Predominance of a novel splenic B cell population in mice expressing a transgene that encodes multireactive antibodies: support for additional heterogeneity of the B cell compartment

Kathleen M. Tumas-Brundage¹, Evangelia Notidis¹, Lynn Heltemes¹, Xianghua Zhang, Lawrence J. Wysocki and Tim Manser¹

Department of Immunology, National Jewish Medical and Research Center and University of Colorado School of Medicine, Denver, CO 80262, USA
¹Kimmel Cancer Institute and the Department of Microbiology and Immunology, Jefferson Medical College, Philadelphia, PA 19107, USA

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Abstract
We generated IgHµδ transgenic mice using a V_H gene that in A/J mice encodes multireactive BCR in the preimmune B cell compartment and is predominantly expressed by a memory B cell subpopulation. Most primary splenic B cells in these mice have a size, cell-surface phenotype and in vitro response profile distinct from mature follicular (B2), marginal zone (MZ) or B1 B cells, but are long-lived and appear to be slowly cycling. They reside in conventional B cell areas of the spleen and mount robust foreign antigen-driven germinal center responses, but do not efficiently differentiate to secretory phenotype. We propose that these qualities result from ongoing, low-avidity BCR–self-ligand interactions and promote entry into the memory pathway. Given these data, and the enormous diversity and characteristic multireactivity of the preimmune antibody repertoire, we also suggest that it may be more appropriate to view the primary B cell compartment as a continuum of functional and phenotypic ‘layers’, rather than as a group of discrete B1, B2 and MZ subsets.

Introduction
B cell development takes place in a ordered series of steps, beginning in the fetal liver or adult bone marrow and culminating in peripheral lymphoid organs. First, the pre-BCR and subsequently the BCR play key roles in regulating progression in this developmental pathway (1–3). Engagement of self-ligands by the BCR can have profound inhibitory effects on development. Depending on the concentration and nature of the ligand, these may include death, receptor editing, developmental arrest or anergy (4). However, inability to express a BCR precludes B cell survival in both the bone marrow (BM) and the periphery (5,6). Moreover, there is mounting evidence that self-ligand engagement by the BCR can result in positive clonal selection, as well as influence the microenvironmental locale and peripheral B cell subset in which a clone resides (7–14).

One view of the primary B cell compartment is that its peripheral subsets collectively create a ‘layered’ immune system, with each subset specialized to perform particular functions (15). In this context, the role of B1 cells seems to be to constitutively secrete IgM with specificities that allow binding to common pathogen structures, promotion of clearance of dead or damaged cells, immunoregulation and priming of the memory B cell response (16–19). The marginal zone (MZ) B cell compartment is thought to be necessary for rapid responses to thymus-independent antigens encountered in the general circulation (20). Finally, follicular (B2)
cells are viewed as the precursors of thymus-dependent responses leading to formation of the germinal center reaction and the memory compartment (21,22). Many primary B cells that do not fulfill the requirements for assignment to the B1, B2 or MZ subsets may well be in transit from the central lymphoid organs to one of these peripheral compartments, or from one peripheral compartment to another. Alternatively, some may represent more stable compartments that constitute yet additional functional ‘layers’ of the immune system (5,23,24).

During the anti-p-azophenylarsonate (Ars) response in A/J mice a particular B cell clonotype that is a minor participant in the primary response comes to dominate anamnestic responses. The BCR expressed by this clonotype is encoded by a single combination of VJh, D, Jh, Vκ and Jκ gene segments (25,26). We term this clonotype and the antibodies it expresses ‘canonical’. Canonical antibodies expressed by primary B cells are multireactive, binding both Ars and a variety of self-antigens, including DNA, with low avidity (27). While, by definition, primary canonical B cells are precursors to memory B cells, the peripheral B cell compartment in which they reside has not been well studied due to their extremely low precursor frequency (28). For this reason we have generated and analyzed transgenic mice using a heavy chain gene construct representative of those expressed by canonical clonotypes prior to the Ars response.

