

# Human T follicular helper and T follicular regulatory cell maintenance is independent of germinal centers

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## Key words

Rituximab, Anti-CD20, GC, T follicular helper cell, T follicular regulatory cell, Regulatory T cell

## Abbreviations

T follicular helper cell (Tfh), Regulatory T cell (Treg), T follicular regulatory cell (Tfr), Germinal center (GC).

## Key points

1. Rituximab treatment results in loss of human germinal center B cells
2. Human T follicular helper and T follicular regulatory cells do not require germinal center B cells for their maintenance

## Abstract

The monoclonal anti-CD20 antibody rituximab depletes B cells in the treatment of lymphoma and autoimmune disease, and contributes to alloantibody depletion in transplantation across immunological barriers. The effects of rituximab on T cells are less well described. T follicular helper cells (Tfh) provide growth and differentiation signals to germinal centre (GC) B cells to support antibody production, and suppressive T follicular regulatory cells (Tfr) regulate this response. In mice, both Tfh and Tfr are absolutely dependent on B cells for their formation and on the GC for their maintenance. Here, we demonstrate that rituximab treatment results in a lack of GC B cells in human lymph nodes without affecting the Tfh or Tfr cell populations. These data demonstrate that human Tfh and Tfr do not require an on-going GC response for their maintenance. The persistence of Tfh and Tfr in following rituximab treatment may permit rapid reconstitution of the pathological GC response once the B cell pool begins to recover. Strategies for maintaining remission after rituximab therapy will need to take this persistence of Tfh into account.

## **Introduction**

In response to infection or immunization with a T-dependent antigen, germinal centers (GC) form within the B cell follicles of secondary lymphoid tissues<sup>1</sup>. GC are clusters of rapidly dividing B cells that are undergoing rounds of somatic hypermutation of their antigen receptor genes. This process of somatic hypermutation is random; therefore, in order to improve the affinity of cells that exit the GC as differentiated cells, selection needs to occur. B cells compete with each other for T cell help within the GC; B cells with high-affinity for antigen can outcompete lower affinity B cells for T cell help. Those B cells that receive help differentiate into antibody secreting plasma cells and memory B cells<sup>2-4</sup>. T cell help within the GC is provided by a subset of CD4<sup>+</sup> T cells, T follicular helper cells (Tfh). Tfh are a specialized subset of CD4<sup>+</sup> helper T cells that migrate into GC and provide help and survival signals to GC B cells, promoting their differentiation into long-lived plasma or memory B cells<sup>5,6</sup>. T cell help is essential for the formation and maintenance of the GC and the response collapses in the absence of Tfh<sup>7</sup>. The survival signals provided by Tfh to those GC B cells with the highest affinity BCR allow these B cell clones to proliferate and differentiate to become the predominant antibody producing cells<sup>8</sup>. Tfh are required for the response to foreign antigens, but in excess they can support autoreactive GC responses, leading to autoimmunity<sup>9,10</sup>. In addition to Tfh, there is another subset of CD4<sup>+</sup> T cells within the GC, follicular regulatory T (Tfr) cells, which has been characterized by our group and others<sup>11-13</sup>.

Tfr cells share phenotypic characteristics with Tfh but are derived from suppressive Foxp3<sup>+</sup> regulatory T cells (Tregs). Tfr co-opt aspects of the Tfh differentiation pathway and upregulate B-cell lymphoma – 6 (Bcl6), the transcriptional repressor that is essential for the formation of Tfh<sup>11,14-16</sup>. This allows Tfr to enter the GC and exert a suppressive function. Within the GC, Tfr cells control the size of the GC response and restrict the outgrowth of non-antigen specific B cell clones<sup>11-13</sup>.

