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Resolution of Three Nonproliferative Immature B Cell Subsets Reveals Multiple Selection Points During Peripheral B Cell Maturation

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Although immature/transitional peripheral B cells may remain susceptible to selection pressures before full maturation, the nature and timing of these selection events remain unclear. We show that correlated expression of surface (s) IgM (sIgM), CD23, and AA4 defines three nonproliferative subpopulations of immature/transitional peripheral B cells. We designate these populations transitional (T) 1 (AA4+/CD23−sIgM high), T2 (AA4+/CD23+sIgM high), and T3 (AA4+/CD23−sIgM low). Cells within all three subsets are functionally immature as judged by their failure to proliferate following sIgM cross-linking in vitro, and their rapid rate of turnover in vivo as assessed by 5-bromo-2′-deoxyuridine labeling. These labeling studies also reveal measurable cell loss at both the T1-T2 and T2-T3 transitions, suggesting the existence of multiple selection points within the peripheral immature B cell pool. Furthermore, we find that Btk-deficient (xid) mice exhibit an incomplete developmental block at the T2-T3 transition within the immature B cell pool. This contrasts markedly with lyn−/− mice, which exhibit depressed numbers but normal ratios of each immature peripheral B cell subset and severely reduced numbers of mature B cells. Together, these data provide evidence for multiple selection points among immature peripheral B cells, suggesting that the B cell repertoire is shaped by multiple unique selection events that occur within the immature/transitional peripheral B cell pool. The Journal of Immunology, 2001, 167: 6834–6840.
along with our examination of the cellular dynamics of each population in wild-type mice, indicate the existence of multiple selection points without detectable proliferative events within the immature-transitional B cell pool.

Materials and Methods

Mice

Eight- to 12-wk-old female BALB/c mice and BALB.xid (also referred to as C.BA/N) congenics were bred and maintained in the Institute for Cancer Research animal facility or purchased from Taconic Farms (Germantown, NY). Germfree BALB/c mice were generated and maintained in the Department of Biology at the University of Pennsylvania and were kindly provided by Dr. J. Cebra (University of Pennsylvania, Philadelphia, PA). Lyn–/– mice were kindly provided by Dr. J. Eriksen (Wistar Institute, Philadelphia, PA).

Cell preparation and staining

Suspensions of BM cells were flushed from tibias and femurs and splenocytes prepared through perfusion of spleens with FACS buffer (PBS containing 0.5% BSA, 1 mM EDTA, and 0.05% sodium azide). Following lysis of RBCs with 0.165 M NH4Cl 2 , cells were washed and then incubated with optimal dilutions of the indicated Abs in 96-well round-bottom plates in medium containing 0.5% BSA, 1 mM EDTA, and 0.05% sodium azide. Following incubation with FACS buffer and then, when appropriate, cells were incubated for 20 min on ice before two final washes with fluorochrome-conjugated streptavidin (SA) to reveal staining by biotinylated Abs.

Abs and flow cytometric analyses

PE and biotin (BI) anti-CD45R/B220 (RA3-6G2), fluorescein (FL) and BI-anti-CD24/HSAsa(30F1), and sIgD (AMS 15.1) and allophycocyanin-AA4 (AA4.1) were generated, purified, and conjugated in our laboratories by standard methods. Commercially obtained Abs used in these studies include FL-and BI-conjugated Fab of goat anti-mouse IgM (Jackson Immunoresearch Laboratories, West Grove, PA) and FL-anti-CD21/CD35 (7G6), IA-4 (39–10–8), CD62L (Mel-14), and CD22 (Lyb-8.2), PE and BI-anti-CD23 (B3B4), and BI-anti-CD138 (syndecan-1; BD Pharmingen, San Diego, CA). Biotinylated Abs were revealed with PerCP or allophycocyanin-Cy7-conjugated SA (BD Pharmingen), and dead cells were excluded with propidium iodide. Analyses were conducted on a dual laser flow cytometer (FACScanCaliber; BD Immunocytometry Systems, San Jose, CA) or a MoFlo cell sorter (Cytometry, Fort Collins, CA) equipped for detection of nine parameters. All flow cytometry data were analyzed by uploading data files into FlowJo (TreeStar, San Carlos, CA).

