The phenotype of HLA-binding B-cells from sensitised renal transplant recipients correlates with clinically prognostic patterns of IFNγ production against purified HLA proteins

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<th>Journal:</th>
<th>Kidney International</th>
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<tbody>
<tr>
<td>Manuscript ID</td>
<td>KI-10-21-1723.R1</td>
</tr>
<tr>
<td>Article Type:</td>
<td>Clinical Investigation</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>04-Feb-2022</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
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<tr>
<td>Keywords:</td>
<td>chronic allograft nephropathy, inflammation, lymphocytes, transplantation</td>
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<td>Subject Area:</td>
<td>Transplantation</td>
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The phenotype of HLA-binding B cells from sensitised renal transplant recipients correlates with clinically prognostic patterns of IFNγ production against purified HLA proteins

**Methods**

1. Flow cytometric detection of HLA-binding B cells using biotinylated HLA Pure™ proteins, matched to known sensitisation status
2. Anti-HLA IFNγ:CD4+ T cell ELispot assay (matched to Pure™ protein)

**Outcomes**

1. Biotinylated antigen specifically binds to B cells and can be used to detect HLA-binding B cells in sensitised renal transplant recipients
2. B cell subset composition and the presence or absence of Tregs influences IFNγ production as follows:

**Antigen presentation by non-B cells:**
- HLA-binding CD27+ cells at higher frequency than HLA-binding CD27- cells
- HLA-binding CD27+ cells at higher frequency than HLA-binding CD27- cells: suppressible by Tregs

**CONCLUSION**

B cell subset composition, particularly HLA-binding cells, critically influences patterns of in vitro anti-HLA IFNγ response in sensitised patients

338x190mm (300 x 300 DPI)
The phenotype of HLA-binding B-cells from sensitised renal transplant recipients correlates with clinically prognostic patterns of IFNγ production against purified HLA proteins.

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The authors acknowledge that the research was funded/supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy’s and St Thomas’ NHS Foundation Trust and King’s College London. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

This study was funded by an MRC/KRUK Clinical Research Training Fellowship for H. Burton (MR/M01813X/1 // JF1/2015), a project grant from Kidney Research UK (RP3/2011) and generous donations from the GSTT Kidney Patients Association.

Running headline: HLA-binding B-cell phenotypes in transplant recipients
Abstract

B-cells play crucial roles in cell-mediated alloimmune responses. In vitro, B-cells can support or regulate indirect T-cell alloreactivity in response to donor antigens on ELISpot; these patterns associate with clinical outcome. Previous reports of associations between B-cell phenotype and function have examined global phenotypes and responses to polyclonal stimuli. We hypothesised that studying antigen-specific B-cells, using samples from sensitised patients, would inform further study to identify novel targets for intervention.

Using biotinylated HLA proteins, which bind HLA-specific B-cells via the B-cell receptor in a dose-dependent fashion, we report the specific phenotype of HLA-binding B-cells and define how they associate with patterns of anti-HLA response in IFN\(\gamma\) ELISpot. HLA-binding class-switched and IgM+CD27+ memory cells associated strongly with B-dependent IFN\(\gamma\) production and appeared not suppressible by endogenous Tregs. When the predominant HLA-binding phenotype was naïve B-cells, the associated functional ELISpot phenotype was determined by other cells present: high numbers of non-HLA-binding transitional cells associated with B-suppressed IFN\(\gamma\) production, especially if Tregs were present. However, high frequencies of HLA-binding marginal-zone precursors (MZP) associated with B-dependent IFN\(\gamma\) production that appeared suppressible by Tregs. Finally, non-HLA-binding MZP may also be able to suppress IFN\(\gamma\) production, though this association only emerged when Tregs were absent from the ELISpot. These novel data provide a foundation on which to further define the complexities of interactions between HLA-specific T- and B-cells, to identify new targets for intervention in new therapies for chronic rejection.

Keywords: chronic allograft nephropathy, inflammation, lymphocytes, transplantation
**Translational Statement**

Patterns of IFNγ production by CD4+ T-cells on ELISpot correlate with clinical outcomes in sensitised renal transplant recipients. Here we show that different B-cell subsets, including HLA-binding B-cells, correlate with these patterns and interact with regulatory T-cells. This has two important ramifications: i) phenotyping B-cells could yield useful prognostic information as a biomarker for patients’ clinical progress and ii) it could form the basis of identifying new treatments targeting specific B-cell subsets to prevent allograft loss.
Introduction

B-cells play diverse roles in the immune response, including antibody production, antigen presentation & T-cell activation, and an immunomodulatory role, linked to IL-10-producing B-cells in experimental models \(^1\)\(^-\)\(^5\) and in humans to transitional B-cells, as defined by high expression of CD24 and CD38 \(^6\), and more recently to unswitched memory (IgM+CD27+) B-cells.\(^7\)

In transplantation, two landmark studies linked operational tolerance to distinct B-cell transcriptional signatures;\(^8\),\(^9\) tolerant patients had higher frequencies of transitional and naïve cells making more IL-10.\(^10\) In the non-tolerant setting, depleting B-cells at the time of transplantation can be detrimental, \(^11\) higher transitional cell frequencies associate with reduced rates of rejection, better graft function and improved graft survival rates, \(^12\) and patients with chronic rejection have fewer T1 transitional B-cells and an increase in class-switched memory B-cells, \(^13\) associating with poorer graft outcomes,\(^14\),\(^15\) all of which suggests that immunomodulation by B-cells plays an important role in promoting graft survival. However, all studies to date have analysed whole B-cell populations, not alloantigen-specific cells, and functional assays, where performed, used polyclonal, rather than antigen-specific, stimuli.

We have previously demonstrated, in 2 cohorts of renal transplant recipients with biopsy-proven chronic rejection, that B-cells play complex roles in indirect CD4+ T-cell responses to donor antigens in vitro.\(^16\),\(^17\) Using enzyme-linked immunosorbent spot (ELISpot) assays to detect anti-donor interferon-\(\gamma\) (IFN\(\gamma\)) production in response to donor antigens, we showed that in many patients the IFN\(\gamma\) response was dependent on the presence of B-cells, but in others B-cells appeared to be suppressing responses. This regulation associated with polarised cytokine production by B-cells toward IL-10 after polyclonal activation by IgG/IgM and by an increased proportion of both T1 and T2 populations.\(^18\) Importantly, we demonstrated that dynamic changes in anti-donor IFN\(\gamma\) production between B-dependency and B-regulation correlated with rate of reduction in estimated glomerular filtration rate (eGFR).\(^17\),\(^18\)
We therefore hypothesised that it was differences in the phenotype of HLA-specific B-cells that determined these patterns in ELISpot. In this new work, we characterise HLA-binding B-cells (HLA-BC) in sensitised renal transplant recipients and investigate their functional role in HLA-specific indirect alloresponses.
Methods

Source of PBMC

PBMC were obtained from patients enrolled in the UK multicentre RituxiCAN-C4 randomised controlled trial (ClinicalTrials.gov NCT00476164), the full details of which have been published or the Antibody Incompatible Transplantation observational study, approved by the West Midlands-Coventry&Warwickshire Research Ethics Committee (16/WM/0370), conforming to the 1964 Declaration of Helsinki. All participants gave written informed consent before inclusion. RituxiCAN-C4 samples were those not required for analysis of endpoints. Samples from the AIT study were from the first 40 patients enrolled at the Guy’s Renal Transplant Clinic by HB between 2016 and 2017, from whom sufficient PBMC could be extracted and HLA proteins available. All HLA-sensitised patients awaiting a transplant, or previous recipients of HLA-incompatible transplants, that were willing to consent to give up to 16 blood samples for analysis, over a period of up to 10 years, were eligible for recruitment.

PBMC from healthy volunteers were obtained as concentrated leucocytes provided by the NHS blood transfusion service as a by-product of the blood donation process (referred to as “cones”).

Antigen preparation

HLA proteins were purchased (Pure Protein LLC, Texas) in unmodified and biotinylated forms. Proteins were selected according to patients’ known donor-specific antibodies (DSA) or HLA-mismatch in samples without DSA. Proteins chosen for each sample are detailed in supp.tables S1&S2. 9 individual proteins enabled analysis of samples from 21 RituxiCAN-C4 patients and 18 AIT patients. Cytomegalovirus glycoprotein-B (CMV-gpB) (Abcam, UK) was biotinylated in-house using the EZ-Link Sulfo-NHS-Biotinylation kit (Thermo Scientific), as per manufacturer instructions.

Flow cytometry
PBMC were thawed, washed, then incubated with biotinylated antigen (30 min on ice). After washing, cells were stained with titrated amounts of fluorochrome-conjugated monoclonal-Ab (anti-human CD4-FITC, CD8-Qdot®605, CD10-APC, CD14-Pacific Blue, CD19-APCeFluor®780, CD19-PerCPeFluor®710, CD24-FITC, CD25-PE, CD27-PECy7, CD38-BUV395, CD45RB MEM55-PE, IgM-BV605, Streptavidin-BV421) in Brilliant Stain Buffer (BD Bioscience, Oxford, UK) for 30 min (4°C). Antibodies were obtained from eBioscience (San Diego, CA), BD Bioscience, Life Technologies (Paisley, UK) and Biolegend (London, UK). Cells were washed, then incubated with Fixable LIVE/DEAD Aqua-fluorescent reactive dye (Life Technologies) for 30 min (4°C). Cells were washed, fixed (1% paraformaldehyde), washed with PBS-5%FCS and stored (4°C) before acquisition within 24h on an LSRII/Fortessa flow cytometer (KCL BRC Flow Cytometry Laboratory), using FlowJo software (Treestar, Ashland, OR).

For unsupervised analyses, CITRUS was run using equal numbers of CD19+ events per sample and these clustering channels: IgM, IgD, CD27, CD45RB MEM55, CD10, CD24, CD38.

**ELISpot assay**

ELISpot plates (Mabtech, Sweden) precoated with primary IFNγ Ab were blocked for 2 hours with ‘complete medium’ (AIM-V/10% human AB serum, Life Technologies) before addition of 4×10^5 responder PBMC/well in 100 μL of complete medium alone or with unbiotinylated HLA (Pure Protein LLC). PBMC were prepared according to standard laboratory protocols; Dynabeads® (Life Technologies) were used to deplete CD8+ cells from all experiments and CD19+ or CD25+ cells when required. Plates were incubated for 24 hours (37°C) then developed according to manufacturer instructions. In some experiments, 10μg/ml ultra-purified anti-HLA-DR (Biolegend) was added.

**MACS cell separation**
For depletion/addback experiments, MACS® technology (Miltenyi Biotech, Germany) isolated cells with either negative (CD19) or positive (CD27, CD10, CD45RB) selection, as per manufacturer instructions.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism. P-values < 0.05 were considered statistically significant. Wilcoxon matched-pairs signed-rank tests were used for non-parametric paired data; Mann-Whitney tests were used for non-parametric unpaired data.
Results

1 - Biotinylated antigen specifically binds to B-cells and can be used for the detection of antigen-binding cells

To preserve patient samples, we used B-cells from healthy donor “cones” and compared binding of a biotinylated model protein antigen (CMV-gpB), to which a significant proportion of people have been sensitised to binding by bovine serum albumin (BSA) to which most people should be naive.

Figure 1A shows that antigen-binding B-cells were only detected when biotinylated CMV-gpB but not controls were incubated with PBMC, demonstrating that events in the antigen-binding gate were not due to B-cells binding non-specifically to streptavidin, BSA, or biotin directly.

To ensure that antigen was binding B-cells directly, PBMC were incubated with blocking anti-CD32 antibody prior to incubation with biotinylated CMV-gpB. Figure 1B demonstrates that blocking CD32 does not affect the antigen-binding B-cell frequency. Blocking with increasing concentrations of Fab anti-IgG/IgM resulted in decreased CMV-gpB binding, confirming that antigen was specifically binding the B-cell antigen receptor (fig.1C). Together these data indicate that biotinylated protein antigen was specifically binding to immunoglobulin receptors on small proportions of B-cells, consistent with binding by antigen-specific B-cells.

2 - HLA-binding B-cells can be detected in sensitised renal transplant recipients and healthy donors

Using increasing concentrations of biotinylated Pure™ HLA proteins incubated with whole PBMC, we demonstrated dose-dependent binding by B-cells but not by other leukocytes within the non-B-cell gate (fig.1D). Figure 1E depicts HLA-BC frequencies in HLA-sensitised recipients (n=27, 8 at >1 timepoint; total sample n=37), expressed as a percentage of all B-cells. The median frequency was 0.0681%; most patients had <0.2% HLA-BC, but in two samples, 1-2.3% circulating B-cells bound HLA. In comparison, healthy donor PBMC (n=10 samples, from 3 cones), exhibited significantly lower HLA-
BC frequencies (0.005%; median=0.022%). There was no relationship between frequency and median fluorescence intensity of HLA-BC (fig.1F).

3 – Antigen-binding B-cell phenotype correlates with IFNγ production in antigen-specific ELISpot

Compared to previous studies, which used whole membrane preparations as a donor antigen source, here we used single HLA proteins, chosen on a patient-specific basis as above, in IFNγ ELISpots.

Serial samples (n=48) from an initial cohort of sixteen patients were tested (supp.table S1). Supp. table S3 summarises interpretations of ELISpot patterns; assays were performed in triplicate under two conditions per sample: with CD19+ cells present, and with CD19+ cells depleted (depletion effect shown in supp.fig.S1A-C).

Donor-specific responsiveness (DSR: antigen-specific IFNγ production by CD8-depleted PBMC) was demonstrated by 12 samples and was further defined by spot counts after CD19+ depletion as “B-dependent” (counts reduced by ≥20% after B-cell depletion; n=9), “B-suppressed” (counts increased by ≥20%; n=2) or “B-independent” (n=1). We confirmed that IFNγ production in these assays was dependent on antigen presentation through HLA class II, as the presence of anti-HLA-DR antibody significantly reduced spot counts in samples with sufficient PBMC (n=3) (fig.1G).

No donor-specific responsiveness (NDSR) was demonstrated in 36 samples, but was further defined after CD19+ depletion, as either “B-suppressed” (anti-donor-reactivity revealed by B-depletion; n=2), or “non-reactive” (NR, n=34). We hypothesised that in B-suppressed samples, specific B-cell subsets inhibited antigen presentation by non-B-cell antigen-presenting cells (APC). Thus, of 48 samples, 14 showed reactivity to HLA whereas 34 were non-reactive under any condition (supp.table S1).

Each sample was tested for the presence of HLA-BC using the same biotinylated proteins as used in ELISpot. 21 of 48 (44%) had detectable HLA-BC. Although a higher proportion of ELISpot-reactive
samples (9/14) had HLA-BC compared to NR samples (12/34), an association between detectable HLA-BC and ELISpot reactivity to the same protein did not meet statistical significance (p=0.1088, Fisher’s test); HLA-BC frequency in reactive and NR samples was not significantly different (supp.fig.S2A). ELISpot reactivity and HLA-BC were seen in DSA-negative samples where proteins were chosen based on donor-recipient mismatch only, suggesting the presence of donor-antigen-specific T- and B-cells despite undetectable DSA.

