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Human B-1 cells take the stage

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Abstract

B-1cells play critical roles in defending against microbial invasion and in housekeeping removal of cellular debris. B-1cells secrete natural antibody and manifest functions that influence T cell expansion and differentiation and in these and other ways differ from conventional B-2 cells. B-1-cells were originally studied in mice where they are easily distinguished from B-2cells, but their identity in the human system remained poorly defined for many years. Recently, functional criteria for human B-1cells were established on the basis of murine findings, and reverse engineering resulted in identification of the phenotypic profile, CD20⁺CD27⁺CD43⁺CD70⁻, for B-1cells found in both umbilical cord blood and adult peripheral blood. Human B-1cells may contribute to multiple disease states through production of autoantibody and stimulation/modulation of T cell activity. Human B-1cells could be a rich source of antibodies useful in treating diseases present in elderly populations where natural antibody protection may have eroded. Manipulation of human B-1cell numbers and/or activity may be a new avenue for altering T cell function and treating immune dyscrasias.

Keywords

B-1; B cells; lymphocytes; antibody

Introduction

The immune system is an intricate and multifaceted device. It is comprised of numerous cell types and structures that respond in a measured way to microbial assaults with actions that arise appropriately, end promptly, and, for the most part, do not damage normal tissue, all the while maintaining tolerance for self structures and symbiotic microorganisms. B cells are the key effectors of serological immunity, responsible for generation of life-saving antibody in response to microbial invasion (and intentional vaccination), and possibly malignant transformation. In the past, B cells have often been envisioned as simple antibody production facilities responding to antigen binding and signals derived from other cell types, although they are clearly capable of presenting antigen and influencing T cells, among other

activities (1). In recent years the marked clinical success of B cell depletion therapy with anti-CD20 antibody in several autoimmune diseases (2–8) has suggested that B cells may be prime movers in some and possibly many immune dyscrasias. This has evoked considerable renewed interest in the physiological and pathological functions and roles of B cells.

B cells are divided into separate populations, each recognized by a particular phenotypic array of surface markers. These include transitional, follicular, germinal center, and memory (isotype switched and unswitched) B cells. Together these populations constitute conventional B cells that react adaptively to new antigenic challenges with antibody responses produced by differentiated plasmablasts and plasma cells that are generally characterized by affinity maturation (9). Separate and apart from these conventional populations of B cells stand B cells that can be classified as elements of the innate immune system: marginal zone (MZ) B cells that specialize in responses to blood-borne pathogens and may represent a type of memory B cell (10, 11), and B-1 cells, that constitutively and spontaneously secrete “natural” antibody and influence other elements of immune activity (12).

Identification of B-1 cells, a relatively small but unique B cell population, emerged from the initial finding over 3 decades ago that malignant cells of human and mouse B cell leukemias and lymphomas incongruously express the T cell antigen, CD5 (13, 14). It was subsequently determined that CD5 expression is a characteristic feature of a small population of normal B cells (15, 16) that may be the cell of origin for, or transform into, B cell malignancy. CD5 expression on CD5⁺ B cells is dim, much less than that of T cells, for which reason CD5⁺ B cells can be identified by flow cytometry with its use of photomultiplier detectors but for all practical purposes cannot be detected by fluorescence microscopy. In the mouse system B-1 cell expression of CD5 correlates with expression of several other distinctive markers (IgM^{hi}IgD^{lo}CD45^{lo}CD23^{lo}–CD43⁺; also mostly CD11b⁺ in the peritoneal cavity and CD9⁺ shared with MZ B cells) such that B-1 cells are readily identifiable and cleanly separable from conventional B (B-2) cells and can thus be individually examined (Table 1). Of note, two kinds of B-1 cells have been described in mice: B-1a cells, with the phenotype noted above; and, B-1b cells, that bear all B-1a cell surface markers except CD5. B-1b cells are regulated separately from B-1a cells, fulfill distinct immune functions, and appear to develop in concert with B-2 cells (17–22). Intensive study in the murine system has demonstrated that B-1a cells (denoted B-1 cells in the foregoing except where specified) are quite different from conventional B-2 cells in many ways, as discussed in the next 5 sections, after which the nature of human B-1 cells is addressed in the remaining sections.

B-1 cells secrete protective natural antibody

Mouse B-1 cells are predominantly located in the peritoneal cavity, but are also found in the pleural cavity, the spleen, and the bone marrow, with little representation in lymph nodes or blood (23, 24). The principal function unique to B-1 cells is spontaneous, constitutive secretion of antibody. This antibody accumulates as baseline or resting immunoglobulin, termed *natural antibody*, that appears in the absence of infection or immunization. It is predominantly IgM, and it is estimated that 80–90% of resting serum IgM, and perhaps 50% of resting serum IgA (the major isotype of switched B-1 cell immunoglobulin) is derived

from B-1 cells (25–27). More specifically, serum IgM is preferentially produced by B-1 cells in the spleen that secrete much more antibody than peritoneal B-1 cells, and by B-1 cells in the bone marrow (28, 29). There is evidence that peritoneal B-1 cells migrate to the spleen under certain activation conditions and upregulate antibody secretion there (30–33), although it is possible that splenic B-1 cells represent a separate, high-secreting subpopulation. It has also been suggested that the splenic environment is critical to B-1 cell generation (34, 35). Recent results suggest that for at least one antigen, and maybe more, B-1 cells in the spleen are activated by immunization in a T-independent fashion to become memory B cells that reside in the peritoneal cavity and, upon re-challenge, return to the spleen where they differentiate to antigen-specific, high-secreting plasma cells (36, 37). These findings raise the possibility that many B-1 cells located in the peritoneum may represent memory cells, a status that had not been thought to exist among B-1 cells.

