B-Cells and Antibodies in Old Humans

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1 Role of B-Cells in Age-Associated Susceptibility to Infection

It has been well established that the efficiency of the immune system declines with increasing age. Immunosenescence causes increased susceptibility to infectious diseases, and infection is, in fact, the third leading cause of mortality in people aged 65 and over [1]. As is clearly apparent from the other chapters of this book, there are many components of the immune system that can change with age, and are crucial to maintaining an effective immune system. The humoral immune system interacts with the other components, both as part of its own development and via its effector mechanisms. The most important function of B-cells is to produce antibodies, the indispensable soluble effectors of many functions. There are a number of different stages of development for B-cells and their antibodies (Fig. 1).

In the primary B-cell response antibodies that recognize pathogen, although not necessarily with high affinity, are rapidly produced. They may include the so-called “polyspecific” antibodies, which have the ability to recognize multiple antigens [2]. The first antibodies are of the IgM isotype and are crucial for opsonizing pathogens, inducing phagocytosis and activating the complement cascade. These
antibody functions, and the rapidity of this primary response, have been shown to play a vital role in protection from extracellular bacterial pathogens [3]. Antibodies afford protection against viral infection by neutralizing the virus particles; binding and blocking key molecules involved in cellular infection. Similarly they can also neutralize toxins. Later maturation of the B-cells in the immune response is slower but results in the generation of more highly specific antibodies, which may be of a different isotype, following a process known as affinity maturation. In addition to the neutralizing and opsonizing functions of antibody, B-cells are also important as modulators of inflammation [4, 5], regulators of the immune response [6] and as antigen presenting cells and activators of T-cells [7–10].

Fig. 1  B-cell development. The humoral immune response is mediated by antibodies produced from plasma cells. These plasma cells are the end point in B-cell development, which is characterized by (a) generation of a huge diversity of different B-cells, each carrying a different antibody gene in the bone marrow and (b) selection processes using the affinity of the membrane-bound form of the antibody (the B-cell receptor) for its antigen as the selection criteria. Diversity is generated by a process of gene rearrangement early on in the development of the cell, in the bone marrow prior to antigen encounter. The selection processes are twofold. Firstly B-cells are selected for survival, or not, on the basis of their antibody recognition—to eliminate inappropriate self-reactivity and encourage reactivity with foreign pathogens. Secondly there is a mutation step in development, and the resultant B-cells carrying improved antibodies are selected—this occurs in the germinal centre of secondary tissues, after encounter with antigen, and serves to increase the affinity of the antibody for the relevant antigen. Both generation of diversity and selection of antibody are complex processes that are crucial for an effective humoral immune system. A clear understanding of these processes, and how they are affected with age, is needed in order to comprehend the etiology of age-related inflammatory and infectious disease.
The elderly are susceptible to infections by a wide variety of pathogens, all of which involve B-cells and antibodies in the normal course of the immune response (Table 1). The lungs are, in common with other mucosal surfaces of the gastrointestinal and genito-urinary tracts, particularly vulnerable to infection by virtue of their exposure to the environment. As is illustrated in Table 1, pulmonary infections are common in older people. The elderly are usually the first to be affected by annual epidemics of respiratory infections, and frequently suffer the worst clinically. Mortality figures attributable to influenza and pneumonia are confused by the fact that influenza is very often followed by a secondary infection—most notably by *Streptococcus pneumoniae*. Some would argue that this confounding factor results in a two to threefold underestimate of influenza mortality [23]. It is also argued that mortality due to influenza is negligible and it is the secondary bacterial infection that causes almost all deaths [24, 25]. Whichever way round, it is generally agreed that older people are the worst affected by these diseases. It has been reported that 90% of all pneumonia and influenza deaths and 88% of respiratory syncytial virus-associated deaths occur in those aged over 65 years [26]. In the oldest old (85 years and over) there was a 32-fold increased chance of mortality from influenza or influenza-associated pneumonia compared with those aged 65–69 years [26]. According to the Department of Health, in the UK there are more than 18,000 hospitalizations resulting from pneumococcal pneumonia each year in those aged 65 years and over [27]. There is also an increased incidence of pneumococcal septicemia in old people associated with *S. pneumoniae* infection [28].