The formation of Tfh and Tfr is dependent on interactions with B cells outside the B cell follicle. Recent data suggests that the initial step in the formation of Tfh is upregulation of the achaete-scute homologue 2 (Ascl2)<sup>17</sup>. This transcription factor induces upregulation of the chemokine receptor CXCR5, the ligand of which, CXCL13, is expressed in the B cell follicle, enabling pre-Tfh to migrate to the border of the B cell follicle. Ascl2 has also been shown to suppress genes associated with other T cell subsets, priming pre-Tfh differentiation down the follicular pathway<sup>17</sup>. Pre-Tfh cells also express Bcl-6, which is both necessary and sufficient for Tfh differentiation<sup>14-16</sup>. In contrast with the role for Ascl-2 in Tfh cells, Tfr cells require NFAT2 for upregulation of CXCR5 and their subsequent migration<sup>18</sup>. At the T-B border Tfh precursors encounter antigen primed B cells, and receive a second round of antigen presentation, enabling them to stabilize Bcl-6 expression, commit to becoming a Tfh cell and migrate into the GC<sup>19,20</sup>. In return, pre-Tfh provide signals to B cells to initiate immunoglobulin isotype class switching and form GCs<sup>21</sup>. In mice, it is clear that the interactions between Tfh, Tfr and GC B cells are reciprocal. Tfh and Tfr both require on-going interactions with GC B cells in order to maintain their phenotype and function, and selective lack of GC B cells during an on-going response leads to a reduction in Tfh numbers<sup>22</sup>. Equally, GC B cell numbers and differentiation depend on support from Tfh, with the GC response collapsing in the absence of Tfh<sup>11-13,22</sup>.

Translating the extensive knowledge of mouse Tfh and Tfr biology into humans has been difficult, in part because obtaining normal human secondary lymphoid tissue is not straightforward, but also because manipulation of the immune responses in humans is rarely possible. In humans, both Tfh and Tfr have been identified in tonsillar GCs, but description of their development and function has been limited<sup>12,23-26</sup>. Here we have taken advantage of the use of the B cell depleting anti-CD20 monoclonal antibody rituximab<sup>27</sup> prior to renal transplantation to assess the effects of this treatment on lymph node GC, Tfh and Tfr cells, and their circulating counterparts.

Because of the limited access to secondary lymphoid tissues from humans, several

groups have examined circulating cells expressing the same surface markers as Tfh, in particular CXCR5, ICOS and PD-1, coined circulating Tfh-like cells (cTfh), that can act as a biomarker for Tfh differentiation in the secondary lymphoid tissues<sup>25,28-30</sup>. cTfh numbers are elevated in patients with autoimmune diseases such as systemic lupus erythematosus<sup>31</sup> and rheumatoid arthritis<sup>32</sup> and appear to correlate with increased antibody levels and disease severity. Circulating Tfh-like cells appear to be a population of memory T cells that are generated during Tfh differentiation. Tfh-like cells do not express Bcl-6 but are able to quickly differentiate into cells capable of supporting antibody production<sup>25,28-30</sup>. In order to compare circulating Tfh-like cells with tissue resident cells, we have obtained paired blood and tissue samples from patients immediately prior to renal transplantation, who have been screened to exclude concurrent infection or malignancy. Some patients undergoing transplantation will have pre-formed antibodies directed against donor antigens, donor specific antibodies (DSA)<sup>33</sup>. These can be antibodies against blood group antigens, or anti-HLA antibodies formed from sensitizing events such as pregnancy. To reduce antibody titers and allow ABO or HLA incompatible transplantation, patients can undergo “desensitization” regimens<sup>33</sup>, often including the anti-CD20 monoclonal antibody rituximab<sup>27</sup>. Rituximab provides rapid and profound depletion of circulating B cells, although pre-B and mature plasma cells do not express CD20 and are thus preserved<sup>34</sup>. In our center, rituximab is given a month before transplantation along with tacrolimus and mycophenolate mofetil (MMF), then followed with five sessions of plasma exchange with the aim of reducing DSA titers by targeting both the antibodies themselves and the cells producing them.

We have taken advantage of the rituximab treatment regimen to examine the importance of B cells in maintaining the Tfh and Tfr populations in human lymph nodes. We show that rituximab in combination with tacrolimus and MMF is an efficient treatment for removing circulating naïve B cells, but not memory B cells, and surprisingly does not significantly alter the number of lymph node B cells. However, rituximab in combination with tacrolimus and MMF leads to an absence of GC B cells in the lymph node of treated patients. Strikingly, despite the lack of GC B cells, Tfh and Tfr are still present, suggesting that, contrary to reports in the mouse, human Tfh and Tfr cells do not require the GC B cell niche for their maintenance.

## **Patients and Methods**

### ***Patients***

This study received ethical approval from the Local Research Ethics Committee.

