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Alemtuzumab Treatment Leads to Delayed Recovery of T Follicular Regulatory Cells, and May Therefore Predispose Patients to *de novo* Donor-Specific Antibody Formation

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Abstract

Background: T follicular helper (Tfh) and regulatory (Tfr) cells are key players in the formation of long-lived antibody responses. Their circulating counterparts, cTfh and cTfr, are often used as biomarkers because longitudinal sampling of secondary lymphoid tissues is unfeasible in clinical studies. This is the first study to track cTfh and cTfr cells following therapeutic lymphocyte depletion with alemtuzumab, an anti-CD52 monoclonal antibody, to infer the influence of this treatment on the germinal centre response.

Methods: Samples from 60 transplant recipients (41 kidney-alone, 19 simultaneous kidney-pancreas (SPK)) were analysed at the time of transplant, and at regular intervals post-transplant for both flow-cytometric immunophenotyping, and Luminex-based donor-specific antibody (DSA) assessment.

Results: Patients treated with alemtuzumab (19 SPK recipients (group A-SPK) and 23 kidney-alone (group A-K) recipients) had a significantly lower ratio of cTfr to cTfh at all time points post-transplant compared to patients treated with basiliximab (group B-K). We found that, despite a high proportion of Tregs in the recovering cell population, cTfr cells did not repopulate in alemtuzumab treated patients, while cTfh cells reconstituted to higher than pre-transplant levels over the 2-year follow-up.

Conclusions: Alemtuzumab has been used as first-line induction immunosuppression and treatment for steroid-resistant rejection in transplantation; our data suggests alemtuzumab-treated patients have a lower cTfr to cTfh ratio even 2 years post-transplant, and may therefore be at higher risk of *de novo* DSA formation.

Keywords

Donor specific antibodies; T follicular helper cell; T follicular regulatory cell

1. Introduction

Alemtuzumab is a monoclonal antibody directed against CD52, a marker found on the majority of lymphoid cells [1]. Administration causes rapid and profound lymphocyte depletion with slow recovery of first B cells, then CD8 T cells and finally CD4 T cells [2-4]. It is widely used to treat autoimmunity, particularly in active relapsing multiple sclerosis (MS) [5]. It is also in routine use as an induction immunosuppression agent in solid organ transplantation [6-9].

While alemtuzumab is an effective immunosuppressant, it is paradoxically associated with the development of secondary autoimmune disease in up to a third of patients [10, 11] In transplant patients the rate of post-alemtuzumab autoimmunity is lower [12]. However, 20%-30% of solid organ recipients will develop *de novo* DSAs post-transplantation [13-15], both shortening graft lifespan and reducing success rates of future transplants [16-18] and there appears to be an increased risk of developing *de novo* donor specific antibodies (DSAs) in those receiving alemtuzumab compared to those receiving alternative therapies despite lifelong immunosuppressive therapy [9, 19, 20].

In order to generate a long-lived antibody response to protein antigen B cells require T cell help [21, 22]. Long-lived antibody secreting plasma cells and memory B cells arise from the germinal centre (GC) response, a specialised microenvironment within the B cell follicles of secondary lymphoid organs (SLOs). Here, GC B cells and specialised subset of CD4 T cells known as T follicular helper (Tfh) cells interact, resulting in the positive selection of high-affinity GC B cells that can differentiate into long-lived plasma cells and memory B cells [23]. Tfh cells express the transcription factor Bcl6, which allows them to upregulate CXCR5 and enter the B cell follicle to support the GC response [24]. They also express high levels of PD-1 and ICOS, secrete IL-21, and contain pre-formed CD40L that can be rapidly expressed on the cell surface to provide CD40 signalling to GC B cells [25].

Uncontrolled GC responses can lead to spontaneous autoimmunity. In the presence of excessive Tfh cells, self-reactive B cells can receive help to produce autoantibodies [26, 27]. Thus, control of the GC response is essential. Regulatory T cells (Tregs) are an important mechanism of

immune control. $CD4^+$ Tregs can either be thymically-derived or peripherally-induced and are characterised by expression of the transcription factor FOXP3 [28, 29]. In addition, Tregs can express transcription factors traditionally associated with other Th subsets alongside FOXP3 and tailor their suppression to the response generated [30-33]. As such Tregs can co-opt the Tfh pathway, expressing BCL6 alongside FOXP3, and upregulating surface markers associated with Tfh, such as CXCR5, ICOS and PD-1, allowing them to enter the B cell follicle, and limit the size of the GC response [32, 34, 35].

The collaboration between B cells, Tfh cells and Tfr cells leads to a high-affinity, highly specific and long-lived antibody response, with the capacity for rapid memory responses. Circulating Tfh-like cells (cTfh) have been identified in both humans and mice, and evidence suggests they are generated both before and during GC formation [36, 37]. They can be seen in the circulation of both mice and humans early after immunisation [38-40], and express lower levels of CXCR5 and PD-1 compared to GC Tfh cells [40, 41]. Those circulating $CXCR5^+CD4^+$ cells expressing PD-1 are thought to represent early memory Tfh, consistent with an active GC response and Tfh cell differentiation in SLOs [36, 40]. cTfh cells can also express different surface receptors and produce cytokines consistent with Th1, Th2 and Th17-like skewing. The surface markers CXCR3 and CCR6 have been used to divide $CXCR5^+$ cTfh into $CXCR3^+$ Th1-like cells, $CCR6^+$ Th17-like cells and $CXCR3^-$ $CCR6^-$ Th2 like cells [39]. A rise in $CXCR3^+$ Th1-like Tfh cells has been associated with antibody production following vaccination [42-44], while increased numbers of $CXCR3^-PD\text{-}1^+CXCR5^+CD4^+$ Tfh cells have been associated with the production of broadly neutralising antibodies (bnAb) in HIV infected individuals [38] and autoantibodies in systemic autoimmune disease [45, 46].

Alterations in cTfh cells have been proposed as biomarkers for GC activity, allowing tracking of tissue responses to antigen through peripheral blood sampling [47]. Circulating cells that resemble Tfr cells (cTfr) have also been identified in humans and mice [40, 48, 49]. These cells express CXCR5, PD-1 and ICOS and FOXP3, the canonical transcription factor associated with Treg cells. In mice cTfr cells have been shown to have a lower suppressive capacity than lymph node (LN) Tfr cells [40], which themselves have similar suppressive capacity to Tregs [50]. In humans cTfr cells are thought, like cTfh, to reflect an on-going GC response [51].

