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Original Article

Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion

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Abstract Natural antibodies are produced at tightly regulated levels in the complete absence of external antigenic stimulation. They provide immediate, early and broad protection against pathogens, making them a crucial non-redundant component of the humoral immune system. These antibodies are produced mainly, if not exclusively, by a subset of long-lived, self-replenishing B cells termed B-1 cells. We argue here that the unique developmental pattern of these B-1 cells, which rests on positive selection by self antigens, ensures production of natural antibodies expressing evolutionarily important specificities that are required for the initial defense against invading pathogens. Positive selection for reactivity with self antigens could also result in the production of detrimental anti-self antibodies. However, B-1 cells have evolved a unique response pattern that minimizes the risk of autoimmunity. Although these cells

respond rapidly and strongly to host-derived innate signals, such as cytokines, and to pathogen-encoded signals, such as lipopolysaccharide and phosphorylcholine, they respond very poorly to receptor-mediated activation. In addition, they rarely enter germinal centers and undergo affinity maturation. Thus, their potential for producing high-affinity antibodies with harmful anti-self specificity is highly restricted. The positive selection of B-1 cells occurs during the neonatal period, during which the long-lived self-renewing B-1 population is constituted. Many of these cells (B-1a) express CD5, although a smaller subset (B-1b) does not express this surface marker. Importantly, B-1a cells should not be confused with short-lived anergic B-2 cells, which originate in the bone marrow in adults and initiate CD5 expression and programmed cell death following self-antigen recognition. In summary, we argue here that the mechanisms that enable natural antibody production by B-1 cells reflect the humoral immune system, which has evolved in layers whose distinct developmental mechanisms generate complementary repertoires that collectively operate to maximize flexibility in responses to invading pathogens. B-2 cells, present in what may be the most highly evolved layer(s), express a repertoire that is explicitly selected against self recognition and directed towards the generation of high-affinity antibody response to external antigenic stimuli. B-1 cells, whose repertoire is selected by recognition of self antigen, belong to what may be earlier layer(s) and inherently maintain production of evolutionarily important antibody specificities that respond to pathogen-related, rather then antigen-specific signals.

Keywords B-1 cells - Immune protection - Natural antibodies - Influenza - Anergy

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Introduction

Serum IgM levels of mice that are reared germfree and receive an "antigen-free" diet have long been known to be similar to those of mice held under conventional or SPF housing conditions [*1*]. Serum IgG and mucosal IgA levels in these mice, in contrast, are greatly reduced. Thus, IgM secretion is by-and-large induced independently of external antigenic stimulation, while IgG and IgA secretion are mainly dependent on antigen stimulation. Since antigen-specific IgM and IgG/A/E responses are also induced in response to antigen challenge, this further suggests that regulation of the constitutive production of "natural" IgM is distinct from that of antigen-induced IgM and other downstream isotypes.

Early studies by Lalor et al. [*2*, *3*], which have recently been confirmed and extended [*4*], were the first to demonstrate that a numerically small B cell subset, termed B-1 cells, is the major source of natural IgM in mice. As outlined below, B-1 cells differ from B-2 (sometimes called conventional) B cells in many ways,

including development patterns [2, 5, 6], activation requirements [7, 8, 9, 10, 11, 12, 13] and receptor-mediated signaling pathways [14]. Thus, the dichotomy in the regulation of natural and antigen-induced antibody secretion could be achieved at the level of the B cell subsets that produce them.

While the pathway of B-1 cell development is incompletely understood and subject of intense debate, there is little doubt that these cells are the major contributor of natural serum antibodies both in mice and humans [5, 6, 7, 15, 16, 17, 18, 19, 20, 21]. An abundance of studies collectively show that B-1 cells are the producers of natural antibodies reactive with self antigens, such as Thy-1 [7] and phosphatidylcholine [15]. Importantly, these cells are also the producers of natural IgM antibodies whose inherent specificities, accumulated in the germline as the "layered" immune system evolved [21, 22, 23], provide the first line of defense against invasion by influenza and other pathogens [38, 39].

The expression of CD5, a surface marker otherwise restricted to T cells, is one of the hallmarks of B-1 cells and underlies their initial identification and characterization [2, 11, 15]. These cells, which reside mainly in the peritoneal and pleural cavities, develop in neonates and persist principally, if not exclusively, by self replenishment thereafter [5]. Later studies identified a second B cell population that shares most of the characteristics of the originally identified B-1 cells, but does not express CD5. The two B-1 subsets are respectively referred to as B-1a ($CD5^+$) and B-1b ($CD5^-$) cells [6, 24].

CD5 is a cell-signaling molecule known to potently suppress signaling through both T and B cell antigen receptors [8, 9]. The presence of CD5 on the B-1a cells has been linked to the failure of these cells to proliferate in response to the cross-linking of IgM that readily induces activation and proliferation in B-2 cells, and to the induction of apoptosis in response to this cross-linking [9]. CD5 has also been demonstrated on anergic B cells in Ig-transgenic mice in which the self antigen to which the transgenic Ig reacts is present [25, 26]. The question of whether these latter CD5-expressing B cells are related to the typical peritoneal and splenic B-1 cells has been roundly debated.

