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Impact of cytokine expression in the pre-implantated donor lung on the development of chronic lung allograft dysfunction subtypes.

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12nd of August, 2013

Dear Professor Kirk,

We would like to thank all of the editors and reviewers for their comments on our manuscript, entitled '**Impact of cytokine expression in pre-implanted donor lung on the development of chronic lung allograft dysfunction subtypes**' (AJT-O-13-00689). We have revised the Manuscript Body, Figures and Tables based on all of the comments of the referees. Each concern has been addressed specifically in the following section, where our response is indicated in *italics*. We hope you find the revised manuscript worthy of publication in ***The American Journal of Transplantation*** and we thank you in advance for your time and consideration.

Sincerely,

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EDITORIAL COMMENTS:

Editor: 1
Comments to the Author:
A nicely written manuscript from the leading group in the world in this field of research. The reviewers have identified several important areas where clarification, expanded discussion, and additional information is needed. For the most part, these suggestions are ones that the authors should easily be able to address. For those that are inherent to the study, a more developed limitation section would be required.

In response to all the comments from editors as well as reviewers, we have revised the manuscript. The changes to the manuscript are yellow highlighted in the Manuscript Body, Figures and Tables.

We have also re-calculated CLAD-free survival as suggested and the redrawn five- and ten-year CLAD-free survival rates are shown (please see Page 10 of Manuscript Body and Figure Legend of Figure 1D).

Editor: 2
Comments to the Author:

1.More detailes of the limitations of this study should be provided in the discussion.

In response to the editor's comment, we have developed and further clarified the limitation section (please see Pages 14 and 15 of Manuscript Body).

2.Reported lack of correlation between acute rejection and BOS is contradictory to the current dogma. It should be discussed.

In response to the editor's comment, we developed the discussion by expanding on possible factors which may affect the statistical ability to evaluate the impact of acute cellular rejection on eventual development of chronic lung allograft dysfunction in this study (please see Page 14 of the Manuscript Body and Figure S1).

3. If data is available on DSA post transplantation it should be taken into consideration in their analysis.

In response to the editor's comment, we have developed the limitation section upon recognition that we were unable to take into account DSA prior to the onset of CLAD, due to insufficiency of the available clinical data (please see Page 15 of the Manuscript Body).

4. Survival outcome need to be reported.

In response to the editor's comment, we have reported the survival outcome of the study cohort (please see Page 9 of the Manuscript Body and Figure 1B and 1C).

REVIEWER COMMENTS:

Reviewer: 1

Comments to the Author
Saito et al., present interesting data linking increased levels of IL-6 transcript in pre-transplant lung graft tissue to increased probability of developing chronic lung allograft dysfunction (CLAD). Although the sample size is small for such studies (although understandable for a single site study involving lung transplant recipients) the authors use of appropriate statistical methods, well-reasoned patient exclusion-inclusion criteria and the reasonable variance in the demographic data help support their conclusions. The study also underscores the highly cited literature that shows early inflammatory events within lung grafts are predictive of poor survival. I only have some moderate concerns.

1. Are the primers used to measure IL-6 mRNA extra-exonic (across introns)? This is important as the method used is appropriate to measure IL-6 mRNA (the RNA term used by authors) and not its non-spliced forms (generally referred to as transcripts). How was RT reaction conducted? Oligo dT or random primers?

In response to the reviewer's comment, we have described detailed information on primers as well as the Reverse Transcription reaction employed to measure cytokine mRNA levels in the Materials and Method section. Each primer pair for IL-6 and IL-1β spanned an intron and reverse transcription reaction was conducted using random hexamers. Please see Page 6 of the Manuscript Body and Table S1.

The relevance of IL-6 mRNA as a proxy for IL-6 activity should be better discussed. IL-6 mRNA transcriptional stability and its relationship to IL-6 activity in autoimmunity has been intensively studied emphasizing why IL-6 RNA levels measured by real time pcr methods should be better described.

In response to the reviewer's comment, we have developed a discussion on the relevance of IL-6 mRNA as a proxy for IL-6 activity. Please see Page 13 of the Manuscript Body.

Also, additional discussion about IL-6 and its potential to drive autoimmune responses in lung transplant recipients (e.g. autoreactive CD4+ IL-17+ T cells and autoantibody production) should be included in the manuscript.

In response to the reviewer's comment, we have developed a discussion on the importance of IL-6 as a potential driver of autoimmunity. Please see Page 13 of Manuscript Body.