Cell sorting

Each indicated B cell subset was isolated on a nine-parameter MoFlo cell sorter (Cytometry) equipped with Summit software and three lasers including an I-90C argon laser tuned to 488 nm and an I-70C Spectrum argon/krypton laser (both from Coherent, Santa Clara, CA) tuned to 647 nm for excitation of allophycocyanin and its derivatives. For experiments examining in vitro proliferation of sorted cells, splenocytes were stained with PE-CD23, BI-B220 (revealed with SA-allophycocyanin-Cy7), allophycocyanin-AA4, and FL-conjugated Fab of goat anti-IgM (μ-chain specific; Jackson Immunoresearch Laboratories) to avoid BCR cross-linking as a consequence of cell sorting.

In vitro proliferation assays

Sorted cells from the indicated populations were incubated at 30,000–50,000 cells/well in triplicate in 96-well flat-bottom plates in medium consisting of OPTI-MEM, to which was added 5% FCS [Irvine Scientific, Santa Ana, CA], 10 mM glutamine, 10 mM HEPES, 0.5 mg/ml gentamicin, and 5 × 10−5 2-ME. Stimuli added included F(ab′)2 goat anti-IgM (μ-chain specific; Jackson Immunoresearch Laboratories) or LPS (Fisher Scientific, Pittsburgh, PA), both at a final concentration of 50 μg/ml as previously described (2). After 48 h, all cultures were pulsed with 1 μCi of [1H1]thymidine and harvested 18 h later for scintillation counting.

Cell cycle analysis

To determine the degree of proliferation in vivo, 50,000 cells from each population were sorted directly into microcentrifuge tubes containing 1 ml of ice-cold 95% ethanol and then stored at −20°C for 24 h. Tubes were then allowed to warm briefly at room temperature before centrifugation and resuspension of cell pellets with PBS containing 1% glucose, 1 mg/ml RNase A, and 50 μg/ml propidium iodide. After a 30-min incubation at room temperature, cells were analyzed on a BD Immunocytometry Systems FACSCalibur utilizing pulse width doublet discrimination.

In vivo 5-bromo-2′-deoxyuridine (BrdU) incorporation

A modification of previously published protocols for assessment of BrdU incorporation was used. Adult BALB/c mice were inoculated with 0.4 mg of BrdU (Sigma-Aldrich, St. Louis, MO) in PBS every 12 h for 0.5–7 days. BM and spleen cells were stained for surface expression of IgM, CD23, and AA4 using standard FACS buffer, washed once with FACS buffer followed by a wash in protein-free PBS, then permeabilized using Fix and Perm (Caltag Laboratories, Burlingame, CA). Subsequently, cells were washed, incubated with DNase I, and then stained with FL-anti-BrdU (BD Biosciences, Mountain View, CA) as previously described (1) before analysis on a BD Immunocytometry Systems FACScalibur.

Results

AA4 expression is restricted to B cells with an immature phenotype

Previous analyses have demonstrated AA4 surface expression on B lineage progenitors in the BM (16, 17). As shown in Fig. 1, this includes recently formed slgMlowslgD+ cells but not mature recirculating slgM+slgD− B cells in adult BM, raising the possibility that AA4 surface expression is down-regulated after migration of immature B cells into peripheral lymphoid tissues. Supporting this, only 16–20% of B220+slgM+ splenocytes were AA4+ and these cells were B220low (Fig. 2). Together, these data suggested that AA4+B220+slgM+ cells correspond to immature-transitional B cells. To test this possibility, we analyzed AA4 and slgD expression on BM B220+slgM+ cells during the earliest phases of radiation-induced autoreconstitution when all B cells are phenotypically and functionally immature (2). As shown, all BM B220+slgM+ B cells were AA4+ with low to undetectable levels of slgD 12 days postirradiation (Fig. 1D), indicating that AA4 surface expression correlates with immaturity.

