T-bet⁺CD27⁺CD21⁻ B cells poised for plasma cell differentiation during antibody-mediated rejection of kidney transplants

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Abstract

Alloimmune responses driven by donor-specific antibodies (DSAs) can lead to antibody-mediated rejection (ABMR) in organ transplantation. Yet, the cellular states underlying alloreactive B cell responses and the molecular components controlling them remain unclear. Using high dimensional profiling of B cells in a cohort of 96 kidney transplant recipients, we identified expanded numbers of CD27⁺CD21⁻ activated memory (AM) B cells that expressed the transcription factor T-bet in patients who developed DSAs and progressed to ABMR. Notably, AM cells were less frequent in DSA+ABMR- patients and at baseline levels in DSA-patients. RNA-seq analysis of AM cells in patients undergoing ABMR revealed these cells to be poised for plasma cell differentiation and to express restricted IGHV sequences reflective of clonal expansion. In addition to T-bet, AM cells manifested elevated expression of IRF4 and Blimp1, and upon co-culture with autologous T follicular helper cells, differentiated into DSA-producing plasma cells in an IL-21 dependent manner. The frequency of AM cells was correlated with the timing and severity of ABMR manifestations. Importantly, T-bet⁺ AM cells were detected within kidney allografts along with their restricted IGHV sequences. This study delineates a pivotal role for AM cells in promoting humoral responses and ABMR in organ transplantation and highlights them as important therapeutic targets.
Introduction

Humoral alloimmunity mediated by anti-human leukocyte antigen (HLA) donor-specific antibodies (DSAs) significantly impedes prolonged survival of allografts after organ transplantation (1-3). Antibody-mediated rejection (ABMR) represents the complex clinical manifestation of deleterious DSA responses, which involve microvascular inflammation, arteritis and complement activation in allograft vessels, and is associated with poor outcome (4,5). We and others have shown that the emergence of proliferating B cells enriched for donor HLA-specificity preceded the onset of ABMR, was predictive of the magnitude of DSA responses and histological damage (6-8) and was associated with increased risk of kidney allograft loss (9). The HLA-specific activated B cells are primarily contained within the memory B cell (MBC) compartment, thereby suggesting a prominent role for antigen-experienced cells in ABMR and warranting their further exploration.

Over the past decade it has become clear that specific inflammatory conditions and antigenic stimulation, particularly in the context of vaccination, autoimmunity and chronic infections, can result in the emergence of MBCs lacking CD21 and expressing the transcription factor T-bet (10,11). Initially described in mice, T-bet-expressing B cells, including age-associated B cells, display multiple common phenotypic and functional attributes conserved in humans such as the overexpression of the integrin CD11c, the activation markers CD86 and CD95, as well as the downregulation of the classical B cell markers CD23, CD24 and CD38. Moreover, these cells can function as potent effectors or display features of exhaustion (12,13). Flu or yellow fever vaccines have been shown to strongly induce CD27⁺CD21⁻ T-bet-expressing B cells that are enriched for vaccine-specific plasma cell precursors (14,15). In lupus and rheumatoid arthritis, such CD27⁺/⁻CD21⁻ T-bet-expressing cells manifest an effector B cell profile with increased potential to differentiate into autoreactive plasma cells after stimulation with toll-like receptor ligand and IL-21 signals (16-18).
malaria, HIV and hepatitis infections, CD27⁻CD21⁻ T-bet-expressing cells dominate the B cell response but display significantly impaired function with diminished ability to secrete antibodies, suggestive of B cell exhaustion (19-23). Conversely, HIV-positive individuals mounting highly efficient IgG1⁺ and IgG3⁺ antibodies displayed increased frequencies of activated CD27⁺CD21⁻ T-bet-expressing cells (24), and individuals lacking these cells failed to elaborate a protective antiviral response (25). Despite these functional differences, T-bet-expressing B cells display shared features of antigen-experienced cells such as isotype-switched IgH loci and somatic hypermutation (26).

The occurrence of T-bet-expressing MBCs and their functional as well as genomic states remain to be explored in the context of organ transplantation and ABMR. It should be noted that we and others have documented that humoral alloimmunity (e.g. DSA generation and ABMR) is dominated by skewed and exaggerated production of IFNg, IL-17 and IL-21 by T follicular helper cells (T₉H) in response to donor-antigens (6,27-28). Based on the above considerations, we undertook a multidimensional and functionally integrated characterization of the B cell responses in patients undergoing ABMR. Importantly, our cohort design enabled these B cell responses to be compared with those in transplant patients that did not undergo ABMR and either remained DSA- or developed DSAs. Our overall approach, encompassing high dimensional flow cytometry and RNA-seq analyses of phenotypically defined B cell subsets in a cohort of 96 kidney transplant recipients, spanned unsupervised exploration of B cell states along with in-depth characterization of particular populations based on prior biological information.

Our results uncover a subset of activated memory (AM) B cells, that are T-bet⁺ CD27⁻CD21⁻ and whose dynamics track with both early ABMR involving pre-formed DSAs as well as with late ABMR associated with de novo generated DSAs. These AM cells expressed IL-21R, were transcriptionally poised for plasma cell differentiation, and displayed
amplification of restricted $IGHV$ sequences consistent with clonal expansion. Such cells differentiated into DSA-producing plasma cells when co-cultured with autologous $T_{FH}$ cells in an IL-21 dependent manner. Importantly, Tbet$^+$ AM cells were detected within kidney allografts of ABMR patients along with their characteristic amplified $IGHV$ sequences, supporting their pathogenic role in allograft rejection.
Results

**Multidimensional profiling of B cell responses in kidney transplant patients**

We enrolled 96 kidney transplant recipients who were systematically screened for circulating DSAs and allograft rejection in the first 24 months post-transplant, and identified three groups: patients who did not manifest DSAs nor experienced ABMR (DSA-, N=48), those who had DSAs but did not undergo ABMR (DSA+ABMR-, N=28) and patients who had DSAs and experienced ABMR (DSA+ABMR+, N=20) (Figure S1A). Their clinical characteristics are shown in Table S1. Although age and gender were comparable across the groups, DSA+ABMR- and DSA+ABMR+ patients had higher rates of re-transplantation as compared to DSA- patients, suggesting their increased prior exposure (memory) to alloantigens (Table S1). Among the DSA+ABMR+ patients, 12 had DSA pre-transplant and all experienced early ABMR (before 3-months). In contrast 8 DSA+ABMR+ patients did not manifest pre-transplant DSAs and underwent late ABMR (after 3-months) (Figure S1B). Of the 20 DSA+ABMR+ patients, 17 displayed concomitant T-cell mediated rejection lesions (mixed ABMR), while 3 were assessed to have pure ABMR lesions.