Longitudinal studies looking at the impact of commonly used immunosuppressive agents on cTfh and cTfr are lacking, despite the relevance of these cells in antibody formation. We have previously described the impact of short-duration rituximab and tacrolimus on both circulating and LN-resident Tfh and Tfr cells [52, 53], supporting the use of circulating cells as biomarkers of LN cell activity. Alemtuzumab is associated with profound CD4 T cell depletion, but also IL-21 dependent [11] *de novo* autoantibody formation, therefore we were interested in the impact of alemtuzumab on Tfh and Tfr cell frequency, and the recovery of these cells following therapeutic lymphocyte depletion. Here we compare the circulating cell phenotypes in transplant recipients treated with alemtuzumab at the time of transplant and show that they have a lower ratio of cTfr cells to cTfh cells at all times post-transplant compared to patients receiving alternative immunosuppression with basiliximab, an anti-CD25 monoclonal antibody.

2. Materials and Methods

2.1 Patients

This study complied with Good Clinical Practice and the Declaration of Helsinki, and received ethical approval from the Local Research Ethics Committee, REC reference 14/SC/0091. Patients provided written informed consent. Kidney and simultaneous pancreas-kidney (SPK) transplant recipients were recruited from May to December 2014 from the Oxford Transplant Centre. Patients with pre-formed DSAs and those undergoing planned pre-transplant desensitisation were excluded, but those with a negative pre-transplant cross-match to donor HLA were approached for study specific consent. Patients were assessed at the time of admission, and only those with no evidence of acute infection went ahead to receive the transplant. Pre-transplant, 50mls of blood was taken immediately after induction of anaesthesia. For follow up blood samples, 45mls of blood was taken at the time of routine clinical sampling at 1, 3, 6, 9, 12, 18 and 24 months post-transplant.

Choice of immunosuppression was by the clinical team and based on perceived 'immunological risk'. Patients receiving an SPK transplant were given alemtuzumab induction and maintenance therapy with tacrolimus and mycophenolate mofetil (MMF) with steroids only at induction. Patients receiving a DCD (donation after cardiac death) kidney, or a living-donor kidney but who had pre-formed HLA antibodies that were not donor specific, or received a poorly HLA-matched kidney were also given alemtuzumab induction and maintenance therapy with tacrolimus and MMF, with the addition of steroids that were rapidly weaned to 5mg or lower per day. Patients receiving a DBD (donation after brainstem death) or well-matched living-donor kidney were given basiliximab induction unless they were considered at risk of non-concordance, in which case they were given alemtuzumab induction. All received tacrolimus with azathioprine maintenance and steroids that were rapidly weaned to 5mg or lower per day.

2.2 Flow Cytometry

Blood samples were prepared over a density gradient as previously described [54]. Samples were cryopreserved in 90% fetal calf serum, 10% DMSO, then thawed and batch-processed for flow cytometry. Defrosted samples were washed, counted and $3x10^6$ cells added to each polypropylene tube.

All samples were incubated with human Fc block (eBioscience) to prevent non-specific binding of antibodies. Samples were incubated for 30 minutes at room temperature with a cocktail of surface antibodies. After washing, streptavidin and live/dead stains were added for 10 minutes at room temperature. After washing, eBioscience Foxp3 staining fixation/permeabilisation buffer was added for a minimum of 45 minutes at 4^oC as per manufacturers' protocol. Intracellular stains were then added and samples incubated for 60 minutes at 4^oC. Antibody cocktails were mixed in brilliant violet stain buffer (BD biosciences). Samples were then run on a BD LSR Fortessa cell analyser.

2.3 Antibodies

Anti-human CD45RA (HI100), CXCR5 biotin (RF8B2), PD-1 (EH12.1), IL-7R (HIL-7R-M21), CXCR3 (IC6), PD-1 (EH12.1) and CXCR3 (1C6/CXCR3) all BD Biosciences, brackets represent clones. Anti-human CCR6 (R6H1) and CD4 (OKT4), all eBioscience. Anti-human Foxp3 (259D), CD25 (M-A251), streptavidin brilliant violet 605, CD4 (OKT4), CXCR5 (J252D4) and CD3 (OKT3) all BioLegend. Invitrogen near-IR fixable live-dead stain kit.

2.4 DSA Assessment

Clotted blood samples were centrifuged at 1500g for 5 minutes to separate serum, which was snap frozen to -80^{o}C. Luminex screening for anti-HLA antibodies followed the same protocols and used the same machines as for clinical assessment. Defrosted serum was spun at 13,000 rpm in a microcentrifuge to pellet impurities, then 10µl incubated for 30 minutes with either LabScreen Mixed beads, HLA class-specific beads (PRA), or single antigen beads (SAB), all OneLambda. Samples were washed 5 times in wash buffer (OneLambda) then incubated with PE-conjugated goat anti-human IgG for 30 minutes, washed again to remove residual antibody and re-suspended in PBS. Samples for SAB assay were pre-treated with EDTA and Adsorb-Out treatment (OneLambda) to avoid the 'prozone' effect [55, 56].

Samples were analysed on a Liquichip 200 machine (Luminex) with one positive and two negative controls per batch; then analysed with HLA-Fusion software using positive and negative cut-off levels determined by the control samples, to avoid inter-lot variation. Samples showing positive on Mixed Screening and/or PRA were screened with SAB. Positive SAB antibody profiles were compared with both donor and recipient genotype to establish whether the identified antibody was donor-specific or a non-specific HLA antibody.

2.5 Statistical Analysis

Statistical analysis was performed using Graph Pad Prism software. Patient samples were analysed as non-parametric data using Kruskal-Wallis test with Dunn's multiple comparison test when comparing the three subgroups at each time point. The Mann-Whitney test was used when comparing two groups. Absolute cell counts for peripheral blood samples were calculated from hospital laboratory lymphocyte counts taken at the same time as sampling.

3. Results

3.1 Recruitment

60 patients were recruited to the study and provided samples for analysis; details are shown in Table 1. At the time of recruitment, patients in our centre received different immunosuppressive regimens depending on their calculated immunological risk of graft rejection. Patients receiving SPK transplants; donation after cardiac death (DCD) organs; those with previous transplants; or those considered at risk of non-concordance with maintenance therapy received induction immunosuppression with alemtuzumab. All other patients received basiliximab at induction. Maintenance treatment was with triple therapy of tacrolimus, an antimetabolite (either MMF or azathioprine) and steroids, which were weaned in all patients to

5mg daily or less. The decision of which induction and maintenance therapy was used for each patient was made by the clinical team.

