Targeting TFH cells is a novel approach for donor-specific antibody desensitization of allograft candidates: an *in vitro* and *in vivo* study

Ning Ma,[1*] Wei-Bing Wu,[1*] Xiang-Yu Zhao,[1] Lan-Ping Xu,[1] Xiao-Hui Zhang,[1] Yu Wang,[1] Xiao-Dong Mo,[1] Yuan-Yuan Zhang,[1] Xiao-Su Zhao,[1] Yu-Qian Sun,[1] Yi-Fei Cheng,[1] Kai-Yan Liu,[1] Ying-Jun Chang[1] and Xiao-Jun Huang[1,2,3]

[1]Peking University People's Hospital & Peking University Institute of Hematology, National Clinical Research Center for Hematologic Disease, Beijing Key Laboratory of Hematopoietic Stem Cell Transplantation; [2]Peking-Tsinghua Center for Life Sciences and [3]Research Unit of Key Technique for Diagnosis and Treatments of Hematologic Malignancies, Chinese Academy of Medical Sciences, Beijing, China

**NM and W-BW contributed equally as first authors.*

Correspondence: Y-J. Chang
rmcyj@bjmu.edu.cn

X-J. Huang
xjhrm@medmail.com.cn

Received: June 6, 2023.
Accepted: October 3, 2023.
Early view: October 12, 2023.

https://doi.org/10.3324/haematol.2023.283698

Abstract

The presence of donor-specific antibodies (DSA) are associated with graft failure either following human leukocyte antigen (HLA)-mismatched allogeneic stem cell transplantation or after organ transplantation. Although targeting B cells and plasma cells have been used for desensitization, there have been reports of failure. T-follicular helper (Tfh) cells assist B cells in differentiating into antibody-secreting plasma cells. We used haploidentical allograft as a platform to investigate the possibility of targeting Tfh cells to desensitize DSA. The quantities of circulating Tfh (cTfh) cell subsets in allograft candidates were abnormal, and these cells, including the cTfh2 and cTfhem cell subsets, were positively related to the production of anti-HLA antibodies. *Ex vivo* experiments showed that the cTfh cells of anti-HLA antibody-positive allograft candidates could induce B cells to differentiate into DSA-producing plasmablasts. The immune synapse could be involved in the assistance of cTfh cells to B cells in antibody production. *In vitro* experiments and *in vivo* clinical pilot studies indicated that targeting cTfh cells with sirolimus can inhibit their auxiliary function in assisting B cells. *Ex vivo* and *in vivo* studies demonstrated the effect of sirolimus and rituximab on DSA desensitization compared with either sirolimus or rituximab alone (60%, 43.75%, and 30%, respectively). Our findings provide new insight into the role of Tfh cells in the pathogenesis of DSA production in HLA-mismatched transplant candidates. Our data also indicate that targeting Tfh cells is a novel strategy for DSA desensitization and combination of sirolimus and rituximab might be a potential therapy. The prospective cohort of this study is registered at http://www.chictr.org.cn as #ChiCTR-OPC-15006672.

Introduction

Donor-specific antibodies (DSA) are anti-human leukocyte antigen (HLA) antibodies in transplant candidates that specifically correspond to mismatched donor antigens.[1-5] The presence of DSA contribute not only to graft failure following organ transplantation,[1,2,6,7] but also to graft failure, including graft rejection, and poor graft function, after HLA-mismatched allogeneic hematopoietic stem cell transplantation (allo-HSCT).[4,5,8,9] Multiple desensitization strategies for DSA targeting B cells and plasma cells have been implemented for patients who have undergone a transplant; however, there have been several instances of failure.[2,10-12] It has been demonstrated that, T-follicular helper (Tfh) cells are a lineage of $CD4^+$ T cells distinguishable by their unique expression of CXCR5, which are required for class-switching and high-affinity antibody generation by B cells, through Tfh cell-dependent B-cell responses.[13-16] The Tfh cell-assisted B cells then differentiate into antibody-secreting plasma cells. Therefore, in addition to targeting B and plasma cells,[10-12] clinical manipulation of Tfh cells may provide a therapeutic intervention for antibody-mediated diseases.[17-19]

The difficulties in accessing lymphoid tissues in humans have led to the analysis of circulating Tfh (cTfh) cells which have proven to be valuable in understanding alterations

in the Tfh-cell response that contribute to antibody production, involved in auto- and allo-immunity.[20-23] These studies suggest that cTfh cells are reliable surrogate indicators of germinal center activity.[17-24] As far as the effects of Tfh cells on production of DSA by B cells are concerned, Mohammed *et al.*[25] showed the crucial role of Tfh cells in promoting DSA responses and antibody-mediated rejection (AMR); they demonstrated that deletion of Tfh cells at the time of transplantation leads to significantly less severe allograft AMR in mouse models of solid organ transplantation. Louis *et al.*[18] found that cTfh cells obtained from AMR patients with positive DSA could promote memory B cells to generate DSA in kidney transplant recipients. Cano-Romero *et al.*[19] reported that for patients who had undergone renal transplant, induction therapy with thymoglobulin drastically eliminated cTfh cells and it took 6 months for these cells to recover, whereas basiliximab did not deplete the cTfh cells. Komagome *et al.*[26] reported that in a liver transplant recipient with refractory DSA-mediated rejection, desensitization with plasma exchange and bortezomib improved liver function tests; additionally, cholestasis in the graft improved after everolimus administration, which might have targeted the cTfh cells. These studies suggest that biotherapeutic targeting of Tfh cells could represent an alternative therapy for DSA desensitization.[17-19,26,27]

Presently, there are no data on the characteristics of cTfh cells and the role of these cells in DSA production by B cells in patients who are candidates for HLA-mismatched allo-HSCT.[4,5,8,9] Furthermore, it is currently unclear whether targeting Tfh cells can successfully desensitize DSA in the HLA-mismatched allo-HSCT candidates.[4,5,8,9] In the current study, using haploidentical HSCT as a platform,[8,28] we showed that cTfh cells, from DSA-positive allografting candidates, are abnormal in terms of quantity and quality and can promote the generation of DSA by B cells. *In vitro* and *in vivo* studies have indicated that targeting Tfh cells can offer an alternative approach for the desensitization of DSA in allografting candidates. In addition, the simultaneous targeting of Tfh and B cells has a synergistic effect on DSA desensitization.

Methods

Study design

Between July 2020 and March 2022, 83 haploidentical allografting candidates were prospectively enrolled (Table 1). Patient sera were collected for DSA testing within 1 month of the initiation of haploidentical hematopoietic HSCT conditioning. This study met the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the Peking University People's Hospital. Informed consent was obtained from all the patients or their guardians and donors.

Detection of donor-specific antibodies

DSA were detected in accordance to our previously reported method.[10,29]

Flow cytometry

The antibodies used are listed in *Online Supplementary Table S1*. More details are included in the *Online Supplementary Appendix*.

Table 1. Cohort patient characteristics.

Characteristics	HD N=20	Anti-HLA antibody-positive group N=40	Anti-HLA antibody-negative group N=43	*P*
Sex: male/female, N	8/12	12/28	21/22	0.216
Age in years, median (range)	35.0 (12-61)	41.5 (13-74)	36.5 (12-65)	0.346
Diagnosis, N AML ALL MDS AA Other	 - - - - -	 22 6 7 3 2	 21 11 5 3 3	0.750
Pregnancy, N Yes No	 8 4	 22 6	 14 8	0.726
Times of transfusion, N (range)	-	8 (4-62)	6 (2-153)	0.526
Median course of disease in months (range)	-	8 (3-43)	8(2-52)	0.823

HD: healthy donor; AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; MDS: myelodysplastic syndrome; AA: aplastic anemia; HLA: human leukocyte antigen.

Cell sorting

Peripheral blood mononucelar cells (PBMC) were stained and sorted using a BD Biosciences FACSAria II cytometer (BD Biosciences). cTfh cells were sorted as $CD3^+$ $CD4^+$ $CD45RA^-$ $CXCR5^+$ cells, while memory B cells were sorted as $CD3^-$ $CD19^+$ $CD27^+$ cells.

Co-cultures

Sorted cTfh cells were co-cultured with sorted autologous memory B cells (5×10^4) in a 1:1 ratio with staphylococcal enterotoxin B (SEB) (1 mg/mL; Toxin Technology) in complete RPMI medium supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin (Life Technologies). After 6 days of co-culture, the cells were stained with antibodies against CD3, CD4, CD19, CD20, CD27, and CD38 before data acquisition on a cytometer. In the drug experiments, the target cells were cultured with the corresponding drug at an appropriate concentration for 1 day (IL-21b,[30] ruxolitinib,[31] sirolimus,[32] CTLA4-Ig [50 μg/mL][33]), then fully washed, and finally co-cultured with other cells in the co-culture system described earlier.

