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Characteristics of natural antibody–secreting cells

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Abstract

Natural IgM plays a critical role in protection from pathogens and the prevention of autoimmunity. While its importance has been shown in many different settings, its origins are incompletely understood. This review focuses on the properties of the natural IgM antibody-secreting cells (ASCs), which arise mainly from the B-1 cell lineage. B-1 cells are generated in multiple waves during development, mostly in the fetal and early postfetal periods. The developmental time points can affect their repertoire: prenatal B-1 cells express a mainly germ line–encoded repertoire, while postnatally developing B-1 cells can express Ig with a greater degree of variation. Spleen and bone marrow, but not the body cavities, are primary sites of natural IgM secretion. Within these tissues heterogeneous populations of IgM ASCs can be found. While some ASCs express classical markers of B-1 lymphocytes, others express those of terminally differentiated plasma cells. A better understanding of the properties of these different natural IgM ASCs could aid their future therapeutic exploitation.

Keywords

B-1 cells; B-1 cell development; Blimp-1; natural IgM

Introduction

Natural IgM, a polyreactive antibody secreted from birth in health and disease, even in the absence of exogenous antigen, is a key part of the immune system. Because it can bind to both self-antigens and to pathogens, natural IgM appears to serve many purposes. This includes its role in facilitating normal B cell development, preventing autoimmune diseases such as arthritis and lupus, and providing early immune defenses against numerous infectious diseases.[1–4] In contrast to other immunoglobulin isotypes, natural IgM is highly evolutionarily conserved. Its presence in all mammals, birds, amphibians, and bony and cartilaginous fish points to key functions in the immune system, not all of which may have been identified to date.[5]

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B-1 cells, a distinct lineage of B cells that primarily develop early in ontogeny, secrete much of the circulating natural IgM. This was shown primarily with neonatal B-1 cell chimeric mice, which are generated by transferring B-1 cells or total peritoneal cavity cells (perc) into congenic newborn mice that differ in their Ig-allotype and which are temporarily depleted of endogenous B cells via allotype-specific anti-IgM treatment. In such mice, typically over 90% of the circulating IgM is B-1 cell derived.[6] Ichikawa *et al.* recently reported the sequestration of autoreactive IgM-secreting cells into the marginal zone (MZ) B cell compartment.[7] Thus, MZ B cells may also contribute to the natural serum IgM pool. However, they are likely much more important as a major source or rapidly produced T-independent IgM in the spleen against blood-derived pathogens, as shown elegantly by Martin *et al.*[8]

In contrast to earlier assumptions, increased sensitivity of flow cytometric assays now demonstrate that B-1 cells are found in most tissues of adult inbred mice, including the peritoneal cavity, pleural cavity, spleen, bone marrow, lymph nodes, and blood.[9] B-1 cells have been divided into two populations based on their expression of CD5. B-1a cells are $CD5^+$ and B-1b cells are $CD5^-$. However, additional heterogeneities have been reported based on differential expression of CD43, CD11b, and Ly49 among peritoneal cavity B-1 cells.[10–13] Whether the B-1 cell pool harbors additional heterogeneities, and if so how this may affect their functions and contribution to the pool of IgM ASCs, have not been systematically studied to date.

Here we review the literature and provide data on the cellular sources of natural IgM and the properties of the cells that secrete it. These ASCs seem to develop primarily from fetal cells and early postnatal bone marrow, and may develop from adult bone marrow under conditions of leukopenia. Natural IgM ASCs are located primarily in the bone marrow and spleen and constitute a heterogeneous set of cells. Mice that lack transcriptional regulators of plasma cell differentiation show levels of serum IgM that are about half of the levels found in wild-type mice, consistent with recent data that some of these B-1 lineage cells are terminally differentiated IgM-producing plasma cells[14]. However, significant amounts of natural IgM, particularly in the spleen, are generated by non-terminally differentiated, classical B-1 lymphocytes[15]. Overall, the current data support a model in which a heterogenous set of B-1 lineage cells provides highly stable and tightly controlled levels of natural serum IgM.

