Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways

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Running title: Delineation of human memory B cells

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ABSTRACT

Multiple distinct memory B cell subsets have been identified in humans, but it remains unclear how their phenotypic diversity corresponds to the type of responses they originate from. Especially the contribution of germinal center-independent responses in human remains controversial. We defined six memory B cell subsets based on their antigen-experienced phenotype and differential expression of CD27 and IgH isotypes. Molecular characterization of their replication history, Ig somatic hypermutation and class-switch profiles demonstrated their origin from three different pathways. CD27-IgG+ and CD27+IgM+ B cells are derived from primary germinal center reactions, and CD27+IgA+ and CD27+IgG+ B cells from consecutive germinal center responses (pathway 1). In contrast, natural effector and CD27-IgA+ memory B cells have limited proliferation and are also present in CD40L-deficient patients, reflecting a germinal center-independent origin. Natural effector cells at least in part originate from systemic responses in the splenic marginal zone (pathway 2). CD27-IgA+ cells share low replication history and dominant Igλ and IgA2 usage with gut lamina propria IgA+ B cells suggesting their common origin from local germinal center-independent responses (pathway 3). Our findings shed light on human germinal center-dependent and – independent B cell memory formation and provide new opportunities to study these processes in immunological diseases.
INTRODUCTION
Antigen-specific memory formation following a primary infection contributes greatly to human health. Immunological memory lies in long-lived T and B cells derived from the initial immune response. Precursor B cells develop from hematopoietic stem cells in the bone marrow and create a unique receptor by V(D)J recombination in their immunoglobulin (Ig) loci. Following antigen recognition, mature B cells proliferate and can further optimize antigen-binding by the introduction of point mutations in the V(D)J exons of their Ig heavy and light chains (somatic hypermutations; SHM) and the subsequent selection for high affinity mutants. Furthermore, the antibody effector functions can be modified by changing the isotype of the IGH constant region from mu to alpha, delta, epsilon or gamma (Ig class switch recombination; CSR). Both processes are mediated by activation-induced cytidine deaminase (AID), which preferentially targets specific DNA motifs.

In addition to antigen recognition via the B cell antigen receptor (BCR), B cells need a second signal to become activated. Activated T cells can provide such a signal via CD40L that interacts with CD40 on B cells. T cell-dependent (TD) B cell responses are characterized by germinal center (GC) formation, extensive B cell proliferation, affinity maturation and Ig CSR. Thus, high-affinity memory B cells and Ig-producing plasma cells are formed. Additionally, B cells can respond to T cell-independent (TI) antigens that either activate via the BCR and another (innate) receptor (TI-1) or via extensive cross-linking of the BCR due to the repetitive nature of the antigen (TI-2). TI responses are directed against blood-borne pathogens in the splenic marginal zone and in mucosal tissues (reviewed in ).

A substantial fraction of B cells in blood of human subjects has experienced antigen and shows hallmarks of memory B cells: SHM of rearranged Ig genes and fast recall responses to antigen. Initially, human memory B cells were identified based on the expression of CD27. IgA and IgG-class switched CD27+ B cells are derived from T cell-dependent
responses in the GC and contain high loads of SHM in their Ig genes.\textsuperscript{16-18} CD27+IgM+ B cells contain less SHM, but show molecular footprints of (early) GC generation.\textsuperscript{19} Interestingly, in contrast to CD27+IgM+IgD- ‘IgM-only’ cells, CD27+IgM+IgD+ ‘natural effector’ B cells are present in patients with CD40 or CD40L-deficiency, indicating that at least part of this subset can be generated independently of T cell help.\textsuperscript{17,20-21} Furthermore, natural effector B cells resemble splenic marginal zone B cells and have a limited replication history as compared with GC B cells (both centroblasts and centrocytes) and CD27+IgD-memory B cells.\textsuperscript{17-18}

More recently, CD27- IgG and IgA class-switched B cells have been described.\textsuperscript{22-24} CD27-IgG+ B cells contain fewer SHM in their Ig genes and have increased IgG3 usage as compared with their CD27+ counterparts.\textsuperscript{22-23} Thus, six B cell subsets have been described to contain genetic hallmarks of B cell memory. This raises the question whether all these subsets show functional characteristics of memory B cells \textsuperscript{25} and whether the phenotypic diversity reflects functional diversity or an origin from different maturation pathways.

