REVIEW

Refinement of humoral immune monitoring in kidney transplantation: the role of "hidden" alloreactive memory B cells

Sergi Luque[1], Marc Lúcia[1,2] & Oriol Bestard[1,3]

1 Experimental Nephrology Laboratory, IDIBELL, Barcelona, Spain
2 Transplant Immunology, Stanford School of Medicine, Stanford, CA, USA
3 Kidney Transplant Unit, Nephrology Department, Bellvitge University Hospital, Barcelona University, Barcelona, Spain

Correspondence
Oriol Bestard MD, PhD, Kidney Transplant Unit, Nephrology Department, Bellvitge University Hospital, Av. Feixa Llarga s/n. 08907. L'Hospitalet de Llobregat, Barcelona. Spain.
Tel.: 0034932607602;
Fax: 0034932607604;
e-mail: obestard@bellvitgehospital.cat

SUMMARY

The advent of novel sensitive assays assessing circulating anti-human leukocyte antigen (HLA) antibodies has allowed recognizing humoral alloimmunity as the main immune-mediated mechanism responsible for allograft rejection and graft loss in kidney transplantation. However, current immune-monitoring techniques, exclusively focusing on circulating anti-HLA antibodies, may underestimate the magnitude of humoral immune response as they exclude the memory B-cell (mBC) pool. Different biological compartments are involved in the intricate mechanisms triggering humoral alloimmune responses even in absence of detectable circulating alloantibodies. Recent studies in animal models as well as in clinical kidney transplantation have shown the key role of this B-cell subset triggering allograft rejection, thus emphasizing the value of recognizing antidonor mBC both as a biomarker of allosensitization and as therapeutic targets. Therefore, considerable efforts are being made among the transplant research community to better understand the role, hierarchy, and impact of mBC in the context of organ transplantation. In this review article, we provide a deep insight into the biology of mBC as well as main evidence of their role orchestrating allograft rejection. Also, we provide a thorough description of main immune-monitoring tools aiming at tracking mBC and a rational for their potential use to refine current humoral immune-risk assessment in kidney transplantation.

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Introduction

Humoral alloimmunity has taken the spotlight in current research in solid organ transplantation, particularly in the kidney transplant setting, as it has been recognized as the major cause of immune-mediated chronic allograft rejection, accounting for more than 50% of kidney allograft losses [1,2]. The emergence of highly sensitive immune assays assessing donor-specific antibodies (DSA), both preformed and *de novo*, has allowed the recognition of specific histological and molecular phenotypes eliciting irreversible allograft damage [3].

Alloantibody formation with donor-antigen specificity is one of the most important traits of the alloimmune response occurring after transplantation aiming at triggering allograft rejection. The formation of plasmablasts

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and plasma cells, responsible for all alloantibody production, is sustained by an exquisite biological process associated with significant changes in the morphology, gene expression profile, and life-span of differentiated antibody-secreting cells (ASCs).

In the setting of solid organ transplantation, the evaluation of the humoral antidonor alloimmune response is exclusively based on detectable circulating anti-human leukocyte antigen (HLA) IgG antibodies using different immune tools. While such assessment has allowed a better understanding of the pathophysiology of kidney allograft rejection and the allosensitization state of transplant patients, its solely analysis may remarkably underestimate the magnitude of the global humoral immune response as it excludes the entire memory B-cell (mBC) compartment. Indeed, tracking antigen-specific mBC has shown to potentially refine the assessment of the immune-sensitization burden, besides circulating antibodies in fields of medicine other than transplantation [4,5]. In this regard, an important interest has arisen within the transplant community to evaluate the memory B-cell compartment, beyond the analysis of circulating alloantibodies [6–11]. Recent studies in animal models of kidney transplantation have shown the key role of this B-cell subset triggering allograft rejection, thus emphasizing the value of recognizing antidonor mBC both as a therapeutic target and as a new biomarker of antidonor allosensitization. Moreover, preliminary studies in human kidney transplant patients using novel immune assays have also highlighted the value of evaluating alloreactive mBC to better identify the degree of activation of the antidonor humoral immune response [12,13]. In this review article, we will tackle the biology and role of mBC, their interactions with other B- and T-cell counterparts and provide some insight into new immune methods capable of accurately tracking circulating mBC in the context of solid organ transplantation.

Memory B cells as a hallmark of adaptive alloimmunity

Circulating high-affinity antibodies represents the effector humoral response that confers long-term protection to invading pathogens. This serological memory consists in pre-existing protective antibodies secreted by long-lived plasma cells (LLPC) from the bone marrow. These pre-existing protective antibodies act as the first layer of defense and are known as *constitutive humoral memory*. In case of re-infection or persistence, a second layer of humoral defense emerge as functional (*reactive humoral memory*) through the activation of antigen-specific mBC, which is typically faster, of greater magnitude, and is formed by antibodies with high antigen affinity and switched isotypes [14].

