HLA-specific memory B cells : the missing link?

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Chapter 6

A memory B cell crossmatch assay for quantification of donor-specific memory B cells in the peripheral blood of HLA-immunized individuals

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ABSTRACT

Humoral responses against mismatched donor HLA are routinely measured as serum HLA antibodies, which are mainly produced by bone-marrow-residing plasma cells. Individuals with a history of alloimmunization but lacking serum antibodies may harbor circulating dormant memory B cells, which may rapidly become plasma cells upon antigen reencounter. Currently available methods to detect HLA-specific memory B cells are scarce and insufficient in quantifying the complete donor-specific memory B cell response due to their dependence on synthetic HLA molecules. We present a highly sensitive and specific tool for quantifying donor-specific memory B cells in peripheral blood of individuals using cell lysates covering the complete HLA class I and class II repertoire of an individual. Using this enzyme-linked immunospot (ELISPOT) assay, we found a median frequency of 31 HLA class I and 89 HLA class II-specific memory B cells per million IgG-producing cells directed at paternal HLA in peripheral blood samples from women (n=22) with a history of pregnancy using cell lysates from spouses. The donor-specific memory B cell ELISPOT can be utilized in HLA diagnostic laboratories as a cross-match assay to quantify donor-specific memory B cells in patients with a history of sensitizing events.

INTRODUCTION

Pre-existence or *de novo* development of donor-specific antibodies (DSAs) directed at mismatched donor human leukocyte antigens (HLA) portends a major risk for adverse outcome of kidney, heart, lung and to some extent liver transplantations (1-4). HLA antibodies may develop in an individual on exposure to allogeneic HLA via blood transfusions, pregnancies or previous transplantations. B cells encountering allogeneic HLA molecules may differentiate into plasma cells producing HLA-specific antibodies as well as becoming circulating dormant memory B cells (5). HLA antibodies produced by plasma cells can directly be detected in serum using complement-dependent cytotoxicity, ELISA, and bead-based assays. Although proven to be extremely informative, HLA antibody analyses in serum do not provide any information on the magnitude of the HLA-specific memory B cell pool (6). Because HLA-specific memory B cells can rapidly differentiate into antibody secreting cells on antigen rechallenge, there is a clinical need to detect these cells in transplant recipients. Therefore, several assays have been developed to quantify memory B cells directed to specific synthetic HLA molecules (7-12). Considering the enormous polymorphism of the HLA system, it is very unlikely to be able to cover the complete HLA repertoire of an individual, even if all combinations of synthetic HLA molecules are used. Consequently, we aimed to develop an assay in which donor HLA containing cell lysates are used as a detection matrix in enzyme–linked immunospot (ELISPOT) assays. In this study, we present a donor-specific ELISPOT assay capable of detecting and quantifying HLA-specific memory B cells using peripheral blood or spleen cell lysates in polyclonally activated peripheral blood samples of alloantigen immunized individuals.

MATERIALS AND METHODS

Cells

Peripheral blood samples from healthy volunteers and spleen samples from deceased organ donors were obtained with informed consent under guidelines issued by the medical ethics committee of Leiden University (Leiden, the Netherlands). Mononuclear cells from peripheral blood or spleen were isolated by Ficoll-Hypaque density gradient centrifugation and kept frozen in liquid nitrogen until further use. A total of 27 peripheral blood samples from HLA-typed healthy women (n=22) with a history of at least one pregnancy were collected and tested against immunizing HLA using donor-specific ELISPOT assays. In addition, peripheral blood samples from 10 men without any history of immunizing events and serum HLA antibodies were assessed for the presence of HLA-specific memory B cells by donor-specific ELISPOT assays.

Human B cell hybridomas producing HLA class I or class II-specific monoclonal antibodies were used for the development and optimization of the donor-specific ELISPOT assays (13, 14).

Lysate preparation

Lysates were prepared from either peripheral blood mononuclear cells (PBMCs) or spleen cells. Mononuclear cells from peripheral blood or spleen samples ($1x10^6$ cells/well in 24-well plates) were stimulated for 7 days with 2 µg/ml phytohemagglutinin (Remel, Dartford Kent, UK) and 60 IU/ml IL-2 (Proleukin; Novartis, Amsterdam, the Netherlands) in Iscove's modified Dulbecco's medium (IMDM; Gibco Invitrogen, Paisley, UK) containing 10% fetal bovine serum (FBS) (Gibco Invitrogen) and 100 U/ml penicillin with 100 µg/ml streptomycin (Gibco Invitrogen). After 7 days, cells were washed with phosphate-buffered saline (PBS) before proceeding with lysate preparation. For HLA class II lysate preparation, cells were first fixed with 1% paraformaldehyde for 5 min at 4°C and washed three times with 0.2% bovine serum albumin (BSA) in PBS. Cell pellets were transferred to microcentrifuge tubes and washed twice with 2% BSA/PBS. Next, cells were resuspended in 2% BSA/PBS (100 µl buffer per $3x10^6$ cells) and incubated with a mouse anti-human pan-HLA class II antibody (IC2) at a concentration of 5 µg/ml for 30 min at 37°C to stabilize the HLA class II molecules (15). Cells were then washed three times with cold PBS to remove the unbound antibody. For HLA class I lysate preparation, cells were directly transferred to microcentrifuge tubes and pelleted without the need for HLA stabilization. After removing the supernatant, 100 µl lysis buffer (diluted 1:10 in distilled water) (Lymphocyte lysing buffer, Immucor, Stamford, CT) was added per $30x10^6$ cells and mixed vigorously. Following a second centrifugation step, supernatants containing the soluble HLA molecules were collected and either used immediately or kept in aliquots at-80°C for further use.

Polyclonal B cell activation

Polyclonal B cell activation was carried out by stimulating PBMCs with an activation cocktail consisting of 2.5 µg/ml Toll-like receptor 7 or 8 (TLR 7/8) agonist (resiquimod [R848]; Sigma-Aldrich, St. Louis, MO) and 1000 IU/ml IL-2 (Proleukin; Novartis) (16). PBMC cultures were carried out in 10% FBS/IMDM for 6 days by seeding $2x10^6$ cells per well in 24-well flat-bottomed plates (Corning Inc., Corning, NY, USA) at 37°C in a 5% CO_2 humidified incubator.

ELISPOT assays

Total IgG ELISPOT assays were performed as described previously (17). For donor-specific ELISPOT assays, 96-well polyvinylidene fluoride ELISPOT plates (Millipore, Billerica, MA) were coated with 5 µg/ml goat anti-human IgG (Jackson Immunoresearch Laboratories Inc., Baltimore, PA) in PBS and incubated overnight at 4°C. Plates were then blocked for at least 1 h with 5% FBS/IMDM at 37°C, after which thoroughly washed activated cells ($2.5x10^5$ cells/well) or hybridoma cells (500 or 1000 cells/well) were added. After overnight incubation at 37°C, plates were washed and incubated for 4 h with HLA class I or class II donor or autologous cell lysates (4 µl lysate in 50 µl PBS per well) at room temperature on a platform shaker. Following washing, horse radish peroxidase-conjugated mouse anti-human β2 microglobulin antibody diluted 1:1000 in 1% BSA/Tween 20/PBS (AbD Serotec/BioRad, Puchheim, Germany) for HLA class I or goat anti-mouse IgG2b antibody diluted 1:8000 in 1% BSA/Tween 20/PBS (Southern Biotech, Birmingham, AL) for HLA class II ELISPOT assays were added to separate wells and incubated for 2 h at room temperature. After washing, plates were incubated with 3,3',5,5'-tetramethylbenzidine substrate (Mabtech, Nacka Strand, Sweden) to visualize the spots. The reaction was stopped by adding cold tap water and, after drying, analysed by an automated ELISPOT reader (Bio-Sys GmbH, Karben, Germany). An overview of the ELISPOT techniques, as well as Luminex data on mouse anti-human β2 microglobulin and IC2 used in ELISPOT assays are provided in Figures S1 and S2, respectively.

Wells with B cell hybridomas were included in every donor-specific ELISPOT assay to validate the lysate quality. In addition, a total IgG ELISPOT assay was performed with every donor-specific ELISPOT assay to verify the B cell activation. Autologous cell lysates served as negative controls and spots, if any, in autologous lysate wells were subtracted from spot counts in wells with donor cell lysates. HLA-specific B cell frequencies were expressed as number of HLA-specific B cells per million IgG producing B cells.

