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Ageing of the B-cell repertoire

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Older people are more susceptible to infection, less responsive to vaccination and have a more inflammatory immune environment. Using spectratype analysis, we have previously shown that the B-cell repertoire of older people shows evidence of inappropriate clonal expansions in the absence of challenge, and that this loss of B-cell diversity correlates with poor health. Studies on response to vaccination, using both spectratyping and high-throughput sequencing of the repertoire, indicate that older responses to challenge are lacking in magnitude and/or delayed significantly. Also that some of the biologically significant differences may be in different classes of antibody. We have also previously shown that normal young B-cell repertoires can vary between different phenotypic subsets of B cells. In this paper, we present an analysis of immunoglobulin repertoire in different subclasses of antibody in five different populations of B cell, and show how the repertoire in these different groups changes with age. Although some age-related repertoire differences occur in naive cells, before exogenous antigen exposure, we see indications that there is a general dysregulation of the selective forces that shape memory B-cell populations in older people.

1. Introduction

The unique processes involved in generation and shaping of the B-cell repertoire are interesting in their own right, but the results of their action can also be exploited to gain insight into immunological processes. The recombination of immunoglobulin heavy chain variable (*IGHV*), diversity (*IGHD*) and joining (*IGHJ*) genes, coupled with the imprecise joining of these and the addition of random nucleotides by the action of terminal deoxynucleotidyl transferase, results in a 'fingerprint' complementarity determining region 3 (CDR-H3) that can be used to identify a B cell and its progeny. This capability enables us to track B-cell families in blood/tissue so we can determine the extent and type of dissemination [1,2]. We can also examine immune responses by building lineage trees to look at the extent of somatic hypermutation and selection acting on the clone and to determine whether class switching occurs within clones [1]. A study of the Ig gene sequences and the preponderance of their use has long been used to gain insight regarding biologically important selective forces acting on the repertoire. This is useful for identification of high-quality antigen-specific antibodies for use in therapeutics and, conversely, for identification of antibodies associated with autoimmune diseases or lymphoid malignancies [3–9]. With the advent of high-throughput sequencing (HTS), we can now study the repertoire on a scale more appropriate to a system where the number of unique rearranged Ig gene sequences per person is estimated to be in the order of 10^8.

Many of the initial studies of B-cell repertoire have been on peripheral blood mononuclear cells (PBMCs) on a small number of individuals [10–13]. As we progress in our understanding we are realizing that inter-individual variability in B-cell repertoire of humans is quite significant and this brings challenges for an expensive approach such as HTS. Furthermore, we have shown that different types of B cell can have very different repertoires [2]. This means that studies such as the influenza/pneumococcal vaccination study that we published

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recently [13], where we looked at the repertoire in whole blood, are limited by the averaging effect of looking at a mixed population. Often it is not possible to obtain sufficient blood in a study to be able to sort enough cells to repeat the sequencing on different subsets, in which case the judicious use of constant region primers would be able to make some distinction between B cells based around the class of antibody [1,2]. The advantage of knowing that different B-cell populations have a different repertoire, is that it implies the selective forces acting on the B-cell repertoire will vary depending on the development pathway of the cell. Therefore, a study of repertoire in different B-cell subsets in health and disease can tell us whether the forces acting on the lifespan of the B repertoire have changed or not.

The older immune system is less effective, showing reduced responses to infection and vaccination, and increased background levels of autoantibodies and inflammatory cytokines [14–18]. We, and others, have previously shown that the numbers of different types of B cells have changed with age [14,19,20]. The $CD27^+IgD^+$ so-called 'IgM memory' cells decrease in older people [20]. Although there is some evidence that some cells in this population are the precursors of $IgG^+CD27^+IgD^-$ memory cells [21], we have shown that the majority of this population must be activated differently as the population has a distinctive pattern of *IGHV* gene use [1,2]. Evidence seems to point towards a large proportion of IgM memory cells being responders to T-independent antigens such as pneumococcal polysaccharide [22–24]. This is still a matter of debate, and the finding that the human B1-like B cells and the IL10-producing B regulatory cells may also be in this $CD27^+IgD^+$ population [25,26] does not help to clarify the situation. The other type of cell that was found to change with age was that which looks in many respects like a normal IgD^- memory cell, but which does not have CD27 expression [19]. The function of these cells is unknown, but they have been postulated to be exhausted memory cells [19]. We have indeed found that many features of these cells are similar to $CD27^+$ memory cells [1], although when it comes to hypermutation levels, and to the CDR-H3 character of IgM^+IgD^- cells in these compartments, we have also shown some key differences [1].

In this study, we sorted cells into different subsets based on CD27, IgD and CD10 staining. As we have previously shown differences in repertoire between $IgM^+IgD^+CD27^-$ (naive) and $IgM^+IgD^+CD27^+$ (IgM memory) cells, we further subdivided the subsets into different classes by using different constant region-specific primers. This enabled us to investigate whether IgM^+ cells without IgD also differed with respect to repertoire as well as facilitating the comparisons between switched cells. We produced a large number of *IGH* sequences from 14 different individuals aged from 21 to 87 years. We report here that there are different repertoire characteristics, even within one class of antibody, in the young individuals. When compared with the old individuals we find multiple age-related differences which together point towards an alteration in selective processes with age.

2. Material and methods

(a) B-cell isolation and cell sorting

The PBMCs were isolated from a total of six young (21–45 years) and eight old (62–87 years), healthy volunteers. PBMCs were isolated using Ficoll-Paque Plus (GE Healthcare) and Leucosep tubes (Grenier Bio-One Ltd). For HTS analysis, $CD19^+$ B cells were positively selected for using the MACS B-cell Isolation Kit (Miltenyi Biotec), stained with $CD10^-$APC, $CD27^-$FITC (Miltenyi Biotec) and IgD^-PE (BD Bioscience PharMingen) at 4°C (15 min) and analysed on a FACSAria (BD Biosciences PharMingen). Populations were defined using single stain controls before smaller gates were drawn for sorting to ensure a pure population. The same gates were used across all donors and the five subsets were separately collected (figure 1*a*) as previously published: $IgD^+CD27^-CD10^+$ transitional, $IgD^+CD27^-CD10^-$ naive, IgD^+CD27^+ IgM memory, IgD^-CD27^+ and IgD^-CD27^- into 180 µl of Sort-Lysis RT buffer (SLyRT), and the number of sorted cells is recorded in the electronic supplementary material, table S1 [2]. SLyRT comprised 150 ng μl^{-1} $pd(N)_6$ (Invitrogen), 2.5 U μl^{-1} RNAse inhibitor (Bioline), 0.13% Triton X-100 (Sigma-Aldrich), 12.5 mM DTT and 500µM each deoxyribonucleotide triphosphate (dNTP) mix (Promega) in 1 × First-Strand RT buffer (Invitrogen) final concentration (i.e. in 200 µl).

