoveries for all follicular B cell transfers typically were 1–2% of the donor inoculum, but sort purities for follicular B cells typically were ~99%, with <0.1% contamination by MZ B cells (unpublished data). These high sort purities, the stepwise differentiation of AA4^{+} T2 and follicular B cells in RAG2$^{-/-}$ hosts, and the maintenance of the follicular phenotype in C57BL/6 hosts argue that selective survival of contaminating MZ B cells is unlikely to account for these data. Overall, these results strengthen the notion that CD21/35^{high} CD23^{+} splenocytes are an intermediate in the differentiation pathway of MZ B cells.

CD21/35^{high} CD23^{+} splenocytes share key characteristics with MZ B cells

If CD21/35^{high} CD23^{+} splenocytes are immediate precursors for MZ B cells, they might share certain functional characteristics with them. One functional characteristic of MZ B cells is their ability to trap the T-independent antigen TNP-Ficoll (22, 23). To assess whether CD21/35^{high} CD23^{+} splenocytes share this characteristic with MZ B cells, we injected C57BL/6 mice i.v. with TNP-Ficoll or PBS. 30 min later, each splenic B cell subset was examined for antigen retention. As shown in Fig. 5 A, follicular B cells and AA4^{+} T2 cells trapped relatively little TNP-Ficoll, whereas MZ and CD21/35^{high} CD23^{+} B cells trapped a relatively large amount of TNP-Ficoll on their surfaces. Thus, CD21/35^{high} CD23^{+} splenocytes mirror MZ B cells with regard to their capacity to trap T-independent antigens.

MZ B cells also express higher levels of the integrins $\alpha_L\beta_2$ and $\alpha_4\beta_7$ than do follicular B cells. Binding of these integrins to their ligands, intercellular adhesion molecule and vascular cell adhesion molecule (VCAM), respectively, is critical for retention of MZ B cells in their anatomic niche (25). Surface expression of these integrins on AA4^{+} T2 cells, follicular B cells, CD21/35^{high} CD23^{+} splenocytes, and MZ B cells are shown in Fig. 5 B. MZ and CD21/35^{high} CD23^{+} B cells exhibit elevated levels of $\alpha_L\beta_2$, whereas AA4^{+} T2 cells express lower levels, similar to follicular B cells. In contrast, $\alpha_4\beta_7$ is expressed at low levels on follicular B cells, at intermediate levels on T2 and CD21/35^{high} CD23^{+} B cells, and at high levels on MZ B cells (25). Thus, CD21/35^{high} CD23^{+} splenocytes may exhibit enhanced binding to intercellular adhesion molecule, but not to VCAM. We also assayed the expression of markers that were reported to differ on MZ B cells and follicular B cells. The glycolipid presentation molecule, CD1d, is expressed at higher level on MZ B cells (26) and, in line with previous findings (27), has intermediate expression on CD21/35^{high} CD23^{+} splenocytes relative to AA4^{+} T2 and follicular B cells. In addition, the adhesion molecule CD9 is expressed preferentially on MZ B cells (28) and shows intermediate levels on CD21/35^{high} CD23^{+} splenocytes. Finally, expression of the Toll-like receptor homologue RP105 was expressed at roughly equivalent levels on MZ and CD21/35^{high} CD23^{+} splenocytes, and was significantly higher on these cells compared with follicular and AA4^{+} T2 B cells.

Finally, we assessed whether CD21/35^{high} CD23^{+} splenocytes exhibit robust proliferation to LPS, which is characteristic of MZ B cells (29). Fig. 6 A shows the proliferation profiles of sorted, carboxyl fluorescein succinimidyl ester (CFSE)–labeled follicular, CD21/35^{high} CD23^{+} and MZ B cells that were stimulated by BCR cross-linking or LPS. Fig. 6 B shows the responder frequencies and proliferative capacities that were calculated from these CFSE profiles (30). Like MZ B cells, CD21/35^{high} CD23^{+} splenocytes show a much stronger response to LPS than do follicular B cells. This greater response is reflected in a larger responder frequency, rather than a greater proliferative capacity. Strikingly, CD21/35^{high} CD23^{+} splenocytes also responded robustly to BCR cross-linking, which distinguishes them from MZ B cells and from AA4^{+} transitional cells, which are nonproliferative to BCR cross-linking (6, 29). Thus, CD21/35^{high} CD23^{+} splenocytes share many characteristics with MZ B cells, but are not functionally equivalent to MZ B cells. Overall, these

