Phenotypically distinct B cell development pathways map to the three B cell lineages in the mouse

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A recent article by Montecino-Rodriguez *et al.* [Montecino-Rodriguez, E., Leathers, H. & Dorshkind, K. (2006) *Nat. Immunol.* 7, 293–301] has distinguished the early progenitors for B-1 cells, which principally develop in neonates, from early progenitors for B-2 cells, which principally develop in adult bone marrow. Here we introduce syndecan-1 (CD138) and MHC class II (I-A) as markers of early B cell development [Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D. & Hayakawa, K. (1991) *J. Exp. Med.* 173, 1213–1225; Hardy fractions *B–D*] and show that the expression of these markers distinguishes the predominant B cell development pathway in neonates from the corresponding predominant pathway in adults (both progenitors are present but differently represented in each case). We show that pre-B cells (Hardy fraction *D*) in the predominant adult pathway express high levels of CD138 and intermediate levels of I-A, whereas the corresponding pre-B cells in the pathway that predominates in neonates do not express either of these markers. As expected, because most of the pre-B cells in adults express CD138, we find that sorted CD138$^+$ adult pre-B cells differentiate to IgM$^+$ B cells *in vitro*. Sorted CD138$^-$ pre-B cells from neonates, the majority subset at this age, also mature to IgM$^+$ cells (without passing through a CD138$^+$ stage). Importantly, our studies here confirm the differential representation of adult and neonatal progenitor populations and further demonstrate that CD138 expression subdivides the adult CD19$^+$, B220–6B2$^{-/low}$ population shown to contain B-1 progenitors in a way consistent with the predominance of B-1b progenitors in adults. Thus, CD138 expression provides a key route to distinguishing early B cell development pathway for what now are clearly three B cell lineages.

B-1 cells | B-2 cells | CD138 | MHC class II | progenitors

Some time ago, our laboratory proposed the existence of three B lymphocyte lineages (B-1a, B-1b, and B-2) in the mouse (1–3). We based this proposal on *in vivo* reconstitution studies demonstrating that progenitors for these lineages are enriched differentially in traditional sites for lymphocyte progenitors, i.e., B-1a progenitors are found principally in fetal liver, neonatal spleen, and neonatal bone marrow (BM), whereas B-1b and B-2 progenitors are found principally in adult BM. In the ensuing years, alternate theories were advanced suggesting that antigen-driven differentiation of mature B cells accounted for the development of these functionally distinct B cells subsets (4–9). However, in a recent paper, Montecino-Rodriguez *et al.* (10) directly demonstrate that B-1a, B-1b, and B-2 belong to three separate lineages by showing that they derive from phenotypically and temporally distinct progenitors, i.e., progenitors for B-2 cells are phenotypically distinct from progenitors for B-1a and B-1b; among progenitors for B-1 cells, B-1a progenitors are found principally in neonates, whereas progenitors for B-1b are found principally in adults.

Studies here provide key support for the existence of the distinct B cell lineages by showing that the predominant B cell development pathway in adults, where B-2 development predominates, is phenotypically distinct from the predominant pathway in neonates, where B-1 development predominates. In essence, we show that syndecan-1 (CD138) and MHC class II (I-A) are both expressed throughout B cell development in the predominant B cell pathway adult BM, beginning when Ig rearrangement is initiated [at Hardy fraction (Fr.) *B* (11)] and persisting thereafter (I-A), or terminating when the B cells reach maturity (CD138). In contrast, neither marker is expressed during early B cell development in the predominant pathway in neonatal BM and spleen, where I-A appears initially on mature B cells (12, 13), and CD138 is not detectable on most of the developing B cells.

Results

CD138 Expression Distinguishes the Predominant Neonatal B Cell Development Pathway from the Predominant Pathway in Adults. To identify the standard subsets in the B cell development pathway, i.e., Fr. *A–F* (11), we analyzed the stained adult or neonatal BM cells according to the scheme shown in Fig. 1. For this analysis, the BM cells are first gated to include only single cells (no doublets) and to include only live cells [propidium iodide (PI)$^-$, Fig. 1]. Next, the single live cells are gated to include B cells and their progenitors, i.e., cells that are B220$^+$ and dump$^-$ (CD3$^-$CD8$^-$F4/80$^-$Gr-1$^-$). Finally, the gated B220$^+$ cells are separated by CD43 expression: the CD43$^+$ population contains pro-B cells (Fr. *A*, *B*, and *C*/*C*′) and the CD43$^-$ population contains pre-B (Fr. *D*), immature B (Fr. *E*), and mature B cells (Fr. *F*).