Table 1  Pathogens found frequently in elderly subjects with respiratory or urinary tract infections. (adapted from [1])

| Organ system          | Pathogen found frequently | B-cell role in immune response to pathogen                                                                 |
|-----------------------|---------------------------|-------------------------------------------------------------------------------------------------------------|
| Respiratory tract     | **Bacteria**              | B-cells are crucial to the TI-II response [11]                                                             |
| (upper and lower)     | *Streptococcus pneumoniae*| Mucosal IgA has a protective role independent of serum antibody levels [12]                                |
|                       | *Hemophilus influenza*    |                                                                                                             |
|                       | *Legionella pneumophila*  | B-cells are required for opsonization [13]                                                                 |
|                       | *Chlamydia pneumoniae*    | Neutralization by antibody [14]                                                                            |
|                       | **Viruses**               | Antibody-mediated neutralization [15,16]                                                                   |
|                       | *Rhinoviruses*            |                                                                                                             |
|                       | *Coronaviruses*           |                                                                                                             |
|                       | *Influenza*               |                                                                                                             |
|                       | *Respiratory syncytial*   |                                                                                                             |
| Urinary tract         | **Bacteria**              |                                                                                                             |
|                       | *Escherichia coli*        | IgA secretion and antigen-specific Ig inhibits attachment of bacteria [17,18]                             |
|                       | *Proteus*                 | An increase in IgM and IgA aids protection [19,20]                                                        |
|                       | *Klebsiella*              |                                                                                                             |
|                       | *Pseudomonas aeruginosa*  | Opsonization [21]                                                                                          |
|                       | *Enterococci*             | Antibody alone not hugely effective, but effective in the presence of complement [22]                    |
It is known that specific antibodies, generated during a T-dependent B-cell response, are crucial for protection against influenza. Ineffective influenza-specific antibody, as assessed by the Haemagglutination inhibition (HI) test, is associated with lowered protection from the disease [29]. Studies have shown that 25% or more of the elderly fail to develop HI titres of a protective level following vaccination [30, 31]. In vivo studies in mice have shown that higher levels of B-cells and IgG2a antibody confer increased levels of protection [32]. It has been said that an age-related decrease in influenza protection can be solely accounted for by the reduced T-cell help available in the diminished elderly T-cell repertoire. However, this does not take into account the fact that the CD4+ T-cells themselves may rely on fully functioning B-cells for their activation [7, 10].

In other areas of humoral immunity the B-cells are even less reliant on T-cells for help. Pneumonia is a bacterial infection, caused by a number of different organisms (e.g. *Streptococcus pneumoniae* [33], *Staphylococcus aureus* [34], *Streptococcus pyogenes* [35]) although *S. pneumoniae* is the major cause [33]. Immunity against *S. pneumoniae* is particularly reliant on a healthy B-cell population. This is because the antigenic portion of *S. pneumoniae* is a capsular polysaccharide and a T-independent type II (TI-II) antigen. Unlike a T-dependent B-cell response, where the maturation of the B-cell antibody relies on T-cell help and therefore any failure to respond could be attributed to a failure of T-cells, the TI-II response is independent of direct T-cell help. Therefore a failure to protect against *S. pneumoniae* is more likely to be a failure ascribable to deficits in the B-cells themselves.

In children a reduced pneumococcal response can be explained by a lack of marginal zone B-cells in the spleen, where the main TI-II responding B-cells are thought to reside. However, older people appear to have a fully functioning splenic marginal zone [36] so the lack of effective pneumococcal protection in the elderly still remains a mystery. One good candidate for further study is the IgM response. It has been shown, in mice, that the classical complement pathway, partially mediated by binding of natural IgM to bacteria, is vital for innate immunity to *S. pneumoniae* [3]. Human studies have also shown that antibody of the IgM isotype is vital in providing efficient protection against *S. pneumoniae* [37], although this has been mainly attributed to “IgM memory,” with mutated IgM genes. The exact roles and relationships between natural antibody, IgM memory and class switched memory in the pneumococcal response remain to be determined.

