Annexin V Binds to Positively Selected B Cells[1]

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Recombinant annexin V (rAnV) has been used in flow cytometry to identify cells undergoing apoptosis, based on its ability to bind to phosphatidylserine, a negatively charged lipid normally restricted to the cytoplasmic face of the plasma membrane but externalized early during apoptosis. When we stained murine bone marrow (BM) cells with fluorescently labeled rAnV, we found that a surprisingly large fraction of BM B cells bearing selectable transgenic Ag receptors bind significant amounts of rAnV, but that these cells are not apoptotic. Here, we show that binding of rAnV to developing B cells in normal mice correlates with B cell receptor-dependent selection events at several stages of development within both B-1 and B-2 cell subsets. In fact, nearly all B-1 B cells and splenic marginal zone B cells bind rAnV, suggesting that the externalization of phosphatidylserine occurs once mature B cells are selected through BCR-mediated signaling. However, this plasma membrane alteration is apparently not shared by all lymphocytes, because we did not find a parallel population of rAnV-binding viable T cells in vivo in normal or TCR transgenic mice. We also show that BM stromal cell lines can influence the extent of rAnV binding by viable BM B cells during coculture in vitro. We suggest that rAnV detects a potentially important membrane alteration that occurs as B cells develop in the BM and are readied for export to the peripheral lymphoid organs and again among mature B cells recruited to the marginal zone or the B-1 compartment. ***The Journal of Immunology,*** **2001, 166: 58–71.**

Developing B lymphocytes must undergo selection at multiple maturational stages, based primarily on their ability to express a functional and self-tolerant B cell receptor (BCR)[5] for Ag (reviewed in Refs. 1 and 2). The genes encoding this receptor, Ig H and L chain genes, are assembled by V(D)J recombination in a highly regulated fashion during the early stages of B cell development (reviewed in Ref. 3). Ig H chain (μ) gene rearrangement precedes L chain (κ or λ) gene rearrangement. Pro-B cells that generate a productive (in-frame) H chain gene assemble a signaling complex known as the pre-BCR (4). The pre-BCR signals the pre-B cell to activate L chain gene rearrangement and inactivate further H chain gene rearrangement, but it is unclear whether the pre-BCR signal requires extracellular ligand (5, 6). If a pre-B cell generates a productive L chain gene rearrangement, it uses the resultant Ig to assemble its BCR. The subsequent stages of B cell maturation can be defined by the expression of surface Ig (sIg). Immature B cells express sIgM, but not sIgD, while mature B cells and most peripheral B cells express both sIgM and sIgD.

There are two subsets of peripheral B cells called B-2 and B-1 (reviewed in Ref. 7). These subsets express distinct Ig repertoires, presumably reflecting their different functions in the immune system (8). B-2 cells are the predominant B cell population in the blood, spleen, and lymph nodes. B-1 cells are largely restricted to the peritoneum and other body cavities and comprise the majority of B cells in these areas. B-1 cells have the capacity for self-renewal and are responsible for secreting most of the IgM present in the serum of unimmunized animals (9). In fact, a large fraction of the B-1 subset is composed of cells with measurable affinity for self Ags with repetitive structures, such as phosphatidylcholine (PC), Ig (rheumatoid factor), and DNA, as well as common bacterial carbohydrate Ags (9–13). Thus, it has been suggested that developing B cells bearing self-specific Ag receptors are actively selected into the B-1 cell repertoire (14), and that mature B-1 cells may be involved in T cell-independent responses to common environmental Ags. Recent evidence supports a model in which B-2 and B-1 cells derive from a single lineage, and that an uncommitted B cell (B-0) is induced to differentiate to a B-1 cell through interaction with T cell-independent Ags in the absence of T cell help (14, 15).

Although the concept of positive selection of T cells developing in the thymus is well established, only recently has the idea that a parallel event occurs during B cell development gained similar acceptance (1, 2). Thymic T cells receive a positive selection signal when their Ag receptor is "tickled" by a low intensity interaction with MHC plus self Ag, but undergo apoptosis if this interaction is too strong (reviewed in Refs. 1 and 16). Tickling of the TCR on mature T cells by MHC^+ self Ag in the periphery is thought to enable the survival of memory T cells (17). Memory B cells might similarly survive if the BCR is tickled by the same self Ags responsible for their emigration from the bone marrow. There are three basic checkpoints at which developing B cells undergo selection (1). First, as noted above, pro-B cells fail to survive

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[5] Abbreviations used in this paper: BCR, B cell receptor; BM, bone marrow; FO, follicular; M, mature (B); MZ, marginal zone; NF, newly formed; nonTg, nontransgenic; PC, phosphatidylcholine; PS, phosphatidylserine; QR, Quantum Red; rAnV, recombinant annexin V; SA, streptavidin; 7AAD, 7-amino-actinomycin D; T1, transitional-1 B; T-2, transitional-2; Tg, transgenic; CDR, complementarity-determining region; HEL, hen egg lysosome; FLP, fragment length polymorphism; RP10, RPMI 1640 supplemented with 10% FBS, 4 mM glutamine, 10 mM HEPES, and antibiotics; WB, wash buffer; FSC, forward scatter; SSC, side scatter; PerC, peritoneal washout cells.

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unless they generate a productive H chain gene rearrangement capable of pairing with surrogate L chains to form a pre-BCR (18, 19). There is evidence that the structure of the H chain variable domain can influence the outcome of pre-BCR signaling (19–22). Second, at a later stage of development, immature B cells expressing a self-specific BCR are induced to edit that receptor by reactivation of the V(D)J recombinase, to undergo apoptosis, or to become anergic (23–25). Those cells with useful BCRs are positively selected to emigrate to the periphery. Finally, the earliest B cell emigrants from the bone marrow arrive in the spleen, where they enter follicles and acquire the ability to join the recirculating pool. BCR-mediated signaling is required for the maintenance (selection) of peripheral B cells (26).

One important subset of mature B cells resides in the marginal zone (MZ) of the spleen, a site bridging the red and the white pulp, where blood filters past macrophages, B cells, and dendritic cells before reaching the red pulp. MZ B cells are both phenotypically and functionally distinct from follicular B cells, expressing higher levels of complement receptors (CR1/2; CD21), but little or no CD23. MZ B cells are more sensitive to B cell mitogens than follicular B cells and differentiate extremely rapidly into Ab-secreting cells upon encounter with Ag (reviewed in Ref. 27). There is strong evidence that an appropriate quality BCR signal is required for B cells to become MZ cells, and that these B cells play an important role in T-independent Ab responses and in Ag retention in the MZ (28, 29). There is also evidence that recruitment to the MZ is a competitive process (30), and that, as for the B-1 compartment, it is dependent upon BCR H and L chain composition (28, 31). Thus, B cell clones are thought to join the long-lived MZ repertoire based on their BCR specificity and the nature of individual BCR-mediated encounters, although it is not yet clear whether this process requires a particular ligand.

Transgenic (Tg) model systems have clearly demonstrated that most self-reactive B cells failing to successfully edit their receptors are negatively selected at an immature stage of development (32, 33), analogous to thymocytes expressing a self-reactive TCR (34). However, the concept that an Ig-dependent positive selection event might also occur within the B cell compartment, either via a cell autonomous mechanism or mediated by ligand(s) in the BM microenvironment, is still a matter of debate (1, 2). At least two lines of evidence suggest that B-2 cells are positively selected. First, DNA sequence analyses have demonstrated that the Ig repertoires of pre-B, immature B, and mature peripheral B cells in normal mice are distinct. While the use of a broad array of V_H genes characterizes B cells at early stages of development, mature splenic B cells are more restricted in their V_H gene usage, suggesting that the expansion of Ig-expressing B cells is ligand dependent (35–37). Second, only a small proportion (10–20%) of newly formed B cells populate the peripheral lymphoid organs, indirectly suggesting that only actively selected cells migrate from the bone marrow or that only a subset of B cells receive signals enabling their survival in the periphery (38–40).

Evidence for Ag-driven positive selection of cells into the B-1 cell lineage has been even more convincing. In normal unimmunized mice PC-specific B cells appear to be exclusively B-1. They are driven to expand from birth, and eventually account for 2–10% of the peritoneal B-1 repertoire in normal adults. IgM specific for PC is encoded predominantly by either of two combinations of V_H and V_L: V_H11 and $V_\kappa9$ (41) or V_H12 and $V_\kappa4$ (42). In the case of V_H12^+ PC-reactive B-1 cells, the V_H12 H chain is further characterized by a complementarity-determining region 3 (CDR3) that is invariably 10 aa in length (42). In mice carrying a PC-specific V_H12-DSP2.9-J_H1 H chain transgene, large numbers of $V_\kappa4^+$ transgene$^+$ PC-specific cells populate the spleen and peritoneum, resembling double ($V_H12/V_\kappa4$) Tg mice provided with both H and L chain genes (43). This similarity between H and H+L chain Tg mice lends credence to the model that V_H12^+ B cells are positively selected into the B-1 cell lineage. Positive selection of self-reactive B cells was also observed in a transgenic mouse expressing an IgH chain gene conferring reactivity to Thy-1 (44).

We recently reported some unexpected results arising from studies aimed at identifying B cells undergoing negative selection in the bone marrow by using the apoptosis marker, annexin V (AnV) (45). AnV is a member of a large family of Ca^{2+} and phospholipid binding proteins (46), and in the presence of physiological concentrations of Ca^{2+}, AnV has high affinity for negatively charged phospholipids, especially phosphatidylserine (PS). The plasma membrane of a healthy cell typically exhibits an asymmetric distribution of its major phospholipids maintained via the activity of aminophospholipid translocase (47). Virtually all the PS and most of the phosphatidylethanolamine (PE) and phosphatidylinositol reside on the inner leaflet of the plasma membrane, with sphingomyelin largely confined to the outer leaflet, and PC distributed equally between both leaflets (48). During the early stages of apoptosis, cells lose their membrane phospholipid asymmetry and expose PS on the outer leaflet of the plasma membrane, triggering their phagocytosis by macrophages that bear PS receptors. Rapid phagocytosis prevents secondary necrosis and inflammation within the surrounding tissue (reviewed in Refs. 49 and 50).

Fluorescently labeled forms of recombinant AnV (rAnV) have been used previously in flow cytometry to identify apoptotic cells (51). We used FITC- or biotin-rAnV to stain BM cells from Tg mice bearing a potentially autoreactive BCR (32). Surprisingly, we found that rAnV stains nearly all the Tg BM B cells in mice on a background lacking the autoantigen, but at levels somewhat lower than those on apoptotic cells. rAnV-binding B cells are also present in nontransgenic (nonTg) mice among both B-1 and B-2 cell populations and do not display any of the other classic physiological changes associated with apoptosis (45). Instead, we show here that rAnV binding to the surface of B cells, both early and late in their developmental progression, correlates with BCR-dependent positive selection events.

