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# A Hard(y) look at B-1 cell development and function*

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## Abstract

A small population of B cells exists in lymphoid tissues and body cavities of mice that is distinct in development, phenotype and function from the majority (B-2) B cell population. This population, originally termed “Ly1” and now “B-1”, has received renewed interest as an innate-like B cell population of fetal-derived hematopoiesis, responsible for natural antibody production and rapid immune responses. Molecular analyses have begun to define fetal and adult hematopoiesis, while cell-fate mapping studies have revealed complex developmental origins of B-1 cells. Together the studies provide a more detailed understanding of B-1 cell regulation and function. This review outlines studies that defined B-1 cells as natural antibody and cytokine-producing B cells of fetal origin, with a focus on work conducted by Randy Hardy, an early pioneer and co-discoverer of B-1 cells, whose seminal contributions enhanced our understand of this enigmatic B cell population.

## Introduction

This review focuses on a small population of B cells, termed B-1 cells, which exists in lymphoid tissues and body cavities of mice and is distinct in development, phenotype and function from the majority B cell population, termed “conventional” or B-2 cells. B-1 cells were identified initially as “Ly-1” B cells, expressing the surface antigen “Ly-1” (mice), or “Leu-1” (humans), now known as CD5. This discovery, nearly 35 years ago (1), was tightly linked to the development of monoclonal antibodies and of multicolor flow cytometry and its application for the analysis of leukocytes. It was the team around Len and Lee Herzenberg at Stanford University, including Kyoko Hayakawa, Randy Hardy and David Parks, who first identified this B cell subset in mice (1).

The discovery of CD5+ B cells was not a chance finding, but rather was spurred by the discovery that CD5 was expressed on most human B-CLL (2, 3) and on many B cell tumors (4). The original goal had been to identify the normal CD5+ B cell counterparts of these tumors, although follow-up studies revealed a likely more complex picture on the precursor relationship between CD5+ B cells and CLL. CD5, whose ligand is still unknown, has been identified as a negative regulator of T and B cell antigen-receptor signaling. CD5 is expressed on all T cells, where it is required for normal thymocyte development. Its expression by B-1 cells has been linked to their inability to proliferate in response to IgM-

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BCR stimulation, while CD5 expression by conventional B cells has been linked to the maintenance of the anergic state (5–7).

While the initial impetus was to find CD5-expressing B cells, it soon became clear that B-1 cells were distinct in many other ways from conventional B cell populations. Indeed in 1992, Kantor et al. and Stall et al. reported on a population of B-1 cells that lacked expression of CD5, but otherwise showed many similar characteristics (8, 9). This included their i) maintenance by self-renewal, ii) ability to survive long-term and expand after adoptive transfer, as compared to the rapid death seen after transfer of conventional B cells, iii) predominance in the pleural and peritoneal cavities of mice, vi) and ability to secrete IgM without foreign antigen-exposure (8, 9). Collectively, the data showed that CD5 expression was insufficient for delineating all cells with the characteristics of “Ly-1” B cells. A new nomenclature was therefore adopted in 1991 (10), in which these early-developing “B-1 cells” were distinguished from later developing, bone marrow-derived conventional “B-2” cells and in which B-1 cells were separated based on their expression or not of CD5 into B-1a and B-1b, respectively.

This review outlines the scientific milestones that have led to our current understanding of B-1a cell development and regulation. I will attempt to highlight major findings made by Randy Hardy, who died recently and who together with Kyoko Hayakawa made some of the most impactful discoveries about this still enigmatic B cell subset. This review is dedicated to his memory.

## B-1a cells are a fetal-derived B cell lineage

The original studies on CD5+ B cells revealed what has turned out to be one of their most important characteristics, namely their relative abundance in young mice and their reduced frequencies as mice age (from 30% in the spleen on day 5 after birth to about 1–2% by 8 weeks) (1). Cell transfer experiments soon demonstrated that adult bone marrow transfer did not fully reconstitute the B-1a cell compartment of lethally-irradiated mice, while transfers of fetal liver as well as newborn spleen and bone marrow were able to do so (11). The selective ability of early but not later developing precursors to replenish fully the B-1a compartment suggested that distinct B cell hematopoietic precursors in fetal and adult compartments give rise to B-1 and B-2 cells, respectively. In the 30-plus intervening years since these original studies, many subsequent data were published in support of and against the hypothesis of an independent lineage of B-1 cells. We refer to prior reviews on that topic for a more comprehensive discussion (12–15).

