Major Histocompatibility Complex Class II Expression Distinguishes Two Distinct B Cell Developmental Pathways During Ontogeny

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Summary

All mature B cells coexpress major histocompatibility complex (MHC) class II molecules, I-A and I-E, which are restriction elements required for antigen presentation to $CD4^+$ T cells. However, the expression of class II during the early stages of B cell development has been unclear. We demonstrate here that there is a difference in the expression of class II during murine B cell development in the fetal liver and adult bone marrow (BM). These differences define two distinct B cell developmental pathways. The Fetal-type (FT) pathway is characterized by pre-B and immature IgM^+ B cells generated in the fetal liver which initially lack all class II expression. In contrast, the Adult-type (AT) pathway is typified by B cells developing in the adult BM which express class II molecules from the pre-B cell stage. In vitro stromal cell cultures of sorted fetal liver and adult BM pro-B cells indicated that the difference in I-A expression during B cell development is intrinsic to the progenitors. In addition, we show that FT B cell development is not restricted to the fetal liver but occurs in the peritoneal cavities, spleens, liver, and BM of young mice up to at least 1 mo of age. The AT B cell development begins to emerge after birth but is, however, restricted to the BM environment. These findings indicate that there are two distinct B cell developmental pathways during ontogeny, each of which could contribute differentially to the immune repertoire and thus the functions of B cell subsets and lineages.

B cell lymphopoiesis is first seen in the fetal liver and eventually shifts to the bone marrow (BM)[1] in the adult (1). In these sites, B cell development occurs in sequential stages, starting from pro-B and differentiating into pre-B and B cells (2). Cells at different stages of maturation can be defined by their cell surface phenotypes, differential gene expressions, and status of Ig gene rearrangements (3). For example, pro-B cells are $B220^{lo}CD43^{hi}$, express the recombination activating genes, RAG-1 and RAG-2, and undergo D-J rearrangement at the Ig H chain genes loci. Pre-B cells are $B220^{int}$ $CD43^{lo}$ and have cytoplasmic μ proteins, and finally, B cells express cell surface Ig (sIgM) and downregulate both the RAG genes expressions (2–4).

There are known differences between fetal liver and adult BM B lymphopoiesis. It has been shown that the enzyme terminal deoxynucleotidyl transferase (TdT) (5) and the precursor lymphocyte–regulated myosin-like L chain (PLRLC) (6) are expressed in pro-B cells in the BM but not in the fetal liver (4). The lack of TdT expression results in a lack of N region additions in the fetal mouse Ig V-D-J junctional sequences as compared with those derived from the adult BM (7, 8). More significantly, recent studies (9, 10) show that fetal liver but not adult BM pro-B cells readily reconstitute $CD5^+$ B cells, strongly suggesting that there are intrinsic developmental differences in the B cell progenitors obtained at distinct time points during ontogeny. However, to date, no phenotypic cell surface markers have been identified which can distinguish between fetal liver and adult BM–derived progenitor cells. Similarly, no phenotypic differences have been observed in the pro- or pre-B cells derived from fetal or adult B cell progenitors. The lack of such distinction complicates a detailed analysis of the role each developmental pathway plays in the derivation and functions of B cell subsets and lineages and their contributions to the immune repertoire. In addition, it is not known when a developmental switch occurs between the fetal and the adult B cell differentiation pathways and to what extent they overlap in ontogenetic timings.

All mature B cells constitutively express MHC class II molecules on their cell surfaces (11, 12). The class II molecules are heterodimeric glycoproteins (13) consisting of an α and β chain which are encoded within the I-A and I-E subregions of the MHC (11). Appropriate expression of these class II molecules is required for proper presentation of peptide an-

[1] *Abbreviations used in this paper:* APC, allophycocyanin; AT, adult type; bi, biotin; BM, bone marrow; Fl, fluorescein; FT, fetal type; PerC, peritoneal cavity.

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tigens to recognition by $CD4^+$ T cells (14). To date, studies indicate that the expression of the class II genes is coordinate (12, 15, 16).

Early studies on the timing of class II expression during B cell development have yielded contradictory results. Studies by Lala et al. (15) and Mond et al. (16) indicated that B cells acquire class II and sIgM concurrently during development. On the other hand, Kearney et al. (17) using immunofluorescence microscopy showed that there was a population of $sIgM^+$ cells in neonatal spleens that lack class II expression. Hammerling et al. (18) also reported the presence of class II^- $sIgM^+$ cells and suggested that they are newly generated B cells. Using long-term BM cultures that presumably mimic normal B cell development in vivo, Dasch and Jones (19) also suggested that $sIgM^+$ cells that lack class II molecules on their cell surfaces were immature B cells. These earlier works contrasted with recent studies by Miki et al. (20) which provided evidence that class II molecules may be expressed at the pre-B cell stage. This group showed that BM pre-B cells treated with anti-class II mAb or obtained from mice transgenic for an antisense I-A construct have diminished B cell development in vitro. These studies, however did not demonstrate directly the stage of B cell development at which I-A is first expressed. Recently, Tarlinton (21) showed, by flow cytometry, that class II molecules were expressed at the pre-B cell stage during B cell development in the BM. However, the report still did not resolve the differences reported earlier on the presence of class II^- IgM^+ cells. All the above studies of class II expression have assumed that B cells follow the same developmental pathway throughout ontogeny. Given the known differences between fetal liver and adult BM B cells and that $sIgM^+$ cells lacking class II had been reported in neonatal spleens (17, 18), we compared class II expression during fetal liver and adult BM B lymphopoiesis.

We find that there is a difference in class II expression during B cell development in the fetal liver and BM. In the fetal liver, pre-B cells and newly generated $sIgM^+$ cells do not express any class II molecules, I-A or I-E. In contrast, class II molecules are found to be expressed on pre-B cells in the adult BM. Using I-A expression on pre-B cells and immature $sIgM^+$ cells as a distinguishing marker between adult BM and fetal liver B cell developmental pathways, we further show that Fetal-type (FT) B cell developmental pathway occurs in many neonatal lymphoid organs. In contrast, the Adult-type (AT) B cell developmental pathway is largely confined to the BM environment. Our results suggest the presence of two distinct B cell developmental pathways during ontogeny. This finding has great implications for the generation of the immune repertoire and also to the derivation of B cell lineages and subsets.

Materials and Methods

Animals. BALB/c, BAB/25, C57BL/6, and CBA/Ca mice were obtained from our animal facility. All adult mice studied were 2–3-mo-old. For timed pregnancies, females were housed in separate cages from the males until mating. The appearance of a vaginal plug after overnight mating was labeled as day 1 of gestation. Age of neonates was given with respect to the day of birth.

