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Phenotypic and functional heterogeneity of human memory B cells

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Abstract

Memory B cells are more heterogeneous than previously thought. Given that B cells play powerful antibody-independent effector functions, it seems reasonable to assume division of labor between distinct memory B cells subpopulations in both protective and pathogenic immune responses. Here we review the information emerging regarding the heterogeneity of human memory B cells. A better understanding of this topic should greatly improve our ability to target specific B cell subsets either in vaccine responses or in autoimmune diseases and organ rejection among other pathological conditions where B cells play central pathogenic roles.

Keywords

B cells; effector memory; central memory; marginal zone; homeostasis

Introduction

Immunological memory represents a highly effective mechanism to ensure quick protection against prevalent infections. B cell memory is generally viewed as supported by two cellular compartments: plasma cells, responsible for the production of antibodies (effector memory) and memory B cells which would represent precursors (central memory) capable of generating and replenishing the plasma cell compartment through a combination still incompletely understood of antigen-dependent and independent mechanisms (1–4). This view is certainly valid when B cell effector functions are limited to the production of antibody. Yet, it is also incomplete as it fails to incorporate important effector and regulatory functions that may be played by “central” memory B cells (antigen-presentation, T cell and dendritic cell regulation and cytokine and chemokine production) (5–7). When these critical B cell functions are considered, it can be postulated that effector memory is mediated by both plasma cells (through the production of antibodies) and by memory B cells (mostly through the production of cytokines). In turn, central memory B cells would be responsible not only for the generation

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and replenishment of plasma cells but also for the generation of distinct subsets of effector B cells. It is also possible that central memory B cells could play distinct regulatory roles.

Of note, the same properties of memory cells that are desirable for protective immune responses (long lifespan, prompt and enhanced responses to activation and ability to stimulate T cells) may be deleterious when it comes to avoiding chronic autoimmunity. Thus, once tolerance is broken in the B cell compartment, autoimmune memory would tend to persist (facilitated by availability of self antigens), could be easily reactivated and could break tolerance in the T cell compartment thereby providing a mechanism for further diversification and amplification (8, 9). Given that substantial autoreactivity can be detected even in the IgG memory compartment of healthy subjects (10), it seems obvious that protective (anti-microbial) and potentially pathogenic (autoreactive) memory B cells must have different properties and be controlled by distinct regulatory mechanisms.

With regard to surface phenotype, substantial heterogeneity amongst human memory B cells has been documented by many studies including our own. Indeed, despite initial descriptions of CD27 as a universal marker of human memory cells, we and others have described memory populations that lack expression of CD27 and may be substantial in SLE and in some infections such as RSV (11–14). As discussed in this review, additional heterogeneity of memory B cells can also be demonstrated on the basis of the expression of CD38, CD21, CD24, CD19, B220, FcRH4 and CD25 (12,15–17).

In addition to surface phenotype, the heterogeneity of human memory B cells can also be documented by: 1) differential regulation in autoimmune diseases and infections; 2) different repopulation kinetics after B cell depletion therapy (BCDT); 3) differential impact of other biological therapies; and 4) different in vivo doubling times and half-life as documented by heavy water labeling (our own preliminary data indicating that CD27[−] memory cells have a much longer doubling time than CD27⁺ memory cells).

Importantly however, the actual functions of memory B cell subsets remains to be understood in terms of their relative participation in protective and autoimmune responses and in terms of their specific effectors and regulatory functions including cytokine production, antigen presentation and ability to differentiate into plasma cells. Also currently unknown is whether specific memory B cell subsets have different participation in central memory (understood as precursors of effector B cells and plasma cells) versus effector memory (defined as cytokine production). It is worth noting however that there is significant literature to support the ability of memory cells to produce abundant cytokines (TNF and lymphotoxin) with patterns different from naïve B cells (IL-10). Different studies also indicate that cytokine production by B cells may be relevant to human autoimmune diseases including SLE and MS. Finally, preliminary evidence indicates that excess production of pro-inflammatory cytokines by B cells may be reversed by BCDT in patients with MS (18).

Memory B cell heterogeneity

As recently discussed by others (19), our understanding of the diversity of memory B cell populations is hampered by pre-conceived notions of their surface phenotype, function and cellular and anatomical origin. Thus, the strict definition of a memory cell as one expressing isotype-switched, somatically mutated antibodies and generated through a T cell-dependent germinal center reaction would exclude important memory responses mediated by unswitched and/or unmutated B cells that at least in some cases may originate through GC-independent pathways (20–30). On the other hand, if a long life-span represents a critical property of memory cells, then the expression of switched, mutated antibodies might not suffice for the identification of memory cells. Similar caveats are applicable to the definition of memory T cell subsets and their life-span (31,32)

Despite these caveats, the heterogeneity of B cell memory responses, while poorly understood, is likely to be substantial. In addition to conventional IgG memory to T cell-dependent (TD) protein antigens generated through the germinal center (GC) selection of follicular B2 cells, mouse IgM memory to T cell-independent type 2 (TI-2) antigens, including pneumococcal polysaccharides, NP-Ficoll and *Borrelia hermsii* antigens, can be provided by B1b cells (33–35). Early studies also indicated that rat MZ B cells can carry IgG memory to TI-1 and TI-2 antigens (36). Recent evidence indicates that, in the mouse, TI-2 antigens can also induce IgG memory responses mediated by B2 cells with a phenotype different from both follicular and marginal zone (MZ) B cells (37). Finally, at least in rodents, marginal zone B cells can provide memory responses, albeit with a relatively short half-life, to T-dependent antigens (38). This study also provided early evidence to advance the concept of IgM memory responses.