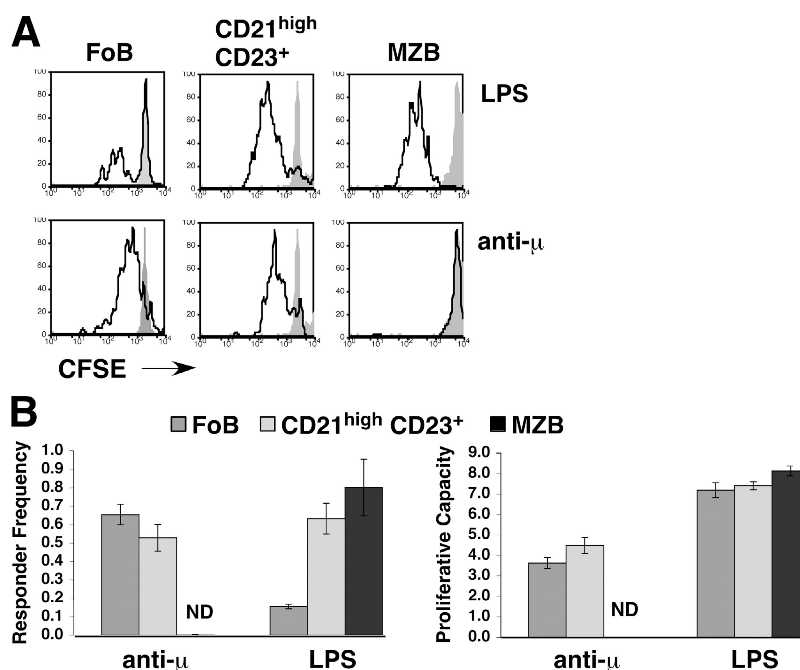


Figure 6. Mitogen responsiveness of purified splenic B cell subsets. Response of sorted CFSE-labeled follicular (FoB), CD21/35^{high} CD23^{+}, and MZ B cells to LPS (5 μg/ml) or BCR-cross-linking (10 μg/ml F[ab′]$_2$ anti-IgM). (A) Plots show resulting CFSE levels on live (DAPI^{-}) B220^{+} cells at day 3 of culture with unstimulated cells in the gray filled line graphs. (B) Responder frequencies and proliferative capacities calculated from the plots in A by the method of Wells et al. (30). ND, no proliferation detected.

similarities strengthen the idea that CD21/35^{high} CD23^{+} splenocytes have initiated the developmental program that distinguishes MZ B cells from other splenic B cells.

DISCUSSION

Nonfollicular CD23^{+} splenic B cells can be divided into at least two subsets (Fig. 1 A): a CD21/35^{int} subset that expresses relatively high levels of AA4, and a CD21/35^{high} subset that expresses low to background levels of AA4. The former AA4^{+} CD21/35^{int} subset corresponds to the T2 population of Allman et al. (6) and includes a fraction of the T2 population of Loder et al. (5), whereas the latter AA4^{low} CD21/35^{high} subset corresponds to a fraction of the T2 population that was defined by Loder et al. (5). This population, which we have referred to as CD21/35^{high} CD23^{+} splenocytes, has many characteristics that distinguish it from the bulk of immature cells and strongly implicate it as a precursor pool for MZ B cells. During the early establishment of the B cell pool, development of this subset is delayed compared with the AA4^{+} pools, T1 and T2; it coincides with the development of follicular B cells; and it immediately precedes initial reconstitution of the MZ B cell pool (Fig. 2). Moreover, BrdU labeling in adults indicates that the CD21/35^{high} CD23^{+} splenocytes turn over relatively slowly, and are generated in small numbers that are sufficient to account for MZ B cell, but not for follicular B cell, production (Fig. 3). Adoptive transfer experiments indicate that CD21/35^{high} CD23^{+} splenocytes are an intermediate in the development of MZ B cells from AA4^{+} T2 splenocytes or follicular B cells (Fig. 4) in RAG2$^{-/-}$ hosts. Taken together, ontogenic relationships, population dynamics, and lineage potentials that were revealed by adoptive transfer argue against the classification of CD21/35^{high} CD23^{+} splenocytes as significant intermediates in the development of follicular B cells, and support a precursor–product relationship with MZ B cells.