13 of 21 (62%) samples, in which the HLA-BC frequency doubled over background (illustrated in supp.figs.S3A-B), underwent further phenotyping (gating strategy and population phenotypes illustrated in supp.fig.S3D). In this setting, background was usually determined by not including any biotinylated antigen but we confirmed that self-antigens, to which patients had no HLA-BC, could also be used to determine ‘background binding’ (illustrated in supp.fig.S3C). Significantly higher frequencies of HLA-binding class-switched-memory B-cells (CSMBC) were seen in the 8 samples with any HLA-specific IFN\(\gamma\) reactivity (fig.2A, p=0.0295), particularly in the 5 DSR samples (fig.2B, p=0.0119). However, the most significant association was between HLA-binding CSMBC frequency and B-dependent DSR (fig.2C, p=0.0088). Importantly, there was no significant association with overall frequencies of CSMBC (fig.2A-C). No other HLA-BC subset significantly associated with ELISpot response. In contrast, samples with evidence of any HLA-specific IFN\(\gamma\) reactivity had significantly lower overall frequencies of transitional B-cells (CD19+CD27-CD24hiCD38hi) compared to NR samples (fig.2D, p=0.0317); differences in HLA-binding transitional B-cell frequencies were not statistically significant. 2/5 patients with samples at multiple timepoints had detectable HLA-BC with variable ELISpot reactivity. In both, the highest frequencies of HLA-binding CSMBC occurred in samples with B-dependent ELISpots, affirming the association described above.

Therefore, the data from this cohort indicate a significant association between HLA-binding CSMBC and any HLA-specific IFN\(\gamma\) production by CD8-depleted PBMC. The association becomes statistically stronger as the functional phenotype on ELISpot is more precisely defined, with IFN\(\gamma\) production
dependent on the presence of B-cells being the most significant. Furthermore, there is an inverse
relationship between HLA-specific ELISpot reactivity and overall transitional B-cell frequency.

4 – Manipulating B-cell subsets in ELISpots using CMV-gpB reveals additional complexity and the
importance of assessing B-cell/Treg interactions

To validate these interpretations, we conducted exploratory ELISpots using cones and the model
antigen CMV-gpB, adding back different magnetic-bead-sorted B-cell fractions to ELISpot wells
(supp.fig.S4). These data, which are mostly descriptive, are related in detail in the supplementary
results, with the caveat that add-back experiments using positively selected subsets must be
interpreted with caution. Several resultant observations challenged our interpretations, primarily
the observation that CD27-negative, non-memory B-cells contained fractions that could both
support IFN\(\gamma\) production, particularly in the absence of regulatory T-cells (Tregs), but also that cells
within CD10+ and CD45RB\(^{MEM55+}\) subpopulations could suppress IFN\(\gamma\) production.

This suggested that the cohort 1 analysis, using a highly-selective definition of antigen-binding B-cells
and measuring PBMC responses without depleting Tregs, might have thwarted a full understanding
of the complexity of the role that B-cell subsets play in HLA-specific IFN\(\gamma\) production.

5 - Exploratory analyses in a second, larger cohort of sensitised transplant recipients

We included two additional conditions in the ELISpot: CD25+cell depletion and CD25+&CD19+cell
depletion, to ask how Tregs interacted with B-cells in indirect alloresponses. This increased the
complexity of ELISpot patterns from four in cohort 1 to nine (supp.table S4), and allowed further
dissection of DSR & NDSR patterns, according to whether they are consistent only with B-
dependency or only with B-suppression (supp.table S4). We also expanded B-cell flow-panels to
permit gating of additional subsets (supp.fig.S3E), and phenotyped all samples with HLA-BC (population phenotypes detailed in supp.fig.S3E).

The second cohort comprised 27 transplant recipients (supp.table S2), from which 30 samples were used in ELISpot and 113 flow comparisons were obtained. Descriptions of these patterns are found in supplementary results.

5a – HLA-BC subsets associated with B-dependent and B suppressed IFNγ production

The different comparisons made for cohort 2 are summarised in Figure 3. In analyses 1&2, associations between B-cell subsets and IFNγ production by CD8-depleted PBMC were assessed as for cohort 1 (supp.figs.S5&S6). In addition, the automated algorithm "cluster identification, characterisation, and regression" (CITRUS) was used to identify populations with significantly different abundances between ELISpot patterns of interest; these unsupervised analyses were compared to manual gating (used for the very low frequency HLA-BC that cannot be characterised by CITRUS).

These analyses confirmed that broadening the definition of an antigen-specific B-cell reduced the strength of the associations between DSR and B-dependent IFNγ production on ELISpot and a high proportion of HLA-binding CSMBC (supp.fig.S5 and supp.fig.S6D), found in cohort 1. Instead, new associations were found between B-dependency and HLA-binding IgM+ memory cells (supp.fig.S6F), and between DSR and B-dependency and IgM+CD27-CD45RBMEM55+cells, a population identified as antigen-experienced marginal-zone precursor (MZP) B-cells (supp.figs.S5I&S6G). Absent IFNγ production associated with high frequencies of HLA-binding CD27- naïve cells (supp.fig.S5G), as did suppression by B-cells (supp.fig.S6H), and suppression also associated with high overall frequencies of transitional cells (supp.fig.S6Ci&l).

As illustrated in figure 3, a major opportunity arising from the additional depletion of CD25+ cells included in the analyses of the ELISpot patterns of cohort 2 was to compare samples where IFNγ
production was consistently B-dependent (patterns 1,2&4, supp.tables S2&S4 (7 samples, 19 flow profiles)) to samples that were consistently B-suppressed (patterns 6,7&8, supp.tables S2&S4 (5 samples, 13 flow profiles)). This is analysis 3 in figure 3.

CITRUS identified several significant differences between these 2 groups (fig.4A-C): consistently B-dependent samples had more abundant IgM+ memory cells (IgM+CD27+CD45RB^MEM55^+, fig.4Ci) and more abundant MZP (fig.4Cii), whereas consistently B-suppressed samples had more abundant naïve cells (supp.fig.S7). Manual gating confirmed that consistently B-dependent samples had higher frequencies of IgM+ memory (fig.4D) and MZP (fig.4E) within both whole B-cells AND HLA-BC when compared to samples showing consistent B-suppression. In contrast, B-suppressed samples had significantly higher frequencies of CD27-(fig.4G) and IgM^{hi}CD27-(fig.4H) HLA-BC compared to B-dependent samples, and within this latter population, a significantly higher frequency of naïve (CD10-CD45RB^MEM55^-) HLA-BC. Samples showing consistent B-suppression also had significantly higher transitional cell (CD24^{hi}CD38^{hi}) frequencies within the whole, but not HLA-binding, population of CD27- B-cells (fig.4I).

In summary, B-dependent IFNγ production therefore strongly associates with HLA-binding by several different subpopulations of antigen-experienced B-cells, including IgM+ memory cells, and CD27-CD45RB^MEM55^+MZP. In contrast, consistent B-cell suppression associates with HLA-binding by predominantly naïve cells, and with higher overall frequencies of transitional cells.

5b – HLA-binding naïve and MZP B-cells associate with IFNγ production that is suppressed by Tregs

We compared B-cell phenotypes in 3 samples that were B-dependent only when CD25+ cells were present (pattern 1, supp.tables S2&S4; 9 flow profiles), to those in 4 samples that were B-dependent only when CD25+ cells were absent (pattern 4, supp.tables S2&S4; 10 flow profiles). This is analysis 4 in figure 3.
CITRUS analysis revealed two distinct memory populations associated with pattern 1 (fig.5A-C): CSMBC (IgM-IgD-CD27+CD45RB<sup>MEM55</sup>+, fig.5Ci) and IgM+ memory cells (IgM+CD27+CD45RB<sup>MEM55</sup>+, fig.5Cii). In contrast, pattern 4 associated with more abundant naïve cells (IgM+IgD+CD27-CD45RB<sup>MEM55</sup>-, fig.5Ciii) and MZP (IgM<sup>hi</sup>CD27-CD45RB<sup>MEM55</sup>+) (fig.5Civ).

Supervised analysis revealed that “pattern 1” samples had significantly higher frequencies of IgM+ memory HLA-BC (fig.5D) compared to those with pattern 4 reactivity and had significantly higher overall CSMBC frequencies (supp.fig.S8C). “Pattern 4” samples had significantly higher frequencies of HLA-binding and total CD27- B-cells (supp.fig.S8D), and within both these populations, significantly higher proportions of IgM<sup>hi</sup>CD27- cells (fig.5E), although further interrogation according to CD10/CD45RB<sup>MEM55</sup> expression revealed no statistically significant associations (figs.5F-I).

These data are consistent with the hypothesis that CD4+ T-cell IFNγ production in the presence of Tregs occurs only when there is a predominance of memory HLA-BC. In contrast, B-dependent IFNγ production that is suppressed when Tregs are present associates with HLA-binding by a predominantly IgM<sup>hi</sup> non-memory B-cell subpopulation, including an MZP subpopulation.

5c – B-cell-mediated suppression and interaction with Tregs – exploratory data from small numbers of samples

This is analysis 5 in figure 3. Because of the paucity of samples, descriptions of these results are presented in the supplementary file, but both CITRUS and supervised analyses support other data presented above that suppression by B-cells is non-antigen specific, and that transitional cells appear more abundant in samples showing B-cell suppression when Tregs are present.
Discussion

A recent meta-analysis of 12 studies, including >1000 patients, confirmed that anti-donor reactivity in the IFN\(\gamma\) ELISpot prior to transplantation is associated with a significantly higher risk of acute rejection,\(^{19}\) thus establishing that the ELISpot test, which detects alloreactive T-cells, has relevance for early clinical graft outcomes. Our previous work has focussed on chronic allograft dysfunction, where we now know that alloimmune pathology is the single biggest cause of renal allograft failure in patients surviving beyond the first-year post-transplantation.\(^{20,21}\) A strong prognostic biomarker for alloimmune injury is the development of de novo DSA against donor HLA\(^{22-27}\) which are associated with a >3x greater risk of graft failure, even after correction for other risk factors associated with graft loss.\(^{25,26}\) Graft failure is usually preceded by progressive graft dysfunction, though rate is highly variable. Although DSA are involved in mediating pathological graft damage,\(^{28}\) evidence also supports the hypothesis that T-cell-mediated effector mechanisms operate,\(^{29-31}\) which is biologically plausible, as DSA production depends on activating multiple components of the recipient immune system, including donor-specific T- and B-cells. We have previously demonstrated the relevance of ELISpot in this context, in two separate cohorts of transplant recipient, by showing strong correlations between ELISpot patterns of anti-donor CD4+T-cell IFN\(\gamma\) production and rates of deterioration in eGFR\(^{16-18}\). Moreover, we showed for the first time that antigen presentation by B-cells associated with a more rapid eGFR decline, but that subgroups of both B- and T-cells actively suppressed donor-specific T-cell alloreactivity in vitro,\(^{16}\) which associated with slower eGFR decline. Patient samples showing active suppression by B- or T-cells had higher transitional cell numbers and CD4+CD25+CD39+Tregs respectively, than samples with no active regulation. These earlier findings formed the foundation of this work, to identify the HLA-BC phenotype associated with these ELISpot patterns.

Studying antigen-specific B-cells is a nascent field, particularly in transplantation. Identifying these cells is fundamentally challenging, due to low frequencies (typically 0.05-0.005% circulating...
lymphocytes) and difficulties determining purity once isolated. Most techniques rely on the principle that antigen is recognised by BCR representing the same specificity as the antibodies secreted, using fluorochrome-conjugated antigen for detection. Although there is a sizeable literature in which antigen-specific B-cells have been detected, subsequent phenotypic analysis is limited. In systemic lupus erythematosus (SLE), B-cells binding double-stranded DNA and antinuclear antigens are found at higher frequencies at earlier maturational stages. In contrast, in rheumatoid disease and ANCA-associated vasculitis, antigen-specific cells are disproportionately found in mature subsets, particularly CSMBC.

In transplantation, several different techniques to detect the presence of HLA-BC have been proposed, including assays that polyclonally stimulate PBMC followed by subsequent detection of either secreted IgG using single antigen beads or detection of HLA-BC in ELISpot using labelled HLA reagents. However, both these techniques bias detection towards a CD27+ or IgG-secreting (i.e., class-switched) phenotype. Other approaches include using HLA-tetramers to detect HLA-BC in sensitised patients with end-stage renal disease, though further characterisation was limited to the memory marker CD27 and CD38 (identifying immunoglobulin-secreting cells). More recently, HLA-coated flow beads were used to detect HLA-BC at greater frequencies in sensitised patients than controls. HLA-BC had higher proportions of non-switched memory cells, with lower CSMBC frequencies.

Despite the inherent challenges, herein we describe the successful identification of HLA-BC in sensitised recipients and healthy donors, using biotinylated Pure HLA proteins. Our major findings are summarised in figure 6. Control experiments demonstrated that antigen specifically binds the BCR. Corresponding Pure HLA proteins were used as an antigen source in ELISpots to assess the contribution of B-cells, and in a second cohort Tregs, to the indirect anti-donor-HLA alloresponse. Though the vast majority of samples had HLA-BC frequencies <0.2%, in two samples 1-2.3% circulating B-cells bound HLA, all of which were IgM+CD27+ cells. Interestingly, these samples were...
from patients who had received rituximab and had very few circulating B-cells, consistent with our
previous description that rituximab selectively spares CD27+ memory cells.\textsuperscript{18,43} However, we didn’t
assess absolute numbers of B-cells in most other samples, which we acknowledge is a weakness of
our methodology.

In both cohorts, there was no correlation between the presence of HLA-BC and DSA, consistent with
earlier reports.\textsuperscript{41} Our finding that isolating then adding back B-cells prior to the ELISpot was
sufficient to turn a non-reactive sample into a reactive one suggests that lack of association between
ELISpot reactivity and presence of HLA-BC might be due solely to the frequency of HLA-BC, relative
to HLA-specific T-cells in any given sample. For some patients with clearly detectable DSA, but no
detectable HLA-BC, the most likely explanation is that the frequency of BC in these patients is too
low to be detectable by our technique, in the relatively small sample of peripheral blood available to
test. Alternatively, it is conceivable that DSA in these patients may be generated predominantly by
plasma cells.

Our predominant hypothesis was that ELISpot patterns of CD4+ T-cell IFN\textsubscript{\gamma} production to Pure\textsuperscript{TM} HLA
proteins, chosen to represent mismatched donor HLA, would strongly correlate with antigen-binding
B-cell phenotype. More specifically, these phenotypes would determine how IFN\textsubscript{\gamma} production
changed when B-cells were depleted. Our finding from the first cohort of an association between
HLA-binding but not overall CSMBC frequency and B-dependent IFN\textsubscript{\gamma} production is consistent with
this hypothesis. Sensitised patients with chronic rejection have increased frequencies of CSMBC with
strong antigen-presenting capacity,\textsuperscript{13} so these new data provide a direct link between a B-dependent
ELISpot and memory B-cell phenotype, specifically for the HLA-BC population. In the larger second
cohort of sensitised recipients, we also found a strong association between HLA-binding IgM+
memory cells and B-dependent IFN\textsubscript{\gamma} production. The importance of these memory cells, which
undergo rapid secondary germinal centre reactions on activation,\textsuperscript{44} has been defined in responses to
Rhesus D\textsuperscript{45} and bacteria such as tetanus,\textsuperscript{45} S.typhi\textsuperscript{46} and Pneumococcus,\textsuperscript{47} but this is the first data
suggesting they might have significance after transplantation. There was no association between HLA-binding CSMBC in cohort 2 and DSR or B-dependent DSR, and the most likely explanation is because we broadened the definition of an HLA-BC for cohort 2, from a stringent definition (samples in which there were at least twice as many B-cells in the HLA-binding gate as were present without antigen) to a less stringent definition (all samples with more B-cells in the HLA-binding gate with antigen than without).