Development and repertoire of natural IgM–secreting cells

Development of natural IgM–secreting cells

Several studies aimed to determine where B-1 precursors are located and when they develop. Montecino-Rodriguez and Dorshkind recently incorporated earlier findings with results from their own studies and developed an integrated model of B-1 cell development. The model predicts that B-1 cells develop in waves, with B-1 cells developing first from the yolk sac (extrafetal) and the para-aortic splanchnopleura, then from the fetal bone marrow and liver, and finally, for a limited number of weeks after birth, from the bone marrow[16] (Fig. 1). This is well supported by the literature, including descriptions of B-1 development from the para-aortic splanchnopleura,[17] the yolk sac,[18] fetal omentum,[19] fetal liver,[20–24]

fetal bone marrow,[23] and the perc[25] and bone marrow[21,26–29] of adults. Interestingly, the first wave of development occurs as early as embryonic day 9 in the mouse, which is before the development of hematopoietic stem cells (HSCs), suggesting the existence of a non-HSC precursor for some B-1 cells. This was recently shown to be true by Kobayashi *et al*., who found B-1 cell precursors in the yolk sac and para-aortic splanchnopleura of mice deficient in HSCs.[18]

While there is overwhelming evidence that B-1 cells develop in the fetus, development of B-1 cells from precursors found in the adult bone marrow has been more controversial, particularly the development of B-1a cells. Soon after the discovery of B-1 cells, it was reported that fetal liver, fetal bone marrow, and perc held B-1 cell precursors, but that adult bone marrow did not generate B-1a cells.[25] More recently, Ghosn *et al*. reconstituted irradiated mice with single adult bone marrow HSCs and found that these mice could generate B-2 and B-1b cells, but not B-1a cells,[29] supporting earlier work by Kantor *et al*. who had shown that the transfer of fetal liver but not adult bone marrow could fully reconstitute a mouse with B-1a cells.[22] In contrast, multiple recent studies reported the generation of $CD5^+$ B cells from adult bone marrow.[21,23,28,30] These studies were all performed by transferring adult bone marrow into severely lymphopenic mice—either SCID mice[21,23,30] or mice that had undergone lethal irradiation.[28] The degree to which B-1 precursors in adult bone marrow generate B-1a and B-1b cells in normal mice is difficult to precisely determine owing to the lack of specific markers. However, the ability to generate B-1 cells from bone marrow precursors was shown to diminish with increasing age of mice,[16] consistent with early studies by Lalor *et al*.[6] Together, the data suggest that the adult bone marrow is not a significant source of B-1a cell development. This is significant, as it would explain the more constrained repertoire of B-1 cells compared to that of conventional B-2 cells.

Given the multiple tissue origins and developmental time points, the pool of B-1 cells is heterogeneous in origin, allowing for the possibility that the term “B-1 cells” covers populations of B-1 cells with distinct properties and/or functions. In fact, several differences have been described between fetal-derived and adult bone marrow–derived cells. This was summarized by Hardy and Hayakawa,[31] who have long maintained the importance of distinguishing primarily fetal from adult B cell developmental origins when discussing the properties of B cells. As they point out, phenotypic changes often used to distinguish B-1 and B-2 cells can be induced on all B cell populations. Indeed, CD5 expression on B-2 cells mark anergic B cells,[32] low/lack of IgD expression is a property of all activated B cells, CD43 expression also identifies antibody-secreting plasmablasts/cells,[33] and CD11b seems to be upregulated temporarily also on B-2 cells in activated lymph nodes (E.E. Waffarn, C. J. Hastey, N. Dixit, Y. S. Choi, S. R. Cherry, U. Kalinke, S. I. Simon and N. Baumgarth, submitted).

Repertoire development

Natural IgM secretion begins at the later stages of fetal development.[34] Consistent with development in this sterile environment, natural IgM appears unaffected by the presence of food antigen or microbiota.[35,36] Bos *et al*. and Haury *et al*. both found that serum IgM levels

and serum IgM specificities were similar between germ-free mice fed low-antigen food and conventionally reared mice.

Because at least some B-1 cells develop at an age when TdT is minimally or not at all expressed, non-template (N) nucleotide insertions during VDJ recombination are rare in the B-1 cells of very young mice. Expression of primarily germ line–encoded specificities might have allowed for the B-1 cell repertoire to undergo evolutionary selection, resulting in cells that bind to self-antigens and cross-react with conserved epitopes on common pathogens.[2,37–40] There is strong evidence to support the need for self-antigen reactivity and binding to develop and maintain B-1 cells. Several groups have developed mice with fixed BCR specificities or mutations in the BCR signaling pathway and have shown that BCR signaling is needed for B-1 cell development and that reductions in BCR-signaling strengths usually results in reduction in B-1 cell frequencies, whereas increases in BCR signaling correlate with increased B-1a cell development.[41]

However, N region insertions are not uncommon among B-1 cells. Kantor *et al*. showed through single-cell PCR analysis of perc cells that up to two-thirds of B-1a cells and an even higher percentage of B-1b cells have N region insertions in either their V–D or D–J junctions in 5-month-old adult mice.[42] More recently, Holodick *et al*. reported that B-1a cells generated from adult bone marrow contain similar levels of N insertions as B-2 cells, compared to the greatly reduced N insertions seen in typical fetal-derived B-1 cells.[21,28] Interestingly, they also found a phenotypically distinct subset of fetal liver B-1 cell precursors that give rise to B-1a cells with several N insertions, despite the lack of TdT in these cells.[21] Because N nucleotide additions are found primarily in the B-1 cells of older mice, they suggest that this population is quiescent early in life but later migrates to the bone marrow.