As outlined below, lineage tracing studies together with the recent identification of B-1 cell restricted precursors in the fetal liver and neonatal bone marrow, that lose *de novo* B-1 cell development potential by about 6 weeks after birth, have provided support for a lineage distinction between B-1 and B-2 cells. Furthermore, recent studies showed that a newly discovered master regulator of fetal but not adult hematopoiesis, Lin 28b, drives B-1 cell development (16, 17). Together the data support the early observations of B-1 cells as belonging to a wave of B cells that develop early in ontogeny and onto which later-developing B-2 cells are “layered upon” (14).

## B-1 cell development occurs early in ontogeny

Indications for the presence of distinct B-1 and B-2 precursors were first provided by studies with embryonic tissues such as the para-aortic splanchnopleura (18) and the fetal liver (11) which, when taken from early embryos, were shown to contain precursors that give rise exclusively to B-1 cells. In contrast, transfer of bone marrow fully reconstituted the B-2 but not the B-1a cell compartment. Two important technological advances eventually led to the identification of distinct precursor populations for B-1 and B-2 cells.

First, the establishment of culture systems that recapitulated B cell development from precursors in tissue culture (19). In support of those efforts Hardy et al. established a fetal liver-derived stromal cell line (ST-2) that is now widely used for these purposes (20). Second, using the ability to generate distinct stages of B cell development *in vitro,* and assessing immunoglobulin gene-rearrangement in the cultured cells by PCR, Hardy and his team developed a flow cytometry-based scheme to distinguish distinct B cell developmental stages. This now widely adopted “Hardy scheme” uses a set of 7 surface markers: AA4.1, CD24, CD43, CD45R (B220), BP-1, IgM and IgD that distinguishes the stages of B cell development from early pre-pro-B cells (“Hardy fraction” A) to mature B cells (Hardy fraction F). Each step of the B cell developmental path identified by phenotype is reflective of a step in the successive rearrangement of immunoglobulin heavy and light chain genes in that cell (20, 21).

Montechino-Rodriquez and Doshkind et al. then used this approach to identify distinct precursor populations for B-1 and B-2 cells in fetal liver and bone marrow. B-1 cell precursors were identified as lacking expression of CD45R (B220) among AA4.1+ CD19+ CD43+ pro B cell precursors, while early B-2 cell precursors expressed CD45R but lacked CD19 (22). Consistent with previous studies (23, 24), follow-up studies then demonstrated that B-1a cell output from bone marrow precursors strongly diminished over time, such that by about 6–8 weeks of life they no longer contribute significantly to the B-1 cell pool (25). The data were consistent with reports that total bone marrow transfer into irradiated adult mice results in only limited B-1a cell reconstitution. However, the extent of B-1 cell reconstitution observed following bone marrow transfer seems to vary greatly from lab to lab, with some finding very poor reconstitution (23, 24), while others find it to be extensive (26) (27). In our hands, and consistent with studies by Lalor et al (23), B-1a cell reconstitution after bone marrow transfer is slow and incomplete, resulting in higher frequencies of B-1b than B-1a in the peritoneal cavity of reconstituted mice. The data by the Dorshkind group (25) would support an overall poor B-1a cell reconstitution potential of bone marrow precursors. The discrepancies may indicate the presence of some unknown stimuli that could reactivate the reconstitution potential of B-1 precursors in the bone marrow. However, such a signal(s), if it exists, has not been identified (23, 26, 27).

## B-1 cells derive from multiple early waves of hematopoietic precursors

The above outlined experiments provided significant evidence for the existence of B-1 cell restricted precursors in embryonic and fetal tissues. Already in 1991, Hardy and Hayakawa argued that the appearance of CD5+ B cells from fetal but not adult pro-B cells represent a “developmental switch in B lymphopoiesis”, similar to that observed for erythropoiesis, and

that these cells are then carried over into the adult long-lived B cell pool (28). They observed that such a developmental switch was consistent with existing data on the preponderance of B-1 cells early but not later after birth and the adoptive transfer data summarized above, and it supported the hypothesis of the layering of immune cell development (14) observed for γδ T cells (29, 30) and macrophages (31).