There were 7 episodes of biopsy-proven acute T-cell mediated rejection (TCMR) in 6 patients over the 2-year study period (see Table 1). All patients experiencing biopsy proven acute rejection were treated with basiliximab induction, and the first episode of rejection in all was within the first three months (4 within the first 7 days, 1 at 3 weeks, 1 at 3 months). In 3 patients (two borderline, one Banff 1B) these responded to pulsed methylprednisolone alone. The other 3 had steroid-resistant rejection and were given alemtuzumab as rescue therapy. In all patients this was given within the first three weeks post-transplant, and prior to the first post-transplant study sample. Following alemtuzumab, one of these patients had a further biopsy at 5 months post-transplant, which showed on-going TCMR. The graft failed shortly after this biopsy and the patient returned to haemodialysis.

Table 1 Table of patient characteristics at recruitment. 60 patients were recruited in total. K= kidney alone transplant, SPK = simultaneous pancreas kidney transplant, DBD = donor after brainstem death, DCD = donor after cardiac death, MMF = mycophenolate mofetil, Aza = azathioprine. HLA = human leucocyte antigen.

	Basiliximab treated	**Alemtuzumab treated**
Number of patients	18	42
Sex M/F	10/8	30/12
Median Age (range)	53 (26-68)	51 (27-74)
Organ transplanted		
Kidney alone (K)	18	23
Kidney + pancreas (SPK)	0	19
Donor type		
Living	10	6
DBD	8	18
DCD	0	18
Maintenance therapy		
Tacrolimus + MMF	10	40
Tacrolimus + Aza	8	2
***De novo* antibody formation**		
No antibody (%)	12 (66.7%)	26 (61.9%)
Non-specific HLA antibody (%)	5 (27.8%)	11 (26.2%)
Donor specific antibody (%)	1 (5.6%)	5 (11.9%)

Patients were therefore split into two groups: Group 1 included those treated with alemtuzumab pre-transplant and within the first month post-transplant. These patients were subgrouped into those receiving a kidney-alone transplant (A-K, n=23), and those receiving an SPK transplant (A-SPK, n=19). Group 2 consisted of basiliximab-treated patients who did not receive alemtuzumab (B-K, n=18).

22 patients developed *de novo* anti-HLA antibodies during the study period. In the majority of patients (16/22, 73%) these were seen early post-transplant and were not donor specific.

In 6 patients (27%) these anti-HLA antibodies were reactive to donor antigens. 3 of these developed within the first 6 months, one at 12 months and two at 24 months post-transplant (Table 2). No antibodies, whether non-specific HLA or DSA, were associated with biopsy-proven rejection episodes. In the patient whose graft failed due to TCMR, DSAs developed at 24 months, 12 months after graft failure.

Table 2 Characteristics of patients developing *de novo* DSAs.

	Age at Tx	**Organ Transplanted**	**Previous transplant**	**Pre Tx Abs**	**Induction**	**Biopsy proven rejection**	**De Novo DSA**	**Date of DSA**	**Graft lost?**
1	38	Kidney	Yes	HLA-DR53 (first donor)	Alemtuzumab*	TCMR day 5, TCMR month 5	HLA-DQ6	24 months	Yes – prior to DSA
2	27	Kidney	No	Nil	Alemtuzumab	Nil	HLA-B8	3 months	No
3	74	Kidney	No	Nil	Alemtuzumab	Nil	HLA-Cw9	24 months	No
4	48	Kidney	Yes - two	Nil	Basiliximab	Nil	HLA-A2**	6 months	No
5	45	Kidney	No	HLA-A34, HLA-DP low grade	Alemtuzumab*	TCMR day 7	HLA-DQ4, HLA-DQ6	3 months	No
6	48	SPK	No	Nil	Alemtuzumab	Nil	HLA-B58	12 months	No

* Both patients received basiliximab induction, but alemtuzumab treatment for their rejection episodes at days 5 and 7 respectively. All subsequent samples were analysed according to alemtuzumab treatment.

** Shared epitope with donor one (failed transplant).

3.2 Induction Immunosuppression Altered cTfh Cell Proportions

The gating strategies [38, 52] for cTfh are shown in Figure 1A & Figure 2A. Early post-transplant, the proportion (Figure 1B) and absolute cell count (Figure 1C) of $CD4^+$ T cells fell in all alemtuzumab treated patients (A-K and A-SPK), consistent with the mechanism of action of this drug. $CD4^+$ T cell counts began to recover from 3 months but remained significantly lower than B-K patients at all time points and did not reach pre-transplant levels. In contrast, $CXCR3^-PD\text{-}1^+CD45RA^-CXCR5^+CD4^+$ cTfh were low pre and early post-transplant, but began to rise as a proportion of $CD45RA^-CXCR5^+CD4^+$ T cells in both (A-K and A-SPK) cohorts from three months and remained significantly higher than B-K patients at all time points post-transplant (Figure 1D, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). The absolute cell count of $CXCR3^-PD\text{-}1^+CD45RA^-CXCR5^+CD4^+$ cTfh was significantly higher in both A-K and A-SPK cohorts compared to B-K pre-transplant. Early post-transplant absolute $CXCR3^-PD\text{-}1^+CD45RA^-CXCR5^+CD4^+$ cTfh cells in both group 1 cohorts fell significantly below the levels seen in B-K (Figure 1E) in keeping with the CD4 lymphopenia seen in Figure 1C but recovered by 12 months as $CD4^+$ cell numbers began to rise. Dunn's multiple comparisons test showed these significant differences were due to differences between alemtuzumab-treated patients (A-K and A-SPK combined) compared to basiliximab-treated patients (B-K) with no significant differences between A-K and A-SPK patients.