Transwell experiments

In the experiment using transwells, we selected cTfh and memory B cells from five healthy donors and placed them on a transwell plate (PET membrane, 0.4 μm, Corning). Specifically, 5×10^4 cTfh was inoculated on the base plate, while the same number of memory B cells were placed in the diaphragm pore. The data were collected after the cells were trained for the same period of time.

Enzyme-linked immunosorbant assay

Details are included in the *Online Supplementary Appendix*.

RNA sequencing and ATAC-sequencing

Details are included in the *Online Supplementary Appendix*.

Treating patients with sirolimus and/or rituximab

This pilot study was conducted at the Peking University People's Hospital. Informed consent was obtained from all the DSA-positive patients defined according to cutoff value of mean fluorescent intensity (MFI) ≥2,000 as previously reported by us[8,10] or their guardians and donors. DSA desensitization treatment flow chart is shown in *Online Supplementary Figure S1*. Sixteen patients were treated with sirolimus alone (orally at a dose of 2 mg/day for 4 weeks) to keep the trough level at 5-20 ng/mL. Ten patients were treated with rituximab (intravenously at a dose of 375 mg/m^2 weekly, for 4 weeks) for DSA desensitization. Eight patients were administered rituximab or sirolimus (*Online Supplementary Table S2*). During transplantation, cyclosporine (CSA), mycophenolate mofetil (MMF), and short-term methotrexate (MTX) was administered to prevent graft-*versus*-host disease GVHD according to our previous study (*Online Supplementary Figure S1*).[34] The MFI of DSA was used to evaluate the efficiency of the different methods for DSA desensitization.

Statistical analyses

Details are included in the *Online Supplementary Appendix*.

Results

Correlation of cTfh with the level of anti-human leukocyte antigen A antibodies in allogeneic hematopoietic stem cell transplantation candidates

Eighty-three haploidentical HSCT candidates were classified as anti-HLA antibody-positive (n=40, group A) and anti-HLA antibody-negative (n=43, group B) candidates defined according to cutoff value of MFI more than 500 as previously reported by others and us,[4,8,29,35] and 20 healthy donors were used as the control group (Table 1) according to previous studies.[18,23] Flow cytometry representation graphs of cTfh and its subsets are shown in *Online Supplementary Figure S2*. The percentage of cTfh cells in $CD3^+CD4^+$ T cells ($P<0.001$) and the absolute cTfh cell counts ($P<0.001$) in group A were higher than in group B (Figure 1A; *Online Supplementary Figure S3A*). The absolute numbers of cTfh1, cTfh2, and cTfh17 subsets in group A were higher than those in group B ($P<0.001$, $P<0.001$, and $P=0.031$, respectively), although only the percentage of cTfh2 cells among cTfh cells in group A was higher than that in group B ($P=0.032$) (Figure 1B, C). The percentage of cTfhem subsets in cTfh cells ($P=0.013$) and absolute cTfhem cell counts ($P<0.001$) in group A were higher than those in group B (Figure 1D). There were no differences in absolute cTfhcm cell counts between group A and group B, although the percentage of cTfhcm cell subsets in cTfh cells ($P=0.004$) in group B was higher than that in group A (Figure 1D).

The percentages of either cTfhem or cTfh2 cell subsets in cTfh cells were positively correlated with the MFI of the anti-HLA antibody (r=0.3942, $P=0.0118$; r=0.3318, $P=0.0365$, respectively) (Figure 1E). The absolute numbers of cTfhem cells was also positively correlated with the MFI of the anti-HLA antibody (r=0.3570, $P=0.0237$) (*Online Supplementary Figure S3C*). Collectively, these observations show that quantities of the cTfh subsets were abnormal and that these cells, including the cTfh2 and cTfhem cell subsets, were positively related to the production of anti-HLA antibodies in allo-HSCT candidates.

Circulating T-follicular helper cells induce B cells to differentiate into donor-specific antibodies

In order to investigate the capacity of cTfh cells to assist B cells in anti-HLA antibody production, cTfh cells were sorted and cultured with autologous memory B cells in superantigen SEB (Figure 2A; *Online Supplementary Figure S4*). *In vitro* experiments showed that cTfh cells of pa-

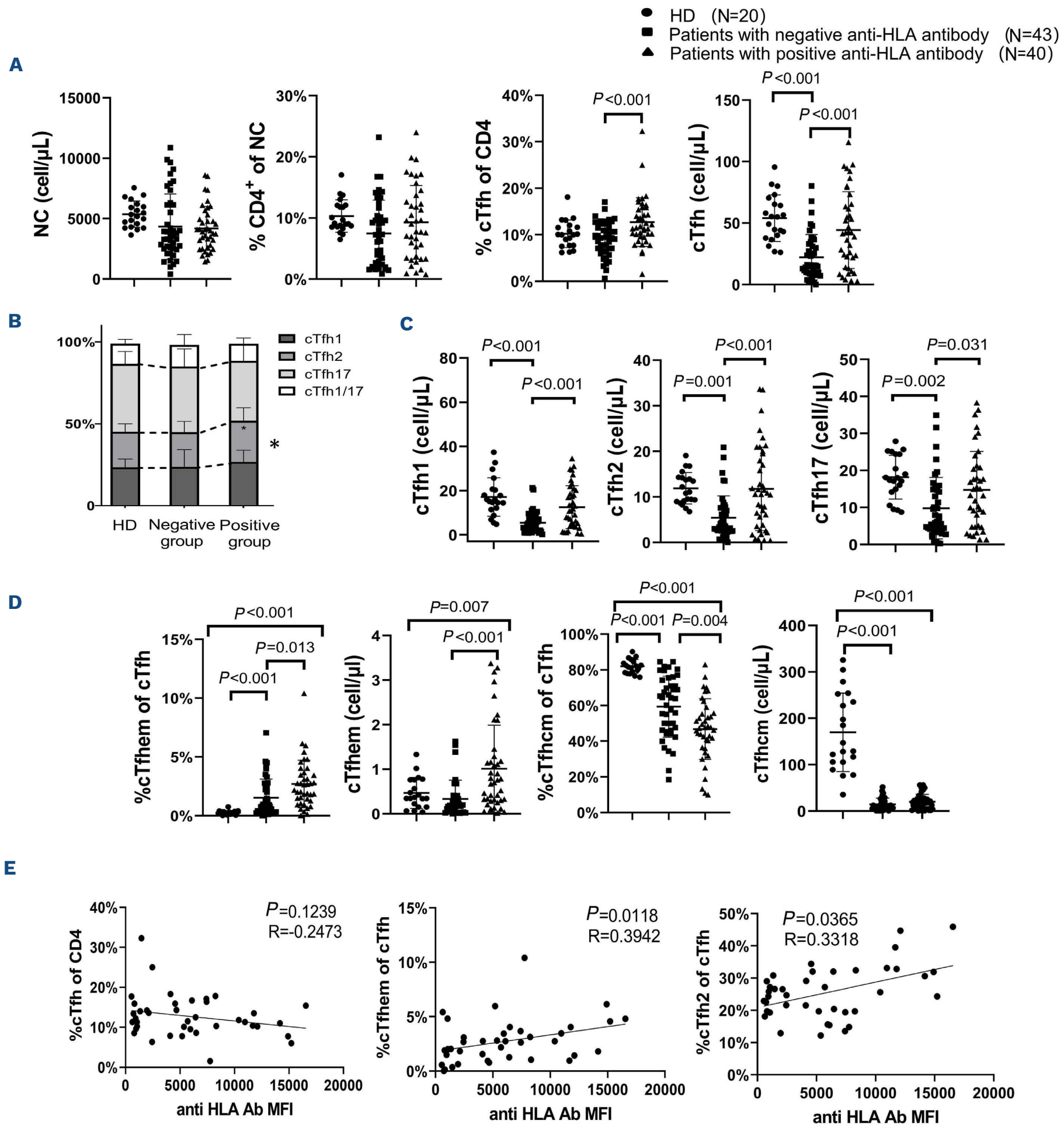

Figure 1. Association of circulating T-follicular helper cells with the titers of anti-human leukocyte antigen antibodies in transplant candidates. The results were analyzed by flow cytometry. (A) The number of nucleated cells (NC) per microliter of peripheral blood in transplant candidates negative for anti-human leukocyte antigen (HLA) antibodies (Ab) (N=43), transplant candidates positive for anti-HLA Ab (N=40), and healthy controls (N=20). The proportion of $CD4^+$ T cells among NC. The proportion of circulating T-follicular helper cells (cTfh, $CD3^+$ $CD4^+$ $CD45RA^-$ $CXCR5^+$) to $CD4^+$ T cells. The absolute number of cTfh cells per microliter. (B) Proportions and absolute numbers of cTfh1 ($CXCR3^+$ $CCR6^-$), cTfh2 ($CXCR3^-$ $CCR6^-$), cTfh17 ($CXCR3^-$ $CCR6^+$), and cTfh1/17($CXCR3^+$ $CCR6^+$) in cTfh cells from different groups. The proportion and absolute numbers of cTfhem ($ICOS^+$ $PD\text{-}1^+$ $CCR7^-$) (C) and cTfhcm ($ICOS^-$ $PD\text{-}1^-$ $CCR7^+$) (D) in different populations. Correlation between cTfh and donor-specific Ab DSA mean fluorescent intensity (MFI) in transplant candidates (E). The Wilcoxon rank-sum test was performed to assess the significance of (A-D), and the Spearman test was applied to (E). *P<0.05. HD: healthy donor.

tients in the anti-HLA antibody positive group could assist autologous memory B cells to produce a higher proportion of plasmablasts than patients in the negative group (P<0.001) (Figure 2B). The supernatant of the co-culture system of anti-HLA antibody-positive allo-HSCT patients contained higher levels of immunglobulin (Ig)M (P=0.043), IgG (P=0.002), CXCL13 (P=0.004), and interleukin (IL)-21 (P=0.011), but no difference in the levels of IgA and IL-2 compared with anti-HLA antibody-negative cases (Figure 2C, D). The percentages of IL-21$^+$ cTfh cells in the co-culture system of the anti-HLA antibody group was higher than that in the negative group (P<0.001) (Figure 2E).

