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B lymphocyte ontogeny and immunoglobulin production

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SUMMARY

In this paper we review different aspects of B cell development on the path from the proB cell to the memory B cell and the plasmocyte. Emphasis is given to the positive and negative selection effects mediated by the changing forms of the surface immunoglobulin (Ig) receptor under successive microenvironments. Positive selection is linked to λ chain expression at the pro- and preB cell stage in fetal liver and bone marrow. Negative selection takes place when surface (s)IgM is being cross-linked by autoantigens before the immature B cell can leave, or after it has left, the bone marrow. After somatic mutation, major expansion becomes possible for B cells with high-affinity sIg receptors. This takes place in the germinal centres of the secondary lymphoid organs in the context of major histocompatibility complex (MHC) restriction and provided the necessary T cell help is given. Kinetic data on B cell replenishment in the rodent models are used to draw a schematic view of an established B cell repertoire.

Keywords selection bone marrow periphery germinal centre pool dynamics

INTRODUCTION

While the short-term effects of intravenous immune globulin (IVIG) administration are due to direct interaction with antigens, autoantigens, idiotypes and idiotopes of the recipients' immunoglobulins (Ig), the long-term effects may be due to an altered activation of the B cell pool. In this respect it might be interesting to review the ontogeny of the B lymphocyte system, especially in terms of the shaping of the active repertoire through the specific interaction of the surface immunoglobulin (sIg) receptor with stromal cells in the bone marrow, autoantigens in the bone marrow or the periphery, and antigens in the context of major histocompatibility complex (MHC) restriction (Fig. 1).

FETAL LIVER AND BONE MARROW

During their proliferation and differentiation in the primary organs, pro- and preB cells require contact with a microenvironment of stromal cells [1]. Interleukin-7 (IL-7) is a major cytokine which costimulates this proliferation [2]. Other cytokines such as insulin-like growth factor-I (IGF-I) [3], IL-6, IL-11, granulocyte–macrophage colony-stimulating factor (GM-CSF) [4] and steel factor [5], are likely to play a role at certain steps. In mice the differentiation stages leading from the proB cell through several preB cell stages to the immature B cell can be differentiated through several CD markers, N-*myc* proto-oncogene expression, and MHC class I expression. Furthermore, the products of the recombination-activating genes, *RAG-1*, *RAG-2*, which encode tissue-specific compounds of the VDJ recombination system [6] but also the terminal desoxynucleotidyl-transferase (TdT), are differentially expressed. The following sequential steps have been proposed for the expression of the surrogate light chain, the p55 chain, the rearrangement of the Ig heavy chain and light chain genes and the expression of their products [7] (Fig. 2).

ProB cells have at their surface the surrogate light (L) chain associated with p55, and heavy (H) chain loci and L chain loci are in germline configuration. At the preB-1 stage, H chain loci are in DJ rearranged form, and L chain loci are in germline configuration. PreB-2 cells have rearranged VDJ at the H chain loci, L chain loci are still in germline configuration, μ chain is to be found in the cytoplasm, and a complex of VDJCμ and surrogate L chain is expressed at the cell surface. Immature B cells have rearranged L chain loci and express μ chains and surrogate L chains, as well as normal L chains. Mature B cells no longer express the surrogate L chain; they often express both μ and δ chains at their surface in association with the L chain. Selection mechanisms operate at the preB cell level. The expression of the productive rearrangements of the H chain loci, either as DJCμ or VDJCμ linked by a disulphide bond to the surrogate L chain, is important for early differentiation along the B lymphocyte lineage pathway. The $\lambda 5$ gene is critical for normal B cell development. Knock-out mice made doubly deficient for $\lambda 5$ [8] have an altered precursor pool and a delayed appearance of Ly-1$^+$ and Ly-1$^-$ B cells. After 1 month Ly-1$^+$ cells are normalized, at 6 months Ly-1$^-$ cells are at 50% of their normal number. However, immune responses to T-independent and T-depen-

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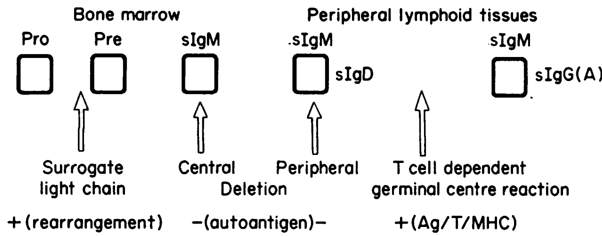

Fig. 1. B cell selections via sIgG receptor(s). Ag, antigen; +, positive selection; −, negative selection; T, T lymphocyte; MHC, major histocompatibility complex.

