Peer Review File
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Reviewer A

Comment 1: M&M: Cardiac histopathology data obtained from a single transplanted patient is insufficient to draw any serious scientific conclusion and should be removed from the study (Subsections Human tissue collection and Immunohistochemistry and immunofluorescence staining in M&M and the paragraphs and figure on this topic in Results).

Reply 1: thank you for your suggestion. Since in the clinical it is hard to obtain re-heart transplantation tissue from multiple patients, and single transplanted patient is insufficient to draw scientific conclusion and we removed the subsection of human tissue collection and immunohistochemistry and immunofluorescence staining. However, we still kept the figure 6A HE results of the CAV patients and CAD patients to further illustrate that the percentages of cTfh were increased as time went by after heart transplantation as illustrated in the new version of figure 6A 6B and 6C.

Changes in the text: we deleted the part B in the figure 6. We also deleted the related part of histopathology data in the M&M, results and discussion part.

Comment 2: spell FVD

Reply 2: we extended FVD to fixable viability dye.

Changes in the text: in the Co-culture of cTfh cells and B cells of methods and materials part, we listed FVD as abbreviation of fixable viability dye.

Comment 3: Please, specify which tests were used for statistical analysis (Kolmogorov-Smirnov, ANOVA follow by appropriate post-tests, Kruskal-Wallis, etc.) in M&M, and following significance levels in the legends of figures.

Reply 3: thank you for your advice, we have added the specific statistic method in the M&M part considering the variables are fitting normal distribution or skewed distribution. ANOVA with post-hoc Bonferroni or Tamhane T2 correction were used to analyzed the multiple group data, however, 2-sample t-test were used to analyzed the groups' difference.

Changes in the text: we have changed the description of statistical analysis to "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. 2 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".

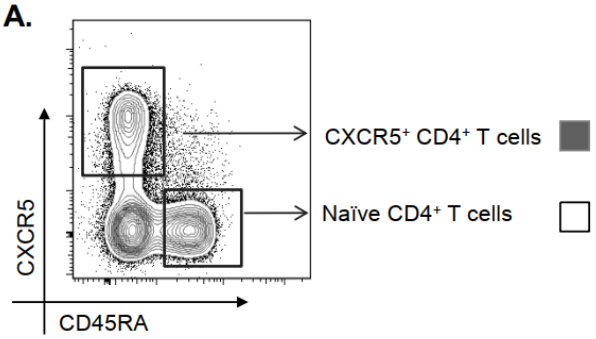
Comment 4: Were isotype controls or Fluorescence minus one (FMO) used to detect non-specific fluorescence in flow cytometry analysis?

Reply 4: in the very beginning of the flow cytometry, we would use the non-stain control and isotype control to detect non-specific fluorescence and gate the cell subsets of different panel especially for some important molecules like CXCR5, Bcl6, Bcl2, Foxp3 and so on. However, we found that our flow antibody is very specific to the surface and nucleus marker and all of them did not have non-specific binding to other proteins. For choosing different antibodies, we first double checked the clone number to the high-impact paper of the antibody, e.g. anti-human CXCR5 we used RF8B2 as reported by 'Forcade E, Kim HT, Cutler C, Wang K, Alho AC, Nikiforow S, Ho VT, Koreth J, Armand P, Alyea EP, Blazar BR, Soiffer RJ, Antin JH, Ritz J. Circulating T follicular helper cells with increased function during chronic graft-versus-host disease. *Blood*. 2016;127:2489-97.' After so many tests, we adjusted our experiment design and found no non-specific fluorescence of the antibody. Meanwhile, in the figure 2D, we used isotype control to detect the baseline of Bcl2 MFI and calculate the MFI between the groups.

Changes in the text: we added all the clone number we used of the flow antibody in the M&M part.