Patients were recruited from the renal transplant live-donor programme, and provided informed consent to participation. 26 patients provided paired blood and tissue samples, 9 women and 17 men. Mean age was 46 (18-69). All patients were either receiving or within 6 months of requiring renal replacement therapy. Patients taking immunosuppressive medication prior to transplant were excluded, except for those undergoing planned desensitization.

Patients undergoing desensitization therapy (n=5) received 500mg of the anti-CD20 monoclonal antibody rituximab one month before transplantation, followed by tacrolimus (dose adjusted for each patient aiming for blood levels 5-15ng/ml), and mycophenolate mofetil (MMF) 1g twice daily. All patients underwent antibody removal with plasma exchange for 5 sessions prior to transplantation, which went ahead only if antibody levels were within locally acceptable limits.

50mls of blood was taken at the time of transplant. Lymphoid tissue was removed to allow access to iliac vessels as part of the routine operative procedure.

### ***Sample preparation***

Blood samples were prepared as previously described<sup>35</sup>. Lymph node samples were made into a single cell suspension and stained for flow cytometry as previously described<sup>11</sup>.

### ***Antibodies***

Anti-human CD45RA clone HI100 (eBioscience), CD127 clone eBioRDR5 (eBioscience), CXCR3 clone IC6 (BD), FoxP3 clone PCH101 (eBioscience), CCR6 clone R6H1 (eBioscience), BCL-6 clone K112-91 (BD), CD57 clone NK-1 (BD), CD4 clone OKT4 (eBioscience), CXCR5 clone RF8B2 (BD) CD20 clone 2H7 (eBioscience), CD19 clone SJ25C1 (eBioscience), CD38 clone HIT2 (BD), IgD clone 1A6-2 (BD), CD27 clone O323 (eBioscience) and Streptavidin AF780 (eBioscience).

### ***Tfh, Tfr and B cell co-cultures***

CD19<sup>+</sup>CD27<sup>+</sup>IgD<sup>-</sup> B cells, CXCR5<sup>+</sup>CD57<sup>+</sup>CD25<sup>-</sup>CD4<sup>+</sup> Tfh cells and CXCR5<sup>+</sup>CD57<sup>+</sup>CD127<sup>-</sup>CD25<sup>+</sup>CD4<sup>+</sup> Tfr cells were flow-sorted from iliac lymph nodes and then cultured in 96-well U-bottom tissue culture plates (1x10<sup>4</sup> of each cell type per well) in the presence of T cell activation and expansion beads (Miltenyi, as per manufacturers instructions). IgA secretion was determined after 5 days by ELISA.

### ***IgA ELISA***

Secretion of IgA was determined by Ig H chain-specific enzyme-linked

immunosorbent assay. Nunc MaxiSorp® flat-bottom 96 well plate (eBioscience) were coated with goat anti-human IgA (Jackson ImmunoResearch). Nonspecific binding sites were blocked with 1% BSA/PBS. Culture supernatants were added to the wells and incubated for 2 h at 37°C. Plates were washed before adding HRP-conjugated donkey anti-human Ig H+L chain (Jackson ImmunoResearch). Bound IgA was visualized with TMB substrate (BioLegend).

### ***Statistical analysis***

Statistical analysis was performed using Prism software package using Mann-Whitney testing. Absolute cell counts for peripheral blood samples were calculated from hospital laboratory lymphocyte counts taken at the same time point as sampling. Absolute cell counts for lymph nodes were calculated from total cell count obtained at the time of processing the lymph node to form a single cell suspension.