Indeed, studies published since have demonstrated the presence of a developmental switch in B cell lymphopoiesis and established that fetal hematopoietic stem cells (HSC) differ significantly from adult HSC. The transcription factor Sox 17 was the first molecular factor identified as being required selectively for the maintenance of fetal but not adult HSC (32). Subsequently, Yuan et al. reported in 2012 on the expression of Lin28b in fetal but not adult HSC and common lymphoid progenitors in both humans and mice (16). Lin 28b is known as a posttranscriptional inhibitor of Let-7 miRNAs, a family of miRNAs expressed in adult but not fetal B cell precursors. This important study showed that ectopic expression of Lin 28b in adult HSC instructed a “fetal HSC” transcriptional program, leading to their preferential reconstitution of various innate-like lymphocytes, including the B-1a cell compartment (16). Hardy et al. also identified Lin 28b as a regulator of fetal hematopoiesis and confirmed that the regulation of Let-7 by Lin 28b was sufficient to instruct fetal/adult B cell development. Furthermore, his group showed that the major target of Let-7 miRNA mediated transcriptional control is the transcription factor Arid3a, which modulates BCR signaling (17). Importantly, the study by Hardy also suggested that ectopic expression of Lin 28b, although sufficient to drive extensive development of B-1a cells from adult bone marrow precursors, was insufficient to reconstitute the normal BCR repertoire of B-1a cells (17). Thus additional differences between fetal and adult B cell development must exist that are independent of Lin28b/Let-7 and mediate development of B-1 cells and affect the B-1 cell repertoire.

A fetal versus adult developmental switch integrates known differences in the development and selection of B-1 and B-2 cells. However, additional complexities in the developmental paths of fetal/neonatal B-1 cells appear to exist. Early B cell precursors are present in multiple tissues, including the embryonic yolk sac, para-aortic splanchnopleura, fetal liver and neonatal bone marrow. In keeping with the hypothesis of a layered immune system, Montecino-Rodriquez and Dorshkind suggested (33) that each of these tissues, at different times in ontogeny, give rise to waves of B-1a cells: A first wave that is derived from non-hematopoietic precursors, potentially developing from hemogenic endothelium in the embryonic yolk sac (34–37); a previously identified wave of B-1 cell development from the AGM region (18) and the fetal liver; and finally an early wave of bone marrow-derived B-1 cells in the neonate (38–40) (Figure 1). A question emerging from these findings is, which of these precursors contribute to the B-1 cell pool that persists into adulthood.

Earlier studies showed that fetal-derived HSC had long-term reconstitution potential after adoptive transfer into lethally irradiated adult recipients (41). Using the receptor tyrosine kinase Flk2, previously shown to be expressed on fetal, but not adult, HSC with long-term multi-lineage reconstitution potential (41), Forsberg and colleagues generated a reporter mouse in which expression of Flk2 would lead to deletion of expression of one fluorochrome and expression of another (42). Using this system they could then follow the

fate of adoptively transferred HSC. The results demonstrated the continued presence of B-1 cells from both Flk2 expressing and non-expressing precursors, not all of which persisted into adulthood. Furthermore they demonstrated the preferential generation of peritoneal cavity B-1 cells from fetal-derived, transient, Flk2-expressing HCS (43). Thus, at least two distinct B cell precursors contribute to B-1 cell development (Figure 1).

## B-1 cell repertoire, selection and maintenance

The repertoire of B-1 cells in body cavities and the spleen reflects their developmental paths. A relatively large fraction of B-1 cells lack non-template-derived nucleotide (N-region) insertions at the VDJ joining ends, consistent with a lack of terminal deoxynucleotidyl transferase (TdT) expression in the fetus (44, 45). TdT induction is controlled by IL-7α receptor signaling, which is expressed at lower levels in fetal-B cell precursors (46) and is down-regulated following expression of a rearranged μ-heavy chain as part of the pre-BCR complex (47). Increased frequencies of N-region containing sequences of peritoneal cavity B-1 cells were reported for aging mice (48).

Hardy and colleagues identified the skewing of the B-1 cell repertoire to mostly autoreactive specificities early in their studies. 1–2% of B-1 cells in the spleen (49) and closer to 10% in the peritoneal cavity were found to bind to the head group of PtC, a cell surface phospholipid expressed on senescent red blood cells. V-gene usage by these PtC-binders was found to be highly restricted, but not monoclonal. In C57BL/6 mice PtC-binding BCR are predominantly encoded by VH11 or VH12 in conjunction with JH1 (49–51), whereas in BALB/c mice PtC-binders are encoded predominantly by VHQ52/JH4 (52). Self-reactivity, predominance and clonal relationship of anti-PtC specific B-1 cells in the body cavities suggested an antigen-specific expansion process (49, 50, 53). Consistent with that, repertoire studies demonstrated continued expansion of B-1a cells with that specificity over the first few months of life, including in mice kept germfree (45).