The mBC population belongs to an adaptive humoral immune response, which is consequent of a first antigen-specific B-cell activation (Fig. 1). B cells have the necessity to obtain two different activation signals once they interact with the antigen. The first signal arises from the binding to surface immunoglobulin of B cells known as B-cell receptor (BCR) with the cognate antigen that leads to an internalization and presentation of antigen peptides and also drives B cells to migrate into the B/T-zone in secondary lymphoid organs. There, a second activation signal takes place, which is triggered by follicular helper T cells (TFH) through the CD40-CD40L costimulatory interplay, among others [15,16]. A portion of activated B cells differentiate into short-lived plasmablasts that generate an early burst of mainly low-affinity IgM antibodies, while another fraction remain as *germinal center-independent mBC* or alternatively, can migrate into B-cell follicles to undergo *germinal center (GC)* selection, a process mainly driven by BCL-6 (B-cell lymphoma 6 protein) signal [17]. In the latter, B cells undergo a positive selection through cognate antigen interaction with TFH cells leading to class switching and somatic hypermutation to ultimately produce high-affinity antibodies. Two different cell differentiation processes might then occur in the light zone of GC; differentiation into either LLPC, driven by specific transcriptions factors such as PRDM1 and IRF4 that upregulate the expression of plasma cell-specific genes and suppress B-cell lymphoma 6 protein (BCL6); or into *germinal center-dependent* (IgG^+) *mBC* where the transcription factor-kB downregulates BCL6 [18,19]. Nevertheless, recent reports have also shown the presence of unswitched IgM^+ mBC [20,21]. Therefore, while the specific function of all these different mBC subsets has not yet been completely characterized, which may vary according to different features such as longevity, affinity to antigen and rapid responsiveness, an increasing body of evidence supports the key role of mBC in maintaining long-lasting humoral immune memory in different clinical settings.

Memory B-cell types and main attributes

During the primary immune response, several types of mBC are generated, which evokes the idea that these memory B cells have distinct functions [22]. Two decades ago, it was hypothesized that there are two distinct