The immune response of the elderly to RSV is less well studied than that against other pulmonary infections. Recent data shows that the senescence accelerated mouse has a severely compromised cellular immune system and produces less virus-specific local IgA in response to RSV infection [38].

Although pulmonary infections of the elderly are the most notable, by virtue of the fact that they cause the most mortality, there are also significant increases in morbidity and mortality from other infections. Bacterial infections of the skin, urinary tract, soft tissue, and gastrointestinal tract are all increased with age [1]. The exact role of the humoral response in this declined protection has yet to be elucidated.
2 Vaccination in the Elderly

Vaccines are an extremely important tool in preventing deaths from infection, and since they are routinely administered as part of a normal health care routine they are the main source of data on immune responses in man. It has been consistently shown that the effectiveness of vaccines is severely diminished in older people. The most commonly studied vaccine is that against influenza. The cellular response, i.e. T-cells and release of cytokines, macrophages and natural killer cells, is decreased with age [39]. In terms of the humoral response the antibody titre, in the form of IgG, is significantly lower [39–41]. While vaccination of the elderly against influenza is widely accepted as a valid health strategy to reduce disease incidence, and studies support this, [42–44] other studies suggest that influenza vaccination does not significantly decrease influenza-related mortality in older people [45, 46]. The age-related reduction in specific antibody production also occurs in response to other vaccines, such as against hepatitis B [47], tetanus and tick-borne encephalitis (TBE) [48]. Data on some of the less common vaccines is more scarce, but gradually becoming available with the advent of an older population which travels more widely. Some travel vaccines, such as hepatitis A, also show a reduced specific antibody response [49], while others such as yellow fever seem to show an undiminished antibody response but have an increased risk of adverse events in the elderly [50].

A possible explanation for a decrease in specific antibody is that the process of affinity maturation is defective. During one study on influenza vaccine it was discovered that an age-related decrease in specific antibody was accompanied by an increase in antibodies against double stranded DNA—indicative of self reactive/polyclonal B-cells [51]. Polyclonal B-cells are often associated with naive B-cells that have not been through the affinity maturation process and are reacting in either a low-affinity manner to specific antigen, or in a non-specific manner by virtue of their innate pattern recognition responses. It was this finding that led to the idea that perhaps humoral immunity in the older person was better represented by the T-independent response. However, as mentioned above, there is a large T-independent component to immune protection against S. pneumoniae and general protection is decreased with age. Cross-reactive antibodies certainly appear to be increased in older people treated with the polysaccharide pneumococcal vaccine [52], although the failure of the vaccine to adequately protect against pneumonia [53–57] implies that they are not adequate compensation for the reduction in specific antibody that is also seen [52].

3 Autoantibodies and Age

There is a well-documented shift towards self-reactive antibody production with age. One of the most common autoantibody types, frequently associated with disease, is antinuclear antibodies (ANAs). These have consistently been found to be
increased in the old (over 65) in the absence of disease; a prospective study showed persistence of these raised levels throughout older life [58]. The significance of this increase has not yet been determined, and attempts to relate these antibodies with general levels of disease and frailty have shown no associations. The Swedish longitudinal NONA immune study [59] showed significantly higher ANA levels in the oldest old (86–95 years) but found there to be no association nor any correlation to other immune risk factors (e.g. CD4/CD8 T-cell ratio, CMV seropositivity). These findings are echoed by a Finnish study, where ANA positivity at the age of 90 did not show any correlation with survival, or with the levels of serum markers of inflammation [60]. It has even been suggested that an increase in ANA antibodies may have beneficial effects by virtue of a possible anti-tumor activity [61].

