Profiling circulating T follicular helper cells and their effects on B cells in post-cardiac transplant recipients

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Background: To evaluate circulating T follicular helper (cTfh) cells and characterize their function in chronic-phase recipients after heart transplantation.

Methods: Participants were divided into healthy control (HC, n=40), preoperative (Pre, n=40), and post-transplantation chronic-phase recipient (1-year, n=40) groups. The percentages of cTfh cell subsets and $CD19^+$ B cell subsets were measured using flow cytometry. *In vitro* co-culture experiments were performed using cTfh cells and B cells isolated by fluorescence-activated cell sorting. Plasma concentrations of IL-21, chemokine ligand 13 (CXCL13), immunoglobulin G1 (IgG1), and immunoglobulin G3 (IgG3) were quantified using enzyme-linked immunosorbent assays (ELISA).

Results: cTfh and programmed cell death protein 1-positive ($PD-1^+$) cTfh cells, the cTfh17/cTfh ratio, and class-switched memory B cells in peripheral blood were significantly increased in the 1-year group versus the HC and Pre groups (P<0.01), whereas the cTfh1/cTfh ratio and number of naïve B cells were significantly decreased in the 1-year group. Co-culture experiments showed that cTfh cells promoted B cell differentiation to plasmablasts. In the 1-year group, cTfh and $PD-1^+$ cTfh cell numbers were positively correlated with plasmablasts in $CD19^+$ B cells (P<0.01). The cTfh17/cTfh ratio was positively correlated with IgG3 concentrations in plasma (P<0.01). The plasma concentrations of interleukin-21 (IL-21) and CXCL13 in the 1-year group were increased compared to the HC and Pre groups (P<0.05). Chronic-phase recipients had increased proportions of $CD4^+CXCR5^+$ and $CD4^+CXCR5^+PD-1^+$ cTfh cells, with a cTfh1-to-cTfh17 subtype conversion. An increased number of cTfh cells was positively correlated with B cell differentiation to plasmablasts, class-switched memory B cells, and greater IgG production.

Conclusions: During the chronic phase, the proportion of cTfh cells increased and enhanced B cell responses. The cTfh-related soluble factors CXCL13 and IL-21 may regulate the immunopathogenesis of chronic immune injury. Thus, cTfh cells may drive long-term immune rejection in chronic-phase recipients after heart transplantation.

Keywords: Heart transplantation; circulating T follicular helper cells; B cells

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Introduction

Heart transplantation is a life-saving treatment used for patients with end-stage heart failure. Due to advancements in medical technologies in the fields of cardiac protection, cardiac surgery, and immunology, the post-transplantation 1-year survival rate is 82%, and the 5-year survival rate is 70%, as reported by the International Society of Heart and Lung Transplantation (ISHLT) (1). As of 2017, more than 144,230 heart transplants had been conducted worldwide. With the development and clinical application of multiple inhibitors of immune rejection, such as adrenal glucocorticoids (2,3), new polyclonal and monoclonal lymphocyte antibodies, and other immunosuppressive drugs (4-6), acute immune rejection of a transplanted heart can be controlled more effectively. Chronic rejection, which causes functional loss of the transplanted heart, has become the primary factor that significantly affects the long-term survival of cardiac transplant patients.

The underlying mechanisms of chronic rejection are multifactorial, although the activation of the innate and adaptive immune systems is the main mediating factor in chronic rejection-linked injury of transplanted hearts. Chronic immune damage can involve multiple T cell subsets (7). T follicular helper (Tfh) cells, which are CXCR5-positive $CD4^+$ T cells, are a recently defined subset of T cells (8-10). These cells are activated in the germinal centers (GCs) of secondary lymphoid organs and have a stronger ability to promote immunoglobulin formation and B cell differentiation compared with CXCR5-negative $CD4^+$ T cells. In 2000, circulating Tfh (cTfh) cells were first identified as a $CD4^+CXCR5^+$ T cell subset in peripheral circulation that play a similar role to GC Tfh cells (11). Recently, the de Graav (12) and Forcade (13) groups revealed the pivotal role of cTfh cells in patients who undergo kidney transplants or who have chronic graft-versus-host disease. Combined with the fact that CXCR5 can be induced during lymphocytic choriomeningitis virus infection (14), exactly how the cTfh subtypes are distributed in the peripheral blood of chronic-phase patients after heart transplant is not well understood.

In this study, we profiled cTfh subtypes and examined the levels of their related cytokines, CXCL13 and interleukin-21 (IL-21), in the peripheral blood of patients after heart transplantation. Our findings offer novel insights that help elucidate the pathological connection between cTfh cells and B cells in chronic-phase recipients after heart transplantation, providing potential therapeutic targets for ameliorating chronic rejection.

Methods

Study population

This study complied with the Declaration of Helsinki (as revised in 2013) and was approved by the review boards of Union Hospital and Tongji Medical College (IORG number: IORG0003571). Informed consent was obtained from all patients and healthy control (HC) subjects. The study participants were divided into the following three groups: the preoperative group (Pre group, n=40), the group of chronic-phase recipients at 1-year post-heart transplantation (1-year group, n=40), and an age and gender-matched HC group (n=40) comprising volunteers. The demographic information of patients and HC subjects is summarized in Table 1, and the detailed perioperative and follow-up data of the 1-year group is shown in Table 2.

After being discharged from our hospital, all patients were admitted to the outpatient department weekly for the 1st month, biweekly until the 3rd month, monthly from the 4th to 12th month, and twice for one year thereafter. Outpatients who had undergone orthotopic heart transplantation over 1 year before at our institute were enrolled in this study. The maintenance immunotherapies used for all patients included tacrolimus (0–90 days, 10 ng/mL; 90 days–1 year, 8–10 ng/mL; >1 year, 5–8 ng/mL), mycophenolate (maintenance dose 1.5–2 g/day), and prednisone. The exclusion criteria for patients in the Pre and 1-year groups were: (I) a history of autoimmune disease (such as rheumatoid arthritis, systemic lupus erythematosus, or rheumatic heart disease); (II) a medical history of malignant tumors, hepatitis virus infection, or human immunodeficiency virus (HIV) infection; (III) fever or cough symptoms, with lung computed tomography (CT) scans showing infectious lesions; (IV) acute rejection symptoms with a biopsy diagnosed with a grade exceeding 2R according to the ISHLT criteria (15); (V) a blood test showing a white blood cell (WBC) count of >9.5 g/L, erythrocyte sedimentation rate (ESR) of >20 mm/h, and C-reactive protein (CRP) level of >8 mg/L. The exclusion criteria for the HC group included: (I) fever, cough, or phlegm in the week before the start of the trial, which were treated with antibiotics; or (II) a previous history of autoimmune diseases, hepatitis virus infection, or HIV infection.

For the 1-year group patient, they would have coronary

Table 1 Demographic and baseline clinical characteristics of participants from the healthy control group, preoperative group, and 1-year group

Factor	HC (n=40)	Pre (n=40)	1-year (n=40)
Male/total	34/40	32/40	30/40
Age (years)	46.00 (45.00–46.75)	44.50 (30.50–56.00)	49.00 (38.00–56.00)
Weight (kg)	67.95±10.56	61.50±11.35	65.16±12.45
Diseases	–	Idiopathic cardiomyopathy, 34/40; ischemic cardiomyopathy, 2/40; *others, 4/40	Idiopathic cardiomyopathy, 34/40; ischemic cardiomyopathy, 2/40; *others, 4/40
Post-operation time (months)	–	–	31.7 (18.9–49.6)

*, other diseases included end-stage valvular heart disease, congenital heart disease, secondary cardiomyopathy, and cardiac tumors.

angiography once per year. For patients who underwent a coronary angiography to detect cardiac allograft vasculopathy (CAV), the diagnosis of CAV included a detectable angiographic lesion including any branch stenosis (including diffuse narrowing), with or without allograft dysfunction (16).

