CONCENSUS STATEMENT

Stratifying the humoral risk of candidates to a solid organ transplantation: a proposal of the ENGAGE working group

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SUMMARY

Detection of circulating antibodies directed against human leukocyte antigen (HLA) molecules, which corresponds to the current definition of 'sensitized patient', has been shown to have a severe impact on both access to transplantation and, if the anti-HLA antibodies are specific to the selected donor, survival of the graft. However, not all donor-specific antibodies (DSA) are equally harmful to the graft and progress in the understanding of humoral memory has led to the conclusion that absence of DSA at transplantation does not rule out the possibility that the patient has a preformed cellular humoral memory against the graft (thereby defining a category of DSA-negative sensitized recipients). Technological progress has led to the generation of new assays that offer unprecedented precision in exploring the different layers (serological and cellular) of alloimmune humoral memory. Based on this recent knowledge, the EuropeaN Guidelines for the mAnagement of Graft rEcipients (ENGAGE) working group to propose an updated definition of sensitization in candidates for solid organ transplantation – one that moves away from the current binary division towards a definition based on homogenous strata with similar humoral risk.

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Introduction

End-stage vital organ failure is a life-threatening condition and the leading cause of premature death worldwide. The World Health Organization currently estimates that the economic burden of vital organ failure represents 25% of total health expenditures and is in rapid expansion. Patients with end-stage vital organ failure depend on solid organ transplantation, which represents their best (often the only) therapeutic option.

For patients on a waiting list for transplantation, the detection of circulating antibodies directed against human leukocyte antigen (HLA) molecules, which corresponds to the current definition of 'sensitized patient', represents a major unmet challenge. Presence of anti-HLA antibodies in the circulation of a candidate to solid organ transplantation indeed detrimentally impacts both access to transplantation and, if the anti-HLA antibodies are specific to the selected donor, survival of the graft [1].

However, the status of sensitized patients encompasses significant heterogeneity. Recent literature suggests that not all DSA are equally harmful to the graft. On the other hand, progress in the understanding of humoral memory has led to the conclusion that absence of DSA at transplantation does not necessarily rule out the possibility that the patient has a preformed cellular humoral memory against the graft (thereby defining a category of DSA-negative sensitized recipients).

New assays have become available that allow humoral memory to be investigated with unprecedented precision. Using these tools, evidence is emerging of homogenous strata with similar humoral risk, prompting the European Society for Organ Transplantation to appoint a group of experts: the EuropeaN Guidelines for the mAnagement of Graft rEcipients (ENGAGE) working group to redefine the notion of sensitized patients.

Humoral memory is complex and multilayered

Because the major histocompatibility complex (HLA, in humans) is highly polymorphic, situations in which a patient is exposed to allogeneic HLA molecules (previous transplants, blood transfusions or pregnancies) can result in the generation of anti-HLA-specific immunoglobulin (Ig)G. This depends on a prototypical T cell-dependent B-cell response [2,3], which occurs in secondary lymphoid organs (spleen and/or draining lymph nodes). The binding of donor HLA molecules to the surface immunoglobulins of recipient allospecific B-cell clones delivers a first signal of activation that leads to the internalisztion of the alloantigen, which is then processed and presented to cognate T follicular helper (Tfh) cells (Fig. 1). This germinal centre (GC) reaction, during which the affinity of the immunoglobulin is increased (by somatic hypermutation) and its heavy-chain isotype modified (by class switch recombination), finally results in the generation of long-lived plasma cells, which reside in the bone marrow [4]. These release the alloantibodies into the circulation, where most are sequestrated due to their size [5]. This 'serological memory' (Fig. 1) represents a major obstacle to transplantation. Preformed DSA can cause hyperacute rejection within minutes of revascularization by binding to the directly accessible antigenic targets expressed by graft microvasculature and fixing complement; antibody-mediated complement activation extensively damages the endothelium integrity and initiates an intravascular coagulation cascade that results in vessel thrombosis and tissue infarction [6].

Recent advances in the field of basic immunology have shown the humoral memory to be heterogeneous and multilayered [7]. Besides serological memory, the humoral response also generates a 'cellular memory' (Fig. 1), represented by memory B cells (MBC) [4]. MBCs persist for long periods and recirculate through the bloodstream and secondary lymphoid organs, awaiting a secondary antigen encounter [8]. Upon re-exposure to the same antigen, MBC form proliferative foci in the subcapsular sinus of lymph nodes and rapidly differentiate into plasma cells [9]. Alternatively, they will re-enter the GC for another round of affinity maturation and class switch recombination. Recent data regarding this functional heterogeneity of MBC suggest it depends on B-cell receptor isotype [10] and/or the expression of the two-cell surface proteins CD80 and PD-L2 [11].

Until recently, Tfh cells were considered as fully differentiated effector cells prone to apoptosis while the GC reaction resolved. However, the existence of memory Tfh cells (Fig. 1) has now been demonstrated in both mice and humans [12–15]. In humans, there is convincing experimental evidence that memory Tfh cells promote B-cell responses after antigen rechallenge [12–15].