Substantial heterogeneity is also apparent among human memory B cells. In fact, while their functional heterogeneity is less well understood, several phenotypic subsets have been recognized in humans due to the advantages conferred by the higher abundance of human memory cells and by the usefulness of CD27 as a marker of human B cell memory. Thus, when defined by the expression of CD27 one important difference between humans and mice is the frequency of memory B-cells which in humans represent 40–60% of all PBL B cells (39). This difference has been attributed to the accumulation of long-lived memory cells during the longer human life-span (39). Also of significant interest is that less than half of all human CD27⁺ memory B cells have undergone isotype switch, while the rest express surface IgM. IgD-only cells that have experienced isotype switch in the GC also exist although they represent a minor fraction of all B cells. Interestingly, it has been reported that these cells are enriched in autoreactivity (40). Their physiological or pathogenic roles however remain to be elucidated.

Whether IgM memory cells represent a homogeneous subset remains controversial. Initially, it was reported that populations of IgM-only and IgM/IgD memory cells could be clearly differentiated (39). However, other reports and our own experience indicate that the vast majority of IgM memory cells also express at least low levels of surface IgD (23–25,41). The actual developmental origin of these subsets of memory cells and their specific role in functional immune responses remain to be elucidated. IgM/IgD memory cells, which may develop through GC-independent pathways, have been proposed to represent the human functional equivalents of B1 and MZ B cells and to represent the critical cellular compartment for protection against infections with encapsulated organisms (20,23–25,28). Significant controversy (reviewed below) still exists regarding the concept that IgM/IgD memory cells may represent a recirculating subset of marginal zone B cells. It has been suggested that IgM-only memory cells may represent B2 follicular precursors of GC-dependent isotype switched memory cells (25). Other studies however have shown that, at least in vitro, IgM-only memory cells do not efficiently undergo isotype switch (42).

Current schemes of classification of human memory B cell populations

By and large, the analysis of human B cell populations by flow cytometry has thus far relied on the expression of 4 major surface markers: CD19, IgD, CD38 and D27. With this four-color approach, two major classification schemes can be produced depending on the relative expression of either IgD and CD38 or IgD and CD27 (Figure 1). Thus IgD/CD38 staining provides the so-called Bm1-Bm5 classification and can be used to identify multiple subsets in the human tonsil including: virgin naïve cells (Bm1: IgD⁺CD38[−]); activated naïve cells (Bm2: IgD⁺CD38⁺); pre-GC cells (Bm2'; IgD⁺CD38⁺⁺); GC cells (Bm3-centroblasts and Bm4-centrocytes, both are IgD[−]CD38⁺⁺); and memory cells (Bm5: IgD[−]CD38^{+/-}). Bm5 memory cells which express levels of CD38 ranging from moderately positive to negative have been further divided into early Bm5 (CD38⁺) and late Bm5 (CD38[−]). While this division is arbitrary and implies a chronological relationship that has never been formally established, it may

nonetheless identify two different subsets of memory cells. Evidence for this contention is provided by the preferential expansion of early Bm5 cells in some clinical situations and by the recognition that CD38⁺ late Bm5 cells are enriched for cells that are CD27⁺ and FcRH4⁺ (12,14). In the peripheral blood the Bm1-Bm5 classification recognizes similar subsets with the exception of GC cells. While this classification has been quite useful and represents an almost mandatory point of reference in the field, it also suffers from significant limitations. For example, Bm1 and Bm2 cells also contain unswitched memory cells and Bm2⁺ also contains transitional cells. Interestingly, while a similar division between early Bm5 and late Bm5 can be established in the peripheral blood, no FcRH4 memory cells (Bm5 or otherwise) are found in this compartment (12,14).

The IgD/CD27 classification builds on the notion of CD27 as a universal marker of human memory B cells to distinguish between memory cells (CD27⁺) and naïve B cells (CD27⁻IgD⁺). In turn, CD27⁺ memory cells can be divided into IgD⁺ (usually together with IgM or alone in a minor fraction of memory cells) and IgD⁻ with the latter subset usually labeled as isotype switched (predominantly IgG⁺ or IgA⁺) although it also contains a small fraction of IgM-only memory cells. In the peripheral blood, transitional cells (in the healthy adult representing less than 2–3% of all CD19⁺ B cells) would also be CD27⁻IgD⁺. In addition, in the secondary lymphoid tissues, 50–70% of all germinal center (GC) cells would stain as CD27⁺IgD⁻ (43,44).

A major drawback of current classifications is their inability to recognize memory cells that lack expression of CD27. Such cells were initially recognized in SLE by ourselves and others as an expansion of cells negative for both CD27 and IgD (45,46). More recently the existence and characteristics of CD27⁻ memory cells have been described by ourselves and others in the peripheral blood and tonsils (11,12,14,47). In the PBL, global CD27⁻ memory B cells can be recognized as double negative for IgD and CD27 that represent less than 5% of all B cells in healthy subjects. Positive identification can be achieved for isotype switched (IgG⁺ or IgA⁺) CD27⁻ cells and all CD27⁻ memory cells stain positive for rhodamine123 or mito-tracker dyes (12,47). In the tonsil, CD27⁻ memory cells also lack expression of IgD and CD27 as well as CD10 and express equivalent levels of CD38 as conventional Bm5 cells (CD38^{+/+}). The actual frequency of CD27⁻ cells among Bm5 cells, although not systematically explored, may have been underestimated as our data and those of others indicate these cells may represent even in healthy subjects 20–50% of all Bm5 cells in the tonsil (Figure 2c) (12,14).