Antibodies. The following rat anti-mouse mAbs: anti-IgM (331.12), anti-B220 (RA3-6B2), and anti-CD43 (S7) were produced and conjugated to biotin (bi) or fluorochromes (fluorescein [Fl]) as described (10, 22, 23). The mouse mAbs: anti-I-A^d (AMS 32.1), anti-I-E (14-4-4s), anti-I-A^b (AF6-120.1), anti-I-A^k (11-5.2), and anti-H-$2K^d$ (SF1-1.1) were obtained from PharMingen (San Diego, CA).

Cell Preparations. Single cell suspensions of BM were prepared by injecting staining medium (deficient RPMI [Irvine Scientific, Santa Ana, CA] containing 10 mM Hepes, 3% FCS, and 0.1% NaN_3) into femurs and tibia using a 1-ml syringe and 26-gauge needle. Peritoneal cells were obtained by injecting staining medium into the peritoneal cavities (PerCs) of neonates and extracting with Pasteur pipettes. Fetal liver and spleen cells were prepared by dissociation between frosted glass slides. All cells were treated with RBC lysing solution (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, and 0.1 mM Na_2 EDTA) to eliminate erythrocytes and resuspended at 2.5×10^7 cells/ml in staining medium (24).

Staining, Flow Cytometry, and Cell Sorting. Cells were incubated on ice with optimal amounts of Fl, PE, allophycocyanin [APC]-conjugated or biotin (bi)-conjugated Abs for 15 min and washed three times with staining medium. Bi-conjugated Abs were revealed with Texas red-avidin. Flow cytometric analyses and cell sorting were conducted using a modified dual laser/dye laser FACStar$^{Plus®}$ (Becton Dickinson & Co., San Jose, CA) equipped with appropriate filters for seven parameter analyses. Samples were kept on ice during sorting. Reanalysis of sorted populations consistently showed purity in excess of 95%. Analyses of cell samples always included propidium iodide (1 μg/ml) for dead cell exclusion. Data were analyzed using FACS/DESK (Stanford University, Stanford, CA) and unless otherwise noted, are presented as 5% probability contour plots.

Culture Conditions. BM stromal layers were established (25) by seeding 3×10^5 BAB/25 BM cells per well of a 24-well tissue culture plate 3 wk before the initiation of cultures. The cultures were fed twice weekly in culture medium (RPMI-1640 supplemented with 5×10^{-5} M 2-ME, 5% FCS, and penicillin/streptomycin) and kept in a humidified 5% CO_2 incubator at 37°C. Only wells with no nonadherent cells were used in the experiments. The FLST2 stromal cell line used was also maintained in standard medium and passaged by treatment with trypsin-EDTA (26). All experimental cultures with stromal layer were performed in 24-well plates with 1 ml culture medium. Typically, sorted cells were seeded at 30,000 cells/well on the stromal layer and cultured for 4 d. The cultures were fed every other day by aspirating off half the medium and refeeding with an equal volume of fresh medium. In certain sets of experiments, supernatant from Con A–stimulated splenic T cells was added to 10% of the volume at the time of initiation of cultures. After cultures, cells were recovered and stained for B220, IgM, I-A, and I-E expression.

Results

Class II Expressions on BM B Cell Populations. B lineage cells in the adult BM can be resolved into different stages of maturation based on their differential expression of B220 and CD43 (leukosialin) (3). In Fig. 1 *A*, flow cytometric analysis of an adult BALB/c BM identifies three populations: pro-B cells ($B220^{lo}$ $CD43^{hi}$); pre-B and immature B cells ($B220^{int}$

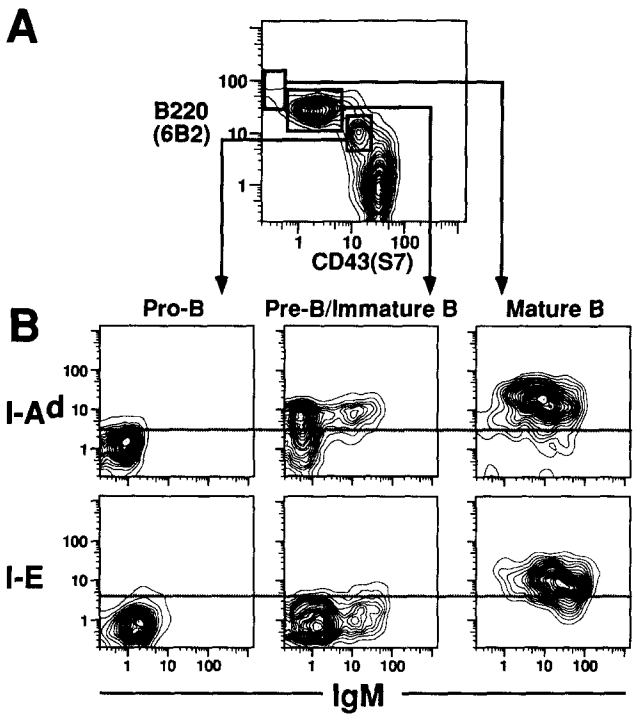

Figure 1. I-A is expressed on pre-B and immature sIgM$^+$ cells in adult BALB/c BM. BM cells from a 2.5-mo-old BALB/c mouse were stained with ^{APC}anti-B220/6B2, ^{Fl}anti-IgM, ^{PE}anti-CD43, and ^{bi}anti-I-A^d (AMS 32.1) or ^{bi}anti-I-E (14-4-4s). (*A*) The boxed areas delineate the different stages of B cell maturation resolved by B220 and CD43/S7 phenotypes as defined by Hardy et al. (3). Pro-B cells are B220^{lo} CD43^{hi}, pre-B/immature B cells are B220^{int} CD43^{lo}, and mature B cells are B220^{hi}CD43$^-$ and represented 6, 23, and 3% of the total BM cells, respectively. The use of a brighter ^{PE}anti-CD43 reagent reveals a low level of CD43 expression on pre-B and immature B cells not seen in previous reports (3). (*B*) Shown are the IgM, and class II, I-A and I-E expressions of the gated populations. The horizontal lines designate the cutoff for positive and negative cells. Data shown are representative of numerous separate analyses (n >10).