We performed detailed analyses on six phenotypically distinct memory B cell subsets, which all appear to display an activated phenotype and molecular signs of antigen recognition. The comparative analyses of replication history, SHM and CSR profiles of these subsets enabled us to trace their origins to three different germinal center-dependent and – independent maturation pathways.
METHODS

Flow cytometric immunophenotyping and purification of B cell subsets from human peripheral blood, tonsil and colon

All peripheral blood from adult donors, childhood tonsil and colon samples were obtained with informed consent following the Declaration of Helsinki and according to the guidelines of the Medical Ethics Committee of Erasmus MC and the Institutional Review Board of Weill Medical College of Cornell University.

Immunophenotyping and cell sorting details are provided in the supplemental Methods.

Hematoxylin-eosin staining

Up to 30,000 cells from each sorted population were applied to poly-L-lysine coated slides and stained with Diff-Quik® Staining Set (Medion Diagnostics). Pictures were acquired on the Axiovert 100 microscope (Zeiss) and the original magnification was 63x.

CD40L-deficient patients

All five CD40L-deficient patients lacked expression of CD40L protein on activated T cells as shown after 5 hour stimulation with PMA (Sigma) and Calcium Ionophore (Sigma). Mutations were detected by exon sequencing of the CD40L gene. Details of the patients are shown in supplemental Table 3.

Sequence analysis of complete IGH gene rearrangements and Ig switch regions

DNA was isolated from each sorted subset with the GenElute Mammalian Total DNA Miniprep Kit and RNA was isolated from Ig-class switched B cell subsets using the GeneElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Complete IGH gene
rearrangements and hybrid switch regions were amplified and analyzed as described in Supplemental Methods.

**Replication history analysis using the KREC assay**

The replication history of sorted B cell subsets was determined with the Kappa-deleting Recombination Excision Circles (KREC) assay as described previously. Briefly, the amounts of coding and signal joints of the *IGK*-deleting rearrangement were measured by RQ-PCR in DNA from sorted B cell populations on an ABI Prism 7000 (Applied Biosystems). Signal joints, but not coding joints are diluted two-fold with every cell division. To measure the number of cell divisions undergone by each population, we calculated the ratio between the number of coding joints and signal joints. The previously established control cell line U698 DB01 (InVivoScribe) contains one coding and one signal joint per genome and was used to correct for minor differences in efficiency of both RQ-PCR assays.

**IgκREHMA**

The frequency of mutated *IGK* alleles was determined with the Igκ restriction enzyme hot-spot mutation assay (IgκREHMA) as described previously. Briefly, PCR was performed on genomic DNA using a HEX-coupled IGKV3-20 intron forward primer and two FAM-coupled IGKJ reverse primers recognizing all five IGKJ gene segments. The PCR products were digested by the KpnI and Fnu4HI restriction enzymes and run on the ABI Prism 3130 XL. Fnu4HI recognizes two adjacent sites in the unmutated gene product in the hot-spot region of IGKV-CDR1. Unmutated gene products can therefore be visualized as 244 or 247-bp HEX-coupled fragments. KpnI cuts the gene product in FR2 downstream of the Fnu4HI sites, resulting in a 262-bp HEX-coupled mutated fragment. The unmutated B cell line CLL-1

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was used as a positive control for complete digestion with Fnu4HI. The digests hardly contained undigested gene products of 481bp, indicating complete digestion by KpnI.

**Statistical analyses**

Statistical analyses were performed with the Mann-Whitney U test, or $X^2$ test as indicated in details in Figure legends. $p$ values < 0.05 were considered statistically significant.
RESULTS

Phenotypic characterization of memory B cell subsets in healthy individuals

To study the diversity in the human B cell compartment, we defined and purified two naive and six memory B cell subsets (Figure 1A). Within the CD19+ B cell compartment, we defined CD38$^{hi}$CD24$^{hi}$ transitional B cells. CD38$^{dim}$CD24$^{dim}$ B cells were subdivided based on the expression of IgM and CD27. Naive mature B cells were defined as CD27-IgM+. CD27+IgM+ B cells were separated into IgD+ ‘natural effector’ B cells and IgD- ‘IgM-only’ B cells. Finally, IgM-negative B cells were separated into 4 class-switched B cell populations based on the expression of IgA, IgG and CD27.

All eight purified subsets had a typical lymphocytic morphology with a large nucleus and little cytoplasm as observed after hematoxylin-eosin staining (Figure 1B). Furthermore, all six memory B cell subsets showed an immunophenotype that was characteristic for activated cells; with increased expression of the B7 family member CD80, TLR-related CD180 and TNF receptor superfamily member TACI as compared with naive B cell subsets (Figure 1C). Additionally, all B cell subsets highly expressed BAFFR, and all memory B cell subsets showed bimodal expression of inhibitory collagen receptor CD305 and were dimly positive for CD95 (data not shown). Thus, all 6 subsets we studied had the phenotype that was reported to be important for fast and powerful memory responses.