Assays currently available to probe serological humoral memory

Various crossmatch (XM) techniques exist to detect preformed DSA before transplantation, either

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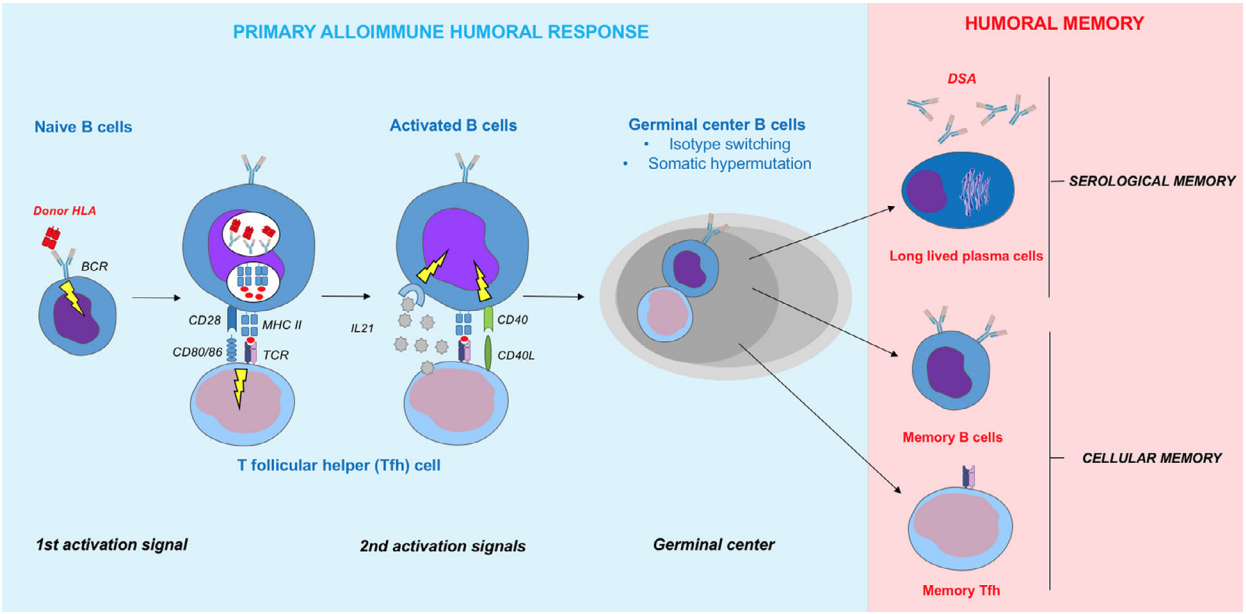


Figure 1 The humoral alloimmune response and its multiple layers of memory.

confronting donor cells and recipients' sera in traditional 'crossmatch tests' or identifying reactions against HLA antigens in sera contributing to current virtual crossmatches. These are divided into cell-based and solid-phase assays, described below (see also Table 1, Fig. 2a).

Cell-based assays

These assays use lymphocytes, because latent T cells express only HLA class I antigens, whereas B cells express both class I and II antigens.

Complement-dependent cytotoxic crossmatch

CDC-XM was introduced in the 1960s [16]. It measures donor lymphocyte lysis produced by recipient's serum and rabbit complement. It can be executed with donor cells or as a pretransplant screening assay to measure the overall level of sensitization in candidates awaiting transplantation, using panels of lymphocytes from a diverse pool of individuals. This way, the assay helps to define HLA antibody specificities and calculate the per cent panel of reactive antibodies (%PRA).

Flow cytometric crossmatch

Flow cytometry (FC) crossmatch was introduced in the 1980s. It measures the amounts of antibodies in recipient's serum which bind to donor lymphocytes using fluorescent-labelled antibodies to T and B cells, as well as labelled antibodies to IgG and/or IgM [17].

This assay can also be used to delineate PRA using pooled target cells to determine the level of sensitization in recipients – an approach that anticipated the current widespread Luminex PRA.

A positive FCXM does not always indicate a detrimental donor-specific humoral memory. Positivity can result from nonspecific antibodies revealed by a positive auto-crossmatch. In the absence of DSA detected by single-antigen bead (SAB), a positive FCXM is not predictive of rejection [18,19], probably because antibodies identified in this situation could recognize antigens not present at the endothelial cell surface.

Solid-phase assays

Solid-phase assays were designed in the 1990s, when purified HLA molecules became available and could be fixed on a solid phase (plaque or beads).

