HLA-specific memory B cells : the missing link?

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Aan mijn ouders

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Chapter 1

General introduction

THE IMMUNE SYSTEM

The immune system has evolved to discriminate between self and pathogenic non-self in order to enable elimination of the foreign intruder. This defense system is devised by innate and adaptive immune responses with an extensive cross-talk between the two. In addition to the defense provided by physical barriers (skin and mucosal epithelium) and soluble mediators such as the complement system, the cellular effectors of the innate immune system (granulocytes, mast cells, macrophages, dendritic cells and NK cells) provide the first line of defense against pathogens through germ-line encoded pattern recognition receptors that sense conserved molecular structures in and on microorganisms (1). Innate immune cells rapidly exert their functions in a generic way against pathogens, whereas cells of the adaptive immune system (T and B lymphocytes) provide a relatively slower but more specific response through their somatically rearranged receptors. A feature unique for the adaptive immune system is the capacity to develop immune memory (2-4). Upon re-encountering the same antigen, the adaptive immune system manifests a much more rapid and stronger response compared to the primary response. Although innate immune cells are known to lack the ability to learn from primary antigenic exposure, recent reports suggest memory cell-like functions also for activated NK cells (5, 6).

An effective immune response depends on the interplay between innate and adaptive immune system components. While B cells can directly recognize epitopes on intact antigens with their B cell receptors, T cells recognize processed peptides of the antigen presented through major histocompatibility complex (MHC) molecules. $CD4^+$ T cells recognize peptides in the context of MHC class II on antigen presenting cells (APCs), such as dendritic cells, macrophages and B cells. $CD8^+$ T cells (cytotoxic T cells) can directly kill target cells upon binding of their T cell receptor (TCR) to MHC class I/peptide complexes, whereas the majority of $CD4^+$ T cells (helper T cells) binding to MHC class II/peptide complex are responsible for providing help to cytotoxic T cells to become fully activated, and B cells for antibody production and class switching. Not all $CD4^+$ T cells are driving immune responses as inhibiting $CD4^+$ T cells, or regulatory T cells have also been described (7).

HLA and ALLOIMMUNE RESPONSES

MHC proteins in humans are encoded by the highly polymorphic human leukocyte antigen (HLA) genes located on chromosome 6p23 region (8). There are 2 classes of HLA molecules, class I and class II, which are heterodimeric transmembrane glycoproteins both functional in presenting peptides to T cells. Extracellular domains of the HLA molecules responsible for peptide binding and interaction with variable regions of the TCRs are highly polymorphic, facilitating the binding of a wide variety of peptides. This extensive polymorphism in HLA alleles ensures that populations will not succumb to new pathogens since a wide range of processed foreign peptides can be presented to the TCR to generate an immune response (9). While contributing to effective adaptive immune responses against a variety of pathogens, the polymorphism of HLA represents a hurdle in allogeneic transplantation. The enormous polymorphism in HLA as evident with currently more than 16000 HLA class I and class II alleles, makes it almost impossible to find a fully HLA-matched unrelated donor for a recipient (10). Disparities in HLA alleles between recipient and donor enhance the recognition of the graft as non-self by the host and may initiate the graft destruction process. The classical way of pathogen encoded antigen recognition by $CD4^+$ T cells also applies to the recognition of alloantigens, namely the indirect allorecognition. Through this pathway, T cells recognize graft MHC alloantigens that are processed into peptides and presented by APCs. A second way of allorecognition, which is unique to transplantation, is the direct recognition of the intact MHC alloantigen on the surface of the donor APCs. While indirect recognition plays an essential role in chronic allograft failure due to the involvement of $CD4^+$ T helper cells in alloantibody generation, the direct pathway of allorecognition is considered to be principal contributor of cytotoxic T cell responses mediating early rejection episodes (11, 12).

In the setting of solid organ transplantation, HLA mismatches between patient and donor evoke both the cellular and the humoral arms of the adaptive immune response in a recipient (13, 14). To prolong the survival of the HLA mismatched grafts, the vast majority of recipients rely on lifelong immunosuppressive treatment. While T cell alloimmunity is effectively kept under control with current immunosuppressive drugs, donor-specific alloantibody (DSA)-driven humoral immune responses are still considered as a risk factor for long term solid organ allograft survival (15-17).

Today, pretransplant risk assessment regarding the donor-specific humoral alloimmune response in a transplant recipient is based on detection of serum antibodies directed to mismatched donor HLA class I (HLA-A,-B,-C) and HLA class II (HLA-DR,-DQ,-DP) antigens (18). Serum HLA antibodies are mainly produced by bone-marrow residing plasma cells. However, dormant memory B cells may also contribute to HLA antibody repertoire of an individual with a history of alloimmunization upon re-encountering the same HLA or as a result of bystander activation (19, 20). Although there are several methods (complement dependent cytotoxicity (CDC), ELISA, bead-based assays) that are commonly used to detect these serum antibodies, none of the currently available assays provide any information on the magnitude of HLA-specific memory B cell responses.

AIM OF THE THESIS

The aim of this thesis was to develop tools to detect and quantify HLA-specific memory B cells in peripheral blood of HLA-immunized individuals and to assess the applicability of the newly developed assays in the setting of clinical transplantation. B cells are mainly known for their merit of antibody production; however, they have a broad range of functions other than antibody production. **Chapter 2** provides an overview on diverse functions of B cells in solid organ transplantation and highlights their roles in generation of humoral immune responses. Humoral alloimmune responses directed at mismatched donor HLA are usually detected as serum HLA antibodies of IgG isotype. Despite the abundant number of ways to detect serum HLA antibodies, methods to detect HLA-specific memory B cells are limited due to the technical challenges associated with the detection of these dormant cells. Various methods to detect HLA-specific memory B cells which are all limited in their capacity to quantify only HLA class I-specific memory B cell responses are described in **chapter 3**. Since memory B cells are dormant cells, polyclonal activation is required for them to be detected *in vitro*. However, it is critical that the polyclonal activation cocktail used does not induce isotype switching in naïve B cells and enables detection of IgG type of antibodies deriving solely from memory B cells. **Chapter 4** describes a polyclonal activation cocktail that can be used to activate isolated B cells in order to detect pre-existing antigen-specific memory B cells without inducing antibody isotype switching. Using this polyclonal activation protocol, an ELISPOT method was developed in which synthetic HLA class II molecules serve as the HLA target as described in **chapter 5**. Methods to detect memory B cells mainly utilize synthetic monomeric/tetrameric HLA molecules which generally do not represent the complete HLA repertoire of an individual. A donor-specific HLA-ELISPOT assay enabling the screening for HLA-specific memory B cells in peripheral blood of immunized individuals using cell lysates as a natural source of both HLA class I and II is described in **chapter 6**. While peripheral blood samples can be used to detect memory B cells *in vitro*, secondary lymphoid organs and bone marrow also contain memory B cells. As described in **chapter 7**, the bone marrow harbours not only plasma cells but also memory B cells specific for the same antigen whereas the two cell types display a different immunoglobulin isotype distribution. While serum HLA antibodies are produced by bone marrow residing plasma cells, memory B cells can contribute to HLA antibody formation upon activation, and these antibodies may not necessarily mirror the antibody specificities produced by bone marrow residing plasma cells. Therefore, determination of HLA antibodies in B cell culture supernatants supplementary to serum HLA antibody detection may provide a more complete picture of the potential humoral alloimmune response in an alloantigen immunized individual. **Chapter 8** elaborates on accurately discriminating HLA antibody specificities found in serum and B cell culture supernatants from kidney transplant recipients.

REFERENCES

1. Alegre ML, Leemans J, Le Moine A, Florquin S, De Wilde V, Chong A, et al. The multiple facets of toll-like receptors in transplantation biology. Transplantation. 2008;86(1):1-9.
2. Gray D. A role for antigen in the maintenance of immunological memory. Nat Rev Immunol. 2002;2(1):60-5.
3. Espinosa JR, Samy KP, Kirk AD. Memory T cells in organ transplantation: progress and challenges. Nat Rev Nephrol. 2016;12(6):339-47.
4. Kurosaki T, Kometani K, Ise W. Memory B cells. Nat Rev Immunol. 2015;15(3):149-59.
5. Cooper MA, Elliott JM, Keyel PA, Yang L, Carrero JA, Yokoyama WM. Cytokine-induced memory-like natural killer cells. Proc Natl Acad Sci U S A. 2009;106(6):1915-9.
6. Paust S, Gill HS, Wang BZ, Flynn MP, Moseman EA, Senman B, et al. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. Nat Immunol. 2010;11(12):1127-35.
7. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol. 2010;10(7):490-500.
8. McCluskey J, Peh CA. The human leucocyte antigens and clinical medicine: an overview. Rev Immunogenet. 1999;1(1):3-20.
9. Little AM, Parham P. Polymorphism and evolution of HLA class I and II genes and molecules. Rev Immunogenet. 1999;1(1):105-23.
10. Heidt S, Witvliet MD, Haasnoot GW, Claas FH. The 25th anniversary of the Eurotransplant Acceptable Mismatch program for highly sensitized patients. Transpl Immunol. 2015;33(2):51-7.
11. Ali JM, Bolton EM, Bradley JA, Pettigrew GJ. Allorecognition pathways in transplant rejection and tolerance. Transplantation. 2013;96(8):681-8.
12. Waanders MM, Heidt S, Koekkoek KM, Zoet YM, Doxiadis, II, Amir A, et al. Monitoring of indirect allorecognition: wishful thinking or solid data? Tissue Antigens. 2008;71(1):1-15.
13. Safinia N, Afzali B, Atalar K, Lombardi G, Lechler RI. T-cell alloimmunity and chronic allograft dysfunction. Kidney Int Suppl. 2010(119):S2-12.
14. Everly MJ, Rebellato LM, Haisch CE, Ozawa M, Parker K, Briley KP, et al. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. Transplantation. 2013;95(3):410-7.
15. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med. 2004;351(26):2715-29.
16. Lodhi SA, Lamb KE, Meier-Kriesche HU. Solid organ allograft survival improvement in the United States: the long-term does not mirror the dramatic short-term success. Am J Transplant. 2011;11(6):1226-35.
17. Loupy A, Hill GS, Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. Nature reviews Nephrology. 2012;8(6):348-57.
18. Tait BD, Susal C, Gebel HM, Nickerson PW, Zachary AA, Claas FH, et al. Consensus guidelines on the testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation. Transplantation. 2013;95(1):19-47.
19. Crotty S, Ahmed R. Immunological memory in humans. Seminars in Immunology. 2004;16(3):197-203.
20. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science. 2002;298(5601):2199-202.

Chapter 2

B cell immunity in solid organ transplantation

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ABSTRACT

The contribution of B cells to alloimmune responses is gradually being understood in more detail. We now know that B cells can perpetuate alloimmune responses in multiple ways: (i) differentiation into antibody-producing plasma cells; (ii) sustaining long-term humoral immune memory; (iii) serving as antigen-presenting cells; (iv) organizing the formation of tertiary lymphoid organs; and (v) secreting pro- as well as anti-inflammatory cytokines. The cross-talk between B cells and T cells in the course of immune responses forms the basis of these diverse functions. In the setting of organ transplantation, focus has gradually shifted from T cells to B cells, with an increased notion that B cells are more than mere precursors of antibody-producing plasma cells. In this review, we discuss the various roles of B cells in the generation of alloimmune responses beyond antibody production, as well as possibilities to specifically interfere with B cell activation.

INTRODUCTION

In the setting of organ transplantation, B cells are primarily known for their ability to differentiate into long-lived plasma cells producing high affinity, class-switched alloantibodies. The detrimental role of pre-existing donor-reactive antibodies at time of transplantation was already described in the 60s of the previous century in the form of hyperacute rejection (1). With the introduction of the complement dependent cytotoxicity (CDC) crossmatch assay by Terasaki and colleagues, the problem of hyperacute rejection was largely eliminated (2, 3). In the decades that followed focus shifted towards T cells and the prevention of cellular rejection. As a consequence, many drugs have been developed to successfully keep T cell immunity in check (4). With T cells largely under control, it is now clear that B cells remain important as precursors of antibody producing plasma cells. However, B cells also give rise to humoral immune memory in the form of memory B cells, process and present alloantigens to T cells, are involved in ectopic lymphoid follicle formation, and modulate T cell responses by secreting cytokines. Reciprocal cognate interactions between T cells and B cells play key roles in the generation of alloimmune responses (5) (Figure 1).

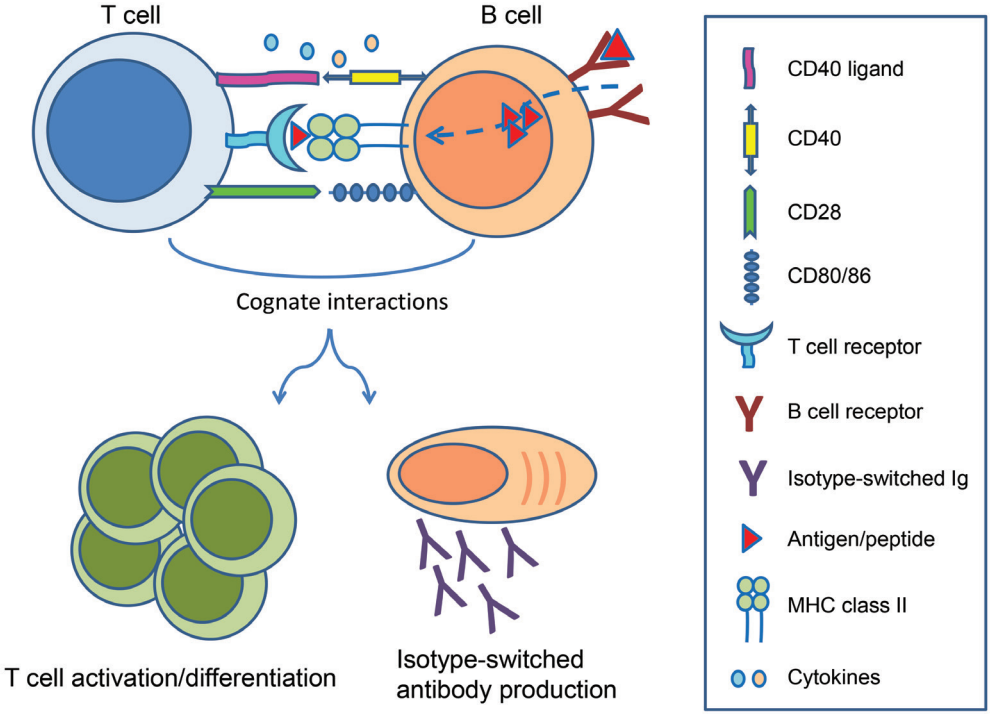

Figure 1. Reciprocal interactions between T cells and B cells. *Following BCR-mediated uptake of protein antigens, activated B cells process and present antigenic peptides in the context of major histocompatibility complex (MHC) class II on their surface to cognate T cells that recognize the MHC-peptide complex through their T cell receptor. Ligation of CD40 ligand and CD28 on T cells to CD40 and CD80/86 on B cells, as well as production of several cytokines enable differentiation of both B cells and T cells into effector and memory subsets. While B cells can become isotype-switched antibody producing plasma cells and memory B cells, T cells can become activated as effectors or differentiate into memory T cells to sustain cellular immune responses.*

In order to understand how B cells contribute to adaptive immune responses, we will first summarize the basics of human B cell development. Afterward, we will focus on the various roles of B cells in the setting of solid organ transplantation by antibody production, alloantigen presentation to T cells, intragraft tertiary lymphoid organ formation, as well as immune regulation. Finally, we will discuss new venues in interfering with B cell activation.

GENERATION OF HUMORAL IMMUNE RESPONSES IN SECONDARY LYMPHOID ORGANS

B cell development in bone marrow

B cells are crucial components of the humoral immune response. They participate in eradication of pathogens by their ability to differentiate into antibody-producing plasma cells, thereby propagating long-term serological immune memory. B cell development encompasses a programmed set of events that initiate in primary lymphoid organs, which advances to a functional maturation stage in secondary lymphoid organs. Development and survival of B cells depend on the cell surface expression of a functional antigen receptor, namely the B cell receptor (BCR), which is a membrane bound immunoglobulin (Ig) molecule in complex with Ig α/β heterodimer signaling molecules (6). In order to generate a functional BCR capable of recognizing a broad range of antigens but not self, the gene segments encoding the BCR go through rearrangements in the bone marrow, by the assembly of variable (V), diversity (D) and joining (J) gene segments at both Ig heavy and light chain loci *via* DNA recombination (7). Newly-formed B cells that express autoreactive BCRs are modified either by receptor editing or deleted by apoptosis. Upon completion of receptor editing, immature B cells with an intact BCR on their cell surface leave the bone marrow as transitional B cells to further continue maturation in the peripheral circulation and secondary lymphoid organs (8).

Modifications of the BCR proceed in germinal centers (GCs) at later stages of B cell differentiation during T cell-dependent immune responses as discussed below. While certain B cell subsets respond to polysaccharide antigens such as non-self blood group antigens by producing natural antibodies independent of T cell help, responses to protein antigens (e.g.; human leukocyte antigen-HLA) develop in the presence of T cell help. Since alloimmune responses are generally directed at protein antigens, we will focus on T cell-dependent follicular B cell responses.

B cell activation in secondary lymphoid organs

Secondary lymphoid organs are located at strategic sites throughout the body and provide the proper environment for T and B cells to come into contact with antigen and interact with each other. Both aspects are essential for the generation of antibody responses. In lymph nodes, B cells form follicles in the cortex just beneath the subcapsular sinus (SCS) of the lymphatic vessel, while T cells are localized in the paracortex adjacent to B cell follicles. The paracortex contains high endothelial venules through which lymphocytes and dendritic

cells enter the lymph node (9). Immature naïve B cells continuously circulate through the peripheral blood, lymph, and enter secondary lymphoid organs in order to gain access into B cell follicles where they can complete their maturation and receive further survival signals. These naïve B cells home to secondary lymphoid organs through chemokines secreted by a network of stromal and follicular dendritic cells (FDC) (10-12). If a B cell does not encounter its specific antigen it detaches from FDC, leaves the lymph node via efferent lymphatics, and continues to recirculate between peripheral blood and secondary lymphoid organs (13) .

Mature naïve B cells can become activated when their BCR engages an intact antigen inside or outside primary B cell follicles. While follicular B cells can recognize antigen presented on the surface of FDC, small soluble antigens can quickly diffuse from SCS into B cell follicles and can directly be recognized by BCRs. Large antigens such as immune complexes and viruses can be transported to B cell follicles by specialized $CD169^+$ macrophages resident at SCS. These macrophages lack phagocytosis ability and can present the antigen in its intact form to B cells (14). The immunological synapse between APC and BCR initiates downstream signaling events and rearrangements of the B cell cytoskeleton. Subsequently, B cells that have acquired and processed antigen move toward the boundaries of T and B cell zones to survey for cognate T cell help. $CD4^+$ T cells in interfollicular and paracortical T cell zones initially interact with cognate antigen presenting dendritic cells and subsequently increase their ability to migrate to B cell follicles.

A mature naïve B cell requires two signals to become activated: the first signal is received through the engagement of its BCR with cognate antigen and the second through cognate interaction with $CD4^+$ T cells, termed as follicular helper T cells (T_{FH}). Upon receiving T cell help at the T-B cell border, B cells can either differentiate into short-lived extrafollicular plasmablasts that produce low-affinity IgM antibodies, or can proceed to go through germinal center (GC) reactions.

Germinal center reactions

Repositioning of antigen-activated T and B cells from the T-B cell zone back to the follicle initiates the GC reaction. During this transient reaction, B cells start to proliferate and consequently trigger the egress of naïve, circulating B cells from the primary follicle. The follicle resolves into light and dark zones harboring B cells at various levels of cell division. Although the exact mechanisms that define the fate of B cells in GC are not entirely understood, signaling through the BCR and interactions with T_{FH} are known to be essential for their survival and differentiation into long-lived plasma cells and memory B cells. B cells present antigen to T_{FH} in GCs for the second time during the course of the humoral immune response. GC B cells with high affinity BCR appear to be most efficient at antigen uptake, processing and presentation to T_{FH} cells as well as being more prone to survival than those with low affinity BCR. Ligation of peptide/MHC class II, CD40, and CD80/86 on B cells with the TCR, CD40L, and CD28 on T cells, respectively, in the presence of cytokines such as IL-2, IL-4,

IL-5, IL-21 appear to be crucial (15-17). The activated B cells undergo clonal expansion, class switch recombination from IgM to IgG, IgA, or IgE, and acquire somatic hypermutations in the variable region of their BCR (18, 19). Affinity-driven selection enables further proliferation and differentiation of B cells with high affinity BCR into long-lived plasma cells and memory B cells (20). While long-lived plasma cells preferentially home to the bone marrow, memory B cells remain quiescent until re-encounter with antigen and recirculate between secondary lymphoid organs and the peripheral blood (21, 22). Generation of rapid antibody responses following antigen re-challenge requires efficient antigen presentation by memory B cells to cognate memory T_{FH}. Upon receipt of this T cell help, memory B cells rapidly differentiate into plasma cells and produce high levels of antigen-specific, mainly IgG type of antibodies.

WHY ARE B CELLS IMPORTANT IN SOLID ORGAN TRANSPLANTATION?

Solid organ transplantation is a life-saving treatment option for patients with end stage organ failure. The level of genetic disparities at HLA class I and II loci between donor and recipient, as well as the ability of the recipient's immune system to respond determine the strength of the immune response to an allograft (23-25). Immune responses directed towards mismatched HLA evoke both the cellular and the humoral arm of the adaptive immune system (26, 27). To prevent immunological rejection of the allograft, patients receive life-long immunosuppressive treatment. Currently available immunosuppressive regimens are centered on T cells and have been successful in curtailing acute cellular rejection. Successful treatment of cellular rejection by targeting T cells with immunosuppressive drugs have reduced acute rejection rates and hence improved short-term graft survival. It is clear that these drugs are insufficient in controlling humoral immune responses since antibody-mediated rejection (ABMR) is the leading cause of chronic allograft failure (28, 29). A growing body of evidence suggests that B cells play essential roles in alloimmunity besides mediating humoral immune responses. Understanding the various functions of B cells and the delicate balance between different B cell subsets may facilitate advances in B cell-targeting immunosuppressive drug development and eventually direct toward understanding the mechanisms involved in allograft tolerance.

SIGNIFICANCE OF ANTIBODY RESPONSES IN SOLID ORGAN TRANSPLANTATION

Antibodies binding to mismatched HLA (or non-HLA) molecules on donor endothelial cells initiate a set of signaling events leading to recruitment of effector cells to the graft endothelium through complement-dependent and -independent pathways. This process results in graft thrombosis and eventually a decline in allograft function. Clinical studies have shown that both pre-existence and *de novo* production of IgG donor-specific antibodies (DSA) are strongly associated with acute and chronic allograft injury in kidney, heart, lung and to some extent, liver transplantation (29-34). On the contrary, studies on IgM and IgA DSA did not reveal any isolated effect of these isotypes on allograft outcome unless they were co-existent with IgG

antibodies (35, 36). This indicates that the above described GC response needs to be active for pathological antibody response to occur in the setting of organ transplantation.

In accordance with several earlier studies, Loupy *et al*. found in a large scale retrospective study on renal transplant recipients that patients developing DSA after transplantation have inferior 5-year graft survival rates compared to those without DSA (37). Among those patients with *de novo* DSA, the capability to fix complement was associated with more severe lesions including microvascular inflammation and C4d deposition. In a recent study, Lefaucher *et al*. investigated the role of complement fixation of HLA-DSA in a cohort of 635 kidney transplant recipients (38). The authors categorized patients into three groups: ABMR-free, acute ABMR, and subclinical ABMR. They found that whereas ABMR-free patients most prominently had $IgG1^+$ DSA lacking C1q fixing capacity, patients with acute ABMR most frequently showed $IgG3^+$ DSA, which was associated with microvascular inflammation, C4d deposition in peritubular capillaries, and inferior graft survival. Interestingly, patients classified as having subclinical ABMR showed $IgG2^+$ and $IgG4^+$ DSA and had predominantly chronic lesions. Results from this study highlight the divergence between acute complement-dependent and chronic complement-independent roles for HLA-specific antibodies in mediating different types of allograft injury.

While circulating antibodies are mainly produced by long-lived plasma cells residing in the bone marrow, local alloantibody production within intragraft tertiary lymphoid organs have also been described (39). Thaunat *et al*. demonstrated the presence of alloantibodies in supernatants of renal cortex tissue cultures, suggestive for local antibody production within the graft. Comparison of HLA antibody specificities and strength of the antibody response revealed differences in serum and supernatant samples from the same patient (39). Several studies have shown the presence of DSA eluted either from core needle biopsy samples or explanted renal tissue of patients with failed allografts, which may be due to absorbance of circulating alloantibodies, but may also be pointing toward local production (40-42). Huibers *et al*. found DSA in lysates of coronary arteries of heart allograft autopsies harboring ectopic lymphoid structures. Interestingly, DSA and non-DSA found in the graft and serum at the time of autopsy were directed only against HLA class II (43). A recent study by Milango *et al*. showed the presence of DSA in both serum and graft eluates at time of nephrectomy in the absence of immunosuppressive treatment. Although HLA-C and -DP mismatches between the recipients and donors were not analyzed, 80% of HLA antibody specificities were found to be directed at mismatched donor epitopes both for HLA class I and II (44).

Currently available methods to detect serum HLA antibodies (discussed elsewhere in this issue of *Frontiers in Immunology*) do not provide any information on the magnitude of HLA-specific memory B cells (45). As described above, these memory B cells can rapidly differentiate into antibody secreting cells upon re-challenge. Memory B cells exert this rapid function upon re-encounter with the immunizing HLA or in response to a non-specific innate stimuli due to their lower activation threshold and constitutive toll-like receptor expression (46-48).

Several reports have shown the presence of additional HLA antibody specificities that are not detected in serum but in the culture supernatants of polyclonally activated peripheral blood B cells from kidney transplant recipients with a history of sensitization (49, 50). Therefore, studying donor-specific B cell responses in the transplant setting is certainly of importance, and several recently developed techniques allow to do so (51-57).

A ROLE FOR B CELLS IN ANTIGEN PRESENTATION TO ALLOREACTIVE T CELLS

Expression of high levels of MHC class II and costimulatory molecules on activated B cells, their capacity to take up antigens by their BCR and ability to clonally expand make B cells also extremely potent APC (58-64). Nonetheless, the APC function of B cells in transplantation setting was initially neglected among others due to murine studies reporting efficient $CD4^+$ T cell priming in B cell deficient mice transplanted with skin or cardiac allografts (65-67). However, it turned out that the developmental absence of B cells may have triggered non-B cell APC to deviate T cell responses toward a Th1 phenotype, potentiating allograft rejection (68).

In order to assess the role of B cells as APC to alloreactive T cells in the transplant setting, Noorchashm *et al*. generated bone marrow chimeric mice lacking either MHC class II or the MHC class II peptide loading machinery, specifically in B cells (69). Both of these chimeras showed prolonged cardiac allograft survival compared to wild type controls, which experienced early T cell-mediated rejection. These results indicate that antigen presentation by B cells is involved in T cell-mediated rejection. However, although the authors observed impaired IgG alloantibody production in addition to a decreased $CD4^+$ T cell division rate, these experiments did not formally answer the question whether B cells are required for T cell differentiation into effector or memory subsets. This question was addressed by Ng *et al*. in an allogeneic skin transplantation model using B cell deficient (μMT) mice. Whereas similar numbers of IFN-γ producing $CD4^+$ and $CD8^+$ T cells compared to wild type were found early after transplantation (effector phase), at a later stage (memory phase) μMT mice showed decreased numbers of alloreactive IFN-γ^+ T cells (70). These data suggest that memory T cell development is dependent on the interaction with B cells. While these studies provided evidence for the contribution of B cells to antigen presentation and T cell differentiation, the impact of alloantibodies on transplant outcome was not formally excluded. It appears that both alloantibodies and B cell-dependent T cell activation are important since Burns and colleagues showed that the enhanced T cell-mediated rejection of murine cardiac allografts upon re-challenge is caused by a combined effect of alloantibodies and memory B cell-dependent activation of T cells (71).

In clinical kidney transplantation, the possible role for B cells as APC in T cell-mediated rejection mainly comes from studies on renal biopsies. A landmark study by Sarwal and colleagues showed dense B cell clusters in biopsies of acute cellular rejections that did not correlate with C4d deposition, but were associated with steroid resistance and inferior graft

survival (72). Since then, several groups confirmed the correlation of graft infiltrating $CD20^+$ B cell clusters with steroid-resistant acute cellular rejection and poor graft survival (73-75) whereas other investigators did not find any prognostic significance of these intragraft B cell clusters neither for treatment sensitivity nor for transplant outcome (76-78). Remarkably, $CD20^+$ B cell clusters were mainly present in cases of T cell-mediated rejections without any association to ABMR, which is suggestive for a significant role of B cells other than antibody production (72-75). Indeed, intragraft $CD20^+$ B cells have been shown to display an activated, mature phenotype as shown by CD79a and HLA-DR expression and are often found in close proximity to $CD4^+$ T cells (75). In an elegant study using cell distance mapping, $ICOS^+CXCR4^+$ T_{FH}-like cells were found in close proximity to B cells in both T cell-mediated or mixed cellular rejection, thereby strongly supporting the concept of antigen presentation by these B cells to alloreactive T cells (79).

B CELLS IN TERTIARY LYMPHOID ORGANS OF CHRONICALLY REJECTED ALLOGRAFTS

Ectopic lymphoid organs resemble canonical secondary lymphoid organs regarding their T and B cell compartmentalization and interaction with dendritic cells, as well as the utilization of chemokine-mediated signaling pathways. In contrast, they display impaired lymphatic drainage and therefore trap the antigen leading to continuous exposure of immune cells to the antigen. *De-novo* formation of lymphoid-like structures as a result of persistent antigen exposure at sites of chronic infection or inflammation in non-lymphoid organs have been described in both autoimmunity and cancer (80, 81).

Upon organ transplantation, an environment containing persisting antigen similar to an autoreactive milieu is created and as a result can lead to tertiary lymphoid organ formation (82). Kerjaschki *et al*. demonstrated proliferating T cells (75%) and B cells (25%) in nodular infiltrates in close proximity with lymphatic vessels in explanted kidney allografts (83). Similarly, Thaunat *et al*. described the presence of lymphoid neogenesis in virtually all allografts explanted due to chronic rejection (39, 84). B cells in these explants were organized into nodules reminiscent of either primary or secondary B cell follicles. Relatively high expression of genes characteristic for GCs were observed in renal secondary B cell follicles indicating a highly activated phenotype for graft infiltrating B cells (39, 85). Furthermore, local B cell proliferation, a characteristic for the GC response, occurs as shown by Ki67 positivity and clonality of infiltrating B cells (83-85). In tertiary lymphoid organs, graft-infiltrating B cells might be contributing to lymphoid angiogenesis by prominent expression of vascular endothelial growth factor-A (86). Organization of the lymphoid infiltrates in the form of ectopic GCs may lead to containment of the alloimmune response within the graft. The aforementioned absence of DSA in circulation or discrepancies in specificities or strength of locally produced and circulating HLA antibodies support this hypothesis (39, 84). It is possible that the infiltrates observed during acute T cell-mediated rejection may represent an early stage of tertiary lymphoid organ development.

B CELLS AS IMMUNE REGULATORS

In addition to their roles in immune activation, (subsets of) B cells may also have regulatory function (87). Several groups have reported B cells with regulatory properties in controlling autoimmunity and inflammation (87-90). A complicating factor in studying regulatory B cells (Bregs) is the lack of a unique marker to define these cells. This has resulted in a wide range of B cell subsets to be identified as Bregs with the ability to secrete IL-10, IL-35 or TGF-β (91-93). In mice, a T cell costimulatory molecule termed as T cell Ig domain and mucin domain (TIM1) was found to be useful for identifying IL-10 producing Bregs (94). In humans, two main subsets of B cells enriched for Bregs have been described: $CD24^{hi}CD38^{hi}$ transitional B cells (89), and $CD24^{hi}CD27^{+}$ B10 cells (95). Whereas IL-10, IL-35 and TGF-β have all been described as effector molecules of Bregs, in the setting of transplantation, the main focus has been on IL-10 producing B cells.

In transplantation, regulatory functions of B cells have mainly been investigated in murine models of allograft tolerance. Ding *et al*., using a mouse model of islet transplantation, demonstrated that TIM1 may also have functional properties in Breg development. They observed prolonged allograft survival in mice treated with an agonistic anti-TIM1 antibody compared to untreated mice (94). Interestingly, in mice depleted of B cells before transplantation, anti-TIM1 treatment accelerated allograft rejection, indicating an important role for B cells in TIM1-mediated tolerance. Transfer of $TIM1^{+}$ B cells into untreated recipients of islets led to prolonged allograft survival. This regulatory effect was defective in $TIM1^{+}$ B cells, showing the dependency of B cells on IL-10 for their regulatory capacity. Shortly after, Lee *et al*. reported 100% long term islet allograft survival in mice treated with a combination of anti-CD45RB and TIM1 (96). They demonstrated prompt rejection of islet allografts if regulatory T cells (Tregs) were depleted before transplantation, implying that Bregs require an interaction with Tregs to induce tolerance. Furthermore, Le-Texier *et al*. have shown the presence of intragraft IgM^{+} B cells in rats with cardiac allograft tolerance compared to the presence of IgG^{+} B cells in allografts showing chronic rejection (97), suggestive for a restriction in B cell activation in the tolerant group. To demonstrate that tolerance was (at least partially) caused by B cells, the authors performed adoptive transfer of splenic B cells from tolerant rats to show allograft tolerance in these secondary mice.

A hint toward a role for B cells in clinical transplantation tolerance came from studies identifying B cell signatures in operationally tolerant kidney transplant recipients who were immunosuppression-free for at least one year with stable graft function (98-100). Microarray analyses on peripheral blood revealed 22 B cell-specific genes that were enriched in tolerant patients compared to those with stable graft function. Furthermore, the CD20 transcript was found to be the only marker higher in urine sediments of tolerant patients. Indeed, three genes (*IGKV4-1, IGLL1,* and *IGKVID-13*) encoding Ig kappa and lambda light chains in the course of B cell differentiation were shown to be predictive of operationally tolerant

patients (98). In an accompanying study six highly overexpressed genes were identified in tolerant patients (*CD79B, TCL1A, SH2D1B, MS4A1, FCRL1,* and *FCRL2*) that were associated with B cell-related pathways (99). Interestingly, expression of *CD79B*, *MS4A1* and *TCL1A* has been shown to be significantly down-regulated in renal transplant recipients with acute rejection (101, 102).

Tolerant patients showed increased peripheral blood B cell numbers and a redistribution of B cell subsets toward a naïve ($IgM^+IgD^+CD27^-$) and transitional ($CD24^{hi}$ $CD38^{hi}$) phenotype with increased expression of IL-10, compared to patients with stable graft function under immunosuppressive treatment (98, 99). The findings on IL-10 competent transitional B cells are in line with the definition of Bregs as descibed by Blair *et al*. (89). Pallier and colleagues confirmed the elevated peripheral blood B cell numbers and found that B cells with a memory phenotype ($IgD^-CD38^{-/+}CD27^+$) were increased (103). Whether these are the B10 cells as described by the group of Tedder remains to be established (95). Compared to patients with stable graft function, the majority of the operationally tolerant patients do not have circulating DSA and have a lower frequency of $CD38^+CD138^+$ plasma cells in the peripheral blood (98, 99, 103). In order to determine whether there was a defect in tolerant patients in generating humoral immune responses, Chesnau *et al*. polyclonally activated purified B cells from operationally tolerant patients *in vitro*. Polyclonally activated B cells proliferated and produced normal levels of IgM and IgG, accompanied by increased levels of IL-10 compared to those with stable graft function (104). In order to asses the inhibitory role of polylconally activated B cells of tolerant patients on autologous $CD4^+CD25^-$ T cells, Chesneau *et al*. blocked IL-10, TGF-β and granzyme B in a T-B cell co-culture system and found that only granzyme inhibitors affected the suppressive effects of B cells (105). However, antigen specificity, a prerequisite for immune regulation, has yet to be demonstrated.

