

The ageing human B cell repertoire: A failure of selection?

Short title: "Ageing B cell repertoire"

Deborah K Dunn-Walters, Faculty of Life Sciences & Medicine, King's College London, UK.

Corresponding author:

Professor Deborah K Dunn-Walters, PhD

Department of Immunobiology

King's College London

Faculty of Life Sciences & Medicine

Guy's Campus

London SE1 9RT

+44 (0)20 7188 3067

deborah.dunn-walters@kcl.ac.uk

www.bcell.org.uk

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Abbreviations:

AID, activation induced cytidine deaminase

BCR, B cell receptor

CDRH, Complementarity determining region heavy chain

CMV, Cytomegalovirus

D, Diversity region of immunoglobulin gene

EBV, Epstein Barr Virus

HTS, high throughput sequencing

J, joining region of immunoglobulin gene

SLE, Systemic Lupus Erythrematosus

V, Variable region of immunoglobulin gene

Abstract

B cells undergo a number of different developmental stages, from initial formation of their B cell receptor (BCR) genes to differentiation into antibody secreting plasma cells. Since the BCR is vital in these differentiation steps, then autoreactive and exogenous antigen binding to the BCR exert critical selection pressures to shape the B cell repertoire. Older people are more prone to infectious disease, less able to respond well to vaccination and more likely to have autoreactive antibodies. Here we review evidence of changes in B cell repertoires in older people, which may be a reflection of age-related changes in B cell selection processes.

Introduction

As with many biological systems, the B cell repertoire exists in a state of equilibrium. On the one hand it is beneficial to have the widest diversity of potential antigen specificities as possible, in order to have the best chance of recognising any potential challenge to the organism. At the same time any B cells with receptors that recognise self have to be eliminated or controlled to avoid potential autoreactive pathology. Evidence from our lab, and others, has been accumulating to suggest that the aged immune system is less efficient as a result of dysregulation of this balance rather than a simple failure to respond.

“B cell repertoire” is a phrase that can be used at both the cellular and molecular level. At the cellular level there are a number of different phases of B cell development, from immature B cells through to memory cells and antibody-secreting plasma cells. Given the finite size of the B cell “space”, and the findings that the total number of B cells changes little throughout most of life, then changes in the different types of B cells represented in the population will have important consequences. At the molecular level, the specificity of an individual B cell is defined by the variable regions of immunoglobulin genes. The functionality of antibodies produced by these cells can be changed by class switching of the constant regions of Ig genes. Thus a study of Ig genes can determine the breadth of the repertoire in terms of B cell receptor/antibody specificity. Since the Ig genes determine the specificity of a B cell, then selective forces imposed by events dependent on antigen specificity will be reflected in the Ig gene repertoire of a population.

Generation of B cell repertoire diversity

Initial B cell Ig gene diversity is created during early B cell development in the bone marrow. The Ig gene locus comprises a number of different types of genes that make up the variable region of the antibody. A process of random gene assortment to recombine *IGHV, IGHD, IGHJ* gene segments for heavy chain (and *IGKV, IGKJ* or *IGLV, IGLJ* segments for kappa or lambda light chains) can facilitate the creation of thousands of different variable regions from just a couple of hundred different gene segments [1]. The subsequent random assortment of heavy and light chains increases the diversity further, to over 4 million different possible combinations (Figure 1). These numbers are hypothetical, since factors such as proximity of gene segments to each other or recombination signal sequence preference can influence gene choice, and this skews the recombination slightly [2]. In addition, and a much greater influence, is the increase in diversity because the joining of the different segments is imprecise and includes random extra nucleotide addition by the action of an enzyme called terminal deoxynucleotidyl transferase (TdT) [3]. Therefore there will likely be many

more than 4 million combinations. However, not all combinations of V(D)J genes will survive early development and enter the mature B cell repertoire. Only the heavy chain rearrangements that are functional and bind effectively with surrogate light chain and kappa or lambda light chain will be able to send back survival signals to ensure the cell progresses further in its development pathway [4]. This is the first selection step in altering the shape of the repertoire (Figure 2). The second influence over the shaping of the B cell repertoire is the process of central tolerance. One of the inevitable consequences of generating such a huge diversity of different B cell specificities is that some heavy/light chain pairs will produce a B cell receptor (BCR) that will recognise self antigens. These will be removed from the repertoire at the immature stage by a poorly-understood process of negative selection [5]. Some cells avoid negative selection by editing their receptors, replacing the light chain with a different light chain in an effort to change the receptor specificity to something more acceptable [6]. Transitional cells, leaving the bone marrow, may be subjected to a further round of negative selection which involves competition for the B cell survival factor BAFF [7]. The surviving mature B cells form the naïve B cell compartment, which is a highly diverse pool available to react against challenge. Upon challenge the naïve cells that recognise the antigen will expand, so the repertoire is again affected by positive selection. Further genetic diversity of the activated B cell repertoire can be introduced by the processes of somatic hypermutation and class switching in the germinal centre of secondary lymphoid follicles [8]. Both these processes are initiated by the deamination of Cytosine to Uracil in DNA by the enzyme Activation induced cytidine deaminase (AID) [9]. B cells carrying Ig genes where hypermutation has resulted in a significant advantage in antigen binding will out-compete other B cells for survival signals in the germinal centre, a selection process reminiscent of Darwinian evolution [8]. The B cells can undergo class switching to change the class of antibody from IgM/IgD to IgG/IgA/IgE, which keeps the variable region binding qualities but changes the potential function of the BCR/antibody.