Data in Fig. 2*A Upper* show that CD138 (a surface proteoglycan that binds to the extracellular matrix and is expressed on plasma cells and pre-B cells) (14) is not expressed or is expressed at low levels in the predominant B cell development subsets (Fr. *A–F*) in neonates (Fig. 2*A Upper*, shaded histograms). In contrast, the predominant B cell development subsets in adults express relatively high levels of CD138, from Fr. *B* onward. In Fr. *F*, which contains a mixture of newly developed B cells and recirculating B cells, approximately half of the cells still express CD138, and some of the others appear to have down-regulated CD138 to different extents (Fig. 2*A Upper*, unshaded histograms). Consistent with the loss of CD138 on mature B cells, none of the B cells in spleen express CD138 (data for splenic IgM$^+$ IgD$^+$ are shown in Fig. 2*A Lower*).

Overall, we detect four levels of CD138 expression. Plasma cells, which have been gated out from the data shown here, express the highest CD138 levels, i.e., ≈10-fold above the CD138 levels expressed on CD138-expressing cells during B cell development. Gating on the Fr. *A–C* (a combined subset in which most of the cells are from Fr. *B*) shows three levels of CD138 expression (CD138^{hi}, CD138^{int}, and CD138$^-$) and varying frequencies of cells at these expression levels depending on the source of the cells (Fig. 2*B*). In adult BM, the frequency of CD138^{hi} is the highest, and the frequency of CD138^{int} is greater than CD138$^-$. In neonates, the reverse is true: The majority of

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Abbreviations: BM, bone marrow; CD138, syndecan-1; Fr., Hardy fraction(s); I-A, MHC class II.

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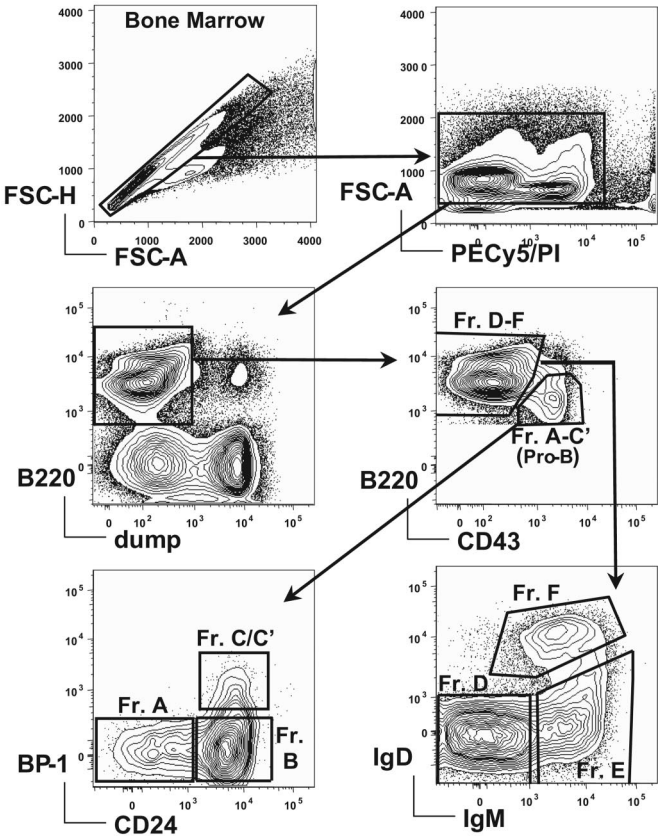

Fig. 1. Successive gating scheme for identifying developing B cell subsets in BM. BALB/cN adult BM cells were stained according to the 10 color-stain combinations described in *Materials and Methods*. (*Bottom Left*) Pro-B cells (Fr. *A–C'*). (*Bottom Right*) Pre-B cells (Fr. *D*).

cells are CD138$^-$ cells, although some CD138^{int} and a few CD138^{hi} cells are detectable.

These striking differences in CD138 expression, which are fixed early in the B cell development, suggests that the three levels of CD138 expression may distinguish the progenitors for B-1a, B-1b, and B2 cells. Data presented in the next section support this hypothesis.

CD138 Expression as a Marker for B Lineage Progenitors. Sorting and transfer studies conducted by Montecino-Rodriguez and colleagues (10) have identified subsets of cells in neonates and adults that are each selectively enriched for one of the lineage progenitors. In addition, Hardy and colleagues, in earlier studies (15–17), have shown similar distinctions between sorted B cell progenitors in fetal liver vs. adult BM. Results from our studies indicate that CD138$^-$, CD138^{int}, and CD138^{hi}, respectively, mark the progenitors for B-1a, B-1b, and B-2 cells.