ANAs are not the only auto-antibodies to increase with age. The study by Xavier et al. [58] also noted an increase in the frequency of anti-ssDNA antibodies, as have other studies [62, 63]. Increases in antibodies against many other auto-antigens have been reported, for example against cardiolipin, dsDNA and rheumatoid factor, [62–65] although, again, there were no associations found with mortality [62]. The Danish study by Andersen-Ranberg et al. [65] did find a correlation between autoantibodies and comorbidity and disability, although this was only for the organ-specific antibodies, indicating that these were more likely a result of age-associated disease.

Although the aetiology of Rheumatoid arthritis (RA) is not yet fully elucidated, it is an age-related inflammatory autoimmune disorder. Coincidentally, as reported above, there is also an increased incidence of rheumatoid factor (RF) with age—regardless of whether the subject has RA or not [62–65]. There has been a decline in incidence of the disease that has been observed over the last 40 years [66] which has been attributed to environmental factors. One possible contributor to this is the gradual decrease in the number of smokers. Recent evidence has shown that the presence of another auto-antibody, anti-cyclic citrullinated peptide (anti-CCP) is associated with smoking and a higher risk of RA [67]. The successful use of therapies such as Rituximab, which utilize an anti-CD20 monoclonal antibody to ablate peripheral B-cells, is ample evidence that B-cells play an important part in the disease process of RA [68]. In addition to the obvious mechanism of depleting auto-antibody producing cells, there is increasing evidence for a role of B-cells in RA as antigen-presenting cells, activating T-cells, and producing and responding to cytokines [69]. A further complication in understanding the role of B-cells is the fact that B-cells have recently been shown to be capable of immunosuppression—including in animals models of arthritis [70, 71].

4 Immunodysregulation of B-cells in Aging

The above observations are all evidence that the humoral immune system is dysregulated in older people. At first glance it would appear that there is no easily identifiable quantitative defect in the humoral immune system with age. However, although the range of B-cell numbers, as a percentage of peripheral blood lymphocytes, varies greatly between individuals, it has been reported that there is a slight decline in the
number of CD19+ B-cells in old age [72–75]. It has also been reported that having a higher number of CD19+ B-cells is associated with better survival [76, 77]. When CD20 is used as a marker for B-cells no age-related change could be found [78]. The number of antibody molecules circulating in the periphery of older adults remains relatively stable [79, 80]. Similarly, studies have been conducted on the ratio of different Ig isotypes in the elderly and most show no significant change during later life [75, 81, 82]; although it has been reported that an increase of the mucosal IgA antibody in the serum can be a predictor of mortality [83]. In general the picture is one of a qualitative change in the antibody repertoire rather than a quantitative one [84].

4.1 Generation of High Affinity Antibodies

Since the lack of high affinity antibodies is a key feature of the older immune system, and our expertise is in the study of Ig genes, we initially investigated the affinity maturation process. Affinity maturation occurs in the germinal centre (GC) and involves the expansion of antigen-specific B-cells, mutation of their Ig genes (resulting in altered antibody function), followed by selection of the B-cells producing the best antibody [85–87]. Contained within the dynamic microenvironment of the GC are B-cells, T-cells, and follicular dendritic cells (FDCs) all in close proximity to allow the exchange of costimulatory molecules and cytokine signaling.