There is evidence to support both sides of this argument. In addition to expressing CD5, B-1 cells and the anergic B cells appear to use similar signaling pathways [14], and to be poised for apoptosis induction when the cognate antigen is encountered [7, 27]. However, the full surface phenotype of the $CD5^+$ B-1 cells differs substantially from that of the $CD5^+$ anergic B cells [7, 26]. Furthermore, short-lived, anergic B cells by definition do not secrete antibodies, while long-lived, self-replenishing B-1 cells clearly perform this function throughout the life of the animal. In fact, these cells even function normally to secrete the transgenic Ig in the transgenic animals in which anergic $CD5^+$ B cells are readily detectable [7].

This review is principally concerned with characterizing the role(s) played by B-1 cells in natural antibody production and the ways in which the inherent immunological specificity embodied in natural antibodies mediates immune protection from pathogen encounter. However, full consideration of this issue also requires some focus on the criteria for proper classification of B cells as belonging to the B-1 or B-2 subsets, since this issue is at the heart of the apparently contradictory data concerning the physiology and function of B cells that express CD5.

We take the view here, in agreement with Hayakawa et al. [*7*], that the published data are best reconciled when the expression of CD5 is accepted as inadequate to identify B cells as belonging to the B-1 subset (i.e., put colloquially, when we accept that "CD5 doth not a B-1 cell make"). With a modern understanding, B-1 cells are more appropriately distinguished from B-2 cells by the combination of phenotypic, developmental and anatomical localization criteria. It is important to recognize, as well, that the two types of B cells have distinctive activation requirements and distinctive signaling pathways; and, that these operate to favor the production, by B-1 cells, of natural antibodies that express inherent immunological specificities.

Inherent specificities in natural antibodies

Natural antibodies are defined as antibodies that circulate in normal individuals in the absence of exogenous antigenic stimulation. They are predominantly IgM, although other isotypes are expressed, and are sometimes (often?) able to bind to more than one self or foreign antigen [*28*]. The repertoire and reactivity pattern of natural antibodies is remarkably stable within each species and even between species [*28*]. This stability is in part explained by their frequent (although clearly not majority) usage of germline-encoded V_H and V_L genes that lack N-region additions [*17*, *29*, *30*, *31*, *32*]. We would argue that, in addition, it reflects the innate usage of a series of V genes that evolutionary pressures have selected into the genome to provide an inherent legacy of specificities suitable for protection against pathogen invasion.

How the seemingly stochastic rearrangement of hundreds of V genes could result in similar frequencies of certain specificities in individual mice of the same strain is incompletely understood. Hardy et al. [*33*] have presented evidence suggesting that the ability to bind to surrogate light chain may play a role in selecting certain IgH rearrangements into the fetal repertoire. In addition, there is increasing evidence for the long-held notion that positive selection of natural antibody-secreting cells occurs via binding to self antigens [*26*, *34*, *35*]. Thus, the natural antibody repertoire seems shaped both by molecular events that occur

during Ig rearrangement and by selection events that follow surface IgM expression. Finally, this repertoire is kept in check by homeostasis mechanisms that keep total serum antibody levels within narrow physiological ranges in healthy individuals and the natural antibody reactivity pattern relatively constant throughout life [*36*].

The broad reactivity of individual natural antibodies, and the broad reactivity of the natural antibody population as a whole, is important because it provides pre-existing antibody reactivity that allows animals to rapidly recognize and protect against pathogens that have not been encountered previously. Although this antibody reactivity tends to be low affinity, it provides key protection during the period between the onset of infection and the emergence of the adaptive immune response [*4*, *10*, *37*, *38*, *39*]. We discuss this initial protective mechanism in the context of influenza infection in the next section of this review.

Other functions of natural antibodies may include the recognition and removal of senescent cells and other self antigens. Indeed, it has been suggested that this physiological "housekeeping" function, rather than the immune function of natural antibodies, has led to their development during evolution [*34*, *40*]. However, although mice deficient in B cells or deficient only in secreted IgM are strongly immunocompromised, they do not show apparent deficits in physiological processes such as the removal of aging or damaged cells or cell products [*41*, *42*]. At a minimum, this suggests that natural antibodies are redundant in these types of processes under non-stress conditions.

Natural antibody protection against influenza infection

Measurable induction of antigen-induced antibodies to infectious agents such as influenza virus can be detected in serum as early as 5 days after infection [*10*]. The timeframe of this adaptive immune response, however, is too slow to prevent major viral replication and consequent virus-induced tissue destruction. Indeed, in the case of influenza virus, peak virus replication is well controlled by the time the virus-induced adaptive antibody response becomes detectable [*10*]. As indicated below, natural antibodies provide the initial protection against this virus infection.

The natural antibodies present in mouse serum prior to influenza virus infection show broad reactivity with a series of influenza A and B strains. While the specific target antigen(s) are currently unknown, earlier work suggested that at least some natural antibodies bind to the hemagglutinin molecule of influenza A [*43*,

44]. Sera from BALB/c, CB.17, C57BL/6 and 129SV/EV and other mice all show reactivity with the influenza A and B strains [*4*, *39*]. In fact, in tests with more than a dozen influenza A and B strains, we were unable to identify a single virus strain to which pre-infection sera do not bind (Baumgarth, unpublished). Prior sub-clinical infection or exposure during housing of the mice is excluded as an explanation for these pre-existing antibody levels because mice are not natural hosts for influenza virus.