EFFECTS OF IMMUNOSUPPRESSIVE TREATMENTS ON B CELLS

In the current practice of kidney transplantation, standard triple immunosuppressive regimen consists of a calcineurin inhibitor (tacrolimus or cyclosporine), a purine analogue (mycophenolic acid-MPA), and corticosteriods as maintenance therapy in addition to a non-depleting anti-CD25 monoclonal antibody as the induction agent (106). Since these agents exert their effects preferentially on T cells, they may abrogate humoral immune responses indirectly by inhibiting the T cell help (107), although some of these also have a direct effect on B cells (108, 109). Drugs specifically interfering with humoral immunity can be classified into several groups: drugs that deplete B cells from the circulation, those that interfere with T-B cell interaction, drugs targeting B cell survival signals and drugs interfering with antibody production or effector function.

Current therapies for (highly) sensitized patients are primarily focused on removal of antibodies before transplantation by plasmapheresis, intravenous immunoglobulins or immunoadsorption (110). Addition of rituximab, a humanized murine CD20 antibody

which depletes circulating CD20$^+$ B cells, to desensitization protocols resulted in improved outcomes in ABO-incompatible transplantation (111-113). Surprisingly, when rituximab was administered to non-sensitized patients as induction therapy, a higher rate of acute rejection was observed compared to controls (114). In addition to its application in treatment of ABMR (115), administration of rituximab led to successful treatment of steroid-resistant acute cellular rejections (116) and resolution of B cell infiltrates in graft (117-120). However, in patients experiencing chronic allograft dysfunction, rituximab treatment was ineffective in resolution of tertiary lymphoid organs despite the successful depletion of circulating B cells (121). Kamburova *et al*. showed long-lasting B cell depletion in patients receiving rituximab as induction agent with repopulating B cells mainly consisting of transitional B cells (122). Similar results were obtained when patients were treated with alemtuzumab, an anti-CD52 monoclonal antibody (123, 124). Although polyclonal activation of purified B cells did not reveal a difference in proliferation or IgM producing cells, a significant decrease in IgG producing cells was observed (123).

Another way of attenuating B cell responses can be achieved by blocking the critical co-stimulatory pathways between T and B cells. A recent study by Chen *et al*. in a mouse model of cardiac allograft transplantation showed that co-stimulation blockade with a high-affinity CTLA-4Ig (belatacept) inhibited memory B cell responses and DSA formation, leading to prolonged graft survival (125). By blocking both CD28-CD80/86 (belatacept) and CD40-CD40L (2C10R4) pathways in a non-human primate model of ABMR, Kim *et al*. showed a decrease in clonal B cell expansion in GCs (126). Combined blockade led to reduced IL-21 production and was strongly associated with reduced DSA levels. Importantly, results of a large phase 3 trial confirmed the efficacy of belatacept in the clinical setting (127). This study revealed a reduction of DSA in the belatacept-treated group with a significant reduced risk of graft loss and death compared to the cyclosporine-treated group.

Several studies have shown increased serum levels of B cell activating factor (BAFF) following treatment with depleting agents in kidney transplant recipients (128, 129), possibly due to a lack of BAFF consuming B cells. BAFF has a critical role in promoting survival, maturation and activation of B cells, as well as maintaining self-tolerance (130). High levels of BAFF have been described in the setting of autoimmunity, and it is conceivable that high BAFF levels could also influence alloimmunity. Indeed, elevated serum BAFF levels were associated with increased risk of developing DSA and ABMR in the setting of kidney transplantation (131-133). Blockade of BAFF, and/or the related molecule called a proliferation inducing ligand (APRIL) may be an additional tool to down regulate humoral alloimmune responses as was suggested by the prolonged survival of cardiac allografts in BAFF-deficient mice (134). Also in a non-human primate ABMR model BAFF/APRIL blockade (atacicept) was able to prevent *de novo* DSA production (135).

Plasma cells are responsible for the continuous production of antibodies and therefore have a high proteasomal activity. Proteasome inhibitors, such as bortezomib, are effective for the

treatment of plasma cell malignancies (136). Bortezomib has been used to treat ABMR and diminish DSA production in sensitized transplant recipients (137-140). However, the inhibitory capacity of proteasome inhibitors is not limited to plasma cells as also naïve and memory B cell proliferation can be affected (141). Therefore, antibody production through plasma cells, as well as the various effects of B cells may be dampened by proteasome inhibition.

CONCLUSION AND REMARKS

B cells contribute to acute and chronic allograft rejection processes by producing DSA. More recently, other functions have been attributed to B cells that may also influence the alloimmune response, such as antigen presentation to T cells, formation of tertiary lymphoid organs, or secretion of regulatory cytokines.

Considering that one third of the patients on the kidney waiting lists are sensitized as a result of previous exposure to allogeneic HLA, memory B cells and their effector functions may play central roles in prospective transplantation outcome of these patients. Upon re-challenge, HLA-specific memory B cells generated during primary immune responses can promptly become high affinity DSA producing plasma cells and may serve as potent APC by their high expression of HLA-DR and costimulatory molecules. In conclusion, a variety of B cell populations with different functions may affect the alloimmune response after transplantation. Future therapies targeting B cells should take into consideration these different functions and the consequence that a simple depletion of all B cells will also interfere in the beneficial effects of certain B cell subpopulations.

REFERENCES

1. Kissmeyer-Nielsen F, Olsen S, Petersen V, Fjeldborg O. Hyperacute rejection of kidney allografts, associated with pre-existing humoral antibodies against donor cells. Lancet. 1966;24(2 (7465)):662-5.
2. Terasaki PI, McClelland JD. Microdroplet Assay of Human Serum Cytotoxins. Nature. 1964;204:998-1000.
3. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. The New England journal of medicine. 1969;280(14):735-9.
4. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med. 2004;351(26):2715-29.
5. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. Transplantation. 2012;93(1):1-10.
6. Reth M. Antigen receptor tail clue. Nature. 1989;338(6214):383-4.
7. Granato A, Chen Y, Wesemann DR. Primary immunoglobulin repertoire development: time and space matter. Curr Opin Immunol. 2015;33:126-31.
8. Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, et al. Human memory B cells originate from three distinct germinal center-dependent and-independent maturation pathways. Blood. 2011;118(8):2150-8.
9. Fu YX, Chaplin DD. Development and maturation of secondary lymphoid tissues. Annu Rev Immunol. 1999;17:399-433.
10. Cyster JG, Ansel KM, Reif K, Ekland EH, Hyman PL, Tang HL, et al. Follicular stromal cells and lymphocyte homing to follicles. Immunol Rev. 2000;176:181-93.
11. Katakai T, Suto H, Sugai M, Gonda H, Togawa A, Suematsu S, et al. Organizer-like reticular stromal cell layer common to adult secondary lymphoid organs. J Immunol. 2008;181(9):6189-200.
12. Reif K, Ekland EH, Ohl L, Nakano H, Lipp M, Forster R, et al. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. Nature. 2002;416(6876):94-9.
13. Schwab SR, Cyster JG. Finding a way out: lymphocyte egress from lymphoid organs. Nat Immunol. 2007;8(12):1295-301.
14. Gaya M, Castello A, Montaner B, Rogers N, Reis e Sousa C, Bruckbauer A, et al. Host response. Inflammation-induced disruption of SCS macrophages impairs B cell responses to secondary infection. Science. 2015;347(6222):667-72.
15. Batista FD, Iber D, Neuberger MS. B cells acquire antigen from target cells after synapse formation. Nature. 2001;411(6836):489-94.
16. Cannons JL, Qi H, Lu KT, Dutta M, Gomez-Rodriguez J, Cheng J, et al. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. Immunity. 2010;32(2):253-65.
17. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. Nature. 2008;455(7214):764-9.
18. McHeyzer-Williams LJ, McHeyzer-Williams MG. Antigen-specific memory B cell development. Annu Rev Immunol. 2005;23:487-513.
19. Tarlinton DM. Evolution in miniature: selection, survival and distribution of antigen reactive cells in the germinal centre. Immunol Cell Biol. 2008;86(2):133-8.

20. Crotty S, Ahmed R. Immunological memory in humans. Semin Immunol. 2004;16(3):197-203.
21. Kunkel EJ, Butcher EC. Plasma-cell homing. Nat Rev Immunol. 2003;3(10):822-9.
22. Agematsu K, Hokibara S, Nagumo H, Komiyama A. CD27: a memory B-cell marker. Immunol Today. 2000;21(5):204-6.
23. Claas FH, van Rood JJ. The hyperimmunized patient: from sensitization toward transplantation. Transpl Int. 1988;1(2):53-7.
24. Duquesnoy RJ. HLA epitope based matching for transplantation. Transpl Immunol. 2014;31(1):1-6.
25. Lin CM, Gill RG. Direct and indirect allograft recognition: pathways dictating graft rejection mechanisms. Curr Opin Organ Transplant. 2016;21(1):40-4.
26. Safinia N, Afzali B, Atalar K, Lombardi G, Lechler RI. T-cell alloimmunity and chronic allograft dysfunction. Kidney Int Suppl. 2010(119):S2-12.
27. Everly MJ, Rebellato LM, Haisch CE, Ozawa M, Parker K, Briley KP, et al. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. Transplantation. 2013;95(3):410-7.
28. Lodhi SA, Lamb KE, Meier-Kriesche HU. Solid organ allograft survival improvement in the United States: the long-term does not mirror the dramatic short-term success. Am J Transplant. 2011;11(6):1226-35.
29. Loupy A, Hill GS, Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. Nat Rev Nephrol. 2012;8(6):348-57.
30. Jeannet M, Pinn VW, Flax MH, Winn HJ, Russell PS. Humoral antibodies in renal allotransplantation in man. N Engl J Med. 1970;282(3):111-7.
31. Lee PC, Terasaki PI, Takemoto SK, Lee PH, Hung CJ, Chen YL, et al. All chronic rejection failures of kidney transplants were preceded by the development of HLA antibodies. Transplantation. 2002;74(8):1192-4.
32. Le Pavec J, Suberbielle C, Lamrani L, Feuillet S, Savale L, Dorfmuller P, et al. De-novo donor-specific anti-HLA antibodies 30 days after lung transplantation are associated with a worse outcome. J Heart Lung Transplant. 2016.
33. Tran A, Fixler D, Huang R, Meza T, Lacelle C, Das BB. Donor-specific HLA alloantibodies: Impact on cardiac allograft vasculopathy, rejection, and survival after pediatric heart transplantation. J Heart Lung Transplant. 2016;35(1):87-91.
34. Kaneku H, O'Leary JG, Banuelos N, Jennings LW, Susskind BM, Klintmalm GB, et al. De novo donor-specific HLA antibodies decrease patient and graft survival in liver transplant recipients. Am J Transplant. 2013;13(6):1541-8.
35. Everly MJ, Rebellato LM, Haisch CE, Briley KP, Bolin P, Kendrick WT, et al. Impact of IgM and IgG3 anti-HLA alloantibodies in primary renal allograft recipients. Transplantation. 2014;97(5):494-501.
36. Arnold ML, Heinemann FM, Horn P, Ziemann M, Lachmann N, Muhlbacher A, et al. 16(th) IHIW: anti-HLA alloantibodies of the of IgA isotype in re-transplant candidates. Int J Immunogenet. 2013;40(1):17-20.
37. Loupy A, Lefaucheur C, Vernerey D, Prugger C, Duong van Huyen JP, Mooney N, et al. Complement-binding anti-HLA antibodies and kidney-allograft survival. N Engl J Med. 2013;369(13):1215-26.
38. Lefaucheur C, Viglietti D, Bentlejewski C, Duong van Huyen JP, Vernerey D, Aubert O, et al. IgG Donor-Specific Anti-Human HLA Antibody Subclasses and Kidney Allograft Antibody-Mediated Injury. J Am Soc Nephrol. 2016;27(1):293-304.

39. Thaunat O, Patey N, Caligiuri G, Gautreau C, Mamani-Matsuda M, Mekki Y, et al. Chronic rejection triggers the development of an aggressive intragraft immune response through recapitulation of lymphoid organogenesis. J Immunol. 2010;185(1):717-28.
40. Martin L, Guignier F, Mousson C, Rageot D, Justrabo E, Rifle G. Detection of donor-specific anti-HLA antibodies with flow cytometry in eluates and sera from renal transplant recipients with chronic allograft nephropathy. Transplantation. 2003;76(2):395-400.
41. Martin L, Charon-Barra C, Bocrie O, Guignier F, D'Athis P, Dautin G, et al. Detection of plasma cells, C4d deposits and donor-specific antibodies on sequential graft biopsies of renal transplant recipients with chronic dysfunction. Transpl Immunol. 2010;22(3-4):110-4.
42. Bachelet T, Couzi L, Lepreux S, Legeret M, Pariscoat G, Guidicelli G, et al. Kidney intragraft donor-specific antibodies as determinant of antibody-mediated lesions and poor graft outcome. Am J Transplant. 2013;13(11):2855-64.
43. Huibers MM, Gareau AJ, Beerthuijzen JM, Siera-de Koning E, van Kuik J, Kamburova EG, et al. Donor-specific antibodies are produced locally in Ectopic Lymphoid Structures in Cardiac Allografts. Am J Transplant. 2016.
44. Milongo D, Kamar N, Del Bello A, Guilbeau-Frugier C, Sallusto F, Esposito L, et al. Allelic and epitopic characterization of intra-kidney-allograft anti-HLA antibodies at allograft nephrectomy. Am J Transplant. 2016.
45. Perry DK, Pollinger HS, Burns JM, Rea D, Ramos E, Platt JL, et al. Two novel assays of alloantibody-secreting cells demonstrating resistance to desensitization with IVIG and rATG. Am J Transplant. 2008;8(1):133-43.
46. Lanzavecchia A, Bernasconi N, Traggiai E, Ruprecht CR, Corti D, Sallusto F. Understanding and making use of human memory B cells. Immunol Rev. 2006;211:303-9.
47. Tangye SG, Avery DT, Deenick EK, Hodgkin PD. Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses. J Immunol. 2003;170(2):686-94.
48. Tangye SG, Avery DT, Hodgkin PD. A division-linked mechanism for the rapid generation of Ig-secreting cells from human memory B cells. J Immunol. 2003;170(1):261-9.
49. Han M, Rogers J, Lavingia B, Stastny P. Peripheral blood B cells producing donor-specific HLA antibodies in vitro. Hum Immunol. 2009;70(1):29-34.
50. Snanoudj R, Claas FH, Heidt S, Legendre C, Chatenoud L, Candon S. Restricted specificity of peripheral alloreactive memory B cells in HLA-sensitized patients awaiting a kidney transplant. Kidney Int. 2015;87(6):1230-40.
51. Mulder A, Eijsink C, Kardol MJ, Franke-van Dijk ME, van der Burg SH, Kester M, et al. Identification, isolation, and culture of HLA-A2-specific B lymphocytes using MHC class I tetramers. J Immunol. 2003;171(12):6599-603.
52. Zachary AA, Kopchaliiska D, Montgomery RA, Leffell MS. HLA-specific B cells: I. A method for their detection, quantification, and isolation using HLA tetramers. Transplantation. 2007;83(7):982-8.
53. Zachary AA, Kopchaliiska D, Montgomery RA, Melancon JK, Leffell MS. HLA-specific B cells: II. Application to transplantation. Transplantation. 2007;83(7):989-94.
54. Zachary AA, Lucas DP, Montgomery RA, Leffell MS. Rituximab prevents an anamnestic response in patients with cryptic sensitization to HLA. Transplantation. 2013;95(5):701-4.

55. Heidt S, Roelen DL, de Vaal YJ, Kester MG, Eijsink C, Thomas S, et al. A Novel ELISPOT Assay to Quantify HLA-Specific B Cells in HLA-Immunized Individuals. Am J Transplant. 2012;12(6):1469-78.
56. Karahan GE, de Vaal YJ, Roelen DL, Buchli R, Claas FH, Heidt S. Quantification of HLA class II-specific memory B cells in HLA-sensitized individuals. Hum Immunol. 2015;76(2-3):129-36.
57. Lucia M, Luque S, Crespo E, Melilli E, Cruzado JM, Martorell J, et al. Preformed circulating HLA-specific memory B cells predict high risk of humoral rejection in kidney transplantation. Kidney Int. 2015;88(4):874-87.
58. Constant S, Schweitzer N, West J, Ranney P, Bottomly K. B lymphocytes can be competent antigen-presenting cells for priming $CD4^+$ T cells to protein antigens in vivo. J Immunol. 1995;155(8):3734-41.
59. Ron Y, Sprent J. T cell priming in vivo: a major role for B cells in presenting antigen to T cells in lymph nodes. J Immunol. 1987;138(9):2848-56.
60. Wilson JL, Cunningham AC, Kirby JA. Alloantigen presentation by B cells: analysis of the requirement for B-cell activation. Immunology. 1995;86(3):325-30.
61. Janeway CA, Jr., Ron J, Katz ME. The B cell is the initiating antigen-presenting cell in peripheral lymph nodes. J Immunol. 1987;138(4):1051-5.
62. Constant SL. B lymphocytes as antigen-presenting cells for $CD4^+$ T cell priming in vivo. J Immunol. 1999;162(10):5695-703.
63. Lanzavecchia A. Pillars article: Antigen-specific interaction between T and B cells. 1985. J Immunol. 2007;179(11):7206-8.
64. Crawford A, Macleod M, Schumacher T, Corlett L, Gray D. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. J Immunol. 2006;176(6):3498-506.
65. Brandle D, Joergensen J, Zenke G, Burki K, Hof RP. Contribution of donor-specific antibodies to acute allograft rejection: evidence from B cell-deficient mice. Transplantation. 1998;65(11):1489-93.
66. Epstein MM, Di Rosa F, Jankovic D, Sher A, Matzinger P. Successful T cell priming in B cell-deficient mice. J Exp Med. 1995;182(4):915-22.
67. Di Rosa F, Matzinger P. Long-lasting CD8 T cell memory in the absence of CD4 T cells or B cells. J Exp Med. 1996;183(5):2153-63.
68. Moulin V, Andris F, Thielemans K, Maliszewski C, Urbain J, Moser M. B lymphocytes regulate dendritic cell (DC) function in vivo: increased interleukin 12 production by DCs from B cell-deficient mice results in T helper cell type 1 deviation. J Exp Med. 2000;192(4):475-82.
69. Noorchashm H, Reed AJ, Rostami SY, Mozaffari R, Zekavat G, Koeberlein B, et al. B cell-mediated antigen presentation is required for the pathogenesis of acute cardiac allograft rejection. J Immunol. 2006;177(11):7715-22.
70. Ng YH, Oberbarnscheidt MH, Chandramoorthy HC, Hoffman R, Chalasani G. B cells help alloreactive T cells differentiate into memory T cells. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2010;10(9):1970-80.
71. Burns AM, Ma L, Li Y, Yin D, Shen J, Xu J, et al. Memory alloreactive B cells and alloantibodies prevent anti-CD154-mediated allograft acceptance. J Immunol. 2009;182(3):1314-24.
72. Sarwal M, Chua MS, Kambham N, Hsieh SC, Satterwhite T, Masek M, et al. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. N Engl J Med. 2003;349(2):125-38.

73. Hippen BE, DeMattos A, Cook WJ, Kew CE, 2nd, Gaston RS. Association of $CD20^+$ infiltrates with poorer clinical outcomes in acute cellular rejection of renal allografts. Am J Transplant. 2005;5(9):2248-52.
74. Tsai EW, Rianthavorn P, Gjertson DW, Wallace WD, Reed EF, Ettenger RB. $CD20^+$ lymphocytes in renal allografts are associated with poor graft survival in pediatric patients. Transplantation. 2006;82(12):1769-73.
75. Zarkhin V, Kambham N, Li L, Kwok S, Hsieh SC, Salvatierra O, et al. Characterization of intra-graft B cells during renal allograft rejection. Kidney International. 2008;74(5):664-73.
76. Kayler LK, Lakkis FG, Morgan C, Basu A, Blisard D, Tan HP, et al. Acute cellular rejection with CD20-positive lymphoid clusters in kidney transplant patients following lymphocyte depletion. Am J Transplant. 2007;7(4):949-54.
77. Doria C, di Francesco F, Ramirez CB, Frank A, Iaria M, Francos G, et al. The presence of B-cell nodules does not necessarily portend a less favorable outcome to therapy in patients with acute cellular rejection of a renal allograft. Transplant Proc. 2006;38(10):3441-4.
78. Bagnasco SM, Tsai W, Rahman MH, Kraus ES, Barisoni L, Vega R, et al. CD20-positive infiltrates in renal allograft biopsies with acute cellular rejection are not associated with worse graft survival. Am J Transplant. 2007;7(8):1968-73.
79. Liarski VM, Kaverina N, Chang A, Brandt D, Yanez D, Talasnik L, et al. Cell distance mapping identifies functional T follicular helper cells in inflamed human renal tissue. Sci Transl Med. 2014;6(230):230ra46.
80. Aloisi F, Pujol-Borrell R. Lymphoid neogenesis in chronic inflammatory diseases. Nat Rev Immunol. 2006;6(3):205-17.
81. Figenschau SL, Fismen S, Fenton KA, Fenton C, Mortensen ES. Tertiary lymphoid structures are associated with higher tumor grade in primary operable breast cancer patients. BMC Cancer. 2015;15:101.
82. Hsiao HM, Li W, Gelman AE, Krupnick AS, Kreisel D. The Role of Lymphoid Neogenesis in Allografts. Am J Transplant. 2016;16(4):1079-85.
83. Kerjaschki D, Regele HM, Moosberger I, Nagy-Bojarski K, Watschinger B, Soleiman A, et al. Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. J Am Soc Nephrol. 2004;15(3):603-12.
84. Thaunat O, Field AC, Dai J, Louedec L, Patey N, Bloch MF, et al. Lymphoid neogenesis in chronic rejection: evidence for a local humoral alloimmune response. Proc Natl Acad Sci U S A. 2005;102(41):14723-8.
85. Cheng J, Torkamani A, Grover RK, Jones TM, Ruiz DI, Schork NJ, et al. Ectopic B-cell clusters that infiltrate transplanted human kidneys are clonal. Proc Natl Acad Sci U S A. 2011;108(14):5560-5.
86. Adair A, Mitchell DR, Kipari T, Qi F, Bellamy CO, Robertson F, et al. Peritubular capillary rarefaction and lymphangiogenesis in chronic allograft failure. Transplantation. 2007;83(12):1542-50.
87. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. Nat Immunol. 2002;3(10):944-50.
88. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J Clin Invest. 2008;118(10):3420-30.

89. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi) CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. Immunity. 2010;32(1):129-40.
90. Duddy M, Niino M, Adatia F, Hebert S, Freedman M, Atkins H, et al. Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. J Immunol. 2007;178(10):6092-9.
91. Matsumoto M, Baba A, Yokota T, Nishikawa H, Ohkawa Y, Kayama H, et al. Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation. Immunity. 2014;41(6):1040-51.
92. Shen P, Roch T, Lampropoulou V, O'Connor RA, Stervbo U, Hilgenberg E, et al. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. Nature. 2014;507(7492):366-70.
93. Lee KM, Stott RT, Zhao G, SooHoo J, Xiong W, Lian MM, et al. TGF-beta-producing regulatory B cells induce regulatory T cells and promote transplantation tolerance. Eur J Immunol. 2014;44(6):1728-36.
94. Ding Q, Yeung M, Camirand G, Zeng Q, Akiba H, Yagita H, et al. Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. J Clin Invest. 2011.
95. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood. 2011;117(2):530-41.
96. Lee KM, Kim JI, Stott R, Soohoo J, O'Connor MR, Yeh H, et al. Anti-CD45RB/anti-TIM-1-induced tolerance requires regulatory B cells. Am J Transplant. 2012;12(8):2072-8.
97. Le Texier L, Thebault P, Lavault A, Usal C, Merieau E, Quillard T, et al. Long-term allograft tolerance is characterized by the accumulation of B cells exhibiting an inhibited profile. Am J Transplant. 2011;11(3):429-38.
98. Newell KA, Asare A, Kirk AD, Gisler TD, Bourcier K, Suthanthiran M, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. J Clin Invest. 2010;120(6):1836-47.
99. Sagoo P, Perucha E, Sawitzki B, Tomiuk S, Stephens DA, Miqueu P, et al. Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. J Clin Invest. 2010;120(6):1848-61.
100. Heidt S, Wood KJ. Biomarkers of Operational Tolerance in Solid Organ Transplantation. Expert Opin Med Diagn. 2012;6(4):281-93.
101. Viklicky O, Krystufkova E, Brabcova I, Sekerkova A, Wohlfahrt P, Hribova P, et al. B-Cell-Related Biomarkers of Tolerance are Up-Regulated in Rejection-Free Kidney Transplant Recipients. Transplantation. 2013;95(1):148-54.
102. Heidt S, Vergunst M, Anholts JD, Reinders ME, de Fijter JW, Eikmans M, et al. B Cell Markers of Operational Tolerance Can Discriminate Acute Kidney Allograft Rejection From Stable Graft Function. Transplantation. 2014.
103. Pallier A, Hillion S, Danger R, Giral M, Racape M, Degauque N, et al. Patients with drug-free long-term graft function display increased numbers of peripheral B cells with a memory and inhibitory phenotype. Kidney Int. 2010;78(5):503-13.

104. Chesneau M, Pallier A, Braza F, Lacombe G, Le Gallou S, Baron D, et al. Unique B cell differentiation profile in tolerant kidney transplant patients. Am J Transplant. 2014;14(1):144-55.
105. Chesneau M, Michel L, Dugast E, Chenouard A, Baron D, Pallier A, et al. Tolerant Kidney Transplant Patients Produce B Cells with Regulatory Properties. J Am Soc Nephrol. 2015;26(10):2588-98.
106. Ekberg H, Tedesco-Silva H, Demirbas A, Vitko S, Nashan B, Gurkan A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. N Engl J Med. 2007;357(25):2562-75.
107. Heidt S, Roelen DL, Eijsink C, Eikmans M, van Kooten C, Claas FH, et al. Calcineurin inhibitors affect B cell antibody responses indirectly by interfering with T cell help. Clin Exp Immunol. 2010;159(2):199-207.
108. Heidt S, Roelen DL, Eijsink C, van Kooten C, Claas FH, Mulder A. Effects of immunosuppressive drugs on purified human B cells: evidence supporting the use of MMF and rapamycin. Transplantation. 2008;86(9):1292-300.
109. De Bruyne R, Bogaert D, De Ruyck N, Lambrecht BN, Van Winckel M, Gevaert P, et al. Calcineurin inhibitors dampen humoral immunity by acting directly on naive B cells. Clin Exp Immunol. 2015;180(3):542-50.
110. Jordan SC, Pescovitz MD. Presensitization: the problem and its management. Clin J Am Soc Nephrol. 2006;1(3):421-32.
111. Donauer J, Wilpert J, Geyer M, Schwertfeger E, Kirste G, Drognitz O, et al. ABO-incompatible kidney transplantation using antigen-specific immunoadsorption and rituximab: a single center experience. Xenotransplantation. 2006;13(2):108-10.
112. Tyden G, Donauer J, Wadstrom J, Kumlien G, Wilpert J, Nilsson T, et al. Implementation of a Protocol for ABO-incompatible kidney transplantation--a three-center experience with 60 consecutive transplantations. Transplantation. 2007;83(9):1153-5.
113. Kahwaji J, Sinha A, Toyoda M, Ge S, Reinsmoen N, Cao K, et al. Infectious complications in kidney-transplant recipients desensitized with rituximab and intravenous immunoglobulin. Clin J Am Soc Nephrol. 2011;6(12):2894-900.
114. Clatworthy MR, Watson CJ, Plotnek G, Bardsley V, Chaudhry AN, Bradley JA, et al. B-cell-depleting induction therapy and acute cellular rejection. N Engl J Med. 2009;360(25):2683-5.
115. Tyden G, Genberg H, Tollemar J, Ekberg H, Persson NH, Tufveson G, et al. A randomized, doubleblind, placebo-controlled, study of single-dose rituximab as induction in renal transplantation. Transplantation. 2009;87(9):1325-9.
116. Becker YT, Becker BN, Pirsch JD, Sollinger HW. Rituximab as treatment for refractory kidney transplant rejection. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2004;4(6):996-1001.
117. Alausa M, Almagro U, Siddiqi N, Zuiderweg R, Medipalli R, Hariharan S. Refractory acute kidney transplant rejection with CD20 graft infiltrates and successful therapy with rituximab. Clin Transplant. 2005;19(1):137-40.
118. Lehnhardt A, Mengel M, Pape L, Ehrich JH, Offner G, Strehlau J. Nodular B-cell aggregates associated with treatment refractory renal transplant rejection resolved by rituximab. Am J Transplant. 2006;6(4):847-51.
119. Steinmetz OM, Lange-Husken F, Turner JE, Vernauer A, Helmchen U, Stahl RA, et al. Rituximab removes intrarenal B cell clusters in patients with renal vascular allograft rejection. Transplantation. 2007;84(7):842-50.

120. Zarkhin V, Li L, Kambham N, Sigdel T, Salvatierra O, Sarwal MM. A randomized, prospective trial of rituximab for acute rejection in pediatric renal transplantation. Am J Transplant. 2008;8(12):2607-17.
121. Thaunat O, Patey N, Gautreau C, Lechaton S, Fremeaux-Bacchi V, Dieu-Nosjean MC, et al. B cell survival in intragraft tertiary lymphoid organs after rituximab therapy. Transplantation. 2008;85(11):1648-53.
122. Kamburova EG, Koenen HJ, van den Hoogen MW, Baas MC, Joosten I, Hilbrands LB. Longitudinal Analysis of T and B Cell Phenotype and Function in Renal Transplant Recipients with or without Rituximab Induction Therapy. PLoS One. 2014;9(11):e112658.
123. Heidt S, Hester J, Shankar S, Friend PJ, Wood KJ. B-Cell Repopulation After Alemtuzumab Induction-Transient Increase in Transitional B cells and Long-Term Dominance of Naive B Cells. Am J Transplant. 2012.
124. Cherukuri A, Salama AD, Carter C, Smalle N, McCurtin R, Hewitt EW, et al. An Analysis of Lymphocyte Phenotype After Steroid Avoidance with Either Alemtuzumab or Basiliximab Induction in Renal Transplantation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2012.
125. Chen J, Wang Q, Yin D, Vu V, Sciammas R, Chong AS. Cutting Edge: CTLA-4Ig Inhibits Memory B Cell Responses and Promotes Allograft Survival in Sensitized Recipients. J Immunol. 2015;195(9):4069-73.
126. Kim EJ, Kwun J, Gibby AC, Hong JJ, Farris AB, 3rd, Iwakoshi NN, et al. Costimulation blockade alters germinal center responses and prevents antibody-mediated rejection. Am J Transplant. 2014;14(1):59-69.
127. Vincenti F, Rostaing L, Grinyo J, Rice K, Steinberg S, Gaite L, et al. Belatacept and Long-Term Outcomes in Kidney Transplantation. N Engl J Med. 2016;374(4):333-43.
128. Bloom D, Chang Z, Pauly K, Kwun J, Fechner J, Hayes C, et al. BAFF is increased in renal transplant patients following treatment with alemtuzumab. Am J Transplant. 2009;9(8):1835-45.
129. Zarkhin V, Li L, Sarwal MM. BAFF may modulate the rate of B-cell repopulation after rituximab therapy for acute renal transplant rejection. Transplantation. 2009;88(10):1229-30.
130. Mackay F, Schneider P. Cracking the BAFF code. Nat Rev Immunol. 2009;9(7):491-502.
131. Thibault-Espitia A, Foucher Y, Danger R, Migone T, Pallier A, Castagnet S, et al. BAFF and BAFF-R levels are associated with risk of long-term kidney graft dysfunction and development of donor-specific antibodies. Am J Transplant. 2012;12(10):2754-62.
132. Banham G, Prezzi D, Harford S, Taylor CJ, Hamer R, Higgins R, et al. Elevated pretransplantation soluble BAFF is associated with an increased risk of acute antibody-mediated rejection. Transplantation. 2013;96(4):413-20.
133. Snanoudj R, Candon S, Roelen DL, Jais JP, Claas FH, Legendre C, et al. Peripheral B-cell phenotype and BAFF levels are associated with HLA immunization in patients awaiting kidney transplantation. Transplantation. 2014;97(9):917-24.
134. Ye Q, Wang L, Wells AD, Tao R, Han R, Davidson A, et al. BAFF binding to T cell-expressed BAFF-R costimulates T cell proliferation and alloresponses. Eur J Immunol. 2004;34(10):2750-9.
135. Kwun J, Page E, Hong JJ, Gibby A, Yoon J, Farris AB, et al. Neutralizing BAFF/APRIL with atacicept prevents early DSA formation and AMR development in T cell depletion induced nonhuman primate AMR model. Am J Transplant. 2015;15(3):815-22.

136. Manasanch EE, Korde N, Zingone A, Tageja N, Fernandez de Larrea C, Bhutani M, et al. The proteasome: mechanisms of biology and markers of activity and response to treatment in multiple myeloma. Leuk Lymphoma. 2014;55(8):1707-14.
137. Everly MJ, Terasaki PI, Hopfield J, Trivedi HL, Kaneku H. Protective immunity remains intact after antibody removal by means of proteasome inhibition. Transplantation. 2010;90(12):1493-8.
138. Perry DK, Burns JM, Pollinger HS, Amiot BP, Gloor JM, Gores GJ, et al. Proteasome inhibition causes apoptosis of normal human plasma cells preventing alloantibody production. Am J Transplant. 2009;9(1):201-9.
139. Everly MJ, Everly JJ, Susskind B, Brailey P, Arend LJ, Alloway RR, et al. Bortezomib provides effective therapy for antibody- and cell-mediated acute rejection. Transplantation. 2008;86(12):1754-61.
140. Woodle ES, Shields AR, Ejaz NS, Sadaka B, Girnita A, Walsh RC, et al. Prospective iterative trial of proteasome inhibitor-based desensitization. Am J Transplant. 2015;15(1):101-18.
141. Mulder A, Heidt S, Vergunst M, Roelen DL, Claas FH. Proteasome inhibition profoundly affects activated human B cells. Transplantation. 2013;95(11):1331-7.

Chapter 3

Detecting the humoral alloimmune response: We need more than serum antibody screening

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ABSTRACT

Whereas many techniques exist to detect human leukocyte antigen (HLA) antibodies in the sera of immunized individuals, assays to detect and quantify HLA-specific B cells are only just emerging. The need for such assays is becoming clear, as in some patients HLA-specific memory B cells have been shown to be present in the absence of the accompanying serum HLA antibodies. Because HLA-specific B cells in the peripheral blood of immunized individuals are present at only a very low frequency, assays with high sensitivity are required. In this review, we discuss the currently available methods to detect and/or quantify HLA-specific B cells, as well as their promises and limitations. We also discuss scenarios in which quantification of HLA-specific B cells may be of additional value, besides classical serum HLA antibody detection.

INTRODUCTION

The humoral immune system is increasingly recognized as being an important player in the immune response towards transplanted organs. Central in the humoral immune system is the B cell, which finds its origin in the bone marrow. Once mature, antigen recognition can trigger naïve B cells to differentiate into antibody producing plasma cells or memory B cells. In the setting of organ transplantation, the antigens recognized are mostly HLA, although development of non-HLA antibodies has been described as well (1-3). The destructive capacity of alloantibodies was already recognized in the 1960s because of the occurrence of hyperacute rejection in many immunized kidney allograft recipients (4). Although the problem of hyperacute allograft rejection has virtually been eliminated by the introduction of cross-match techniques (5) and detailed pre-transplant antibody analysis (6), *de novo* alloantibody production post-transplantation is likely to be involved in most other types of allograft rejection (7-12).