We profiled PBMCs and sera from cross-sectional blood samples collected at the time of the following immunological events: (i) detection of post-transplant DSAs for DSA+ABMR- patients and (ii) detection of ABMR in the presence of DSAs for DSA+ABMR+ patients. For DSA- patients, the blood samples were analyzed at matched time points with those from DSA+ABMR- and DSA+ABMR+ patients (Figure S1B). Longitudinal analyses of PBMC and sera from representative patients were also performed (see Methods). Using multidimensional approaches, we analyzed the phenotypic, transcriptional and functional profiles of B cells as well as their dynamics in the three groups of transplant recipients. Healthy control (HC) subjects served as a control group (Table S2).
Emergence of circulating MBCs in patients developing post-transplant DSAs and ABMR

We used high-dimensional flow cytometry analyses of PBMCs to evaluate the frequencies of the major circulating B cell subsets (29) (transitional, naive, MBCs and plasmablasts) among the transplant groups and HCs (Figure S2A). We observed a significant increase in the frequencies of total B cells in DSA+ABMR+ patients as compared to DSA- patients and HCs, that was due to higher frequencies of MBCs and plasmablasts. Moreover, we observed a concomitant decrease in the frequencies of transitional B cells in DSA+ABMR+ patients (Figure S2B).

MBCs associated with ABMR are heterogeneous and include expanded T-bet-expressing subsets

To investigate the phenotypic states of MBCs including the testing of our hypothesis pertaining to T-bet-expressing cells, we performed unbiased high-dimensional t-SNE analyses and created consensus t-SNE maps on MBCs (gated as in Figure S2A) and based on the expression of 20 markers (Figure 1A). We observed prominent differences in MBC profiles among the various groups (Figure 1B), with a cluster of T-bet-expressing cells that had downregulated CD21 markedly distinguishing DSA+ABMR+ from other patients and HCs. T-bet+ cells also co-expressed CD11c, CD19 and CD20, as well as Ki67, CD95 and CXCR3. Conversely, these cells downregulated CD24, CD38 and CXCR5, and therefore, were highly distinctive from T-bet MBCs. Interestingly, T-bet+ cells displayed disparate expression of CD27 and IgD (Figure 1A). We next used SPADE clustering to further resolve MBC subpopulations (Figure 1C). Among the 12 SPADE clusters, high T-bet expression along with low CD21 robustly demarcated cluster 11 and 12 from all other clusters (Figure 1D) (Table S3). Cluster 11 was further distinguished from cluster 12 by higher expression of IL-21R, CXCR3 and IgD. These
two clusters in addition to the T-bet\textsuperscript{low} cluster 7 were significantly expanded in DSA+ABMR+ patients (Figure 1E) (Figure S3). The frequencies of T-bet\textsuperscript{+} CD21\textsuperscript{-} cells could represent up to 40\% of MBCs in these patients (Figure 1F). We note that MBCs, when compared to naive, transitional and plasmablast compartments, exhibited the highest fractions of T-bet\textsuperscript{+} cells (Figure S4). Thus, consistent with our hypothesis, DSA+ patients undergoing ABMR manifest increased frequencies of MBCs expressing high levels of T-bet.

**Expansion of T-bet-expressing AM and TLM cells during ABMR**

Based on the above clustering analyses and previous studies (14-15, 30), we used a targeted gating approach on MBCs to further segregate CD21\textsuperscript{-} B cells based on their expression of CD27 into: CD27\textsuperscript{+}CD21\textsuperscript{-} activated memory (AM) and CD27\textsuperscript{-}CD21\textsuperscript{-} tissue-like memory (TLM) cells. Their resting memory (RM) counterparts were identified as CD27\textsuperscript{+}CD21\textsuperscript{+} cells. A significant expansion in AM cell frequencies (Figure 2A) and their absolute counts (Figure S5A) were observed in DSA+ABMR+ patients. Cellular expansion was observed at a lesser extent for TLM cells in these patients. Notably, the expression of T-bet, and that of CD11c, were restricted to AM and TLM cells and not detected in RM cells. The expression of T-bet was significantly increased in both AM and TLM cells in DSA+ABMR+ patients, but was the highest in the latter subset (Figure S5B). AM cells were more heterogeneous, comprising of SPADE clusters 4, 7, 11 and 12, as compared to TLM cells that resided in clusters 11 and 12 (Table S3). The AM cluster 7, and the common AM/TLM cluster 11 were substantially enriched in DSA+ABMR+ patients, which distinguished them from the other patient groups (Figure S3). We next evaluated the functional potential of AM and TLM cells by analyzing the expression of activating and inhibitory receptors. IL-21R, a critical regulator of activated B cells (31), was found to be co-expressed with T-bet (Figure 2B) and upregulated in AM cells from DSA+ABMR+ patients (Figure 2C). In addition to IL-21R, the expression of CD40, a
crucial co-receptor that permits efficient response to CD40L costimulatory signals provided by T_{FH} cells, was increased in AM cells in DSA+ABMR+ patients and markedly reduced on TLM cells (Figure 2C). In contrast, TLM cells displayed increased expression of the inhibitory receptors CD72, FcRL5 and CD32b in these patients. These results suggested that AM cells, unlike TLM cells, were functionally poised to interact with cognate T_{FH} cells.

**AM cells are poised for plasma cell differentiation and enriched for distinct IGHV genes during ABMR**

We next undertook RNA-seq analyses of sorted RM, AM and TLM subsets (Figure S6) to analyze the transcriptional programs underlying the phenotypic states of each MBC subset in DSA+ABMR+ patients at the time of rejection. When compared to RM cells, the AM gene expression programs were significantly enriched in molecular pathways associated with B cell effector functions including cell activation, cell division, somatic hypermutation and Ig production (Figure 3A, Table S4). In striking contrast, TLM cells were enriched in molecular pathways reflecting negative regulation of B cell activation and cell division (Figure 3B, Table S5). TBX21, which encodes for T-bet, was expressed in both AM and TLM cells, but at higher levels in the latter, consistent with the flow cytometry data (Figure 3C). However, compared to TLMs, AM cells uniquely upregulated IL2RG, reflecting their increased potential to respond to IL-21, and displayed elevated expression of the plasma cell genes IL6R, MZB1 and XBP1 (Figure 3C). Importantly, IRF4 and Blimp1, which are key transcription factors required for plasma cell differentiation in response to IL-21 (32), were expressed at higher levels at both transcript and protein levels in AM cells (Figure 3C-3D). Analysis of putative promoter/enhancer regions of differentially expressed genes (DEGs) that were delineated by a comparison of AM with RM cells from DSA+ ABMR+ patients, using HOMER for matching T-bet binding motifs [-1000, +150 bps flanking TSS], revealed that 308 DEGs contained
TBX21 motifs around their TSS, with some genes containing multiple sites. Of genes containing TBX21 binding motifs, 176/308 (57.1%) genes were up-regulated in AM cells, while 132/308 (42.9%) were down-regulated. Of note, TBX21-motif-containing DEGs included FCRL3, FCRL5, JCHAIN, MCL1, TCF7 and IL7R, all of which were upregulated in AM cells (not shown). TLM cells upregulated numerous genes associated with B cell exhaustion (LILRB1, LILRB2) and inhibitory function (FCRL3, FCRL5, CD72, FCG2RB, PTPN22) (23), while RM cells expressed genes related to quiescence and central memory state (SELL, CCR7, TCF7) (Figure 3C).