**Methods**

**Mice**

The two transgene constructs used are shown schematically in Fig. 1(A). The μδ construct contains the heavy chain VDJ coding and promoter regions from the A/J canonical anti-Ars hybridoma 36-65, with the heavy chain intronic enhancer and the BALB/c μ and δ constant region genes and substantial amounts of 3′ flanking DNA. The 36-65 VDJ lacks somatic mutations. The HS1234 construct (29) contains four 3′ α heavy chain enhancer elements (including the 1, 2, 3b and 4 DNase I hypersensitive regions) and was included to increase the probability that the transgenic heavy chain V gene was subjected to normal hypermutation. This issue is currently being investigated and will be the subject of a future report. Transgene inserts were purified and injected in equimolar amounts into the male pronuclei of fertilized FVB/NJ eggs. Initial PCR and Southern blotting screens revealed six founders with the μδ transgene out of which five also had the HS1234 transgene. Two founder lines (Ars20 and Ars30) were obtained that consistently passed both transgene constructs to their progeny. The Ars20 line contains four or five copies of the μδ construct, while the Ars30 line contains one copy. Founder mice were backcrossed to the A/J strain. Litters were screened for the presence of μδ and HS1234 transgenes as well as for the presence of A/J IgH alleles at the endogenous heavy chain loci. The screening for the A/J IgH alleles was done by Southern blot analysis probing for a restriction fragment length polymorphism near the μ switch region. Most mice used in the reported studies were maintained on a mixed A/J×FVB background. All A/J mice used were 8–12 weeks old and obtained from Jackson Laboratory (Bar Harbor, ME). All mice were housed under specific pathogen-free conditions, and given autoclaved food and water. The use of mice in these studies was conducted in compliance with Institute guidelines and all protocols using animals were approved by the Institutional Animal Care and Use Committee.

**Flow cytometry**

Cells were isolated from lymphoid organs of 10- to 20-week-old, naive transgenic, age-matched transgene-negative littersmates or normal A/J mice. Staining of cells was done using rat whole IgG and mouse whole IgG to block nonspecific binding. One or several of the following reagents were used: polyclonal rat anti-mouse IgD–biotin (Fisher Scientific Pittsburgh, PA), mouse anti-mouse IgM–phycoerythrin (PE) (clone 1B4B1; Southern Biotechnology Associates, Birmingham, AL), donkey anti-mouse IgM–FITC (Jackson ImmunoResearch, West Grove, PA), anti-IgD–biotin (clone AF4-73.3), anti-CD43–biotin (clone S7), anti-I-A^k–biotin (clone 11-5.2b), anti-CD95–biotin (Fas clone Jo2), anti-CD80–biotin, rat anti-mouse IgD–biotin (clone 11-26, Southern Biotechnology Associates), PE– and FITC–anti-B220 (clone RA3-6B2), PE–anti-CD1d (clone 1B1), FITC–anti-IgG3 (clone DS-1), FITC–anti-CD3 (clone 145-2C11), FITC–anti-CD24 (clone HSA clone M1/69), FITC–anti-CD40 (clone HM40-3), FITC–anti-CD21/CD35 (clone 7G6), FITC–anti-CD86 (clone GL-1), allopurinol–anti-CD62L (clone Mel-14), FITC–anti-CD23 (clone B3B4), FITC– streptavidin, PE–streptavidin, and R670–streptavidin (Gibco/BRL, Rockville, MD), and analyzed immediately or fixed in 1% paraformaldehyde. In some experiments PE–anti-CD5 (clone 53-7), anti-CD24–biotin (clone 30-F1), FITC–anti-BP-1 or PE–anti-BP-1 were used (all kind gifts of Dr R. R. Hardy). The anti-idiotypic antibodies 107 and E4 were purified from ascites and biotinylated using standard methods. Unless indicated otherwise, all antibodies were obtained from PharMingen (San Diego, CA). Analyses were done on a Coulter Epics Elite or
on a Becton Dickinson FACStar plus using either the WinMDI 2.7/2.8 programs from the Scripps Institute (San Diego, CA) website or FlowJo (Tree Star, San Carlos, CA) software.