Materials and Methods

Mice

Tg mice expressing the Ig H+L chain genes from the 3-83 BCR that confers reactivity to the mouse MHC class I Ags H-2K^b and -K^k (52) were provided by Dr. David Scott (American Red Cross, Rockville, MD) and maintained on an H-2^d background by continuous backcrosses to BALB/c mice (National Cancer Institute, Frederick, MD). To obtain H-2$^{b\times d}$ and H-2$^{k\times d}$ Tg mice, H-2^d Tg males were bred to either C57BL/6 (H-2^b) or B10.A (H-2^k) females obtained from The Jackson Laboratory (Bar Harbor, ME). IgH Tg mice (53) were obtained from Drs. Michel Nussenzweig (Rockefeller University, New York, NY) and Phil Leder (Harvard University, Boston, MA). V_H12 Tg mice (43), which carry a rearranged Ig H chain gene from a PC-specific B-1a clone, were provided by Dr. Stephen Clarke (University of North Carolina, Chapel Hill, NC). V_H12 Tg mice were maintained as heterozygotes by continuous backcrosses to C57BL/6 mice. Control nonTg mice were offspring from these same matings. Mice were maintained in specific pathogen-free conditions in our facility, and unless otherwise noted were used in experiments at 4–20 wk of age.

Reagents

Recombinant human AnV was cloned by PCR from a human placental cDNA library, expressed in *Escherichia coli*, then purified and conjugated to FITC or biotin as previously described (54). Our reagent gave results identical with those using commercially prepared versions of FITC-rAnV (Clontech, Palo Alto, CA) or biotin-rAnV (PharMingen, San Diego, CA). TUNEL assays were performed with a kit (Roche, Indianapolis, IN), according to the manufacturer's instructions. 7AAD and Quantum Red (QR)-streptavidin (SA) were purchased from Sigma (St. Louis, MO). Anti-B220-PE (clone RA3-6B2), anti-CD5-PE (clone 53-7.3), anti-CD8-FITC

(clone 53-6.7), anti-CD4-PE (clone GK1.5), and anti-CD19-biotin (clone 1D3) mAbs were obtained from PharMingen (San Diego, CA). Goat anti-mouse IgM-PE and goat anti-mouse IgD-biotin were purchased from Southern Biotechnology Associates (Birmingham, AL). Anti-CD43-biotin (clone S7) and anti-B220-biotin (clone RA3-6B2) were prepared in our laboratory. Both FITC- and biotin-conjugated versions of 54.1, the mAb that recognizes the 3-83 Id, were gifts from Terri Grdina (American Red Cross).

Cell lines

The H-2^d stromal cell line S17 (55) was obtained from Dr. Ken Dorshkind (University of California, Los Angeles, CA), and op42 (56), an H-$2K^b$-bearing stromal cell line, was provided by Dr. Paul Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK). Both stromal cell lines were cultured at 37°C in a 5% CO_2 incubator in RPMI 1640 supplemented with 10% FBS, 4 mM glutamine, 10 mM HEPES, and antibiotics (RP10).

Cell preparation and purification

BM cells were isolated from femurs and tibias by careful disruption in PBS using a mortar and pestle. Cells were resuspended, depleted of bone fragments by passive sedimentation, and pelleted at 1000 × *g*. Splenocytes were obtained by crushing spleens between glass slides, then resuspending and pelleting as described for BM. Peritoneal cavity washout cells (PerC) were obtained by repeatedly flushing the peritoneum with PBS and collecting the cells in a 5-cc syringe with an 18-gauge needle. To avoid the potential loss or gain of $rAnV^+$ cells, BM and spleen samples were not depleted of RBC by Ficoll treatment or hypotonic lysis; instead, RBC and dead cells were gated out electronically after flow cytometric analysis. All cells were resuspended in FACS wash buffer (FACS WB; HBSS, 1% BSA, and 10 mM HEPES buffer, pH 7.4) at a concentration of 20–30 × 10^6 c/ml before staining. To enrich for B cells, BM or spleen preparations were stained with anti-CD19-biotin followed after a wash by SA-conjugated magnetic beads, then passed over a MiniMacs separation column, according to the manufacturer's instructions (Miltenyi Biotec, Sunnyvale, CA).

Cell staining and flow cytometry

For staining with FITC-rAnV, 1–1.5 × 10^6 cells were transferred to 5-ml tubes and washed with 1 ml of FACS WB and 2 mM $CaCl_2$ or with WB and 2 mM EGTA, then pelleted at 1000 × *g*. Cells were then incubated on ice for 20 min in the presence of saturating amounts of the appropriate PE- and/or biotin-conjugated mAbs and FITC- or biotin-rAnV in a total volume of 100 μl of FACS WB with Ca^{2+} or EGTA. Cells were washed with 1.5 ml of WB and Ca^{2+} or EGTA, pelleted, then resuspended in 100 μl WB and Ca^{2+} or EGTA containing a saturating amount of QR-SA. After another 20-min incubation on ice, the cells were washed and pelleted as before, then resuspended in 0.5 ml of WB and Ca^{2+} or EGTA and analyzed on a FACScan using CellQuest software (Becton Dickinson, Mountain View, CA). Detectors for forward (FSC) and side (SSC) light scatter were set on a linear scale, whereas logarithmic detectors were used for all three fluorescence channels (FL-1, FL-2, and FL-3), except for cell cycle analyses, where FL-3 was used on a linear scale. Compensation for spectral overlap between FL channels was performed for each experiment using single-color-stained cell populations. Wherever possible, instrument settings were saved to disk and used again with slight modifications if necessary in related experiments. All data were collected ungated to disk and were analyzed using CellQuest software. Unless otherwise noted, RBC and dead cells were excluded by electronically gating data on the basis of FSC vs SSC profiles; a minimum of 10^4 cells of interest were analyzed further. Cell sorting was performed at 4°C using a FACStar^{Plus} (Becton Dickinson) with LYSYS II or CellQuest software or using a Coulter EPICS Elite flow cytometer (Miami, FL). To ensure retention of rAnV binding throughout the sort and reanalysis, $CaCl_2$ was added to the sheath tank to a final concentration of 2 mM before sorting. To avoid the formation of calcium/phosphate precipitates during sorting, the standard PBS-based sheath fluid was replaced with HBSS (lacking phenol red) buffered with 10 mM HEPES. Approximately 10^3 sorted cells of each type were reanalyzed for purity. TUNEL staining of BM cell cultures was performed using a kit (Roche) after surface labeling the cells with anti-B220-PE and rAnV-biotin plus QR-SA, followed by fixation and permeabilization, according to the manufacturer's instructions. For cell cycle analyses, BM cells were first stained with FITC-rAnV and anti-B220-PE, then fixed and permeabilized as for TUNEL staining, and incubated for 15 min on ice with 10 μg/ml 7-AAD before analysis.

Stromal cell cultures

To determine the relative viability of purified B cells upon culture with stromal cells, unseparated or column-enriched $CD19^+$ nonTg or 3-83 Ig Tg BM B cells were cultured for 40–48 h on plates with the adherent H-2^d stromal cell line S17 (55) or the H-$2K^b$ stromal cell line op42 (56). Stromal cells were plated 2 days before use at a density of 3–6 × 10^4 cells/well in 24-well plates in RP10 and were essentially confluent when the B cells were added. After culture, the B cells were harvested by gentle resuspension, counted, and stained with FITC-rAnV, anti-B220-PE, and 7AAD or were stained with anti-B220-PE and subjected to the TUNEL assay. Stained cells were analyzed by flow cytometry; collected data were later reanalyzed by gating on $B220^+$ cells.

In vitro stimulation of splenocytes

Spleen cells from C57BL/6 mice were cultured in 24-well flat-bottom plates for 2 days in 2 ml of RP10 alone or in RP10 supplemented with 25 μg/ml LPS (Sigma), 25 μg/ml LPS and 10 ng/ml rmIL-4 (R&D Systems, Minneapolis, MN), 5 μg/ml Con A (Vector, Burlingame, CA), or 0.5 ng/ml PMA and 500 ng/ml ionomycin (Calbiochem, La Jolla, CA). Two days later, cells were harvested, washed twice with HBSS and 1% BSA, and stained as described for flow cytometry.

PCR-FLP

DNA was isolated from sorted cells by lysing them in the presence of SDS and EDTA, incubating overnight with proteinase K, adding 20 μg of glycogen as carrier, and performing one phenol:$CHCl_3$ and one $CHCl_3$ extraction, followed by precipitation in ethanol/0.3 M ammonium acetate. For reading frame usage analysis of sorted pro-B cell populations, DNA from 1–4 × 10^4 cells was first amplified (20 cycles of 94°C for 1 min and 66°C for 2.5 min, followed by one cycle of 72°C for 10 min) with a primer downstream of J_H3 (JHA, 5′-TGCCTCAGACTTCAAGCTTCAGTTCT GG-3′) and a degenerate V_HJ558 gene family-specific primer (VH558-FR1, 5′-ARGCCTGGGRCTTCAGTGAAG-3′). A portion of this reaction (1 μl of 25 μl total) was used in a second round of amplification (20–25 cycles under the same conditions as used in the first round) using VH558-FR3 (5′-CTGACWTCTGAGRACTCYGCRGTCYATT-3′) and an end-labeled primer downstream of J_H2 (JHB3; 5′-ACACACATTTCCCCCC CAACAAA-3′). Similar results were obtained using primers downstream of J_H1 or J_H3. For ^{32}P end labeling of the oligonucleotide primers, T4 polynucleotide kinase (New England Biolabs, Beverly, MA) was used according to the manufacturer's instructions. The labeled oligos were purified on a QiaQuick spin column (Qiagen, Valencia, CA) according to the manufacturer's instructions. Three microliters of the second PCR were analyzed on a 6% acrylamide sequencing gel alongside a 1-bp ladder. The sequencing gels were subsequently analyzed using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) or were visualized by autoradiography. PCR-FLP assays of IgH rearrangements in sorted immature B cell subsets were similar, except that the first PCR contained JHA and a mix of VH558-FR1 and VH12-FR1 (5′-TACCTGCTCTATTACTGGTTTCC-3′). The second PCR contained VH186.2-FR3 (5′-AGCAGCCTGACATCT GAGGACTC-3′) or VH12-FR3a (5′-CCAGTTCTTTCTGCAATTGAAC TC-3′) and a primer just downstream of J_H1 (JHB6-xba, 5′-GGCTCTA GAGTGTGGCAGATGGCCTGACA-3′).