Blood sample collection

Overnight-fasted blood samples (6 mL) from each participant were collected into heparin-treated anticoagulation tubes. Each blood sample (3 mL) was then carefully added to human lymphocyte-separation solution (3 mL) in a plastic centrifuge tube, followed by centrifugation at 500 ×g for 20 min at 4 ℃ to separate the plasma, mononuclear cells, and erythrocytes.

Human tissue collection and H&E staining

A post-cardiac transplant patient diagnosed with CAV by coronary angiography was admitted to the cardiovascular department of our institute for repeat heart transplantation, because of progressive deterioration in cardiac function. Myocardial tissue was collected during the operation. Tissue samples were either frozen in liquid nitrogen until use, or fixed with formalin and embedded in paraffin. Hematoxylin and eosin (H&E) staining was performed on 4-μm-thick cardiac tissue sections according to a standard protocol.

Cell staining and flow cytometry

Single-cell suspensions were stained for 30 min using the following fluorophore-conjugated antibodies: FITC anti-CD3 (BD Biosciences Cat# 555332, RRID:AB_395739), APC/Cy7 anti-CD4 (BD Biosciences Cat# 341095, RRID:AB_400218), Percp-Cy5.5 anti-CXCR5 (BD Biosciences Cat# 562781, RRID:AB_2313576), PE anti-PD-1 (BD Biosciences Cat# 560795, RRID:AB_2033989), PE/Cy7 anti-CCR6 (BD Biosciences Cat# 560620, RRID:AB_1727440), APC anti-CCR6 (BD Biosciences Cat# 560619, RRID:AB_1727439), PECy7 anti-CXCR3 (BD Biosciences Cat# 560831, RRID:AB_2033944), APC anti-CXCR3 (BD Biosciences Cat# 565223, RRID:AB_2687488), FITC anti-CD19 (BD Biosciences Cat# 555412, RRID:AB_395812), BV421 anti-CD27 (BioLegend Cat# 302823, RRID:AB_10900425), APC anti-CD38 (BD Biosciences Cat# 555462, RRID:AB_398599), PECy7 anti-IgD (BD Biosciences Cat# 555776, RRID:AB_396111), and PE anti-CD138 (BD Biosciences Cat# 552026, RRID:AB_394323). For intracellular staining, cells were permeabilized using the Foxp3 Staining Buffer Set (eBioscience), then stained with PECy7 anti-Ki67 (BD Biosciences Cat# 561283, Clone B56), PE anti-Foxp3 (BD Biosciences Cat# 560082, RRID:AB_1645509), and PE anti-Bcl2 (BD Biosciences Cat# 340576, RRID:AB_400061). All samples were analyzed with an LSRFortessa flow cytometer (Beckton Dickinson), and data were processed with FlowJo software, version 10 (TreeStar, Inc.).

Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed to determine the levels of human plasma IL-21, CXCL13, immunoglobulin (IgG), immunoglobulin G1 (IgG1), and immunoglobulin G3 (IgG3) according to the protocols provided by the manufacturers. The following ELISA kits were used: human IL-21 ELISA kit (BioLegend Cat# 433808, RRID:AB_10663410), human CXCL13 ELISA kit (R&D Systems, USA), human IgG1 ELISA kit, and human IgG3 ELISA kit (eBioscience, USA).

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Table 2 Perioperative and follow-up data of chronic-phase recipients after heart transplantation (1-year group)

Factor	Values
Recipient preoperative factor	
Undergoing surgery again (n)	4/40
History of diabetes (n)	8/40
History of hypertension (n)	6/40
Kidney injury (n)	8/40
Liver injury (n)	8/40
Nervous system injury (n)	2/40
Donor factor	
Age (years)	37.00 (27.50–44.25)
Male (n)	35/40
Weight (<0.8)	7/40
PRA ≥10% (n)	0/40
Lymph toxicity test (%)	7.00 (5.75–8.00)
Cold ischemia time (min)	293.00 (148.00–442.00)
Compatible, but different blood type (n)	0/40
Recipient perioperative factors	
Mechanical assistance* (n)	5/40
Postoperative pathological arrhythmia (n)	8/40
In-hospital infection (n)	23/40
In-hospital pericardial effusion (n)	23/40
Nervous system complications (n)	0/40
Kidney complications (n)	2/40
Outpatient follow-up factor	
Tacrolimus blood concentration (mg/mL)	7.85 (6.48–9.25)
Pericardial or pleural effusion (n)	8/40
Arrhythmia (n)	6/40

*, mechanical assistance refers to intra-aortic balloon counter-pulsation or extracorporeal membrane oxygenation. PRA, panel reaction antibody. The blood tacrolimus concentration; abundances of white blood cells, granulocytes, and lymphocytes; and patients with pericardial or pleural effusion, or arrhythmia were determined on the day of blood sampling.

Co-culture of cTfh cells and B cells

WBCs were isolated as described above and analyzed with a FACS Aria flow cytometer (Beckton Dickinson) to obtain CD4$^+$CXCR5$^-$ T cells, CD4$^+$CXCR5$^+$ cells, and CD19$^+$CD27$^+$ memory B cells (mB) (4×10^5/mL of each cell type). The following fluorescently labeled antibodies were used for fluorescence-activated cell sorting (FACS) analysis: APC anti-CXCR5, APCCy7 anti-CD4, PE anti-CD27, PECy7 anti-CD19, and BV421 anti-CD38 (all from BD Bioscience).

Cells were maintained in RPMI 1640 medium supplemented with 50% fetal bovine serum (FBS). For co-culture experiments, 1×10^5 T and B cells were added to wells of a 96-well plate and co-cultured in 200 μL complete medium containing 10% FBS and 0.2 μg/mL staphylococcal enterotoxin B (SEB). Half of the medium was changed every 2 days to keep the SEB concentration constant. After 6 days of culture, co-cultured cells were subjected to flow cytometry, based on positive staining against the markers fixable viability dye (FVD), CD4, CXCR5, CD19, CD27, and CD38 to reveal changes in the B cell subtype.

Statistical analysis

Unless otherwise stated, continuous variables conforming to a normal distribution were articulated as a mean ± standard deviation and analyze by ANOVA with post-hoc Bonferroni or Tamhane T2 correction. Two-group data were analyzed by a 2-sample t-test. Variables fitting a skewed distribution, which were reported as the median [inter-quartile range (IQR)], were analyzed by the Mann-Whitney test. Categorical variables were presented as counts followed by percentages in parentheses and analyzed by the Chi-square test. Correlations between two variables were evaluated using Pearson's correlation coefficient, except in cases of non-normal data distribution, where Spearman's test was used. Data were analyzed with GraphPad Prism software, version 6.04 for Windows (GraphPad Software, Inc.), differences with $P<0.05$ were statistically significant.

Results

The percentage of cTfh cells was elevated 1-year post-heart transplantation

A total of 120 subjects (each group, n=40) were included in this study. After gender and age matching, the baseline characteristics of the three groups were comparable (*Table 1*). We first evaluated CXCR5 expression in CD4$^+$ T cells. As shown in *Figure 1A* and *B*, the 1-year group had significantly higher CXCR5 expression in their CD4$^+$ T cells compared with the HC and Pre groups (22.95%±1.17%

vs. 17.20%±0.80% and 18.03%±0.87%, P<0.001 and P=0.0012, respectively), but no significant difference was found between the HC and Pre group.

Programmed cell death protein 1 (PD-1) and inducible T cell costimulator (ICOS), which are expressed on cTfh cells, show better function of promoting B cell differentiation and maturation *in vitro* (13,17), defined by the CD4⁺CXCR5⁺PD-1⁺ T cells (polarized cTfh). As shown in *Figure 1A* and *C*, the percentage of CD4⁺CXCR5⁺PD-1⁺ T cells among CD4⁺ T cells was also elevated in the 1-year group (4.08%±0.33% *vs.* 2.52%±0.19% and 2.52%±0.19%, P<0.001, respectively). These results demonstrated that the percentage of cTfh cells increased significantly during the chronic phase in cardiac transplant recipients.