HLA antibody detection

Serum samples were screened for the presence of HLA antibodies by Luminex using Lifecodes Lifescreen Deluxe kit (Immucor Transplant Diagnostics, Stamford, CT). Positive samples were further tested using LabScreen HLA class I and class II single antigen beads (One Lambda, Canoga, CA) to identify HLA antibody specificities. Serum and PBMC samples were collected

at the same time period.

Statistical analysis

The Mann-Whitney *U* test was used for comparisons of the results between groups. The Tukey method was used to find the outlier values in HLA class I and class II-specific B cell frequencies in samples from nonimmunized men and to define the donor-specific ELISPOT assay cut-off using the following formula: third quartile+(1.5 x interquartile range). Statistical level of significance was defined as $p<0.05$.

RESULTS

Cell lysates can be used to detect HLA-specific antibody producing cells

Human B cell hybridomas producing monoclonal HLA antibodies were utilized to develop and optimize the donor-specific memory B cell ELISPOT assays. We have previously shown that synthetic HLA molecules could be used as detection agents to capture HLA antibody-producing cells with high sensitivity and specificity (10). Using the same approach, we used cell lysates as detection matrix for quantification of HLA-specific B cell hybridomas. Initially, we tested whether each HLA specificity in single lysates were recognized by corresponding specificity of HLA antibody-producing hybridoma cells. To this end, we prepared several lysates positive for different HLA molecules. As exemplified in Figure 1A, an HLA class I PBMC lysate positive for HLA-A2, HLA-B7, and HLA-B13 was tested against three different hybridoma cells producing antibodies directed at either HLA-A2, HLA-B7, or HLA-B13.

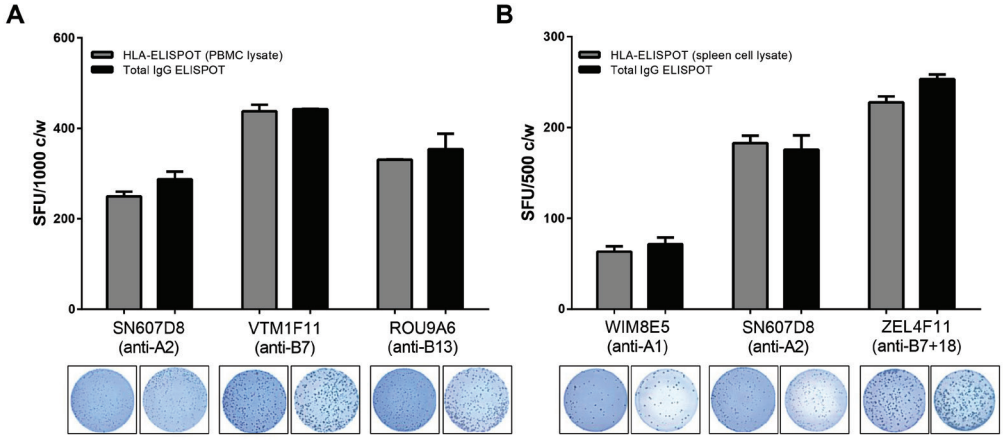

Figure 1. Lysates can be used to detect HLA-antibody producing cells with high sensitivity. *(A) One PBMC lysate (positive for HLA-A2, HLA-B7 and HLA-B13) was tested against three different hybridomas producing HLA antibodies of the corresponding specificity. Comparable numbers of spots were found for each HLA in the lysate using donor-specific enzyme-linked immunospot (ELISPOT; gray bars) and concurrent total IgG ELISPOT assays (black bars). (B) All antibody-producing cells were detected using spleen cell lysates (positive for HLA-A1, HLA-A2, HLA-B7 and HLA-B18). Error bars represent the mean±SD of triplicate wells. Results are representative of experiments performed with four different lysates. SFU, spot forming units.*

All antibody-producing cells from each corresponding hybridoma were detected with high sensitivity using the same lysate as evident by comparable number of spots between donor-specific and total IgG ELISPOT assays (Figure 1A). Similar results were found when a class I lysate positive for HLA-A1, HLA-A2, HLA-B7 and HLA-B18 was prepared from spleen mononuclear cells and tested against hybridoma cells producing corresponding specificity of HLA antibodies, indicating the potential of using cell lysates covering multiple HLA specificities in quantifying HLA-specific B cells (Figure 1B).

To verify lysate stability with freezing and thawing, we analyzed spot numbers obtained in donor-specific ELISPOT assays after freezing and thawing at several time-points. As exemplified in Figure 2A, spot counts comparable to total IgG ELISPOT assays were found by using a donor-specific ELISPOT assay when a freshly made or 4-day frozen HLA-A1$^+$ lysate was utilized to detect HLA-A1 antibody-producing cells. Similarly, HLA-A1 antibody-producing cells were still detected with high sensitivity when 2 or 3-year frozen class I lysates were used, as evident by comparable spot counts between donor-specific and total IgG ELISPOT assays. Likewise, similar spot counts were obtained when short-term (1 day) or longer-term (36 days) frozen class II lysates were used to detect HLA-DR11 antibody-producing hybridoma cells, suggesting that long term storage did not affect HLA class I or HLA class II stability in the lysates (Figure 2B).

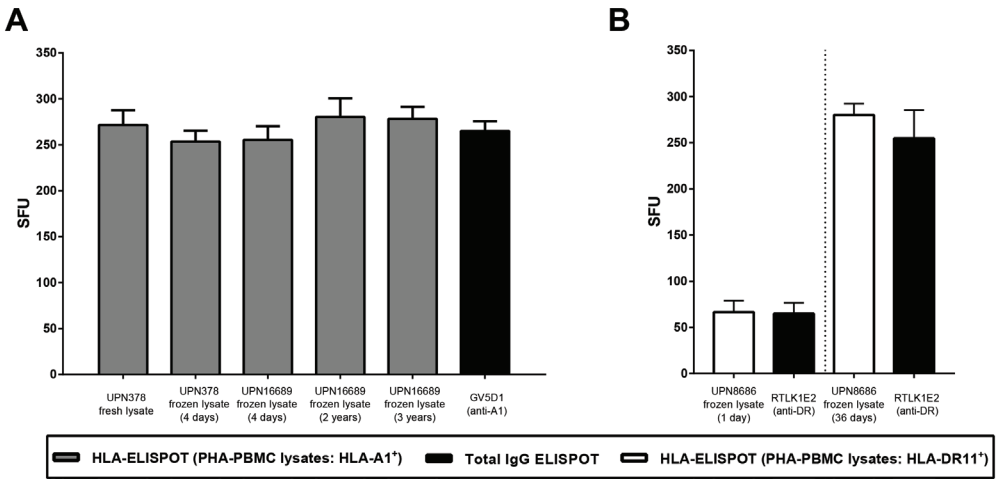

Figure 2. HLA in lysates remain stable upon freezing-thawing. *(A) Comparable numbers of spots were obtained when HLA-A1$^+$ lysates prepared from PHA stimulated PBMC samples were used immediately after preparation (fresh) or frozen for 4 days up to 3 years (gray bars) and then used to detect HLA-A1 antibody-producing cells (GV5D1, black bar). (B) Similarly, all HLA-DR11 antibody-producing cells were detected at high sensitivity similar to total IgG ELISPOT assay (RTLK1E2, black bars) when HLA-DR11$^+$ cell lysates (white bars) frozen for short (1 day) or longer term (36 days) were used. Error bars represent the mean±SD of triplicate wells. Results are representative of experiments performed with four different lysates. ELISPOT, enzyme-linked immunospot; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; SFU, spot forming units.*

Because the cell lysates contain various HLA antigens, we next questioned whether B cell hybridomas were detected with correct specificity in donor-specific-ELISPOT assays. To this aim, we prepared PBMC lysates from HLA-A1$^+$ and HLA-A1$^-$ individuals and tested these lysates against HLA-A1 antibody-producing hybridoma cells. Clear spot formation was found when an HLA-A1$^+$ lysate was used, whereas no significant spot formation was observed with HLA-A1$^-$ lysates. Likewise, when lysates positive for HLA-B13 or lacking HLA-B13 were used to quantify HLA-B13 antibody-producing cells, spot counts with HLA-B13$^+$ lysates were comparable to total IgG spots while no spots were formed when HLA-B13$^-$ lysates were used (Figure 3A). Similar results were found for HLA class II lysates prepared from PBMCs or spleen mononuclear cells to detect hybridoma cells (RTLK1E2) producing anti HLA-DR3, -DR8, -DR11, -DR12, -DR13, and -DR14 (Figure 3B). Use of lysates containing HLA-DR11 and-DR15, or HLA-DR12 and-DR13 resulted in spot formation whereas no spots were observed when HLA-DR7 and -DR9, or HLA-DR9 and -DR10-containing lysates were used, confirming that spots were detected with correct specificity in donor-specific ELISPOT assays.