Mouse B-1 cell natural antibody is distinctive and differs from conventional B-2 cell adaptive antibody in many ways, including repertoire, structure, and development. Natural antibody tends to be polyreactive, autoreactive, and anti-microbial at relatively modest affinity (38–41). B-1 cell natural antibody is highly effective, such that animal recovery from a number of experimental infections depends on the joint action of B-1 and B-2 cell-derived antibodies as mice with only B-1 cells or only B-2 cells do poorly (22, 42–45). The success of B-1 cell natural antibody may depend, in part, on polyreactivity, which provides the means for a single antibody to heterologate different, each possibly widely spaced, surface antigens, thereby increasing effective avidity (46). Alternatively, or in addition, B-1 cell natural antibody may succeed as a result of the specific nature of putative conformational changes in the Fc region following antigen binding, in that effector function typically depends on engagement of other cell types and immune elements. Because B-1 cell natural antibody is secreted spontaneously and constitutively, it forms a pre-existing shield against infection that provides protection during the lag period required for germinal center formation and adaptive antibody production (Fig 1). A major component of natural antibody recognizes phosphorylcholine (PC), a key and invariant constituent of Gram positive microbial pathogen membranes, including the *Streptococcus pneumoniae* bacterial cell wall, and is often encoded by V_HS107.1 (45, 47).

Anti-PC antibodies do not just recognize PC on pneumococci, as PC is also present on a number of other bacterial pathogens, apoptotic cell membranes, and oxidized lipids (48). Another important component of natural antibody recognizes phosphatidylcholine (PtC), a key constituent of senescent red blood cell membranes, that is encoded primarily by V_H11 and V_H12 (49, 50). These and other examples of autoreactivity have given rise to the concept that a second, parallel function of B-1 cell natural antibody lies in housekeeping or homeostatic activity that speeds elimination of dead and dying cells and cellular debris. In so doing, potentially inflammatory and/or toxic molecules are removed before damage in the form of immune cell priming or direct tissue injury can occur. This anti-inflammatory effect can involve deposition of complement and suppression of accessory cell function (51). Thus, mice lacking natural IgM antibody are prone to accelerated development of autoantibodies and more severe autoimmune disease (52), presumably because antigens and inflammation associated with apoptotic cell debris stimulate B-2 cell responses when not properly cleared

in a timely fashion. Further, B-1 cell-derived natural IgM has been shown to be protective against atherosclerosis in a mouse model of cardiovascular disease, presumably as a result of efficient disposal of oxidized low density lipoprotein (LDL) and similar pathogenic lipids (53–55). Thus natural antibody produced by B-1 cells is considered to fulfill two important functions: immediate defense against microbial pathogens and housekeeping removal of cellular debris.

The structure of B-1 cell natural antibody differs markedly from B-2 cell adaptive antibody in being more germline-like. In comparison to B-2 cell antibody, B-1 cell antibody contains little or no somatic hypermutation and much reduced, or non-existent, N-region addition (49, 56–58). This means B-1 cell antibodies more or less exactly reflect germline $V_H D_H J_H / V_L J_L$ sequences. A good example of this is the prototypic, pneumococcal-protective anti-PC antibody, T15, that is completely germline and contains no somatic mutation or N-addition (47). Somatic mutation and N-addition would be expected to increase repertoire diversity by altering antibody structure, but at the same time could change the original binding specificity of the germline sequence. In fact, enforced N-addition in transgenic mice results in loss of T15 antibody and loss of protection against pneumococcal infection (59). Notably N-region addition is determined during $V_H-D_H-J_H$ and V_L-J_L joining early in B cell development and requires the enzyme terminal deoxynucleotidyltransferase (TdT), which adds non-templated nucleotides (60); somatic mutation occurs in the course of antigen-triggered expansion of mature B-2 cells during residence in transient, specialized germinal center structures (61). Because B-1 cell antibodies tend to reflect sequences delineated in the genome largely without alteration, and because B-1 cell antibodies play a critical role in anti-microbial defense, it has been suggested that the B-1 cell repertoire is “tuned” over evolutionary time, obeying Darwinian precepts such that sequences functioning to promote survival are retained (47). This then differs from the situation with B-2 cell antibodies in which the principal criterion for retention is affinity. In this view B-1 cell antibodies may represent the very best antibodies for functional protection against some infectious pathogens and, by extension, the very best antibodies for disposing of apoptotic cellular constituents.

B-1 cells constitute a separate B cell lineage

The origin of B-1 cells has been the subject of much debate over many years' time, involving two distinct hypotheses of lineage versus differentiation. Early adoptive transfer experiments indicated that adult bone marrow does not reconstitute the B-1 cell population; instead, reconstitution only occurs if/when B-1 cells (e.g., peritoneal B-1 cells) are transferred along with bone marrow cells (62). However, both B-1 and B-2 cells are generated after adoptive transfer of fetal liver cells. Moreover, cells from omental tissue give rise predominantly to B-1 cells (63). These results suggest that B-1 cells only arise early in ontogeny and cannot be generated later in life. The early appearance of B-1 cells is the reason this population is designated “B-1” and conventional B cells that arise later are termed “B-2” (64). Consistent with an early, restricted origin for B-1 cells is the special property of self-renewal, in which mature, surface immunoglobulin-bearing B-1 cells give rise to their own progeny, unlike B-2 cells that derive from immature, immunoglobulin-negative progenitors (65, 66). All together these findings suggest that B-1 cells emerge early

in the life of the animal as a separate and distinct lineage that maintains itself through self-renewal without further input from other sources. The sequential timing of B-1 and B-2 cell development suggests an ontological “switch” in development from production of B-1 cells to production of B-2 cells (67). Similar changes in T cell development (from $\gamma\delta$ to $\alpha\beta$ antigen receptors) and red blood cell development (from fetal to adult hemoglobin) support the hypothesis that a generalized ontological switch involving multiple hematopoietic lineages occurs around the time of birth (68). Lin28b may dictate that switch, and results with enforced expression of Lin28b support the notion that ontological switching occurs (69).