Cellular Repertoire of B cells

Mature B cells in the periphery are not of a uniform type. As well as there being a distinction between naïve and memory, and between B cells of different classes of BCR/antibody, there are further distinctions of B cells by their phenotype. Classical memory cells produced in the T-dependent germinal centre response are recognised by the presence of memory marker CD27 on their surface in conjunction with the loss of IgD in favour of IgG/IgA/IgE [10]. The presence of CD27 on the surface of an IgD positive B cell distinguishes a population of so-called "IgM memory" cells [11]. While this population contains some cells that are precursors to the classical switched memory cells (IgD-, CD27+) [12], a larger proportion of this compartment is thought to be activated by a

different pathway, possibly in a T-independent antibody response to antigens such as bacterial polysaccharides [13-15]. TLR signalling pathways, TLR10 in particular, are thought to be important for the development and/or survival of "IgM memory" cells [16]. Regulatory B cells are also present in the "IgM memory" population [17]. Another population separated by IgD/CD27 is the population that is negative for both markers and which seems to comprise memory cells [18]. However, there are also reports that this population contains tissue-trafficking B cells that can produce granzyme B [19] and some of the IgA+ CD27- cells may be T-independent responders, carrying polyreactive BCRs which readily bind to bacterial antigens [20], so the population is likely of mixed function.

Age-related changes

The older immune system is less able to respond effectively to infectious challenge. Pneumococcal disease is perhaps the most well known instance of immune failure. The very young and the old have less resistance to *S. pneumoniae*, older people being three times more susceptible to pneumonia than young adults and having a greater morbidity and risk of mortality [21]. The 23 valent pneumococcal polysaccharide vaccine is also less efficient at protecting the very young and the old, although in the case of the young this has been circumvented by conjugating the polysaccharide to a protein antigen [22]. There are many more infections, with perhaps a lower profile, that increase in incidence and morbidity with age [23], for example urinary tract infections are up to 20x more common in older people [24]. Recent data on the mortality rates from the recent Ebola virus disease epidemic in West Africa also shows a greater mortality in the very young and the older population compared to young adults [25].

One of the most common ways of measuring the extent of an immune response is by measuring antibody efficiency after immunisation. Whether this is done by ELISA, or by functional studies such as opsonophagocytic or hemagglutination inhibition assays, it is clear that the efficacy of the human antibody response is decreased with age [26,27]. The total number of B cells remains constant throughout most of life [28]. Only in extreme old age does a decline occur [29], long after the loss of immune system efficacy is first seen. Levels of serum and salivary antibody are quite variable between individuals but there has been no consistently reported increase/decline in antibody quantity with age [30]. Hence it is generally accepted that it is the quality of the B cell/antibody repertoire that changes.

Cellular repertoire

There are some age-related changes in the peripheral blood B cell composition. There is a general decrease of memory and plasma cells as defined by CD27 and CD38 surface markers [31]. The IgM memory compartment has been reported to decrease with age [32,33], and may well be responsible for the decreased reactivity to polysaccharide antigens and increased susceptibility of older people to bacterial infections such as pneumococcal disease [21, 33,34]. Particularly in light of the fact that removal of IgM from serum shows a decrease in opsonophagocytic activity [35] and ELISA analysis of polysaccharide-specific IgM shows an age-related decrease after pneumococcal vaccination [36]. Furthermore, we have also shown that there is a great deal of variability in IgM memory cells with respect to the quantity of IgM on the surface of the cell. In particular we could identify two populations that differed in a number of respects – one that was IgM medium, IgD hi CD27+ and the other being IgM hi, IgD medium CD27+. The latter population was increased in age at the expense of the IgD hi population [32]. So the repertoire of B cells available to respond to T-independent challenge may well be compromised in old age.