Recent studies suggest that peripheral B cell maturation is a stepwise process that correlates with up-regulation of CD23 (3). An assessment of the relative expression of these surface proteins therefore allows the resolution of two subsets of transitional (T) peripheral B cells designated T1 (slgMhighCD23−) and T2 (slgMlowCD23+) from conventional mature B cells (slgM+CD23+) (3). To test whether AA4+ B cells correspond to these or additional subsets, we assessed slgM and CD23 levels on B220+AA4+ and B220+AA4− B cells splenocytes. As shown in Fig. 2, three subsets of B220+AA4+ cells in adult spleen can be identified.

FIGURE 1. AA4 expression on newly formed BM B cells. BM cells from a control 8-wk-old BALB/c mouse and an age-matched mouse given 500 rad of whole-body irradiation 12 days previously were stained with PE-anti-B220, FL-anti-IgD, allophycocyanin-anti-AA4.1, and BI-anti-IgM revealed with SA-PerCP before analysis on a dual-laser FACScalibur. Data are representative of three separate experiments consisting of at least three mice per experiment.
based on differential sIgM and CD23 levels. We designate these populations T1 (sIgM$^{\text{high}}$/CD23$^{+}$), T2 (sIgM$^{\text{high}}$/CD23$^{+}$), and T3 (sIgM$^{\text{low}}$/CD23$^{+}$). Each of these T subsets constitutes 1–2% of all splenocytes in BALB/c mice housed in pathogen-free and germ-free facilities (Table I).

Since progression to functional maturity in the B2 lineage correlates with down-regulation of CD24/HSA and up-regulation of sIgD, MHC class II, complement receptor type 2-complement receptor complex CD21/CD35, and CD22 at levels comparable to mature B cells progressively increase from T1 through T3. Significantly, these data also show that each AA4$^{+}$ subset is distinct from B cells expressing a CD23$^{-}$/sIgM$^{\text{high}}$ marginal zone (MZ) phenotype which express CD21/CD35 and CD22 at levels comparable to mature B cells, since among AA4$^{+}$ splenic B cells only the T1 population is CD23$^{-}$ and these cells express relatively low levels of CD21/CD35 and CD22 (Fig. 3).

Consistent with data from Loder et al. (3), cells in T1 also lacked expression of MEL-14/CD62L, suggesting that cells within the T1 population do not effectively migrate into lymph nodes. Indeed, although the T2 and T3 populations were readily detected among lymph node B cells (both ranging from 3.0 to 3.5% of B cells in an adult BALB/c lymph node), we were unable to detect cells with the T1 phenotype in these preparations (data not shown).

Three subsets of functionally immature peripheral B cells

Assessment of B cell functional maturity can be performed by examining the in vitro proliferative response to BCR cross-linking (2, 20). Whereas mature, follicular B2 cells exhibit a robust proliferative response to this stimulus, immature B cells do not. To verify their functional immaturity, we applied this criterion to sorted subpopulations of B220$^{+}$AA4$^{+}$ cells. To preclude inadvertent receptor cross-linking during cell purification, all sIgM staining for functional studies was performed using μ-chain specific, monomeric Fab Abs.

Although AA4$^{+}$ sIgM$^{\text{low}}$/CD23$^{+}$ mature B cells readily proliferated following maximal stimulation with anti-IgM Abs in vitro, we did not detect measurable proliferation in any of the three AA4$^{+}$ splenic subsets after identical stimulation (Fig. 4A). In contrast, all three transitional populations readily proliferated to LPS (Fig. 4B). Interestingly, levels of LPS-induced proliferation were consistently lower in T1 cells compared with all downstream CD23$^{-}$ populations. Regardless, the inability of purified AA4$^{+}$ B cell subsets to proliferate in response to BCR cross-linking, coupled with their predominance during the earliest phases of radiation-induced autoreconstitution indicates that they are functionally immature.