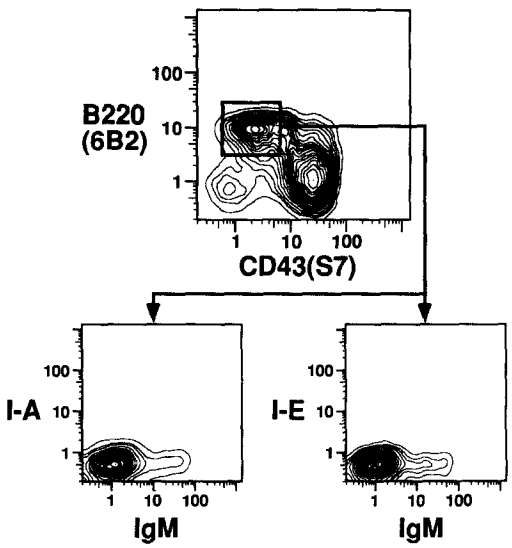

Figure 2. Fetal liver-derived pre-B and immature sIgM$^+$ B cells initially do not express class II antigens. Fetal liver cells from a day 19 BALB/c fetus were stained as described in Fig. 1. (*Top*) Contour plot of B220 vs. CD43 expression. (*Bottom*) Class II expression on pre-B and immature IgM$^+$ B cells (B220$^+$, CD43^{dull}; gated population). (*Boxed area*) Approximately 33% of the total cells in the day 19 fetal liver. Data shown were representative of several (n = 5) independent analyses in BALB/c mice and were reproducible in various other strains.

CD43^{lo}), and mature B cells (B220^{hi} CD43$^-$). Mature B cells are further distinguished from immature B cells by their higher expression level of IgD and CD23 (data not shown). Fig. 1 *B* shows the expression of IgM, I-A and I-E for each of these populations. Pro-B cells (*left*) do not express any sIgM nor class II antigens on their cell surfaces. The class II molecule, I-A is first expressed on pre-B cells (*middle*). In 3 mo adult BM, ~30% of the pre-B cells are I-A$^-$ and 70% are I-A$^+$. Thus the expression of I-A subdivides the pre-B cell compartment. All sIgM$^+$ B cells in the BM express I-A on their cell surfaces. As seen in the middle and right panels of Fig. 1, immature B cells express a lower level of I-A (intensity = 7) compared with mature B cells (intensity = 20). These observations confirm recent data by Tarlinton (21) which showed that class II is first expressed during the pre-B cell stage in the BM.

The I-A$^-$ pre-B cells in the adult BM represent an earlier stage of maturation than the I-A$^+$ pre-B. When sorted I-A$^-$ pre-B cells were cultured for 2 d, only 10% of the B cells in culture remained as I-A$^-$ pre-B and ~67 and 23% became I-A$^+$ pre-B and I-A$^+$ IgM$^+$ B cells, respectively (data not shown). Similar culturing of I-A$^+$ pre-B cells yielded only I-A$^+$ pre-B and I-A$^+$ IgM$^+$ B cells, (44 and 56%, respectively). Thus the data supported a sequential order of maturation of pre-B cells from a I-A$^-$ to a I-A$^+$ stage and finally to I-A$^+$ IgM$^+$ B cells.

The Tarlinton study used a rat anti-mouse class II Ab that could not distinguish between I-A or I-E. Using individual anti-I-A mAbs, we found similar patterns of I-A expression on adult BM B cells from CBA/Ca and C57BL/6 mice (data not shown). However, we now report differences in cell surface expression of I-E during BM B cell development in different mice strains. In BALB/c mice, I-E is not detected on the cell surface of pre-B or newly generated immature B cells, but rather is first expressed on mature B cells (Fig. 1 *B*). In contrast, in the adult BM of CBA/Ca mice, I-E, like I-A, is first expressed on pre-B cells (data not shown). The reason for this difference in I-E expression between the two strains of mice is not known. The anti-I-E Ab used was made against Eα of the k haplotype but cross-reacts with Eα of the d, p, and r haplotypes (27). It is unlikely that the difference in I-E staining on BM pre-B and immature B cells is due to the preferential staining of one haplotype with the anti-I-E Ab since the Ab detects equivalent levels of I-E antigens on splenic IgM$^+$ B cells in BALB/c and CBA/Ca mice (data not shown).

Fetal Liver Pre-B and Immature B Cells Do Not Express Class II Molecules. In the fetal liver, B cell maturation occurs in a single wave (28) with pro-B cells emerging at day 14 of

gestation, and sIgM$^+$ B cells first detectable at day 18 or 19 of gestation. Fig. 2 shows a flow cytometric profile of a day 19 fetal liver stained with anti-B220, anti-CD43, anti-IgM, and anti-I-A or anti-I-E. Unlike the profiles in the adult BM, distinct populations of B cells in various stages of maturation are not well resolved in the fetal liver. However, most of the B220$^+$ cells at this time point are stromal independent for growth (28) and are thus beyond the pro-B cell stage of differentiation. Cells within the gated B220$^+$ CD43$^+$ fraction are lymphoid in morphology and in addition, more than 80% of the sorted B220$^+$ Ig$^-$ cells stained for cytoplasmic μ (data not shown) and are thus pre-B cells.

With respect to class II, a totally different pattern of expression is seen during fetal B lymphopoiesis. Both Ig$^-$ pre-B and Ig$^+$ immature B cells do not express I-A or I-E (Fig. 2). This is in striking contrast to the situation in the adult BM where I-A can be detected on the cell surface of pre-B and immature B cells (Fig. 1). This lack of class II expression during fetal liver B lymphopoiesis is not strain dependent since it is also seen on C57BL/6 and CBA/Ca mice (data not shown).

The Difference in I-A Expression during Fetal Liver and Adult BM B Lymphopoiesis Is Intrinsic to the B Cell Progenitors. There are several possibilities that could explain the lack of I-A expression during B cell development in the fetal liver: (*a*) Differences in the fetal and adult stromal microenvironment influences class II expression on developing B cells; (*b*) the presence of T cells or their factors are required for class II expression; and/or (*c*) the difference is due to the intrinsic properties of progenitor cells derived at distinct ontogenetic timings.

To address these possibilities, pro-B cells were sorted from either 3-mo-old BALB/c BM or day 16 BALB/c fetal liver (Fig. 3) and cultured for 4 d on the FLST2 fetal liver–derived stromal line (26), which has been shown to support B cell differentiation from very early stages of development (3). The pro-B cells found in the adult BM and day 16 fetal liver are at an equivalent stage of differentiation since most of these cells are undergoing D-J rearrangement and are also dependent on stromal contact for growth and differentiation (3, 9).

The BM pro-B cells gave rise to both I-A$^+$ pre-B and I-A$^+$ IgM$^+$ B cells in culture. These cells were I-E$^-$ (data not shown). In contrast, the fetal liver pro-B cells gave rise to both pre-B and sIgM$^+$ cells that were I-A$^-$ and I-E$^-$. This does not appear to be a simple difference in the timing of I-A expression since after 8 d of culture there were still no I-A$^+$ pre-B or immature B cells in the fetal liver pro-B cultures (data not shown). Similar culturing of fetal liver and BM pro-B cells on heterogeneous BM stromal cells yielded identical differences (data not shown). Thus, the differences in B cell development observed in our in vivo analyses (Figs. 1 and 2) are completely reproduced in in vitro cultures. The data indicate that the stromal microenvironment is not responsible for the differences in class II expression during B cell development at these two stages of ontogeny.