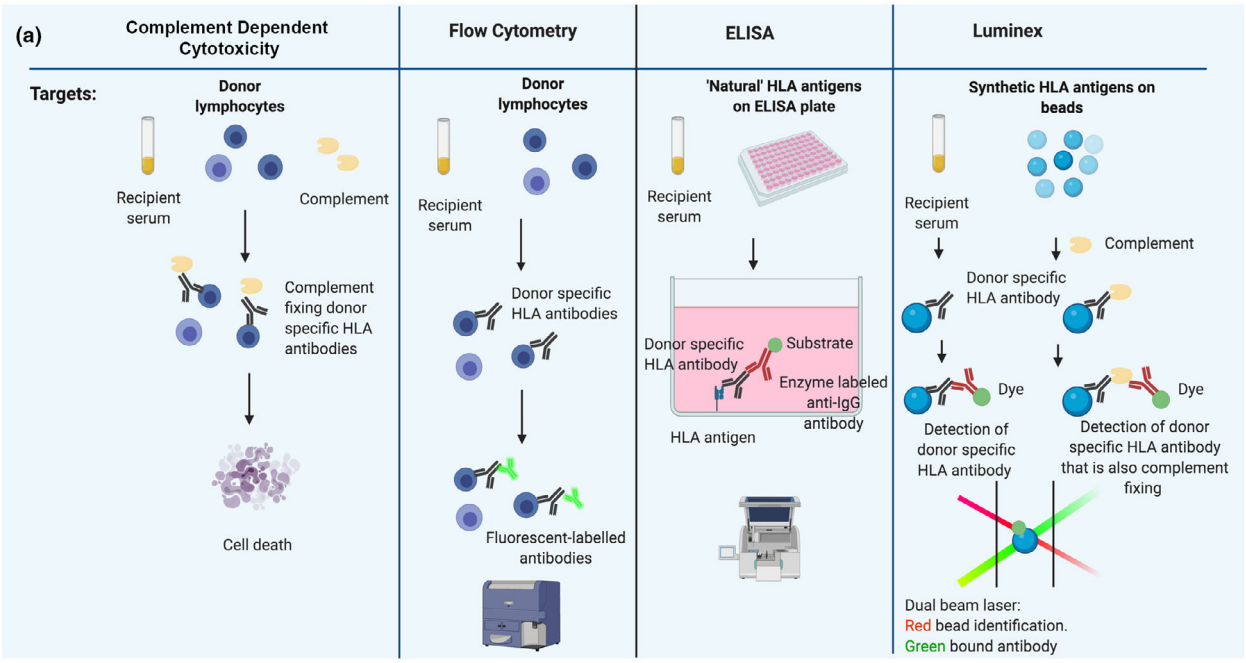
Enzyme-linked immunosorbent assay

ELISA extended the application of a routine test for a new indication [20], using purified HLA molecules as targets on plaques. It was not widely adopted because bead assays quickly showed many more advantages.

Table 1. Available assays evaluating alloreactive serological memory.

Platform	Antigen detection systems	Assay output	Advantages	Limitations	References
Cellular assays to detect donor-specific HLA antibodies on recipients' serum					
Complement-dependent cytotoxicity	Donor lymphocytes, rabbit complement	Proportion of donor lymphocytes dead by cytotoxicity, assessed visually	• PPV +++ of hyperacute rejection • Cell death % and serum dilution correlate with amount of circulating HLA-DSA	• Low sensitivity for low-level or noncomplement-fixing antibody • With T cells, only proves HLA class I antibody • Changes needed to distinguish IgM/IgG • FP results by non-HLA or auto-antibody • Subjectivity and interobserver variability in results	Patel *et al*. 1969 [16]
Flow cytometry	Donor lymphocytes, fluorescent-labelled antibody to T/B cells and to IgG	Amount of antibody bound to lymphocytes with fluorescent anti-human IgG antibody. Result expressed as mean channel shift of fluorescence emitted by lymphocytes incubated with neat vs case serum	• More sensitive than CDC • Detects low-level DSA • Predictive of early AMR	• Treatment with anti-T/B-cell antibody may interfere with output • Threshold for positive reactions depends on each lab • Positivity can be due to nonspecific antibody confirmed by a positive auto-XM • In absence of DSA by SAB, a positive FC XM is not predictive of rejection	Bray *et al*. 2011 [17] Couzi *et al*. 2009 Schinstock *et al*. 2017 [47]
Solid-phase assays to detect donor-specific HLA antibodies					
ELISA	HLA molecules from platelet donors or EBV-transformed cells on a microtitre plate	Detection of antibody attached to HLA by anti-IgG, measured with an ELISA	• First assays to use capture HLA proteins, enabling testing without donor cells	• Low sensitivity and specificity	Schlaf *et al*. 2010 [20]
Bead-based assays on Luminex	HLA purified antigen on plastic beads, fluorescent-labelled antibody to IgG. Beads can have a mix (screening test) or individual HLA (SAB)	Beads' fluorescence assessed on standard flow cytometer or Luminex platform, able to read ≥100 beads with different HLA proteins to semi-quantify the amount of bound antibody as MFI per bead	• More sensitive/specific than CDC and FC in sensitized persons • Less FP than ELISA, especially for class II antibody • Reliable virtual XM without donor cells in minutes	• Interpretation requires expertise • Relies on complete donor HLA typing, preferably high-resolution • Significant variations between laboratories and kits • Lack of consensus regarding level of clinically relevant antibody • FP reactions for denatured conformation of HLA on bead surface not correlated with AMR	Pei *et al*. 1999 [20] Reed *et al*. 2013 [21] Visentin *et al*. 2015 [28] Tait *et al*. 2016 [28]

AMR, antibody-mediated rejection; CDC, complement-dependent cytotoxicity; DSA, donor-specific antibodies; EBV, Epstein–Barr virus; ELISA, enzyme-linked immunosorbent assay; FC, flow cytometry; FP, false positive; HLA, human leukocyte antigen; Ig, immunoglobulin; MFI, mean fluorescence intensity; PPV, positive predictive value; PRA, panel of reactive antibodies; SAB, single-antigen bead; XM, crossmatch.