Another binding-specificity of B-1a cells, extensively studied by Hayakawa, Hardy and colleagues, is a carbohydrate epitope on the thymocyte glycoprotein “Thy-1” recognized by a natural IgM (54). Elegant studies by that group showed that mice lacking this self-antigen also lacked B-1 cells of that specificity (55, 56). This led them to propose that B-1 cells, similar to T cells, are positively selected based on their ability to bind to self-antigens. Further supporting strong self-antigen binding as a driver of B-1a cell development were studies that showed the lack of B-1a cell development in CD19-deficient mice (57), as well as in mice lacking either other co-stimulatory molecules (reviewed in (13)), or components of the NFkB signaling cascade (58), all defects that reduce BCR signaling. Thus, heavy usage of germline-encoded BCR, and the presence of relatively large clones of distinct, self-reactive B-1a cells develop in all mouse strains to shape the B-1 cell repertoire, possibly following BCR-stimulation by self-antigens.

Hardy and his group further aimed to understand the mechanisms for the repertoire skewing and distinct VH usage of B-1 compared to B-2 cells, which led them to study pre-BCR signaling (59). In pro/pre B cells the surrogate light chains, V-preB and lamda5, are expressed as binding-partners for the rearranged μ-heavy chain, with which they form the

pre-BCR (60). Pairing of the rearranged μ-heavy chain with surrogate light chains was shown to be critical for conventional B cell development in the bone marrow. In contrast, using a B-1a cell-restricted VH11 transgene, the dominant VH gene associated with PtC-binding, Hardy's group demonstrated that VH11 μ-heavy chains did not pair with surrogate light chains, yet they still enabled B-1 cell development (59). The data thus suggested that B-1 and B-2 cell development rely on distinct pre-BCR signals. Altered pre-BCR signaling was also suggested to play a role in the development of B-1 cells expressing a BCR specific for the hapten Arsonate in A/J mice (61). Such differential requirement for pre-BCR signaling provides a potential further distinction between fetal and adult-developing B cells that could lead to strong differences in VH-gene usage among B-1 and B-2 cells and explain their repertoire differences.

Together, the existing data suggest that self-reactivity acts as a positive selection step for B-1a cell development and/or expansion. Yet, positive selection for self-reactivity is anathema to our understanding of adaptive T and B cell development, a process that is expected to result in the development of a broadly reactive repertoire of antigen-receptors devoid of, or at least greatly curtailed for, self-reactivity. Nevertheless, the evidence for positive selection of B-1 cells is overwhelming as is the finding of B-1 cell-derived spontaneous "natural" IgM production that contributes broadly self-reactive natural antibodies. This points not only to a distinction between fetal and adult B cell development but also to fundamental differences between B-1 and B-2 cell function.

## Antigen-exposure modulates the B-1 cell pools

Despite the restricted *de novo* development of B-1 cells in adulthood, dramatic and dynamic changes of the B-1a cell repertoire in ontogeny have been documented for B-1a cells from peritoneal cavities and spleens via next generation sequencing (45). Importantly, those studies confirmed that the *de novo* appearance of B cell clones is restricted to the early phase of ontogeny, but also provided evidence that strong ongoing selection and/or clonal expansion shapes the repertoire of the adult B-1 cell pool. Similar repertoire changes among B-1a cells seem to develop over time even when mice were reared in the absence of microbiota, as the B-1a cell repertoire of SPF- and germfree-housed mice were indistinguishable (45).

In apparent contrast to the above studies with gnotobiotic mice, evidence for direct effects of foreign antigen-exposure on the B-1 cell repertoire came from studies by Kearney and colleagues. They showed that injection of α1-3 dextran permanently altered the repertoire of carbohydrate-reactive B cells when given within a short window after birth, but not later (summarized in (62)). This antigen is known to activate B-1 cells (63), and is expressed on *Aspergillus fumigatus* as well as house dust mites, While immunization did not change anti-α1-3 dextran serum IgM levels, it expanded the repertoire of polysaccharide-specific B cells and protected the mice from increased airway reactivity and signs of TH2-mediated airway disease (64). In addition, recent studies suggested that a portion of mainly B-1 cell-derived maternal IgG3 antibodies binds to gut microbiota and is induced in a TLR-dependent manner (65). While this could be explained by cross-reactivity of certain B-1 cells induced originally to self-antigens, or DAMPS, significant changes are reported in the B-1a cell

repertoire of mice around the time of weaning, when food-antigens and the gut microbiota undergoes extensive change (45).