Mice are not natural hosts of influenza virus because the appropriate cellular receptor for virus entry is not present in the murine upper respiratory tract. However, mice can be infected with influenza by pulmonary delivery of the virus to the lower respiratory tract, which has appropriate receptors for virus entry. Using this infection route, we compared the source(s) of the natural and adaptive antibodies to influenza virus and defined the role each of these types of antibodies plays in protecting the animal against invasion by the pathogen. Importantly, we showed with this model that the presence of natural antibodies to influenza virus has profound effects on survival [*39*].

To identify the source of the natural antibodies to influenza, we used allotype-chimeric mice in which we transferred B-1 cells from BALB/c mice into newborn aallotype-congenic CB.17 strain (and vice versa), treated with allotype-specific anti-IgM. After the chimeras were fully reconstituted, the mice were infected with influenza virus. Allotype-marked antibodies to the virus were measured at appropriate times for pre-immune sera and post-infection sera. Results showed that the natural antibodies to the influenza virus were almost exclusively produced by the B-1 cells [*39*].

To dissect the effects mediated by natural antibodies from the effects mediated by antibodies produced in the adaptive response to the virus, we monitored the consequences of influenza infection in radiation chimeras in which the B-1 cells (producers of natural antibodies) were derived from gene-targeted mice unable to produce the secreted form of IgM. The B-2 cells in these chimeras were derived from allotype-congenic bone marrow taken from fully competent mice. In essence, we found that infection in the absence of natural IgM resulted in increased viral replication in the lung and in markedly decreased survival of the infected mice [*39*]. Death presumably was a consequence of tissue damage induced early during infection in the absence of natural antibodies.

Studies with other pathogens have similarly shown that the lack of natural antibodies has severe consequences during overwhelming sepsis and other infections [*37*, *38*]. In addition, studies with other organisms have shown that B-1 cells produce natural antibodies reactive with pathogens, such as salmonella and *E. coli*, and can be expected to play a role in protection against infection by these organisms [*4*, *5*].

Collectively, our influenza studies demonstrate that preexisting natural antibodies derived from B-1 cells ameliorate disease outcome following infection. However, the presence of natural antibodies is not sufficient to provide complete immune protection. This failure may be due to the relatively low levels of natural antibodies of a given specificity that are present in serum and secretions, to the specificity/avidity of the natural antibodies for their targets, or to the limitations on the effector functions mediated by the natural antibodies, e.g., neutralization, opsonization, complement binding, etc. In any event, data from these studies clearly show that natural antibodies are broadly specific and provide the first line of defense, albeit limited, against viral infection.

Interestingly, we did not find an increase in the levels of natural antibodies reactive with influenza virus as the disease progressed [*4*]. Although the adaptive antibody response to the pathogen increased dramatically, the natural antibody titer remained relatively constant. This might suggest that antigenic stimulation cannot induce B-1 cells to produce antibodies. However, studies with other antigens indicate that B-1 cells do indeed have a limited, but important, ability to respond to immunization. We discuss this apparent contradiction in detail below.

There are some key practical aspects to our findings with this influenza model. Influenza virus strains, like some other viruses, bacteria and protozoa, undergo constant antigenic drift and occasional antigen shifts that can cause devastating pandemics [*45*]. The 1918 influenza virus pandemic that is estimated to have caused 40 million deaths worldwide is a well-known example of such a shift. The emergence of a new influenza virus pandemic, through the appearance of natural (or man-made) re-assortants of two influenza virus strains, is regarded by many as one of the main public health concerns we are currently facing [*46*]. Could such a pandemic be stopped, or at least reduced, by quickly boosting natural antibody titers, even if we lack exact information on what virus strain is causing the outbreak? To address this question one needs to consider the activation requirements and the mechanisms regulating natural antibody-producing B cells.

Natural and autoreactive antibodies are produced by B-1 cells

The presence and production of natural antibodies, as indicated above, are intimately linked to the function of a relatively small subset of B cells, termed B-1 cells. B-1 cells originating in fetal and neonatal liver are readily detectable in neonates, as are the natural antibodies produced by these cells. Very few B-2 cells are present in neonates, which mount very poor adaptive immune

responses. The presence of the B-1 cells and the natural antibodies in mice and humans at the time of birth [*5*, *36*] strongly suggest that B-1 cells are the source of the natural antibodies.

Adoptive transfer studies, such as the influenza virus study described above [*39*] and short-term depletion studies of B-1 cells in neonates, have provided direct evidence supporting this argument. [*2*, *3*, *5*, *47*]. Results of studies with mutated and gene-targeted mice in which B-1 development is impaired similarly show that B-1 cells are the major, if not exclusive, source of natural IgM. In essence, when B-1 cells are missing, serum IgM levels are low; when B-1 are present, serum IgM levels are normal (summarized in [*11*]). In contrast, the presence or absence of B-2 cells does not influence serum IgM levels, allowing the conclusion that most, if not all, natural antibodies are produced by B-1 cells.