Immunohistochemistry
Spleens were isolated from naive or immunized mice, frozen and cryosections prepared as previously described (30). Sections were stained with one of the following sets of reagents: (i) biotin–107 or biotin–E4, branched streptavidin–alkaline phosphatase (Dako, Glostrup, Denmark) and horseradish peroxidase (HRP)–peanut agglutinin (PNA) (EY Laboratories, San Mateo, CA); (ii) biotin–PNA (EY Laboratories), branched streptavidin–alkaline phosphatase and HRP–(Fab′)2 fragment of donkey anti-mouse IgM (Jackson ImmunoResearch); or (iii) anti-B220 ascites (clone RA3-6B2), streptavidin–alkaline phosphatase-conjugated (Fab′)2 fragment mouse anti-rat Ig (Jackson ImmunoResearch) and HRP–anti-CD4 (clone GK1.5 coupled to HRP using standard methods).

Hybridomas
Hybridomas were generated from lipopolysaccharide (LPS)- and dextran sulfate-stimulated spleens cells from naive Ars20 line mice (10 weeks of age) as previously described (31). Twenty randomly chosen wells from each fusion were subcloned by limiting dilution. Supernatants from the subclones were subjected to quantitative Ars, E4, 107, anti-mouse κ, and single- and double-stranded DNA ELISAs as well as antinuclear antibody assays as previously described (31). Prior to addition to the ELISA plates, samples were treated with 100 mM β-mercaptoethanol for 30 min at room temperature to reduce IgM to a monomeric form, thus reducing the non-specific background in the assays (32). In the evaluation of Ars and DNA binding activity, the 36-65 and 3H9 mAb were used respectively as ELISA standards. Values obtained in Ars, and single- and double-stranded DNA binding assays were normalized to those obtained in anti-κ assays. Only those mAb displaying normalized binding values >10% produced by the positive control mAb standard were considered ‘positive’.

Vk amplification and sequencing
Total RNA was prepared from 107 + hybridomas as previously described (33). RNA was used for RT-PCR using the S′ primer univ 5LK which has been shown to amplify all known Vk genes (34) and the 3′ primer SalI CK 5′-GCAGCTGTGGA-TGGTGGGAAG-3′. Since the hybridoma fusion partner, Sp2/0, expresses a sterile Vk mRNA (35), we modified the RT-PCR protocol as follows: after reverse transcription, only five cycles of PCR were done followed by digestion of the products with the restriction enzyme Tatf (MBI Fermentas, Amherst, NY) per the manufacturer’s instructions. Tatf cleaves the PCR product generated from the Sp2/0 fusion partner into small fragments that cannot be amplified in subsequent PCR reactions. Of all known mouse Vk gene segments, the Tatf restriction site is found in central regions of only 20 out of 109 segments (36). After a 1 h digestion with Tatf, samples were ethanol precipitated. Precipitated products were pelleted, and washed twice with 70% ethanol. Pellets were resuspended in PCR buffer containing univ 5LK and SalI CK primers (see above) and 30 cycles of PCR were performed. PCR products were directly sequenced using an internal Cκ primer.

In vitro proliferation assays
B cells were purified from the spleens of mice as previously described (37). Cells were plated in 96-well dishes at 1–2×106 cells/ml and stimulated with LPS (Difco; Detroit, MI), goat anti-mouse IgM(Fab′)2 (Pierce, Rockford, IL) or anti-mouse CD40 (clone FGK45, a gift of Dr Anton Rolink) as previously described (37). After 48 h in culture, cells were pulsed for 12–16 h with [3H]thymidine (New England Nuclear Life Sciences, Boston, MA), harvested and 3H incorporation evaluated.

Serology and ELISAspot assays
ELISA assays were performed as previously described (31). ELISAspot assays were performed using Multiscreen 96-well plates (Millipore, Bedford, MA). Wells were coated overnight at 4°C with 10 µg/ml of goat anti-mouse Ig or Ars–BSA. After extensive washing the plates were blocked with 10% FCS/RPMI 1640 and dilutions of cells then added in the same medium. After incubation at 37°C for 6 h, the plates were washed with PBS/0.5% Tween 20 and dilutions of biotinylated 107, E4 or rat anti-mouse κ mAb were then added in PBS/1% BSA. After incubation overnight at 4°C, the plates were washed with TBS/0.5% Tween 20 and dilutions of streptavidin–alkaline phosphatase were added in TBS. After incubation for 2 h at room temperature, the plates were washed with TBS/0.5% Tween 20 and the spots developed by the addition of Fastblue BB. After washing and drying the spots were scored at ×5–10 magnification using a stereomicroscope.