Following antigenic stimulation, selected B-cells migrate and converge on the GC FDCs, making contact with their long processes [88] and differentiating into centroblasts. The FDCs are the stromal cells of the GC and play a key role in regulating the humoral immune response [89]. Unlike antigen presenting cells (APCs), FDCs present intact antigen–antibody complexes on their cell surface [88], in the form of immune complexes which are highly immunogenic, and assist GC B-cell proliferation [90–92]. Proliferating GC B-cells are known as centroblasts. During centroblast proliferation, in the dark zone of the GC, hypermutation of the immunoglobulin (Ig) genes encoding antibody occurs. The B-cells move into the light zone, as centrocytes, and will die through apoptosis unless they receive rescue signals conditional on efficient recognition of the antigen by the newly formed B-cell receptor. Rescue signals are provided by FDCs and T-cells [93]. The helper T-cells in the GC are a particular subset of CD4+ T-cells, expressing CD57. These cells have unique characteristics that have yet to be fully elucidated [94]. Since FDC and T-cell help is limiting there is competition between B-cells and therefore selection of those B-cells with the highest affinity for antigen occurs. The resulting B-cells can switch the class of their antibody, from IgM to IgG/IgA/IgE, and this also requires T-cell help. B-cells with high affinity antibody differentiate into either memory B-cells, to provide for an efficient recall response, or plasma cells to secrete antibody. We have addressed the possible age-related changes in the GC reaction in three main areas: proliferation of B-cells, hypermutation of the Ig genes, and selection of high-affinity, antigen-specific, antibodies.
4.2 Proliferation

A defect in B-cell proliferation would have severe consequences for the GC reaction, since the loss of cells due to deleterious mutations acquired by hypermutation is extremely large and the pool of B-cells required to counter this is therefore also large. For some cell types proliferating cells can reach replicative senescence—where the telomeres at the ends of the chromosomes erode at each division and therefore there is a limit to the amount of proliferation one cell line can undergo set by the length of the telomere [95]. It has been shown that telomere length decreases with age in T-cells, and to a lesser extent in B-cells [96, 97]. However, we do not believe that the proliferative capacity of B-cells in the GC is impaired in this way as a result of old age. Telomerase, the enzyme that elongates telomeres, is upregulated in the GC, being high in centroblasts and higher still in centrocytes. This results in B-cells leaving the GC for the periphery with substantially longer telomeres than when they first entered, up to 4 kb longer as determined by Southern blotting [98]. Further to this, memory B-cells have telomeres on average 2 bp longer than naïve B-cells [97].

There has been much debate as to whether the overall size and number of GCs decrease with age. Several studies have pointed to this though they have all been conducted in rodent models [99–101]. Immunohistochemical studies measuring the size and overall number of B-cell follicles in human spleen, Peyer’s patches [36] and lymph nodes [78] have not shown any age-related difference. However, there have been two studies of human tonsil, performed by flow cytometry rather than measuring individual GC sizes, which have both reported a decrease in GC B-cells with age [99, 102]. Tissue specific differences may account for these discrepancies and further work would be needed to clarify the issue.

4.3 Hypermutation of B-Cells

As outlined above, somatic hypermutation occurs following activation of the B-cells by antigen and entry into the GC reaction. The mutations introduced are generally point mutations, though some insertions and deletions may occur, and tend to be in areas containing hotspot motifs [103–105].

There is conflicting opinion regarding whether there is a quantitative change in hypermutation in the ageing individual. Reports have indicated no change [106–108], a decrease [109, 110] or increase [99, 111, 112] in mutation with increasing age. The fact that these studies do not agree is hardly surprising as they do not take into account patient health history i.e. prior immune responses. The tissue origin of samples can also make a significant difference to the number of mutations observed, for example we have shown consistently that B-cells of mucosal origin have a higher level of mutations than those from, say, spleen or blood [113].

We addressed these issues by attempting to quantitate the frequency of hypermutation in individual B-cell GC expansions. We microdissected histologically-defined
areas of GC from the spleen and Peyer’s patch follicles of young and old humans so that only the mutations in that particular GC reaction were counted [114, 115]. Individual B-cell expansions were identified by their Ig gene characteristics; by identifying Ig gene sequences that have the same CDR3 region we can identify related B-cell clones (Fig. 2, see later for a more detailed explanation of Ig gene rearrangement). Furthermore, we can draw a lineage tree of individual B-cell clonal expansions (Fig. 3) by analyzing the order of accumulation of mutations in the hypermutation process [114, 115]. In this way we look at the number of mutations that occurred within that particular clonal expansion, and can compare lineage trees from subjects of different ages. We have shown that there was no difference in the frequency of mutation occurring in human GC reactions in the spleen and Peyer’s patch with age.