(b) High-throughput sequencing and data analysis

cDNA synthesis was performed by adding 500U SuperScript III reverse transcriptase (Invitrogen) to the 180 µl of SLyRT buffer containing the sorted cells. The following RT reaction was performed: 42°C (10 min), 25°C (10 min), 50°C (60 min) and 72°C (15 min). The Ig genes were amplified as in Wu *et al.* [2]. Briefly, Ig genes were amplified using a semi-nested isotype-specific PCR. A 25 µl PCR1 reaction containing 6.25 µl of cDNA, 0.625U Phusion DNA polymerase (NEB, UK), 200 µM each dNTP, 41.75nM each of 5′ *IGHV* gene family primer and 250 nM constant region primer (for either IgA, IgG or IgM), was run for 15 cycles of 98°C (10 s); 58°C (15 s); 72°C (30 s), after a hot start of 98°C for (30 s), ending with final extension of 72°C for 5 min. A second nested PCR was then performed using 2 µl of PCR1 product, 0.5 U Phusion DNA polymerase, 200 µM each dNTPs, 41.75 nM each of 5′ *IGHV* gene family and 250 nM nested constant region primer. All primers contained matched multiplex identifiers (MID) and 20 cycles of 98°C (10 s); 58°C (15 s); 72°C (30 s) were carried out before final extension at 72°C for 5 min. PCR products were purified and the Roche 454 Titanium platform was used for HTS by LGC Genomics. All five cell populations underwent PCR reactions with IgM C-region primers while the IgD^-CD27^- and IgD^-CD27^+ sorted populations underwent additional IgG and IgA C-region-specific PCR reactions, thus enabling subdivision of these cellular populations into individual classes.

Downstream data clean-up and processing were carried out as previously published [2]. Briefly, Ig gene usage and the CDR-H3 junction regions were determined using High-V-QUEST [27]. ProtParam was used to determine the physico-chemical properties of the CDR-H3 peptide between the conserved first (cysteine) and last amino acid (tryptophan) [28]. Annotated and cleaned data were combined and subsequent analyses performed in Excel (Microsoft) using Mann–Whitney *U* tests and Spearman's correlations. Sequence data are available upon request.

3. Results

(a) Immunoglobulin repertoire of B-cell subsets

To determine whether there are repertoire similarities between different types of memory cells, and whether these are affected by age, we compared the Ig gene usage of different subclasses of Ig genes within five different cell types. Cells were sorted by CD27, IgD and CD10 expression as shown in figure 1 ($IgD^+CD27^-CD10^+$ transitional, $IgD^+CD27^-CD10^-$ naïve, $CD27^+IgD^+$IgM memory, $CD27^+IgD^-$ and $CD27^-IgD^-$).

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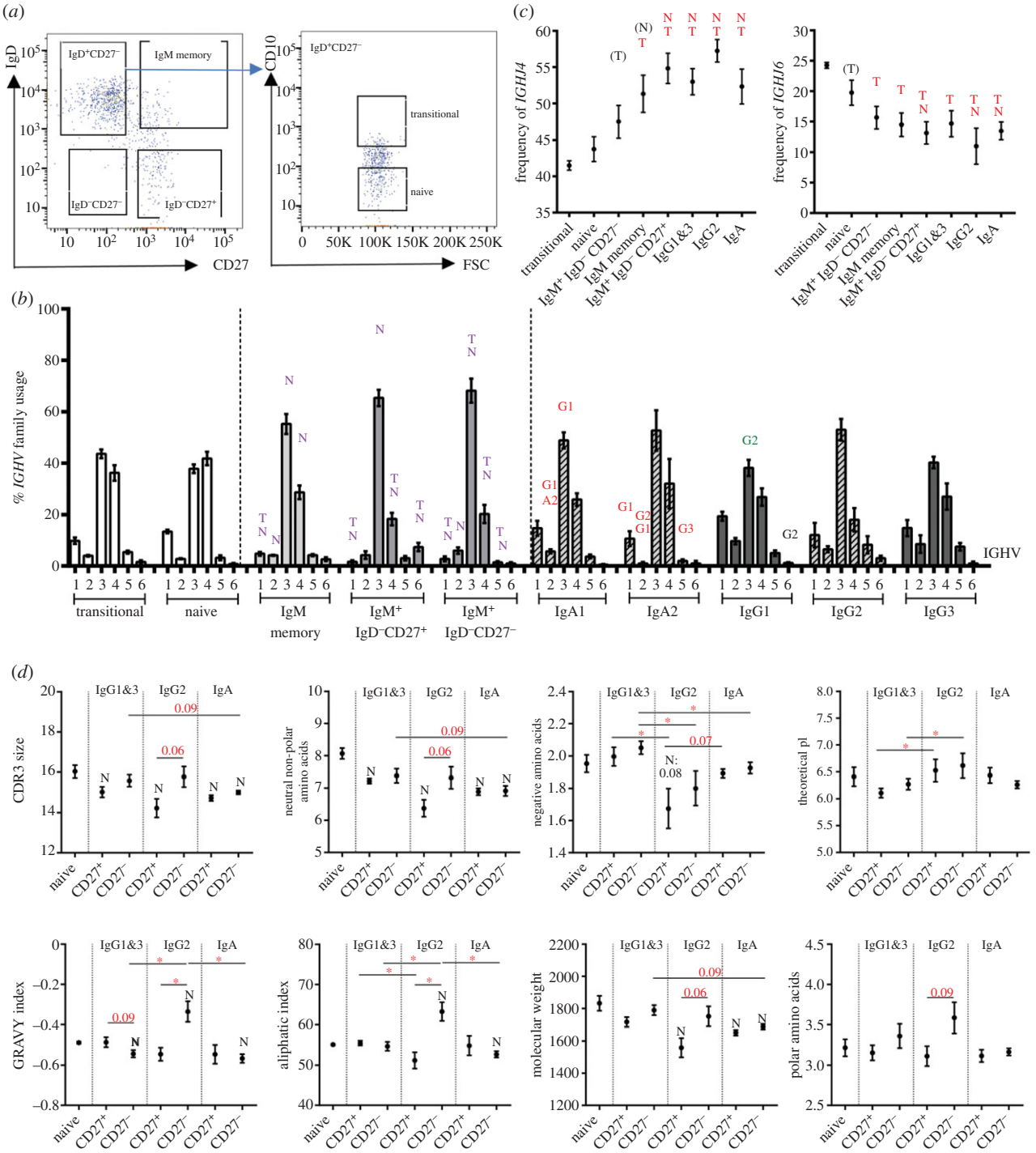