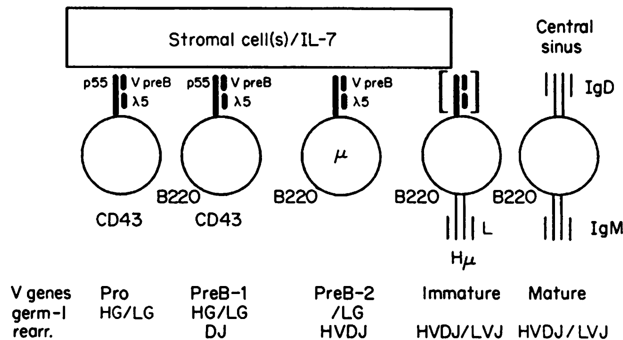

Fig. 2. Model for surrogate chain, heavy chain and light chain expression at different B cell development stages in the bone marrow.

dent antigens develop normally. Positive selection of the cells that have successfully rearranged their μ chain probably occurs through binding of the μ H chain–surrogate L chain complex to a ligand in the environment.

CENTRAL AND PERIPHERAL DELETION

Autoreactive B lymphocytes are probably continuously produced in the primary lymphoid organs. Negative selection has been shown in several mouse models. The treatment of immature B cells from normal mice with tolerogens or antibodies specific for IgM [9], and the generation of transgenic mice bearing functional, rearranged Ig genes encoding autoantibodies [10], have documented that B cells can be tolerized to self-antigens through probably more than one mechanism. Experiments using transgenic mice have shown that clonal deletion is one mechanism which explains the counterselection of autoreactive B cells *in vivo* [11,12]. In the central deletion situation, B cells that encounter cell-membrane associated self-antigens (capable of cross-linking) binding to their sIg receptors in the bone marrow with high affinity are eliminated. That the ongoing mechanism is not simply programmed cell death of autoreacting B cells has been shown recently [13,14]. Indeed, doomed autoreactive B cells have the possibility to escape deletion, by a mechanism called 'receptor editing'. This consists of a secondary modification of the Ig receptor specificity (at the IgM^+ immature B cell level) by expressing endogenously encoded Ig variable L chain genes through activation of the *RAG-1* and *RAG-2* genes. This has been shown using the 3-83 $\mu\delta$ line mouse. This mouse is transgenic for IgM and IgD anti-MHC class I Ig genes (recognizing H-2K molecules of all allelic forms except for d and f). Using different MHC (H-2K) backgrounds the following observations have been made.

In the H-2^d/3-83 $\mu\delta$ mice (non-deleting), B cells are abundant and are essentially monoclonal with respect to specificity. Over 95% of the B cells bear the 3-83 clonotype, as detected with a specific monoclonal antibody [15].

In contrast, in F_1 crosses between 3-83 $\mu\delta$ mice and H-2^k or H-2^b mice, which express the autoantigen in the whole body and of course also in the bone marrow (centrally deleting), autoreactive B cells are eliminated and their number in peripheral organs is drastically decreased. However, an unexpectedly large number of variant B cells escapes deletion and, even more surprisingly, they frequently express a λ L chain. Thus, cells bearing λ L chains must arise by the rearrangement and expression of endogenous λ L chain genes because the transgenic L chain is κ.

Several important differences were found when comparing the centrally deleting mice to a peripherally deleting model. The latter was obtained by crossing the 3-83 $\mu\delta$ mice with MT-K^b transgenic mice [16], which express H-$2K^b$ exclusively at the periphery (hepatocytes, exocrine pancreatic cells, kidney tubules); their autoreactive B cells do not encounter antigen in the bone marrow, but only after having entered the circulation. The peripherally deleting mice usually have fewer B cells than age-matched centrally deleting mice. The peripherally deleting mice lack the large increase in λ-bearing B cells seen in the centrally deleting mice. There is an increase in Ig L chain rearrangement activity in the bone marrow of centrally deleting mice. This was assessed by measuring the levels of *RAG-1* and *RAG-2* recombinase gene mRNA, as well as by quantifying Ig gene rearrangement excision products in bone marrow DNA. These findings permit us to speculate that any antigen present in the bone marrow and capable of cross-linking the B cell receptors with sufficient affinity can induce recombinase expression and secondary L chain rearrangement in $sIgM^+$ B cells. Given successful rearrangement and specificity modification, this receptor editing would allow the cells to escape apoptosis. B cells that encounter autoantigen only in the periphery cannot rely on this salvage mechanism and their deletion proceeds by a mechanism of rapid programmed cell death.