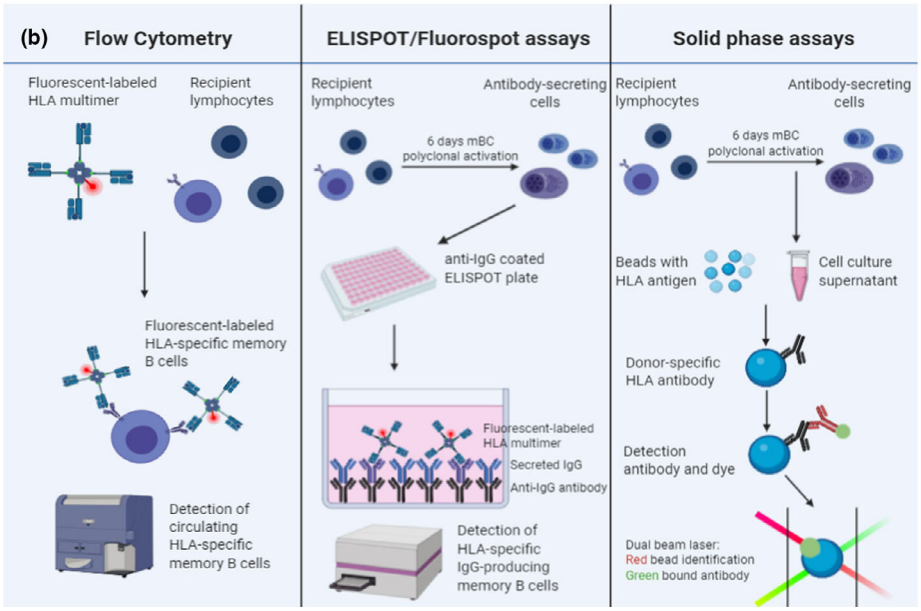


Figure 2 Available assays to evaluate humoral alloimmune (a) serological memory and (b) cellular memory.

Bead-based assays (Luminex)

SAB-based assays measure the amounts of antibodies present in a given serum and can identify purified class I or II phenotype (A/B/C or DR/DQ/DP) antigens adhered to plastic beads with fluorescent-labelled antibodies to IgG (or IgM or IgA, if preferred). Beads' fluorescence can be assessed using either a standard flow cytometer or the newer Luminex platform, semi-quantifying the amounts of bound antibodies as mean fluorescence intensity per bead (MFI) [21,22].

There are different types of test, offering considerable versatility to detect antibodies to a mix of HLA antigens on the surface of the beads (screening tests with pooled HLA class I or II on a few beads, or mix tests with several antigens on the bead-simulating cells) or to individual antigens present in a person's serum (SAB). Some

test modifications have arisen in an attempt to better assess functionality of HLA antibodies:

- SAB tests have incorporated a way to evaluate the complement-binding capacity of antibodies, either in the form of C1q or through the physiological generation of split products C3d or C4d. Kits are available from two different vendors to test for C1q and C3d binding antibodies. Importantly, the absence of complement-binding capacities does not rule out the harmful potential of DSA [23–25].
- Assessment of IgG subclasses thought to be associated with different severity of antibody-mediated rejection (AMR) because of their different potential to activate the classic complement pathway [26] and the Fcγ receptors of innate immune effectors [27]. Current reagents do not meet the criteria for scientifically sound interpretation, so the test is not routinely used in the clinical setting.

SAB tests allow for a reliable virtual crossmatch that does not require viable donor cells; instead, they rely on complete HLA typing of the donor and current antibody assessment of the recipient, along with assortment of HLA antigens on the beads of the kit, especially for noncommon antigens.

SAB assays have many limitations (Table 1) reviewed in [28]. In particular, intact HLA molecules coexist with denatured HLA molecules (also called β_2-microglobulin free HLA class I heavy chain) at the microbeads surface. The assay can therefore identify antibodies directed against denatured class I HLA antigens displaying cryptic epitopes [29], which appear irrelevant because they are associated with a low risk of AMR [30]. They are usually associated with a negative FCXM and are more frequently directed against HLA-Cw antigens [31].

Assays currently available to probe cellular humoral memory

Studies on HLA-specific B cells are evolving from evaluating the ability of B cells to produce HLA antibodies *in vitro* to quantifying HLA-specific B cells by flow cytometry and enzyme-linked immunosorbent spot (ELISpot; Table 2, Fig. 2b). The difference in the activation of B cells or the affinity of antibodies generated by different *in vitro*-activated MBC and plasma cells may determine the pathogenicity of resulting donor-reactive antibodies [32]. Human blood CXCR5+CD4+ T cells, which share functional properties with Tfh cells and appear to represent their circulating memory compartment [15], also probably contribute to the cellular humoral memory.

Flow cytometry

It is possible to quantify peripheral HLA-specific B cells with FC by combining staining for CD19 with fluorescent HLA tetramers. Patients without circulating HLA antibodies in pretransplant sera, but with high frequencies of peripheral HLA-specific B cells, could be at higher risk of developing anti-HLA antibodies post-transplant [33,34]. Although the test can detect circulating anti-HLA B cells, it suffers from some technical limitations and the functionality of these cells has yet to be fully understood.

ELISpot

Solid-phase ELISpot is designed for enumeration of MBC or plasmablasts secreting specific antibodies. It employs circulating MBC from immunized individuals, which are incubated in antigen-coated polystyrene plates (the antigen of interest) after a 6-day *in vitro* polyclonal activation. After removal of the cells, bound secreted antibodies are visualized by means of an immunoenzyme procedure. Spots form where antibody production has occurred and can be enumerated to establish a frequency of antigen-specific IgG-producing plasmablasts, which may be provided as a ratio over the total polyclonal IgG-producing plasmablasts [35–41].

The high sensitivity of the assay could be useful for detecting low-frequency HLA-specific circulating MBCs. The main limitations of the assay are that it is time-consuming (6 days of stimulation), costly and requires a high number of peripheral B cells to allow assessment of a large HLA repertoire.