## **Results**

### *Rituximab effectively removes naïve circulating B cells but not memory B cells.*

The impact of rituximab on peripheral and tissue resident B cells has been of considerable interest since this B cell-depleting therapy has been shown to be efficacious in treating hematological malignancies and autoimmune diseases. Many studies have examined the effect of both single and multiple doses of rituximab and shown variable removal of different B cell subsets<sup>36-40</sup>. Here, we compared blood and lymph node samples from patients receiving a 500mg dose of rituximab (RTX) one month prior to antibody-incompatible kidney transplantation, with those from patients not receiving rituximab (no RTX). This single dose effectively removed CD20<sup>+</sup> B cells within the peripheral blood, although a proportion of CD19<sup>+</sup>CD20<sup>-</sup> B cells were still detectable (Figure 1A-C). We characterised these cells in more detail using flow cytometry to divide them into IgD<sup>+</sup>CD27<sup>-</sup> naïve B cells, IgD<sup>+</sup>CD27<sup>+</sup> memory cells, IgD<sup>-</sup>CD27<sup>+</sup> memory cells, IgD<sup>-</sup>CD27<sup>-</sup> cells and IgD<sup>-</sup>CD27<sup>hi</sup>CD38<sup>hi</sup> plasma cells. There were differences in these subsets between untreated patients (Fig 1D) and those who had received rituximab (Fig 1E). In particular we found that naïve, IgD<sup>+</sup> memory and plasma cells were significantly reduced in rituximab treated patients compared to control (Figs 1F-H). In contrast, IgD<sup>-</sup> memory and IgD<sup>-</sup>CD27<sup>-</sup> cells showed relative preservation (Figs 1I, 1J), consistent with previous reports<sup>41-43</sup>. IgD<sup>-</sup>CD27<sup>-</sup> cells have previously been characterised as IgG<sup>-</sup>CD27<sup>-</sup> memory B cells<sup>44</sup> suggesting that the memory B cell population in the peripheral blood is not as severely affected following rituximab treatment as naïve B cells. Interestingly, these memory B cell populations

are reduced in the peripheral blood of patients with end-stage renal failure compared to healthy controls (Supplementary figure 1).

*Tissue resident B cells are more resistant to removal by rituximab*

Flow cytometric analysis of lymphocytes from iliac lymph nodes showed no reduction in the number of CD19<sup>+</sup>CD20<sup>+</sup> B cells in rituximab-treated patients compared to controls (Figure 2A-C). This relative preservation of tissue B cells has been previously described<sup>43</sup>. However, we noted a complete absence of CD38<sup>hi</sup>Bcl6<sup>+</sup> GC B cells in rituximab-treated patients (Figure 2D-F). CD38<sup>hi</sup>Bcl6<sup>+</sup> GC B cells in untreated patients expressed higher levels of CD20 (Figure 2G) compared to CD38<sup>lo</sup>Bcl6<sup>-</sup> B cells, suggesting they might be preferential targets for rituximab-mediated removal. Having said that, whether this loss of GC B cells is due to direct depletion by rituximab, or is indirect and due, for example, to a failure of GC generation secondary to loss of a pre-GC B cell population, cannot be determined from these data. These data show that rituximab, in combination with tacrolimus and MMF, has no overall effect on B cell numbers in the iliac lymph node, but GC B cells are not detectable following this treatment combination.

*Memory B cells are preserved in the lymph nodes of rituximab treated patients*

Despite the preservation of total B cell number in the lymph node compared to peripheral blood we assessed whether there was a change in the composition of the B cell population following rituximab treatment. We compared B cell subsets in the lymph node between untreated (Fig 3A) and rituximab treated (Fig 3B) patients, as we had previously in the blood (Figure 1). There was a significant reduction in IgD<sup>+</sup>CD27<sup>-</sup> naïve B cells and IgD<sup>+</sup>CD27<sup>+</sup> memory cells in rituximab treated patients and controls (Figs 3C, 3D), together with preservation of IgD<sup>-</sup>CD27<sup>+</sup> memory and IgD<sup>-</sup>CD27<sup>-</sup> cells in the lymph node (Figs 3E, 3F) of both patient groups. We did not find a reduction in IgD<sup>-</sup>CD27<sup>hi</sup>CD38<sup>hi</sup> plasma cells in the lymph node, consistent with their low expression of CD20 (Fig 3G). In addition, rituximab treated patients had an unusual population of CD19<sup>lo</sup>CD20<sup>o</sup> cells, which consisted largely of memory B cells (Fig 3H).