CD45RA is a surface marker expressed on naïve cells but downregulated by activated/memory T cells. Lack of CD45RA expression is often used to gate cTfh cells, as they are memory cells. However, a genetic variant exists, in which CD45RA cannot be downregulated and is expressed alongside CD45RO [57-59], in these patients loss of CD45RA cannot be used to distinguish naïve from memory cells. Three patients in this study had this gene variant and therefore could not be analysed through CD45RA gating as activated $CD4^+$ T cells still express this cell surface receptor; therefore $CXCR3^-CXCR5^+PD\text{-}1^+CD4^+$ cTfh, which have also been shown to correlate with tissue-resident Tfh cells [52, 60], were assessed in all 60 patients (Figure 2A). Similar to Figure 1D, the proportion of these cTfh cells was significantly higher in both A-K and A-SPK patients than in B-K patients from 3 months post-transplant (Figure 2B). In both A-K and A-SPK patients, the absolute count of $CXCR3^-CXCR5^+PD\text{-}1^+CD4^+$ cTfh cells was significantly higher pre-transplant, but fell post-transplant below that of B-K patients until 18 months post-transplant, in keeping with the early CD4 lymphopenia. B-K patients saw a steady rise in $CXCR3^-CXCR5^+PD\text{-}1^+CD4^+$ cTfh cells post-transplant to above pre-transplant levels (Figure 2C). From 18 months post-transplant there were no significant differences between the two groups. Again, all significant differences were due to differences between alemtuzumab-treated (A-K and A-SPK combined) patients when compared to B-K with no significant differences between A-K and A-SPK patients compared to each other.

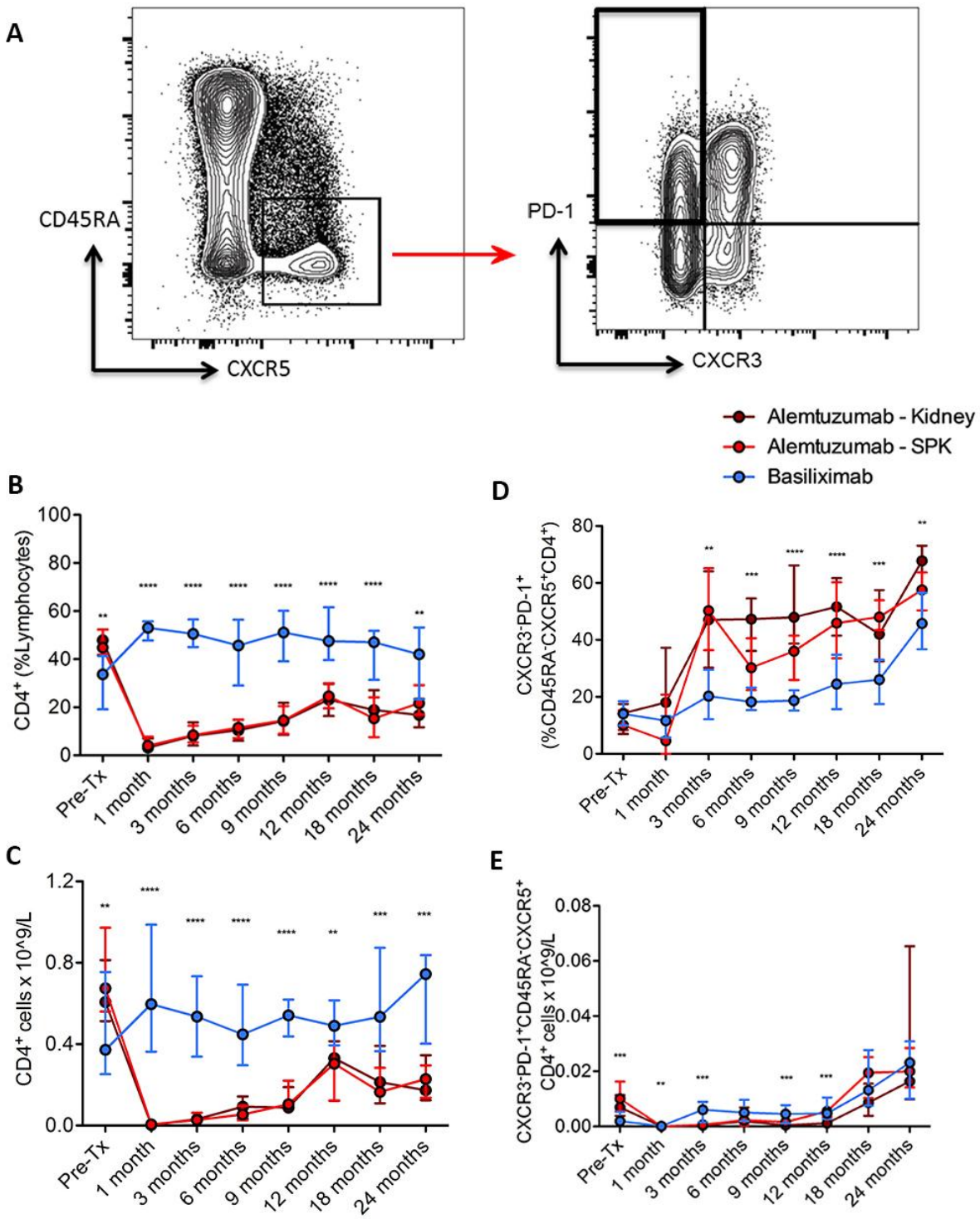

Figure 1 Circulating Tfh. A) Gating strategy, cells were gated on live $CD4^+$ and then further divided as shown into $CXCR3^-PD\text{-}1^+CD45RA^-CXCR5^+CD4^+$ Tfh. All $CD4^+$ cells shown as (B) proportion of lymphocytes and (C) absolute cell count. $CXCR3^-PD\text{-}1^+CD45RA^-CXCR5^+CD4^+$ Tfh as (D) proportion of B cells, and (E) absolute cell count, shown as mean and SD for each treatment group. Alemtuzumab n=42 (23 kidney, 19 SPK), Basiliximab n=18. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