Excluding the contribution of food antigens to the observed repertoire changes at various times after birth is experimentally very challenging. Even sterile food can contain PAMPS or other structural components that may influence B-1 cells in a TLR-dependent manner, without the presence of actual microbiota. This would be consistent with the findings by Koch and colleagues, who showed that maternal IgG2b and IgG3 antibody-development to gut microbiota requires TLR-signaling (65). On the other hand, weaning is also the time that the Flk2+ HSC, which give rise preferentially to B-1 cells, seem to be lost (43). Their disappearance could alter the B-1 cell pools. Future studies will be required to understand more fully the extent to which self and foreign antigens shape the B-1 cell repertoire during and after development and selection, and to understand what triggers B-1 cell clonal expansion.

Once established, B-1 cell numbers seem to be maintained through self-renewal, i.e. slow turnover. This was first suggested by studies demonstrating that mature IgM+ CD5+ B-1 cells from the peritoneal cavity were shown to fully reconstitute all B-1 cell compartments after adoptive transfer, a stem cell-like behavior (11, 23, 24, 66). The findings by Beaudin et al., which provided evidence for a developmentally-restricted HSC with preferential B-1 cell reconstitution potential (43), seem to support those early data by also suggesting that once established, the maintenance of the peripheral B-1 cell pool is achieved at the level of self-renewal rather than de novo development. If correct, then the continued presence of B-1 cells from those time-restricted HSC precursors would not require the continued presence of the HSC, but rather signals that trigger self-renewal of the mature B-1 cells. Identification of the signals and the biological context that cause B-1 cell expansion and/or maintenance should be an important future research goal for the field.

In this context it is interesting to note that the transfer of peritoneal cavity B-1 cells into neonatal mice rendered otherwise temporarily B-deficient, caused a near complete arrest of subsequent B-1 cell reconstitution from the bone marrow (23). Thus, B-1 cells or their products, particularly natural IgM antibodies, might be considered candidates for providing feedback regulation. Indeed, mice genetically engineered to lack secreted, but not membrane-bound IgM (sIgM), were originally reported to harbor increased frequencies of peritoneal cavity B-1a cells (67, 68), suggesting that the presence of secreted IgM might control B-1 cell development. Similarly, a recent study suggested that natural antibody production regulates the size of the B-1 cell compartment (69). However, with regards to the former study, multiple lines of evidence demonstrated that the accumulating CD5+ B cells in the body cavities of sIgM−/− mice, previously identified as B-1a, were not B-1 cells, but anergic B cells: Phenotypic analysis showed that they lacked surface expression of CD43, a hallmark of many albeit not all B-1 cells (70), that they did not express high levels of CD19, characteristic of B-1a cells, and they nearly completely lacked cells binding to liposomes containing phosphatidylcholine (PtC), a predominant B-1a cell specificity encoded by VH11 or VH12, genes that were not expressed by peritoneal cavity B cells in sIgM−/− mice (71). Functional analyses confirmed that most CD5+ B cells in the sIgM−/− mice were anergic B-2 cells (71). Conclusions from the earlier studies that reported expansion of B-1a cell

populations in the absence of sIgM (67, 68) were thus likely based on the somewhat subtle phenotypic differences between B-1a cells and anergic B cells. B-1 cell frequencies were either unaffected or even enhanced in mice that lacked the FcμR (72–74), although $FcμR^{-/-}$ mice had increased B-1 cell-derived serum IgM-levels. Therefore, sIgM does not appear to provide a direct negative feedback signal for B-1 cell development and/or expansion.

## B-1 cell regulation and functions

### Natural antibody production by B-1 cells

As outlined above, early work on B-1 cells, including by Hardy and colleagues, recognized that a major function of B-1 cells is the generation of “natural antibodies”, mainly IgM and IgG3 that react with both self (anti-Thy-1, anti-PtC) as well as foreign antigens (antigens on *S. pneumonia*, Influenza, *Borrelia hermsii*, *Salmonella* among others, reviewed in (12)). However, the exact mechanisms that induce and control natural antibody production remain incompletely understood. In contrast to B-2 cells, which require foreign antigen and often CD4 T cell help, for activation to antibody secretion, B-1 cells apparently “spontaneously” differentiate into natural antibody-forming cells (AFC). Given the observed dynamic changes to the repertoire outlined above, it seems that BCR-engagement to self- and/or environmental antigens drives these processes. This is supported by studies with gene-targeted mice that show enhanced BCR signaling and increased serum IgM levels in mice with deletions of a repressor of BCR-signaling, such as the two members of the sialic acid-binding immunoglobulin (Ig)-like lectin family, CD22 and Siglec G (75), as well as CD72 (13). Furthermore, B cell specific deletion of the FcμR, which was shown to increase BCR expression and BCR tonic signaling due to enhanced BCR transport from the trans-Golgi (72), resulted in increased numbers of CD138+ B-1 plasma cells in the spleen and higher concentrations of serum IgM (72). In all cases, however, both B-1 cell development and B-1 cell differentiation were enhanced. On the other hand, genetic ablation of IL-5Rα, or the lack of IL-5, resulted in selective reductions in natural serum IgM and IgG3 levels as well as reduced B-1 cell frequencies (76, 77). Given that injection of IL-5 induces strong IgM production by B-1 cells *in vivo* (76–78) and drives plasma cell differentiation of B-2 cells *in vitro* (79), it is tempting to speculate that IL-5 enhances B-1 cell differentiation to antibody-secreting cells. It will be important to identify the cellular source for this IL-5 and the biological processes by which IL-5 production regulates natural antibody production *in vivo*.