*Loss of the GC after rituximab treatment did not reduce Tfh within the lymph node.*

In mice there is a positive correlation between the number of GC B cells and the number of Tfh cells<sup>45</sup>, because Tfh maintenance requires sustained antigenic stimulation from GC B cells<sup>22</sup>. We were therefore interested in whether the lack of GC B cells in rituximab treated patients reduced GC Tfh numbers. Although CXCR5 and ICOS are routinely used to identify T cells with B helper capacity, we were specifically interested in those T cells that would be located within the GC. CD57 has been shown to be a marker of T cells within the GC, and CXCR5<sup>+</sup>CD57<sup>+</sup>CD4<sup>+</sup> T cells have been demonstrated to be Tfh in both phenotype and function<sup>45</sup>. Surprisingly, there was no difference in the proportion or total number of CXCR5<sup>+</sup>CD57<sup>+</sup>CD4<sup>+</sup> Tfh between rituximab-treated patients and controls (Figure 4A-C). Tfh can be divided into Th1-like, Th2-like and Th17-like subsets, each with different capacity for B cell help<sup>29</sup>. To determine if loss of the GC affected these Tfh subsets differentially we divided cells into CXCR3<sup>+</sup>CCR6<sup>-</sup>Th1-like Tfh, CXCR3<sup>-</sup>CCR6<sup>-</sup>Th2-like Tfh and CXCR3<sup>-</sup>CCR6<sup>+</sup>Th17-like Tfh. We found no differences in these subsets in rituximab-treated patients. (Figure 4D, E). Furthermore, we did not see any alteration in the circulating CXCR5<sup>+</sup>CD4<sup>+</sup> Tfh-like population in the blood of the same patients (Figure 4F, G). These data suggest that rituximab treatment does not reduce GC Tfh in humans, despite completely eliminating GC B cells.

*Rituximab did not alter Tregs or Tfr numbers within the lymph node.*

Suppressive Tfr cells have been previously described within the GC<sup>11-13</sup>. These cells derive from Foxp3<sup>+</sup> precursors, and in mice are absolutely dependent on B cells and the GC for their formation<sup>11,12,46</sup>. Rituximab has been described to alter proportions and function of Tregs in humans<sup>47,48</sup>; we therefore determined whether either the total Treg pool or the Tfr subset was altered after rituximab treatment. There was no difference in FOXP3<sup>+</sup>CD127<sup>-</sup>CD4<sup>+</sup> Treg in the lymph node (Figure 5A-C) or CXCR5<sup>+</sup>CD57<sup>+</sup>FOXP3<sup>+</sup>CD127<sup>-</sup>CD4<sup>+</sup> Tfr cells (Figure 5D-F), between treated and untreated patients, suggesting that maintenance of the Tfr cell pool does not require an on-going GC response in humans. To confirm that CXCR5<sup>+</sup>CD57<sup>+</sup>FOXP3<sup>+</sup>CD127<sup>-</sup>CD4<sup>+</sup> Tfr cells function as suppressors of B cell responses, we performed co-culture assays of Tfh cells (CXCR5<sup>+</sup>CD57<sup>+</sup>CD25<sup>-</sup>CD4<sup>+</sup>) with memory B cells (CD27<sup>+</sup>IgD<sup>-</sup>CD19<sup>+</sup>) in the presence or absence of CXCR5<sup>+</sup>CD57<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup>CD4<sup>+</sup> Tfr cells. Tfr cells inhibited the production of T-dependent IgA by memory B cells after five days of culture (Figure 5G). Together, these data demonstrate that these cells have a surface phenotype and function consistent with Tfr cells, and they do not require an on-going GC for their maintenance.

## Discussion

In this study we show that rituximab treatment combined with tacrolimus and MMF led to a profound reduction in the numbers of circulating naïve and IgD<sup>+</sup> memory B cells with relative preservation of IgD<sup>-</sup>CD27<sup>+</sup> memory B cells, which has been well described in previous studies<sup>43,49</sup>. The effects of rituximab on tissue resident cells have been investigated and it is clear that even a complete lack of circulating cells does not necessarily reflect deficiency of these cells in the tissues<sup>49,50</sup>. In our cohort, rituximab, in combination with tacrolimus and MMF, did not reduce the total B cell numbers in the lymph node, but did result in the absence of CD38<sup>hi</sup>Bcl6<sup>+</sup>CD19<sup>+</sup> GC B cells and CD19<sup>+</sup>IgD<sup>+</sup> naïve B cells, while leaving the CD27<sup>+</sup>CD19<sup>+</sup> memory B cell compartment intact. Although loss of GC B cells by this treatment has not been described in humans previously, it has been suggested that rituximab can reduce the number of GC B cells in cynomolgus monkeys, based on an altered histological appearance of follicles with a reduction in the number of CD19<sup>+</sup> cells, rather than using specific GC markers<sup>49</sup>.