The reason we changed the definition was because depletion/add-back experiments suggested that a population of CD27- B-cells could also support IFNγ production, particularly where CD25+T-cells were depleted, and in the second cohort we found that HLA-binding by CD27- cells associated with B-dependency, particularly HLA-binding by naïve cells and a population found within the IgMhiCD27-pool. This interesting subset comprises classical naïve B-cells, CD10+ transitional cells, and B-cells with antigen experience, expressing CD45RBMEM55. HLA-binding by CD45RBMEM55+ cells seemed to associate with B-dependent responses.

CD45RBMEM55 is a relatively novel marker expressed by memory B-cells, including a subpopulation of IgMhiCD27- B-cells, but not naïve cells. Detailed analysis suggests that IgMhiCD27-CD45RBMEM55+ cells are precursors to marginal-zone B-cells (MZB) separate from memory cells. Recently published work identified a bifurcation of human B-cell maturation from the T1 stage along two trajectories: IgMlo cells, selectively recruited to gut and spleen lymphoid tissue, and IgMhi cells, resulting in the peripheral B-cell compartment. IgMhiT2 cells share transcriptomic features with MZB; these cells were reduced in frequency in severe SLE, possibly suggesting a protective role for MZB in autoimmunity, though it remains unclear whether collapse of the MZB axis in SLE is a cause or consequence of inflammation. The role of MZB is still being defined, but recent work suggests they are the main IgM producers in humans, producing both TNFα & IL-10. Our new data, to the best of our knowledge, are the first suggesting that HLA-specific MZP may play a role in antigen presentation of HLA in sensitised transplant patients.
Our attempts to dissect whether HLA-BC subsets are differentially susceptible to Treg suppression strongly indicates that CD27+ memory cells support B-dependent responses when Tregs are present; though we did not formally show they are insensitive to regulation by Tregs, this is one interpretation. Alternatively, samples with predominantly memory HLA-BC could be the same samples with fewer Tregs. Work is underway to distinguish these two possibilities.

In contrast, predominant HLA-binding by naïve cells/MZP associated with suppression of IFNγ production by Tregs. However, HLA-binding naïve cells also strongly associated with B-suppressed patterns, if abundant transitional cells were present. These data support our previous findings that higher transitional cell frequencies strongly associate with regulation of IFNγ production by B-cells, but here we show this association is with overall transitional cell frequency, not with HLA-binding transitional cells and that regulation appears dependent on there being insufficient numbers of memory HLA-BC alongside a higher frequency of naïve HLA-BC. Whether these naïve cells play an active role in mediating regulation by transitional cells is not clear. These data provide a link between established correlations between higher transitional cell frequencies and better graft outcomes, and our published work correlating B-suppressed ELISpot responses with stabilisation of graft function in patients with chronic rejection.

Only a few samples could be used to assess the relationship between Tregs and putative suppression of IFNγ production by B-cells, so our data is highly exploratory and therefore described in the supplementary file. It suggests that transitional cells operate in the presence of Tregs, but that other, rare populations of non-HLA-binding MZP may also suppress when Tregs are absent.

In summary, we have defined HLA-BC phenotypes and their associations with patterns of HLA reactivity on IFNγ ELISpot, which are known to correlate with rates of functional deterioration in patients with chronic rejection. As expected, memory HLA-BC associated strongly with B-dependent IFNγ production that was not suppressed by endogenous Tregs. Where the predominant HLA-binding phenotype was naïve B-cells, the associated ELISpot phenotype appeared to be determined...
by other cells: high overall transitional cell frequencies associated with B-suppressed IFN\(\gamma\) production, especially if Tregs were present, whereas high frequencies of HLA-binding MZP associated with B-dependent IFN\(\gamma\) production, particularly when Tregs were absent, hinting that the involvement of these cells is suppressible by Tregs. Finally, non-HLA-binding MZP may be able to suppress IFN\(\gamma\) production, though this only emerges when Tregs are depleted.

These data provide a novel foundation on which to build further studies to examine the complexities of interactions between HLA-specific T- and B-cells in sensitised patients, to identify new targets for intervention and future therapies for chronic rejection.
Disclosure

No conflicts of interest to declare.
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Acknowledgements

The authors would like to thank the patients, nursing staff and physicians at Guy’s Hospital.
Figure legends

Figure 1: Biotinylated proteins can be used to detect antigen-binding B-cells.

A-F: Flow cytometric assessment of the ability of B-cells from healthy donors or HLA-sensitised renal allograft recipients to bind streptavidin BV421 after incubation with different biotinylated protein antigens. Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values.

A: In healthy volunteers, only B-cells pre-incubated with a biotinylated CMV-gpB protein, but not biotinylated BSA, nor non-biotinylated proteins bind the streptavidin BV421.

B-C: In healthy volunteers, blocking FcγRIIB with an anti-CD32 monoclonal antibody does not reduce the frequency of B-cells that bind biotinylated CMV-gpB (B), whereas competitive incubation with increasing doses of an anti-human immunoglobulin Fab to block the B-cell receptor does inhibit binding (C).

D: PBMC from sensitised transplant recipients were incubated with increasing doses of biotinylated HLA (chosen to correspond to the patients’ known sensitisation status) or control media (“0μg/ml”), prior to assessment of streptavidin-BV421 binding to CD19+ cells and CD19- cells. Only CD19+ cells displayed significantly higher frequencies of HLA-binding after incubation with biotinylated HLA (black unfilled bars; ****p<0.0001); there was no difference between HLA-incubated CD19- cells and control cells not incubated with HLA (grey bars; p=ns).

E: PBMC from healthy donor buffy coats (‘cones’ n=3) or HLA-sensitised transplant recipients (‘patients’ n=27) were incubated with different biotinylated HLA class I antigens, chosen, in the case of patients, on the basis of their sensitisation history. 8 patients provided multiple samples for testing. Each dot is a single sample, and bars indicate median with interquartile range of the proportion of B-cells that bind streptavidin-BV421. HLA-binding B-cells are detected at higher
frequencies in sensitised patients compared to cones from healthy individuals (**p=0.0002, Mann-Whitney test).

F: To illustrate that there is no correlation between the frequency of HLA-binding B-cells amongst total CD19+ cells, and Median Fluorescence Intensity (MFI) of the HLA-binding B-cells (linear regression line with 95% confidence interval shown; $R^2 = 0.07$). Black dots = patient samples; red dots = healthy cones.

G: ELISpot assays were performed using PBMC depleted of CD8+ cells from 3 sensitised renal transplant recipients as described in methods and repeated with the addition of antibodies to HLA-DR. In all 3 experiments, blocking HLA class II resulted in a substantial reduction in the number of IFNγ-producing cells, which just failed to reach statistical significance ($p=0.0708$, paired t test).

**Figure 2: Cohort 1: correlations between B-cell phenotypes and patterns of reactivity to HLA Pure proteins on ELISpot.**

Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values. HLA-binding B-cell phenotypes are denoted by unfilled bars; whole B-cell subset phenotypes are denoted by grey bars.

A: Samples showing antigen-reactive T-cell activity on ELISpot (defined as showing evidence antigen-specific IFNγ production under any condition – see supplementary table S3) have significantly higher frequencies of HLA-binding class-switched memory (IgM-CD27+) B-cells, compared to samples showing no antigen-reactive T-cell activity ($^*p=0.0295$). There is no difference between frequencies of class-switched memory B-cells in the whole B-cell population (i.e., non-HLA-binding).

B: Samples showing donor-specific reactivity (DSR, defined as IFNγ production by CD8-depleted PBMC – see supplementary table S3), have significantly higher frequencies of HLA-binding class-switched memory B-cells compared to samples showing no donor-specific reactivity (NDSR, defined...
as no IFNγ production by CD8-depleted PBMC (*p=0.0119). There is no difference between frequencies of class-switched memory B-cells in the whole B-cell population (i.e., non-HLA-binding).

C: Samples showing B-dependent DSR (defined as spot count reduction of at least 20% when CD19+ cells depleted from CD8-depleted PBMC) have higher frequencies of HLA-binding class-switched memory B-cells compared to samples that do not show B-dependency (***p=0.0088). There is no difference between frequencies of class-switched memory B-cells in the whole B-cell population (i.e., non-HLA-binding).

D: Samples showing evidence of antigen-reactive T-cells on ELISpot (defined as in A) have lower frequencies of transitional (CD19+CD27-CD24hiCD38hi) B-cells in whole B-cell population (i.e., non-antigen-binding) than those showing no antigen-reactivity. (*p=0.0317). This is not seen in HLA-binding B-cells.

**Figure 3: Overview of the analyses undertaken for cohort 2.** Each box details the ELISpot conditions (blue) and results (red) along with the B cell subsets found to associate with the ELISpot pattern described (red), either the overall or HLA-binding subset where indicated. Numbers of samples analysed for each group are listed; several comparisons are limited by small sample sizes as shown. The different comparisons undertaken and described in the text are in green.

Definitions: DSR = IFNγ by CD8-depleted PBMC; NDSR = no IFNγ production by CD8-depleted PBMC; B-dependence = reduction in spot count of ≥20% after B-cell depletion; B-suppression = increase in spot count of ≥20% after B-cell depletion; Analysis 3: As analysis 2, but includes samples where both B-cells and CD25+ Tregs have been depleted from CD8-depleted PBMC.

*These 13 flow profiles included 3 from AIT017 (supp. table S2) that are not included in analysis 5 (hence analysis 5 only refers to 10 flow profiles)
Figure 4: Cohort 2: Consistent B-dependency, not influenced by CD25+ T-cells, correlates with memory B-cell subsets, whilst consistent B-suppression correlates with naïve and transitional B-cells.

Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values. HLA-binding B-cell phenotypes are denoted by unfilled bars; whole B-cell subset phenotypes are denoted by grey bars. Consistent B-dependence = patterns 1, 2 and 4; consistent B-suppression = patterns 6, 7 and 8.

A-C: CITRUS analysis of B-cell phenotype identifies seven groups of cell clusters, two of which are illustrated here (annotated i and ii in A), that significantly correlate with consistent B-dependent phenotype in ELISpot. B shows expression of individual markers by each of the clusters. C shows phenotype of group i and ii, comparing samples showing consistent B-dependency on ELISpot (red) to samples showing consistent B-suppression on ELISpot (blue).

D: Samples showing consistent B-dependency have a significantly higher frequency of IgM+ memory cells (IgM+CD27+) within both HLA-binding (**p=0.0024) and whole B-cell populations (**p=0.0003) compared to samples showing consistent B-suppression.

E: Samples showing consistent B-dependency have a significantly higher frequency of marginal-zone precursors (CD10−CD45RBMEM55+−) within both HLA-binding (**p=0.0023) and whole IgMhiCD27− fraction of B-cells (****p<0.0001) compared to samples showing consistent B-suppression.

F: Samples showing consistent B-dependency have a significantly higher frequency of a rare CD10+CD45RBMEM55+ subset of B-cells within the IgMhiCD27− population (i.e., non-antigen-binding) compared to samples showing consistent B-suppression (**p=0.0067). There is no difference between frequencies of these cells that bind HLA.

G: Samples showing consistent B-suppression have a significantly higher frequency of HLA-binding CD27− cells (**p=0.0015) compared to samples showing consistent B-dependency. There is no
difference between frequencies of these B-cells in the whole B-cell population (i.e., non-HLA-binding).

H: Samples showing consistent B-suppression have a significantly higher frequency of HLA-binding IgM^{hi}CD27^- cells (*p=0.02) compared to samples showing consistent B-dependency. There is no difference between frequencies of these B-cells in the whole B-cell population (i.e., non-HLA-binding).

I: Samples showing consistent B-suppression have a significantly higher frequency of CD24^{hi}CD38^{hi} transitional cells within the CD27^- population of B-cells (i.e., non-antigen-binding) compared to samples showing consistent B-dependency (**p=0.0069). There is no difference between frequencies of HLA-binding transitional B-cells.

J: Samples showing consistent B-suppression have significantly higher frequencies of both HLA-binding (**p=0.0013) and non-HLA-binding (**p=0.0044) CD10^-CD45RB^{MEM55^-} cells within the IgM^{hi}CD27^- compartment compared to samples showing consistent B-dependence.

Figure 5: Cohort 2: ELISpot patterns where the presence or absence of CD25+ cells influences B-dependent reactivity have significantly different B-cell subset compositions.

Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values. HLA-binding B-cell phenotypes are denoted by unfilled bars; whole B-cell subset phenotypes are denoted by grey bars.

A-C: CITRUS analysis identifies two groups of cells that significantly correlate with B-dependence that only occurs in the presence of CD25+ cells (pattern 1) and two groups that significantly correlate with B-dependence that is suppressed by the presence of CD25+ cells (pattern 4).

D: Pattern 1 (B-dependent in the presence of CD25+ cells) associates with higher frequencies of HLA-binding IgM+ memory cells (*p=0.0123).
E: Pattern 4 (B-dependent in the absence of CD25+ cells) associates with higher frequencies of HLA-binding IgM\(^{\text{hi}}\)CD27- (*p=0.0124) and overall IgM\(^{\text{hi}}\)CD27- cells (****p<0.0001).

F-I: There were no significant associations between patterns 1 or 4 and any of the four IgM\(^{\text{hi}}\)CD27-subsets (defined as transitional CD10+CD45RB\(^{\text{MEM55}}\), CD10+CD45RB\(^{\text{MEM55}}\), CD10-CD45RB\(^{\text{MEM55}}\), MZP; and naïve CD10-CD45RB\(^{\text{MEM55}}\)), in both the HLA-binding and non-HLA-binding populations.

Figure 6: Summary of the major associations described for different B cell subsets.

Figure 6 is based around the gating strategy used to define CD19+ B cells (A) and beyond that, subsets based on CD27 expression either with IgM (B) or without (C). The IgM\(^{\text{hi}}\)CD27- subset in B is further defined by CD10 and CD45RB\(^{\text{MEM55}}\) expression (D). The CD27- fraction in C is further defined by CD38 and CD24 expression (E). The table (F) details the associations between the subsets defined in B-E (HLA-binding and total populations) with ELISpot patterns.
The phenotype of HLA-binding B-cells from sensitised renal transplant recipients correlates with clinically prognostic patterns of IFNγ production against purified HLA proteins.