Importantly, besides differentiating into alloantibody-producing plasma cells, increasing evidence suggests that B cells also play a role as antigen presenting cells during T cell mediated rejection. In a murine cardiac transplantation model, the selective absence of major histocompatibility complex (MHC) class II on B cells resulted in prolonged graft survival (13). Moreover, in clinical transplantation it has been shown that B cells are present in allografts undergoing T cell mediated rejection, in the absence of alloantibodies or complement deposition (14, 15). In addition, B cells with regulatory properties have been described (16, 17). In animal models, regulatory B cells have been shown to be able to induce long-term graft survival (18, 19). Importantly, B cells with regulatory properties may be involved in the induction or maintenance of clinical operational tolerance (20, 21). Whether antigen-specificity plays any role in regulation mediated by B cells remains to be determined.

Novel insights into the impact of the humoral immune response on the outcome of organ transplantation have resulted in a renewed interest in B cell research. However, despite the availability of many tools to study alloantibodies and phenotypical characteristics of B cells, reliable assays for analysis of alloantigen-specific B cells are only just emerging. This review will provide a comprehensive overview of the current techniques to study HLA-specific B cells and discuss their potential clinical application. Because most work have been performed in the field of kidney transplantation, we will focus on the renal transplant setting and will briefly discuss the utility of memory B cell techniques in other transplant settings.

ANTIBODY PROFILE OF *EX VIVO* ACTIVATED B CELLS

Luminex single antigen bead (SAB) assays are used routinely in many laboratories to determine the fine specificity of HLA antibodies in the sera of immunized individuals. This sensitive way of antibody detection is obviously not restricted to serum samples. Perry and colleagues were the first to describe single antigen flow bead analysis of concentrated supernatants from cultures of enriched $CD138^+$ plasma cells that had been isolated from the bone marrow of sensitized individuals (22). In these assays, the specificities found in the culture supernatants greatly overlapped with the specificities found in serum, supporting the notion that serum antibodies are mainly produced by bone marrow-residing plasma cells.

Both antibody-producing plasma cells as well as long-lived memory B cells originate from activated naïve B cells that have undergone class switch and somatic hypermutation in germinal centers upon recognition of cognate antigen (23, 24). Whereas plasma cells are antibody production factories residing in bone marrow, memory B cells circulate through secondary lymphoid organs and peripheral blood. Upon secondary encounter with the same antigen, memory B cells can rapidly respond and become plasma cells, instantly producing high affinity, class-switched antibodies. Whereas it is clear that circulating antibodies are mainly produced by plasma cells, it remains uncertain whether serum antibody levels and frequencies of memory B cells correlate well for most antigens (25). Studies on humoral responses against viral antigens have yielded contradictory results (26-31), indicating the importance of considering the memory B cell compartment alongside serum antibody levels. Memory B cells can be activated *in vitro* to become antibody producing cells, allowing for studying memory B cell specificity and frequency, as has been extensively shown by Lanzavecchia and colleagues (26, 32).

Han *et al*. described a method to determine HLA antibody specificities of *ex vivo* activated B cells (33). After CD40-driven polyclonal B cell activation, supernatants were concentrated and assayed by Luminex SAB assays. In 13 out of 16 transplant recipients tested, they found donor-specific antibodies (DSA) in B cell supernatants. A total of 50 DSA were detected, of which 35 matched those found in serum, and 11 DSA were detected in serum, but not in the supernatant of activated B cells. The latter may be due to limited sampling of B cells from the periphery or low precursor frequencies. Interestingly, 4 specificities were exclusively detected in the supernatant of activated B cells. These may potentially represent antibodies that were absorbed by the graft and thereby led to negative results in serum DSA tests (34, 35). However, other explanations are also possible. A recent study by Snanoudj *et al*. describes HLA antibody levels in supernatants of activated B cells from patients awaiting a kidney transplant, some after rejection of a previous transplant (Snanoudj *et al*., in press). Also here, a proportion of antibodies found in the supernatants were not detected in the serum. Because in the majority of patients no transplant was left *in situ* or no previous transplant had been performed at all, antibody absorption can be ruled out. This leaves the

possibility that memory B cells may be present in the circulation in the absence of antibody production of the same specificity.

We have performed similar experiments in which supernatants from isolated B cells, activated in a CD40-driven fashion, were tested for the presence of HLA antibodies alongside serum samples.

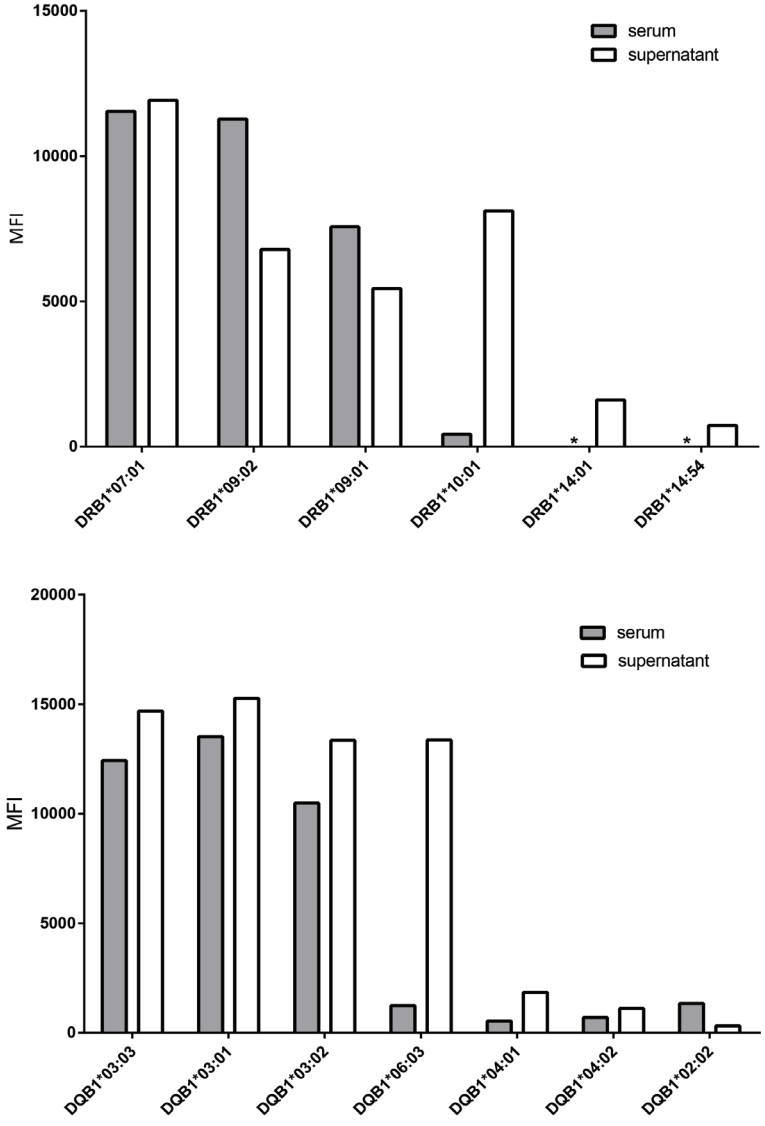

Figure 1. HLA class II antibody specificities in serum and B cell culture supernatants detected by Luminex SAB assay. *B cells isolated from the peripheral blood of an immunized patient awaiting a kidney transplant were polyclonally activated. Ten-fold concentrated supernatants from these cultures were tested for presence of HLA class II antibodies, as well as the sera from the same bleeding date. Among the antibodies detected, HLA-DRB1*10:01 and –DQB1*06:03 were detected in the B cell culture supernatants, whereas these specificities were undetectable in the serum. This patient had no detectable HLA class I antibodies in the serum, nor in the B cell culture supernatant. MFI: mean fluorescence intensity, *: No antibody detected.*

Figure 1 shows an example of an immunized patient on the waiting list for a kidney transplant of whom we tested the serum for HLA antibody specificities, as well as the supernatant of polyclonally activated B cells from the same bleeding date. Several HLA class II antibodies were detected in both the serum and B cell culture supernatant. Most strikingly, despite the absence of HLA-DRB1*10:01 and -DQB1*06:03 antibodies in the serum, the B cell culture supernatants showed strong positive reactions for these HLA specificities as detected by Luminex SAB assay. This clearly shows that memory B cells towards certain HLA antigens can be present, despite undetectable levels of serum antibodies towards these antigens. Potentially, such memory B cells may become activated if a transplant is performed with a donor organ expressing these mismatched antigens. This is illustrated by a study of Zachary and colleagues who described a cohort of kidney transplant recipients (KTR) that had circulating HLA-specific memory B cells in the absence of HLA antibodies pre-transplant (36). In 13 out of 16 patients (81%), they observed HLA antibody formation with specificities corresponding to those pre-existing memory B cells within 2 months after transplantation.

Obviously, alternative techniques exist to determine antibody specificities in both serum and cell culture supernatants, such as the enzyme-linked immunosorbent assay (ELISA) or complement-dependent cytotoxicity (CDC) assay, both of which Mulder and colleagues have used previously to detect HLA-specificity of B cells cultured in limiting dilution (discussed below) (37). However, the high level of sensitivity and specificity makes Luminex SAB analysis particularly useful for comparison of serum and culture supernatant antibody reactivity. Clearly, while using Luminex SAB analysis of B cell culture supernatants is useful to determine the HLA antibody profile of the peripheral memory B cell pool, it does not provide information on the frequency of HLA-specific memory B cells. Therefore, assays that can be used to quantify the HLA-specific B cell compartment would provide an important addition to this test.

FLOW CYTOMETRY BASED ASSAYS FOR HLA-SPECIFIC B CELL QUANTIFICATION

The introduction of HLA tetramer technology has revolutionized the way in which antigen-specific T cells can be quantified. For this purpose, immunodominant peptides are loaded into HLA molecules of interest, allowing for precise determination of antigen-specific $CD8^+$ T cell frequencies by flow cytometry (38). Besides analysis of T cells responsive against, for example, viral or tumor-associated peptides, HLA tetramers can also be used to study alloreactive T cells (39). Because HLA-specific B cells bind epitopes on foreign HLA molecules in their native confirmation by means of their B cell receptor (BCR), HLA tetramers could potentially also be used for quantification of alloreactive B cells.

Mulder and colleagues were the first to show that HLA tetramers could indeed be used for detecting, quantifying and isolating HLA-specific B cells (40). When human B cell hybridomas which specifically produce anti-HLA monoclonal antibodies (mAbs) of defined specificity (41), were incubated with tetrameric HLA complexes, a clear tetramer-positive cell

population could be detected by flow cytometry, showing proof of principle. Moreover, HLA-specific B cells from pregnancy-immunized individuals, isolated by cell sorting after tetramer staining, could be activated to produce HLA-specific antibodies.

Zachary and colleagues followed up on this work by using tetramer staining to quantify HLA-specific B cells in KTR (42, 43). In their initial studies, B cell frequencies directed towards single HLA specificities were found to consist up to 5.5% of $CD19^+$ B cells (42). Importantly, antibody-negative, but tetramer-positive individuals were more prone to produce antibodies of the tetramer specificity early after transplantation compared to tetramer-low or-negative individuals, indicating that the presence of memory B cells in absence of serum HLA antibodies may give rise to rapid antibody formation post-transplant (43). These findings have been extended in a recent report, where HLA-specific B cell frequencies in sensitized kidney transplant recipients who were antibody negative at time of transplantation were determined by tetramer staining. In patients who had not been treated with rituximab induction therapy, the presence of pre-transplant HLA-specific B cells was associated with post-transplant antibody production to the tetramer antigen tested (36). Subsequent B cell reconstitution after rituximab appeared to be accompanied by a delayed return of donor-specific memory B cells compared to third party HLA-specific B cells (44).

A potential caveat of B cell identification by HLA tetramers is binding of B cells to streptavidin-phycoerythrin, leading to non-specific staining of B cells (40, 45). This potentially results in an overestimation of alloreactive B cell frequencies. Because up to a staggering 6% of total $CD19^+$ B cells have been described to bind HLA tetramers of single specificity in patients without the presence of circulating HLA antibodies (36), this possibility should certainly not be ruled out. This technical issue may be overcome by immunomagnetic depletion with streptavidin beads prior to staining (40). Alternatively, one may use identical tetramers conjugated to 2 different fluorochromes as advocated by Chen and colleagues, who used tetramer staining in an experimental animal setting of B cell alloreactivity (46).

ELISPOT ASSAYS FOR QUANTIFYING HLA CLASS I-SPECIFIC B CELLS

Tetramer-based B cell staining is a straightforward means of quantifying HLA-specific B cells. However, for T cells, it has previously been shown that flow cytometric detection of low level responses is less sensitive than enzyme-linked immunosorbent spot (ELISPOT) assays (47). It is likely that for low-level B cell responses the same holds true, although this needs to be formally established. Furthermore, without further sorting and *in vitro* activation of the tetramer stained cells, the isotype and antibody producing capacity of the HLA-specific B cells remains unknown. To overcome these issues, alternative strategies for HLA-specific B cell quantification have been established. One of the first techniques published for estimating the precursor frequency of B cells with HLA specificity was established in our laboratory (37). For this B cell precursor frequency (BCPF) assay, supernatants from polyclonally activated B cells cultured in limiting dilution fashion were tested for the presence of HLA-specific B cells, allowing

for calculation of the HLA-specific B cell precursor frequency. At the time of development of this particular assay, the CD40L-driven B-cell activation protocol applied included a poorly defined supplement of cytokines from phytohaemagglutinin activated T cells, which precluded standardization that would normally be required for a clinical test.

The ELISPOT platform is characterized by its high sensitivity (47) and may therefore be particularly useful for detecting low-frequency (HLA-specific) B cells. The first report on applying ELISPOT assays in the setting of humoral alloimmunity was by Fan *et al*. who used ABO-specific ELISPOT assays to detect blood group tolerance in the setting of infant heart transplantation (48). By using this assay, the authors could show that tolerance in this setting was caused by elimination of blood group-specific B cells.

Subsequently, Perry *et al*. developed an ELISPOT assay for the quantification of bone marrow-residing plasma cells producing HLA antibodies (22). Here, enriched plasma cells from bone marrow aspirates were directly plated onto HLA-coated plates in the presence of PHA and exogenous cytokines. Whereas antibody-secreting cells were detected in bone marrow aspirates from immunized individuals, no antibody secreting plasma cells were found in peripheral blood from the same individuals, confirming that the vast majority of antibody producing cells reside in the bone marrow. Although this approach is very informative with respect to the presence or absence of antibody secreting plasma cells, bone marrow aspirates will not be a regularly available cell source for recipients of solid organ transplants. In contrast, peripheral blood can be routinely analyzed, and harbors memory B cells (25, 49). However, detection of HLA-specific memory B cells from the periphery has different requirements in comparison to plasma cells, most notably because B cells require short-term *in vitro* preactivation to produce antibodies.

By combining the ability of HLA-specific antibodies to bind to synthetic HLA molecules and polyclonal B cell activation in a CD40-driven culture system, we were able to develop the first HLA-specific memory B cell ELISPOT assay (50). For this assay, isolated B cells from peripheral blood are precultured with either L-CD40L cells or recombinant αCD40 mAb in combination with a cytokine cocktail consisting of IL-2, IL-10, IL-21, and CpG DNA (51). This activation protocol solely leads to antigen-specific IgG spot formation in the memory B cell population, and is therefore a suitable tool to quantify pre-existing memory B cells (52). Moreover, by CDR3 fragment analysis, we were able to show that there was no change in B cell polyclonality due to *in vitro* activation (52).

After activation, B cells are transferred to ELISPOT plates that have been precoated with streptavidin, followed by biotinylated synthetic HLA class I molecules (Figure 2). We could demonstrate, by using B cell hybridomas, that these synthetic HLA molecules allowed for the detection of all HLA-antibody producing cells of corresponding specificity, while no spots were detected when testing HLA-antibody producing cells of other specificity. In both pregnancy-immunized individuals, as well as sensitized patients awaiting a kidney transplant, we could detect HLA class I-specific memory B cells in the circulation.

All of the aforementioned techniques use recombinant HLA molecules for detecting either HLA-specific B cells directly or the antibodies they produce. These recombinant HLA molecules are generally stabilized by a single peptide in the peptide-binding groove. Our group has previously shown that peptide selectivity exists for certain HLA-specific monoclonal antibodies to bind to HLA molecules (53), potentially influencing the sensitivity of assays using recombinant HLA molecules. However, data from the group of Zachary, and our own data on the level of polyclonal B cell responses suggest that in a polyclonal response towards foreign HLA, peptide selectivity of single clones is not visible due to the activity of the remaining clones (42, 50).

Regardless, one may still argue that using actual donor HLA molecules for the detection of donor-specific memory B cells will be more clinically relevant. Recently, Lynch *et al*. made the first step towards detecting memory B cells directed at the full spectrum of donor HLA class I molecules (54). For the detection of donor-specific HLA class I-specific memory B cells in ELISPOT format, they isolated donor fibroblasts, which were subsequently cultured as monolayers. Autologous and third party fibroblasts were used as negative controls. Once confluent, polyclonally activated B cells were added to these fibroblast monolayers. By applying this method to a cohort of nine KTR that were HLA antibody negative at time of transplant, the authors showed an increase in donor-specific B cells in all patients. This occurred despite the absence of circulating HLA-specific antibodies after transplantation and provides a novel insight on the possible magnitude of the humoral alloimmune response after clinical transplantation.

Although of interest to get a better understanding of the humoral alloimmune response, the fibroblast-based assay is likely not useful for regular clinical use since it requires an extensive culture period of allogeneic and autologous fibroblasts and appears to suffer from rather high background signals.

ELISPOT ASSAYS FOR QUANTIFYING HLA CLASS II-SPECIFIC B CELLS

All current assays focusing on quantifying the number of HLA-specific B cells have been limited to determining levels of HLA class I-specific B cells. However, the majority of antibodies developing after transplantation appear to be directed against HLA class II molecules (55-57). Therefore, we have recently developed an ELISPOT assay enabling the detection and quantification of HLA class II-specific memory B cells in the peripheral blood of sensitized individuals (Karahan *et al.*, submitted). For this assay, similar to the HLA class I-specific ELISPOT, isolated B cells are pre-cultured with a CD40-driven polyclonal activation cocktail. Activated B cells are then transferred to anti-human IgG mAb-coated ELISPOT plates after which HLA class II-specific antibody secreting memory cells are detected by biotinylated HLA class II molecules (Figure 2).

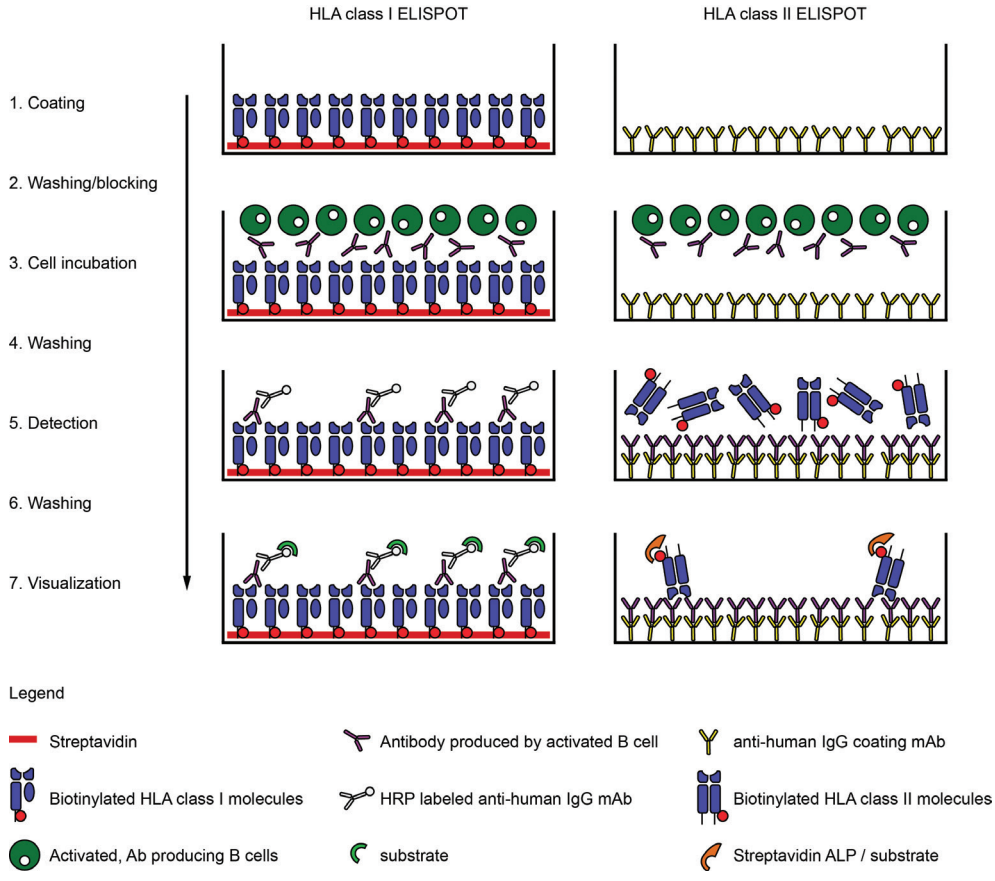

Figure 2. Schematic overview of HLA class I- and HLA class II-specific memory B cell ELISPOT assays adapted from Heidt et al. *(89). (Left panel) ELISPOT plates are coated with streptavidin followed by incubation of biotinylated HLA class I molecules to saturate the plate with the HLA molecules of interest. After blocking of the ELISPOT plate, activated, antibody producing B cells are added. After an overnight incubation, unbound antibodies and the B cells are washed away, leaving bound antibodies that are the footprints of HLA-class I antibody-producing cells. Bound antibodies are detected by an HRP-labeled anti-human IgG mAb. Finally, using a substrate, each HLA class I antibody producing cell can be visualized as a single spot. (Right panel) ELISPOT plates are coated with anti-human IgG mAb. After blocking of the ELISPOT plate, activated, antibody producing B cells are added. After an overnight incubation, cells are washed away and leaving the antibodies produced by the activated B cells bound to the plate. Among these antibodies captured in the plate, the ones that are HLA-specific can be detected by adding biotinylated HLA class II molecules. Finally, using streptavidin-ALP and a proper substrate, each HLA class II antibody-producing cell can be visualized as a single spot. HRP indicates horse radish peroxidase; ALP, alkaline phosphatase; IgG, immunoglobulin G.*

Using this method in peripheral blood samples of pregnancy-immunized women, we found memory B cell frequencies ranging from 25 to 756 per 10^6 B cells, specific for the immunizing paternal HLA class II molecules. We did not detect any spots in non-immunized males, which confirmed that spots detected in pregnancy-immunized women were produced by memory B cells.

Based on the very sensitive and practical ELISPOT platform, HLA class I- and class II-specific ELISPOT assays provide unique tools to monitor and quantify the HLA-specific memory B cell pool in sensitized transplant recipients in a highly specific and reproducible manner.

WHAT WILL BE THE FUTURE OF DONOR-SPECIFIC MEMORY B CELL ASSAYS?

Determining the level of HLA- or donor-specific memory B cells can be performed either before transplantation for risk stratification, or after transplantation for immunological monitoring. In both settings, we foresee that assays to quantify the HLA-specific memory B cell pool will aid clinical decision making in the future.

As discussed above, in the setting of renal transplantation, there are situations in which candidates for transplantation have no detectable DSA at time of transplantation, regardless of the presence of donor-specific memory B cells in the circulation (36). Especially for patients with antibody positivity in historical sera, or husband to wife and child to mother combinations, determining the potential to develop an accelerated humoral rejection is of great importance. In retransplant candidates with the previous allograft still *in situ*, serum HLA antibodies may be undetectable due to antibody absorption by the failed allograft. It has been shown that upon graft nephrectomy, these antibodies become detectable in the serum, while previously undetectable (58). Such humoral immunization may be detected by circulating HLA-specific memory B cells even before the graft is surgically removed.

Another group of patients for which pretransplant donor-specific memory B cell quantification may be of importance are highly sensitized KTR who have been treated with desensitization protocols and B cell depleting therapies (59). Association of increased levels of post-transplant DSA with the incidence and severity of antibody mediated rejection in patients with low levels of pretransplant DSA after desensitization treatment implies a role for the relevance of memory B cell responses in antibody production and eventually on clinical outcome (60). Although desensitization protocols including low/high dose IVIg, plasmapheresis, or immunoadsorption are effective to remove alloantibodies from the serum, little is known about the effects of B cell-depleting therapies on the function of memory B cells particularly in the repopulating B cell compartment after the therapy (44).

As discussed above, HLA antibody formation in the presence of the allograft may remain undetected due to antibody absorption, leaving alloreactive memory B cells as a possible surrogate marker for the humoral alloimmune response. Because many of the HLA antibodies that are detected after transplantation are directed at HLA class II antigens (55-57), it

will be of interest to see the kinetics of both HLA class I and class II-specific B cells after transplantation, and their relation to graft outcome. Interestingly, environmental factors may play a role in amplifying the donor-specific humoral immune response after transplantation. It has been shown for both sensitized patients on the transplant waiting list, as well as for patients transplanted following desensitization that the occurrence of infections or other proinflammatory events result in an increase in the breadth and strength of HLA-specific antibodies (61). This is likely due to a burst of pro-inflammatory cytokines upon recognition of pathogen-associated molecular patterns or damage-associated molecular patterns. In line with this hypothesis, *de novo* HLA antibody production in KTR has been reported after seasonal influenza vaccination (62). These results advocate that monitoring the humoral immune system after transplantation is of great importance.

Longitudinal monitoring of HLA-specific B cell frequencies after transplantation may help to understand not only the effect of pro-inflammatory events, such as described above but also including cellular rejection, on the magnitude and durability of the humoral memory response. Finally, these observations may be transformed into monitoring algorithms with the ultimate aim to tailor immunosuppressive treatments in cases where B-cell memory compartment should be targeted. For clinical validation of HLA-specific ELISPOT assays, we are currently conducting a multi-center trial in the Netherlands. To this aim, blood samples from kidney retransplant patients are taken before transplantation, as well as 3 and 6 months after transplantation to be tested in HLA-specific memory B cell ELISPOT assays. This will provide information on the kinetics of donor-specific memory B cells in the early phase after transplantation in a high-risk renal transplant cohort.

The assays described here have potential use beyond the renal transplant setting. It is now well established that DSA are associated with inferior graft survival and increased acute rejection of thoracic transplants (63-66). Ventricular assist devices, which are a bridge to transplantation in cardiac transplant candidates, are considered as risk factors for HLA immunization (67, 68). Similar to kidney transplantation, plasmapheresis, IVIg and B cell depleting approaches are commonly used to remove circulating antibodies and/or suppress antibody responses in sensitized cardiac patients (68). In case of IVIg treatment, serum HLA antibody screening by Luminex bead assays may result in incorrect interpretation due to the interference of IVIg with the beads (69), a problem circumvented by using memory B cells as source of HLA antibodies *ex vivo* (33). Furthermore, following B cell depletion, assays capable of detecting HLA-specific memory B cells may be informative to determine whether alloreactive B cells reemerge.

The liver has long been considered as being resistant to antibody-mediated rejection possibly due to its capacity to absorb antibodies and secrete soluble HLA class I molecules (70-72). In fact, combined liver-kidney transplants enable transplantation of highly sensitized patients, because the preimplanted liver protects the kidney by antibody absorption (73). However, despite the protective effects of the liver by clearing HLA class I antibodies, there are multiple

reports showing that persisting anti-HLA class II antibodies may be detrimental to the kidney graft (74-76). In addition, there is increasing evidence that DSA could eventually lead to chronic rejection and inferior liver allograft survival (77-79). In case antibody absorption by the liver precludes proper serum antibody identification, assays for quantifying HLA-specific B cells may help detecting the humoral alloimmune response.

Presence of DSA in recipients undergoing unrelated hematopoietic stem cell (HSCT) or cord blood transplantation has been associated with engraftment failure, decreased patient survival, increased occurrence of acute graft *versus* host disease (GvHD), and decreased neutrophil and platelet recovery (80-84). For the latter, many patients need platelet transfusions as a supportive treatment. There are several reports showing that platelet transfusion refractoriness can be a consequence of HLA antibodies produced by donor cells in patients undergoing HSCT from antibody-positive multiparous female donors (85-88). In order to achieve better management of post-transplant platelet refractoriness in patients transplanted from donors with an immunization history such as pregnancy, antibody specificities produced by HLA-specific memory B cells from the donor may be defined in advance by testing the culture supernatants from polyclonally activated donor B cells by Luminex bead assays (33).

In conclusion, several assays for the detection and quantification of HLA-specific memory B cells have been developed over the last few years. The task ahead is to determine their applicability in the various clinical transplantation settings. We envisage that, besides serum HLA antibody detection, HLA-specific memory B cell assays will serve as additional tools to determine risk factors for individual patient management.

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REFERENCES

1. Opelz G. Non-HLA transplantation immunity revealed by lymphocytotoxic antibodies. Lancet. 2005;365(9470):1570-6.
2. Jackson AM, Lucas DP, Melancon JK, Desai NM. Clinical relevance and IgG subclass determination of non-HLA antibodies identified using endothelial cell precursors isolated from donor blood. Transplantation. 2011;92(1):54-60.
3. Dragun D, Muller DN, Brasen JH, Fritsche L, Nieminen-Kelha M, Dechend R, et al. Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection. N Engl J Med. 2005;352(6):558-69.
4. Kissmeyer-Nielsen F, Olsen S, Petersen V, Fjeldborg O. Hyperacute rejection of kidney allografts, associated with pre-existing humoral antibodies against donor cells. Lancet. 1966;24(2 (7465)):662-5.
5. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. The New England journal of medicine. 1969;280(14):735-9.
6. Susal C, Roelen DL, Fischer G, Campos EF, Gerbase-DeLima M, Honger G, et al. Algorithms for the determination of unacceptable HLA antigen mismatches in kidney transplant recipients. Tissue Antigens. 2013;82(2):83-92.
7. Itescu S, Tung TC, Burke EM, Weinberg A, Moazami N, Artrip JH, et al. Preformed IgG antibodies against major histocompatibility complex class II antigens are major risk factors for high-grade cellular rejection in recipients of heart transplantation. Circulation. 1998;98(8):786-93.
8. Pelletier RP, Hennessy PK, Adams PW, VanBuskirk AM, Ferguson RM, Orosz CG. Clinical significance of MHC-reactive alloantibodies that develop after kidney or kidney-pancreas transplantation. Am J Transplant. 2002;2(2):134-41.
9. Girnita AL, McCurry KR, Iacono AT, Duquesnoy R, Corcoran TE, Awad M, et al. HLA-specific antibodies are associated with high-grade and persistent-recurrent lung allograft acute rejection. J Heart Lung Transplant. 2004;23(10):1135-41.
10. Di Filippo S, Girnita A, Webber SA, Tsao S, Boyle GJ, Miller SA, et al. Impact of ELISA-detected anti-HLA antibodies on pediatric cardiac allograft outcome. Hum Immunol. 2005;66(5):513-8.
11. Girnita AL, Duquesnoy R, Yousem SA, Iacono AT, Corcoran TE, Buzoianu M, et al. HLA-specific antibodies are risk factors for lymphocytic bronchiolitis and chronic lung allograft dysfunction. Am J Transplant. 2005;5(1):131-8.
12. Piazza A, Poggi E, Ozzella G, Borrelli L, Scornajenghi A, Iaria G, et al. Post-transplant donor-specific antibody production and graft outcome in kidney transplantation: results of sixteen-year monitoring by flow cytometry. Clin Transpl. 2006:323-36.
13. Noorchashm H, Reed AJ, Rostami SY, Mozaffari R, Zekavat G, Koeberlein B, et al. B cell-mediated antigen presentation is required for the pathogenesis of acute cardiac allograft rejection. J Immunol. 2006;177(11):7715-22.
14. Sarwal M, Chua MS, Kambham N, Hsieh SC, Satterwhite T, Masek M, et al. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. N Engl J Med. 2003;349(2):125-38.
15. Steinmetz OM, Panzer U, Kneissler U, Harendza S, Lipp M, Helmchen U, et al. BCA-1/CXCL13 expression is associated with CXCR5-positive B-cell cluster formation in acute renal transplant rejection. Kidney International. 2005;67(4):1616-21.

16. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi) CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. Immunity. 2010;32(1):129-40.
17. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood. 2011;117(2):530-41.
18. Le Texier L, Thebault P, Lavault A, Usal C, Merieau E, Quillard T, et al. Long-term allograft tolerance is characterized by the accumulation of B cells exhibiting an inhibited profile. Am J Transplant. 2011;11(3):429-38.
19. Lee KM, Kim JI, Stott R, Soohoo J, O'Connor MR, Yeh H, et al. Anti-CD45RB/anti-TIM-1-induced tolerance requires regulatory B cells. Am J Transplant. 2012;12(8):2072-8.
20. Sagoo P, Perucha E, Sawitzki B, Tomiuk S, Stephens DA, Miqueu P, et al. Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. J Clin Invest. 2010;120(6):1848-61.
21. Newell KA, Asare A, Kirk AD, Gisler TD, Bourcier K, Suthanthiran M, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. J Clin Invest. 2010;120(6):1836-47.
22. Perry DK, Pollinger HS, Burns JM, Rea D, Ramos E, Platt JL, et al. Two novel assays of alloantibody-secreting cells demonstrating resistance to desensitization with IVIG and rATG. Am J Transplant. 2008;8(1):133-43.
23. Shlomchik MJ, Weisel F. Germinal center selection and the development of memory B and plasma cells. Immunological reviews. 2012;247(1):52-63.
24. Kuppers R. B cells under influence: transformation of B cells by Epstein-Barr virus. Nat Rev Immunol. 2003;3(10):801-12.
25. Crotty S, Aubert RD, Glidewell J, Ahmed R. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. Journal of Immunological Methods. 2004;286(1-2):111-22.
26. Lanzavecchia A, Bernasconi N, Traggiai E, Ruprecht CR, Corti D, Sallusto F. Understanding and making use of human memory B cells. Immunol Rev. 2006;211:303-9.
27. Blanchard-Rohner G, Galli G, Clutterbuck EA, Pollard AJ. Comparison of a limiting dilution assay and ELISpot for detection of memory B-cells before and after immunisation with a protein-polysaccharide conjugate vaccine in children. J Immunol Methods. 2010;358(1-2):46-55.
28. Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, Ahmed R. Cutting edge: long-term B cell memory in humans after smallpox vaccination. Journal of immunology. 2003;171(10):4969-73.
29. Tuaillon E, Tabaa YA, Petitjean G, Huguet MF, Pajeaux G, Fondere JM, et al. Detection of memory B lymphocytes specific to hepatitis B virus (HBV) surface antigen (HBsAg) from HBsAg-vaccinated or HBV-immunized subjects by ELISPOT assay. J Immunol Methods. 2006;315(1-2):144-52.
30. Amanna IJ, Carlson NE, Slifka MK. Duration of humoral immunity to common viral and vaccine antigens. N Engl J Med. 2007;357(19):1903-15.
31. Leyendeckers H, Odendahl M, Lohndorf A, Irsch J, Spangfort M, Miltenyi S, et al. Correlation analysis between frequencies of circulating antigen-specific IgG-bearing memory B cells and serum titers of antigen-specific IgG. Eur J Immunol. 1999;29(4):1406-17.
32. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science. 2002;298(5601):2199-202.
33. Han M, Rogers J, Lavingia B, Stastny P. Peripheral blood B cells producing donor-specific HLA antibodies in vitro. Human Immunology. 2009;70(1):29-34.