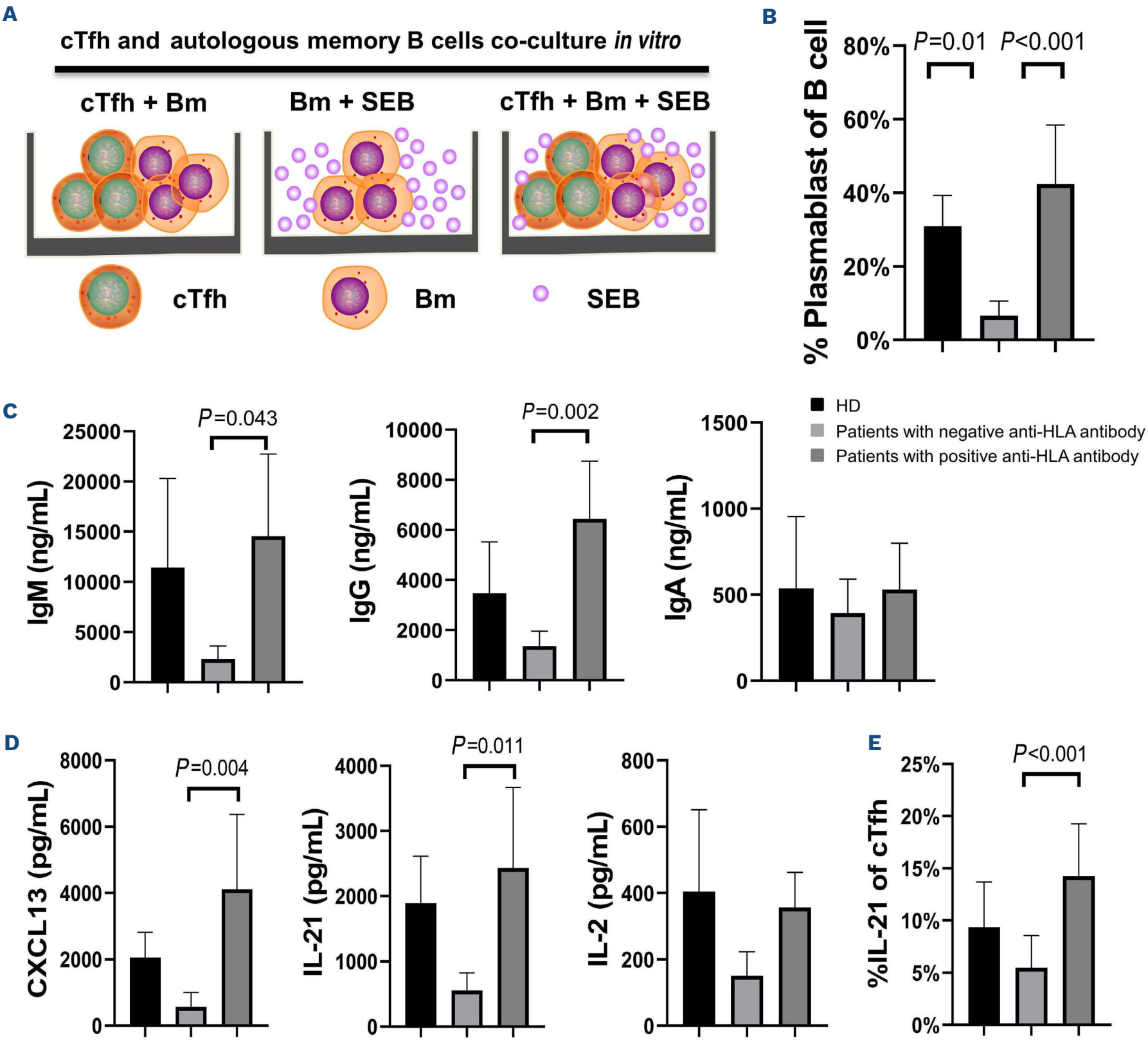

Figure 2. Circulating T-follicular helper cells aid donor-specific antibody production by memory B cells in allogeneic hematopoetic stem cell transplantation candidates with positive anti-human leukocyte antigen antibodies. (A) *In vitro* culture pattern of circulating T-follicular helper (cTfh) and autologous memory B cells. Peripheral blood mononucelar cells (PBMC) was isolated from PB of healthy donors, and transplant candidates positive and negative for anti-human leukocyte antigen (HLA) antibody (N=5). The cTfh and memory B (Bm) cells ($CD3^-CD19^+CD27^+$) were then isolated by flow cytometry and finally co-cultured in the presence of staphylococcal enterotoxin B (SEB) (1 mg/mL) for 6 days. (B) The transformation ratio of Bm cells to plasmablast cells ($CD3^-$ $CD20^-$ $CD27^+$ $CD38^+$) was detected by flow cytometry. (C) The immunoglobulin (Ig) level was detected by enzyme-linked immunosorbant assay (ELISA) in the supernatant. (D) The levels of CXCL13, interleukin (IL)-21 and IL-2 were also detected by ELISA in the supernatant. (E) Flow cytometry was used to detect the expression of IL-21 in peripheral blood cTfh cells of different populations (N=10) after 4 hours of phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation. Wilcoxon rank sum test was performed to assess the significance in (B-E). HD: healthy donor.

In order to explore whether the presence of anti-HLA antibodies in the supernatant after co-culture, anti-HLA antibodies were tested. The results showed that only the supernatant of the co-culture system of anti-HLA antibody-positive allo-HSCT patients contains class I and/or class II anti-HLA antibodies (*Online Supplementary Figure S5*). In addition, the profiles of anti-HLA antibodies produced by memory B cells *in vitro* were consistent with the *in vivo* analysis (*Online Supplementary Figure S5*), except for some differences in the MFI. Collectively, these observations show that the cTfh cells of anti-HLA antibody-positive allo-HSCT candidates induce B cells to differentiate into DSA-producing plasmablasts.

Contribution of immune synapse to circulating T-follicular helper cells in assisting B cells

In order to investigate whether cTfh assists memory B cells in antibody production through the direct contact pathway, we sorted cTfh and memory B cells from five healthy donors for Transwell experiments (Figure 3A). Compared to the control group, the percentages of plasmablast produced by cTfh cell-assisted autologous memory B cells in the experimental group decreased significantly (P=0.007; Figure 3B). The levels of IL-21 (P=0.032), CXCL13 (P=0.014), IgG (P=0.011), and IgM (P=0.024) in the supernatant of the experimental group were also significantly lower than that in the control (Figure 3C, D). These results suggest that the direct-contact pathway plays an important role in cTfh cell-assisted antibody production by B cells.

Subsequently, immunosynaptic-associated adhesion molecules and co-stimulatory molecules (mainly LFA-1, ICOS, CD40L, and CD28 on cTfh and CD86, CD80, CD40, ICOSL, and ICAM-1) expressed in cTfh cells and memory B cells in patients positive (n=10, group C) and negative (n=10, group D) for anti-HLA antibody were determined. The results showed higher expression of CD28 (P=0.036) and ICOS (P=0.029) in cTfh cells in patients of group C than in group D (Figure 4A); as well as higher expression of CD86 (P=0.003) and ICAM-1 (P=0.031) in memory B cells (Figure 4B). These results suggest that immune synapses may be involved in the assistance of B cells by cTfh cells in anti-HLA antibody-positive patients.

We investigated whether the inhibition of molecules related to immune synapses could affect the auxiliary effect of cTfh cells on antibody production by B cells. The sorted cTfh cells were co-incubated with autologous memory B cells *in vitro* in the presence of CTLA-4 Ig. The CTLA-4 Ig inhibition of CD28 effectively reduced the percentages of plasmablast (P=0.011), IL-21 (P=0.006), CXCL13 (P=0.002), IgG (P<0.001), IgM (P<0.001), and IgA (P=0.018) in B cells compared to that in the control group (Figure 4C-E).