Results

Recombinant AnV binds to viable bone marrow B cells bearing a selectable BCR

We have shown previously that our preparations of FITC- or biotin-labeled rAnV clearly demarcate apoptotic B cells in vitro, using as a control a temperature-sensitive Abelson virus-transformed cell line, 103-bcl2, that can be induced to undergo apoptosis upon shifting the cells to the nonpermissive temperature (45, 54). Based on published reports describing the utility of rAnV to identify apoptotic cells (reviewed in Ref. 51), our original goal was to use this reagent to track apoptotic B cells undergoing negative selection in vivo. To do this, we obtained mice expressing transgenes encoding both the H and L chains of an Ab (clone 3-83) specific for the MHC class I molecule H-2K (52). The 3-83 Ab binds to H-$2K^k$ with high affinity and to H-$2K^b$ with moderate affinity, but does not bind with appreciable affinity to H-$2K^d$ (57, 58). Thus, in H-2^d mice bearing the 3-83 transgenes, nearly all the immature BM B cells and mature peripheral B cells express the Tg

BCR and stain with the 3-83 Id-specific mAb 54.1 (59). Development of Ig Tg B cells is also accelerated, and there are very few pro- and pre-B cells present in H-2^d 3-83 Tg BM (32) (data not shown). However, in H-2^b or H-2^k 3-83 Tg mice, B cell development is arrested at an immature stage, and those Tg B cells remaining are $IgM^{low}IgD^-$ (32). Nemazee and colleagues have shown that a subset of these arrested BM B cells can undergo receptor editing (60).

To quantify apoptosis in Tg mice on a negatively selecting background (i.e., in H-2^b and H-2^k 3-83 Tg mice), we stained BM cells from these mice and from H-2^d 3-83 Tg mice with FITC-rAnV. Contrary to expectation, instead of identifying a large population of Tg B cells undergoing apoptosis in the H-$2^{b\times d}$ and H-$2^{k\times d}$ Tg mice, we found that most of the "live-gated" $B220^+$ cells in the H-2^d Tg BM bind rAnV (Fig. 1). These Tg B cells bind rAnV at intermediate levels ($rAnV^{int}$), significantly higher than background staining in the absence of Ca^{2+}, but lower than the levels found on dead or dying cells (based on ungated cell profiles; see below). Furthermore, a substantial proportion (~40–50%) of B cells in both nonTg BM and H-$2^{b\times d}$ and H-$2^{k\times d}$ Tg BM also bind rAnV at intermediate levels (Fig. 1).

We addressed the binding specificity of rAnV by preincubating FITC-rAnV with liposomes prepared from a panel of phospholipids (61) before staining BM cells (45). Recombinant AnV binding

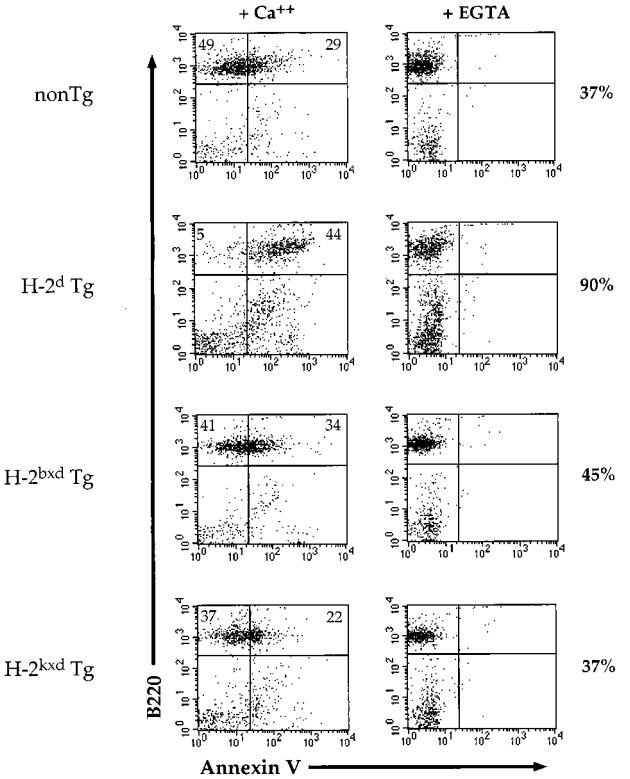

FIGURE 1. A large proportion of viable BM B cells bearing selectable H+L Ig Tg BCRs stain with FITC-rAnV. BM cells collected from H-$2^{k\times d}$ nonTg (*top row*) or from H-2^d (*second row*), H-$2^{b\times d}$ (*third row*), or H-$2^{k\times d}$ (*fourth row*) 3-83 Ig Tg mice were stained with FITC-rAnV and anti-B220-PE in the presence of $CaCl_2$ (*left panels*) or EGTA (*right panels*) and analyzed by flow cytometry. B220 (*y*-axis) vs rAnV (*x*-axis) profiles are shown, with the percentage of cells in each of the upper quadrants indicated; the percentage of $B220^+$ cells that bind FITC-rAnV in the presence of $CaCl_2$ is listed in the *far right column*. Data were gated on viable lymphocytes based on their light scatter characteristics, as shown in Fig. 4*A*, below. Separate analyses confirmed that nearly all the $B220^+rAnV^+$ cells in this gate exclude 7AAD (data not shown).

to viable (7-amino-actinomycin D $(7AAD)^-$) nonTg or H-2^d 3-83 Tg BM B cells was strongly inhibited by PS-containing liposomes, partially inhibited by phosphatidylinositol (another negatively charged phospholipid), but virtually unaffected by PC, phosphatidylethanolamine, phosphatidic acid, or sphingomyelin. Furthermore, rAnV binding was unaffected by pretreatment of the B cells with pronase (45). Thus, rAnV is binding in a specific fashion to negatively charged phospholipids, especially PS, exposed on the surface of BM B cells. However, we should note that although rAnV binding may simply reflect a change in the distribution of PS, it is also possible that rAnV may be recognizing a more general alteration in membrane structure (45).

Given the presence of substantial numbers of Tg Id^+ B cells in the peripheral lymphoid organs of H-2^d 3-38 Tg mice (32) (data not shown), it seemed unlikely that all the $rAnV^{int}$ cells in the BM of these mice represent cells undergoing apoptosis. However, to test whether other MHC molecules, for example class Ib complexes (reviewed in Ref. 62), might trigger apoptosis of 3-83 Tg B cells in BALB/c (H-2^d) mice, we crossed the 3-83 transgene onto the β_2-microglobulin-deficient background (63). $B220^+$ BM cells from H-2^b β_2-microglobulin$^{-/-}$ 3-83 Tg mice also bind rAnV at intermediate levels (45). In addition, we used ligation-mediated PCR (64) to examine sorted $rAnV^+7AAD^-$ 3-83 Tg BM B cells for dsDNA breaks in the Igκ locus associated with receptor editing. Although receptor editing was clearly evident in unsorted Tg BM cells from the H-$2^{k\times d}$ and H-$2^{b\times d}$ mice, we found no evidence for ongoing κ rearrangement in the H-2^d Tg BM (data not shown), as predicted from the literature (24). Furthermore, we confirmed our initial suspicions that most rAnV-binding B cells in Ig Tg and nonTg BM are not dying by testing $rAnV^{int}$ B cells in a wide variety of assays for apoptosis (45). As described previously (45), we found that nearly all $rAnV^{int}$ B cells lack internucleosomal DNA cleavage, and they do not exhibit a loss of mitochondrial transmembrane potential (65) or caspase activity, even though the latter is one of the earliest detectable events during apoptosis (45). We have also shown that the affinity for rAnV is not an idiosyncrasy of B cells in the 3-83 Tg mice, because B cells in nonTg mice as well as in several other Ig Tg mouse strains, including anti-hen egg lysosome (anti-HEL) Ig Tg mice (66), also stain in a similar fashion with FITC-rAnV (Fig. 1) (45).

Recombinant AnV does not bind to positively selected T cells

To investigate whether rAnV binding might reflect a general membrane alteration on all lymphocytes undergoing selection, we obtained anti-H-Y TCR Tg mice, a well-characterized model of T cell positive and negative selection (34). We compared FITC-rAnV staining levels on viable double negative, double positive, and mature SP thymocytes from female or male anti-H-Y TCR Tg mice (Fig. 2). Although anti-H-Y TCR Tg T cells are subject to negative selection in male mice, substantial numbers of $rAnV^+$ cells do not accumulate, probably because apoptotic thymocytes are rapidly engulfed by resident macrophages (67). Furthermore, we found that rAnV does not bind to anti-H-Y TCR Tg thymocytes undergoing positive selection (i.e., in H-2^b SCID females; Fig. 2). Thus, PS exposure on nonapoptotic cells does not occur for all developing lymphocytes in vivo, but is instead a feature that is specific to the B cell lineage.

The altered membrane structure on rAnV-binding BM B cells is influenced by coculture with stromal cell lines

Although it is clear that rAnV-binding B cells isolated directly ex vivo are not apoptotic (45), we reasoned that they still might be at the very earliest stage of apoptosis, undetectable by our other assays and might not survive well after several days culture in vitro.

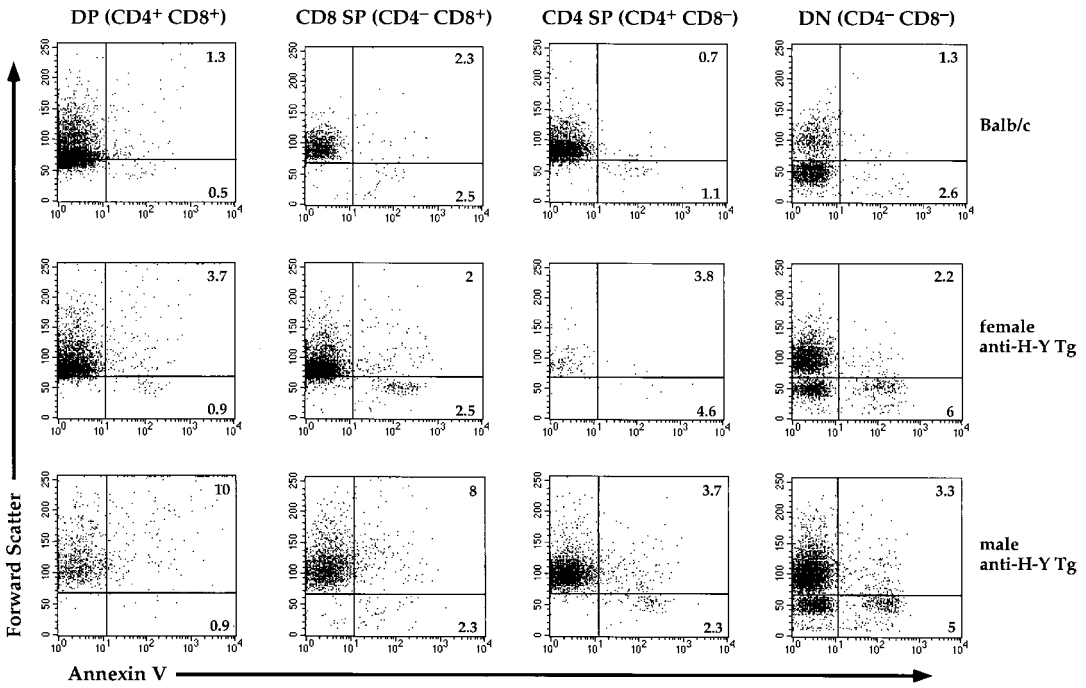

FIGURE 2. Unlike their B cell counterparts, positively selected T cells do not bind rAnV. Thymocytes isolated from a 14-wk-old BALB/c (*top row*) or a 6-wk-old female H-2^b SCID anti-H-Y + D^b TCR Tg (*middle row*) mouse or from a male littermate (*bottom row*) were stained with anti-CD8-FITC, anti-CD4-PE, and rAnV-biotin followed by QR-SA and analyzed by flow cytometry. The FSC vs rAnV profiles are shown for gated double-positive (CD4$^+$CD8$^+$), CD8 single-positive (SP; CD4$^-$CD8$^+$), CD4 SP (CD4$^+$CD8$^-$), or double-negative (CD4$^-$CD8$^-$) cells, and the percentage of cells in each right quadrant is indicated; no size/viability gates were used. CD4 SP cells in the male TCR Tg thymus were actually double-positive^{dull} cells that fell into the gate defined using the BALB/c control.