34. Mohanakumar T, Waldrep JC, Phibbs M, Mendez-Picon G, Kaplan AM, Lee HM. Serological characterization of antibodies eluted from chronically rejected human renal allografts. Transplantation. 1981;32(1):61-6.
35. Ishida H, Hirai T, Kohei N, Yamaguchi Y, Tanabe K. Significance of qualitative and quantitative evaluations of anti-HLA antibodies in kidney transplantation. Transpl Int. 2011;24(2):150-7.
36. Zachary AA, Lucas DP, Montgomery RA, Leffell MS. Rituximab prevents an anamnestic response in patients with cryptic sensitization to HLA. Transplantation. 2013;95(5):701-4.
37. Mulder A, Kardol MJ, Kamp J, Uit Het Broek C, Schreuder GM, Doxiadis, II, et al. Determination of the frequency of HLA antibody secreting B-lymphocytes in alloantigen sensitized individuals. Clinical and experimental immunology. 2001;124(1):9-15.
38. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, et al. Phenotypic analysis of antigen-specific T lymphocytes. Science. 1996;274(5284):94-6.
39. Moris A, Teichgraber V, Gauthier L, Buhring HJ, Rammensee HG. Cutting edge: characterization of allorestricted and peptide-selective alloreactive T cells using HLA-tetramer selection. J Immunol. 2001;166(8):4818-21.
40. Mulder A, Eijsink C, Kardol MJ, Franke-van Dijk ME, van der Burg SH, Kester M, et al. Identification, isolation, and culture of HLA-A2-specific B lymphocytes using MHC class I tetramers. J Immunol. 2003;171(12):6599-603.
41. Mulder A, Kardol MJ, Arn JS, Eijsink C, Franke ME, Schreuder GM, et al. Human monoclonal HLA antibodies reveal interspecies crossreactive swine MHC class I epitopes relevant for xenotransplantation. Mol Immunol. 2010;47(4):809-15.
42. Zachary AA, Kopchaliiska D, Montgomery RA, Leffell MS. HLA-specific B cells: I. A method for their detection, quantification, and isolation using HLA tetramers. Transplantation. 2007;83(7):982-8.
43. Zachary AA, Kopchaliiska D, Montgomery RA, Melancon JK, Leffell MS. HLA-specific B cells: II. Application to transplantation. Transplantation. 2007;83(7):989-94.
44. Kopchaliiska D, Zachary AA, Montgomery RA, Leffell MS. Reconstitution of peripheral allospecific $CD19^{+}$ B-cell subsets after B-lymphocyte depletion therapy in renal transplant patients. Transplantation. 2009;87(9):1394-401.
45. Pape KA, Taylor JJ, Maul RW, Gearhart PJ, Jenkins MK. Different B cell populations mediate early and late memory during an endogenous immune response. Science. 2011;331(6021):1203-7.
46. Chen J, Yin H, Xu J, Wang Q, Edelblum KL, Sciammas R, et al. Reversing Endogenous Alloreactive B Cell GC Responses With Anti-CD154 or CTLA-4Ig. Am J Transplant. 2013.
47. Karlsson AC, Martin JN, Younger SR, Bredt BM, Epling L, Ronquillo R, et al. Comparison of the ELISPOT and cytokine flow cytometry assays for the enumeration of antigen-specific T cells. J Immunol Methods. 2003;283(1-2):141-53.
48. Fan X, Ang A, Pollock-Barziv SM, Dipchand AI, Ruiz P, Wilson G, et al. Donor-specific B-cell tolerance after ABO-incompatible infant heart transplantation. Nature Medicine. 2004;10(11):1227-33.
49. Bleesing JJ, Souto-Carneiro MM, Savage WJ, Brown MR, Martinez C, Yavuz S, et al. Patients with chronic granulomatous disease have a reduced peripheral blood memory B cell compartment. J Immunol. 2006;176(11):7096-103.
50. Heidt S, Roelen DL, de Vaal YJ, Kester MG, Eijsink C, Thomas S, et al. A Novel ELISPOT Assay to Quantify HLA-Specific B Cells in HLA-Immunized Individuals. Am J Transplant. 2012;12(6):1469-78.

51. Heidt S, Roelen DL, Eijsink C, van Kooten C, Claas FH, Mulder A. Effects of immunosuppressive drugs on purified human B cells: evidence supporting the use of MMF and rapamycin. Transplantation. 2008;86(9):1292-300.
52. Karahan GE, Eikmans M, Anholts JD, Claas FH, Heidt S. Polyclonal B cell activation for accurate analysis of pre-existing antigen-specific memory B cells. Clin Exp Immunol. 2014;177(1):333-40.
53. Mulder A, Eijsink C, Kester MG, Franke ME, Kardol MJ, Heemskerk MH, et al. Impact of peptides on the recognition of HLA class I molecules by human HLA antibodies. J Immunol. 2005;175(9):5950-7.
54. Lynch RJ, Silva IA, Chen BJ, Punch JD, Cascalho M, Platt JL. Cryptic B Cell Response to Renal Transplantation. Am J Transplant. 2013.
55. Campos EF, Tedesco-Silva H, Machado PG, Franco M, Medina-Pestana JO, Gerbase-DeLima M. Post-transplant anti-HLA class II antibodies as risk factor for late kidney allograft failure. Am J Transplant. 2006;6(10):2316-20.
56. Wiebe C, Gibson IW, Blydt-Hansen TD, Karpinski M, Ho J, Storsley LJ, et al. Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant. Am J Transplant. 2012;12(5):1157-67.
57. Everly MJ, Rebellato LM, Haisch CE, Ozawa M, Parker K, Briley KP, et al. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. Transplantation. 2013;95(3):410-7.
58. Marrari M, Duquesnoy RJ. Detection of donor-specific HLA antibodies before and after removal of a rejected kidney transplant. Transpl Immunol. 2010;22(3-4):105-9.
59. Doxiadis, II, Claas FH. Transplantation of highly sensitized patients via the acceptable mismatch program or desensitization? We need both. Curr Opin Organ Transplant. 2009;14(4):410-3.
60. Burns JM, Cornell LD, Perry DK, Pollinger HS, Gloor JM, Kremers WK, et al. Alloantibody levels and acute humoral rejection early after positive crossmatch kidney transplantation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2008;8(12):2684-94.
61. Locke JE, Zachary AA, Warren DS, Segev DL, Houp JA, Montgomery RA, et al. Proinflammatory events are associated with significant increases in breadth and strength of HLA-specific antibody. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2009;9(9):2136-9.
62. Katerinis I, Hadaya K, Duquesnoy R, Ferrari-Lacraz S, Meier S, van Delden C, et al. De novo anti-HLA antibody after pandemic H1N1 and seasonal influenza immunization in kidney transplant recipients. Am J Transplant. 2011;11(8):1727-33.
63. Morrell MR, Pilewski JM, Gries CJ, Pipeling MR, Crespo MM, Ensor CR, et al. De novo donor-specific HLA antibodies are associated with early and high-grade bronchiolitis obliterans syndrome and death after lung transplantation. J Heart Lung Transplant. 2014;33(12):1288-94.
64. Kim M, Townsend KR, Wood IG, Boukedes S, Guleria I, Gabardi S, et al. Impact of pretransplant anti-HLA antibodies on outcomes in lung transplant candidates. Am J Respir Crit Care Med. 2014;189(10):1234-9.
65. Fine NM, Daly RC, Shankar N, Park SJ, Kushwaha SS, Gandhi MJ, et al. The role of donor-specific antibodies in acute cardiac allograft dysfunction in the absence of cellular rejection. Transplantation. 2014;98(2):229-38.
66. Peng DM, Law YM, Kemna MS, Warner P, Nelson K, Boucek RJ. Donor-specific antibodies: can they predict C4d deposition in pediatric heart recipients? Pediatr Transplant. 2013;17(5):429-35.

67. Picascia A, Grimaldi V, Casamassimi A, De Pascale MR, Schiano C, Napoli C. Human leukocyte antigens and alloimmunization in heart transplantation: an open debate. Journal of cardiovascular translational research. 2014;7(7):664-75.
68. Velez M, Johnson MR. Management of allosensitized cardiac transplant candidates. Transplant Rev (Orlando). 2009;23(4):235-47.
69. Ravindranath MH, Terasaki PI, Pham T, Jucaud V, Kawakita S. Therapeutic preparations of IVIg contain naturally occurring anti-HLA-E antibodies that react with HLA-Ia (HLA-A/-B/-Cw) alleles. Blood. 2013;121(11):2013-28.
70. Lobo PI, Spencer C, Douglas MT, Stevenson WC, Pruett TL. The lack of long-term detrimental effects on liver allografts caused by donor-specific anti-HLA antibodies. Transplantation. 1993;55(5):1063-6.
71. Hubscher SG. Antibody-mediated rejection in the liver allograft. Curr Opin Organ Transplant. 2012;17(3):280-6.
72. McMillan RW, Gelder FB, Zibari GB, Aultman DF, Adamashvili I, McDonald JC. Soluble fraction of class I human histocompatibility leukocyte antigens in the serum of liver transplant recipients. Clin Transplant. 1997;11(2):98-103.
73. Olausson M, Mjornstedt L, Norden G, Rydberg L, Molne J, Backman L, et al. Successful combined partial auxiliary liver and kidney transplantation in highly sensitized cross-match positive recipients. Am J Transplant. 2007;7(1):130-6.
74. O'Leary JG, Gebel HM, Ruiz R, Bray RA, Marr JD, Zhou XJ, et al. Class II alloantibody and mortality in simultaneous liver-kidney transplantation. Am J Transplant. 2013;13(4):954-60.
75. Dar W, Agarwal A, Watkins C, Gebel HM, Bray RA, Kokko KE, et al. Donor-directed MHC class I antibody is preferentially cleared from sensitized recipients of combined liver/kidney transplants. Am J Transplant. 2011;11(4):841-7.
76. Reichman TW, Marino SR, Milner J, Harland RC, Cochrane A, Millis JM, et al. Acute humoral rejection in an ABO compatible combined liver-kidney transplant--the kidney is not always protected. Am J Transplant. 2009;9(8):1957-60.
77. Kozlowski T, Rubinas T, Nickeleit V, Woosley J, Schmitz J, Collins D, et al. Liver allograft antibody-mediated rejection with demonstration of sinusoidal C4d staining and circulating donor-specific antibodies. Liver Transpl. 2011;17(4):357-68.
78. O'Leary JG, Kaneku H, Susskind BM, Jennings LW, Neri MA, Davis GL, et al. High mean fluorescence intensity donor-specific anti-HLA antibodies associated with chronic rejection Postliver transplant. Am J Transplant. 2011;11(9):1868-76.
79. Musat AI, Agni RM, Wai PY, Pirsch JD, Lorentzen DF, Powell A, et al. The significance of donor-specific HLA antibodies in rejection and ductopenia development in ABO compatible liver transplantation. Am J Transplant. 2011;11(3):500-10.
80. Zhu H, He J, Cai J, Yuan X, Jiang H, Luo C, et al. Pre-existing anti-HLA antibodies negatively impact survival of pediatric aplastic anemia patients undergoing HSCT. Clin Transplant. 2014;28(11):1225-33.
81. Ansari M, Uppugunduri CR, Ferrari-Lacraz S, Bittencourt H, Gumy-Pause F, Chalandon Y, et al. The clinical relevance of pre-formed anti-HLA and anti-MICA antibodies after cord blood transplantation in children. PLoS One. 2013;8(8):e72141.
82. Takanashi M, Atsuta Y, Fujiwara K, Kodo H, Kai S, Sato H, et al. The impact of anti-HLA antibodies on unrelated cord blood transplantations. Blood. 2010;116(15):2839-46.

83. Ruggeri A, Rocha V, Masson E, Labopin M, Cunha R, Absi L, et al. Impact of donor-specific anti-HLA antibodies on graft failure and survival after reduced intensity conditioning-unrelated cord blood transplantation: a Eurocord, Societe Francophone d'Histocompatibilite et d'Immunogenetique (SFHI) and Societe Francaise de Greffe de Moelle et de Therapie Cellulaire (SFGM-TC) analysis. Haematologica. 2013;98(7):1154-60.
84. Spellman S, Bray R, Rosen-Bronson S, Haagenson M, Klein J, Flesch S, et al. The detection of donor-directed, HLA-specific alloantibodies in recipients of unrelated hematopoietic cell transplantation is predictive of graft failure. Blood. 2010;115(13):2704-8.
85. Nakazawa Y, Saito S, Hasegawa Y, Yanagisawa R, Sakashita K, Kamijo T, et al. A possible role for the production of multiple HLA antibodies in fatal platelet transfusion refractoriness after peripheral blood progenitor cell transplantation from the mother in a patient with relapsed leukemia. Transfusion. 2007;47(2):326-34.
86. Hatakeyama N, Hori T, Yamamoto M, Inazawa N, Iesato K, Miyazaki T, et al. Platelet transfusion refractoriness attributable to HLA antibodies produced by donor-derived cells after allogeneic bone marrow transplantation from one HLA-antigen-mismatched mother. Pediatric transplantation. 2011;15(8):E177-82.
87. Fasano RM, Mamcarz E, Adams S, Donohue Jerussi T, Sugimoto K, Tian X, et al. Persistence of recipient human leucocyte antigen (HLA) antibodies and production of donor HLA antibodies following reduced intensity allogeneic haematopoietic stem cell transplantation. British journal of haematology. 2014;166(3):425-34.
88. Taniguchi K, Yoshihara S, Maruya E, Ikegame K, Kaida K, Hayashi K, et al. Donor-derived HLA antibody production in patients undergoing SCT from HLA antibody-positive donors. Bone Marrow Transplant. 2012;47(10):1338-42.
89. Heidt S, Eikmans M, Roelen DL, Claas FH. Novel strategies for immunological monitoring of kidney transplant recipients: from microRNA to alloantibodies. Clin Transpl. 2013:257-67.

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.

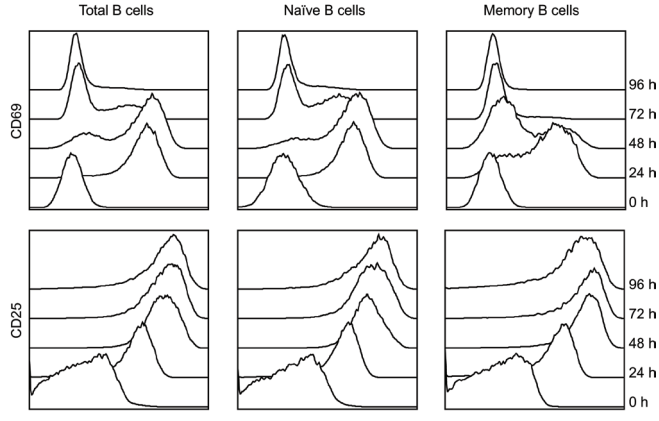


Figure 1. Immunophenotyping for cell surface activation markers. *Total, naïve and memory B cells were monitored for the expression of CD69 and CD25 before (0 h) and several times (24-96 h) after polyclonal activation. All cells up-regulated both activation markers as early as 24 h following activation.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

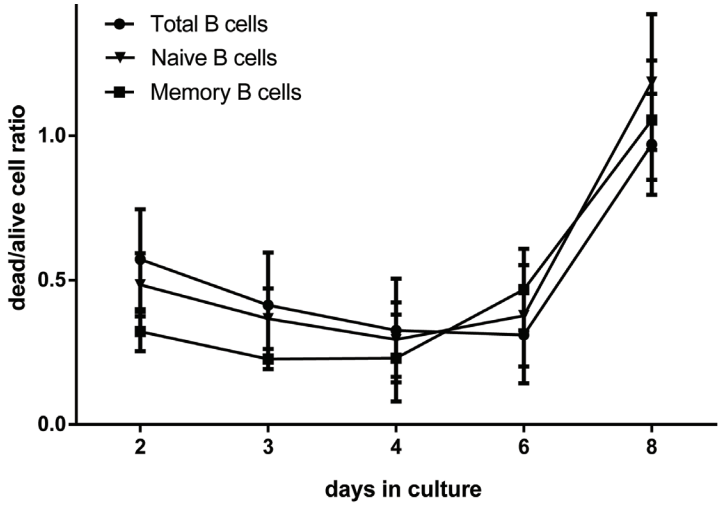


Figure 2. Dead : alive cell ratios among B cell subsets upon polyclonal activation. *Total, naïve and memory B cells were activated polyclonally for varying lengths of time. At each time point, cells were harvested and cell viability was determined by 7-aminoactinomycin D (7-AAD) exclusion by flow cytometry. 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.

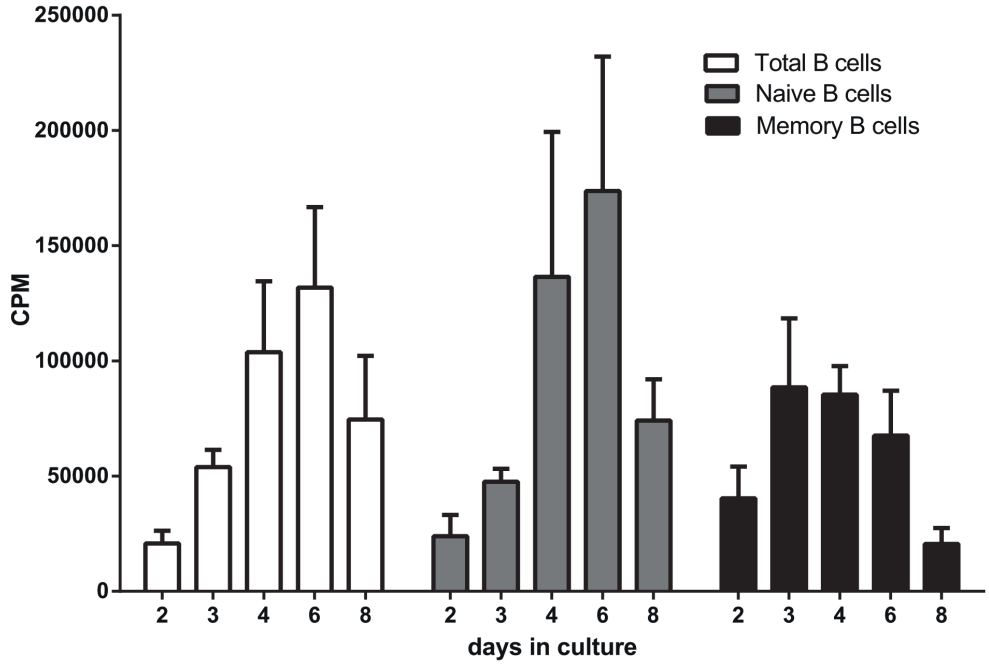
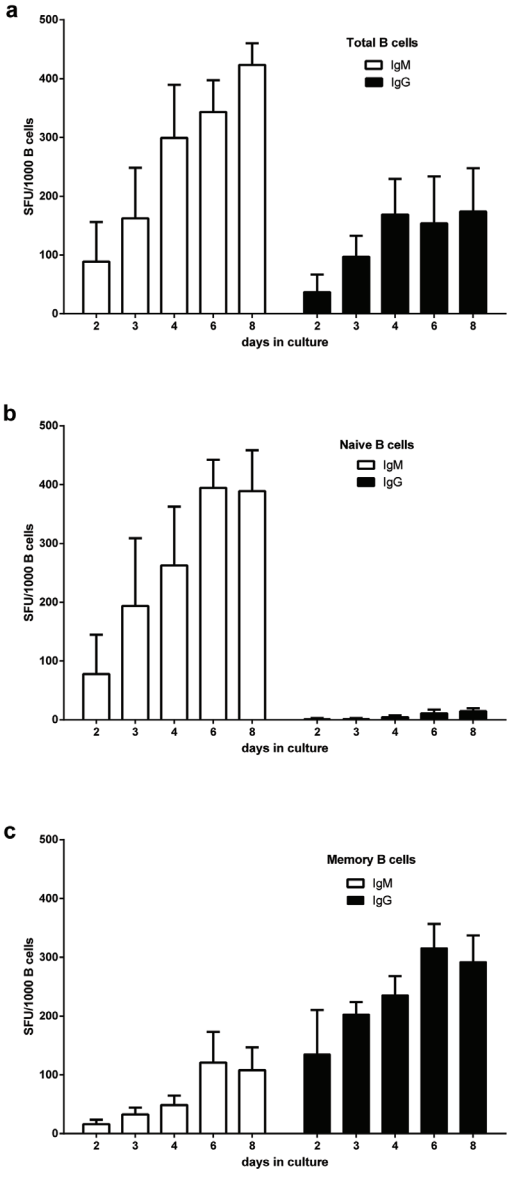


Figure 3. Proliferation kinetics of B cells upon polyclonal activation. *Total, naïve and memory B cells were activated polyclonally for varying lengths of time. Cell proliferation for each fraction was measured by tritiated thymidine ([^{3}H]-TdR) incorporation. Results are expressed as mean counts 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.

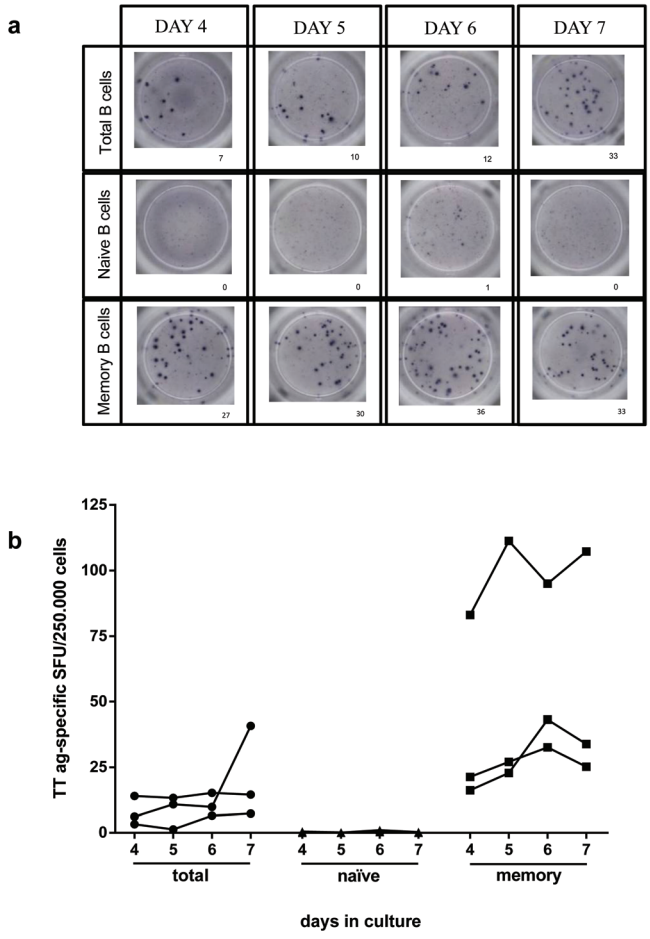


Figure 5. Tetanus toxoid (TT) antigen-specific spots are not detected in naïve B cells upon polyclonal activation. *(a) Activated naïve B cells did not produce TT antigen-specific spots, whereas memory cells produced a robust number of TT specific spots. Total, naïve and memory B cells were activated 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.

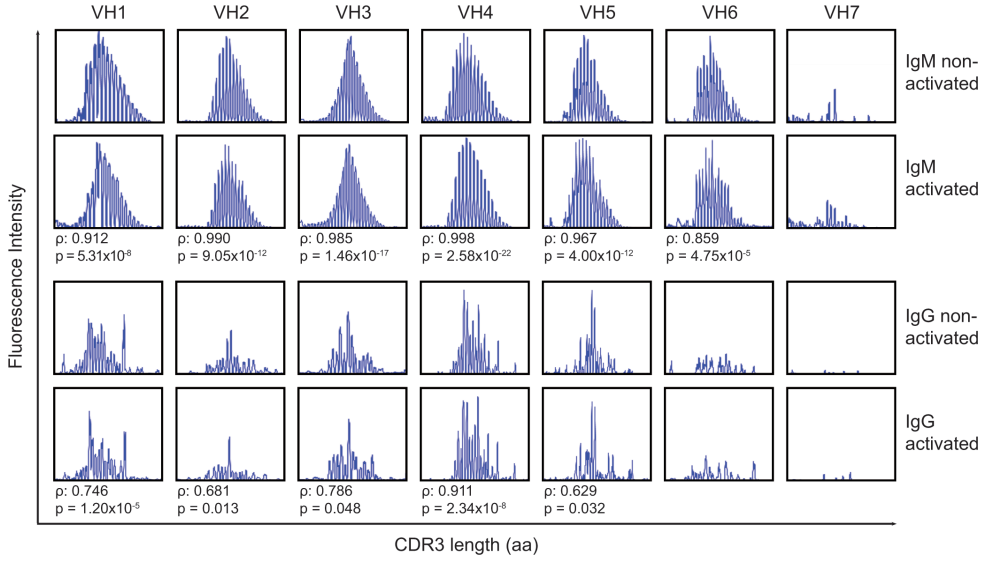


Figure 6. B cell receptor CDR3 length distribution is not significantly altered upon polyclonal B cell activation. *Each panel shows the CDR3 length distribution profile for one of seven V_H families, as determined for non-activated B cells as well as 6-day activated B cells. The x-axis indicates CDR3 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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REFERENCES

1. Kyu SY, Kobie J, Yang H, Zand MS, Topham DJ, Quataert SA, et al. Frequencies of human influenza-specific antibody secreting cells or plasmablasts post vaccination from fresh and frozen peripheral blood mononuclear cells. Journal of Immunological Methods. 2009;340(1):42-7.
2. Crotty S, Ahmed R. Immunological memory in humans. Semin Immunol. 2004;16(3):197-203.
3. Halliley JL, Kyu S, Kobie JJ, Walsh EE, Falsey AR, Randall TD, et al. Peak frequencies of circulating human influenza-specific antibody secreting cells correlate with serum antibody response after immunization. Vaccine. 2010;28(20):3582-7.
4. Weiss GE, Ndungu FM, McKittrick N, Li S, Kimani D, Crompton PD, et al. High efficiency human memory B cell assay and its application to studying Plasmodium falciparum-specific memory B cells in natural infections. J Immunol Methods. 2012;375(1-2):68-74.
5. Titanji K, De Milito A, Cagigi A, Thorstensson R, Grutzmeier S, Atlas A, et al. Loss of memory B cells impairs maintenance of long-term serologic memory during HIV-1 infection. Blood. 2006;108(5):1580-7.
6. Ren S, Fengyu, Zuo S, Zhao M, Wang X, Chen Y, et al. Inhibition of tumor angiogenesis in lung cancer by T4 phage surface displaying mVEGFR2 vaccine. Vaccine. 2011;29(34):5802-11.
7. Tait BD, Susal C, Gebel HM, Nickerson PW, Zachary AA, Claas FH, et al. Consensus guidelines on the testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation. Transplantation. 2013;95(1):19-47.
8. Mulder A, Kardol MJ, Kamp J, Uit Het Broek C, Schreuder GM, Doxiadis, II, et al. Determination of the frequency of HLA antibody secreting B-lymphocytes in alloantigen sensitized individuals. Clinical and experimental immunology. 2001;124(1):9-15.
9. Zachary AA, Kopchaliiska D, Montgomery RA, Leffell MS. HLA-specific B cells: I. A method for their detection, quantification, and isolation using HLA tetramers. Transplantation. 2007;83(7):982-8.
10. Wadia PP, Herrera ND, Abecassis MM, Tambur AR. Mycophenolic acid inhibits maturation and function of human dendritic cells and B cells. Human Immunology. 2009;70(9):692-700.
11. Heidt S, Roelen DL, de Vaal YJ, Kester MG, Eijsink C, Thomas S, et al. A Novel ELISPOT Assay to Quantify HLA-Specific B Cells in HLA-Immunized Individuals. Am J Transplant. 2012;12(6):1469-78.
12. Lynch RJ, Silva IA, Chen BJ, Punch JD, Cascalho M, Platt JL. Cryptic B cell response to renal transplantation. Am J Transplant. 2013;13(7):1713-23.
13. Banchereau J, Rousset F. Growing human B lymphocytes in the CD40 system. Nature. 1991;353(6345):678-9.
14. Rousset F, Garcia E, Banchereau J. Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. J Exp Med. 1991;173(3):705-10.
15. Huggins J, Pellegrin T, Felgar RE, Wei C, Brown M, Zheng B, et al. CpG DNA activation and plasma-cell differentiation of $CD27^-$ naive human B cells. Blood. 2007;109(4):1611-9.
16. Giordani L, Sanchez M, Libri I, Quaranta MG, Mattioli B, Viora M. IFN-alpha amplifies human naive B cell TLR-9-mediated activation and Ig production. J Leukoc Biol. 2009;86(2):261-71.
17. Fecteau JF, Neron S. CD40 stimulation of human peripheral B lymphocytes: distinct response from naive and memory cells. J Immunol. 2003;171(9):4621-9.
18. Ettinger R, Sims GP, Fairhurst AM, Robbins R, da Silva YS, Spolski R, et al. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. J Immunol. 2005;175(12):7867-79.

19. Nagumo H, Agematsu K, Kobayashi N, Shinozaki K, Hokibara S, Nagase H, et al. The different process of class switching and somatic hypermutation; a novel analysis by CD27(-) naive B cells. Blood. 2002;99(2):567-75.
20. Heidt S, Roelen DL, Eijsink C, van Kooten C, Claas FH, Mulder A. Effects of immunosuppressive drugs on purified human B cells: evidence supporting the use of MMF and rapamycin. Transplantation. 2008;86(9):1292-300.
21. Heidt S, Hester J, Shankar S, Friend PJ, Wood KJ. B-Cell Repopulation After Alemtuzumab Induction-Transient Increase in Transitional B cells and Long-Term Dominance of Naive B Cells. Am J Transplant. 2012.
22. Eikmans M, Rekers NV, Anholts JD, Heidt S, Claas FH. Blood cell mRNAs and microRNAs: optimized protocols for extraction and preservation. Blood. 2013;121(11):e81-9.
23. Silva HM, Takenaka MC, Moraes-Vieira PM, Monteiro SM, Hernandez MO, Chaara W, et al. Preserving the B-cell compartment favors operational tolerance in human renal transplantation. Mol Med. 2012;18:733-43.
24. Lim A, Lemercier B, Wertz X, Pottier SL, Huetz F, Kourilsky P. Many human peripheral VH5-expressing IgM^+ B cells display a unique heavy-chain rearrangement. International immunology. 2008;20(1):105-16.
25. Berek C, Berger A, Apel M. Maturation of the immune response in germinal centers. Cell. 1991;67(6):1121-9.
26. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long-term humoral immunity. Nature. 2003;421(6920):282-7.
27. Manz RA, Arce S, Cassese G, Hauser AE, Hiepe F, Radbruch A. Humoral immunity and long-lived plasma cells. Curr Opin Immunol. 2002;14(4):517-21.
28. Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood. 2003;101(11):4500-4.
29. Jiang W, Lederman MM, Harding CV, Rodriguez B, Mohner RJ, Sieg SF. TLR9 stimulation drives naive B cells to proliferate and to attain enhanced antigen presenting function. Eur J Immunol. 2007;37(8):2205-13.
30. Eriksen AB, Indrevaer RL, Holm KL, Landskron J, Blomhoff HK. TLR9-signaling is required for turning retinoic acid into a potent stimulator of RP105 (CD180)-mediated proliferation and IgG synthesis in human memory B cells. Cell Immunol. 2012;279(1):87-95.
31. Tangye SG, Avery DT, Deenick EK, Hodgkin PD. Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses. J Immunol. 2003;170(2):686-94.
32. Ireland SJ, Blazek M, Harp CT, Greenberg B, Frohman EM, Davis LS, et al. Antibody-independent B cell effector functions in relapsing remitting multiple sclerosis: clues to increased inflammatory and reduced regulatory B cell capacity. Autoimmunity. 2012;45(5):400-14.
33. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science. 2002;298(5601):2199-202.
34. Crotty S, Aubert RD, Glidewell J, Ahmed R. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. Journal of Immunological Methods. 2004;286(1-2):111-22.

35. Han M, Rogers J, Lavingia B, Stastny P. Peripheral blood B cells producing donor-specific HLA antibodies in vitro. Human Immunology. 2009;70(1):29-34.
36. Gupta S, Agrawal S, Gollapudi S. Increased activation and cytokine secretion in B cells stimulated with leptin in aged humans. Immun Ageing. 2013;10(1):3.
37. Kamburova EG, Koenen HJ, Boon L, Hilbrands LB, Joosten I. In Vitro Effects of Rituximab on the Proliferation, Activation and Differentiation of Human B Cells. Am J Transplant. 2011.

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.

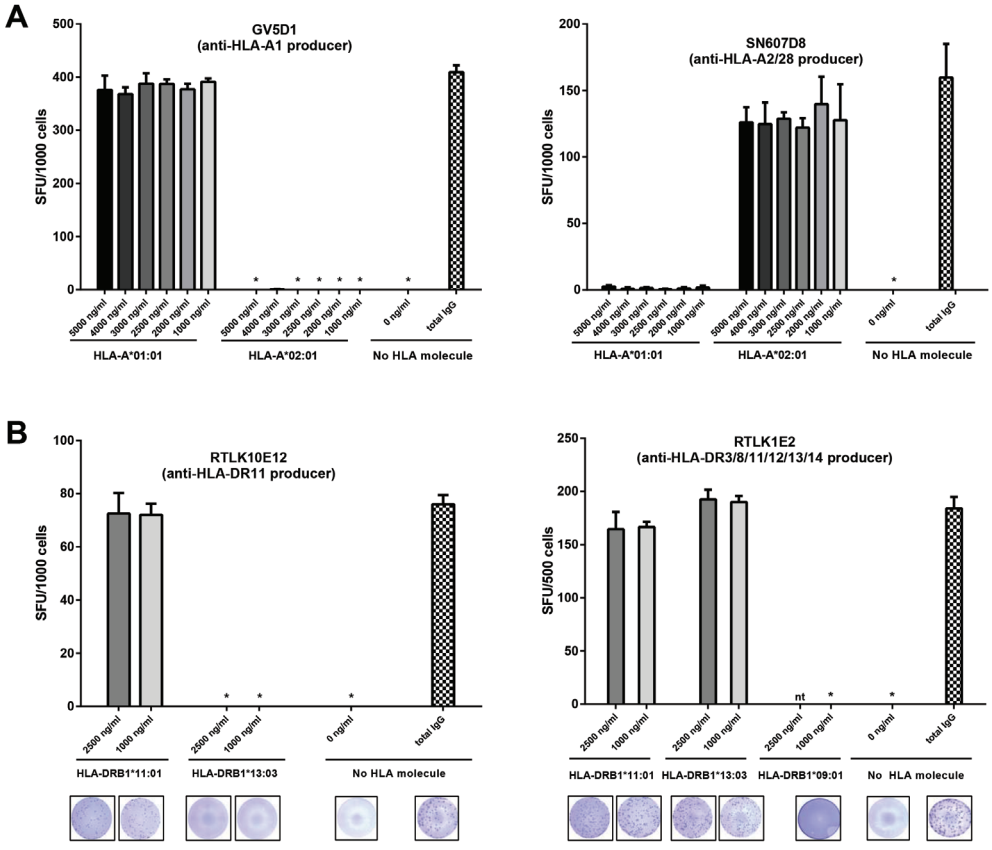

Figure 1. Soluble HLA molecules can be used as detection agents in ELISPOT assays. *Comparable number of spots were detected by using 1000 ng/ml biotinylated HLA molecules as detection agents in the HLA-ELISPOT and total IgG ELISPOT assay (checkered bars) within the same experiment (A) Biotinylated HLA-A*01:01 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%).

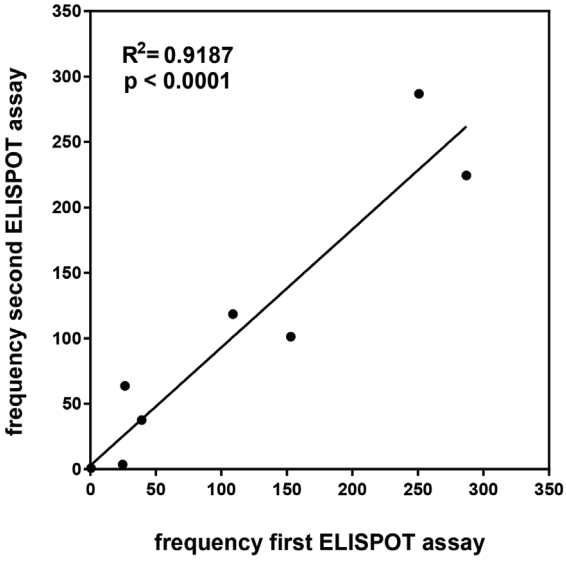

Figure 2. Reproducibility of the HLA class II-specific ELISPOT assay. *PBMCs from three pregnancy-immunized individuals were tested twice in separate experiments for the presence/absence of HLA class II-specific B cells against immunizing and self HLA-DRB1/HLA-DQB1 molecules. Each sample was tested against 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).