Montecino-Rodriguez and colleagues (10) demonstrated the presence of B-2 progenitors among AA4.1$^+$CD19$^-$ cells that express intermediate levels of the "CD45R" (B220-6B2) determinant detected by the B220/RA3–6B2 monoclonal antibody (circular gates, Fig. 3). This subset, which is basically Fr. *A* with AA4.1$^-$ cells excluded and a proportion of CD19^{low} Fr. *B* included, is mainly present in adult BM.

In contrast, the B-1a progenitors, which are found mainly in neonatal BM, and the B-1b progenitors, which are found mainly in adult BM, are located in an AA4.1$^+$CD19$^+$ subset similar to Fr. *B* with respect to expression of these markers but containing cells that only express minimal levels of the B220-6B2 determinant (designated as B220-6B2$^{lo/neg}$). These cells, Montecino-Rodriguez and colleagues (10) show, express other B220 isoforms and hence are committed to the B lineage. Consistent with this commitment, and with the similarity of these subsets to Fr. *B*, we find that the cells in both subsets express CD24 at levels typical for Fr. *B*.

With hindsight, the low expression of B220-6B2 on B-1

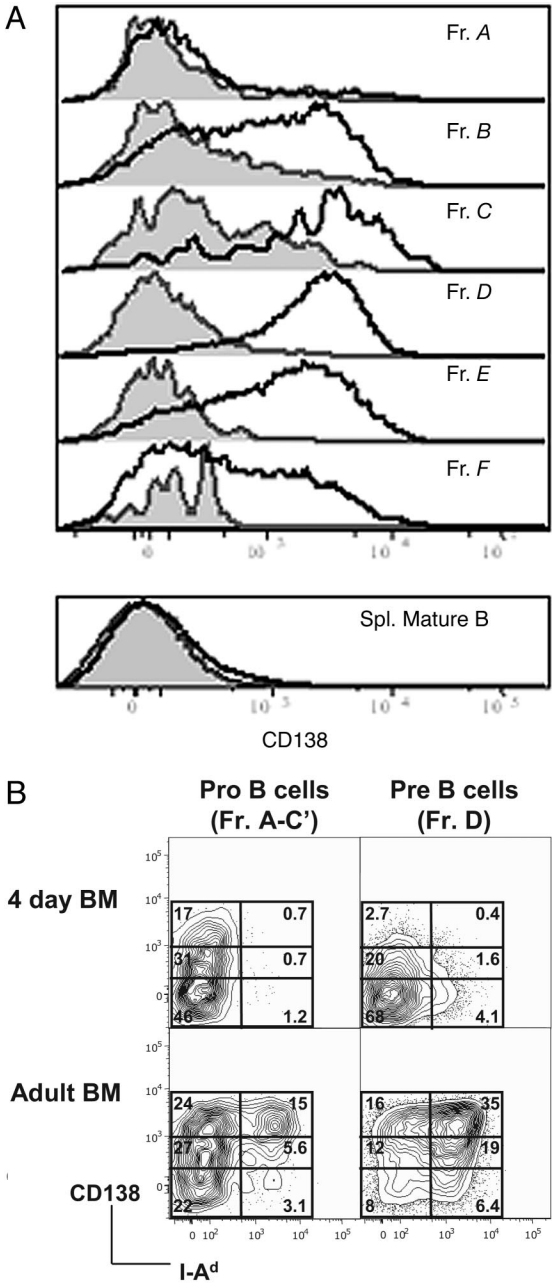

Fig. 2. CD138 expression varies in adult and neonatal developing B cells. (*A Upper*) Developing B cells (Fr. *A–F*) are gated based on the surface expression as shown in Fig. 1, and CD138 expression in each fraction is plotted as histogram. Shaded histograms, CD138 expression in neonatal fractions; unshaded histograms, CD138 expression in adult fractions. Note that pre-B cells all express CD138 and that even in Fr. *F*, which contains mature B cells, approximately half the cells still express CD138. (*A Lower*) In contrast, none of the mature B cells in spleen (adult or neonate) express CD138. (*B*) CD138 and I-A expression distinguish neonatal from adult pro-B and pre-B cells in BALB/c BM. Pro-B cells (B220 6B2$^+$ CD43$^+$ IgM$^-$ IgD$^-$) and pre-B cells (B220 6B2$^+$ CD43$^-$ IgM$^-$ IgD$^-$) are defined according to the gating scheme shown in Fig. 1. BALB/cN mice express the I-A^d haplotype.