Ig repertoire selection in memory B cell subsets

To study whether the memory B cell subsets showed molecular signs of antibody selection, we sequenced IGH gene rearrangements from sorted fractions of healthy adult donors and compared these with naive B cell subsets from adult blood as well as with GC B cells from childhood tonsils. In the detailed gene segment usage analysis we focused on two most
commonly used IGHV subgroups: IGHV3 and IGHV4. All subsets showed diverse usage of IGHV3 subgroup genes with IGHV3-23, IGHV3-21 and IGHV3-30 predominating (Figure 2A). Naive mature B cells showed dominant usage of the IGHV4-34 and IGHV4-59 genes (Figure 2B), likely resulting from increased recombination frequency due to highly efficient recombination signal sequences. Importantly, IGHV4-34 was hardly used in memory B cell subsets, indicating selection against this inherently autoreactive gene.

Of the three complementarity determining regions (CDR), the VDJ-junction encoded CDR3 region is the most dominant in establishment of antigen binding specificity. Long IGH-CDR3s are associated with auto- and polyreactivity. We observed diverse IGH-CDR3 sizes in transitional and naive mature B cells with a median of 17 amino acids (Figure 2C). The median size was slightly reduced to 16 in both centroblasts and centrocytes. All memory B cell subsets had significantly (p<0.05) shorter IGH-CDR3s (median of 14-15 amino acids) as compared with naive mature B cells. Thus, all six memory B cell subsets showed comparable signs of Ig repertoire selection.

**Distinct degrees of replication history and SHM in memory B cell subsets**

Typical hallmarks of memory B cells are extensive antigen-induced proliferation and SHM. We previously showed that GC B cells in tonsils from young children have undergone ~8 cell cycles, by calculating the ratio between genomic coding joints and signal joints on kappa-deleting recombination excision circles (KREC) of the IGK-deleting rearrangement. This replication history was similar in childhood CD27+IgD- B cells, but clearly higher in adulthood CD27+IgD- cells, probably due to consecutive GC reactions. Proliferation of GC B cells was accompanied by SHM of their Ig loci and further enrichment of mutated IGKV3-20 alleles in memory B cells, both in children and adults. We quantified the replication history, frequency of mutated nucleotides in rearranged IGHV genes and the frequency of
mutated IGKV3-20 alleles in naive B cells and all six memory B cell subsets. As shown before, transitional B cells did not undergo proliferation since their release from bone marrow, whereas naive mature B cells underwent ~2 cell cycles in absence of SHM (Figure 3). Conventional adult CD27+IgG+ and CD27+IgA+ B cells underwent the highest number of cell divisions (~10) with high levels of SHM. Both proliferation and SHM levels were clearly higher than in GC B cells from childhood tonsils. This might suggest additional proliferation and mutation in consecutive GC reactions.

IgM-only and CD27-IgG+ B cells underwent ~9 cell divisions, had similar SHM levels in rearranged IGHV genes as GC B cells, but increased frequencies of mutated IGKV3-20 alleles. The characteristics of both subsets suggest an origin from primary GC responses followed by selection for mutated IGKV3-20.

Finally, natural effector and CD27-IgA+ B cell subsets showed less proliferation as compared with GC B cells (Figure 3A). Natural effector B cells showed only 7 cell cycles, whereas the IGHV mutation loads were similar to GC B cells and these cells were enriched for mutated IGKV3-20 alleles. These proliferation and SHM levels were clearly higher than those observed for natural effector cells in childhood tonsil. Still, these results indicate that a substantial fraction of this population had been generated independently from a GC. Finally, we observed only 4 cell divisions for CD27-IgA+ B cells. Interestingly, the IGHV gene mutation loads were increased as compared with GC B cells, whereas the frequency of mutated IGKV3-20 alleles was similar. These results indicate a GC-independent origin of CD27-IgA+ B cells, but with high AID activity generating high SHM levels and IgA class-switching. Still, these cells lacked selection for mutated IGKV3-20 alleles.
**Targeting and selection of SHM in rearranged IGHV genes**

We analyzed type and targeting of SHM in the memory B cell subsets to obtain insight into the activity of AID, POLζ and UNG. Neither the SHM targeting nor the nucleotide substitution spectra and transition/transversion ratios were significantly different between the memory B cell subsets and centrocytes (supplemental Table 1 and supplemental Figure 1). Furthermore, the targeting of specific nucleotides in motifs was largely similar between subsets (supplemental Table 2). Thus, we conclude that the differences in mutation frequencies did not result from altered AID, UNG and Polζ activities, rather they likely reflect the duration of exposure to these enzymes.