Comparison of the proliferative and anti-apoptotic capabilities of cTfh cell subtypes in the HC, Pre, and 1-year groups

Peripheral blood cTfh cells can be divided into three subtypes based on the expression patterns of the surface molecules, CXCR3 and CCR6: cTfh1 (CXCR3⁺CCR6⁻), cTfh2 (CXCR3⁻CCR6⁻), and cTfh17 (CXCR3⁻CCR6⁺). cTfh2 and cTfh17 have a stronger ability to promote B cell differentiation and antibody class switching (18). As shown in *Figure 2A* and *B*, we found that cTfh1 cells accounted for a smaller proportion of cTfh cells in the 1-year group than in the HC and Pre groups (14.44%±1.09% *vs.* 18.25%±1.28% and 18.58%±1.26%, P=0.027 and P=0.015, respectively). Furthermore, the 1-year group had significantly more cTfh17 (26.25%±1.58%) than the HC group (20.58%±1.38%, P=0.012) and the Pre group (19.60%±1.29%, P=0.002).

Next, we investigated the proliferative and anti-apoptotic abilities of cTfh cells in these three groups by examining the expression of the nuclear proteins Ki67 (a mitotic marker) and Bcl-2 (an apoptosis inhibitor). As shown in *Figure 2C* and *D*, the percentages of Ki67⁺ cTfh cells in the HC, Pre, and 1-year groups were 4.54%±0.27%, 4.36%±0.30%, and 2.90%±0.23%, respectively. We also found that the proliferative capacity of cTfh cells was correlated significantly with the proportion CD4⁺CXCR5⁺PD-1⁺ T cells (*Figure 2C*, r=0.49, P=0.002). No significant difference was found in the Bcl2-expression levels between groups, suggesting that cTfh cells in the patients of these groups exhibited comparable anti-apoptotic abilities.

We then compared the levels of circulating T follicular regulatory cells (cTfr), which are positive for both CXCR5 and Foxp3, in peripheral blood samples from patients in each group. The cTfr/cTfh ratio was significantly lower in the 1-year group than in the HC and Pre groups (*Figure 2E*, 14.85%±0.74% *vs.* 19.27%±0.94% and 18.73%±1.09%, P<0.001 and P=0.017, respectively). No significant difference was observed in the proportion of cTfr cells among total CD4⁺ T cells between these groups (*Figure 2E*). We found that the proportion of cTfh cells were negatively correlated with the cTfr/cTfh ratio (r=−0.371, P=0.018) and cTfr/CD4⁺ T cell ratio (r=−0.43, P=0.006) in the 1-year group, but were not related to the number of Treg (CD4⁺Foxp3⁺) cells (*Figure 2F*).

Stimulation of B cell differentiation by cTfh cells in vitro

To examine whether the elevation of cTfh cells could stimulate B cell differentiation *in vitro*, we isolated CD4⁺CXCR5⁻ T cells, CD4⁺CXCR5⁺ cells, and CD19⁺CD27⁺ mB cells via FACS. These cells were uniformly translucent, and the purity obtained by flow cytometry exceeded 95% (*Figure 3A,B*). We co-cultured these cells based on the following groupings: mB alone, the CD4⁺CXCR5⁺ + mB group, and the CD4⁺CXCR5⁻+ mB group. After 6 days of culture, the percentage of CD19⁺CD38^hi plasmablasts per well was obtained using flow cytometry. The proportion of mB-derived plasmablasts in the CD4⁺CXCR5⁺ + mB group accounted for 37.63%±3.22% of the CD19⁺ B cells, which was significantly higher than the CD4⁺CXCR5⁻ + mB group, in which the proportion was 21.50%±4.13% (*Figure 3C,D*, P=0.001), indicating that cTfh cells could promote the differentiation of mB cells into plasmablasts.

Distribution of B cell subpopulations and the correlation between cTfh cells and B cells in chronic-phase recipients after heart transplantation

Based on our *in vitro* experimental results, we examined whether cTfh cells could promote B cell differentiation in chronic-phase recipients after heart transplantation. Flow cytometry was used to delineate different B cell subsets as shown in *Figure 4A* and *B*, including CD19⁺CD27⁻IgD⁺ naïve B cells, CD19⁺CD27⁻IgD⁺CD38⁺⁺ transitional B cells, CD19⁺CD27⁺IgD⁺ non-class-switched memory B cells, CD19⁺CD27⁺IgD⁻ class-switched memory B cells, and CD19⁺IgD⁻CD27^hiCD38^hi plasmablasts (19). Among these subsets, the proportion of naïve B cells and transitional B cells in CD19⁺ B cells was significantly decreased in the 1-year

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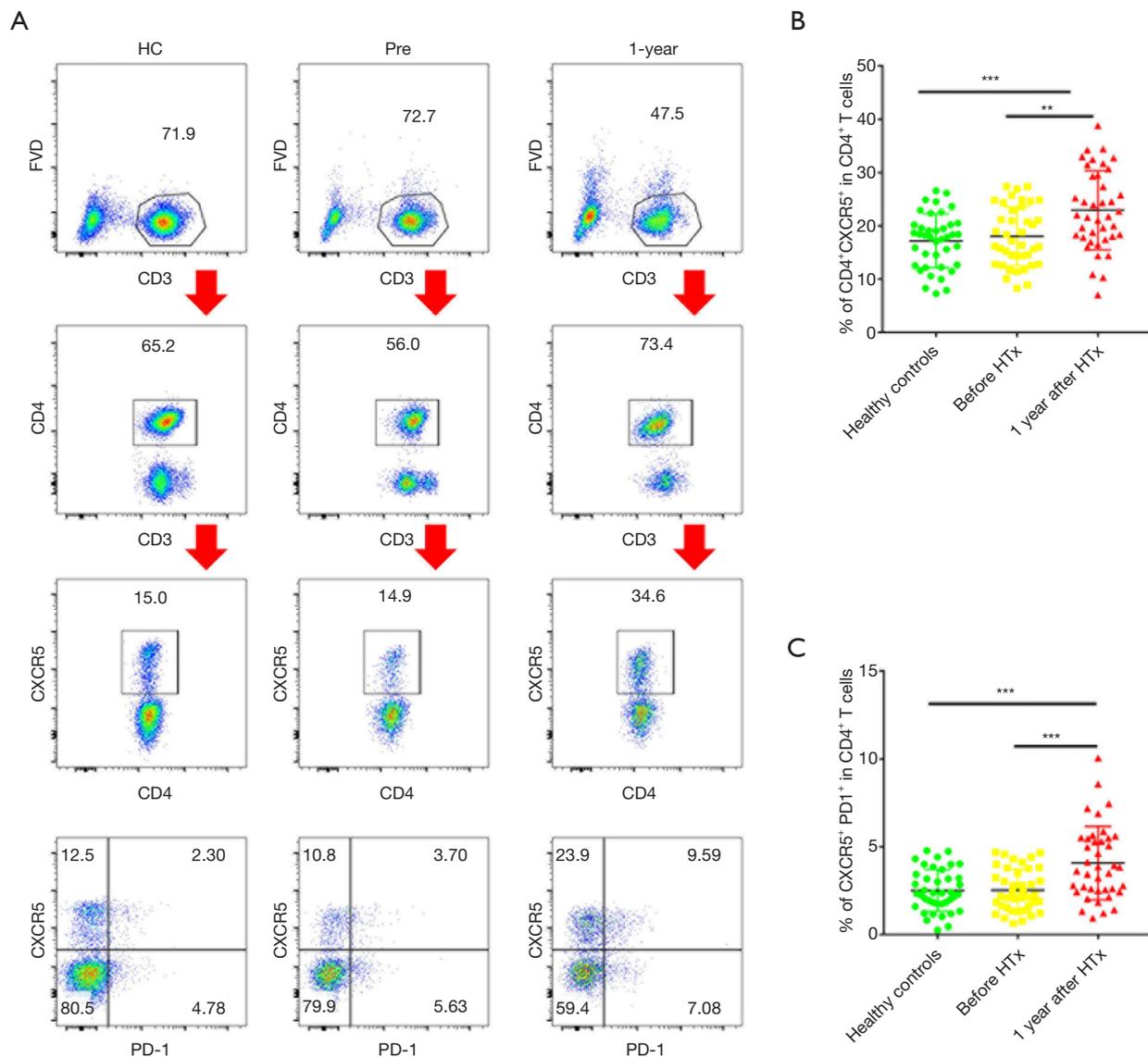
Figure 1 CD4+CXCR5+PD-1+ cTfh cells were elevated in the blood of patients 1-year after heart transplantation (HTx, 1-year) compared to patients before HTx (Pre) and healthy control (HC) subjects. (A) Gating strategy to detect CD4+CXCR5+PD-1+ cTfh cells in the HC (n=40), Pre (n=40) and 1-year (n=40) groups; (B) the percentage of CD4+CXCR5+ T cells within CD4+ T cells among the 3 groups; (C) the percentage of CXCR5+PD-1+ T cells within CD4+ T cells among the 3 groups. The data are presented as the mean ± SEM. **, P<0.01; ***, P<0.001. NS, not significant.