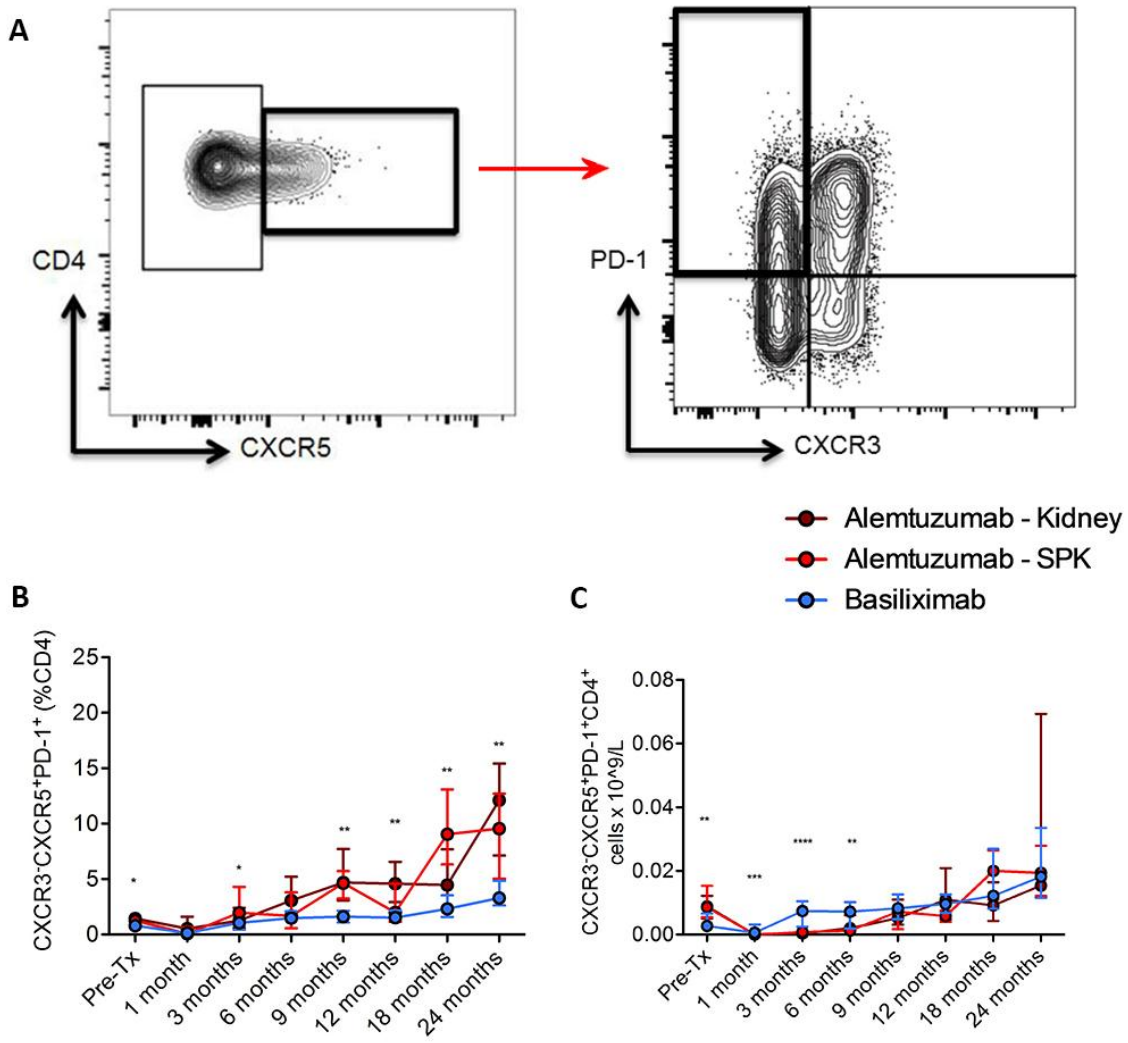

Figure 2 Circulating Tfh – alternative gating strategy. A) Gating strategy for $CXCR3^{-}CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ Tfh, samples were gated on live $CD4^{+}$ cells then as shown. $CXCR3^{-}CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ cells shown as (B) proportion of $CD4^{+}$ T cells and (C) absolute cell count. Shown as mean and SD for each treatment group. Alemtuzumab n=42 (23 kidney, 19 SPK), Basiliximab n=18. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

3.3 Tregs and cTfr Cells were also Altered with Induction Regimen

In autoimmunity, treatment with alemtuzumab leads to a high proportion of Tregs in the recovering CD4 population [61]. This provides the rationale for alemtuzumab use as an induction agent in transplantation, as Tregs are important for induction of tolerance in animal models of transplantation [62]. We assessed Tregs (gating strategy Figure 3A) and found a similar pattern, with a significantly higher proportion of $IL\text{-}7R^{lo}Foxp3^{+}CD4^{+}$ T cells in both A-K and A-SPK patients early post-transplant, returning to mirror B-K levels by 12 months (Figure 3C). Despite this high proportion, the absolute count of $IL\text{-}7R^{lo}Foxp3^{+}CD4^{+}$ Tregs was significantly lower in both A-K and A-SPK patients at all times points other than 12 months (Figure 3D) reflecting the broad lymphopenia seen following alemtuzumab treatment.