As organ rejection is known to involve the expansion of dominant B cell clones with characteristic IGHV usage (33,34), we examined the differential expression of V\textsubscript{H} germ line (IGHV) genes in RM, AM and TLM subsets in DSA+ABMR+ patients. Strikingly, AM and TLM subsets from DSA+ABMR+ patients significantly differed from the DSA+ABMR- and DSA- groups (Figure S7A-B) by the selective expression of specific IGHV genes, reflecting a distinct IGHV gene usage (Table 1, Table S6). Notably, the elevated expression of the IGHV3-7, IGHV3-15 and IGHV3-74 genes was selective to AM subset in DSA+ABMR+ patients, while IGHV3-23 was also elevated in RM and TLM subsets, albeit to a lesser extent (Figure 3E). In addition to differential IGHV usage, TLM cells from DSA+ABMR+ patients were enriched for ZBTB32, DOK3 and FCRL4 (Figure S7B) reflecting a more pronounced inhibitory state as compared to TLM cells from DSA+ABMR- and DSA- groups. Thus, the transcriptional state of AM cells in ABMR patients is readily distinguishable from that of their TLM and RM counterparts and suggests that many of these cells are poised to undergo differentiation into plasma cells in response to IL-21 signaling. Furthermore, the increased expression of selective IGHV genes within the AM compartment suggests expansion of dominant clones associated with ABMR.
**AM cells can be induced by CD40L and IL-21 stimulation in vitro**

Given that AM cells in DSA+ABMR+ patients display upregulated CD40 and IL-21R, we tested if CD40L and IL-21, that are T<sub>FH</sub>-derived signals, could promote their generation in vitro. Naive B cells from HCs were isolated (Figure S6) and stimulated with a combination of signals including anti-IgM for five days (Figure S8A). While anti-IgM and soluble CD40L (sCD40L), resulted in increased levels of T-bet in naive B cells, IL-21 was required for its maximum expression (Figure S8B). The combination of IL-21 with anti-IgM and sCD40L resulted in higher induction of CD27<sup>-</sup> among T-bet<sup+</sup> cells (Figure S8C). IL-21 was also necessary for optimal activation of B cells (CD71<sup+</sup>), and their maximal expression of CD11c and IL-21R. These T-bet<sup+</sup>CD27<sup+</sup>CD11c<sup+</sup>IL-21R<sup+</sup> cells closely resembled AM cells present in blood of DSA+ABMR+ patients (Figure S8D). The ability to induce T-bet expression was not unique to IL-21, as IFN-g was also able to do so (Figure S9A-B). Notably, however, when combined with anti-IgM and sCD40L, IFN-g preferentially induced T-bet<sup+</sup> cells that were CD27<sup+</sup>, thus resembling TLM cells (Figure S9C). Unlike IL-4 and IL-17, IFN-g could also induce expression of CD11c and IL-21R, albeit at lesser extent than IL-21 (Figure S9D). Thus, compared to IFN-g, IL-21 is a stronger inducer of AM-like cells in the presence of CD40-mediated stimulation. These data, along with the forementioned analyses and our earlier study (6), suggest that the AM cells detected in DSA+ABMR+ patients likely arise from coordinated interactions with cognate T<sub>FH</sub> cells in the context of allograft responses.

**AM cells are responsive to cT<sub>FH</sub> cells and differentiate into DSA-producing plasma cells in an IL-21 dependent manner**

To directly test the above hypothesis, we analyzed the effect of autologous T<sub>FH</sub> cells in promoting the differentiation of AM cells into plasma cells that secrete DSAs. This involved use of a 6-day co-culture system with sorted circulating T<sub>FH</sub> (cT<sub>FH</sub>) and MBC subsets from
individual patients and stimulating the former with SEB. We note that AM cells did not differentiate into plasma cells in the absence of cTFH cells or SEB in co-cultures (data not shown). AM cells from DSA+ABMR+ patients manifested enhanced activation (CD71+) and more pronounced differentiation into plasma cells as compared to those from DSA- or DSA+ABMR- patients and HCs (Figure 4A-4B). Accordingly, the amounts of total IgG produced by AM cells were substantially higher in DSA+ABMR+ patients (Figure 4C). Importantly, antigen-specific IgG (DSAs) were specifically detected only in co-cultures of AMs with cTFH cells in these patients, but not in DSA+ABMR- group (Figure 4D). This indicates an enrichment for donor antigen-specific cells in AM cells from DSA+ABMR+ patients as compared to those of DSA+ABMR- group. The production of IgGs and DSAs by AM cells was regulated by IL-21 signaling, as the levels of T_{FH}-derived IL-21 in the co-cultures was increased in DSA+ABMR+ patients and conversely the inhibition of IL-21 signaling resulted in significant reduction of secreted IgGs including IgG3 subclass, and DSAs (Figure 4E-4H). We note that TLM cells failed to differentiate into plasma cells and generate DSAs. Thus, AM cells from DSA+ABMR+ patients manifest increased capacities to undergo activation and differentiation into plasma cells through their interactions with T_{FH} cells in an IL-21 dependent manner. Furthermore, they contain alloreactive clones which when activated differentiate into plasma cells that secrete DSAs.

Magnitude and dynamics of AM cell expansion correlate with ABMR manifestations and timing

We next evaluated whether the frequencies of AM cells correlated with the amplitude of blood cTFH, plasmablast and DSA responses. AM frequencies significantly correlated with those of activated Ki67+ICOS+ cTFH and plasmablasts (Figure S10A-B). Also, higher DSA levels in serum samples paralleled increased AM frequencies, further suggesting that expansion of these
cells reflect the strength of the donor-specific response in vivo (Figure S10C). Furthermore, AM frequencies significantly correlated with IgG3 DSA levels in DSA+ABMR+ patients (Figure S10D). These correlations between AM cells with cT\textsubscript{FH}, plasmablasts and DSAs were stronger for T-bet\textsuperscript{+} IL-21R\textsuperscript{+} cells as compared to the T-bet\textsuperscript{-} IL-21R\textsuperscript{+} AM subset (Figure 5A-5D). Importantly, DSA+ABMR+ patients with high T-bet\textsuperscript{+} IL-21R\textsuperscript{+} AM frequencies (>1.79\%) also manifested more microvascular inflammation and intimal arteritis lesions (Figure 5E).