An alternative to this paradigm was advanced by the finding that CD5 expression is induced on conventional, B-2 cells after stimulation by BCR crosslinking (70). This suggests that CD5⁺ B-1 cells are generated by stimulation of B cells at a relatively mature stage (71). Strong support for this idea comes from the construction of immunoglobulin transgenic mice in which the BCR is fixed as a typical anti-phosphatidylcholine specificity; B cells in these mice (V_H12V_K4 and V_H11V_K9) are overwhelmingly phenotypic B-1 cells (72, 73), implying that the events responsible for producing B-1 cells occur subsequent to immunoglobulin rearrangement. The failure of B-1 cell development in animals with dysfunctional mediators of BCR signaling, such as *xid* animals with mutant Btk (23, 24, 74), lends further support to the idea that BCR signaling is necessary for B-1 cell development, which occurs after the BCR is expressed. Along the same lines, mutation of SHP tyrosine phosphatase involved in modulating BCR signaling cascades leads to increased numbers of B-1 cells (27, 75, 76). Because the B-1 cell repertoire is selected for certain specificities like PtC and PC, taken together these results infer that B-1 cells arise not from a separate lineage but from a differentiative process triggered by ligand interactions with B cell receptors capable of recognizing certain self-antigens (77).

This controversy regarding lineage and differentiation was resolved by the discovery of a specific progenitor that gives rise to B-1 cells, but not B-2 cells, and phenotypes as lineage-negative (lin⁻) CD45^{lo/-}CD19⁺, in contradistinction to the conventional B-2 cell developmental pathway in which CD45 expression precedes expression of CD19 (78, 79). This lin⁻CD45^{lo/-}CD19⁺ B-1 cell progenitor arises from yolk sac and para-aortic splanchnopleura tissues and is abundantly present in mouse fetal liver (80); although a small number of B-1 cell progenitors are found in adult bone marrow, these do not appear to function as efficiently as phenotypically similar progenitors present earlier in life (81). B-1 cell progenitors are also present, albeit quiescent, in adult spleen but can respond to inflammation (80, 82). Although it cannot be said with certainty that every B-1 cell derives from the identified progenitor, these findings clearly indicate that at least some, and likely most if not all, B-1 cells constitute a separate lymphocyte lineage derived from a distinct, surface immunoglobulin negative progenitor. Commitment to the B-1 cell lineage appears to occur prior to the acknowledged CD45^{lo/-}CD19⁺ B-1 cell progenitor, at least as early as the common lymphocyte precursor (CLP) stage (18, 81).

These results also support the notion that B cell development occurs in waves, with B-1 cells generated initially and B-2 cells after that. However, B-1 cells can be divided based on expression or lack thereof for CD11b, CD25, PD-L2, and PC-1, among other surface

antigens (17, 83–85, 191), and thus it remains possible that a particular subpopulation could be generated through a distinct pathway separate from the identified B-1 cell progenitor. Of note, earlier work had indicated that developing B-1 cells are affected differently than developing B-2 cells by strong BCR binding of self-antigens (86–89); whereas B-2 cells are deleted, B-1 cell development is promoted (88, 90, 91). Along the same lines, strong signaling for B-1 cell development may be produced by the BCR autonomously in the absence of antigen (92). This positive response specifically in B-1 cells may explain the necessity for intact BCR signaling during B-1 cell development and, along with other functional differences between B-1 and B-2 cells, can now be ascribed to the distinct lineages of which these cells are the mature, differentiated end products.

B-1 cell natural antibody changes with advancing age

Although reduced in number and function, bone marrow B-1 progenitors do, in fact, generate B-1 cells after adoptive transfer (93–96). However, the B-1 cells produced by adult bone marrow, though authentic in phenotype and function, differ in one important respect from the native B-1 cells that populate young animals: bone marrow-derived B-1 cells express antibodies that contain abundant N-region additions, in contrast to native B-1 cells from young animals (94, 95). In fact, the number of bone marrow-derived B-1 cells that lack N-addition at both the D_H–J_H and V_H–D_H junctions approximates the very low level present in native B-2 cells from young animals (94). At the same time, among wild-type mice, B-1 cells in older animals express antibodies that contain much more N-addition as compared to B-1 cells in younger animals (56, 94); in other words, as mice age, the level of N-addition in B-1 cell antibody increases. Taken together, these results infer that bone marrow progenitors give rise to mature B-1 cells which over time become part of the native B-1 cell pool. In other words, the early B-1 cell population is not completely perpetuated (via self-renewal) without change, but rather appears to accumulate bone marrow immigrants that, with increasing age, alter the overall composition of the native B-1 cell pool. Such changing composition may actually amount to erosion of beneficial germline-determined specificities because the abundance of N-addition in bone marrow-derived B-1 cell antibodies can alter CDR3 combining site structures. If germline-like natural antibody is evolutionarily tuned for efficacy, then with increasing age B-1 cell natural antibody may become less and less effective in carrying out its key functions of microbial defense and debris disposition. This process could play a role in the increased susceptibility of older individuals to a variety of diseases whose incidence is age-related, such as the aforementioned pneumococcal pneumonia and cardiovascular disease. Going further, the existence of human natural antibodies against pathogenic amyloid peptides and tumor-associated gangliosides and other antigens (97–100) raises the possibility that erosion of the B-1 cell repertoire could degrade defense against neurodegenerative and neoplastic diseases.