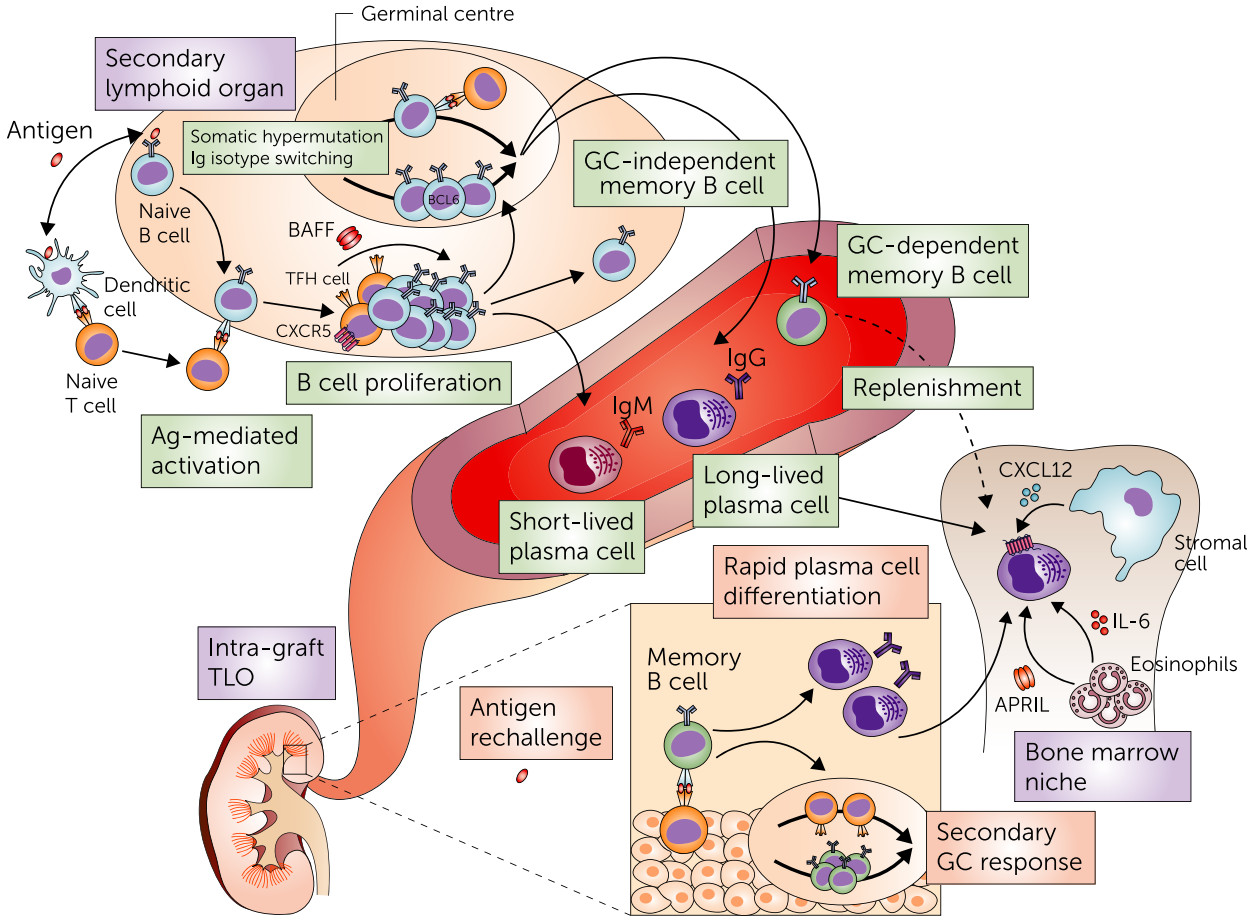

Figure 1 Activation of the humoral memory B-cell immune response in the context of kidney transplantation. After first antigen challenge, naïve B and T cells migrate toward B-cell follicles in secondary lymphoid organs enabling B cells to receive helper signals from cognate T cells. Rapidly, short-lived IgM plasma cells were released into bloodstream while others activated B cells develop into germinal center (GC)-independent mBC. Alternatively, activated B cells will enter into germinal centers and undergo somatic hypermutation and immunoglobulin isotype class switching. As a result GC-dependent mBC and long-lived IgG plasma cells are released into bloodstream. In case of antigen rechallenge, mBC will trigger a rapid plasma cell differentiation and produce an efficient secondary GC response in secondary lymphoid organs (SLO) through memory TFH costimulation signals. Eventually, d-sp mBC might enter into the allograft and form ectopic GC so-called tertiary lymphoid organs (TLO). Finally, long-lived plasma cells are recruited in the bone marrow niche through the expression of different chemokines such as CXCL12 by stromal cells and other important plasma cell survival factors such APRIL (a proliferation-inducing ligand) and IL-6 secreted by hematopoietic cells, mainly eosinophils. Memory B-cell (mBC) might occupy empty bone marrow niches after secondary activation replenishing plasma cell pool.

types of memory B cells (IgM^+ and IgG^+ cells) presenting different functions during antigen rechallenge [23]. In spite of the absence of appropriate markers and methods to distinguish IgM^+ naive B cells from IgM^+ memory B cells, two groups have recently addressed this question having reached a similar conclusion; upon antigen rechallenge, IgG^+ memory B cells preferentially differentiate into plasmablasts, whereas IgM^+ memory B cells proliferate more and enter into germinal center reaction [20,21]. A more recent study has proposed that other markers such as CD80 and programmed cell death 1 ligand 2 (PDL2) are more functionally relevant to mBC. Double-positive cells differentiate into plasmablasts upon restimulation, whereas $CD80^-$ $PDL2^-$ more likely enter the germinal center reaction [24]. These features are depicted in Fig. 2.

Moreover, new studies have highlighted the need to functionally characterize each isotype of mBC [25] showing that the origin, the function, and their longevity could differ between cells expressing different antibody isotypes. These mechanisms used by the humoral memory system seem to be similarly to a stem cell-based mechanism, which requires bifunctionality to efficiently make effector cells upon re-encountering antigens and simultaneously continue to maintain the responsive memory state.

	Naive B-cell	IgM mBC	GC-independent IgG mBC	GC-dependent IgG mBC
BCR Type	IgD + / IgM +	IgD ± / IgM +	IgD – / IgG +	IgD – / IgG +
Secondary response				
Plasmablast conversion	–	–	+	+
GC response	–	+	–	–
Surface Phenotype				
CD27	–	+	+	+
PDL-2	–	–	++	++
CD80	–	–	+	+
Afinity	Low	Low	Low	High
Somatic Hypermutation	–	±	–	+
Molecular traits				
Bcl-6	–	–	–	+
CD40	–	+	+	+
IL-21	–	?	–	+

Figure 2 Memory B-cell types and main attributes. Comparative features of IgM memory B-cell (mBC), GC-dependent IgG mBC and GC-independent IgG mBC based on their phenotype, Ag rechallenge plasticity and molecular traits.

Although virus-specific mBC can be activated in the absence of T cells [26], T-cell help is a strict requirement for the reactivation of mBC [26,27]. Accumulating evidence has shown that TFH cell-derived, CXC-chemokine receptor 5 (CXCR5)-expressing memory T cells exist in secondary lymphoid tissues or in the circulation and that they have a crucial role in helping B-cell activation [28,29]. Importantly, a recent study showed that loss of TFH cells abolished the reactivation of mBC to differentiate into plasma cells [30], which clearly highlights the requirement for TFH cells for efficient recall antibody responses.

Interplay between T follicular helper cells and memory B cells

The germinal center (GC) response consists of antigen-specific B cells undergoing repeated rounds of somatic hypermutation of the BCR, and the selection is helped by TFH cells [31]. TFH help in GC reactions involves many costimulation molecules including CD40L, IL-21, IL-4, and CXCL13 [32]. Recent studies have shown that the mechanisms involved in GC-dependent or GC-independent fate differentiation of activated B cells are directly related to a persistent contact with TFH cells [33]. In fact, high-affinity B cells display higher major histocompatibility complex (MHC) levels to interact with TFH cells, thus facilitating the entrance in the GC cycle and the upregulation of BCL-6 for the subsequent maintenance of GC [34], whereas if T-B interactions are scarce, B cells will more likely develop into GC-independent mBC.