As we have reported in SLE, CD27⁻ memory cells are comparable to conventional CD27⁺ cells in their isotype expression (either IgM, IgG or IgA), their inability to extrude rhodamine123 or similar dyes and their ability to proliferate in response to stimulation only with CpG DNA. Of note, these culture conditions trigger upregulation of CD27. Moreover, IgG⁺CD27⁻ memory cells express significant levels of somatic hypermutation with genetic features consistent with antigen selection. Interestingly, we have found however that the average rate of mutation in IgG⁺CD27⁻ memory cells is somewhat lower than in their CD27⁺ counterparts. Our unpublished results also indicate that a significant percentage of IgM⁺CD27⁻ memory cells are unmutated. Whether these unmutated cells encode antibodies of importance for protection against infections remains to be determined (48). Interestingly, thus far our studies have documented two situations in which CD27⁻ memory cells are significantly expanded and represent a large percentage of all PBL memory B cells: patients with active SLE (in whom these cells may become in some cases the largest memory subset) (12) and healthy subjects exposed to RSV (Figure 3).

Many questions remain to be addressed about CD27⁻ memory cells including their origin and their ability to differentiate into plasma cells (12). As we have discussed elsewhere, these cells are likely to derive either from extra-follicular responses or from incomplete germinal center

reactions. Alternatively, they could represent the progeny of activated CD27⁺ memory cells that have lost CD27 expression perhaps after engaging CD70 on activated T cells. Mitigating against this possibility is the observation that on average, the level of somatic hypermutation observed in CD27[−] memory cells is lower than in CD27⁺ cells. It is also unlikely that CD27[−] memory cells represent an intermediate step in the differentiation of CD27⁺ cells into plasma cells given that plasma cells express high levels of CD27 and that the ability of memory cells to differentiate into plasma cells appears to correlate with CD27 upregulation (49). Moreover, at least a subset of CD27[−] memory cells (expressing the inhibitory FcRH4 receptor) are impaired in their ability to generate plasma cells in culture (14).

Substantial additional complexity of human memory cells as revealed by multi-chromatic flow cytometry

In addition to the aforementioned classification schemes, human B cell subsets can also be defined by the surface expression levels of CD24 and CD38 (naïve, memory, GC, transitional B cells and plasmablasts). However, how the subsets defined by one classification scheme relate to those subsets defined by other schemes is not often clear. Hence, we have developed six different 10-color flow cytometry panels which provide a comprehensive phenotypic analysis of human B cell subsets and reveal additional complexity of human memory B cells (Figure 2 and Table 1). As mentioned previously, IgD[−] B cells consist of CD27⁺ and CD27[−] memory B cells, and both subsets comprise switched and IgM-only memory B cells. With the exception of a few percents of them being CD38⁺⁺, B cells in both memory subsets are approximately evenly distributed between the CD38⁺ early Bm5 and CD38[−] late Bm5 subpopulations (Figure 2A). On the other hand, both early Bm5 and late Bm5 subsets in the periphery comprise a vast majority of CD27⁺ cells and a minor population of CD27[−] cells (Figure 2A). This is in contrast to what has been observed in tonsil where 20–50% of Bm5 are CD27[−] (Figure 2C). It is interesting to note that in the Bm3+4, early Bm5 and Bm5 subpopulations, the ratio of CD27⁺ to CD27[−] cells remains quite constant, possibly reflecting a developmental relationship among the corresponding subsets within these subpopulations.

The analysis of additional surface markers permits the identification of additional subpopulations of memory cells within both CD27⁺ and CD27[−] cells. This is illustrated here by multicolor staining of peripheral blood B cells and analysis of the expression patterns of CD24/CD38 in both subsets (Figure 2A). In contrast to the even distribution of CD27⁺ and CD27[−] cells between CD38⁺ and CD38[−] subsets, the expression patterns of CD24 are quite different in that the majority of CD27⁺ cells are CD24⁺ while the majority of CD27[−] cells are CD24[−], a trend that is in contrast to the expression of B220 (Figure 2B). The B220 glycoform of CD45R (as detected by mAb RA3-6B2) is almost universally expressed by human naïve B cells but only in about 20% of all CD27⁺ memory cells (12, 50). Yet, our preliminary results (Figure 2B) indicate that B220⁺ cells represent 50–60% of CD27[−] memory cells and are largely restricted to the isotype-switched compartment (12). Indeed, a detailed isotype analysis within the CD27⁺ and CD27[−] subsets reveals that the IgM-only cells in both subsets are a distinctive population of CD24⁺B220[−] cells. On the contrary, IgG memory B cells in both subsets are heterogeneous in the expression of CD24 and B220, suggesting that the switched memory B cells might be further divided into finer subsets (Figure 2B). Collectively, in the periphery less than 20% of the CD27⁺ cells are B220⁺ while about 50% of the CD27[−] cells are B220⁺ (Figure 2B). The same distributions of B220⁺ cells in the CD27⁺ and CD27[−] compartments of Bm5 memory cells are also observed in the tonsil (Figure 2C). Our data indicating that 90% of Bm3 and Bm4 germinal center cells (of which 60% are CD27⁺) are B220⁺ (not shown) and that a large fraction of CD27[−] memory cells are B220⁺ suggest that B220 is not lost during the germinal center reaction and would seem to refute an inverse developmental relationship between the expression of these two markers (50). Hence, the significance of the differential expression of B220 in human memory B cells remains to be understood but it could be of major

functional relevance given the critical role of CD45 in lymphocyte activation and tolerance and the essential role of CD45-expressing B cells in the induction of T cell tolerance by anti-CD45RB antibodies (51, 52). Understanding the significance of these findings will require to first elucidate the relationship between the expression of B220 and: 1) the different pathways of memory cell development (GC-dependent and independent); 2) different memory responses (T-dependent and T-independent); 3) the division of labor between B220⁺ and B220⁺ memory cells in terms of their effector functions (both antibody-dependent and independent) and their relative participation in protective versus autoimmune responses (12). One particularly intriguing possibility is that B220 expression could be lost during differentiation into resting (long-lived) central memory cells and upregulated during activation and generation of (short-lived) effector memory B cells (32, 53–55). This model would be consistent with the upregulation of B220 observed in activated mouse and human T cells before they undergo apoptosis (55–57). It would also account for the expansion of B220⁺ memory cells we have observed in active SLE if one assumes abnormal activation and/or impaired apoptosis of effector memory B cells, a model currently tested in our laboratory.