Figure 1. Immunoglobulin repertoire of B-cell subsets from young donors. (*a*) Illustration of sorting strategy used to identify populations of transitional ($IgD^+CD27^-CD10^+$), naive ($IgD^+CD27^-CD10^-$), IgM memory (IgD^+CD27^+), IgD^-CD27^+ and IgD^-CD27^- populations. (*b*) Frequency of *IGHV* family usage within transitional, naive, IgM memory cells, $IgM^+IgD^-CD27^+$, $IgM^+IgD^-CD27^-$ and all switched isotypes within IgD^- cells (IgA1, IgA2, IgG1, IgG2, IgG3). (*c*) Gene usage of *IGHJ4* (left) and *IGHJ6* (right) within all cell types. N denotes a comparison with naive cells and T a comparison with transitional cells. Letters indicate $p < 0.05$ and letters within parentheses indicate $p < 0.09$. (*d*) CDR-H3 properties within naive, IgG1&3 (IgG1 and IgG3 combined data), IgG2 and IgA cells from either IgD^-CD27^+ or IgD^-CD27^- cells: CDR3 length, average number of neutral non-polar amino acids, average number of negative amino acids, theoretical pI, grand average hydrophobicity (GRAVY) index, aliphatic index, molecular weight and average number of polar amino acids. Significant differences between a gene family frequency in one cell compared with another (i.e. IGHV1 in IgM memory cells compared with IGHV1 in transitional cells, T) are denoted by a letter. The letter corresponds to the cells type being compared such that for IgM memory cells there is a significant difference between *IGHV1* usage in IgM memory cells versus transitional cells denoted by 'T' above the *IGHV1* column. T, transitional; N, naive; G1, IgG1; G2, IgG2; G3, IgG3; A1, IgA1; A2, IgA2. $n = 4-6$ young donors. *$p < 0.05$ by Mann–Whitney *U* test, bars are s.e.m.

A total of 112 886 sequences were obtained from six young and eight old individuals. After CDR-H3 clustering to identify related sequences, we identified 49 770 unique VDJ gene rearrangements that were categorized according to class and subclass of antibody as shown in table 1.

Key differences in the repertoire between different groups of cells from young donors seem to be focused on the relative use of *IGHV1* and *IGHV3* family genes. In agreement with our previous observations [1,2], IgM^+ memory cells (composed of IgM memory, $IgM^+IgD^-CD27^+$, $IgM^+IgD^-CD27^-$

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Table 1. Number of unique VDJ rearrangements generated by HTS for each cell type.

		unique sequences (young)	unique sequences (old)
transitional	IgM	4345	4367
naive	IgM	1386	4398
IgM memory	IgM	9302	10 176
classical memory	IgA	89	84
($CD27^+IgD^-$)	IgA1	816	621
	IgA2	133	157
	IgG1	594	175
	IgG1/3	65	32
	IgG2	198	163
	IgG3	108	28
	IgM	773	430
double negative	IgA	76	55
($CD27^-IgD^-$)	IgA1	888	764
	IgA2	102	76
	IgG1	1458	625
	IgG1/3	163	88
	IgG2	99	63
	IgG3	323	87
	IgM	2142	3321
	total	23 060	25 710

are characterized by increased usage of *IGHV3* family genes at the expense of *IGHV1* and *IGHV4* families when compared with naive cells in the young sequences (figure 1*b*). Conversely, the class switched B cells, when analysed in combination, are characterized by an increase in *IGHV1* and decreased *IGHV3* (data not shown) [2]. However, this latter observation has since been shown to be an averaged effect across class switched cells as the *IGHV* repertoires between different classes of cells in young individuals differ from each other [1]. Even within the same class of antibody there are differences: IgG1 and IgG3 share the same distribution of *IGHV* family usage, but this is different to the *IGHV* family repertoire in IgG2. The IgG2 repertoire has more in common with the IgA1 and IgA2 repertoires than with the other IgG subclasses, having more *IGHV3* and less *IGHV1*. The IgG2/IgA1/IgA2 pattern of *IGHV* gene usage also shows similarity with the IgM memory repertoire (figure 1*b*). Hence, it would appear that the selective forces acting on the IgG1 and IgG3 antibody repertoire differ from the other types of memory cells.

Although the *IGHD* repertoire only varies slightly between cells (data not shown) the *IGHJ* repertoire varies significantly between naive and memory cells. There is an increased frequency usage of *IGHJ4* within memory cells compared with antigen-inexperienced cells, which occurs at the expense of *IGHJ6* (figure 1*c*). This will have an impact on the CDR-H3 region, which makes the largest contribution to the antigen binding site of the antibody. We have previously shown that the memory B-cell repertoire has, on average, a significantly shorter CDR-H3 region than naive cells [1,2]. This is also true for different types of IgM memory cells [1]. In view of the *IGHV* gene usage differences between different subclasses of antibody, we analysed the physico-chemical characteristics of CDR-H3 in IgG1 and IgG3 compared with IgG2 and IgA in both IgD^-CD27^+ and IgD^-CD27^- cells. In agreement with previous findings [1,2,13,25,29], these CDR-H3 regions from switched cells are shorter than in naive cells in all cases except for $IgD^-CD27^-IgG^+$ cells (figure 1*d*). There is also a decrease in the number of neutral non-polar amino acids within the CDR-H3 region in all switched memory cells, except for $IgD^-CD27^-IgG^+$ cells, compared with naive cells. We found that IgA and IgG2 have a similar *IGHV* repertoire (figure 1*b*), and can also be distinguished from IgG1 and IgG3 by the CDR-H3 biophysical properties of theoretical pI and a lower average count of negative amino acids (figure 1*d*). Strikingly, we found that IgG2 cells had very different CDR-H3 properties dependent on whether they were from the IgD^-CD27^- or IgD^-CD27^+ population. IgG2 IgD^-CD27^- cells were distinguished from IgG2 IgD^-CD27^+, IgA and IgG1 and IgG3 by having a higher aliphatic index and GRAVY index (figure 1*d*). Additionally, they also show trends towards longer CDR3 lengths, more neutral polar and non-polar amino acids and a larger molecular weight than IgG2 IgD^-CD27^+ cells. By contrast, no statistically significant differences were seen between $CD27^+$ or $CD27^-$ populations for the IgG1 and IgG3 and IgA cells. These data suggest that different subclasses of antibody can be distinguished by the physico-chemical properties of their CDR-H3 regions as well as the Ig gene usage, with the IgG2 IgD^-CD27^- population being particularly distinct from the other groups.