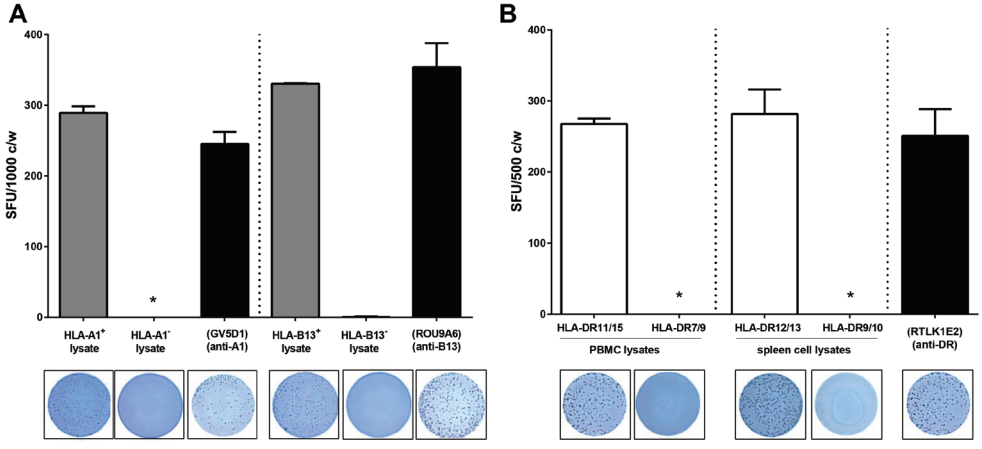

Figure 3. Lysates prepared from peripheral blood mononuclear cells (PBMCs) or spleen cells can be used to detect HLA antibody-producing cells with correct specificity. *(A) A PBMC lysate containing HLA-A1 or HLA-B13 only gave spot formation with only an HLA-A1 or HLA-B13 specific antibody-producing hybridoma, whereas no spot formation was observed with a PBMC lysate lacking either HLA-A1 or HLA-B13 (gray bars). (B) Spot formation was observed only when an HLA-DR3, -DR8, -DR11, -DR12, -DR13 or DR14-specific hybridoma was tested against PBMC or spleen cell lysates containing HLA-DR11 and -DR15, or HLA-DR12 and -DR13 (white bars) and not against lysates containing HLA-DR7 and -DR9, or HLA-DR9 and -DR10. In all cases, number of spots detected by donor-specific ELISPOT assays were comparable to those detected by total IgG ELISPOT assays (black bars). (*): No SFU detected. Error bars represent the mean±SD of triplicate wells. Results are representative of experiments performed with four different lysates. SFU: spot forming units.*

Paternal HLA-specific B cells can be detected in peripheral blood of women with a history of pregnancy

Having shown that the donor-specific ELISPOT allowed for quantification of HLA antibody-producing hybridoma cells, we aimed to determine whether HLA-specific memory B cells directed at paternal HLA could be detected in peripheral blood samples from women with history of pregnancy using paternal (n=14) or paternal-like (n=21) cell lysates sharing the immunizing HLA antigens with the spouses (Table S1). A total of 24 HLA class I and 27 HLA class II-specific assays were performed using donor or donor-like cell lysates with peripheral blood samples from parous women (Table 1).

As shown in Figure 4, memory B cells could be visualized as single spots in donor-specific ELISPOT assays when polyclonally activated PBMC samples from women with a history of pregnancy were tested against cell lysates containing paternal HLA class I or class II molecules. As expected, no significant spot formation was found against self HLA class I or class II molecules, confirming the specificity of the assay.

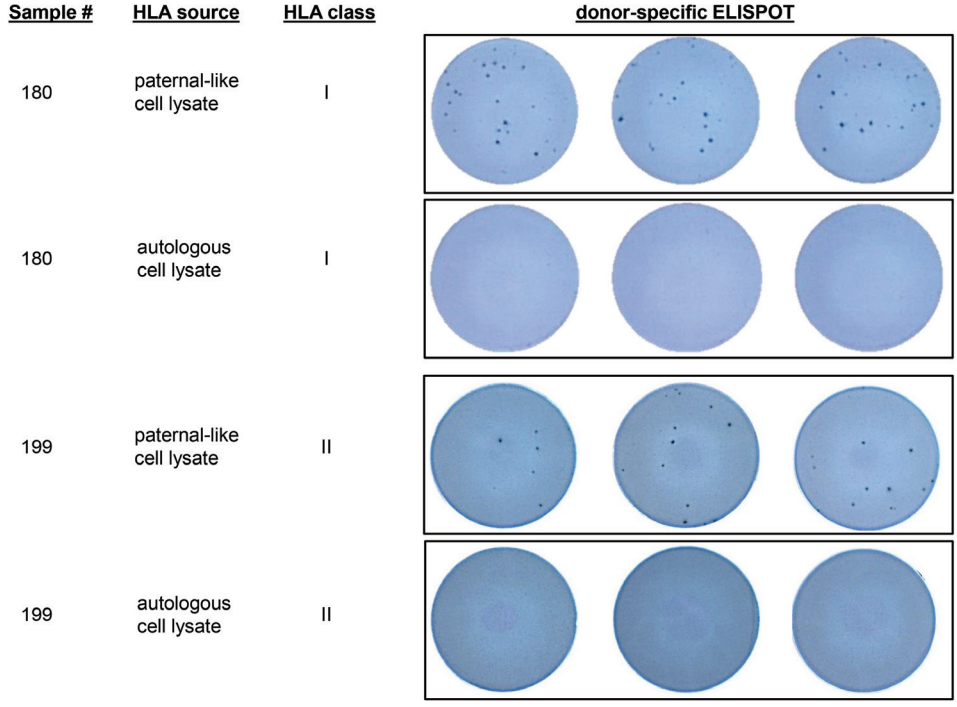

Figure 4. Representative example of a donor-specific memory B cell ELISPOT assay. *HLA class I-specific memory B cells were detected in a pregnancy immunized woman (sample 180) when tested against a cell lysate (PBMC/paternal-like donor 3) that contains immunizing HLA-B*07:02. Similarly, HLA class II-specific memory B cells were detected in another pregnancy-immunized woman (sample 199) when tested against a cell lysate (PBMC/paternal-like donor 10) containing HLA-DRB1*04 and HLA-DQB1*03 using donor-specific ELISPOT assays. No significant spot formation was observed when tested against autologous cell lysates. Donor-specific memory B cell frequencies per total million IgG$^+$ cells were 401 for HLA class I in sample 180 and 137 for HLA class II in sample 199.*

We found significantly higher HLA class I-specific memory B cells (median frequency: 202, range: 0-802) in women with serum HLA class I antibodies compared to those without serum HLA class I antibodies (median frequency:0, range:0-8) and nonimmunized men (median: 0, range: 0-25) (p<0.0001) (Figure 5A). Similarly, HLA class II-specific memory B cell frequencies were significantly higher in group of women with serum HLA class II antibodies (median frequency: 137, range: 0-1050) compared to women without serum antibodies (median frequency: 1, range: 0-19) (p<0.05) and nonimmunized men (median frequency: 0, range: 0-26) (p<0.0001) (Figure 5B). HLA-specific memory B cell frequencies did not differ between women without serum HLA antibodies and nonimmunized men (p-value not significant).