It is unlikely that the lack of class II expression on fetal pre-B/immature B cells is due to a lack of T cell factors or help. Culturing of day 16 fetal liver pro-B cells with supernatants from the EL4 T cell line or Con A activated splenic T cells did not yield any I-A$^+$ B cells (data not shown). Furthermore, class II$^-$ pre-B cells and immature IgM$^+$ B cells are found in the spleens of mice up to 5–6 wk of age when the level of splenic T cells have reached the adult equivalent. These data strongly suggest that the differences in class II expression during B cell development are intrinsic to the B cell progenitors present in the fetal liver and adult BM. This difference would allow us to distinguish between FT and AT B cell developmental pathways.

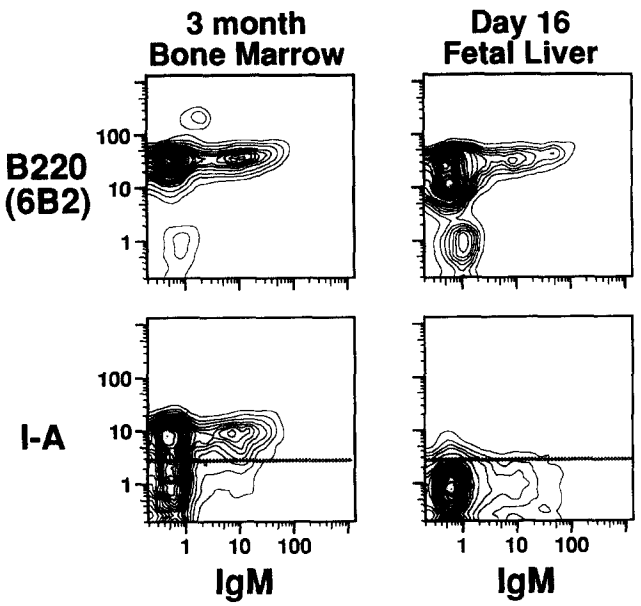

Figure 3. The difference in class II expression on pre-B and newly generated IgM$^+$ B cells is intrinsic to the developmental program of pro-B cells derived at distinct ontogenetic timings. Cells from BALB/c day 16 fetal liver and 3-mo-old adult BM were stained as in Fig. 1 except that both ^{bi}anti-I-A and ^{bi}anti-I-E were used simultaneously. Pro-B cells were sorted as B220^{lo}, CD43^{hi}, IgM$^-$, and class II (I-A and I-E)$^-$ and cultured for 4 d on the FLST2 stromal cells. Typically, 30–50,000 cells were cultured in a single well. The relative cell recoveries after a 4-d culture were 2–2.5 times the input cell numbers for both BM and fetal liver pro-B cells. Cells recovered from the cultures were stained with ^{APC}anti-B220, ^{Fl}anti-IgM, ^{PE}anti-I-A, and ^{bi}anti-I-E. The cells presented in the IgM vs. I-A contour plots were pregated for B220$^+$ cells in order to eliminate contaminating stromal cells. Approximately 13 and 18% of the B220$^+$ cells in the fetal liver and BM cultures were IgM$^+$, respectively, and of the B220$^+$ IgM$^-$ cells in the adult BM cultures, 62% were I-A$^+$ and 38% were I-A$^-$. Data shown were representative of individual five adult BM and three fetal liver pro-B cultures.

FT B Cell Developmental Pathway Occurs in Many Neonatal Lymphoid Organs and Extends to Adulthood. Analyses of spleen cells from mice at various time points after birth revealed the presence of a population of B220$^+$ IgM$^-$ cells (Fig. 4 *A*, population *a*). Histologic studies indicated that these are pre-B cells as ~75–80% of these B220$^+$ IgM$^-$ cells stained positive for cytoplasmic μ (data not shown). These pre-B cells did not express I-A or I-E. As seen in Fig. 4 and summarized in Table 1, I-A$^-$ pre-B cells can be detected up to 1 mo of age in murine spleens, well beyond the stage of fetal ontogeny. During the first month the spleen increased more than 200-fold in cellularity from 0.4 to 88 $\times$ 10^6 cells. Although the

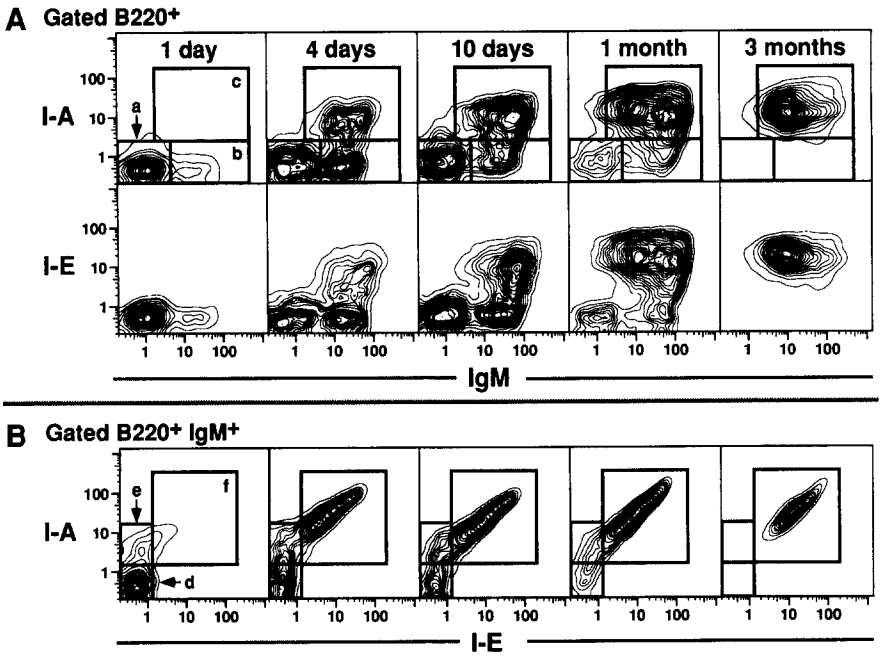

Figure 4. Presence of I-A$^-$ pre-B cells in murine spleens during neonatal ontogeny. Spleen cells were obtained from BALB/c mice at the ages indicated and stained with ^{APC}anti-B220, ^{bi}anti-IgM, ^{PE}anti-I-A, and ^{Fl}anti-I-E. (*A*) The contour plots show the expression of IgM vs. I-A or I-E on B lineage (gated for B220$^+$) cells. Cells in population a are IgM$^-$ pre-B cells. Population b cells are IgM$^+$ I-A or I-E negative. Cells in population c coexpress IgM and I-A or I-E. (*B*) Correlation of I-A and I-E on IgM$^+$ B cells. Spleen cells were gated on B220 and IgM (cutoff, 4 in *A*). Three populations are gated: (*d*) Class II$^-$; (*e*) I-A^{lo} I-E$^-$; and (*f*) I-A and I-E double positive.

percentage of I-A$^-$ pre-B cells (calculated as %B220$^+$ – %B220$^+$ IgM$^+$ from Table 1) decreased, the absolute numbers actually increased during this period (from 0.04 × 10^6 cells at day 1 to 5.3 × 10^6 cells at 1 mo) and then decrease such that at 3 mo the levels were equivalent to background.