Ex vivo cultures

Ex vivo culture is a strategic simplification of ELISpot, similarly based on the *ex vivo* polyclonal expansion of HLA-specific MBC (6-day stimulation) to testing of supernatants with SAB. This is an easy, low-cost way to identify all antibody types but is less sensitive than ELISpot, especially for low antibody titres [42–44].

Ex vivo cultures have also been used to stimulate circulating (i.e. memory) Tfh of transplant recipients in presence of patient's own serum (containing relevant concentration of immunosuppressive drugs) [45]. This 'residual activatability' of the recipient's circulating Tfh has been shown to correlate with transplant recipients' ability to respond to a protein vaccine [45]. Further studies are warranted to determine whether this test

Table 2. Available assays evaluating alloreactive cellular memory.

Platform	Antigen detection systems	Assay output	Advantages	Limitations	References
Antigen (HLA)-specific B-cell assays					
Flow cytometry	Surface cell markers, HLA-specific tetramers	Detection of circulating MBC numbers bearing a HLA-specific B-cell receptor	• Easy detection of B cells harbouring a HLA-specific B-cell receptor	• Nonspecific B-cell binding to fluorochromes • Low sensitivity to detect low frequencies (does not quantify MBC capable of secreting alloantibodies)	Mulder *et al*. 2003 [31] Zachary *et al*. 2007 [32]
ELISpot/ FluoroSpot assays	HLA monomers, fluorescent-labelled multimerized HLA monomers, donor-derived cell lysates, expanded donor fibroblasts	Detection of circulating HLA-specific ASC, either circulating plasmablasts or MBC capable of rapidly switching to an ASC-like phenotype	• Precise enumeration of HLA-specific MBC capable of secreting antibodies (also low frequencies) • Provides frequency (strength) of alloresponse, as it may be extrapolated to the total clonable MBC of a given individual	• Needs 6-day polyclonal stimulation • Needs all synthetic HLA molecules to assess entire HLA repertoire • Expensive and labour intensive	Heidt *et al*. 2012 [33] Perry *et al*. 2008 [39] Karahan *et al*. 2015 [34] Lynch *et al*. 2013 [38] Lúcia *et al*. 2015 [37] Luque *et al*. 2018 [36] Luque *et al*. 2019 [37]
Solid-phase assays	Supernatants of polyclonally expanded MBC	Detection of anti-HLA antibodies using solid-phase assays in supernatants of *ex vivo* polyclonally expanded HLA-specific MBC	• Simple detection of anti-HLA Ab released by MBC	• Indirect quantification (assumes all ASC secrete a constant amount of IgG) • Low sensitivity for low antibody titres (effect of dilution) • Needs 6-day polyclonal stimulation	Han *et al*. 2009 [40] Snanoudj *et al*. 2015 [42] Karahan *et al*. 2019 [41]
Single-cell RNA sequencing	Bar-coded tetramers with oligonucleotide-conjugated antibodies and RNA sequencing	Simultaneous measurement of the protein and gene expression of antigen-specific B cells	• Concomitant assessment of multiple antigen specificities with transcriptional profiling	• Costly and complex • Requires computational analysis	Bentzen *et al*. 2016 [87] Bentzen *et al*. 2018 [88]
Non-antigen-specific assays					
Next-generation sequencing	Full-length sequencing of IGHV genes encoding the V,D,J complementary determining region 3	Detection of B-cell repertoire rearrangement of variable IgG regions	• Assessment of clonality and diversity of IgG B-cell repertoire	• No antigen-specific information • Costly and complex • Requires computational analysis	Pineda *et al*. 2019 [89] Moore *et al*. 2020 [90]
Flow cytometry	Surface cell markers on circulating (memory) Tfh, directly or after *ex vivo* stimulation	Assessment of number/ residual activatability of circulating (memory) Tfh	• Explore Tfh, simple and cheap	• No antigen-specific information • Lack of validation	Cano-Romero *et al*. 2019 [80] Dahdal *et al*. 2018 [43]

ASC, antibody-secreting cells; ELISpot, enzyme-linked immune absorbent spot; HLA, human leukocyte antigen; Ig, immunoglobulin; MBC, memory B cell(s); Tfh, T follicular helper cells.

could identify transplant patients at higher risk of developing DSA under immunosuppression.

Not all preformed DSA present at the time of transplant are the same

The pathogenic potential of DSA varies considerably: although preformed DSA are a major cause of accelerated graft loss due to post-transplant AMR [46–49], their presence is not always a strict contraindication to the procedure [50]. The current challenge is therefore to identify clinically relevant preformed anti-HLA antibodies by analysing their molecular targets (allowing identification of DSA) while also evaluating quantitative and qualitative characteristics.

The mechanisms by which DSA induce graft damages are now well understood (Fig. 3) [51]. DSA binding to the directly accessible antigenic targets expressed by graft microvasculature can trigger the classic complement cascade and/or recruit Fcγ receptor-expressing innate immune effectors, which promote damage to the graft vasculature through the release of lytic enzymes (antibody-dependent cell-mediated cytotoxicity; ADCC). Though not essential for the development of chronic endothelial damage, complement activation is generally understood to strongly accelerate the rejection process. This concept is at the basis of the seminal work by Patel and Terasaki, who demonstrated as early as 1969, using CDC-XM, that the ability of preformed DSA to activate (rabbit) complement *ex vivo* predicts hyperacute rejection and graft failure [16].