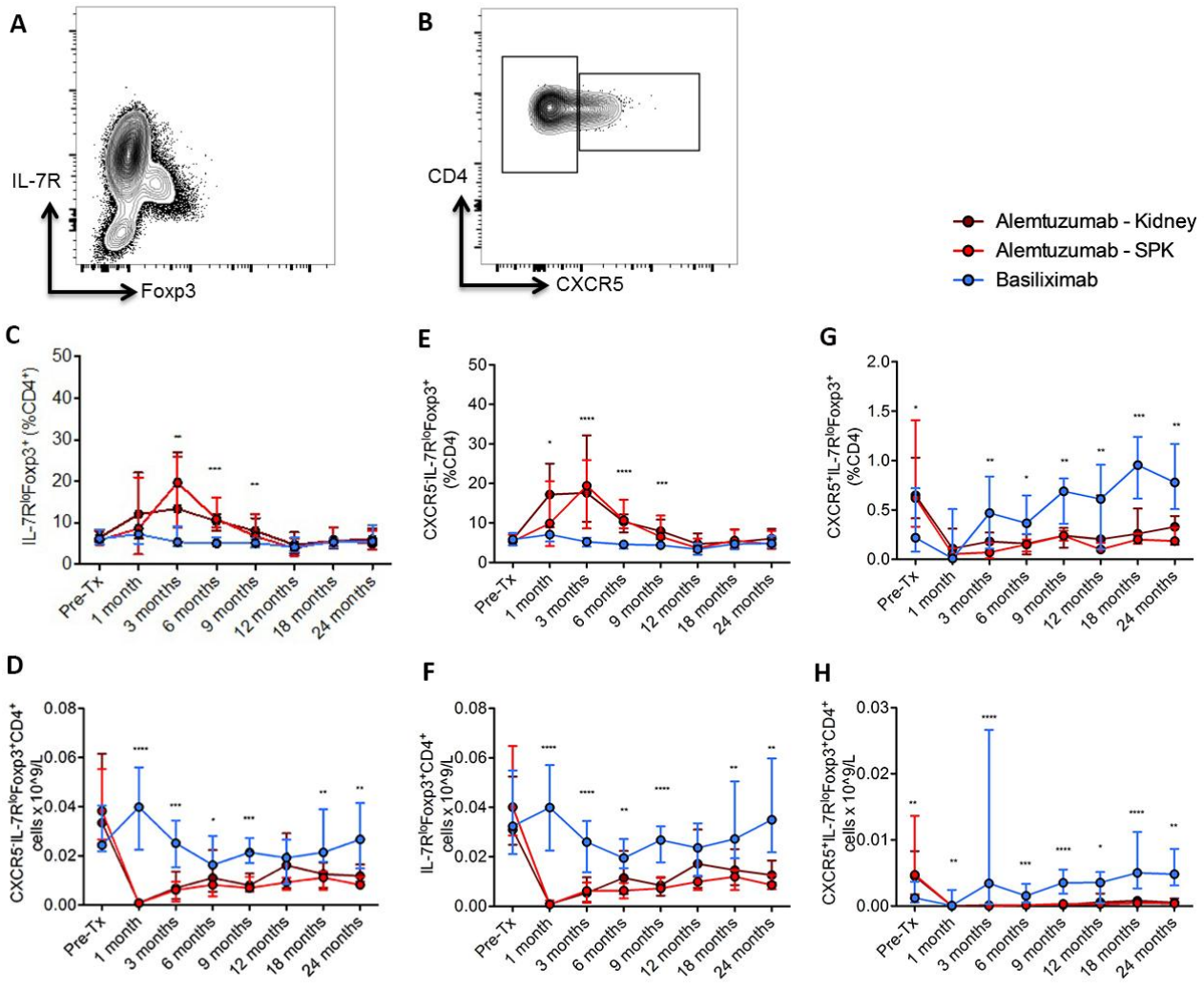

Figure 3 Circulating regulatory T cells. A) Samples were gated on lymphocytes, live $CD4^+$ then IL-$7R^{lo}Foxp3^+$ Tregs. B) IL-$7R^{lo}Foxp3^+$ cells were divided into $CXCR5^-$IL-$7R^{lo}Foxp3^+CD4^+$ Tregs and $CXCR5^-$IL-$7R^{lo}Foxp3^+CD4^+$ Tfr. IL-$7R^{lo}Foxp3^+CD4^+$ Tregs as (C) proportion of $CD4^+$ T cells and (D) absolute cell count. $CXCR5^-$IL-$7R^{lo}Foxp3^+CD4^+$ regulatory T cells as (E) proportion of $CD4^+$ T cells and (F) absolute cell count, shown as mean and SD for each treatment group. $CXCR5^+$IL-$7R^{lo}Foxp3^+CD4^+$ Tfr as (G) proportion of $CD4^+$ T cells and (H) absolute cell count, shown as mean and SD for each treatment group. Alemtuzumab n=42 (23 kidney, 19 SPK), Basiliximab=18. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Tregs are dependent on IL-2 signalling, which blocks the differentiation of both Tfh and Tfr cells. It is thought that consumption of IL-2 by Tregs allows Tfh cells to develop, but as Tfr develop from Tregs, high levels of IL-2 prevent up regulation of Bcl6 by maintaining high levels of Blimp1 expression, and hence block Tfr cell development. There have been no previous descriptions of CXCR5 expression on Tregs following alemtuzumab treatment; we therefore split IL-$7R^{lo}Foxp3^+CD4^+$ Tregs into $CXCR5^+$ and $CXCR5^-$ (gating seen in Figure 3B) to compare cTfr cells and non-follicular Tregs. Interestingly $CXCR5^-$IL-$7R^{lo}Foxp3^+CD4^+$ Tregs followed the well-described pattern in both A-K and A-SPK patients with a significantly higher proportion seen early post-

transplant, returning to mirror B-K levels by 12 months (Figure 3E). As with all $CD4^+$ cells, absolute numbers of $CXCR5^-IL\text{-}7R^{lo}Foxp3^+CD4^+$ Tregs in A-K and A-SPK patients dropped significantly below B-K patients early post-transplant, but rose to mirror B-K counts by 12 months (Figure 3F). However, $CXCR5^+IL\text{-}7R^{lo}Foxp3^+CD4^+$ cTfr cells remained significantly lower in both A-K and A-SPK patients, both in proportion (Figure 3G) and absolute cell count (Figure 3H) compared to B-K patients at almost all time points post-transplant, despite being significantly higher in this group prior to transplant. As previously, Dunn's multiple comparisons test showed these significant differences were due to differences between group 1 (A-K and A-SPK combined) patients compared to B-K patients with no significant differences between A-K and A-SPK patients.

3.4 cTfr and cTfh Cell Frequencies were not Significantly Different in Patients Developing de novo DSA

Given the association of Tfh cells with antibody formation, we assessed whether there were any differences in patients developing *de novo* DSA compared to those who did not. During the course of the study only 6/60 (10%) of patients developed *de novo* DSA, so while there was a trend towards a higher proportion of both $CXCR3^-PD\text{-}1^+CD45RA^-CXCR5^+CD4^+$ cTfh cells (Figure 4A) and $CXCR3^-CXCR5^+PD\text{-}1^+CD4^+$ cTfh cells (Figure 4B) in patients who developed *de novo* DSA compared to those who did not, these differences did not reach statistical significance. There was a similar trend towards a lower proportion of $CXCR5^+IL\text{-}7R^{lo}Foxp3^+CD4^+$ cTfr cells in patients developing *de novo* DSA compared to those who did not (Figure 4C), but again this did not reach statistical significance due to the low number of patients developing *de novo* DSA.

3.5 Alemtuzumab Treated Patients Have a Low cTfr: cTfh Ratio Compared to Basiliximab Treated Patients

The ratio of $CXCR5^+IL\text{-}7R^{lo}Foxp3^+CD4^+$ cTfr to $CXCR5^+PD\text{-}1^+CD4^+$ cTfh is thought to be important in antibody production [63]. In patients developing *de novo* DSA there was a trend towards a lower ratio of cTfr to cTfh cells compared to those who did not develop *de novo* DSAs (Figure 4D), although due to the small number of patients developing *de novo* DSAs, the study was underpowered to identify if this was statistically significant. Comparing the ratio of cTfr to cTfh between treatment groups we found the ratio was significantly lower in both A-K and A-SPK patients when compared to B-K patients at all times points post-transplant, reflecting the persistent low levels of cTfr cells in alemtuzumab treated patients despite a recovering cTfh population (Figure 4E). There were no significant differences between the two alemtuzumab-treated cohorts on Dunn's multiple comparisons test.