That the T3 subset as a population is also refractory to BCR engagement is of particular importance, since the minimal parameter utilized to distinguish T3 from mature B cells is differential AA4 expression. Using conventional staining (IgM, IgD, B220, and CD24/HSA), T3 B cells have often likely been included within mature B cell gates as they express higher levels of B220 and lower levels of CD24/HSA and sIgM than all other immature B cells (Figs. 1–3). The functional immaturity observed in this population serves to complement assertions based on cell surface
staining and ensure the absence of contaminating mature B cells in our gating and analysis of this novel subset.

Peripheral maturation is not accompanied by a proliferative burst in vivo

Loder et al. (3) previously reported that 17% of immature CD23\(^{+}\)/H11001 sIgM high (T2) B cells in conventional mice are in the G2-M phase of the cell cycle, suggesting that peripheral B cell development is associated with a proliferative burst analogous to surrogate L chain selection of developing B lineage progenitors in the BM. To quantify the extent and distribution of basal proliferation within each immature AA4\(^{+}\) subset, we sorted each population and mature B cells directly into ethanol, then assessed the DNA content of the purified cells. Fig. 5 illustrates representative data from four such experiments. As shown, we were consistently unable to detect evidence of significant proliferation within any subset examined, including cells sorted from T2 (AA4\(^{+}\)CD23\(^{+}\)/sIgM\(^{+}\)). These data provide unambiguous evidence for the lack of significant proliferation at each immature stage of peripheral B cell maturation.

T1-T3 transitional subsets exhibit sequential labeling and unique turnover rates as determined by continuous in vivo BrdU labeling

Although several studies suggest that survival and maturation of immature peripheral B cells can be modulated by negative and positive selection events (3, 12, 13), our ability to subdivide immature splenic B cells into three subpopulations allows us to examine at higher resolution the stage and timing of such events. To probe for evidence of selection events governing transitions in peripheral B cell development, we assessed the cellular dynamics of each population by continuous in vivo BrdU labeling.

A representative BrdU staining profile following 4 days of continuous BrdU administration for mature B cells and each transitional subset is illustrated in Fig. 6, and the turnover rates of BM and splenic immature subsets are depicted in Fig. 7. As shown, each peripheral immature subset defined by AA4 expression exhibited a rapid rate of turnover relative to mature AA4\(^{-}\) B cells, with cells in T1 and T2 achieving >90% labeling by days 4 and 5, respectively. Supporting the notion that CD23\(^{+}\) cells are derived from less mature CD23\(^{-}\) B cells, labeling kinetics for all AA4\(^{+}\) peripheral B cell subsets were delayed compared with each immature BM subset, and labeled cells accumulated in T2 with delayed kinetics compared with those in T1. Furthermore, cells in T3 also exhibited rapid turnover, albeit with an even greater delay in labeling kinetics and a reduction in the overall labeling rate compared with cells in T1 and T2.

Extrapolations of production rates from these data are found in Table II. Interestingly, rates of cell entry into each population decreased significantly from T1 to T2 and to a lesser extent from T2 to T3. In contrast, we were unable to detect evidence for cell loss coincident with movement of cells from T3 to the mature B cell pool, suggesting that in normal adult mice selection events resulting from negative or failed positive selection are restricted to cells within T1 and T2.
The xid mutation in Btk results in a specific disruption of peripheral B cell development within the transitional B cell pool

Previous reports have shown that mice possessing mutations of the Tec family kinase Btk exhibit reduced numbers of mature B cells (21–24). Furthermore, other studies have proposed that the immature B cell compartment of these mice is intact, and have placed the site of this developmental defect at the transition from immature to mature B cell (3). However, as shown in Fig. 8, we find that an examination of each AA4+/H11001 B cell subset in xid mice reveals a 10-fold diminution in the relative and absolute number of T3 phenotype cells, whereas the T1 and T2 subsets exhibit only 2-fold reductions in total cellularity (Fig. 8 and Table III).