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The authors acknowledge that the research was funded/supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy’s and St Thomas’ NHS Foundation Trust and King’s College London. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

This study was funded by an MRC/KRUK Clinical Research Training Fellowship for H. Burton (MR/M01813X/1 // JF1/2015), a project grant from Kidney Research UK (RP3/2011) and generous donations from the GSTT Kidney Patients Association.

Running headline: HLA-binding B-cell phenotypes in transplant recipients
Abstract

B-cells play crucial roles in cell-mediated alloimmune responses. In vitro, B-cells can support or regulate indirect T-cell alloreactivity in response to donor antigens on ELISpot; these patterns associate with clinical outcome. Previous reports of associations between B-cell phenotype and function have examined global phenotypes and responses to polyclonal stimuli. We hypothesised that studying antigen-specific B-cells, using samples from sensitised patients enrolled in two clinical studies, would inform further study to identify novel targets for intervention.

Using biotinylated HLA proteins, which bind HLA-specific B-cells via the B-cell receptor in a dose-dependent fashion, we report the specific phenotype of HLA-binding B-cells and define how they associate with patterns of anti-HLA response in IFNγ ELISpot. HLA-binding class-switched and IgM+CD27+ memory cells associated strongly with B-dependent IFNγ production and appeared not suppressible by endogenous Tregs. When the predominant HLA-binding phenotype was naïve B-cells, the associated functional ELISpot phenotype was determined by other cells present: if high numbers of non-HLA-binding transitional cells, then this associated with B-regulated-suppressed IFNγ production, especially if Tregs were present. However, if high frequencies of HLA-binding marginal-zone precursors (MZP) were present, these associated with B-dependent IFNγ production that appeared suppressible by Tregs. Finally, non-HLA-binding MZP may also be able to suppress IFNγ production, though this association only emerged when Tregs were absent from the ELISpot. These novel data provide a foundation on which to further define the complexities of interactions between HLA-specific T- and B-cells, to identify new targets for intervention in new therapies for chronic rejection.

Keywords: chronic allograft nephropathy, inflammation, lymphocytes, transplantation
Translational Statement

Patterns of IFNγ production by CD4+ T-cells on ELISpot correlate with clinical outcomes in sensitised renal transplant recipients. Here we show that different B-cell subsets, including HLA-binding B-cells, correlate with these patterns and interact with regulatory T-cells. This has two important ramifications: i) phenotyping B-cells could yield useful prognostic information as a biomarker for patients’ clinical progress and ii) it could form the basis of identifying new treatments targeting specific B-cell subsets to prevent allograft loss.
Introduction

B-cells play diverse roles in the immune response, including antibody production, antigen presentation & T-cell activation, and an immunomodulatory role, linked to IL-10-producing B-cells in experimental models \(^1\text{-}^5\) and in humans to transitional B-cells, as defined by high expression of CD24 and CD38 \(^6\), and more recently to unswitched memory (IgM+CD27+) B-cells.\(^7\)

In transplantation, two landmark studies linked operational tolerance to distinct B-cell transcriptional signatures;\(^8\text{-}^9\) and tolerant patients had higher frequencies of transitional and naïve cells making more IL-10.\(^10\) In the non-tolerant setting, depleting B-cells at the time of transplantation can be detrimental, \(^11\) higher transitional cell frequencies associate with reduced rates of rejection, better graft function and improved graft survival rates, \(^12\) and patients with chronic rejection have fewer T1 transitional B-cells and an increase in class-switched memory B-cells, \(^13\) associating with poorer graft outcomes,\(^14,15\) all of which suggests that immunomodulation by B-cells plays an important role in promoting graft survival post-transplantation. However, all studies to date have analysed whole B-cell populations, not alloantigen-specific cells, and functional assays, where performed, used polyclonal, rather than antigen-specific, stimuli.

We have previously demonstrated, in 2 cohorts of renal transplant recipients with biopsy-proven chronic rejection, that B-cells play complex roles in indirect CD4+ T-cell responses to donor antigens in vitro.\(^16,17\) Using enzyme-linked immunosorbent spot (ELISpot) assays to detect anti-donor interferon-\(\gamma\) (IFN\(\gamma\)) production in response to donor antigens, we showed that in many patients the IFN\(\gamma\) response was dependent on the presence of B-cells, but in others B-cells appeared to be suppressing responses. This regulation associated with polarised cytokine production by B-cells toward IL-10 after polyclonal activation by IgG/IgM and by an increased proportion of both T1 and T2 populations.\(^18\) Importantly, we demonstrated that dynamic changes in anti-donor IFN\(\gamma\) production between B-dependency and B-regulation correlated with rate of reduction in estimated glomerular filtration rate (eGFR).\(^17,18\)
We therefore hypothesised that it was differences in the phenotype of donor-HLA-specific B-cells that determined these patterns in ELISpot. In this new work, we characterise HLA-binding B-cells (HLA-BC) in sensitised renal transplant recipients and investigate their functional role in HLA-specific indirect alloresponses.
Methods

Source of PBMC

PBMC were obtained from patients enrolled in the UK multicentre RituxiCAN-C4 randomised controlled trial (ClinicalTrials.gov NCT00476164), the full details of which have been published or the Antibody Incompatible Transplantation observational study, approved by the West Midlands-Coventry&Warwickshire Research Ethics Committee (16/WM/0370), conforming to the 1964 Declaration of Helsinki. All participants gave written informed consent before inclusion. RituxiCAN-C4 samples were those not required for analysis of endpoints. Samples from the AIT study were from the first 40 patients enrolled at the Guy’s Renal Transplant Clinic by HB between 2016 and 2017, from whom sufficient PBMC could be extracted and HLA proteins available. All HLA-sensitised patients awaiting a transplant, or previous recipients of HLA-incompatible transplants, that were willing to consent to give up to 16 blood samples for analysis, over a period of up to 10 years, were eligible for recruitment.

PBMC from healthy volunteers were obtained as concentrated leucocytes provided by the NHS blood transfusion service as a by-product of the blood donation process (referred to as “cones”).

Antigen preparation

HLA proteins were purchased (Pure Protein LLC, Texas) in unmodified and biotinylated forms. PHLA proteins were selected from a panel of 9 specificities available, according to patients’ known donor-specific antibodies (DSA) or HLA-mismatch in samples without DSA. Proteins chosen for each sample are detailed in supp.figtables S1&S2. 9 individual proteins enabled analysis of samples from 21 RituxiCAN-C4 patients and 18 AIT patients. Cytomegalovirus glycoprotein-B (CMV-gpB) (Abcam, UK) was biotinylated in-house using the EZ-Link Sulfo-NHS-Biotinylation kit (Thermo Scientific), as per manufacturer instructions.

Flow cytometry
PBMC were thawed, washed, then incubated with biotinylated antigen (30 min on ice). After washing, cells were stained with titrated amounts of fluorochrome-conjugated monoclonal-Ab (anti-human CD4-FITC, CD8-Qdot®605, CD10-APC, CD14-Pacific Blue, CD19-APCeFluor®780, CD19-PerCPeFluor®710, CD24-FITC, CD25-PE, CD27-PECy7, CD38-BUV395, CD45RB MEM55-PE, IgM-BV605, Streptavidin-BV421) in Brilliant Stain Buffer (BD Bioscience, Oxford, UK) for 30 min (4°C). Antibodies were obtained from eBioscience (San Diego, CA), BD Bioscience, Life Technologies (Paisley, UK) and Biolegend (London, UK). Cells were washed, then incubated with Fixable LIVE/DEAD Aqua-fluorescent reactive dye (Life Technologies) for 30 min (4°C). Cells were washed, fixed (1% paraformaldehyde), washed with PBS-5%FCS and stored (4°C) before acquisition within 24h on an LSRII/Fortessa flow cytometer (KCL BRC Flow Cytometry Laboratory), using FlowJo software (Treestar, Ashland, OR).

For unsupervised analyses, CITRUS was run using equal numbers of CD19+ events per sample and these clustering channels: IgM, IgD, CD27, CD45RB MEM55, CD10, CD24, CD38.

**ELISpot assay**

ELISpot plates (Mabtech, Sweden) precoated with primary IFNγ Ab were blocked for 2 hours with ‘complete medium’ (AIM-V/10% human AB serum, Life Technologies) before addition of 4×10^5 responder PBMC/well in 100 μL of complete medium alone or with unbiotinylated HLA (Pure Protein LLC). PBMC were prepared according to standard laboratory protocols; Dynabeads® (Life Technologies) were used to deplete CD8+ cells from all experiments and CD19+ or CD25+ cells when required. Plates were incubated for 24 hours (37°C) then developed according to manufacturer instructions. In some experiments, 10μg/ml ultra-purified anti-HLA-DR (Biolegend) was added to wells at 10μg/ml.

**MACS cell separation**
For depletion/addback experiments, MACS® technology (Miltenyi Biotech, Germany) isolated cells with either negative (CD19) or positive (CD27, CD10, CD45RB) selection, as per manufacturer instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Prism. P-values < 0.05 were considered statistically significant. Wilcoxon matched-pairs signed-rank tests were used for non-parametric paired data; Mann-Whitney tests were used for non-parametric unpaired data.
Results

1 - Biotinylated antigen specifically binds to B-cells and can be used for the detection of antigen-binding cells

To preserve patient samples, we used B-cells from healthy donor “cones” and compared binding of a biotinylated model protein antigen (CMV-gpB), to which a significant proportion of people have been sensitised to binding by bovine serum albumin (BSA) to which most people should be naive.

Figure 1A shows that antigen-binding B-cells were only detected when biotinylated CMV-gpB but not controls were incubated with PBMC, demonstrating that events in the antigen-binding gate were not due to B-cells binding non-specifically to streptavidin, BSA, or biotin directly.

To ensure that antigen was binding B-cells directly, PBMC were incubated with blocking anti-CD32 antibody prior to incubation with biotinylated CMV-gpB. Figure 1B demonstrates that blocking CD32 does not affect the antigen-binding B-cell frequency. Blocking with increasing concentrations of Fab anti-IgG/IgM resulted in decreased CMV-gpB binding, confirming that antigen was specifically binding the B-cell antigen receptor (figure 1C). Together these data indicate that biotinylated protein antigen was specifically binding to immunoglobulin receptors on small proportions of B-cells, consistent with binding by antigen-specific B-cells.

2 - HLA-binding B-cells can be detected in sensitised renal transplant recipients and healthy donors

Using increasing concentrations of biotinylated Pure™ HLA proteins incubated with whole PBMC, we demonstrated dose-dependent binding by B-cells but not by other leukocytes within the non-B-cell gate (fig.1D). Figure 1E depicts HLA-BC frequencies in HLA-sensitised recipients (n=27, 8 at >1 timepoint; total sample n=37), expressed as a percentage of all B-cells. The median frequency was 0.0681%; most patients had <0.2% HLA-BC, but in two samples, 1-2.3% circulating B-cells bound HLA. In comparison, healthy donor PBMC (n=10 samples, from 3 cones), exhibited significantly lower HLA-
BC frequencies (0-0.05%; median=0.022%). There was no relationship between frequency and median fluorescence intensity of HLA-BC (fig.1F).

3 – Antigen-binding B-cell phenotype correlates with IFNγ production in antigen-specific ELISpot

Compared to previous studies, which used whole membrane preparations as a donor antigen source,16,17 here we used single HLA proteins, chosen on a patient-specific basis as above, in IFNγ ELISpots.

Serial samples (n=48) from an initial cohort of sixteen patients were tested (supp.table S1). Supp. table 2-S3 summarises interpretations of ELISpot patterns; assays were performed in triplicate under two conditions per sample: with CD19+ cells present, and with CD19+ cells depleted (cell-depletion effect shown in supp.fig.S1A-C).

Donor-specific responsiveness (DSR: antigen-specific IFNγ production by CD8-depleted PBMC) was demonstrated by 12 samples and was further defined by spot counts after CD19+ depletion as “B-dependent”(counts reduced by ≥20% after B-cell depletion; n=9), “B-suppressed”(counts increased by ≥20%; n=2) or “B-independent”(n=1). We were able to confirmed that IFNγ production in these assays was dependent on antigen presentation through HLA class II, as the presence of an anti-HLA-DR antibody significantly reduced spot counts in samples with sufficient numbers of PBMC remaining (n=3) (fig.Figure 1G).

No donor-specific responsiveness (NDSR) was demonstrated in 36 samples, but was further defined after CD19+ depletion, as either “B-suppressed”(anti-donor-reactivity revealed by B-depletion; n=2), or “non-reactive”(NR, n=34). We hypothesised that in B-suppressed samples, specific B-cell subsets inhibited antigen presentation by non-B-cell antigen-presenting cells (APC). Thus, of 48 samples, 14 showed reactivity to HLA-proteins whereas 34 were non-reactive under any condition (supp.table S1).
Each sample was tested for the presence of HLA-BC using the same biotinylated proteins as used in ELISpot. 21 of 48 (44%) had detectable HLA-BC. Although more a higher proportion of ELISpot-reactive samples (9/14) had HLA-BC compared to NR samples (12/34), an association between detectable HLA-BC and ELISpot reactivity to the same protein did not meet statistical significance (p=0.1088, Fisher’s test). Comparing actual and the frequency of HLA-BC frequency frequencies between reactive and NR samples also failed to demonstrate a difference was not significantly different (supp.fig.S2A). ELISpot reactivity and HLA-BC were seen in DSA-negative samples where HLA proteins were chosen based on donor-recipient mismatch only, suggesting the presence of donor-antigen-specific T- and B-cells despite undetectable DSA.

13 of 21 (62%) samples, in which the HLA-BC frequency doubled over background (illustrated in supp.figs.2A3A-B&B), underwent further phenotyping (gating strategy and population the phenotypes of the populations studied are illustrated in supp.fig.2CS3D). In this setting, background was usually determined by not including any biotinylated antigen but we confirmed that self-antigens, to which patients had no HLA-BC, could also be used to determine ‘background binding’ (illustrated in supp.fig.-S3C). Significantly higher frequencies of HLA-binding class-switched-memory B-cells (CSMBC) were seen in the 8 samples with any HLA-specific IFNγ reactivity (fig.2A, p=0.0295), particularly in the 5 DSR samples (fig.2B, p=0.0119). However, the most significant association was between HLA-binding CSMBC frequency and B-dependent DSR (fig.2C, p=0.0088). Importantly, there was no significant association with overall frequencies of CSMBC (fig.2A-C). No other HLA-BC subset significantly associated with ELISpot response. In contrast, samples with evidence of any HLA-specific IFNγ reactivity had significantly lower overall frequencies of transitional B-cells (CD19+CD27-CD24hiCD38hi) compared to NR samples (fig.2D, p=0.0317); differences in HLA-binding transitional B-cell frequencies were not statistically significant. 2/5 patients with samples at multiple timepoints had detectable HLA-BC with variable ELISpot reactivity. In both, the highest frequencies of HLA-binding CSMBC occurred in samples with B-dependent ELISpots, affirming the association described above.
Therefore, the data from this cohort indicate a significant association between HLA-binding CSMBC and any HLA-specific IFNγ production by CD8-depleted PBMC. The association becomes statistically stronger as the functional phenotype on ELISpot is more precisely defined, with IFNγ production dependent on the presence of B-cells being the most significant. Furthermore, there is an inverse relationship between HLA-specific ELISpot reactivity and overall transitional B-cell frequency.