The other major change in the peripheral blood B cell population is an increase in IgD- memory cells lacking the activation marker CD27 [37]. These cells are also increased in chronic viral infection, and in autoimmune conditions such as SLE [38-40]. Studies, looking at levels of somatic hypermutation in conjunction with replication history in normal and CD40 ligand-deficient patients, suggest a T-independent origin for these cells, at least for the CD27-IgA+ subset [20]. In our lab we also see lower levels of somatic hypermutation in the CD27- cells in the population as a whole, but we do find a very similar B cell repertoire between the CD27- and CD27+ populations and evidence of members of the same clonal expansion of B cells differing by CD27 expression [18]. This would indicate that lack of CD27 on the cell surface is not always a feature of lineage. Taking together this information, coupled with the fact that older people have more autoreactive antibody in their serum, it is tempting to speculate that an increase in this population of cells may be partially a result of downregulated activation markers as part of a peripheral tolerance mechanism. i.e. an insufficient numbers of appropriate T cells in a T-dependent reaction may result in some members of a B cell clone missing out on the CD40 interactions required to maintain activation status. There is as yet no evidence to support this and further characterisation of the function of CD27- memory B cells will be required to properly interpret the observed age-related changes.

The environment of B cells is paramount to their survival and function. Information on tissue-resident B cells is scarce. Although there is evidence in mice that germinal centres decrease in size and number with age [41], and there is a report of human tonsillar germinal centre changes [42], previous studies on human splenic, lymph node and gut associated follicles did not show any

significant age-related changes in the size of the different B cell compartments [43,44]. The bone marrow environment is crucial for early B cell development and undergoes substantial age-related changes, with decreased cellularity and increased fatty tissue [45]. Whether this causes decreased naïve B cell output, as in mice, has not been formally shown but is assumed in many hypotheses of ageing lymphocyte studies. A decrease in the numbers of antigen specific plasma cells in the bone marrow has recently been reported and will likely affect the quality of serum antibody [31].

Immunoglobulin gene repertoire

Since the quality of the immune response with age is called into question, studies of Ig gene repertoire have been undertaken to look for indications that the repertoire might change with age [46]. None of our studies have indicated that the mechanism of somatic hypermutation of memory cells is altered in any way. Within single germinal centres the rate of hypermutation is unaltered, and the AID targeting motifs do not vary [43,47]. Our first indication that a change in selection of the B cell repertoire might occur was in a study of individual germinal centres. B cells were microdissected out of germinal centres and their Ig sequences were assembled into lineage trees to look at how the high affinity B cells were selected. We found that that the B cell selection processes in the gut appeared to weaken with age, although there was no equivalent change in the splenic germinal centres [47]. Later studies of repertoire diversity, using spectratype analysis of the Ig CDRH3 region, indicated that an increase in expanded clones of B cells occurs with age and correlates with ill health and poor survival in the very old [48].

The advent of high throughput sequencing (HTS) has enabled a more detailed and comprehensive study of changes in the Ig gene repertoire. An HTS study of identical twin pairs indicates that Ig gene segment usage has a strong genetic determinant and is quite resilient to environmental influences. So any observed changes in Ig gene segment use with age or disease may be confounded by inter-individual variation. The CDRH3 region, however, is much more reflective of environmental effects [36, 49]. Further confounders of HTS results are the variations in Ig gene repertoire that can be seen between different subsets of B cells [13]. Since, as mentioned above, the composition of B cell subtypes in the blood may change with age, then repertoire changes seen in whole blood may partially reflect the change in cellular repertoire. Nevertheless, there is a lot of useful information to be gained from whole peripheral blood analyses, especially from studies looking at immune challenge. HTS of the repertoire before and after vaccine challenge has highlighted the diversity of an immune response at all ages, with many different types of genes expanding upon challenge [50, 51]. It has shown that older people have switched memory cells with a higher load of mutation prior to vaccination, and confirms in detail that some people have pre-expanded population of B cells in

the repertoire [51]. Where relative proportions of IgM versus IgG antibodies change after challenge to reflect creation of more IgG memory, this is not always as apparent in older people [51]. Detailed analyses of the different subclasses of antibody are possible and have indicated that some age-related changes are class-specific. In particular the IgM and IgA polysaccharide response in the elderly is diminished, coincident with a reduced expansion of IgM and IgA genes in the repertoire [36,50]. Highlighting the fact that different types of antibody can be selected by different antigens and this selection may fail with age. Chronic viral infections such as CMV and EBV can have a selective effect on the B cell repertoire, affecting the presence of clonal expansions (EBV) or the levels of mutation of antibodies (CMV), so it is useful to know the donor status with respect to these viruses in future studies on ageing [52].