(b) Alterations in immunoglobulin repertoire within switched subclasses with age

Within our data we cannot compare the relative use of different classes of antibody, as they are isolated using different C region primers in separate PCR reactions. We can, however, determine the relative use of different subclasses of antibody, as all sequences of one class are amplified with the same primer and we distinguish the subclasses using sequence motifs upstream of the primer. The proportions of different subclasses in the repertoire are affected by age. At all ages there is a preponderance of IgA1 over IgA2 in the blood in both the $CD27^+$ and $CD27^-$ memory populations. The relative amounts do not change with age in the $CD27^-$ memory cells, but in the $CD27^+$ memory cells there is a significantly greater proportion of IgA2 in the older group (figure 2*a*). In the $CD27^-$IgG population again there is not much age-related difference, except perhaps a slight increase in IgG1. However, the largest age-related differences are in the $CD27^+$ memory population, where the proportion of IgG2 increases significantly at the expense of IgG1&3 (figure 2*a*).

The differences in *IGHV* family use between IgG1&3 and IgG2 that we saw in the young are no longer present in the older samples (figure 2*b*). This seems to be mainly because of the fact that old IgG1&3 genes use a similar repertoire to that of IgG2, having a significantly increased use of *IGHV3* compared with the young IgG1&3 samples. This change seems to be a result of many smaller changes at the individual gene levels, as there were not many individual genes that were significantly changed with age (figure 2*c*).

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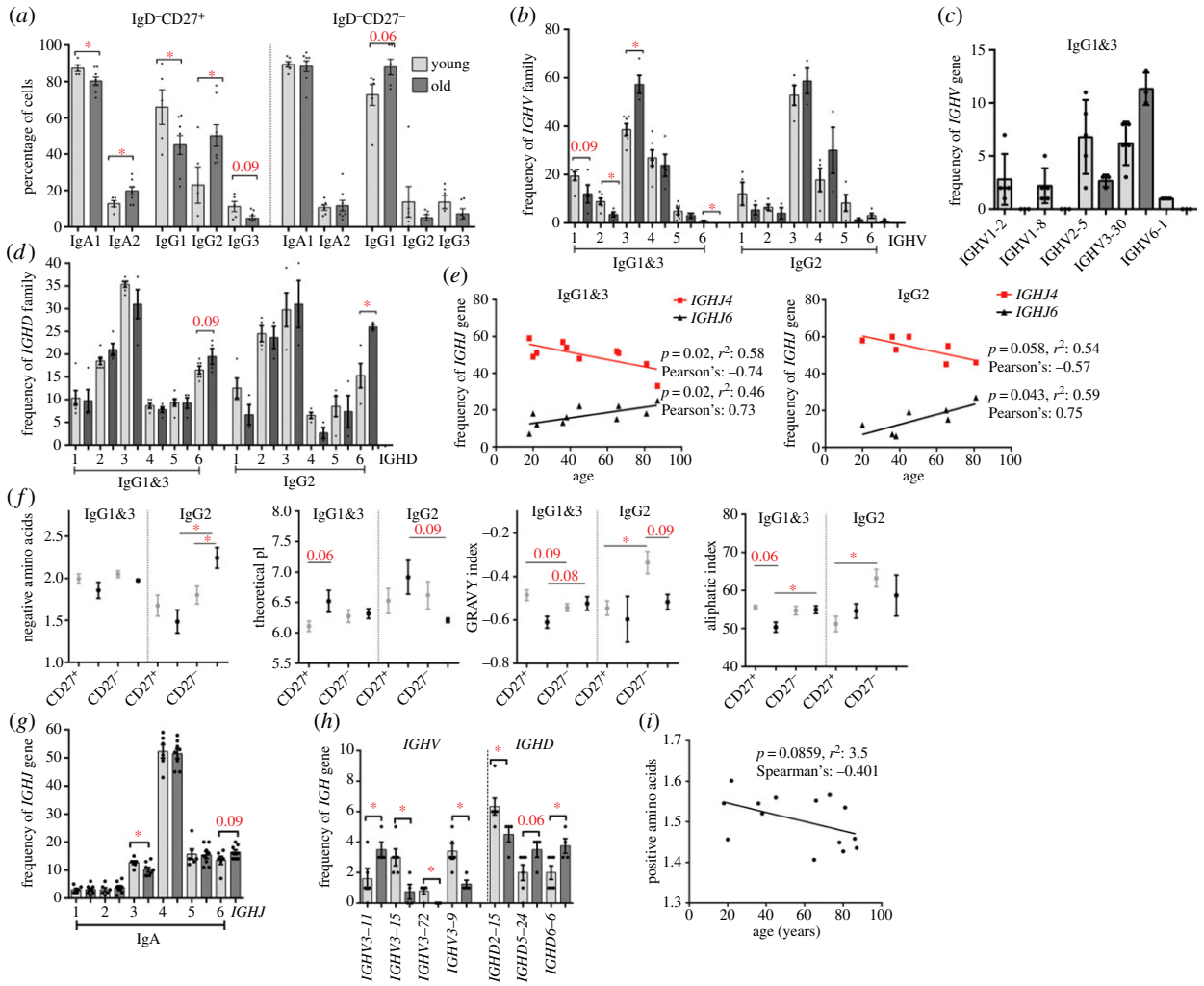

Figure 2. Age-related alterations in immunoglobulin repertoire within subclasses. Light grey, young ($n = 6$, 21–45 years) and dark grey/black, old ($n = 8$, 65–87 years) donors. (*a*) Age-related change in the relative proportions of IgA1 and IgA2 sequences and in the relative proportions of IgG1, IgG2, IgG3 sequences within $CD27^+$ and $CD27^-$ memory subsets. (*b*) Frequency of *IGHV* usage within IgG1&3 and IgG2 cells from young and old donors. (*c*) Frequency of individual *IGHV* gene usage within IgG1&3 cells from young and old donors. (*d*) Frequency of *IGHD* usage in IgG1&3 and IgG2 cells from young and old donors. (*e*) Correlation of *IGHJ4* (squares) and *IGHJ6* (triangles) gene usage with age in IgG1&3 (left) and IgG2 (right) cells. (*f*) Average number of negative amino acids, the theoretical pI, GRAVY index and aliphatic index from IgG1&3 and IgG2 cells in young and old donors. (*g*) Frequency of *IGHJ* usage within IgA cells from young and old donors. (*h*) Age-related changes in the gene usage of individual *IGHV* and *IGHD* genes in IgA cells. (i) Correlation of the average number of positive amino acids with age from IgA cells. $*p < 0.05$ by Mann–Whitney *U* test, bars are s.e.m. Spearman's correlations were calculated where stated.