The studies by Holodick *et al*. suggested that the phenotype and location of the B-1a precursors determine the specificity of IgM that the mature cell secretes. Developmentally early B-1 cells are generated in the absence of foreign antigen and tend to express BCRs and secrete IgM that differ little from germ line sequences. B-1 cells that form later in life, such as in adult bone marrow, express TdT and may be in an environment with a larger variety of antigens available to support their development. From this, it would follow that large differences in the repertoire of natural serum IgM ASCs in germ-free compared to normally housed mice should exist. This is not currently supported by evidence in the literature.[43,44] There is some evidence, however, that local IgM production in the intestines can be affected by the microbiota,[45] pointing to marked differences between mucosal and systemically elaborated IgM and the need to differentiate between truly natural versus antigen-induced IgM production.

Source of natural IgM

Tissue location of natural IgM ASCs

The peritoneal cavity is rich in B-1 cells, and much current work on B-1 cells has focused on these cells. However, while perc B-1 cells are capable of secreting large amounts of IgM with LPS stimulation, they do not spontaneously secrete significant amounts of IgM per cell

compared to spleen and bone marrow cells.[15,46,47] This is also consistent with gene expression analysis of perc B-1 cells, which showed a lack of mRNA expression for secreted IgM.[48] In addition, functional evidence comes from the work of Watanabe *et al*. They showed that *BCR*-transgenic mice expressing an anti-erythrocyte BCR that segregated into the B-1 cell lineage would succumb to hemolytic anemia only, after peritoneal cavity B-1 cells were induced to migrate to lymphoid organs, but not when they were present only in the peritoneal cavity. This indicates that even if perc B-1 cells generated small amounts of IgM, this IgM is maintained locally and does not enter the circulation.[49] Based on these lines of evidence by others and results from our own extensive investigations (Fig. 2), we conclude that most natural IgM production occurs outside the body cavities.

Van Oudenaren *et al*. showed that IgM ASCs in germ-free mice were found primarily in the spleen and, to a lesser extent, the bone marrow at a young age (6–8 weeks), with ASC numbers in the bone marrow increasing as the mice aged (40+ weeks), although they did not further characterize these cells.[47] Choi *et al*. showed, using fluorescence-activated cell sorting(FACS) sorting/enzyme-linked ImmunoSpots (ELISPOTs) spontaneous IgM ASCs in the spleen and bone marrow with the phenotype $CD19^{hi}IgM^{hi}IgD^{lo}CD43^{+}$ (i.e., the characteristics of B-1 cells[15]). These cells were found to be perc donor–derived in newborn Ig-allotype chimeric mice. Contributions by both spleen and bone marrow to serum IgM was further supported by Wardemann *et al*., who found that serum IgM was reduced but not absent in splenectomized and asplenic mice.[50]

Cellular sources of natural IgM

Early studies by Lalor *et al*. showed that cells of B-1 origin are responsible for the secretion of natural IgM.[6] They depleted the B cells in a neonatal mouse, supplied allotype-disparate sorted perc B-1 cells, and allowed the B-2 cells to regenerate. The recipients of FACS-sorted B-1 cells developed normal levels of IgM, which were secreted mostly by the donor B-1 cells. Multiple groups later confirmed the findings that B-1 cells secrete natural IgM.[51–53] We have further shown that low-pressure FACS–sorted phenotypic B-1 cells ($CD19^{+}IgM^{+}IgD^{low/-}CD43^{+}$) in both the spleen and bone marrow secrete natural IgM (Fig. 3), confirming our previous study[15] and supporting the findings by Lalor *et al*. that B-1 cells are responsible for natural IgM secretion.

This body of work was recently challenged by Reynolds *et al*., who described a population of natural IgM ASCs that could be transferred into $Rag1^{-/-}$ mice with total perc but not with purified perc B-1a or B-1b cells.[14] The BCRs of the IgM ASCs identified by Reynolds *et al*. showed frequent usage of VH11 and VH12 and expressed N-nucleotide additions less frequently than B-2 cells. Thus, their Ig repertoire appeared similar to that described for B-1 cells. Since multiple earlier studies clearly demonstrated the ability of natural IgM secretion to be reconstituted with FACS-sorted B-1a cells,[6,53] we believe it likely that the population identified by Reynolds *et al*. is a population of terminally differentiated B-1 cells (see below). The lack of reconstitution of serum IgM levels in $Rag1^{-/-}$ mice with FACS-purified B-1 cells observed by them might be due to the much higher pressures used to sort cells on the new generation FACS machines compared to those used by Lalor *et al*. and others. This may have damaged the cells in their ability to reconstitute the mice. Reynolds *et al*. may