Cell cycle and BrdU-labeling analyses
Splenic B cells were isolated from mice as described above. Ethanol-fixed and RNase-treated cells were stained with propidium iodide (PI) immediately after isolation, and 24, 48 and 72 h after culture with LPS (25 µg/ml), anti-mouse IgM [F(ab′)2, 10 µg/ml]. anti-CD40 (FGK45, 10 µg/ml) or media alone as previously described (37), and analyzed using a Coulter Epics Profile II and Elite software.

Ars20 transgene-positive and control littermates were injected i.p. with 0.6 mg BrdU (Sigma) in 0.2 ml PBS at 12-h intervals for 11 days. Three mice from each group, plus an unlabeled mouse from each group, were sacrificed on days 4, 8 and 11. The cells obtained from each mouse were stained for surface markers as described above and washed in cold PBS. Incorporated BrdU was analyzed by flow cytometry according to Allman et al. (38).

Adoptive transfer experiments
Donor B cells were isolated from the pooled spleens of two to four Ars20 transgene-positive mice as described above. Following a modified protocol of Garside et al. (39), 2.5–3.0×106 Ars20 splenic B cells were injected i.v. into one uninjured (A/J×FVB/N) F1 mouse. The day prior to administration of the cells, recipient mice were bled to obtain background serum antibody levels. The day after cells were administered, some mice were injected i.p. with 100 µg Ars–keyhole limpet hemocyanin (KLH) in either complete Freund’s adjuvant as an alum precipitate. For serological analysis, these
Additional heterogeneity of the B compartment

mouse were bled at 1-week intervals. For immunohistochemistry analysis, these mice were bled at day 8 or 12 after initial immunization and their spleens frozen for cryosectioning. Unimmunized mice were sacrificed at various times after cell transfer (up to 3 weeks) and their spleens frozen for histology.

**Results**

Lines of transgenic mice were generated using the µδ and HS1234 constructs shown in Fig. 1(A) (see Methods for details). The transgene constant regions are Igha allotype and the transgene(s) were back crossed to A/J mice (Igh e allotype). In adult mice in which both endogenous IgH loci were of A/J origin, transgene expression and allelic exclusion were evaluated using IgMa- and IgDe-specific mAb. As shown in Fig. 1(B), IgDδ–, IgMδ– spleen cells were absent in a mouse of the Ars20 transgenic line, but a large population of IgMδ+ cells was detected only a very minor fraction (<2%) of which were also weakly IgDδ+, indicating extremely good transgene exclusion of endogenous IgH expression. Analogous results were obtained using several independent lines of mice and other lymphoid organs (data not shown).

The frequency of B cells expressing canonical BCR is substantially increased in transgenic mice

The anti-idiotypic mAb 107 is specific for a subset of canonical antibodies, including the 36-65 (transgene heavy chain donor) mAb, containing rare V-D and D-J junctional amino acids (40,41). Figure 2 (upper panels) demonstrates that the vast majority of Ars20 B220+ cells are 107+. The few 107- cells presumably express light chains that preclude idiotope expression. Similar results were obtained from mice of other transgenic lines. In subsequent analyses we focused on the Ars20 line, in which uniform genetic transmission of both transgene constructs was always observed, suggesting they had co-integrated. A second anti-idiotypic antibody, E4, which is rather specific for V regions composed of the canonical heavy and light chain combination (42), stained subpopulations of splenic and lymph node Ars20 B cells (Fig. 2, bottom panels). Such B cells are extremely rare in A/J mice prior to Ars immunization.