Consistent with previous reports showing variable but incomplete depletion of B cells in lymph nodes, we found no significant reduction in the number of B cells in the lymph node<sup>40,43,50,51</sup>. However we did find a significant reduction in the number of naïve and IgD<sup>+</sup> memory cells in the lymph node, consistent with changes observed in the peripheral blood, though less profound. The residual cells in the lymph node showed a higher proportion of IgD<sup>-</sup>CD27<sup>+</sup> memory B cells and IgD<sup>-</sup>CD27<sup>-</sup> memory B cells, which have been previously described as being resistant to removal by one dose of rituximab<sup>43</sup>. The mechanism by which memory cells are resistant to removal by one dose of rituximab is unclear, although several have been postulated. B cell depletion by rituximab is dependent on antibody-dependent cellular cytotoxicity (ADCC), thus anatomical variation in Fc receptor expression may limit cell depletion, and an FcγRIIIa polymorphism has been associated with poor response to rituximab in lymphoma patients<sup>52</sup>. However, there is little evidence addressing which mechanisms predominate outside the setting of hematological malignancies, where there is derangement of the normal environment including depletion of complement and proportionately low numbers of effector cells, due to the expansion of malignant cells, thus limiting ADCC<sup>52</sup>.

We did not find a reduction in the number of plasma cells in the lymph node, which do not express CD20 and are therefore not targeted by rituximab. The absence of

plasmablasts in the peripheral blood of rituximab treated patients may reflect the absence of an on-going GC response due to the lack of GC B cells, as circulating plasma cells are short lived outside of their bone marrow niche<sup>53</sup>.

The effects of rituximab on T cells have been long debated, with suggestions that regulatory T cell numbers and function may be increased following rituximab treatment for autoimmune diseases<sup>47,48,54</sup> but other group have described reductions in Treg numbers<sup>37</sup>. Alterations in other subsets of CD4 T cells have also been suggested<sup>47</sup>, however despite these previous findings, and the absence of GC B cells in all rituximab treated patients we found no alterations in Tfh or Tfr cell numbers. This observation is in contrast to what has observed in mice, where depletion of GC B cells results in the loss of Tfh cells<sup>22</sup>. However, data from patients with X-linked agammaglobulinaemia, who have an almost complete arrest of B cell differentiation in the bone marrow resulting in very few peripheral B cells, have a markedly reduced population of peripheral blood cTfh cells compared to healthy controls<sup>55</sup>, consistent with a failure of formation, or maintenance, of Tfh in the absence of B cells in humans. Taken together with our observations, this suggests that, in humans, B cells are required for Tfh formation, but the GC is not necessary for their maintenance.

That on-going interactions with GC B cells are dispensable for the persistence of GC-T cells may occur because human Tfh and Tfr can be maintained without interactions with B cells, or that such interactions can be provided by non-GC B cells, such as the memory B cells we show persist despite rituximab therapy. Either of these possibilities is intriguing, the latter because the maintenance of GC-T cells by memory B cells has not been described in humans or mice previously. It has, however, been suggested that memory B cells are able to support lymph-node resident memory Tfh-like cells in mice<sup>56,57</sup>, raising the possibility that the Tfh cells identified in this study may represent a lymphoid reservoir of memory Tfh cells similar to those described in mice<sup>56</sup>, rather than a *bona fide* effector population. This seems unlikely, however, as CD57<sup>+</sup>CXCR5<sup>+</sup>CD4<sup>+</sup> cells are prone to apoptosis<sup>58</sup>, which is not a characteristic of persisting memory cells.

Alternatively, it is possible that our assessment at a single time point reflects repopulation of cells from circulating Tfh-like cells, rather than persistence despite an absence of GC cells. This would seem unlikely, however, as it would involve migration and *de novo* differentiation of lymph node Tfh and Tfr cell populations in the absence of a GC response.

