HLA-specific memory B cells : the missing link?

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Chapter 4

Polyclonal B cell activation for accurate analysis of pre-existing antigen-specific memory B cells

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ABSTRACT

The enzyme-linked immunospot (ELISPOT) assay is a widely used tool for enumeration of antigen-specific memory B cells in several disciplines such as vaccination, cancer immunotherapy and transplantation. For the accurate estimation of antigen-specific memory B cell frequencies, a well-defined B cell activation protocol is pivotal. Here, we aimed to characterize a polyclonal B cell activation protocol to facilitate optimal monitoring of antigen-specific memory B cell frequencies. Total, naïve and memory B cells were polyclonally activated with an α-CD40 monoclonal antibody, cytosine-phosphate-guanine (CpG) oligodeoxynucleotide (ODN) 2006, interleukin (IL)-2, IL-10 and IL-21. Polyclonal activation of B cells resulted in equal cell death ratios in naïve and memory B cells. When tested in an antigen-specific system, immunoglobulin (Ig)G spots were only detected in the memory fraction. There was no change in B cell polyclonality due to *in vitro* activation. Our data show that the current polyclonal activation protocol may be used reliably to estimate the frequency of memory B cells in ELISPOT assays.

INTRODUCTION

The extent of humoral immune responses can be characterized by quantifying the antigen-specific memory compartment using *in vitro* assays. Quantification of the humoral immune response is of particular significance in many disciplines such as vaccination, cancer immunotherapy and transplantation. In the field of vaccination, it is important to characterize the normal immune response to a pathogen as well as to monitor the protective response elicited by vaccination in means of immunological memory (1, 2). Measuring the memory B cell response is crucial to evaluate the efficacy of the vaccine and eventually to identify the risk groups that will not benefit from the vaccine in infectious diseases (3-5) or cancer immunotherapy (6).

In solid organ transplantation, detecting and quantifying memory B cells capable of producing donor-directed anti-human leukocyte antigen (HLA) antibodies in a patient will potentially aid in defining the post-transplant immunological risk (7). Currently available methods detecting anti-HLA antibodies in the serum do not provide any information on the magnitude of the memory response.

Quantification of humoral immune response in sensitized individuals by detection of HLA-specific B cells has previously been done by us and others (8, 9). However, there are only a few studies aiming at the detection and enumeration of the relatively low levels of HLA-specific memory B cells (10, 11). Our group has recently developed an HLA specific B cell enzyme-linked immunospot (ELISPOT) assay which allows for the quantification of memory B cell frequencies directed towards defined HLA molecules (11). This technique was recently adapted by Lynch and colleagues to detect B cell memory towards donor-specific HLA class I on cultured fibroblasts from donor origin (12).

Both naïve and memory B cells can differentiate into antibody secreting cells (ASC) upon antigen-specific stimulation (2). *In vitro*, polyclonal activation can result in the antigen-independent differentiation of B cells into ASC (13, 14). There are several protocols for the polyclonal activation of human B cells most of which favour isotype switching or plasma cell differentiation of naïve B cells, particularly after long-term cultures (15-19). However, to estimate the actual frequency of antigen-specific memory B cells in a patient, an activation protocol that does not induce IgG production in naïve B cell population is preferred. In the present study, we aimed to determine the kinetics of *in vitro* human B cell activation upon a previously defined activation protocol (20, 21). Here, we report the distinct proliferation kinetics and antibody production patterns of naïve and memory B cells and show that the current polyclonal B cell activation protocol can be used for specifically enumerating memory B cell frequencies using techniques such as the ELISPOT assay.

MATERIALS AND METHODS

Peripheral blood B cell isolation

Peripheral blood was obtained with informed consent from blood bank donors under guidelines issued by the Medical Ethics Committee of the Leiden University Medical Center (Leiden, the Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Hypaque (Pharmacy LUMC, Leiden, the Netherlands) density gradient centrifugation and frozen in liquid nitrogen until further use. After thawing, B cells were isolated by negative selection using the EasySep Human B cell enrichment kit (Stem Cell Technologies, Grenoble, France) according to the manufacturer`s instructions. The purity of B cells was found to be >98% as assessed by CD19 positivity measured by flow cytometry (FCM).

