HLA-specific memory B cells : the missing link?

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Chapter 5

Quantification of HLA class II-specific memory B cells in HLA-sensitized individuals

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ABSTRACT

For the quantification of HLA-specific memory B cells from peripheral blood of sensitized individuals, a limited number of methods are available. However, none of these are capable of detecting memory B cells directed at HLA class II molecules. Since the majority of antibodies that occur after transplantation appears to be specific for HLA class II, our aim was to develop an assay to detect and quantify HLA class II-specific memory B cells from peripheral blood.

By using biotinylated soluble HLA class II molecules as detection agent, we were able to develop an HLA class II-specific memory B cell ELISPOT assay. The assay was validated using B cell-derived hybridomas that produce human monoclonal antibodies directed at specific HLA class II molecules. In pregnancy-immunized females, we found memory B cell frequencies ranging from 25 to 756 spots per 10^6 B cells specific for the immunizing paternal HLA class II molecules, whereas in non-immunized males no significant spot formation was detected.

Here, we present a novel ELISPOT assay for quantifying HLA class II-specific memory B cells from peripheral blood. This technique provides a unique tool for monitoring the HLA class II-specific memory B cell pool in sensitized transplant recipients.

INTRODUCTION

Pre-existing as well as *de novo* produced HLA antibodies represent an important risk factor for adverse transplantation outcome (1). In the last decade, major advances in HLA antibody detection have enabled a clear definition of antibody specificities in the serum of transplant recipients, and thereby improved the prediction of immunologically low and high-risk patients (2-4). However, none of the currently available methods to detect serum HLA antibodies provide information on the presence or absence of HLA-specific memory B cells. Since serum antibodies are mainly produced by bone marrow residing plasma cells, serum antibody levels may not be representative for the size of the peripheral memory B cell pool (5, 6). Upon a re-encounter with antigen, memory B cells can rapidly differentiate into antibody secreting cells to drive anamnestic responses (7). Therefore, while serum antibody levels may be undetectable prior to transplant, accelerated antibody mediated rejection can occur in case memory B cells directed at donor HLA antigens are present (8). Furthermore, apart from antibody production, memory B cells are potent cytokine producing cells (9) and can also function as antigen presenting cells (10), potentially driving the rejection process by activating alloantigen specific T cells. Determining the level of HLA- specific memory B cells pre-transplant would benefit the risk assessment for early humoral rejection, whereas monitoring these cells post-transplant may provide a better understanding on how B cells may affect transplant outcomes in other ways than by antibody production (11).

As a result of the technical challenge of detecting and enumerating the relatively low numbers of HLA-specific memory B cells in peripheral blood, only a few studies exist aiming at quantification of HLA-specific memory B cells in sensitized individuals (12-18). Assays determining HLA antibody production capacity by *in vitro* polyclonal B cell activation (12, 19), as well as HLA class I tetramer staining of $CD19^+$ B cells (13-15) have previously been described by us and others. Though useful, these assays are limited to the detection of HLA class I-specific memory responses in sensitized individuals. Importantly, recent evidence suggests that the predominance of HLA antibodies developing post kidney transplantation is directed at HLA class II (2, 20, 21). Therefore, we aimed at developing an assay capable of detecting and quantifying HLA class II-specific memory B cells from peripheral blood. Previously, we have developed an HLA class I-specific B cell ELISPOT assay by combining the ability of polyclonally activated B cells to produce HLA antibodies *in vitro* with the potential of these antibodies to bind HLA tetramers (17). In the current study, we adapted and validated this technique for the detection of HLA class II-specific memory B cells. We show that this newly developed assay allows for the quantification of HLA class II-specific memory B cells in sensitized individuals.

MATERIALS AND METHODS

Subjects

The study population consisted of pregnancy immunized women and non-immunized males. Pregnancy immunized individuals (n=6) were assessed for the presence of peripheral HLA class II-specific memory B cells against a total of 18 immunizing and/or self HLA class II molecules. For the same purpose, B cells isolated from peripheral blood mononuclear cells (PBMCs) of non-immunized healthy males (n=3) were tested against a total of 6 self as well as non-self HLA class II molecules.

Cells

Peripheral blood was obtained with informed consent under guidelines issued by the Medical Ethics Committee of the Leiden University Medical Center (Leiden, the Netherlands). 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 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.