group compared with the HC and Pre groups. In addition, the 1-year group had a higher number of class-switched memory B cells compared with the HC and Pre groups (20.50%±1.49% *vs.* 16.31%±1.32% and 14.19%±1.20%, P=0.039 and P=0.002, respectively). This finding suggests that the abundance of memory B cells gradually increased, whereas naïve B cells gradually decreased over time compared with pre transplantation group.

Through correlation analysis, we found that the CD4+CXCR5+PD-1+ cTfh/CD4+ T cell ratio (r=0.329, P=0.037) and the CD4+CXCR5+PD-1+ cTfh/cTfh ratio (r=0.417, P=0.007) were closely related to the percentage of peripheral plasmablasts (*Figure 4C*), as was the proliferative capacity of cTfh cells (r=0.384, P=0.014). These observations suggest that cTfh cell activation and proliferation can promote plasma cell-like changes in
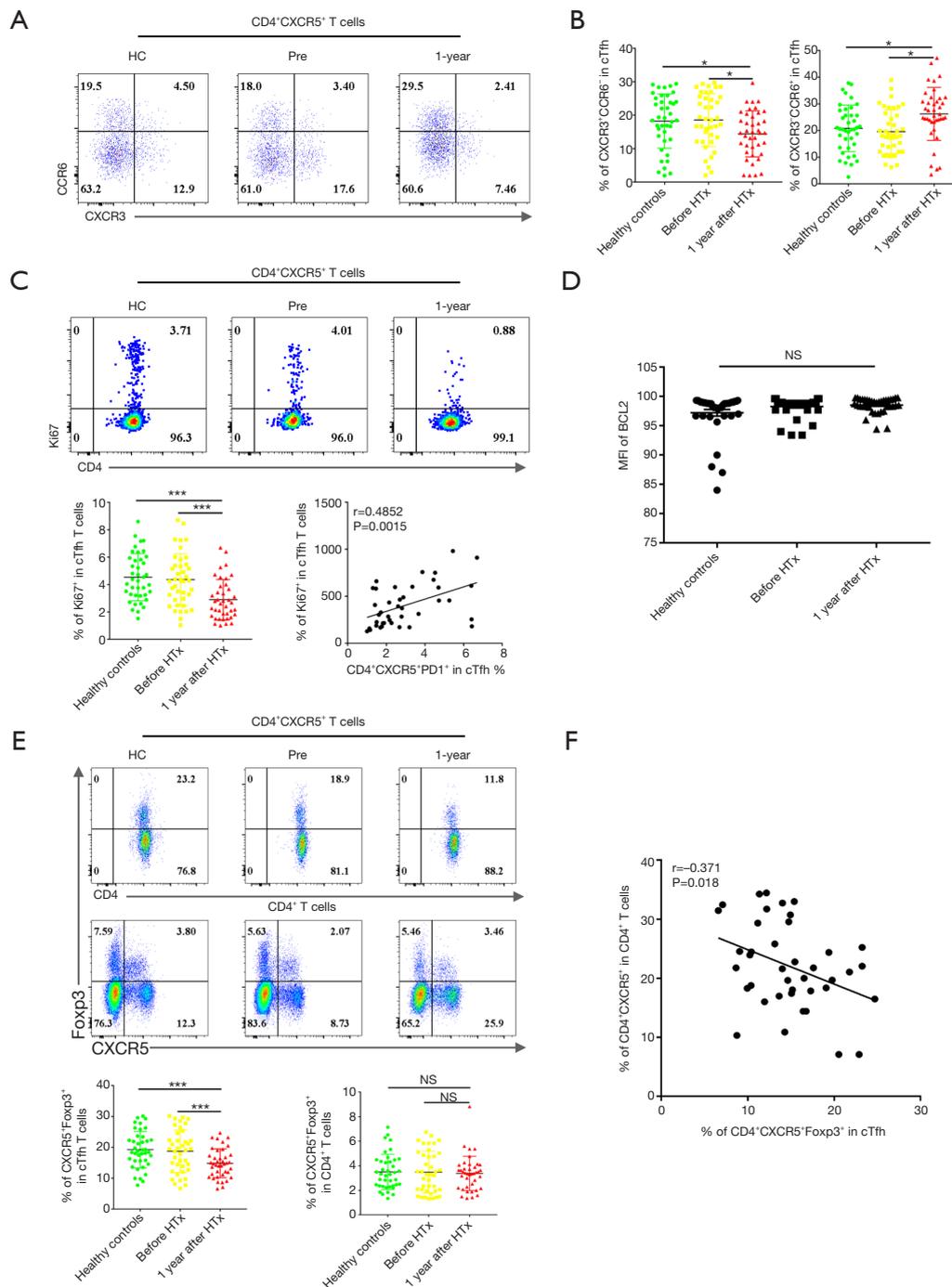
Figure 2 Comparison of the cTfh subtypes, proliferative and anti-apoptotic capabilities, and regulatory cTfh cells among the HC, Pre, and 1-year groups. (A) The relative abundances of CD4$^+$CXCR5$^+$CXCR3$^+$CCR6$^-$ (cTfh1), CD4$^+$CXCR5$^+$CXCR3$^+$CCR6$^-$ (cTfh2), and CD4$^+$CXCR5$^+$CXCR3$^-$CCR6$^+$ (cTfh17) cell populations were determined; (B) the proportion of cTfh1 cells and cTfh17 cells within CD4$^+$ T cells among the 3 groups (n=40/group); (C) comparison of Ki67 expression within cTfh cells among the 3 groups. The correlation between the proportion of CD4$^+$CXCR5$^+$PD-1$^+$ cTfh cells and Ki67 expression in the 1-year group is shown; (D) comparison of Bcl-2-expression levels among the 3 groups; (E) flow-contour plots of Foxp3$^+$ cells within cTfh cells and a comparison of Foxp3 expression among the 3 groups; (F) correlation between peripheral blood cTfh cells and follicular T regulatory (cTfr) cells in the 1-year group. The data are presented as the mean ± SEM. *, P<0.05; ***, P<0.001. NS, not significant.

