

Differential development of progenitor activity for three B-cell lineages

(B-1 cells/Ly-1 B cells/B cell development/fluorescence-activated cell sorter)

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ABSTRACT Cell-transfer studies presented here distinguish three murine B cell lineages: conventional B cells, which develop late and are continually replenished from progenitors in adult bone marrow; Ly-1 B cells (B-1a), which develop early and maintain their numbers by self-replenishment; and Ly-1 B “sister” (B-1b) cells, which share many of the properties of Ly-1 B cells, including self-replenishment and feedback regulation of development but can also readily develop from progenitors in adult bone marrow. The sequential emergence of these lineages, the time at which their progenitors function during ontogeny, and the distinctions among their repertoires and functions suggest that evolution has created a layered immune system in which the immune response potential of each successive lineage is adapted to its particular niche.

Earlier studies identified two distinct murine B-cell lineages: conventional B cells, which are predominant in adult spleen and lymph nodes, and Ly-1 B cells [now called B-1 cells (1)], which are predominant in the peritoneal and pleural cavities (2). These B-cell lineages were initially distinguished by multiparameter fluorescence-activated cell sorter (FACS) analyses of lethally irradiated recipients reconstituted with Igh-C allotype congenic cells (3). In adults, conventional B cells are replenished only from Ig⁻ progenitors, whereas B-1 cells are replenished by transfer of mature Ig⁺ cells from the peritoneal cavity (4).

B-1 cells are disproportionately represented in the production of autoantibodies (5) and B-cell neoplasms (6). They tend to use a restricted set of variable-region (V) genes (7–10) and to use N-region insertions less often than conventional B cells (11). These repertoire differences may arise from differences in the diversity-generating mechanisms in individual lineages (12), to the different times that lineages develop (3), and/or to selection by particular antigens (13–16).

The independent development of the B-cell lineages has been verified by Hardy and Hayakawa (33, 38) and Solvason *et al.* (17). Fetal liver (FL) (day 13) reconstituted all B-cell populations, whereas the mesodermally derived fetal omentum, which had been shown (18) to be a source of lymphocytes in the mouse, reconstituted only B-1 cells. Cografting fetal omentum (H-2K^{b,d}) with fetal thymus (H-2K^d) shows that fetal omentum can yield H-2K^{b,d}-positive T cells. This fact suggests some progenitors in omentum are not yet committed to the B lineage (17).

In this study, we characterize the progenitor capacity of FL and adult bone marrow (BM) for three kinds of mature B cells: conventional B cells, B-1a (CD5⁺ Ly-1 B) cells, and B-1b (CD5⁻ Ly-1 B “sister”) cells. Conventional B cells are distinguishable from both kinds of B-1 cells by their anatom-

ical location, their functional characteristics, and a series of FACS-detectable cell-surface markers (for review, see refs. 19 and 20). B-1a and B-1b cells are very similar; however, B-1a cells, the major B-1 population, express detectable levels of surface CD5, whereas B-1b cells do not. Within experimental limits, each of these B-1-cell populations can replenish itself but not the other (19, 21).

We show here that significant progenitor activity for B-1b cells is present in fetal and adult animals, whereas progenitor activity for B-1a cells is readily detectable in FL but is largely missing or nonfunctional in adult BM. We further show (i) that the failure to detect progenitor activity for B-1a cells in adult BM is not due to the presence of inhibitors or the absence of inducers that regulate B-1a development, (ii) that the progenitors for conventional B cells are already distinct from progenitors for the B-1 subsets in 14-day FL, and (iii) that the distinctive development of the three B-cell populations depends on properties inherent in their progenitors and cannot be explained solely by differential selection.

Taken together, these studies indicate that B-1a and B-1b cells belong to separate developmental lineages and that both lineages are distinct from the conventional B lineage. We discuss these findings in the context of our recent hypothesis that these B-cell lineages reflect the existence of an evolutionarily layered immune system in which the immune response potential of each successive lineage is adapted to particular challenges (22).

MATERIALS AND METHODS

Transfer Experiments. BALB/cHz (Igh-C^a haplotype) and allotype congenic BAB/25 (Igh-C^b) mice were used. Recipients were x-irradiated (650 rads; 1 rad = 0.01 Gy) 1 day before transfer of 2×10^6 FL [13 and 14 day based on observation of the last vaginal plug and confirmed by fetal characteristics (23)] or 2×10^6 BM (femur and tibia) cells. FACS-sorted BM cells were transferred in numbers equivalent to that found in 2×10^6 unsorted BM cells.