EMIGRATION KINETICS AND LIFE SPAN OF THE RAPIDLY RENEWING BONE MARROW LYMPHOCYTES

Of the total small lymphocyte population in the murine marrow, 75–95% are rapidly renewing. This value decreases with age [17]. The loss of newly formed small lymphocytes represents local cell death and export from the bone marrow. Those cells that escape deletion accumulate in sinusoidal spaces near the central sinus, from where they are released into the periphery. There is a continuous migration of newly formed small lymphocytes from the marrow sinusoids through the bloodstream to the peripheral lym-

phoid tissues, particularly the spleen and lymph nodes. Intramyeloid labelling with [^{3}H]thymidine provides direct *in vivo* evidence that small lymphocytes leave the bone marrow in the first 1–2 days after their production, and that this traffic includes sIgM$^+$ cells [18–20]. In murine rapidly renewing small lymphocytes (most of which are B cells), cells aged 4 days or less account for about one-third of all small lymphocytes in the spleen, one-quarter of those in the lymph nodes and one-twentieth of those in the blood [21].

The spleen collects newly formed B lymphocytes from the bloodstream with high efficiency. Most of the cells are taken up during their first passage through the spleen [22]. After deleting all B cells by sustained anti-IgM and anti-IgD treatment, the abrupt cessation of suppression is followed by a rapid reappearance of immature B lymphocytes, mainly sIgM$^+$, sIgD$^-$ cells in the red pulp of the spleen within 24 h, and mature sIgM$^+$, sIgD$^+$ cells in the white pulp by 48 h [23]. The results of intramyeloid labelling, lymphocyte transfer and repopulation experiments all indicate a lag of 1–2 days between the time newly formed small lymphocytes enter the spleen and the lymph nodes, respectively.

Newly formed primary B lymphocytes may be activated in the peripheral lymphoid tissues if the appropriate antigenic and accessory signals happen to be present during the cells' short life span. In the mature animal the fraction of newly formed B cells that enters the long-lived pool at any time must be small. It has been estimated that no more than a few per cent of the lymphocytes produced in the bone marrow can enter the circulating pool [17]. However, in young postnatal mice that have a limited recirculating pool initially and are exposed abruptly to environmental antigens, the fraction of newly formed marrow lymphocytes entering the long-lived pool is probably substantial.

THE GERMINAL CENTRE RESPONSE

During humoral immune responses, B cells which specifically interact with antigen through their sIg receptor are activated. Through a complex differentiation process that takes place in the B cell areas of the peripheral lymphoid organs, plasma cells and long-lived memory cells are generated. With some antigens, such as bacterial surface polysaccharides, the B cell differentiation process is independent of T cell help. In these responses IgM-secreting plasma cells are generated. Memory cells do not seem to be established. The B cell response to proteins is much more complex. The protein antigen has to be processed by antigen-presenting cells (macrophages, follicular dendritic cells, B cells). In association with class II MHC antigens, peptides are recognized by T helper cells. The interaction of T helper cells with antigen-activated B cells and antigen-presenting cells, leads to a complex spatial and sequential series of proliferation and differentiation, giving rise to somatically mutated and selected progeny of both plasmacytes and memory B cells.