We next evaluated the relationship between the dynamics of the AM cell response and the timing to ABMR onset post-transplant. We found that patients with late (after 3-months post-transplant) DSA+ABMR+ displayed significantly more T-bet\textsuperscript{+}IL-21R\textsuperscript{+} AM cells than those with early (before 3-months) occurring DSA+ABMR+ (Figure S11A). Notably, T-bet\textsuperscript{+}IL-21R\textsuperscript{+}AM cells were predominantly IgD\textsuperscript{+} in late DSA+ABMR+ group, while being mostly IgD\textsuperscript{-} in the early forms (Figure S11B). We next performed longitudinal analyses of samples collected pre-transplant, and at 1, 3, 6, 12 months post-transplant. Both IgD\textsuperscript{-} and IgD\textsuperscript{+} T-bet\textsuperscript{+}IL-21R\textsuperscript{+} AM cells were present at low frequencies pre-transplant and were induced post-transplant (Figure S11C). Importantly, the dynamics of IgD\textsuperscript{+} cell expansion paralleled with late onset of DSA+ABMR+, while that of IgD\textsuperscript{-} T-bet\textsuperscript{-}IL-21R\textsuperscript{+} AM cells coincided with early DSA+ABMR+ occurrence. Consistent with this timing, IgD\textsuperscript{-} cells contained more proliferating (Ki67\textsuperscript{+}) cells than IgD\textsuperscript{+} T-bet\textsuperscript{-}IL-21R\textsuperscript{-} AM cells (Figure S11D). Clustering analyses confirmed the higher frequencies of the IgD\textsuperscript{+} T-bet\textsuperscript{+} IL-21R\textsuperscript{+} cells (cluster 11) in late DSA+ABMR+, compared to predominance of the IgD\textsuperscript{-} T-bet\textsuperscript{+/-} IL-21R\textsuperscript{+/-} cells (cluster 4 and 7) in early forms (Figure S12A-B). We note that the three DSA+ABMR+ patients with pure ABMR displayed similar distribution of cluster 4, 7 and 11 than the other 17 patients with mixed ABMR (Figure S12A). Thus, the temporal dynamics of expansion of the AM subset, enriched in T-bet\textsuperscript{-}IL-21R\textsuperscript{+} cells, coincided with the timing of ABMR onset post-transplant and their increased frequencies were associated with the severity of ABMR manifestations.
**AM cells and their restricted IGHV sequences are detected within kidney allografts of patients with ABMR**

Using multiplex immunofluorescence staining, we investigated whether AM cells could be detected within kidney allografts of patients. We detected the presence of CD20+ CD27+ T-bet+ cells, consistent with an AM phenotype, within the interstitial inflammatory infiltrate of allografts from patients with acute (Figure 6A) or chronic (Figure S13) forms of DSA+ABMR+, and these cells represented 1 ± 0.8 cells per mm² of tissue. We did not detect any of these cells in allografts from DSA+ABMR- nor DSA- patients (Figure S13). We next determined whether the AM-specific molecular signatures, including select IGHV genes, previously defined in circulating AM cells were also found within kidney allografts. We therefore recruited 21 additional kidney transplant patients from our Transplant Institute with available allograft biopsy samples (Table S7) and performed RNA-seq analysis on these samples (DSA- N=7, DSA+ABMR- N=2, DSA+ABMR+ N=12). The RNA-seq profile of allografts from DSA+ABMR+ patients markedly differed from that of DSA+ABMR- and DSA- groups (Figure S14A). This distinct transcriptional profile was due to the increased expression of the hallmark molecular features of AM cells TBX21 (T-bet), MS4A1 (CD20), CD27 and IL21R in DSA+ABMR+ patients compared to the other patient groups (Figure S14B). Additionally, 6 of the 9 AM-specific IGHV genes, including IGHV3-7, IGHV3-15 and IGHV3-74, previously found increased in circulating AM cells (Figure 3E), were also significantly upregulated in allografts from DSA+ABMR+ patients (Figure 6B). We note that IGHV3-23 and 2 of the 3 TLM-specific IGHV (IGHV1-69, IGHV3-11) genes were also upregulated in allografts of these patients as compared to DSA- group (Table 1). Thus, circulating AM cells and their molecular signatures could be detected within allografts of patients undergoing ABMR.
Discussion

The phenotypic states and functional roles of T-bet-expressing, antigen-experienced B cells have previously been analyzed in multiple clinical settings with a focus on their protective responses in viral and bacterial infections and their pathogenic potential in autoimmune diseases but not in organ transplantation (35,36). This study uncovers the emergence of these cells in the context of pathogenic alloimmune responses directed against organ transplants. As noted in other disease settings in which T-bet-expressing B cell responses have been analyzed, alloimmunity in organ transplantation is dominated by a sustained type-1 (IFN-γ) and TFH (IL-21) cell driven inflammatory environment (27,37). Consistent with studies in other disease contexts, the T-bet-expressing B cells in ABMR patients were heterogeneous and comprised of expanded AM as well as TLM cells. However, our extensive phenotypic, molecular and functional analyses strongly suggest that the T-bet-expressing AM cells, unlike TLM cells, are the source of pathogenic alloreactive humoral responses in ABMR.

We provide the following lines of evidence that T-bet-expressing AM B cells are pathogenic drivers in patients undergoing ABMR and can be functionally distinguished from their TLM cell counterparts: (i) AM cells express higher levels of the B cell costimulatory receptors IL-21R and CD40, (ii) manifest increased expression of IRF4 and Blimp1 that are required for plasma cell differentiation, (iii) are proliferating and display amplification of restricted IGHV sequences consistent with their clonal expansion in vivo during ABMR, (iv) AM cells from ABMR patients preferentially differentiate into plasma cells when co-cultured with autologous TFH cells in an IL-21 dependent manner, (v) uniquely generate DSAs, (vi) temporal dynamics of expansion of AM cells coincide with early ABMR involving pre-formed DSAs and with late ABMR associated with de novo generated DSAs, (vii) AM cell frequency is correlated with the pathogenic IgG3 DSA isotype involved in ABMR and is predictive of
the severity of histological lesions, and (viii) AM cells were detected within kidney allografts of ABMR patients, along with their characteristic amplified IGHV sequences.