The role of TFH cells in transplantation has partly been defined in animal models. B-cell production of high-affinity alloantibody requires T-cell help provided by the interaction between T-cell receptors and class II MHC-peptide complexes. In a mouse model without class II MHC B-cell expression, allogeneic skin grafts failed to stimulate IgG alloantibody production [35]. Studies of peripheral TFH in human transplantation are scarce. Recently, de Graav *et al.* [36] suggested that patients with pre-existing DSA had higher numbers of circulating TFH cells at 3 months compared with those without. Moreover, in the transplantation setting, inflammatory intragraft infiltrates have been described as becoming organized into ectopic lymphoid tissue known as tertiary lymphoid organ (TLOs), which may itself trigger a humoral response [37]. TLOs are well documented in allografts, both in animal models and in human, but their role in the pathogenesis of allograft rejection still remains to be clearly defined, as it is still unclear whether they have independent functions from spleen and secondary lymphoid organs (SLOs). Evidence from renal biopsy studies has suggested the presence of B cells within allografts in some follicle-like structures [38] colocalizing with T cells, which appear to be producing immunoglobulin within the kidney itself. In addition, Thaunat *et al.* [37] reported that inflammatory intragraft infiltrates during chronic

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rejection result in the generation of functional ectopic GCs allowing the differentiation of mBC into plasmablasts. Altogether, these evidences support the premise that the ability to generate and sustain superior TFH and germinal center responses may favor the generation of alloantibodies that will ultimately lead to allograft rejection.

Dynamics and plasticity of memory B cells and antibody-secreting cells

The maintenance of serum antibody level after immunization, also defined as *serological memory*, is the desirable output of vaccination, because it provides immediate protection against pathogens or toxins. The dynamics of mBC and ASCs during secondary immune responses is a key process occurring during a humoral response as mBC can be activated to proliferate and differentiate either in an antigen-independent way by microbial products, cytokines, or alternatively through bystander T-cell help.

Human studies have shown that the peak level of serum antibodies that is reached following acute infection or immunization declines initially over a period of a few months, but serum antibodies are then maintained at a constant level for decades and eventually for a lifetime in the absence of additional antigenic stimulation. A striking example is the fact that serum antibodies to vaccinia virus, as well as vaccinia virus-specific mBC, may still be detected more than 50 years after vaccination [39,40]. Interestingly, Lanzavecchia and colleagues showed how in the absence of antigenic stimulation mBC stay in a dynamic equilibrium with plasma cells and antibodies, but on day 6 and 7 after booster immunization, large numbers of mBC are generated in an antigen-dependent way. Some of these cells enter the bone marrow in part by normal turnover or by displacing old plasma cells, while most die by day 10. On day 12, there is a large population of LLPC that are rescued in the bone marrow [41].

Plasmablasts are capable of migrating into bone marrow where they benefit of specialized niches that procure an appropriate environment for their differentiation and survival as LLPC [42]. Upon antigen re-encounter, mBC will react quickly and differentiate into plasmablasts expressing high levels of CXC-chemokine receptor 4 (CXCR4), thus allowing the homing process into bone marrow driven by its ligand the chemokine CXC-chemokine ligand 12 (CXCL12) secreted by stromal cells [43]. Eosinophils are proposed to act as secondary components of the bone marrow plasma cell niche, secreting the important plasma cell survival factors APRIL (a proliferation-inducing ligand) and interleukin-6 (IL-6) and promoting plasma cells survival [44,45]. Taking into account these evidences, targeting precursor B cells to prevent the replenishment of nondesirable plasma cell into bone marrow should also be considered as a possible treatment option in the field of transplantation.

Main immune assays tracking alloreactive memory B cells

Important advances have been made in the last years in the development of novel and sensitive immune assays aiming at evaluating circulating mBC in context of organ transplantation. While some of these assays are strictly circumscribed as research tools, some others have shown to have potential room in clinical transplantation if further validated in larger, controlled clinical trials.

Multicolor Flow cytometry technology

Initial attempts were performed using multicolor flow cytometry technology in order to enumerate circulating B cells and classify them into different B-cell subsets including transitional B cells, naïve B cells, plasma cells, and mBC. This analysis has mainly been based on four cell surface markers expression: CD19, IgD, CD38, and CD27. Two major categorization schemes have been put forward depending on the relative expression of either IgD or CD38 (named Bm1-Bm5 classification) from tonsils samples [46] and an alternative classification, using IgD and CD27 staining, based on the fact that CD27 is a marker that discriminates mBC ($CD27^+$) and naïve B cells ($CD27^-IgD^+$) [47,48] although some controversy exist since $CD27^-$ mBC description has been published [49]. Additional B-cell markers have been proposed such as CD24 and CD10, highlighting the impossibility to combine all of them and create a consensus for a B-cell phenotyping panel [50]. To overcome this limitation, a "Standardizing immunophenotyping for the Human Immunology Project" has been suggested to homogenize the B-cell immunophenotype using an eight-color panel with CD19 or CD20 for B cells, CD38 for plasmablasts and transitional B cells, CD24 for transitional B cells, and IgD/CD27 for naïve and mBC [51].

MHC-tetramer-based Flow cytometry technology

Taking advantage of the introduction of the HLA tetramer technology to quantify antigen-specific T cells [52,53], recent studies show the feasibility of tracking HLA-specific B cells binding epitopes of foreign HLA

molecules in their original conformation through their BCR [54–56]. This technology uses streptavidin–biotin complexes of HLA molecules conjugated to a fluorescent protein. Then, tetramer-binding B cells can be accurately enumerated by flow cytometry and allow a rapid quantization of B-cell response to the given HLA antigen. Importantly, while this technique allows for easy quantification of HLA-specific B cells harboring an HLA-specific BCR using flow cytometry readouts, it does not really enumerate frequencies of HLA-specific mBC capable of releasing antibodies. Nevertheless, as a significant number of B cells may recognize non-HLA fractions of the HLA tetramers, this assay may lead to nonspecific binding and thus overestimate the results of this assay [20]. In addition, while this technique may easily enumerate HLA-specific B cells harboring a particular BCR using flow cytometry readouts, it does not ensure that these mBC will differentiate into an ASC capable of releasing antigen-specific antibodies.