Several groups have investigated the characteristics of CD21/35^{high} CD23^{+} splenocytes and have interpreted their findings in the context of follicular B cell development. However, our data suggest that many of the characteristics that are attributed to late transitional B cells in these studies may reflect events that are associated with MZ, rather than follicular B cell, development. For instance, the reported upregulation of survival molecules among CD21/35^{high} CD23^{+} splenocytes in response to BCR signaling that was observed by Su and Rawlings (14) and Petro et al. (15), may reflect BCR-mediated selection events that are associated with prolonged survival of MZ B cells, which unlike follicular B cells, persist after cessation of B cell production in the BM (31). Likewise, the exquisite sensitivity of CD21/35^{high} CD23^{+} splenocytes to levels of the prosurvival cytokine, BLyS (32, 33), most likely reflects a more stringent requirement for BLyS in the development and maintenance of MZ cells and their precursors as compared with follicular B cells. Similarly, many discrepancies in the literature can be clarified by interpreting the CD21/35^{high} CD23^{+} splenocyte as a MZ B cell precursor. In certain lymphopenic mice, such as IL-7R$\alpha^{-/-}$ and *xid* mice, MZ B cells and CD21/35^{high} CD23^{+} splenocytes are maintained, whereas AA4^{+} transitional and

follicular B cells are diminished severely (1, 34; and unpublished data). Likewise, overexpression of the *c-fos* proto-oncogene results in enhanced frequencies of MZ B cells as well as CD21/35^{high} CD23^{+} splenocytes, whereas the number of earlier transitional cells and follicular B cells is unchanged (35). Conversely, mice with a targeted disruption of the G protein α inhibitory subunit exhibit decreased MZ and CD21/35^{high} CD23^{+} B cell pools, and have increased numbers of follicular B cells (36). These results are consistent with our proposed precursor–product relationship, and support coordinate maintenance or expansion of MZ B cells and their direct precursors.

Although CD21/35^{high} CD23^{+} splenocytes share several characteristics with MZ B cells, these cells are not merely a subset of MZ B cells, because CD21/35^{high} CD23^{+} splenocytes do not seem to be functionally equivalent to MZ B cells. First, CD21/35^{high} CD23^{+} splenocytes do not express high levels of the integrin $\alpha_4\beta_7$ (Fig. 4 A), which binds to VCAM and is required to maintain MZ B cells in their anatomic niche (25). This difference in integrin expression may contribute to the localization of CD21/35^{high} CD23^{+} splenocytes to the follicle, rather than the MZ (5, 37). Second, CD21/35^{high} CD23^{+} splenocytes respond robustly to stimulation by BCR cross-linking and LPS treatment, whereas MZ B cells are unresponsive to stimulation through the BCR but are robustly responsive to LPS (15, 38). The enhanced frequencies of LPS-responsive cells in the MZ and CD21/35^{high} CD23^{+} pools correlated with increased surface levels of the toll-like receptor homologue, RP105, a component of the RP105/MD-1 complex that is associated with LPS responsiveness (39). Thus, it is tempting to speculate that LPS responsiveness for all splenic B cells—regardless of their subset designation—may relate directly to surface densities of RP105 on individual cells within each population. Third, unlike MZ B cells, the development of CD21/35^{high} CD23^{+} splenocytes seems to be independent of CD19, because CD19$^{cre/cre}$ mice lack MZ B cells (40), but contain normal frequencies of CD21/35^{high} CD23^{+} splenocytes (unpublished data). The characteristics of MZ B cells and CD21/35^{high} CD23^{+} splenocytes indicate functional distinctions between these cells, and support a paradigm where developmental cues that act on the CD23^{+} CD21/35^{high} population initiate or complete full differentiation of MZ B cells.

It is possible that a small fraction of follicular B cells derive from CD21/35^{high} CD23^{+} splenocytes during steady-state B cell development. When transferred into lympho-sufficient hosts, CD21/35^{high} CD23^{+} splenocytes give rise to cells with a follicular surface phenotype, although their sIgM and CD21/35 levels are, on average, greater than those of follicular B cells (Fig. 4 A). Whereas this result suggests a degree of plasticity among CD21/35^{high} CD23^{+} splenocytes, it is difficult to ascertain clear precursor–product relationships from these experiments, which typically are characterized by poor donor cell survival that likely is due to the failure of donor cells to compete effectively with host cells for critical microenvironmental niches. Neither the AA4^{+} T2 (Fig. 4 B) nor T1 subsets (not depicted) yielded MZ B cells upon adoptive transfer into lympho-sufficient hosts, which suggested that all donor cells failed to gain access to limiting factors that are required uniquely for MZ B cell differentiation. Although the nature of these factors is not entirely clear, they likely include the Notch2 ligand Delta-Like-1 (41), and the antiapoptotic cytokine BLyS (32, 33). Likewise, because each population tested gave rise to MZ-like B cells when transferred into RAG2$^{-/-}$ hosts, we further suggest that resources that are required for MZ B cell generation are available more readily in lymphopenic environments, which can promote development of MZ B cells from multiple precursor populations (Fig. 4).