4 – Manipulating B-cell subsets in ELISpots using CMV-gpB reveals additional complexity and the importance of assessing B-cell/Treg interactions

To validate these interpretations, we conducted exploratory ELISpots using cones and the model antigen CMV-gpB, adding back different magnetic-bead-sorted B-cell fractions to ELISpot wells (supp.fig. S43). These data, which are mostly descriptive, are described in detail in the supplementary results, with the caveat that add-back experiments using positively selected subsets must be interpreted with caution. Several resultant observations challenged our interpretations, primarily the observation that CD27-negative, non-memory B-cells contained fractions that could both support IFNγ production, particularly in the absence of regulatory T-cells (Tregs), but also that cells within CD10+ and CD45RBMEM55+ B-cell subpopulations could suppress IFNγ production.

This suggested that the cohort 1 analysis, using a highly-selective definition of antigen-binding B-cells and measuring PBMC responses without depleting Tregs, might have thwarted a full understanding of the complexity of the role that B-cell subsets play in HLA-specific IFNγ production.

5 - Exploratory analyses in a second, larger cohort of sensitised transplant recipients

We included two additional conditions in the ELISpot: CD25+ cell depletion and CD25+&CD19+ cell depletion, to ask how Tregs interacted with B-cells in indirect alloresponses. This increased the
complexity of ELISpot patterns from four in cohort 1 to nine (supp.table 3S4), and allowed further
dissection of DSR & NDSR patterns, according to whether they are consistent only with B-
dependency or only with B-suppression (supp.table 3S4). We also expanded B-cell flow-panels to
permit gating of additional subsets (supp.fig.2D3E), and phenotyped all samples with HLA-BC (the
phenotype of the population phenotypes studied is also detailed in supp.fig.5S3E).

The second cohort comprised 27 transplant recipients (supp.table 4S2), from which 30 samples were
used in ELISpot and 113 flow comparisons were obtained. Descriptions of these patterns are found
in supplementary results.

5a – HLA-BC subsets associated with B-dependent and B suppressed IFNγ production

The different comparisons made for cohort 2 are summarised in Figure 3. In analyses 1 & 2, a
Associations between B-cell subsets and IFNγ production in the presence of Tregs by CD8-depleted
PBMC (supp.figs 5&6) were assessed as for cohort 1 (supp.figs.6S5&7S6), as a quality control
assessing the impact of broadening antigen-specific B-cell definitions. In addition, the automated
algorithm "cluster identification, characterisation, and regression" (CITRUS) was used to identify
populations with significantly different abundances between ELISpot patterns of interest; these
unsupervised analyses were compared to manual gating (used for the very low frequency HLA-BC
that cannot be characterised by CITRUS).

These analyses confirmed that broadening the definition of an antigen-specific B-cell reduced the
strength of the associations between DSR and B-dependent IFNγ production on ELISpot and a high
proportion of HLA-binding CSMBC (supp.fig.6S5 and supp.fig.7S6D), found in cohort 1. Instead, a
continued association between CSMBC and B-dependent IFNγ production was confirmed
(supp.fig.6C&D) and we identified new associations were found between B-dependency and HLA-
binding IgM+ memory cells (supp.fig.7S6F), and between DSR and B-dependency and IgM+CD27-
CD45RBMEM55+cells, a population identified as antigen-experienced marginal-zone precursor (MZP) B-
cells (supp.figs.6S5I &7S66G). Absent IFNγ production associated with high frequencies of HLA-
binding CD27- naïve cells (supp.fig.55G), as did suppression by B-cells (supp.fig.76H), and
suppression also associated with high overall frequencies of transitional cells (supp.fig.76Cii&l).

As illustrated in figure 35, a major opportunity arising from the additional depletion of CD25+ cells
included in the analyses of the ELISPOT patterns of with cohort 2 was to compare samples where
IFNγ production was consistently B-dependent (patterns 1, 2&4, supp.tables 32&S4 (7 samples, 21
flow profiles)) to samples that were consistently B-suppressed (patterns 6, 7&8, supp.tables
32&S4 (5 samples, 13 flow profiles)). This is analysis 3 in figure 35.

CITRUS identified several significant differences between these 2 groups (fig.43A-C): consistently B-
dependent samples had more abundant IgM+ memory cells (IgM+CD27+CD45RBMEM55+, fig.43Ci) and
more abundant MZP (fig.43Cii), whereas consistently B-suppressed samples had more abundant
naïve cells (supp.fig.87). Manual gating confirmed that consistently B-dependent samples had
higher frequencies of IgM+ memory (fig.43D) and MZP (fig.43E) within both whole B-cells AND HLA-
BC when compared to samples showing consistent B-suppression. In contrast, B-suppressed samples
had significantly higher frequencies of CD27-(fig.43G) and IgMhiCD27-(fig.43H) HLA-BC compared to
B-dependent samples, and within this latter population, a significantly higher frequency of naïve
(CD10-CD45RBMEM55-) HLA-BC. Samples showing consistent B-suppression also had significantly
higher transitional cell (CD24hiCD38hi) frequencies within the whole, but not HLA-binding, population
of CD27- B-cells (fig.43l).

In summary, B-dependent IFNγ production therefore strongly associates with HLA-binding by several
different subpopulations of antigen-experienced B-cells, including IgM+ memory cells, and CD27-
CD45RBMEM55+MZP. In contrast, consistent B-cell suppression associates with HLA-binding by
predominantly naïve cells, and with higher overall frequencies of transitional cells.

5b – HLA-binding naïve and MZP B-cells associate with IFNγ production that is suppressed by Tregs
We compared B-cell phenotypes in 3 samples that were B-dependent only when CD25+ cells were present (pattern 1, supp.tables 3S2&84; 11.9 flow profiles), to those in 4 samples that were B-dependent only when CD25+ cells were absent (pattern 4, supp.tables 3S2&84; 10 flow profiles).

This is analysis 4 in figure 35.

CITRUS analysis revealed two distinct memory populations associated with pattern 1 (fig.54A-C): CSMBC (IgM-IgD-CD27+CD45RBMEM55+, fig.54Ci) and IgM+ memory cells (IgM+CD27+CD45RBMEM55+, fig.54Cii). In contrast, pattern 4 associated with more abundant naïve cells (IgM+IgD+CD27-CD45RBMEM55-, fig.54Ciii) and MZP (IgMhiCD27-CD45RBMEM55+) (fig.54Civ).

Supervised analysis revealed that “pattern 1” samples had significantly higher frequencies of IgM+ memory HLA-BC (fig.54D) compared to those with pattern 4 reactivity and had significantly higher overall CSMBC frequencies (supp.fig.8C8S8C). “Pattern 4” samples had significantly higher frequencies of HLA-binding and total CD27- B-cells (supp.fig.8D8S8D), and within both these populations, significantly higher proportions of IgM+CD27- cells (fig.54E), although further interrogation according to CD10/CD45RBMEM55 expression revealed no statistically significant associations (figs.54F-I).

These data are consistent with the hypothesis that CD4+ T-cell IFNγ production in the presence of Tregs occurs only when there is a predominance of memory HLA-BC. In contrast, B-dependent IFNγ production that is suppressed when Tregs are present associates with HLA-binding by a predominantly IgMhi non-memory B-cell subpopulation, including an MZP subpopulation.

5c – Confirmation of the importance of transitional cells for B-cell-mediated suppression and preliminary evidence that a MZP sub-population can suppress under certain conditions

interaction with Tregs – exploratory data from small numbers of samples

We compared 3 samples showing a B-suppressed pattern with CD25+ cells present (pattern 7, supp.tables 3&4; 7 flow profiles), with 1 sample showing a B-suppressed pattern only with CD25+
cells absent (pattern 6, supp.tables 3&4; 3 flow profiles). This is analysis 5 in figure 35. Because of
the paucity of samples, description of these results are presented in the supplementary file, but both
CITRUS and supervised analyses support other data presented above that suppression by B-cells is
non-antigen specific, and that transitional cells appear more abundant in samples showing B-cell
suppression when Tregs are present.

CITRUS analysis (fig.5A-C and supp.fig.9) revealed abundant naïve cells (IgM+IgD+CD27-
CD45RB<MEM55<, fig.5Ci), transitional cells (IgM+CD27+CD38+CD45RB<MEM55<CD10+, fig.5Cii) and
the rare B-cell population with the phenotype IgM+CD27+CD45RB<MEM55<CD10+CD10+, fig.5Ciii), associating
with B-suppression when Tregs were present, whereas MZP (IgM+CD27+CD10+CD45RB<MEM55<)
fig.5Civ) were more abundant in samples with B-suppression only when Tregs were absent. In the
supervised analysis, there were no significant differences in HLA-binding subpopulations (figs.5D-F),
but the whole B-cell findings were consistent with the CITRUS results. This data supports the
hypothesis that non-HLA-binding CD10+ transitional cells, and potentially a rare population of CD27-
CD10+CD45RB<MEM55< cells, are involved in suppressing antigen presentation by non-B-cell APC when
Tregs are present, whereas non-HLA-binding MZP may suppress antigen presentation by non-B-cell
APC when Tregs are absent.
Discussion

A recent meta-analysis of 12 individual studies, including >1000 patients, confirmed that anti-donor reactivity in the IFN\(\gamma\) ELISPOT\(_{pot}\) prior to transplantation is associated with a significantly higher risk of acute rejection post-transplantation,\(^{19}\) thus establishing that the ELISPOT\(_{pot}\) test, which detects alloreactive T-cells, has relevance for early clinical graft outcomes. Our previous work has focussed on chronic allograft dysfunction, where we now know that alloimmune pathology is the single biggest cause of renal allograft failure in patients surviving beyond the first-year post-transplantation.\(^{19,20,21}\) A strong prognostic biomarker for alloimmune injury is the development of de novo DSA against donor HLA\(^{22-26}\) which are associated with a >3x greater risk of graft failure, even after correction for other risk factors associated with graft loss.\(^{24,25,26}\) Graft failure is usually preceded by progressive graft dysfunction, though rate is highly variable. Although DSA are involved in mediating pathological graft damage,\(^{28}\) evidence also supports the hypothesis that T-cell-mediated effector mechanisms operate,\(^{29-31}\) which is biologically plausible, as DSA production depends on activating multiple components of the recipient immune system, including donor-specific T- and B-cells. Our previous work We have previously demonstrated the relevance of ELISPot in this context, in two separate cohorts of transplant recipient, by showing demonstrated strong correlations between ELISPot patterns of anti-donor CD4+T-cell IFN\(\gamma\) production and rates of deterioration in eGFR\(^{16-18}\), further supporting this hypothesis. Moreover, we showed for the first time that antigen presentation by B-cells associated with a more rapid eGFR decline, but that subgroups of both B- and T-cells actively suppressed donor-specific T-cell alloreactivity in vitro,\(^{16}\) which associated with slower eGFR decline. Patient samples showing active suppression by B- or T-cells had higher transitional cell numbers and CD4+CD25+CD39+Tregs respectively, than samples with no active regulation. These earlier findings formed the foundation of this work, to identify the HLA-BC phenotype associated with these ELISpot patterns.
Studying antigen-specific B-cells is a nascent field, particularly in transplantation. Identifying these cells is fundamentally challenging, due to low frequencies (typically 0.05-0.005% circulating lymphocytes) and difficulties determining purity once isolated. Most techniques rely on the principle that antigen is recognised by BCR representing the same specificity as the antibodies secreted, using fluorochrome-conjugated antigen for detection. Although there is a sizeable literature in which antigen-specific B-cells have been detected, subsequent phenotypic analysis is limited. In systemic lupus erythematosus (SLE), B-cells binding double-stranded DNA and anti-nuclear antigens are found at higher frequencies at earlier maturational stages. In contrast, in rheumatoid disease and ANCA-associated vasculitis, antigen-specific cells are disproportionately found in mature subsets, particularly CSMBC.

In transplantation, several different techniques to detect the presence of HLA-specific B-cells have been proposed, including assays that polyclonally stimulate PBMC followed by subsequent detection of either secreted IgG antibodies using single antigen beads or detection of HLA-specific B-cells in an ELISpot using labelled HLA class I or II reagents. However, both these techniques bias detection towards a CD27+ or IgG-secreting (i.e., class-switched) phenotype. Other approaches have included using HLA-tetramers to detect HLA-BC in sensitised patients with end-stage renal disease, though further characterisation was limited to the memory marker CD27 and CD38 (identifying immunoglobulin-secreting cells). More recently, HLA-coated flow beads were used to detect HLA-BC at greater frequencies in sensitised patients than controls. HLA-BC had higher proportions of non-switched memory cells, with lower CSMBC frequencies, amounting to a ten-fold difference in their ratio between HLA-binding and all B-cells.

Despite the inherent challenges, herein we describe the successful identification of HLA-BC in sensitised recipients and healthy donors, using biotinylated PureTM HLA proteins. Our major findings are summarised in figure 6. Control experiments demonstrated that antigen specifically binds the
BCR. Corresponding Pure™ HLA proteins were used as an antigen source in ELISpots to assess the contribution of B-cells, and in a second cohort Tregs, to the indirect anti-donor-HLA alloresponse. Though the vast majority of samples had HLA-binding cellBC frequencies <0.2%, in two samples 1-2.3% circulating B-cells bound HLA, all of which were IgM+CD27+cells. Interestingly, these samples were from patients who had received rituximab and had very few circulating B-cells. This is consistent with our previous description that rituximab selectively spares CD27+ memory cells.\textsuperscript{18,40-43}

However, we didn’t assess absolute numbers of B-cells in most other samples, which we acknowledge is a weakness of our methodology. In both cohorts, there was no correlation between the presence of HLA-BC and DSA, consistent with earlier reports.\textsuperscript{41-43} Our finding that isolating then adding back B-cells prior to the ELISpot was sufficient to turn a non-reactive sample into a reactive one suggests that lack of association between ELISpot reactivity and presence of HLA-BC might be due solely to the frequency of HLA-BC, relative to HLA-specific T-cell in any given sample. For some patients with clearly detectable antibodiesDSA, but no detectable HLA-BC, the most likely explanation is that the frequency of BC in these patients is too low to be detectable by our technique, in detection of this very rare population has been limited by the relatively small sample of peripheral blood available to test. Alternatively, it is conceivable that DSA in these patients may be being generated predominantly by plasma cells.

Our predominant hypothesis was that ELISpot patterns of CD4+T-cell IFN\textgreek{y} production to Pure™ HLA proteins, chosen to represent mismatched donor HLA, would strongly correlate with antigen-binding B-cell phenotype. More specifically, these phenotypes would determine how IFN\textgreek{y} production changed when B-cells were depleted. Our finding from the first cohort of an association between HLA-binding but not overall CSMBC frequency and B-dependent IFN\textgreek{y} production is consistent with this hypothesis. Sensitised patients with chronic rejection have increased frequencies of CSMBC with strong antigen-presenting capacity,\textsuperscript{13} so these new data provide a direct link between a B-dependent ELISpot and memory B-cell phenotype, specifically for the HLA-BC population. In the larger second
cohort of sensitised recipients, we also found a strong association between HLA-binding IgM+ memory cells and B-dependent IFNγ production. The importance of these memory cells, which undergo rapid secondary germinal centre reactions on activation, has been defined in responses to Rhesus D and bacteria such as tetanus, S. typhi and Pneumococcus, but this is the first data suggesting they might have significance after transplantation. There was no association between HLA-binding CSMB in cohort 2 and DSR or B-dependent DSR, and the most likely explanation is because we broadened the definition of an HLA-binding B cell for cohort 2, from a stringent definition (samples in which there were at least twice as many B-cells in the HLA-binding gate as were present without antigen) to a less stringent definition (all samples with more B-cells in the HLA-binding gate with antigen than without).