FACS Analyses. Reagents, protocols, FACS instrumentation, and software have been described (24). Reagents used are fluorescein anti-Igh-6a and -6b, allophycocyanin anti-Ly-1 and anti-Mac 1, and biotin anti-Igh-5a and -5b followed by Texas red-avidin. Percentages are reported per total number of live lymphocytes. Plots have 5% probability contours. Three B-cell populations from normal BALB/c peritoneal cells (PerC) are identified in Fig. 1. Conventional B cells are identified by a broad, positive IgM and tight, bright IgD FACS profile (IgM⁺IgD^{br}). All B-1 cells are IgM^{br} and low to moderate for IgD (IgD^{lo}). B-1 cells are also Mac1⁺ in peritoneum; conventional B cells are Mac1⁻ (19, 24). Gating

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Abbreviations: BM, bone marrow; FACS, fluorescence-activated cell sorter; FL, fetal liver; PerC, peritoneal cells; HSC, hematopoietic stem cells; V, variable region.

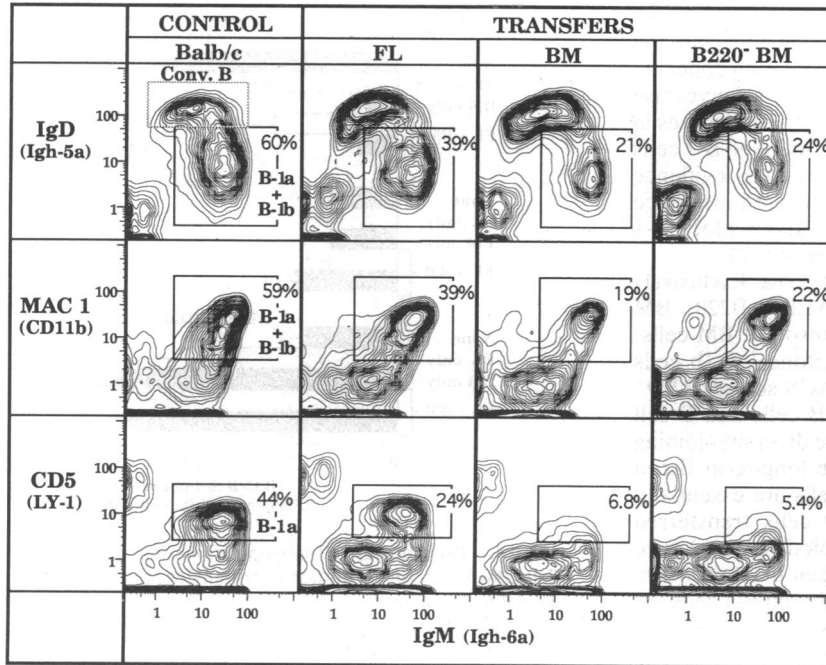


FIG. 1. Peritoneal B cells from a normal BALB/c and recipients of FL, BM, and B220⁻ BM. Representative plots are shown for an untreated BALB/c PerC and BAB recipients that received BALB/c day 14 FL, adult BM, or FACS-sorted B220⁻ BM 8 weeks before analysis. Reanalysis of the sorted cells yields <1% B220⁺ cell contamination (see *Materials and Methods* for definitions of B-cell populations).

on the IgM/IgD and IgM/Mac 1 plots yields comparable values of B-1 cells. The B-1 cells are divided into B-1a (CD5⁺) and B-1b (CD5⁻) populations. Thus, the number of B-1b cells is obtained from the difference of total B-1 cells and B-1a cells. Direct gating on CD5⁻IgM^{br} cells is avoided because of overlap with conventional B cells.

RESULTS

FL Reconstitutes All Three Mature B-Cell Populations. FL on gestational day 13 or 14 is an active hematopoietic organ that regularly reconstitutes splenic T cells and normal levels of conventional B cells 8 weeks after transfer (data not shown). FACS analyses of recipient peritoneal cells show that FL reconstitutes all three B-cell populations (Fig. 1). PerC are analyzed because donor-derived B-1 cells are well represented in this anatomical location and, hence, most accurately measured there. Total number of B cells recovered in FL-recipient PerC is normal. However, the relative frequencies of the B-cell populations differ: normal numbers of B-1b cells are recovered; roughly half the B-1a cells are recovered; and the reduction in B-1 numbers is compensated by an increase in conventional B cells (Fig. 2).