Because of the absence of GC B cells and the reports that rituximab can distort follicular architecture<sup>49</sup>, we have used CD57 as a marker of GC-Tfh cells, in combination with the Tfh/Tfr surface receptor CXCR5. Although the function of CD57 on Tfh cells is not well understood, it is clear that it identifies the GC-located Tfh cells<sup>59</sup>. It has been shown that CXCR5<sup>+</sup>CD57<sup>-</sup> cells have similar B helper capacity to CXCR5<sup>+</sup>CD57<sup>+</sup> cells *in vitro* and that ICOS expression is more important for the identification of cells that can support antibody production<sup>60</sup>. CXCR5 and ICOS have therefore become the classical markers used to identify cells with effector Tfh function, which may reflect GC-Tfh and Tfh cells that act to support antibody production outside the follicle. It has also been shown that CXCR5<sup>+</sup>CD57<sup>+</sup> cells are Tfh, located in the GC and expressing ICOS and Bcl6, the key transcription factor for Tfh differentiation<sup>45,61</sup>.

Whether these cells are effector or memory populations, the preservation of these Tfh and Tfr cells following rituximab, tacrolimus and MMF treatment, is likely to result in the persistence of T cells that are able to support an allo- or autoreactive B cell response once the B cell pool recovers. Consistent with this, after a single dose of rituximab to treat autoimmunity, disease flare and autoantibody production are common after repopulation of the B cell pool<sup>42,62,63</sup>. Together, our results suggest that additional treatment targeting Tfh may be required to reduce relapse risk following rituximab treatment for autoimmune disease or in the setting of antibody incompatible transplantation.

## **Acknowledgements**

This work was funded by a Wellcome Trust Programme Grant (083650/Z/07/Z) and a Lister Prize Fellowship to KGCS and supported by the National Institute of Health Research Cambridge Biomedical Research Center. EFW was supported by an Addenbrooke's Charitable Trust research fellowship; MAL was supported by a NHMRC Overseas Biomedical Fellowship, then by the Biotechnology and Biological Sciences Research Council.

## **Authorship**

Contribution statement: E.F.W. performed research, analyzed data, and wrote the paper. E.C.J performed research. O.S. performed research. J.A.B. provided help in establishing the study, obtained clinical samples and reviewed the manuscript.

D.R.W.J. provided help in establishing and undertaking this study and reviewed the manuscript. M.E. designed research and reviewed the manuscript. M.A.L. designed research, performed research, analyzed data, and wrote the paper. K.G.C.S designed research, and wrote the paper.

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## Figure legends

**Figure 1: Rituximab effectively depletes naïve circulating B cells but not memory B cells** (A) Flow cytometric contour plots of CD19 and CD20, and numbers of CD20<sup>+</sup> B cells (B), CD19<sup>+</sup> B cells (C) on peripheral blood lymphocytes taken from patients that have not (control patients, top) or have (lower) been treated with rituximab. D) Flow cytometric contour plot of naïve, IgD<sup>+</sup> memory, IgD<sup>-</sup> memory, plasma cell and CD27<sup>-</sup>IgD<sup>-</sup> subsets of CD19<sup>+</sup> cells and MFI of CD38 on these subsets in a representative untreated patient. E) Flow cytometric contour plot and MFI of CD38 of the same subsets in a representative rituximab treated patient. Bar graphs of peripheral blood IgD<sup>+</sup>CD27<sup>-</sup>CD19<sup>+</sup> naïve B cells (F) IgD<sup>+</sup>CD27<sup>+</sup>CD19<sup>+</sup> memory B cells (G), CD38<sup>hi</sup>IgD<sup>-</sup>CD27<sup>hi</sup>CD19<sup>+</sup> plasma (H), IgD<sup>-</sup>CD27<sup>+</sup>CD19<sup>+</sup> memory B cells (I) and cells IgD<sup>-</sup>CD27<sup>-</sup>CD19<sup>+</sup> B cells (J). For all bar graphs one symbol represents one individual, and the height of the bar represents the mean.