To test the survival capacity of purified rAnV^{int} B cells, we cultured unseparated or immunoaffinity-purified CD19$^+$ BM cells from H-2^d 3-83 Ig Tg mice or nonTg littermates on the H-2^d BM stromal cell line S17 (55) or the H-2K^b-expressing BM stromal cell line op42 (56). We then assessed the viability of the B220$^+$ cells 2 days later using either a combination of rAnV and 7AAD staining (Fig. 3*A*), or the TUNEL assay (Fig. 3*B*). In contrast to cell populations cultured at 37°C in the absence of stroma, both nonTg and 3-83 Tg B cells cocultured with S17 remained largely viable by both FACS assays (Fig. 3 and data not shown). However, a substantial fraction of 3-83 Tg cells that were cultured on the stromal line op42 did undergo apoptosis, as expected (Fig. 3) (60). The percentages of input cells recovered from each culture condition were comparable (no stroma, 52% for Tg and 30% for nonTg; S17, 37% for Tg and 29% for nonTg; op42, 38% for Tg), arguing against the possibility that the stromal cells might actively phagocytose a majority of the rAnV^{int} B cells. Thus, multiple experimental criteria confirm that the majority of rAnV$^+$ BM B cells we detect in both nonTg and Ig Tg mice are not apoptotic (Fig. 3) (45).

Interestingly, the levels of FITC-rAnV fluorescence on the viable B cells from 3-83 Tg or nonTg BM consistently decreased ~2-fold upon coculture with either S17 or op42 stromal cell lines (Fig. 3*A*, compare the mean fluorescence intensities of the live cells in the *lower left quadrants*). The relative decrease in rAnV binding on B cells in the stromal cell cocultures probably does not reflect the loss of a signal provided in vivo, because BM B cells cultured in the absence of stromal cells maintain high levels of exposed PS (Fig. 3, *upper panels*). Given the short time span of the assay and the recovered cell counts (see above), it is also unlikely that this change in rAnV staining levels reflects a rapid outgrowth of the small number of rAnV$^-$ input cells and a coincident disappearance of the rAnV^{int} cells. Instead, the stroma present in the cocultures seems to have induced the reversal of membrane alteration on the rAnV^{int} B cells that had been prematurely removed from their normal in vivo microenvironment (see *Discussion*).

The proportion of cells binding rAnV consistently differs between B cell subsets

Having established that rAnV can recognize B cells that alter their membrane in a process distinct from apoptosis, we next investigated whether there might be a pattern of rAnV binding on B cells at various stages of development. We stained BM and spleen from nonTg mice for three-color flow cytometric analysis with FITC-rAnV and pairs of several mAbs useful for distinguishing well-defined immature and mature B cell subsets (Fig. 4, *A* and *B*) (68–71). When we analyzed these cells, we did, in fact, observe a trend whereby the proportion of each BM B cell subpopulation that binds rAnV increases as the cells mature, but consistently decreases once the cells exit the BM and continue their maturation in the periphery (Fig. 4*C*). A relatively small proportion (30 ± 8.5%) of the pro-B cell (B220$^+$CD43$^+$) compartment in the BM binds rAnV, while the proportion of cells that bind rAnV steadily expands among increasingly mature sIg$^+$ BM B cells (reaching a high of 68 ± 9%; Fig. 4*C*). This trend was also apparent if we focused on the levels of B220 and heat-stable Ag surface expression to define the stages of BM B cell maturation (data not shown). Interestingly, the levels of rAnV binding dropped fairly sharply on the mature recirculating IgM^{low}D^{high} BM B cell subset (42 ± 10%; Fig. 4*B*). When we examined splenic B cells for rAnV staining among the peripheral B cell subsets, fractions III (IgM^{high}IgD^{low}), II (IgM^{high}IgD^{high}), and I (IgM^{low}IgD^{high}), we found that the proportion of rAnV-binding cells steadily decreased as the cells transited to the next maturational stage (Fig. 4*C*). Thus, a

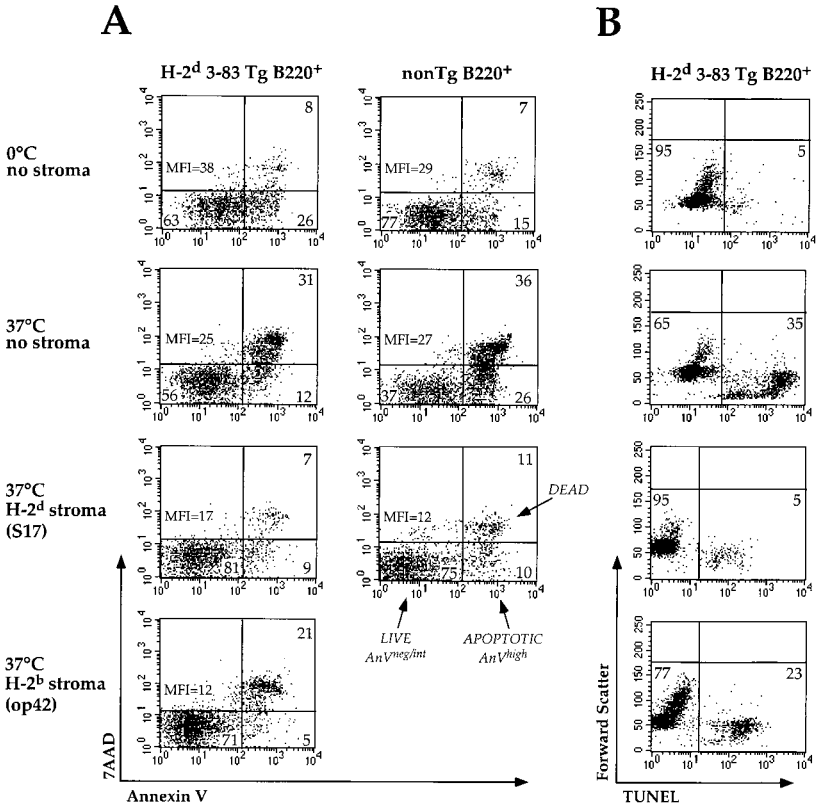

FIGURE 3. rAnV^{int} BM B cells survive well in vitro upon coculture with stromal cells. Enriched CD19$^+$ BM cells from H-2^d 3-83 Ig Tg (65% B220$^+$/58% rAnV$^+$) mice or nonTg (74% B220$^+$/36% rAnV$^+$) littermates were resuspended in RP10 and were left on ice (*top row*), cultured at 37°C in the absence of stroma (*second row*) or cultured at 37°C on H-2^d S17 (*third row*) or H-2^b op42 (*bottom row*) stromal cell layers, as described in *Materials and Methods*. *A*, After 2 days, cells were harvested, counted, and then stained with FITC-rAnV, anti-B220-PE, and 7AAD and analyzed on a FACScan. The rAnV vs 7AAD profiles for all (live and dead) B220$^+$ cells in each culture are shown. Note that the markers have been set differently than in other figures, well above the background staining in the presence of EGTA, to distinguish vital rAnV$^{-/int}$ cells from the bona fide apoptotic rAnV^{high} populations. The percentage of cells falling into each quadrant and the mean fluorescence intensity (MFI) of FITC-rAnV on the viable cells (rAnV$^{-/int}$ 7AAD$^-$) are indicated. For comparison, the starting B220$^+$ population (day 0) contained an average of 5% AnV^{high}7AAD$^-$ (apoptotic) and 10% AnV^{high}7AAD$^+$ (dead) cells. Similar results were obtained using whole unseparated Tg or nonTg BM and gating on the B220$^+$ cells after culture. *B*, 3-83 Tg cells cultured as described in *A* were stained with anti-B220-PE then fixed, permeabilized, and subjected to the TUNEL assay. FSC vs TUNEL FACS profiles for gated B220$^+$ cells are shown. A good correlation between rAnV^{high} cells and TUNEL$^+$ cells was also found using nonTg BM B cells (data not shown).

larger fraction of those cells thought to represent recent emigrants from the BM (IgM^{high}D^{low} or B220^{low}heat-stable Ag^{high}) bind rAnV than do the more mature peripheral B cell subsets, a portion of which probably return to the BM bearing reduced levels of PS (Fig. 4*B*; IgM^{low}IgD^{high}).

However, the combination of IgM and IgD Abs does not adequately separate all known mature B cell populations. In particular, it is now known that fraction III is quite heterogeneous and includes MZ B cells, some B-1 cells, and other activated B cells, in addition to newly formed (NF) and transitional B cells (70, 72). Thus, we also analyzed rAnV binding to transitional 1 (T1) and T2 and mature (M) B cells, as defined by IgM and CD21 costaining (Fig. 4*B*) (70). We also stained CD43$^-$ spleen cells (negatively selected on a magnetic bead column; >95% B220$^+$) with rAnV and Abs to CD21 and CD23 to distinguish NF, follicular (FO), and MZ B cells (Fig. 4*B*) (28, 29). As shown in Fig. 4*C*, the T2 population contains a higher fraction of rAnV-binding cells than do the T1 or mature B subsets. More strikingly, however, an average of 87% of the MZ B cells are rAnV$^+$, significantly higher than their NF and FO counterparts (Fig. 4*C*). Thus, PS is apparently gradually reinternalized on B cells as they exit the BM or shortly thereafter, but is again externalized on mature B cells recruited to the MZ by a BCR-mediated interaction.