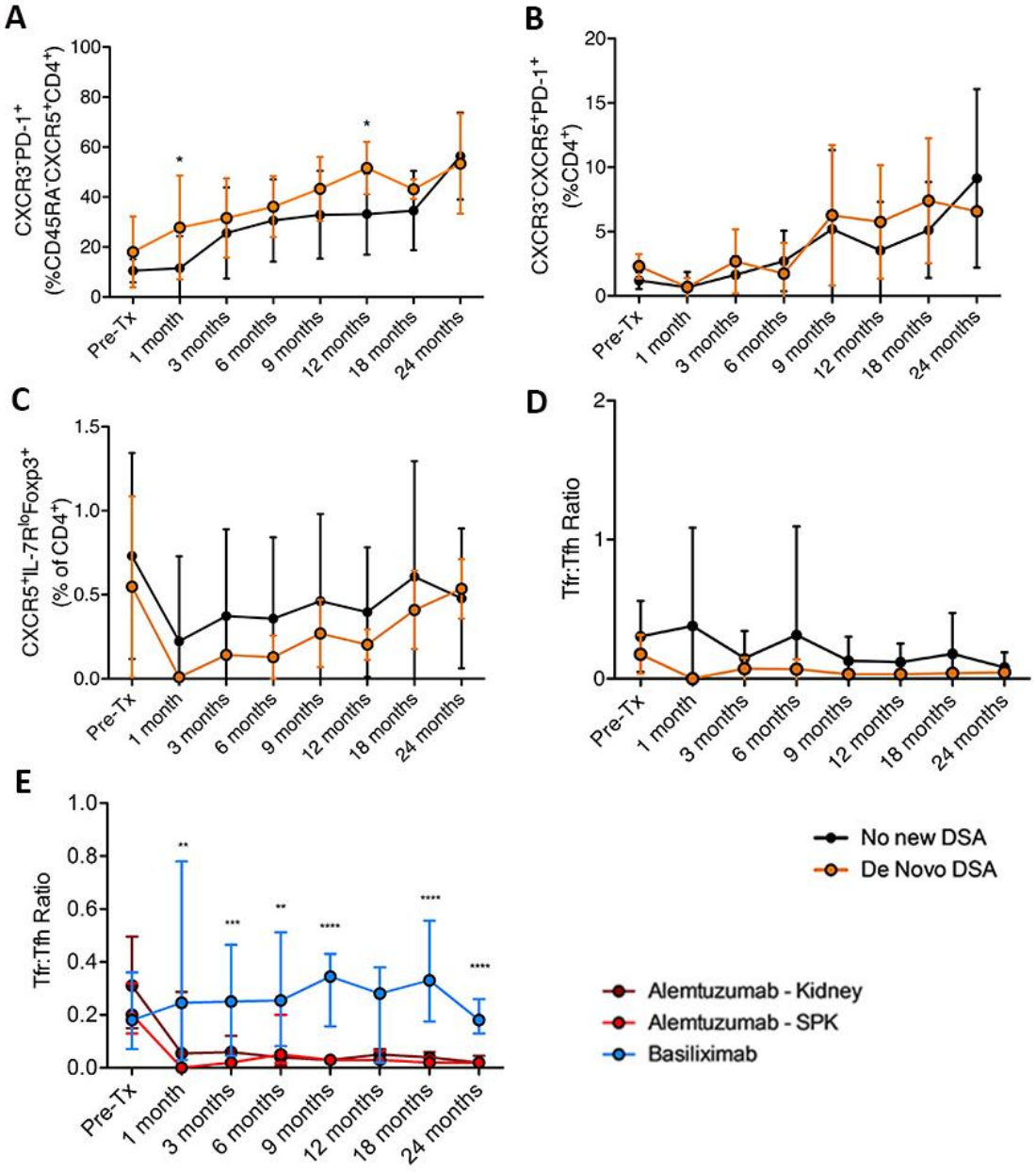

Figure 4 Association of circulating cells with *de novo* DSA. A) $CXCR3^{-}PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ cTfh cells showing significantly higher $CXCR3^{-}PD\text{-}1^{+}$ cTfh at 1 month (p=0.026) and 12 months (p=0.032) in patients with *de novo* DSA formation compared to those with no new donor-directed HLA antibodies (no new DSA). B) $CXCR3^{-}CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ cTfh and (C) $CXCR5^{+}IL\text{-}7R^{lo}Foxp3^{+}CD4^{+}$ cTfr in patients with *de novo* DSA formation compared to those with no new DSA. D) Ratio of $CXCR5^{+}IL\text{-}7R^{lo}Foxp3^{+}CD4^{+}$ cTfr to $CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ cTfh in patients with *de novo* DSA compared to those with no new DSA. Shown as mean + SD, *de novo* DSA n=6, no new DSA n=55. Analysed with Mann Whitney testing, only significant differences shown. E) Ratio of $CXCR5^{+}IL\text{-}7R^{lo}Foxp3^{+}CD4^{+}$ cTfr to $CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ cTfh by treatment group, alemtuzumab n=42 (23 kidney, 19 SPK), basiliximab n=18. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

4. Discussion

We and others have previously shown that cTfh cells correlate with LN resident Tfh cells [44, 52, 53], and in particular we have shown through sequencing that PD-1^{+}CXCR5^{+} and PD-1^{++}CXCR5^{+} cTfh cells are circulating biomarkers of genuine LN Tfh cells that correlate with antibody production [64], supporting their use as a biomarker. Several studies have also looked at cTfh in transplantation; one study suggested increased numbers of CXCR5^{+}CD4^{+} cells post-transplant in patients with pre-formed DSA compared to those without, but no differences in those developing *de novo* DSA [65]. More recently increased numbers of CXCR5^{+}CD4^{+} cells with low PD-1 expression have been described in a small cohort of patients with chronic rejection, compared to those with stable renal function [66]. However, despite these early studies in transplantation, there is little data looking at the impact of commonly used immunosuppressive agents on cTfh and cTfr. Alemtuzumab induces rapid and profound depletion of all lymphocytes, with slow recovery of first B cells, then CD8 T cells and finally CD4 T cells [2-4]. There has been a particular interest the recovery of CD4 T cells, because different subsets appear to recover at different intervals, with much higher proportions of Tregs in the recovering cell population compared to other subsets [61]. We have shown that, while there is a high proportion of Tregs in the recovering cell population post-transplant, the proportion of cTfr cells remains low for 2 years following a single treatment with alemtuzumab. In contrast, we have shown that the proportion of cTfh cells drops early after administration of alemtuzumab, in keeping with profound depletion of all CD4 T cell subsets, but recovers slowly over the next two years, rebounding above pre-administration levels in this patient cohort, leading to a low cTfr:cTfh ratio in these patients for 2 years following a single treatment with alemtuzumab.