Not all aspects of the repertoire are differentially altered between the different IgG subclasses. Despite the differential change in CDR-H3 characteristics, the gene segments that form a large part of this region seem to change with age in a similar fashion across all IgG genes. Analysis of *IGHD* and *IGHJ* usage in the old showed similar increases in *IGHD6* and *IGHJ6*, with a decrease in *IGHJ4* (figure 2*d*,*e*). Additionally, the distinctive properties of the CDR-H3 regions seem to be altered with age. There is no longer a significant difference between IgG2 from $CD27^+$ or $CD27^-$ cells in the old, when looking at the GRAVY and aliphatic index, which was the most distinctive characteristic of IgG2 $CD27^-$ cells in the young. Additionally in the young, IgG2 for both cells types had a lower average number of negative amino acids and a higher theoretical pI compared with both IgG1&3 cells and despite this still being true within IgG2 $CD27^+$ cells, it is not the case for IgG2 $CD27^-$ cells from the old. This results in differences between IgG2 $CD27^+$ and $CD27^-$ cells in the old that were not present in the young, in terms of the average number of negative amino acids and the theoretical pI, and highlights that IgG2 $CD27^-$ is most affected by age (figure 2*f*).

IgA also shows a trend towards increasing *IGHJ6* with age, although this is less marked than in the IgG repertoire and occurs at the expense of *IGHJ3* rather than *IGHJ4* (figure 2*g*). Despite the fact that there are no significant differences in *IGHV* or *IGHD* family usage, IgA does show small variations with age in individual gene usage. *IGHV3–11* and *IGHD6–6* are seen to increase with age whereas *IGHV3–15*, *IGHV3–72*, *IGHV3–9* and *IGHD2–15* decrease (figure 2*h*). In additional analysis of the IgA CDR-H3 region identified an age-related decrease in the occurrence of positive amino acids (figure 2*i*).

(c) Age-related alterations in antigen-inexperienced cells

Age-related changes in memory cells may depend on the transitional and naive cell repertoire from which the memory cells develop. Analysis of the antigen-inexperienced repertoire did not show a clear change in *IGHV* family use by the tests of significance that we use, although there does appear to be a trend towards increased *IGHV1* and *IGHV5* at the expense of *IGHV3* (figure 3*a*). We did identify a significant decreased use of

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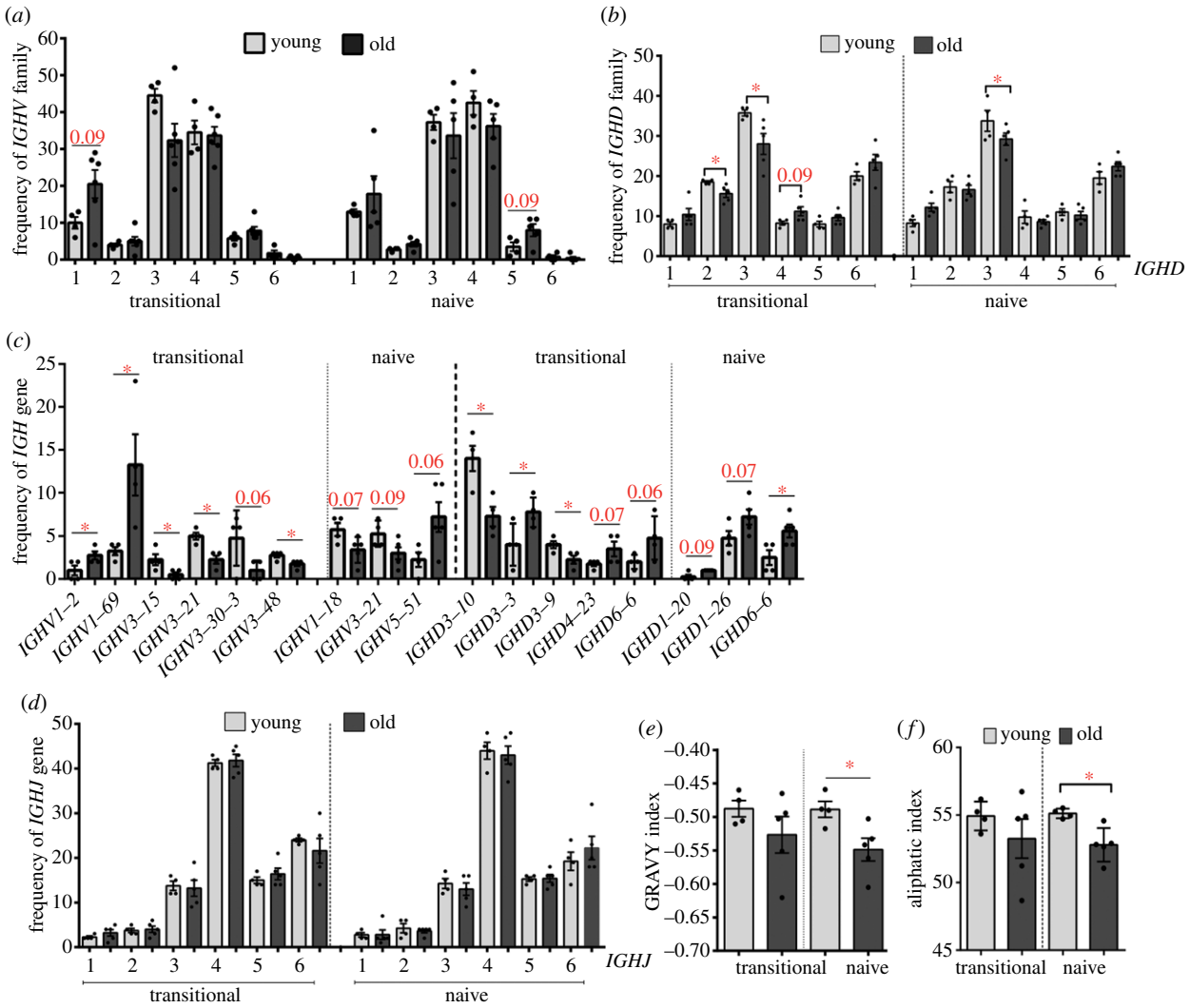

Figure 3. Age-related alterations in antigen-inexperienced cells. Frequency of *IGHV* (*a*) and *IGHD* (*b*) usage within transitional and naive cells from young (light grey) and old (dark grey) donors. (*c*) Frequency of individual *IGHV* and *IGHD* gene usage within transitional and naive cells from young (light grey) and old (dark grey) donors. (*d*) Frequency of *IGHJ* gene usage. (*e*) Average GRAVY index and aliphatic index from transitional and naive populations from young and old donors. $n = 4$ young (light grey) and $n = 5$ old donors (dark grey), s.e.m., Mann–Whitney *U* tests were carried out such that $*p < 0.05$ and $p < 0.09$ is written above.