To confirm that these splenic I-A$^-$ pre-B cells were in the FT developmental pathway, splenic B220$^+$ IgM$^-$ I-A$^-$ cells were sorted from day 1 to day 12 neonatal spleens, cultured on the FLST2 stromal line, and analyzed 4 d later for the expression of I-A on B cells arising in the cultures. The I-A$^-$ pre-B cells gave rise to immature sIgM$^+$ cells that did not express any class II antigens (data not shown).

1 d after birth, 78% of the splenic IgM$^+$ B cells are I-A$^-$ and I-E$^-$ (Table 1 and Fig. 4 *B*, population *d*). This confirms

Table 1. *B Cell Development in the Spleen of Young Animals*

Age	N*	Total No. of spleen cells × 10^6	Percentage of total cells‡		Percentage of B220$^+$IgM$^+$§		
			B220$^+$	B220$^+$IgM$^+$	I-A$^-$I-E$^-$	I-A^{lo}I-E$^-$	I-A$^+$I-E$^+$
			%			%	
1 d	6	0.4 ± 0.1	14 ± 0.9	2.5 ± 1.0	78 ± 5.6	11 ± 3.5	11 ± 5.3
4 d	3	6.7 ± 1.8	28 ± 9.1	17 ± 6.3	41 ± 20	16 ± 7.2	43 ± 18
10 d	5	25 ± 9.0	38 ± 5.2	21 ± 5.1	15 ± 2.2	16 ± 3.4	69 ± 1.8
1 mo	5	88 ± 10	44 ± 2.1	38 ± 1.9	6 ± 1.5	8 ± 2.3	86 ± 2.5
3 mo	3	95 ± 14	43 ± 1.0	42 ± 1.1	0.3 ± 0.2	0.9 ± 1.0	99 ± 1.3

* Number of animals tested.

‡ Values are based on gates shown in Fig. 6. The percentage of B220$^+$IgM$^+$ cells was calculated as the number of cells in populations b and c divided by the total number of spleen cells. The percentage of pre-B cells can be determined from %B220$^+$ – %B220$^+$IgM$^+$.

§ Values are determined from the gated populations shown in Fig. 6: I-A$^-$I-E$^-$, population d; I-A^{lo}I-E$^-$, population e; I-A$^+$I-E$^+$, population f and are expressed as the percentage of B220$^+$IgM$^+$ cells.

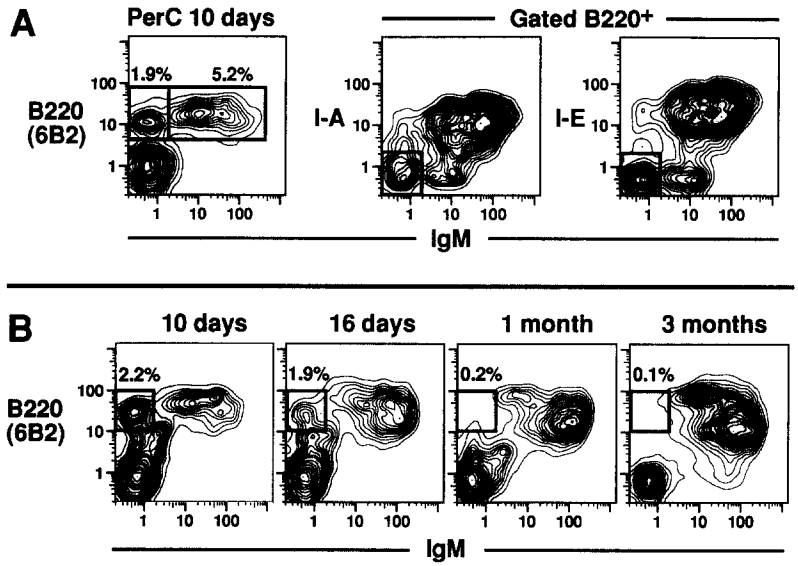

Figure 5. The PerC is a site of B lymphopoiesis in neonatal mice. PerC cells obtained from BALB/c mice have been gated for lymphocytes by forward and side scatter. (*A*) (*Left*) IgM vs. B220 expression. The gates identify the pre-B and IgM$^+$ B cell populations. (*Right*) Class II expression on the B220$^+$ (cutoff >4) cells. The gates denote the class II$^-$ pre-B cells. (*B*) IgM vs. B220 contour plots of peritoneal cells from mice of different ages. The average number of cells recovered are: 10 d, (5.8 ± 1.2) × 10^5 (n = 5); 16 d, (1.3 ± 0.3 × 10^6 (n = 2); 1 mo (1.5 ± 0.5) × 10^6 (n = 2); and 3 mo (6.3 ± 1.3) × 10^6 (n = 3). The frequency of B cells (and pre-B) as a percentage of total cells shown in the plots is: 10 d, 7.5% (2.2%); 16 d, 31.1% (1.9%); 1 mo, 22.2% (0.2%), and 3 mo, 64% (0.1%).

early data by Kearney et al. (17) which showed the presence of B cells that lacked class II expression in neonatal mice. As with the I-A$^-$ pre-B cells, these I-A$^-$ IgM$^+$ immature B cells can be detected out to 1 mo of age and are absent in 3-mo-old mice. In 1-d-old mice, a small population of the IgM$^+$ B cells appears to express very low levels of I-A in the absence of I-E (Fig. 4 *B*, population *e*), again suggesting that in BALB/c mice, I-A may be expressed briefly before I-E. At this time there is no distinct population of I-A$^+$ I-E$^+$ IgM B cells (Fig. 4 *B*, population *f*). In the first week after birth there was a major increase in the number of splenic IgM$^+$ B cells expressing both class II antigens, and by 3 mo of age, essentially all splenic B220$^+$ cells coexpress IgM, I-A, and I-E.