Generally, a high ratio of replacement versus silent mutations (R/S) in IGHV-CDRs is regarded as a molecular sign of affinity maturation. Nevertheless a clear cut-off value, which would reflect antigenic selection, remains difficult or even impossible to define.36 We found accumulation of replacement mutations in CDR1 and CDR2 of rearranged IGHV genes in all analyzed subsets (supplemental Figure 2). IGHV-CDR R/S ratios were similar between all memory B cell subsets ranging between 3.3 and 4.0, except for CD27-IgG+ B cells which had a slightly lower IGHV-CDR R/S ratio of 2.3 (supplemental Table 1).

Alignment of rearranged IGHV genes revealed the existence of recurrent amino acid changes (i.e., the same amino acid replacement at the same position) in all except the natural effector, transitional and naive mature B cell subsets. In centrocytes, we identified a cluster of five sequences with identical VDJ gene usage and closely similar if not identical IGH-CDR3s (always of identical length), pointing to their common ancestry. In addition to recurrent mutations, the sequences exhibited a different number and distribution of SHM, indicating that the process of antigen-driven clonal expansion was also accompanied by intraclonal diversification.
IgG and IgA subclass distribution in class-switched memory B cell subsets

In addition to differential CD27 expression, both IgG+ and both IgA+ memory B cell subsets varied in their replication history and SHM levels (Figure 3). This suggests different origins and functions for the CD27+ and CD27- B cell subsets. Since the constant region of an antibody molecule is important for its function and the human IGH locus contains 4 IGHG and 2 IGHA constant genes (Figure 4A), we studied the Ig subclass usage in sequenced IGH transcripts. We found a dominant use of IGHG2 (51%) and IGHG1 (40%), and low IGHG3 and IGHG4 in CD27+IgG+ cells (Figure 4B). In contrast, CD27-IgG+ cells showed a dominant use of IGHG1 (63%) and IGHG3 (31%) with little IGHG2 and no IGHG4. Thus, the CD27-IgG+ cells showed a dominant use of IGHM-proximal IGHG3 and IGHG1 regions (94%), whereas this was reduced to only 47% in CD27+IgG+ cells (P<0.0001). Ig CSR to distal constant genes can occur indirectly via an IGHM-proximal gene. Analysis of hybrid switch regions (Sµ-Sγ2) in genomic DNA of sorted populations indeed revealed that 24% of junctions had remnants of Sγ3 or Sγ1, whereas this was only 9% for Sµ-Sγ1 junctions (Figure 4C,D). Furthermore, the IGHV genes in IGHG2 and IGHG4 transcripts contained higher SHM loads than IGHG1 and IGHG3 (supplemental Figure 3A). The (indirect) switching to downstream IGHG genes accompanied by increased SHM frequencies suggest more prolonged AID activity in CD27+IgG+ cells, potentially reflecting multiple GC reactions.

The IgA+ memory B cell subsets also showed differential subclass usage: CD27-IgA+ memory B cells contained significantly more IGHA2 transcripts (33%) than CD27+IgA+ memory B cells (19%; Figure 4B) (P<0.05). Even though IGHA2 is the most downstream constant gene in the human IGH locus (Figure 4A), only 4% of hybrid Sµ-Sα2 regions contained remnants of more proximal S regions (Figure 4C,D), suggesting that most of the switching towards IGHA2 occurred directly from Sµ. No evidence for indirect class
switching was found in IGHM-IGHA1 switch regions. Furthermore, there was no difference in the mutation frequencies between IGHA1 and IGHA2 transcripts (supplemental Figure 3B). These results imply that switching towards IGHA2 occurs mainly directly from Sμ and the molecular differences between CD27+IgA+ and CD27-IgA+ memory B cells most likely reflect their generation via separate response pathways, rather than consecutive GC reactions as observed for CD27-IgG+ versus CD27+IgG+ memory B cells.

T cell-independent generation of B cell memory in CD40L-deficient patients

Replication history analyses indicated a GC-independent origin of natural effector and CD27-IgA+ B cells. To demonstrate that these subsets can be generated in the absence of the T cell help, we analyzed their presence in five CD40L-deficient patients (supplemental Table 3). We found a clear population of natural effector B cells in CD40L deficient patients, confirming previous observations that at least part of the blood natural effector B cell population can be generated independently from T cell help.\textsuperscript{17,20-21,38} Still, this subset was approximately three times reduced in number as compared to age-matched healthy controls (Figure 5), highlighting the fact that in healthy controls a major part of this subset has a germinal center origin. More importantly, blood of CD40L-deficient patients also contained CD27-IgA+ memory B cells and their numbers were similar as compared to healthy controls (Figure 5). Thus, in addition to natural effector cells, T cell-independent humoral responses in human can generate IgA-class switched memory B cells.