Germinal centre (GC) reactions develop in immune responses where T helper cells are involved [24]. Principally, GC arise in three situations [25]. The first is during a primary response when no antigen-specific antibody exists and T cell help is limited. In this situation, virgin B cells become activated in extrafollicular sites, probably in the vicinity of T cells and interdigitating cells. Subsequently they localize to the follicular dendritic cells (FDC) and give rise to the GC response [26]. In the second instance, during a secondary exposure, antigen is rapidly complexed with circulating high-affinity antibody and trapped on the plasma membrane of a FDC, where the specific B cell encounters the antigen through its surface receptor. T cell help is not limited and a GC is formed rapidly (within 3 days). B cell clones with higher Ig affinity are produced. Higher titres of IgG or IgA versus IgM are produced. The third situation is during the maintenance phase of the immune response; when the levels of antibody decrease to a critical threshold, a further round of antigen-specific B cell proliferation is induced [27]. The immune complexes trapped and retained for long periods by FDC will stimulate antigen-specific cells into antibody production in a cyclical fashion. When antibody is maximally available the covering of all antigenic sites of the immune complexes by the FDC shuts down the GC reaction. When circulating antibody titres naturally decrease, antibodies from the complexes on the FDC diffuse and antigen is exposed for a new round of stimulation.

Immunohistochemical characterization of the GC

When there is free antigen, B cells are recruited from outside into T cell-dependent (Td) antibody responses [28]. Analysis of the sites of B cell activation in Td antibody responses shows that B cells proliferate both in T cell-rich zones (containing T helper cells, B cells and interdigitating cells) [29] of secondary lymphoid tissues, and in follicles (containing follicular dendritic cells and recirculating sIgM$^+$, sIgD$^+$ small B cells) [30]. The GC precursor cells, as they occur in Td antibody responses, appear to be found among the sIgM$^+$, sIgD$^+$ recirculating B cells.

Proliferation occurs in both sites in the period immediately following antigen administration, but B cell proliferation in T zones ceases about the time virgin B cell recruitment ends [31]. Proliferation in follicles continues throughout the response. T cell zones are rich in T cells and in interdigitating cells. Interdigitating cells show high constitutive expression of class II MHC and are highly efficient in processing antigen and in presenting peptides to T helper cells. Recirculating B cells migrate through the outer layers of T zones on the way to and from follicles [30]. During primary splenic responses to hapten–protein conjugates, antibody production typically starts toward the end of the first week after immunization. This is preceded by the appearance of small numbers of hapten-specific B cells in the T zones [29].

B cell proliferation in the follicles starts at about the time antibody production begins; it falls to low levels by 3 weeks after immunization. The slowness, the variability and relatively small size of primary responses are attributable to lack of T cell help and not paucity of hapten-specific B cells. This can be optimized by priming mice with a carrier protein before challenging with a carrier–hapten conjugate. The magnitude and uniformity of the hapten-specific follicular response after carrier priming has assisted the analysis of the sequential events involved in GC formation in follicles. Different phases can be distinguished in GC formation [32].

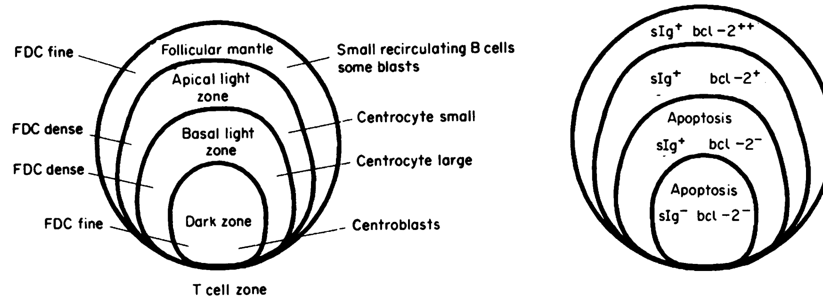

Fig. 3. Model for germinal centre.

Exponential proliferation of B blasts. On day 3 of bihapten–carrier challenge and after an exponential proliferation of B blasts in the FDC network, 6–31% of the follicles are found to contain blasts of a single specificity, while the remainder of the follicles are of mixed specificity. Statistically, 12% of the follicles would be expected to be monospecific if each follicle was colonized by three hapten-specific blasts. The number of blasts in 3-day-old follicles is about $1-1{\cdot}5 \times 10^4$ cells; from this it can be deduced that the cell cycle of these primary B blasts lasts about 3 h. During the exponential growth of the B blasts in the GC formation, the small recirculating B cells are excluded from the follicle centre and form the follicular mantle that surrounds the GC.