Recombinant AnV binds to nearly all B-1 cells

Given the developmentally regulated patterns of rAnV binding throughout B cell development in normal mice (Fig. 5) and our observation that a large fraction of Ig Tg B cells bearing a selectable BCR bind rAnV (Fig. 1) (45), we postulated that this reagent might be identifying positively selected B cells. To investigate this possibility, we first analyzed BM, spleen, and peritoneal washout cells (PerC) from nonTg mice and from V_H12 Tg mice in which the rearranged IgH transgene efficiently mediates positive selection of cells into the CD5$^+$ B-1a cell subset (43). These cells were stained for three-color flow cytometric analysis with FITC-rAnV and mAbs specific for CD5 and B220. As shown in Fig. 5, a large majority (80–95%) of the B-1a cells (B220$^+$CD5$^+$) in the peritoneum (*upper panels*), spleen (*lower panels*), and BM (data not shown) of both nonTg and V_H12 Tg mice bound significant levels of rAnV, analogous to what we had observed with 3-83 and anti-HEL Ig Tg BM (Fig. 1) (45). Similar to the rAnV^{int} BM B cells from those mice, rAnV^{int} CD19$^+$ cells purified from V_H12 Tg spleen are not apoptotic by multiple criteria (data not shown) (45). Interestingly, in both nonTg and V_H12 Tg mice, the proportion of cells binding rAnV in the B-1a lineage is greater than that in the B-2 population (B220$^+$CD5$^-$) from the same individual (Fig. 5).

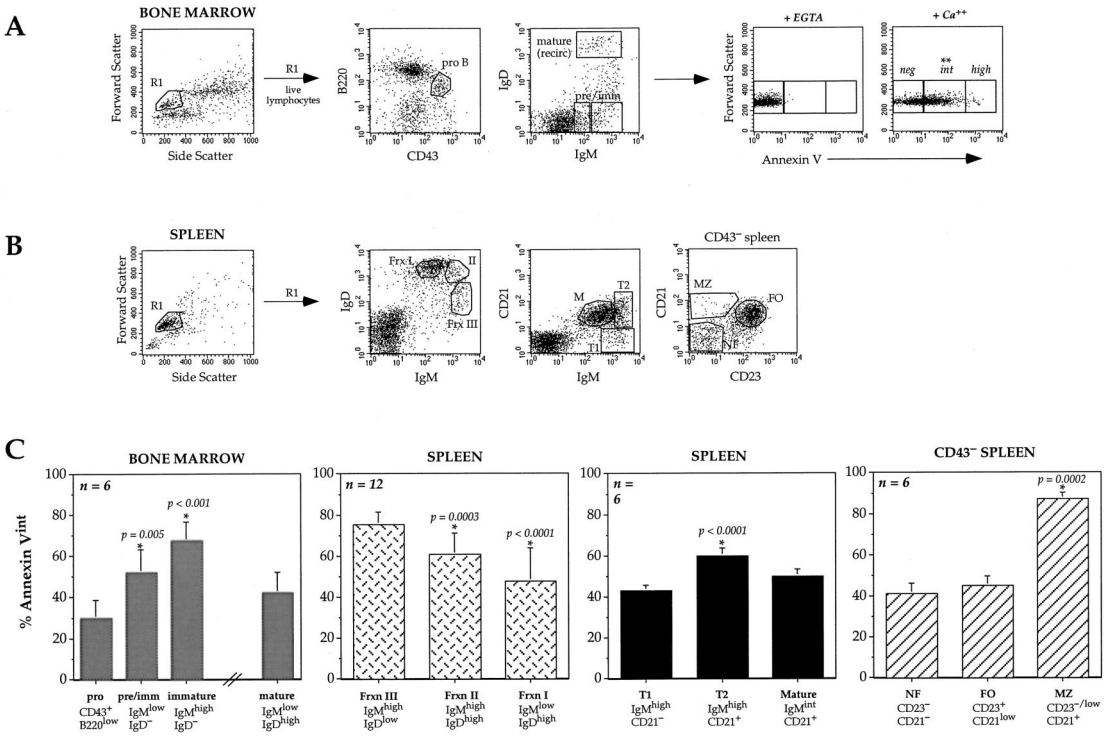

FIGURE 4. The proportion of cells binding rAnV consistently differs between B cell subsets. *A*, Gates used to identify BM B cell subsets. BM cells isolated from C57BL/6 or BALB/c mice (4 wk to 8 mo of age) were stained with FITC-rAnV and either anti-B220-PE plus anti-CD43-biotin followed by QR-SA or anti-IgD-PE plus anti-IgM-biotin followed by QR-SA. The percentage of cells binding rAnV at intermediate levels (**) among live-gated (R1) pro-B ($B220^+CD43^+$), immature B ($IgM^{low/high}$ IgD^-), and mature recirculating B cells (IgM^{high} IgD^{high}) were determined using the AnV gating shown. *B*, Gates used to define splenic B cell subsets. Unseparated splenocytes (first three plots) were stained with FITC-anti-IgD or -CD21, anti-IgM-PE, and rAnV-biotin followed by QR-SA and were analyzed by flow cytometry. The combination of IgD and IgM was used to define fractions III, II, and I (68); IgM and CD21 costaining was used to identify T1, T2, and M cells (70). NF, FO, and MZ B cells (28) were defined by isolating T cell-depleted splenocytes (the flow-through of a MACS column using splenocytes stained with anti-CD43-coated magnetic beads) and staining these cells with FITC-anti-CD21, anti-CD23-PE, and rAnV-biotin followed by QR-SA. $rAnV^{int}$ cells among each R1-gated (live) subset were defined using markers similar to those shown in A (**). *C*, The percentages of $rAnV^{int}$ cells among each BM and splenic B cell subpopulation are plotted as the mean and SD of the data obtained from 6–12 mice (*n*). Asterisks indicate values significantly different ($p < 0.05$, by Student's unpaired *t* test) from the first set of data on each graph. At least 100,000 total events were collected for each sample, and a minimum of 1,500 gated events were assessed for rAnV binding.

Even more striking is the comparison of rAnV levels on either B cell subset to those on T cells (Fig. 5; $B220^-CD5^+$). Unlike the staining pattern on B cells, rAnV binding to viable resting T cells is rarely above the background staining seen in the absence of Ca^{2+}, even in the BM microenvironment (Fig. 5 and data not shown).

Recombinant AnV binding B cells are not actively cycling

Because many of the B cell subsets exhibiting extensive AnV binding either contain dividing cells or are the successors of cells that have divided recently (Figs. 4 and 5), we began to address the issue of whether AnV binding is a consequence of cell division. We stained bone marrow cells from C57BL/6 mice with anti-B220 and rAnV-FITC, fixed and permeabilized the cells, then stained their DNA with 7AAD before flow cytometric analysis (Fig. 6). Analysis of gated $B220^+AnV^-$ vs $B220^+AnV^{int}$ B cells did not reveal any notable differences in the distribution of cells in the G_0/G_1 or G_2/M/S phases of the cell cycle. This suggests that if PS is exposed during cell division, it remains externalized once the cells have exited the cell cycle.

Recombinant AnV binding increases on T cells activated in vitro, but remains essentially unchanged on activated B cells

Although resting T cells stained ex vivo are predominantly AnV^- (Figs. 2 and 7*A* and data not shown), we questioned whether T cells activated in vitro might externalize PS and bind rAnV. We also asked whether we might be able to alter the rAnV binding properties of B cells if we deliberately forced them into cycle. To address these questions, we isolated splenocytes from C57BL/6 mice; cultured them for 2 days with LPS, LPS plus IL-4, PMA plus ionomycin, or Con A; and compared the AnV staining patterns of each cell type with that of unstimulated control cell cultures (Fig. 7*A*). We compared the fraction of cells falling in the AnV^-, AnV^{int}, and AnV^{high} regions among live-gated $B220^+$ B or $CD3^+$ T cells (Fig. 7 and data not shown) for each culture condition. We found that the extent of AnV binding to B cells remained essentially unchanged under all stimulation conditions, except for the LPS plus IL-4 treatment (86% $AnV^{int/high}$ compared with 59% for the unstimulated cells). Interestingly, both Con A and PMA/ionomycin stimulation induced a substantial increase in AnV-binding cells within the $CD3^+$ T cell population (82 and 81% $AnV^{int/high}$, respectively, compared with 20% for the unstimulated cells; Fig. 7*B*). Whether these AnV-binding T cells expose PS on the pathway leading to activation-induced cell death or are instead mimicking the events occurring in B cells in vivo remains to be determined (see *Discussion*).

Recombinant AnV marks selected pro-B and immature B cells

To more rigorously test the idea that rAnV staining marks positive selection of B cells, we undertook a molecular analysis of the IgH

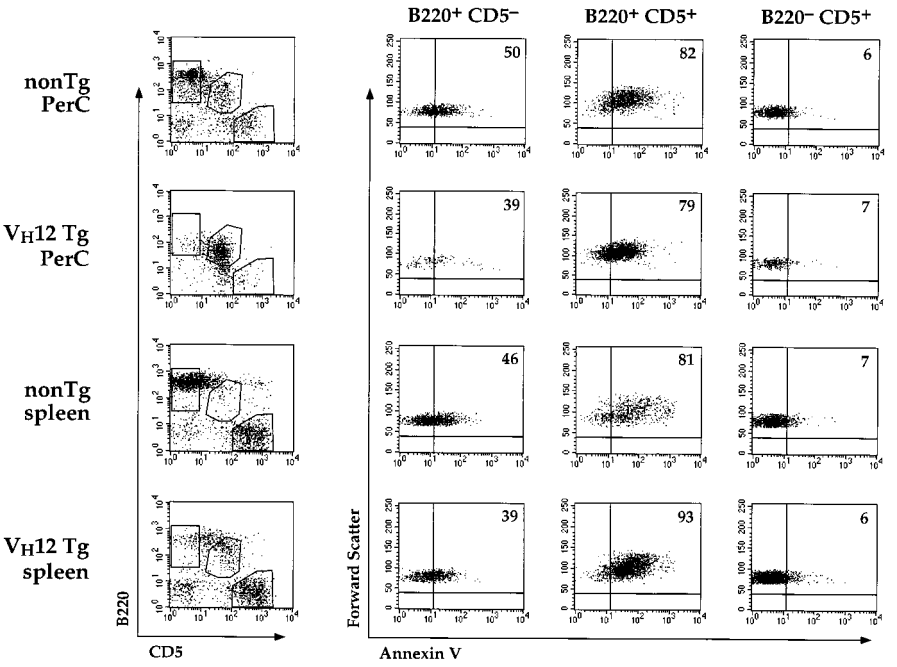

FIGURE 5. Recombinant AnV binds to a large fraction of B-1a cells from nonTg and V_H12 Tg mice. PerC and splenocytes isolated from a V_H12 Tg or a nonTg littermate were stained with FITC-rAnV, anti-CD5-PE, and anti-B220-biotin followed by QR-SA and were analyzed on a FACScan. The FSC vs rAnV profiles are shown for gated $B220^+CD5^-$ (B-2), $B220^+CD5^+$ (B-1a), and $B220^-CD5^+$ (T) cells, as indicated. Note that the level of rAnV binding to the T cells is significantly lower than that to either B cell subset. For comparison, the fluorescence level of FITC-rAnV on gated dead cells is $\sim$1–2 $\times$ 10^3 on the logarithmic *x*-axis scale (data not shown). Similar results were obtained in three independent experiments.

repertoire of $rAnV^-$ vs $rAnV^+$ B cells from nonTg mice. We looked for differences in cell populations using PCR-fragment length polymorphism (PCR-FLP) analysis of DNA from FACS-sorted BM cells (73). This assay consists of a PCR step using primers that amplify across the VDJ junction, followed by separation of the amplified products on a denaturing polyacrylamide gel, allowing an assessment of both CDR3 length heterogeneity and the distribution of in-frame and out-of-frame rearrangements within a population of cells.