In order to optimize and validate the HLA class II-specific memory B cell ELISPOT assay, previously described human B cell hybridomas producing class I and class II anti-HLA monoclonal antibodies (mAbs) were used (22): GV5D1 (IgG, anti-HLA-A1/9), SN607D8 (IgG, anti-HLA-A2/28), RTLK10E12 (IgG, anti-HLA-DR11) and RTLK1E2 (IgG, anti-HLA-DR3/8/11/12/13/14).

Biotinylated soluble HLA class I and class II molecules

Synthetic biotinylated HLA class I monomers were constructed around various peptide sequences (Table 1) as described previously (17, 23). For HLA class II, constructs were made by deletion of the transmembrane domains of the alpha and beta chains of HLA-DR and HLA-DQ molecules and replacing a 7 amino acid linker followed by leucine zipper ACIDp1 for alpha chains and leucine zipper BASEp1 for beta chains. These constructs were cloned into the mammalian expression vector pcDNA3.1(–) and expressed in B-lymphoblastoid cell lines (B-LCL) by transfection, as described elsewhere (24). Secreted HLA class II molecules were used in biotinylated form. Table 1 shows the list of biotinylated HLA class I and class II molecules used.

Table 1: Biotinylated HLA class I and class II molecules

HLA	coupled HLA allele	clone designation	peptide sequence
DRB1*01:01	DRA1*01:01	C5B6	-
DRB1*04:05	DRA1*01:01	#3.14	-
DRB1*07:01	DRA1*01:01	D3C2	-
DRB1*09:01	DRA1*01:01	C2G5	-
DRB1*11:01	DRA1*01:01	H9/III	-
DRB1*13:03	DRA1*01:01	#4.8	-
DRB1*15:02	DRA1*01:01	B12	-
DQB1*02:01	DQA1*02:01	E12G8	-
DQB1*03:01	DQA1*02:01	C6E3	-
DQB1*03:02	DQA1*02:01	C9A8	-
DQB1*03:03	DQA1*02:01	E2C9	-
DQB1*06:02	DQA1*01:01	C3F10	-
A*01:01	-	-	EVDPIGHLY
A*02:01	-	-	YLEPGPVTA

Cell cultures

Purified B cells were activated for 6 days at $2.5x10^5$ cells/well in 24-well flat bottom plates (Corning Incorporated, Corning, NY). 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 500 ng/ml α-CD40 mAb (R&D systems, Minneapolis, MN, USA), 2.5 µg/ml Toll-like receptor-9 (TLR-9) ligand oligodeoxynucleotides (ODN)-2006 CpG (Hycult Biotechnology, Uden, the Netherlands), 600 IU/ml IL-2 (Proleukin, Amsterdam, the Netherlands), 25 ng/ml IL-10 (R&D systems, Minneapolis, MN, USA) and 100 ng/ml IL-21 (Gibco Invitrogen), as described previously (25), and cultured at 37°C in a 5% CO_2 humidified incubator. At day 6, supernatants were collected and 10-fold concentrated using 0.5 ml centrifugal filter units (Amicon Ultra, Merck Millipore Ltd), and frozen at-20°C until further use. Cells were harvested and thoroughly washed.

ELISPOT assays

We performed total IgG ELISPOT assays as previously described (26). For the HLA class II-specific ELISPOT assays, we coated 96-well ELISPOT plates (Millipore, Billerica, MA, USA) with 5 µg/ml goat anti-human IgG (Jackson Immunoresearch Laboratories Inc., Baltimore, PA, USA) in phosphate buffered saline (PBS) and incubated overnight at 4°C. Following blocking

for at least 1 h with 5% FCS/IMDM at 37°C, thoroughly washed activated B cells ($2.5x10^5$ cells/well) or hybridoma cells (500 or 1000 cells/well) were added to each plate. After overnight incubation at 37°C, plates were washed and incubated for 4 h with biotinylated HLA class II molecules (1000 ng/ml) at room temperature on a platform shaker. Following washing, plates were incubated with streptavidin-conjugated alkaline phosphatase (Sigma-Aldrich) for 1 h at room temperature. BCIP/NBT substrate (Mabtech, Nacka Strand, Sweden) was added to visualize the spots. The reaction was stopped by cold tap water and after drying, analyzed by an automated ELISPOT reader (Bio-Sys GmbH, Germany).

To correct for non-specific background spots, we included wells without adding the HLA molecules at the detection step. Spots, if any, were subtracted from the spot numbers counted in the wells with HLA molecules added. The ratio of HLA class II-specific B cells per total input B cells was calculated as described previously (17).