Adult BM Fully Reconstitutes Conventional B Cells in the Recipient Spleen and Peritoneum. Total number of splenic T cells and conventional B cells routinely returns to normal levels or above in BM recipients (data not shown). However, although conventional B cells comprise 10–20% of the lymphocytes in normal BALB/c PerC, they represent 50–60% of the PerC lymphocytes in BM recipients (Figs. 1 and 2). This increased frequency of conventional B cells mainly reflects the failure to reconstitute normal numbers of B-1a cells.

BM Reconstitutes B-1b Cells Better than B-1a Cells. BM usually reconstitutes the combined B-1a plus B-1b population (as measured by their common FACS phenotype, Fig. 1) to ≈20% of normal. Earlier evidence indicated that BM reconstitutes B-1 cells very poorly (5); however, most B-1 cells reconstituted from BM are B-1b cells that were not considered in the earlier studies. As before, BM reconstituted B-1a cells to less than one-tenth their level in untreated mice (Fig. 2). B-1b cells, in contrast, are reconstituted moderately well by adult BM—i.e., to half normal levels.

This reversal of the normal B-1a/B-1b ratio occurs consistently: B-1a cells always predominate in normal animals; however, B-1b cells are more abundant than B-1a cells in every BM recipient analyzed. Thus, B-1b cells represent 20–30% of the peritoneal B-1 cells in normal mice and 30–40% of B-1 cells in FL recipients, but B-1b cells represent 70–80% of B-1 cells in recipients of adult BM.

B-1b cells also predominate over B-1a cells in anti-μ-treated animals in which depleted B cells are repopulated from endogenous progenitors (21). Murine B cells can be temporarily depleted by injecting the neonate with anti-IgM (25). This depletion can be maintained into adulthood by repeated injections. Once the injections are terminated and the antibody is allowed to decay, conventional B cells return rapidly to normal levels. B-1 cell levels return more slowly and eventually stabilize with a preference for B-1b over B-1a cells similar to that shown here for BM transfers (21).

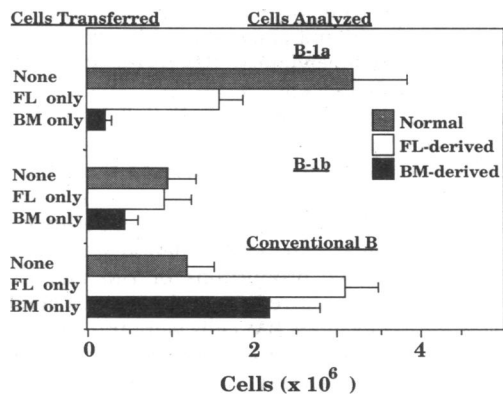


FIG. 2. Total B-cell subpopulations in peritoneum. The total number of each B-cell population is shown for normal BALB/c mice, recipients of BALB/c 13-day and 14-day FL transfer (no difference seen between the two sources) and recipients of adult BALB/c BM. BAB to BALB/c transfers yield equivalent results. The average number of cells and 1 SD from 8–14 recipients is indicated. Mice were analyzed 8–10 weeks after transfer; similar results are obtained 4–7 mo after transfer. B220⁻ BM transfers are very similar to unsorted BM transfers and yield ($n = 3$) $2.8 \pm 0.5 \times 10^5$ B-1a, $6.1 \pm 0.9 \times 10^5$ B-1b, and $2.1 \pm 0.4 \times 10^6$ conventional B cells.

Reconstitution of B-1 cells in irradiated recipients occurs most readily when BM is transferred alone—i.e., in the absence of mature B-1 cells. When transferred with congenic PerC, which contain self-replenishing B-1 cells, BM gives rise to a small B-1 population—i.e., <5% of PerC lymphocytes in BM-derived B-1 cells, and 50–60% are PerC-derived B-1 cells (data not shown). This result is consistent with evidence showing that allotype-congenic B-1 cells injected into mice treated with anti- μ allotype antibodies selectively block B-1 cell development from endogenous progenitors (21).