have identified a new, fetal-derived subpopulation of B cells that secrete natural antibodies, possibly similar to those identified by Kobayashi *et al*. originating from the yolk sac.[18] However, given the body of literature demonstrating that B cells from the peritoneal cavity can reconstitute, at least based on frequencies, the natural IgM ASCs in both bone marrow and spleen, it appears likely that the IgM ASCs identified by Reynolds *et al*. are of B-1 cell origin.

IgM secretion by B-1a versus B-1b cells

Previous studies in B-1 neonatal chimeric mice have shown clearly that B-1 cells are an important source of natural IgM;[6] however, the precise contributions of B-1a or B-1b cells are currently unclear. Data in the literature indicate that B-1a and B-1b subsets can perform both functions, depending on the context of the infection/insult. In addition to the early studies by Lalor *et al*.,[6] more recently Haas *et al*. and Holodick *et al*. reported that B-1a cells secrete natural IgM. Holodick *et al*. showed by ELISPOT that sorted B-1a cells from both the peritoneal cavity and bone marrow form IgM ASCs.[28] Haas *et al*. compared *Cd19*$^{-/-}$ mice, which lack B-1a cells but have B-1b cells, and CD19-overexpressing mice, which have B-1a cells but lack B-1b cells,[52] and found that the serum of *Cd19*$^{-/-}$ mice was deficient in natural IgM and that the serum of CD19-overexpressing mice had IgM levels equal to that of controls.

B-1b cells were also reported to secrete natural IgM. Ohdan *et al*. examined the source of natural antibodies, in particular those against Galα1-3Galβ1-4GlcNAc (Gal) carbohydrate residues.[46] They sorted several populations of B cells and showed that sorting B-1b cells from the spleen enriched for both total IgM ASCs and for anti-Gal IgM ASCs, compared to sorting for total B-1 cells or for B-2 cells. They also found a reservoir of non-secreting anti-Gal B-1b cells in the perc. Gil-Cruz *et al*. reported that mice with only B-1b cells (no B-1a cells and limited B-2 cells) were able to produce serum IgM, although whether the amount of IgM was comparable to wild-type mice was not stated.[54]

B-1a cells were reported to respond to several pathogens, including *S. pneumoniae* (T15 idiotype),[50,55,56] influenza virus,[53] and *Francisella tularensis*,[57] and B-1b cells reportedly respond to infections with antigen-specific Ig responses to *Salmonella typhimurium*,[54] *B. hermsii*,[58,59] and *S. pneumonia*.[52] Collectively, the literature on B-1a and B-1b cells suggests that both populations are capable of secreting natural IgM in steady state and may respond to foreign stimuli with differentiation to antigen-specific Ig ASCs.

Studies by Ohdan *et al*. and Gil-Cruz *et al*. support the hypothesis that B-1b cells make up the majority of the natural IgM ASCs, but that B-1a cells are also capable of natural IgM secretion.[46,54] Our recent studies show that both B-1a and B-1b cells from the spleen and bone marrow are able to secrete IgM (Fig. 3). This appears to contradict work of Haas *et al*., who reported an IgM deficiencies in the *Cd19*$^{-/-}$ mice, which lack B-1a but not B-1b cells. However, they reported data only on IgM binding to *S. pneumoniae*, the capsular polysaccharide PPS-3, and phosphorylcholine (PC), not total IgM levels. It would be of interest to more fully evaluate the repertoire of these mice to determine whether shifts in the repertoire of natural IgM occur, since the repertoire of B-1a and B-1b cells appears distinct.[46,55,60–62] Thus, while it is clear that phenotypic B-1 lymphocytes contribute

strongly to the production of natural IgM, the relative contributions of B-1a and B-1b cells to the pool of natural IgM–producing cells, however, remains to be clarified further.

Differentiation state of natural antibody–secreting cells

B-1 cells in the spleen and bone marrow share some of the defining characteristics of plasma cells, namely their expression of CD43, the secretion of antibodies, and their potential for long life spans. However, they have properties not shared by plasma cells: typical B-1 cells do not express CD138; they maintain an ability to self-renew and thus proliferate; and they retain high expression of the BCR and its co-receptors, characteristics typically blocked by the transcription factors involved in plasma cell formation.