Comment 5: It is not clear whether the expression of CXCR5+ only or CXCR5 plus PD-1 markers were used to define cTfh cells.
Reply 5: as illustrated in the paper 'Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, Manku H, Vyse TJ, Roncador G, Huttley GA, Goodnow CC, Vinuesa CG, Cook MC. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. Arthritis Rheum. 2010;62:234-44' which first define cTfh, $CXCR5^+CD4^+$ was enough to define cTfh cells. However, in germinal center, Tfh were more polarized with high expression of PD-1, ICOS and Bcl6. In peripheral, $CXCR5^+PD\text{-}1^+CD4^+$ cells had been shown to have stronger function in promoting B cell maturation and differentiation as shown in the paper 'Szabo K, Papp G, Szanto A, Tarr T, Zeher M. A comprehensive investigation on the distribution of circulating follicular T helper cells and B cell subsets in primary Sjogren's syndrome and systemic lupus erythematosus. Clin Exp Immunol. 2016;183:76-89.' All in all, $CXCR5^+CD4^+$ could define cTfh, $CXCR5^+PD\text{-}1^+CD4^+$ cells could be called more activated or more polarized cTfh.
Changes in the text: we changed the first sentence of second result paragraph to '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 $CD4^+CXCR5^+PD\text{-}1^+$ T cells (polarized cTfh)' to shown that $CD4^+CXCR5^+PD\text{-}1^+$ cTfh cells are polarized cTfh.

Comment 6: Naïve CD4+ T cells are known to transiently express CXCR5 upon activation. Was there any attempt to distinguish naïve CD4+ T cells from cTfh , which stably express this marker (i.e., the analysis of the expression of CD45RA, CD45RO+ and/or CD27 markers)?
Reply 6: thank you for your question. In CD3/CD28 activation culture condition, naïve $CD4^+$ T cells would witness CXCR5 upregulation. However, in human peripheral blood, we could use CXCR5 to define cTfh. After germinal center response, germinal center Tfh come to the next stage of memory Tfh, it has been reported that the circulating Tfh cells are memory status of germinal center Tfh, and $CD45RA^-$ cells should be pre-gated before choosing $CD4^+CXCR5^+$ cTfh cells. However, in the 'Immunity' paper 'Circulating CCR7loPD-1hi CXCR5+ $CD4^+$ T Cells Indicate Tfh Cell Activity and Promote Antibody Responses upon Antigen Reexposure', the flow of Figure S1A showed that CXCR5 was almost expressed in the $CD45RA^-$ PBMCs, while barely expressed in the $CD45RA^+$ cells as shown in the following figure.



So in order to eliminate the effect of naïve $CD4^+$ T cells in the PBMC, CD45RA should be stained and analyzed. However, among the groups, it was still comparable when we only used the $CD4^+CXCR5^+$ without CD45RA to define cTfh which is also the same with other paper like 'De Graav GN, Dieterich M, Hesselink DA, Boer K, Clahsen-van Groningen MC, Kraaijeveld R, Litjens NH, Bouamar R, Vanderlocht J, Tilanus M, Houba I, Boonstra A, Roelen DL, Claas FH, Betjes MG, Weimar W, Baan CC. Follicular T helper cells and humoral reactivity in kidney transplant patients. Clin Exp Immunol. 2015;180:329-40'.
Changes in the text: None.

Comment 7: Fig. 2C shows statistically significant decreased Ki67+ cTfh cells in the group of 1-year post-Tx patients compared to HC and pre-Tx groups. Please, state that important statistical information in the text.
Reply 7: thank you so much for your suggestion. There were 2 possible reasons for this phenomenon. First, compared with HC and pre-Tx group, 1-year group patient ordinarily have oral administration of

immunosuppressive drugs like tacrolimus, glucocorticoid and mycophenolate mofetil. Upon using tacrolimus, cell proliferation could be affected because protein phosphatase is closely linked with T cell proliferation and metabolism. Second, cTfh is the memory status in the peripheral blood after germinal center response, memory cells are always in the quiescent or stemness status, it might have high expression of Ki67 after re-exposure to the antigen.
Changes in the text: we added 'cTfh might exist in the peripheral blood as the memory status of germinal center Tfh, thus this could also account for the fact that they had lower expression of Ki67' in the second paragraph in the discussion part.

Comment 8: Correlation data between different parameters are shown for the group of 1 year post-Tx patients only. However, the data for the other groups is critical to establish the true biological relevance of the findings.
Reply 8: Truly the correlation analysis should be listed and analyzed also in the other 2 groups. After our calculation, we found that the proportion of cTfh with IL-21, CXCL13, IgG3 and plasmablast also share the same trend in the other 2 groups. These results also prove the findings in the 1-year group. Our in vitro cTfh and B cell co-culture experiment also prove this phenomenon.
Changes in the text: we added 'no matter in the 1 year group but also in the HC and Pre group' after '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' in the paragraph 5 of the discussion part.