IGHD2 and *IGHD3* in older cells (figure 3*b*). Analysis of individual genes, rather than family usage, identified significant increases in *IGHV1–2* and *IGHV1–69* usage and a significant decrease in *IGHV3–15*, *IGHV3–30–3* and *IGHV3–48* usage within old transitional cells (figure 3*c*). Analysis of individual *IGHD* genes highlighted a decreased use of *IGHD3–9* and *IGHD3–10* alongside an increased use of *IGHD3–3* and *IGHD6–6* with age. No age-related changes were seen within *IGHJ* gene usage (figure 3*d*). Consistent with these gene changes, together with the fact that *IGHD* genes make up a large part of the CDR-H3 region, age-related changes in hydrophobicity and aliphatic index were also seen in antigen-inexperienced cells (figure 3*e*,*f*).

(d) Alteration in antigen selection pressures on *IGHD* and *IGHJ* usage within older memory cells

The above data indicate that age affects the immunoglobulin switched repertoire in a variety of different ways. In combination with our recent data identifying changes in IgM^+ repertoire with age [25], our findings could be indicative of changes in exogenous antigen selection pressures with age. However, we have also shown that exogenous antigen-inexperienced B cells may also have a changed repertoire in older people, so some changes in memory cells may be a reflection of an altered repertoire in the first instance. To address this, we looked at the relative frequencies of immunoglobulin gene use in naive versus memory cells. Analysis of *IGHD* family and *IGHJ* gene (DJ) combination frequencies between antigen-inexperienced cells (transitional and naive), IgM^+ memory cells and switched memory cells identified that not only is there a biased use of gene combinations, even in antigen-inexperienced cells (naive and transitional), but that this selection can change as cells become memory (figure 4*a*). The choice of DJ combination gene impacts the CDR-H3 length, as they compose 85% of the CDR-H3, and it is clear that even within a particular size DJ combination size there are biases towards specific combinations (figure 4*a*). In the young group, four combinations increase in both IgM^+ and switched memory cells upon antigen contact (D1J4, D1J3, D1J5, D2J4) with six also decreasing in both (D4J6, D3J3, D3J5, D5J6, D6J6, D3J6). Interestingly, D2J6 is the only combination to be altered in one type of memory but not the other as it is significantly decreased in

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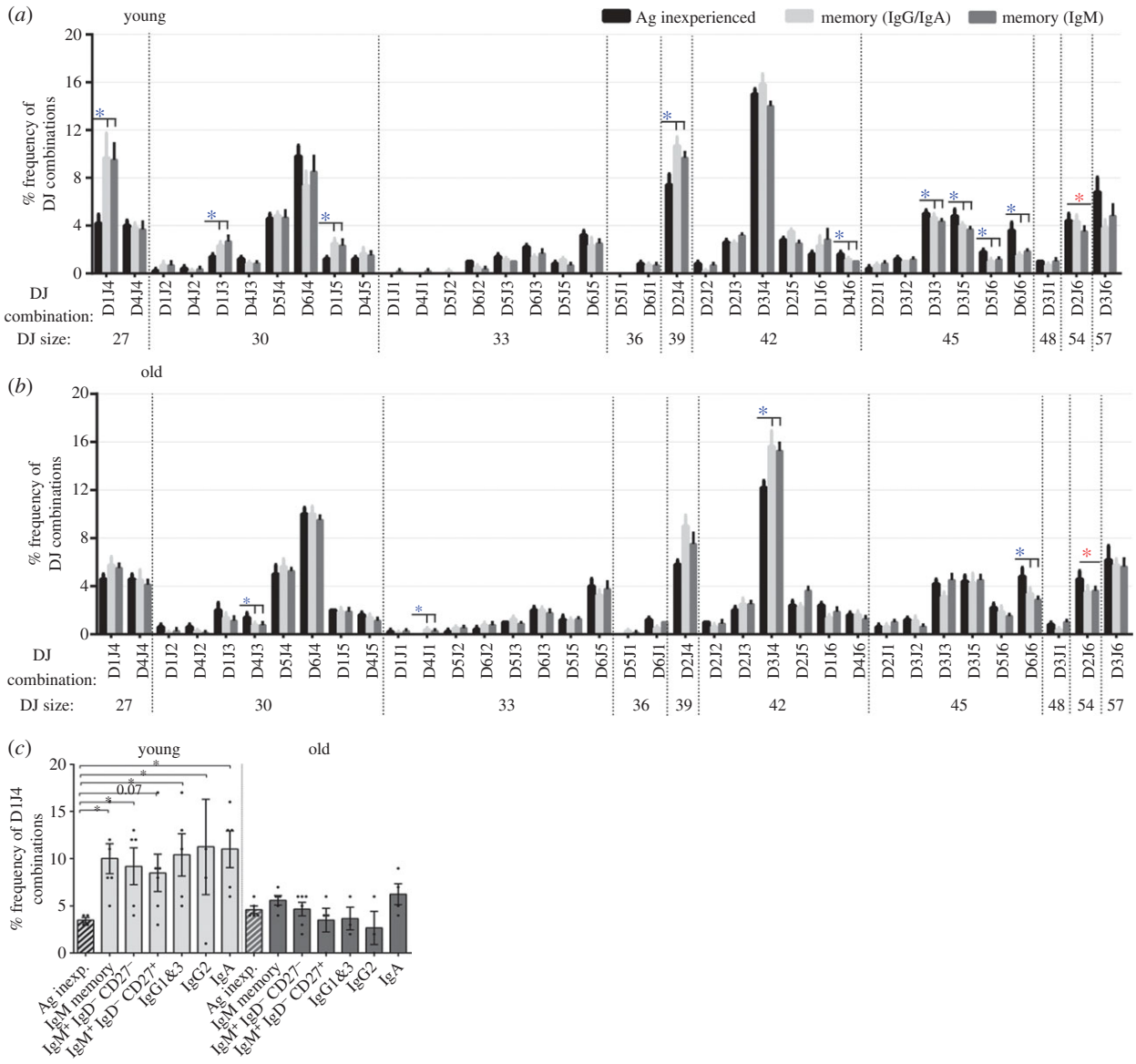

Figure 4. Alteration in antigen selection pressures within old memory cells. Frequency usage of all *IGHD* and *IGHJ* combinations within antigen-inexperienced cells (naive and transitional cells) in black, IgM positive memory (IgM memory cells, $CD27^{+}IgD^{-}IgM^{+}$ and $CD27^{-}IgD^{-}IgM^{+}$) in dark grey, and switched cells (IgG1&3, IgG2 and IgA) in light grey from young (*a*) and old (*b*) donors. (*c*) Frequency usage of D1J4 combination within all cells from young and old donors. $n = 6$ young (light grey) and $n = 8$ old donors (dark grey). $^{*}p < 0.05$ by Mann–Whitney *U* test; bars are s.e.m. (Online version in colour.)