We performed PCR-FLP analyses with DNA from $rAnV^-$ or $rAnV^{int}$ pro-B ($B220^+CD43^+$) cells sorted from BALB/c BM using a degenerate PCR primer that recognizes the large J558 family of V_H genes and a primer that anneals downstream of the J_H1 or J_H2 gene segments. As noted above, pro-B cell survival and maturation require the production of an in-frame Ig H chain gene rearrangement. Interestingly, we reproducibly found that while the $rAnV^-$ pro-B cell population bore many out-of-frame IgH rearrangements, the $rAnV^+$ pro-B cells were enriched for in-frame rearrangements (Fig. 8*A* and data not shown).

We next sorted immature (IgM^+D^-) BM B cells from BALB/c mice based on rAnV binding and performed PCR-FLP analysis with primers that amplify $V_H186.2$ (a large subfamily of J558) to DJ_H1 and V_H12 to DJ_H1 rearrangements (Fig. 8*B*). Not surprisingly, given that the $V_H186.2$ family is large and is characterized by no known CDR3 length preferences in selected B cells, the PCR-FLP analyses of $V_H186.2$ to DJ_H1 rearrangements did not reveal any obvious differences between the $rAnV^-$ and $rAnV^+$ immature B cells (Fig. 8*B*, *upper panel*). DNA sequence analysis of the IgH repertoire in $rAnV^-$ vs $rAnV^+$ populations at various stages of B-2 cell development will ultimately be required to identify potential differences among cells in this very diverse compartment. However, when we analyzed rearrangements of the much smaller V_H12 family, we obtained a striking result. As noted above, the predominant V_H12-containing H chain, found paired with a $V\kappa4^+$ L chain in the peritoneal anti-PC B-1 cell subset, consists of a V_H12-D_H-J_H1 rearrangement containing a 10-aa CDR3 (42). In three independent experiments, we found that $rAnV^+$ immature B cell populations consistently contain a dominant V_H12 to DJ_H1 rearrangement that comigrates with the product from the V_H12 transgene and with the corresponding positively selected V_H12 H chains in control nonTg PerC, day 6 spleen, and adult spleen (Fig. 8*B*, *lower panel*). Conversely, the V_H12 rearrangements present within the $rAnV^-$ population are more diverse and more closely resemble the pattern found in unsorted BM samples (Fig. 8*B*). Thus, rAnV marks a subset of immature BM B cells selected based on the structure of its surface Ig.

Discussion

Recombinant AnV binds to nonapoptotic B cells in a developmentally regulated fashion

We have previously reported our surprising finding that the phospholipid-binding protein AnV, generally a reliable marker of PS externalization during apoptosis, binds at appreciable levels to viable B cells (45). We showed that the bulk of B cells that bind rAnV at these so-called intermediate levels (higher than background staining in the absence of Ca^{2+}, but lower than that on dead or dying cells) are not apoptotic by multiple criteria (45). Instead, we found that PS is mobilized to co-cap with IgM on anti-IgM-treated B cells and to colocalize with a marker of lipid rafts, dynamic membrane microdomains thought to function as platforms for the attachment of proteins whose localization and clustering may be critical for signal transduction (74–77). Furthermore, because we could disrupt normal signaling through IgM by sequestering PS on $rAnV^+$ B cells, we suggested that PS might play a role in BCR-mediated signaling events (45).

Here, we have described another unexpected facet of our studies using rAnV by showing that the externalization of PS on viable B cells is regulated during development, and that this membrane remodeling event largely depends upon the expression by each B cell of a selectable BCR. Recombinant AnV binding clearly increases on B cells as they traverse the pro- to pre-B and immature to mature B transitions as assessed by both cell surface (Figs. 4 and 5) and molecular genetic analyses (Fig. 8). Direct IgH repertoire analyses of sorted $rAnV^+$ and $rAnV^-$ B cell subsets in normal

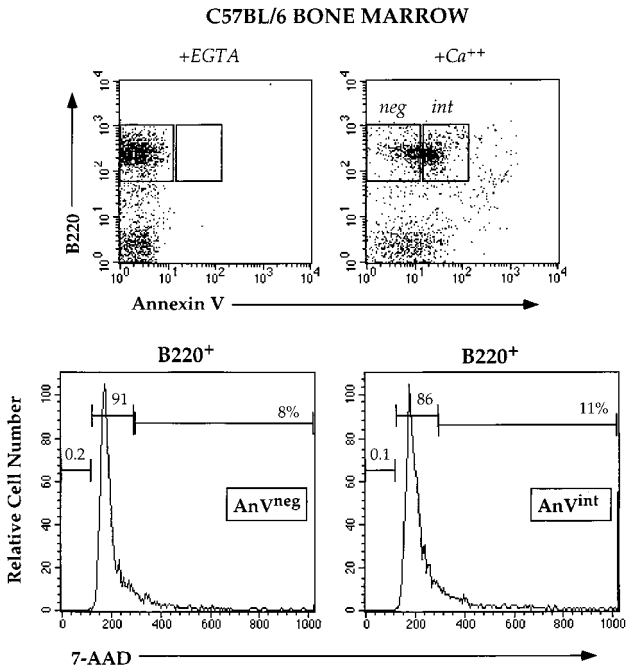

FIGURE 6. Recombinant AnV binding B cells are not actively cycling. BM cells from a C57BL/6 mouse were stained with FITC-rAnV and anti-B220-PE, then fixed, permeabilized, stained with 7-AAD, and analyzed on a FACScan with FL-3 on a linear scale. Data were gated on live lymphocytes, as described in Fig. 4*A*, then further gated on $B220^+AnV^-$ and $B220^+AnV^{int}$ cells and analyzed for 7-AAD levels, as indicated. Markers are set on 7-AAD histograms to delineate subdiploid, G_0/G_1, and G_2/M/S phase cells; the corresponding percentages are shown. Data are representative of results from three experiments. Similar results were obtained using BM cells from H-2^d 3-83 Tg mice.

mice strongly suggest that changes in the plasma membrane leading to rAnV binding correlate with the generation of an in-frame H chain gene rearrangement in pro-B cells (Fig. 8*A*) and with a positive selection signal in immature B cells (Fig. 8*B*).

Our data reveal a general trend whereby the proportion of each BM B cell subset that can bind rAnV increases as progenitor cells mature, but steadily decreases once the cells exit the BM and continue their maturation in the periphery (Fig. 4). Because a larger fraction of those cells thought to represent cells about to exit from the BM (i.e., immature $IgM^{high}IgD^-$) bind rAnV than do the early splenic (NF/T1) B cell subsets (Fig. 4), this membrane remodeling event might play a role in cell trafficking to the peripheral lymphoid organs (Fig. 9). However, it is not yet clear what induces the steady loss of rAnV binding sites on increasingly mature (FO/fraction I) splenic B cells, or what induces the re-externalization of PS as the cells are recruited to the MZ (Fig. 4). These changes may be the result of changes in the expression or activity of enzymes known to alter lipid asymmetry, such as the scramblase and aminophospholipid translocase (see Ref. 49 for review).

Is PS externalization linked to cell cycle status?

In general, the immature and mature B cell subsets that exhibit the highest proportion of positive staining by rAnV either contain dividing cells or are the successors of cells that have divided recently (i.e., immature $IgM^{high}IgD^-$ BM B, B-1, and MZ B cells; Figs. 1, 4, and 5). Because early selection processes in the BM are accompanied by proliferation (78), it is possible that this membrane alteration occurs when B cells divide. Thus, we were initially led to consider whether PS is externalized as a consequence of cell division. When we directly analyzed the cell cycle status of normal or H-2^d 3-83 Ig Tg BM B cells, we found that the vast majority of cells binding rAnV at intermediate levels are in G_0/G_1 (Fig. 6 and data not shown). Furthermore, the proportion of B cells binding rAnV does not increase significantly if B cells are forced into cell cycle in vitro (Fig. 7). It is also important to note that although nearly 95% of the B-1 and MZ B populations bind rAnV (Figs. 4 and 5), a much smaller fraction of these cells is actually cycling at any given time (72, 79). Thus, at least for the B cell lineage, our data suggest that if the externalization of PS requires cell division, these PS residues remain exposed once the cell has exited the cell cycle.

Interestingly, T cells (which are rAnV negative in vivo in normal and TCR Tg mice; Fig. 2 and data not shown) stimulated in vitro with Con A or PMA/ionomycin exhibit an increase in rAnV binding (Fig. 7). However, whether this form of PS exposure represents a situation comparable to that occurring on B cells in vivo or instead reflects the early stages of T cell apoptosis (via activation-induced cell death) remains an open question. Preliminary confocal microscopy experiments support the former possibility, because PS colocalizes with Thy-1 molecules on Con A blasts, or with TCR-CD3 complexes on splenocytes stimulated briefly with soluble anti-CD3 mAb (data not shown). These data suggest that, analogous to its role in B cells (45), PS may also play a role as a component of lipid rafts in activated T cells.

Why do B cells alter their membrane lipid distribution during development?

B cells develop in intimate contact with a highly structured stromal cell environment that provides both specific adhesion contacts with the B cells and factors required for their growth and differentiation (80). Stromal cells are also important mediators of tolerance induction of sIg^+ B cells. As B-lineage cells mature, they migrate in close proximity to stromal cells within the marrow. The earliest stem cells lie in the subendosteum, just adjacent to the bone surface, and as maturation proceeds the B cells move toward the central axis of the marrow cavity where they wait in sinuses for export to the periphery. Later stages of maturation become less dependent on stromal cells, and final transition from immature to mature B cell can actually occur either in the BM or in secondary lymphoid organs such as the spleen.