We further explored the differences in the transcriptome of cTfh cells between patients positive and negative for anti-HLA antibodies. The top 70 differential genes are shown in *Online Supplementary Figure S6A*. ATAC-sequencing data showed that 609 genomic regions exhibited greater chromatin accessibility and 3,778 genomic regions exhibited reduced accessibility in patients with positive anti-HLA antibody (*Online Supplementary Figure S6B*). Pathway enrichment analysis of the RNA-sequencing results revealed that the upregulated genes were mainly involved in positive regulation of synaptic transmission (*Online Supplementary Figure S6C*). Notably, not only was the BAIAP3 gene upregulated, but the degree of chromatin accessibility of the BAIAP3 molecule located on chromosome 16 was also increased in the cTfh cells of patients positive for anti-HLA antibody (*Online Supplementary Figure S6D*). The association of BAIAP3 with post-synaptic neurotransmission[36] and our results suggest a correlation between BAIAP3 gene expression and the positive regulation of synaptic transmission in the cTfh cell of anti-HLA antibody positive patients.

In summary, our observations suggest that the immune synapse may contribute in the assistance of B cells in antibody production by cTfh cells.

In vitro targeting circulating T-follicular helper cells inhibits their auxiliary function on B cells

We investigated the effects of ruxolitinib, sirolimus and IL-21 blocking antibody (IL-21b) on the function of cTfh cells in assisting B cells *in vitro*. The sorted cTfh cells were treated with the drugs for 24 hours, after which the drugs were eluted and the cells were incubated with autologous memory B cells, the ratio of cTfh cells to memory B cells was 1:1 according to previous studies[18,20,21] (Figure 5A). All three drugs were found to inhibit the differentiation of B cells into plasmablasts in five healthy donors (Figure 5B), and there was a significant decrease in the levels of IgG, IgA, and IgM, as well as IL-21 and CXCL13, in the experimental group compared with those in the control group (Figure 5C, D). As previously reported,[22] the percentage of $ICOS^+$ $CD28^+$ cTfh cells among all the cTfh cells was used to reflect the intensity of immune synaptic activity. Targeting cTfh cells with the three drugs significantly decreased the percentage of $ICOS^+$ $CD28^+$ cTfh subsets in the cTfh cells (Figure 5E). Overall, *in vitro* experiments indicated that targeting cTfh cells inhibited their auxiliary function in antibody production by B cells.

In vivo targeting of circulating T-follicular helper cells with sirolimus reduces donor-specific antibody levels in allografting candidates

Based on the literature[32] and our *in vitro* experiments, a pilot study was performed to investigate whether sirolimus can reduce DSA levels by suppressing cTfh cells in allo-SCT candidates. Sixteen transplant candidates positive for DSA were enrolled (*Online Supplementary Table S2*). A 50% reduction in a patient's DSA MFI was defined as an effective treatment; however, the effective rate of sirolimus treatment was 43.75% for all patients (Figure 6A; *Online Supplemen-*

tary Figure S7A-E). The levels of IL-21 (P=0.002) and CXCL13 (P=0.002) after 2 weeks of sirolimus treatment decreased significantly compared with levels prior to treatment (Figure 6B, C). Following sirolimus administration, we observed a significant decrease in the percentages of cTfhem cells (P=0.01) and ICOS$^+$CD28$^+$ cTfh cells (P<0.001) (Figure 6D-F). Collectively, these observations suggest that *in vivo* targeting of cTfh cells could provide a novel approach for the desensitization of DSA-positive allografting candidates.

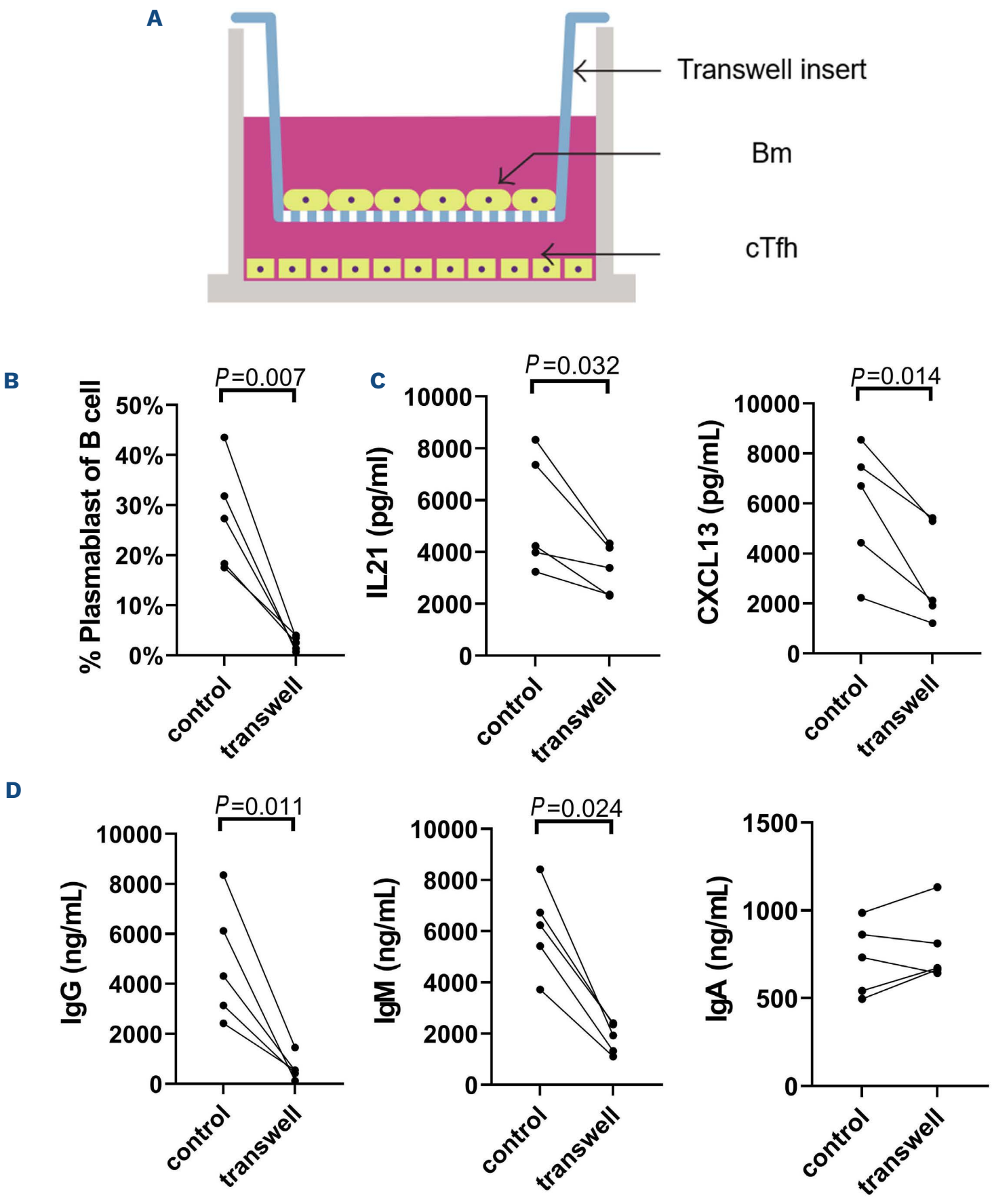

Figure 3. Involvement of direct contact between circulating T-follicular helper cells and memory B cells for antibody production. (A) Model diagram of Transwell experiment. After sorting, circulating T-follicular helper (cTfh) and memory B (Bm) cells from healthy donors (N=5) were co-cultured in the presence of diaphragm. (B) The transformation ratio of Bm cells to plasmablast cells was detected by flow cytometry. The levels of interleukin (IL)-21 and CXCL13 (C) were detected by enzyme-linked immunosorbant assay (ELISA) in the supernatant. The immunoglobulin level (D) was detected by ELISA in the supernatant. Paired *t* test was performed to assess the significance in (B-D).