Blimp-1 expression

Blimp-1 is an important transcription factor driving plasma cell formation, but its role in B-1 cells is not fully resolved. Tumang *et al*. reported that perc B-1 cells expressed Blimp-1 and XBP-1 mRNA at very low levels similar to that of naive B-2 cells, but that they, in contrast to B-2 cells, could be rapidly induced to secrete IgM.[63] From that, they concluded that B-1 cells do not require Blimp-1 for natural IgM secretion. Interestingly, they also found that PAX-5a and BCL-6, which are usually highly expressed when Blimp-1 and XBP-1 are repressed, were expressed at very low levels in B-1 cells, similar to the levels seen in activated B-2 cells. This supports the idea that B-1 cells are more activated than naive B-2 cells. This is, however, in contrast to findings by Fairfax *et al*., who found Pax-5 mRNA levels to be similar between perc B-1 cells and B-2 cells.[64] Additionally, as discussed in the previous section, perc B-1 cells secrete very little natural IgM without stimulation, so this work does not reveal whether the majority of natural IgM, secreted by cells in the spleen and bone marrow, is dependent on Blimp-1.

Two groups found Blimp-1 to be necessary for natural IgM secretion. Savitsky *et al*. found that mice with B cell–specific deletion of Blimp-1 expression (conditional knockout with Cre-driven by the *Cd19* promoter), had reduced serum IgM levels, and that perc cells from these mice secreted less IgM in culture, both before and after LPS stimulation.[65] IgM antibodies of the T15 idiotype, secreted by B-1a cells, were also reduced in those mice. These results agree with another B cell–specific Blimp-1 gene (*Prdm1*) knockout model, the $Prdm1^{\Delta Ex1A}$ mice, which have reduced IgM serum levels.[66] B cells lack Blimp-1 expression in these mice, due to a deletion of exon 1 of *Prdm1*, which is used as transcriptional start site exclusively by B cells.

Fairfax *et al*. used mice that express GFP under the *Prdm1* promoter to examine Blimp-1 expression in perc B-1 cells, compared to naive B cells, marginal zone B cells, and bone marrow ASCs.[64] They found that some, but not all, perc B-1 cells express Blimp-1, though at much lower levels than bone marrow ASCs. They also stimulated perc B-1 cells with LPS for 3 days and then sorted GFP^+ versus GFP^- cells and found that many of the GFP^+ but none of the GFP^- cells were secreting IgM by ELISPOT. Despite the small percentage of GFP^{low} cells seen in perc B-1 cells before LPS stimulation, they did not find any IgM ASCs among non-stimulated perc B-1 cells.

The conflicting reports on the role of Blimp-1 and thus the need for terminal differentiation of natural IgM–secreting cells requires further studies and should be expanded to the spleen and bone marrow, where the majority of B-1 IgM ASCs are located. The behavior of perc B-1 cells after LPS stimulation is reflective of B-1 cells that have been activated to secrete IgM, but whether this is replicated by the behavior of the truly natural IgM ASCs is unclear. Furthermore, two separate mouse strains that lack Blimp-1 expression in their B cells have reduced, but not absent, serum IgM, indicating that Blimp-1 enhances but is not necessary for natural IgM secretion. Whether Blimp-1 enhances the secretory ability of all B-1 cells or is needed for secretion by a subset remains to be determined. The former would be most consistent with data from B-2 cells, where Blimp-1 expression was shown to drive enhanced antibody secretion in cells.[67] Interestingly, Castro *et al*. recently showed that natural IgM ASCs in nurse sharks are Blimp-1 independent, whereas the induced ASCs required Blimp-1 expression, providing a precedent for the existence of Blimp-1–independent natural IgM ASCs.[68]

CD138 expression

There is also some debate over whether B-1 cells express CD138, a plasma cell marker supported by expression of Blimp-1.[69] Odhan *et al*. reported splenic Gal-binding IgM ASCs with a B-1b phenotype as $CD138^{-}$.[46] In apparent contrast, in addition to Blimp-1 (GFP) upregulation, Fairfax *et al*. saw CD138 upregulation of LPS-stimulated perc B-1 cells.[64] They found that some GFP^{+} cells were initially $CD138^{-}$, but became $CD138^{+}$ over time. CD138 expression was also seen in activated splenic B-1a cells by Yang *et al*. and Holodick *et al*.[70,71] Holodick *et al*. reported minor shifts in VH usage between $CD138^{+}$ and $CD138^{-}$ B-1a cells, as well as increased N region additions among the $CD138^{+}$ cells, indicating a possible developmental heterogeneity between these cells. The data indicate that at least some of IgM-secreting cells are differentiated, plasmablast/plasma cell like.