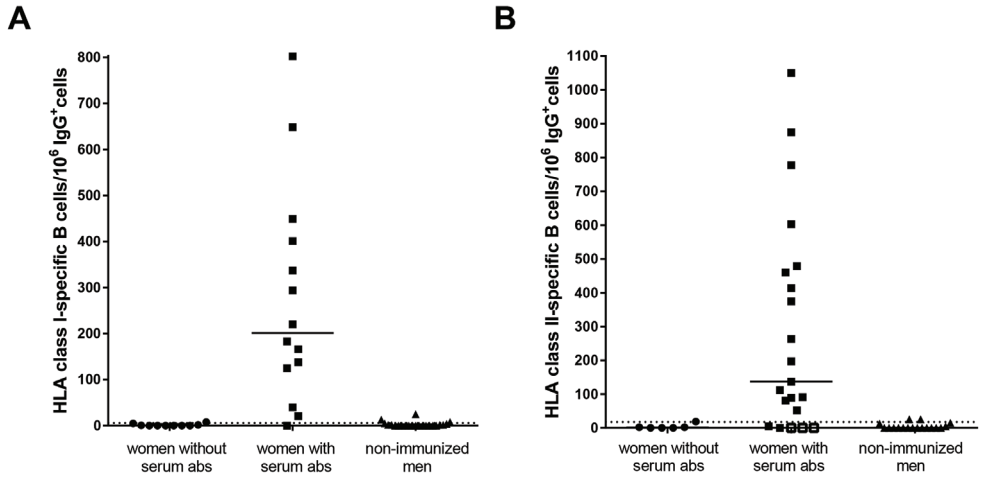

Figure 5. Pregnancy immunized women harbour HLA-specific memory B cells directed at immunizing paternal HLA. *(A) The number of HLA class I and (B) HLA class II-specific B cells per total million IgG⁺ cells were significantly higher in peripheral blood samples from women with serum antibodies (median HLA class I: 202 and median HLA class II: 131) compared to those women without serum antibodies (median HLA class I: 0 and median HLA class II: 1) and non-immunized men (median HLA class I and II: 0) (p<0.05). Lines represent the median values. Dotted lines represent the cutoff values for donor-specific ELISPOT assays. Three open squares (□) in (B) represent the absence of HLA-specific memory B cells in one sample (203) tested with three different lysates. No donor-specific HLA antibodies were found in culture supernatants for this sample. Abs: antibodies.*

HLA ELISPOT assay cut off

To define a cutoff value for positive responses in donor-specific ELISPOT assays, polyclonally activated PBMC samples from men without any history of sensitizing events were tested against cell lysates that were at least one-locus mismatched for HLA class I and/or class II. ELISPOT assays were performed testing nine men against two different lysates and one men against four different lysates for class I and six men with three different lysates for class II. The median frequencies of HLA class I-specific (range: 0-25) and class II-specific (range: 0-26) B cells per million IgG⁺ cells were found to be 0 in samples from nonimmunized men tested against non-self HLA containing lysates. To establish a cutoff for positive responses in donor-specific ELISPOT assays, we first defined the outlier values separately for HLA class

I and II frequencies in nonimmunized men. Accordingly, we established HLA-specific B cell frequencies > 6 (for class I) and >18 (for class II) per million IgG^+ cells as positive.

When we classified pregnancy-immunized women into 2 groups as women with and without serum HLA antibodies and applied the donor-specific ELISPOT assay cutoff, we found that all except two (sample 148, HLA class I frequency: 0; sample 203, HLA class II frequency: 0) in the serum antibody-positive group had paternal HLA-specific memory B cell frequencies above the cutoff for both class I and class II (Table 1). Interestingly, one sample (sample 201) in the HLA class II serum antibody-positive group was found to have no HLA-DR12-specific memory B cells when a lysate expressing HLA-DRB1*12/13 was used, despite lysates bearing other HLA specificities and serum antibodies giving positive reactions (Table S1). In the serum HLA antibody-negative group, all except 2 samples (sample 159, HLA class I frequency: 8, sample 151/207.3, HLA class II frequency: 19) had HLA-specific memory B cell frequencies below the donor-specific ELISPOT assay cutoff.

Table 1: Characteristics of the study population

Sample#	HLA-source (lysate)	HLA locus tested	HLA mismatch[c]	Children#	Time (year)[d]	Frequency[e]	Serum antibodies
147	PBMC/paternal	class 1	yes	2	12.3	21	yes
148	PBMC/paternal	class 1	yes	2	20.6	0	yes
149	PBMC/paternal	class 1	yes	2	7.8	138	yes
150	PBMC/paternal	class 1	yes	2	3.7	0	no
151/207.3	PBMC/paternal	class 1	yes	3	13.2	0	no
		class 2	yes			19	no
152	PBMC/paternal	class 1	yes	1	17.8	5	no
153/207.1	PBMC/paternal	class 1	yes	2	11.9	0	no
		class 2	yes			2	no
154/206.3	PBMC/paternal	class 1	yes	2	25.1	0	no
		class 2	yes			2	no
155/207.2	PBMC/paternal	class 1	yes	3	28.8	2	no
		class 2	no			0	no
156	PBMC/paternal	class 1	yes	2	18.9	40	yes
157/206.1	PBMC/paternal	class 1	yes	2	48.5	0	no
		class 2	yes			0	no
158	PBMC/paternal	class 1	yes	1	23.7	1	no
159	PBMC/paternal	class 1	yes	3	23.3	8	no
160/206.2	PBMC/paternal	class 1	yes	1	0.5	0	no
		class 2	yes			0	no

Table 1: *Continued*

Sample#	HLA-source (lysate)	HLA locus tested	HLA mismatch[c]	Children#	Time (year)[d]	Frequency[e]	Serum antibodies
106[a]	PBMC/paternal-like[1]	class 1	yes	2	24.5	648	yes
109	PBMC/paternal-like[2]	class 1	yes	1	10.4	220	yes
109	PBMC/paternal-like[3]	class 1	yes			125	yes
109	splenocytes/paternal-like[4]	class 1	yes			183	yes
111	PBMC/paternal-like[5]	class 1	yes	2	0.3	294	yes
112[b]	PBMC/paternal-like[6]	class 1	yes	1	2	802	yes
118[a]	PBMC/paternal-like[7]	class 1	yes	2	1.4	166	yes
118[a]	splenocytes/paternal-like[8]	class 1	yes			337	yes
180	PBMC/paternal-like[3]	class 1	yes	1	12	401	yes
180	PBMC/paternal-like[9]	class 1	yes			449	yes
199	PBMC/paternal-like[10]	class 2	yes	1	39.9	137	yes
199	PBMC/paternal-like[11]	class 2	yes			89	yes
199	splenocytes/paternal-like[12]	class 2	yes			81	yes
201.1[b]	PBMC/paternal-like[13]	class 2	yes	1	1.3	263	yes
201.1[b]	PBMC/paternal-like[14]	class 2	yes			1050	yes
201.1[b]	PBMC/paternal-like[15]	class 2	yes			460	yes
201.1[b]	splenocytes/paternal-like[16]	class 2	yes			0	yes
201.2[b]	PBMC/paternal-like[13]	class 2	yes	1	23.5	52	yes
201.2[b]	PBMC/paternal-like[14]	class 2	yes			374	yes
201.2[b]	PBMC/paternal-like[15]	class 2	yes			197	yes
201.2[b]	splenocytes/paternal-like[16]	class 2	yes			5	yes
202	PBMC/paternal-like[17]	class 2	yes	1	14.1	875	yes
202	PBMC/paternal-like[18]	class 2	yes			112	yes
202	PBMC/paternal-like[19]	class 2	yes			479	yes
202	splenocytes/paternal-like[20]	class 2	yes			414	yes
203[a]	PBMC/paternal-like[17]	class 2	yes	2	2.7	0	yes
203[a]	PBMC/paternal-like[19]	class 2	yes			0	yes
203[a]	splenocytes/paternal-like[20]	class 2	yes			0	yes
205	PBMC/paternal-like[14]	class 2	yes	2	10	603	yes
205	PBMC/paternal-like[15]	class 2	yes			91	yes
205	PBMC/paternal-like[21]	class 2	yes			777	yes

PBMC, peripheral blood mononuclear cell.
[a] These samples were sampled at different times from the same individual.
[b] These samples were sampled at different times from the same individual.
[c] HLA mismatches between the women and the lysate donors are defined for HLA-A, -B, -C, -DR and -DQ loci.
[d] Time between the sampling and the last delivery.
[e] Donor-specific memory B cell frequency per million IgG-producing cells.
[1-21] Each number represents a different lysate donor.