B-1 cells are also known to produce autoreactive antibodies, including antibodies to cell membrane components such as phosphorylcholine (PC) [*48*], phosphatidylcholine (PtC) [*15*, *49*]; to carbohydrate epitopes [*50*]; to immunoglobulins (rheumatoid factor) [*51*]; to intracellular molecules such as single-stranded DNA [*51*]; and, to cell surface molecules such as Thy1 (CD90), an antigen expressed on most thymocytes and peripheral T cells [*26*, *52*, *53*]. Since many of the self-antigen determinants or closely related molecules are also present on pathogens, there is reason to suspect that many, if not all, natural antibodies may be both autoreactive and reactive with antigens expressed by pathogens. This is consistent with data from hybridoma studies with autoreactive antibodies, which demonstrate a high degree of cross-reactivity with microbial and other antigens [*5*, *54*, *55*, *56*]. Indeed, if self antigens play a major role in shaping the B-1 repertories (see below), then all natural antibodies can be expected to react with cognate self antigens, i.e., to be autoreactive.

Recognizing B-1 cells and their antibody-producing progeny

Some of the apparent uncertainty [*35*] as to whether B-1 cells are indeed the only, or even the major, source of natural antibodies may stem from difficulties in unequivocally identifying B-1 cells. There currently exists no single marker, or even combination of markers, that is expressed uniquely on all B-1 cells or on their progeny antibody-secreting cells. CD5, the marker that initially was used to distinguish B-1 from B-2 cells, was later shown to be expressed on B-1a but not B-1b cells. Other markers expressed on both B-1a and B-1 b have proven similarly elusive. For example, CD11b (Mac-1), which is expressed at low levels on both B-1a and B-1b in the peritoneal and pleural cavities, is usually not detectable on splenic B-1 cells.

Relatively few studies have addressed the changes in marker expression that occur when B-1 cells differentiate to antibody-producing cells. Some antibody-secreting cells derived from B-1a cells (notably some of the plaque-forming cells that produce antibodies to PtC) continue to express CD5 [*15*]. However, CD5 expression is for the most part extinguished during B-1a differentiation to antibody-secreting cells, since the expression of this marker has not been reported on splenic and intestinal plasma cells known to be derived from B-1a cells [*57*]. Thus, a proportion of the natural antibody-secreting progeny of B-1a, perhaps the majority, can be expected to have terminated CD5 expression, i.e., to be $CD5^{-}$. Thus, they will share the $CD5^{-}$ phenotype of B-1b progeny, which are also known to secrete natural antibodies [*58*].

In humans, natural antibody-producing $CD5^{+}$ and $CD5^{-}$ cells have been isolated [*59*]. The latter cells resembled B-1b cells in that they expressed CD11b and were further distinguished from conventional B cells by low expression of CD45RA, which also tends to be low on murine B-1 cells [*15*]. The human natural antibody-secreting cells that did not express surface CD5 in this study were shown to contain CD5 mRNA [*59*], suggesting that the $CD5^{-}$ cells were derived from $CD5^{+}$ B cells. Thus, it should be emphasized that both in man and in mouse, neither the expression of CD5 nor the expression of CD11b can be used to identify the origins of natural antibody-producing cells.

In mice, as indicated above, Ig allotype chimeras in which the B-1 and B-2 cells express different Ig allotypes have been used to advantage in defining the origins of natural and antigen-stimulated antibody-producing cells [*3*, *60*]. Consistent with data presented in these initial studies, our recent influenza studies show that >80% of the serum IgM and much smaller fraction of IgG1, IgG2a and IgA antibodies are made by B-1 cells (Fig. 1) [*4*]. Importantly, >80% of the serum IgM from non-infected mice that bound to influenza virus was B-1 cell derived [*4*, *39*].

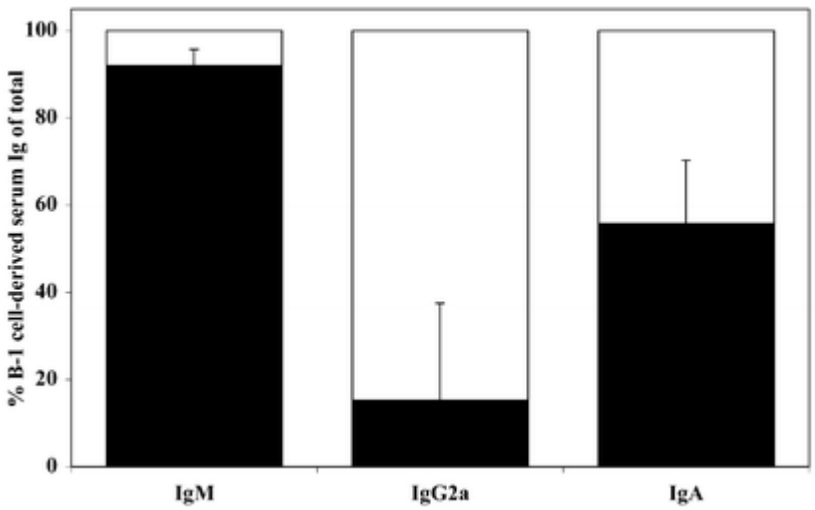

Fig. 1 B-1 cells strongly contribute to the serum antibody pool. Allotype chimeric mice were created by injecting CB.17 (Igh-b) mice from birth for 6 weeks with allotype-specific anti-IgM^b. Congenic Ig-allotype-mismatched peritoneal cavity wash-out cells from BALB/c (Igh-a) mice were provided on day 2 after birth as source of B-1 cells, as described [*4*]. Three months after end of antibody-treatment mice were bled and contributions of donor B-1 (*black*) cells on total serum IgM, IgG2a and IgA levels were determined by ELISA. At that time, donor B-1 cells made 80–95% of total B-1 cell pool in the chimeric mice as assessed by flow cytometric analysis (not shown)