B-1 Cells Reconstituted from Adult BM Arise Exclusively from B220⁻ Progenitors. FACS-sorted BALB/c B220⁻ BM cells reconstitute B cells equivalently to unsorted BM cells, with the characteristic preference for peritoneal B-1b cells over B-1a cells (Fig. 1). In contrast, FACS-sorted B220⁺ [RA3-6B2 (26)] BM cells, which include B cells and B-cell precursors that have rearranged at least one diversity-joining (DJ) region (27), do not contribute to the long-term B-cell reconstitution. Progeny of these B220⁺ cells are essentially undetectable—i.e., BALB/c B220⁺ BM cells transferred with BAB B220⁻ BM cells (as a hematopoietic source) gave rise to <0.5% of the cells in recipient spleen or peritoneum (data not shown). Thus, conventional B cells and B-1 cells are both reconstituted by B220⁻ progenitors from adult BM. Nevertheless, as studies presented below indicate, these B-cell progenitors are most likely distinct.

Cotransfer Studies Confirm Progenitor Differences in FL and Adult BM. Because B-1a cells readily develop from FL in adoptive transfers, their limited reconstitution from adult BM is not due to conditions inherent in the recipient environment. However, before a definitive statement can be made about the lack of progenitors for B-1a cells in BM, studies are required to determine whether cells transferred with B-1a progenitors could modulate progenitor activity. This question was partially addressed by previous cotransfer studies with BM and neonatal liver; however, we now know that neonatal liver contains mature, self-replenishing B-1 cells (3) that could have clouded the earlier conclusion. The following study resolves the issue: BM does not inhibit FL reconstitution of B-1 cells and FL does not enhance BM reconstitution of B-1 cells.

In a series of cotransfer experiments, we reconstituted irradiated recipients with mixtures of day-14 FL from BAB mice and BM from adult BALB/c mice. Data from these studies (Fig. 3) eliminate the possibility that BM contains cells that limit the development of B-1 cells from their progenitors: FL and BM reconstitute the same proportion of peritoneal B-1a, B-1b, and conventional B cells when transferred together or independently; BM reconstitutes the B-1b cells better than B-1a cells, as in the separate transfers described above; and the ratio of B-1b cells to B-1a cells is equal to the ratio seen when BM is transferred alone.

The percentage of total B cells derived from BM or FL varied substantially among recipients—i.e., BM-derived B cells accounted for 25–90% of the B cells in peritoneum and 20–97% of B cells in spleen. This variation was not a linear function of the relative proportions of BM and FL cells transferred because it occurred when either equal numbers of each type or six times more BM cells were transferred. Such variability is consistent with the low-to-moderate number of hematopoietic stem cells (HSC) transferred (28).

FL Reconstitutes Conventional B and B-1 Cells Independently. In 9 of 13 BM and FL cotransfer recipients, FL reconstitutes the same proportion of B-1a, B-1b, and conventional B cells as it does when transferred independently. The four remaining recipients were in groups that received the 6-fold excess of BM cells. BM-derived B cells in these mice gave rise to their usual distribution of B-1a, B-1b, and conventional B cells; however, substantial deviations occurred in the FL-derived B cells. (These four mice are not included in the FL averages in Fig. 3.) In one of the four mice, there were essentially no FL-derived B