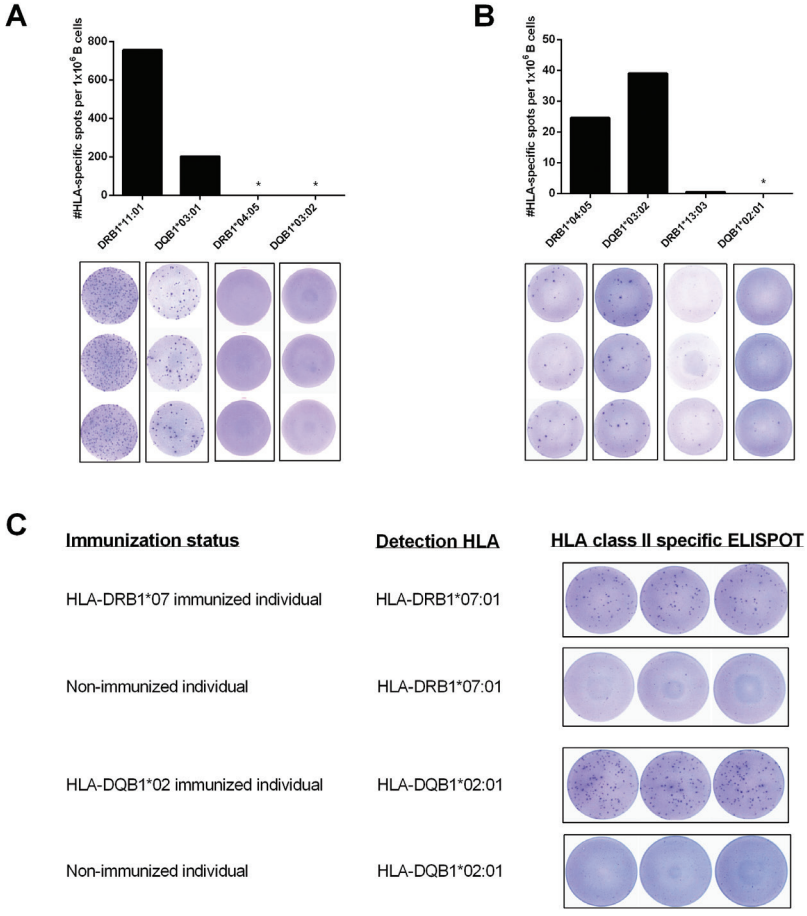

Figure 3. The HLA class II-specific ELISPOT assay is highly specific. *B cells from two pregnancy-immunized individuals sampled 4 months after delivery (3A, donor 3) and 15 years after delivery (3B, donor 4) had different HLA-specific memory B cell frequencies for different HLA molecules. Spots were 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).

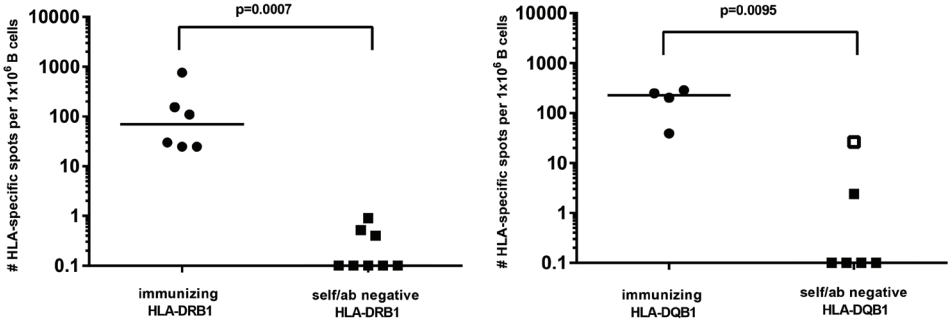

Figure 4. Frequencies of HLA class II-specific memeory B cells detected by ELISPOT. *The number of spots directed at immunizing (A) HLA-DRB1 and (B) HLA-DQB1 molecules was significantly higher than the number of spots against self or antibody negative HLA molecules. The open dot in (B) is the unexpectedly 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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REFERENCES

1. Terasaki PI, Ozawa M. Predicting kidney graft failure by HLA antibodies: a prospective trial. Am J Transplant. 2004;4(3):438-43.
2. Everly MJ, Rebellato LM, Haisch CE, Ozawa M, Parker K, Briley KP, et al. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. Transplantation. 2013;95(3):410-7.
3. Wiebe C, Nickerson P. Posttransplant monitoring of de novo human leukocyte antigen donor-specific antibodies in kidney transplantation. Curr Opin Organ Transplant. 2013;18(4):470-7.
4. Tait BD, Hudson F, Cantwell L, Brewin G, Holdsworth R, Bennett G, et al. Review article: Luminex technology for HLA antibody detection in organ transplantation. Nephrology (Carlton). 2009;14(2):247-54.
5. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long-term humoral immunity. Nature. 2003;421(6920):282-7.
6. Manz RA, Arce S, Cassese G, Hauser AE, Hiepe F, Radbruch A. Humoral immunity and long-lived plasma cells. Curr Opin Immunol. 2002;14(4):517-21.
7. Berek C, Berger A, Apel M. Maturation of the immune response in germinal centers. Cell. 1991;67(6):1121-9.
8. Zachary AA, Lucas DP, Montgomery RA, Leffell MS. Rituximab prevents an anamnestic response in patients with cryptic sensitization to HLA. Transplantation. 2013;95(5):701-4.
9. Lund FE. Cytokine-producing B lymphocytes-key regulators of immunity. Current Opinion in Immunology. 2008;20(3):332-8.
10. Noorchashm H, Reed AJ, Rostami SY, Mozaffari R, Zekavat G, Koeberlein B, et al. B cell-mediated antigen presentation is required for the pathogenesis of acute cardiac allograft rejection. J Immunol. 2006;177(11):7715-22.
11. Sarwal M, Chua MS, Kambham N, Hsieh SC, Satterwhite T, Masek M, et al. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. N Engl J Med. 2003;349(2):125-38.
12. Mulder A, Kardol MJ, Kamp J, Uit Het Broek C, Schreuder GM, Doxiadis, II, et al. Determination of the frequency of HLA antibody secreting B-lymphocytes in alloantigen sensitized individuals. Clinical and experimental immunology. 2001;124(1):9-15.
13. Mulder A, Eijsink C, Kardol MJ, Franke-van Dijk ME, van der Burg SH, Kester M, et al. Identification, isolation, and culture of HLA-A2-specific B lymphocytes using MHC class I tetramers. J Immunol. 2003;171(12):6599-603.
14. Zachary AA, Kopchaliiska D, Montgomery RA, Leffell MS. HLA-specific B cells: I. A method for their detection, quantification, and isolation using HLA tetramers. Transplantation. 2007;83(7):982-8.
15. Zachary AA, Kopchaliiska D, Montgomery RA, Melancon JK, Leffell MS. HLA-specific B cells: II. Application to transplantation. Transplantation. 2007;83(7):989-94.
16. Wadia PP, Herrera ND, Abecassis MM, Tambur AR. Mycophenolic acid inhibits maturation and function of human dendritic cells and B cells. Human Immunology. 2009;70(9):692-700.
17. Heidt S, Roelen DL, de Vaal YJ, Kester MG, Eijsink C, Thomas S, et al. A Novel ELISPOT Assay to Quantify HLA-Specific B Cells in HLA-Immunized Individuals. Am J Transplant. 2012;12(6):1469-78.
18. Lynch RJ, Silva IA, Chen BJ, Punch JD, Cascalho M, Platt JL. Cryptic B cell response to renal transplantation. Am J Transplant. 2013;13(7):1713-23.

19. Han M, Rogers J, Lavingia B, Stastny P. Peripheral blood B cells producing donor-specific HLA antibodies in vitro. Human Immunology. 2009;70(1):29-34.
20. Wiebe C, Gibson IW, Blydt-Hansen TD, Karpinski M, Ho J, Storsley LJ, et al. Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant. Am J Transplant. 2012;12(5):1157-67.
21. Campos EF, Tedesco-Silva H, Machado PG, Franco M, Medina-Pestana JO, Gerbase-DeLima M. Post-transplant anti-HLA class II antibodies as risk factor for late kidney allograft failure. Am J Transplant. 2006;6(10):2316-20.
22. Mulder A, Kardol M, Regan J, Buelow R, Claas F. Reactivity of twenty-two cytotoxic human monoclonal HLA antibodies towards soluble HLA class I in an enzyme-linked immunosorbent assay (PRA-STAT). Human Immunology. 1997;56(1-2):106-13.
23. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, et al. Phenotypic analysis of antigen-specific T lymphocytes. Science. 1996;274(5284):94-6.
24. McMurtrey C, Lowe D, Buchli R, Daga S, Royer D, Humphrey A, et al. Profiling antibodies to class II HLA in transplant patient sera. Hum Immunol. 2014;75(3):261-70.
25. Karahan GE, Eikmans M, Anholts JD, Claas FH, Heidt S. Polyclonal B Cell Activation for Accurate Analysis of Pre-Existing Antigen-Specific Memory B Cells. Clin Exp Immunol. 2014.
26. Heidt S, Roelen DL, Eijsink C, van Kooten C, Claas FH, Mulder A. Effects of immunosuppressive drugs on purified human B cells: evidence supporting the use of MMF and rapamycin. Transplantation. 2008;86(9):1292-300.
27. Dosenovic P, Chakrabarti B, Soldemo M, Douagi I, Forsell MN, Li Y, et al. Selective expansion of HIV-1 envelope glycoprotein-specific B cell subsets recognizing distinct structural elements following immunization. J Immunol. 2009;183(5):3373-82.
28. Freitas MC, Rebellato LM, Ozawa M, Nguyen A, Sasaki N, Everly M, et al. The role of immunoglobulin-G subclasses and C1q in de novo HLA-DQ donor-specific antibody kidney transplantation outcomes. Transplantation. 2013;95(9):1113-9.
29. Worthington JE, Martin S, Al-Husseini DM, Dyer PA, Johnson RW. Posttransplantation production of donor HLA-specific antibodies as a predictor of renal transplant outcome. Transplantation. 2003;75(7):1034-40.
30. Tambur AR, Leventhal JR, Friedewald JJ, Ramon DS. The complexity of human leukocyte antigen (HLA)-DQ antibodies and its effect on virtual crossmatching. Transplantation. 2010;90(10):1117-24.
31. Tambur AR, Rosati J, Roitberg S, Glotz D, Friedewald JJ, Leventhal JR. Epitope analysis of HLA-DQ antigens: what does the antibody see? Transplantation. 2014;98(2):157-66.

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.

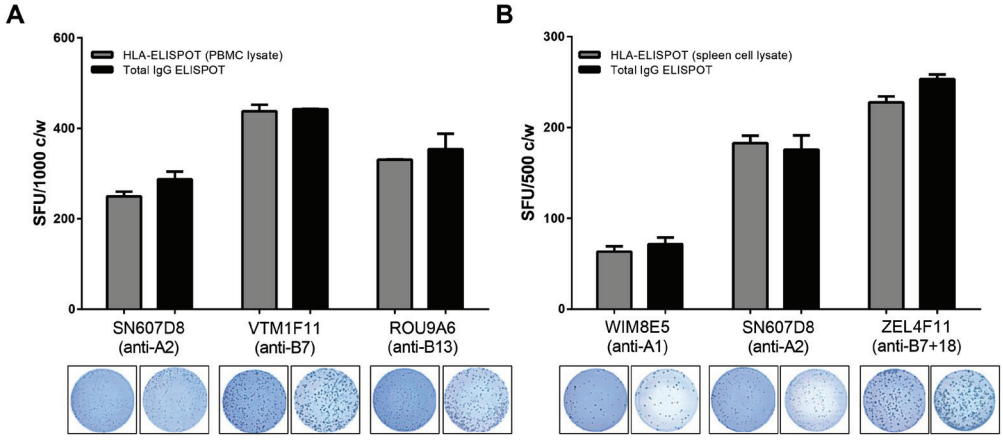

Figure 1. Lysates can be used to detect HLA-antibody producing cells with high sensitivity. *(A) One PBMC lysate (positive for HLA-A2, HLA-B7 and HLA-B13) was tested against three different hybridomas producing HLA antibodies of the corresponding specificity. Comparable numbers of spots were found for 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).

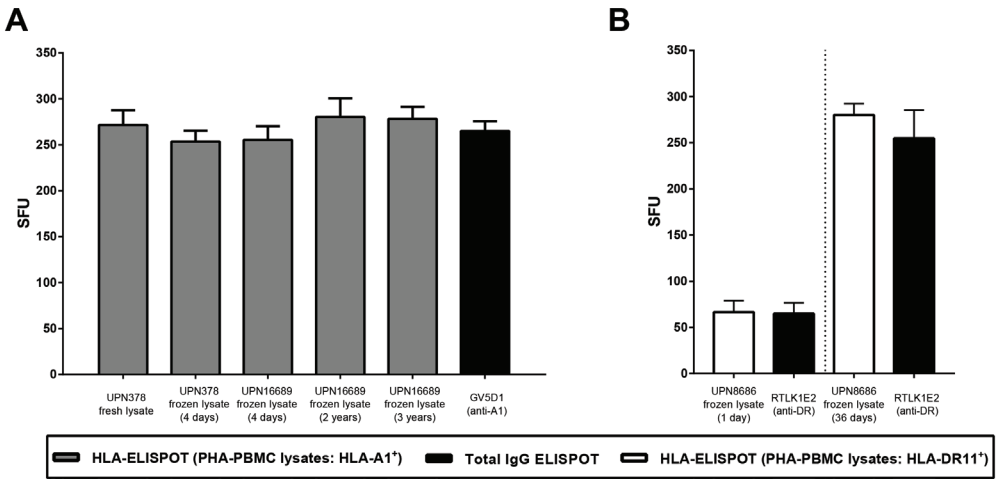

Figure 2. HLA in lysates remain stable upon freezing-thawing. *(A) Comparable numbers of spots were obtained when HLA-A1$^+$ lysates prepared from PHA stimulated PBMC samples were used immediately after preparation (fresh) or frozen for 4 days up to 3 years (gray bars) and then used to detect HLA-A1 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.

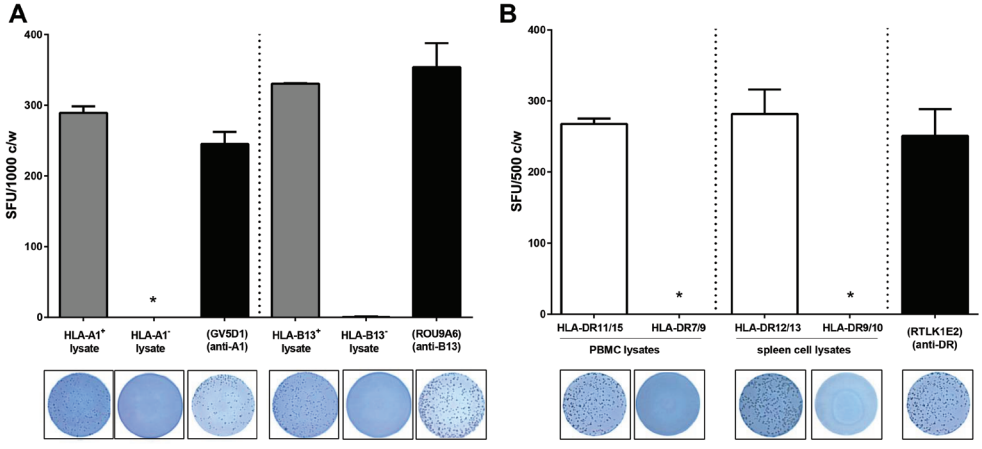

Figure 3. Lysates prepared from peripheral blood mononuclear cells (PBMCs) or spleen cells can be used to detect HLA antibody-producing cells with correct specificity. *(A) A PBMC lysate containing HLA-A1 or HLA-B13 only gave spot formation with only an HLA-A1 or HLA-B13 specific antibody-producing hybridoma, 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.

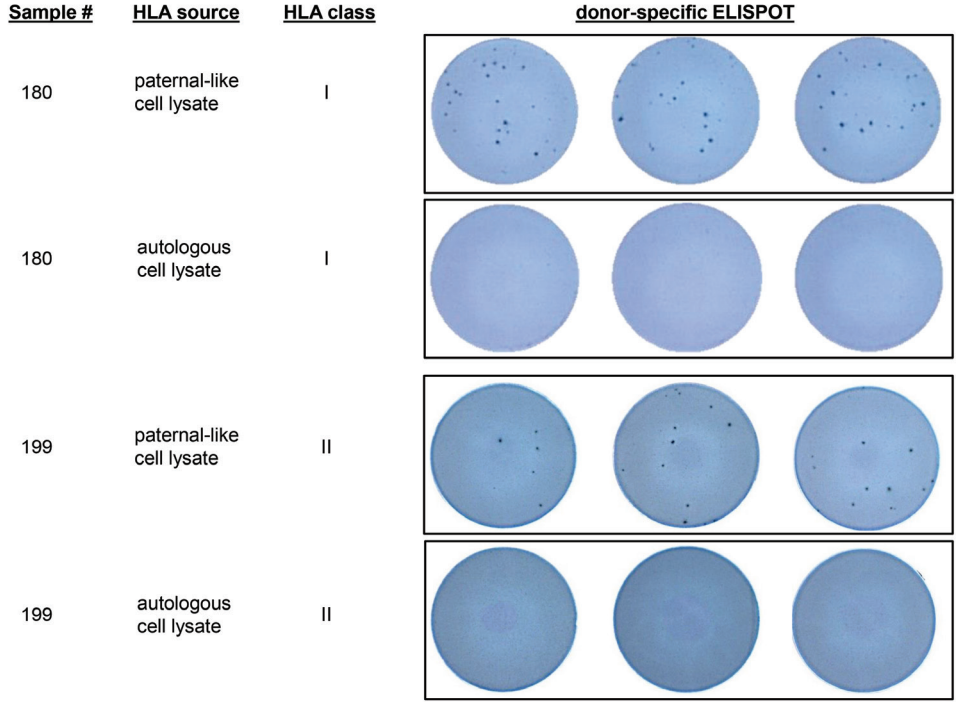

Figure 4. Representative example of a donor-specific memory B cell ELISPOT assay. *HLA class I-specific memory B cells were detected in a pregnancy immunized woman (sample 180) when tested against a cell lysate (PBMC/paternal-like donor 3) that contains immunizing HLA-B*07:02. Similarly, HLA class II-specific 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).

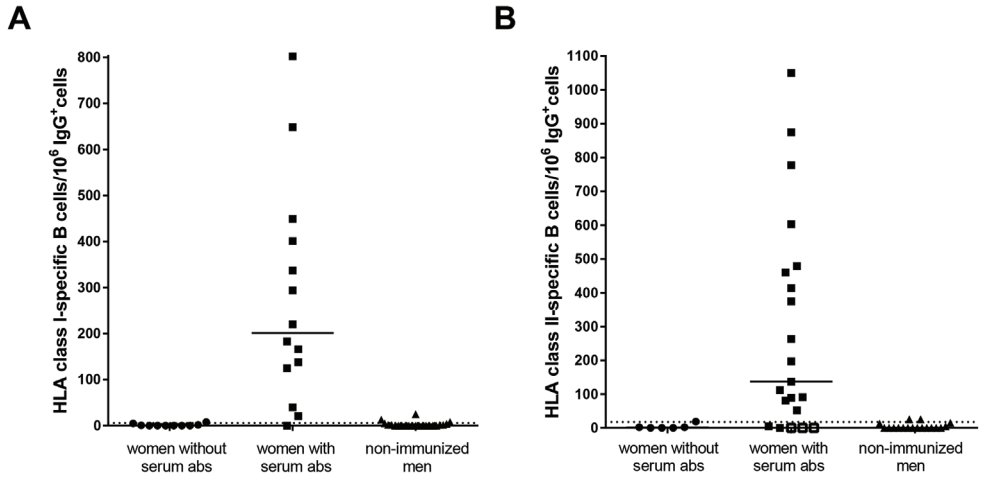

Figure 5. Pregnancy immunized women harbour HLA-specific memory B cells directed at immunizing paternal HLA. *(A) The number of HLA class I and (B) HLA class II-specific B cells per total million IgG⁺ cells were significantly higher in peripheral blood samples from women with serum antibodies (median 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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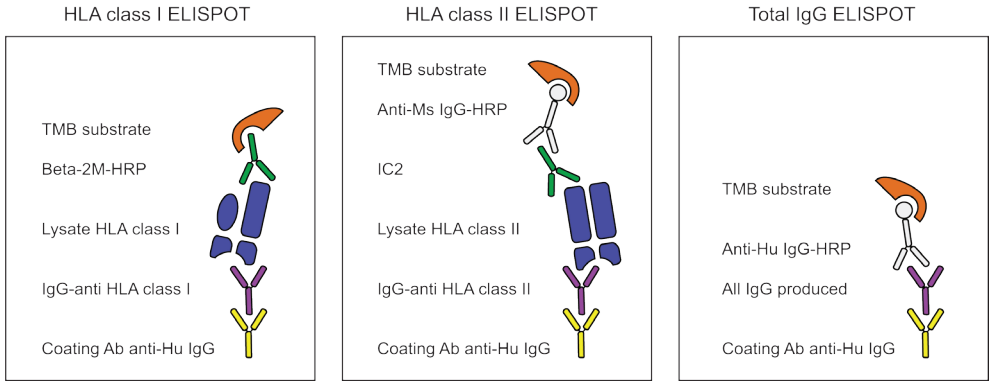
REFERENCES

1. Loupy A, Hill GS, Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. Nature reviews Nephrology. 2012;8(6):348-57.
2. Tran A, Fixler D, Huang R, Meza T, Lacelle C, Das BB. Donor-specific HLA alloantibodies: Impact on cardiac allograft vasculopathy, rejection, and survival after pediatric heart transplantation. J Heart Lung Transplant. 2016;35(1):87-91.
3. Le Pavec J, Suberbielle C, Lamrani L, Feuillet S, Savale L, Dorfmuller P, et al. De-novo donor-specific anti-HLA antibodies 30 days after lung transplantation are associated with a worse outcome. J Heart Lung Transplant. 2016; 35:1067-1077.
4. Kaneku H, O'Leary JG, Banuelos N, Jennings LW, Susskind BM, Klintmalm GB, et al. De novo donor-specific HLA antibodies decrease patient and graft survival in liver transplant recipients. Am J Transplant. 2013;13(6):1541-8.
5. Kurosaki T, Kometani K, Ise W. Memory B cells. Nat Rev Immunol. 2015;15(3):149-59.
6. Karahan GE, Claas FH, Heidt S. Detecting the humoral alloimmune response: we need more than serum antibody screening. Transplantation. 2015;99(5):908-15.
7. Mulder A, Eijsink C, Kardol MJ, Franke-van Dijk ME, van der Burg SH, Kester M, et al. Identification, isolation, and culture of HLA-A2-specific B lymphocytes using MHC class I tetramers. J Immunol. 2003;171(12):6599-603.
8. Zachary AA, Kopchaliiska D, Montgomery RA, Leffell MS. HLA-specific B cells: I. A method for their detection, quantification, and isolation using HLA tetramers. Transplantation. 2007;83(7):982-8.
9. Heidt S, Roelen DL, de Vaal YJ, Kester MG, Eijsink C, Thomas S, et al. A Novel ELISPOT Assay to Quantify HLA-Specific B Cells in HLA-Immunized Individuals. Am J Transplant. 2012;12(6):1469-78.
10. Karahan GE, de Vaal YJ, Roelen DL, Buchli R, Claas FH, Heidt S. Quantification of HLA class II-specific memory B cells in HLA-sensitized individuals. Hum Immunol. 2015;76(2-3):129-36.
11. Lucia M, Luque S, Crespo E, Melilli E, Cruzado JM, Martorell J, et al. Preformed circulating HLA-specific memory B cells predict high risk of humoral rejection in kidney transplantation. Kidney Int. 2015;88(4):874-87.
12. Zachary AA, Kopchaliiska D, Montgomery RA, Melancon JK, Leffell MS. HLA-specific B cells: II. Application to transplantation. Transplantation. 2007;83(7):989-94.
13. Mulder A, Kardol MJ, Arn JS, Eijsink C, Franke ME, Schreuder GM, et al. Human monoclonal HLA antibodies reveal interspecies crossreactive swine MHC class I epitopes relevant for xenotransplantation. Mol Immunol. 2010;47(4):809-15.
14. Duquesnoy RJ, Marrari M, Tambur AR, Mulder A, Sousa LC, da Silva AS, et al. First report on the antibody verification of HLA-DR, HLA-DQ and HLA-DP epitopes recorded in the HLA Epitope Registry. Hum Immunol. 2014;75(11):1097-103.
15. van Lochem EG, Bakker A, Snijder S, Aarts M, de Gast GC, Goulmy E. Epstein-Barr virus infection abrogates the stimulatory capacity of B cells to a major histocompatibility complex class-II-restricted proliferative T-cell clone. Hum Immunol. 1995;42(2):137-44.
16. Pinna D, Corti D, Jarrossay D, Sallusto F, Lanzavecchia A. Clonal dissection of the human memory B-cell repertoire following infection and vaccination. Eur J Immunol. 2009;39(5):1260-70.

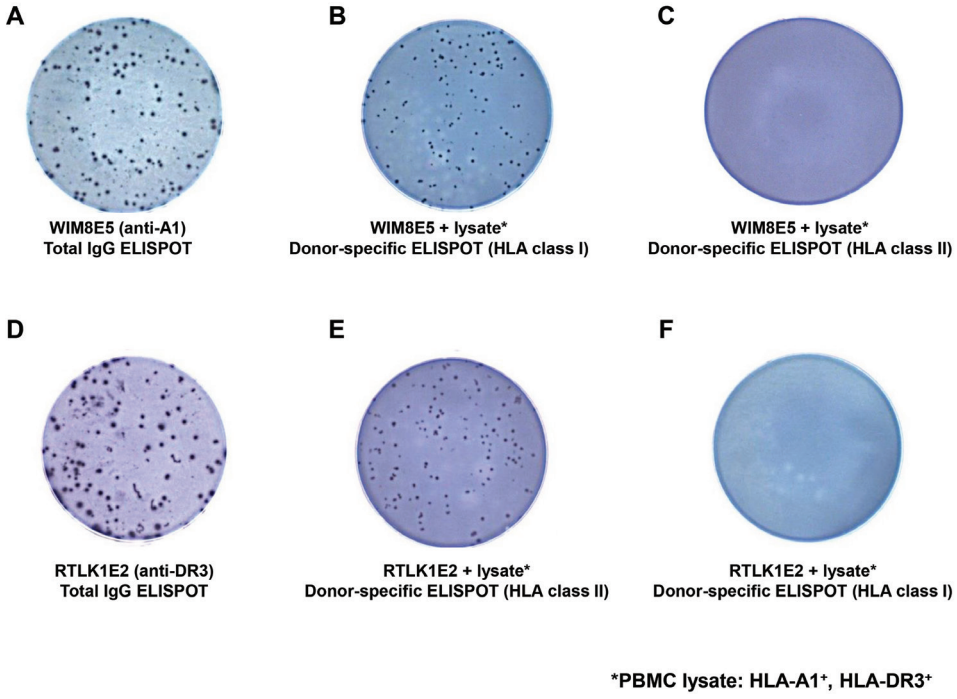
17. Heidt S, Roelen DL, Eijsink C, van Kooten C, Claas FH, Mulder A. Effects of immunosuppressive drugs on purified human B cells: evidence supporting the use of MMF and rapamycin. Transplantation. 2008;86(9):1292-300.
18. Han M, Rogers J, Lavingia B, Stastny P. Peripheral blood B cells producing donor-specific HLA antibodies in vitro. Human Immunology. 2009;70(1):29-34.
19. Lynch RJ, Silva IA, Chen BJ, Punch JD, Cascalho M, Platt JL. Cryptic B cell response to renal transplantation. Am J Transplant. 2013;13(7):1713-23.
20. Gerald Schlaf IPaWA. Detection of Post-Transplant Anti-HLA Donor-Specific Antibodies through the Use of Stored Donors` Cell Lysates and Solid Phase-Based Cross-Matching. Journal of Clinical and Cellular Immunology. 2015;6(6):9.
21. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science. 2002;298(5601):2199-202.
22. Gorden KK, Qiu X, Battiste JJ, Wightman PP, Vasilakos JP, Alkan SS. Oligodeoxynucleotides differentially modulate activation of TLR7 and TLR8 by imidazoquinolines. J Immunol. 2006;177(11):8164-70.
23. Triulzi DJ, Kleinman S, Kakaiya RM, Busch MP, Norris PJ, Steele WR, et al. The effect of previous pregnancy and transfusion on HLA alloimmunization in blood donors: implications for a transfusion-related acute lung injury risk reduction strategy. Transfusion. 2009;49(9):1825-35.
24. Hyun J, Park KD, Yoo Y, Lee B, Han BY, Song EY, et al. Effects of different sensitization events on HLA alloimmunization in solid organ transplantation patients. Transplant Proc. 2012;44(1):222-5.
25. van Kampen CA, Versteeg-van der Voort Maarschalk MF, Langerak-Langerak J, van Beelen E, Roelen DL, Claas FH. Pregnancy can induce long-persisting primed CTLs specific for inherited paternal HLA antigens. Hum Immunol. 2001;62(3):201-7.
26. Picascia A, Grimaldi V, Sabia C, Napoli C. Comprehensive assessment of sensitizing events and anti-HLA antibody development in women awaiting kidney transplantation. Transpl Immunol. 2016;36:14-9.
27. Mulder A, Eijsink C, Kester MG, Franke ME, Kardol MJ, Heemskerk MH, et al. Impact of peptides on the recognition of HLA class I molecules by human HLA antibodies. J Immunol. 2005;175(9):5950-7.
28. Grenzi PC, de Marco R, Silva RZ, Campos EF, Gerbase-DeLima M. Antibodies against denatured HLA class II molecules detected in luminex-single antigen assay. Hum Immunol. 2013;74(10):1300-3.
29. Burns JM, Cornell LD, Perry DK, Pollinger HS, Gloor JM, Kremers WK, et al. Alloantibody levels and acute humoral rejection early after positive crossmatch kidney transplantation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2008;8(12):2684-94.
30. Lefaucheur C, Viglietti D, Bentlejewski C, Duong van Huyen JP, Vernerey D, Aubert O, et al. IgG Donor-Specific Anti-Human HLA Antibody Subclasses and Kidney Allograft Antibody-Mediated Injury. J Am Soc Nephrol. 2016;27(1):293-304.

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

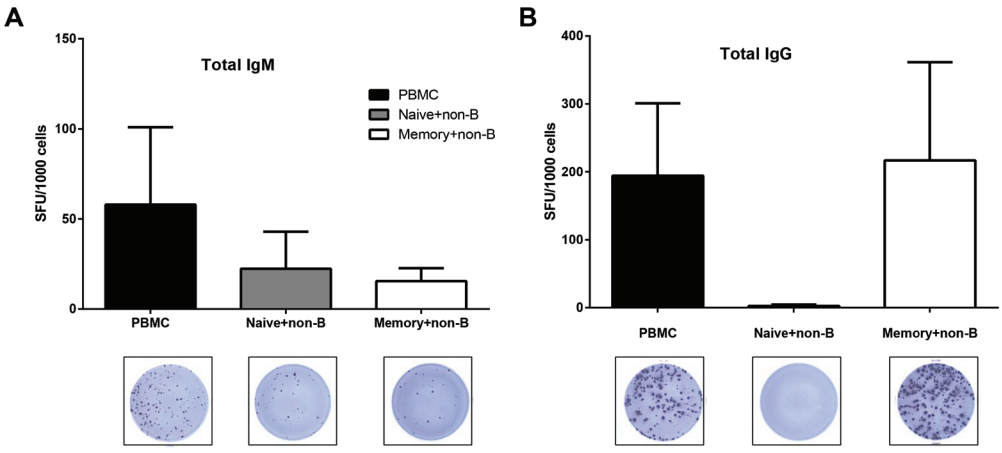
Isolation of naïve, memory and non-B cells from PBMC

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

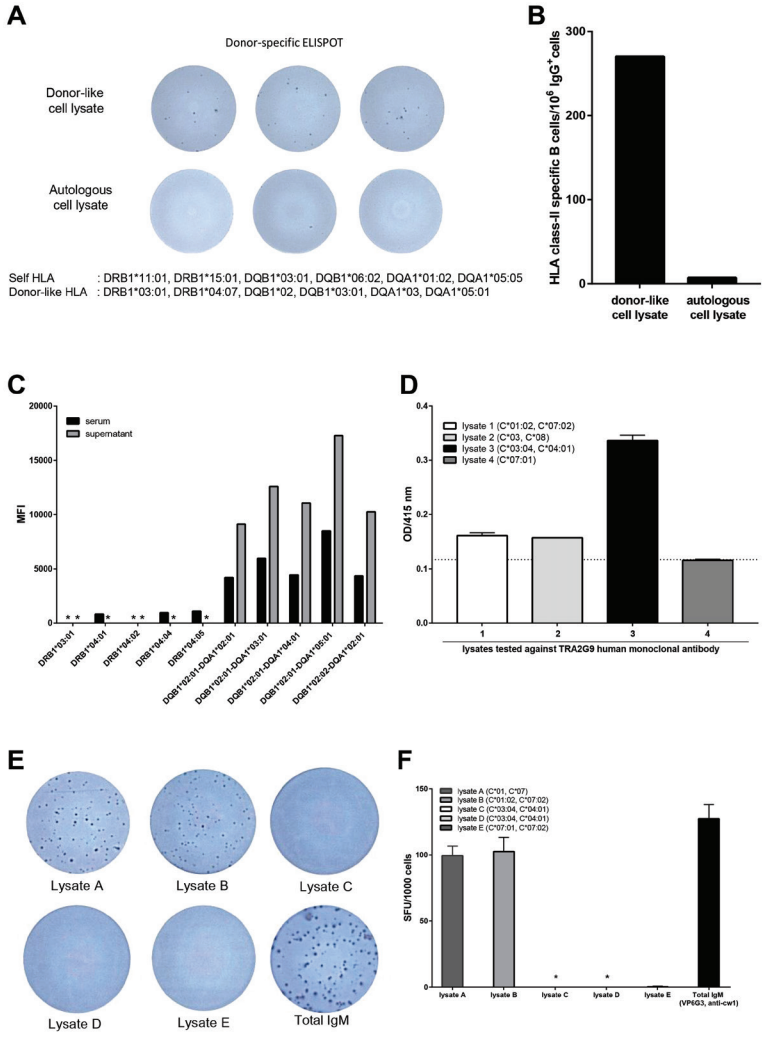
In vitro, polyclonal activation of peripheral blood B cells results in expansion and differentiation of B cells into antibody secreting cells (21). For an accurate estimation of pre-existing memory B cells, a polyclonal activation that does not induce isotype switching in the naïve B cell compartment 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*

Chapter 7

Bone marrow plasma cells and memory B cells display a divergent immunoglobulin isotype distribution

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ABSTRACT

Bone marrow transplantation is a curative treatment for various hematological disorders. In some instances, the transfer of humoral immunity from donor to recipient is observed, either in the form of protective immunity, or as detrimental immune responses such as auto- or allo-immunity. This could be due to the transfer of plasma cells, memory B cells, or both. By using highly sensitive ELISPOT methods, we characterized the bone marrow-residing plasma cells, as well as memory B cells present in the bone marrow and in peripheral blood. By using tetanus toxoid as a model antigen, we detected both IgG-producing plasma cells and memory B cells specific for the same antigen in bone marrow samples. IgG subclass distribution was similar for memory B cells in bone marrow and peripheral blood. However, immunoglobulin isotype distributions were distinctively different between plasma cells and B cells derived from the same bone marrow.

These data indicate that both cell types can contribute to humoral immune reconstitution after bone marrow transplantation with partially overlapping, but also different properties.