One of the limitations of the allotype-chimeric system is that up to 20% of the B-1 cells in the irradiation chimeras mice express the same allotype as the B-2 cells. This is because roughly half of the B-1b population, and a very small proportion of the B-1a population, in these chimeras tends to be reconstituted from the adult bone marrow used as a source of B-2 cells. In addition, both B-1a and B-1b cells tend to survive the radiation doses used to deplete the host cells [*5*]. Similarly, in allotype chimeras made by neonatal depletion of host B-1 by anti-allotype treatment, some of the target B-1 cells also survive. Thus, antibodies produced by the host-derived B-1 cells can not be fully distinguished from antibody production by conventional B cells and vice versa [*4*, *5*].

Nevertheless, taken in conjunction with data from the mutant and gene-targeted mice discussed above, current data are consistent with virtually all of the serum IgM in non-immunized mice, and therefore all influenza-binding natural IgM, being produced by B-1 cells. We believe this is the case and, by the same token, we believe that B-1 cells secrete the vast majority of, if not all, natural antibodies.

Antigen selection mechanisms differentially influence the B-1 and B-2 repertoires

A variety of studies, beginning with the first study to identify B-1 cells [*16*], have shown that the antibodies produced by B-1 cells react with self antigens or antigens that can readily be revealed on, or in, cells. Initially, these self-reactive antibodies were thought to be distinct from the natural antibodies that react with invading pathogens. However, evidence demonstrating that monoclonal antibodies reactive with self antigens also react with pathogens has led to the recognition that the natural antibody repertoire may indeed be composed entirely of autoantibodies that also react with pathogens [*18*, *50*, *61*, *62*]. Importantly, this dual reactivity may underlie the developmental differences that have evolved to assure the distinctive properties of the B-1 and B-2 repertoires.

De novo development of B-2 cells from unrearranged progenitors begins late in

the neonatal period and persists as the major B cell developmental pathway in the bone marrow throughout life. De novo generation of B-1 cells, in contrast, is largely restricted to the fetal and neonatal period, during which progenitors are initially present in the liver and can later be found in spleen and bone marrow [*5*, *55*]. Very few B-1a cells develop from progenitors in adult bone marrow. However, significant numbers of B-1b cells can be derived from this source [*2*, *5*, *6*, *11*, *55*].

In the adult, the B-1 subset is maintained by self replenishment except for possibly a few B-1b cells that develop from bone marrow. De novo development of B-2 cells, in contrast, begins late in the neonatal period and persists in the bone marrow throughout life, thus continually replenishing the B-2 pool with newly generated cells.

The selective forces that operate on these two development pathways are quite distinct. B-1 cells are positively selected on the basis of their ability to bind to self antigens. In mice that carry an Ig transgene that binds to Thy-1, a pan T cell surface antigen, the B-1 population expressing this transgene is expanded in normal mice but not in Thy-1 gene-targeted mice [*7*]. Thus, the pool of self-reactive B-1 cells that "see" their cognate antigen is preserved and expanded, while the pool of potentially self-reactive B-1 cells that do not "see" their cognate self antigen remains as a tiny component of the repertoire.

Self-reactive B-2 cells, in contrast, have quite a different fate. These cells are negatively selected (i.e., eliminated) when they encounter cognate antigen. This negative selection occurs at the final stages of B cell development and results in apoptosis, use of a different light chain, receptor editing, or the appearance of anergic B cells that migrate to the spleen [*7*, *27*]. The induction of anergy has a curious property in that it results in the expression of CD5 on the anergized B cells, which otherwise express a typical immature B-2 phenotype [*7*]. This finding likely explains previously reported antigen-stimulated "conversions" of B-2 cells into what appeared to be B-1 cells (as judged by the expression of CD5) [*63*].

For example, the expression of CD5 on anergic B cells (which are short-lived and fated to die by apoptosis) could explain why Ig transgenic mice that express an antibody specificity usually only found in the B-1 repertoire have such poor ability to generate B cells from the bone marrow [*63*]. As in the Hayakawa transgenic mice, some $CD5^+$ B cells were found in the spleen, but the overall frequencies of B cells were exceedingly low. It would be interesting to test whether, rather than being B-1 cells, those CD5-expressing cells that do develop from the bone marrow in these transgenic mice are mainly short-lived anergic CD5-expressing B-2 cells, as suggested by Hayakawa's studies [*7*].