The reason we changed the definition was because depletion/add-back experiments suggested that a population of CD27- B-cells could also support IFNγ production, particularly where CD25+ T-cells were depleted, and in the second cohort we found that HLA-binding by CD27- cells associated with B-dependency, particularly HLA-binding by naïve cells and a population found within the IgMhiCD27- pool. This interesting subset comprises classical naïve B-cells, CD10+ transitional cells, and B-cells with antigen experience, expressing CD45RBMEM55. HLA-binding by CD45RBMEM55+ cells seemed to associate with B-dependent responses.

CD45RBMEM55 is a relatively novel marker expressed by memory B-cells, including a subpopulation of IgMhiCD27- B-cells, but not naïve cells. Detailed analysis suggests that IgMhiCD27-CD45RBMEM55+ cells are precursors to marginal-zone B-cells (MZB) separate from memory cells. Recently published work identified a bifurcation of human B-cell maturation from the T1 stage along two trajectories: IgMhi cells, selectively recruited to gut and spleen lymphoid tissue, and IgMlo cells, resulting in the peripheral B-cell compartment. IgMhiT2 cells share transcriptomic features with MZB; these cells were reduced in frequency in severe SLE, possibly suggesting a protective role for MZB in autoimmunity, though it remains unclear whether collapse of the MZB axis in SLE is a cause or
consequence of inflammation.\textsuperscript{50, 51} The role of MZB is still being defined, but recent work suggests
they are the main IgM producers in humans, producing both TNF\(\alpha\) & IL-10.\textsuperscript{54, 52} Our new data, to the
best of our knowledge, are the first suggesting that HLA-specific MZP may play a role in antigen
presentation of HLA in sensitised transplant patients.

Our attempts to dissect whether HLA-BC subsets are differentially susceptible to Treg suppression
strongly indicates that CD27+ memory cells support B-dependent responses when Tregs are present;
though we did not formally show they are insensitive to regulation by Tregs, this is one
interpretation. Alternatively, samples with predominantly memory HLA-BC could be the same
samples with fewer Tregs. Work is underway to distinguish these two possibilities.

In contrast, predominant HLA-binding by naïve cells/MZP associated with suppression of IFN\(\gamma\)
production by Tregs. However, HLA-binding naïve cells also strongly associated with B-suppressed
patterns, if abundant transitional cells were present. These data support our previous findings that
higher transitional cell frequencies strongly associate with regulation of IFN\(\gamma\) production by B-cells,\textsuperscript{18}
but here we show this association is with overall transitional cell frequency, not with HLA-binding
transitional cells and that regulation appears dependent on there being insufficient numbers of
memory HLA-BC alongside a higher frequency of naïve HLA-BC. Whether these naïve cells play an
active role in mediating regulation by transitional cells is not clear. These data provide a link
between established correlations between higher transitional cell frequencies and better graft
outcomes,\textsuperscript{12} and our published work correlating B-suppressed ELISpot responses with stabilisation of
graft function in patients with chronic rejection.\textsuperscript{17}

Only a few samples could be used to assess the relationship between Tregs and putative suppression
of IFN\(\gamma\) production by B-cells, so our data is\textsuperscript{preliminary, highly exploratory and therefore described in
the supplementary file}. It suggests that transitional cells do operate in the presence of Tregs, but
surprisingly that when Tregs were depleted, B-regulation associated with other, rare populations
of non-HLA-binding MZP-P may also suppress when Tregs are absent. Whether this is due to a rare
MZP population retaining CD10 expression is not certain. This population has been documented elsewhere but not discussed. The role of this intriguing population and its relationship with transitional CD10+CD45RB\textsuperscript{mem} cells and CD10-CD45RB\textsuperscript{mem}+ MZP is yet to be defined.

In summary, we have defined HLA-BC phenotypes and their associations with patterns of HLA reactivity on IFN\textgamm; ELISpot, which are known to correlate with rates of functional deterioration in patients with chronic rejection. As expected, memory HLA-BC associated strongly with B-dependent IFN\textgamm; production that was not suppressed by endogenous Tregs. Where the predominant HLA-binding phenotype was naïve B-cells, the associated ELISpot phenotype appeared to be determined by other cells: high overall transitional cell frequencies associated with B-suppressed IFN\textgamm; production, especially if Tregs were present, whereas high frequencies of HLA-binding MZP associated with B-dependent IFN\textgamm; production, particularly when Tregs were absent, hinting that the involvement of these cells is suppressible by Tregs. Finally, non-HLA-binding MZP may be able to suppress IFN\textgamm; production, though this only emerges when Tregs are depleted.

These data provide a novel foundation on which to build further studies to examine the complexities of interactions between HLA-specific T- and B-cells in sensitised patients, to identify new targets for intervention and future therapies for chronic rejection.
Disclosure

No conflicts of interest to declare.
References


Acknowledgements

The authors would like to thank the patients, nursing staff and physicians at Guy’s Hospital.
Figure legends

**Figure 1: Biotinylated proteins can be used to detect antigen-binding B-cells.**

A-F: Flow cytometric assessment of the ability of B-cells from healthy donors or HLA-sensitised renal allograft recipients to bind streptavidin BV421 after incubation with different biotinylated protein antigens. Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values.

A: In healthy volunteers, only B-cells pre-incubated with a biotinylated CMV-gpB protein, but not biotinylated BSA, nor non-biotinylated proteins bind the streptavidin BV421.

B-C: In healthy volunteers, blocking FcγRIIB with an anti-CD32 monoclonal antibody does not reduce the frequency of B-cells that bind biotinylated CMV-gpB (B), whereas competitive incubation with increasing doses of an anti-human immunoglobulin Fab to block the B-cell receptor does inhibit binding (C).

D: PBMC from sensitised transplant recipients were incubated with increasing doses of biotinylated HLA (chosen to correspond to the patients’ known sensitisation status) or control media (“0 μg/ml”), prior to assessment of streptavidin-BV421 binding to CD19+ cells and CD19- cells. Only CD19+ cells displayed significantly higher frequencies of HLA-binding after incubation with biotinylated HLA (black unfilled bars; ****p<0.0001); there was no difference between HLA-incubated CD19- cells and control cells not incubated with HLA (grey bars; p=ns).

E: PBMC from healthy donor buffy coats (‘cones’ n=3) or HLA-sensitised transplant recipients (‘patients’ n=27) were incubated with different biotinylated HLA class I antigens, chosen, in the case of patients, on the basis of their sensitisation history. 8 patients provided multiple samples for testing. Each dot is a single sample, and bars indicate median with interquartile range of the proportion of B-cells that bind streptavidin-BV421. HLA-binding B-cells are detected at higher
frequencies in sensitised patients compared to cones from healthy individuals (***, p=0.0002, Mann-Whitney test).

F: To illustrate that there is no correlation between the frequency of HLA-binding B-cells amongst total CD19+ cells, and Median Fluorescence Intensity (MFI) of the HLA-binding B-cells (linear regression line with 95% confidence interval shown; R^2 = 0.07). Black dots = patient samples; red dots = healthy cones.

G: ELISPOT assays were performed using PBMC depleted of CD8+ cells from 3 sensitised renal transplant recipients as described in methods and repeated with the addition of antibodies to HLA-DR. In all 3 experiments, blocking HLA class II resulted in a substantial reduction in the number of IFNγ-producing cells, which just failed to reach statistical significance (p=0.0708, paired t test).

Figure 2: Cohort 1: correlations between B-cell phenotypes and patterns of reactivity to HLA Pure proteins on ELISPOT.

Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values. HLA-binding B-cell phenotypes are denoted by unfilled bars; whole B-cell subset phenotypes are denoted by grey bars.

A: Samples showing antigen-reactive T-cell activity on ELISPOT (defined as showing evidence antigen-specific IFNγ production under any condition – see supplementary table 253) have significantly higher frequencies of HLA-binding class-switched memory (IgM-CD27+) B-cells, compared to samples showing no antigen-reactive T-cell activity (*p=0.0295). There is no difference between frequencies of class-switched memory B-cells in the whole B-cell population (i.e., non-HLA-binding).

B: Samples showing donor-specific reactivity (DSR, defined as IFNγ production by CD8-depleted PBMC – see supplementary table 253), have significantly higher frequencies of HLA-binding class-switched memory B-cells compared to samples showing no donor-specific reactivity (NDSR, defined...
as no IFNγ production by CD8-depleted PBMC (*p=0.0119). There is no difference between
frequencies of class-switched memory B-cells in the whole B-cell population (i.e., non-HLA-binding).

C: Samples showing B-dependent DSR (defined as spot count reduction of at least 20% when CD19+
cells depleted from CD8-depleted PBMC) have higher frequencies of HLA-binding class-switched
memory B-cells compared to samples that do not show B-dependency (**p=0.0088). There is no
difference between frequencies of class-switched memory B-cells in the whole B-cell population (i.e.,
non-HLA-binding).

D: Samples showing evidence of antigen-reactive T-cells on ELISpot (defined as in A) have lower
frequencies of transitional (CD19+CD27−CD24hiCD38hi) B-cells in whole B-cell population (i.e., non-
antigen-binding) than those showing no antigen-reactivity. (*p=0.0317). This is not seen in HLA-
binding B-cells.

**Figure 3: Overview of the analyses undertaken for cohort 2.** Each box details the ELISpot conditions
(blue) and results (red) along with the B cell subsets found to associate with the ELISpot pattern
described (red), either the overall or HLA-binding subset where indicated. Numbers of samples
analysed for each group are listed; several comparisons are limited by small sample sizes as shown.
The different comparisons undertaken and described in the text are in green.

Definitions: DSR = IFNγ by CD8-depleted PBMC; NDSR = no IFNγ production by CD8-depleted PBMC;
B-dependence = reduction in spot count of ≥20% after B-cell depletion; B-suppression = increase in
spot count of ≥20% after B-cell depletion; Analysis 3: As analysis 2, but includes samples where both
B-cells and CD25+ Tregs have been depleted from CD8-depleted PBMC.

*These 13 flow profiles included 3 from AIT017 (supp. table 4S2) that are not included in analysis 5
(hence analysis 5 only refers to 10 flow profiles)
Figure 43: Cohort 2: Consistent B-dependency, not influenced by CD25+ T-cells, correlates with memory B-cell subsets, whilst consistent B-suppression correlates with naïve and transitional B-cells.

Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values. HLA-binding B-cell phenotypes are denoted by unfilled bars; whole B-cell subset phenotypes are denoted by grey bars. Consistent B-dependence = patterns 1, 2 and 4; consistent B-regulation suppression = patterns 6, 7 and 8.

A-C: CITRUS analysis of B-cell phenotype identifies seven groups of cell clusters, two of which are illustrated here (annotated i and ii in A), that significantly correlate with consistent B-dependent phenotype in ELISpot. B shows expression of individual markers by each of the clusters. C shows phenotype of group i and ii, comparing samples showing consistent B-dependency on ELISpot (red) to samples showing consistent B-regulation suppression on ELISpot (blue).

D: Samples showing consistent B-dependency have a significantly higher frequency of IgM+ memory cells (IgM+CD27+) within both HLA-binding (**p=0.0024) and whole B-cell populations (***p=0.0003) compared to samples showing consistent B-regulation suppression.

E: Samples showing consistent B-dependency have a significantly higher frequency of marginal-zone precursors (CD10-CD45RB<sup>MEM55</sup>+CD<sup>M</sup>hiCD27-) within both HLA-binding (**p=0.0023) and whole IgM<sup>hi</sup>CD27-fraction of B-cells (****p<0.0001) compared to samples showing consistent B-regulation suppression.

F: Samples showing consistent B-dependency have a significantly higher frequency of a rare CD10<sup>-</sup>CD45RB<sup>MEM55</sup>+ subset of B-cells within the IgM<sup>hi</sup>CD27- population (i.e., non-antigen-binding) compared to samples showing consistent B-regulation suppression (**p=0.0067). There is no difference between frequencies of these cells that bind HLA.
G: Samples showing consistent B-regulation suppression have a significantly higher frequency of HLA-binding CD27- cells (**p=0.0015) compared to samples showing consistent B-dependency.

There is no difference between frequencies of these B-cells in the whole B-cell population (i.e., non-HLA-binding).

H: Samples showing consistent B-regulation suppression have a significantly higher frequency of HLA-binding IgM^hiCD27- cells (*p=0.02) compared to samples showing consistent B-dependency.

There is no difference between frequencies of these B-cells in the whole B-cell population (i.e., non-HLA-binding).

I: Samples showing consistent B-regulation suppression have a significantly higher frequency of CD24^hiCD38^hi transitional cells within the CD27- population of B-cells (i.e., non-antigen-binding) compared to samples showing consistent B-dependency (**p=0.0069). There is no difference between frequencies of HLA-binding transitional B-cells.

J: Samples showing consistent B-regulation suppression have significantly higher frequencies of both HLA-binding (**p=0.0013) and non-HLA-binding (**p=0.0044) CD10^-CD45RB^MEM55^- cells within the IgM^hiCD27- compartment compared to samples showing consistent B-dependence.

**Figure 54**: Cohort 2: ELISpot patterns where the presence or absence of CD25+ cells influences B-dependent reactivity have significantly different B-cell subset compositions.

Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values. HLA-binding B-cell phenotypes are denoted by unfilled bars; whole B-cell subset phenotypes are denoted by grey bars.

A-C: CITRUS analysis identifies two groups of cells that significantly correlate with B-dependence that only occurs in the presence of CD25+ cells (pattern 1) and two groups that significantly correlate with B-dependence that is suppressed by the presence of CD25+ cells (pattern 4).
D: Pattern 1 (B-dependent in the presence of CD25+ cells) associates with higher frequencies of HLA-binding IgM+ memory cells (*p=0.0123).

E: Pattern 4 (B-dependent in the absence of CD25+ cells) associates with higher frequencies of HLA-binding IgM\(^\text{hi}\)CD27- (*p=0.0124) and overall IgM\(^\text{hi}\)CD27- cells (**p<0.0001).

F-I: There were no significant associations between patterns 1 or 4 and any of the four IgM\(^\text{hi}\)CD27- subsets (defined as transitional CD10+CD45RB\(^\text{MEM55}^-\); CD10+CD45RB\(^\text{MEM55}^+\); CD10-CD45RB\(^\text{MEM55}^-\)MZP; and naive CD10-CD45RB\(^\text{MEM55}^+\)), in both the HLA-binding and non-HLA-binding populations.