The expression of the inhibitory FcR homolog 4 (FcRH4) has been shown to be mainly restricted to the early Bm5 and late Bm5 regions in the tonsil, and the majority of FcRH4⁺ cells are CD27⁺ (14). Our previous (12) and current results also demonstrate that there is greater than 20% of Bm5 cells in the tonsil that express FcRH4, which in turn are evenly distributed between the B220⁺ and B220⁺ subpopulations (Figure 2C). Our results also show that there is a great variability in the distribution of FcRH4⁺ cells between the CD27⁺ and CD27⁺ memory compartments. On the one hand, the majority of FcRH4⁺ cells are CD27⁺ as previously reported by us and others (12,14). On the other hand, over two thirds of FcRH4⁺ cells can be found in the CD27⁺ subset as well (Figure 2C). This variability might be attributed to sample variations, or it can reflect the dominance of a particular subset (FcRH4⁺CD27⁺ or FcRH4⁺CD27⁺) during an immune response.

Anatomical distribution of human memory B cell subsets: The conundrum of the human spleen marginal zone and its equivalents

One of the main challenges in the study of human memory B cells is to understand their localization, phenotype and function of different subsets in the locales where they are generated in the course of orchestrated immune responses, where they reside as selected resting memory cells (in both cases presumably in the secondary lymphoid tissues although the contribution of the bone marrow needs to be more thoroughly assessed), and where they are recruited or generated as activated effector cells to fight incoming infections (non-lymphoid organs) (19, 58–60). Similarly, it is important to understand the participation and function of specific memory B cell subsets in the tertiary (ectopic) organized lymphoid tissues which are frequently formed in multiple organs (including the kidneys, pancreas, lungs, thyroid, CNS, synovium and salivary glands) in autoimmune or infectious diseases (61–63). A related outstanding issue is whether the memory B cells that recirculate in the peripheral blood are representative of the tissue-bound populations at least as a representative reflection of the immune repertoire available to the host. The latter issue carries significant experimental implications since, due to ethical and practical restrictions, most human studies of (auto)-immune responses are performed with blood samples.

Much remains to be learned about these critical topics. Significant information however can be gleaned from the analysis of tissue-bound and circulating memory B cells. In rodents, memory B cells are typically thought to localize to the marginal zone although a newly described subset of mouse unmutated memory cells can also be substantially found within the follicle (38,64) (30). Most human memory B cells appear to be located in two likely inter-related anatomical areas: the marginal zone and the sub/intra-epithelial surfaces (65–71)

although, by analogy with the mouse, other locations need to be investigated with markers that may identify specific subpopulations (30).

Initially recognized as an anatomical compartment, the marginal zone is most often invoked however as a developmental and functional entity. Thus, the distinctive functional characteristics of marginal zone B cells include their critical participation in T-independent responses through a brisk, powerful and short-lived generation of IgM-secreting plasmablasts due to their pre-activated phenotype (72,73). MZ B cells also display heightened sensitivity to stimulation through at least some TLR ligands (74,75). Under this definition, MZ cells have been viewed as cells expressing a pre-selected repertoire of B cell receptors enriched for self-reactivity and reactivity to some bacterial antigens whose main role is to provide a first line of defense against overwhelming blood-borne infections and possibly to participate in house keeping functions including the clearance of apoptotic cells. Hence, MZ B cells have been postulated to represent an innate, evolutionarily selected “memory” as opposed to the conventional adaptive memory cells generated in the germinal centers by T-dependent antigens. This would be consistent with evidence from multiple mouse models indicating that the development of these MZ B cells producing natural IgM (auto)-antibodies is differentially regulated from follicular B cells as a binary cell fate decision that is likely to occur at the transitional B cell stage. The nature of MZ B cell precursors and the contribution of genetic factors and BCR signaling strength to MZ B cell fate determination have been recently reviewed and shall not be discussed further (76–79).

There is significant evidence nonetheless, indicating that the MZ is a much more complex compartment that is also the main repository of memory cells including cells responsible for isotype-switched secondary responses to some T-independent antigens (36,80). In addition, the mouse MZ also houses memory cells derived from recirculating and high-affinity germinal center cells in response to T-dependent stimulation (38,81–83). Although these findings could be interpreted simply as the anatomical MZ housing both memory cells with and without MZ-like function, resident MZ cells generated in a T-dependent response may also have the ability to respond in a typical fashion to subsequent challenge with polysaccharide antigens (83). Finally, at least a subpopulation of MZ cells has the potential to generate GC-derived memory cells in response to T-dependent antigens (84).

Of course, virtually all this information derives from studies performed in mice and rats. Where does that leave us when it comes to understanding the human MZ? Interestingly, the complex, heterogeneous pattern observed in animals seems to fit the complexity also observed in humans. The human MZ as an anatomical compartment is well identified in the spleen although it bears some significant differences with its mouse equivalent including the absence of a marginal sinus and metallophilic macrophages and the presence of an inner and outer marginal zone surrounded by a large perifollicular area (85–87). In addition, the human MZ, at least in some studies, may be interrupted by the T cell zone (69). Of significant importance, the human marginal zone does not develop during the first few of years of life and its absence correlates with the inability of infants to respond to T-independent antigens and their corresponding susceptibility to infections by encapsulated organisms (88). Similarly, the absence of the spleen increases dramatically the susceptibility of adult subjects to these infections and compromises their ability to respond to vaccination with polysaccharide antigens (89,90). Interestingly, the anatomical or functional absence of the spleen correlates with the absence of circulating IgM memory cells and serum anti-polysaccharide IgM antibodies and with the susceptibility of these patients to infections with encapsulated organisms such as streptococcus pneumoniae (23,26,28). These observations are in keeping with the role proposed in rodents for marginal zone B cells as critical IgM responders to T-independent antigens and essential sentinels against blood-borne antigens. Interestingly however, it has been suggested that