Naïve and memory B cell separation

For some experiments, isolated total B cells were further sorted on fluorescence activated cell sorter (FACS) AriaII (BD Biosciences, Breda, the Netherlands) into $CD3^-CD19^+IgD^+CD27^-$ naïve and $CD3^-CD19^+IgD^-CD27^+$ memory B cells using the following monoclonal antibodies (clone): CD3-Pacific Blue (UCHT1), CD19-allophycocyanin (APC-cyanin 7 (Cy7) (SJ25C1), IgD-phycoerythrin (PE) (IA6-2; all from BD) and CD27- fluorescein isothiocyanate (FITC) (CLB-CD27/1, 9F-4; Sanquin, Amsterdam, the Netherlands). Cell sorting purity for both fractions was more than 95% after the sorting.

Cell cultures

Cell cultures were carried out in Iscove's modified Dulbecco`s medium (IMDM) (Gibco Invitrogen, Paisley, UK) containing 10% fetal calf serum (FCS) (Gibco Invitrogen), supplemented with 50 µM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands), 2 mM L-glutamine (Gibco Invitrogen), ITS (5 µg/ml insulin, 5 µg/ml transferrin, sodium selenite 5 ng/ml, (Sigma-Aldrich) and 100 U/ml penicillin with 100 µg/ml streptomycin (Gibco Invitrogen). Cells were activated with an activation cocktail consisting of 500 ng/ml α-CD40 monoclonal antibody (R&D systems, Minneapolis, MN, USA), 2.5 µg/ml Toll-like receptor-9 (TLR-9) ligand oligodeoxynucleotides (ODN)-2006 cytosine-phosphate-guanine (CpG) (Hycult Biotechnology, Uden, the Netherlands), 600 IU/ml interleukin (IL)-2 (Proleukin, Amsterdam, the Netherlands), 25 ng/ml IL-10 (R&D systems), 100 ng/ml IL-21 (Gibco Invitrogen), as described previously (21) and cultured at 37°C in a 5% CO_2 humidified incubator. Total, naïve and memory B cell fractions were cultured separately for various lengths of time by seeding $1x10^4$ cells/well in 96-well U-bottomed plates (BD Falcon, Breda, the Netherlands). In experiments aiming to detect antigen specific responses, B cell fractions were activated at $2.5x10^5$ cells/well in 24-well flat bottomed plates (Corning Incorporated, Corning, NY). Cell viability at each harvest time point was defined by 7-aminoactinomycin D

(7-AAD) exclusion (BD Biosciences) by FCM, as well as eosin staining using light microscopy.

Analysis of activation markers

Total, naïve and memory B cells were monitored for the expression of activation markers before and after polyclonal activation at various time points using the following monoclonal antibodies (clone): CD25-PE-Cy7 (M-A251), (BD Biosciences) and CD69-ECD (TP1.55.3, Beckman Coulter). 7-AAD exclusion and side scatter (SSC) were used to gate on living cells. Cells were acquired on an LSRII flow cytometer (BD Biosciences) and analysed using FlowJo software (v.10, Tree Star Incorporated, Ashland, OR).

Proliferation assay

B cell proliferation was measured by pulsing with 1 µCi tritiated thymidine ([^{3}H]-TdR; Amersham International, Amersham, UK) per well for the last 18 h of each culture time point. [^{3}H]-TdR incorporation was measured using a liquid scintillation counter (Perkin-Elmer, Groningen, the Netherlands). Results are expressed as the mean counts per minute (cpm) of triplicate wells.

ELISPOT assays

Total IgM and IgG-producing cell numbers were measured by ELISPOT assays, as described previously (20). In brief, 96-well ELISPOT plates (Milipore, Billerica, MA, USA) were coated with either goat α-IgM or α-IgG antibodies (both from Jackson Immunoresearch Laboratories, Inc., Baltimore, PA, USA) diluted in phosphate-buffered saline (PBS) and incubated overnight. To detect antigen- specific responses, plates were coated with 1 Lf/ml tetanus toxoid (TT) antigen (Dutch Vaccine Institute, Bilthoven, the Netherlands) diluted in PBS. Plates were blocked for at least 1 hr with 5% FCS/IMDM, after which activated B cells were added in various cell concentrations for 6 h at 37°C in a 5% CO_2 humidified incubator. After washing, biotinylated goat α-IgM and α-IgG antibodies (both from Invitrogen) were added in the appropriate wells and incubated at 4°C overnight. Following washing, plates were incubated with streptavidin-conjugated alkaline phosphatase (Sigma-Aldrich) for 1 h at room temperature. 5-Bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (Mabtech, Nacka Strand, Sweden) was added to visualize the spots. The reaction was stopped by cold tap water and after drying the plates were analysed by an automated ELISPOT reader (AID, Strassberg, Germany).