While AM and TLM cells share the expression of T-bet and IL-21R, fundamental differences exist in their differentiation states and effector functions. AM cells are heterogeneous, preferentially express the key costimulatory receptor CD27, which is acquired through germinal center (GC) responses (38,39). It is therefore likely that majority of AM cells represent recent GC emigrants. Moreover, most of T-bet+ AM cells from early ABMR (sensitized patients) lack IgD suggestive of switched memory B cells, while T-bet+ AM cells from late ABMR (de novo DSA patients) display elevated frequencies of unswitched IgD+ memory B cells that likely reflect an earlier stage in the alloreactive humoral response. Notably, AM cells appear to be poised for plasma cell differentiation (14). Our RNA-seq and flow cytometry analyses suggest a molecular basis for the poised effector state of AM cells, as they express higher levels of the transcription factors IRF4, and Blimp1, which are required for plasma differentiation (32, 40-41) as well as increased IL6R, MZB1 and XBP1, three key genes modulating plasma cell differentiation (14,42). Analysis of putative promoter/enhancer regions from AM cells of DSA+ABMR+ patients suggests that Tbet (TBX21) directly binds to promoter regions of a large set of genes in AM cells and either activates or represses their transcription. We note that AM cells express low levels of CD38 and high levels of CD20 thereby supporting our overall conclusion that these cells represent plasma cell precursors in the context of pathogenic alloimmune responses. Therefore, concomitant targeting of the AM cell compartment and their plasma cell progeny could provide an optimal means of dampening the humoral response during ABMR.

Strikingly, AM cells were selectively enriched for IgH transcripts containing VH germ line sequences that have been previously documented to predominate during organ rejection including IGHV3-7, IGHV3-15, IGHV3-74 and IGHV3-23 (33, 43-44). This specific IGHV
gene usage likely reflects responses to a common pool of highly immunogenic alloantigens, and potentially auto- and recall-antigens. These could induce the emergence of dominant B cell clones of varying specificities that may drive organ rejection. While B cell clones with dominant *IGHV*3-7, *IGHV*3-15, *IGHV*3-74 and *IGHV*3-23 germ line genes were previously detected in blood of patients with organ rejection, our study reveals that these clones appear to be differentially distributed among B cells subsets. The *IGHV*3-23 gene, which is documented across several studies (33, 43-45), involved in superantigen recognition, was found to be expressed by the three MBC subsets (RM, AM and TLM cells). In contrast the *IGHV*3-7, *IGHV*3-15 and *IGHV*3-74 genes were unique to AM cells. Importantly, our study is the first to document a memory subset (AM)-specific V<sub>H</sub> gene usage, in circulating and allograft infiltrating B cells in patients undergoing ABMR. It is likely that these infiltrating AM cells bearing pathogenic *IGHV* genes may participate to the DSA production *in situ* in kidney allografts. In contrast, AM cells from DSA+ABMR- patients, which lacked these pathogenic *IGHV* genes were less likely to produce DSAs in vitro when stimulated with cT<sub>FH</sub> cells. Thus, this strongly suggests the deleterious role of specific B cell clones within the AM cell compartment during ABMR and suggest their value for immune monitoring as predictive biomarkers for ABMR. However, it remains to be resolved whether AM cell function and pathogenic potential are similar according to the timing (early vs late) of ABMR occurrence and its clinical presentation (pure versus mixed ABMR).

TLM cells in ABMR patients were substantially different from their AM cell counterparts, as they displayed lower levels of the costimulatory molecules CD27 and CD40, and were hyporesponsive to T<sub>FH</sub>-cell help. TLM cells have been recently shown to be localized in the extrafollicular zone of lymph nodes and virtually absent from the lymphatic circulation (26). It is tempting to speculate that TLM cells are mainly generated through the extrafollicular pathway, as they have been shown to display poor affinity maturation, to generate short-lived
plasma cells and to strongly respond to TLR stimulation and IFN-γ, a cytokine mainly found outside of GCs (46). On the other hand, the common IGHV3-23 gene usage between AM and TLM cells and the T-bethi expression of TLM cells compared to the T-betint status of AM cells, suggests that these two subsets may be partly clonally related and that TLM cells may arise from further differentiation of AM cells under persistent antigenic stimulation. As with exhausted CD8 T cells, these exhausted-like B cells lose classical markers of effectors cells and acquire an inhibitory expression profile, including CD72 and the inhibitory IgG receptors CD32b and FcRL5 that are associated with impaired effector function (47).

One of the unexpected findings of this study was the expansion of TLM cells during ABMR, coincident with AM cells. Given their functional properties, TLM cells would be expected to be generated at low levels during a highly efficient antibody response such as in ABMR. A likely explanation could be that these cells arise concomitantly to counterbalance the hyperactivation state of their AM counterparts in the context of chronic activation. Indeed, TLM cells during HIV infection were reported to be involved in a regulatory loop triggered by the binding of circulating IgG3 and C1q (also present in excess during ABMR) onto their surface IgM, which conveyed a strong BCR-mediated inhibitory signal to these cells (48).

IL-21 is the principal cytokine of TFH cells and is a major regulator of B cell-mediated immunity. It induces potent B cell activation, drives their differentiation into plasma cells and favors the generation of class-switched antibodies of IgG3 isotype (49,50). We and others have shown that IL-21 is a major cytokine produced by donor-specific cTFH cells and that increased IL-21 production was predictive of ABMR (6, 27-28, 51). Additionally, IL-21R blockade resulted in reduced plasma cell differentiation in vitro in a coculture model of TFH-B cells stimulated with donor antigen, and it could delay skin allograft rejection in vivo in mice (52,53). Here, we show that AM cells specifically upregulated IL-21R in ABMR and that IL-21 was required for the differentiation of these cells into plasma cells that secrete DSAs.
Importantly, IL-21R along with T-bet, were induced on naive B cells upon their activation by IL-21 and CD40 stimulation. Thus, in concert with our earlier study demonstrating the emergence of cT\(\text{FH}\) cells and increased IL-21 production in patients undergoing ABMR (6), we now delineate a pivotal role for AM B cells in promoting the pathogenic humoral responses. In so doing, we propose alloreactive AM B cells and the IL-21 pathway as novel therapeutic targets to promote the durability of allografts in organ transplantation.
Methods

Study design

This study was performed on samples from patients who underwent kidney transplantation between January 2013 and December 2017 at University of Pittsburgh Medical Center and who were recruited to participate in a biorepository initiative at Thomas E. Starzl Transplantation Institute (STI). All patients signed a written informed consent (IRB no. PRO12030552; PRO17020318).

A total of 530 patients were screened for the following immunological events: presence of post-transplant DSA and biopsy-proven ABMR. We identified 48 patients who developed DSA in the first 24 months post-transplant and had available PBMCs, defining two study groups: patients with ABMR (DSA+ABMR+, N=20) and patients without ABMR (DSA+ABMR-, N=28). Forty-eight age- and gender-matched patients with no DSA or ABMR (DSA-) in the first 24 months post-transplant were selected to form the third study group. In addition, 17 age- and gender-matched healthy controls (HC) from the STI Human Immunology Program were also enrolled. The flow chart of the study design is depicted in Figure S1A. Clinical data of the study patients were extracted from the prospective database of the STI biorepository.