IgM^{+} memory cells (IgM memory, $IgD^{-}CD27^{-}IgM^{+}$ and $IgD^{-}CD27^{+}IgM^{+}$ cell populations) and not in the switched cells.

In the young, there were ten DJ gene combinations that are seen to significantly change their frequency between antigen-inexperienced and memory cells, whereas in the elderly only five DJ combinations change. This suggests a possible reduction of antigen selection with age. An example of this is shown for D1J4 usage, where in the young the frequency of use is increased in all types of memory cells whereas in the old this selection does not occur and no change is seen (figure 4*b*,*c*).

4. Discussion

It would appear that the selective forces acting on the IgG1 and IgG3 antibody repertoire differ from the other types of memory cells. This could be owing to a change in positive selection, with a different type of antigen initiating class switching to IgG1&3. As IgM memory and IgG2 are known to respond to polysaccharide antigens, then we could hypothesize that *IGHV3* genes are more likely to bind polysaccharides [30–32]. The repertoire shift could also be owing to a shift in negative selection. If we assume that peripheral tolerance mechanisms constantly remove potentially autoreactive cells from the repertoire in an immune response then a decrease in use of a particular gene family (in this case *IGHV1*) could reflect the autoreactive potential of the family. *IGHV1* gene use has been previously reported in autoimmune disorders [3–9]. Cells receiving more survival help in the immune response may bypass the tolerance mechanism and show increased use of *IGHV1* genes. Much more information as to antigenic preferences of *IGHV* genes would be required to test these hypotheses.

Our repertoire analysis of $CD27^{-}$memory cells would agree that many features are similar to the classical $CD27^{+}$ memory cells, but we have found differences. In particular, there are strong differences in the CDR-H3 region of IgG2

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cells depending on whether they express CD27 or not. A difference in repertoire, especially one involving the major antigen binding region of the antibody, would imply a difference in the antigen-driven selective forces acting on it. If the $CD27^-$ group of IgG2 memory cells were an exhausted subset of the $CD27^+$ memory cells we would expect them to have the same repertoire. Given that they are not the same, we therefore hypothesize that perhaps the $CD27^-$ IgG2 memory cells have undergone a downregulation of the activation marker CD27 because of some repertoire-related quality. The most obvious quality would be autoreactivity, given that this group of cells is increased in autoimmune conditions and in ageing where there is also a correlation with increased autoreactive antibodies [29].

It is interesting that some of the most significant differences between types of cells that are seen in the young group are not apparent in the old group. Apart from a slight change in the proportion of IgA1 versus IgA2 use in the older group, all the changes are in the IgG subclasses. There is greater use of IgG2 than IgG1 and IgG3 in the old, and the IgG1/3 genes that do exist have an *IGHV* gene family use that looks more like the IgG2 family. In general, there seems to be a skewing towards the IgG2/IgM memory-type repertoire, which is thought to arise mainly as a result of T-independent activation. This would perhaps fit with the known decrease in T cell numbers with age, reducing the amount of T cell help available for T-dependent activation. However, it is difficult to reconcile the observations of an altered repertoire within the IgG1/3 populations without also hypothesizing that the helper cell effects on class switching are separated from their effects on helping a B cell of a particular specificity to survive.

We did see some age-related changes in the transitional and naive repertoires that were supposedly unaffected by exogenous antigen, and therefore some of the age-related changes that we see may well be a result of an altered repertoire in the bone marrow. Changes in transcription factors affecting early B-cell development have been shown, and may well affect the repertoire [15]. However, the overall picture seems to be of a change in selective forces with age. Unsurprisingly, given its importance in antigen binding, the CDR-H3 region perhaps shows this more than individual *IGHV* gene usage. There is a very clear increase in use of *IGHJ4* at the expense of *IGHJ6* in the transition between naive and memory cells, and this selection decreases with age in all IgG subclasses (figure 2*e*). Some of the striking physico-chemical differences between $CD27^+$ and $CD27^-$ IgG2 memory CDR-H3 regions disappear with age, whereas others appear (figure 2*f*), and clearly the selective forces acting to determine the use of particular *IGHD*/*IGHJ* combinations are changed with age across many types of memory.

In summary, there are some major differences in repertoire between different cell types, and between different classes and subclasses of antibody. It is important to bear this in mind in repertoire studies, as averaging effects could mean that important information may be missed in a study of mixed populations. The old individuals lose some of these repertoire distinctions; this likely reflects a difference in the selective forces acting on the older repertoire. Antigen history between different individuals will always vary, but the fact that a number of older individuals show the same change in repertoire implies a more intrinsic effect of something other than antigen history.

Ethics. Written consent was obtained in accordance with the Declaration of Helsinki after approval from the Guy's Hospital Research Ethics Committee (REC 08/H0804/57).

Authors' contributions. Y.-C.W. performed experiments, D.K. performed bioinformatics analysis of sequences, V.M. performed data analysis of sequences and wrote the paper, D.D.-W. performed data analysis of sequences, designed the experiments and wrote the paper.

Competing interests. We declare we have no competing interests.