We can identify differences in gene usage during B cell repertoire development in young subjects. As well as our studies showing differences between different mature B cell subsets [13] we can see changes in gene use between immature and naïve B cells [data not shown]. Notable is a decrease in use of IGHV3 family between immature and naïve, and between naïve and class switched memory, although IGHV3 family use increases in IgM memory cells [13]. In view of the fact that immunoglobulin repertoire changes with the type of B cell, we have conducted a study of repertoire in people of different ages after sorting cells with respect to IgD and CD27 expression, and according to subclass usage. Some differences can be seen in older people, such as an increased use of IgA2 over IgA1, and of IgG2 over IgG1 and IgG3 in CD27+ memory cells. Moreover, a repertoire distinction that existed between IgG2 and IgG1/3 in younger subjects was no longer seen in older individuals, the low *IGHV1* family/high *IGHV3* family characteristic of IgG2 in the young being the predominant pattern for all IgG in older samples [53]. This, and other changes in CDRH3 characteristics, indicates that the activation factors involved in repertoire selection in the young have changed with time such that older people may have a repertoire more influenced by IgG2-type activation. Since IgG2 is the subclass involved in T-independent responses, such as to polysaccharide antigen, then one hypothesis would be that the older repertoire is selected in a more T-independent fashion.

Aside from measuring expansions of genes, or usage of gene segments, a study of the CDRH3 region of antibodies is critical. The CDRH3 has the largest influence over the antigen binding site and therefore is the most likely to be affected by positive or negative selection events [49]. Both the spectratype and the high throughput sequencing methods have shown that the repertoire of antibodies upon challenge has a smaller CDRH3 size [36,50,52], implying that smaller CDRH3 have an advantage in the response. This appears to be independent of specific antigen, since it is a general

feature of memory cells compared with naïve cells [36, 52-54]. Peptide analysis also shows an increased hydrophilic characteristic after challenge [50]. While these differences occur at all ages, the challenge-related decrease in size is less marked in the samples from older individuals, implying that the selection of repertoire for smaller CDRH3 size is diminished with age [36]. Hydrophobicity of CDRH3 after challenge does not appear to change with age in a broad overview of sequences, although when cells are separated by CD27 and IgG subclass before sequencing there are some significant age-related differences in the physical characteristics of the CDRH3, particularly CD27-IgG2 cells [52,53].

At first sight it might seem that a decrease in CDRH3 size is an advantage for positive selection during challenge by exogenous antigen. However, an alternative explanation is that a larger, hydrophobic, CDRH3 may be a disadvantage for survival in the face of negative selection mechanisms that ensure tolerance towards self antigens. This latter hypothesis was prompted by the observations, using both sanger sequencing and HTS methods, that naïve B cells have shorter, less hydrophobic, CDRH3 regions than immature B cells in the bone marrow [data not shown, 55]. HTS analysis of DNA sequences that can be divided into productive versus non-productive sequences has also shown that there is a general selection for shorter hydrophilic sequences in the functional repertoire [56]. So there is a selection against longer hydrophobic CDRH3 regions coincident with the negative selection processes of central tolerance. The older samples show a greater CDRH3 size in the naïve repertoire, indicating that these putative negative selection processes are also less effective with age [52,53]. Coupled with the fact that older people have more autoreactive antibodies we therefore propose the hypothesis that at all stages of B cell development, including during a response to exogenous challenge, there are negative selection tolerance processes that are less efficient with age, and where the size and physicochemical characteristics of immunoglobulin CDRH3 are major determinants of the tolerance process. These changes in selection may be partially responsible for the altered repertoire in different B cell subsets of the aged repertoire.

In summary, there are both positive and negative selection processes in the immune system which largely depend on antigen specificity and therefore are reflected in the repertoire of Ig genes in the B cell population. Knowledge of the mechanisms of selection processes and how they affect the repertoire, together with repertoire comparisons between people of different ages, should help elucidate the age related changes in B cell function that contribute to immune senescence.

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Figure legends

Figure 1. Generation of antibody diversity. A number of different genes exist in three loci in the genome, for heavy chain (IGH), kappa light chain (IGK) and for lambda light chain (IGL). Gene rearrangement occurs between the variable (IGHV), diversity (IGHD) and joining (IGHJ) regions of heavy chain such that one of each type of gene is brought together with the help of Recombination activating genes (RAG1 and RAG2). Similarly the light chain genes are rearranged, either kappa or lambda, but without any diversity regions. Random recombination of heavy and light chain genes results in 9516 or 520 different combination possibilities respectively. Random assortment of heavy and light chain gene rearrangements further increases the number of possibilities, in a multiplicative manner, to 4.9×10^6 . Inaccurate joining of the V(D)J regions further increases the possibility for diversity. Antibody CDR3 regions are at the junction of the different genes and therefore the CDR3 regions have the highest diversity.

Figure 2. Selection events in B cell development. B cells undergo a number of developmental stages in the bone marrow and in the periphery. At various stages there will be positive selection to enrich for B cells with attributes useful for binding exogenous antigens (green arrows) and negative selection to ensure that autoreactive B cells do not survive (red arrows).