It is well established through multiple studies that, at least when defined by the expression of CD27 and/or CD148, human memory cells localize to the anatomical marginal zone and perifollicular area. Furthermore, the large majority of B cells contained in the anatomical MZ express CD27 and display somatic hypermutation of their rearranged antibody genes (91–93). Consistent with an earlier report using CD148, we have reported that the human spleen contains a population of B cells with the surface phenotype of murine MZ B cells ($CD21^+CD23^-IgM^+IgD^-$) which are almost equally split between cells expressing either IgM, IgG or IgA (44,69) (and Cappione et al, unpublished results). Similar results have been recently reported using a more strict definition of MZ phenotype based on high expression of CD27 and CD21, the latter being a feature shared with mouse MZ cells (60,74). Indeed, this report has proposed that MZ B cells are represented by $CD21^{high}CD23^-$ cells and universally express high levels of CD27. Under this definition, IgA^+ B cells would not be represented within the MZ population as these cells were split between IgG^+ (30%) and $IgM^{high}IgD^+$ cells (70%). $CD21^{high}$ MZ cells had a phenotype defined as $TACI^{high}BAFFR^{high}CD95^{high}$. Of note, in this work the assignment of high expression of these markers was based on relative increase over $CD23^+CD21^+$ follicular cells and $CD21^{low/-}CD23^-$ cells. Relative increase over isotype controls was not provided. $CD27^{high}$ MZ cells were also proposed to express high levels of B220 but the actual levels shown were rather modest and may have included $B220^+$ and $B220^-$ populations. Similarly, modest levels of expression of CD148 was preferentially observed in $CD27^{high}$ MZ cells and appeared to be restricted to IgM^+ cells, a result in keeping with previous observations (69). Consistent with other studies, MZ B cells were shown to be histologically $CD1c^+$ but the expression of this marker was not assessed in the different subsets by flow cytometry. Finally, and in contrast with some, but not all studies of MZ B cells in mice and humans, these $CD21^{high}$ MZ B cells did not appear to express significant levels of CD80/86 (69,94). Of significant interest however, IL-21 and BAFF were synergistic in their ability to induce powerful plasma cell differentiation and antibody production from $CD21^{high}IgG^+$ MZ B cells, which were shown to reside in the outer edge of the MZ in close association with T cells and DC, but not from unswitched MZ memory B cells. Accordingly, it was postulated that these cells could be responsible for maintaining serological memory through polyclonal Ag-independent responses induced by soluble factors produced from neighbor T cells and DCs (60).

Marginal zone equivalents have also been defined in other human lymphoid organs including the sub-epithelial and intra-epithelial areas of the tonsil, the dome region of the Peyer's patches the subcapsular sinus of lymph nodes and the thymic medulla (95–97). As in the spleen, the evidence available indicates that memory B cells accumulate in these areas at least when defined by the expression of CD27 and the presence of somatic hypermutation. However, different studies have defined these memory cells on the basis of different surface markers and with different combinations of histological and flow cytometry techniques. This makes it difficult to correlate the different populations identified in different studies and to establish the actual heterogeneity of tissue-bound memory cells. That they are heterogeneous however, in ways that may resemble spleen cells, is well established. Indeed, the phenotype of MZ B cells outside the spleen has been most extensively studied in the tonsil. Here, the sub-epithelial B cells (initially purified for flow cytometry analysis through Ficoll density gradients) are also heterogeneous and contain almost equal numbers of resting, largely unmutated IgM^+ cells ($CD23^-CD38^+CD24^+$) and activated, generally mutated IgG^+ cells ($CD24^+CD38^-CD80^+CD95^+$). CD27 was found expressed at high levels in most activated cells and at a lower level in most resting cells expressing IgM-only. In contrast, low levels of CD27 were found in only a fraction of IgM^+IgD^{low} cells. More recent studies have confirmed that isotype switched (both IgG^+ and IgA^+) also accumulate with the sub-epithelial and intra-epithelial areas of the human tonsil. These elegant studies indicate that these isotype-switched, mutated yet polyreactive B cells may derive from in situ differentiation of IgM-expressing B

cells induced to express AID by interaction with either BAFF-expressing DCs or TSLP-expressing ECs (58).

Interestingly, sub/intra-epithelial tonsil B cells have been found to express the Fc-related inhibitory receptor IRTA1 (immunoglobulin superfamily receptor translocation-associated 1). In this study, IRTA1 (also known as Fc receptor homologue 4 or FcRH4), was reported to be selectively expressed by these B cells underneath and within the tonsil epithelium and dome epithelium of Peyer patches but not in the spleen or lymph nodes MZ even though reactive lymph node monocytoid B cells were strongly IRTA1⁺. Consistent with a memory phenotype, IRTA1⁺ cells expressed CD27 and mutated antibody genes (98). On the other hand, using different antibodies against FcRH4, these memory cells appear to be largely CD27⁻ and confined to the tonsil but absent from the spleen peripheral blood and bone marrow, findings consistent with our own studies (12,14).

Another potential population of tissue bound memory cells is represented by large B cells identified within the interfollicular T-cell-rich zones of normal human lymph nodes which also bear morphologic resemblance to the large (asteroid) B cells found in the thymic medulla (99–101). Interestingly, these cells also lacked expression of CD27 even though they were somatically mutated. The relationship between these cells and FcRH4⁺ memory cells or the IRTA1⁺ monocytoid B cells found in reactive lymph nodes remains to be established (99, 102).