Blood samples

PBMCs and sera were prospectively collected and banked at pre-transplant, 1-, 3-, 6-, 12- and 24-month post-transplant and at the time of clinically indicated kidney allograft biopsies. The presence of DSAs in sera was systematically assessed at these time points. Surveillance protocol allograft biopsies were performed at 3- and 12-month post-transplant (Figure S1B). We analyzed cross-sectional PBMCs and serum samples banked from the blood collected at
the time of the immunological events of interest: (i) detection of post-transplant DSA for DSA+ABMR- patients and (ii) detection of ABMR in presence of DSA for DSA+ABMR+ patients. In DSA- patients, the blood samples were analyzed at the time of a protocol biopsy and their time points were matched with those from DSA+ABMR- and DSA+ABMR+ patients (Figure S1B, Table S2). Patients for whom cryopreserved PBMC samples were available at time points considered (pre-transplant, 1-, 3-, 6- and 12-month post-transplant) were included in the longitudinal flow cytometry analysis in Figure S11C.

**Detection and characterization of DSAs**

The presence of anti-HLA antibodies with known reactivity against the donor HLA molecules (DSAs) in sera was systematically assessed at indicated time points described above. Pan-IgG anti-HLA -A, -B, -C, -DRB1/3/4/5, -DQB1, -DQA1, and -DPB1 DSAs were assessed in sera using single-antigen flow bead assays (SAB) (One Lambda Thermo Fisher) on a Luminex platform (Luminex Corp.) according to manufacturer’s protocol. DSA-positive sera were tested for the presence of C1q-binding using the C1q-modified SAB assay (One Lambda Thermo Fisher) and IgG subclasses were tested using the SAB assay, substituting PE-conjugated anti-human IgG1, IgG2, IgG3 and IgG4 secondary antibodies for anti-human IgG. Normalized mean fluorescence intensity (MFI) cut-offs for positive results were MFI >1000 for the pan-IgG and IgG subclass assays, and MFI >500 for the C1q assays (5).

**Kidney allograft histology**

Kidney allograft tissues were fixed in formalin and stained with Masson’s trichrome and periodic acid–Schiff. Allograft biopsies were scored and graded from 0 to 3, and diagnosis of ABMR was histologically defined using the international Banff 2017 criteria and was reviewed by an expert clinical transplant pathologist. Lesions of T-cell mediated rejection (TCMR) were
also defined according to Banff criteria (54). DSA- and DSA+ABMR- patients did not show signs of ABMR or TCMR at the time of sampling. All 20 ABMR cases were acute and C4d-positive, with the exception of one case of chronic active ABMR (cg>0) and one case of C4d-negative ABMR.

**Spectral flow cytometry**

Spectral flow cytometry is a fluorochrome-based system that allows analysis of protein expression of more than 20 parameters simultaneously with single-cell resolution and minimal signal overlap between channels. Full details of the antibodies used are given in Table S8. Briefly, 1-2 million PBMCs were thawed and incubated with a mixture of antibodies diluted in 75% phosphate buffered saline (PBS) and 25% Brilliant Violet Buffer (BD Biosciences) for 30 min at 4°C. Cells were surface-stained in Fc receptor blocking media (10% fetal calf serum (FCS) PBS). Then PBMCs were washed, fixed and permeabilized with fixation/permeabilization buffer (eBioscience) for 40 min at 4°C, washed with permeabilization buffer (eBioscience) and incubated in the dark for 30 min at 4°C with intracellular antibodies, washed before acquisition on Aurora spectral flow cytometer (Cytek).

**High-dimensional flow cytometry data analysis**

The flow cytometry data were first curated with FlowJo software (Tree Star) to exclude debris, dead cells and doublets, and MBCs were identified by gating for further downstream analyses. Single-cell data were normalized and analyzed simultaneously using Cytobank software (55). T-distributed stochastic neighbor embedding (t-SNE) analysis makes a pairwise comparison of cellular phenotypes to optimally plot similar cells close to each other and reduces multiple parameters into two dimensions (t-SNE X, t-SNE Y) (56). Data from Flow Cytometry Standard (FCS) files were normalized, downsampled then concatenated to create t-SNE maps. To run t-
SNE algorithm, we applied the following settings: 3,000 iterations, perplexity of 30 and theta of 0.5. Cell clusters were determined by the spanning-tree progression analysis of density-normalized events (SPADE) (57) algorithm using 12 as target number of nodes without downsampling events. The cell clusters identified by SPADE were overlaid on the consensus t-SNE maps for visualization and a heatmap was generated to delineate specific phenotypic patterns.

**Cell sorting**

PBMCs were thawed, stained and sorted on a BD Biosciences FACSaria II cytometer. Naive B cells were sorted as CD3−CD19−CD38loCD27−CD21+IgD+, RM cells as CD3−CD19−CD38loCD27−CD21+, AM cells as CD3−CD19−CD38loCD27−CD21+, TLM cells as CD3−CD19−CD38loCD27−CD21+ and cT<sub>FH</sub> as CD19−CD3+CD4+CD45RO+CXCR5+ cells.

**Co-cultures**

Sorted cT<sub>FH</sub> were co-cultured with sorted autologous RM, AM or TLM cells (2x10⁴) at 1:1 ratio with staphylococcal enterotoxin B (SEB) (1µg/ml, Toxin technology) in RPMI (Gibco) supplemented with 10% FCS, 100IU/ml penicillin, 100mg/ml streptomycin (Life Technologies), 1M HEPES buffer (Corning) and L-glutamine. Some co-cultures were supplemented with mouse IL-21R-Fc (R&amp;D systems) or isotype-matched control (R&amp;D systems). After 6 days of co-culture, cells were stained with CD4, CD19, CD27, CD38 and CD71 antibodies before acquisition on cytometer. DSAs were detected in supernatants after 6 days of co-culture using Luminex SAB assay.

**IgG ELISA**
Total IgG production was measured in co-culture supernatants using Human IgG total ELISA kit (eBioscience) and IgG3 production was measured with IgG Subclass Human ELISA kit (eBioscience).

**IL-21 assay**

Supernatants from co-cultures were collected and analyzed for the presence of IL-21 by Cytometric Bead Array (BD Biosciences) according to the manufacturer's protocol. All events were acquired using a Fortessa (BD Biosciences) cytometer.