In animal models a decreased ratio of Tfr:Tfh cells has been associated with generation of an effective antibody response, with this ratio shifting from helper function early in an immune response, then regulation to curtail the response as the antigen is cleared [40, 49, 67]. In humans, where only circulating cells are routinely accessible for analysis, it is not clear whether the ratio of cTfr to cTfh is relevant in disease, studies suggest that a low cTfr:cTfh ratio is associated with active inflammatory disease [48, 68], auto-antibody [69] or alloantibody production [70] but other studies suggest that cTfr increase similarly to cTfh in the context of an active GC response following vaccination [51]. While the number of patients developing *de novo* DSAs in our cohort was very low, and hence the study was underpowered to detect significant differences between cell subsets in these patients, there was also a trend towards a low cTfr:cTfh cell ratio in these patients, although caution must be used in interpreting this data.

The low cTfr:cTfh cell ratio in both patients receiving alemtuzumab, and suggestion of a similar pattern in those developing *de novo* DSAs, appears to support previous clinical studies showing alemtuzumab treated patients are at higher risk of developing *de novo* DSAs than those receiving basiliximab induction and provides a potential explanation for this phenomenon [9, 19, 20]. However, in addition to the small number of patients developing *de novo* DSAs in this study, there were a number of potential confounding factors, including different immunosuppression regimens, different HLA mismatch, different types of transplant as well as different types of donor, and previous transplants. It is therefore difficult to draw any firm conclusions about the cause of *de novo* DSA formation in such a small number of patients. It is possible that maintenance immunosuppression could have contributed to these changes, however all patients received tacrolimus maintenance therapy, and we have previously shown this has a greater impact on Tfh and cTfh than Tfr and cTfr cells [52]. The majority of patients in this study received MMF maintenance, and the numbers receiving azathioprine in each group (n=2 in A-K cohort, n=8 in B-

K) were too small to allow detection of meaningful differences. Further studies are required to determine if there is an impact of maintenance therapy on these circulating cells.

In transplant recipients, alemtuzumab-based induction leads to reduced acute rejection compared to basiliximab [6, 12], but may lead to higher rates of *de novo* DSA formation [9, 19, 20]. The 3C study compared basiliximab induction to alemtuzumab induction and showed a significantly reduced rate of TCMR in the alemtuzumab-treated cohort; however there was a non-significant trend towards a higher rate of antibody mediated rejection, although patients were not screened for HLA antibodies post-transplant [19]. Our data suggest that an increased risk of *de novo* DSA formation with alemtuzumab induction may be related to uncontrolled Tfh responses, with rising cTfh but persistently low cTfr in the recovering cell population.

Evidence suggests that the balance of cTfh and cTfr cells in the circulation reflects the balance of Tfh and Tfr cells within SLOs. A 'normal' ratio of cTfh to cTfr is yet to be determined, although in the SLO a ratio of 1:1 is thought to be representative of the resting state [40, 49, 67]. It has been shown that a low ratio of Tfr to Tfh cells is permissive of autoimmune disease, and while our data did not reach statistical significance the trends reported here suggest that a low ratio may be permissive to alloantibody formation; a finding that would need to be tested in a larger cohort in which a higher number of patients develop *de novo* DSAs.

We postulate that, with a high proportion of $CXCR5^{-}IL\text{-}7R^{lo}Foxp3^{+}$ Tregs following alemtuzumab treatment, the consumption of IL-2 by these Tregs would permit Tfh cell development, but a lack of $CXCR5^{+}IL\text{-}7R^{lo}Foxp3^{+}$ Tfr cells would prevent control of these Tfh responses, and support antibody formation. In the context of transplantation there is a large amount of non-self antigen present and being constantly trafficked to SLOs as lymphatics regenerate [71], therefore alloreactive B and T cells would be activated and excessive Tfh cells may provide support for alloantibody formation. In our patient cohort, a decreased ratio of Tfr:Tfh cells following alemtuzumab induction therapy may contribute to an increased risk of donor-specific antibody development compared to patients receiving basiliximab induction, where the ratio of Tfh to Tfr cells remains stable. Avoiding other risk factors for DSA formation, such as lower CNI levels, would be of greater importance in these patients, particularly given our previous work showing that tacrolimus has more of an impact on Tfh than Tfr cells [52].

Alemtuzumab is frequently used as induction immunosuppression in transplantation but is known to be associated with an increased risk of *de novo* DSA formation. The data presented here suggest that there is an imbalance between Tfr and Tfh cells in the recovering CD4 cell population following alemtuzumab treatment, but the number of patients that developed DSA in this study was limited meaning this study was underpowered to detect statistically significant differences. A balance in favour of Tfh cells is more permissible to antibody formation, and may explain the increased risk of *de novo* DSA as well as the increased rate of antibody mediated autoimmune disease in patients receiving alemtuzumab.

Abbreviations

Aza: azathioprine; bnAb: broadly-neutralizing antibody; DSA, donor-specific antibody; GC: germinal center; HLA: human leucocyte antigen; IL: interleukin; LN: lymph node; MMF: mycophenolate mofetil; MS: multiple sclerosis; PRA: panel reactive antibody; SAB: single antigen bead; SLO: secondary lymphoid organ; SPK: simultaneous pancreas-kidney (transplant); TCMR: T cell mediated rejection; Tfh: T follicular helper cell (when prefaced with c indicates a circulating cell); Tfr: T follicular regulatory cell (when prefaced with c indicates a circulating cell); Treg: regulatory T cell.

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Author Contributions

E.F.W. designed and performed research, analysed data and wrote the paper. M.A.L. designed research and wrote the paper. K.J.W. designed research and wrote the paper.

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Competing Interests

The authors declare no conflict of interests.

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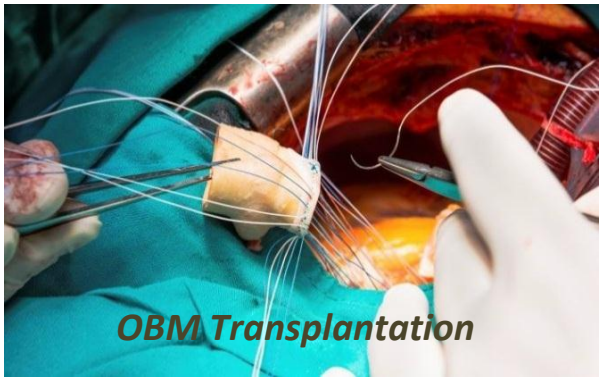

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