CD27-IgA+ memory B cells resemble colon lamina propria IgA+ B cells

T cell-independent responses have been demonstrated to generate IgA-producing B cells in the lamina propria of human gut.\textsuperscript{39-40} Furthermore, these IgA+ B cells showed predominant use of IGHA2.\textsuperscript{41} These similarities with blood CD27-IgA+ memory B cells encouraged us to
study whether these cells had been generated in similar responses. First, we analyzed the replication history of IgA2+ B cells isolated from colon lamina propria. Similar to CD27-IgA+ B cells, these cells had proliferated less than GC B cells in childhood tonsils and significantly less than GC-derived CD27+IgA+ memory B cells in adult blood (Figure 6A). Additionally, as it was previously suggested that a broad Igλ repertoire may be beneficial for responses in the human gastrointestinal tract, we analyzed the κ/λ light chain isotype ratios of blood B cell subsets by flow cytometry. We found a high frequency of Igλ+ cells (80%) within the CD27-IgA+ B cell subset as compared to both CD27+IgA+ cells (55%) and naive mature B cells (45%; Figure 6B). Sequence analysis of IGLV-IGLJ rearrangements revealed fewer mutations in CD27-IgA+ than in CD27+IgA+ memory B cells, despite similar IGLV and IGLJ gene usage, and IGL-CDR3 size and composition (Figure 6C and supplemental Table 4). The molecular similarities between CD27-IgA+ B cells and gut lamina propria IgA-producing B cells suggest a common origin of these cells from local responses in the gastrointestinal tract.

**Model of memory B cell generation from three distinct pathways**

Here we demonstrate by molecular analysis of Ig genes that six distinct memory B cell subsets can be identified based on their IgH isotype and expression of CD27. To recapitulate our findings, we propose a modified scheme of memory B cell generation (Figure 7): CD27-IgA+ B cells and natural effector B cells (at least in part) are derived from local and systemic GC-independent responses, respectively; CD27-IgG+ and CD27+IgM+ B cells are derived from primary GC, and CD27+IgG+ and CD27+IgA+ B cells (at least in part) from secondary GC responses.
DISCUSSION

In this study, we set out to relate distinct memory B cell subsets to the diverse humoral response types that have been documented in the literature. We defined 6 memory B cell subsets with phenotypic and molecular signs of antigen encounter. Detailed comparative analysis of their Ig genes, comparison with tissue-derived B cell subsets, and analysis of memory B cell subsets in CD40L-deficient patients allowed us to distinguish three unique maturation pathways: GC-independent local and systemic responses, and GC-dependent responses. Furthermore, we delineated primary and consecutive phases of GC responses.

The CD27+IgA+ and CD27+IgG+ subsets are generally regarded as true B cell memory.25 Whereas this qualification is somewhat controversial for CD27+IgM+ subsets and CD27- class-switched subsets, our results strongly support these to be true memory B cells based on the: 1) high expression of activation and co-stimulatory molecules; 2) selection against inherently autoreactive VH domain characteristics; 3) extensive replication history as compared with naive B cells; 4) SHM profiles of Ig heavy and light variable genes with high R/S ratios in VH-CDRs. Despite these common features of B cell memory, we found clear quantitative differences in proliferation, SHM and CSR processes between these subsets. We conclude that these differences reflect different origins and maturation pathways prior to becoming memory B cells. Consequently, these differences justify dividing the memory B cell compartment into subsets.

Of the six memory B cell subsets, the CD27+IgG+ and CD27+IgA+ B cells had the highest degrees of proliferation and SHM. Interestingly, these levels were higher than those of GC B cells from childhood tonsil. We previously observed increased proliferation and SHM in CD27+IgD- cells from adults as compared to children and concluded that in adults at least part of these cells had undergone additional immune responses upon secondary or tertiary antigen encounter.18 Our current results showed similar additional proliferation and
SHM for both CD27+IgA+ and CD27+IgG+ B cells. Furthermore, the increased proliferation was accompanied by increased usage of distally located *IGHG2* and *IGHG4* genes with signs of indirect CSR. Thus, these results support the concept that at least part of the CD27+IgA+ and CD27+IgG+ B cell subsets in healthy adults has undergone multiple immune responses.