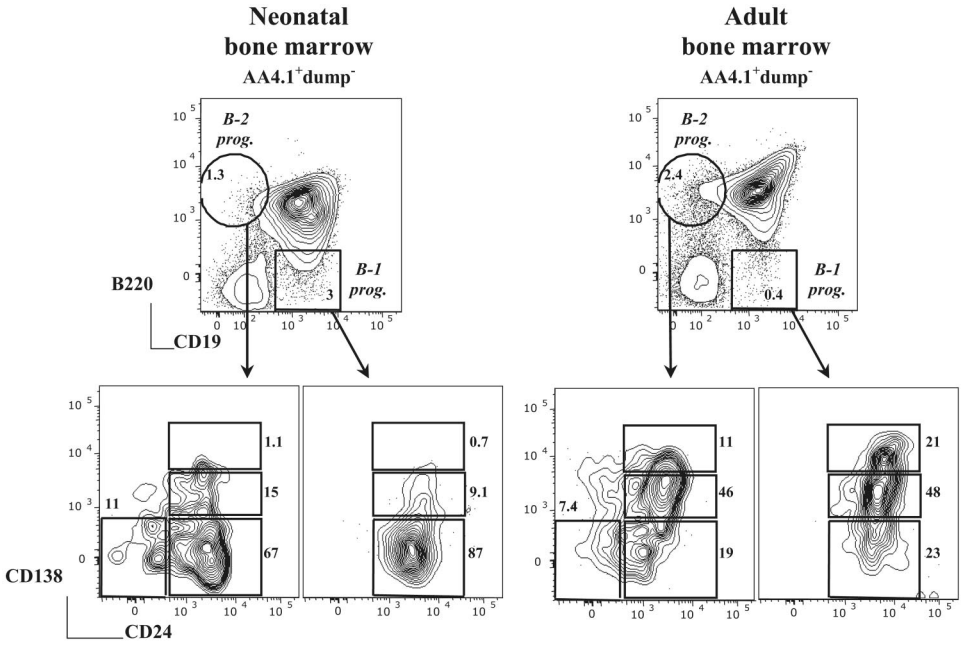

Fig. 3. CD138 expression separates B-1 progenitors in adult and neonate BM. BM cells from BALB/cN neonates (*Left*) or adults (*Right*) were gated initially for AA4.1+ cells that were dump$^-$ (CD3$^-$ CD8$^-$ Gr-1$^-$ F4/80$^-$). The gated cells were further gated to include cells expressing CD19+ B220- expression, i.e., the B-1 progenitors from each source (*Lower Middle*). (*Lower*) The vast majority of the B-1 progenitors in neonatal BM do not express CD138 (*Lower Left*), whereas the B-1 progenitors in adult BM express CD138 at three distinct levels (*Lower Right*), likely representing progenitors for each of the lineages. Circular gates (*Lower Middle*) define the phenotype of progenitors for B-2 cells.

progenitors is not surprising. Although this determinant is readily detectable on mature B-1 cells, it is usually expressed at lower levels on these cells than on B-2 cells (18, 19). In fact, the B220-6B2 monoclonal antibody was adopted for general use for B cell identification in our laboratory (and elsewhere) specifically because, although its expression overlaps on B-1 and B-2 cells, it provides an opportunity to estimate whether B-1 or B-2 cells predominate in a given B cell population (18).

Results from our studies with the progenitor subsets defined by Montecino-Rodriguez and colleagues (10) strongly support the idea that CD138 expression distinguishes early-stage B lineage progenitors. Data in Fig. 3 show that CD138^{hi} cells are enriched in the B-2 progenitor subset. CD138$^-$ cells, in contrast, constitute ≈90% of the neonatal subset that gives rise to B-1a cells; CD138^{int} constitute ≈50% of the adult subset that predominantly gives rise to B-1b cells.

Analysis of this latter subset (AA4.1$^+$, CD19$^+$, and B220-6B2$^{-/lo}$ BM from adult BM) is complicated by the presence of substantial numbers of cells at all three CD138 levels (Fig. 3). Although approximately half the cells express CD138^{int}, a quarter is CD138^{hi}, and the remainder is CD138$^-$. The CD138$^-$ cells in this subset likely reflect the presence of progenitors for B-1a cells, which this adult subset also reconstitutes (although to a lesser extent that B-1b). The CD138^{hi} cells, in contrast, likely represent contamination from the previously defined AA4.1$^+$, CD19$^+$ Fr. *B*, which expresses intermediate levels of B220-6B2 but does not otherwise differ from the B220-6B2$^{-/lo}$ subset defined by Montecino-Rodriguez.