Also poorly understood in humans is the ability of specific tissue memory cell subsets to recirculate, the features that determine their ability to egress from lymphoid organs and recirculate and whether or not recirculating memory cells are imprinted for migration to specific locations by their encounter with antigen (either in the tissue or in the peripheral blood). These important questions, which apply to all memory subsets, are particularly relevant for marginal zone B cells which in the mouse are considered a sessile, non-recirculating population whose localization in the marginal zone appears to be determined by the ability of the sphingosine 1-phosphate (S1P) receptor 1 (S1P₁) to counteract the follicular chemoattraction of CXCL13 and by the ability of the integrins LFA-1 (α L β 2) and α 4 β 1 to retain MZ B cells (103,104). Indeed, the combined inhibition of these integrins results in recirculation of MZ B cells (104). These and other markers of importance for the egress, migration and retention, remain largely unexplored in human memory B cells although interesting information is emerging regarding the homing of gut targeted anti-viral memory B cells and plasma cells (105–107).

Yet, it has been proposed that human peripheral blood IgM⁺IgD⁺CD27⁺ B cells may represent a recirculating MZ subset which develops in a GC-independent fashion but still expresses a mutated Ig repertoire (20,25), an observation consistent with the absence of these cells from the peripheral blood of splenectomized patients (23). PBL IgM⁺IgD⁺CD27⁺ cells have an almost identical surface phenotype to their spleen counterparts (IgM^{high}IgD^{low}CD23⁻CD21⁺CD1c⁺) although with lower levels of CD21 expression. In addition, these cells appear to share gene expression profiles and contain the same clones as spleen cells in response to immunization with bacterial polysaccharide (25).

The concept of recirculating MZ B cells, originally proposed through the study of X-linked hyper-IgM patients lacking GCs, is also supported by the expansion of anti-pneumococcal polysaccharide IgM⁺IgD⁺ B cells observed in the PBL of immunized subjects (108). It is also supported by our observation in normal subjects that autoreactive IgM⁺9G4 B cells accumulate within the spleen MZ and the peripheral blood IgM⁺IgD⁺CD27⁺ populations despite their inability to participate in GC reactions (44,109). Still, it is fair to say that the MZ equivalence of peripheral blood IgM⁺IgD⁺CD27⁺ B cells remains in dispute despite early suggestions that

the human spleen outer MZ contains IgM⁺IgD⁺ cells that could represent recirculating B cells (92,93,95) and the confirmation by other groups including our own of the existence of IgM⁺IgD⁺ MZ B cells (25,44,67,110). Whether or not they represent circulating MZ cells, the question still remains whether these cells should be considered as bona fide memory cells or just a subset of pre-diversified B cells that mediate protection against T-independent antigens (25). However, recent studies strongly indicate that these cells closely resemble IgG⁺ memory cells in terms of morphology and in vitro behavior and that they may be able to participate in affinity-selected T-dependent responses (29,111). Similarly, it also remains to be established whether the resident IgG⁺ MZ B cells (44,60) have the ability to recirculate as suggested by the expression of CD1c in a small fraction of peripheral blood IgG⁺CD27⁺ cells (25). With regard to CD1c expression, our results confirm that the IgM⁺IgD⁺CD27⁺ unswitched memory population in the peripheral blood exhibits the highest fraction of CD1c-expressing cells (Figure 4). In addition, both the IgD⁺CD27⁻ naïve and IgD⁻CD27⁺ memory subsets also contain a significant fraction of CD1c-expressing cells, albeit at lower levels. In contrast, CD1c is virtually absent in IgD⁻CD27⁻ DN memory cells. It is of interest to note that the fraction of CD1c-expressing cells is substantially decreased in CD27⁺ subsets in SLE patients (Figures 4). Whether this reflects splenic hypofunction, relative expansion of isotype-switched memory cells, retention within the splenic marginal zone and/or active participation of MZ B cells in germinal center reactions in SLE remains to be determined (26,84).

Kinetics and homeostasis of memory B cell subsets: Lessons from clinical situations and BCDT

The importance of elucidating human memory B cell heterogeneity is highlighted by our findings and those of others that certain memory subsets are preferentially expanded in particular clinical situations. Moreover, such studies have provided insight into the potential origin of diverse subsets and their dynamic generation in peripheral lymphoid tissue.

As shown by different groups, SLE patients display an expansion of switched memory in the peripheral blood which as previously discussed, is accompanied by an striking contraction of unswitched (marginal zone) memory B cells and distinct from healthy controls (Figure 4 and Figure 5) (46,112). Moreover, in contrast to healthy subjects, in SLE both CD27⁺ and CD27⁻ memory cells are predominantly found in the CD38⁺ early Bm5 compartment, a finding recapitulated in lymphoid tissue (Figure 6A). In addition, B220⁺ memory cells contribute a larger fraction of the CD27⁺ and CD27⁻ compartment in SLE patients than in healthy subjects (12). Additional studies will be required to elucidate whether the predominance of early Bm5 cells in SLE represents activation of Bm5 cells with upregulation of CD38 perhaps as a first step in the generation of the plasmablast expansion frequently observed in SLE (113). Similarly, whether expanded B220⁺ memory cells represent activated autoreactive B cells will require further study (56,114). Other abnormalities of interest in Lupus memory B cells include the recent description of a subset of memory B cells expressing high levels of surface CD19 and enriched for anti-Smith autoreactivity (15).

As previously mentioned, we have recently demonstrated an expansion of IgD⁻CD27⁻ memory B cells in the peripheral blood of SLE patients which correlated with disease activity, nephritis, and titers of anti-double stranded-DNA antibodies (12). Interestingly, these CD27⁻ memory cells are also substantially expanded in the peripheral blood of patients exposed to RSV, a finding that suggests their participation in at least the early phases of protective memory responses (Figure 3B). Notably, we have reported that the CD27⁻ memory expansion in SLE resolves after B cell depletion therapy (BCDT) even in patients in whom a failure to fully interrupt GC reactions was suggested by rapid re-accumulation of post-GC CD27⁺ memory (46). The potential GC-independent origin of this memory subset is further supported by the

observation that DN cells did not decrease in SLE patients in whom productive T-cell dependent GC reactions were disrupted with anti-CD154 antibodies (45).