Chronically signaling B-1 cells stimulate and regulate T cell responses

Beyond natural antibody secretion and its key role in infection remediation and apoptotic housekeeping, B-1 cell activities affect other elements of the immune system in both stimulatory and suppressive ways. B-1 cells strongly induce activation and proliferation of naive T cells across an allogeneic barrier, indicating that B-1 cells are efficient antigen

presenting cells, as effective as dendritic cells (101–103). Among mouse B-1 cells, the PD-L2⁺ subpopulation is particularly adept at stimulating T cell expansion and particularly engaged in producing autoantibodies (104). T cell stimulation by B-1 cells is evident directly *ex vivo* without any activation or further manipulation of B-1 cells, and can be attributed at least in part to constitutively elevated expression of the co-stimulatory molecule, CD86 and, to a lesser extent, CD80 (101, 102). Upregulated expression of CD86 joins several other characteristics of “resting” B-1 cells that are similar to features of activated B-2 cells, including upregulated expression of phosphorylated ERK, phosphorylated STAT3, NF-AT, and CD44 (105–109). Constitutively phosphorylated ERK would seem incongruous because ERK phosphorylation occurs downstream of BCR signaling, yet it has long been known that BCR signaling is impaired in B-1 cells; in B-1 cells, unlike B-2 cells, BCR triggering fails to induce cellular proliferation or NF- κ B activation (110–112). Further, signaling mediators between BCR and ERK are not constitutively phosphorylated in B-1 cells, further clouding the origin of pERK. However, BCR signaling intermediates are indeed responsible for constitutive ERK phosphorylation in B-1 cells, as evidenced by recent work showing that various signalosome inhibitors quickly eliminate phosphoERK, and that phosphatase inhibitors result in accumulation of phosphorylated forms of BCR signaling intermediates (113). It is speculated, then, that B-1 cells are chronically signaling as a result of self-recognizing BCR specificities or autonomous BCR activity, but that signaling for phosphorylation of most intermediates is exactly matched by upregulated phosphatase activity, except in the case of ERK, and so becomes apparent only in the presence of phosphatase inhibition. This more active physiology and enhanced signal modulation may explain, as a form of tachyphylaxis, why acute BCR triggering has little effect on NF- κ B activation. Because BCR signaling induces CD86 expression (114), these studies suggest a mechanism for baseline upregulated expression of CD86 by B-1 cells, and indeed inhibition of the signalosome element, PI-3K, produces a rapid decline in B-1 cell CD86 which is reversed upon removal of the inhibitor (113). Thus, in this case, lineage and BCR structure/specificity appear to encode destiny, producing chronic signaling that leads to increased CD86 expression that contributes to strong antigen presentation and efficient T cell stimulation.

Beyond stimulating T cell expansion, B-1 cells can influence T cell function. B-1 cells induce differentiation of naive, CD4⁺ T cells to IL-17–expressing pro-inflammatory Th17 cells, whereas B-2 cells induce T cell differentiation to regulatory T (T_{reg}) cells (102, 104, 115). Although no direct mechanism for B-1 cell-induced Th17 cell differentiation is known, co-stimulatory molecules CD80 and CD86 play a role (albeit a smaller role than in stimulation for T cell expansion), as does CD44 through binding of osteopontin (115). It is also possible that cytokine secretion by B-1 cell–stimulated T cells, or by B-1 cells themselves, could play a role (102, 104). Induction of Th17 cell differentiation is not unique to B-1 cells, as B-2 cells can do the same, but only after activation, which upregulates CD80, CD86 and CD44, among other molecules (115).

These stimulatory effects contrast with B cell mediated suppression, identified early on as a key element in recovery from experimental autoimmune encephalomyelitis (EAE) (116). The beneficial effects of B cells in modulating EAE result from IL-10 (117). Inasmuch as B-1 cells have long been known to secrete IL-10 (118), the property of T cell regulation by

B cells would appear to be another unique characteristic of B-1 cells, not shared with unstimulated B-2 cells. However, it is not clear that regulatory B cells secreting IL-10 and B-1 cells are synonymous groupings. IL-10 secreting B cells have been reported to express CD5 and high level CD1d, and to consist mostly of B-1 cells but to also overlap phenotypically with some MZ B cells (119). Alternatively, IL-10 secreting B cells have been reported to express CD23 and CD21 and high level CD1d, and to consist primarily of T2-MZ precursor B cells (120). Adding to confusion regarding identity is the possibility of other mechanisms for B cell mediated suppression, including secretion of TGF- β ; expression of FasL, TRAIL and PD-L2; and production of adenosine (84, 121–126). Although it is clear that B cells can exert suppression, and it seems likely that B-1 cells are responsible through IL-10 secretion, the role of other actors and other mechanisms remains uncertain, including the role of B-2 cells following activation.

Considering that B-1 cells are capable of presenting antigen to T cells and stimulating T cell expansion, inducing Th17 cell differentiation, and suppressing T cell activity, they would appear to be in a position to heavily influence the nature and direction of T cell responses, depending on whether stimulatory or suppressive activities dominate (Fig. 2). It may be speculated, then, that excessive or inappropriate B-1 cell activity may be involved in the pathophysiology of autoimmune dyscrasias by presenting autoantigen or stimulating effector T cell expansion, inducing Th17 cell differentiation, and/or failing to suppress T cell function at the proper time and place. The role of B-1 cells could even include production of pathological autoantibodies, if the infrequent events of isotype switching and somatic mutation are determinative for the infrequent occurrence of autoimmune dyscrasias. Evidence implicating B-1 cells in some autoimmune animal models supports this connection (101, 103, 104, 127).