2. Table 2: The column title NO CLAD vs BOS vs RAS is confusing. Do you mean BOS vs No CLAD and RAS? P-values where each comparison is clearly denoted should be included Table 2.

We intended to indicate by using "No CLAD vs. BOS vs. RAS" that the p-values were calculated by Kruskall-Wallis ANOVA across the three study groups.

In response to the reviewer's comment, we have changed the column title "No CLAD vs. BOS vs.

RAS" to "Three subgroups (Kruskall-Wallis ANOVA)" to clarify this. We have also carefully revised annotation of Table 2. Please see Table 2.

3. The finding of what appears to be marked differences of IL-1b transcript expression in Table 2 is not discussed. It is quite interesting as IL-1b is major mediator of sterile injury and is a strong driver of IL-6 expression.

We think this is quite an important point. In response to the reviewer's comment, we have further developed discussion on the potential role of IL-1β as a stimulating factor of IL-6 production in CLAD development. Please see Pages 13 to 14 of the Manuscript Body.

4. The PGD criteria (T=0) should be clearly stated in the methods section as a key finding is the independence of IL-6 levels from PGD. Is this T=0 measurement similar to how immediate early PGD is defined by other institutions?

In response to the reviewer's comment, we have added to the Materials and Methods section the definition of Grade 3 PGD T-zero in the in accordance with ISHLT PGD grading criteria. Grade 3 PGD T-zero was defined as a combination of PaO2/FiO2 ratio below 200 mmHg and radiographic infiltration consistent with pulmonary edema within 6 hours of reperfusion.

We have also calarified that we employed PaO2/FiO2 ratio at ICU arrival as a surrogate factor for Grade 3 PGD T-zero for this study cohort in the Materials and Methods section and the Discussion section. Please see Pages 9 and 15 of the Manuscript Body.

5. On page 16 has "ENREF" next to ref 39.

In response to the reviewer's comment, we have deleted "ENREF" which was unformatted in the previous manuscript.

Reviewer: 2

Comments to the Author
This is a well-written manuscript from the Toronto group, evaluating long term effects of donor gene expression profiles. The current study builds on an impressive body of work by this group in classifying RAS, and evaluating gene expression in donor specimens for risk of early events. The authors demonstrate that IL-6 gene expression in the donor associates with BOS, but not with RAS. These findings fit with a hypothesis that inflammation and epithelial cell injury beginning in the donor can predispose to early BOS. Strengths include the survival analysis conditioning on 3 month survival, the long follow up time, and the well-detailed methods for assessing BOS association, and choosing the IL-6 cutoff. However, there are some issues, which, if addressed, would make the study even stronger:

Major:
1) The multivariable analyses are well-detailed and include many potential confounders. However, in the introduction, the authors highlight several clinical factors that have likely causal associations with CLAD. These may be important causal mediators or confounding variables in this study, but are incompletely included in the analyses. In particular, as listed by the authors, improved accounting for AR, PGD, CMV, and GERD would be helpful to better understand the main findings.

a. While ACR is addressed, the method of inclusion into the models should be more precise. Was this a time-varying covariate? If not, why not?

We acknowledge 'acute cellular rejection (ACR)' as a time-dependent covariate. We first intended to analyze ACR by landmarking analysis using categorical variable 'ACR with Grade A≥2 within 6 months after transplantation'.

In response to the reviewer's comment, we have conducted a time-dependent Cox regression analysis with treating Grade A2-4 ACR as a time-dependent variable. Please see Pages 9 and 11 of the Manuscript Body and Table 3.

b. Analyses of IL-6 by donor CMV status should be reported.

In response to the reviewer's comment, we have reported IL-6 expression according to donor CMV status in the Results section. IL-6 expression levels were not significantly different between donor CMV serology (i.e. positive vs. negative, p=0.905). Please see Page 11 of the Manuscript Body.

c. If available, CMV infection post-transplant, but prior to CLAD should be included. If not available, it should be discussed in the limitations section.

In response to the reviewer's comment, we have developed the limitation section upon recognition that we were unable to take into account post-transplant CMV pneumonitis prior to CLAD or BOS due to insufficiency of the available clinical data of the study cohort. Please see Page 15 of the Manuscript Body.

d. GERD is not assessed as a confounder. At the very least, this should be in the discussion section.

In response to the reviewer's comment, we have developed the limitation section upon recognition that we were unable to take into account post-transplant GERD prior to CLAD or BOS due to insufficiency of the available clinical data of the study cohort. Please see Page 15 of the Manuscript Body.