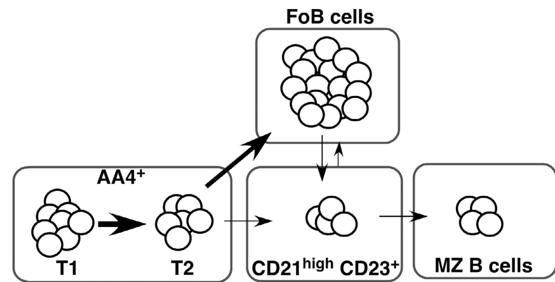


Figure 7. Model for peripheral B cell maturation. The first cells to colonize the spleen are the AA4^{+} T1 subset, which yields the AA4^{+} T2 subset that represents the common precursor of follicular and MZ B cells. CD21^{high} CD23^{+} splenocytes are the immediate precursor of MZ B cells, and might arise from AA4^{+} T2 cells, follicular B cells, or both. It also is possible that CD21^{high} CD23^{+} splenocytes make a small contribution to the follicular pool, or that this subset is in dynamic equilibrium with the follicular subset. The relative thickness of the arrow represents the estimated flux from one compartment to another based on BrdU-labeling kinetics.

Given our data, together with genetic data from the Aiolos- and Notch-deficient mice (9–11), we propose that CD21/35^{high} CD23^{+} splenocytes be termed marginal zone B cell precursors. A similar suggestion was made recently by Pillai et al. (42). We further propose a model of peripheral B cell development, in which AA4^{+} T2 splenocytes are the common precursor of follicular and MZ B cells and CD21/35^{high} CD23^{+} splenocytes are the immediate precursor of MZ B cells (Fig. 7). In addition to clarifying developmental relationships among splenic B cells, clear resolution of MZ B cell precursors will allow better characterization of the requirements for MZ versus follicular B cell development. Studies which compare the gene expression profiles of AA4^{+} transitional populations, versus CD21/35^{high} CD23^{+} and MZ B cells, should be especially useful in defining the molecular requirements that signal MZ B cell development.

MATERIALS AND METHODS

Mice. 6–10-wk-old C57BL/6 (Ly5^{B6}), congenic B6.Ly5^{SJL} (often termed B6.Ly5.2), and RAG2$^{-/-}$mice were obtained from Jackson ImmunoResearch Laboratories or the National Cancer Institute. All live animal experiments were performed according to protocols that were approved by the Office of Regulatory Affairs of the University of Pennsylvania in accordance with guidelines set forth by the National Institutes of Health.

Antibodies. Antibodies were purified and labeled in our laboratory or purchased from the indicated vendor. Fluorescein-labeled antibodies included monoclonal anti-B220 (RA3-6B2) and polyclonal Fab fragments of goat anti–mouse IgM (μ-chain specific, Jackson ImmunoResearch Laboratories). PE-labeled antibodies included AA4 (AA4.1), CD23 (B3B4, BD Biosciences), IgD (11–26, Southern Biotechnology Associates, Inc.), heat stable antigen (30F1), CD43 (S7), and anti-Ly5^{SJL} (A20, BD Biosciences). Biotin (BI)-conjugated antibodies included anti-Ly5^{SJL} (BD Biosciences), anti-Ly5^{B6} (104, BD Biosciences), CD1d (1B1, BD Biosciences), CD23 (BD Biosciences), and B220. PE-Cy5.5-anti-IgM (331.12), CD21/35 (7G6), and APC-conjugated anti-CD21/35, IgM (331.12), AA4, and B220 were prepared with standard methods in our laboratory. PE-Cy5.5 anti-Ly5^{B6} (104), PE-Cy7 anti-Ly5^{SJL} (A20) and B220, and APC-Cy7 B220 were purchased from eBioscience. All BI-conjugated antibodies specific for integrin family members were purchased from BD Biosciences. Streptavidin (SA)-conjugated reagents included SA-PE-TexasRed (Caltag), and SA-PE-Cy5.5 and SA-PE-Cy7 (eBioscience).