Complementarity determining region 3 (CDR3) fragment analysis

Non-activated and activated B cells were preserved in RNAlater solution (Qiagen, Venlo, the Netherlands) following isolation and harvesting. Total RNA was extracted by using the NucleoSpin miRNA kit (Bioké, Leiden, the Netherlands), according to manufacturer's

instructions. Complementary DNA synthesis was carried out by using SuperScript III reverse transcriptase (Life Technologies), as previously described (22). Polymerase chain reaction (PCR) assays were performed with each of seven forward primers covering the different VH1-7 chains in combination with a fluorophore-labelled reverse primer specific for the constant region of IgM (μ) or IgG (γ). Primer sequences have been described in a previous paper (23). Amplified fragments were separated and visualized using a 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). Genescan 400HD (ROX) size standard (Applied Biosystems) was used for accurate determination of the size of the DNA fragments. Fragment analysis was performed using Peak Scanner software (version 1; Applied Biosystems).

Statistics

Correlations between the distributions of VH families before and after activation of B cells for IgM and IgG were analysed by Spearman's rank test using Graphpad Prism software (version 6.02). The statistical level of significance was defined as $P<0.05$.

RESULTS

Both naïve and memory B cells are activated upon polylonal activation

In order to assess whether our activation protocol lead to activation of both naïve and memory B cells, we assessed the expression of the activation markers CD69 and CD25 on both subsets, as well as on total B cells. As shown in figure 1, all B cell fractions up-regulated CD69 and CD25 by day 1 of polyclonal activation. The percentage of the early activation marker CD69 decreased to baseline levels by day 2 in the memory fraction, followed by naïve B cells at day 4. The percentage of $CD25^+$ cells remained constantly high in both naïve and memory B cell fractions.

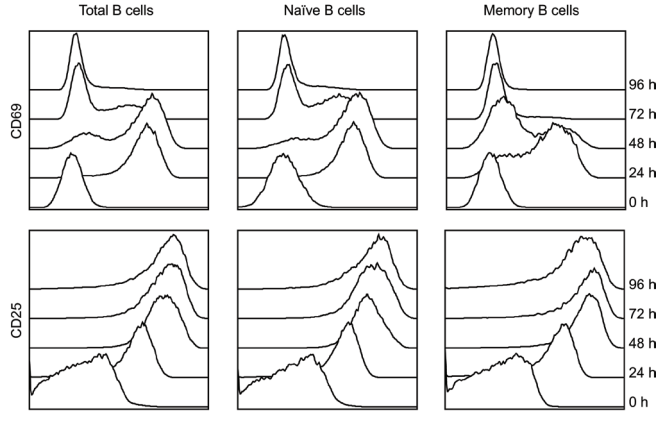


Figure 1. Immunophenotyping for cell surface activation markers. *Total, naïve and memory B cells were monitored for the expression of CD69 and CD25 before (0 h) and several times (24-96 h) after polyclonal activation. All cells up-regulated both activation markers as early as 24 h following activation. Results are representative of 2 experiments with different donors.*

Naïve and memory B cells have similar cell death kinetics upon polyclonal activation

Polyclonal B cell activation is accompanied by a certain degree of cell death (20). We wanted to know whether the induction of cell death by activation is equally distributed among naïve and memory B cells. Upon polyclonal activation, there was no difference in the proportion of dead cells among the total, naïve and memory B cell populations throughout the culture period. In time, the dead : alive cell ratio increased sharply in all fractions after day 6 (Figure 2). We obtained similar results when assessing cell death by eosin exclusion using light microscopy (data not shown).