I-A$^-$ pre-B cells were also present in the neonatal livers up to at least the second week of birth (the last point we were able to readily isolate lymphocytes from this organ). Like the pre-B cells found in the neonatal spleens, the I-A$^-$ pre-B cells stained for cytoplasmic μ and gave rise to class II$^-$ IgM$^+$ cells in the FLST2 stromal cell cultures, indicating that they originate from the FT progenitor cells (data not shown).

Similar flow cytometric analyses of the PC cells from young mice also revealed the presence of I-A$^-$ pre-B cells (Fig. 5). At 10 d of age (the earliest time point tested) ~40% of the total B lineage cells were B220$^+$ IgM$^-$ pre-B cells that were positive for cytoplasmic μ. Like the pre-B cells found in the spleens and livers, most of these cells do not express any de-

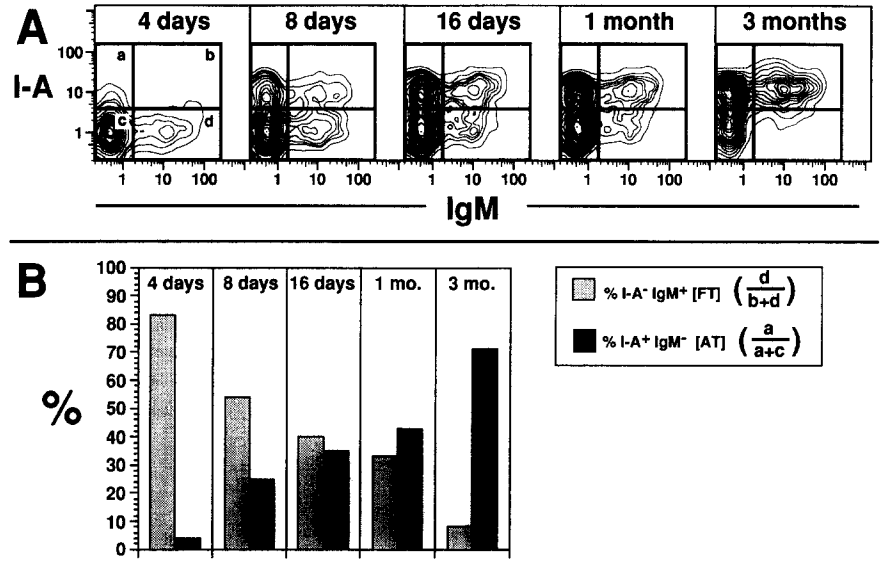

Figure 6. Developmental switch from FT to AT B cell differentiation pathway occurs in the young BM. BM cells were obtained from BALB/c mice at the ages indicated and stained as in Fig. 1. (*A*) Contour plots of IgM vs. I-A expression is shown for pre-B/immature B cells (B220^{int} CD43^{lo}), gated as in Fig. 1. These cells are divided into four populations: (*a*) I-A$^+$ IgM$^-$ pre-B cells; (*b*) I-A$^+$ IgM$^+$ immature B cells; (*c*) I-A$^-$ IgM$^-$ pre-B cells; (*d*) I-A$^-$ IgM$^+$ immature B cells. (*B*) Graph showing the switch from FT to AT B cell developmental pathways in the BM over age. The values plotted are the percentage of IgM$^+$ immature B cells that are I-A$^-$ (FT) and the percentage of IgM$^-$ pre-B cells that are I-A$^+$ (AT). These values are calculated as shown in *B* based upon the populations defined in *A*. The number of cells recovered from femur and tibia of one hind limb of mice of different ages are: 4 d, (1.1 ± 0.1) × 10^6 (n = 4); 8 d, (4.6 ± 0.9) × 10^6 (n = 3); 16 d, (1.1 ± 0.2) × 10^7 (n = 3); 1 mo (2.2 ± 0.3) × 10^7 (n = 2); and 3 mo, (2.7 ± 0.1) × 10^7 (n = 3). The average percentages of pre-B and sIgM$^+$ cells found in the BM are respectively: 4 d, 27.2%, 4.6%; 8 d, 23.6%, 4.5%; 16 d, 31.9%, 9.5%; 1 mo, 30.4%, 10.5%; 3 mo, 20.9%, 8.1%.

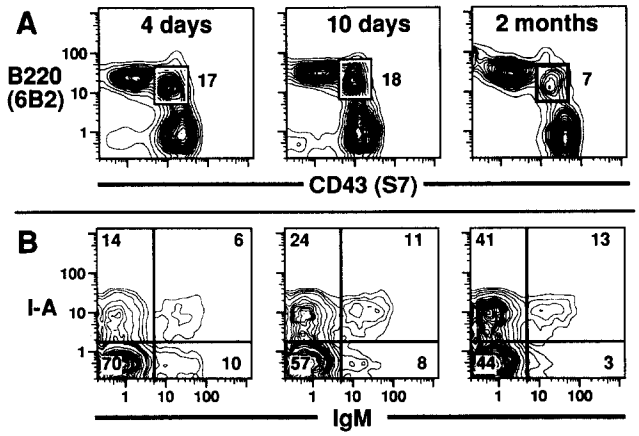

Figure 7. Young BM pro B cells contain a mixture of precursor cells that differentiates along both FT and AT developmental pathways. (*A*) BM from BALB/c mice of different ages were stained as in Fig. 1 *A*. Pro-B cells (cells in gates) were sorted and cultured on FLST2 stromal cells. (*B*) After 4 d of culture, cells were recovered and stained as in Fig. 3. The relative cells recoveries were similar to those in Fig. 3. The cells presented in the IgM vs. I-A contour plots were pregated for B220$^+$ cells. Percentages of total cells for each population are shown in the figures. The data are representative of three independent sets of culturing experiments, each with multiple mice of different ages.

tectable levels of cell surface class II antigens (Fig. 5 *A*). These pre-B cells were readily detected up to 2.5 wk of age in this location (Fig. 5 *B*). When these peritoneal pre-B cells were sorted and cultured for 4 d, they generate IgM$^+$ cells at a frequency ($\sim$20% of B220$^+$ cells in culture) comparable to the pre-B cells derived from neonatal spleens, livers, or adult BM. These IgM$^+$ cells were I-A$^-$ (data not shown).

AT B Cell Developmental Pathway Begins to Emerge after Birth but is Confined to the BM. In the neonatal liver, spleen, and PerC there was little evidence of AT B cell development as indicated by the absence of I-A$^+$ pre-B cells. Since the BM is the site of AT development in the adult, we investigated B cell development in BM at various ages (Fig. 6). As in the adult BM, pro-B, pre-B, and immature B cells could be distinguished by B220 and CD43 staining in the young neonatal BM (Fig. 7 *A*), indicating that a steady state level of B lymphopoiesis is already occurring in this location.