HLA typing and anti-HLA antibody detection

HLA-DRB1 and-DQB1 genotyping was performed by PCR-SSO technique, whereas HLA-DQA1 genotyping was performed by PCR-SSP. HLA class II antibody specificities, both in sera and B cell culture supernatants from pregnancy immunized individuals were identified by Luminex using LabScreen HLA class II single antigen beads (One Lambda, Canoga, USA). Samples from non-immunized males were screened for anti-HLA antibodies using Lifecodes Lifescreen Deluxe kit (Immucor Transplant Diagnostics, Stamford, USA).

Statistics

The Mann-Whitney *U* test was used for comparison of the results for immunizing and self-HLA class II molecules. Linear regression analysis and Pearson correlation tests were performed to analyse the ELISPOT reproducibility as well as the calculation of coefficient variation (% CV) from duplicate measurements. Statistical level of significance was defined as $p< 0.05$.

RESULTS

Soluble HLA molecules can be used as detection agents in ELISPOT assays

We have previously described an HLA class I-specific B cell ELISPOT assay in which HLA molecules of interest were directly coated onto ELISPOT plates. When we attempted a similar strategy for the secreted HLA class II molecules, we observed high background levels and large, blurry spots (data not shown), similar to a previously described HIV-specific ELISPOT assay (27). To overcome this problem, we determined whether HLA molecules could be used at the detection level instead. To this aim, we first tested HLA class I monomers as detection agent in our established HLA class I-specific ELISPOT system for detection of HLA-specific B cell hybridoma cells. Since we expected lower HLA monomer concentrations to be

sufficient at the detection stage compared to coating, we tested several concentrations of HLA class I monomers.

When hybridoma cells were tested for spot formation against their corresponding HLA class I molecules used in the detection phase of the assay, similar spot numbers were found in the HLA-specific ELISPOT compared to the total IgG ELISPOT (Figure 1A). Spot numbers remained comparable for all monomer concentrations tested. The detection of HLA spots was specific, since for an irrelevant HLA class I molecule spot numbers ranged from 0 to 2 per 10^3 cells (Figure 1A).

Having shown that comparable number of spots could be detected in total IgG ELISPOT and HLA class I-specific ELISPOT assay with lower concentrations of HLA molecules at the detection step compared to monomer coating, we tested whether HLA class II molecules could also be used in the detection phase of the ELISPOT assay. To this aim, we tested two concentrations (1000 ng/ml and 2500 ng/ml) of HLA-DRB1*11:01 and HLA-DRB1*13:03 molecules for the detection of the anti-HLA-DR11 producing hybridoma cells (RTLK10E12). We found no spots against the irrelevant HLA-DRB1*13:03, whereas a clear spot formation against HLA-DRB1*11:01 was visible, with comparable spot numbers when using HLA class II molecules at both concentrations (Figure 1B, left panel). Importantly, the number of spots detected against HLA-DRB1*11:01 were comparable to those detected by total IgG ELISPOT, indicating that all cells producing anti-HLA-DR11 antibodies were detected. Additionally, we confirmed the specificity of the HLA class II-specific ELISPOT assay with another hybridoma (RTLK1E2) producing antibodies against an epitope shared by HLA-DR3/8/11/12/13/14. Expectedly, we were able to detect spots against specific HLA molecules HLA-DRB1*11:01 and HLA-DRB1*13:03, and no spots against the irrelevant HLA class II molecule HLA-DRB1*09:01 (Figure 1B, right panel). Comparable numbers of HLA-DRB1*11:01 and HLA-DRB1*13:03 specific spots and total IgG spots within the same experiment assured that all HLA class II antibody producing hybridoma cells were detected by the HLA class II-specific ELISPOT assay with corresponding specificities.