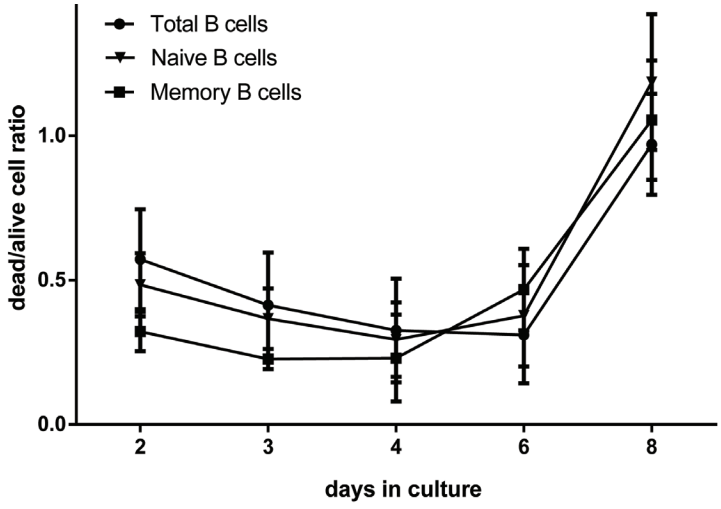


Figure 2. Dead : alive cell ratios among B cell subsets upon polyclonal activation. *Total, naïve and memory B cells were activated polyclonally for varying lengths of time. At each time point, cells were harvested and cell viability was determined by 7-aminoactinomycin D (7-AAD) exclusion by flow cytometry. Dead : alive cell ratios for each time point in all B cell fractions were obtained from percentages of dead (7-AAD positive) and alive (7-AAD negative) cells. Dead : alive cell ratios were similar among total, naïve and memory B cells. Results are expressed as mean ± standard deviation (s.d.) of seven independent experiments performed with a minimum of three and a maximum of seven donors for each time point.*

Naïve B cells proliferate stronger upon polyclonal activation compared to memory B cells

To address the proliferation kinetics of the naïve and memory B cell subsets, an equal number of total, as well as sorted naïve and memory B cells, were polyclonally activated and analysed for proliferation by [^{3}H]-TdR incorporation on consecutive days. Upon polyclonal activation, the total B cell fraction proliferated increasingly up to day 6, after which the proliferation gradually declined (Figure 3, white bars). Similarly, the naïve B cell fraction increasingly proliferated up to day 6, with a steep increase from day 3 to day 4. After day 6, a steep decline in the proliferation was also observed (Figure 3, grey bars). Finally, memory B cells started proliferating earlier upon activation compared to naïve B cells, with the peak proliferation at day 3, after which proliferation gradually declined (Figure 3, black bars). The maximum proliferation of the two B cell fractions at their respective peak proliferation days was 1.96 fold higher for naïve B cells compared to memory B cells.