To determine whether this defect is specific to Btk or rather a more general consequence of BCR signal perturbation, we examined the immature B cell compartment of mice deficient for the Src family kinase, Lyn. A recent comparison of Btk and Lyn function revealed that these proteins function independently of each other in BCR signaling (25), suggesting that mutations in these proteins might also exert unique effects on peripheral B cell development. To avoid the complicating autoimmune splenomegaly found in aged lyn−/− mice, we limited our analysis to 10- to 12-wk-old animals. In addition, to preclude inclusion of activated cells in our analysis, we gated on cells lacking expression of the B cell activation marker CD138/syndecan-1. Twelve to 15% of splenic B cells in lyn−/− mice were CD138/syndecan-1+ (data not shown).

In our analysis and as previously reported, lyn−/− mice possess normal numbers and ratios of pro-, pre-, and immature B cells in the BM (data not shown) (26). By contrast, transitional and mature B cell subsets in the spleen are markedly reduced in number. Moreover, although the mature B cell compartment is 10- to 20-fold reduced, AA4+ immature subsets are each reduced 2- to 4-fold (Table III). Importantly, and in direct contrast to the xid phenotype, we did not detect a selective depletion of any immature subset in Lyn-deficient mice, indicating that Lyn is likely not required for specific developmental transitions among immature peripheral B cells (Fig. 8B and Table III).

**Discussion**

Our data reveal four novel aspects of peripheral B cell development. First, we show that immature/transitional peripheral B cells can be subdivided into three subpopulations based on AA4 surface expression and differential expression of CD23 and sIgM. All three subpopulations are functionally immature as reflected by their high degree of turnover and their failure to proliferate following sIgM cross-linking in vitro. Moreover, down-regulation of surface AA4, rather than down-regulation of sIgM levels, or up-regulation of sIgD expression, correlates with acquisition of functional maturity in developing peripheral B cell (Figs. 2–4). Second, all three subsets clearly lack significant levels of proliferation in vivo (Fig. 5), indicating that maturation of peripheral transitional B cells is neither dependent on nor accompanied by entry of developing cells into the cell cycle. Third, the cellular dynamics of these populations suggest detectable cell loss within both T1 and T2 or during the transition of cells in each population into more mature compartments (Fig. 7B and Table I). Finally, although defects in peripheral B cell maturation in xid and lyn−/− mice have been reported previously, we find that these mutations have markedly different effects on peripheral B cell maturation, with the xid mutation effecting a developmental step (T2 to T3) within the immature B cell pool, and lyn−/− mice exhibiting normal ratios of each immature subset and highly depressed numbers of phenotypically mature B cells (Fig. 8 and Table III). Thus, while previous studies suggested that btk mediates the immature to mature developmental transition (3), the increased resolution of these populations afforded herein allows us to conclude that normal btk function is required for developmental events within the immature peripheral B cell pool.

Several observations made herein and elsewhere support a developmental sequence in which cells within T1 give rise to each downstream immature and mature B cell subset. First, our BrdU-labeling experiments (Figs. 6 and 7), which allow an examination of precursor-product relationships at steady state in vivo, clearly support this model. Second, newly formed B cells in the BM are also CD23− (8), and cells within this pool become sIgMhigh before migrating to the periphery (1, 6). Moreover, Carsetti and colleagues (3) previously demonstrated that CD23− sIgMhigh B cells yield CD23+ sIgMhigh cells following adoptive transfer. Thus, together these observations support the notion that AA4+ CD23+ sIgMhigh B cells (T1) give rise to cells in each immature and mature subset described herein, and thus provide a model for examining the cellular basis for peripheral B cell development.

In contrast to a previous report (3), we failed to measure significant proliferation in vivo within CD23+ sIgMhigh (AA4+) T2 cells. Although the reason for this discrepancy is not immediately apparent, we are confident that isolation of defined subpopulations by electronic cell sorting before DNA content analysis as illustrated herein provides a clear picture of the cell cycle status of any given cell population. Regardless, our data argue against the notion of...
that peripheral B cell development is accompanied by a proliferative burst within T2. Therefore, although btk is clearly required for the efficient development of cells in T2 into T3 and mature B cells, btk may not function to dampen proliferation within T2 as previously suggested (3).