Formation of the dark and light zones within the follicle centre (Fig. 3). When the FDC network becomes filled with B cell blasts (expressing sIg) the period of exponential growth ends. At this stage very profound changes occur in the follicle centre, resulting in the formation of the typical structure of the fully developed GC. Most of the B cell blasts are lost from the FDC network. A new zone of blasts appears at the part of the follicle nearest the T zone; these blasts, termed 'centroblasts' [33], express little or no surface or cytoplasmic Ig. The area they occupy is called the dark zone. Centroblasts are in rapid cell cycle but they do not increase in number because they continually give rise to centrocytes. Centrocytes are located in the dense central part of the FDC network [34]. The part which harbours the centrocytes forms the light zone of the GC. By *in vivo* labelling studies [35] it has been shown that centrocytes derive from centroblasts and that centrocytes themselves are not dividing. Centrocytes within single follicles are oligoclonal [36]. They are sIg^+. Different experimental approaches point to the conclusion that centrocytes undergo selection on the basis of affinity for antigen held on FDC. Those cells that receive antigen-dependent signals in the light zone subsequently differentiate to become plasma cells [37] or memory cells [38]. Cells that are not selected die by apoptosis and are eliminated by macrophages.

Hypermutation in Ig V region genes in GC

T cell-dependent affinity maturation of antibody responses is attributable to the emergence of B cell clones that have undergone somatic mutations in their rearranged Ig V region genes. Analysis of cDNA sequences of V_H and V_L chains from mouse hybridomas, made on different days after immunization, shows that on day 7 after primary immunization there are almost no mutations in the rearranged H and L variable genes [39]. One week later (day 14) most hybridomas still have similar but no longer identical sequences, suggesting a high degree of somatic mutation. Hybridomas obtained 3 days after a secondary immunization show a further increase in the mutation observed in the initial V_H and V_L chain combinations, but also a very important shift to different V_H and V_L chain combinations. Recently, specifically isolated GC cells showed essentially the same pattern of increase in mutations in their Ig variable genes at sequential moments after immunization [40,41]. The increase in somatic mutations from day 10 to day 14 after primary immunization was seen essentially in the cells that had switched from IgM to IgG.

Fate of cells produced in GC

Selection versus apoptosis. It has long been known that there is a high death rate within GC. No part of the GC is free of apoptosis, but this is particularly noticeable in the upper part of the dark zone and in the base of the light zone. It was suggested that cells might be selected within GC by interaction with antigen held on FDC. Direct evidence has been obtained [42] by showing that cells isolated from human tonsil GC, doomed to die by apoptosis when cultured at 37°C, could be rescued by incubation with anti-Ig-coated sheep red blood cells. These cells start to express bcl-2 oncogene within 4 h of such treatment. There is now clear evidence implicating the expression of the protein encoded by the bcl-2 oncogene with cell selection and survival in GC. A translocation between a site on chromosome 18, adjacent to the *bcl-2* gene, and the *IgH* genes on chromosome 14 is frequently found in centroblastic/centrocytic lymphomas. Bcl-2 encodes a 2-kD protein that is located mainly in the mitochondria [43] and cytosol [44]. While this protein is not expressed in appreciable amounts in GC cells, it is found in most if not all small lymphocytes and extrafollicular B cell blasts. Expression of bcl-2 has been implicated in cell survival [45] in Burkitt's lymphoma cell lines, which have many of the features of centroblasts. These cells enter apoptosis if cultured at low serum concentrations but become serum-independent after transfection of bcl-2.

Differentiation signals for GC cells. Three types of signals can prolong GC cell survival and induce their differentiation [41]. First, exposure to the anti-CD40 antibody [46] induces cells to leave the cell cycle and to acquire the phenotype of small lymphocytes (sCD23 and an increase in sIg). This is consistent with the production of memory B cells, which are known to be produced in GC. Bcl-2 expression is also induced in centrocytes through the CD40-derived signal. When IL-4 is added together

with CD40 antibody, the cells remain in cell cycle and the sIg and CD23 expression of these cells is greater than that of cells treated with CD40 alone. This phenotype resembles extra-follicular B blasts that are characteristic of the established phase of Td antibody responses. Second, a combination of the recombinant fragment (25 kD) of the CD23 molecule plus rIL-1α also prevents GC cells from entering apoptosis. There is cytoplasmic expression of Ig and bcl-2, in addition to the development of endoplasmic reticulum. These are the features of plasmablasts [38]. Third, some GC cells respond to IL-2 [47] and continue to proliferate over several days with low sIg expression. They continue to resemble centroblasts in that they do not express bcl-2. A second effect of IL-2 is on a small fraction of GC cells which express sIgM. These cells start to produce cytoplasmic IgM but fail to express bcl-2. These cells may reflect the IgM-producing plasma cells found in tonsil GC. The relevance of these *in vitro* differentiation pathways to the *in vivo* situation remains to be defined.