Functional quantification of alloantigen-specific memory B cells

As antigen-specific ASCs do not generally circulate in the periphery or at very low levels (0.1–1%) and antigen-specific mBC do not secrete antibodies, *in vitro* differentiation of circulating mBC onto an ASC-like phenotype capable of secreting antibodies and preserving the original BCR repertoire can be achieved using different antigen-independent polyclonal activation methods. Most common antigen-independent activators used are CpG [a Toll-like receptor (TLR) 9 agonist], pokeweed mitogen (PWM), and Staphylococcus aureus Cowan (SAC) often combined with CD40-ligand (CD40L) and/or cytokines such as interleukin (IL-) 2 and IL-10 [57]. More recently, the use of the TLR7/TLR8 agonist R848 plus IL-2 has proven to efficiently activate and differentiate mBC onto ASCs [58,59]. Of note, to efficiently obtain sufficient ASC numbers, 5- to 7-day *in vitro* stimulation of either purified B cells or PBMCs is required. During an ongoing immune response, antigen-specific mBC are present at very low frequencies in the circulation; approximately one of 2500–100 000 [60]. Therefore, to detect antigen-specific mBC in an immune-monitoring assay, *in vitro* polyclonal activation/expansion is required.

Quantification of anti-HLA antibodies of expanded memory B-cell cultures

Some studies interrogate antigen-specific mBC by analyzing the presence of anti-HLA antibodies of expanded mBC culture supernatants using HLA-coated multiplex beads into a solid-phase assay platform [61,62]. While this method seems a feasible approach to potentially translate into clinical practice for defining the mBC compartment, it has two major caveats: (i) It does not quantify the frequency of HLA-specific IgG-producing mBC in a functional manner and (ii) the relatively low titers of certain HLA-specific antibodies not found in expanded mBC cultures might lead to false-negative results, regardless the use of highly sensitive solid-phase assay platforms.

Memory B-cell Enzyme-linked immunosorbent spot (ELISPOT) assay

To accurately quantify the frequency of HLA-specific mBC, the most sensitive technical approach is to use a B-cell enzyme-linked immunosorbent spot (ELISPOT) assay. This assay was first described in 1983 by Czerkinsky *et al.* [63] and has been established as a reliable method for detecting of IgG-producing B cells. The assay has also been further developed for the detection of antigen-specific plasmablasts and mBC [4,5,64]. In the transplantation field, first efforts to introduce the ELISPOT assay were performed through the development of an HLA-specific B-cell ELISPOT assay consisting in purified B cells and CD40L cell line stimulation with synthetic HLA monomers as a detection matrix [13]. The same group recently refined their original method using a new ELISPOT assay approach consisting in PBMC or spleen cell lysates from donors as a HLA targets. They assessed 22 healthy women with a history of pregnancy with cell lysates of the respective husbands and 10 males without any prior immunizing events. As a result, 50% of women with previous pregnancy harbored mBC directed to paternal HLA antigens [65]. With the aim to better characterize the role of donor-specific mBC in human transplantation, our group recently reported a novel HLA-specific B-cell ELISPOT assay approach to quantify HLA-specific mBC from peripheral blood [12]. This method comprehends a 6-day polyclonal stimulation of B cells through the TLR7/TLR8 agonist R848 plus IL-2 from unfractioned PBMC and ASC detection through fluorescent labeled multimerized class I and class II HLA molecules. With the use of this assay, a significantly higher sensitivity to detect low class I or class II HLA-sp mBC frequencies was observed as compared to HLA monomers. Despite that a 6-day culture is required to activate mBC onto ASC, this functional immune assay represents a promising tool for an accurate evaluation of circulating mBC in the context of organ transplantation.

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Next-Generation Sequencing to track alloreactive memory B cells

The advent of high-throughput next-generation sequencing (NGS) allows for large-scale identification and characterization of functional antibodies. This powerful technology has been used in the vaccinology [66], autoimmune [67], infection [68], and cancer fields [69]. So far, in transplantation, the usage of NGS technologies has only focused on high-resolution HLA typing, but has not been applied yet to study alloreactive B-cell populations or alloantibodies. Antibodies are composed of two immunoglobulin chains, the heavy and light chain (IgH and IgL). Each B cell expresses a single-antigen specificity determined during B-cell development due to the segment rearrangement of the genes encoding the variable regions (V,D,J) of IgH and IgL. At the single cell level, one can obtain the full-length sequences for the paired chains expressed in an individual mBC. This high-fidelity analysis is critical for a functional characterization of antibody repertories. Prior to sequencing, a polyclonal activation, similar to those described in section 3, is usually required for overcoming the quiescent state of mBC and increasing the transcription of Ig genes [70]. These NGS methodologies open the door to bioinformatics identification of clonal antibody families, clonal expansions, and recombinant expression of DSA that can be used for subsequent mechanistic studies investigating their pathogenic function.