Serum antibody levels in Ars20 mice

Total serum Ig levels were found to be ~75% lower in naive Ars20 mice as compared to non-transgenic littermates. More strikingly, these mice had very low (<10 µg/ml) serum titers of 107+ antibodies and undetectable levels of E4+ antibodies (data not shown). These results stood in marked contrast to the data presented in Fig. 2, showing that most splenic and lymph node B cells express 107+ BCR and a subpopulation of these also expresses E4+ BCR.

Peripheral B and T cell compartments in Ars20 mice

Quantitative flow cytometric analysis of B and T cell populations demonstrated a somewhat reduced number of total B (B220+ or IgM+) cells in the spleen (average 35% reduction) and a more substantial reduction in the lymph nodes (>50% reduction) in Ars20 mice compared to non-transgenic littermates. Visual examination of Peyer’s patches revealed on average only two or three Peyer’s patches per Ars20 mouse, as compared to 8–10 in control littermates. Analysis of the T cell compartment in spleen and lymph nodes with a variety of mAb specific for activation and differentiation revealed no numerical or phenotypic abnormalities (data not shown).

The majority of splenic Ars20 B cells do not have a follicular (B2) phenotype

In the spleen and lymph nodes of adult Ars20 mice, the mature, resting B cell population was substantially reduced. When Percoll gradients were done on either whole or T-depleted splenocytes, >30% of B cells in naive A/J mice or non-transgenic littermates pelleted through a ρ = 1.079 density layer (i.e. small, resting B cells). However, on average 3-fold fewer splenic B cells from Ars20 mice located to this position in the gradients, with most banding at the interface of the ρ = 1.079 and 1.066 layers. Flow cytometry also revealed a deficiency of splenic B cells with low forward and side light scatter values in Ars20 mice (data not shown).
Additional heterogeneity of the B compartment

**Fig. 3.** Cell-surface phenotype of Ars20 transgenic B cells. Spleen, lymph node and peritoneal cells from three Ars20 mice and three littermates were isolated, pooled, stained with various combinations of mAb fluorescent conjugates and analyzed by flow cytometry, all as described in Methods. In these analyses data obtained from the live lymphocyte gate of littermate cells was compared with data obtained from both the live lymphocyte and either $10^7$/H11001 or E4$/H11001$ gates of Ars20 cells. In each panel the intersection of the cross-hairs marks the center of the distribution of the major stained population of cells in the corresponding littermate samples, to allow better visualization of the differences or similarities observed in the Ars20 samples. There were too few E4$^+$ cells in the lymph node and peritoneal samples for accurate gating and so these data are not shown.

Figure 3 shows that the majority of Ars20 splenic B cells express elevated levels of slgM and slgD characteristic of a subpopulation of splenic B cells present in littermates. Moreover, major subpopulations of either $10^7^+$ or E4$^+$ cells in the spleen express elevated levels of CD21/35, CD24 and CD1d, but low levels of CD23, also characteristic of subpopulations present in littermates. In littermates, CD21/35$^{high}$CD1d$^{high}$CD23$^{low}$ slgM$^{high}$CD21/35$^{high}$ and CD24$^{high}$ subpopulations account for 10–15% of splenic B cells. In contrast, among both $10^7^+$ and E4$^+$ B cells in adult Ars20 mice these subpopulations make up 50–75%. In addition, Ars20 splenic B cells in general express elevated levels of CD21/35, CD1d and CD24, and reduced levels of CD23, as compared to the major subpopulation of littermate splenic B cells. Ars20 splenic B cells expressing $10^7^+$ or E4$^+$ BCR are also deficient in a subpopulation of CD62L (L-selectin), and generally increased levels of MHC class II and CD40 on Ars20 splenic B cells (data not shown). While levels of CD69, CD86 (B7-2), CD43 (S7), CD95 (Fas) and B220 also appeared to be slightly elevated on these cells; this may result from their increased size.