Interestingly, the *IGHV* gene mutation frequency was clearly higher in CD27+IgA+ as compared with CD27+IgG+ B cells. Since the targeting of mutations was similar, AID and UNG activities seemed unaffected. Rather, CD27+IgA+ B cells might have experienced prolonged AID and UNG activities. Since IgA class-switching mostly occurs in mucosa-associated lymphoid tissues, this difference might reflect the location of the immune response. Still, despite these higher mutation loads, we found no differences in replacement mutation patterns in *IGHV* genes or the frequency of mutated *IGK* alleles, suggesting similar selection mechanisms for both CD27+IgA+ and CD27+IgG+ B cells.

We conclude that IgM-only and CD27-IgG+ B cells are derived from primary GC responses. This was based on their highly similar replication history and *IGHV* gene mutation loads as compared to GC B cells from childhood tonsil, and is further supported by the dominant usage (>90%) of the *IGHM*-proximal *IGHG1* and *IGHG3* genes in CD27-IgG+ B cells. In contrast to *IGHV* gene mutation loads, the frequencies of mutated *IGKV* alleles were increased in both subsets as compared with GC B cells. We previously found this increased frequency in tonsillar CD27+IgD- memory B cells. Since this occurred in the absence of additional proliferation, it likely reflects positive selection for the mutated hotspot in memory B cells rather than additional mutations.

IgM responses are initiated early in primary infection. Dogan et al. described that following primary immunization of mice, IgM+ memory B cells were formed that upon secondary challenge could class-switch towards IgG1+ cells. Furthermore, clonally related IgM+ and IgG+ B cells were found in human GCs and peripheral blood. Thus, as
compared with CD27+IgA+ and CD27+IgG+ memory B cells, CD27+IgM+ memory B cells are early GC-emigrants that did not undergo class-switching.\textsuperscript{45} Still, two issues have hampered proper studies on IgM+ memory B cells in recent years. Firstly, the CD27+IgM+IgD- and CD27+IgM+IgD+ subsets have not always been separated, despite evidence that only the CD27+IgM+IgD+ subset contains cells that have been generated independent from GC (Figure 5B).\textsuperscript{17,21} Secondly, often the CD27+IgD- population is not further subdivided. As a consequence, this is a mixed population of Ig-class switched and IgM+ memory B cells. Our results demonstrate that this has no major implications, since these subsets all seem GC-dependent. However, it should be noted that the CD27+IgD-subsets contain a substantial fraction of IgM+ memory B cells, particularly in young children, and therefore it should be avoided to address these as “Ig-class switched memory”.

The low SHM loads in CD27-IgG+ B cells as compared with CD27+IgG+ B cells have lead to speculations on the origin of these cells: from T cell-independent responses or first wave from a GC reaction.\textsuperscript{22-23} We found that the replication history and SHM levels of CD27-IgG+ B cells highly resemble GC B cells. Furthermore, CD27-IgG+ B cells were hardly detectable in CD40L-deficient patients and they have dominant use of IgM-proximal IgG3 and IgG1 subclasses. Thus, we conclude that, similar to CD27+IgM+ cells, CD27-IgG+ cells are derived from primary GC-dependent responses.

Several studies have shown an expansion of both CD27+IgM+IgD- and CD27-IgG+ memory B cells in autoimmune diseases.\textsuperscript{17,22-23} Interestingly, CD27-IgG+ B cells dominantly use IgG1 and IgG3, which are potent activators of the complement system and inducers of antibody-dependent cell-mediated cytotoxicity.\textsuperscript{46} Thus, our observations of the different IgG subclass usage in CD27+IgG+ versus CD27-IgG+ B cells suggest a potential role of CD27-IgG+ cells in autoimmunity. Still, additional studies need to address whether many CD27-
IgG+ B cells carry an autoreactive BCR or whether other mechanisms result in deregulation of CD27-IgG+ B cells in patients with an autoimmune disease.

In contrast to the other memory B cell subsets, natural effector and CD27-IgA+ B cells showed limited proliferation as compared with GC B cells and were present in CD40L-deficient patients. Thus, we concluded that these cells can be generated independent from T cell help. It is debated whether CD27+IgM+IgD+ natural effector B cells in healthy adults are generated from germinal center responses or independently of T cell help in the splenic marginal zone.\textsuperscript{17,19-21} We describe reduced replication history and SHM levels in natural effector B cells as compared to IgM-only memory B cells. Since IgM-only memory B cells highly resemble germinal center B cells on the molecular level, we conclude that in healthy adults part of the natural effector B cells can be generated outside of a germinal center. Thus, the natural effector B cell subset in healthy individuals is likely a mixed population of GC-derived and splenic marginal zone-derived memory B cells. Interestingly, a recent study described the presence of CD27+CD43+CD20+ B1 cells in umbilical cord blood and in adult peripheral blood.\textsuperscript{47} It is possible that the T cell-independent characteristics ascribed to CD27+IgM+IgD+ natural effector B cells are specific for the CD43+ fraction. This should be further investigated.