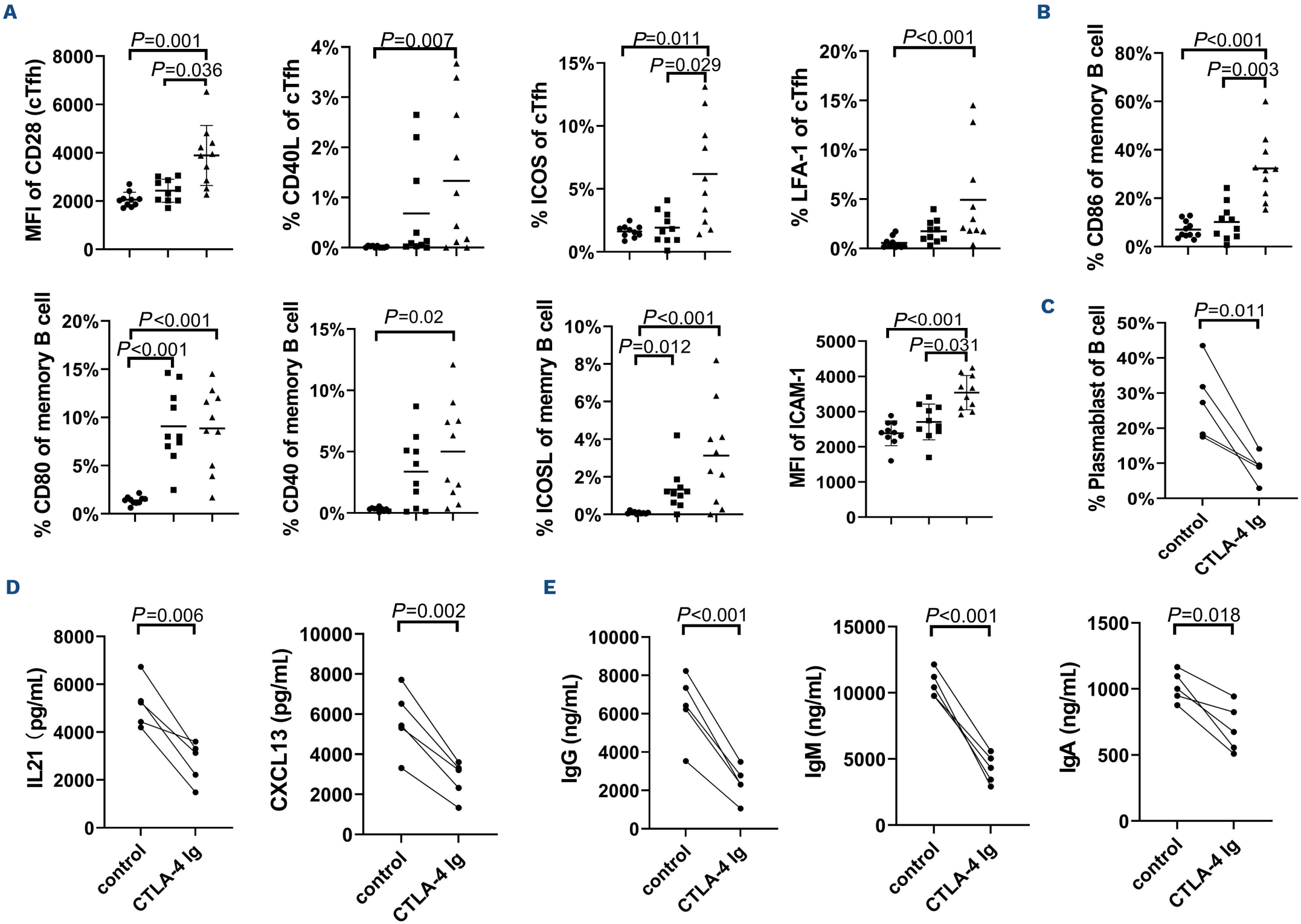

Figure 4. Expression of immune synapse molecules on circulating T-follicular helper and memory B cells in allogeneic hematopoetic stem cell transplantation candidates with positive anti-human leukocyte antigen antibodies. (A) The expression levels of immune synapse-related molecules on circulating T-follicular helper (cTfh) were detected using flow cytometry in different groups (N=10). (B) Expression levels of immune synapse-related molecules in memory B cells (N=10). Peripheral blood samples from healthy donors (N=5) were used for the *in vitro* experiments. CTLA-4-immunoglobulin (Ig)-binding ligands CD80 and CD86 were used to prevent them from binding to CD28. The ratio of plasmablast cells was detected (C), and interleukin (IL)-21, CXCL13 (D), and Ig were detected in the supernatant (E). The Wilcoxon rank-sum test was used to assess the significance of (A, B), and a paired *t* test was used to assess the significance of (C-E). MFI: mean fluorescence intensity.

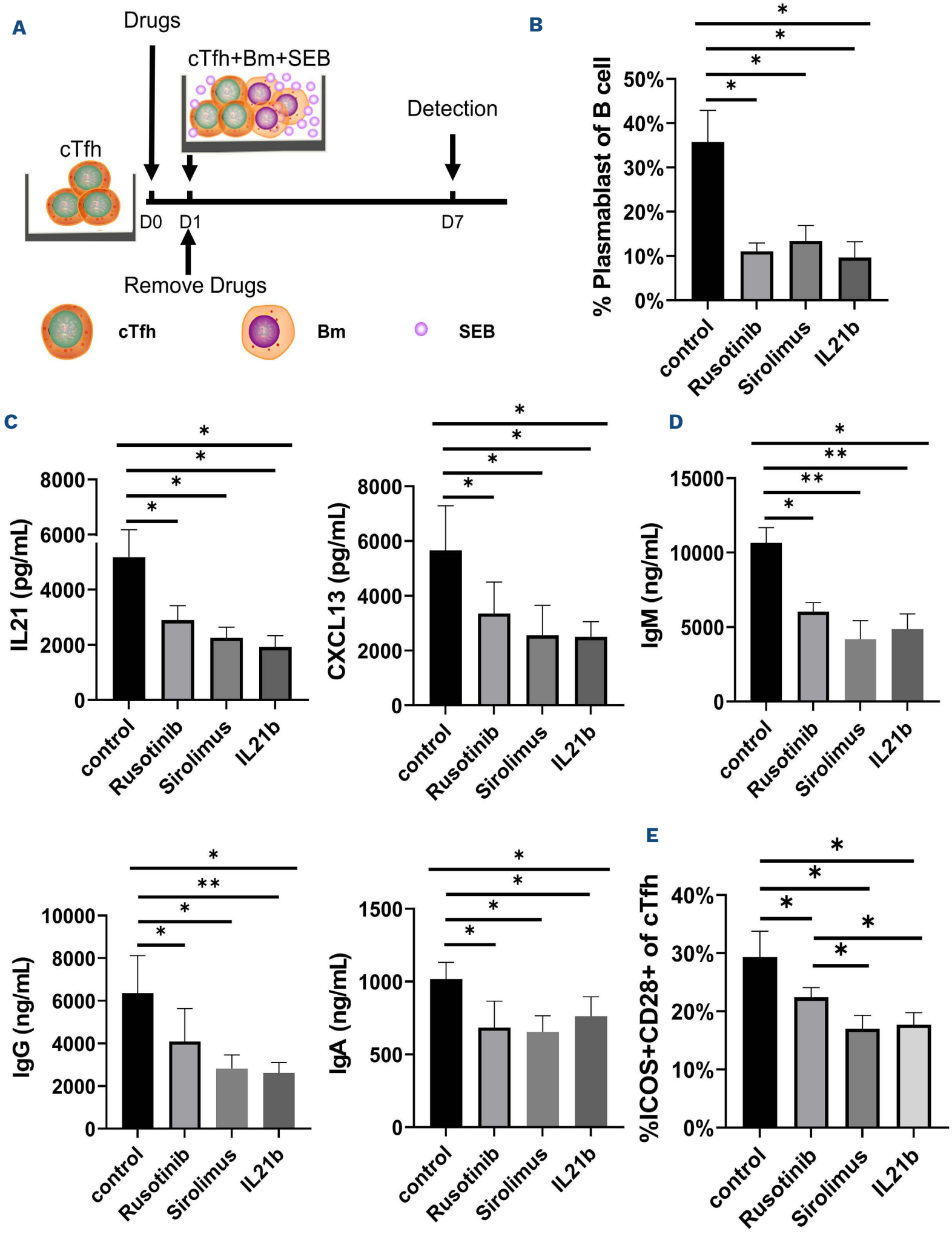

Figure 5. Inhibition of circulating T-follicular helper cell function and reduction in antibody production *in vitro* by immunosuppressive drugs. (A) Model diagram of *in vitro* drug experiment, circulating T-follicular helper (cTfh) from healthy donors (N=5) were sorted, treated with drugs, and then co-cultured with autologous memory B (Bm) cells. (B) The proportion of plasmablast cells in Bm cells. (C) The change of immunoglobulin (Ig) level in the supernatant. (D) The supernatant interleukin (IL)-21 and CXCL13 were detected by enzyme-linked immunosorbant assay. (E) Proportion of $ICOS^+$ $CD28^+$ subgroup in cTfh cells. Wilcoxon rank sum test was performed to assess the significance in (B-E). **P*<0.05; ***P*<0.01. SEB: staphylococcal enterotoxin B.