Role of alloreactive memory B cells in experimental transplantation

According to evidence reported in other fields of medicine, in organ transplantation, there are two different scenarios where mBC may have a pivotal role: (i) in the context of chronic antibody-mediated rejection (ABMR), where persistently activated mBC lead to a low but progressive DSA formation related to insufficient immunosuppression exposure; and (ii) in highly sensitized patients receiving a new graft harboring alloantigens previously recognized by the host, thus triggering a rapid and robust secondary DSA response after antigen re-exposure. In both scenarios, the implications of the different B-cell effector immune subsets, both mBC and LLPC are still not very well characterized. Different reports using diverse models of organ transplantation aiming at reproducing these different scenarios have provided interesting new insight into mechanisms of humoral allograft rejection.

Using MHC-tetramer tracking flow technology combined with function analyses employing ELISPOT assays, Purtha *et al.* [71] recently reported that after clearance viral infection in mice, serum antibodies produced by LLPCs were specific for a single dominant neutralizing epitope, whereas mBC had the ability to respond against both wild-type virus and homologous or heterologous viral variant. This suggest that exclusive stratification of patients through sera DSA alone could significantly underestimate the donor-specific (d-sp) alloreactivity, having important deleterious implications for subsequent transplants as pre-existing mBC responses would not be taken into account. Kwun *et al.* [72] in a mice model of chronic rejection of fully mismatched heart transplantation showed how the increase in allospecific B-cell frequencies was in line with the development of circulating DSA and thus, allograft injury. More recently, Chong and colleagues reported a series of works providing new mechanistic explanations on the key role of mBC triggering humoral rejection and how such immune response may be targeted by specific therapeutic strategies favoring allograft acceptance, particularly at the CD28-CD80/CD86 costimulation level [73–75]. In a first approach, Chen *et al.* [73] elegantly described in a sensitized mouse model of heart transplantation using cell enrichment and MHC class I tetramers that during recall responses, d-sp mBC preferentially differentiate into ASC, whereas in the primary response, d-sp mBC differentiated into germinal center cells. Additionally, treatment with CTLA-4Ig prior and after transplantation effectively abrogated B-cell responses and heart allograft rejection in sensitized recipients, despite fundamental differences in B-cell fates in sensitized versus naïve recipients, emphasizing the key role of B-T interaction to avoid germinal center formation. The adoptive transfer of d-sp mBC into naïve mice translated into a rapid increase in IgG-DSA suggesting the capacity of these mBC triggering class switching alloantibody response in presence of naïve T cells. Furthermore, the same group tested whether early versus delayed CTLA4-Ig treatment (either at day 7 or 14 after active sensitization) could prevent alloreactive B-cell effector immune responses and thus rescue allografts from ABMR despite prior mBC formation. Notably, they observed that alloantibody production could be effectively inhibited only when treatment was initiated by day 7 after sensitization. Conversely, mice treated at day 14 postsensitization, although alloantibody levels did not change, the percentage of allospecific mBC decreased regardless CTLA-4Ig treatment, suggesting that most germinal center B cells were already

completely differentiated into ASC by day 14 after sensitization. Moreover, a minimal effect on endogenous $CD4^+$ T cells was observed, indicating that costimulation blockade with CTLA4-Ig effectively abrogates TFH cells and germinal center B-cell interactions. Likewise, but in a more complex model of allotransplantation using nonhuman primates, Kim *et al.* [76] showed that costimulation blockade (either through CTLA4-CD80/CD86 or CD40-CD40L pathways) was able to abrogate GC formation, thus preventing DSA production.

Interestingly, although no mechanistic studies have been performed in human transplantation, robust indirect evidence of the effect of costimulation blockade abrogating alloreactive B-cell activation comes from a belatacept-based trial, in which in a 7-year follow-up assessment, kidney transplant patients on belatacept therapy displayed significantly lower levels of *de novo* DSA as compared to patients treated with a calcineurin inhibitors (CNI)-based immunosuppressive regimen [77].

Assessment of alloreactive memory B cells in human transplantation

In human transplantation, there are a number of clinical situations in which the role of humoral memory beyond circulating alloantibodies may be clearly observed: Highly suggestive lesions of humoral rejection are frequently observed despite no detectable HLA-DSA [78]; retransplant patents display worse allograft outcomes regardless of preformed HLA-DSAs [79]; anti-HLA antibodies fluctuate in the serum of patients on the waiting list and particularly in those undergoing transplantectomy [80].

With the introduction in the last years of novel immune assays tracking mBC, a number of interesting studies have been reported, highlighting the role of such B-cell subset facilitating allograft rejection in the human transplantation (Table 1).

Initially, efforts aiming at characterizing mBC made by Mulder *et al.* [54] using either human B-cell hybridomas or HLA-specific B cells from pregnancy-immunized individuals [81], show the intimate interplay between the BCR of alloantigen-specific B cells and streptavidin–biotin complexes of HLA tetramers conjugated to a fluorescent protein. Zachary and colleagues [55,56] went one step further by adding the CD27 and CD38 markers to accurately enumerate tetramer-specific-binding mBC and plasma cells. Of note, they found higher HLA tetramer mBC frequencies prior to transplant surgery in kidney transplant patients who generated HLA-DSA after transplantation as compared to those that did not, denoting pre-existing d-sp mBC before transplantation and therefore their eventual resistance to conventional immunosuppression, as this feature was particular evident among transplant patients not receiving CD20 monoclonal antibodies. Likewise, but using HLA-coated multiplex beads, circulating HLA bead B-cell-specific counts has also been reported, Ahmed AKL and colleagues [82] showed in a small cohort of kidney transplant patients that those with poor graft outcomes and circulating alloantibodies displayed significantly higher frequencies and polyreactivity against both class I and class II HLA antigens than patients with good graft function.