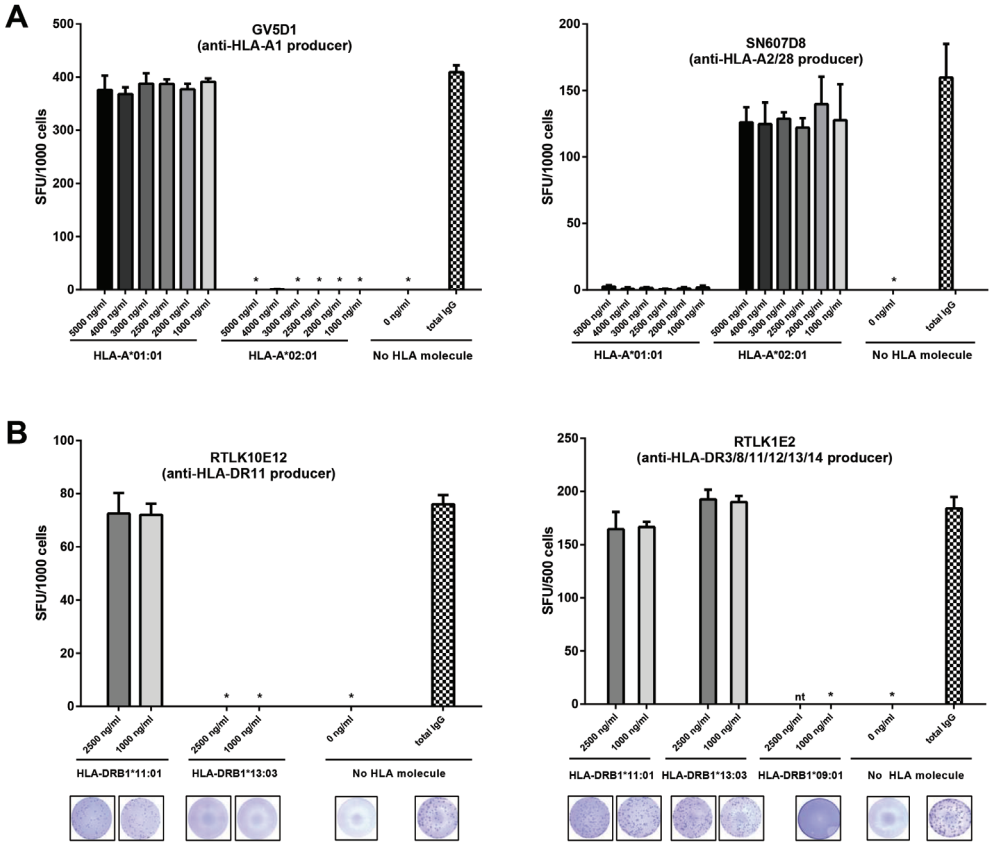

Figure 1. Soluble HLA molecules can be used as detection agents in ELISPOT assays. *Comparable number of spots were detected by using 1000 ng/ml biotinylated HLA molecules as detection agents in the HLA-ELISPOT and total IgG ELISPOT assay (checkered bars) within the same experiment (A) Biotinylated HLA-A*01:01 and A*02:01 molecules at varying concentrations were used as detection agent to detect anti-HLA-A1 (GV5D1, left panel) and anti-HLA-A2 (SN607D8, right panel) producing hybridoma cells, respectively. (B) HLA class II mAb-producing hybridoma cells RTLK10E12 (left panel) and RTLK1E2 (right panel) were tested against relevant and irrelevant HLA class II molecules. Spots were detected against the corresponding specificity of HLA molecules whereas no spots were detected against irrelevant HLA specificities or when no soluble HLA molecules were added. Representative results of one of 2 experiments performed with different HLA mAb-producing cells are shown. Error bars represent standard deviation of triplicate wells. SFU: spot forming units: *: no HLA-specific spots detected, nt: not tested.*

The HLA class II-specific ELISPOT assay detects HLA specific B cells in a reproducible fashion

The reproducibility of the ELISPOT assay was determined by repeating the experimental procedures with frozen PBMCs of the same bleeding date from 3 pregnancy-immunized individuals at different days. Reproducibility was tested by quantifying HLA class II-specific B cells against immunizing HLA class II molecules, as well as self HLA-DRB1 and HLA-DQB1 molecules. Figure 2 shows that the HLA class II-specific memory B cell ELISPOT assay is highly reproducible (R^2=0.9187, p<0.0001) (% CV: 28%).