B-1 cells are distinguished from B-2 cells in other ways

B-1 cells differ from resting B-2 cells in a number of characteristics beyond surface antigen expression, natural antibody secretion, chronic intracellular signaling, and, T cell stimulation, differentiation and suppression. Some additional features that have been reported to distinguish B-1 cells in comparison to B-2 cells include larger overall size; enhanced survival *ex vivo*; resistance to Fas-mediated apoptosis; unique expression of gene transcripts and proteins; distinct signaling characteristics, including responsiveness to phorbol ester and increased intracellular Ca⁺⁺; and, developmental independence from BAFF/BLys and IL-7 (Table 2) (108, 109, 111, 128–142). These findings have resulted for the most part from comparisons between peritoneal B-1 cells and splenic B-2 cells, and it should be noted that splenic B-1 cells are not in every respect the same as peritoneal B-1 cells, (73, 106). However, the range and extent of B-1 cell characteristics that differ from B-2 cells are consistent with the current view that B-1 cells represent a separate lineage borne of a distinct progenitor.

Human CD5⁺ B cells are not synonymous with human B-1 cells

Following the initial finding that human chronic lymphocytic leukemia (CLL) malignant B cells express CD5, the presumed normal counterpart, a CD5-expressing B cell, was

identified in healthy individuals (143). Human CD5⁺ B cells were reported to produce polyreactive autoantibodies much like mouse B-1 cells, to be expanded in autoimmune rheumatoid arthritis, and to represent 15–30% of circulating B cells and up to 75% of umbilical cord blood B cells (144–149). However, it is now clear that CD5 is not an exclusive marker of B-1 cells in many species, especially in *Homo sapiens*, where CD5 is expressed on pre-naïve, transitional, and activated B-2 cell populations (150–154). This somewhat promiscuous expression of CD5 on various B cell populations may explain the relatively large numbers of circulating CD5⁺ B cells identified in early human studies.

The uncertainty regarding markers for B-1 cells in non-Mus species raised the possibility that this population is a limited idiosyncrasy and possibly a feature not found in the wild but restricted to inbred strains of mice (155), and therefore of little clinical significance. This minimalist view was counterbalanced by the implied role of CD5⁺ B cells in human disease and the important physiological functions extrapolated for human B-1 cells on the basis of murine studies.

Human B-1 cells are identified through a functional reverse-engineering strategy

To identify human B-1 cells, the typical discovery vector from recognizing phenotype to ascertaining function, which served to identify and characterize mouse B-1 cells, was reversed. In this approach, several distinctive functional features of mouse B-1 cells were collected as a set of index characteristics that any putative human B-1 cell population should fulfill to be validated as such. These key features are: (1)spontaneous IgM secretion; (2)efficient T cell stimulation; and, (3)chronic intracellular signaling. Spontaneous IgM secretion, assessed by ELISPOT assay, was used for initial screening and CD20 was used to mark B cells because it is lost during mature B cell differentiation (156), unlike CD19, and thus insures that immunoglobulin-secreting plasmablasts and plasma cells are excluded. Various populations of sort-purified CD20⁺ B cells expressing different combinations of surface markers were isolated and tested for function in vitro, beginning with umbilical cord blood, because in mice B-1 cells predominate early in ontogeny. A particularly interesting, albeit small, population of CD27⁺ B cells was identified that had previously been noted as infrequently present in cord blood (157). Cord blood CD27⁺ B cells are not memory B cells, because immunoglobulin sequencing shows a dearth of somatic mutation, unlike adult peripheral blood CD27⁺ B cells (158). Instead, cord blood CD27⁺ B cells spontaneously secrete IgM and fulfill the other criteria for B-1 cell status (159, 160). They also express CD43, a surface antigen that is expressed on mouse B-1 cells (161). Thus, umbilical cord blood B cells that co-express CD20, CD27, and CD43 fulfill the three-fold criteria for B-1 status and hence by that functional definition can be said to represent human B-1 cells.

The same phenotype, CD20⁺CD27⁺CD43⁺, recognizes a small number of B cells in adult peripheral blood samples. However, unlike cord blood, expression of CD27 and CD43 in adult blood do not coincide; rather, CD43 identifies a small subset within the overall CD27⁺ B cell population, heretofore presumed to consist entirely of memory B cells. This previously unrecognized population of CD20⁺CD27⁺ B cells that co-express CD43 spontaneously secretes IgM, efficiently stimulates T cells, and evidences chronic

intracellular signaling, and thus parallels its cord blood counterpart in fulfilling the criteria for B-1 cell status (Table 1) (159, 160). These CD20⁺CD27⁺CD43⁺ B cells preferentially bind PC, as well as the DWEYS peptide mimotope of DNA (162), two typical mouse B-1 cell specificities, unlike CD20⁺CD27⁺CD43⁻ memory B cells and CD20⁺CD27⁻CD43⁻ naive B cells, which fail to do so, lending further support to their designation as B-1 cells. Of note, CD43 is inducible on conventional B-2 cells following some forms of stimulation, but this is not the mechanism for CD43 expression on B-1 cells because stimulated, CD43⁺ B-2 cells always express typical activation markers CD69 and CD70, whereas CD43⁺ B-1 cells express neither (159, 160). Unlike the B-1 cell marker CD43, CD5 is found on the majority of, but not all, human B-1 cells, whereas most CD5⁺ B cells are other than B-1 cells, in keeping with the different human B cell types known to express CD5 (159, 160).

As noted above, CD27 has been considered to constitute a marker for memory B cells in the human system (158, 163). The finding that a portion of CD27⁺ B cells are B-1 cells raises questions regarding the validity of conclusions reached during studies of what is now shown to be a heterogeneous population. The observation that CD27⁺CD43⁺ B-1 cells secrete antibody that recognizes PC, a major antigenic determinant of pneumococci, suggests the possibility that previous work indicating that IgM⁺CD27⁺ memory B cells are responsible for controlling pneumococcal infection (164, 165) may actually represent the activity of B-1 cells co-existing with memory B cells in the CD27⁺ B cell pool. Thus, it may be necessary to re-evaluate the features of memory B cells, particularly IgM memory B cells, after removal of CD27⁺ B cells that also express CD43.