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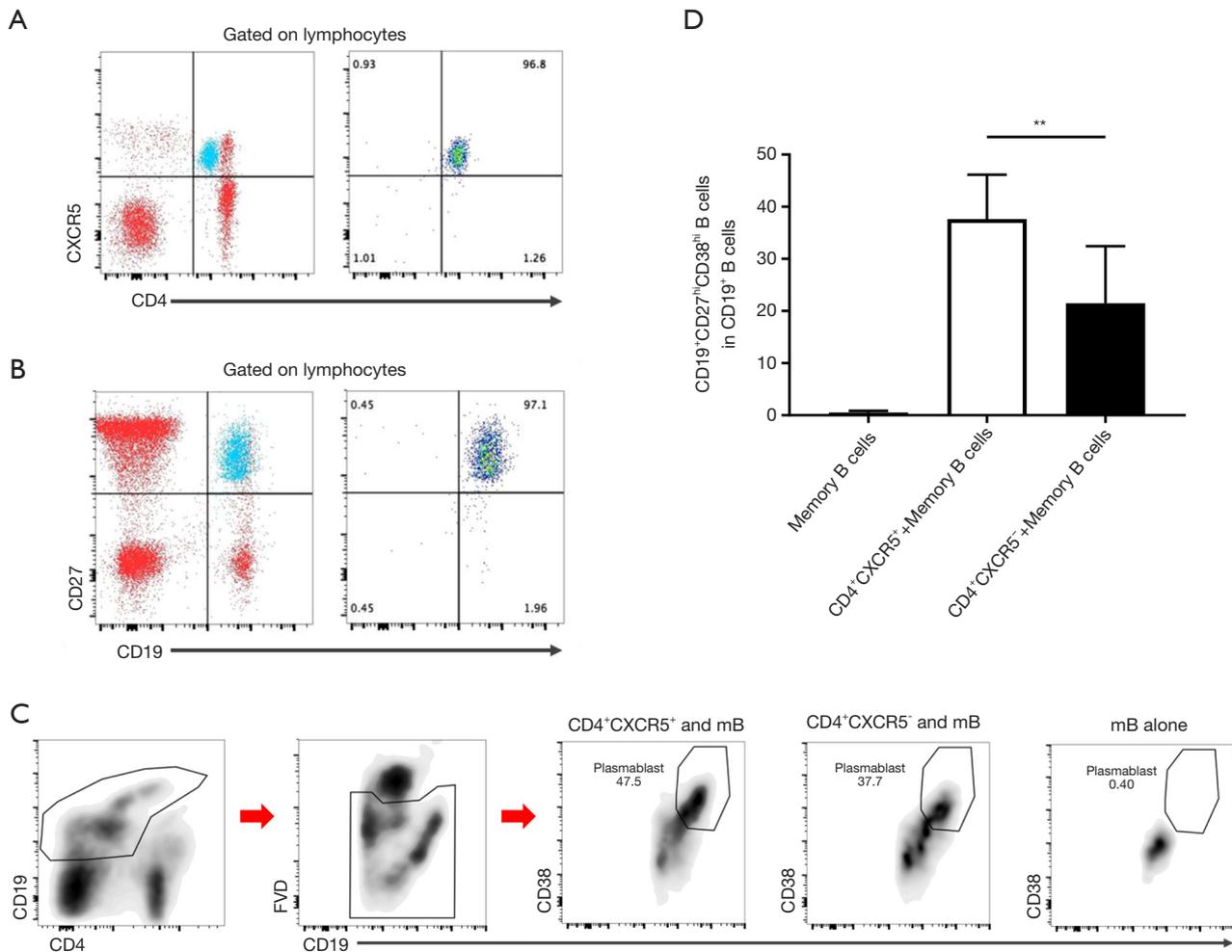
Figure 3 cTfh cells promote B cell differentiation to plasmablasts *in vitro*. (A) FACS analysis of CD4+CXCR5+ cTfh and CD4+CXCR5− T cell subtypes. The blue dots represent cells after sorting, and the red dots represent cells before sorting; (B) FACS-based sorting of CD19+CD27+ mB cell subtypes. The blue dots represent cells after sorting, and the red dots represent cells before sorting; (C) flow cytometric gating strategy used for CD19+CD27hiCD38hi B cells after co-culture for 6 days in a 96-well plate; (D) the percentage of CD19+CD27hiCD38hi B cells within CD19+ B cells in the CD4+CXCR5+ T cell (n=5) and CD4+CXCR5+ T cell group (n=5). The data are presented as the mean ± SEM. **, P<0.01. NS, not significant.

peripheral blood B cells.

Cytokine concentrations and correlations with cTfh cells and B cell subsets between groups

IL-21 is a cytokine produced by cTfh cells that regulates B cell differentiation and proliferation as well as promoting auto-antibody production. Compared with the HC and Pre groups, the IL-21 protein and mRNA expression levels in PBMCs were significantly higher in the 1-year group

(*Figure 5A*). IL-21 production was positively correlated with the proportions of cTfh cells (r=0.407, P=0.009) and CD4+CXCR5+PD-1+ cTfh cells (r=0.394, P=0.018) among CD4+ T cells.

The CXCR5 ligand CXCL13 can be secreted by immune cells, endothelial cells, dendritic cells, and macrophages. The CXCL13–CXCR5 axis can promote the migration of cTfh cells and B cells to lymph node follicles and enhance the responses of these two cell populations. We measured the plasma CXCL13 levels in the three groups
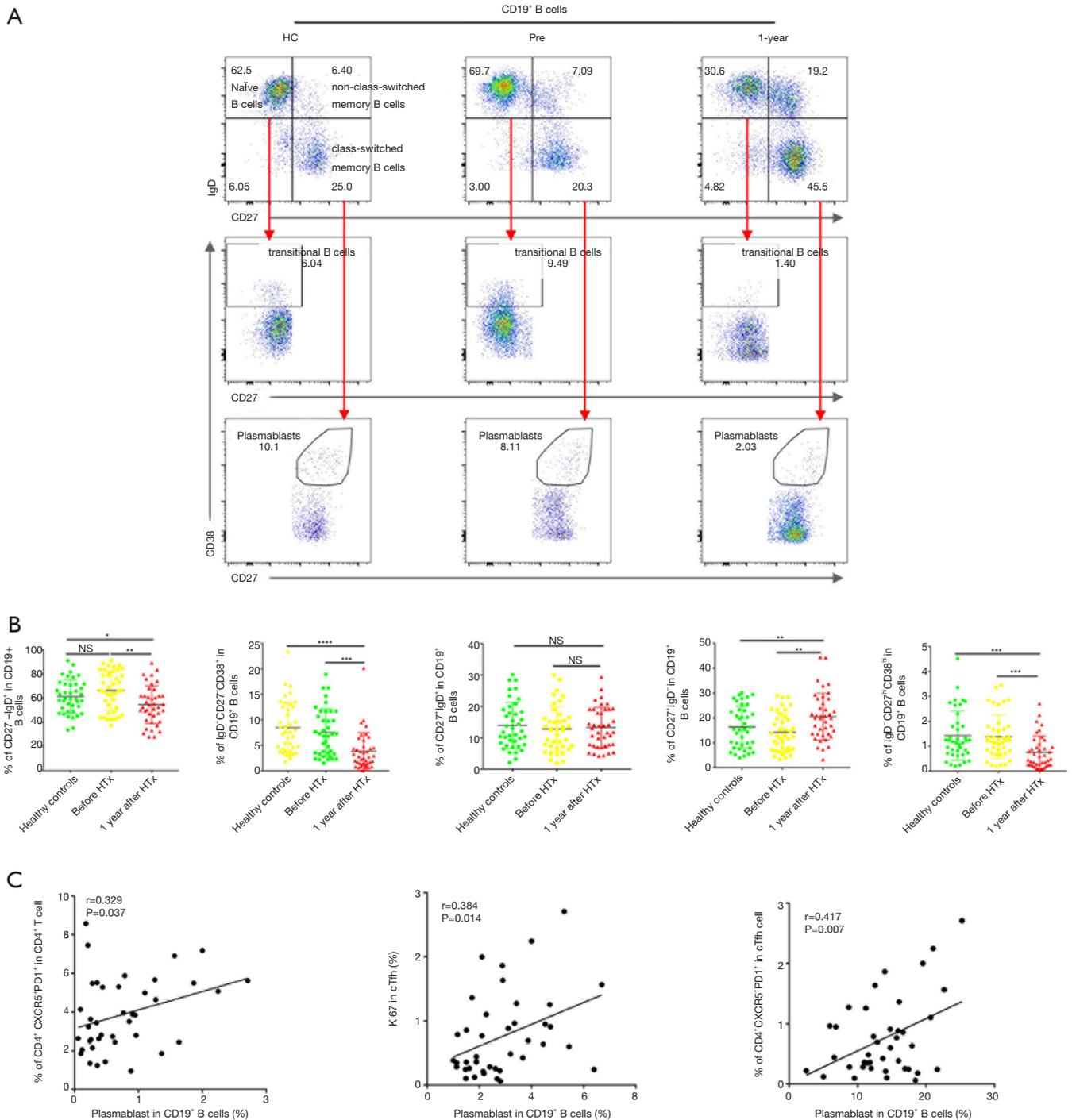
Figure 4 Comparison of B cell subsets among the 3 treatment groups, and the correlation between cTfh cells and B cells in the 1-year group. (A) Gating strategy used to display CD19$^+$IgD$^+$CD27$^-$ naive B cells, CD19$^+$IgD$^+$CD38$^+$CD27$^-$ transitional B cells, CD19$^+$IgD$^+$CD27$^+$ non-class-switched memory B cells, CD19$^+$IgD$^-$CD27$^+$ class-switched memory B cells, and CD19$^+$IgD$^-$CD27^{hi}CD38^{hi} plasmablasts in the 3 groups (n=40/group); (B) the percentage of naive B cells, transitional B cells, non-class-switched memory B cells, class-switched memory B cells, and plasmablasts in CD19$^+$ B cells among the 3 group; (C) the correlations between the ratio of plasmablasts to CD4$^+$CXCR5$^+$PD-1$^+$ cTfh cells (%) within CD4$^+$ T cells, Ki67 (%) in cTfh cells, and CD4$^+$CXCR5$^+$ PD-1$^+$ cTfh cells (%) in the cTfh cells in the 1-year group. The data are presented as the mean ± SEM. *, P<0.05; **, P<0.01; ***, P<0.001. NS, not significant.