Ex vivo and *in vivo* experiments on the combination of rituximab and sirolimus for antibody reduction

In previous studies,[2,10] rituximab had a significant desensitization effect on DSA; therefore, we further explored whether sirolimus and rituximab have a combined effect on antibody production. Using an *ex vivo* experiment, we found that the combination of sirolimus and rituximab significantly inhibited the differentiation of B cells into plasmablasts and decreased the levels of IgG, IgM, and IgA as opposed to sirolimus and rituximab used independently (Figure 7A). In the clinical study, we showed that the effective rates of the combination of sirolimus and rituximab as well as sirolimus or rituximab used independently in desensitizing DSA-positive patients were 60%, 43.75%, and 30%, respectively (Figure 7B–F), when a 50% reduction in a patient's DSA MFI was defined as effective treatment. Collectively, the *in vitro* and

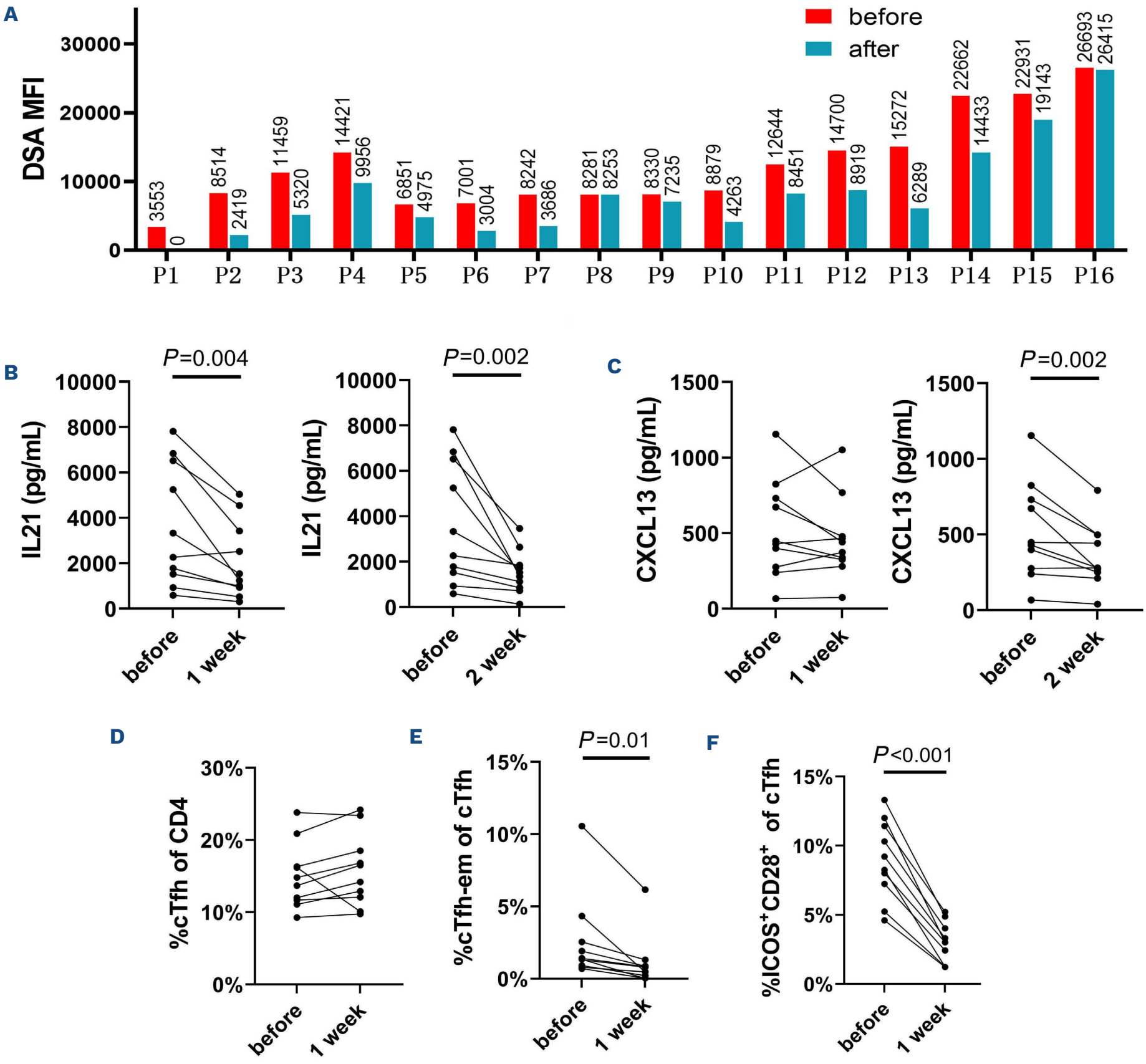

Figure 6. Inhibition of circulating T-follicular helper cell function and reduction in donor-specific antibody level *in vivo* by sirolimus. Donor-specific antibody (DSA)-positive candidates for transplant (N=16) were administered sirolimus regularly after enrollment and their measured blood concentrations were within the range specified in the instructions. (A) Decline in the DSA mean fluorescent intensity (MFI) per patient at the end of the observation period. During the first and second weeks of treatment, serum levels of interleukin (IL)-21 (B) and CXCL13 (C) were measured. Changes in the proportion of circulating T-follicular helper (cTfh) subsets after sirolimus administration (D-F). A paired *t* test was performed to assess the significance in (B-F).

in vivo studies demonstate the efficiency of DSA desensitization using a combination of sirolimus and rituximab as well as sirolimus or rituximab used independently. The infection rates of COVID-19 of patients treated with sirolimus or rituximab either alone or incombination were 75%, 60%, and 50%, respectively. Other side effects are listed in *Online Supplementary Table S3*.

Discussion

This study demonstrated that cTfh cells from candidates undergoing allografting aided B cells in producing DSA, which suggests a potential role of the immune synapse between cTfh cells and B cells. Moreover, we observed that targeting cTfh cells can inhibit the DSA secretion by B cells, in the *in vitro* and *in vivo* studies. As per our knowledge, these results present the first evidence that targeting Tfh cells independently or targeting both the Tfh and B cells can control humoral allogeneic immunity in patients with positive DSA who are candidates for HLA-mismatched allo-HSCT.

The cTfh cells contain two functionally distinct subgroups:[13,20-22] efficient helpers (cTfh2 and cTfh17 cells) and non-efficient helpers (cTfh1 cells). cTfh2 and cTfh17 cells secrete IL-21 and induce B cells to proliferate, differentiate into antibody-producing cells, and undergo class-switching to IgG and IgA, whereas cTfh1 cells lack these characteristics.[13,21,22] We found that the cTfh2 cells were correlated with the level of anti-HLA antibodies in the candidates for HLA-mismatched allo-SCT. This was consistent with the function of cTfh2 cells, as previously demonstrated.[37,38] Moreover, we also observed an association between the percentages and absolute numbers of cTfhem cell subsets and the level of anti-HLA antibodies. Danger *et al.*[39] and La Muraglia *et al.*[17] observed a similar association in organ transplantation settings. Except for the results reported by others in different clinical settings, such as vaccination,[40] viral infection,[24,37] autoimmune disease[31] and organ transplantation,[17,19] our results provide further evidence suggesting that the activated $ICOS^+PD\text{-}1^+$ populations (the cTfhem subsets) may represent the most efficient helpers among the cTfh cells.

In the present study, we observed that cTfh cells induced B cells to differentiate into DSA-producing plasmablasts, which was in accordance with the observation of Louis *et al.*[18] In contrast to the study by Louis *et al.*,[18] our results suggest the probable involvement of the immune synapse in the assistance of B cells by cTfh cells during the production of anti-HLA antibodies. This observation was based on the following findings: first, higher expression of CD28 and ICOS on cTfh cells, as well as CD86 and ICAM on B cells in DSA-positive patients was observed. Second, *in vitro* experiments demonstrated that blocking CD28 on cTfh cells with CTLA-4 Ig significantly reduced the percentage of plasmablasts in B cells, as well as the antibody levels. Third, patients with positive anti-HLA antibodies had genes that were upregulated in expression, such as *BAIAP3*, which were mainly involved in the positive regulation of synaptic transmission. These observations suggest that targeting immune synapse-related molecules could be a novel strategy for inhibiting cTfh cell function, although further studies are required.

The important role of cTfh cells in DSA production, demonstrated in our study, prompted us to investigate drugs that can effectively inhibit DSA function *in vitro*. We found that *in vitro* treatment of cTfh cells with ruxolitinib,[31] sirolimus[32] or IL-21b[30] effectively inhibited the differentiation of plasmablasts from B cells and decreased the levels of CXCL13 and antibodies. Based on previous studies reported by others and our group that showed the effects of rituximab in desensitizing DSA either in the haploidentical HSCT modality[10,11] or in renal transplant settings,[2,12] we further demonstrated the synergistic effect of sirolimus and rituximab in DSA desensization *ex vivo*. Our results indicate that targeting cTfh cells or B cells can inhibit antibody production by B cells. Overall, *in vitro* experimental results indicate that targeting cTfh cells can inhibit their assistance in antibody production by B cells, which provides evidence for the clinical use of cTfh cell-targeted drugs in candidates for HLA-mismatched allografting with positive DSA.