The recent study by Reynolds *et al*. reported differentiated plasmablast/plasma cell–like IgM ASCs in the bone marrow. These cells were characterized as $IgM^{+}IgD^{-}CD138^{+}CD43^{+}CD5^{-}$, $B220^{-}$ or low, similar to the typical phenotype attributed to plasma blasts or B-1b cells, the latter usually lacking expression of CD138. While the data demonstrate very convincingly that a population of fetal-derived $CD138^{+}$ IgM ASCs is present in the bone marrow, it is unclear whether these cells are differentiated B-1 cells, B-2 cells, or a novel subpopulation of B cells. In addition, it is unclear whether the terminally differentiated cells are truly natural IgM secretors or whether they are the outcome of microbe-stimulated B-1 cell activation. Yang *et al*. showed that LPS simulation caused a migration of B-1a cells from the perc to the spleen and their subsequent upregulation of CD138.[71] While two studies reported the presence of a small percentage of $CD138^{+}$ B-1a IgM-secreting cells in the spleen of naive mice, these might have been B-1a cells activated by foreign antigens.[70,71] Examination of germ-free mice for the presence of $CD138^{+}$ IgM ASCs would help to determine whether these cells are natural or induced secretors.

Given the above summarized data, it appears clear that some but not all B-1 cell–derived IgM arises from full differentiated Blimp-1^{hi} $CD138^{+}$ ASCs.

Conclusions

Recent studies by us and others show that at least three phenotypically distinct populations of spontaneous IgM ASCs: B-1a, B-1b, and CD138^{+}, B-1–derived, terminally differentiated plasmablasts/cells, which exist in spleen and bone marrow but not the perc. All three contribute significantly to the pool of natural serum IgM. The current literature is compatible with the idea that B-1 cells develop in waves, in which the earliest wave contributes both B-1a and B-1b cells, while the latest wave from the adult bone marrow contributes preferentially B-1b cells. Early- and later-developing B-1 cells may differ in their Ig repertoire, which could affect the quality of the natural serum IgM during ontogeny. The Dorshkind lab found several B-1 and B-2 cell– restricted precursors in adult bone marrow, but also pro-B cell populations that could give rise to both B-1 and B-2 cells.[23] Determining their respective contributions to the pool of natural IgM ASC would be of interest.

The differences in spontaneous IgM secretion from perc versus spleen and bone marrow B-1 cells demonstrate clear functional differences among B-1 populations. Whether this is due to inherent differences or is imprinted by the environment, or both, remains to be investigated. The recent study by Reynolds *et al.*[14] clearly shows a population of IgM ASCs in the bone marrow that expresses a typical plasma cell phenotype, which can be reconstituted from peritoneal cavity B cells and appear to be of B-1 cell origin. However, those cells are only one of numerous populations secreting IgM[15] (Fig. 3). We and others showed that CD138^{+} and CD138^{-} IgM ASCs also exist in the spleen.[46,70,71] Their relationship to the bone marrow ASCs is unknown.

Studies in B cell–specific Blimp-1–deficient mice reveal that Blimp-1 enhances but is not required for IgM secretion.[65] This is consistent with studies in *Irf4*$^{-/-}$ mice[72], which raises the question of whether secretion by all or only some B-1 cells is enhanced by Blimp-1 expression. Based on the reports that both CD138^{+} and CD138^{-} B-1 cells secrete IgM, it is likely that dependency on Blimp-1 for secretion will show the same variability. Given the above-described functional and phenotypic heterogeneity among B-1 cells, we favor the hypothesis that some of the early-developing B-1 cells contribute to natural IgM production in the absence of terminal differentiation, while perhaps the later-developing B-1 cells preferentially differentiate to IgM plasma blasts/cells in a Blimp-1–dependent manner.

Materials and methods

Mice

Nine-week-old female Blimp-1-YFP mice (originally provided by Michel Nussenzweig) were bred and kept under specific pathogen–free housing conditions in microisolator cages for the duration of the experiments. All procedures and experiments were approved by the Animal Use and Care Committee of the University of California, Davis.

Flow cytometry

For FACS sorting, single-cell suspensions of spleen and bone marrow were stained with the following antibody conjugates, after RBCs were lysed with ACK and FC receptors were

blocked with anti-CD16/32 antibody (2.4G2): CD19–Cy5PE (1D3), CD90.2–Pacific blue, IgM–APC (331), IgD–Cy7PE (11–26), CD43–PE (S7), CD5–biotin (53-7.8), and Streptavidin–Qdot 605 (Life Technologies, Grand Island, New York). Dead cells were stained with Live/Dead Fixable Violet stain (Invitrogen, Carlsbad, CA). Sorting was done using FACSAria (BD Bioscience, San Jose, CA, USA) equipped as described with lasers and optics for 13 color data acquisition[73]. Data analysis was done using FlowJo software.