We propose that the change we observed in membrane structure on BM B cells that accompanies differentiation (i.e., the expression of a selectable receptor) may in part explain how a cell progresses to the next maturational microenvironment (Fig. 9). PS^+ pro-/pre-B cells with a functional pre-BCR might detach from the outer zone of the BM and begin to migrate toward the central axis. Immature B cells may be less dependent upon stromal cells because of their altered membrane structure, and this change in membrane asymmetry may also play a role in B cell export from the BM. Our data suggest that the overall density of exposed PS may be important for export of immature B cells from the BM, because FITC-rAnV staining increases as B cells mature in nonTg BM (Fig. 4) and is brightest on Tg B cells expressing a functional BCR in a permissive (nonnegatively selecting) environment (Fig. 1) (45). Additional changes in adhesion molecule or chemokine receptor expression, influenced in part by stromal cells and their products, are likely to occur concomitantly with PS exposure (1, 80, 81). It remains to be determined whether plasma membrane remodeling is a crucial occurrence, or simply a byproduct of these other maturational events.

Given that the levels of rAnV binding on viable B cells from 3-83 Tg or nonTg BM consistently decreased upon coculture with either the S17 or op42 stromal cell lines (Fig. 3*A*), it seems likely

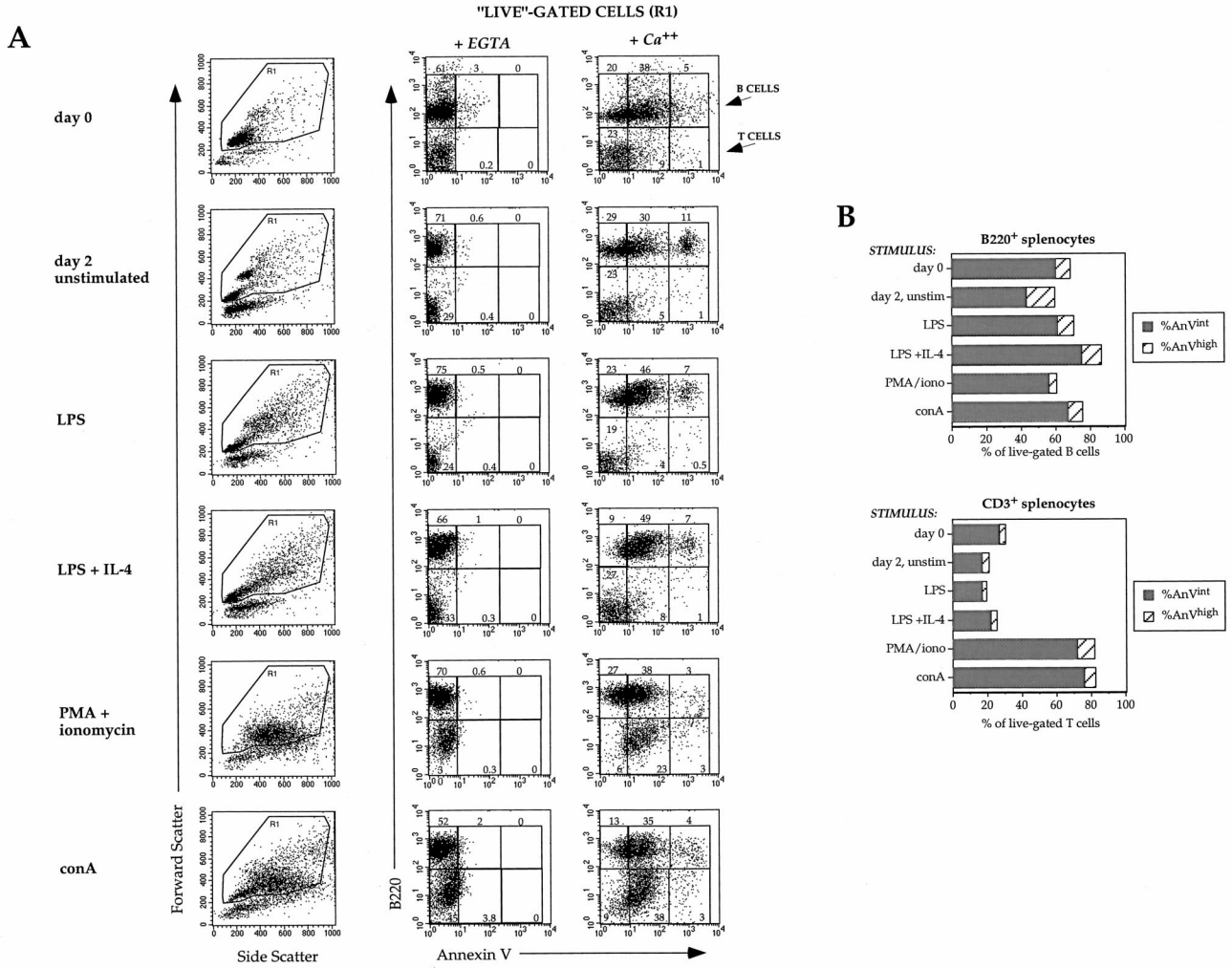

FIGURE 7. Recombinant AnV binding increases on T cells activated in vitro, but remains essentially unchanged on activated B cells. *A*, C57BL/6 splenocytes were cultured for 2 days with LPS, LPS and IL-4, PMA and ionomycin, or Con A, then harvested, washed, stained with FITC-rAnV, anti-CD3-PE, and anti-B220-TC in the presence of 2 mM EGTA or $CaCl_2$, and analyzed by flow cytometry. The rAnV staining patterns of each live-gated (R1) cell type were compared with those of unstimulated control cell cultures analyzed on day 0. The percentages of R1-gated cells falling into each box are shown. *B*, The fraction of cells in the AnV^{int} (shaded) and AnV^{high} (hatched) regions among R1-gated $B220^+$ B (*upper panel*) or $CD3^+$ T (*lower panel*) cells are plotted for each culture condition. Data are representative of results from three independent experiments. Similar results were obtained if the B and T cells were purified before culture on a CD43 MACS column (as $CD43^+$ T or $CD43^-$ B cells).

that stroma present in these cultures induced the reversal of PS distribution on the $rAnV^{int}$ immature B cells that had been prematurely removed from their normal in vivo microenvironment. It thus remains of further interest to determine whether PS exposure accompanies an alteration in the adhesion properties of B cells. Because thymocytes do not alter their membrane in this manner, it may be that the externalization of PS during B cell development is dependent upon the presence of unique BM stromal cell cues, signals not provided by thymic stroma.

Our finding that nearly all B-1 and MZ B cells bind rAnV (Figs. 4 and 5) is compelling in light of the recent advances in understanding the functional relatedness of these two subsets (reviewed in Ref. 72). It has been postulated that these two B cell subsets have evolved to provide first-line responses for gut/peritoneum and blood-borne Ags, and that restriction of the Ig repertoire of these compartments may ensure rapid development of short term responses to a limited number of conserved Ags. It has also been reported recently that Pyk-2-deficient mice lack MZ B cells (29). The authors suggest that the absence of Pyk-2 affects MZ B cell localization to their specialized anatomical niches, possibly due to a compromised response to one or several chemokines or to the dysfunction in the adhesion to some matrix component or stromal cells. It will be interesting to test whether mature B cells in these mice bind rAnV in a manner similar to those in normal mice to begin to address whether externalized PS promotes improved cell adhesion and/or motility of B cells in addition to its apparent role in BCR signaling (45).

The link between positive selection and rAnV binding in B-2 vs B-1 cell lineages

In the 3-83 Ig Tg model system, where the Tg BCR mediates B cell selection into the conventional B-2 population in mice lacking $H\text{-}2K^{b\ or\ k}$ molecules (i.e., $H\text{-}2^d$ or MHC class $I^{-/-}$), we found that nearly all the Tg 3-83 Id^+ B cells bind rAnV (Fig. 1) (45). Those B cells arrested at the immature $IgM^{low}IgD^-$ stage in $H\text{-}2^b$ and $H\text{-}2^k$ Tg mice actually bind lower levels of rAnV than their $H\text{-}2^d$ counterparts (Fig. 1). Interestingly, the few splenic B cells that have escaped negative selection in $H\text{-}2^{b\ or\ k}$ Tg mice by replacing the Tg IgL chain with an L chain encoded by an endogenous Ig

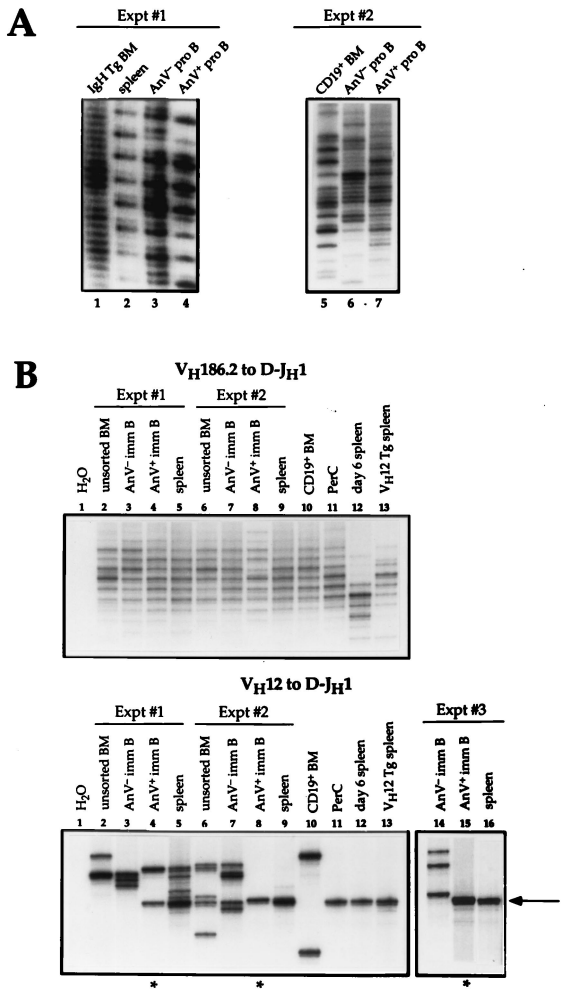

FIGURE 8. IgH chain repertoire analyses reveal that rAnV marks positively selected B cells. *A*, Reading frame usage analysis of V_H558 to DJ_H2 rearrangements in $rAnV^-$ and $rAnV^+$ wild-type pro-B cells. PCR-FLP assays were performed as described in *Materials and Methods* with degenerate primers specific for the largest V_H gene family (J558) and a primer that anneals downstream of J_H2, using DNA isolated from the cells indicated. Unrearranged DNA is not amplified under these conditions. In-frame rearrangements predominate in the spleen and display peak intensity every third nucleotide (*lane 2*), while out of frame rearrangements, which predominate in IgH Tg BM (*lane 1*), migrate between the in-frame bands. Pro-B cells were isolated from BALB/c mice by staining pre-enriched $CD19^+$ BM (*lane 5*) with FITC-rAnV, anti-B220-PE, and anti-CD43-biotin followed by QR-SA, and then sorting the $B220^+CD43^+$ subset into $rAnV^-$ (80–93% pure; *lanes 3* and *6*) and $rAnV^+$ (67–80% pure; *lanes 4* and *7*) cells. Similar results were obtained in four independent experiments, two of which are shown. *B*, PCR-FLP analysis of $V_H186.2$ to DJ_H1 and V_H12 to DJ_H1 rearrangements in whole BM (*lanes 2* and *6*), FACS-sorted $rAnV^-IgM^+IgD^-$ immature BM B (80–90% pure; *lanes 3*, *7*, and *14*), sorted $rAnV^+IgM^+IgD^-$ immature B (63–75% pure; *lanes 4*, *8*, and *15*), and from spleen cells (*lanes 5*, *9*, and *16*) isolated from BALB/c mice, in three independent experiments. Control samples included column-enriched $CD19^+$ BM (*lane 10*), PerC (*lane 11*), day 6 spleen (*lane 12*), and V_H12 Tg spleen (*lane 13*). The V_H12 H chain with a conserved 10-aa CDR3 is indicated by an arrow. Note the consistent presence of the selected V_H12 H chain within the $rAnV^+$ immature BM B cell population (asterisks).