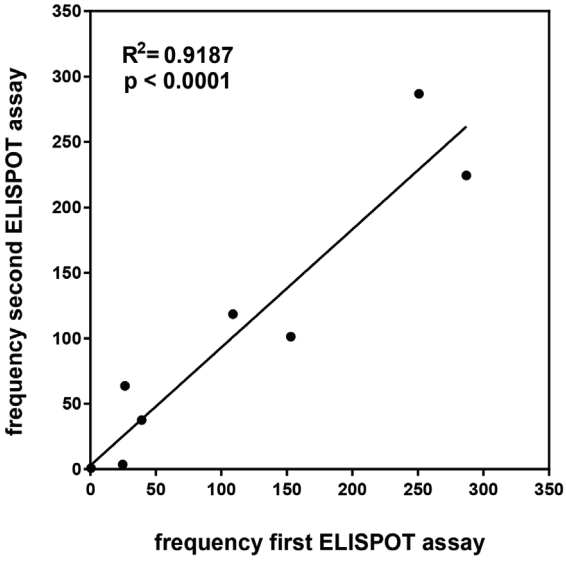

Figure 2. Reproducibility of the HLA class II-specific ELISPOT assay. *PBMCs from three pregnancy-immunized individuals were tested twice in separate experiments for the presence/absence of HLA class II-specific B cells against immunizing and self HLA-DRB1/HLA-DQB1 molecules. Each sample was tested against two HLA-DRB1 and two HLA-DQB1 molecules. The frequency of HLA class II-specific B cells against a total of five self-HLA class II molecules was zero. Coefficient of variation (% CV): 28%.*

To assure that spots detected in the ELISPOT assay were truly formed against HLA class II molecules, we tested supernatants from the B cell pre-culture by the Luminex class II single antigen bead assay (19). In all cases where we found spots against immunizing HLA molecules, we also detected the corresponding specificity of HLA antibodies in the supernatants of the B cell cultures. No antibodies were detected against self-HLA molecules. Furthermore, B cell culture supernatants from non-immunized individuals were also found to be negative for HLA class II antibodies by the Luminex assay.

Detection of HLA-DR specific memory B cells in HLA immunized individuals

Having established the HLA class II-specific ELISPOT methodology, we wanted to determine the frequency of HLA class II-specific memory B cells in the peripheral blood of pregnancy-immunized individuals. Therefore, we tested B cells from pregnancy-immunized individuals against immunizing HLA class II molecules and self-HLA class II molecules. As negative control, B cells from non-immunized individuals were tested against self and non-self HLA class II molecules.

In six pregnancy-immunized individuals (three immunized against DRB1*04, two immunized against DRB1*07 and one immunized against DRB1*11), we detected spots against immunizing HLA-DRB1 molecules at frequencies ranging from 25 to 756 spots per 10^6 B cells (median=69) (exemplified in Figure 3A and 3B). HLA class II-specific B cell frequencies showed a wide range, likely representing different levels of immunization and time since immunization (range: 3-180 months). Importantly, when we tested PBMCs from non-immunized males, the frequency of the spots against self or non-self HLA class II molecules ranged from 0-2 per 10^6 B cells (Figure 3C).