In contrast to splenic B cells, Fig. 3 shows that $10^7^+$ cells present in the lymph nodes of Ars20 mice display a phenotype consistent with mature follicular cells, in that the majority are slgM$^{low}$CD21/35$^{low}$CD24$^{low}$CD1d$^{int}$CD23$^{high}$ (the levels of slgD on these cells is also similar to littermate lymph node B cells, data not shown). In the peritoneum, Ars20 $10^7^+$ B cells reveal staining profiles consistent with the presence of mainly slgM$^{high}$ cells. However, these cells are deficient in CD21/35$^{low}$CD23$^{low}$ cells (Fig. 3), indicating a deficiency in conventional B1 cells as was observed in the spleen. In addition, while $10^7^+$ cells are abundant in the peritoneum and clearly present in the lymph nodes, E4$^+$ cells are rare in both these locales.

**In vivo** life span and cell cycle activity of Ars20 splenic B cells

Neither the IgD$^+$ or $10^7^+$ splenic compartments, or the E4$^+$ subpopulation, appeared to have substantially different intermediate-term *in vivo* BrdU labeling rates as compared to the IgD$^+$ splenic compartment in transgene-negative littermates (Fig. 4A). PI cell cycle analyses revealed that the
of this mitogen. In contrast, Ars20 splenic B cells proliferated to comparable extents as compared to control B cells in response to agonistic anti-CD40 treatment (Fig. 5B) and were hypo-responsive to stimulation via cross-linking of their BCR with anti-μ (Fig. 5C). These data were corroborated using PI cell cycle analyses (data not shown).

ELISAspot analyses of secretion of anti-Ars, 107+ and E4+ antibodies were also performed on Ars20 and control A/J splenic B cell cultures stimulated for 3–5 days with LPS. An ~5-fold increase in Ars+ ELISAspots was obtained from Ars20 B cells as compared to controls. The frequency of E4+ ELISAspots was also increased (to ~0.4% of input B cells) among Ars20 B cells. 107+ antibody forming cells (AFC) were undetectable in littermate cultures, but were detected at a frequency of ~1–2% of total input B cells in Ars20 cultures. However, these frequencies of AFC obtained from transgenic B cells were extremely low given the precursor frequencies indicated in Fig. 2 and the average size of the spots the Ars20 AFC produced was small (data not shown).

**Ars20 transgenic B cells home to the follicles of the spleen**

Figure 6 shows the results of immunohistochemical staining of three parallel spleen sections from a representative naive Ars20 mouse and a non-transgenic littermate. In the transgenic sections, 107 stained the vast majority of cells in the follicles and MZ. Staining of parallel sections with B cell (anti-B220, blue)- and T cell (anti-CD4, red)-specific reagents showed that the overall lymphoid microenvironmental organization of the spleen was normal in the Ars20 mouse (Fig. 6, bottom panels). B cell location was also assessed using anti-IgM (red staining; Fig. 6, middle panels). The overall anti-IgM staining pattern in the Ars20 spleen is analogous to the staining pattern seen in the non-transgenic mouse. Staining of parallel sections with E4 revealed that canonical BCR-expressing B cells were present throughout the follicles and MZ. Identical results were obtained for several other Ars20 mice (data not shown).

**Ars20 splenic B cells efficiently participate in germinal center reactions**

Small numbers of Ars20 B cells were injected into syngeneic, unirradiated A/J×FVB/nJ mice, followed by immunization of the chimeric mice with Ars–KLH. At various times thereafter mice were bled and spleens taken for histological analysis. Figure 7(C) shows that, as expected from the in vitro studies presented above, only low levels of 107+ serum antibodies were produced by the chimeric mice and only at late stages of the primary response. In contrast, the immunohistochemistry analysis revealed abundant germinal centers that stained with 107 or both 107 and E4 at both days 8 and 12 after immunization (a day 12 example is shown in Fig. 7A and B). At neither of these time points were substantial numbers of 107+ or E4+ cells observed outside of germinal centers. If chimeric mice generated in this way were not immunized with Ars–KLH, 107+ and E4+ cells could be observed throughout the MZ and follicular regions of the white pulp, and the number of these cells remained stable for 3 weeks after transfer (the latest time point examined, data not shown).
A large percentage of Ars20 B cells have multireactive BCR