4.4 Selection of High Affinity B-Cells and Class Switching

Lineage tree construction can furnish information on the affinity maturation dynamics by measurement of lineage tree shape parameters. The shape of the lineage tree can help indicate the degree of selection that has taken place. For instance, a ‘pruned’ tree (few branches) indicates high selection pressure whereas a ‘bushy’ tree (many branches), indicates less selection (Fig. 3). Since a failure of adequate selection
could result in the production of a population of cells with low affinity, such as is seen in the elderly, we investigated lineage trees from GC reactions in samples from patients of different ages for selection differences. We found a significant decrease in the degree of selection pressure acting on GC B-cells in the Peyer’s patch of the gut (but not the spleen). These data were confirmed by further analysis of the distribution of mutations within the Ig gene. A high level of replacement mutations in the complementarity-determining areas of the gene (relative to the more conserved Framework areas, Fig. 2) is expected in a selected Ig gene, and is indeed seen in the younger Peyer’s patch GC samples but not the old [114, 115].

An explanation for these apparent changes in selection is still elusive, but several factors could contribute. It may be solely a failure of the quality of B-cells in terms of specificity or signaling function. However, since FDCs and T-cells are important in the selection process they are also good candidates to investigate for the failure of selection pressure.

There is a well-documented age-related decline in thymus size and a reduced T-cell output. Homeostatic regulation in the face of reduced levels of naïve T-cells causes skewing of the T-cell repertoire which may reduce the availability of appropriate T-cell help for B-cells. Immunohistochemically stained human tissue sections have illustrated changes in T-cell populations in B-cell follicles [36,102]. The CD8+ T-cell numbers decline with age resulting in an increased CD4+/CD8+ ratio. Since it is CD4+ cells that are important in the affinity maturation process the significance of these findings is not known. There is, as yet, no information on whether the GC-specific T helper cells (CD4+ CD57+) are changed with age. CD40 ligand on GC
T-cells interacts with CD40 expressed on B-cells and this relationship is critical to T-cell dependent activation of B-cell proliferation, memory formation and class-switch recombination in the GC. Aged CD4 T-cells in mice have shown reduced CD40L expression [116] and in these animals there is a decrease in IgG levels reminiscent of the decreased IgG production in response to influenza vaccination in humans [40,41].

It has been suggested that the function of FDCs declines with increasing age [101, 117, 118]. Defects may be intrinsic to the FDCs themselves, or may be a failure of the FDC-B-cell interactions. FDCs have Fc receptors (FcR) and complement receptors 1 and 2 (CR1 and CR2) on their surface which retain antigen as immune complexes [119], and these interactions are crucial for the signaling and activation of antigen-specific B-cells. The immune complexes coat the FDCs to form bodies known as iccosomes. Aged FDCs have been reported to produce few to none of these iccosomes [117]. This may be due to the apparent down-regulation of FDC-FcγRII expression by FDC-bound immune complexes demonstrated in the GCs of old mice [120]. The resulting decrease in immune complex retention and presentation to B-cells would lead to lowered B-cell activation in the GC.

Although there is clearly a role for accessory cell failure in the age-related changes in GC responses, changes intrinsic to the B-cell itself are also responsible. The key enzyme in affinity maturation of B-cells is Activation Induced Cytidine Deaminase (AID) which is directly responsible for both hypermutation of Ig genes and class switching. Class switch recombination, from IgM to either IgG, IgA or IgE isotypes, creates antibodies with the same antigen specificity but different effector functions (e.g. complement fixing, secretory, opsonizing). AID expression is regulated by the E2A-encoded transcription factor E47. It has been shown, in mice, that E47 and AID expression is reduced in old B-cells [121], and that this reduction is due to a failure in the CD40 signaling pathway (indicative of T-dependent interactions) and the BAFF signaling pathway (indicative of T-independent reactions) [122]. Preliminary results also suggested that there was a similar decrease of E47 and AID in human peripheral blood B-cells [121].