Based on the results of *in vitro* experiments, and considering the evidence that sirolimus has been used in allo-SCT patients for GVHD prophylaxis.[41,42] we performed a pilot study to investigate the efficiency of sirolimus in desensitizing DSA of candidates for HLA-mismatched allografting. We observed that the efficacy rate of sirolimus as a sole agent was 43.75% in all patients. The decrease in the level of DSA was accompanied by the following changes: i) a decrease in the levels of IL-21 and CXCL13 and ii) a decrease in the percentages of cTfhem and $ICOS^+CD28^+$ cTfh cells. Thus, the results of our *in vivo* study and those of other studies[17,19,25] indicate that targeting Tfh cells can provide effective strategies for DSA desensitization in both allo-HSCT and organ transplant settings. In our study, although the role of other immune cells in reducing DSA could not be completely excluded,[43-46] the results of our study and the data reported by other researchers[17-20] support the conclusion that, in terms of reducing the DSA of actual patients, cTfh cells might be more important than other T cells, even when considering the complex interaction between Tfh cells and other T/B cells of patients with positive DSA *in vivo*. Additionally, the comparison between sirolimus and rituximab in DSA desensitization as well as whether treating patients with combined sirolimus and rituximab is better than using either sirolimus or rituximab alone will be investigated in future studies.

In summary, we demonstrated for the first time that there were quantity and quality abnormalities in cTfh cells characterized by an increase in the cTfh2 and cTfhem subsets, and the enhanced ability of cTfh cells in assisting B cells in DSA production, in HLA-mismatched transplant candidates.

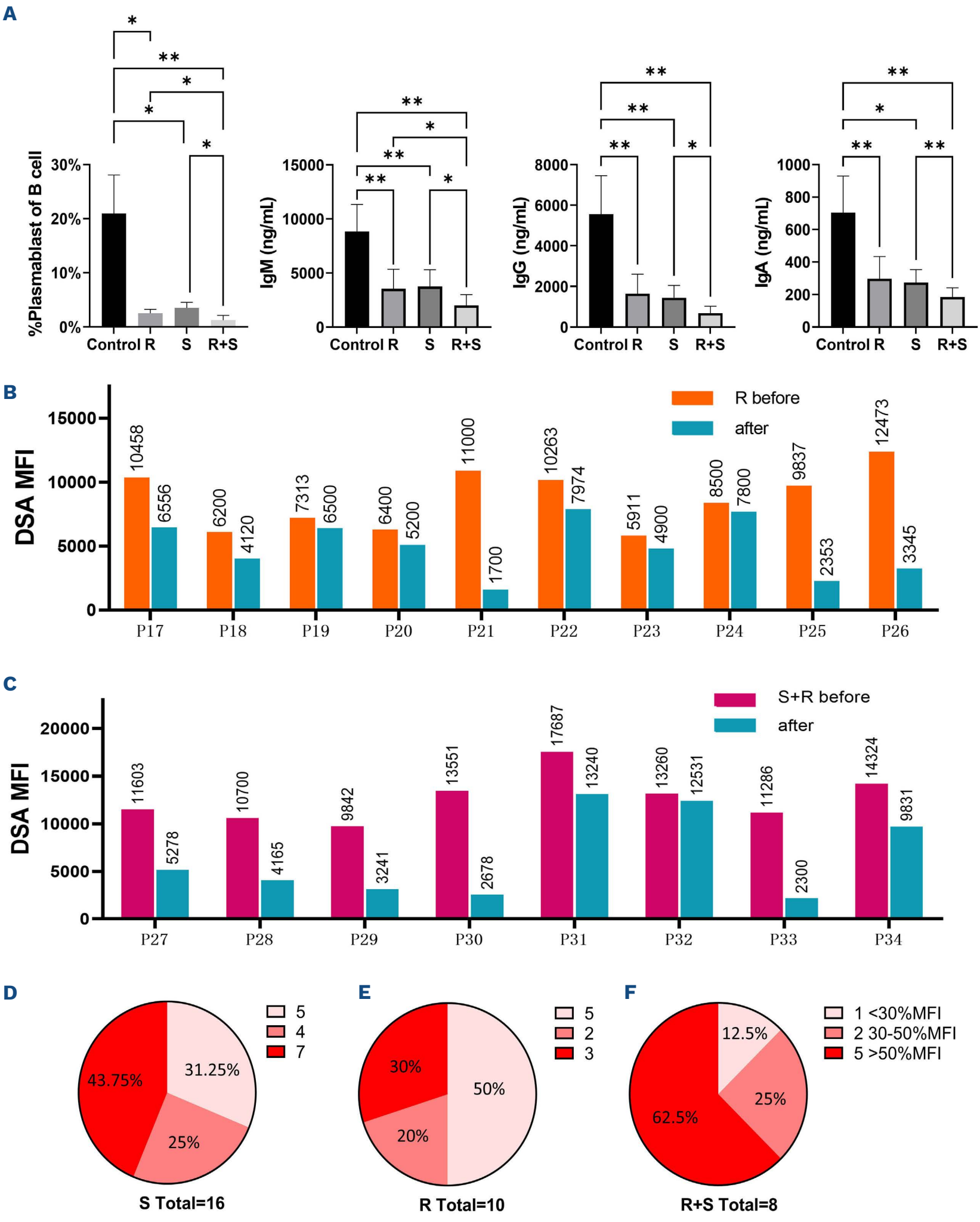

Figure 7. Synergistic effects of sirolimus and rituximab on donor-specific antibody desensitization *ex vivo* and *in vivo*. Cells from healthy donors were used for *in vitro* experiments (N=5), and the indices after co-culturing in different drug groups were analyzed (A). (B) Comparison of donor-specific antibody (DSA) mean fluorescent intensity (MFI) before and after treatment in patients who were administrated with rituximab (R) (N=10). (C) Comparison of the DSA MFI in patients in the sirolimus (S) and R groups (N=8) before and after treatment. (D-F) Comparison of the therapeutic effects in different experimental drug groups. One-way ANOVA (Welch test) was performed to assess the significance in (A). The χ^2 test was used for rate comparison and the Bonferroni test was used for the pairwise control test (D-F). **P*<0.05; ***P*<0.01. R+S: sirolimus and rituximab.

In vitro and *in vivo* experiments indicated that targeting Tfh cells independently or both Tfh and B cells can provide novel methods for DSA desensitization. Further studies are required to identify potential agents for inhibiting cTfh cells. Prospective studies that are clinically focused, conducted in multiple centers, and encompassing a large-sample are warranted to investigate the effectiveness of the agents that target cTfh cells and aid in desensitizing DSA in candidates undergoing allografting.

Disclosures

No conflicts of interest to disclose.

Contributions

X-JH and Y-JC designed the study. X-JH and Y-JC conceived the project and drafted the manuscript. All authors contributed to data interpretation and manuscript preparation. All authors approved the final version of the manuscript.

Funding

This work was partly supported by grants from the Beijing Municipal Science and Technology Commission (no. Z221100007422008), the National Key Research and Development Program of China (no. 2022YFA1103300), and Bethune Charitable Foundation (no. 2020-Z-07).

Data-sharing statement

The data that support the findings of this study are available upon reasonable request from the corresponding author.