Determination of human B-1 cell frequencies can be difficult

Although confocal microscopy has verified the existence of B cells that co-express CD20, CD27 and CD43 (160), enumeration of B-1 cells by flow cytometry can be difficult, depending as it does on multiple parameters, a partial listing of which includes efficiency of antibody binding; brightness of fluorescence signals; sensitivity of fluorescence detection; nature and hierarchy of gating strategy, and competency of computerized algorithms for data analysis (166). For these reasons, results from different laboratories using different staining panels, different gates, different instruments, and different software may well differ, above and beyond differences in donor characteristics. A particularly difficult issue is posed by expression of CD27 and CD43 on most normal T cells, as well as other non-B cell types (167, 168). Because peripheral blood contains many more T cells than B cells, any stochastic tendency of (non-B-1) B cells to form doublets with another lymphocyte is likely to produce B:T doublets rather than B:B doublets. B:T doublets would express CD20, contributed by the B cell constituent, and CD27/CD43, contributed by the T cell constituent. In other words, a B:T doublet would express CD20, CD27 and CD43, and could easily be confused with an authentic CD20⁺CD27⁺CD43⁺ B-1 cell (169). In fact, B:T doublets can form under certain conditions. The most effective counter measure is taken before cytometry to avoid doublets in the first place, by briefly agitating samples just prior to analysis, by including EDTA in cell suspensions, and by examining samples at low concentrations (160, 166). If present, B:T doublets can be removed from consideration by area/height or pulse-width doublet discrimination, keeping in mind that doublet discrimination is geared around small, spherical cells so that excessively tight gating may exclude large normal cells such as

B-1 cells (160, 166). B:T doublets can also be removed from consideration by staining for, and excluding, all CD3⁺ cells (160, 166). However, the presence of substantial fractions of CD3⁺CD20⁺ doubly positive events, and/or the presence of substantial fractions of doublet excluded cells, suggests suboptimal initial cell preparation and the likelihood that results obtained will not be truly representative of the sample at hand.

There is much individual and age-related variability among healthy donors in the fraction of circulating CD20⁺ B cells that are B-1 cells (166), and so large numbers of determinations are required to arrive at normal values for any given population. At present the frequency of B-1 cells among B cells in peripheral blood samples from normal individuals is estimated to vary from 1–9% and to decline with advancing age (159, 160, 170). Although 1–9% covers a wide range (and some values are still higher and some lower than these limits), it is important to keep in mind that thus far no tissue reservoir for human B-1 cells has been identified, unlike the situation in mice where B-1 cells are concentrated in coelomic cavities. And with or without a reservoir, the endogenous and/or environmental factors that regulate the free circulation of B-1 cells on a day to day, week to week, or longer basis are at present unknown.

Since the first publication delineating the phenotype of human B-1 cells, several studies have appeared in which CD20⁺CD27⁺CD43⁺ cells were examined. These investigations report the frequency of CD20⁺CD27⁺CD43⁺ cells among B cells obtained from adult peripheral blood as 2.2% ($n=8$), approximately 1–2.5% ($n=5$), and 4.1% ($n=33$) (169, 171, 172). The latter two studies included assay of CD5 expression, finding markedly contrasting results, with 65.6% of B-1 cells, and 11.5% of B-1 cells, positive for CD5 (171, 172). Despite variable results regarding CD5 staining, these studies are consistent in supporting the new phenotypic identity of human B-1 cells. Further support derives from study of the response to *Borrelia* infection in immunodeficient NSG mice engrafted by human hematopoietic stem cells (human immune system, or HIS, mice). B-1b cells are responsible for resolving *Borrelia* infection in normal, wild-type (WT) mice (173). HIS mice counteract *Borrelia* infection, unlike unengrafted NSG mice, and contain CD20⁺CD27⁺CD43⁺ B cells, suggesting that stem cell derived human B-1 cells in HIS mice fulfill a function carried out by murine B-1b cells in WT mice (174).

Human B-1 cell antibodies may have therapeutic benefit

As noted earlier, natural antibodies are pivotal in defending against microbial pathogens and in disposing of cellular debris and molecular toxins. And because natural antibodies tend to reflect sequences found in the genome, it is likely that they are subject to evolutionary selection. In other words, natural antibodies produced by B-1 cells are likely to be optimized for effectiveness in doing what they do (which is likely to be different than being optimized for binding affinity). However, in mouse studies B-1 cell antibodies change with advancing age in a way that erodes germline configuration (94, 95). Moreover, in humans B-1 cell numbers decline with advancing age (159). Taken together these results suggest that protection afforded by B-1 cell-derived natural antibody declines with age; this in turn may increase susceptibility to several diseases that are more common in the elderly. In both human and mouse studies, natural antibodies have been implicated in protection against

illnesses ranging from microbial infection to atherosclerosis to neurodegenerative diseases to cancer, and intravenous immunoglobulin has been used therapeutically in some of these dyscrasias (22, 55, 98, 175–177). One could speculate then that passive immunization with specific natural antibodies, against PC, oxidized LDL, tau, amyloid, or NeuGcGanglioside, by supplying repertoire elements that are missing or deficient, might prevent or treat one or more diseases of aging. Methods for generating human monoclonal antibodies from individual cells by single cell PCR/expression cloning have been described (178) and can be used to obtain antibodies from members of small cell populations, such as B-1 cells. Generation of completely human antibodies from B-1 cells or other infrequent B cell populations with the goal of passive immunization may well constitute the next phase of monoclonal antibody therapy and bypass the need for, and issues associated with, “humanizing” antibodies obtained from other species. Alternatively, as more is learned about human B-1 cell development, and the potential role and behavior of a putative lineage-specific human B-1 cell progenitor, it may be possible to upregulate B-1 cell numbers and/or function as individuals age and thereby maintain a “young” natural antibody protective/homeostatic shield.