DISCUSSION

We present, for the first time, a highly specific and sensitive tool to screen for donor-specific memory B cells in peripheral blood on polyclonal activation using PBMC or spleen cell lysates as the HLA targets representing the complete HLA class I and class II repertoire of an individual. Using these cell lysates, antibody-producing cells in the peripheral blood directed specifically to the allogeneic HLA contained in the lysate can be quantified in an ELISPOT format. Currently available methods to quantify HLA-specific memory B cells in peripheral blood of alloantigen-immunized individuals are scarce and rely on synthetic HLA molecules (7-12, 18). While B cell detection by HLA tetramers using flow cytometry is a simple method to quantify the percentage of HLA-specific B cells; however, to ensure that these HLA-tetramer positive B cells have the capacity to produce HLA antibodies, further sorting and culturing steps are required (7, 8). By combining the ability of polyclonally activated B cells to produce HLA antibodies *in vitro* with the potential of these antibodies to bind synthetic HLA molecules, HLA-specific ELISPOT assays, enable quantification of HLA-specific memory B cells (9, 10). Although HLA-specific ELISPOT assays allow for detection and quantification of both HLA class I and class II-specific memory B cells by utilization of monomeric HLA molecules, they are limited to certain HLA specificities that are commercially available restricting the clinical applicability. Previously, Lynch *et al.* used donor-derived fibroblasts as the HLA targets to show the presence of donor-specific memory B cells directed toward donor HLA class I using ELISPOT (19). Difficulties in obtaining the donor material and long culture time of fibroblasts (30 days) limit the applicability of this assay in routine clinical monitoring. Furthermore, because fibroblasts express only HLA class I molecules, this assay cannot be used to quantify the full donor HLA-specific memory B cell population.

Unlike the latter technique, the current assay allows for the quantification of both donor HLA class I and class II-specific memory B cells. It is vital that every HLA specificity expressed by the donor cells is represented in the lysate to serve as a specific detection matrix to be able to detect the complete repertoire of donor-specific memory B cells. Availability of a wide variety of HLA-A, -B and -DR-specific antibody-producing hybridoma cells in our laboratory enabled us to show that the donor-specific ELISPOT assay is highly specific and equally sensitive to the total IgG ELISPOT assay. Although we have tested various HLA specificities using B cell hybridomas and peripheral blood of pregnancy-immunized women in the lysate-based ELISPOT assay, further validation is warranted. Previous research on the use of donor cell lysates in ELISA format suggests that many, if not all, specificities can be detected in lysates (20), and this is confirmed by our findings so far. Importantly, we excluded the possibility that complexes of HLA class I and class II molecules in the lysate preparations could lead to false-positive signals (Figure S3).

Polyclonal activation of peripheral blood B cells leads to differentiation of B cells into antibody-

producing cells and allows for the detection of memory B cells *in vitro* (21). The assay we present here includes a 6-day preculture period in which PBMC samples are activated with a polyclonal activation cocktail that consists of TLR7/TLR8 agonist (R848) in combination with IL-2 and results in the preferential activation of memory B cells (16, 22). This activation protocol does not induce class switching from naïve B cells as evident by the lack of IgG spots from naïve B cell fractions (Figure S4). The presence of IgG spots exclusively in the memory B cell fraction ensured that HLA-specific B cell frequencies obtained by the current donor-specific ELISPOT assays were accurate estimations of HLA-specific memory B cell pools in the peripheral blood.

Alloimmunization through pregnancy is known to induce alloantibodies in 30-50% of the women against the mismatched paternal HLA expressed on fetal cells, and these HLA antibodies can be detected in serum for decades after delivery (23-26). Using the donor-specific ELISPOT assay, we found that 50% of women with a history of at least one pregnancy harbored memory B cells directed to paternal HLA in their peripheral blood sampled at a median time of 14 years after the last delivery in addition to the presence of serum HLA antibodies, demonstrating the long-term persistence of HLA-specific memory B cells.

In the present study, using the donor-specific ELISPOT assays, we found median frequencies of 31 and 89 HLA class I and class II-specific B cells per million IgG producing cells, respectively, in women with a history of pregnancy, whereas the median frequency of HLA-specific memory B cells in men without any history of immunization was 0 for both class I and class II. Using the outlier cutoff calculated from HLA-specific B cell frequencies in peripheral blood of nonimmunized men, we found that two women (sample 159 and 151/207.3) with a history of pregnancy had HLA-specific B cells in the absence of serum antibodies, suggesting that HLA-specific memory B cells may be present in the absence of serum antibodies. Interestingly, in two samples that were obtained at different time points from a single pregnancy-immunized woman after delivery (sample 201.1 and 201.2), we could not detect HLA-DR12-specific memory B cells when HLA-DR12 containing cell lysates were used, which might be due to peptide selectivity of the HLA antibody (27), or reactivity with denatured HLA class II molecules on the beads (28).

Although we could not formally test whether HLA-DQ antibody-producing cells were detected due to the lack of appropriate B cell hybridomas, we did observe spot formation in samples from pregnancy-immunized women (samples 201.1, 201.2, and 202) when lysates containing self or antibody-negative HLA-DR were used, suggesting that these spots are due to sole HLA-DQ specificity (Figure S5A-C). Furthermore, by ELISA, we could demonstrate that HLA-C is present in the lysate preparations and can be used to detect HLA-C specific memory B cells in ELISPOT format (Figure S5D-F).

In the current study, we tested PBMC samples from women with a history of immunization for the presence of paternal HLA-specific B cells using paternal cell lysates from spouses.

This can readily be translated to HLA diagnostic laboratories as a “memory B cell crossmatch

assay'' since it allows for donor-specific memory B cell detection in PBMC samples of patients using cell lysates prepared from candidate donors in the setting of solid organ transplantation, in particular for patients with a history of sensitizing events. A particularly important group of transplant recipients for this assay are women receiving a transplant from their spouses, in whom sensitization in the absence of circulating antibodies may occur. In addition, patients undergoing desensitization therapies for a prospective solid organ transplantation need to be closely monitored for DSA development during the therapy as well as in the post-transplantation period. Quantification of donor-specific memory B cells in addition to serum antibody detection in this group of high risk patients may aid better risk stratification and provide a comprehensive picture of the entire donor-specific B cell response (29). Furthermore, longitudinal monitoring of patients for the presence of donor-specific memory B cells may, as such, provide insight in the evolution of the humoral alloimmune response upon an organ transplantation.

In summary, here we demonstrate, for the first time, a method which allows for detecting and quantifying donor-specific memory B cells in peripheral blood of individuals with a history of alloimmunization. While the current assay can be utilized to quantify IgG isotype of DSA-producing cells, it can also be used to detect antibody producing cells of IgM isotype or IgG subclasses (30). This donor-specific memory B cell ELISPOT assay can serve as a memory B cell crossmatch assay to estimate the frequency of donor HLA-specific memory B cells, which have the capacity to produce HLA antibodies in a transplant recipient.