The expression of V_H12 in the B-1 repertoire of certain mouse strains provides another example of the selection of cells into the repertoire based on reactivity with self antigen. In this case, the frequency of cells expressing a variant form of V_H12 (present in C57BL/10 and CB.17 mice) increases virtually linearly throughout life. However, the increase is restricted to cells expressing V_H12 antibodies with specificity for PtC. B-1 cells expressing V_H12 antibodies that do not react with this antigen stay at roughly the same frequency from neonatal life onward.

Importantly, the increase in B-1 cells expressing V_H12 as the animals age is not due to newly developed bone marrow immigrants. Rather, it is due to the continued expansion of the V_H12 anti-PtC population present during the neonatal period ([*64*], and Wilshire, Baumgarth and Herzenberg, unpublished). (Note that this V_H12 gene is the same as the V_H12 used in transgenic and other studies in which the origins of B-1 cells have been examined [*18*, *19*, *20*, *65*, *66*].)

Early developmental mechanisms also differentially influence B-1 and B-2 repertoires

In addition to late-acting selection based on antigen encounter, mechanisms operant during or prior to the pre-B cell stage in the B-1 and B-2 pathways differentially influence V_H and V_L usage in the two subsets [*33*, *67*]. The development of B cells during fetal and neonatal life appears to require a number of distinct selection/signaling events that mitigate towards inclusion of the V genes that predominate in the B-1 repertoire [*33*, *67*]. In contrast, development mechanisms operant during B cell development in the bone marrow skew the initial repertoire towards inclusion of V_H and V_L typical of the B-2 subset [*67*].

In particular, production of immunoglobulin μ heavy chain and subsequent assembly with a surrogate light chain to form the pre-B cell receptor complex is a critical checkpoint for B cell (B-2) development in the bone marrow. Hardy's group, studying fetal B cell development, also sees the formation of this complex as a critical checkpoint [*33*, *67*]. However, while the complex formation is required for promoting pre-B cell progression in adult bone marrow, formation of complex tends to inhibit further development of pre-B cells in fetal liver. Thus, at least some B-1 cells specifically fail to develop because heavy-chain pairing with surrogate light-chain is successful.

This distinction in the response to the strength of V_H pairing with surrogate light chain can explain how certain V_H genes tend to enter the B-2 repertoire, whereas others selectively enter the B-1 repertoire [*33*]. Thus, it introduces a developmental mechanism that has evolved to enable the expression of unique subsets of V_H genes in the B-1 and B-2 repertoires [*33*, *67*]. Further, it provides a framework within which the evolution of V_H genes that code for autoreactivity can occur and be expressed by a B cell subset whose responses to signaling and other properties have evolved to enable safe production of these antibodies.

The developmental pathways for B-1 and B-2 also differ with respect to sensitivity signaling through various receptors and pathways. The development of a variety of gene-targeted mouse strains has revealed selective developmental dependencies that include the greater dependence of B-2 development on the presence of IL-7 [*68*], the greater dependence of B-1 on signaling through CD19, to name but one example (summarized in [*11*]). This differential sensitivity during B cell development is also visible in studies with mature B cells, which show much the same differences.

Limiting the risk of autoimmunity due to B-1 antibody production

One of the most intriguing differences between mature B-1 and B-2 cells is the well-known failure of B-1a cells to respond to IgM cross-linking (at least as indicated by proliferation and NF-κB activation [*12*]). The lack of a B-1a response to IgM cross-linking has been taken as meaning that antigen itself, while it is crucial for the development and selection of B-1 cells, is not sufficient to trigger B-1 cell antibody responses. Recent studies have implicated negative signaling by the co-expressed CD5 molecule, which also down-modulates signaling through the T cell receptor on developing and mature T cells, in this failure to respond [*8*, *9*] (B-1b have not been studied).

Indeed, the earliest studies of B-1 cells demonstrated that they typically produce little or no response to immunization protocols with antigens that readily stimulate production of large amounts of high-affinity antibody by B-2 cells [*2*, *15*]. Nevertheless, B-1a cells clearly can respond to certain antigenic stimuli, e.g., α(1, 3)dextran, PC and even dinitrophenol (DNP), when presented on an appropriate carrier or in an appropriate immunization vehicle [*2*, *15*, *50*, *62*].

The antibody responses of B-1 cells are quite distinct from those of B-2 cells. For example, B-1 cells are exclusively responsible for producing antibodies to PC

that express the T15 idiotype [*48*, *50*, *69*]. Infection with *Streptococcus pneumoniae*, *Salmonella typhimurium* or immunization with PC on a carrier molecule, usually keyhole limpet hemocyanin, in adjuvant induces a strong IgM, IgA and IgG3 anti-T15 response [*62*, *69*]. However, this immunization does not induce typical B cell memory. Even after secondary immunization [*70*], there is no affinity maturation or memory response associated with the T15 idiotype [*61*]. Thus, B-1 cells of appropriate specificity can respond to infection and immunization by antibody production and limited isotype switching, but do not produce high-affinity antibodies that might efficiently react with the self antigens that selected these antibodies into the B-1 repertoire.