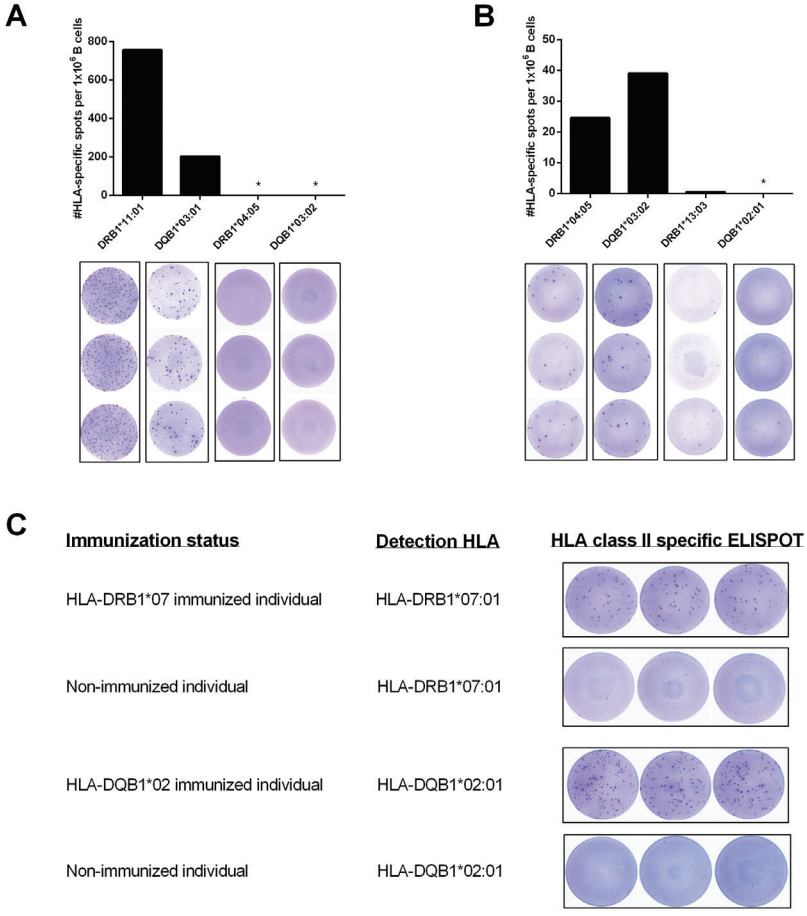

Figure 3. The HLA class II-specific ELISPOT assay is highly specific. *B cells from two pregnancy-immunized individuals sampled 4 months after delivery (3A, donor 3) and 15 years after delivery (3B, donor 4) had different HLA-specific memory B cell frequencies for different HLA molecules. Spots were detected against the HLA of the child; however no significant spots were detected against self-HLA molecules. (C) HLA class II-specific memory B cells were detected in HLA immunized individuals but not in non-immunized males. A frequency of 109 spots were detected in samples from a DRB1*07 immunized individual (donor 5) against HLA-DRB1*07:01 molecule whereas in a non-immunized individual (donor 9) no spots were detected against the same HLA molecule. Similarly, a frequency of 251 spots was detected in a DQB1*02 immunized individual (donor 5) against HLA-DQB1*02:01 molecule while the frequency of HLA-DQB1*02:01 specific B cells in a non-immunized individual (donor 7) was 2 per 10^6 B cells. *: no HLA-specific spots detected.*

The significantly higher number of HLA-specific B cells directed at immunizing HLA-DRB1 molecules (25 - 756 spots per 10^6 B cells) compared to self or non-immunizing HLA-DRB1 molecules (0- 1 spots per 10^6 B cells) in immunized individuals demonstrated the specificity of the assay (p= 0.0007, Figure 4A and Table 2).

Detection of HLA-DQ specific memory B cells in HLA immunized individuals

Similar to HLA-DR specific memory B cells, we tested B cells from four immunized individuals for the detection of HLA-DQB1-specific B cells (two immunized against DQB1*03:01, one immunized against DQB1*03:02 and one immunized against DQB1*02:01). In these individuals we found frequencies of HLA-DQB1 specific cells against immunizing HLA-DQB1 ranging from 39 to 287 spots per 10^6 B cells (median= 227), (Figure 4B and Table 2).

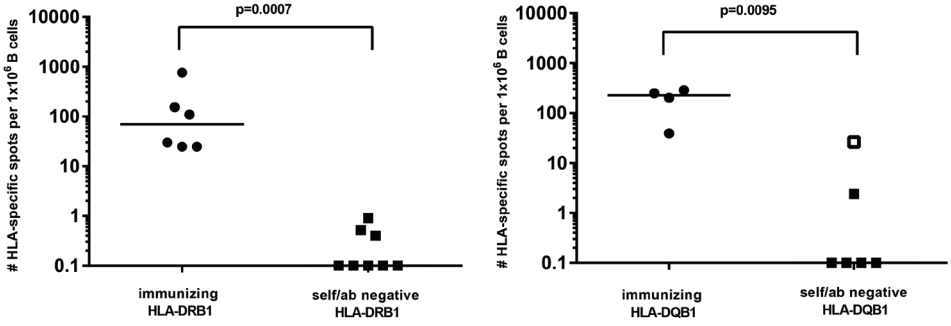

Figure 4. Frequencies of HLA class II-specific memeory B cells detected by ELISPOT. *The number of spots directed at immunizing (A) HLA-DRB1 and (B) HLA-DQB1 molecules was significantly higher than the number of spots against self or antibody negative HLA molecules. The open dot in (B) is the unexpectedly high frequency of 27 spots per 10^6 B cells for a self HLA-DQB1 molecule. Bars indicate the median in each group. ab: antibody.*

Table 2: Characteristics of the study population

Donor	Immunization status	Sampling time after last delivery	Self/child HLA	Serum ab[a]	Detection HLA molecule	Frequency[b]	MFI supernatant[c]
1	immunized	12 years	unknown	yes	DRB1*04:05	25	1848
2	immunized	10 years	Self: DR9, DR15	yes	DRB1*07:01	30	7139
3	immunized	4 months	Self: DRB1*04, DQB1*03:02, DQB1*04:02	yes	DRB1*11:01	756	16615
			(Child: DRB1*04, DRB1*11, DQB1*03:01, DQB1*04:02)	yes	DQB1*03:01	203	15967
				no	DRB1*04:05	0	0
				no	DQB1*03:02	0	0
4	immunized	15 years	Self: DRB1*03:01, DRB1*13:01, DQB1*02, DQB1*06:03	yes	DRB1*04:05	25	3415
			(Child: DRB1*04, DRB1*13:01, DQB1*03:02, DQB1*06:03)	yes	DQB1*03:02	39	6250
				no	DRB1*13:03	1	34
				no	DQB1*02:01	0	0
5	immunized	2 years	Self: DRB1*11:01, DRB1*15:02, DQB1*03:01, DQB1*06:02	yes	DRB1*07:01	109	11991
			DQA1*01:02, DQA1*05:05	yes	DQB1*02:01	251	12923
				no	DRB1*15:02	0	0
				no	DQB1*03:01	27	1621*
6	immunized	3 months	Self: DRB1*13:01, DRB1*15, DQB1*06:02, DQB1*06:03	yes	DRB1*04:05	153	9346
			(Child: DRB1*04:02, DRB1*13:01, DQB1*03:01, DQB1*06:03)	yes	DQB1*03:01	287	19337
				no	DRB1*15:02	0	0
				no	DQB1*06:02	0	0