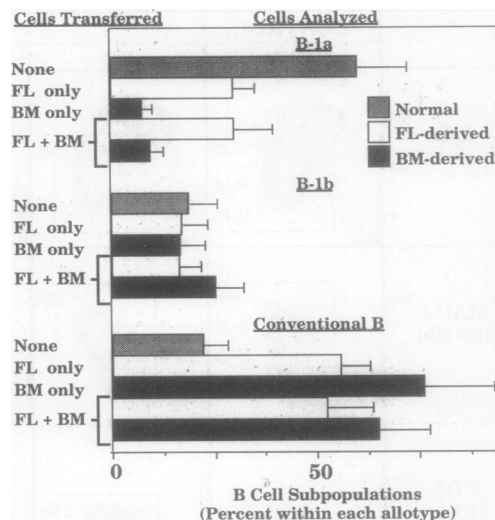


FIG. 3. Peritoneal B-cell subpopulations after cotransfers of FL and adult BM. B-cell reconstitution in cotransfer recipients is evaluated by calculating the a-allotype (BM-derived) B-1a, B-1b, and conventional B-cell populations as percent of total a-allotype B cells and by calculating b-allotype (FL-derived) B-1a, B-1b, and conventional B-cell populations as percent of total b-allotype B cells. To facilitate comparison, data from Fig. 2 are also presented in this format. Analyses were done 8–11 weeks after transfer. Two ratios of cells were used: (i) 5×10^5 BM plus 5×10^5 FL (three BALB/c and three BAB recipients) and (ii) 8.5×10^5 BM plus 1.5×10^5 FL (three BALB/c and four BAB recipients). All 13 mice are included in calculations for BM-derived cells. Nine of 13 mice are included in FL calculations (see text). BALB/c and BAB recipients were both used to facilitate detection of low levels of radiation-resistant host Ly-1 B cells that sometimes survive irradiation. The distributions of BM-derived B-cell populations in BAB and BALB/c cotransfer recipients are indistinguishable, indicating little contribution from the endogenous cells in BALB/c recipients.

cells (<3%). In the remaining three mice, the FL-derived B-1 cells had the normal B-1a/B-1b ratio but failed to reconstitute significant numbers of conventional B cells in either PerC or spleen (Fig. 4).

The B cells attributed to the FL donor could have been radiation-resistant host B-1 cells in one of these three recipients; however, in the other two recipients, the donor/host allotype combination forces us to conclude that the reconstituted cells came from the FL and not from the host. The B-1 cells do not appear clonal by the FACS criteria described (6), suggesting the reconstituted population uses multiple V genes and arises from progenitors that were not yet rearranged. Thus, although FL usually gives rise to both B-1 and conventional B cells in adoptive recipients, the development of these B-cell lineages from progenitors proceeds independently. Mature conventional B cells do not inhibit *de novo* B-cell production from adult BM (24); however, it is always possible that a complex feedback mechanism blocks conventional B but not B-1 cell genesis from FL in these cotransfer recipients. We favor the simpler conclusion that separate progenitors give rise to each of these types of B cells.

DISCUSSION

Webster's Dictionary defines lineage as "descent in a line from a common progenitor" (37). Developmental biologists adhere to this definition; however, there is often considerable discussion, particularly with respect to the immune system, as to what characteristics define a lineage and its progenitor. This definition is often made on practical grounds: in the broadest sense, all cells in a given animal can be assigned to a single lineage because the zygote is the ultimate progenitor;

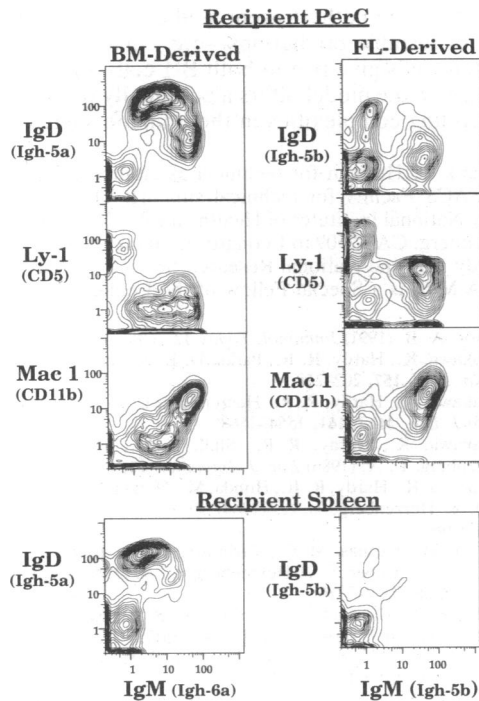


FIG. 4. Recipient peritoneal and splenic B cells after cotransfer of FL and BM. The B-1 cells in this mouse are all derived from the FL. Cells injected were 8.5×10^5 BALB/c BM cells and 1.5×10^5 BAB FL cells. Most cells of the opposite allotype were excluded from each PerC profile to increase the number of contours for the specific allotype of interest. For this recipient 19% of peritoneal B cells and <5% of splenic B cells are derived from FL.

at the other extreme, the progeny of a single, newly arisen B cell can be treated as a lineage because such B cells are distinguished from each other by specific V-gene rearrangements. By and large, however, developmental lineages are defined as deriving from relatively undifferentiated progenitors that have, at least, a limited capacity for self-renewal and give rise to progeny committed to differentiate into cells with particular functional characteristics.