INTRODUCTION

Allogeneic and autologous hematopoietic stem cell transplantation (HSCT) are curative therapies for a wide range of disorders and malignancies affecting the immune system (1). Conditioning regimens administered before HSCT to eradicate malignant host cells lead to pancytopenia in almost all HSCT recipients. Therefore, red blood cell and platelet transfusions may be administered to patients in the pre- and post-transplantation period (2). Moreover, T and B lymphocyte reconstitution may be prolonged compared to the recovery of the innate immune cells leading to immuno-incompetence in these patients (3).

In contrast to the dominant repopulation of effector memory T cells expanding from mature T cells present in the donor bone marrow, immune restoration of B cells after HSCT has been reported to resemble normal B cell ontogeny (3). Indeed, the majority of repopulating B cells are transitional and naïve B cells with a minority of cells gradually differentiating into memory B cells upon immune challenge (4). A limited B cell repertoire resulting from the lack of somatically hyper-mutated B cells as well as the loss of protective humoral immunity acquired through childhood vaccinations render stem cell transplant recipients at risk for developing severe infections (5). Functional restoration of the B cell compartment may take up to 2 years (6) and during this recovery process most patients require immunoglobulin (Ig) replacement therapies and vaccinations (7, 8). However, B cell memory may be transferred by bone marrow transplantation (BMT), as anecdotally reported for a patient with leucocyte adhesion deficiency in whom transfer of donor memory B cells upon pre-BMT vaccination was found (9). With regard to antibodies, early studies have indicated that antigen-specific protective humoral immunity could be adoptively transferred from immune donors to recipients by allogeneic BMT (10-12). In addition to the transfer of humoral immunity to microbial antigens, there is also evidence that autoimmunity and allergy can be transferred via donor marrow cells (13, 14). Recently, several case reports have suggested that donor-derived HLA antibody production could be transferred to recipients of HSCT from HLA-immunized donors (15-17). From these reports, it is not clear whether plasma cells, memory B cells, or both, are responsible for the transfer of this pre-existing humoral immunity from donor to recipient.

In the present study, we aimed to characterize the bone marrow (BM) residing plasma cell and memory B cell compartments. Our data indicate that both memory B cells and plasma cells specific for the same antigen are present in bone marrow aspirates. While bone marrow residing plasma cells predominantly produce IgA and IgG antibodies, bone marrow B cells produce mainly IgM and IgG antibodies upon polyclonal activation resembling the isotype distribution of peripheral blood B cells.

Upon BMT, bone marrow residing plasma cells and memory B cells are potentially complementary in their contribution to humoral immune reconstitution. While overlapping specificities exist, the immunoglobulin isotype distributions of these cell types are distinctively different.

MATERIALS AND METHODS

Bone marrow and peripheral blood samples

Human bone marrow was drawn from punctures of the posterior iliac crests of ten individuals (female/male: 4/6). Of these samples, four were taken from individuals undergoing autologous BMT due to vascular disorders (median age: 79 y, range: 73-83 y), one was derived from a surplus clinical sample of a BMT donor (age: 28 y) and five were commercially obtained (Lonza, Maryland, USA) (median age: 28 y, range: 25-37 y). For 3 out of these 5 commercially obtained bone marrow samples we also had access to parallel blood samples. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Hypaque (Pharmacy LUMC, Leiden, the Netherlands) density gradient centrifugation. All bone marrow samples (and paired peripheral blood samples) were used fresh. Surplus clinical samples (n=5) were obtained from individuals with informed consent under guidelines issued by the Medical Ethics Committee of the Leiden University Medical Center (Leiden, the Netherlands).

Isolation of plasma cells from bone marrow aspirates

Bone marrow aspirates were either lysed with ammonium-chloride-potassium (ACK) lysing buffer (Life Technologies-Gibco, USA) for 5 min and centrifuged (n=4) or mononuclear cells were isolated by Ficoll Hypaque (pharmacy LUMC, Leiden, the Netherlands) density gradient centrifugation (n=6). Lysed bone marrow aspirates or isolated mononuclear cells of the bone marrow samples were suspended in Iscove's modified Dulbecco's medium (IMDM) (Gibco Invitrogen, Paisley, UK) containing 10% fetal bovine serum (FBS) (Gibco Invitrogen), supplemented with 50 µM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands), 2 mM L-glutamine (Gibco Invitrogen) and 100 U/ml penicillin with 100 µg/ml streptomycin (Gibco Invitrogen) and filtered through a 70 µm cell strainer (BD Falcon, Belgium). Hereafter, plasma cells were enriched using the EasySep CD138$^+$ plasma cell positive selection kit (Stem Cell Technologies, Grenoble, France), according to manufacturer's instructions. Positive selection of plasma cells enabled us to analyse the plasma cell-depleted B cell containing cell fraction from the same bone marrow sample.

Cell cultures

Polyclonal activation of bone marrow B cells and peripheral blood B cells was carried out in 10% FBS/IMDM with an activation cocktail consisting of 2.5 µg/ml Toll-like receptor (TLR) 7/8 agonist (Resiquimod-R848, Sigma) and 1000 IU/ml IL-2 (Proleukin, Novartis, Amsterdam, the Netherlands). The plasma cell-depleted cell fraction and PBMCs were cultured for 6 days by seeding 2x10^6 cells/well in 24-well flat-bottomed plates (Corning Inc., Corning, NY, USA) at 37^{0}C in a 5% CO_2 humidified incubator. Supernatants from the activated bone marrow and peripheral blood B cells at day 6 of culture were collected and stored at-20^{0}C for further use.

The sample workflow is schematically represented in Figure 1.

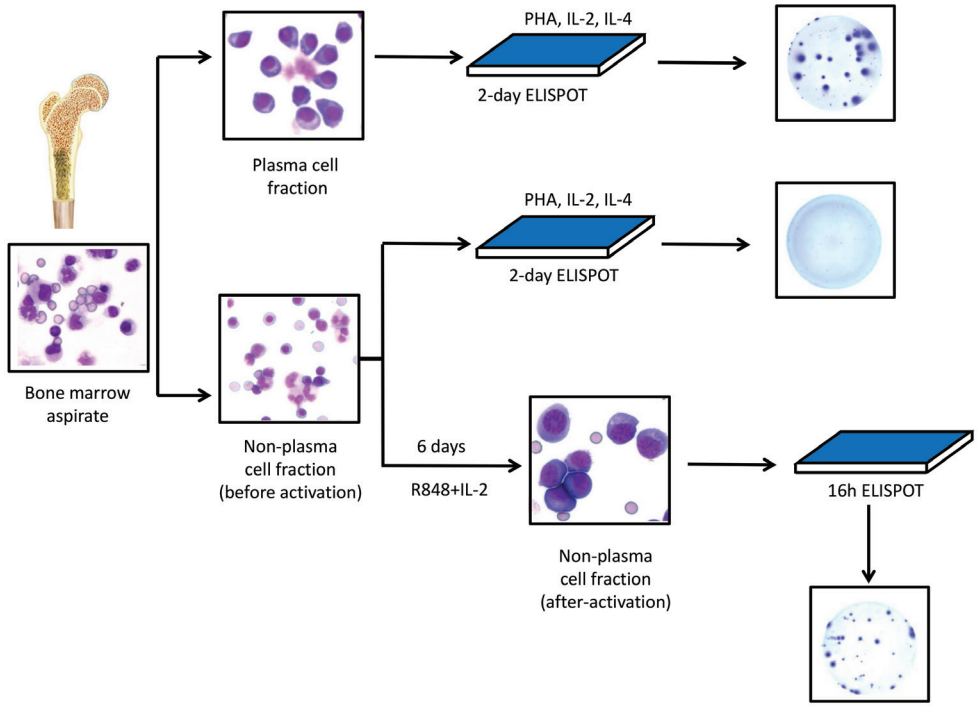

Figure 1. Workflow of processing bone marrow aspirates. *Plasma cells were enriched by CD138 positive selection upon lysing or mononuclear cell isolation of bone marrow aspirates. Plasma cell morphology was confirmed by the large cell size and eccentric localization of the nucleus in cytospin preparations. Positively selected plasma cells, as well as depleted non-plasma cell populations were incubated in ELISPOT plates for 2 days after which spontaneous antibody secreting cell (ASC) counts were assessed (n=10). Additionally, the depleted non-plasma cell fraction was polyclonally activated for 6 days and transferred to ELISPOT plates for ASC assessment (n=7).*

Morphologic assessment of bone marrow cells

Unseparated bone marrow cells, enriched plasma cells, and non-plasma cells (before and after polyclonal activation) were centrifuged onto slides (Thermo-Scientific, Braunschweig, Germany) and fixed with methanol (Merck, Darmstadt, Germany) at 4^0C for 3 min. Following fixation, slides were stained for 2 min with May-Grunwald (Merck) and 20 min with Giemsa (Merck), washed with tap water and air-dried. Visualization was performed using an Axioskop 40 microscope (Zeiss, Oberkochen, Germany).

Flow cytometry

Flow cytometry was performed according to standard protocols using the following antibodies (clone): IgD (IA6-2), CD3 (UCHT1), CD19 (J3119), CD27 (1A4CD27) and CD45 (J33) (all from Beckman Coulter, Woerden, the Netherlands), CD38 (HIT2) (eBioscience, San Diego, CA) and 7-AAD cell viability solution (BD Biosciences, Breda, the Netherlands).

B cell ELISPOT assays

Upon polyclonal activation, the frequencies of tetanus toxoid (TT), IgG subclass, as well as total IgM, IgG, and IgA antibody-secreting cells (ASC) were quantified by enzyme-linked immunosorbent spot (ELISPOT) assays. ELISPOT plates (Millipore, Billerica, MA, USA) were coated with 5 Lf/ml TT antigen (Dutch Vaccine Institute, Bilthoven, the Netherlands) to detect TT-specific ASC. For total immunoglobulin isotype analysis goat anti-human IgM (1.8 μg/ml), IgG (5 μg/ml) or IgA (2.5 μg/ml) (Jackson-Immunoresearch Laboratories, Inc., Baltimore, PA, USA) were used for coating. All coating antibodies were diluted in phosphate-buffered saline (PBS) and incubated overnight. Plates were blocked with 5% FBS/IMDM for at least 1h at 37^0C after which cells of interest were plated at a concentration of $2.5x10^5$ cells/well for TT-specific ELISPOT and 500 cells/well for total IgM, IgG, IgA ASC detection. After incubation for 16h at 37^0C in a 5% CO2 humidified incubator, either biotinylated (Novex Life Technologies, USA) or horse radish peroxidase (HRP)-conjugated (Southern Biotech, Birmingham, AL, USA) goat α-IgM, α-IgG or α-IgA antibodies were added to the appropriate wells for 2h at RT. Following washing, plates were incubated with streptavidin-conjugated alkaline phosphatase (Sigma-Aldrich) if biotinylated detection antibodies were used. Spots were visualized by addition of 5-Bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT), or 3, 3', 5, 5' tetramethylbenzidine (TMB) substrate (Mabtech, Nacka Strand, Sweden), where appropriate. The reaction was stopped by the addition of cold tap water and, after drying, the plates were analysed by an automated ELISPOT reader (Bio-Sys GmbH, Karben, Germany).

Plasma cell ELISPOT assays

To quantify the spontaneous TT-specific IgG and total IgM, IgG, IgA producing plasma cells, ELISPOT plates were coated as described above. Cells were plated at a concentration of $2.5x10^5$ cells/well for TT antigen-specific ELISPOT and 500 cells/well for the detection of immunoglobulin isotypes. Cells were incubated for 48h in the presence of 10% FBS/IMDM supplemented with ITS (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (Sigma-Aldrich), 200 IU/ml IL-2 (Proleukin,Novartis, Arnhem, the Netherlands), 50 IU/ml IL-4 (Gibco) and 2 μg/ml phytohaemagglutinin (PHA) (Remel, Dartford Kent, UK), as described elsewhere (18). Detection and visualization steps were performed as mentioned above.

IgG subclass ELISA

Supernatants from polyclonally activated bone marrow and peripheral blood B cells were tested for IgG1, IgG2, IgG3 and IgG4 levels by enzyme-linked immunoassay (ELISA) (Novex, Life Technologies, USA) following the manufacturer's description. The lower level of detection for IgG subclasses was: IgG1: 0.43 μg/ml; IgG2: 0.17 μg/ml; IgG3: 0.042 μg/ml; IgG4: 0.024 μg/ml.

Statistics

Spot forming unit (SFU) counts are expressed as the median of results from different experiments. The non-parametric Wilcoxon matched pairs signed rank test was used for all comparisons. Statistical level of significance was defined as $p<0.05$.

RESULTS

Bone marrow contains both plasma cells and memory B cells

Bone marrow, in addition to being a primary lymphoid organ, provides a microenvironment where billions of lymphocytes recirculate every day (19). In order to gain insight into antibody secreting cell composition of the bone marrow aspirates, we first enriched these samples for plasma cells. Upon enrichment, the median percentage of plasma cells ($CD27^+CD38^{high}$ in $CD19^+$ lymphocytes) in bone marrow aspirates increased from 6.1% to 87.2% (Figure 2A). Cells in these enriched fractions exhibited typical plasma cell morphology with a relatively large cell size and eccentric localization of the nucleus as demonstrated by cytospins (Figure 1). Flow cytometric analysis on non-plasma cell fractions revealed that the bone marrow (n=5) contained both $CD3^+$ T cells (median: 64.2%, range: 55.1-80.2%) and $CD19^+$ B cells (median: 19.7%, range: 6.3-30.2%). $CD19^+$ B cells in non-plasma cell fractions were composed of IgD^+CD27^- naïve B cells (median: 42%, range: 29-54%) and IgD^-CD27^+ memory B cells (median: 13%, range: 10-25%) in addition to IgD^+CD27^+ unswitched memory B cells (median: 7%, range: 4-12%) and IgD^-CD27^- exhausted memory B cells (median: 37%, range: 21-55%) (Figure 2B).

We next investigated whether we could activate these bone marrow B cells *in vitro*. To this aim, we polyclonally activated the B cells from the non-plasma cell fractions (devoid of plasma cells) for 6 days and assessed their phenotype and morphology. The median percentage of $CD19^+CD27^+CD38^{high}$ plasmablasts increased from 0.2% before activation to 38.2% at day 6 of polyclonal activation (Figure 2C). In addition, the B cells gained a plasma cell-like morphology as shown in cytospins (Figure 1).

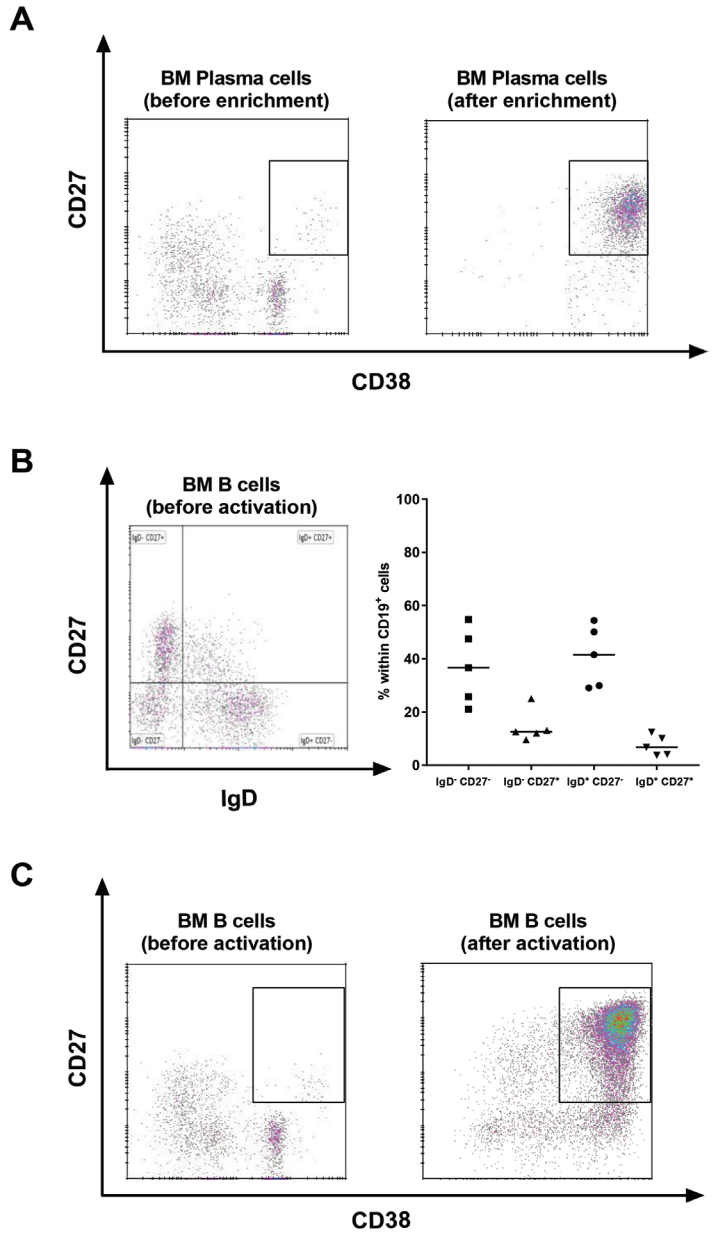

Figure 2. Plasma and B cell composition of bone marrow aspirates. *(A) Enrichment of $CD27^+$ $CD38^{high}$ plasma cells within $CD19^+$ lymphocytes from bone marrow samples. (B) The composition of $CD19^+$ B cells in the nonplasma cell fractions (BM B cells) are as follows: IgD^-CD27^- (median 37%, range: 21-55%); IgD^-CD27^+ (median 13%, range: 10-25%); IgD^+CD27^- (median 42%, range: 29-54%); IgD^+CD27^+ (median 7%, range: 4-12%). (C) Polyclonal activation of B cells in non-plasma cell fraction of bone marrow samples resulted in an increment in the percentage of $CD27^+CD38^{high}$ cells within $CD19^+$ lymphocytes. Horizontal lines represent median values. BM: bone marrow.*

Bone marrow harbours both TT-specific plasma cells and memory B cells

In order to gain insight into the presence of antigen-specific plasma cells and memory B cells in the bone marrow, we directly assessed plasma cells *ex vivo* as well as polyclonally activated bone marrow B cells in TT-specific ELISPOT assays (n=3, median age: 28 y, range: 25-35 y). We found TT-specific bone marrow plasma cells in 2 of the 3 individuals tested at a frequency of 106 and 382 TT-specific cells per 10^5 IgG producing cells (Figure 3A). Furthermore, we found TT-specific bone marrow memory B cell frequencies varying from 2 to 85 per 10^5 IgG producing cells (Figure 3B). No spot formation was found in non-stimulated cells from the same non-plasma cell fractions (data not shown). Polyclonally activated paired peripheral blood B cells showed TT-specific memory B cell frequencies at a range of 10 to 133 per 10^5 IgG producing cells. We found no differences in the bone marrow and peripheral blood TT-specific memory B cell frequencies (p=NS) (Figure 3B).

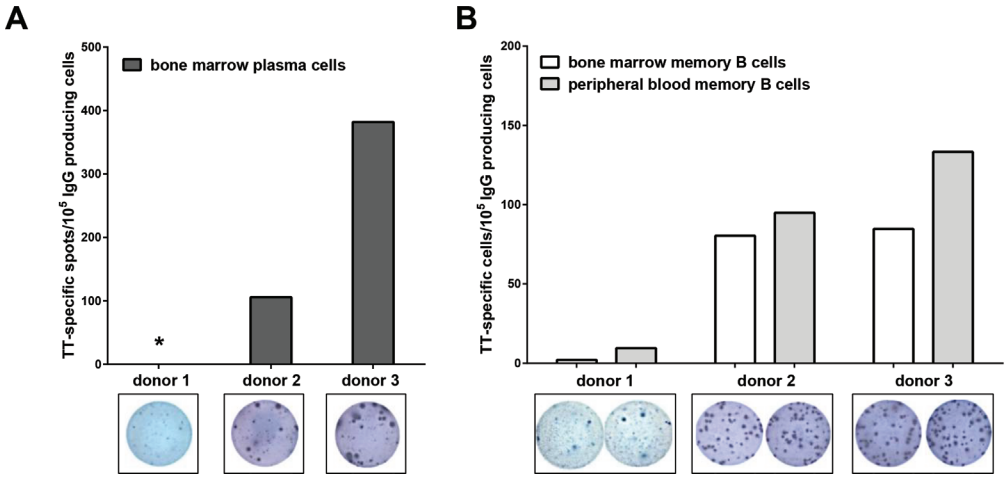

Figure 3. Frequencies of TT-specific memory and plasma cells in paired bone marrow and peripheral blood. *(A) TT-specific plasma cell frequencies in bone marrow samples. (B) TT-specific memory B cell frequencies in bone marrow and peripheral blood samples. Representative pictures are shown from a total of 3 experiments performed with different samples. TT: tetanus-toxoid.*

Bone marrow memory B cells and peripheral blood memory B cells display similar IgG subclass distribution

We next analyzed the B cell culture supernatants from paired bone marrow and peripheral blood samples for IgG subclass distribution. As shown in Figure 4, IgG1 was most dominant in the supernatants of both bone marrow memory B cells (5.7 µg/ml) and peripheral blood memory B cells (16 µg/ml), followed by IgG3 (0.7 µg/ml vs 1.1 µg/ml), IgG2 (0.4 µg/ml vs 0.8

μg/ml) and IgG4 (0.2 vs 0.3 μg/ml). No significant differences were found for any of the IgG subclass antibody concentrations between bone marrow and peripheral blood memory B cells (p=NS).

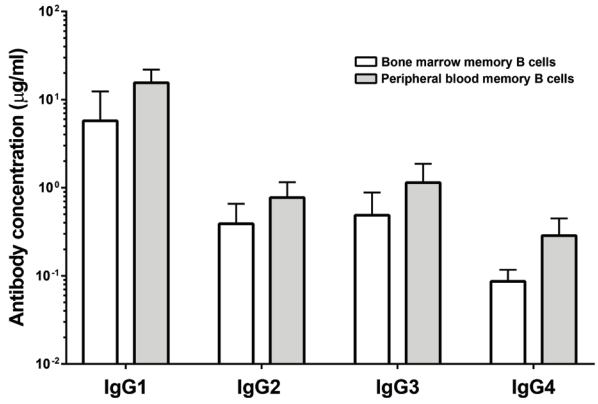

Figure 4. Distribution of IgG subclass antibody production in paired bone marrow and peripheral blood samples. *Supernatants from polyclonally activated bone marrow B cells and peripheral blood B cells showed same IgG subclass distribution with the following order: IgG1>IgG3>IgG2>IgG4. Results are expressed as the mean±SD of experiments performed with 3 different samples.*

Bone marrow plasma cell derived immunoglobulins display a different isotype distribution compared to bone marrow and peripheral blood B cells

In order to determine the isotypes of antibodies produced by plasma cells, we directly analyzed enriched bone marrow plasma cell antibody production *ex vivo*. Plasma cells spontaneously produced IgM, IgG and IgA when incubated for 2 days in ELISPOT plates (Figure 5A-left panel). Enriched bone marrow-derived plasma cells predominantly produced IgA (median SFU/500 cells: 23, range: 7-47) and IgG (median SFU/500 cells: 19, range: 11-51), and to a lesser extent IgM (median SFU/500 cells: 5, range: 1-22) (Figure 5B, n=8). Bone marrow B cells did not produce any IgM, IgG or IgA spots prior to activation (Figure 5A-middle panel), whereas upon polyclonal activation, these cells produced predominantly IgG (median SFU/500 cells: 82; range: 14-98) and IgM (median SFU/500 cells: 43, range: 8-82), and to a lesser extent IgA spots (median SFU/500 cells: 9; range: 0-24) (Figure 5A-right panel, 5C, n=7).

For the three paired bone marrow and PBMC samples we also had the opportunity to analyze bone marrow and peripheral blood B cell isotype distribution in a paired fashion. When analyzed separately we observed that the polyclonally activated bone marrow B cells (Figure 5D) and peripheral blood B cells (Figure 5E) from the same individuals showed predominance of IgM (BM: 38% vs PBMC: 30%) and IgG antibody producing cells (BM: 52% vs PBMC: 57%) and to a lesser extent IgA (BM: 10% vs PBMC: 13%).

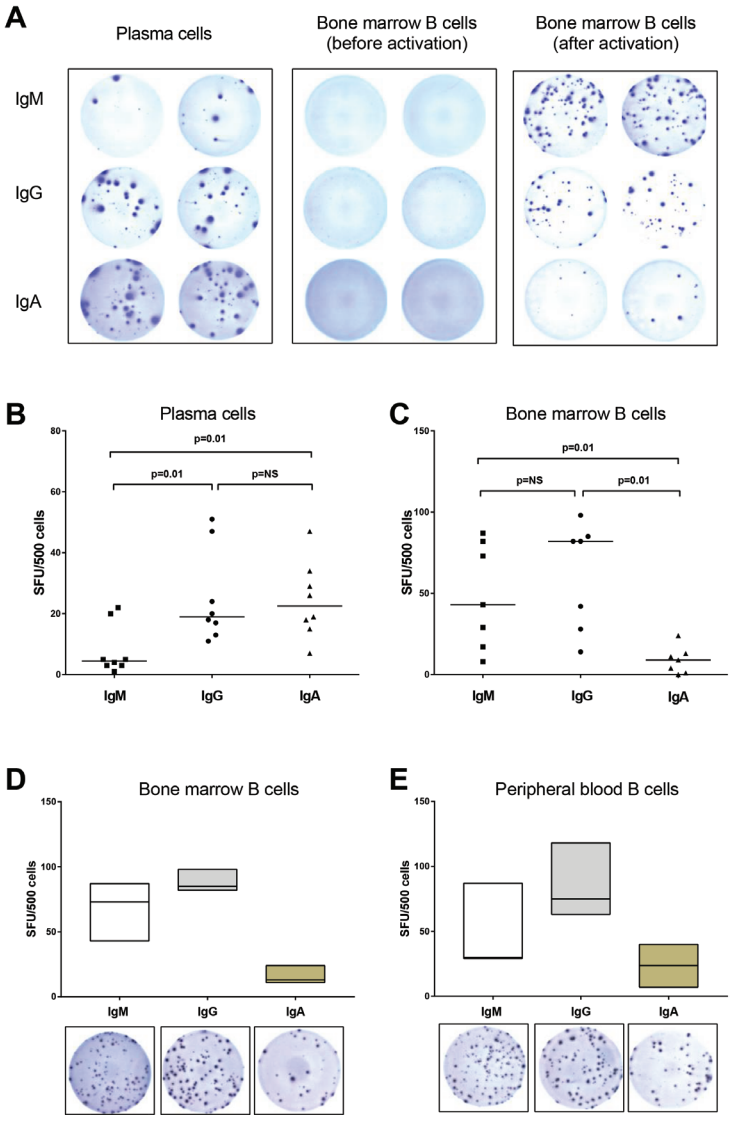

Figure 5. Characterization of the enriched plasma cell fractions, bone marrow and peripheral blood B cells for immunoglobulin production. *(A) Plasma cells were positively selected from bone marrow aspirates and immediately seeded onto ELISPOT plates for 2-day incubation. IgG and IgA producing cells constituted the majority of the antibody producing cells in the plasma cell fractions (left panel) whereas no spot formation was observed in bone marrow B cell fractions before activation (middle panel). When B cells were transferred to ELISPOT plates following 6-day polyclonal activation, comparable number of IgM and IgG spots were observed whereas IgA spot formation was scarce (right panel). (B) Significantly lower number of IgM spots (median SFU: 5) were found compared to IgG (median SFU: 19) (p=0.01) and IgA spots (median SFU: 23) (p=0.01) in enriched plasma cell fractions (n=8). (C) The number of IgM (median SFU: 43) and IgG (median SFU: 82) spots in polyclonally activated non-plasma cell fractions were significantly higher than the number of IgA spots (median SFU: 9) (p=0.01) (n=7). (D) Polyclonally activated B cells from paired bone marrow and (E) peripheral blood samples mainly produced IgM and IgG, and lower numbers of IgA spots. SFU: spot forming unit. Horizontal lines represent median values.*

DISCUSSION

Successful humoral memory depends on both pre-existing protective antibodies produced by plasma cells, as well as pathogen-experienced memory B cells in case circulating antibody levels are not sufficient (20). We here show that both levels of humoral immunity are present in bone marrow aspirates and that, while similarities exist, these compartments are not mirror images. Transfer of antigen-specific memory B cells via BMT may help immuno-compromised recipients to mount an effective, protective humoral immune response early after BMT and may also contribute to the pool of repopulating cells throughout the immune reconstitution period. Several studies have shown that the immune status of a bone marrow donor may affect the recipient's immune response to certain antigenic challenges after transplantation. In accordance, enhanced recipient antibody responses to infectious agents can be achieved if donors are vaccinated before the bone marrow is harvested (21, 22). Indeed, Lausen *et al*., described the transfer of donor Haemophilus influenza type b capsular polysaccharide-specific memory B cells that could be re-activated after antigen recall in a BMT recipient (9).

We tested the presence of TT-specific B cell memory in bone marrow from donors of whom we did not know the immunization history. TT was used as a model antigen because it is universally included in childhood vaccination schedules and evokes T cell-dependent B cell responses giving rise to high affinity antibodies and immunological memory. The fact that we found TT-specific memory B cells in all bone marrow samples analyzed suggests that indeed passive transfer of protective B cell memory may occur upon BMT. Whether these bone marrow derived B cells survive after infusion into the patient and become circulating ASC upon re-encounter with their cognate antigen remains to be studied.

We have recently shown that the current activation protocol did not induce any class switching in the naïve B cell population during the 6-day culture period which was evident by IgG spot production exclusively in memory B cell fractions. In the current study while plasma cells from the bone marrow produced predominantly IgG and IgA, the proportion of the IgA producing cells was remarkably low in B cells from both the bone marrow and the periphery. This relatively lower number of IgA memory B cells in bone marrow compared to IgG might be due to the differences in the homing and trafficking properties of these cells (24, 25). Furthermore, both bone marrow and peripheral blood B cell fractions contained IgM producing cells that were lacking in the plasma cell compartment. These data suggest that bone marrow plasma cells and memory B cells may differentially contribute to humoral immunity after BMT.

The assays described here may aid in estimating the magnitude of the memory B cell response of the donor before transplantation either in bone marrow harvest or peripheral blood samples to help clinicians understand the level of donor-derived antibody responses that may emerge in recipients after BMT. This information may be useful in scheduling the vaccination of the donor or recipient for certain antigens. Furthermore, screening of donors

with a history of immunization but lacking serum HLA antibodies for the presence of HLA-specific memory B cells may be informative to avoid platelet transfusion refractoriness in recipients by allowing for selection of acceptable platelet donors.

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REFERENCES

1. Ljungman P, Bregni M, Brune M, Cornelissen J, de Witte T, Dini G, et al. Allogeneic and autologous transplantation for haematological diseases, solid tumours and immune disorders: current practice in Europe 2009. Bone Marrow Transplant. 2010;45(2):219-34.
2. Radia R, Pamphilon D. Transfusion strategies in patients undergoing stem-cell transplantation. Expert review of hematology. 2011;4(2):213-20.
3. Storek J. Immunological reconstitution after hematopoietic cell transplantation- its relation to the contents of the graft. Expert Opin Biol Ther. 2008;8(5):583-97.
4. Mackall C, Fry T, Gress R, Peggs K, Storek J, Toubert A, et al. Background to hematopoietic cell transplantation, including post transplant immune recovery. Bone Marrow Transplant. 2009;44(8):457-62.
5. Glas AM, van Montfort EH, Storek J, Green EG, Drissen RP, Bechtold VJ, et al. B-cell-autonomous somatic mutation deficit following bone marrow transplant. Blood. 2000;96(3):1064-9.
6. Storek J, Wells D, Dawson MA, Storer B, Maloney DG. Factors influencing B lymphopoiesis after allogeneic hematopoietic cell transplantation. Blood. 2001;98(2):489-91.
7. Storek J, Dawson MA, Lim LC, Burman BE, Stevens-Ayers T, Viganego F, et al. Efficacy of donor vaccination before hematopoietic cell transplantation and recipient vaccination both before and early after transplantation. Bone Marrow Transplant. 2004;33(3):337-46.
8. Sullivan KM, Storek J, Kopecky KJ, Jocom J, Longton G, Flowers M, et al. A controlled trial of long-term administration of intravenous immunoglobulin to prevent late infection and chronic graft-vs.-host disease after marrow transplantation: clinical outcome and effect on subsequent immune recovery. Biol Blood Marrow Transplant. 1996;2(1):44-53.
9. Lausen BF, Hougs L, Schejbel L, Heilmann C, Barington T. Human memory B cells transferred by allogenic bone marrow transplantation contribute significantly to the antibody repertoire of the recipient. J Immunol. 2004;172(5):3305-18.
10. Witherspoon RP, Schanfield MS, Storb R, Thomas ED, Giblett ER. Immunoglobulin production of donor origin after marrow transplantation for acute leukemia or aplastic anemia. Transplantation. 1978;26(6):407-8.
11. Wimperis JZ, Brenner MK, Prentice HG, Reittie JE, Karayiannis P, Griffiths PD, et al. Transfer of a functioning humoral immune system in transplantation of T-lymphocyte-depleted bone marrow. Lancet. 1986;1(8477):339-43.
12. Lum LG, Noges JE, Beatty P, Martin PJ, Deeg J, Doney KC, et al. Transfer of specific immunity in marrow recipients given HLA-mismatched, T cell-depleted, or HLA-identical marrow grafts. Bone Marrow Transplant. 1988;3(5):399-406.
13. Waters AH, Metcalfe P, Minchinton RM, Barrett AJ, James DC. Autoimmune thrombocytopenia acquired from allogeneic bone-marrow graft: compensated thrombocytopenia in bone marrow donor and recipient. Lancet. 1983;2(8364):1430.
14. Hallstrand TS, Sprenger JD, Agosti JM, Longton GM, Witherspoon RP, Henderson WR, Jr. Long-term acquisition of allergen-specific IgE and asthma following allogeneic bone marrow transplantation from allergic donors. Blood. 2004;104(10):3086-90.

15. Hatakeyama N, Hori T, Yamamoto M, Inazawa N, Iesato K, Miyazaki T, et al. Platelet transfusion refractoriness attributable to HLA antibodies produced by donor-derived cells after allogeneic bone marrow transplantation from one HLA-antigen-mismatched mother. Pediatric transplantation. 2011;15(8):E177-82.
16. Taniguchi K, Yoshihara S, Maruya E, Ikegame K, Kaida K, Hayashi K, et al. Donor-derived HLA antibody production in patients undergoing SCT from HLA antibody-positive donors. Bone Marrow Transplant. 2012;47(10):1338-42.
17. Fasano RM, Mamcarz E, Adams S, Donohue Jerussi T, Sugimoto K, Tian X, et al. Persistence of recipient human leucocyte antigen (HLA) antibodies and production of donor HLA antibodies following reduced intensity allogeneic haematopoietic stem cell transplantation. British journal of haematology. 2014;166(3):425-34.
18. Perry DK, Pollinger HS, Burns JM, Rea D, Ramos E, Platt JL, et al. Two novel assays of alloantibody-secreting cells demonstrating resistance to desensitization with IVIG and rATG. Am J Transplant. 2008;8(1):133-43.
19. Zhao E, Xu H, Wang L, Kryczek I, Wu K, Hu Y, et al. Bone marrow and the control of immunity. Cell Mol Immunol. 2012;9(1):11-9.
20. Kurosaki T, Kometani K, Ise W. Memory B cells. Nat Rev Immunol. 2015;15(3):149-59.
21. Saxon A, Mitsuyasu R, Stevens R, Champlin RE, Kimata H, Gale RP. Designed transfer of specific immune responses with bone marrow transplantation. J Clin Invest. 1986;78(4):959-67.
22. Parkkali T, Kayhty H, Hovi T, Olander RM, Roivainen M, Volin L, et al. A randomized study on donor immunization with tetanus-diphtheria, Haemophilus influenzae type b and inactivated poliovirus vaccines to improve the recipient responses to the same vaccines after allogeneic bone marrow transplantation. Bone Marrow Transplant. 2007;39(3):179-88.
23. Karahan GE, de Vaal YJ, Krop J, Wehmeier C, Roelen DL, Claas FH, et al. A memory B cell crossmatch assay for quantification of donor-specific memory B cells in the peripheral blood of HLA-immunized individuals. Am J Transplant. 2017.
24. Kunkel EJ, Butcher EC. Plasma-cell homing. Nat Rev Immunol. 2003;3(10):822-9.
25. Williams MB, Rose JR, Rott LS, Franco MA, Greenberg HB, Butcher EC. The memory B cell subset responsible for the secretory IgA response and protective humoral immunity to rotavirus expresses the intestinal homing receptor, alpha4beta7. J Immunol. 1998;161(8):4227-35.