References

1. Girnita AL, Duquesnoy R, Yousem SA, et al. HLA-specific antibodies are risk factors for lymphocytic bronchiolitis and chronic lung allograft dysfunction. Am J Transplant. 2005;5(1):131-138.
2. Vo AA, Lukovsky M, Toyoda M, et al. Rituximab and intravenous immune globulin for desensitization during renal transplantation. N Engl J Med. 2008;359(3):242-251.
3. Lefaucheur C, Louis K, Morris AB, et al. Clinical recommendations for posttransplant assessment of anti-HLA (human leukocyte antigen) donor-specific antibodies: a sensitization in transplantation: assessment of risk consensus document. Am J Transplant. 2023;23(1):115-132.
4. Ciurea SO, Thall PF, Wang X, et al. Donor-specific anti-HLA Abs and graft failure in matched unrelated donor hematopoietic stem cell transplantation. Blood. 2011;118(22):5957-5964.
5. Cutler C, Kim HT, Sun L, et al. Donor-specific anti-HLA antibodies predict outcome in double umbilical cord blood transplantation. Blood. 2011;118(25):6691-6697.
6. Bouquegneau A, Loheac C, Aubert O, et al. Complement-activating donor-specific anti-HLA antibodies and solid organ transplant survival: a systematic review and meta-analysis. PLoS Med. 2018;15(5):e1002572.
7. Gochi F, Chen-Yoshikawa TF, Kayawake H, et al. Comparison of de novo donor-specific antibodies between living and cadaveric lung transplantation. J Heart Lung Transplant. 2021;40(7):607-613.
8. Chang YJ, Zhao XY, Xu LP, et al. Donor-specific anti-human leukocyte antigen antibodies were associated with primary graft failure after unmanipulated haploidentical blood and marrow transplantation: a prospective study with randomly assigned training and validation sets. J Hematol Oncol. 2015;8:84.
9. Ruggeri A, Rocha V, Masson E, et al. Impact of donor-specific anti-HLA antibodies on graft failure and survival after reduced intensity conditioning-unrelated cord blood transplantation: a Eurocord, Societe Francophone d'Histocompatibilite et d'Immunogenetique (SFHI) and Societe Francaise de Greffe de Moelle et de Therapie Cellulaire (SFGM-TC) analysis. Haematologica. 2013;98(7):1154-1160.
10. Chang YJ, Xu LP, Wang Y, et al. Rituximab for desensitization during HLA-mismatched stem cell transplantation in patients with a positive donor-specific anti-HLA antibody. Bone Marrow Transplant. 2020;55(7):1326-1336.
11. Ciurea SO, Cao K, Fernandez-Vina M, et al. The European Society for Blood and Marrow Transplantation (EBMT) Consensus Guidelines for the detection and treatment of donor-specific anti-HLA antibodies (DSA) in haploidentical hematopoietic cell transplantation. Bone Marrow Transplant. 2018;53(5):521-534.
12. Crespo M, Zarraga S, Alonso A, et al. Monitoring of donor-specific anti-HLA antibodies and management of immunosuppression in kidney transplant recipients: an evidence-based expert paper. Transplantation. 2020;104(8 Suppl 2):S1-S12.
13. Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular helper T cells. Annu Rev Immunol. 2016;34:335-368.
14. Chen Z, Cui Y, Yao Y, et al. Heparan sulfate regulates IL-21 bioavailability and signal strength that control germinal center B cell selection and differentiation. Sci Immunol. 2023;8(80):eadd1728.
15. Liu D, Yan J, Sun J, et al. BCL6 controls contact-dependent help delivery during follicular T-B cell interactions. Immunity. 2021;54(10):2245-2255.
16. Quast I, Dvorscek AR, Pattaroni C, et al. Interleukin-21, acting beyond the immunological synapse, independently controls T follicular helper and germinal center B cells. Immunity. 2022;55(8):1414-1430.
17. La Muraglia GM, 2nd, Wagener ME, Ford ML, Badell IR. Circulating T follicular helper cells are a biomarker of humoral alloreactivity and predict donor-specific antibody formation after transplantation. Am J Transplant. 2020;20(1):75-87.
18. Louis K, Macedo C, Bailly E, et al. Coordinated circulating T follicular helper and activated B cell responses underlie the onset of antibody-mediated rejection in kidney transplantation. J Am Soc Nephrol. 2020;31(10):2457-2474.
19. Cano-Romero FL, Laguna Goya R, Utrero-Rico A, et al. Longitudinal profile of circulating T follicular helper lymphocytes parallels anti-HLA sensitization in renal transplant recipients. Am J Transplant. 2019;19(1):89-97.
20. He J, Tsai LM, Leong YA, et al. Circulating precursor CCR7(lo) PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. Immunity. 2013;39(4):770-781.
21. Morita R, Schmitt N, Bentebibel SE, et al. Human blood CXCR5(+) CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion.

Immunity. 2011;34(1):108-121.
22. Walker LSK. The link between circulating follicular helper T cells and autoimmunity. Nat Rev Immunol. 2022;22(9):567-575.
23. Forcade E, Kim HT, Cutler C, et al. Circulating T follicular helper cells with increased function during chronic graft-versus-host disease. Blood. 2016;127(20):2489-2497.
24. Juno JA, Tan HX, Lee WS, et al. Humoral and circulating follicular helper T cell responses in recovered patients with COVID-19. Nat Med. 2020;26(9):1428-1434.
25. Mohammed MT, Cai S, Hanson BL, et al. Follicular T cells mediate donor-specific antibody and rejection after solid organ transplantation. Am J Transplant. 2021;21(5):1893-1901.
26. Komagome M, Maki A, Nagata R, et al. Refractory acute antibody mediated rejection in liver transplant after desensitization of preformed donor specific antibody-validity of bortezomib and everolimus: a case report. Transplant Proc. 2022;54(1):147-152.
27. Louis K, Macedo C, Metes D. Targeting T follicular helper cells to control humoral allogeneic immunity. Transplantation. 2021;105(11):e168-e180.
28. Huang XJ, Zhu HH, Chang YJ, et al. The superiority of haploidentical related stem cell transplantation over chemotherapy alone as postremission treatment for patients with intermediate- or high-risk acute myeloid leukemia in first complete remission. Blood. 2012;119(23):5584-5590.
29. Ma N, Guo JP, Zhao XY, et al. Prevalence and risk factors of antibodies to HLA according to different cut-off values of mean fluorescence intensity in haploidentical allograft candidates: A prospective study of 3805 subjects. HLA. 2022;100(4):312-324.
30. Bucher C, Koch L, Vogtenhuber C, et al. IL-21 blockade reduces graft-versus-host disease mortality by supporting inducible T regulatory cell generation. Blood. 2009;114(26):5375-5384.
31. Ricard L, Jachiet V, Malard F, et al. Circulating follicular helper T cells are increased in systemic sclerosis and promote plasmablast differentiation through the IL-21 pathway which can be inhibited by ruxolitinib. Ann Rheum Dis. 2019;78(4):539-550.
32. Caballero-Velazquez T, Sanchez-Abarca LI, Gutierrez-Cosio S, et al. The novel combination of sirolimus and bortezomib prevents graft-versus-host disease but maintains the graft-versus-leukemia effect after allogeneic transplantation. Haematologica. 2012;97(9):1329-1337.
33. Pistillo MP, Tazzari PL, Palmisano GL, et al. CTLA-4 is not restricted to the lymphoid cell lineage and can function as a target molecule for apoptosis induction of leukemic cells. Blood. 2003;101(1):202-209.
34. Lu DP, Dong L, Wu T, et al. Conditioning including antithymocyte globulin followed by unmanipulated HLA-mismatched/haploidentical blood and marrow transplantation can achieve comparable outcomes with HLA-identical sibling transplantation. Blood. 2006;107(8):3065-3073.
35. Susal C, Wettstein D, Dohler B, et al. Association of kidney graft loss with de novo produced donor-specific and non-donor-specific HLA antibodies detected by single antigen testing. Transplantation. 2015;99(9):1976-1980.
36. Kim H, Kim J, Lee H, et al. Baiap3 regulates depressive behaviors in mice via attenuating dense core vesicle trafficking in subsets of prefrontal cortex neurons. Neurobiol Stress. 2022;16:100423.
37. Locci M, Havenar-Daughton C, Landais E, et al. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. Immunity. 2013;39(4):758-769.
38. Boswell KL, Paris R, Boritz E, et al. Loss of circulating CD4 T cells with B cell helper function during chronic HIV infection. PLoS Pathog. 2014;10(1):e1003853.
39. Danger R, Chesneau M, Delbos F, et al. CXCR5(+)PD1(+)ICOS(+) Circulating T follicular helpers are associated with de novo donor-specific antibodies after renal transplantation. Front Immunol. 2019;10:2071.
40. Mudd PA, Minervina AA, Pogorelyy MV, et al. SARS-CoV-2 mRNA vaccination elicits a robust and persistent T follicular helper cell response in humans. Cell. 2022;185(4):603-613.
41. Shaw BE, Jimenez-Jimenez AM, Burns LJ, et al. National Marrow Donor Program-sponsored multicenter, phase II trial of HLA-mismatched unrelated donor bone marrow transplantation using post-transplant cyclophosphamide. J Clin Oncol. 2021;39(18):1971-1982.
42. Pidala J, Walton K, Elmariah H, et al. Pacritinib combined with sirolimus and low-dose tacrolimus for GVHD prevention after allogeneic hematopoietic cell transplantation: preclinical and phase I trial results. Clin Cancer Res. 2021;27(10):2712-2722.
43. Kumar D, Nguyen TH, Bennett CM, et al. mTOR inhibition attenuates cTfh cell dysregulation and chronic T-cell activation in multilineage immune cytopenias. Blood. 2023;141(3):238-243.
44. Gallon L, Traitanon O, Yu Y, et al. Differential effects of calcineurin and mammalian target of rapamycin inhibitors on alloreactive Th1, Th17, and regulatory T cells. Transplantation. 2015;99(9):1774-1784.
45. Tresoldi E, Dell'Albani I, Stabilini A, et al. Stability of human rapamycin-expanded CD4+CD25+ T regulatory cells. Haematologica. 2011;96(9):1357-1365.
46. Yin Y, Mitson-Salazar A, Wansley DL, Singh SP, Prussin C. Rapamycin preferentially inhibits human IL-5(+) T(H)2-cell proliferation via an mTORC1/S6 kinase-1-dependent pathway. J Allergy Clin Immunol. 2017;139(5):1701-1704.