While a correlation between complement activation observed in the CDC-XM *ex vivo* and DSA-induced complement activation in the graft *in vivo* is conceptually coherent, it has yet to be conclusively demonstrated. The fact that 30% and 50%, respectively, of 3- and 12-month protocol graft biopsies show positive staining for C4d in patients with preformed DSA but negative CDC-XM, might cast some doubt on the hypothesis [50,52]. However, C4d staining is known to imperfectly explore the downstream part of the complement cascade (i.e. the formation intragraft C5b9 membrane attack complex and its regulatory mechanisms).

IgG's ability to trigger classic complement activation is linked with its titre, since recruitment of C1q (the first molecule of the cascade) requires IgG hexamerization [53]. The quantity of DSA can be estimated by the MFI value in SAB testing. Since 2010, the post-transplant risks of AMR and graft loss are generally recognized as increasing with the strength of baseline DSA MFI [54,55]. Although there is no general consensus regarding the MFI cut-offs that should be used to stratify the risk of AMR and graft loss (largely because MFI values vary among laboratories and commercial suppliers), the STAR working group recently recommended a MFI cut-off of 1400 [56]. Interestingly, Visentin et al recently reported that the exact same threshold of MFI was associated with positivity of T-cell FCXM for class I DSA [57], and several studies have reported that the ability of DSA identified by SAB to bind *ex vivo* donor cells in FCXM is a good predictor of subsequent AMR lesions and graft loss (in 50% and 30% of recipients, respectively [49,58–60]). Altogether, these data suggest that optimal performance of FCXM in identifying pathogenic DSA depends on both higher specificity (elimination of false positivity due to denatured HLA molecules on SAB) and lower sensitivity (so that only DSA with high titre are detected).

Beyond the quantitative aspect, IgGs have several qualitative properties that have been shown to modulate their pathogenicity. The γ heavy chain has distinct isotypes endowed with different abilities to bind C1q [26] and the Fcγ receptors of innate immune effectors [27]. Patients usually exhibit a mixture of noncomplement-fixing IgG2/IgG4 DSA and complement-fixing IgG1/IgG3 DSA [61]. One-year post-transplant immunodominant IgG3 DSA are associated with more clinically patent ('acute') AMR, whereas immunodominant IgG4 DSA could induce subclinical AMR [62], suggesting different injury pathways perhaps associated with intragraft complement activation. The glycosylation status of the Fc region of DSA has been shown to be highly variable among patients [63]. Although, theoretically, this property could also modulate the ability of DSA to recruit C1q and innate immune effectors [64], evidence for its clinical impact remains scarce [63].

In search of a single assay to evaluate the quantitative and qualitative properties of DSA while also determining pathogenicity, SABs were modified to analyse the ability of DSA to fix C1q and C3d. During the first year after transplantation, patients with complement-activating DSA have an increased MVI with more CD68+ monocyte/macrophage and natural killer (NK) cells, more NK-selective transcripts, more chemokine transcripts (CCL4 and CXCL11), more γ-interferon-inducible genes and a higher rate of C4d deposition (64% vs. 18%) compared with patients with noncomplement-fixing DSA. This specific histomolecular kidney allograft rejection phenotype could be abrogated by complement inhibition with eculizumab, suggesting that these assays reliably reflect intragraft complement activation [65].

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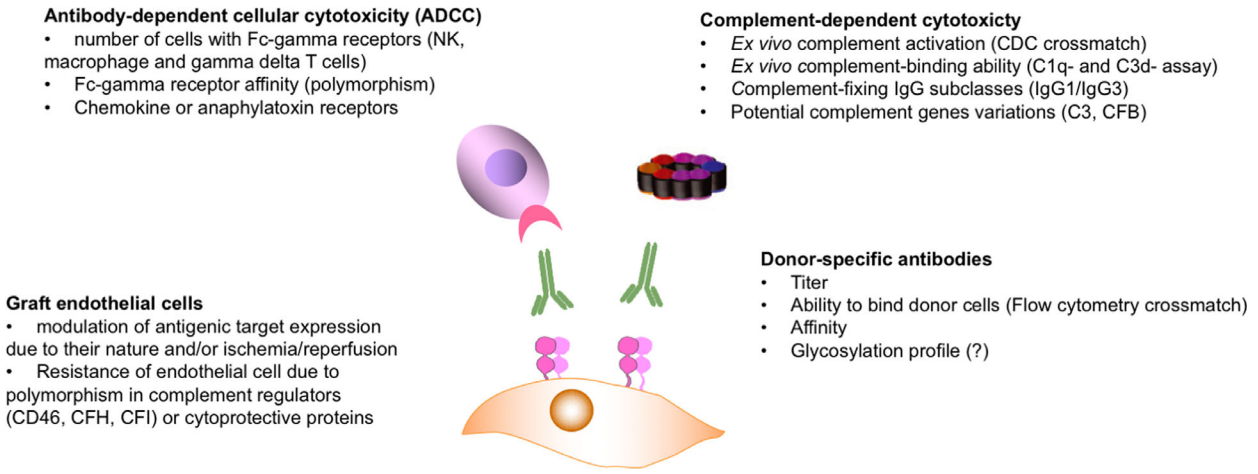