Collectively, the data cited above provide good reason to believe that the expression of CD138 on developing B cells (Fr. *B–E*) will prove to distinguish reliably between the progenitors for each of the B cell lineages. However, in the absence of data from sorting and transfer studies with this marker, these findings must still be considered provisional.

I-A Also Distinguishes the Predominant B Cell Development Pathway in Adults and Neonates. Previously, I-A expression has been shown to be expressed from Fr. *D* during B cell development in adults, only to be expressed on mature (IgM$^+$ IgD$^+$) B cells in neonatal animals (12, 13). In a previous study (20), we detected I-A expression earlier than Fr. *D* in adults but were unable to obtain a clear view of the expression of this marker on the early subsets. Our current methods resolve these subsets better and show that cells expressing I-A expression become detectable in Fr. *B* in adults, mainly in the CD138^{hi} population and increase substantially in frequency in Fr. *D* (see Fig. 2*B Lower*). Neonates, in contrast, have virtually no detectable cells expressing I-A in pre-B cells and only a few cells (if any) expressing I-A in pre-B cells (Fig. 2*B Upper*) Comparison of CD138 and I-A expression in the predominant adult pathway indicates that CD138 is expressed on Fr. *B* before I-A is expressed, and that both markers are expressed by the majority of the cells in Fr. *D* (Fig. 2*B Lower right*).

CD138$^-$ Pre-B Cells (Fr. *D*) from Neonates Differentiate to IgM$^+$ Cells *in Vitro* Without Passing Through a CD138$^+$ Stage. To evaluate whether CD138 is transiently expressed during B-1 development, we sorted and cultured the predominant pre-B cell subsets in adult and neonatal BM, i.e., CD138^{hi} from adults and CD138$^-$ from neonates and determined how/whether CD138 expression changes as these two types of pre-B cells mature (Fig. 4). Over 30% of the cultured pre-B cells were recovered as live cells after 36 h in culture in the absence of stromal or cytokine support; 30–40% of these cells had differentiated to IgM$^+$ B cells in all cultures. The IgM$^+$ B cells, derived from the sorted neonatal pre-B cells, retained their CD138$^-$ phenotype and did not pass through a CD138$^+$ expression as they matured. Among the IgM$^+$ B cells derived from the adult (CD138^{hi}) pre-B cells, approximately one-third had down-regulated CD138 expression; the remainder were still CD138^{hi}, despite the initiation of surface Ig production (Fig. 4).

Preliminary studies with sorted pre-B cells cultured for 90 h indicates that ≈80% of the CD138$^+$ pre-B cells sorted from adult BM express IgM and that the majority of these cells down-