rearrangement bind increased levels of rAnV relative to the arrested Tg BM B cells (data not shown) (45). This suggests that the edited cells receive a positive signal through their modified BCR, allowing both a membrane remodeling event and export to the periphery. Similarly, B-1a cells in both nonTg and V_H12 Tg mice also bind rAnV at appreciable levels (Fig. 5). These data support our hypothesis that rAnV binding and positive selection are linked, because B-1 cells have been described as a subset that is more intensely positively selected than conventional naive B-2 cells (1 HEL Ig Tg strain (66), provided similar results in our rAnV flow cytometry assays (45). Notably, the anergic B cells in anti-HEL/soluble HEL double Tg mice also bind high levels of rAnV (45), again consistent with the idea that this membrane alteration occurs on B cells that have received a BCR signal (in this case, a tolerogenic signal).

It is interesting to note that even mature peritoneal and splenic B-1 as well as mature MZ B cells retain the ability to bind rAnV (Fig. 5). This distinguishes them from follicular B-2 cells, which tend to revert to a relatively normal plasma membrane phospholipid arrangement as they mature (Figs. 4 and 5) and may reflect in part the different anatomical distributions of B-1 and MZ B cells. It is possible that the PS receptors expressed on macrophages and other cells throughout the body influence the trafficking of PS^+ B cells. B-1 cells may receive frequent positive selection signals through recurrent interactions with their cognate self-Ags or via their constitutively active STAT3 protein (82, 83). Both B-1 and MZ B cells are also known to differ dramatically from follicular B-2 cells in their capacity to more rapidly respond to BCR signals (72, 79). It has been argued that, in contrast to B-2 and B-1b ($CD5^-$) cells, the progenitors of B-1a ($CD5^+$) cells are abundant in the fetal omentum and liver, but are absent from adult BM (84–86). However, we have shown that immature (IgM^+IgD^-) B cells that bind rAnV in adult BALB/c BM include apparent B-1 cell precursors carrying a rearranged canonical anti-PC V_H12^+ Ig H chain (Fig. 8*B*). Although PS externalization is a prominent feature of mature B-1 cells (Fig. 5), we do not believe that the canonical V_H12 rearrangements in $rAnV^+$ BM belong to mature recirculating B-1 cells, because our sort gates on immature B cells excluded those with even low levels of IgD expression. Because we do not know whether the selected V_H12 H chains from sorted $rAnV^+$ immature (IgM^+IgD^-) BM B cells have paired with $V_\kappa4^+$ L chains, it remains possible that these cells were positively selected at the pre-B stage, but do not bind PC. In fact, Clarke and colleagues have postulated a positive selection step at the pre-BI to pre-BII transition of BM B cells that express this selected V_H12 H chain (21).

The nature of positive selection within the B cell lineage is not well understood. Current models postulate that B cell specificities are selected through interactions with endogenous or environmental Ag, because the IgH repertoire constricts at some point(s) between the pre-B cell stage and the mature IgD^+ splenic B cell stage (reviewed in Refs. 1 and 2). Recent reports have demonstrated that continued BCR expression is required for the survival of mature B cells (26), and that signaling involving Syk is required for B cell maturation beyond the immature peripheral B cell stage (87). Thus, a BCR that can provide an adequate survival signal probably mediates the positive selection event that converts an immature to a mature B cell. After an immature B cell is selected to exit the BM, its BCR may continue to signal its survival and further maturation through repeated contact with Ag.

In contrast to the clear link between PS exposure and positive selection of B-1 cells (Figs. 5 and 8*B*), a correlation between PS externalization and positive selection of follicular B-2 cells has proven more difficult to substantiate at the molecular level (Fig. 8*B*), largely because of the increased complexity of the Ig repertoire of the B-2 population. It is not yet clear whether positive selection based on BCR specificity acts on immature B-2 cells in the BM, or whether the Ig repertoire narrows at a later stage of B-2

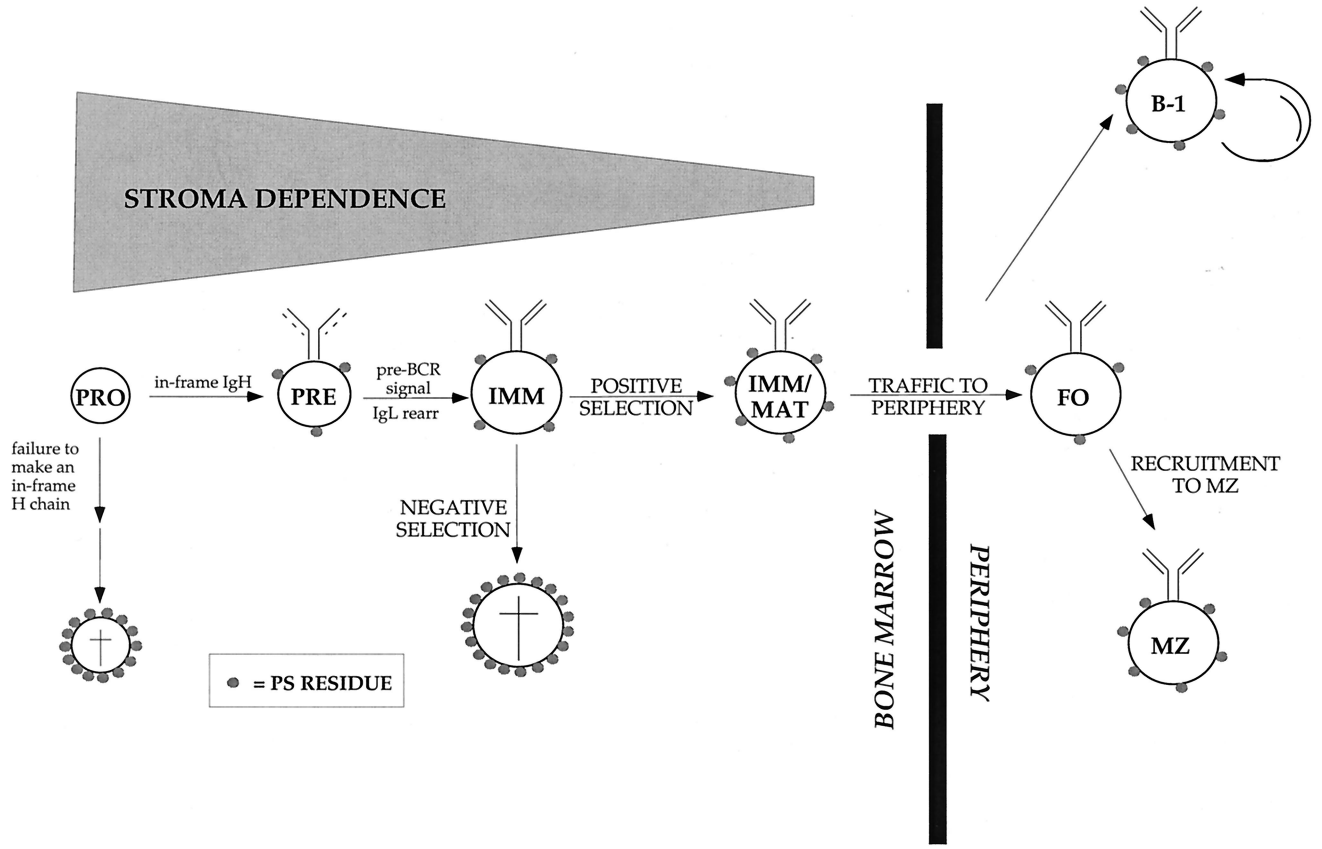

FIGURE 9. Altered membrane structure on developing B cells may affect their trafficking patterns and allow export of positively selected cells to the peripheral lymphoid organs and subsequent recruitment to the MZ. B cell development is critically dependent upon generation of a functional BCR, and PS exposure correlates well with the passage of B cells through successive stages of development and a decreasing dependence on stromal cells. Pro-B cells capable of generating a functional pre-BCR begin to expose low levels of PS on the outer leaflet of their plasma membrane as they concomitantly detach from the outer zone of the BM and begin to migrate toward the central axis. Immature B cells expressing a functional, but non-self-reactive, BCR bind increasing amounts of rAnV, and those cells that receive a positive selection signal bear the highest levels of externalized PS before transiting to the spleen. Mature splenic B cells gradually revert to lower levels of PS (Fig. 4), as observed in BM cultures (Fig. 3), until recruited to the MZ by Ag encounter, probably due to changes in the activity of the scramblase and/or aminophospholipid translocase. As is true for MZ B cells, nearly all self-renewing B-1 B cells in the spleen and peritoneal cavity bind rAnV (Fig. 5).

cell development, perhaps shortly after their arrival in the periphery (36). However, the fact remains that rAnV binds to BM B-2 cells from two independent strains of Tg mice expressing rearranged IgH+L genes encoding BCRs already proven capable of mediating positive selection, because these transgenes were isolated from hybridomas of mature B-2 clones (Fig. 1) (45).

Clearly, a substantially higher fraction of immature BM B cells binds rAnV than actually progresses to join the mature peripheral B cell pool. Estimates of survival of B cells attempting this developmental transition range from 10 to 20% (38–40). Because in some cases 70–90% of the immature BM B cell subset binds rAnV (Figs. 1 and 4) (45), it is apparent that although events associated with positive selection lead to PS externalization, all rAnV-binding B cells are not guaranteed successful emigration to the periphery.

We have previously shown that PS externalized on viable B cells serves as a component of lipid rafts and colocalizes with the BCR after IgM ligation (45). Thus, the increasing levels of PS exposure during B cell maturation may not be merely coincidental, but, rather, may be an important step in the critical process of rewiring the B cell to alter the signaling threshold of its Ag receptor or some quality of this signal. The potential link between PS exposure and B cell activation, proliferation, and adherence suggests that rAnV may represent a useful tool to identify B cell clones selected by Ag during a normal immune response or even to detect self-reactive clones mediating autoimmunity.

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