**B cell activation**

Sorted naive B cells (1x10^5 in 200μl per well) were plated and activated with goat anti-human IgM F(ab’)2 (10 μg/ml, Jackson ImmunoResearch), recombinant human CD40L (500 ng/ml, Enzo life sciences), IL-21 (100 ng/ml, Gibco), IFN-g (50 ng/ml, R&D systems), IL-4 (25 ng/ml, R&D systems) and IL-17 (50ng/ml, R&D systems). Cells were cultured in complete RPMI and incubated at 37°C for 5 days. Cells were harvested stained with CD19, CD38, T-bet, CD27, CD11c, IL-21R and CD71 antibodies before acquisition on cytometer.

**RNA-seq**

Total RNA was extracted from MBC subsets (RM, AM and TLM cells) and formalin-fixed paraffin-embedded (FFPE) sections of kidney allograft biopsies. RNA was isolated using miRNeasy Mini Kit (Qiagen). cDNA synthesis and amplification were performed with SMARTer Stranded Total RNA-Seq Kit v2 – Pico Input Mammalian (Takara). Libraries were sequenced on an Illumina NextSeq 500 using 75-bp paired-end reads. The paired-end reads were checked for quality and adapters using FastQC (v0.11.7). These quality trimmed reads were later mapped against the Ensembl human reference genome (GRCh38 version 91) using
HISAT2 mapper (v2.1.0). Counts for genes were generated using HT-Seq (v0.11.2) on the mapped files. Bioconductor R (v3.8) package EdgeR (v3.24.1) was used to analyze differential gene expression.

Gene ontology (GO) analyses were performed on DEGs between RM, AM and TLM cells from DSA+ABMR+ patients (with P-values ≤ 0.054), using topGO (v2.34.0) in R using the human ENSEMBL database library EnsDb.Hsapiens.v86 (v2.99.0). The classic Fisher’s test with elimination algorithm using default P-value < 0.01 from topGO was applied to DEG sets to conditionally enrich for leaf/terminal nodes.

**Data sharing information**

GEO accession number for RNA-seq: GSE155670.

**Multiplex immunofluorescence of kidney allografts**

FFPE blocks of kidney allograft biopsies from transplant patients were cut (4µm), underwent sequential rehydratation and antigen retrieval in citrate PH6 solution buffer. The sections were incubated overnight with anti-CD20 (mouse, M0755, Dako), anti-CD27 (armenian hamster, ab219779, Abcam) and anti-T-bet (rabbit, ab150440, Abcam) antibodies. After washing, the sections were incubated 30min with secondary anti-mouse (AF488), anti-armenian hamster (AF568) and anti-rabbit (Cy5) antibodies. Nuclei were counterstained with DAPI and slides were mounted using prolong media (Thermo Fisher). Sections were also stained with hematoxylin and eosin for histological evaluation. Each experiment was performed concomitantly with a positive control (section from kidney allograft removed due to incurable ABMR or human spleen) and a negative control (section from kidney allograft incubated with secondary antibody without primary antibody). Images were acquired on a Zeiss Axio Scan
instrument. We used the number of CD20+ CD27+ T-bet+ (triple-positive) cells per ten consecutive high-power fields for cell quantification.

Statistics

Mean ± SD or SEM values and frequencies are provided for the description of the continuous and categorical variables, respectively. The means and proportions were compared using t-test and chi-squared test (or the Mann–Whitney U test and Fisher’s exact test if appropriate, respectively). Multiple groups were analyzed by Kruskal-Wallis test or one-way ANOVA with Tukey post-hoc test for adjustment for multiple comparisons. Values of P<0.05 were considered statistically significant, and all tests were two-sided. Analyses were performed using GraphPad Prism version 8, Cytobank (http://www.cytobank.org), Partek Flow (http://www.partek.com/partek-flow/) and R software (R Development Core Team, Vienna, Austria).

Study approval

The study protocol was approved by the University of Pittsburgh institutional review board (IRB no. PRO12030552; PRO17020318). Subjects provided written informed consent prior to inclusion in the study.
Author contributions

KL and DM designed the study; KL, EB, CM and XG performed the experiments; KL, EB, LL, AC, BR, UC, GC, DS and HS analyzed the data; CM and EB provided clinical data; PR provided the pathology data; AZ provided the tissue typing data and analyzed the DSA results; KL, DM, HS and CL drafted and revised the paper. All authors approved the final version of the manuscript.
Acknowledgments

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References


45. Lai L, et al. Composition and diversity analysis of the B-cell receptor immunoglobulin heavy chain complementarity-determining region 3 repertoire in patients with acute


Figures and figure legends
Figure 1. High-dimensional flow cytometry analyses of MBCs in kidney transplant patients.
(A) t-SNE projections were generated using a concatenated file of N=79,200 memory B cells (MBCs) from HC (N=4), DSA- (N=20), DSA+ABMR- (N=20) and DSA+ABMR+ (N=20) patients; panels display expression levels of indicated markers (MFI). (B) t-SNE projections of MBC densities in the four groups using N=19,800 cells from each group, shown in panel A. (C) t-SNE map overlaid with 12 MBC clusters delineated by SPADE clustering of the concatenated file, as in panel A. (D) Heatmap showing the expression of markers for each MBC cluster according to transformed MFI ratio. (E) Stacked bar plot showing MBC cluster distribution based on SPADE clustering as in panel C. Clusters 3, 4, 6, 7, 9, 11 and 12 are significantly different in their proportions across the indicated groups. Kruskal-Wallis with Dunn’s post-test. (F) Representative examples of flow cytometry analysis and dot plot of percentages of CD21^T-bet^+ cells in CD38^{lo} B cells are displayed; HC (N=17), DSA- (N=48), DSA+ABMR- (N=28) and DSA+ABMR+ (N=20) patients. Kruskal-Wallis with Dunn’s post-test. **P < 0.01; ***P < 0.001; ****P < 0.0001. Each dot represents one subject and horizontal lines are mean values ± SEM.
Figure 2. Identification of three distinct MBC subsets and analysis of their activation and inhibitory receptors by flow cytometry
(A) Representative examples of flow cytometry analysis and dot plots of percentages of resting memory (CD27⁺CD21⁺, RM), activated memory (CD27⁺CD21⁻, AM) and tissue-like memory (CD27⁺CD21⁻, TLM) subsets in CD38lo B cells are displayed; HC (N=17), DSA- (N=48), DSA+ABMR- (N=28) and DSA+ABMR+ (N=20) patients. (B) Representative examples of flow cytometry analysis of T-bet and IL-21R in RM, AM and TLM subsets are displayed. (C) Representative examples of flow cytometry histograms and bar plots of percentages of IL-21R⁺ in RM, AM and TLM subsets are displayed; sample size as in panel A. Percentages of CD40⁺, CD72⁺, FcRL5⁺ cells and MFI values of CD32b expression in RM, AM and TLM subsets are displayed; HC (N=9), DSA- (N=12), DSA+ABMR- (N=4) and DSA+ABMR+ (N=3) patients. Kruskal-Wallis with Dunn’s post-test for panel A and C. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Each dot represents one subject and horizontal lines are mean values ± SEM.
Figure 3. Transcriptional profiling of MBC subsets