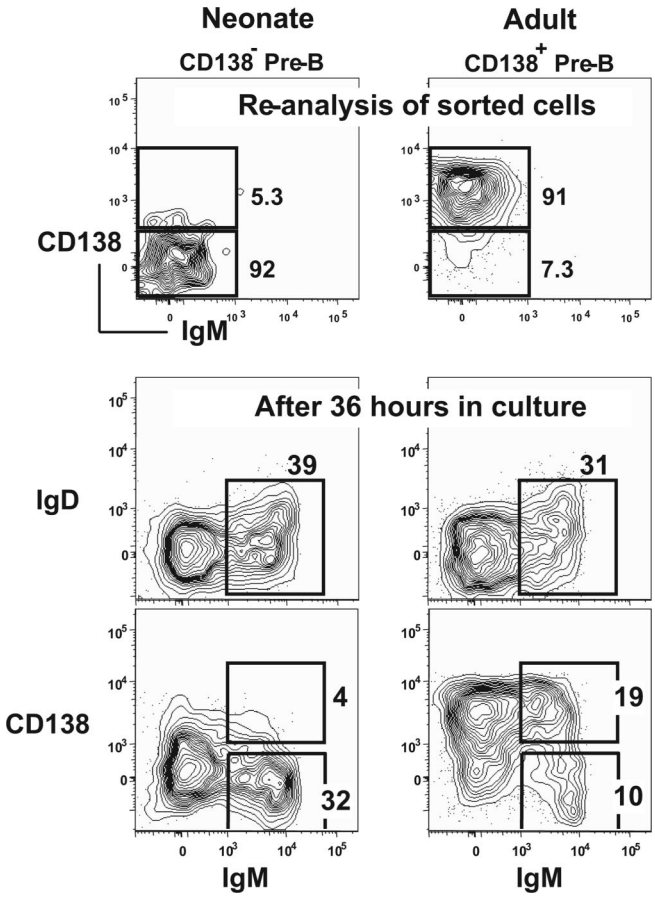

Fig. 4. Sorted neonatal and adult pre-B cells can develop into IgM$^+$ B cells. (*Upper*) Reanalysis of the sorted neonatal (CD138$^-$) and adult (CD138$^+$) pre-B cells (B220 6B2$^+$ CD43$^-$ IgM$^-$ IgD$^-$) from BALB/cN BM. (*Lower*) Sorted neonatal CD138$^-$ cells and adult CD138$^+$ pre-B cells shown in *Upper* were cultured for the indicated time and analyzed for the appearance of surface Ig.

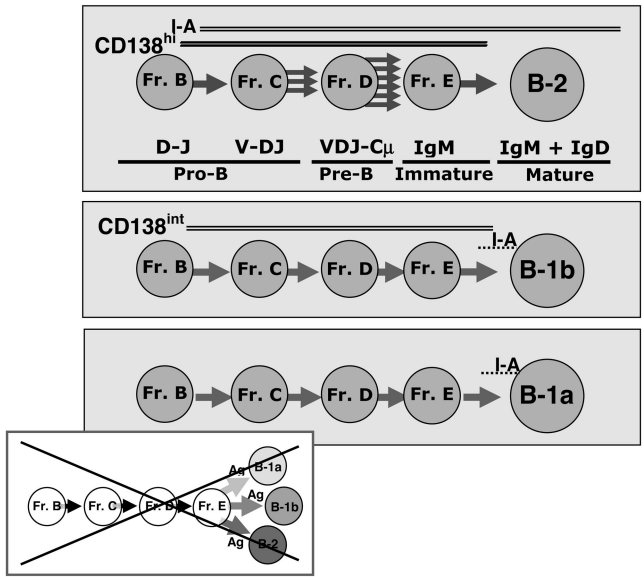

Fig. 5. B cell development in the layered immune system in the mouse. The expression of syndecan-1 (CD138) and MHC class II (I-A) distinguishes B cell development pathways in the mouse. Data presented here show that these pathways correspond to the three recently demonstrated B cell developmental lineages. The times at which these lineages appear during development and the differences in their functional properties and antibody repertoires suggest that they evolved sequentially to create a layered immune system progressively adapted to meet increasing needs for protection against pathogen attack. (*Inset*) The single-lineage B cell development hypothesis is inconsistent with evidence presented and discussed here.

regulate CD138 expression and up-regulate IgD (data not shown). The expression of IgD, however, does not appear to coordinate with the down-regulation of CD138 (data not shown).

Discussion

We show here that CD138 and I-A expression distinguishes the predominant B cell development pathway in adult mice from the predominant pathway in neonates. The expression of CD138 begins early in B cell development (at Fr. *B*) and continues until B cells mature and leave the marrow; however, the frequency of CD138$^+$ cells among developing B cells differs strikingly between adults and neonates. In adult BM, most (>70%) of the developing B cells express CD138; in neonatal BM and spleen, CD138 is only expressed on a minority (<10%) of these cells. The I-A expression pattern is qualitatively similar to the CD138 pattern, except that I-A expression begins somewhat later during development and persists when the mature B cells leave the BM.

Quantitative differences in CD138 expression further distinguish the early B cell progenitors (Fr. *A* and *B*) in animals of different ages. Using the staining and gating criteria that Montecino-Rodriguez *et al.* (10) used to sort progenitors for the B-1a, B-1b, and B-2 subsets from adult and neonatal BM, we show here that CD138$^-$, CD138^{int}, and CD138^{hi} cells predominate, respectively, in the subsets in which B-1a, B-1b, and B-2 progenitors were shown to predominate (Fig. 2). These findings support and extend evidence from the demonstrations by the Montecino-Rodriguez and Hardy groups (refs. 10 and 15, respectively), based on the sorting and transferring of cells early in B cell development pathway, that independent B cell progenitors give rise to B-1a, B-1b, and B-2 cells. Thus, collectively, there is strong support for the conclusion that these phenotypically and functionally distinct B cells belong to distinct developmental lineages (see Fig. 5).