Interestingly, B-1 cells are capable of extremely rapid response to antigenic challenge in a model of T cell-mediated contact sensitivity in which antibody produced by B-1 cells is crucial to initiation of the T cell response [*71*, *72*]. Within 24 h of skin sensitization with oxazalone or other haptens, B-1 cells migrate to the draining lymph nodes. In the presence of IL-4 produced by CD1-restricted NK T cells, the migrated B-1 cells begin secreting IgM specific for the immunizing hapten [*71*, *72*, *73*, *74*]. This participation of B-1 cells, "helped" by IL-4-producing NK T cells, introduces a new function for the inherent B-1 repertoire and a surprising role for B cells in what has long been considered purely a T cell response.

These findings contrast with results from the influenza virus infection studies discussed above, in which we showed that, although B-1 cells produce significant levels of natural antiviral antibodies, serum levels of these antibodies do not increase after viral infection [*4*]. Since the infection in this model is mainly restricted to the respiratory tract, we (Y. S. Choi and N. Baumgarth) have recently reopened these studies to determine whether viral infection stimulates local anti-viral antibody secretion by B-1 cells. Preliminary data indeed indicate that influenza virus infection triggers B-1 cells to migrate to regional lymph nodes and that IgM anti-viral antibody production by B-1 cells is induced at this site by day 5 after influenza virus infection.

Despite this long history of responsiveness to antigens like dextran and PC, the ability of B-1 cells to respond to antigenic stimulation, albeit in restricted fashion, is often overlooked. In contrast, the responses of these cells to stimulation with LPS and other "non-specific" B cell stimuli is well known. LPS, for example, stimulates rapid migration of B cells from the peritoneal cavity to the spleen and a rapid polyclonal Ig response that includes (or may be restricted to) IgM production by B-1 cells [*57*, *75*]. In addition, in one of the best-studied B-1 autoantibody systems, LPS stimulates rapid production of antibodies reactive with bromelain-treated mouse erythrocytes, i.e., with PtC in the erythrocyte membrane. Finally, in mice carrying a transgene encoding efficient autoantibodies to red blood cells, LPS triggers activation of peritoneal B-1 cells

and the induction of autoantibody-mediated hemolytic anemia in previously healthy mice [*75*].

In vitro cytokine and mitogen studies with B-1 cells show that despite their inability to respond to anti-IgM cross-linking, they respond as well or better than B-2 cells to other types of B cell stimulation. For example, LPS and IL-4 both stimulate strong B-1 proliferative responses that, in fact, are equivalent to splenic B-2 responses under the same conditions (Fig. 2). Furthermore, B-1 cells are superior to B-2 cells in their ability to proliferate when stimulated with phorbol esters [*12*] and IL-5 (Fig. 2). The superior response to IL-5 most likely occurs because B-1 cells constitutively express the IL-5R [*76*]. Consistent with that finding, increased frequencies of B-1 cells (but not B-2 cells), and increased autoantibody levels in serum are found in mice overexpressing IL-5 [*77*], while B-1 cells and serum IgM are decreased in mice that lack IL-5 or IL-5R [*78*, *79*].

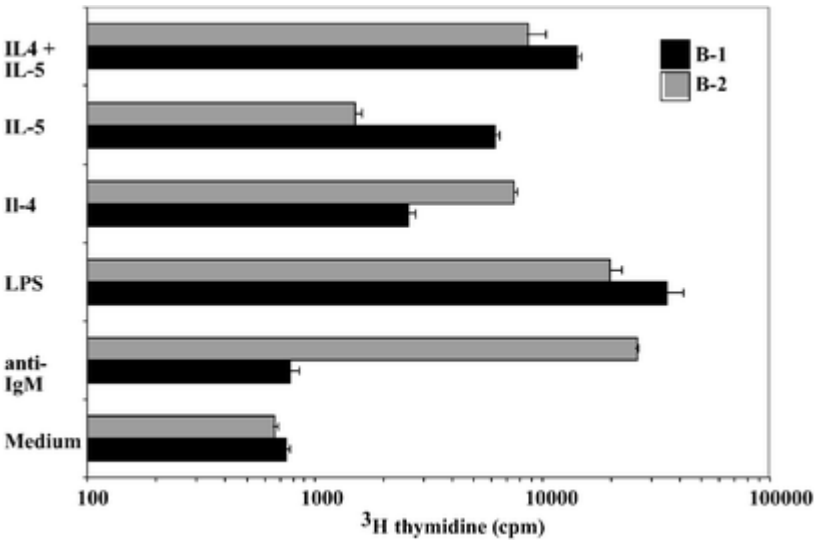

Fig. 2 B-1 cells and B-2 cells respond with proliferation to various stimuli. B-1 cells were purified from peritoneal cavity wash out of BALB/c mice by negative selection over a magnetic column using biotinylated antibodies to GR-1, F4/80, CD3, CD4, CD8, CD23 and streptavidin-coated magnetic beads. Purification of splenic B-2 cells was done similarly using biotinylated antibodies to the following: GR-1, F4/80, CD3, CD4, CD8 and CD5. Purity of cells was >92%; 1×10^5 cells were incubated for 48 h in triplicate with the indicated stimuli or with medium alone. Anti-IgM (10 μg/ml), LPS (10 μg/ml), IL-4 (100 U/ml), IL-5 (320 U/ml). Note the strong responses of B-1 cells to LPS and IL-5

In situations where B-1 cells respond to antigens, they mainly produce low-affinity antibodies that react with repeated epitopes on antigen molecules. The responsiveness to these antigens may be explained by the B-1 V gene repertoire, which is likely to be selected for these kinds of reactivities. The tendency to produce low-affinity antibodies may be explained in part by the fact that B-1 cells seem to be excluded from germinal centers, where somatic

hypermutation and affinity maturation occurs. In other words, the mechanisms that shape the B-1 repertoire and control their responses may have specifically evolved to prevent these natural antibody-producing cells from producing high-affinity responses to their target antigens. The expression of CD5, and perhaps other negative regulatory molecules on the B-1 cells is likely to reflect this evolutionary commitment since CD5 tends to down-regulate signaling through the BCR.