Human B-1 cells may regulate immune responses

Regulatory B cells can modulate immune responses (126, 179, 180). Several candidates for putative human B_{reg} cells that express IL-10 have been suggested, including in particular B cells that are CD24^{hi}CD38^{hi} and B cells that are CD24^{hi}CD27⁺ (181–183). The recognition early on that B-1 cells are a key source of IL-10 in the mouse system suggested the possibility that human B-1 cells might similarly express and secrete IL-10.

Two subpopulations of human B-1 cells have been defined on the basis of CD11b expression: CD11b[−]B-1 cells are the predominant population whereas CD11b⁺ B-1 cells are the minor population (184). CD11b[−]B-1 cells secrete IgM robustly (and constitutively) in comparison to the much smaller amounts constitutively secreted by CD11b⁺ B-1 cells; CD11b⁺ B-1 cells stimulate T cell expansion strongly in comparison to the much weaker stimulation produced by CD11b[−]B-1 cells. This dichotomy is similar to the functional differences between PC-1hi and PC-1lo murine B-1 cells (191). Although the possibility that CD11b⁺ B-1 cells represent B cell: monocyte doublets has been raised (185), confocal microscopy, light microscopy and flow cytometry results support the existence of human B-1 cells expressing CD11b (184, 186). The impression that CD11b⁺ B-1 cells could be monocytes/macrophages may derive at least in part from the many similarities between B-1 cells and myeloid cells delineated in the mouse system that include phagocytosis (and antigen presentation) and have led to the idea that the two derive from the same developmental pathway and share elements of lineage (187–189).

Both CD11b[−] and CD11b⁺ B-1 cells, along with naive mature and true memory B cells, have been examined for IL-10 expression and secretion (190). Among these four B cell populations, the very small pool of CD11b⁺ B-1 cells expresses and secretes IL-10 at baseline, in the absence of any stimulation, in contrast to all other B cells. Moreover, CD11b⁺ B-1 cells suppress naive CD4⁺ T cell activation as judged by expression of TNF-α and do so in an IL-10-dependent manner, unlike CD11b[−]B-1 cells or naive or memory B

cells, which do not suppress. Thus, human B-1 cells, like their murine counterparts, are capable of suppressing immune responses via production of IL-10.

These results on B-1 cell-generated IL-10 and immunosuppression, combined with B-1 cell-induced T cell stimulation, indicate that human B-1 cells, like mouse B-1 cells, manifest potentially countervailing tendencies toward immune regulation with respect to B:T interaction. In view of the therapeutic efficacy of B cell depletion therapy in a variety of autoimmune diseases, it may be speculated that human B-1 cells play a role in pathogenesis or pathopersistence through dysfunctional or skewed immunoregulation, beyond the potential production of autoantibodies. Excessive B-1 cell activity in terms of driving T cell expansion (and, extrapolating from mouse B-1 cell function, also in terms of inducing proinflammatory Th17 cell differentiation), or diminished B-1 cell activity in terms of IL-10 secretion and immunosuppression, could contribute to inflammation and disease. In fact, in a small survey of lupus patients, CD11b⁺ B-1 cells were found to be increased (184). In other words, deletion or immunoregulation of B-1 cells, rather than total B cell depletion, may constitute a means to influence the course of autoimmune and other dyscrasias in which the immune system plays a role.

Concluding remarks

Building on the remarkable foundation provided by decades of research on mouse B-1 cells by many investigators, the anonymity with which human B-1 cells eluded serious scrutiny over the last 30 years has now been lifted by elucidation of the human B-1 cell phenotypic profile. Human B-1 cells share functional characteristics with mouse B-1 cells by definition; in addition, human and mouse B-1 cells share expression of CD43 and for most, CD5, although other surface antigens differ. There are many questions about human B-1 cells yet to be resolved, from simple (e.g., what is the normal level of B-1 cells in healthy individuals) to complex (e.g., how does the B-1 cell antibody repertoire compare to that of B-2 cells). However, previous work with mouse B-1 cells and early work with human B-1 cells suggests that a fuller understanding of B-1 cells in ill patients and healthy donors may favorably impact prevention and treatment of disease. Human B-1 cells are likely to be a rich source of fully humanized antibodies obtained for prevention and/or treatment of microbial pathogens and degenerative diseases associated with the accumulation of toxic molecules. Moreover, through production of autoantibody and through countervailing effects on T cells, human B-1 cells may instigate, perpetuate or exacerbate autoimmune and inflammatory diseases. Thus, a full and complete understanding of B-1 cell physiology and pathology is likely to contribute to new insights into human illness and to open new avenues for treatment of autoimmune dyscrasias and other diseases of humankind.

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Figure 1. B-1 cells secrete protective natural antibody

B-1 and B-2 cells fulfill different functions in generating serological immunity as shown in the right and left panels. B-2 cells express immunoglobulin molecules that are junctionally diverse as a result of N-region addition. Following antigen/microbial/vaccination activation, B-2 cells undergo multiple steps to produce antibody secreting cells that require substantial periods of time to develop after initial exposure. Specific B-2 cells that bind antigen are selected to develop germinal centers during which marked B cell expansion is accompanied by somatic hypermutation (and isotype switching), resulting in further repertoire diversity and selection on the basis of antigen-binding affinity. Differentiation to antibody secreting plasma cells (and memory B cells) completes the process. In contrast, B-1 cells express immunoglobulin molecules that are less diverse as a result of minimal N-region addition and minimal somatic hypermutation. Although many B-1 cell antibodies manifest modest affinity, they are often polyreactive, and the latter feature may assist in functional efficacy. B-1 cells constitutively and spontaneously secrete the range of antibodies (mostly IgM) they express, without the need for stimulation or activation (although immunoglobulin secretion may be increased by certain TLR agonists). This “natural” antibody constitutes the bulk of normal baseline or resting IgM. The B-1 cell repertoire represents a pre-existing anti-microbial shield and functions to dispose of cellular debris and toxic molecules. Because B-1 cell antibodies closely mirror germline sequences, the B-1 cell repertoire is considered to have been optimized for survival over evolutionary time. Thus B-1 cell antibodies are selected for function whereas B-2 cell antibodies are selected for affinity. Erosion of the natural antibody repertoire with increasing age may underlie susceptibility of older individuals to certain infectious and degenerative diseases. Because B-1 cell antibodies are

often autoreactive, imposition of somatic hypermutation and isotype switching could result in production of typical pathogenic autoantibodies.