To better understand the regulation of natural antibody production and development of B-1 cell-AFC we studied the phenotype and developmental paths of IgM and IgG3-secreting B-1 cells. Consistent with previous studies (reviewed in (80)), we found spontaneous IgM production to be restricted to bone marrow and spleen in both SPF-housed as well as germfree mice (81, 82). The study identified two distinct populations of natural AFC: B-1 cell-derived CD19- CD43+ IgM+ plasma cells (B-1PC) and CD19+ CD43+ IgM+ B-1 cells (82). Both cell populations contributed significantly to natural antibody production. While B-1PC was the dominant IgM-AFC population in the bone marrow (67% of IgM-AFC), they contributed only about 25% of IgM-AFC in the spleen. Intriguingly, a subset of B-1 cells expressed J-chain and intracellular IgM, but neither expressed nor required Blimp-1 for maximal IgM or IgG3 secretion.

Further analysis suggested that these B-1 cells develop into AFC without terminal differentiation (82), which is consistent with reduced but not absent serum IgM levels and normal serum levels IgG3 in mice with a B cells-specific deletion of *prdm1*, the gene encoding Blimp-1 and shown to be a master regulator of B cell differentiation. The above-described effects of IL-5 on natural IgM and IgG3 may only affect the Blimp-1 dependent arm of natural antibody production, as IL-5 induces Blimp-1 expression (79), which could explain why natural IgM and IgG3 production is reduced but not gone in the absence of IL-5. The data may also explain previous contradictory results regarding the need for Blimp-1 in natural antibody secretion (83–85). Interestingly, a similar lack of Blimp-1 expression was noted also for a subset of IgM-secreting AFC in the shark (86). Lack of terminal differentiation might allow B-1 cells to rapidly adjust to changing needs for natural antibody production and/or it may enable continued self-renewal.

### Cytokine production by B-1 cells

B-1 cells were shown to regulate immunity by cytokine secretion. A subset of body cavity B-1 cells appears to constitutively express IL-10, which may imbue them with regulatory functions (87). B-1 cell-derived IL-10 production has been associated with attenuated responses to infection with Leishmania (88, 89) and artherosclerosis (90), and it appears to enhance B-1 cell expansion via induction of proliferation (91). The term “innate response activator (IRA) B cell” was coined by Swirski and colleagues for B-1a cells from the body cavities that migrated in response to inflammatory cues and accumulated in the spleen and lung early in sepsis. These cells generated not only IgM, but also GM-CSF and IL-3, importantly affecting the inflammatory responses (92–94).

Indeed, migration of B-1 cells from the body cavities to secondary lymphoid tissues and their activation to IgM secreting cells is a common outcome of activation of peritoneal and pleural cavity B-1 cells in response to cytokines and various PAMPS (95–99) (Figure 2). Whether all B-1a cells can respond to inflammatory cues with cytokine production remains to be determined. Together the data indicate the functional heterogeneous nature of the peripheral B-1 cell pool, which serve to both attenuate inflammatory responses and control infections by generating broadly reactive natural antibodies as well as cytokines.