It might seem counter-intuitive to guarantee the presence of B (B-1) cells that constitutively produce antibodies reactive with invading pathogens, and then restrict the responses that these B cells can produce to the pathogens. This apparent paradox, however, is readily resolved by remembering that positive selection by self antigens plays a major role in shaping the B-1 repertoire. Unbridled antibody production by these cells could result in serious autoimmune problems. Indeed, B-1 cells have been shown to be involved in certain autoimmune diseases [*13*, *16*, *80*], and could wreak havoc if they were not normally kept under control.

The propensity to create autoimmune problems is offset, however, by the strong advantage of having a subset of B cells evolved to constitutively produce an inherent repertoire of antibodies reactive with potential pathogens. We have shown that having these antibodies present at the first encounter with an infective agent provides immediate immune protection that controls much of the infection. This first line of defense provides the protection necessary to allow the activation, clonal expansion and differentiation of the very small numbers of antigen-specific B-2 cells that are present in naïve hosts and ultimately provide effective long-lasting immunity against the invading pathogen.

We have demonstrated the importance of natural IgM antibodies in the initial protection against influenza infection. However, we could expect similar protection from natural IgA and IgG antibodies. For example, IgA antibodies present on mucosal surfaces could decrease the frequency and levels of exposure to infectious agents well before the adaptive immune system could begin to control the infection. Indeed, under half of the plasma cells in the gastrointestinal lamina propria are derived from B-1 cells [*56*, *81*] as are many of the IgA^+ cells in the respiratory tract (N. Baumgarth, L.A. Herzenberg, unpublished).

Concluding remarks

Current data indicate that the immune system regulates the development of two functionally distinct B cell subsets by enabling B-1 cell development during

fetal and neonatal life, and B-2 cell development from late neonatal life onwards. Recognition of self antigens is key to the positive selection of the B-1 cells, but results in negative selection of B-2 cells via induction of apoptosis or anergy [*7*, *27*]. This results in the presence of two subsets of B cells in the adult, one of which (B-2) is responsible for producing high-affinity antibodies and generating memory cells that provide lasting protection against pathogen invasion. The other subset (B-1), is responsible for producing natural antibodies and short-term low-affinity antibody responses that provide more immediate protection against pathogens, and a broader range of protection than the B-2 repertoire is suited to provide.

There has been considerable confusion as to whether B-1 cells respond to antigenic stimulation. Some of this confusion may be rooted in the expectation that B-1 cells should respond similarly to B-2 cells under various assay and immunization conditions. Many studies reporting negative results were done using antigens and adjuvants that were designed to elicit the high-affinity responses obtainable from B-2 cells in late primary and in secondary antibody responses. Other studies were done using IgM cross-linking conditions that similarly were developed to obtain optimal responses with B-2 cells. Studies with most immunization protocols used today basically reveal the effects of presenting antigen in the context of adjuvant-induced inflammation. Direct examination of the responses stimulated by pathogens, in contrast, provides the potential for understanding B-1 (and B-2) cell responses in their native context.

Considering B cell function in this context is key to understanding the distinction between the two B cell subsets. B-1 cells have a particular niche that evolution has granted them. We have suggested that they belong to layer(s) of a system that continued to evolve and eventually added the layer(s) that include the B-2 population, with its more complex developmental mechanisms and its stronger, high-affinity response capability [*22*]. In this model, B-2 cells would have the "luxury" of developing in an environment in which B-1 cells already were adapted to providing basic protection against pathogen invasion. In essence, the maintenance of B-1 cells as constitutive producers of natural antibodies that provide the first line of defense against pathogen invasion allows the development of the B-2 cells, which are slower to respond but produce much more efficient (high-affinity) antibody responses.

This concept is consistent with the way in which the B-1 and B-2 repertoires are shaped during ontogeny. The recognition of self antigens by B-1 cells as they develop during fetal and neonatal life positively selects for self reactivity and the production of the natural antibodies that can also react with antigens on a variety of pathogens [*7*, *26*]. In contrast, the selection against reaction with self selects a B-2 repertoire that appears as the animal matures, and readies it to fight pathogens that escape the first line of defense provided by natural antibodies. Furthermore, the introduction of affinity-maturation mechanisms and

immunological memory into the B-2 response armory enables production of high-affinity antibodies to the foreign antigens and rapid responses to attempts at reinvasion by these pathogens [*27*]. Thus, although the presence of two distinctly different types of B cells may at times appear counter-intuitive to the immunologist, the development and synergistic action of these B cells is highly logical from the standpoint of the survival of the species.

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