Hybridomas were generated from the LPS and dextran sulfate
stimulated spleen cells of two Ars20 mice. Of 28 randomly
selected 107+ hybridomas, nine produced anti-Ars anti-
bodies. All of these also bound single- and double-stranded
DNA. In total, 19 of the hybridomas produced mAb that bound
single-stranded DNA, double-stranded DNA or both. Most of
the Ars-binding mAb bound Ars less well than did the 36-65
mAb, and most of the DNA-binding mAb bound this ligand
less well than the 3H9 mAb (43), attesting to the low avidity
of these binding reactions. Four of the mAb (two with unknown
antigen specificities, one with a specificity for single- and
double-stranded DNA, and one with a specificity for Ars, and
single- and double-stranded DNA) were analyzed in an ANA
assay. All of the mAb uniformly stained the cytoplasm and
also stained nuclei in a punctate pattern (data not shown).

Analysis of Vκ family usage in the hybridomas demonstrated
that all hybridomas analyzed appeared to co-express single
light chain genes with the transgene encoded heavy chain.
These genes were members of seven of the 18 known Vκ
families. The predominant Vκ family was the Vκ9A/B family,
found to be expressed in one-third of the hybridomas ana-
lyzed, and there was a predominance of Jκ5 gene segment
usage by all hybridomas. However, the expression of a
particular Vκ or Vκ-Jκ did not correlate with a particular
antibody specificity.

Discussion

Most splenic Ars20 B cells, including canonical clonotypes,
are functionally and phenotypically distinct from mature follic-
ular (B2) B cells and B1 cells. Comparison of the data we
have obtained with previously published data on splenic B
cells subsets (20,44–47) indicates that Ars20 splenic B cells
bear the strongest resemblance to MZ B cells. They are
slgM high CD21/35 high CD23 low and express high levels of the
CD1d non-classical MHC antigen. They also display increased
size, appear to be slowly cycling and have a heightened
response to LPS but are hyporesponsive to anti-IgM. In
addition, Ars20 B cells are in general inefficient at seeding the
lymph nodes and Peyer’s patches. However, they efficiently
colonize both the splenic follicles and MZ, properties reminis-
cent of the 'T2' transitional population recently defined by
Carsetti et al. (44). Indeed, the in vitro response characteristics
of these cells might well be explained by developmental 'immaturity'.

Nonetheless, Ars20 splenic B cells have unique character-
istics as well. They do not produce high levels of secreted
antibodies, either spontaneously, or after in vitro or in vivo
stimulation in contrast to MZ B cells (20). In addition, elevated
levels of slgD is a characteristic of most Ars20 B cells. This
is not a general property of T2, MZ or B1 B cells (20,44).

Fig. 5. In vitro proliferative responses of Ars20 splenic B cells. Splenic
B cells were purified from three Ars20 mice and three littermates or
three A/J mice. Pooled cells from each type of mouse were then
stimulated with the indicated concentrations of (A) LPS, (B) agonistic
anti-CD40 mAb or (C) goat anti-mouse IgM F(ab')2 fragments in vitro
for 48 h, pulsed with [3H]thymidine for 12–18 h and [3H] incorporated
into DNA measured, all as described in Methods. Ars20 splenic B
cells were consistently hyperproliferative to high concentrations of
LPS as compared to either littermate or A/J splenic B cells, but
depending on the batch of LPS used they sometimes displayed
similar proliferation levels to low (<1 µg/ml) concentrations of this
mitogen. (A) The results of one experiment, where A/J splenic B cells
were used as a control, in which hyperproliferation was observed at
both high and low concentrations of LPS. In this experiment, fewer
c.p.m. were incorporated into DNA than in the anti-IgM and anti-
CD40 experiments since less [3H]thymidine had been added to the
cultures.
While CD1d\textsuperscript{high} follicular B cells express high levels of slgD, they are slgM\textsuperscript{low}CD23\textsuperscript{high} (45,46). These elevated levels of IgD do not appear to result from the fact that the Ars20 transgene is present in multiple copies (four or five) since B cells in the Ars30 line, which carry only one copy of the \(\mu\delta\) transgene, display this same property (L. Heltemes and T. Manser, unpublished observations). Also, the B cells present in the lymph nodes of Ars20 mice express normal levels of slgD and the levels of slgD expression on most Ars20 splenic B cells is similar to a subpopulation of splenic B cells observed in littermates. Since most Ars20 splenic B cells have a decreased buoyant density, appear to be slowly cycling and express levels of other markers consistent with a ‘partially activated’ phenotype, we favor the interpretation that the elevated levels of slgD result from ongoing, low-avidity engagement of self-ligands. These elevated slgD levels may also inhibit tolerance induction, as has previously been suggested to be the case for follicular B cells (48).