ELISAs

IgM production was measured by sandwich ELISA as previously described.[73] Briefly, 96-well plates (Maxisorb, Nalgene Nunc, Rochester, NY) were coated with 1.125 μg/ml of anti-IgM (Southern Biotech, Birmingham, Alabama, CA). Non-specific binding was blocked with 5% dry milk/PBS and either culture supernatants or a purified IgM standard (Southern Biotech, Birmingham, Alabama, CA) were serially diluted with PBS and added to the plate. Binding was revealed by biotinylated anti-IgM (Southern Biotech, Birmingham, Alabama, CA) followed by Streptavadin–Horseradish Peroxidase (Vector Labs, Burlingame, C), and developed with 3,3′,5,5′-tetramethylbenzidine (TMB).

Total IgM in each culture well was normalized to the number of expected IgM ASCs/well, calculated as μg IgM per culture well/average numbers of ASCs (by ELISPOT) among 5×10^5 cells. By ELISPOT, ~ 2000 cells/10^6 total cells in the spleen and ~ 500 cells/10^6 total cells in the BM are IgM ASCs.[15] In the perc, we estimate at least 10^4 cells/10^6 total cells produce these tiny spots.

Cell cultures

Tissues were processed in a BSC, cell counts were performed using Trypan Blue to identify live cells, and 5×10^5 cells per well were plated into 96-well plates in complete RPMI 1640 media. Cells were cultured overnight, then centrifuged at 500g for 5 minutes, and supernatants were collected and stored at −20 °C until use.

ELISPOT

Total IgM ASCs were enumerated by ELISPOT as previously described.[74] Briefly, 5 μg/ml of anti-IgM (331) were coated onto 96-well plates (Multi-Screen HA Filtration, Millipore, Bedford, MA, USA). Plates were blocked with PBS/4% bovine serum albumin, cells were sorted directly into wells with complete RPMI 1640 media, and were incubated overnight at 37 °C in a 5% CO_2 chamber. IgM binding was revealed with biotinylated anti-IgM (Southern Biotech, Birmingham, Alabama, CA) followed by Streptavadin–Horseradish Peroxidase (Vector Labs, Burlingame, CA), and developed with 3-amino-9-ethylcarbazole (Sigma Aldrich, St. Louis, MO). Spots were counted with the help of a stereomicroscope.

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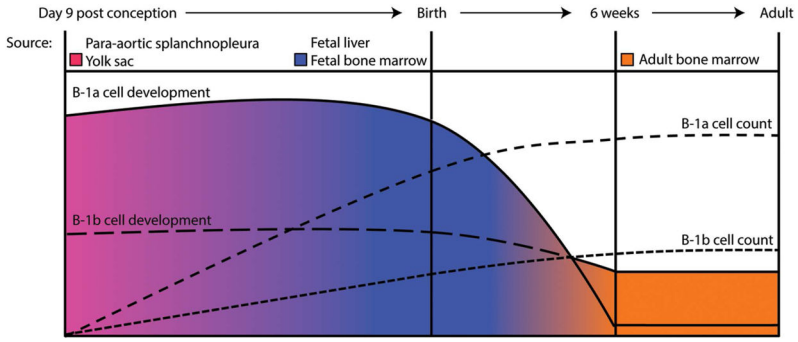

Figure 1.

Multiple sources of B-1 cells contribute to the B-1a and B-1b cell pools. B-1 development begins by embryonic stage day 9.5 in the para-aortic splanchnopleura and yolk sac of the mouse fetus (magenta). HSCs in the fetal liver and bone marrow (blue) contribute later to their development and until 4–6 weeks after birth. The extent to which bone marrow precursors contribute to the maintenance of B-1 cells pools, particularly to B-1b cell generation (orange) from then on is unresolved in normal mice. B-1 cells seem to be maintained at least in part through self-renewal.