Studies presented here focus on the ontogeny of B-cell lineages that are descended initially from self-replenishing HSC and, more immediately, from self-replenishing lymphoid progenitors that also yield T cells. We have characterized the development of three mature B-cell populations—B-1a, B-1b, and conventional B—and shown that these populations most likely arise from distinct, independent progenitors. The following four points assign these B-cell populations to separate lineages:

(i) Progenitors for B-1 cells are already distinct from progenitors for conventional B cells by day 14 of fetal life. Data from the cotransfers of FL and adult BM remove one of the key obstacles to concluding that the progenitors for B-1 cells and conventional B cells are independent. These data show that the set of B-cell progenitors in FL and adult BM differ, as manifested by their differential reconstitution of the three mature B-cell phenotypes. B-1 cell development from fetal progenitors proceeds equivalently in the presence or absence of BM cells, and the presence of FL cells does not reveal cryptic B-1 progenitor activity in adult BM. Thus, we eliminate the possibility that unknown factors in BM block development of B-1 cells from progenitors or that unknown factors derived from FL cells are required for progenitor development.

The cotransfer studies also provide additional evidence demonstrating the independence of the B-cell lineage progenitors by showing that, in some recipients, FL reconstitutes B-1 cells but not conventional B cells. These findings

are consistent with data showing that cells from fetal omentum reconstitute B-1 cells but not conventional B cells (17). In fact, the cells in FL suspensions that give rise to B-1 cells could be contributed by omental-like mesodermal tissue that surrounds the liver and is contiguous with the omentum (23). Because the bulk of the FL, which apparently contains the progenitors for conventional B cells, derives largely from the endoderm, progenitors for B-1 and conventional B cells could actually belong to lineages that separate early in ontogeny.

(ii) Persistence into adulthood distinguishes B-1b from B-1a progenitors. We have shown that the progenitors for B-1a cells are abundant in FL but are rare in adult BM. In contrast, progenitors for B-1b cells persist readily into adulthood. This differential persistence is reflected in data from earlier studies showing that adult BM reconstitutes the B-1a ($CD5^+$) cells poorly (3), whereas B-1 progenitors that persist in adult animals treated neonatally with anti-IgM antibodies preferentially give rise to B-1b cells (21). Data presented here show that adult BM preferentially reconstitutes B-1b cells.

We have not directly shown that the reconstituted B-1b cells in these recipients are identical in all respects to the B-1b cell population found in the normal peritoneum; however, all existing data, including preliminary evidence that BM-derived B-1 cells self-replenish (unpublished work), are consistent with this view. Thus, current evidence best supports the conclusion that B-1a and B-1b cells represent two closely related lineages that derive from distinct progenitor cells with different potentials for surviving into adulthood. Alternatively, the B-1b-phenotype cells could arise from two B-cell progenitors—one that is most active early in ontogeny and also produces B-1a cells and one that is active later in ontogeny and also produces conventional B cells.

(iii) B-cell lineage reconstitutions reflect the inherent developmental potential of the progenitors. In principle, stimulatory or microenvironmental factors in the host, rather than the inherent developmental potential of the donor cells, could control whether transferred progenitors develop into B-1 cells or conventional B cells. For example, as Wortis and colleagues (14) have suggested, the joint stimulation with a T-cell-independent type II antigen and interleukin 6 could trigger B-1a development from a subset of conventional B cells. Similarly, progenitor migration to certain sites could favor development of one kind of B cell over the other.

Data from the cotransfers of BM and FL cells rule out these kinds of possibilities. Were development into conventional B cells or B-1 cells controlled by either the sites at which progenitors land or by the stimulation(s) they receive, the progeny of coinjected progenitors from BM and FL would be proportionately represented in all B-cell populations. This is clearly not so because progenitors from BM largely fail to reconstitute B-1a cells in the same animals in which progenitors from FL readily reconstitute this population. Therefore, the developmental potential of these progenitors must be fixed before transfer and thus be inherent rather than determined solely by stimulatory or other factors in the recipient.