Ars20 B cells also display intriguing phenotypic parallels with B cells present in a \(V_{H}81X\) Ig heavy chain transgenic line of mice previously characterized by Kearney \textit{et al.} (49).
and a line of V\(_H\) ‘knockin’ mice expressing the T15 V\(_H\) gene generated by Rajewsky et al. (50). The V\(_{\mu}B1\) transgenic B cells have specificities for multiple intracellular autoantigens and display a phenotype consistent with self-antigen-mediated activation (51). Interestingly, the most autoreactive subpopulation of these B cells preferentially resides in the splenic MZ; and has a morphologic and cell-surface phenotype similar to that of MZ B cells in normal mice (13). The V\(_{\mu}T15\) ‘knockin’ B cells display some of the phenotypic qualities of ‘T2’ B cells (50), and the T15 V\(_H\) gene confers specificity for the common self-ligand phosphatidylcholine. The in vitro reactivity of many of the Ars20 mAb we isolated makes intracellular nucleic acid-rich components one of the likely candidates for a group of self-ligands that might influence the developmental fate of many Ars20 B cells in vivo.

When small numbers of Ars20 splenic B cells were transferred to non-irradiated, syngeneic recipients, immunization with Ars-KLH resulted in the widespread participation of the 107\(^+\), and 107\(^{*}E4^+\) (canonical) subpopulations in the germinal center response, but not in the AFC response. This indicates that Ars-specific Ars20 B cells, including canonical clonotypes, are efficient precursors to the memory B cell pathway. We have previously shown that canonical clonotypes do not give rise to a periarteriolar lymphoid sheath, or red pulp AFC ‘focus’ response, but are first observed in germinal centers during the anti-Ars response of AJ mice (30). Differentiation to AFC phenotype appears to require substantially higher levels and probably qualitatively distinct types of T cell ‘help’ as compared to differentiation via the germinal center pathway to memory phenotype (52,53). The self-reactivity of most Ars20 B cells may shunt them into the germinal center/memory B cell pathway, by inducing a state of differentiation that precludes the receipt of the T cell help requisite for development into antibody-secreting cells. Once in the germinal center microenvironment, BCR somatic hypermutation and phenotypic selection processes may result in such clonotypes losing their autoreactivity, and gaining increased affinity for Ars. We have previously argued that this is how canonical clonotypes come to be the predominant contributors to the anti-Ars anamnestic serum antibody response (31).

Collectively, our data are consistent with the idea that the quality and quantity of BCR self-ligand engagement dramatically influence primary B cell developmental fate in the periphery (11,54). Since BCR cannot be monospecific and, in fact, the primary B cell compartment is characterized by the expression of multireactive BCR (16,55,56), it is likely that many stable and functional members of this compartment are subjected to such self-antigen-mediated ‘regulation’. Given the enormous diversity of the preimmune antibody repertoire, this reasoning suggests that it may be more appropriate to view this compartment as a continuum of functional and phenotypic ‘layers’, rather than as a group of discrete B1, B2 and MZ subsets.

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Abbreviations

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<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AFC</td>
<td>Antibody forming cells</td>
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<tr>
<td>Ars</td>
<td>p-azophenylarsonate</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MZ</td>
<td>marginal zone</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
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<tr>
<td>PNA</td>
<td>peanut agglutinin</td>
</tr>
</tbody>
</table>

References

Additional heterogeneity of the B compartment


54 Carsetti, R. 2000. The development of B cells in the bone marrow is controlled by the balance between cell-autonomous mechanisms and signals from the microenvironment. J. Exp. Med. 191:5.