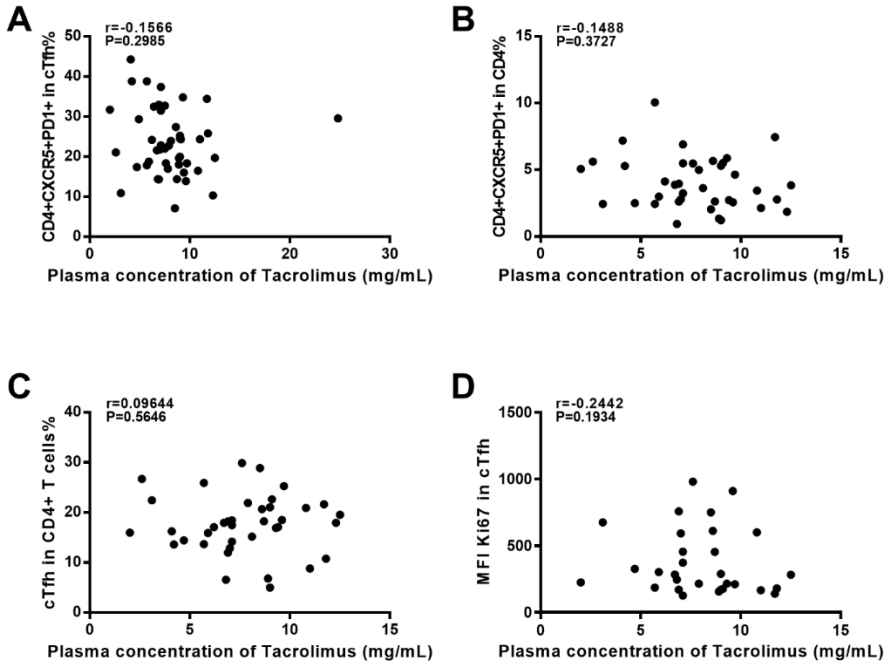
Comment 9: Please, add the phenotype of cells in the Y-axis of Fig. 3D.
Reply 9: the Y-axis of the Figure 3D is % of $CD19^{+}CD27^{hi}CD38^{hi}$ B cells within $CD19^{+}$ B cells.
Changes in the text: we changed the title of Y-axis of Figure 3D.

Comment 10: There are some sentences that left me wondering what the authors were trying to say. For example, the sentences "the abundance of memory B cells gradually increased, whereas that of the naïve B cells gradually decreased over time…" (considering that the analysis was performed only once at 1 year post-Tx), "these observations suggest that the increase in the plasma CXCL13 level was likely related to the transformation of cTfh to Tfh17…" and "subtype conversion from cTfh1 to cTfh17…" .
Reply 10: for the first sentence, after fully development and maturation in the bone marrow and spleen, B cells became naïve B cells. Upon antigen encounter, naïve B cells transit into germinal center B cells, which further differentiate into memory B cells and plasmablasts after germinal center response. The store of memory B cells would help the second antigen response, in our finding, for the 1-year group compared with the pre group, the proportion of naïve B cells decreased which leads to the memory B cells accumulation.
For the 'subtype conversion from cTfh1 to cTfh 17', as cTfh could be divided into different subtypes like Tfh1, Tfh2 and Tfh17 according to CXCR3 and CCR6 expression. Compared with cTfh1 cells, cTfh2 and cTfh17 cells had a relative stronger ability to promote B cell differentiation and antibody class switching. So this sentence appears to illustrate that this kind of subtype transition was linked with more plasmablasts and IgG3 production.
Changes in the text: we changed the last sentence of the paragraph 7 of the result part to '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.'

Comment 11: Neither has it been clear the inclusion of patients with CAV that were subjected to post-Tx follow-up studies during subsequent years in M&M.
Reply 11: thank you for your question. 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 12^{th} month, and twice for one year thereafter. For the 1-year group patient after heart transplantation, they would have coronary angiography once per year for screening of CAV.
Changes in the text: we added '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 12^{th} month, and twice for one year thereafter' at the beginning of the second paragraph of M&M. And we also

added ‘For the 1-year group patient, they would have coronary angiography once per year’ at the beginning of the third paragraph of M&M.