The kinetics of memory B cell reconstitution after BCDT is further instructive regarding memory B cell heterogeneity. Thus, SLE patients with shorter clinical responses post-rituximab experienced faster and more pronounced peripheral blood expansion of post-GC IgD⁻CD27⁺ memory B cells than of IgD⁺CD27⁺ memory B cells, again consistent with the distinct origin of these cell populations (115–117). On the other hand, rituximab treated patients with prolonged clinical remissions had a delay of several years in both IgD⁻CD27⁺ and IgD⁺CD27⁺ memory B cell reconstitution (Figure 5). Finally, memory B cell subpopulations in the tonsils of these same patients were altered with a striking decrease in the FcRH4⁺CD27⁻ memory B cell population several years after treatment (Figure 6A) (117). Interestingly, the majority of memory B cells in the lymphoid tissue after BCDT and reconstitution were found in the late Bm5 compartment, in striking contrast to pre-treatment (Figure 6A). Overall, our results suggest disruption in the generation of tissue based memory after BCDT although the mechanisms of this effect remain to be elucidated. We speculate that profound and lasting B cell depletion may alter the lymphoid architecture and follicular dendritic cells, the latter a critical cell population for primary follicle formation and generation of memory within the GC reaction. This may occur because of the elimination of LT α and TNF α produced by B cells. We further postulate that such alterations in memory subsets have important clinical ramifications given that this Fc receptor homologue is normally expressed on one third of Bm5 memory B cells and appears to define a functionally distinct subpopulation of mucosal associated memory that secretes high levels of immunoglobulin in response to T cell cytokines and is poised to undergo plasma cell differentiation.

As another example of the clinical relevance of understanding memory B cell homeostasis and heterogeneity and conversely, of the opportunity offered by targeted biological treatments to expose the individuality of different memory B cell subsets, we have recently found that TNF blockade alters B cell populations in human peripheral blood in rheumatoid arthritis with decreases in both IgD⁻CD27⁺ and IgD⁺CD27⁺ memory (Figure 6B) (118). This suggests a convergence of mechanisms between BCDT and TNF blockade, with both seemingly divergent approaches causing a reduction in peripheral blood memory B cells. Given an accompanying reduction in FDC networks and GC phenotype cells in the peripheral lymphoid tissue with TNF blockade, this effect appears to be mediated via inhibition of FDCs and B cell entrance into the GC reaction. Moreover, B cell depletion may mimic TNF blockade by eliminating LT α bearing and TNF secreting B cells. With either therapeutic intervention, the effects on the memory B cell compartment may be profound as well as instructive regarding regulation of B cell memory. Particularly intriguing is the observation that recirculating memory B cells are more profoundly impacted by anti-TNF therapy than tissue-based memory cells. This observation suggests that the dynamic memory cell output of the GC is heterogeneous and that biological treatments currently used in the clinic may represent useful tools to dissect such heterogeneity.

Immuno-modulatory and effector functions of human memory B cells

While usually overshadowed by the production of antibodies, the ability of B cells to play important antibody-independent functions is well documented (5–7,119). These antibody-independent functions prominently include cytokine and chemokine production and antigen presentation. Through these functions B cells can profoundly influence the formation and organization of secondary lymphoid tissues and T cell development, activation and function (120–125). Moreover, antibody-independent B cell functions can contribute either to the development or to the prevention or resolution of autoimmune diseases (5,6,119,126–133).

In humans very little information is available in this important area although there is ample evidence of the cytokine producing ability of human B cells and IL-10 production by B cells has been implicated in the pathogenesis of SLE (134–136). Moreover, several studies indicate that human memory B cells produce different cytokines from naïve B cells with the latter subset secreting IL-10 and the former TNF α and lymphotoxin, at least when the cells are sequentially stimulated through the BCR and CD40 (137). Of significant interest, this balance, which is perturbed in multiple sclerosis patients by decreased production of IL-10 by naïve B cells, appeared to be attenuated after B cell deletion and reconstitution and disease amelioration (18).

Virtually nothing however is known regarding cytokine production and other division of labor among the newly emerging memory B cell subsets. Yet, a recent study has suggested that human CD27⁺ memory B cells can be split on the basis of the expression of CD25 with CD25⁺ cells (60%) producing more IL-10 but less IL-2 than CD25⁻ B cells (17). However, although in this study only 10–20% of naïve B cells were CD25⁺, cytokine studies were performed with total B cells only differentiated by the expression of CD25 and therefore, a definitive assignment of the source of IL-10 could not be established. It is also worth noticing that in this study B cells were stimulated with CpG DNA in contrast to the previously mentioned studies in which memory B cells were poor producers of IL-10 (18). It is therefore plausible that such discrepancy could be explained by invoking that different stimulatory conditions would induce the production of different cytokines by memory B cells.

Summary

Experimental evidence continues to accumulate indicating that B cell memory is rather complex and includes T-cell dependent and independent memory, isotype-switched and unswitched, mutated and unmutated memory B cells. It may also include a “natural” memory at least in part provided by conventional MZ B cells, several adaptive memory populations and possibly adaptive memory B cells that acquire marginal zone-like properties. Functionally, effective and appropriate (i.e., protective and non-autoimmune) B cell memory is likely to depend on central and effector/regulatory memory populations with different ability to produce cytokines and to regulate different T cell subsets. Our knowledge of these critical issues is at best rudimentary in the human. Yet, substantial complexity of human memory B cells is strongly supported by phenotypic, functional and clinical studies. In the future, we should aim to improve our understanding of the heterogeneity of human memory B cell subsets, their role in regulatory and effector functions and their differential participation in protective and pathogenic responses. Such knowledge will be instrumental for our ability to design more effective and safe strategies to specifically stimulate, inhibit or target for deletion specific memory subsets (123,138,139). Using these strategies, we should be better able to enhance the efficacy of vaccines and to improve the outcome of multiple autoimmune and inflammatory diseases (6,139–144).