RNA-seq analysis of the three sorted MBC subsets was performed in three patients per group; DSA- (N=3), DSA+ABMR- (N=3) and DSA+ABMR+ (N=3). GO analysis of differentially
expressed genes (DEGs) in AM versus RM (A), or TLM versus RM subset (B) from DSA+ABMR+ group (N=3). (C) Heatmap generated by hierarchical clustering of selected genes in AM, RM and TLM subsets from DSA+ABMR+ group (N=3). (D) Representative examples of flow cytometry histograms and bar plots of MFI values of IRF4 and Blimp1 expression in naive (CD27\textsuperscript{−}CD21\textsuperscript{+}IgD\textsuperscript{+}), RM, AM, TLM and plasmablast (CD24\textsuperscript{−}CD38\textsuperscript{hi}) subsets from DSA+ABMR+ group (N=20). Repeated measures one-way ANOVA with Tukey correction. *P < 0.05; ***P < 0.001. Each dot represents one subject and horizontal lines of bars are mean values ± SEM. (E) Violin plots showing the expression levels of selected V\textsubscript{H} germ line genes in AM, RM and TLM subsets from indicated patient groups; DSA- (N=3), DSA+ABMR- (N=3) and DSA+ABMR+ (N=3). CPM, counts per million. Kruskal-Wallis with Dunn’s post-test. *P < 0.05; **P < 0.01; ***P < 0.001. Each dot represents one subject and horizontal lines are median values ± SEM.
Figure 4. Analysis of co-cultures of MBC subsets with autologous cT_{FH} cells

Co-culture of the sorted MBC subsets with autologous cT_{FH} (CD3^+CD4^+CD45RO^+CXCR5^+) cells in presence of SEB (6 days). (A) Representative examples of flow cytometry histograms
and bar plots of percentages of CD71^+ cells in RM, AM and TLM co-cultures after 6 days are displayed; HC (N=3), DSA- (N=3), DSA+ABMR- (N=3) and DSA+ABMR+ (N=6) patients. **(B)** Representative examples of flow cytometry histograms and bar plots of percentages of CD27^+CD38^+ plasma cells in RM, AM and TLM co-cultures after 6 days are displayed; HC (N=4), DSA- (N=3), DSA+ABMR- (N=3) and DSA+ABMR+ (N=7) patients. **(C)** ELISA analysis of total IgG in supernatants of RM, AM and TLM co-cultures after 6 days is displayed; HC (N=4), DSA- (N=3), DSA+ABMR- (N=3) and DSA+ABMR+ (N=7) patients. **(D)** Luminex analysis of DSAs in supernatants of RM, AM and TLM co-cultures after 6 days is displayed; HC (N=4), DSA- (N=3), DSA+ABMR- (N=3) and DSA+ABMR+ (N=7) patients. **(E)** Cytometric bead array analysis of IL-21 in supernatants of AM co-cultures after 6 days is displayed; HC (N=3), DSA- (N=3), DSA+ABMR- (N=3) and DSA+ABMR+ (N=5) patients. IL-21R-Fc or isotype-matched control were added to AM co-cultures of DSA+ABMR+ (N=7) patients. Total IgG (F), IgG3 (G) and DSA (H) in supernatants of AM co-cultures were measured after 6 days. Kruskal-Wallis with Dunn’s post-test for panel A, B, C, D and E. Wilcoxon matched-pairs signed rank test for panel F, G and H. *P < 0.05; **P < 0.01. Each dot represents one subject and horizontal lines of bars are mean values ± SEM.
Figure 5. Correlation of frequencies of AM subsets with disease manifestations of ABMR

Spearman correlation analysis of percentages of T-bet^IL-21^ AM and T-bet^IL-21^AM subsets with percentages of blood (A) Ki67^ICOS^cTfh (CD3^CD4^CD45RO^CXCR5^) cells, (B) plasmablasts (CD19^CD24^CD38^hi) and (C) DSA MFI levels measured in serum by Luminex are displayed; HC (N=17), DSA- (N=48), DSA+ABMR- (N=28) and DSA+ABMR+ (N=20). (D) Heatmap showing Spearman correlation coefficients of percentages of T-bet^IL-21^ AM and T-bet^IL-21^AM subsets with MFI levels of: class I, class II, sum of class I plus II, C1q-binding and IgG subclasses of DSAs measured in serum from DSA+ABMR+ patients, by Luminex. Bold squares indicate correlations with P<0.05. DSA class I and II analyses were performed for N=20, DSA IgG subclass analysis was performed for N=18 and DSA C1q-
binding analysis was performed for N=19 patients. (E) DSA+ABMR+ patients were stratified into two subgroups based on the median percentage of T-bet^IL-21^AM cells <1.79% (low) and >1.79% (high) in the DSA+ABMR+ group. Histologic Banff scores of kidney allograft lesions were evaluated at the time of ABMR; microvascular inflammation = g^+ptc Banff score and intimal arteritis = v Banff score. Mann-Whitney U test. *P < 0.05. Each dot represents one subject and horizontal lines of bars are mean values ± SEM.
Figure 6. AM cells and their restricted IGHV sequences within kidney allografts of patients with ABMR

(A) Representative multiplex immunofluorescence staining performed on a kidney allograft biopsy from a patient with acute DSA+ABMR+, at the time of ABMR episode. Arrows indicate CD20⁺ CD27⁺ T-bet⁺ (triple-positive) cells. Scale bars indicate 50µm.

(B) RNA-seq analysis of kidney allograft biopsies was performed; DSA⁻ (N=7), DSA⁺ABMR⁻ (N=2) and DSA⁺ABMR⁺ (N=12). Violin plots showing the expression levels of selected IGHV genes in kidney allograft biopsies. CPM, counts per million. Kruskal-Wallis with Dunn’s post-test. *P < 0.05; **P < 0.01. Each dot represents one subject and horizontal lines are median values ± SEM.
Tables
Table 1. \(V_H\) germ line genes differentially expressed in blood and allografts of DSA+ABMR+ versus DSA- group

<table>
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<th>Gene Symbol</th>
<th>P Value (DSA+ ABMR+ vs DSA-)</th>
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<th>Allograft (N=12 vs N=7)</th>
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P values in bold are those of upregulated genes in DSA+ABMR+ versus DSA- group

*indicates \(IGHV\) genes previously reported to be involved in organ rejection (33, 43)

#indicates P values for downregulated genes in DSA+ABMR+ versus DSA- group