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(*Figure 5B*). The 1-year group had significantly higher expression levels of the CXCL13 (130.80±17.26 pg/mL) compared to the HC and Pre groups (59.29±4.00 and 63.43±3.99 pg/mL, P<0.0001, respectively). We also found that plasma CXCL13 levels were positively correlated with the percentage of cTfh17 cells (r=0.340, P=0.031) and plasmablasts (r=0.500, P=0.001), but negatively correlated with the percentage of non-class-switched memory B cells (r=–0.345, P=0.025). Collectively, these observations suggest that the increase in plasma CXCL13 was most likely related to the transformation of cTfh cells to cTfh17 cells, and the migration of memory B cells to the lymph nodes.

We also measured the concentrations of IgG1 and IgG3 in the 1-year group using ELISA (*Figure 5C*). Both IgG subsets were significantly increased in the 1-year group, and IgG3 concentration was positively correlated with the proportion of plasmablasts among $CD19^+$ B cells (r=0.359, P=0.022) and cTfh17 cells (r=0.428, P=0.005).

Presence of cTfh cells in patients with CAV

CAV is a major manifestation of the impaired immune system in chronic-phase recipients after heart transplantation and is the main heart transplantation-related complication affecting long-term survival (20). We examined coronary tissue samples from patients who underwent repeat heart transplantation due to chronic immune rejection. Compared to normal coronary tissue samples, graft vascular stenosis was evident, and was concentric due to the combination of intimal hyperplasia and insufficient external remodeling (*Figure 6A*).

We then analyzed the clinical data of patients in the three treatment groups based on the postoperative duration. We found that the proportion of cTfh cells was higher in patients during the third and fourth years after heart transplantation compared to the first year after surgery (*Figure 6B*). The incidence rate of CAV increases with increasing time after heart transplantation, and our results showed that compared with recipients without angiogram-diagnosed CAV, patients with CAV did not have higher percentages of cTfh cells (*Figure 6C*).

Discussion

Chronic rejection is a major cause of organ transplant failure over time. Currently, limited therapeutic means are available for preventing chronic graft rejection (21). Multiple immunocompetent cells (including innate immune cells and adaptive immune cells) participate in the chronic immune response after heart transplantation. As the key factor linking natural and humoral immunity, T cells indirectly recognize donor major histocompatibility complex (MHC) molecules after transplantation and play important roles in the development of chronic immune damage (22).

Consistent with previous reports (13,23,24), in this study, we found that the proportion of cTfh cells and $CD4^+CXCR5^+PD-1^+$ cTfh cells among $CD4^+$ T cells was increased in the 1-year group compared with the HC and Pre groups. Additionally, the transcription factor Bcl-6 was not expressed on cTfh cells in each group, suggesting that these cells were not the memory form of lymph node Tfh cells reported previously (23). Our findings were in agreement with other previous transplantation-related reports (11,25). Furthermore, the proliferative capacity of cTfh cells in the 1-year group was significantly lower than that in the Pre group, which was likely due to the application of an immunosuppressant. cTfh might exist in the peripheral blood as the memory status of GC Tfh, thus this could also account for the fact that they had lower expression of Ki67. Previous findings suggested that Tfh cells expressing PD-1 and ICOS exhibit stronger function of promoting B cell differentiation (26). In line with this observation, we found that cTfh cell activation and proliferative capacity were closely related. Also this changes were not due to the blood concentration changes of tacrolimus, we found neither the cTfh proportion nor the $CD4^+CXCR5^+PD-1^+$ T cell proportion was related with the tacrolimus (data not shown). This also shown that in the chronic phase of heart transplantation where the alloantigen persisted, cTfh instead of Th1 cells plays vital roles in long-term T cell response.