(iv) Taken together, the evidence cited above defines three B-cell lineages. Predetermined differences in progenitor developmental potential could reflect the initial expression of particular homing receptors. Alternatively, or in addition, such differences could reflect the existence of inherent differences in the capacity to rearrange and express particular immunoglobulin V genes, which would render the cells selectively responsive to certain antigens. In any event, our data indicate that the progenitor activity for conventional B cells is distinct from those for all B-1 cells and that the progenitor activity for B-1a cells is distinct from those for B-1b cells. Therefore, all three types of progenitors must be distinct. By definition, these distinct progenitors (as yet not cleanly isolated from one another) posit the existence of, at least, three murine B-cell lineages.

The Layered Immune System. The developmental pattern of the B-cell lineages could be interpreted strictly within the framework of B-cell development; however, there is good reason to hypothesize that the B-cell progenitors that we have identified arise from distinct pluripotent stem cells that also give rise to progenitors for distinct T-cell lineages (22). The data supporting this idea point to the existence of a layered immune system in which evolution has added developmental lineages capable of progressively more complex functions.

The parallel developmental patterns and repertoires exhibited by T- and B-cell populations/lineages suggest that B-1 cells and early $\gamma\delta$ T cells represent the most primitive "layer" of the vertebrate immune system. $V\gamma 3$ T-cell development occurs during late fetal life and does not occur after birth; $V\gamma 4$ and other $\gamma\delta$ T-cells develop mainly during late fetal and early neonatal life (29–31). Furthermore, HSC from FL but not adult BM have progenitor capacity for $V\gamma 3$ T cells (32). Finally, these early $\gamma\delta$ T cells migrate to a restricted set of tissues, predominately the mucosal epithelium.

The population of the B-cell lineages also occurs sequentially, with some overlap, during development (3, 4): B-1a cells appear first, B-1b cells follow shortly thereafter, and conventional B cells begin to appear during the neonatal period. Recently, Hardy and Hayakawa (33) showed that HSC purified from neonatal liver, but not HSC purified from adult BM, readily repopulate B-1a cells. B-1 cells, like the early $\gamma\delta$ T cells, have a restricted tissue distribution; they reside in the peritoneal and pleural cavities and participate in mucosal immunity (34).

A feedback mechanism, perhaps mediated by cytokines, limits the emergence of B-1 cells from their progenitors at about the time the mice are weaned (2–3 weeks of age), forcing these cells to persist subsequently by self-replenishment (24). This feedback regulation of B-1-cell development and the maintenance of B-1 cells by self-replenishment in adults are also observed for avian B cells (ref. 35 and the references therein). It is unknown whether this apparently primitive regulatory mechanism also controls the development of early $\gamma\delta$ T cells.

$\alpha\beta$ T cells, like conventional B cells, appear around birth and become predominant as the animal matures. Both $\alpha\beta$ T cells and conventional B cells, which circulate throughout the animal and predominate in the secondary lymphoid organs, are replenished throughout life by *de novo* differentiation from stem cells in the BM.

Functional considerations suggest that B-1 cells and early $\gamma\delta$ T cells, by nature of their repertoire and anatomical location, may create a first line of defense against invading pathogens. B-1 cells produce a restricted set of low-affinity, broad-specificity germ-line antibodies that react with ubiquitous microorganisms, whereas conventional B cells produce a large, more diverse set of antibodies capable of specific high-affinity interactions with particular pathogens. Similarly, the repertoire of the early $\gamma\delta$ T cells is considerably more restricted than the diverse repertoire of $\alpha\beta$ T cells (36). Thus, the functional distinctions among immune layers is visible both phylogenetically and ontogenically.

These data suggest that the evolution of the immune system brought a series of stem cells into existence that sequentially give rise to lymphocytes that are similar to their predecessors but have added (or lost) functional capabilities. Because the evolutionary success of the latest layer depends on its ability to introduce a selective advantage, each new layer can be expected to increase the sophistication of the immune system with respect to its ability to efficiently protect the animal against challenges within its environment.

This concept of an evolutionarily layered immune system presents a framework within which the B cells we have defined here can be organized and related to the T-cell

lineages defined in other recent studies. This model makes a number of testable predictions—e.g., that the earliest lymphoid stem cells give rise to both B-1 cells and fetal-type $\gamma\delta$ T cells. Thus, the model offers a potentially productive route for unraveling complexities in the immune system.

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