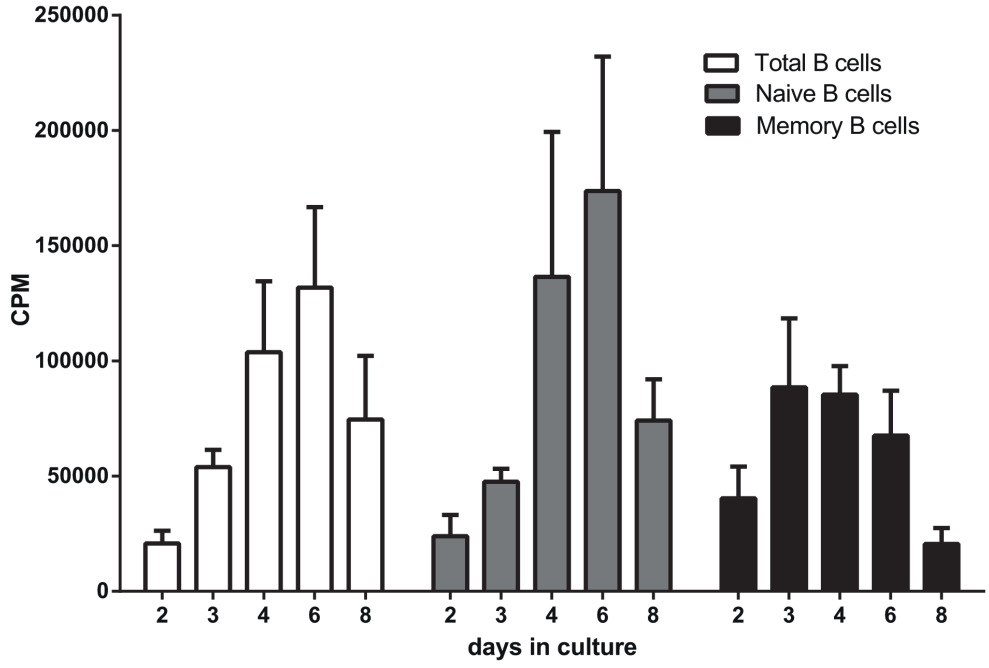
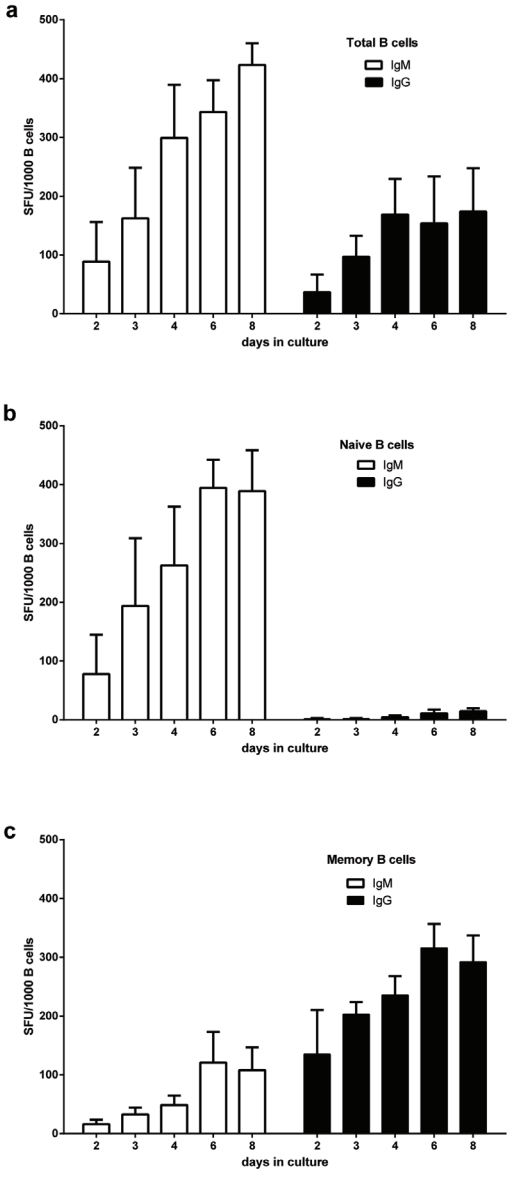


Figure 3. Proliferation kinetics of B cells upon polyclonal activation. *Total, naïve and memory B cells were activated polyclonally for varying lengths of time. Cell proliferation for each fraction was measured by tritiated thymidine ([^{3}H]-TdR) incorporation. Results are expressed as mean counts per minute (cpm) ± standard deviation (s.d.) of seven independent experiments performed with a minimum of three and a maximum of seven donors for each time point. Total B cells (white bars); naïve B cells (grey bars); memory B cells (black bars).*

The frequency estimation of antigen-specific memory B cells in unseparated B cells is not affected by IgG induction in the naïve B cell compartment

Next, we wanted to address the kinetics of antibody production, and whether polyclonal activation induced any IgG-producing cells from the naïve B cell population. The kinetics of antibody production was comparable between naïve and memory B cells, having a similar tendency to increase during the course of the activation. Representative for the naïve and memory B cell subset distribution in peripheral blood, the number of IgM spots at each harvest day of the culture of the total B cell fraction was higher than the number of IgG spots. Antibody production could be detected as early as day 2 after polyclonal activation (Figure 4a).



IgM spot production was mainly by naïve B cells (Figure 4b), but was also detected in the sorted memory population. Virtually all IgG spots were produced by memory B cells (Figure 4c) with a low, yet detectable number of IgG spots formed in the naïve B cell fraction. When determining the frequency of antigen-specific memory B cells, it is pivotal that one does not measure the IgG production induced in the naïve B cell population. In order to test whether IgG production induced in the naïve B cell compartment, if any, would affect the frequency estimation of antigen-specific memory B cells, we set up a TT antigen-specific IgG B cell ELISPOT assay. Total B cells, as well as sorted naïve and memory B cell fractions were polyclonally activated for the detection of TT antigen-specific IgG spots.