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Abbreviations

RSV, respiratory syncytial virus; BCDT, B cell depletion therapy; MZ, marginal zone; S1P, sphingosine 1-phosphate; S1P₁, S1P receptor 1..

Figure 1. Classification of human B cell subsets based on the expression of IgD/CD38 or IgD/CD27 Mononuclear cells from human tonsil or peripheral blood were stained with mouse monoclonal antibodies against human CD19, IgD, CD27 and CD38, and CD19⁺ cells were gated for analysis. The nomenclatures for B cell subsets defined by each scheme are also depicted.

Figure 2. Multicolor flow cytometry provides a comprehensive phenotypic analysis of human B cell subsets and reveals additional complexity of human memory B cells

Ten-color flow cytometry panels were developed to stain the mononuclear cells from peripheral blood (A, B) and tonsil (C) with antibodies against human CD19, CD3, IgD, IgM, IgG, CD1c, CD10, CD24, CD27, CD38, CD45(B220), and FcRH4. Cells were also stained with LIVE/DEAD Fixable Aqua Dead Cell Stain. Live CD19⁺ B cells were gated for analysis. (A) IgD⁺CD27⁺ switched memory, IgD⁺CD27⁻ DN memory, Bm3+4, early Bm5 and late Bm5 cells (blue dots) from the peripheral blood were superimposed on the total CD19⁺ B cells (red dots) to demonstrate where these subsets defined by one classification scheme would fall under other classification schemes. (B) The expression levels of CD24 and B220 in the IgM and IgG fractions of IgD⁺CD27⁺ and IgD⁺CD27⁻ memory cells in the periphery were depicted. IgG memory cells, in contrast to IgM memory cells, exhibited heterogeneous expression levels of CD24 and B220. (C) The expression levels of CD27, B220 and FcRH4 in the early Bm5, late Bm5 and Bm5 as a whole from the tonsil were depicted in various bivariate plots, which revealed the heterogeneity of these memory B cells.

Figure 3. IgD⁻CD27⁻ DN memory cells are expanded in patients with active SLE and healthy subjects challenged with Respiratory Syncytial Virus (RSV)

(A) Our previous results demonstrated an expansion of IgD⁻CD27⁻ DN memory cells in the periphery of active SLE patients (12). Graphs show the representative IgD/CD27 expression profiles from a normal subject and an active SLE patient. (B) Seven healthy subjects were inoculated with RSV A2 at day 0, and the fractions of IgD⁻CD27⁻ DN memory cells in the peripheral blood B cells were followed for a period of up to 28 days. The expansion of IgD⁻CD27⁻ cells occurred as early as 4 days after RSV infection and peaked by day 8 for all but one subject, for whom the expansion was observed at day 12. By day 28, the levels of DN cells waned to the pre-infection levels. Although the antigen specificities of these expanded DN memory cells remain to be elucidated, the waxing and waning of these cells in patients exposed to RSV suggests a role for these cells in protective memory responses. Each line in the graph represents an individual subject.

Figure 5. The reconstitution profiles of memory B cells in SLE patients after rituximab treatment reflect the outcomes

The fractions of $\text{IgD}^-\text{CD27}^+$ switched memory and $\text{IgD}^+\text{CD27}^+$ unswitched memory cells in the CD19^+ PBL were determined from a cohort of SLE patients before and after rituximab treatment. Pre-treated patients displayed an expansion of switched memory and an accompanying reduction of unswitched memory B cells, compared to healthy controls. Patients with shorter clinical responses to rituximab exhibited memory profiles after reconstitution similar to the pre-treated group. Patients with prolonged clinical remissions experienced a delay in both switched and unswitched memory B cell reconstitution.

Figure 6. B cell depletion and TNF α blockade alter the B cell homeostasis and provide a means to dissect the heterogeneity of memory B cells

(A) In contrast to healthy subjects, memory B cells were predominantly found in the early Bm5 compartment of the tonsil in SLE patients. After BCDT and reconstitution, the majority of memory B cells were found in the late Bm5 compartment, and the FcRH4⁺CD27⁻ cells in the whole Bm5 compartment were drastically reduced. (B) RA patients treated with anti-TNF α exhibited decreases in the IgD⁻CD27⁺ switched and IgD⁺CD27⁺ unswitched memory B cell populations in the peripheral blood, compared to the healthy control and RA patients treated with methotrexate

Table 1
The phenotypes of human memory B cell subsets in the peripheral blood.

unswitched memory	IgD+	CD27+	CD38(-), ^a	CD1c(-),+	CD24+	B220(-),+
Memory IgM IgG	IgD-	CD27+	CD38-+,	CD1c(-),+	CD24-,(+) CD24+ CD24-,(+)	B220(-),+ B220- B220(-),+
CD27 ⁺ memory IgM IgG	IgD-	CD27-	CD38-+,	CD1c-	CD24(-),+ CD24+ CD24-,(+)	B220-,(+) B220- B220-,(+)
early Bm5 memory	IgD-	CD27-,(+)	CD38+	CD1c(-),+	CD24-,(+)	B220(-),+
late Bm5 memory	IgD-	CD27-,(+)	CD38-	CD1c(-),+	CD24-,(+)	B220(-),+

^a denotes heterogeneous expression levels with the dominant population in parenthesis.