Table 2: ***Continued***

Donor	Immunization status	Sampling time after last delivery	Self/child HLA	Serum ab[a]	Detection HLA molecule	Frequency[b]	MFI supernatant[c]
7	non-immunized	-	DQB1*02:01, DQB1*06:04	nt[d]	DQB1*02:01	2	0
				nt	DQB1*03:03	0	0
8	non-immunized	-	DRB1*11:01, DRB1*15:01	nt	DRB1*11:01	0	0
				nt	DRB1*09:01	1	0
9	non-immunized	-	DRB1*07:01, DRB1*13:02	nt	DRB1*07:01	0	0
				nt	DRB1*01:01	0	0

[a] HLA antibodies in the serum belonging to the same date of cell collection for ELISPOT experiments, determined by Luminex single antigen bead assay (for donor 5, serum data is 6 months after cell collection).
[b] Total number of HLA-specific spots per 10^6 B cells.
[c] B cell culture supernatants were tested by Luminex for the presence of HLA antibodies using HLA class II single antigen beads in samples of pregnancy immunized individuals.
[d] Sera from non-immunized males were not available for testing (nt=not tested).
**In contrast to all other supernatants, in this particular supernatant only one out five HLA-DQB1*03:01 beads showed reactivity. Positive reaction was only found for HLA-DQB1*03:01 bead that is coupled to HLA-DQA1*02:01. All the remaining HLA-DQB1*03:01 beads coupled to other HLA-DQA1 molecules were negative.*

In addition, we found no spots against the self HLA-DQB1 molecules in 3 out of 4 individuals. Strikingly, in individual 5 (Table 2), we detected HLA-specific B cells against the self-HLA molecule HLA-DQB1*03:01 at an unexpectedly high frequency of 27 per 10^6 B cells (Figure 4B, open dot). Since all but one of the synthetic HLA molecules available for testing in the ELISPOT setting were composed of an HLA-DQB1 chain coupled to DQA1*02:01, reactivity could potentially be directed towards the DQA1 chain or the combination of self DQB1 and non-self DQA1. Therefore, we typed this individual for HLA-DQA1, resulting in the HLA-DQA1 types DQA1*01:02, DQA1*05:05. When we elaborated on this finding, we found detectable mean fluorescence intensity (MFI) values by Luminex class II single antigen bead assay in serum (MFI: 1307; signal to background ratio: 3) and culture supernatant (MFI: 1621; signal to background ratio: 11) samples for one particular bead coated with self HLA-DQB1*03:01 and non-self HLA-DQA1*02:01. The remaining four DQB1*03:01 coated beads coupled to DQA1 molecules other than HLA-DQA1*02:01 were negative. All of the beads coated with self HLA-DQA1*01:02 and-DQA1*05:05 were negative as well. Altogether, these data suggest that this rather high frequency against self HLA-DQB1*03:01 in one sample was resulting from an HLA-specific B cell population against non-self HLA-DQA1*02:01 allele that was coupled to the soluble HLA-DQB1*03:01 molecule used in the ELISPOT assay.

DISCUSSION

Current available methods to quantify HLA-specific memory B cells in alloantigen-sensitized patients are limited by their ability to detect solely HLA class I-specific B cells (13, 15, 17). According to our knowledge, in this study we present for the first time an assay capable of specifically quantifying HLA class II-specific memory B cells in sensitized individuals. The assay is based on a very sensitive and practical ELISPOT platform, allowing for detection of low level of HLA class II-specific memory B cells from peripheral blood.