The unique expression of AA4 among splenic B cells also allows a clear resolution of immature B cells and mature B cells expressing a so-called MZ phenotype. Indeed, while MZ and T1 B cells are both CD23−, unlike MZ B cells, cells within T1 clearly express relatively low levels of CD21/CD35 and CD22 (Fig. 3). Moreover, T1 B cells exhibited unique low levels of LPS-induced proliferation, and thus differ from MZ B cells which undergo a hyperproliferative response following LPS stimulation (27 and our unpublished observations).

Several studies indicate that signals mediated through the BCR govern efficient peripheral B cell maturation. For instance, genetic deletion of the tyrosine kinase syk blocks peripheral B cell maturation and entry into B cell follicles (15), suggesting that syk activity is important for development of B cells within the immature peripheral B cell pool. Likewise, we find that btk mediates a developmental step within this compartment as revealed by the specific reduction of cells within T3 in xid mice (Fig. 8 and Table III). Together, these findings suggest the existence of multiple developmental checkpoints during peripheral B cell maturation. Consistent with this notion, our BrdU studies reveal appreciable cell loss within both T1 and T2 but not T3 (Fig. 7 and Table II), suggesting that these mutations might result in pronounced cellular attrition within T1 and/or T2 rather than during the transition of immature B cells into the mature long-lived B cell pool.

Currently the mechanisms underlying the migration of recently formed B cells from the BM to the periphery are unknown. Our findings, along with previous analyses demonstrating AA4 expression on the earliest and all subsequent B cell precursors in adult BM (16, 17), are consistent with the notion that AA4 expression is maintained throughout B cell development until 2–4 days after recently formed sIgM+ B cells enter peripheral lymphoid organs. Given that AA4 bears homology with members of the L-selectin family of homing receptors (28), it is tempting to speculate that AA4 may play a key role in immature B cell egress from the BM and/or entry into peripheral lymphoid tissues.

Rolink et al. (4) reported that immature peripheral B cells selectively express a 130- to 140-kDa cell surface protein identified by the 493 Ab. We suggest that 493 binds to AA4 and cite two lines of evidence in support of this hypothesis. First, both 493 and AA4.1 precipitate a 130- to 140-kDa cell surface protein (4, 28). Second, expression patterns for 493 and AA4.1 are remarkably similar; in the BM both Abs stain all B lineage subsets except for the mature sIgMhigh population and in the spleen both Abs stain immature B cells exclusively. Since we have been unable to block AA4.1 staining with 493 supernatant, we further suggest that these Abs recognize noncompeting determinants on the AA4 molecule.

The clear resolution of three immature splenic B cell subsets from other peripheral B cells provides the means to address a number of unresolved issues regarding selection and survival of newly emerging peripheral B cells. For instance, although several studies indicate that self-Ag-mediated negative selection is operative in the BM (6–11), whether negative selection of immature B cells occurs in vivo in the periphery of conventional mice remains unclear. Likewise, the frequency of receptor editing events in BM vs peripheral immature B cells is also unknown. Recently, Sandel and Monroe (12) provided evidence that receptor editing occurs primarily in the BM, whereas like affinity interactions in immature peripheral B cells result in apoptosis rather than editing. In addition, Yu et al. (29) recently proposed that germinal center B cells exhibiting evidence for receptor editing are derived solely from immature peripheral B cells. Although consistent with data demonstrating that immature peripheral B cells are receptive to CD40-CD40L ligand-mediated interactions required for formation of germinal centers (4), these experiments raise questions regarding the overall and relative contribution of each immature B cell subset to the germinal center reaction and the generation of the memory B cell pool.

Finally, understanding the mechanisms underlying the development, selection, and migration of recently formed B cells as well as mechanisms controlling life span and migration of mature B cells will require the clear identification of each relevant B cell subpopulation. AA4, in conjunction with varying levels of sIgM, CD21, and CD23 expression, readily allow the resolution of these populations and should thus aid in future experiments designed to address these issues.

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