Chapter 8

Comparison of HLA antibody specificities by plasma cells and memory B cells in repeat kidney transplant candidates

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Preliminary data

ABSTRACT

Kidney transplant recipients with pre-existing or *de novo* donor HLA specific antibodies pose a higher risk for developing antibody mediated rejection (ABMR) which remains to be the major cause of graft loss in kidney transplantation. Pretransplant risk assessment regarding the humoral alloimmune response in a transplant candidate is based on detection of donor HLA specific antibodies in serum that are mainly produced by bone marrow residing plasma cells. However, in patients with a history of alloimmunization, circulating memory B cells can also contribute to HLA antibody production upon re-challenge. In this regard, activated B cell culture supernatants can be used to screen for HLA antibodies produced by memory B cells. In this preliminary study, we aimed at comparing HLA antibody specificities in plasma samples and B cell culture supernatants from repeat transplant candidates (n=105). Our results showed that HLA antibodies detected in plasma samples and B cell culture supernatants display various specificity patterns. In addition to concurrent presence or absence of certain HLA antibody specificities in both sample sources, some HLA antibodies were detected only in plasma samples or solely in culture supernatants. Furthermore, in some cases where HLA antibodies were concurrently present in both plasma and culture supernatant, the hierarchy of HLA antibody specificities were reversed. These results suggest that identifying HLA antibody specificities produced by memory B cells in addition to serum antibody profiles in high risk transplant recipients may enable better risk estimation by providing a more complete picture of the humoral alloimmune response.

INTRODUCTION

In kidney transplantation, pre-existence or *de novo* development of donor directed HLA antibodies of IgG isotype is associated with (hyper)acute and chronic rejection (1). Serum samples are routinely screened for the presence of HLA antibodies before and after transplantation as the only measure of HLA-specific humoral alloimmune responses. Upon antigen recognition, naïve B cells can differentiate into short-lived plasmablasts and produce mainly low affinity antibodies of IgM isotype, or undergo germinal center reactions where somatic hypermutation, isotype switching and affinity maturation take place (2). B cells that have undergone germinal center reactions differentiate into memory B cells and/or plasma cells (3). While plasma cells mainly migrate to the bone marrow and become long-lived bone marrow resident cells that continuously produce antibodies, memory B cells circulate through the body and become an antibody secreting cell upon an antigenic re-challenge or possibly bystander activation during an infection (4, 5).

Which proportion of B cells going through germinal center reactions is committed to become plasma cells or memory B cells is unknown. Indeed, while for some viral antigens the correlation of serum antibodies *versus* antigen-specific memory B cell frequencies are known (6), it is not clear whether immunization against a certain HLA molecule will always result in generation of both memory B cells and plasma cells, and whether the ratio of these cells is different in individuals or even per HLA antigen.

In order to detect HLA antibodies deriving from memory B cells, *in vitro* polyclonal B cell activation is required. Our group was one of the pioneers in showing that memory B cells from pregnancy immunized women could be polyclonally activated *in vitro* to produce HLA antibodies (7). Later on, Han *et al*., showed that certain HLA antibody specificities derived from memory B cells were only present in B cell culture supernatants while being absent in serum samples from transplant recipients (8). Recently, Snanoudj *et al*., also showed that certain HLA antibodies were present in culture supernatants in a more restricted specificity pattern compared to serum antibodies (9). Altogether, these data suggest that B cell culture supernatants can be used to screen for HLA antibody specificities produced by HLA-specific memory B cells, and that the antibody specificity patterns detected are not identical to those from serum.

In this preliminary study, we aimed at comparing the HLA antibody repertoire of HLA-specific memory B cells and plasma cells in samples from repeat transplant candidates.

MATERIALS AND METHODS

Subjects

Peripheral blood samples were collected prior to transplantation from repeat kidney transplant candidates (n=105) from 4 transplant centers in the Netherlands, and from healthy controls (n=102). Mononuclear cells were isolated by Ficoll Hypaque (Pharmacy LUMC, Leiden, the Netherlands) density gradient centrifugation and kept frozen in liquid nitrogen until further use. Samples were obtained with informed consent under guidelines issued by the Medical Ethics Committee of the Leiden University Medical Center (Leiden, the Netherlands) under protocol number P13.025. (http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=4695).

Polyclonal activation of B cells

Polyclonal B cell activation was carried out by stimulating peripheral blood mononuclear cells (PBMCs) with an activation cocktail consisting of 2.5 µg/ml Toll-like receptor (TLR) 7/8 agonist (Resiquimod-R848) (Sigma) and 1000 IL-2 IU/ml (Proleukin, Novartis, Amsterdam, the Netherlands) (10). PBMC cultures were carried out in 10% FBS/IMDM for 6 days by seeding $2x10^6$ cells/well in 24-well flat-bottomed plates (Corning Inc., Corning, NY, USA) at 37^0C in 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. Cork, Ireland), and frozen at -20^0C until further use. To determine the extent of B cell activation, total Immunoglobulin G (IgG) ELISPOT assays were performed as previously described (11).

Flow cytometry

Flow cytometry was performed according to standard protocols using the following antibodies (clone): CD19 (J3119), CD27 (1A4CD27) (both from Beckman Coulter, Woerden, the Netherlands) and CD38 (HIT2) (eBioscience, San Diego, CA, USA).

HLA antibody detection

Serum/plasma samples and concentrated B cell culture supernatants were screened for the presence of HLA antibodies by Luminex using Lifecodes Lifescreen Deluxe kit (Immucor Transplant Diagnostics, Stamford, USA). Positive samples were further tested using LabScreen HLA class I and class II single antigen beads (One Lambda, Canoga, USA) to identify HLA antibody specificities.

Statistical analysis

The Mann-Whitney *U* test was used for comparisons of the results between groups, $p< 0.05$ was considered statistically significant.

RESULTS

Characterization of in vitro polyclonal activation of PBMC samples from patients and controls

PBMC samples were collected from repeat transplant candidates at the day of transplantation (n=38; 36%), one day before the transplantation (n=53; 51%) or at various time points before the transplantation (n=14, 13%; median: 24 days, range: 3-660 days). Samples were obtained after the first transplantation in 76 patients, after the second transplantation in 22 patients and following third transplantation in 7 patients.

Upon polyclonal activation, the median percentage of B cells in patient samples increased from 4% (range: 0.1-25%) before activation to a median of 45% (range: 0.3-73%) (Figure 1A). In addition, the percentage of $CD27^{+}CD38^{hi}$ within the $CD19^{+}$ B cells (plasmablasts) increased from a median of 0.3% (range: 0-6.3%) before activation to 42% (range: 0.4-85%) at day 6 of the culture (Figure 1B). Polyclonal activation of B cells from patients resulted in a median of 64 IgG spots per 1000 plated cells (range: 0-317 SFU/1000 cells) (Figure 1C).

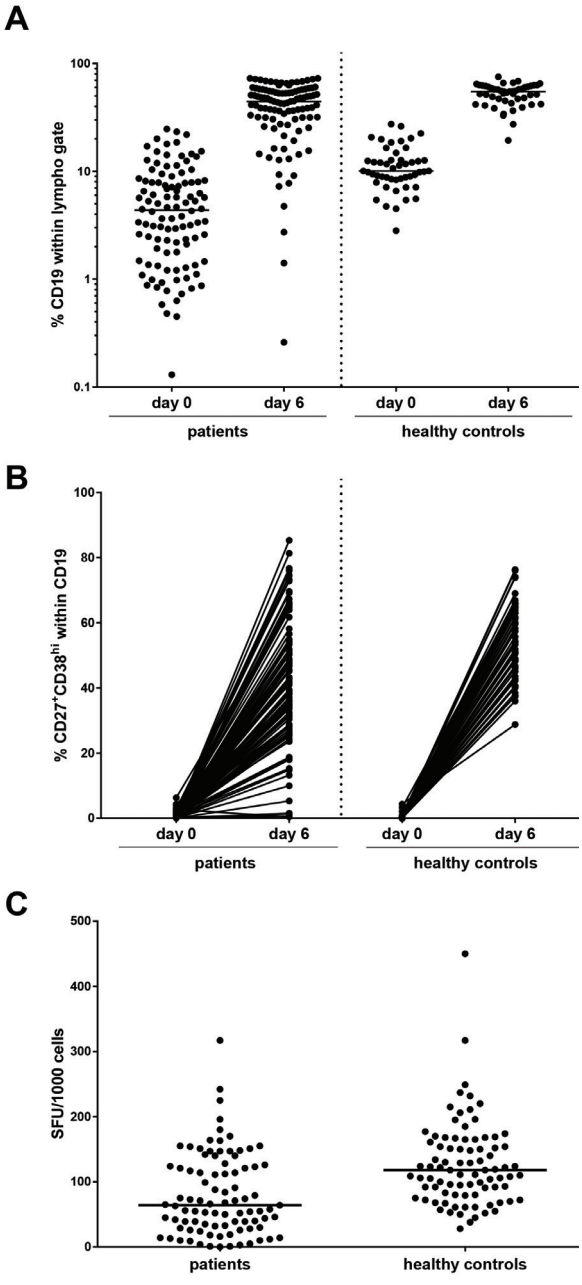

Figure 1. Characterization of polyclonal activation in patient samples. *(A) Patients have significantly lower percentage of $CD19^{+}$ cells before and after polyclonal activation, (B) lower percentage of $CD19^{+}CD27^{+}CD38^{hi}$ cells upon activation and (C) lower number of IgG spots compared to healthy controls.*

The percentage of $CD19^+$ B cells in patient samples was significantly lower than that of healthy controls before (median: 10%; range: 3-27%, $p<0.0001$) and after (median: 55%; range: 19-75%, p=0.001) polyclonal activation (Figure 1A). Likewise, at day 6 of the pre-culture phase, the percentage of $CD27^+CD38^{hi}$ B cells was significantly lower in patients (median 42%, range: 0.4-85%) compared to healthy controls (median: 54%; range: 29-76%, $p<0.0001$) (Figure 1B). In accordance with these findings, significantly lower spot counts were observed in samples derived from patients (median SFU: 64; range: 0-317) compared to healthy controls (median SFU: 118; range: 28-450 SFU/1000 cells) upon polyclonal activation ($p<0.0001$) (Figure 1C).

Distribution of HLA antibody specificities in culture supernatants and plasma samples

HLA antibody analysis in 10-fold concentrated supernatants revealed that 24/105 (23%) patients had evidence for circulating memory B cells directed at the mismatched HLA of the previous donors whereas no HLA antibodies were detected in supernatants of 81/105 (77%) patients. Among those with detectable supernatant HLA antibodies (n=24), 6 (25%) samples had HLA class I antibodies only, 11 (46%) had class II antibodies only and 7 (29%) samples had antibodies directed at both HLA class I and II. While 23% of the patients had HLA antibodies in their culture supernatants, serum HLA antibodies were present in 52% (n=55) of the patients. HLA antibodies detected in plasma samples and 10-fold concentrated B cell culture supernatants displayed various patterns. As exemplified in Figure 2, some HLA antibody

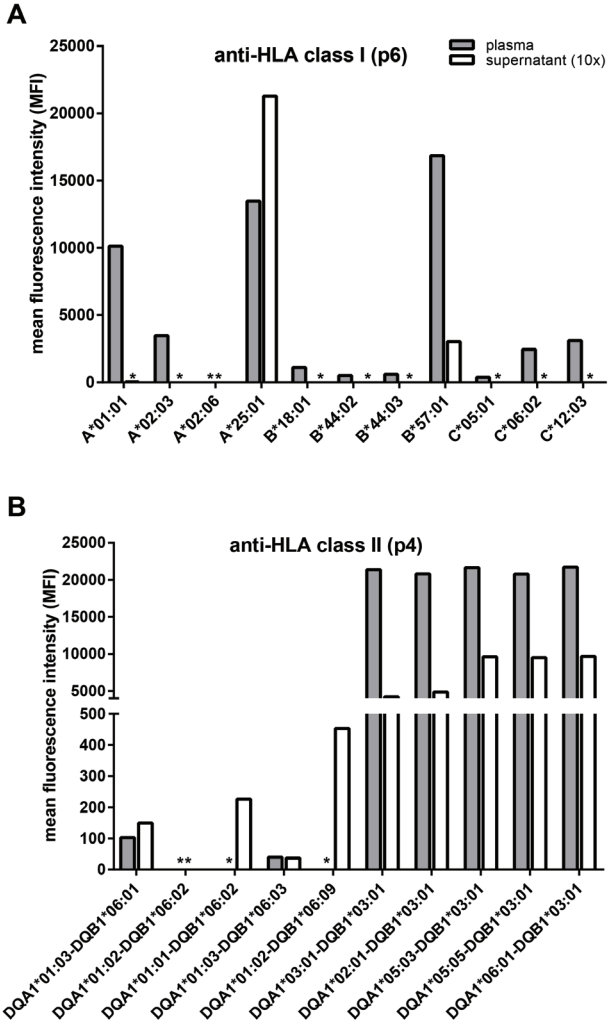

Figure 2. HLA antibody patterns in plasma and (10x) culture supernatants. *Distribution of HLA antibodies directed to previous graft donor mismatch (A) HLA class I (p6) and (B) HLA class II (p4) in 2 different repeat transplant candidates.*

specificities were present only in plasma samples whereas some were present both in the plasma and culture supernatants. Interestingly, as shown for anti HLA-A*25:01 and B*57:01 in figure 2A, the hierarchy of the HLA antibody specificities were reversed in culture supernatants in comparison to plasma samples. Furthermore, while there were no antibodies detected against certain HLA mismatches both in the plasma and culture supernatants, some HLA antibody specificities (e.g. DQA1*01:01/DQB1*06:02 or DQA1*01:02/DQB1*06:09) were found only in B cell culture supernatants (Figure 2B).

Emergence of HLA antibody specificities in culture supernatants upon further concentration

The fact that supernatant HLA antibodies were detected in only 44% of patients with serum antibodies (n=24/55) leaves the possibility that IgG levels in the supernatants are too low for proper HLA antibody detection. We therefore compared the concentration of IgG in culture supernatants to plasma samples obtained at the same bleeding date in a smaller group of transplant recipients (n=28). Healthy individuals with (n=4) or without (n=4) a history of alloimmunization were included as controls. Similar to the larger cohort, 50% of the patients in this group (n=14/28) had HLA antibodies in plasma and among those with plasma HLA antibodies 42% (n=6/14) of the patients had detectable HLA antibodies in B cell culture supernatants (Table 1). As summarized in Table 1, supernatant IgG concentrations of patient samples were significantly lower than that of healthy controls while plasma IgG concentrations did not differ between these two groups.

Table 1: Demographics of patients and controls

	Patients	Controls	p value
n	28	8	
Gender			
female	14	4	
male	14	4	
Age (years)	44 (20-69)	47 (39-62)	0.4
Number of previous tx (n)			
1	19	n/a	
2	7		
3	2		
Immunosuppressive treatment (n)			
Yes	21	n/a	
No	7		
Supernatant IgG concentration (10x) (µg/ml)	19 (0-144)	94 (14-135)	0.001
Total IgG SFU (1000 cells/well)	66 (0-242)	127 (64-249)	0.02
%CD19 (day 0)	2 (0-23)	12 (7-20)	0.0003
%CD19 (day 6)	40 (0-72)	55 (47-63)	0.03
Supernatant HLA antibody status (n)			
only class I +	3	0	
only class II +	2	1	
both +	1	3	
both -	22	4	
Plasma IgG concentration (µg/ml)	4284 (884-15519)	7291 (3043-8420)	0.08
Plasma HLA antibody status (n)			
only class I +	4	0	
only class II +	6	0	
both +	4	4	
both -	14	4	

To determine whether HLA antibody concentration was affecting the ability to detect the antibodies, we diluted the plasma samples from 8 patients to the level of IgG present in 6-day culture supernatants. As shown in Table 2, all HLA class I antibodies disappeared when the plasma IgG levels were as low as that of supernatants whereas the majority of HLA class II positivity persisted despite the dilution of plasma samples. To formally prove that low IgG concentrations in the supernatants were causing the lack of HLA antibody detection, we aimed to increase the IgG content in culture supernatants further by culturing for a longer period

and more extensively concentrating the supernatants. To this aim, we polyclonally activated 2 patient samples (p25 and p26) for 6, 10 and 20 days and followed the IgG concentration in the supernatants, as well as the total IgG producing cell counts at each time point. As expected, longer culture periods resulted in higher IgG concentrations. However, total IgG producing B cell counts became undetectable at day 20 as evident by lack of spots in ELISPOT assays, most likely due to cell death. We next concentrated the culture supernatants obtained at day 10 (IgG p25: 9 µg/ml and p26: 21 µg/ml) up to 356 µg/ml (p25) and 1153 µg/ml (p26) and tested for the presence of HLA antibodies.

Table 2: Presence of HLA antibodies in culture supernatants and plasma samples

	supernatant (10x)		plasma (neat)		plasma (diluted)		
patient #	class 1	class 2	class 1	class 2	class 1	class 2	dilution
p1	+	-	+	+	-	-	1:146
p2	+	-	+	-	-	-	1:232
p3	+	-	+	-	-	-	1:299
p4	-	+	+	+	-	+	1:295
p5	-	+	-	+	-	+	1:61
p6	+	+	+	+	-	+	1:699
p25	-	-	+	-	-	-	1:307
p26	-	-	-	+	-	+	1:87

Interestingly, although IgG levels in these culture supernatants were still lower than in the corresponding neat plasma samples, we found that certain HLA antibodies (directed to Bw4 epitopes) detected in neat plasma samples were emerging in supernatant sample p25 upon further concentration. However, some strong HLA antibodies present in plasma samples such as HLA-B*07:02 and B*82:01 did not appear in the supernatant at all despite further concentration (Figure 3A). Similarly, in sample p26, while some HLA-DR and HLA-DP antibody specificities emerged in supernatants upon further concentration, HLA-DQ antibodies were equally detectable in both diluted plasma samples and further concentrated supernatants (Figure 3B).

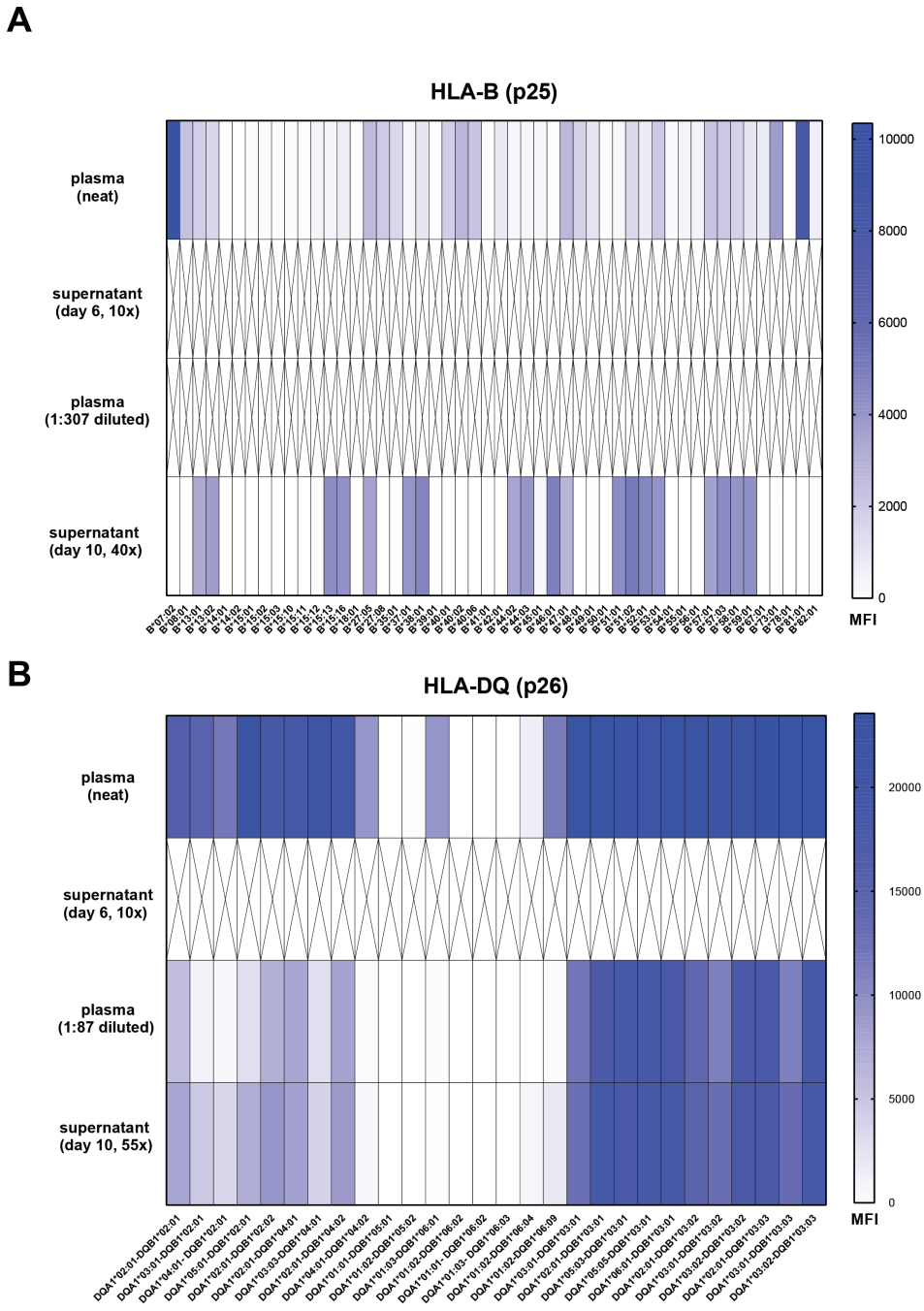

Figure 3. Emergence of HLA antibodies in culture supernatants upon further concentration. *(A) HLA-B antibody specificities as detected by Luminex single antigen bead assays in neat and diluted plasma samples in comparison to concentrated culture supernatants from patient p25. (B) HLA-DQ directed antibody specificities in samples from patient p26.*

DISCUSSION

In clinical kidney transplantation, rapid donor-specific antibody production deriving from an anamnestic response of memory B cells may contribute to antibody mediated rejection (ABMR) episodes (12, 13). Repeat transplant candidates, patients undergoing desensitization therapy or women receiving grafts from their spouses or children comprise a high risk patient group for developing ABMR since they may harbor silent HLA-specific memory B cells which can differentiate into antibody producing plasma cells upon re-challenge (14).

Currently, humoral immune responses in the setting of clinical kidney transplantation are only measured by means of serum HLA antibodies which are mainly produced by bone-marrow residing plasma cells. However, exposure to foreign HLA via blood transfusions, pregnancies or previous transplantations may result in the formation of dormant memory B cells which become antibody secreting cells after re-encounter with the immunizing antigen (4).

HLA antibody specificities deriving from memory B cells can be detected in culture supernatants of activated B cells. While in the study of Han *et al*., IgG isotype of HLA antibodies in 8-14 day culture supernatants were found in 13 of 16 transplant patients tested covering 50 HLA specificities, the majority (70%) of these antibody specificities overlapped with those in found in serum and only 4 (8%) of them were detected solely in culture supernatants. Interestingly, in addition to IgG antibodies, the authors detected 24 DSA of IgM isotype with almost half of them (45%) present only in B cell culture supernatants. Recently, in 10-day B cell culture supernatants that were 30-fold concentrated, Snanoudj *et al*., found HLA-specific antibodies in 18 out of 39 (50%) transplant recipients among whom 5 (13%) patients had HLA antibody specificities detected only in B cell culture supernatants (9). The differences among these studies may lie on the different B cell activation cocktails used as well as the differences in concentrations of B cell supernatants, which may result in appearance of some HLA specificities otherwise undetected. So far there is no established way on how much to concentrate B cell culture supernatants. As a consequence, the detectability of HLA specificities in culture supernatants is not standardized and may be related to the concentration of IgG in the culture supernatant.

Here we show that HLA antibodies derived through polyclonal activation of memory B cells were detected in 23% of 10-fold concentrated culture supernatants from repeat transplant candidates whereas approximately 52% of the serum samples from these recipients were positive for HLA antibodies. One explanation for this discrepancy between the incidence of HLA antibodies in serum and culture supernatants from patients could be the significantly lower amount of IgG in patient samples compared to controls. Appearance of HLA antibodies in samples with higher IgG concentrations (Figure 3) suggests that relatively lower IgG amounts may have affected the detectability of the HLA-antibodies in our culture supernatants. An alternative explanation for this discrepancy is that immunization against a certain HLA which results in HLA-specific plasma cell formation, leading to serum HLA antibodies, might not

always be associated with concurrent HLA-specific memory B cell generation. Interestingly, we observed persistence of HLA class II antibodies in diluted plasma samples whereas HLA class I antibodies were undetectable. This may possibly explain the predominance of HLA class II antibodies in patients who do not respond well to ABMR treatment modalities involving removal of DSA and elimination of B cells/plasma cells (15, 16).

In order to accurately compare the HLA antibody specificities deriving from plasma cells in serum and reactivation of memory B cells in culture supernatants, ideally similar IgG concentrations in both sources need to be achieved. We are currently working on isolating IgG from culture supernatants by Protein G affinity purification to reach an IgG concentration that allows for a more standardized HLA antibody comparison in serum and culture supernatants. Presence of serum DSA has long been a barrier for transplantation of highly immunized patients. With the advances in immunosuppressive agents and HLA antibody testing, a considerable percentage of these patients can now be transplanted following desensitization treatments that lead to a decrease or complete disappearance of DSA titers. Some patients, even without DSA following desensitization, can manifest with ABMR early in the post transplantation period (13, 17). Identification of HLA antibody specificities with a memory profile in the absence or presence of serum antibodies in patients with DSA before transplantation may help clinicians to discriminate the ones that are more prone to develop ABMR post transplant. It is clear that not every highly immunized patient can benefit from desensitization treatments. Determination of the discrepancies in HLA antibody specificities deriving from memory B cells and plasma cells in immunized transplant recipients may allow for developing strategies to exclude some of the so called unacceptable HLA antibody specificities from the antibody profile of patients with very broad immunization.

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REFERENCES

1. Terasaki PI. Humoral theory of transplantation. Am J Transplant. 2003;3(6):665-73.
2. Gray D. A role for antigen in the maintenance of immunological memory. Nat Rev Immunol. 2002;2(1):60-5.
3. Kurosaki T, Kometani K, Ise W. Memory B cells. Nat Rev Immunol. 2015;15(3):149-59.
4. Crotty S, Ahmed R. Immunological memory in humans. Seminars in Immunology. 2004;16(3):197-203.
5. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science. 2002;298(5601):2199-202.
6. Amanna IJ, Carlson NE, Slifka MK. Duration of humoral immunity to common viral and vaccine antigens. N Engl J Med. 2007;357(19):1903-15.
7. Mulder A, Kardol MJ, Kamp J, Uit Het Broek C, Schreuder GM, Doxiadis, II, et al. Determination of the frequency of HLA antibody secreting B-lymphocytes in alloantigen sensitized individuals. Clinical and experimental immunology. 2001;124(1):9-15.
8. Han M, Rogers J, Lavingia B, Stastny P. Peripheral blood B cells producing donor-specific HLA antibodies in vitro. Human Immunology. 2009;70(1):29-34.
9. Snanoudj R, Claas FH, Heidt S, Legendre C, Chatenoud L, Candon S. Restricted specificity of peripheral alloreactive memory B cells in HLA-sensitized patients awaiting a kidney transplant. Kidney Int. 2015;87(6):1230-40.
10. Pinna D, Corti D, Jarrossay D, Sallusto F, Lanzavecchia A. Clonal dissection of the human memory B-cell repertoire following infection and vaccination. Eur J Immunol. 2009;39(5):1260-70.
11. Heidt S, Roelen DL, Eijsink C, van Kooten C, Claas FH, Mulder A. Effects of immunosuppressive drugs on purified human B cells: evidence supporting the use of MMF and rapamycin. Transplantation. 2008;86(9):1292-300.
12. Zachary AA, Lucas DP, Montgomery RA, Leffell MS. Rituximab prevents an anamnestic response in patients with cryptic sensitization to HLA. Transplantation. 2013;95(5):701-4.
13. Burns JM, Cornell LD, Perry DK, Pollinger HS, Gloor JM, Kremers WK, et al. Alloantibody levels and acute humoral rejection early after positive crossmatch kidney transplantation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2008;8(12):2684-94.
14. Karahan GE, Claas FH, Heidt S. Detecting the humoral alloimmune response: we need more than serum antibody screening. Transplantation. 2015;99(5):908-15.
15. Walsh RC, Everly JJ, Brailey P, Rike AH, Arend LJ, Mogilishetty G, et al. Proteasome inhibitor-based primary therapy for antibody-mediated renal allograft rejection. Transplantation. 2010;89(3):277-84.
16. Everly MJ, Everly JJ, Arend LJ, Brailey P, Susskind B, Govil A, et al. Reducing de novo donor-specific antibody levels during acute rejection diminishes renal allograft loss. Am J Transplant. 2009;9(5):1063-71.
17. Gloor JM, Winters JL, Cornell LD, Fix LA, DeGoey SR, Knauer RM, et al. Baseline donor-specific antibody levels and outcomes in positive crossmatch kidney transplantation. Am J Transplant. 2010;10(3):582-9.

Chapter 9

Summary and general discussion

SUMMARY AND GENERAL DISCUSSION

Solid organ transplantation is a life-saving treatment option for patients with end stage organ failure. The effect of HLA matching between the recipient and the organ donor on graft and patient survival has been shown in large scale studies (1, 2). Despite this beneficial effect of HLA matching, it is virtually impossible to find HLA fully-matched organ donors for most of the patients due to the high-level polymorphism in HLA system. As a consequence, patients are transplanted with partially compatible or even fully mismatched organs, made possible by lifelong immunosuppressive treatment. While currently available immunosuppressive agents are mainly targeting T cells, and thus successful in decreasing T cell mediated rejections, the humoral arm of the alloimmune response remains largely uncontrolled leading to antibody mediated recognition of non-self-proteins and carbohydrates on transplanted organs. Among these targets, mismatched HLA most strongly evokes the formation of donor specific antibodies (DSA) (3). Although alloantibodies to other polymorphic targets and several autoantibodies have also been reported in the field of transplantation (4), donor directed HLA antibodies remain the best described correlates of rejection and graft outcome in kidney, heart, lung and to some extent liver transplantation (5-8). Furthermore, the presence of DSA in recipients of allogeneic hematopoietic stem cell transplantation (HSCT) from haploidentical or mismatched unrelated donors has been associated with higher rates of graft failure (9-11).

HLA-specific humoral alloimmune response

HLA antibodies can develop upon exposure to allogeneic HLA via blood transfusions, pregnancies or previous transplantations. As discussed in **chapter 2**, antibody formation to protein antigens such as HLA is a T cell dependent multi-step process taking place in secondary lymphoid organs. In the context of solid organ transplantation, several studies have shown that B cell activation can occur directly in the graft and lead to local antibody production as a result of lymphoid neogenesis and formation of tertiary lymphoid organs (12, 13). Complete activation of naive B cells occurs upon recognition of the alloantigen via the B cell receptor (BCR) and through subsequent interactions with cognate $CD4^+$ T helper cells. Following germinal center reactions, mutated clones with higher affinity are positively selected to differentiate into long-lived memory B cells that continuously circulate between the periphery and secondary lymphoid organs, and into plasma cells majority of which home to bone marrow to maintain serum antibody titers (14).

Pre-existence or *de novo* production of donor HLA specific antibodies produced by plasma cells is associated with the risk of developing hyperacute, acute and chronic antibody mediated rejection (ABMR) in transplant recipients (3). In patients who have a history of alloimmunization, ABMR may develop as a result of rapid DSA formation deriving from an anamnestic response of pre-existing memory B cells (15-17).

Monitoring HLA-specific humoral alloimmune responses

Serum is the only source screened in HLA diagnostic laboratories before and after transplantation for the presence of DSA in patients (18). However, not only plasma cells but also dormant memory B cells can contribute to HLA antibody production (Figure 1).

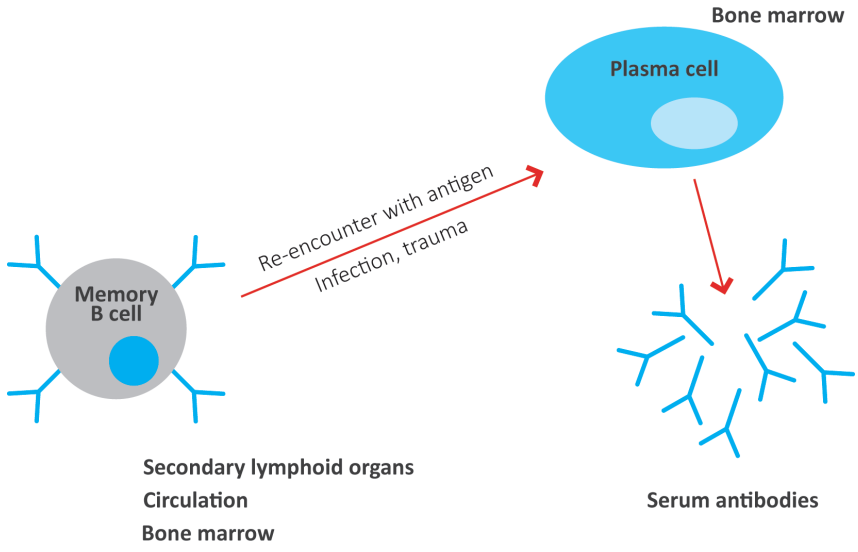

Figure 1. Both long-lived plasma cells and memory B cells contribute to HLA antibody pool. *Serum HLA antibodies are mainly produced by bone marrow residing plasma cells and serum samples are periodically screened in HLA diagnostic laboratories for the presence of HLA antibodies. However, bone marrow residing plasma cells are not the only source of HLA antibodies since silent memory B cells can as well become antibody secreting cells upon antigen-rechallenge. Memory B cells continuously circulate between the secondary lymphoid organs, bone marrow and peripheral blood and need to be polyclonally activated in order to be detected in vitro.*

As exemplified in **chapter 3**, some patients with a history of alloimmunization may harbor HLA-specific memory B cells in the absence of serum antibodies (15). For detection purposes, these dormant HLA-specific memory B cells need to be polyclonally activated *in vitro* to be able to differentiate into antibody secreting cells. Several protocols exist to activate B cells *in vitro*. However, it is critical that the polyclonal activation protocol in use does not induce isotype switching in the naïve B cell population during the culturing process and thereby enables the accurate detection of preexisting memory B cells. Data presented in **chapter 4** characterizes an anti-CD40 driven polyclonal activation protocol that can reliably be used to detect HLA-specific memory B cells in peripheral blood samples since the IgG type of tetanus toxoid (model antigen) specific spots were only found in the memory B cell fraction and not within the naïve B cells. Likewise, the polyclonal activation cocktail comprising of TLR7/8 agonist (R848) in combination with IL-2 used in chapters 6, 7 and 8 does not induce any isotype switching in the naïve B cell population and gives rise to even higher levels of IgG

production compared to the anti-CD40 driven protocol (data not shown) (19).