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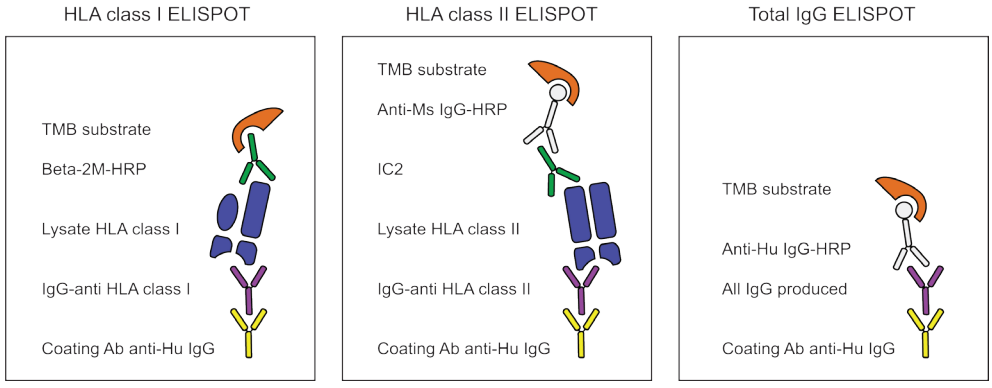
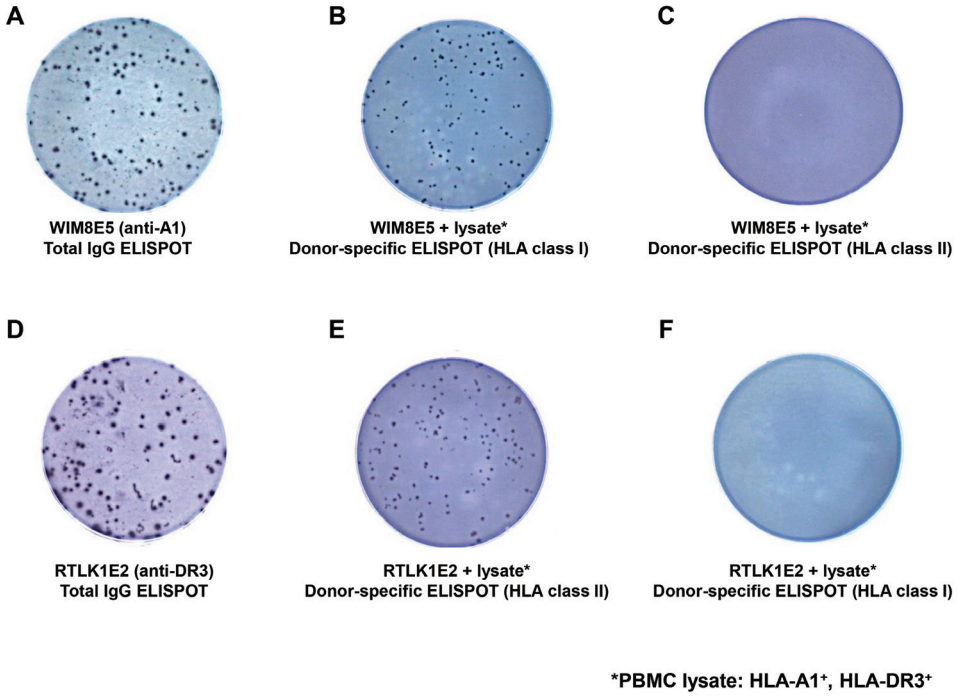
SUPPLEMENTARY DATA



Supplementary figure 1. Schematic overview of ELISPOT techniques. *Left panel: overview of HLA class I lysate-based ELISPOT technique, middle panel: overview of HLA class II lysate-based ELISPOT technique, right panel: overview of total IgG ELISPOT technique. Beta-2M: beta-2 microglobulin, anti-Hu: anti-human, anti-Ms: anti-mouse.*

Supplementary figure 2: Luminex single antigen bead assay data on HLA-specific detection antibodies. *Upper panel: pan HLA class I antibody (beta-2 microglobulin), lower panel: pan HLA class II antibody (IC2).*



Supplementary figure 3: Lysates do not contain complexes of HLA class I and class II molecules. *In order to test whether HLA class I in the lysate could be complexed to HLA class II or vice versa, (A) we incubated ELISPOT plates with HLA-A1 antibody producing hybridoma cells (WIM8E5), and added HLA-A1$^+$ DR3$^+$ PBMC lysate followed by either HLA class I or HLA class II-specific detection antibodies. (B) For the HLA-A1 specific hybridoma, we found significant spot formation when the detection antibody was HLA class I-specific, (C) whereas no spot formation was found when the HLA class II-specific detection antibody was used. (D) In parallel, we incubated ELISPOT plates with HLA-DR3 antibody producing hybridoma cells (RTLK1E2), followed by adding the same lysate and detection antibodies for either HLA class I and II. (E) Similarly, spot formation was observed when HLA-DR3 antibody producing hybridoma cells (RTLK1E2) were detected using the same lysate for the HLA class II specific hybridoma with HLA class II-specific detection (F), whereas no spot formation was observed when HLA class I-specific detection was applied.*

Methods for supplementary figure 4

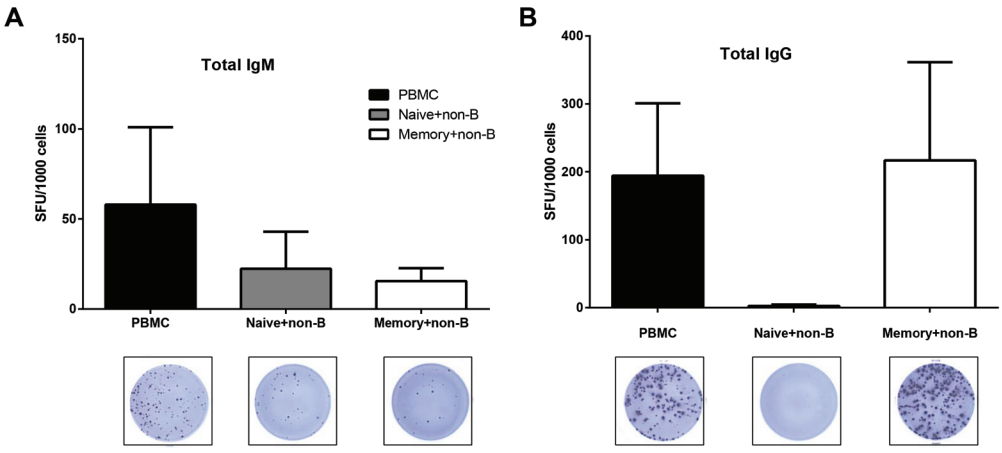
Isolation of naïve, memory and non-B cells from PBMC

B cells were isolated from PBMCs (n=3) by negative selection using EasySep Human B cell enrichment kit (Stem Cell Technologies). These B cells were sorted into $CD19^{+}IgD^{+}CD27^{-}$ naive, $CD19^{+}IgD^{-}CD27^{+}$ memory B cells, whereas non-B cells were directly sorted from PBMCs into $CD19^{-}CD20^{-}$ cells on fluorescence activated cell sorter (FACS) Aria II (BD Biosciences) using the following monoclonal antibodies (clone): CD19-APC.Cy7 (SJ25C1), IgD-PE (IA6-2) (both from BD Biosciences), CD20 (2H7) (e-Bioscience), CD27 (CLBCD27/1, 9F4) (Sanquin, Amsterdam, the Netherlands). Cell sorting purity for all fractions was more than 98%. Total IgM and IgG ELISPOT assays were performed with sorted and unsorted cells, as described elsewhere (17).