By profiling the cTfh cell subtypes, we found that the proportion of cTfh17 cells was increased, and the proportion of cTfh1 cells was decreased, whereas cTfh2 cells exhibited enhanced anti-apoptotic ability in the 1-year group (data not shown). Collectively, these findings suggest that in chronic-phase recipients after heart transplantation, transplantation-related injury can trigger an increase in the number of cTfh17 cells and enhance the survival of cTfh2 cells. These phenotypic changes from cTfh1 to cTfh17 are highly likely to drive more B cell differentiation towards plasmablasts, followed by increased antibody production (27,28). However, the exact relationship between these changes still need further *in vitro* sorting and Tfh17 and B cell coculture experiment validation.
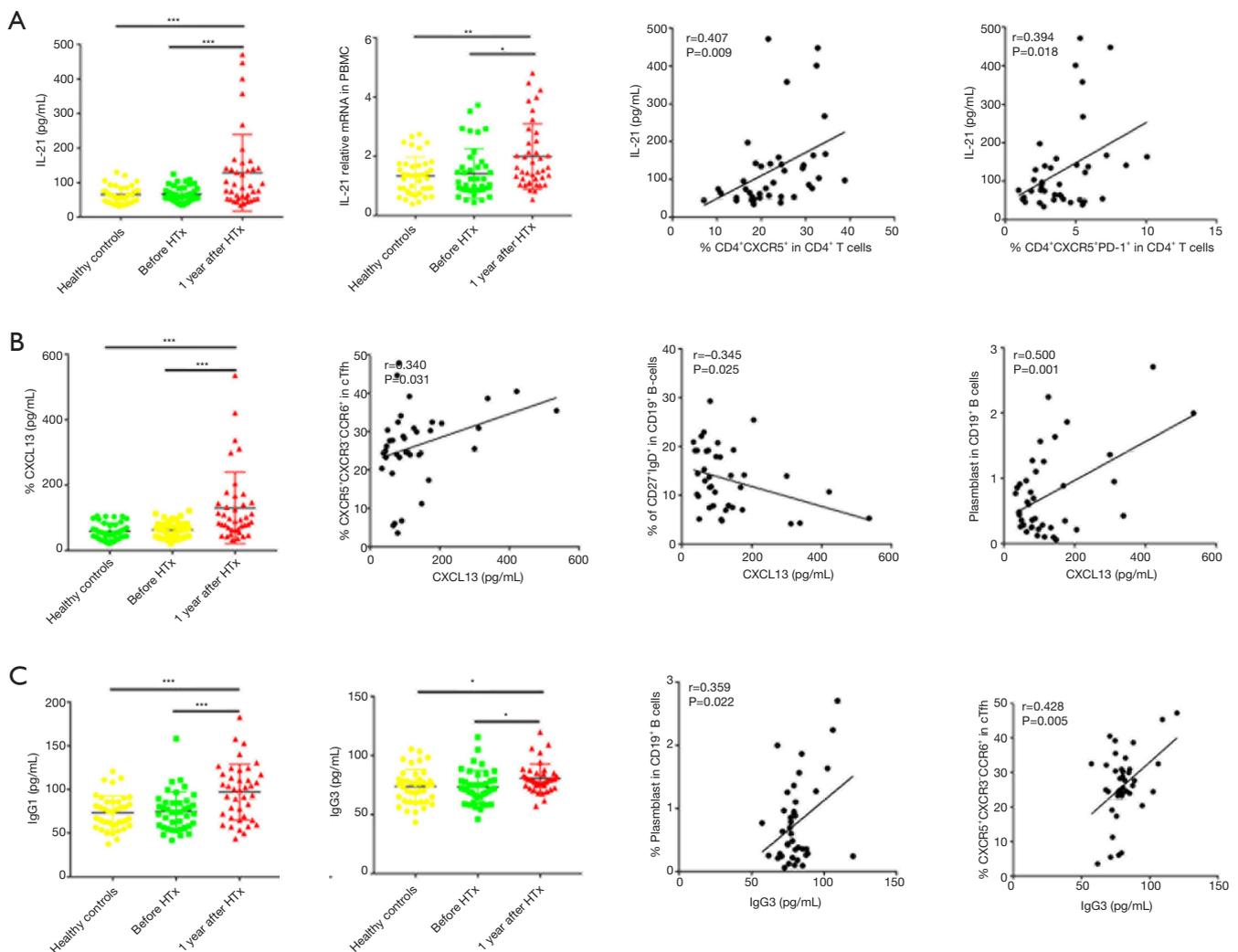
Figure 5 Cytokine concentrations and their correlation with cTfh cells and B cell subsets among the three treatment groups. (A) Comparison of the relative plasma IL-21 protein concentrations (pg/mL) and mRNA-expression levels in the 3 groups (n=40/group). Correlation between the IL-21 concentration and the % cTfh cells within CD4+ T cells and the % CD4+CXCR5+PD-1+ cTfh cells within CD4+ T cells in the 1-year group; (B) comparison of the plasma CXCL13 concentration (pg/mL) in the 3 groups. The correlation between the CXCL13 concentration and % cTfh17 within CD4+ T cells, % non-class-switched memory B cells within CD19+ B cells, and % plasmablasts within CD19+ B cells in the 1-year group; (C) comparison of the IgG1 and IgG3 concentrations (pg/mL) in the three groups. Correlation between the IgG3 concentration and % plasmablasts within CD19+ B cells and % cTfh17 within cTfh cells. The data are presented as the mean ± SEM. *, P<0.05; **, P<0.01; ***, P<0.001. NS, not significant.

In contrast to cTfh cells, cTfr cells prevent the production of alloreactive B cells (29-31). The loss of peripheral blood cTfr cell function has also been etiologically linked to autoimmune disease (32). Consistent with this, we found a negative correlation between peripheral blood cTfr cells and cTfh cells, suggesting that cTfr cells may be important regulators of cTfh cell levels, not only in the GC, but also in the peripheral blood. Meanwhile, for the HC and Pre group, we also witnessed cTfr/cTfh negatively correlated with cTfh/CD4+ T cells (data not shown), which further illustrated no matter in the no heart transplant status but also in the transplant status, cTfr might also participate in the process of cTfh response. However, further *in vitro* coculture experiment

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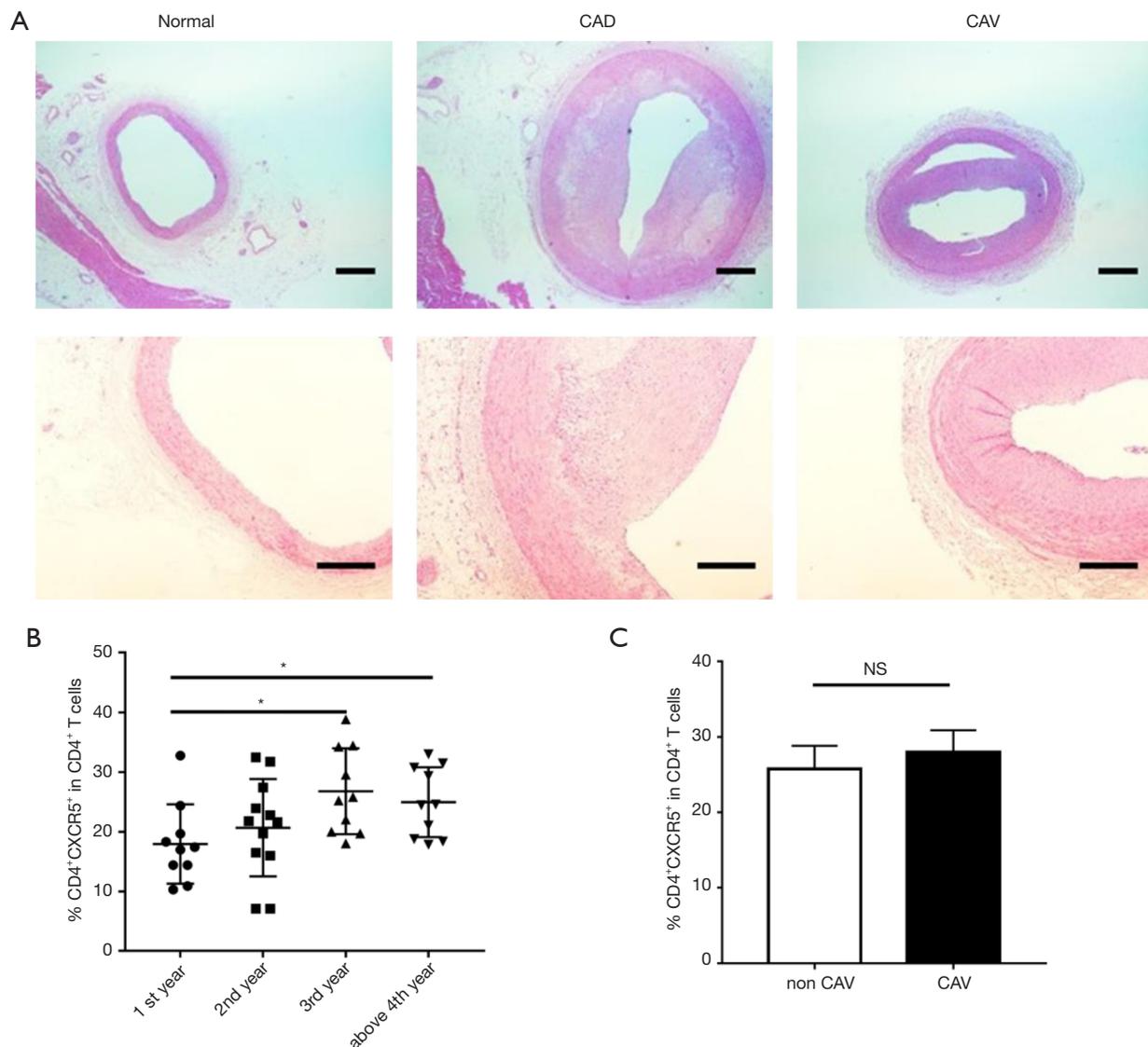
Figure 6 The percentage of cTfh cells were closely related to cardiac allograft vasculopathy (CAV). (A) Hematoxylin and eosin (H&E) staining showing normal, coronary artery disease (CAD), and CAV tissue. Magnification: top panels, 40×; bottom panels, 100×; (B) follow-up analysis of the percentage of cTfh cells within CD4+ T cells at 1, 2, 3, or 4 years after heart transplantation; (C) the percentage of cTfh cells within CD4+ T cells in non-CAV and CAV patients. The data are presented as the mean ± SEM. *, P<0.05. NS, not significant.