Current knowledge on the fate of B cells after germinal center suggests that following germinal center reactions, B cells can pursue a pathway where they simultaneously become plasma cells and memory B cells or alternatively, they may leave the germinal center as memory B cells and then gradually differentiate into plasmablasts and plasma cells (20). Nevertheless, in both cases, memory B cells can serve as potential precursors of terminally differentiated plasma cells. Therefore, quantification of these precursors, i.e., HLA specific memory B cells, may extend the understanding of potential HLA specific humoral immune responses that can be elicited upon antigen re-challenge. This line of information may particularly be useful in risk assessment for patients who have antibodies in the historical serum but do not have detectable DSA at time of transplantation as well as those undergoing desensitization therapies. In this respect, HLA-ELISPOT assays allow for quantification of circulating memory B cells directed at mismatched HLA class I and/or class II. Previously, using recombinant HLA class I monomers as the HLA targets (21) and as described in **chapter 5**, using synthetic HLA class II molecules as the detection matrix, we were able to determine HLA specific memory B cell frequencies in peripheral blood samples from women with a history of pregnancy. These assays are of value in selected cases whereby the presence or level of B cell memory against specific HLA antigens needs to be determined. However, these HLA monomer-based assays are limited by the availability of commercial HLA molecules and even if all combinations are used, the HLA repertoire of an individual can never be completely represented. To overcome this restriction, we have developed a donor-specific HLA-ELISPOT assay using cell lysates instead of synthetic HLA monomers as a natural source of both HLA class I and class II, which is described in **chapter 6**. Using this highly sensitive donor-specific HLA-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 of women with a history of pregnancy using cell lysates from their spouses. The possibility to include autologous cell lysates covering the complete self HLA repertoire allowed for the interpretation of spouse-specific positive responses relative to self and served as an excellent intra-assay negative control. This assay 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 peripheral blood samples of patients both in the setting of solid organ transplantation and HSCT. However, it should be noted that peripheral blood memory B cells comprise only a fraction of the memory B cell pool in an individual. Bone marrow, spleen and lymph nodes in addition to intragraft tertiary lymph nodes can as well harbor memory B cells (22).

Long lived plasma cells and memory B cells are the two mediators of serological memory (23). Whereas it has long been accepted that long-lived plasma cells preferentially reside in niches in bone marrow (24), there are only a few reports on the presence and function of memory B cells in bone marrow (25, 26). Data presented in **chapter 7** clearly presents that bone marrow samples contain both memory B cells and plasma cells specific for the same antigen. Interestingly, while memory B cells residing in or circulating through bone marrow

have similar immunoglobulin isotypes compared to peripheral blood B cells, plasma cells enriched from the same bone marrow samples display a distinct isotype distribution.

Conclusions and future prospects

Transplant recipients with a history of alloimmunization comprise a high-risk patient group for developing ABMR. Pre-transplant immunological risk assessment in these patients is solely based on the presence of serum HLA antibodies. However, currently available methods to detect HLA antibodies in serum do not provide any information on the size of HLA-specific memory B cell pool or memory B cell-derived HLA antibody specificities. Considering that the absence of serum DSA does not reflect the absence of B cell immunity against the graft, repeat transplantation candidates, women receiving grafts from their spouses as well as patients undergoing desensitization treatments may benefit from the assays described in this thesis. In this context, our lysate based HLA-ELISPOT assay can be used to estimate the HLA-specific memory B cell load in high-risk patients prior to transplantation of an organ from a living donor. In addition, utilization of monomer-based HLA-ELISPOT assays in samples from patients on the waiting list for an organ from a deceased donor can aid in quantification of memory B cells directed at a certain HLA antigen. Both assays can be performed in conjunction with B cell supernatant analysis.

Antibody mediated rejection remains to be the major cause of graft loss in the field of kidney transplantation (27-29). Therefore, it is very important to make an inventory of the HLA antibodies that are present in a patient before transplantation in order to define the safest donor candidates. Currently available single antigen bead technology enables extensive determination of HLA antibodies present in the serum of transplant recipients. However, particularly for highly sensitized patients, the broad range of HLA antibodies present in the serum makes it virtually impossible to find an acceptable donor. Nonetheless, it is still a matter of debate whether all HLA antibodies detected by single antigen bead assays are clinically relevant. As described in **chapter 8**, our current focus is towards detection of HLA antibodies using single antigen bead assays in culture supernatants which commonly present with a more restricted HLA antibody profile compared to serum samples. Defining discrepancies in HLA antibody specificities found in serum samples and those detected in B cell culture supernatants in a standard and validated way might be useful in excluding some HLA antibodies from highly immunized patients` antibody profiles. This approach needs further validation but can be supplementary in increasing the possibility of transplantation for highly immunized patients. Defining whether a patient with a history of alloimmunization has generated HLA-specific memory in the presence or absence of plasma cells, i.e. serum antibodies, may be useful in determining clinically relevant HLA antibodies since not all patients with pre-transplant DSA develop antibody mediated rejections (16, 30). This line of information may enable better risk stratification by providing a more complete picture of the humoral alloimmune response in patients undergoing desensitization treatments.

REFERENCES

1. Opelz G, Wujciak T, Dohler B, Scherer S, Mytilineos J. HLA compatibility and organ transplant survival. Collaborative Transplant Study. Rev Immunogenet. 1999;1(3):334-42.
2. Heidt S, Witvliet MD, Haasnoot GW, Claas FH. The 25th anniversary of the Eurotransplant Acceptable Mismatch program for highly sensitized patients. Transpl Immunol. 2015;33(2):51-7.
3. Terasaki PI. Humoral theory of transplantation. Am J Transplant. 2003;3(6):665-73.
4. Dragun D, Philippe A, Catar R. Role of non-HLA antibodies in organ transplantation. Current Opinion in Organ Transplantation. 2012;17(4):440-5.
5. Loupy A, Hill GS, Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. Nature reviews Nephrology. 2012;8(6):348-57.
6. Tran A, Fixler D, Huang R, Meza T, Lacelle C, Das BB. Donor-specific HLA alloantibodies: Impact on cardiac allograft vasculopathy, rejection, and survival after pediatric heart transplantation. J Heart Lung Transplant. 2016;35(1):87-91.
7. Le Pavec J, Suberbielle C, Lamrani L, Feuillet S, Savale L, Dorfmuller P, et al. De-novo donor-specific anti-HLA antibodies 30 days after lung transplantation are associated with a worse outcome. J Heart Lung Transplant. 2016.
8. Kaneku H, O'Leary JG, Banuelos N, Jennings LW, Susskind BM, Klintmalm GB, et al. De novo donor-specific HLA antibodies decrease patient and graft survival in liver transplant recipients. Am J Transplant. 2013;13(6):1541-8.
9. Spellman S, Bray R, Rosen-Bronson S, Haagenson M, Klein J, Flesch S, et al. The detection of donor-directed, HLA-specific alloantibodies in recipients of unrelated hematopoietic cell transplantation is predictive of graft failure. Blood. 2010;115(13):2704-8.
10. Yoshihara S, Maruya E, Taniguchi K, Kaida K, Kato R, Inoue T, et al. Risk and prevention of graft failure in patients with preexisting donor-specific HLA antibodies undergoing unmanipulated haploidentical SCT. Bone Marrow Transplant. 2012;47(4):508-15.
11. Ciurea SO, de Lima M, Cano P, Korbling M, Giralt S, Shpall EJ, et al. High risk of graft failure in patients with anti-HLA antibodies undergoing haploidentical stem-cell transplantation. Transplantation. 2009;88(8):1019-24.
12. Thaunat O, Field AC, Dai J, Louedec L, Patey N, Bloch MF, et al. Lymphoid neogenesis in chronic rejection: evidence for a local humoral alloimmune response. Proc Natl Acad Sci U S A. 2005;102(41):14723-8.
13. Wehner JR, Fox-Talbot K, Halushka MK, Ellis C, Zachary AA, Baldwin WM, 3rd. B cells and plasma cells in coronaries of chronically rejected cardiac transplants. Transplantation. 2010;89(9):1141-8.
14. Radbruch A, Muehlinghaus G, Luger EO, Inamine A, Smith KG, Dorner T, et al. Competence and competition: the challenge of becoming a long-lived plasma cell. Nat Rev Immunol. 2006;6(10):741-50.
15. Zachary AA, Lucas DP, Montgomery RA, Leffell MS. Rituximab prevents an anamnestic response in patients with cryptic sensitization to HLA. Transplantation. 2013;95(5):701-4.
16. Burns JM, Cornell LD, Perry DK, Pollinger HS, Gloor JM, Kremers WK, et al. Alloantibody levels and acute humoral rejection early after positive crossmatch kidney transplantation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2008;8(12):2684-94.
17. Montgomery RA, Cozzi E, West LJ, Warren DS. Humoral immunity and antibody-mediated rejection in solid organ transplantation. Semin Immunol. 2011;23(4):224-34.

18. Tait BD, Susal C, Gebel HM, Nickerson PW, Zachary AA, Claas FH, et al. Consensus guidelines on the testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation. Transplantation. 2013;95(1):19-47.
19. Pinna D, Corti D, Jarrossay D, Sallusto F, Lanzavecchia A. Clonal dissection of the human memory B-cell repertoire following infection and vaccination. Eur J Immunol. 2009;39(5):1260-70.
20. Kurosaki T, Aiba Y, Kometani K, Moriyama S, Takahashi Y. Unique properties of memory B cells of different isotypes. Immunol Rev. 2010;237(1):104-16.
21. Heidt S, Roelen DL, de Vaal YJ, Kester MG, Eijsink C, Thomas S, et al. A Novel ELISPOT Assay to Quantify HLA-Specific B Cells in HLA-Immunized Individuals. Am J Transplant. 2012;12(6):1469-78.
22. Giesecke C, Frolich D, Reiter K, Mei HE, Wirries I, Kuhly R, et al. Tissue distribution and dependence of responsiveness of human antigen-specific memory B cells. J Immunol. 2014;192(7):3091-100.
23. Kurosaki T, Kometani K, Ise W. Memory B cells. Nat Rev Immunol. 2015;15(3):149-59.
24. Manz RA, Radbruch A. Plasma cells for a lifetime? Eur J Immunol. 2002;32(4):923-7.
25. Paramithiotis E, Cooper MD. Memory B lymphocytes migrate to bone marrow in humans. Proc Natl Acad Sci U S A. 1997;94(1):208-12.
26. Mayo MS, Paramithiotis E, Cooper MD. B lymphocyte migration to the bone marrow of humans is not random. Stat Med. 1999;18(2):223-31.
27. Einecke G, Sis B, Reeve J, Mengel M, Campbell PM, Hidalgo LG, et al. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. Am J Transplant. 2009;9(11):2520-31.
28. El-Zoghby ZM, Stegall MD, Lager DJ, Kremers WK, Amer H, Gloor JM, et al. Identifying specific causes of kidney allograft loss. Am J Transplant. 2009;9(3):527-35.
29. Schinstock C, Stegall MD. Acute Antibody-Mediated Rejection in Renal Transplantation: Current Clinical Management. Curr Transplant Rep. 2014;1(2):78-85.
30. Gloor JM, Winters JL, Cornell LD, Fix LA, DeGoey SR, Knauer RM, et al. Baseline donor-specific antibody levels and outcomes in positive crossmatch kidney transplantation. Am J Transplant. 2010;10(3):582-9.

Chapter 10

Nederlandse Samenvatting

Türkçe Özet

Abbreviations

Curriculum Vitae

Publications

NEDERLANDSE SAMENVATTING

Het immuunsysteem beschermt het lichaam tegen lichaamsvreemde pathogenen. Dit verdedigingssysteem is opgebouwd uit aangeboren (aspecifiek) en adaptieve immuun responsen met uitgebreid overlap tussen beiden. Het aangeboren deel van het immuunsysteem vormt de eerstelijnsafweer tegen micro-organismen en is opgebouwd uit fysieke barrières (huid en slijmvlies epitheel), oplosbare bestanddelen zoals het complement systeem, en cellulaire effectorcellen (granulocyten, mest cellen, macrofagen, dendritische cellen en natural killer cellen). Cellen van het aangeboren immuunsysteem komen snel in actie tegen pathogenen, terwijl cellen van het adaptieve immuunsysteem (T en B cellen) een relatief langzame maar meer specifieke respons verzorgen. Een uniek kenmerk van het adaptieve immuunsysteem is het vermogen om geheugen te vormen tegen pathogenen. Hierdoor kan er een snellere en sterkere immuunrespons optreden als het lichaam opnieuw aan het zelfde pathogeen wordt blootgesteld. Speciale eiwitten genaamd humaan leukocytenantigenen (HLA) bevinden zich op het celoppervlak en spelen een belangrijke rol in adaptieve immuunresponsen. Het HLA systeem is erg polymorf, wat betekent dat er vele verschillende allelen voorkomen, zowel in individuele personen maar met name ook in de populatie. De functie van HLA moleculen is het presenteren van (lichaamsvreemde) eiwitten aan T cellen van het adaptieve immuunsysteem, en verschillende HLA moleculen kunnen verschillende eiwitten presenteren. Het uitgebreide polymorfisme in het HLA biedt zekerheid dat gehele populaties niet ten ondergaan aan nieuwe, onbekende pathogenen, omdat op populatie niveau er zo goed als zeker eiwitten van het betreffende pathogeen gepresenteerd kunnen worden om een immuunrespons te genereren. Terwijl HLA polymorfisme bijdraagt aan effectieve adaptieve immuunresponsen tegen een verscheidenheid aan pathogenen, kunnen de verschillen in HLA tussen individuen leiden tot problemen bij transplantatie. Het afweersysteem van de patiënt herkent de vreemde HLA antigenen op het donororgaan en kan hiertegen reageren alsof het om een pathogeen gaat.

Orgaantransplantatie is een levensreddende behandelingsmethode voor patiënten die het eindstadium van orgaanfalen hebben bereikt. Verscheidene studies hebben het gunstige effect van HLA overeenkomst tussen ontvanger en donor beschreven. Echter het enorme HLA polymorfisme maakt het haast onmogelijk om een volledig HLA-identiek niet-gerelateerde donor voor een ontvanger te vinden. HLA mismatches tussen patiënt en donor kunnen dan leiden tot activatie van zowel het cellulaire als het humorale adaptieve immuunsysteem in de ontvanger. Om overleving van een HLA-gemismatchte transplantaat mogelijk te maken, is de ontvanger afhankelijk van immunosuppressieve (afweer onderdrukkende) medicatie. Hoewel de reactiviteit van T cellen effectief onder controle gehouden kan worden door hedendaagse immunosuppressieve geneesmiddelen, zijn HLA-antistoffen geproduceerd na activatie van B-cellen een risicofactor voor een vroegtijdig verlies van een orgaantransplantaat.

HLA antistoffen kunnen ontstaan door blootstelling aan cellen of weefsels met lichaamsvreemd

HLA, zoals het geval is bij bloedtransfusies, zwangerschap of eerdere transplantaties. Zoals besproken in **hoofdstuk 2** spelen B cellen een essentiële rol bij de productie van HLA antilichamen. Volledige activatie van naïeve B cellen treedt op als er vreemde HLA antigenen worden herkend, waarbij interacties met T-helper cellen ook een rol spelen. Specifieke interacties tussen immuuncellen, die plaatsvinden in secundaire lymphoide organen, leiden ertoe dat sommige B cellen positief geselecteerd worden om óf te differentiëren in lang levende memory B cellen die continu circuleren tussen de periferie en de secundaire lymfoïde organen, óf in plasmacellen waarvan het merendeel zich concentreert in het beenmerg om de serum antistof titers te behouden. IgM antilichamen worden voornamelijk geproduceerd door naïeve B-cellen, terwijl IgG wordt geproduceerd door memory B-cellen. Na activatie door een T-helpercel kan een naïeve B cel een verandering ondergaan en IgG produceert (isotype switching).

Transplantatie patiënten, die al eerder een afweerreactie hebben gemaakt tegen het vreemde HLA van een orgaandonor hebben een verhoogd risico op het ontwikkelen van antistof-gemedieerde afstoting, wat een negatieve invloed kan hebben op de overleving van het transplantaat. In diagnostische HLA laboratoria wordt vóór en na transplantatie het serum van patiënten getest op de aanwezigheid van HLA-specifieke antistoffen. Serum antistoffen worden voornamelijk geproduceerd door plasmacellen die zich bevinden in het beenmerg. Daarnaast kunnen echter ook memory B cellen een rol spelen bij de antilichaamproductie tegen het donororgaan. Tot dusver is de rol van deze cellen verwaarloosd in de diagnostiek. Zoals beschreven in **hoofdstuk 3**, hebben sommige patiënten met een geschiedenis van allo-immunisatie circulerende HLA-specifieke memory B cellen in de afwezigheid van serum antistoffen. Hoewel er een aantal verschillende methoden (complement dependent cytotoxicity (CDC), ELISA, bead-based assays) gebruikt worden voor het detecteren van serum antistoffen, geeft geen van deze testen informatie over de aanwezigheid en omvang van het HLA-specifieke memory B cel compartiment. Het doel van dit proefschrift was om technieken te ontwikkelen voor het detecteren en kwantificeren van HLA-specifieke memory B cellen in het perifeer bloed van HLA-geïmmuniseerde individuen en de relevantie van deze assays vast te stellen voor klinische transplantatie.

Omdat memory B cellen pas actief worden na stimulatie, is polyklonale activatie in het laboratorium noodzakelijk om deze memory B cellen *in vitro* te kunnen detecteren. Hierbij is het essentieel dat deze polyklonale activatie cocktail niet tot leidt tot isotype switching in naïeve B cellen veroorzaakt, waardoor deze dezelfde functionele karakteristieken krijgen als memory B cellen, het geen zou leiden tot vals positieve reacties. **Hoofdstuk 4** beschrijft een polyklonale activatie cocktail die gebruikt kan worden om geïsoleerde B cellen te activeren om op deze manier reeds bestaande antigeen-specifieke memory B cellen te detecteren zonder antistof isotype switching te induceren. Door gebruik te maken van dit polyklonale activatie protocol is een ELISPOT methode ontwikkeld waarin synthetische HLA klasse II moleculen dienen als HLA doelwit, zoals beschreven in **hoofdstuk 5**. Methoden

om memory B cellen te detecteren maken voornamelijk gebruik van monomeer/tetrameer HLA moleculen die over het algemeen niet het volledige HLA repertoire van een individu representeren. Een donor-specifiek HLA-ELISPOT assay, die screening van HLA-specifieke memory B cellen in perifeer bloed van geïmmuniseerde individuen mogelijk maakt door gebruik te maken van cellysaten als natuurlijke bron van HLA klasse I en II, is beschreven in **hoofdstuk 6**. Terwijl perifere bloedmonsters gebruikt kunnen worden om memory B cellen *in vitro* te detecteren, bevatten secundaire lymfoïde organen en beenmerg ook memory B cellen. Zoals beschreven in **hoofdstuk 7**, herbergt het beenmerg niet alleen plasmacellen maar ook memory B cellen specifiek voor hetzelfde antigeen, terwijl deze twee celtypen een verschillend immunoglobuline isotype distributie laten zien. Hoewel serum HLA antistoffen geproduceerd worden door plasmacellen die zich in het beenmerg concentreren, kunnen memory B cellen ook bijdragen aan de productie van HLA antistoffen bij activatie. Deze antistoffen zijn niet per definitie een afspiegeling van de antistof specificiteiten geproduceerd door plasma cellen in het beenmerg. Daarom zou het bepalen van HLA antistoffen in B celkweek supernatanten, aanvullend aan het detecteren van serum HLA antistoffen, een completer beeld kunnen geven van de potentiële humorale allo-immuunrespons. **Hoofdstuk 8** wijdt uit over het nauwkeurig bepalen van HLA antistof specificiteiten die gevonden zijn in serum en B celkweek supernatanten van patiënten die een tweede of opeenvolgende transplantatie ondergaan.

Immunologische risicobeoordelingen in patiënten is momenteel alleen gebaseerd op de aan- of afwezigheid van serum HLA antistoffen, die echter kunnen verschillen van antistof specificiteiten afkomstig van memory B cellen. Gezien het feit dat de afwezigheid van donor-specifieke HLA antistoffen in serum niet per definitie de afwezigheid van B cel immuniteit tegen het transplantaat reflecteert, zouden patiënten die mogelijk donor HLA-specifieke memory B cellen bezitten, zoals patiënten met een tweede of opeenvolgende transplantatie, vrouwen die transplantaten ontvangen van hun partner of kind, en patiënten die desensitisatie behandelingen ondergaan, mogelijk voordeel kunnen ondervinden van de assays die beschreven staan in dit proefschrift.

TÜRKÇE ÖZET

Bağışıklık sistemi vücudu, doğal ve kazanılmış immün yanıtlar aracılığıyla, öz olmayan patojenik mikroorganizmalara karşı korur. Doğal bağışıklık, mikroorganizmalara karşı ilk savunma hattını sağlar ve fiziksel bariyerler (deri ve mukozal epitel), kompleman sistemi gibi solubl aracılar ile efektör hücrelerden (granülositler, mast hücreleri, makrofajlar, dendritik hücreler ve doğal öldürücü hücreler) oluşur. Doğal bağışıklık sisteminin hücreleri patojenlere karşı hızlı bir savunma uygularken, kazanılmış bagışıklık sisteminin hücreleri (T ve B lenfositler) nispeten daha yavaş fakat daha spesifik bir yanıt verir. Kazanılmış bağışıklığın en önemli özelliği yabancı bir antijen ile ikinci kez karşılaşıldığında, ilkine göre çok daha güçlü ve hızlı bir yanıt verebilmesi, diğer bir deyişle daha önceden karşılaştığı patojenleri hatırlayabilmesidir. Kazanılmış bağışık yanıtın oluşmasına, insan lökosit antijenleri (Human Leukocyte Antigens-HLA) adı verilen ve bir popülasyondaki her bir bireyde birbirinden tamamen farklı olabilen (polimorfizm) özel proteinler aracılık eder. HLA sistemindeki bu çeşitlilik sayesinde, farklı patojenik antijenler T hücresine sunulabilmekte ve oluşan immün yanıt sayesinde toplumların patojenlere yenik düşmesi engellenmektedir. HLA sisteminin bu oldukça polimorfik yapısı çeşitli patojenlere karşı etkin immün yanıt oluşturulmasına katkıda bulunurken, allojeneik (akraba veya akraba dışı) transplantasyonlara bir engel oluşturmaktadır.

Solid organ transplantasyonu, son dönem organ yetmezliği olan hastalar için hayat kurtarıcı bir tedavi seçeneğidir. Pek çok çalışma, hasta ve verici arasındaki HLA uyumunun greft ve hasta sağkalımı üzerine faydalı etkisini göstermiştir. Ancak, HLA sistemindeki çeşitlilik nedeniyle bir hasta için HLA tam uyumlu bir organ vericisi bulmak neredeyse imkansızdır. Hasta ve verici arasındaki HLA uyumsuzlukları, hastada kazanılmış immün yanıtın hem hücresel (T hücresi) hem de hümoral (B hücresi) kollarını tetikleyerek nakil edilen organın reddine yol açabilir. HLA uyumsuz greftlerin ömrünü uzatmak için hastaların çoğunluğu immünosupresif tedavi almaktadır. T hücresinin reaksiyonları mevcut immünsupresif ilaçlarla etkin bir şekilde kontrol edilebilirken, HLA antikoru aracılı (B hücresi tarafından yönlendirilen) hümoral immün yanıtlar halen uzun dönem solid organ sağkalımı için bir risk faktörü olarak düşünülmektedir.

Doğal yollarla oluşan kan grubu antikorlarının aksine HLA antikorları kan transfüzyonları, gebelikler veya önceki transplantasyonlar yoluyla yabancı HLA'ya maruz kalınması sonucu gelişebilir (alloimmünizasyon). **Bölüm 2**'de tartışıldığı üzere B hücreleri hümoral alloimmün yanıt vasıtasıyla alloimmüniteye (hastanın nakil edilen organa verdiği bağışık yanıt) aracılık ederler. Naif B hücrelerinin aktivasyonu alloantijenin tanınması ve yardımcı T hücreleriyle etkileşim sonucu gerçekleşir. İkincil lenfoid organlarda meydana gelen bazı özel reaksiyonlar sonucu (germinal merkez reaksyonları) bazı B hücreleri uzun ömürlü hafıza hücreleri ve plazma hücrelerini oluşturmak üzere seçilirler. Hafıza B hücreleri ikincil lenfoid organlar ve periferik kan arasında latent fazda dolaşırken, serum antikor seviyelerini korumakla görevli plazma hücrelerinin çoğu kemik iliğine yerleşir.

Alloimmünizasyon geçmişi olan hastalar, allogreft sağkalımını etkileyebilecek olan antikor

aracılı rejeksiyon geliştirmek açısından yüksek riskli hasta grubunu oluştururlar. Serum, HLA tanı laboratuarlarında nakil öncesi ve sonrası donöre karşı yönlenmiş HLA antikorlarının tespitinde kullanılan tek kaynaktır. Serum antikorları çoğunlukla kemik iliğinde yerleşmiş olan plazma hücreleri tarafından üretilir. Ancak sadece plazma hücreleri değil hafıza B hücreleri de HLA antikor üretimine katkıda bulunabilir. **Bölüm 3**'te örneklendiği gibi, allimmünizasyon öyküsü olan bazı hastalar serumlarında HLA antikoru tespit edilmemiş dahi olsa HLA-spesifik hafıza B hücreleri barındırabilir.

Serum HLA antikorlarını saptamak için kullanılan pek çok yöntem (komplemana bağlı sitotoksisite-CDC, ELISA, boncuk bazlı testler) olmasına rağmen, bu yöntemlerin hiçbiri HLA-spesifik hafıza B hücresi yanıtının büyüklüğü hakkında herhangi bir bilgi sağlamamaktadır. Bu tez çalışmasının amacı, HLA'ya karşı immünize edici bir hikayesi olan bireylerin periferik kanında HLA-spesifik hafıza B hücrelerini saptayabilen yöntemler geliştirmek ve yeni geliştirilen bu yöntemlerin klinik transplantasyon ortamında uygulanabilirliğini değerlendirmektir.

Latent haldeki hafıza B hücrelerinin *in vitro* ortamda tespit edilebilmeleri için poliklonal aktivasyonları gereklidir. Ancak, kullanılan poliklonal aktivasyon kokteylinin naif B hücrelerinde izotip değişimine neden olmaması ve yalnızca hafıza B hücrelerinden kaynaklanan IgG tipi antikorların saptanmasına izin vermesi kritik bir öneme sahiptir. **Bölüm 4,** antijen-spesifik hafıza B hücrelerinin tespitinde kullanılabilecek böylesi bir poliklonal aktivasyon kokteylini tarif etmektedir. **Bölüm 5**'te ise izole B hücreleri bu kokteyl kullanılarak aktive edilmiş ve sınıf II HLA-spesifik antikor salgılayan hafıza hücrelerini sentetik HLA molekülleri kullanılarak tespit edebilen ELISPOT yöntemi geliştirilmiştir.

HLA-spesifik hafıza hücrelerinin tespitinde genelde monomerik ya da tetramerik HLA molekülleri kullanılmaktadır. Allel sayısının 16000'den fazla olduğu HLA sisteminde, bir bireye ait HLA repertuarının yalnızca bir kaç spesifiteye özgü bu sentetik moleküllerle temsili imkansızdır. HLA kaynağı olarak sınırlı sayıda mevcut olan bu sentetik moleküller yerine hücre lizatlarının kullanıldığı donör HLA-spesifik ELISPOT yöntemi **bölüm 6**'da anlatılmıştır. Periferik kan örnekleri hafıza hücrelerinin *in vitro* tespiti için yaygın olarak kullanılmaktadır ancak kemik iliği ve ikincil lenfoid organlarda da hafıza hücresi olabileceği unutulmamalıdır. **Bölüm 7**'de gösterildiği üzere kemik iliği sadece plazma hücrelerini değil aynı antijene özgü hafıza B hücrelerini de barındırmakta olup iki hücre tipi birbirinden farklı bir immünglobulin izotip dağılımı sergilemektedir.

Serumdaki HLA antikorları kemik iliğinde yerleşmiş bulunan plazma hücreleri tarafından üretilmekte olup, aktive oldukları takdirde HLA-spesifik hafıza B hücreleri de HLA antikor üretimine katkıda bulunabilmektedir. Ancak hafıza hücresi ve plazma hücresi kaynaklı HLA antikor spesifiteleri her zaman tamamen örtüşmemektedir. Bu sebeple, alloimmünizasyon geçmişi olan bireylerde serum HLA antikorlarına ek olarak, B hücre kültürü supernatantlarında HLA antikor analizi, potansiyel hümoral alloimmün yanıt hakkında daha kapsamlı bilgi verebilir. **Bölüm 8**, daha önceden en az bir böbrek nakli hikayesi olan ve yeni bir nakle hazırlanan

hastalarda serum ve B hücre kültürü supernatantlarındaki HLA antikor spesifitelerinin doğru bir şekilde karşılatırılmasına yönelik preliminer verileri sunmaktadır.

Organ nakline hazırlanan hastalarda immünolojik risk değerlendirmesi yalnızca serum HLA antikorlarına göre yapılmaktadır ancak bu antikor spesifiteleri hafıza B hücresi kaynaklı HLA antikor spesifitelerinden farklı olabilir. Bir hastanın serumunda HLA antikorunun yokluğu o bireyde grefte karşı yönlenmiş B hücre immünitesinin olmadığı anlamına gelmez. Bu sebeple, tekrar nakil bekleyen hastalar, eşlerinden ya da çocuklarından organ nakline hazırlanan kadınlar ve desensitizasyon tedavisi gören hastalar gibi potansiyel olarak HLA-spesifik hafıza hücreleri barındırabilecek olan bireyler bu tez çalışmasında tarif edilen yöntemlerden ek fayda sağlayabilirler.

ABBREVIATIONS

^{3}H-TdR	^{3}H-tritiated thymidine
7-AAD	7-aminoactinomycin D
ABMR	Antibody-mediated rejection
ALP	Alkaline phosphatase
APC	Antigen presenting cell
APRIL	A proliferation inducing ligand
ASC	Antibody secreting cells
BAFF	B cell activating factor
BCIP/NBT	5-bromo-4-chloro-3-indolyl-phosphate/ nitro blue tetrazolium
BCPF	B cell precursor frequency
BCR	B cell receptor
B-LCL	B-lymphoblastoid cell lines
BMT	Bone marrow transplantation
Bregs	Regulatory B cells
BSA	Bovine serum albumin
CDC	Complement-dependent cytotoxicity
CDR	Complementarity determining region
CpG	Cytosine-phosphate-guanin
CPM	Counts per minute
CTLA4	Cytotoxic T lymphocyte associated protein 4
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DSA	Donor-specific antibody
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FCM	Flow cytometry
FCS	Fetal calf serum
FDC	Follicular dendritic cells
GC	Germinal center
GvHD	Graft versus host disease
HEV	High endothelial venules
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
HSCT	Hematopoietic stem cell transplantation
Ig	Immunoglobulin
IL	Interleukin

IMDM	Iscove`s modified Dulbecco`s medium
ITS	Insulin-transferrin-sodium selenite
KTR	Kidney transplant recipients
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MPA	Mycophenolic acid
OD	Optic density
ODN	Oligodeoxynucleotide
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PRA	Panel reactive antibody
PVDF	Polyvinylidene fluoride
R848	Resiquimod
RNA	Ribonucleic acid
SAB	Single antigen bead
SCS	Subcapsular sinus
SD	Standard deviation
SFU	Spot forming units
SSC	Side scatter
T_{FH}	Follicular T helper cells
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
Tregs	Regulatory T cells
TT	Tetanus toxoid

CURRICULUM VITAE

Gonca Emel Karahan was born on the 12th of November 1978 in Istanbul, Turkey. After graduating from Beşiktaş Atatürk Anadolu High School in 1996, she started her studies of Biomedical Sciences in Cerrahpaşa Medical Faculty, Istanbul University.

From her graduation in June 2000 until December 2011, she worked at the diagnostic HLA laboratory of the Department of Medical Biology, Istanbul Medical Faculty. During this period, she received her MSc degree (in 2003) and PhD degree in Medical Biology (in 2010) under supervision of Prof Dr. Mahmut Çarin and Prof. Dr. Fatma Savran Oğuz on research focused on the detection of HLA antibodies in kidney transplant recipients.

From January 2012 until July 2017 she has worked as a PhD student under supervision of Dr. Sebastiaan Heidt and Prof. Dr. Frans Claas at the Department of Immunohematology and Blood Transfusion of the Leiden University Medical Center where the research that is presented in this thesis was carried out. Since July 2017, she is working as a researcher in the same group.

LIST OF PUBLICATIONS

Karahan GE, Seyhun Y, Oguz FS, Kekik C, Onal AE, Yazici H, Turkmen A, Aydin AE, Sever MS, Eldegez U, Carin MN. Impact of HLA on the underlying primary diseases in Turkish patients with end-stage renal disease. Ren Fail 2009; 31: 44-9.

Karahan GE, Seyhun Y, Oguz F, Kekik C, Onal E, Caliskan Y, Bakkaloglu H, Yazici H, Turkmen A, Aydin AE, Sever MS, Eldegez U, Carin MN. Anti-HLA antibody profile of Turkish patients with end-stage renal disease. Transplant Proc 2009; 41: 3651-4.

Karahan GE, Kekik C, Oguz FS, Onal AE, Bakkaloglu H, Caliskan YK, Yazici H, Turkmen A, Aydin AE, Sever MS, Eldegez U, Carin MN. Association of HLA phenotypes of end-stage renal disease patients preparing for first transplantation with anti-HLA antibody status. Ren Fail 2010; 32: 380-3.

Iyibozkurt AC, Kalelioğlu I, Gursoy S, Corbacioglu A, Gurelpolat N, **Karahan GE**, Saygili H, Bengisu E. Evaluation of serum levels of interleukin-10, interleukin-11 and leukemia inhibitory factor in differentiation of eutopic and tubal ectopic pregnancies. Clin Exp Obstet Gynecol 2010; 37: 217-20.

Ozkok A, Caliskan Y, Sakaci T, Erten G, **Karahan G**, Ozel A, Unsal A, Yildiz A. Osteoprotegerin/RANKL axis and progression of coronary artery calcification in hemodialysis patients. Clin J Am Soc Nephrol 2012; 7: 965-73.

Seyhun Y, Ozdilli K, Oguz F, **Karahan G**, Onal E, Turkmen A, Eldegez U, Nane I, Caliskan Y, Bakkaloglu H, Carin M. Human leukocyte antigen and major histocompatibility complex class I-related chain A antibodies after kidney transplantation in Turkish renal transplant recipients. Transplant Proc 2012; 44: 1660-6.

Ozkok A, Kekik C, **Karahan GE**, Sakaci T, Ozel A, Unsal A, Yildiz A. FGF-23 associated with the progression of coronary artery calcification in hemodialysis patients. BMC Nephrol 2013; 14: 241.

Karahan GE, Eikmans M, Anholts JD, Claas FH, Heidt S. Polyclonal B cell activation for accurate analysis of pre-existing antigen-specific memory B cells. Clin Exp Immunol 2014; 177: 333-40.

Karahan GE, de Vaal YJ, Roelen DL, Buchli R, Claas FH, Heidt S. Quantification of HLA class II-specific memory B cells in HLA-sensitized individuals. Hum Immunol 2015; 76: 129-36.

Karahan GE, Claas FH, Heidt S. Detecting the humoral alloimmune response: we need more than serum antibody screening. Transplantation 2015; 99: 908-15.

Karahan GE, Caliskan Y, Ozdilli K, Kekik C, Bakkaloglu H, Caliskan B, Turkmen A, Sever MS, Oguz FS. High soluble CD30 levels and associated anti-HLA antibodies in patients with failed renal allografts. Int J Artif Organs 2017; 39: 547-552.

Karahan GE, Claas FH, Heidt S. B Cell Immunity in Solid Organ Transplantation. Front Immunol 2017; 7: 686.

Karahan GE, de Vaal YJH, Krop J, Wehmeier C, Roelen DL, Claas FHJ, Heidt S. A Memory B Cell Crossmatch Assay for Quantification of Donor-Specific Memory B Cells in the Peripheral Blood of HLA-Immunized Individuals. Am J Transplant 2017; Mar 30.