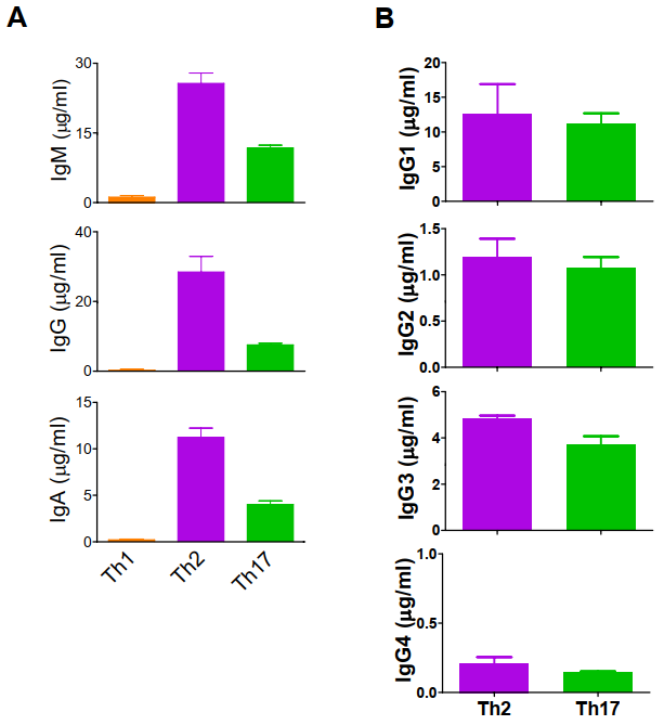
Comment 12: Finally, which would be the effect of chronic tacrolimus treatment on the profile of the cTfh population, as it inhibits calcineurin, a protein phosphatase required for T-lymphocyte activation? Please, discuss.
Reply 12: thank you for your question and consideration. Tacrolimus treatment would inhibit calcineurin, a protein phosphatase required for T-lymphocyte activation. In the 1-year group, tacrolimus would affect T cell activation and proliferation as shown in the comment 7 with Ki67 down-expressing. In order to further illustrate the intrinsic relationship between plasma concentration of tacrolimus, we used correlation analysis to explore the result. As shown in the following figure, we found that neither proportion of cTfh in $CD4^+$ T cell nor $CD4^+CXCR5^+PD\text{-}1^+$ in $CD4^+$ T cells was correlated with plasma tacrolimus concentration.



So from the results above, we got to know though tacrolimus might truly affect the Ki67 expression of cTfh, it would not affect the proportion or number of cTfh in 1-year group patient. This result is in accordance with the fact that CXCR5 was upregulated when the antigen persistence which demonstrated the status of T cell exhaustion (Greczmiel, Ute, and Annette Oxenius. "The janus face of follicular T helper cells in chronic viral infections." *Frontiers in Immunology* 9 (2018): 1162.) This is also the same with the virus infection, alike CD8 T cells, virus-specific CD4 Th1 cell responses are gradually downregulated but instead, follicular T helper (T_{FH}) cell differentiation and maintenance is strongly promoted during chronic infection. So we could conclude that Tfh or cTfh might play vital roles no matter in virus infection but also in chronic phase of heart transplantation which would not be affected by tacrolimus.
Changes in the text: we added ‘Also these changes were not due to the blood concentration changes of tacrolimus, we found neither the cTfh proportion nor the $CD4^+CXCR5^+PD\text{-}1^+$ 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’ to the end of the second paragraph of the discussion part to further illustrate that the change of cTfh was due to the antigen itself but not other immunosuppressive drugs.

Reviewer B
Comment 1: The authors mentioned increased cTfh17 cells well correlated with plasma IgG3. In this article, the authors performed co-culture of cTfh cells and CD19+ B cells, so, I recommend that, if possible, co-culture between isolated Tfh17 cells and CD19+ (CD27+) B cells are performed and IgG3 concentration of culture