The CD27-IgA+ memory B cell subset was the smallest population we studied and, to our knowledge, we showed for the first time that these cells can be derived independent from T cell help. TI IgA responses have been observed both in human and mouse, both systemically in the splenic marginal zone and locally in the gastrointestinal system.\textsuperscript{40,48-49} Potential mediators of CD40-independent IgA CSR are BAFF and APRIL.\textsuperscript{39} Since blood CD27-IgA+ B cells and gut lamina propria IgA-producing B cells were highly similar in their limited replication history, and dominant IgA2 and \(\lambda\) light chain isotypes, we conclude that these cells have been generated in similar responses. Although the anatomic location of TI
CSR towards IgA in human gut remains controversial,\textsuperscript{40,50} on the basis of our findings, we can state that CD27-IgA+ memory B cells resemble IgA+ cells from the gut lamina propria and seem to be a blood counterpart of this IgA-producing population. Even though analysis of the memory B cell compartment showed that CD27-IgA+ B cells seem completely TI, we cannot exclude that in physiological conditions a minor fraction of CD27-IgA+ B cells is generated in a primary immune response analogous to CD27-IgG+ B cells.

The human memory B cell compartment is more complex than originally thought and actually consists of diverse subsets that have originated from functionally distinct responses. Interestingly, differential expression of CD27 was the key to identification of the diverse subsets. The function of CD27 on B cells remains unclear. CD27-CD70 interactions can trigger plasma cell differentiation and provide negative feedback signals. Thus CD27+ and CD27- memory B cells might function differently. Still, the similar upregulation of many other co-stimulatory molecules on these cells might compensate for the lack of CD27. Alternatively, the differential CD27 expression might reflect the different types of responses in which the cells have been generated and thus represents a useful marker to discriminate between these subsets.

Different levels of memory B cell responses seem to reflect the phylogenetic evolution of the immune system from local TI responses, via systemic TI responses to most advanced TD responses in the GC. These different origins suggest unique physiological functions in protection against pathogens.

In this study we dissected the human memory B cell compartment into 6 distinct subsets. Molecular analysis of these memory B cells in healthy controls, and comparison with memory B cells from CD40L-deficient patients and colon lamina propria B cells enabled us to delineate their origin from three different maturation pathways: local and systemic T cell-independent responses, and primary or secondary GC responses. Since these B cell subsets
are present in blood, our studies provide new opportunities to analyze these processes in patients with (auto)inflammatory conditions, B cell immunodeficiencies, nodal and extranodal B cell malignancies.
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FIGURE LEGENDS (7)

Figure 1. Isolation and phenotypic characterization of peripheral blood memory B cell subsets. (A) Gating strategy to identify 2 naive and 6 memory B cell subsets based on expression of CD24, CD38, CD27 and IgH isotypes. (B) Hematoxylin-eosin staining of sorted subsets revealed a typical lymphocytic morphology with large nucleus (purple) and little cytoplasm (pink) (63x magnification; bars, 5μm). (C) All 6 memory B cell subsets showed upregulation of CD80, CD180 and TACI as compared with naive B cells. Expression levels are shown in black and isotype controls as filled, grey histograms. Red lines indicate mode expression levels for each molecule on naive mature B cells.

Figure 2. Selection against the IGHV4-34 gene and long IGH-CDR3s in all six memory B cell subsets. (A) Frequencies of the most commonly used IGHV3 genes in cloned IGH gene rearrangements. Differences between each memory B cell subset as compared with naive mature B cells were statistically analyzed with the X2 test. (B) Frequencies of the most commonly used IGHV4 genes in cloned IGH gene rearrangements. An arrow indicates IGHV4-34 gene usage in naive mature B cells. Differences between each memory B cell subset as compared with naive mature B cells were statistically analyzed with the X2 test. *, p<0.05; **, p<0.01. (C) IGH-CDR3 size distributions. All individual sizes are indicated for each subset as grey dots with red lines representing the median values. The dashed and dotted lines represent median values for centroblasts (n=67) and centrocytes (n=55), respectively. Differences between each memory B cell subset as compared with naive mature B cells were statistically analyzed with the Mann-Whitney test. **, p<0.01; ***, p<0.001.