Previously, we have utilized an HLA class I-specific memory B cell assay in which PBMCs depleted of $CD2^+$ T cells from sensitized individuals were polyclonally activated *in vitro* and assayed in HLA class I monomer-coated ELISPOT plates (17). Recently, Lynch *et al*. showed the presence of donor-specific memory B cells directed towards donor HLA in the absence of donor-specific serum IgG antibodies in kidney transplant recipients by ELISPOT using donor-derived fibroblasts as the HLA target (18). Although this is an elegant method to track donor specific B cell responses, the source of donor material and long culture time of fibroblasts (30 days) pose a potential barrier for its application in routine clinical monitoring. Furthermore, considering that fibroblasts express only HLA class I molecules, such an assay does not allow for the estimation of HLA class II-specific B cell frequencies.

Despite recent advances in transplantation, the long-term outcome of transplanted organs remains affected by chronic rejection. Using highly sensitive and specific Luminex single antigen bead assays, several groups reported high incidences of post-transplantation *de novo* donor-specific antibody (DSA), particularly directed at mismatched HLA class II molecules

(2, 20). These class II antibodies were shown to be a risk factor for late kidney allograft failure (21, 28). In a re-transplant cohort of 112 adult recipients, Worthington *et al*. showed a detrimental role for DSA directed at HLA class II, resulting in late graft damage, while class I DSA seemed to be associated more with early graft failure (29). Since there may be a difference in the development of humoral immunity towards HLA class I and class II in time after transplantation, assays to analyse both the HLA class I- and class II-specific memory B cell compartments are of particular interest.

In the current HLA class II-specific ELISPOT assay, we used highly purified B cells from PBMCs for the detection of HLA class II-specific memory B cells. We have recently shown that the current polyclonal B cell activation protocol could reliably be used for estimating the percentage of pre-existing antigen-specific B cells, since no antigen-specific IgG spots were detected in naïve B cells following a 6-day activation (25). The finding that no spots were detected in non-immunized individuals against self or non-self HLA class II molecules in the present study further assured that the detected spots were formed by memory B cells. Regarding the ELISPOT phase of the assay, we chose to use HLA class II molecules as the detection matrix instead of the coating agent. Besides the fact that clear spot formation was only observed when this strategy was used, it has the additional benefit that a lower concentration of HLA molecules can be used (27).

By using this method, we found up to 756 HLA class II-specific B cells (median: 130) per 10^6 B cells in HLA immunized individuals. Previously, we have found a median frequency of 43 (range: 0-182) HLA class I-specific B cells per 10^6 B cells in pregnancy immunized individuals by HLA class I-specific ELISPOT (17). Whether HLA class II immunization leads to more profound memory formation compared to HLA class I needs to be further elucidated. In a follow-up study, we plan to determine the level of immunization for both HLA class I and class II within patients immunized by both pregnancy and transplantation.

Similar to solid phase antibody assays, we observed that HLA-DQ molecules may give rise to unexpected results due to the polymorphic nature of the HLA-DQA1 chain in addition to the HLA-DQB1 chain. Tambur *et al*., (30) have shown the presence of DQ antibodies directed at Luminex beads coated with self HLA-DQB1 combined with a non-self HLA-DQA1 and pointed out the underestimation of the role of HLA-DQA1 in the detection of antibodies in Luminex assays. Similarly, in the present study, we found spots against a self HLA-DQB1*03:01 molecule in one sample from a pregnancy-immunized individual, which are likely due to antibody producing memory B cells directed at the non-self DQA1*02:01 allele coupled to a self HLA-DQB1*03:01 molecule. Whether this individual produced antibodies directed against an epitope solely on the DQA1*02:01 chain or an epitope formed by the combination of non-self DQA1*02:01 and self HLA-DQB1*03:01 is beyond the scope of this paper (31). Regardless, our results indicate that in future studies, soluble HLA-DQ molecules comprising of several combinations of HLA-DQB and HLA-DQA chains should be tested in order to overcome this limitation.

The high reproducibility rate of the assay and availability of biotinylated soluble HLA class II molecules covering a wide range of HLA class II specificities support the utilization of the assay in the clinical setting. A volume of 10-15 ml peripheral blood sample from the patients which is routinely collected for bio-banking in many transplant centers is sufficient to perform the assay. It should be noted that even if all combinations of the available soluble HLA molecules are used, the donor specific HLA-repertoire may not be completely represented. In conclusion, we have developed a highly specific, sensitive and reproducible ELISPOT assay enabling the quantification of the HLA class II-specific memory B cells from peripheral blood, which may become a useful tool for pre-transplant risk assessment in sensitized transplant recipients.

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