Han *et al.* [61] assessed the presence of mBC in transplant patients, multiparous women, and sensitized individuals after multiple transfusions through the analysis of mBC-expanded supernatants cultures. Authors showed that HLA-specific antibodies were only detected in sensitized subjects but not in nonsensitized patients, being the most of them against mismatched donor HLA antigens. Strikingly, a number of patients showed HLA-DSA in the expanded mBC cultures but not in the serum, suggesting a different origin for these alloantibodies, coming either from circulating mBC or from plasma cells residing in the bone marrow. Using the same technical approach in a group of highly sensitized individuals, Snanoudj *et al.* [62] elegantly reported a more restricted epitope reactivity of mBC alloantibodies as compared to circulating alloantibodies in patients with an important sensitization background, suggesting that strong sensitizing immune events may elicit long-lasting humoral immune responses triggered by antigen-specific mBC.

The development and fine-tuning of the B-cell ELISPOT assay has led to a number of interesting reports tracking alloreactive mBC in human organ transplantation. Perry *et al.* [83] first reported the presence of bone marrow residing HLA-specific plasma cells as well as circulating IgG-producing cells from peripheral blood using an ELISPOT assay and confirmed that most ASC reside exclusively in the bone marrow. Subsequently, Heidt and colleagues were able to quantify class I and class II HLA-sp circulating mBC responses in HLA sensitized women with previous pregnancies and in a small group of kidney transplant patients against previously exposed HLA antigens, which were harbored in previous kidney allografts [13,84]. Recently, our group using a novel B-cell ELISPOT assay approach evaluated 70 highly HLA sensitized patients as well as 16 kidney transplant recipients undergoing acute ABMR for the presence of circulating d-sp mBC [6]. Interestingly, a broad range of HLA-sp mBC frequencies were detected

Table 1. Main studies in kidney transplantation evaluating memory B cells (mBC).

Ref	Author	Platform	Assay	Sample type	Sample number	Study conclusions
54	Mulder *et al.* (2003)	FACS	Class I human leukocyte antigen (HLA) tetramers	PBMC	3 sensitized women	Development of a method for detection of HLA A2-specific B cells in peripheral blood in sensitized women by pregnancy
56	Zachary *et al.* (2007)	FACS	Class I HLA tetramers discerning between CD27 and CD38 population	PBMC	72 subjects: 6 healthy controls and 66 end-stage renal disease patients	They found higher HLA mBC frequencies at pre-tx time in kidney transplant patients who generated donor-specific antibodies (DSA) after transplant compared those did not.
83	Perry *et al.* (2008)	ELISPOT	CD138 + antibody-secreting cells (ASC) ELISPOT and sera SAFB	Bone marrow aspirates, Sera	22 samples: 4 nonsensitized, 9 sensitized at baseline and 9 sensitized post-PP/IVIG	ASC ELISPOT was always positive in sensitized subjects and negative in nonsensitized subject, those demonstrates no detectable antibody in sera or *in vitro* ASC culture by SAFB
61	Han *et al.* (2009)	FACS, Luminex	CD27 FACS, SAFB of supernatants cultures	PBMC. supernatants culture	36 subjects: 16 allograft recipients, 12 sensitized patients, 3 multiparous women and 5 healthy subjects	HLA antibody–producing B cells were detected in sensitized subjects. In 13 of 16 allograft recipients, DSA were observed. The majority of antibody-producing B cells developed from $CD27^+$ mBC.
13	Heidt *et al.* (2012)	ELISPOT	Colorimetric and monomeric HLA determination	PBMC	42 subjects: 20 sensitized patients, 14 healthy donors and 8 kidney recipients	Development of HLA-specific B-cell ELISPOT assay consisting in purified B cells and CD40L cell line stimulation with synthetic HLA monomers as the detection matrix.
85	Lynch *et al.* (2013)	ELISPOT	Colorimetric ELISPOT with donor fibroblasts as target	PBMC, Donor fibroblasts	9 subject undergoing first kidney transplant (8 living donor, 1 deceased donor)	Although none of the nine subjects had detectable DSA, all exhibited increases in the frequency of DSA- secreting cells eight weeks after transplantation.

Table 1. Continued.