### 4.5 Diversity of the B-Cell Repertoire

Evidence from our lineage tree studies on individual GCs indicated that in some instances the founder B-cells of a GC may have already been mutated. This occurred more often in the older samples and led us to postulate that B-cells which have previously been through the affinity maturation process might be being re-used in subsequent immune responses. If the starting population of B-cells has already been modified in response to a different antigen, then its ability to effectively change to accommodate a new antigen may be compromised. This could partially explain the compromised selection noted above. Naive B-cells are characterized by their IgD expression, and memory B-cells are characterized by having mutated Ig genes and expressing CD27 on their surface. It has been shown in mice that the older B-cell
population is made up of a greater number of B-cells carrying mutated Ig genes—i.e. memory B-cells [123]. Observations of an increased number of CD27+ B-cells in humans concur with this [124,125]. A change in serum IgD, which may also reflect an increase in the proportion of IgD-, memory, B-cells, has also been noted [84]. It is now well established, in mice, that naïve B-cell output into the periphery decreases with age [see p. 395 Scholz et al.]. There is, as yet, no evidence that human bone marrow B-cell output decreases with age, although it is known that children reconstitute B-cell function after bone marrow transplants more rapidly than adults do [126]. Therefore, if the overall number of B-cells is not drastically reduced, and there are less naïve cells being produced, an increased proportion of memory B-cells is a logical conclusion [127]. Since B-cell memory appears to be maintained by proliferation [125] it is possible that proliferating memory B-cell clones make up for any shortfall in immunological space caused by lower naïve B-cell input. However, a decrease in the number of memory B-cells with age has also been reported [128], so it would seem that this issue is still not completely resolved.

Our postulation, that GC reactions in the older samples were using “second hand” B-cells, lead us to further investigate B-cell diversity. A diverse and functional repertoire of antibodies is essential to produce an effective humoral immune response. If the repertoire of B and plasma cells is reduced, then the ability to recognize foreign antigen is severely compromised. B-cell diversity and antibody specificity are defined during the early stages of B lymphocyte differentiation, where the Ig genes are formed. The remarkable way in which gene segment rearrangement forms a complete Ig gene from different segments (Fig. 2) results in millions of different B-cells, each with a unique Ig sequence capable of producing antibody with distinctive specificity. Briefly, the Ig molecule consists of both heavy and light chains. There are three types of gene segments, variable (V), diversity (D, heavy chain only) and joining (J). The segments are randomly recombined to generate a V(D)J for the heavy chain (Fig. 2) or VJ for the light chain. Thus a germline repertoire of just 165 different V, D or J genes can result in a possible 8,116 different gene rearrangements. Combination of the heavy and light chains results in a possible 2,643,840 combinations. The region where the junctions join together is further diversified by an incomplete joining process. Addition and deletion of nucleotides by terminal deoxynucleotidy transferase (TdT) activity at these joints leads to junctional diversity. The VDJ joining region of the heavy chain, the CDR3 region, is so highly variable that it can be considered to be a fingerprint for that particular gene and the B-cell (and its progeny) that carries it.

There have been a number of studies which have looked for an age-related change in diversity by investigating the gene segment usage in Ig genes. The studies vary in design (looking at specific gene families only, or at specific isotypes, or only in response to a particular challenge) which may account for some of the discrepancies between them. The earliest report is probably the most comprehensive in terms of VH repertoire, although limited in the number of different subjects used (five old and one young) [106]. They showed an increase in usage of certain IGHV genes, in particular of the IGHV4 family [106]. However, this has since been contradicted. In another sequencing-based study of the IGHV4 repertoire in elderly human tonsil
Kolar et al. did not find any change [99]. The IGHV repertoire has also been analyzed, in a small group of individuals, using a family-specific PCR-based approach. This showed consistency in the IGHV repertoire between samples of the same individual at time points 10 years apart [107]. IGHV family specific studies alone may not pick up functionally significant differences in the repertoire. Although the IGHV3 family usage in response to pneumococcal polysaccharide vaccination showed no overall difference between the elderly and young adults, there was a significant loss of focus in the elderly response as evidenced by a loss of oligoclonality [52]. Furthermore, in the same experiments, a difference in Ig light chain usage was observed [129].