**Figure 5:** Cohort 2: ELISpot patterns where the presence or absence of CD25+ cells influences B-regulation have significantly different B-cell subset compositions.

**Figure 6:** Summary of the major associations described for different B cell subsets.

**Figure 6** is based around the gating strategy used to define CD19+ B cells (A), and beyond that subsets based on CD27 expression either with IgM (B) or without (C). The IgM\(^\text{hi}\)CD27- subset in B is further defined by CD10 and CD45RB\(^\text{MEM55}^+\) expression (D). The CD27- fraction in C is further defined by CD38 and CD24 expression (E). The table (F) details the associations between the subsets defined in B-E (HLA-binding and total populations) with ELISpot patterns.

Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values. HLA-binding B-cell phenotypes are denoted by unfilled bars; whole B-cell subset phenotypes are denoted by grey bars.

A-C: CITRUS analysis identifies one group of cells that significantly correlate with B-regulation that only occurs in the presence of CD25+ cells (pattern 7) and three groups that significantly correlate with B-regulation that is suppressed by the presence of CD25+ cells (pattern 6).

D-E: Pattern 7 (B-regulation in the presence of CD25+ cells) associates with higher overall frequencies of IgM\(^\text{hi}\)CD27- cells (D, *p=0.0263) and IgM\(^\text{hi}\)CD27-CD10+CD45RB\(^\text{MEM55}^+\) transitional cells (E, *p=0.0127).
F: Pattern 6 (B-regulation in the absence of CD25+ cells) associates with higher overall frequencies of IgM<sup>hi</sup>CD27−CD10−CD45RB<sub>MEM55</sub>−MZP cells (**p=0.0007).

Supplementary figure 1: Representative flow cytometry plots illustrating the effect of cell depletion on lymphocyte subsets used in ELISpot assays.

A: Frequencies of CD8+, CD19+ and CD25+ (including CD25<sup>hi</sup>) cells in whole PBMC. Dynabeads effectively deplete CD8+ cells (B), CD8+ and CD19+ cells (C), CD8+ and CD25<sup>hi</sup> cells (D) and CD8+, CD19+ and CD25<sup>hi</sup> cells (E). Comparison of in.

(A) C: (B)C. Statistical comparison

Supplementary figure 2: Illustrative flow cytometry plots depicting HLA-binding B-cell detection and gating strategies. All plots were pre-gated on single, live CD19+ cells.

A and B: Two patient samples illustrating the detection of HLA-binding B-cells. The presence of HLA-binding B-cells was confirmed by an increased frequency seen in samples incubated with biotinylated antigen compared to the “background” frequency in controls (A). If this frequency at least doubled, further phenotyping of HLA-binding B-cells was undertaken (B). Illustrative s

C&E: Gating strategy for further phenotyping of live CD19+ cells: D – (cohort 1; E – additional phenotyping of cohort 2. D: CD19+ B cells stained with IgM, CD27, CD38 and CD24 to define the subsets illustrated. E: additional staining with CD45RB<sub>MEM55</sub> and IgD to define the subsets illustrated).

D: Gating strategy for further phenotyping of live CD19+ cells (cohort 2). Note that transitional cells can be defined as either CD27−CD24<sup>hi</sup>CD38<sup>ki</sup>, or IgM<sup>hi</sup>CD27−CD10+CD45RB<sub>MEM55</sub>−.

Supplementary figure 3: A series of ELISpot experiments examining the effect of depleting and adding back various B-cell subsets, as indicated for each bar. A threshold of 10 spots per million
CD4+ cells normalised for background was set as the threshold for defining activity, as indicated by the horizontal red line.

Supplementary figure 4: Representative flow cytometric data confirming that the depleted CD25+ fraction is CD4+ and CD19-. 3.13% of the CD25+ depleted fraction were CD4-negative cells; one third of these were CD8+; the remaining two thirds were CD4-negative, CD8-negative, CD19-negative and CD14-negative and could not be characterised further.

Supplementary figure 5: Cohort 2: patterns of ELISpot reactivity in the presence of CD19+ and CD25+ cells by CD8-depleted PBMC correlate with specific B-cell subset frequencies.

Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values. HLA-binding B-cell phenotypes are denoted by unfilled bars; whole B-cell subset phenotypes are denoted by grey bars. DSR = donor-specific reactivity; NDSR = no DSR.

A-C: CITRUS analysis of B-cell phenotype identifies six groups of cell clusters, two of which are further illustrated here (annotated i and ii in A), that significantly correlate with DSR on ELISpot. B shows expression of individual markers by each of the cell clusters. C shows phenotype of group i and ii, comparing samples showing DSR on ELISpot (red) to samples showing NDSR on ELISpot (blue).

D: Samples showing DSR have a significantly higher frequency of class-switched memory (IgM-CD27+) B-cells in the whole population (i.e., non-antigen-binding) compared to samples showing NDSR (**p=0.0281). There is no difference between frequencies of HLA-binding class-switched memory B-cells.

E: The same associations with class-switched memory cells are seen when they are defined as IgD- IgM-: higher overall frequencies of IgD-IgM- cells associate with DSR (**p=0.0039), but there is no difference between the HLA-binding B-cells.
F: DSR samples also have significantly higher overall frequencies of IgM+ memory cells than NDSR samples (**p=0.0028), but no differences are seen in the HLA-binding populations of these cells.

G: Samples showing NDSR have significantly higher frequencies of CD27− cells B-cells in the whole population (i.e., non-antigen-binding) compared to samples showing DSR (**p=0.0036). There is no difference between frequencies of HLA-binding class-switched memory B-cells.

H: Samples showing NDSR have significantly higher frequency of IgM^{hi}CD27− cells B-cells in the whole population (i.e., non-antigen-binding) compared to samples showing DSR (**p=0.0018). There is no difference between frequencies of HLA-binding class-switched memory B-cells.

I: Supplementary figure 6: Cohort 2: in the presence of CD25+ cells, B-dependency correlates with memory B-cell subsets, whilst B-suppression correlates with naïve and transitional B-cells.

Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values. HLA-binding B-cell phenotypes are denoted by unfilled bars; whole B-cell subset phenotypes are denoted by grey bars.

A-C: CITRUS analysis of B-cell phenotype identifies four groups of cell clusters, two of which are further illustrated here (annotated i and ii in A), that significantly correlate with a B-dependent DSR pattern in ELISpot. B shows expression of individual markers by each of the cell clusters. C shows phenotype of group i and ii, comparing samples showing B-dependent DSR on ELISpot (red) to samples showing evidence of B-cell suppression in the presence of Tregs (blue).

D: Samples showing B-dependent DSR have a significantly higher frequency of class-switched memory (IgM−CD27+) B-cells in the whole population (i.e., non-antigen-binding) compared to samples showing evidence of B-cell suppression in the presence of Tregs (∗p=0.0125). There is no difference between frequencies of HLA-binding class-switched memory B-cells.
E: Samples showing evidence of B-cell suppression in the presence of Tregs have significantly higher proportions of naïve B-cells (IgM$^{hi}$CD27$^{-}$) in both the HLA-binding (*p=0.011) and whole B-cell (i.e., non-antigen-binding) populations (****p<0.0001) compared to samples showing B-dependent DSR.

F: Samples showing B-dependent DSR have a significantly higher frequency of IgM memory (IgM$^{+}$CD27$^{+}$) B-cells in both the HLA-binding (**p=0.0012) and whole B-cell (i.e., non-antigen-binding) populations (*p=0.0367) compared to samples showing evidence of B-cell suppression in the presence of Tregs.

G: Samples showing B-dependent DSR have a significantly higher frequency of HLA-binding marginal-zone precursors (CD10$^{-}$CD45RB$^{MEM55}$) within the IgM$^{hi}$CD27$^{-}$ fraction of B-cells compared to samples showing evidence of B-cell suppression in the presence of Tregs (**p=0.0059). There is no difference between frequencies of these B-cells in the whole B-cell population (i.e., non-HLA-binding).

H: Samples showing evidence of B-cell suppression in the presence of Tregs have significantly higher proportions of non-memory B-cells (CD27$^{-}$) in both the HLA-binding (***p=0.0003) and whole B-cell populations (*p=0.0253) compared to samples showing B-dependent DSR.

I: Samples showing evidence of B-cell suppression in the presence of Tregs have a significantly higher frequency of transitional B-cells (CD10$^{+}$CD45RB$^{MEM55}$) within the IgM$^{hi}$CD27$^{-}$ fraction of whole B-cells (i.e., non-antigen-binding) compared to samples showing B-dependent DSR (**p=0.0041). There is no difference between frequencies of HLA-binding transitional B-cells.

J: No differences are seen in CD10$^{+}$CD45RB$^{MEM55}$ cell frequency between Bdep and Bsupp samples.

K: Samples showing evidence of B-cell suppression in the presence of Tregs have significantly higher frequencies of naïve CD10$^{-}$CD45RB$^{MEM55}$-HLA-binding B-cells within the IgM$^{hi}$CD27$^{-}$ subpopulation compared to samples showing B-dependence in the presence of Tregs (*p=0.0129). There is no difference however between frequencies of non-HLA-binding B-cells.
Supplementary figure 7: Additional CITRUS results discriminating consistent B-dependence and consistent B-suppression. To complement the data included in main Figure 4.

Supplementary figure 8: Additional CITRUS results discriminating patterns 1 and 4. To complement the data included in main Figure 5.

Supplementary figure 9: Additional CITRUS results discriminating patterns 6 and 7.
Figure 1

190x338mm (300 x 300 DPI)
Figure 2

190x338mm (300 x 300 DPI)
Figure 3

190x338mm (300 x 300 DPI)
Figure 4

190x338mm (300 x 300 DPI)
Figure 5 – Analysis 4 Cohort 2

190x338mm (300 x 300 DPI)
**Figure 6**

190x338mm (300 x 300 DPI)

*The working hypothesis, for B-suppression (defined as ≥20% increase in number of IgM spots when B-cells are depleted) is that antigen presentation is by non-B cells such as monocytes or dendritic cells.*
Supplementary results

Manipulating B-cell subsets in ELISpots using CMV-gpB reveals additional complexity and the importance of assessing B-cell/Treg interactions

These exploratory ELISpots using cones and the model antigen CMV-gpB, were conducted to validate the phenotypic correlations found in the patient samples from cohort 1. We magnetic-bead sorted various B-cell fractions and added back to ELISpot wells (supp.fig.S4).

The caveat with these data is that functional assays using positively selected subsets must be interpreted with caution. Firstly, in two samples that were NR to CMV-gpB, re-addition of either whole CD19+ cells (supp.fig.S4A) or re-combined CD27+/CD27-fractions (supp.fig.S4B) to CD19-depleted wells resulted in antigen-specific responses, suggesting that responses are partly due to the proportions of B-cells present, potentially explaining why some patients varied between DSR/NDSR. Secondly, in one B-dependent sample, spot counts increased to those in CD8-depleted PBMC when whole CD19+B-cells (and re-combined CD27+/CD27-fractions) were added back to CD19-depleted wells (supp.fig.S4C), but also when only CD27-cells were added back (but not CD27+ alone), an observation inconsistent with findings from cohort 1.

In these experiments, we also depleted CD25+cells, as reported previously\textsuperscript{51-53} (effect shown in supp.fig.S1D-E). In 3 of 4 cones where reactivity was only revealed after CD25+ depletion, additional CD19+ depletion reduced spot counts, indicating B-dependence (supp.fig.S4D-H).

In all 3, adding back CD19+cells or only CD27-cells restored spot counts. In contrast, adding back CD27+cells only restored spot counts in 1 sample (supp.fig.S4D). To explain these findings, we assessed the impact of CD25+ depletion (supp.fig.S10): only CD25\textsuperscript{hi} cells were depleted; >95% of the depleted fraction was CD4+ and none were CD19+, confirming that B-
cells were not inadvertently lost. These data suggest that a CD27- B-cell subpopulation can
support IFNγ production by antigen-specific T-cells.

In the fourth sample, reactivity was only revealed following CD19+ depletion, suggesting
that a B-cell subpopulation was suppressing IFNγ production. Adding back CD27-cells
suppressed the response (supp.fig.S4G), suggesting that a putative Breg population was
CD27-. Adding back whole CD19+cells suppressed the response, as did CD27-CD10+cells,
and CD27-CD45RBMEM55+ cells, but not the CD27-CD10-CD45RBMEM55- fraction (supp.fig.S4H),
suggesting that suppressing IFNγ production is a property of a subpopulation(s) found
within CD27-, CD10+ and CD45RBMEM55+ B-cells.

**Exploratory analyses in a second, larger cohort of sensitised transplant recipients**

15/50 (30%) ELISpots displayed reactivity, under any condition, to HLA. Of these, 7 with DSR
could be further defined as B-dependent DSR (n=4) which either became non-reactive after
CD25+ depletion (pattern 1, supp.table S4; n=3), or the B-cells adopted a suppressive
phenotype after CD25+ depletion (pattern 3; n=1). 3 were B-suppressed DSR, which either
became B-dependent after depletion of CD25+cells (pattern 5; n=2), or became non-
reactive after CD25+ depletion, (pattern 7; n=1). Of the remaining 8 NDSR samples, 3 were
B-suppressed (i.e., showed reactivity after CD19+ depletion); 2 of these became non-
reactive after CD25 depletion (pattern 7) and in the third, B-cells maintained an apparent
suppressive phenotype after CD25-depletion (pattern 8). In the remaining 5 NDSR samples,
HLA responses only became apparent after CD25+ depletion. 4 were B-dependent (pattern
4), and 1 showed B-suppression (pattern 6). Thus, the inclusion of CD25-depletion allowed

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*N.B. No samples tested in this cohort showed B-dependence in both the presence and absence of CD25+ cells (i.e., pattern 2 in supp.table 4).*
definition of samples where B cell phenotypes were apparently dependent on the presence or absence of Tregs.

HLA-BC were detected flow-cytometrically in 49/113 samples (43.4%). As in cohort 1, there was no significant association between ELISpot reactivity and the presence of HLA-BC (p=0.1642, Fisher’s test). Multiple flow cytometric profiles are available for correlation with each ELISpot, as for each patient a range of biotinylated HLA Pure™ protein concentrations were incubated with multiple PBMC samples.

B-cell-mediated suppression and interaction with Tregs – exploratory data from small numbers of samples

We compared 3 samples showing a B-suppressed pattern with CD25+ cells present (pattern 7, supp.tables S2&S4; 7 flow profiles), with 1 sample showing a B-suppressed pattern only with CD25+ cells absent (pattern 6, supp.tables S2&S4; 3 flow profiles).