Ref	Author	Platform	Assay	Sample type	Sample number	Study conclusions
62	Snanoudj *et al.* (2015)	Luminex	SAFB of s-HLA Ab and SN-HLA Ab	PBMC, supernatants culture	69 subjects in WL: 18 s-HLA Ab neg, 12 s-HLA Ab pos w/o immunization, 39 s-HLA Ab pos with immunization	SN-HLA-Abs were detected only in patients with classical immunizing events. Antibody repertoire was more restricted in mBC culture supernatants than in serum among highly sensitized patients.
84	Karahan *et al.* (2015)	ELISPOT, Luminex	Colorimetric and monomeric HLA determination	PBMC, supernatants culture	9 subjects: 6 Pregnancy-immunized women and 3 nonimmunized healthy males	Development of HLA-specific class II B-cell ELISPOT assay consisting in purified B cells and α-CD40 mAb stimulation strategy with soluble HLA class II monomers as a detection agent
12	Lúcia *et al.* (2015)	ELISPOT, Luminex	Fluorescent and multimeric HLA determination	PBMC, supernatants culture	70 subjects: 36 patients in WL (26 sens, 10 nonsens), 16 kidney recipients undergoing antibody-mediated rejection (ABMR), 14 kidney recipients with stable graft function and 4 healthy donors.	Presence of HLA-sp mBC frequencies in sensitized individuals was observed, contrary of nonsensitized subjects. Moreover, high frequencies of donor-specific (D-sp) mBC were detected in kidney transplant recipients undergoing ABMR fitting with DSA levels in sera and also before transplantation in spite of no presence of corresponding DSA.
65	Karahan *et al.* (2017)	ELISPOT	Colorimetric and cell lysate determination	PBMC, cell lysate	32 subjects: 22 healthy women with a history of pregnancy and 10 males without any history of immunizing events	Screening of d-sp mBC in peripheral blood using PBMC or spleen cell lysates as the HLA targets. 50% of women with a previous pregnancy harbored memory B cells directed to paternal HLA

Table 2. Suitable clinical settings for immune-monitoring allospecific memory B cell (mBC) in kidney transplant patients.

Clinical setting	Main goal	Guided therapeutic strategy
	Before transplantation	
Waiting List	Un-mask "hidden" humoral sensitization in absence of detectable donor-specific antibodies (DSA) Assess presence or persistence of peripheral donor-specific (d-sp) mBC prior and after desensitization programs	Refine donor organ allocation Confirm preventive treatment efficacy
At the time of Transplantation	Assess the presence of d-sp mBC frequencies regardless circulating DSA	Intensification of B-cell induction Immunosuppression
	After transplantation	
Stable kidney allografts	Monitor the advent of *de novo* mBC responses	Optimize maintenance immunosuppression Perform/avoid surveillance allograft biopsy
In the presence of allograft dysfunction and/or histological lesions suggestive of humoral-mediated injury	Identify the d-sp humoral immune effector mechanism of graft damage	Provide B-cell target rescue immunosuppression
After Rejection rescue therapy	Assess treatment efficacy	Minimize additional immunosuppressive treatment

among highly sensitized patients in the waiting list and most strikingly, high frequencies were observed both at the time of acute ABMR but also prior at transplantation in the same patients, regardless of detectable HLA-DSA. Furthermore, the higher the d-sp mBC frequency observed, the more severe histological rejection was found. Of note, and differently from a previously published report [85], stable kidney transplant patients without circulating HLA-DSA did not show detectable circulating d-sp mBC frequencies, suggesting that the functional measurement of d-sp mBC may serve as predictive biomarker of allograft rejection and not as a widespread phenomenon in all patients after transplantation. In a similar approach, our group has recently carried out a new study evaluating d-sp mBC frequencies in a large number of kidney transplant patients at the time of biopsies for cause showing different histological immune-mediated phenotypes according to the Banff score classification. Interestingly, patients with histological allograft lesions highly suggestive of chronic ABMR showed similar level of d-sp mBC in peripheral blood, regardless of detectable circulating HLA-DSA, although with significantly lower frequencies than patients with acute ABMR. Interestingly, stable kidney transplants showing preserved allograft parenchyma and the majority of patients showing histological lesions of interstitial fibrosis/tubular atrophy (IF/TA) without HLA-DSA did not show circulating d-sp mBC frequencies [86]. Altogether, these findings strongly suggest that measuring circulating alloreactive mBC using different immune assays is feasible and may improve current humoral immune monitoring to assess the risk of transplant rejection. Indeed, there are a number of suitable scenarios in which the knowledge of the presence of alloreactive mBC would be highly recommended (Table 2). For instance, in the pretransplant setting, patients on the waiting list for retransplantation or in husband to wife or children to mother living-kidney donor transplants could "un-mask" hidden mBC immune responses not detected by circulating HLA-DSA. In addition, in the presence of circulating HLA-DSA, detectable d-sp mBC could further highlight the importance of specifically targeting such B-cell subset as main responsible for HLA-DSA formation. Furthermore, after transplantation, the assessment of circulating d-sp mBC could potentially anticipate the advent of circulating HLA-DSA and therefore allograft damage. Importantly, the evaluation of d-sp mBC could help avoiding surveillance biopsies or, conversely, identify the main effector immune mechanism responsible for graft lesions. Lastly, an accurate assessment of circulating mBC after rescue therapies after rejection episodes

as well as prior or after desensitization programs could provide key clinical information to modulate the type and duration of therapy.

Conclusions

ABMR is the final stage of an intricate biological process in which different B-cell subsets play a key role triggering severe allograft injury. An increasing body of evidence suggests a main role of alloreactive mBC driving humoral rejection after kidney transplantation. Besides the assessment of circulating DSA, the advent of new sensitive immune-monitoring tools allowing a functional assessment of antidonor mBC, both prior and at different time points after transplantation, may provide more insight into the main mechanisms of humoral rejection and help a better stratification of "at-risk" patients and thus even guide treatment decision-making. Importantly, larger observational, multicenter studies are warranted to further confirm recent preclinical and preliminary clinical data in the context of kidney transplantation.

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Conflict of interest

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