Figure 4. Immunoglobulin (Ig)M and IgG production kinetics of activated B cells. *(a) Total, (b) naïve and (c) memory B cells were activated polyclonally for varying lengths of time. At each time point, 1x10^3 activated B cells were transferred to individual enzyme-linked immunospot assay (ELISPOT) plates for IgM (white bars) and IgG (black bars) spot detection. Results are expressed as mean ± standard deviation (s.d.) of seven independent experiments performed with a minimum of three and a maximum of seven donors for each time point. SFU=spot forming unit.*

As shown in figure 5, TT-antigen specific spots could be detected in the total B cell population. There was a high inter-individual variation in the number of TT specific B cells within the memory population, as one might expect. Furthermore, we did not observe any spots in the naïve B cell fraction whereas clear spot formation was observed in the memory B cell fraction, indicating that antigen specific IgG spots were only produced by the pre-existing memory B cells.

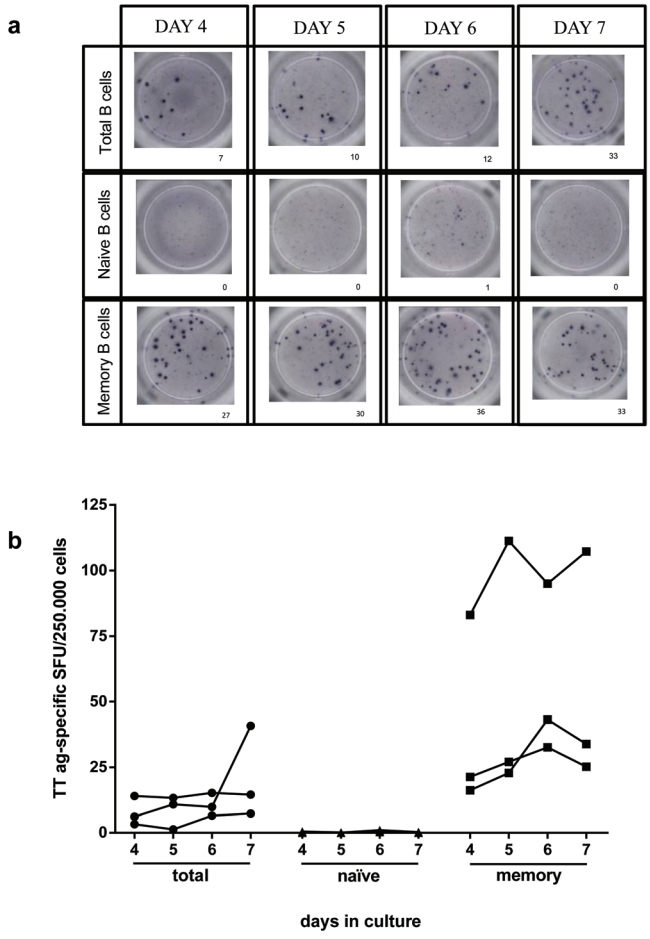


Figure 5. Tetanus toxoid (TT) antigen-specific spots are not detected in naïve B cells upon polyclonal activation. *(a) Activated naïve B cells did not produce TT antigen-specific spots, whereas memory cells produced a robust number of TT specific spots. Total, naïve and memory B cells were activated polyclonally for varying lengths of time. At each time point, 2.5x10^5 activated B cells were transferred to TT-antigen coated ELISPOT plates. Representative results of one of the three independent experiments are shown. (b) Cumulative data on TT-antigen specific spot forming units (SFU) per 2.5x10^5 B cells. Total B cells (●), naïve B cells (▲) and memory B cells (■) are shown separately. Data from three independent experiments with different donors are shown.*