Fig. 6 *A* shows the expression of IgM and I-A on B220^{int} CD43^{lo} BM cells (pre-B and immature B cells) at various time points after birth. In young mice, this population contained both I-A$^+$ and I-A$^-$ pre-B cells (Fig. 6 *A*, populations *a* and *c*, respectively) and I-A$^+$ and I-A$^-$ IgM$^+$ immature B cells (Fig. 6 *A*, populations *b* and *d*, respectively), suggesting the simultaneous presence of the two distinct B cell developmental pathways. Whereas it is difficult to determine the exact contribution of each developmental pathway, in Fig. 6 *B* the percentage of total immature B cells (populations *b* + *d*) which are I-A$^-$ (population *d*) is given as an approximation of the FT pathway. This is probably an underestimate since some of the I-A$^+$ immature B cells may be FT B cells that have begun to express I-A. Similarly, the percentage of total pre-B cells (populations *a*+c) which are I-A$^+$ (population *a*) is given as a measure of the AT pathway. Again, this is an underestimate since AT pre-B cells are initially I-A$^-$. The value of 70% I-A$^+$ pre-B cells in a 3 mo BM probably represents close to 100% AT development since there is no clear population of I-A$^-$ immature B cells at this time point (Fig. 6 *A*).

It is clear that the FT B cell developmental pathways is the major B cell developmental pathway in the BM at birth (Fig. 6 *B*). In a 4-d-old BM, the vast majority of the IgM$^+$ immature B cells (82%) do not express I-A whereas only 5% of the pre-B cells present are I-A$^+$. As the mice age, there is an increase in the frequency of I-A$^+$ pre-B cells and a decrease in the percentage of I-A$^-$ IgM$^+$ cells (Fig. 6 *B*) indicating a gradual switch from one pathway to the other, with the AT differentiation pathway becoming the predominant pathway in older mice.

The I-A$^-$ IgM$^+$ B cells in the BM could be due to FT B cells developing in other organs (e.g., liver, spleen, or peritoneal cavity) and migrating into the BM location, or they could be generated de novo in the BM together with B cells differentiating along the AT B cell developmental pathway. To address this possibility, B220^{lo} CD43^{hi} pro-B cell were sorted from neonatal mice BM and cultured on the FLST2 stromal line (Fig. 7). Pro-B cells sorted from a day 4 or day 10 newborn BM cultured for 4 d gave rise to both I-A$^+$ pre-B cells indicative of AT development, as well as I-A$^-$ IgM$^+$ immature B cells indicative of FT development. Similar to the in vivo analysis of pre-B and immature B cells in the neonatal BM (Fig. 6), pro-B cells derived from older mice gave rise to a greater frequency of I-A$^+$ pre-B and I-A$^+$ IgM$^+$ B cells. In cultures of 4–6-d-old BM pro-B cells, 60% of the IgM$^+$ B cells were I-A$^-$ whereas in cultures of 2–3-mo-old BM pro-B cells, only 20% were I-A$^-$ (Fig. 7). The culturing experiments confirm that the pro-B population present in young BM actually contains a mixture of precursor cells derived from both the FT and AT developmental pathways and that as the animals age, the dominant population of pro-B cell precursors shifts from FT to AT. Hence, in contrast to other neonatal lymphoid organs where FT B cell developmental pathway predominates, the BM is the only location where both the developmental pathways coexist early in ontogeny (Fig. 6).

Discussion

We demonstrate that there is a basic difference in fetal and adult B cell ontogeny with respect to MHC class II antigens, considered basic to B lymphocyte function and immunoregulation. We have distinguished two distinct B cell developmental pathways: the AT typified by B cells developing in the adult BM which never express their surface antigen receptors in the absence of I-A expression, and the FT in which developing B cells first expressed their surface Ig in the absence of any MHC class II antigens. Furthermore, we demonstrate that this difference in B cell development is likely to be intrinsic to progenitor cells arising at distinct ontogenetic timings. This difference allows us for the first time to phenotypically distinguish pre-B and immature B cells arising from

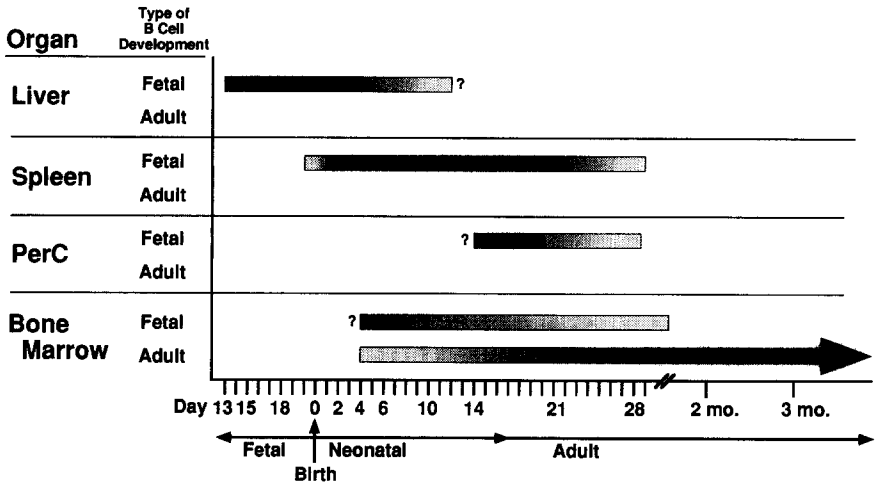

Figure 8. Time course for FT and AT B cell developmental pathways occurring in various organs. See text for description. The intensity of each line is an approximate indication of the relative level of B lymphopoiesis in the organ at that time.

progenitor cells of distinct developmental pathways. Finally, based upon these distinctions we have shown that FT B lymphopoiesis (at least from the pre-B cell stage) occurs in various lymphoid organs and extends to at least 1 mo of age whereas AT B lymphopoiesis is restricted to the BM environment.

In addition to the difference in the expression of class II in FT and AT B cell development, we were surprised to find that in BALB/c mice, AT pre-B cells expressed I-A in the absence of I-E both in vivo (Fig. 1) and in in vitro cultures (Fig. 3). This is in striking contrast to previous studies using in vitro transcription systems or macrophage cell lines which indicated that the I-A and I-E class II genes were coordinately regulated (11,12,29). Yet, in CBA/Ca mice, I-A and I-E were coordinately expressed in both the FT and AT developmental pathways (data not shown). It is not clear why there is such a difference in cell surface expression of I-E on pre-B and immature B cells in the BM of mice of different genetic backgrounds. The difference could be due to epitope or glycosylation differences during different stages of B cell development. For example, at the pre-B cell stage, the E_α chain may associate with A_β (or another stage-specific chain) and the anti-I-E^k mAb might be able to recognize this combination in CBA/Ca but not BALB/c. Alternatively, it could simply reflect differences in the regulation of I-E expression influenced by the genetic background of mice.