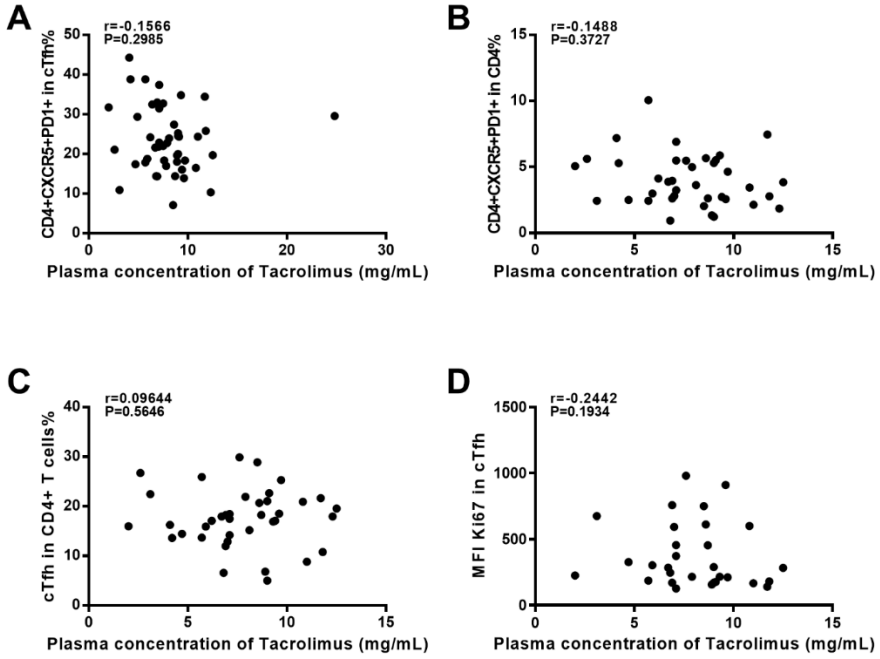
supernatant is measured. It will be a direct evidence that Tfh17 cells promote IgG3 production from B cells.
Reply 1: thank you so much for your suggestion. In the 1-year group, we truly found that proportion of cTfh17 were positively correlated with the IgG3 concentration. As shown in the paper 'Morita, Rimpei, et al. "Human blood CXCR5+ CD4+ T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion." *Immunity* 34.1 (2011): 108-121.', Tfh17 was found to promote memory B cell differentiation and antibody production compared with Tfh1 as shown in the following figure. While this is the truth, the point I listed in Figure 3 was to recheck the function of cTfh, and make reasonable hypothesis in the following part about 1-year group correlation analysis.



Changes in the text: in the discussion part, we added the sentence 'However, the exact relationship between these changes still need further in vitro sorting and Tfh17 and B cell coculture experiment validation.' To the end of the third paragraph in the discussion part to illustrated it is better to use in vitro experiment for validation.

Comment 2: The authors pointed out that cTfh and cTfr cells were good negative correlated. Tfr cells are important cells inhibited Tfh cells function. Tfr cells are noticed in autoimmunity, allergy, infection and cancer. How about the correlation in pre group and HC. Under immunosuppressive condition in 1-year group, % Tfr cells reduction and negative correlation between Tfh cells and Tfr cells were showed, so I look forward to more detailed discussion about the role of Tfr cells in this situation.
Reply 2: thank you for you advice and question. In order to explore the correlation between cTfr/cTfh and cTfh/CD4, we perform correlation analysis and found that no matter in the HC group but also in the pre-HTx group, the cTfr/cTfh was negative correlated with cTfh/CD4 (r=-0.6925 and -0.6513). This result further illustrated that no matter in the quiescent status (no heart transplantation) or activated status (heart transplantation), cTfr could also inhibit the function of cTfh in peripheral blood. Tfr display a unique transcriptional overlapping that of both Tfh and Treg, notably with combined expression of Bcl6, Foxp3 and Blimp1. It originated from Treg precursors dan regulate GC responses through interaction with Tfh. Also in the peripheral, it play vital role in regulating cTfh functions, thus the impairment of cTfr would cause severe autoimmune disease. This result is in accordance with our result that in the 1-year group, we found the relative lower number of the cTfr, which might account for the increasing amount of cTfh.
Tacrolimus treatment would inhibit calcineurin, a protein phosphatase required for T-lymphocyte activation. In the 1-year group, tacrolimus would affect T cell activation and proliferation as shown in the comment 7 with

Ki67 down-expressing. In order to further illustrate the intrinsic relationship between plasma concentration of tacrolimus, we used correlation analysis to explore the result. As shown in the following figure, we found that neither proportion of cTfh in $CD4^+$ T cell nor $CD4^+CXCR5^+PD\text{-}1^+$ in $CD4^+$ T cells was correlated with plasma tacrolimus concentration.