Results for supplementary figure 4

IgG spots are produced only by memory B cells upon polyclonal activation

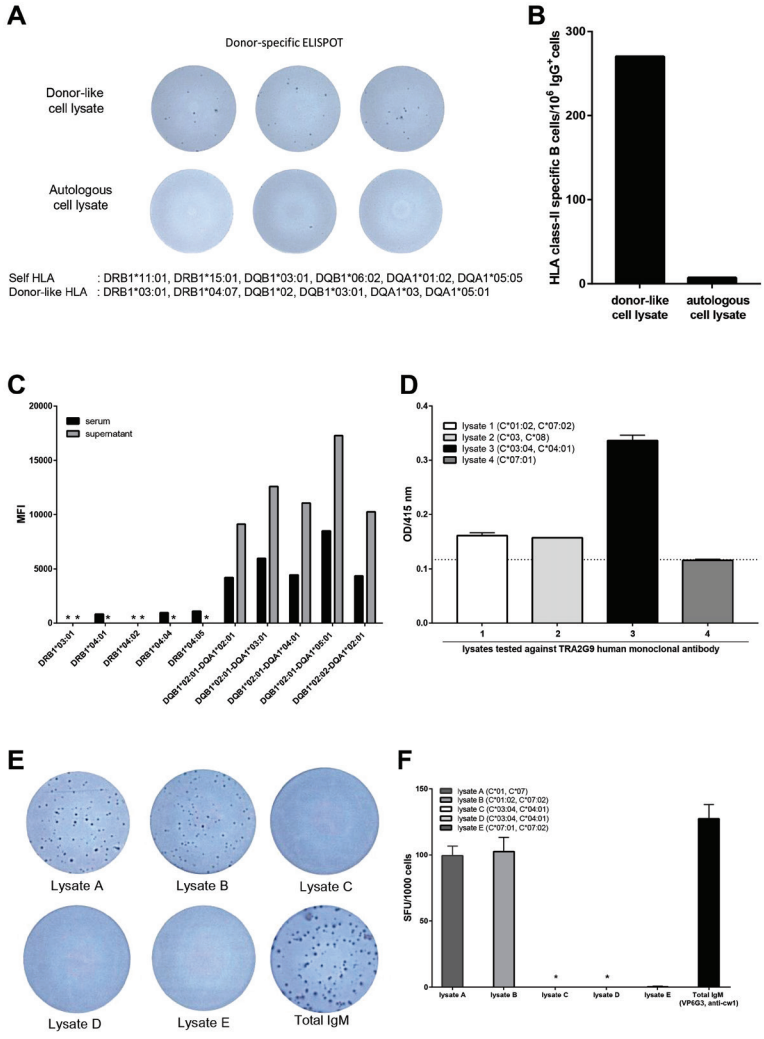
In vitro, polyclonal activation of peripheral blood B cells results in expansion and differentiation of B cells into antibody secreting cells (21). For an accurate estimation of pre-existing memory B cells, a polyclonal activation that does not induce isotype switching in the naïve B cell compartment is necessary. In order to accurately determine pre-existing IgG^{+} memory B cell frequencies, we wanted to assure that the current activation protocol was not inducing any class switching in the naïve B cell population during the 6-day culture period. We designed an experimental setting wherein sorted naïve or memory B cells were combined with sorted non-B cells in a certain ratio (1:9, at a concentration of $2x10^{6}$ cells/well) before polyclonal activation. To this aim, we used highly purified naïve B cells and memory B cells in order to assess the Ig spot production after 6-day polyclonal activation of these cells. Following activation, we found high numbers of IgG spots (median SFU: 244) in the memory B cell population compared to naïve B cell population wherein almost no IgG spot formation was observed (median SFU:1). These results assured that IgG spots were produced only by memory B cells upon polyclonal activation with the current stimuli (Supplementary figure 4).



Supplementary figure 4: IgG spots are produced only by memory B cells upon 6-day in vitro polyclonal activation. *PBMC were sorted into naïve ($CD19^+CD27^-IgD^+$) and memory B cells ($CD19^+CD27^+IgD^-$). Each naïve and memory B cell was combined with non-B cells ($CD19^-CD20^-$) at a ratio of 1 to 9 and polyclonally activated with R848 and IL-2 for 6 days and then transferred to ELISPOT plates at a concentration of 1000 cells per well for the detection of total IgM and IgG producing cells. PBMC from the same donor were treated in the same manner as a control. (A) Total IgM producing cells were observed both in the naïve and memory B cell populations whereas (B) IgG spot production was observed only in memory B cells. Results are expressed as mean±SD of 3 independent experiments performed with 3 different donors. SFU: spot forming units.*

Methods for supplementary figure 5E, 5F

Total Immunoglobulin M (IgM) ELISPOT assays were performed as previously described (17). For donor-specific ELISPOT assays, 96-well PVDF ELISPOT plates (Millipore, Billerica, MA, USA) were coated with 1.8 µg/ml goat anti-human IgM (Jackson Immunoresearch Laboratories Inc., Baltimore, PA, USA) in PBS and incubated overnight at 4°C. Plates were then blocked for at least 1 h with 5% FBS/IMDM at 37°C after which thoroughly washed hybridoma cells (VP6G3) producing HLA-Cw1 antibodies were added at a concentration of 1000 cells/well. After overnight incubation at 37°C, plates were washed and incubated for 4 h with HLA class I cell lysates (4 µl lysate in 50 µl PBS/well) at room temperature on a platform shaker. Following washing, horse radish peroxidase (HRP)-conjugated mouse anti-human beta-2 microglobulin antibody diluted 1:1000 in 1% BSA/Tween 20/PBS (AbD Serotec/BioRad, Puchheim, Germany) was added and incubated for 2 h at room temperature. After washing, plates were incubated with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Mabtech, Nacka Strand, Sweden) to visualize the spots. The reaction was stopped by adding cold tap water and after drying, analysed by an automated ELISPOT reader (Bio-Sys GmbH, Karben, Germany).



Supplementary figure 5: Detection of HLA-DQ and HLA-C antigens in lysate preparations. *(A) HLA-DQ-specific memory B cells can be detected in the lysate-based ELISPOT assay. PBMC from a pregnancy-immunized woman (sample #201.1) was tested against a PBMC donor-like lysate (#13) lacking antibody positive HLA-DR specificities in order to detect solely HLA-DQ-specific memory B cells using the donor-specific memory B cell ELISPOT assay. (B) A frequency of 263 donor-specific memory B cells per million IgG producing cells were detected against the HLA class II present in the lysate whereas only background spots were observed against autologous lysate. (C) Isolated HLA-DQ2 directed antibodies were detected in serum and culture supernatants harvested upon 6-day polyclonal activation by Luminex single antigen bead assays, confirming that spots detected were specific for HLA-DQ. (D) HLA-C molecules are present in lysate preparations. Lysates containing different HLA-C antigens were coated onto ELISA plates by anti-beta-2 microglobulin coating antibody and then tested against a human monoclonal antibody (TRA2G9) recognizing HLA-C*01:02, C*03:02, C*03:03, C*03:04, C*04:01, C*14:01. Reactivity of the monoclonal antibody with corresponding HLA-C antigens in lysates 1, 2 and 3 could be detected using a goat-anti-human IgM antibody. OD: optic density. (E) HLA-C molecules can be detected in ELISPOT format. The HLA-Cw1-specific IgM producing hybridoma VP6G3 was tested against lysates containing various HLA-C molecules (lysate A: C*01, C*07, lysate B: C*01:02, C*07:02, lysate C: C*03:04, C*04:01, lysate D: C*03:04, C*04:01 and lysate E: C*07:01, C*07:02). Only lysates containing HLA-C*01 gave rise to spot formation. (F) Quantification of HLA-C specific ELISPOT assay.*

Supplementary table 1: HLA typing and serum HLA antibody MFI values of the study population

Sample#	Source	HLA type / (serum MFI)
147	female	A3, A28, B*15:01, B35, Cw9, Cw4
lysate	partner	A3 (0), A*11:01 (2807), B*07:02 (12769), B*15:01 (0) Cw10 (0), C*07:02 (0)
148	female	A*02:01, A*11:01, B*15:01, B*56:01, C*01:02, C*03:04
lysate	partner	A*03:01 (0), A*24:02 (1022), B*15:01 (0), B*55:01 (0), C*03 (0, 0, 0)
149	female	A*01, A*02, B*37, B*39, C*06, C*12
lysate	partner	A*24 (1314, 1424), A*32 (3868), B*07 (2544), C*07 (5067)
150	female	A*02:01, A*32:01, B*08:01, B*40:02, C*02:02, C*07:01
lysate	partner	A*01:01, A*68:01, B*08:01, B*51:01, C*07:01, C*15:02
151/207.3	female	A1, A31, B8, B60, Cw10, Cw7, DR4, DQ8
lysate	partner	A*01, A*03, B*07, B*08, C*07, DRB1*15, DQB1*06
152	female	A*02:01, A*11:01, B*08:01, B*40:01, C*03:04, C*07:01
lysate	partner	A*01, B*08:01, B*35, C*07:01
153/207.1	female	A*02, A*03, B*27, B*57, C*02, C*06, DRB1*12:01, DRB1*16:01, DQB1*03:01, DQB1*05:02
lysate	partner	A*01, A*29, B*08, B*44, C*07, C*16, DRB1*03, DRB1*07, DQB1*02