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Figure 2.

Chronically signaling B-1 cells stimulate and regulate T cell responses. B-1 cells affect immune system function in several ways beyond antibody production. B-1 cells strongly present antigen and stimulate T cell proliferation, which depends on baseline enhanced expression of CD86, absent any stimulation or activation. Upregulated CD86 in turn depends on chronic signaling that is presumably generated by B cell receptor binding to self-antigen, but could represent antigen independent activity. Further, B-1 cells promote CD4 T cell differentiation to pro-inflammatory IL-17-containing Th17 cells, a process that also depends on CD86. Whereas these activities promote inflammation, B-1 cells also produce and secrete immunosuppressive IL-10. Thus, B-1 cells manifest a full suite of immune system modulatory activities that include T cell expansion, inflammation, and immunosuppression. As such, B-1 cells express countervailing activities, the balance of which may regulate the direction of normal T cell immune responses and, under pathologic conditions, may contribute to autoimmunity.

Table 1

Phenotypic properties of mouse and human B cell subsets

Phenotypic properties						
Mouse				Human		
B-1	FO	MZ	Memory	B-1	Naive	Switched memory
CD5	+	-	-	CD5	+/-*	+/-**
CD9	++/-*	-	++	ND		
CD11b	++/- ^a	-	-	CD11b	++/-**	-
CD19	+++	++	++	CD19	++	++
CD21	+	++	++	CD20	++	++
CD23	+/-**	+++	+	+/-**		
CD25	+/-**	-	ND	CD27	++	++
CD38	+++	++	++			
CD43	++	-	-	CD43	++	-
CD44	++	-	++			
CD45 ^b	+	++	++	CD45 ^b	++	++
CD80	+++	-	+	+/-**		
CD86	+/-**	-	+	CD86	++/-**	++/-**
CD273 ^c	+/-*	-	-	+/-**		
IL-5R	+	-	ND	ND		
IgM	+++	+	+++	IgM	++/-*	+++
IgD	+	+++	+	IgD	++/-*	++
						-

Phenotypic properties are shown for peritoneal B-1a (B-1), splenic follicular (FO), splenic marginal zone (MZ) and memory B (memory) cells from BALB/c mice. Phenotypic properties are shown for human peripheral B cell subsets, wherein B-1 cells are CD27⁺CD43⁺, mature naive B cells are CD27⁻IgD⁺, IgM memory B cells are CD27⁺IgD⁺, and switched memory B cells are CD27⁺IgD⁻. Phenotypic markers for all cells are described for cells taken *ex vivo* and unstimulated. + = low expression; ++ = moderate expression; +++ = high expression; - = no expression; ND = not determined.

* 50% or more of the population express the indicated marker.

** 50% or more of the population lack expression of indicated marker.

^a CD11b characterizes most B-1 cells in the peritoneum; absence of CD11b defines a distinct subset of CD5⁺ B-1 cells

27-DL
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B24_q

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Table 2

Functional properties of mouse and human B cell subsets*

Functional properties	Mouse		Human	
	B-1	FO	B-1	Naive
Natural antibody	yes	no	yes	no
Few N additions	yes	no	ND	no
skewed repertoire	yes	no	ND	no
Anti-PtC ^a antibody	yes	no	ND	ND
Anti-PC ^b antibody	yes	no	yes	no
Signaling				
Chronic signaling ^c	yes	no	yes	no
Constitutive pERK	yes	no	ND	ND
Constitutive pSTAT3	yes	no	ND	ND
Constitutive NFAT	yes	no	ND	ND
Allogeneic T cell stimulation	yes	no	yes	no
Induction of Th17 differentiation	yes	no	ND	ND
Spontaneous IL-10 secretion	yes	no	yes	no
Sensitivity to Fas induced apoptosis^d	no ^e	yes	ND	yes
Response to phorbol ester	yes	no	ND	yes
Unique gene/protein expression of:				
Elfin	yes	no	ND	ND
Annexin II	yes	no	ND	ND
S100A6	yes	no	ND	ND
Transgelin 2	yes	no	ND	ND
Development				
Specific progenitor	yes	yes	ND	yes
BAFF dependent	no	yes	ND	yes
IL-7 dependent	no	yes	ND	no
TSLP responsive	yes	no	ND	yes
Btk dependent	yes	no ^f	ND ^g	yes
CD19 dependent	yes	no ^f	ND	ND
Proliferation in response to:				
LPS	yes	yes	ND	no
Anti-IgM	no	yes	ND	no
CD40L	yes	yes	ND	no

Functional properties are shown for mouse peritoneal B-1a (B-1) and splenic follicular (FO) B cells. Functional properties are shown for human circulating B-1 and mature naive B cells as noted in the footnote to Table 1. Functional properties are described for cells taken *ex vivo* and unstimulated, except as noted. ND = not determined

^aPhosphatidylcholine

^bPhosphorylcholine

^c All B cells require a degree of tonic signaling for continued viability, separate from chronic signaling

^d Assessed after stimulation and upregulation of CD95 (Fas) expression

^e Sensitive but reduced in comparison to FO B cell

^g Most likely yes, because Btk mutant XLA patients have little resting serum immunoglobulin and few if any circulating B cells