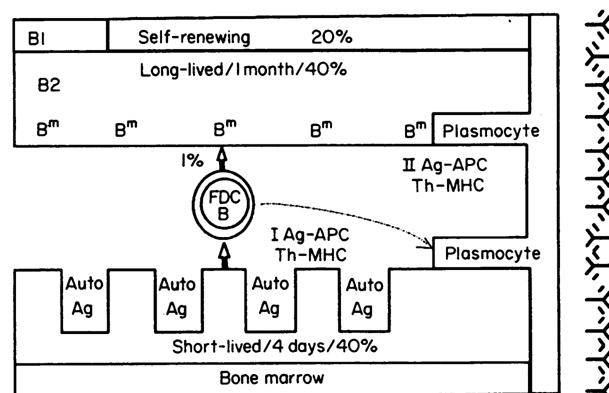

Fig. 4. Synoptic view of the B cell pool dynamics. Percentages indicated are very rough percentages of the total B cell pool. Th, T helper; APC, antigen-presenting cell; B^m, memory B cell; IAg, primary antigenic stimulation; IIAg, secondary antigenic stimulation.

LIFE SPAN OF MEMORY CELLS AND PLASMACYTES

During the splenic anti-hapten responses, small numbers of anti-hapten memory B cells appear in the follicular mantle, but larger numbers are seen to colonize the marginal zone [48]. Hapten-binding memory cells can be found in both these compartments throughout Td antibody responses, and have been found more than a year after secondary immunization [49]. The life span of these cells is no more than a few weeks [48]; consequently it is likely that they are renewed from the few blasts which proliferate in follicles throughout the Td antibody response. Following the transient appearance of memory B cells in follicles after secondary challenge, there is a gap of 1 day before hapten-specific B blasts are seen in the follicles. These increase in number, peaking 3–4 days after immunization. However, it is not clear whether memory B cells undergo further somatic mutation in their IgV regions in secondary responses. It is possible that mutations in B cells occur only during the 3-week period when they are proliferating in follicles after their virgin progenitor was induced to form a GC.

When animals previously immunized with a hapten–carrier conjugate are challenged, there is a selective loss of memory B cells capable of recognizing that hapten from the marginal zone. Following a transient appearance in follicles, they accumulate in T cell zones. The anti-hapten blast reaction in T zones after secondary immunization is quantitatively far greater than in the primary response but lasts no more than 48 h. Many of the blasts in the T zones migrate to the red pulp, where they differentiate to become plasma cells. These plasma cells live only 3 days [50]. They are lost from the spleen within a week after secondary challenge. Long-term antibody production is maintained in the bone marrow where the IgG- and IgA-producing plasma cells have a life span of about a month [51,52]. Some antigen-specific plasma cells can be found in the lymph nodes at the periphery of the GC by day 5 after immunization. These cells can form a continuous trail between the GC and the medullary cords, suggesting that they originated in the GC. A significant proportion of GC cells (characterized by their high affinity for peanut agglutinin; PNA) migrates through blood and homes to the bone marrow [53]. The role of the bone marrow as an antibody-producing site is far more important during secondary responses [54] (Fig. 4). Upon secondary immunization, immune complexes form rapidly. Most are trapped by macrophages but some are transported to FDC cells. Some of the antigen released by the FDC cells under icosomal form will be taken up by GC B cells, which process it before presentation to T cells. The T cells will provide the B cells with the needed growth and differentiation factors to become antibody-forming cells [55]. These will migrate from the lymph nodes and can be found as PNA^{hi} blasts in the thoracic duct and blood as early as 3 days after secondary challenge [56]. The PNA^{hi} cells enter the bone marrow sinuses, possibly through a receptor binding their surface galactose residues. Bone marrow contains factors that are capable of stimulating GC B cells to produce high levels of IgG and possibly high levels of other isotypes as well.

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