of cTfh and cTfr are needed to provide direct evidence of cTfr regulation on cTfh. Under normal conditions, cTfh cells secrete IL-21 (33,34), which promotes plasma cell formation in both humans and mice (35,36). In this study, we found that IL-21 mRNA levels in both PBMCs and plasma were up-regulated in chronic-phase recipients after heart transplantation. This finding suggests that IL-21 may play a vital role in cTfh cell-mediated immune injury. However, the downstream effectors of IL-21 have

yet to be elucidated. CXCL13, a ligand for CXCR5, plays an important role in promoting the migration of cTfh and B cells into lymph nodes (37,38). The concentration of plasma CXCL13 in the 1-year group was significantly higher than that in the HC and Pre groups, suggesting that lymphocytes or peripheral immune cells secreted more CXCL13 in chronic-phase recipients after heart transplantation. An increase in plasma CXCL13 often results in a decreased proportion of cTfh cells among

CD4⁺ T cells by promoting cTfh cells to migrate to the lymphoid organs (13). However, in the 1-year group, we found an increase in both circulating cTfh cells and CXCL13 levels, indicating that cTfh cells were strongly mobilized. In the future, we could block CXCL13-CXCR5 axis *in vitro* culture experiment to further test its functions on Tfh mobilization and recruiting.

The implication of antibody-mediated rejection (AMR), mediated by B cells in graft vascular disease caused by chronic immune injury after transplantation, has been well documented (39). Also, cTfh cells have been shown to play an important role in AMR development (40). In this study, we co-cultured cTfh and mB cells collected from HC peripheral blood, and found that cTfh cells promoted mB cell differentiation into plasmablasts. Our findings support the notion that the functional relationship between T and B cells potentially contributes to AMR development. In this study, we also unveiled a significant correlation between the number of plasmablasts and the activation and proliferative ability of cTfh cells, and that the cTfh cell proliferative capacity was related to the proportion of transitional B cells no matter in the 1 year group but also in the HC and Pre group. We speculate that cTfh cells not only directly promote B cell maturation, but also secrete cytokines to activate B cells localized in lymphoid organs to mediate humoral immune responses. Through GC responses, memory B cells and plasmablasts are produced, and the decreased percentage of plasmablasts may reflect their migration to the bone marrow for long-term antibody production. The higher percentage of class-switched memory B cells is in accordance with the elevated cTfh cell proportions. In addition, conversion of cTfh subtype from cTfh1 to cTfh17 may be linked with IgG3 secretion from plasmablasts.

We also examined the potential correlation between CAV (which is the major complication during chronic immune injury of allografts) and changes in the cTfh subtypes, but did not observe any definite correlations. Thus, our observations are not consistent with those of previous reports (41). This discrepancy may due to the small sample size of our study, or other immune cells or clinical factors affecting the number of cTfh cells. Hence, the correlation between the cTfh subtypes and CAV in chronic-phase recipients after heart transplantation needs to be further clarified in future studies. Also, due to the lack of enough re-heart transplant CAV tissues, whether Tfh could directly infiltrated the tissue remains to be evaluated by myocardium digestion and flow analysis.

Conclusions

All the phenomenon is summarized in the *Figure 7*. We demonstrated that chronic-phase recipients after heart transplantation had increased proportions of CD4⁺CXCR5⁺ and CD4⁺CXCR5⁺PD-1⁺ cTfh cells among CD4⁺ T cells, with a subtype conversion observed from cTfh1 to cTfh17. The increased levels of cTfh cells were positively correlated with the activation and transformation of B cells to plasmablasts and class-switched memory B cells, as well as greater IgG production. cTfh-related soluble factors (chemokines and cytokines) such as CXCL13 and IL-21 may also participate in the immunopathogenesis of chronic immune injury. Thus, our study suggests that cTfh cells are an important factor in the long-term immune rejection observed in chronic-phase recipients after heart transplantation.

Footnote

Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-3027

Peer Review File: Available at http://dx.doi.org/10.21037/atm-20-3027

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-3027). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The protocol for the research project has been approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology (IORG No: IORG0003571) and conforms to
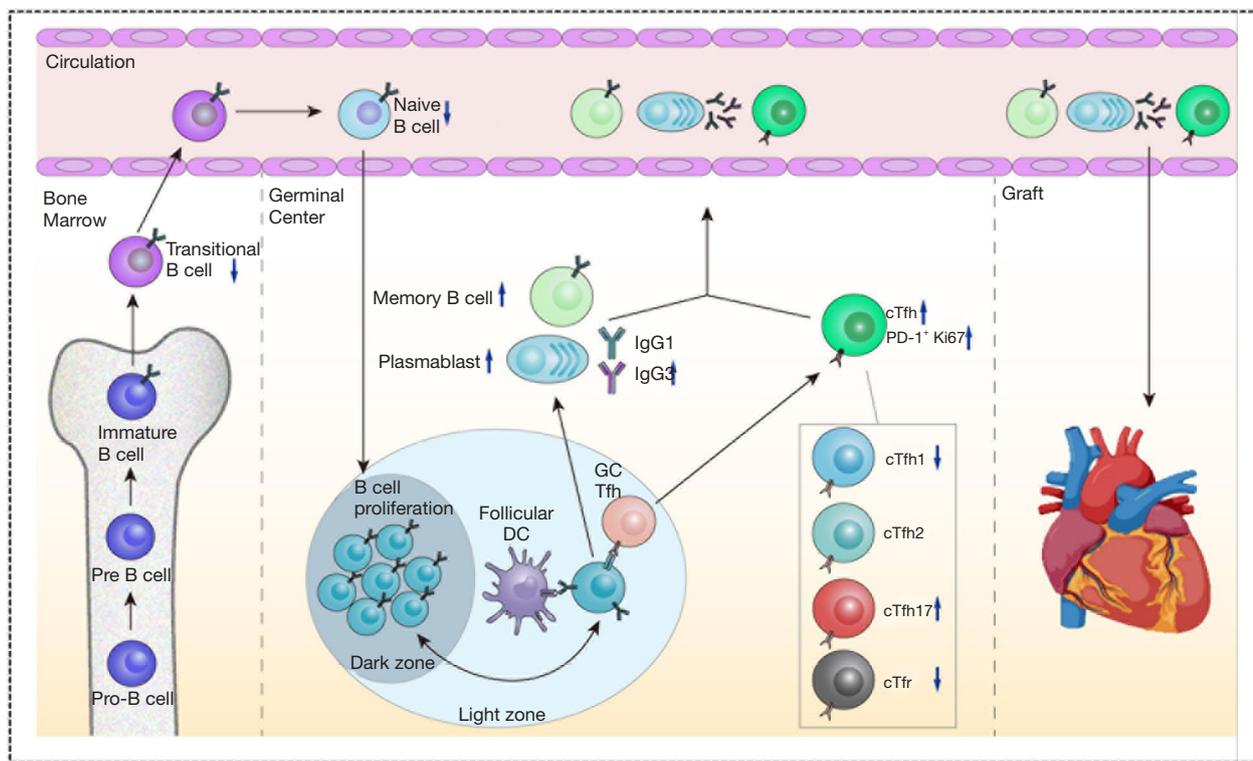
Figure 7 Schematic model illustrating the outcomes of T and B cell responses in chronic-phase recipients after heart transplantation. The proportion of CD4$^+$CXCR5$^+$PD-1$^+$ cTfh cells was significantly increased in the 1-year group, with subtype conversion from cTfh1 to cTfh17. These changes might contribute to B cell differentiation from naïve B cells to class-switched memory B cells or plasmablasts. The higher percentage of the cTfh-related cytokines IL-21 and CXCL13, or B cell-related antibodies IgG1 and IgG3, also demonstrated the immunopathology of cTfh cells during the chronic phase after heart transplantation. cTfh cells may also directly infiltrate the myocardium after migration to enhance antibody-mediated rejection by secreting cytokines or direct contact with B cells.

the provisions of the Helsinki Declaration as revised in 2013. Informed consent was obtained from all patients and healthy control (HC) subjects.

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