CITRUS analysis (supp.fig.S9) revealed abundant naïve cells (IgM+IgD+CD27-CD45RB\textsuperscript{MEM55-}, supp.fig.S9Ci), transitional cells (IgM\textsuperscript{hi}CD27-CD24+CD38\textsuperscript{hi}CD45RB\textsuperscript{MEM55-}CD10+, supp.fig.S9Cii) and the rare B-cell population with the phenotype IgM+CD27-CD10+CD45RB\textsuperscript{MEM55+}(supp.fig.S9Ciii), associating with B-suppression when Tregs were present, whereas MZP (IgM+CD27-CD10-CD45RB\textsuperscript{MEM55}, supp.fig.S9Civ) were more abundant in samples with B-suppression only when Tregs were absent. In the supervised analysis, there were no significant differences in HLA-binding subpopulations (supp.fig.S9D-F), but the whole B-cell findings were consistent with the CITRUS results. This data supports the hypothesis that non-HLA-binding CD10+ transitional cells, and potentially a rare population of CD27-CD10+CD45RB\textsuperscript{MEM55+} cells, are involved in suppressing antigen presentation by non-B-cell APC when Tregs are present, whereas non-HLA-binding MZP may suppress
antigen presentation by non-B-cell APC when Tregs are absent. Caution is however required, since the data are derived from comparison of only a few samples.

**Supplementary References**


Supplementary figure S1: Representative flow cytometry plots illustrating the effect of cell depletion on lymphocyte subsets used in ELISpot assays.
A: Frequencies of CD8+, CD19+ and CD25+ (including CD25^{hi}) cells in whole PBMC.

Dynabeads effectively deplete CD8+ cells (B), CD8+ and CD19+ cells (C), CD8+ and CD25^{hi}
cells (D) and CD8+, CD19+ and CD25^{hi} cells (E).
Supplementary figure S2: Comparison of HLA-BC frequencies in samples with reactive and non-reactive ELISpot results.

(A) Cohort 1; (B) Cohort 2. Statistical comparison by Mann-Whitney testing.
Supplementary figure S3

A

0µg/ml bromelainated B^*44:02

HLA-binding 0.0693%

“Background”

Greater frequency than background: HLA-binding B cells present. However, no further phenotyping undertaken as frequency has not doubled.

HLA B44

1µg/ml bromelainated B^*44:02

HLA-binding 0.1039%

Greater frequency than background: HLA-binding B cells present.

B

0µg/ml bromelainated A^*01:01

HLA-binding 0.0431%

“Background”

Greater frequency than background AND more than doubled in frequency: further phenotyping of HLA-binding B cells undertaken.

HLA A1

2µg/ml bromelainated A^*01:01

HLA-binding 0.0686%

A1 = self antigen; the frequency of cells in the HLA-binding gate is less than half that in the sample tested against B44 below.

HLA A1

The same patient, known to be sensitized to B44. The frequency of cells in the HLA-binding gate is more than double that in the sample tested against self antigen (A1) above.

C

D

E

Lymphocytes

Single cells

Live cells

CD19+ cells

IgM+ CD27+ memory

IgM+ memory and MZ

CD27+ Class-switched memory

Transitional

CD19+ CD45RA+

IgM- memory and MZ

CD27+ Class-switched memory

Transitional

CD19+ CD45RA+

IgM- memory and MZ

CD27+ Class-switched memory

Transitional

CD19+ CD45RA+
**Supplementary figure S3**: Illustrative flow cytometry plots depicting HLA-binding B-cell detection and gating strategies. All plots were pre-gated on single, live CD19+ cells.

A and B: Two patient samples illustrating the detection of HLA-binding B-cells. The presence of HLA-binding B-cells was confirmed by an increased frequency seen in samples incubated with biotinylated antigen compared to the “background” frequency in controls (A). If this frequency at least doubled, further phenotyping of HLA-binding B-cells was undertaken (B).

C: Illustrative samples from a patient with HLA antibodies to B44, also tested against the self-antigen A*01:01. The frequency of cells in the B44-binding gate is more than double that in the A1-binding gate.

D&E: Gating strategy for further phenotyping of live CD19+ cells: D – cohort 1; E – additional phenotyping of cohort 2. D: CD19+ B cells stained with IgM, CD27, CD38 and CD24 to define the subsets illustrated. E: additional staining with CD45RB<sup>MEM55</sup> and IgD to define the subsets illustrated.
Supplementary figure S4
Supplementary figure S4: A series of ELISpot experiments examining the effect of depleting and adding back various B-cell subsets, as indicated for each bar. A threshold of 10 spots per million CD4+ cells normalised for background was set as the threshold for defining activity, as indicated by the horizontal red line.
Supplementary figure S5 – Analysis 1 cohort 2

A

B

CD10

IgD

IgM

CD45RB

CD27

CD24

CD38

C

DSR

NDSR

CD45RB

CD27

CD24

CD10

IgD

IgM

CD38

Background

Cluster
Supplementary figure S5 cont.

**Supplementary figure S5: Cohort 2: patterns of ELISpot reactivity by CD8-depleted PBMC correlate with specific B-cell subset frequencies.**

Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values. HLA-binding B-cell phenotypes are denoted by unfilled bars; whole B-cell subset phenotypes are denoted by grey bars. DSR = donor-specific reactivity; NDSR = no DSR.

A-C: CITRUS analysis of B-cell phenotype identifies six groups of cell clusters, two of which are further illustrated here (annotated i and ii in A), that significantly correlate with DSR on
ELISpot. B shows expression of individual markers by each of the cell clusters. C shows phenotype of group i and ii, comparing samples showing DSR on ELISpot (red) to samples showing NDSR on ELISpot (blue).

D: Samples showing DSR have a significantly higher frequency of class-switched memory (IgM-CD27+) B-cells in the whole population (i.e., non-antigen-binding) compared to samples showing NDSR (**p=0.0281). There is no difference between frequencies of HLA-binding class-switched memory B-cells.

E: The same associations with class-switched memory cells are seen when they are defined as IgD-IgM:- higher overall frequencies of IgD-IgM- cells associate with DSR (**p=0.0039), but there is no difference between the HLA-binding B-cells.

F: DSR samples also have significantly higher overall frequencies of IgM+ memory cells than NDSR samples (**p=0.0028), but no differences are seen in the HLA-binding populations of these cells.

G: Samples showing NDSR have significantly higher frequencies of CD27- cells B-cells in the whole population (i.e., non-antigen-binding) compared to samples showing DSR (**p=0.0036). There is no difference between frequencies of HLA-binding class-switched memory B-cells.

H: Samples showing NDSR have significantly higher frequency of IgMhiCD27- cells B-cells in the whole population (i.e., non-antigen-binding) compared to samples showing DSR (**p=0.0018). There is no difference between frequencies of HLA-binding class-switched memory B-cells.

I: Samples showing DSR have significantly higher frequencies of HLA-binding IgMhiCD27-CD45RBMEM55+ B-cells compared to samples showing NDSR (*p=0.0419). There is no difference between overall frequencies of IgMhiCD27-CD45RBMEM55+ B-cells.
Supplementary figure S6 – Analysis 2 cohort 2

A

B

C

D

E

F

G

H

I

J

K
Supplementary figure S6: Cohort 2: in the presence of CD25+ cells, B-dependency correlates with memory B-cell subsets, whilst B-suppression correlates with naïve and transitional B-cells.

Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values. HLA-binding B-cell phenotypes are denoted by unfilled bars; whole B-cell subset phenotypes are denoted by grey bars.

A-C: CITRUS analysis of B-cell phenotype identifies four groups of cell clusters, two of which are further illustrated here (annotated i and ii in A), that significantly correlate with a B-dependent DSR pattern in ELISpot. B shows expression of individual markers by each of the cell clusters. C shows phenotype of group i and ii, comparing samples showing B-dependent DSR on ELISpot (red) to samples showing evidence of B-cell suppression in the presence of Tregs (blue).

D: Samples showing B-dependent DSR have a significantly higher frequency of class-switched memory (IgM-CD27+) B-cells in the whole population (i.e., non-antigen-binding) compared to samples showing evidence of B-cell suppression in the presence of Tregs (*p=0.0125). There is no difference between frequencies of HLA-binding class-switched memory B-cells.

E: Samples showing evidence of B-cell suppression in the presence of Tregs have significantly higher proportions of naïve B-cells (IgM\text{hi}CD27-) in both the HLA-binding (*p=0.011) and whole B-cell (i.e., non-antigen-binding) populations (****p<0.0001) compared to samples showing B-dependent DSR.

F: Samples showing B-dependent DSR have a significantly higher frequency of IgM memory (IgM+CD27+) B-cells in both the HLA-binding (**p=0.0012) and whole B-cell (i.e., non-
antigen-binding) populations (*p=0.0367) compared to samples showing evidence of B-cell suppression in the presence of Tregs.

G: Samples showing B-dependent DSR have a significantly higher frequency of HLA-binding marginal-zone precursors (CD10-CD45RB^MEM55^+) within the IgM^{hi}CD27^- fraction of B-cells compared to samples showing evidence of B-cell suppression in the presence of Tregs (***p=0.0059). There is no difference between frequencies of these B-cells in the whole B-cell population (i.e., non-HLA-binding).

H: Samples showing evidence of B-cell suppression in the presence of Tregs have significantly higher proportions of non-memory B-cells (CD27-) in both the HLA-binding (***p=0.0003) and whole B-cell populations (*p=0.0253) compared to samples showing B-dependent DSR.

I: Samples showing evidence of B-cell suppression in the presence of Tregs have a significantly higher frequency of transitional B-cells (CD10^CD45RB^MEM55^-) within the IgM^{hi}CD27^- fraction of whole B-cells (i.e., non-antigen-binding) compared to samples showing B-dependent DSR (***p=0.0041). There is no difference between frequencies of HLA-binding transitional B-cells.

J: No differences are seen in CD10^CD45RB^MEM55^+ cell frequency between Bdep and Bsupp samples.

K: Samples showing evidence of B-cell suppression in the presence of Tregs have significantly higher frequencies of naïve CD10^CD45RB^MEM55^- HLA-binding B-cells within the IgM^{hi}CD27^- subpopulation compared to samples showing B-dependence in the presence of Tregs (*p=0.0129). There is no difference however between frequencies of non-HLA-binding B-cells.
Supplementary figure S7: Additional CITRUS results discriminating consistent B-dependence and consistent B-suppression. To complement the data included in main Figure 4.
Supplementary figure S8 – Analysis
4 Cohort 2

A

B

C

D

The International Society of Nephrology (http://www.isn-online.org/site/cms)
Supplementary figure S8 cont.
**Supplementary figure S8**: Additional CITRUS results discriminating patterns 1 and 4. To complement the data included in main Figure 5.
Supplementary figure S9 – Analysis 5 Cohort 2

A

B

CD10

IgD

IgM

CD45RB

CD27

CD24

CD38

C

Pattern 6

Pattern 7

CD45RB

CD27

CD24

CD10

IgD

IgM

CD38

D

E

F

The International Society of Nephrology (http://www.isn-online.org/site/cms)
Supplementary figure S9 cont.

G
Supplementary figure S9 H
Supplementary figure S9 H cont.
**Supplementary figure S9:** Cohort 2: ELISpot patterns where the presence or absence of CD25+ cells influences B-suppression have significantly different B-cell subset compositions.

Box and whisker plots: boxes indicate the interquartile range with a line at the median; whiskers indicate the minimum and maximum values. HLA-binding B-cell phenotypes are denoted by unfilled bars; whole B-cell subset phenotypes are denoted by grey bars.

A-C: CTRUS analysis identifies one group of cells that significantly correlate with B-suppression that only occurs in the presence of CD25+ cells (pattern 7) and three groups that significantly correlate with B-suppression that is suppressed by the presence of CD25+ cells (pattern 6).

D-E: Pattern 7 (B-suppression in the presence of CD25+ cells) associates with higher overall frequencies of IgMhiCD27- cells (D, *p=0.0263) and IgMhiCD27-CD10+CD45RBMEM55- transitional cells (E, *p=0.0127).

F: Pattern 6 (B-suppression in the absence of CD25+ cells) associates with higher overall frequencies of IgMhiCD27-CD10-CD45RBREM55+ MZP cells (**p=0.0007).

G and H: Additional CTRUS results discriminating patterns 6 and 7.
Supplementary figure S10: Representative flow cytometric data confirming that the depleted CD25+ fraction is CD4+ and CD19-. 3.13% of the CD25+ depleted fraction were CD4-negative cells; one third of these were CD8+; the remaining two thirds were CD4-negative, CD8-negative, CD19-negative and CD14-negative and could not be characterised further.
### Supplementary table S1

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*In the absence of detectable DSA, the Pure™ protein used in ELISPOT was identical to a mismatched donor HLA. This was not applicable for the three cone samples tested.

DSR = donor-specific reactivity, defined as IFNγ spot count ≥ 25 spots per million CD4+ cells in CD8-depleted wells; NDSR = no donor-specific reactivity, defined as IFNγ spot count < 25 spots per million CD4+ cells in CD8-depleted wells; NR = non-reactive under any conditions.

For definitions of B-dependent, B-suppressed and B-independent please see supplementary table S3.

†Further phenotyping was undertaken if the frequency of HLA-binding cells doubled compared to the control (see supplementary figure S1B).

‡Staining for transitional cells was not available for these samples.
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**Notes:**
- eGFR: Estimated Glomerular Filtration Rate
- Albuminuria: urine protein measured in mg/minute
- Serum Lipids: cholesterol and triglycerides

*Data and analysis conducted by the research team.*
## Supplementary table S3

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<td>Not exist</td>
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*Definitions:*
- Spot counts meeting threshold for antigen-reactive IFNγ production. Threshold determined by background: If any unstimulated well (i.e. no antigen) has ≥50 spots (i.e. “high background”), the spot count in stimulated wells (i.e. with antigen) must have ≥5 spots per 1x10⁶ CD4+ cells greater than background; if all unstimulated wells have <50 spots, the spot count normalised for background must be ≥10 spots per 1x10⁶ CD4+ cells.
- Spot counts fail to reach the thresholds described for ‘-’.
- B-dependent: Decrease in spot count of ≥20% when CD19+ cells depleted.
- Breg: Increase in spot count of ≥20% when CD19+ cells depleted.
- Treg: Increase in spot count of ≥20% when CD25+ cells depleted.
The phenotype of HLA-binding B cells from sensitised renal transplant recipients correlates with clinically prognostic patterns of IFN-γ production against purified HLA proteins.

**Methods**

1. Flow cytometric detection of HLA-binding B cells using biotinylated HLA Pure™ proteins, matched to known sensitisation status

2. Anti-HLA IFN-γ CD4+ T cell ELISpot assay (matched to Pure™ protein)

**Outcomes**

1. Biotinylated antigen specifically binds to B cells and can be used to detect HLA-binding B cells in sensitised renal transplant recipients

2. B cell subset composition and the presence or absence of Tregs influences IFN-γ production as follows:

   - **Antigen presentation by B cells:**
     - HLA-binding CD27+ cells at higher frequency than HLA-binding CD27- cells
     - HLA-binding CD27- cells at higher frequency than HLA-binding CD27+ cells: suppressible by Tregs

   - **Antigen presentation by non-B cells:**
     - Transitional B cells associate with suppression of antigen presentation by non-B APC in presence of Treg:
     - MZP B cells associate with suppression of antigen presentation by non-B APC in absence of Treg:

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**Abbreviations:**
- APC: antigen presenting cell
- CD27: memory cell marker
- HLA: human leucocyte antigen
- IFN-γ: interferon gamma
- MZP: marginal zone precursor
- PBMC: peripheral blood mononuclear cells
- Treg: regulatory T cell

*Burton 2021*