Figure 3 Immuno-pathophysiology of DSA-mediated damages informs the prediction of antibody-mediated rejection and graft loss. During antibody-mediated rejection (AMR), the first targets of donor-specific antibodies (DSA) are endothelial cells, which are directly accessible to circulating DSA and express donor's human leukocyte antigen (HLA) molecules. DSA binding to HLA molecule can trigger the classic complement cascade and/or recruit Fcγ receptor-expressing innate immune effectors, which in turn promote damage to the graft vasculature via antibody-dependent cell-mediated cytotoxicity. The pathogenicity of DSA is routinely evaluated with their titre (MFI or dilution) or their ability to bind donor cells (by flow cytometry crossmatch). *Ex vivo* complement binding can be evaluated with the C1q and/or C3d assays. Analysis of complement-fixing IgG subclasses or complement genetic variations, number of innate immune effectors (e.g. circulating CD16+ cells), and polymorphism of Fcγ receptors could all help to better stratify the risk for AMR, but these tools are not yet routinely available. Measurement of DSA affinity and glycosylation profile is not yet available. Finally, the characteristics of the target graft endothelial cells (e.g. level of expression of HLA molecules, stress-induced ligands or expression level of complement regulators or cytoprotective proteins) probably influence the pathogenicity of the DSA but are beyond the scope of this article.

However, despite the fact that post-transplant C1q- and C3d-fixing DSA are associated with a worse graft survival [66], day-zero C1q- or C3d-DSA are not predictive of hyperacute rejection [59,67–69].

Finally, another important measure of the pathogenicity of preformed DSA is their persistence after transplantation. In the absence of antibody-depleting therapy, day-zero DSA MFIs are stable or decrease during the first 2 weeks post-transplant in 75% of patients [70], resolving spontaneously in ~50% in the first 3 months [71] and in ~65% at 12 months [72]. Inhibition of the memory humoral response by the maintenance immunosuppressive regimen could partly explain this phenomenon. In contrast, the increase in DSA MFI during the first 15 days after transplantation is strongly associated with an early clinical AMR [70,73], although no universal threshold has been identified [74,75]. The persistence of DSA is also associated with acute clinical AMR during the first 3 months [72]; because AMR is a continuous process, their persistence could lead to chronic transplant glomerulopathy and AMR on 1-year protocol biopsies [52,72,76–78], and ultimately to lower graft survival [71,72]. Therefore, prediction of DSA post-transplant evolution is crucial to determine their pathogenicity. The number of day-zero DSA and HLA-DQ DSA, and high MFI, are markers of post-transplant persistence. A MFI value of 3780 units could be an optimal cut-off for predicting this persistence (specificity, 73.2%; sensitivity, 65.3%), whereas DSA with a MFI <1400 units are usually cleared. Moreover, the modulation of immunosuppression with the addition of rituximab could help to inhibit the humoral memory response [79,80]. This may result from an impact on the cellular memory, explained in the next section.

Absence of circulating DSA doesn't always mean absence of humoral memory

Currently, assessment of anti-HLA humoral immune response is based solely on evaluation of serum anti-HLA antibodies. However, the exclusive assessment of circulating anti-HLA IgG antibodies does not fully illustrate the complete humoral memory alloimmune response occurring in distinct clinical settings of organ transplantation. Despite accurate assessment of serum anti-HLA antibodies using current sensitive immune techniques, misleading interpretations can occur in many clinical situations – such as constant fluctuations of serum antibodies over time in sensitized patients waiting for a kidney transplant [81], or worse allograft outcomes in patients with previous sensitizing events

(e.g. women receiving a living-donor kidney transplant from the husband or children) even in the absence of circulating DSA [82]. New tools are urgently needed to refine evaluation of anti-HLA humoral immune responses [56,83].

Using HLA-specific tetramers to identify allospecific MBC, Zachary et al observed that higher frequencies of circulating donor HLA-specific MBC prior to transplantation was associated with higher risk to generate HLA-DSA after kidney transplantation [34]. Independent studies [42,44] relying on a different approach (the analysis of MBC-expanded supernatant cultures) identified MBC in the circulation of transplant patients, multiparous women and sensitized individuals after multiple transfusions, in the absence of detectable circulating DSA. A similar observation has been made by a Barcelona group, who reported a broad range of HLA-specific MBC frequencies among highly sensitized patients on the waiting list, even in the absence of serum anti-HLA antibodies [37]. Most importantly, the presence of preformed anti-donor MBC frequencies highly correlated with the risk of acute AMR, when they could also be functionally detected using FluoroSpot assay. Similar data were recently reported in the context of a pilot clinical trial among highly sensitized kidney transplant patients showing preformed DSA. Interestingly, the authors reported a higher incidence of AMR among patients with the presence of both DSA and also donor-reactive MBC assessed by the presence of DSA in B-cell expanded supernatant [43]. Finally, in a recent prospective follow-up cohort of kidney transplant patients with for-cause and surveillance kidney graft biopsies [39], anti-donor MBC were clearly observed in patients subsequently developing subclinical AMR – crucially, before detection of serum DSA. Altogether, these data strongly suggest that the assessment of circulating donor (HLA)-specific MBC may significantly improve characterization of the donor-specific humoral immune response of kidney transplant patients both before and after transplantation.

As a surrogate biological marker of an allogeneic humoral alloimmune response, the assessment of circulating Tfh cells has been postulated as an interesting approach to further understand the mechanisms of alloantibody formation. Although a body of evidence exists in experimental transplantation showing the key role of Tfh cells driving antigen-specific humoral alloimmune responses, data in clinical transplantation are scarce. Some recent reports suggest that patients with circulating DSA and previous alloantigen exposure display higher numbers of circulating CD4+CXCR5+ Tfh, even before kidney transplantation [84]. Furthermore, some authors have reported that high frequencies of IL-21-producing T cells, suggested to be circulating Tfh, may also be associated with a higher risk of allograft rejection [85]. Finally, recent reports have demonstrated that immunosuppressive drugs do not entirely block Tfh functions [2] and that the residual activatability of recipient's Tfh is closely correlated with antibody response following immunization with protein antigens [45].