Other studies of B-cell diversity have concentrated on the CDR3 region. This, being the most variable region of the gene and having importance in antigen binding, has traditionally been an area used to define monoclonality and oligoclonality in pathology [130]. However, due to the cumbersome nature of sequencing and identifying V-D-J regions, the numbers of patients studied have generally been low, or limited to particular subsets of genes. For example, one study by Xue et al. [131] looked at D and J

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**Fig. 4** Three different spectratype profiles. All are from old individuals (>88 years). Black bars represent the percentage of cdr3 regions of each different length. The red line represents the mean distribution for the young controls. The blue line shows the best fit for the individual sample data shown. A shows some B-cell repertoire restriction, B shows normally distributed cdr3 lengths, C shows an individual with very restricted B-cell repertoire. Approximately one-third of the old blood samples analyzed show restricted IgH repertoire along the lines of the spectratype in C.
region usage as determined by sequencing and found no difference between younger and older samples. However, they had only seven young and seven old samples and only studied the CDR3 regions of IGHV5 family IgM genes. A more tractable method of looking at CDR3 diversity was also employed by them, using PCR to amplify all CDR3 regions and look at the spread of different sized fragments. This method of spectratyping has also been used in the analysis of T-cell repertoires [132–134] and enables the study of a much greater number of samples. We performed B-cell spectratyping on samples from peripheral blood of 33 old and 24 young subjects. The old samples are from the Swedish NONA Immune Longitudinal Study [135], from patients over 86 years of age. Preliminary data has shown that the B-cell repertoire is indeed restricted in a subgroup (approximately one third) of older people (Fig. 4).

4.6 Association of Monoclonal B-Cell Expansions With Age

Skewed B-cell spectratypes of the kind we have observed may have a number of aetiologies. It may indeed be true that a decreasing naïve B-cell output in the face of homeostatic mechanisms to keep the total number of B-cells the same has resulted in the repertoire being increasingly made up of antigen-experienced expansions of cells. Alternatively there may be pathological monoclonal expansions of B-cells, such as are seen in leukemia or lymphoma. Usually, these are diagnosed conditions, and individuals with this sort of medical history are excluded from studies on B-cell diversity. However, it might be possible that a pre-clinical condition exists in some people. An increase in monoclonal expansions of B-cells, both of CD5+ and CD5- phenotype, has previously been reported in older people [136]. Monoclonal gammopathy of undetermined significance (MGUS) is a predominant plasma-cell disorder [137] and has been shown to increase with age in both humans [137,138] and mouse [139]. It is characterized by an increase in presence of serum monoclonal Ig. MGUS is not found in young subjects, is prevalent in around 2% of over 50s and has been reported to vary in the elderly from 11% to 38% [138,140]. There is an association between MGUS and onset of multiple myeloma or related malignant condition with average risk assessed at about 1% per year [141]. Questions still remain as to what significance these populations have in the aging human. Obviously there is the possibility that MGUS accounts for some of the observed repertoire restriction with increasing age. However, our data does not suggest a high prevalence of such monoclonal expansions, and the restricted repertoires often have a more oligoclonal appearance.

5 Summary

We have outlined the different factors that are involved in making and maintaining an effective humoral immune response and how these may be affected by increasing age. It is clear that the ability to produce high affinity antibody with age is diminished
but there are many possible explanations as to why this might be. We have identified the most likely areas as being a decrease in the ability to select B-cells producing high affinity antibodies, and a decrease in the available repertoire in the first instance.

Many of the studies on B-cells in old age are carried out in mice and the data in humans is sadly lacking. Hopefully this situation will change in the future and maybe the advent of the use of B-cell depletion therapies for the treatment of autoimmune disease can help provide more human data on B-cell dynamics in individuals of different ages.

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