Changes in the text: in the 4th paragraph of the discussion part, we focused on the cTfr function and discussed the changes and reason of cTfr. We added 'Meanwhile, for the HC and Pre group, we also witnessed cTfr/cTfh negatively correlated with cTfh/$CD4^+$ T cells, 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 of cTfh and cTfr are needed to provide direct evidence of cTfr regulation on cTfh' in that paragraph to further illustrated the changes of cTfr in the HC and Pre group. In order the eliminate the effect of immunosuppressive drugs, we added 'Also these changes were not due to the blood concentration changes of tacrolimus, we found neither the cTfh proportion nor the $CD4^+CXCR5^+PD\text{-}1^+$ 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' to the end of the second paragraph of the discussion part to further illustrate that the change of cTfh was due to the antigen itself but not other immunosuppressive drugs.

Reviewer C

Comment 1: It is important to note that whether the Tfh and B cells infiltrated to the heart tissue. The authors only show the CD3 staining, which is far from enough. The co-staining of CD4/CXCR5, CD4/Bcl6, Bcl6/Foxp3, Bcl6/IL-17, Bcl6/IFNg should be performed on heart tissue.

The different types of B cells, including plasma cell should be analyzed by histology in heart tissue.

Reply 1: thank you for your suggestion. Since in our department we only have 3 patients who suffered from secondary heart transplantation due to the CAV. So clinically it is hard to obtain re-heart transplantation tissue from multiple patients, and single transplanted patient is insufficient to draw any scientific conclusion and we removed the subsection of human tissue collection and immunohistochemistry and immunofluorescence staining. However, we still kept the figure 6A HE results of the CAV patients and CAD patients to further illustrate that the percentages of cTfh were increased as time went by after heart transplantation as illustrated in the new version of figure 6A 6B and 6C. This is also the question reviewer A came up with, he suggested me that I should delete all the immunohistochemistry and immunofluorescence staining results due to the relative lower number of the cases. However, from this single sample staining, we found Tfh could directly infiltrate into the CAV heart tissue.

Further, whether Tfr, Tfh1, Tfh2, Tfh3 could also participate in the pathological progress should also be verified. Graft infiltrating cells were also needed by digestion and flow analysis instead of immunofluorescence staining. Changes in the text: we deleted the part B in the figure 6. We also deleted the related part of histopathology data in the M&M, results and discussion part. In order to demonstrate this limitation of the heart tissue of our study, we added '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' at the end of 6th paragraph in the discussion part.

Comment 2: The author hypothesized that CXCl13 may play a role for rejection in heart. Could the author stain the heart tissue and determine what kind of cells express the receptor of CXCL13 ? Do Muscle, B cell or other cells attracte Tfh to the heart via CXCl13?
Relpy 2: thank you so much for your suggestion. Same with the above question, due to the limited number of re-heart transplant myocardium tissue, single sample is not sufficient to draw any scientific conclusions and we have removed the subsection of this part in M&M, results and discussion part. CXCL13, also known as B lymphocyte chemoattractant (BLC) or B cell-attracting chemokine 1 (BCA-1), often elicits its effects by interacting with CXCR5 which could control the organization of B cells and Tfh cells. According to the paper 'Havenar-Daughton, Colin, et al. "CXCL13 is a plasma biomarker of germinal center activity." *Proceedings of the National Academy of Sciences* 113.10 (2016): 2702-2707', CXCL13 could be secreted by Tfh, dendritic cell and monocytes in the secondary lymph nodes. Germinal center Tfh acts as the main robust producers of CXCL13 which can recruit B cells. Plasma CXCL13 has been proposed to serve as a biomarker of autoimmune disease such as rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome and Myasthenia Gravis. It is a very sensitive marker of germinal center response. In order to further prove its value in heart transplantation, in the future we could block CXCL13-CXCR5 axis in the Tfh response and monitor the functional changes of Tfh. Changes in the text: in order to demonstrate the limitation of plasma concentration of CXCL13 and further research on its function to Tfh in the rejected heart tissue, we added 'In the future, we could block CXCL13-CXCR5 axis in vitro culture experiment to further test its functions on Tfh mobilization and recruiting' to the end of 4th paragraph in the discussion part.