We had proposed the existence of the three B cell lineages some time ago (1, 2, 21), based on evidence from reconstitution studies in which B220$^-$ progenitors were sorted from adult BM at a stage before initiation of rearrangement and were cotransferred with B cell progenitors from fetal liver into lethally irradiated recipients. Six weeks (or more) after transfer, B-2 cells were reconstituted equivalently from both fetal and adult progenitors. B-1a cells, however, were reconstituted almost exclusively from the fetal progenitors, indicating that these progenitors are well represented in neonates but are rare in adult BM. B-1b cells were well reconstituted from fetal sources but could also be recovered in reasonable numbers from adult progenitors, indicating that the progenitors for B-1a and B-1b were distinct and suggesting that B-1b progenitors are likely to also be distinct from the progenitors for B-2 cells.

Hardy and Hayakawa (15) extended this work by showing that pro-B cells (B220$^+$CD43$^+$CD24$^+$) sorted from fetal liver selectively reconstitute B-1 cells (mainly B-1a) in adoptive recipients whereas pro-B cells with the same phenotype sorted from adult BM principally give rise to B-2 cells (15). This work demonstrated clearly that the commitment to develop into B-1 vs. B-2 cells occurs at the pro-B stage or earlier and hence that the early B cell progenitors in fetal vs. adult mice are inherently distinct. This inherent distinction meets the basic criterion for assigning the fetal and adult progenitors and their progeny to distinct developmental lineages. Even if the environment (fetal liver vs. adult BM) is responsible for the developmental distinction

between the two types of progenitors, the inherent differences in the progenitors and their progeny means that they still should still be assigned to distinct lineages.

Nevertheless, without the direct demonstration that the two types of progenitors coexist at the same site and can be physically isolated from one another, the findings from these early studies left room for alternate hypotheses (4–9). Thus, until the Montecino-Rodriguez group published their findings demonstrating the isolation of phenotypically distinct progenitors for B-1 and B-2 cells from adult BM, the B lineage issue had not been definitively settled (22).

The alternate hypotheses proposed over the years place B-1a, B-1b, and B-2 within the same lineage, arguing that these phenotypically distinct B cells all derived from the same progenitor but are triggered late in differentiation, after IgH rearrangement is complete, to assume one of the B cell (lineage) phenotypes in response to stimulation and selection with distinctive antigens. Our findings here, coupled with the findings from Montecino-Rodriguez and colleagues (10) demonstrate otherwise. In essence, we show that the B cell development pathways diverge phenotypically at least as early as Fr. *B* and thus clearly before the V to DJ rearrangement. In addition, Montecino-Rodriguez and colleagues show definitively, by sorting and transferring phenotypically distinct subsets of early B cell progenitors from BM, that this lineage commitment occurs at a stage well before IgH rearrangement and expression that could be used for antigen-dependent selection. Thus, the idea that antigen recognition determines whether developing B cells become B-1a, B-1b, or B-2 cells now has been shown to be incorrect.

Locating B-1a, B-1b, and B-2 cells in separate developmental lineages does not, of course, rule out key roles for antigen in negative or positive selection, or in determining the persistence of B cells expressing particular receptors. Instead, the recognition that the developmental pathways for these lineages differ provides a context for thinking about how/whether different antigen-dependent mechanisms operate to determine the lineage repertoires.

Hardy, for example, has proposed that the VDJ rearrangements that form good VpreB/λ5 pre-BCR complexes and positively select B-2 cells are actually toxic to developing B-1 cells, which can only pass this developmental checkpoint if VpreB/λ5 pre-BCR complex formation fails (23). This inherent mechanism would explain evidence presented earlier by Hardy's group (15), which showed clearly that the committed B-1 progenitors in fetal liver maintained their developmental commitment to the unique B-1 repertoire, even in adult adoptive recipients (15).

In addition, the mechanism Hardy (23) proposes would explain otherwise perplexing data in which the mating of a transgenic mouse strain carrying a fully rearranged B-1a expressed gene [V(H)12] with a transgenic strain carrying a B-2 expressed gene [V(H)B1–8 or V(H)glD42] resulted in differentiation and positive selection of B-2 cells but the selective loss of B-1 cells that express both V_H transgenes (9). Interpreted in the context of the Hardy hypothesis, the B-1 cells in these mice would be expected to be lost because the expression of the transgenic B-2 IgH would be toxic to the developing B-1 cells, despite the coexpression of a B-1 transgene that would otherwise enable selection and development of B-1 cells.