## B-1 cells and CLL

Continuing from the earliest studies that aimed to identify the CD5+ non-malignant counterparts to the CD5+ B-CLL, Hardy, Hayakawa et al continued to investigate the potential link between B-1 cells and the development of this common leukemia emerging in aging individuals. B-CLL is characterized not only by CD5 expression but also by a skewed BCR repertoire encoding auto- and poly-reactive antibodies (100), all features they have in common with B-1 cells. In their most recent studies the group identified a non-mutated BCR CDR3-region in B-1 cells of mice that encoded binding to myosin IIA, a specificity recurrent among human B-CLL (101). Interestingly, they observed that B-1 cells with that specificity could expand greatly with age and eventually developed into CLL. The data suggest that the inherent ability of B-1 cells for self-renewal, and a constant activation trigger provided by self-antigen recognition may predispose B-1 cells and their potential

homologues in humans towards malignant transformation. In addition to the studies outlined above that demonstrate the importance in BCR-signaling for B-1 cell activation and natural antibody production, the data further suggest that the presence of self-antigens provide potent and continued stimulation for B-1 cells including after development and selection, driving clonal expansion, differentiation and potentially, in rare instances, malignant transformation.

## Human Orthologs of murine B-1 cells

Early studies demonstrated that similar to mice, human cord blood and fetal spleens also contain higher frequencies of CD5+ B cells than adult spleen and blood (102). In 1987 Hardy et al. (103) and Casali et al. (104) simultaneously reported on the presence of CD5 positive B cells in human blood that dominated the cord blood B cell population of neonates and they reported an increase of CD5+ cells in patients with autoimmune diseases. Furthermore, we now understand that in humans, as in mice, a developmental switch from fetal to adult-like B cell hematopoiesis occurs that is controlled by Lin28b/Let7 (16), thus strongly suggesting that human fetal-derived B-1 cell orthologues exist. However, given the much higher frequencies of CD5+ B cells in adult humans (17% of PBMC) versus mice (0.5% of PBMC), and the fact that not all B-1 cells in mice express CD5, it is clear that CD5 alone does not mark B-1 cells. Rather CD5 expression seems to mark B cells having a certain degree of autoreactivity both, among fetal-derived B-1 cells and postnatal-derived (anergic) B-2 cells (6).

The exact nature and the phenotype of the human B-1 cell orthologs, however, has remained a topic of much study and controversy. This includes the most recent efforts by the group of Dr. Thomas Rothstein that led them to propose that a population of PBMC with the phenotype CD20+ CD27+ CD43+ and an ability to spontaneously secrete self-antigen reactive IgM is the human B-1 cell (105). However, the results of that study have been vigorously questioned (106–108) and further studies are required. The discovery of the Lin 28b-dependency of human fetal B cell development (16) might facilitate such studies.

## Conclusions

The work by Randy Hardy, Kyoko Hayakawa and their team has provided multiple seminal contributions to the field of B cell and B-1 cell biology. Their original findings of a fetal-derived B cell population that persists into adulthood and their continued quest to understand and define these cells in the context of their development has moved the field of B-1 cell biology in a way few others have done. They remained steadfast in their quest even at times when the sheer existence of these cells was questioned. Despite the continued advances made, much remains to be explored about the mechanisms and signals that control B-1 cell activation and differentiation and to more fully understand the breadths of their functions in health and disease.

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I apologize for not being able to include all relevant work, due to space limitations.

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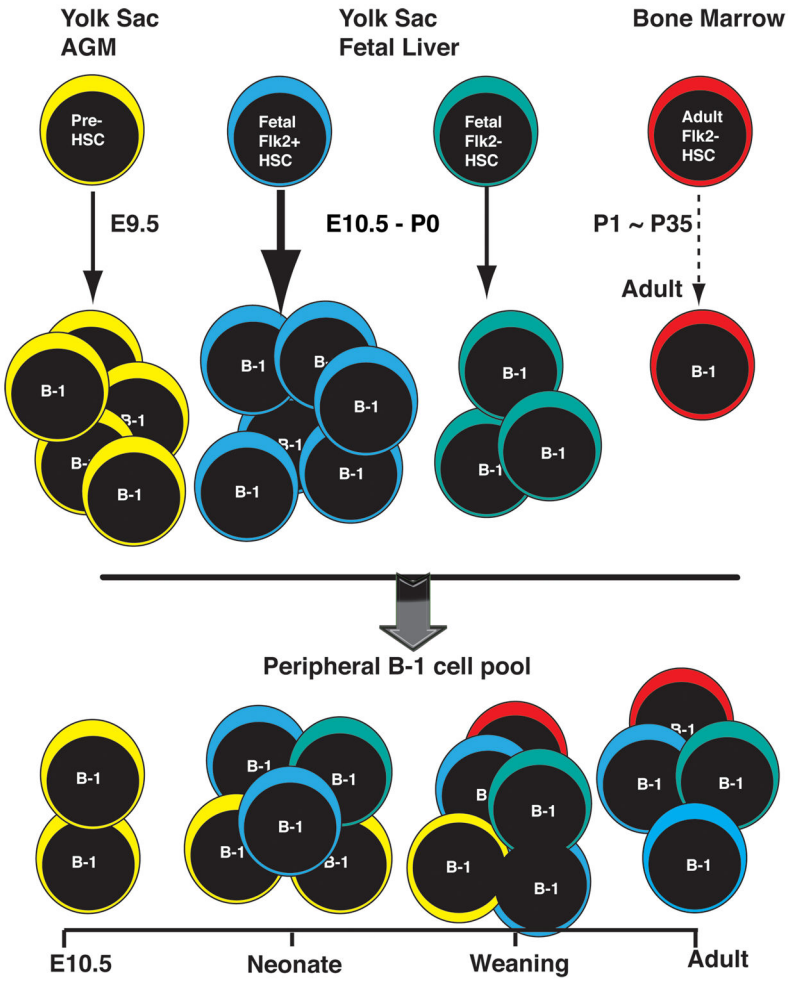