Cell preparation and staining. BM cells were flushed from tibias and femurs, and splenocytes were prepared through perfusion of spleens with FACS buffer (PBS containing 0.5% BSA 1 mM EDTA and 0.05% sodium azide). After lysis of RBCs with ACT (BioWhittaker), 10^6 cells were washed and then incubated with optimized dilutions of antibodies in 96-well round-bottom plates in a final volume of 50 μl. After 30 min on ice, plates were washed twice with FACS buffer then, when appropriate, cells were incubated for 20 min on ice with an optimal dilution of fluorochrome-conjugated SA in 25–40 μl, then washed and resuspended in FACS buffer.

Flow cytometry and cell sorting. Analyses were performed on a dual-laser flow cytometer (FACSCalibur, Becton Dickinson), a four-laser 10-color LSR II (Becton Dickinson), or a three-laser 8-color MoFlo cell sorter (DakoCytomation). All flow cytometry data were analyzed by uploading files into FlowJo 4.6 (TreeStar, Inc.). Data collected on the LSR II or MoFlo sometimes were subjected to the data transformation algorithm in FlowJo that allows negative cell populations to be viewed as symmetric clusters. Cells were sorted on a MoFlo cell sorter; cell suspensions were applied at a sheath pressure of 60 psi, and a drop delay frequency of ∼98,000 drops/s. This resulted in sorting rates of 28–30,000 cells/s with abort rates of 10–12%.

In vivo cell cycle analysis. To determine the degree of proliferation in vivo, splenocytes were surface stained as described above, washed in PBS, then fixed and permeabilized using the Fix & Perm kit from Caltag. Cells were washed twice and resuspended in 1 ml PBS before addition of DAPI at a final concentration of 10 μg/ml. Cells were incubated for ≥30 min at room temperature before analysis on the LSR II using a violet (405 nm) laser for DAPI excitation. Doublets were excluded by gating on the area and width parameters of the DAPI signal.

Adoptive transfers. B cell subsets were sorted into RPMI plus 5% FBS according to the gating strategies shown in Fig. 1, washed in PBS, and resuspended in 400 μl PBS per recipient before adoptive transfer by way of the retro-orbital sinus.

BrdU incorporation. Continuous in vivo BrdU labeling was performed as described previously (6) with the addition of the appropriately conjugated antibodies. In brief, adult C57BL/6 mice were inoculated i.p. with 0.5 mg BrdU (Sigma-Aldrich) in PBS every 12 h for up to 8 d. BM and spleen cells were stained with PE-CD23, PE-Cy5.5 CD21/35, PE-Cy7 B220, APC-Cy7 anti-IgM (331), and APC-AA4.1 in standard FACS buffer, washed twice with protein-free PBS, then permeabilized using "Fix and Perm" (Caltag). Cells were washed, incubated with DNaseI, washed, and stained with fluorescein–anti-BrdU (Becton Dickinson) before analysis on an LSR II.

TNP-Ficoll binding assay. Mice were inoculated by way of the retro-orbital sinus with 500 μl of 1 mg/ml TNP-Ficoll (Biosearch Technologies) suspended in PBS, and were killed 30 min later. Splenocytes were prepared and stained as in Fig. 1 with inclusion of BI-conjugated anti-TNP-Ficoll (BD Biosciences) revealed with SA-PE Cy7.

Stimulation of sorted B cells. Sorted B cells were CFSE-labeled with a 2-min incubation in 5 μM CFSE diluted in PBS, and washed and plated at 20,000 cells/well in media (RPMI 1640, 10% FBS [Hyclone], 1% nonessential amino acids [Invitrogen], 1% OPI [Invitrogen], 100 U/ml gentamicin, and 50 μM 2-mercaptoethanol) supplemented with 10 μg/ml anti-IgM (μ-chain specific, $F[ab]_2$ fragment, Jackson ImmunoResearch Laboratories) or 5 μg/ml LPS (*Escherichia coli*, Sigma-Aldrich). At day 3, CFSE levels were assessed by flow cytometry using DAPI to exclude dead cells.

Construction of BM chimeras. The indicated hosts were maintained on water containing a Bactrim suspension (400 mg sulfamethoxazole and 80 mg trimethoprim/500 ml water) for 1 wk before, and 3 wk after, lethal (900 rad) irradiation. Hosts were irradiated 1 d before retro-orbital injection of 2×10^6 B-lineage depleted BM cells. Depletions were performed on LD depletion columns (Miltenyi Biotec) using BI-anti-B220 and SA microbeads (Miltenyi Biotec).

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