Figure 3. Discrimination of GC-dependent and -independent B cell maturation pathways based on quantitative analysis of the replication history and SHM levels. (A)
Replication history of 2 naive and 6 memory B cell subsets as measured with the KREC assay. Three different levels of extensive proliferation in memory B cell subsets in contrast to naive B cells (blue) could be identified: lower than GC (yellow bars), similar to GC (orange bars) and increased as compared with GC (red bars). Bars represent mean values with SEM. In the whole figure dashed and dotted lines represent values for centroblasts and centrocytes, respectively. Differences between each memory B cell subset as compared with centrocytes were statistically analyzed with the Mann-Whitney test. (B) Frequency of mutated nucleotides in rearranged *IGHV* genes. All individual data points are shown as grey dots with red lines indicating the median value. Differences between each memory B cell subset as compared with centrocytes were statistically analyzed with the Mann-Whitney test. ***, p<0.001. (C) Frequency of mutated *IGKV3-20* genes as measured with the IgκREHMA assay. Bars represent mean values with SEM. Differences between each memory B cell subset as compared with centrocytes were statistically analyzed with the Mann-Whitney test. *, p<0.05.

**Figure 4. Molecular analysis of Ig class switching in IgA+ and IgG+ memory B cell subsets.** (A) Schematic representation of the constant region of the human *IGH* locus. (B) Distribution of IgA and IgG receptor subclasses usage in *IGH* rearrangements of class-switched memory B cell subsets. Total number of analyzed sequences is indicated in the center of each plot. Differences in the distribution were statistically analyzed with the $X^2$ test and were found significant for both CD27+IgG+ vs CD27-IgG+ (P<0.0001) and CD27+IgA+ vs CD27-IgA+ (P<0.05) B cell subsets. (C) Frequency of Sμ-Sα and Sμ-Sγ rearrangements bearing remnants of indirect class-switching. Number of analyzed sequences is given in brackets. (D) Examples of direct and sequential class switching; a piece of Sγ3 sequence in the Sμ-Sγ2 junction is indicated boxed in red font.
Figure 5. Germinal center-independent generation of natural effector and CD27-IgA+ memory B cells. (A) Memory B cell subset distribution was analyzed in 5 CD40L-deficient patients (age 1-13yr) and 50 healthy controls (age 1-5yr). Representative FACS plots of B cell subsets. (B) Absolute cell numbers of 6 memory B cell subsets. Bars represent mean values with SEM. Statistical significance was calculated with the Mann-Whitney test. *, p < 0.05; **, p < 0.01, ***, p < 0.001.

Figure 6. CD27-IgA+ memory B cells resemble colon lamina propria IgA+ B cells. (A) Replication history in naive mature, IgA+ memory B cell subsets and CD19+IgA2+ B cells isolated from human colon lamina propria as measured with the KREC assay. Bars represent mean values with SEM. Statistical significance was calculated with the Mann-Whitney test. (B) Igκ and Igλ isotype distribution of naive mature and IgA+ memory B cell subsets as determined with flow cytometric analysis. (C) Frequency of mutated nucleotides in rearranged IGLV gene segments. All individual data points are showed as grey dots with red lines indicating the median value. Statistical significance was calculated with the Mann-Whitney test. **, p < 0.01.

Figure 7. Model of human memory B cell generation from germinal center-dependent and -independent pathways. Six purified memory B cell subsets showed differential levels of proliferation and BCR maturation. Ig class switching profiles and immunophenotyping of blood of CD40L deficient patients supported delineation of these six subsets from T cell dependent and –independent maturation pathways. CD27-IgA+ and natural effector B cells can be derived independently from T cell help, most likely locally in the gastrointestinal tract and from systemic responses in splenic marginal zone, respectively. The molecular profiles
of CD27-IgG+ and CD27+IgM+ memory B cells resembled those of primary GC cells, whereas CD27+IgG+ and CD27+IgA+ memory B cells has increased proliferation and SHM levels suggestive of further maturation in consecutive GC response.
Figure 2
Figure 3
Figure 4
CD40L deficiency

**Figure 5**
**Figure 6**
Figure 7

TI responses in gut

marginal zone in spleen

germinal center

transitional

naive mature

proliferation

BCR maturation

CD27-IgA+

natural effector

CD27-IgG+

CD27+IgM+

CD27+IgA+

CD27+IgG+

CD27+IgM+
Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways

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