**Figure 1. B-1 cells arise in waves from multiple precursors**

Recent evidence suggests that multiple precursors give rise to waves of B-1 cells emerging mainly during embryonic development and at or shortly after birth. Earliest B-1 cell precursors have been found in the pre-HSC compartment of the embryonic yolk sac and are giving rise only to B-1 but not B-2 cells (36). Lineage-tracing studies indicated the presence of at least two distinct fetal HSC present in yolk sac and fetal liver, differing in expression of Flk2 (43). Flk2-expressing HSC have the highest B-1 cell reconstitution potential, but are transiently expressed only between E10.5 and about 2 weeks after birth. Adult HSC in the bone marrow have poor B-1 reconstitution potential. The distinct contributions of each of these heterogeneous precursor populations shape the peripheral B-1 cell pool and could shape also the functionality of this cell population.

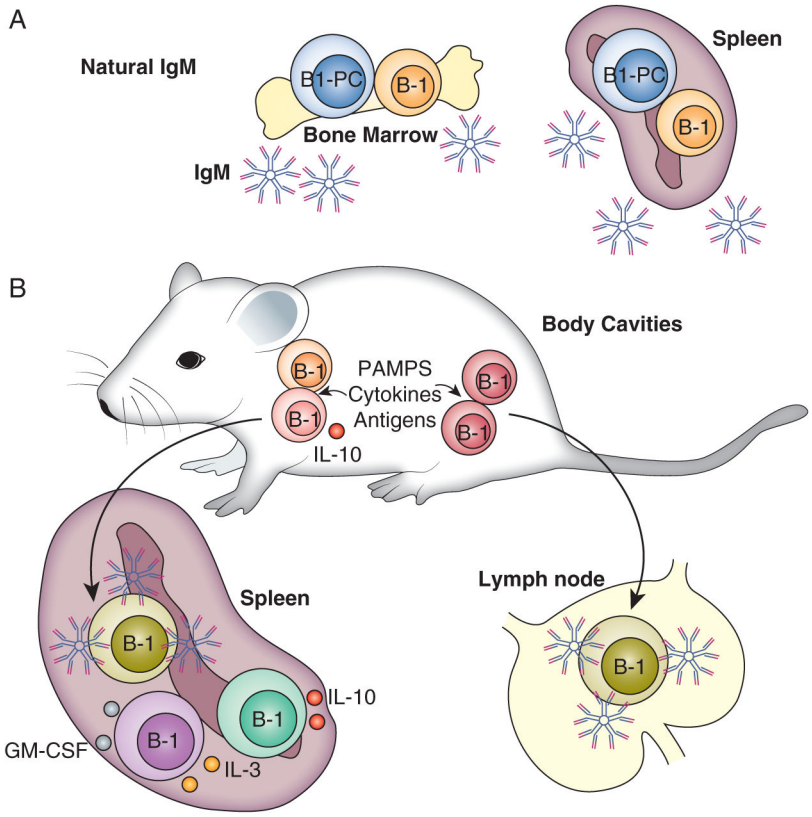


**Figure 2. Distribution and function of B-1 cells**

(**A**) In steady state B-1 cells and B-1 cell-derived plasma cells in the spleen and bone marrow generate natural antibody, mostly IgM (81, 82). (**B**) B-1 cells make the majority B cell population in the peritoneal and pleural cavities. There they do not secrete but are activated by various innate signals, such as LPS (98, 109), IL-5, IL-10 (78) and Type I IFN (96) to rapidly migrate to secondary lymphoid tissues, such as lymph nodes and spleen, where they begin to secrete antibodies and/or cytokines (92, 96).