The activation protocol does not affect the polyclonality of activated B cells

Another important aspect of an activation protocol to determine the frequency of antigen-specific memory B cells is that there is no preferential expansion of certain clones. We addressed this issue by determining the B cell receptor (BCR) repertoire of B cells by immunoscope methodology (23, 24). In order to define if the repertoire diversity in IgM and IgG remained similar before and after polyclonal activation, we performed CDR3 fragment analysis on non-activated and 6-day activated B cells. As depicted in figure 6, there was a strong correlation between the distribution of the peaks in all V_H gene families before and after activation for IgM, indicating the persistency of the repertoire diversity. Concerning IgG, V_H3 and V_H4 represent the majority of all rearrangements whereas the smallest gene families such as V_H6 and V_H7 are rarely used (24). In the present study, the distribution of the peaks for the commonly used V_H3 and V_H4 were significantly correlated before and after activation for IgG. The correlation analysis could not be performed for V_H6 and V_H7 due to their rarity in usage.

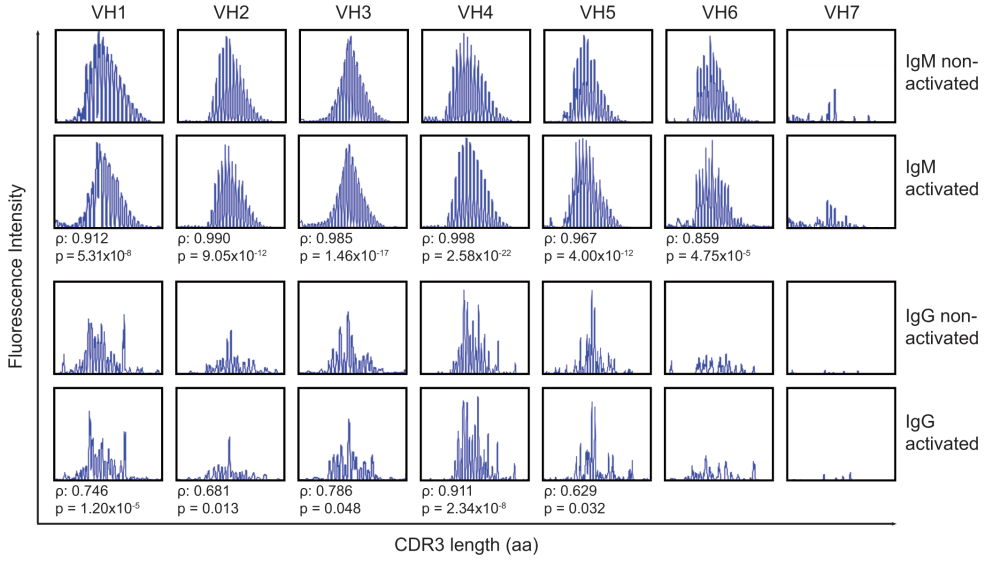


Figure 6. B cell receptor CDR3 length distribution is not significantly altered upon polyclonal B cell activation. *Each panel shows the CDR3 length distribution profile for one of seven V_H families, as determined for non-activated B cells as well as 6-day activated B cells. The x-axis indicates CDR3 length (amino acids), and the y-axis displays fluorescence intensity of polymerase chain reaction (PCR) products. Representative profiles of three independent experiments with different donors are shown. ρ= Spearman's rank correlation coefficient; CDR3=complementarity determining region 3; aa= amino acids.*

DISCUSSION

In vivo, memory B cells provide a more rapid and higher-affinity antibody response compared to primary responses initiated by naïve B cells (25). While serum antibodies are mainly produced by bone marrow-residing plasma cells (26, 27), their serum levels may not be representative for the peripheral memory B cell pool. Indeed, Lynch *et al*. recently showed the presence of donor-specific memory B cells in the absence of donor-specific serum IgG antibodies in kidney transplant recipients (12). These, and other data (11) made clear that, *in vitro* polyclonal B cell activation may be used to provide a snap shot of the B cell memory pool at a given time-point. By focussing on defined antigens, such as viral antigens or HLA molecules, the actual frequency of antigen-specific memory B cells can be determined.