Previous studies have demonstrated that there are significant differences in B cells developing at the two distinct ontogenetic sites, the fetal liver and the adult BM. Notably, TdT (responsible for N region insertions) (5) and the PLRLC (6) are expressed in pro-B cells in the adult BM but not in the fetal liver (4). However since there have been no phenotypic markers to distinguish the FT and AT B cell development, it has not been possible to determine whether or not the FT development is in fact restricted to fetal ontogeny. These studies clearly demonstrate that the FT B cell lymphopoiesis occurs in a variety of organs and extends well into adulthood.

Taken together, our data suggest a pattern of B cell differentiation during ontogeny as shown in Fig. 8. The first major B lymphopoietic site is the liver where B lineage cells are first detected by day 14–15 of gestation and are generated until at least 10 d after birth. Some low level B lymphopoiesis may continue in the liver past this point but it is difficult to obtain clean single cell populations for analysis. However up to day 10 after birth (the last point analyzed) all of the pre-B cells in the liver were I-A^- indicating an absence of any AT B cell development at this site (data not shown).

After birth we were able to identify pre-B cells in the neonatal spleen and in the peritoneal cavity. As in the liver, all of these were FT I-A^- pre-B cells (Figs. 4 and 5). In the PerC the pre-B cells were detectable out to 3 wk of age. In the spleen, such pre-B cells were readily detectable out to 1 mo (Fig. 4 and Table 1). Although the percentage of FT I-A^- pre-B cells decreases over this time, the absolute numbers actually increase during the first month and then dramatically decrease. Again, the absence of any detectable I-A^+ pre-B cells indicates that both the PerC and spleen are sites restricted to FT B cell development during this time period.

The situation in the neonatal BM is quite different. At day 4 after birth, essentially all of the pre-B and immature B cells were I-A^- (Fig. 6), demonstrating the predominance of the FT pathway at this site. Two pieces of data indicate that approximately at day 4 after birth, AT B cell development first emerges: (*a*) at 8 d, I-A^+ (AT) pre-B cells were first detected in the BM (Fig. 6); and (*b*) 4-d FLST2 cultures of pro-B cells sorted from 4-d-old BM gave rise to both I-A^+ (AT) pre-B cells and I-A^- (FT) immature B cells demonstrating the presence of both developmental pathways (Fig. 7). Thus, by 4 d although AT pre-B have not yet been generated, AT pro-B cells are already present. Both in vivo flow cytometric analysis and in vitro cultures demonstrated that from 4 d the BM is shifting from a site of FT to AT B cell development (Figs. 6 and 7). By 3 mo of age, there is little evidence of any significant FT B cell development in the BM which is now the major site of B cell lymphopoiesis for the life of the animal.

Our studies show for the first time that the two developmental pathways are not separated during ontogeny but rather coexist within the animal for at least the first month of life,

that is until young adulthood. What is striking in this view of B cell development is the apparent compartmentalization of the two B cell developmental pathways. During the first month, when both developmental pathways are active, we can find little evidence of AT B lymphopoiesis in the liver, spleen, or PerC although we can readily detect I-A$^-$ (FT) pre-B cells. On the other hand, during this period the BM is clearly a site for both types of development.

It is possible that during the first month the I-A$^-$ pre-B cells present in the PerC, spleens, and livers were generated from pro-B cells present first in the liver and then in the BM. If this is true, it would mean that FT pre-B cells are capable of migrating to other locations such as the spleen and PerC whereas the AT I-A$^+$ pre-B cells are restricted to the BM. The seeding of FT pre-B cells to these various locations early in development could be indicative of an intrinsic expansion program to populate specific organs with cells of this developmental lineage. While these organs lack AT pre-B cells, they are presumably also being filled with AT mature B cells from the BM during this period.

Our results support the concept of (at least) two developmentally and functionally distinct B cell lineages within the immune system (10, 30–32): one generated during fetal life and one in the adulthood which begins to emerge after birth. Developmentally, it has been shown that embryonic (33), fetal omentum (34), and fetal liver (9, 35) B cell progenitors preferentially give rise to B-1a (CD5$^+$) B cells. Given our earlier studies (10) and those of Hardy and Hayakawa (9), it is reasonable to assume that the B-1a (and possibly B-1b) lineage(s) are a part of what we have defined here as the FT developmental pathway. As expected, sorted I-A$^-$ pre-B cells from young spleens can give rise to CD5$^+$ B cells in FLST2 cultures (Lam, K.-P., and A. M. Stall, manuscript in preparation). Further studies are needed to determine whether fetal-derived B-1a lineage is equivalent to the fetal developmental pathway described here or whether B-1 (a and b) cells represent one (or more) subsets or waves of B cells generated within this B cell developmental pathway. Since the in vitro stromal cell culture system is limited in its ability to generate and maintain mature B cell populations, transfer studies will be required to determine the total reconstructive potential of the I-A$^-$ pre-B cells found in neonatal spleens and the types of B cells to which they give rise.

One question raised by these studies is the relationship of the FT B cells generated in the fetal liver to those generated 2–4 wk after birth. Is there a single homogeneous developmental lineage throughout this period or is the FT developmental lineage itself comprised of distinct developmental sublineages? It is known that fetal liver–derived B cells have V gene repertoires skewed towards the D-proximal families (36) and with few N region additions. Using I-A expression as a FT lineage marker, similar analyses of V gene usage and N region additions for FT B cells generated in the spleens, livers, and PerC would be possible.

The functional significance of generating a population of IgM$^+$ B cells during fetal and neonatal development which initially lack class II expression remains to be elucidated. It is known that the fetal repertoire contains a high frequency of B cells that are low affinity, polyreactive, and that often recognize self-antigens (37, 38). It is interesting to note that the period during which the developing fetal/neonatal IgM$^+$ B cells lack class II antigens corresponds exactly to the period when newly generated B cells are exquisitely sensitive to tolerance induction (39, 40). Altered expression of class II may alter the threshold of tolerance induction or clonal deletion of these B cells. Thus, the inability of B cells developing in the early immune environment to present to CD4$^+$ T cells may influence the selection of such self-reactive B cells and in addition, may play a role in the generation of T-independent versus T-dependent responses.

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