Conclusion: A proposal to stratify the humoral risk of candidates for solid organ transplantation

In recent decades, although the definition of 'sensitization' in transplantation has hardly changed, more precise investigation of the humoral alloimmune response has made us aware of significant heterogeneity in the pathogenicity of DSA and has demonstrated that humoral memory extends beyond the mere serological memory compartment. The detection of preformed anti-HLA antibodies in the serum of a patient on the waiting list is the most robust biomarker currently available to ascertain the presence of humoral alloimmune memory; but this evaluation alone now seems insufficient to decipher the complete landscape of humoral alloimmune memory, which ultimately determines individual risk of AMR and graft loss before transplantation.

This lack of definition of homogenous strata for humoral risk in transplantation represents a major obstacle to therapeutic progress: patients with very different profiles are currently mixed together [86], making it extremely difficult to judge the benefit of any new intervention.

The ENGAGE working group proposes that to circumvent this problem, data from the patient's past 'immunological' history should be integrated with the SAB assay and the FCXM method, enabling the risk of AMR and graft loss to be stratified into five distinct categories before transplantation (Fig. 4).

This change in the definition of the humoral risk shall be understood as a first step, which will require incremental updates when the tools allowing for the identification of the effectors of humoral cellular memory, and a more precise evaluation of (i) DSA-induced intragraft complement activation and (ii) immune effector cells recruitment will become available.

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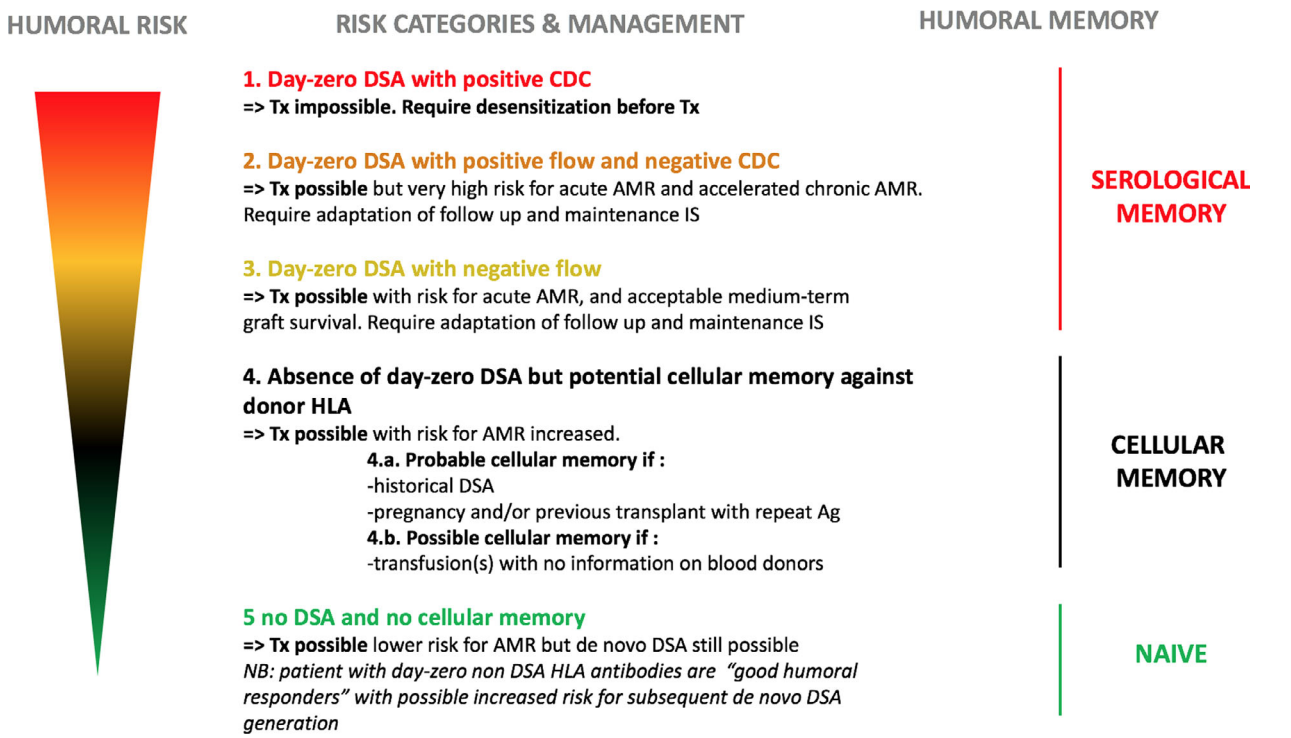

Figure 4 ENGAGE's proposal for categorization of the humoral risk of solid organ transplant candidates.

Authorship

OT was Chairman of the Working Group and drafted the first version of the MS. OB, LC, MC and NK wrote specified sections of the MS. All authors reviewed/provided input into the entire manuscript.

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Conflicts of interest

The authors declare no potential conflicts of interest.

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Ethical approval

This article does not report a study in human subjects; therefore, ethical approval was not required.

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