The accuracy of such assays is in part dependent on the activation protocol used. Several protocols exist to activate B cells polyclonally. Variations in cytokines, source of CD40 ligation, if any, and the inclusion of a BCR trigger have been described (13, 15, 28-32). These variations, as well as differences in cell source and B cell purity may cause different kinetics of naïve *versus* memory B cells, both in terms of proliferation and antibody production. In this study, we were able to demonstrate the distinct proliferation kinetics and antibody production patterns of naïve and memory B cells in response to a previously defined *in vitro* polyclonal activation protocol based on CD40 ligation using an α-CD40 monoclonal antibody in combination with CpG DNA and B cell activating cytokines (20, 21). Our main interest is on HLA-specific memory B cells in the context of (solid organ) transplantation. As HLA-specific memory B cells are present at extremely low frequencies (11), our protocol includes a B cell purification step prior to activation to increase assay sensitivity. For B cell activation, we have selected the CD40-CD40L pathway as surrogate for T cell help in combination with ODN2006 CpG and IL-21, which are potent drivers of B cells into antibody-secreting plasma cells (18, 33). In our hands, this activation protocol was superior to several other protocols, including B cell receptor ligation, *Staphylococcus aureus* and pokeweed mitogen (data not shown). Additionally, the high variability of the commercially available form of pokeweed mitogen, as described by Crotty *et al*., may render standardization difficult (34).

Many *in vitro* B cell assays to assess immunoglobulin production require prolonged stimulation of B cells up to 14 days (8, 35). Such long B cell activation periods invariably lead to a certain degree of cell death, and may therefore influence clonal distribution. In the present study, dead:alive cell ratios between naïve and memory B cells, as well as in total B cells were indistinguishable at all times through the culture process. When we analysed the CDR3 length distribution, we found no statistically significant differences before and after activation, indicating that the clonality of activated B cells at 6 days of culture is not significantly different from the starting population.

Flow cytometric analyses showed that both naïve and memory B cells were activated by our activation protocol. Expression of CD69 and CD25, generally considered as markers for B cell activation (36, 37), was up-regulated in all fractions 1 day after polyclonal activation. These data suggest that the current activation protocol is potent enough to activate both the naïve and memory B cells.

A reliable estimate of the size of an antigen-specific B cell pool requires that the culture protocol does not induce significant levels of isotype switching *in vitro*. As many polyclonal activation strategies do induce a certain degree of isotype switching, particularly after long term cultures varying between 7 to 12 days (15-19), we aimed to determine whether the selected activation protocol in combination with an incubation time of maximally 8 days induced any IgG production in the naïve B cell population. Using total IgM and IgG ELISPOT analysis, very low, yet detectable numbers of IgG spots were found in the naïve B cell cultures. The detection of a small proportion of cells in the naïve B cell fraction that were capable of producing IgG could either be due to the inability to yield sufficient high purity cells in FACS (average sorting purity: 98% for naïve B cells) or due to activation-induced isotype switching of a small number of naïve B cells. Whether or not the presence of a small number of IgG producing cells in sorted naïve B cell fractions would influence the frequency estimation of antigen-specific memory B cells, we used TT as a model antigen for ELISPOT. Using antigen-specific B cell ELISPOT assays we did not observe any spots in polyclonally activated sorted naïve B cells, suggesting that the induction of IgG in naïve B cells, if any, is not interfering significantly with the estimation of antigen-specific memory B cells. Furthermore, our activation protocol may also be used to study naïve B cell responses, as it also results in a potent activation of naïve B cells into IgM-producing cells.

In conclusion, we were able to characterize peripheral blood B cells upon polyclonal activation for the purpose of determining the frequency of antigen-specific memory B cells. Polyclonal activation using α-CD40, TLR9 triggering, IL-2, IL-10 and IL-21 can be used reliably to estimate the frequency of antigen-specific memory B cells in ELISPOT assays without the interference of IgG producing cells in the naïve B cell population and with retained polyclonality of the total B cell population.

These findings are particularly important in assays aiming at estimating the frequency of antigen specific memory B cells, such as the HLA-specific memory B cell ELISPOT assay. Whereas in this study we used TT as a model antigen, previously we have used a similar activation protocol for the quantification of HLA-specific memory B cells (11). We are currently performing a clinical study in order to validate the clinical usefulness of the HLA-specific B cell ELISPOT assay. In this Dutch multicenter study, purified B cells of kidney transplant recipients are polyclonally activated with the present culture system and tested in HLA-specific B cell ELISPOT assays at several time-points before and after transplantation. These assays will be complemented by detailed analysis of the HLA-specific antibody repertoire.

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