Supplementary table 1: ***Continued***

Sample#	Source	HLA type / (serum MFI)
154/206.3	female	A*02:01, A*11:01, B*15:01, B*56:01, C*01:02, C*03:04, DRB1*04:01, DRB1*16, DQB1*03:02, DQB1*05:02
lysate	partner	A*01:01, A*11:01, B*07:02, B*51:01, C*04:01, C*07:02, DRB1*04:07, DRB1*15:01, DQB1*03:01, DQB1*06:02
155/207.2	female	A*11:01, A*25:01, B*15:01, B*18:01, C*03:03, C*12:03, DRB1*08, DRB1*13:01, DQB1*04:02, DQB1*06:03
lysate	partner	A*02, A*02, B*39, B*40, C*03, C*07, DRB1*08, DRB1*13, DQB1*04, DQB1*06
156	female	A*03:01, A*26:01, B*07:02, B*40:01, C*03:04, C*07:02
lysate	partner	A*03 (0), A*26 (0), B*40 (0, 0), B*53:01 (12843), C*03 (0, 0, 0), C*04:01 (1393)
157/206.1	female	A*02:01, A*26:01, B*37:01, B*40:01, C*03:04, C*06:02, DRB1*04, DRB1*10, DQB1*03:02, DQB1*05:01
lysate	partner	A*03, A*11, B*07:02, B*56:01, C*01, C*07:02, DRB1*04, DRB1*15, DQB1*03:02, DQB1*06:02
158	female	A2, A24, B60, Cw3
lysate	partner	A*11, A*33, B*51, B*52, C*12, C*14
159	female	A*02, A*24, B*07, B*35, C*04, C*07
lysate	partner	A*02, A*03, B*40, C*03
160/206.2	female	A*02, A*29, B*07, B*52, C*12, C*15, DRB1*04, DRB1*15, DQB1*03, DQB1*06
lysate	partner	A*02, A*03, B*35, B*44, C*02, C*04, DRB1*11, DRB1*16, DQB1*03, DQB1*05

Supplementary table 1: *Continued*

Sample#	Source	HLA type / (serum MFI)
106[a]	female	A2, B62, Cw4
lysate	1	A*01:01 (9631), A*03:01 (3033), B8 (6652), B27 (2431, 975), C*02:02 (47), C*07:01 (-)
109	female	A*03:01, B*07:02, B*37:01, C*06:02, C*07:02
lysate	2	A*02:01 (4896), A*11:01 (138), B*35:01 (7007), B*51:01 (5802), C*01:02 (264), C*04:01 (209)
lysate	3	A*02:01 (4896), A*03:01 (165), B*07:02 (190), B*51:01 (5802), C*02:02 (104), C*07:02 (124)
lysate	4	A2 (4896), A28 (2406, 3258, 3244), B62 (3268), B35 (7006), Cw9 (6957), Cw4 (209)
111	female	A*01, A*02:01, B*37:01, B*55:01, C*03:03, C*06:02
lysate	5	A*25:01 (20703), A*68:02 (21534), B*14:02 (170), B*18:01 (446), C*08:02 (-), C*12:03 (9177)
112[b]	female	A2, B51, B7, Cw7
lysate	6	A*30:01 (4784), A*31:01 (4829), B*13:02 (12825), B*47:01 (0), C*06:02 (0)
118[a]	female	A2, B62, Cw4
lysate	7	A*02:01 (314), A*24:02 (7882), B*07:02 (14894), B*27:05 (11486), C*01:02 (4399), C*07:02 (179)
lysate	8	A*01:01 (10730), A*03:01 (11221), B*07:02 (14894), B*35:01 (123), C*04:01 (24), C*07:02 (179)
180	female	A*02 (HLA typing is not available for other loci)
lysate	3	A*02:01 (0), A*03:01 (0), B*07:02 (12223), B*51:01 (0), C*02:02 (1023), C*07:02 (0)
lysate	9	A*02:01 (0), A*30:01 (0), B*13:02 (12840), B*40:01 (12945), C*03:04 (747), C*06:02 (0)

Supplementary table 1: ***Continued***

Sample#	Source	HLA type / (serum MFI)
199	female	DRB1*03:01, DRB1*13:01, DQB1*02, DQB1*06:03
lysate	10	DRB1*04:01 (11388), DRB1*04:04 (12529), DQB1*03:01 (5283, 5519, 8571, 8510, 7644), DQB1*03:02 (5443, 5274, 9975)
lysate	11	DRB1*04:02 (11202), DRB1*11:01 (5059), DQB1*03:01 (5283, 5519, 8571, 8510, 7644), DQB1*03:02 (5443, 5274, 9975)
lysate	12	DRB1*04:01 (11388), DQB1*03:01 (5283, 5519, 8571, 8510, 7644), DQB1*03:02 (5443, 5274, 9975)
201.1[b]	female	DRB1*11:01, DRB1*15:01, DQB1*03:01, DQB1*06:02
lysate	13	DRB1*03:01 (0), DRB1*04:07 (-), DQB1*02 (4183, 5954, 4453, 8502, 4342), DQB1*03:01 (0, 597, 0, 0, 0)
lysate	14	DRB1*07:01 (15364), DRB1*09:01 (10892), DQB1*02:02 (4342), DQB1*03:03 (391, 0, 0)
lysate	15	DRB1*07 (15364), DRB1*11 (0, 0), DQB1*02 (4183, 5954, 4453, 8502, 4342), DQB1*03:01 (0, 597, 0, 0, 0)
lysate	16	DRB1*12:01 (6473), DRB1*13:01 (0), DQB1*03:01 (0, 597, 0, 0, 0) , DQB1*06:03 (0)
201.2[b]	female	DRB1*11:01, DRB1*15:01, DQB1*03:01, DQB1*06:02
lysate	13	DRB1*03:01 (246), DRB1*04:07 (-), DQB1*02 (2088, 2666, 2119, 3659, 2225), DQB1*03:01 (499, 626, 770, 869, 843)
lysate	14	DRB1*07:01 (6956), DRB1*09:01 (4115), DQB1*02:02 (2225), DQB1*03:03 (395, 155, 849)
lysate	15	DRB1*07 (6956), DRB1*11 (0, 336), DQB1*02 (2088, 2666, 2119, 3659, 2225), DQB1*03:01 (499, 626, 770, 869, 843)
lysate	16	DRB1*12:01 (1655), DRB1*13:01 (0), DQB1*03:01 (499, 626, 770, 869, 843) , DQB1*06:03 (1306)
202	female	DR3, DR12, DQ2, DQ7
lysate	17	DRB1*01:01 (19607), DRB1*04:01 (12290), DQB1*03:01 (239, 66, 200, 53, 204), DQB1*05:01 (7225)
lysate	18	DRB1*03:01 (7), DRB1*11 (129, 155), DQ2 (4, 150, 10, 0, 0), DQ5 (7225, 4513)
lysate	19	DRB1*01:01 (19607), DRB1*14:54 (382), DQB1*05:01 (7255), DQB1*05:03 (-)

Supplementary table 1: *Continued*

Sample#	Source	HLA type / (serum MFI)
lysate	20	DRB1*09:01 (6711), DRB1*10:01 (7731), DQB1*03:03 (337, 366, 1537), DQB1*05:01 (7225)
203[a]	female	DRB1*13:01, DQ6
lysate	17	DRB1*01:01 (20618), DRB1*04:01 (12459), DQB1*03:01 (861, 711, 1439, 1208, 1238), DQB1*05:01 (1148)
lysate	19	DRB1*01:01 (20618), DRB1*14:54 (616), DQB1*05:01 (1148), DQB1*05:03 (-)
lysate	20	DRB1*09:01 (12847), DRB1*10:01 (13337), DQB1*03:03 (583, 591, 1569), DQB1*05:01 (1148)
205	female	DR9, DR15, DQ2, DQ6
lysate	14	DRB1*07:01 (17708), DRB1*09:01 (11), DQB1*02:02 (4293), DQB1*03:03 (5812, 0, 0)
lysate	15	DRB1*07 (17708), DRB1*11 (470, 479), DQB1*02 (4627, 67, 15, 223, 4293), DQB1*03:01 (0, 6880, 0, 0, 28)
lysate	21	DRB1*03 (4881, 4569), DRB1*07 (17708), DQB1*02 (4627, 67, 15, 223, 4293)

**Mean fluorescence intensity (MFI) values in parenthesis are given for each single antigen bead corresponding to that HLA specificity contained in the lysate*