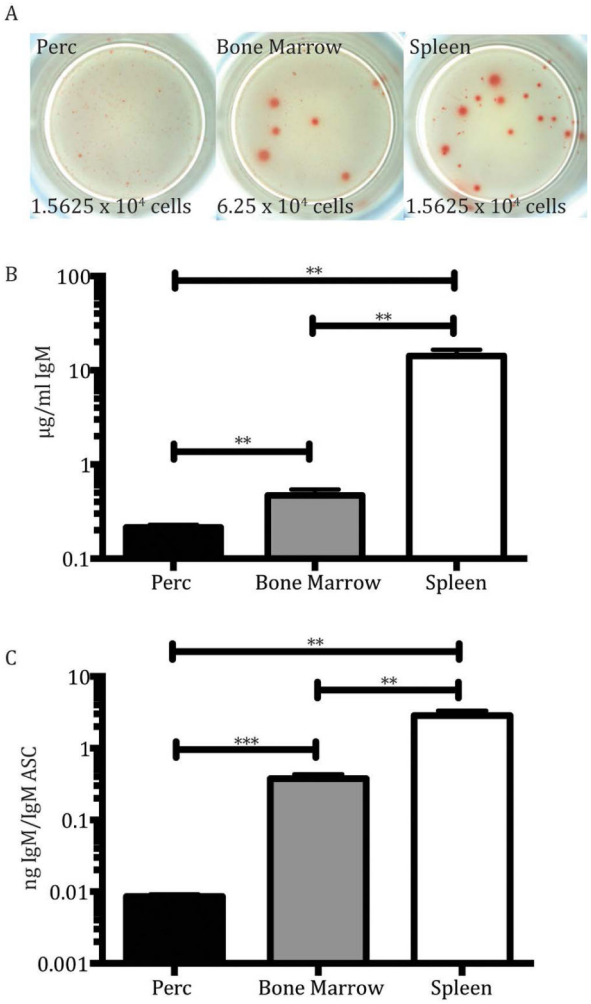

Figure 2.

The spleen and bone marrow are major sites of IgM production compared to the peritoneal cavity. Total spleen, perc, and bone marrow cells were cultured overnight in ELISPOT and cell culture plates, respectively. (A) ELISPOT analysis shows many very small spots formed by peritoneal cells, compared to the less frequent larger spots formed by cells in the spleen and bone marrow. (B) IgM was measured in the supernatants of three culture wells for each tissue (n = 3 mice each). (C) IgM in the supernatants was normalized to the mean number of IgM ASCs as determined by ELISPOT. Spleen cells produced the most IgM, while perc cells produced the least, both overall and per cell. Samples were compared using the unpaired Student's t-test.

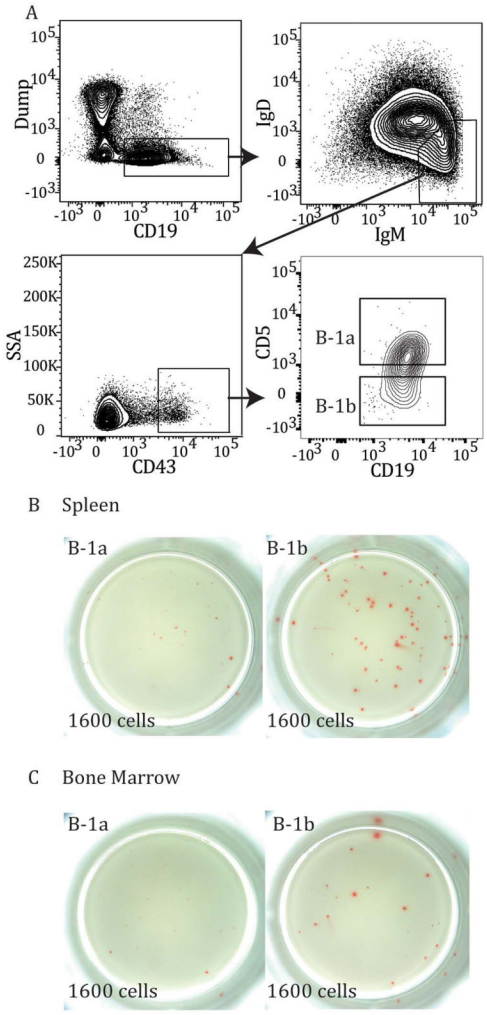

Figure 3.

Sorted B-1a and B-1b cells secrete natural IgM. B-1 cells, defined as $CD19^{+}IgM^{hi}IgD^{low/neg}CD43^{+}CD5^{+/-}$ were sorted as in (A) and their secretion was analyzed by ELISPOT. B-1b cells and B-1a cells from the (B) spleen (n = 11–12 each) and (C) bone marrow secrete IgM (n = 4 each). Number indicates total input cells per well.

Table 1

Characteristics of IgM-secreting cells in various tissues

Tissue source	Contribution to serum IgM	ASC characteristics
Peritoneal cavity	Little/none	Cells can differentiate to Blimp-1 expressing plasmablasts upon stimulation and migration to secondary lymphoid tissues
Spleen	Yes	**1** Typical $CD19^{hi}CD43^{+}CD138^{-}$ B-1a/b cells **2** Small number of $CD19^{hi}CD43^{+}CD138^{+}$ B-1a
Bone marrow	Yes	**1** Typical $CD19^{hi}CD43^{+}CD138^{-}$ B-1a/b cells **2** $CD19^{lo/neg}$ $CD5^{-}CD43^{+}CD138^{+}$ plasmablasts/cells