**Figure 2: GC B cells are not detectable after rituximab treatment.** (A) Contour plots of CD19 and CD20 on lymphocytes from iliac lymph node of rituximab treated patients and controls. The number (B) and proportion (C) of CD20<sup>+</sup> B cells in the lymph nodes of rituximab treated patients and controls. Contour plots (D), the percentage (E) and total number (F) of CD38<sup>+</sup>Bcl-6<sup>+</sup>CD19<sup>+</sup> GC B cells in the lymph nodes of rituximab treated patients and controls. (G) Mean fluorescence intensity of CD20 on CD38<sup>+</sup>Bcl-6<sup>-</sup> non-GC B cells and CD38<sup>+</sup>Bcl-6<sup>+</sup> GC B cells from control patients. (H) Mean fluorescence intensity of CD20 on CD19<sup>+</sup> lymph node B cells from rituximab treated patients and controls. In bar graphs, one symbol represents one individual, and the height of the bar represents the mean.

**Figure 3: Characterisation of CD19<sup>+</sup> B cells in the lymph node suggests the cells resistant to removal are memory cells.** Contour plots of cell subsets and MFI of CD38 on CD19<sup>+</sup> cells from iliac lymph nodes of controls (A) and rituximab treated patients (B). Bar graphs of lymph node CD27<sup>+</sup>IgD<sup>+</sup>CD19<sup>+</sup> naïve B cells (C) CD27<sup>+</sup>IgD<sup>+</sup>CD19<sup>+</sup> memory B cells (D), CD27<sup>hi</sup>CD38<sup>hi</sup>IgD<sup>-</sup>CD19<sup>+</sup> plasma cells (E), CD27<sup>+</sup>IgD<sup>-</sup>CD19<sup>+</sup> memory B cells (F) and CD27<sup>+</sup>IgD<sup>-</sup>CD19<sup>+</sup> B cells (G). For all bar graphs one symbol represents one individual, and the height of the bar represents the mean. (H) Detailed analysis of CD19<sup>lo</sup>CD20<sup>lo</sup> cells alone in rituximab treated patients.

**Figure 4: Loss of the GC after rituximab treatment did not reduce Tfh numbers in the lymph node or peripheral blood.** Flow cytometric contour plots (A), proportion (B) and total number (C) of CXCR5<sup>+</sup>CD57<sup>+</sup>CD4<sup>+</sup>Tfh from iliac lymph nodes taken prior to kidney transplantation in rituximab-treated patients and controls. Contour plots (D) and quantitation (E) of CXCR3<sup>+</sup>CCR6<sup>-</sup>Th1-like Tfh (top left quadrant gate, panel D), CXCR3<sup>+</sup>CCR6<sup>-</sup>Th2-like Tfh (lower left quadrant gate, panel D) and CXCR3<sup>+</sup>CCR6<sup>+</sup>Th17-like lymph node Tfh cells (lower right quadrant gate, panel D). Contour plots (F) and bar graphs (G) of peripheral blood CXCR5<sup>+</sup>CD4<sup>+</sup> Tfh-like cells from the same patients in A-E. In graphs, one symbol represents one individual, the height of the bar represents the mean.

**Figure 5: Rituximab did not alter Treg or Tfr numbers within the lymph node.** Flow cytometric contour plots (A) and quantitation of the proportion (B) and total number (C) of FOXP3<sup>+</sup>CD127<sup>-</sup>CD4<sup>+</sup>Treg from iliac lymph nodes taken prior to kidney transplantation in rituximab-treated patients and controls. Contour plots (D), proportion (E) and total number (F) of CXCR5<sup>+</sup>CD57<sup>+</sup>FOXP3<sup>+</sup>CD127<sup>-</sup>CD4<sup>+</sup>Tfr from iliac lymph nodes. In graphs, one symbol represents one individual, and the height of the bar represents the mean. (G) IgA production in CD19<sup>+</sup>CD27<sup>+</sup>IgD<sup>-</sup> B cells cultured alone, co-culture of B cells with CXCR5<sup>+</sup>CD57<sup>+</sup>CD25<sup>-</sup>CD4<sup>+</sup> Tfh cells or co-culture of B cells with Tfh and CXCR5<sup>+</sup>CD57<sup>+</sup>CD127<sup>-</sup>CD25<sup>+</sup>CD4<sup>+</sup>Tfr flow-sorted from iliac lymph nodes. Data in (G) are a representative biological replicate of three independent biological replicates. Height of the bars represent the mean of 4 technical replicates, and the error bars show the standard deviation (n.d. = not detected above background).