The evidence for selection of antibodies with particular specificities within the B-1 lineage is quite strong (reviewed in ref. 24). For example, Hayakawa *et al.* (25) have shown quite clearly that the presence of B-1 cells producing antibodies to a T cell autoantigen depends on the expression of the autoantigen. However, the importance of antigenic selection in shaping the final B-1 repertoire in no way negates the existence of earlier developmental mechanisms that which V_H genes are expressed in the initial repertoires expressed by the B cell lineages.

The presence of distinctive antibody repertoires in three lineages is, of course, fully compatible with their developmental distinctions. In fact, it is likely that this functional distinction underlies what we have suggested is the sequential evolution of the lineage in response to progressively more sophisticated immune challenges (1, 21, 26, 27). Thus, although B-1a and B-1b cells tend to produce autoreactive and innate antibody responses, the putatively more highly evolved B-2 cells produce antibodies to a wider variety of antigens and, when stimulated, differentiate to long-lived memory B cells capable of rapidly responding with high affinity responses to secondary antigenic challenges.

Further studies are required to characterize the B lineage progenitors discussed here and to identify the stem cell(s) and intermediate progenitors from which these progenitors derive. Now, with the definitive demonstration that the B lineage compartment is subdivided into (at least) three lineages, studies aimed at understanding the developmental origins and functional properties that distinguish these lineages can begin in earnest.

Materials and Methods

Mice and Tissue Preparation. BALB/cN and C.B-17 mice were bred and maintained at the Department of Comparative Medicine at Stanford University. BM cells were extruded from femurs of adult or 3- to 4-day-old neonatal mice by using staining medium (RPMI medium 1640 + 3% newborn calf serum)-filled syringes, then washed, filtered through nylon mesh, and resuspended at 25×10^6 cells per ml. Single-cell suspensions of spleens were prepared by first grinding the spleens with frosted slides and then by gentle passage through nylon mesh. The red blood cells were lysed by incubation with chilled 0.14 M NH_4Cl and 20 mM Tris (pH 7.4) for 2 min. These splenic cells were then washed with staining medium and resuspended at 25×10^6 cells per ml.

FACS Analysis and Sorting. Cell suspensions from BALB/cN or C.B-17 adult and neonate BM or spleen were prestained with anti-CD16/CD32 mAb to block FcγRII/III receptors and stained on ice for 15 min with the following fluorochrome-conjugated antibodies in a ten-color staining combination: FITC-CD19 (1D3) or FITC-I-A^d (AMS-132.1); PE-CD43 (S7); biotin-CD138 (281–2); PECy5.5-CD24 (30F1); PECy7-IgD (11–26); Alexa594-BP-1 (6C3); APC-B220 (RA3–6B2), referred to here as B220–6B2; APCCy5.5-AA4.1; APCCy7-IgM (331); and, as a "dump reagent" with which to gate out unwanted cells, a mixture of cascade blue-conjugated CD4 (GK1.5), CD8a (53–6.7), Gr-1 (8C5), and F4/80. After being stained with this stain set, the cells were washed with staining medium and stained again on ice for 15 min with streptavidin-CyChrome (BD Pharmingen), a second-step reagent that reveals biotin-coupled antibodies. The fluorochrome-conjugated antibodies were either purchased from BD Pharmingen or custom conjugated in the Herzenberg laboratory (Stanford, CA).

After washing away excess reagents, the stained cells were resuspended in staining medium containing 10 μg/ml propidium iodide. Cells that stained with propidium iodide (dead cells) were excluded from analysis and sorting in all cases. Cells were analyzed and sorted on the Stanford shared FACS facility Hi-Dimensional FACS (Hi-D FACS) instruments, either on the FACSAria with DiVa electronics (Becton Dickinson) or on a hybrid instrument ("Flasher II") in which a FACS II bench is coupled to FACS DiVa electronics. Staining protocols were designed with FACSXPERT (http://sciencexperts.com), and data were analyzed with FLOWJO software (http://treestar.com).

Cell Culture. Between 5×10^4 and 8×10^4 sorted pre-B cells from adult or neonatal BM were cultured in 96-well plates in 300 μl

of deficient Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 10^{-5} M 2-mercaptoethanol, 2 mM glutamine, and 100 μg/ml penicillin and streptomycin. Cells were cultured for 36 or 96 h at 37°C in 7% CO_2 ambient oxygen incubators before FACS analysis. No stromal lines were used, and no cytokines were added.

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