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References

1. Wu YC, Kipling D, Dunn-Walters DK. 2011 The relationship between CD27 negative and positive B cell populations in human peripheral blood. *Front. Immunol.* **2**, 81. (doi:10.3389/fimmu.2011.00081)
2. Wu YC, Kipling D, Leong HS, Martin V, Ademokun AA, Dunn-Walters DK. 2010 High-throughput immunoglobulin repertoire analysis distinguishes between human IgM memory and switched memory B-cell populations. *Blood* **116**, 1070–1078. (doi:10.1182/blood-2010-03-275859)
3. Baseggio L *et al*. 2010 CD5 expression identifies a subset of splenic marginal zone lymphomas with higher lymphocytosis: a clinico-pathological, cytogenetic and molecular study of 24 cases. *Haematologica* **95**, 604–612. (doi:10.3324/haematol.2009.011049)
4. Clausen B *et al*. 1998 Clonally-related immunoglobulin VH domains and nonrandom use of DH gene segments in rheumatoid arthritis synovium. *Mol. Med.* **4**, 240–257.
5. Darzentas N *et al*. 2010 A different ontogenesis for chronic lymphocytic leukemia cases carrying stereotyped antigen receptors: molecular and computational evidence. *Leukamia* **24**, 125–132. (doi:10.1038/leu.2009.186)
6. Fraser N, Rowley G, Field M, Stott D. 2003 The VH gene repertoire of splenic B cells and somatic hypermutation in systemic lupus erythematosus. *Arthritis Res. Ther.* **5**, R114–R121. (doi:10.1186/ar627)
7. García-Muñoz R, Panizo C, Bendandi M, Llorente L. 2009 Autoimmunity and lymphoma: is mantle cell lymphoma a mistake of the receptor editing mechanism? *Leuk. Res.* **33**, 1437–1439. (doi:10.1016/j.leukres.2009.05.008)
8. Shin E *et al*. 1994 Variable regions of Ig heavy chain genes encoding antithyrotropin receptor antibodies of patients with Graves' disease. *J. Immunol.* **152**, 1485–1492.
9. Van Es J, Aanstoot H, Gmelig-Meyling F, Derksen R, Logtenberg T. 1992 A human systemic lupus erythematosus-related anti-cardiolipin/single-stranded DNA autoantibody is encoded by a somatically mutated variant of the developmentally restricted 51P1 VH gene. *J. Immunol.* **149**, 2234–2240.
10. Wang X, Stollar B. 1999 Immunoglobulin VH gene expression in human aging. *Clin. Immunol.* **93**, 132–142. (doi:10.1006/clim.1999.4781)
11. Boyd SD *et al*. 2010 Individual variation in the germline Ig gene repertoire inferred from variable region gene rearrangements. *J. Immunol.* **184**, 6986–6992. (doi:10.4049/jimmunol.1000445)
12. Boyd SD *et al*. 2009 Measurement and clinical monitoring of human lymphocyte clonality by massively parallel VDJ pyrosequencing. *Sci. Transl. Med.* **1**, 12ra23. (doi:10.1126/scitranslmed.3000540)
13. Wu YC, Kipling D, Dunn-Walters DK. 2012 Age-related changes in human peripheral blood IGH repertoire following vaccination. *Front. Immunol.* **3**, 193. (doi:10.3389/fimmu.2012.00193)

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14. Ademokun A, Wu YC, Dunn-Walters D. 2010 The ageing B cell population: composition and function. *Biogerontology* **11**, 125–137. (doi:10.1007/s10522-009-9256-9)
15. Dunn-Walters DK, Ademokun AA. 2010 B cell repertoire and ageing. *Curr. Opin. Immunol.* **22**, 514–520. (doi:10.1016/j.coi.2010.04.009)
16. Kared H, Camous X, Larbi A. 2014T cells and their cytokines in persistent stimulation of the immune system. *Curr. Opin. Immunol.* **29**, 79–85. (doi:10.1016/j.coi.2014.05.003)
17. Fulop T, Le Page A, Fortin C, Witkowski JM, Dupuis G, Larbi A. 2014 Cellular signaling in the aging immune system. *Curr. Opin. Immunol.* **29**, 105–111. (doi:10.1016/j.coi.2014.05.007)
18. Pawelec G, Goldeck D, Derhovanessian E. 2014 Inflammation, ageing and chronic disease. *Curr. Opin. Immunol.* **29**, 23–28. (doi:10.1016/j.coi.2014.03.007)
19. Colonna-Romano G, Bulati M, Aquino A, Pellicano M, Vitello S, Lio D, Candore G, Caruso C. 2009 A double-negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people. *Mech. Ageing Dev.* **130**, 681–690. (doi:10.1016/j.mad.2009.08.003)
20. Shi Y, Yamazaki T, Okubo Y, Uehara Y, Sugane K, Agematsu K. 2005 Regulation of aged humoral immune defense against pneumococcal bacteria by IgM memory B cell. *J. Immunol.* **175**, 3262–3267. (doi:10.4049/jimmunol.175.5.3262)
21. Dogan I, Bertocci B, Vilmont V, Delbos F, Megret J, Storck S, Reynaud C-A, Weill J-C. 2009 Multiple layers of B cell memory with different effector functions. *Nat. Immunol.* **10**, 1292–1299. (doi:10.1038/ni.1814)
22. Kruetzmann S *et al*. 2003 Human immunoglobulin M memory B cells controlling *Streptococcus pneumoniae* infections are generated in the spleen. *J. Exp. Med.* **197**, 939–945. (doi:10.1084/jem.20022020)
23. Weller S *et al*. 2001 CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. *Proc. Natl Acad. Sci. USA* **98**, 1166–1170. (doi:10.1073/pnas.98.3.1166)
24. Weller S *et al*. 2004 Human blood IgM 'memory' B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood* **104**, 3647–3654. (doi:10.1182/blood-2004-01-0346)
25. Martin V, Wu Y-C, Kipling D, Dunn-Walters DK. In press. Age-related aspects of human IgM+ B cell heterogeneity. *Ann. NY Acad. Sci*.
26. Khoder A *et al*. 2014 Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. *Blood* **124**, 2034–2045. (doi:10.1182/blood-2014-04-571125)
27. Brochet X, Lefranc MP, Giudicelli V. 2008 IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. *Nucleic Acids Res.* **36**, W503–W508. (doi:10.1093/nar/gkn316)
28. Wilkins MR *et al*. 1999 Protein identification and analysis tools in the ExPASy server. *Methods Mol. Biol.* **112**, 531–552.
29. Ademokun A, Wu YC, Martin V, Mitra R, Sack U, Baxendale H, Kipling D, Dunn-Walters DK. 2011 Vaccination-induced changes in human B-cell repertoire and pneumococcal IgM and IgA antibody at different ages. *Aging Cell* **10**, 922–930. (doi:10.1111/j.1474-9726.2011.00732.x)
30. Lucas A, Moulton K, Tang V, Reason D. 2001 Combinatorial library cloning of human antibodies to *Streptococcus pneumoniae* capsular polysaccharides: variable region primary structures and evidence for somatic mutation of Fab fragments specific for capsular serotypes 6B, 14, and 23F. *Infect. Immun.* **69**, 853–864. (doi:10.1128/IAI.69.2.853-864.2001)
31. Zouali M. 1995 B-cell superantigens: implications for selection of the human antibody repertoire. *Immunol. Today* **16**, 399–405. (doi:10.1016/0167-5699(95)80009-3)
32. Baxendale H, Johnson M, Stephens R, Yuste J, Klein N, Brown J, Goldblatt D. 2008 Natural human antibodies to pneumococcus have distinctive molecular characteristics and protect against pneumococcal disease. *Clin. Exp. Immunol.* **151**, 51–60. (doi:10.1111/j.1365-2249.2007.03535.x)