2) The conditional survival analysis is a nice way to account for competing risks of early mortality. However, not all mortality due to the competing risk of early allograft dysfunction will be captured by this approach. Additional sensitivity analyses conditioning on 1 year survival would be interesting to present.

In response to the reviewer's comment, we have also conducted a time-dependent Cox regression model treating ACR as a time-dependent covariate conditioning on 1-year survival. Please see Table S2.

3) There are several weaknesses that cannot be addressed by the study design. In the limitations section, the authors do a nice job of addressing several of these, including lack of PGD scores and the small sample size. Yet, this limitations section could be more fully developed to include discussion of other limitations, including

a. The prior publication of these same subjects from this single center, and thus the potential limited generalizability of the findings.

In response to the reviewer's comment, we have further developed the limitation section to emphasize that the presented results are based on a single-center study with relatively small sample size and limited generalizability. Please see Page 14 of the Manuscript Body.

b. The limited number of transcripts measured.

In response to the reviewer's comment, we have further developed the limitation section to emphasize that we measured only six cytokines and there might be other culprit cytokines other than IL-6 and IL-1β. Please see Pages 14 to 15 of the Manuscript Body.

c. While the conditional survival analysis is justified, some discussion about how the inclusion criteria (excluding 47 subjects who died within 30 days, and an additional 13 who did not have TLC) would affect the ability to evaluate causal mediators (such as PGD) is warranted.

In response to the reviewer's comment, we have expanded the limitation section focusing on the possible effect of our inclusion criteria of study population on the statistical ability to evaluate casual mediators such as ACR. Please see Page 14 of the Manuscript Body.

d. The study has no validation population. I fell that this is OK, given the unique position of this group. However, given that the cutoffs were derived to best fit the data and that several transcripts were measured, some discussion of need for further validation is warranted.

In response to the reviewer's comment, we have added to the limitation section a comment that our findings have not been tested yet on a validation population to comment on the generalizability of the IL-6 cutoff value for predicting the incidence of CLAD and BOS. Please see Page 15 of the Manuscript Body.

Minor:

1) I would move the p-values in the tables (e.g. table 3) to the right side of the tables, to be a little more conventional.

In response to the reviewer's comment, we have moved the p-values to the right side. Please see Table 3 and Table S2.

2) While the paragraph speculating on IL-6 and Th17 effects is interesting, some balanced discussion of the pleiotropic effects of IL-6 is warranted to maintain appropriate circumspection.

In response to the reviewer's comment, we have further expanded the discussion on the pleiotropic effects of IL-6. Please see Page 13 of the Manuscript Body.

3) There is an unformatted reference on Page 16, line 4 "ENREF_39"

In response to the reviewer's comment, we have deleted "ENREF" which was unformatted in the previous manuscript.

Reviewer: 3

Comments to the Author
This well written manuscript analyzes data from a previous study, with updated PFTs to determine the relationship between donor lung cytokine gene expression (determined earlier) and incidence of CLAD, BOS, and RAS.

The observation that donor IL-6 mRNA levels at the time of transplant is a significant risk for the development of BOS but not RAS may be related to the small number of patients in their series with RAS. This is a limitation of their study, that should be included in the Discussion. The authors allude to small sample size as a general limitation; this is more specific.

In response to the reviewer's comment, we have further developed the limitation section to clarify the recpgnition that the small sample size could influence the analysis of the RAS group. Please see Page 14 of the Manuscript Body.

Another peculiar finding is that only IL6 seemed to be a "culprit" cytokine. Because signaling pathways are not specific for IL6, one would expect other cytokines to be implicated. That IL6 was so different from other cytokines implies that this too was related to sample size, or there were other reasons, such as the Th17 amplification mechanism alluded to in the Discussion. This could be mentioned in the Discussion.

In response to the reviewer's comment, we have expanded the limitation section acknowledging that the small sample size could influence the analysis on comparison of cytokine expression among BOS, RAS and No CLAD. We have also expanded the discussion related to the Th17 pathway. Please see Page 14 of the Manuscript Body.

Although the mathematically calculated threshold for donor IL6 threshold is 0.124, it would appear from Supplemental Figure 1 that the threshold may be lower. Is it a more continuous variable? Presumably to determine this would require more patients. Did the authors see a relationship between the amount of mRNA for IL6 and the onset of BOS?

In response to the reviewer's comment, we have re-calculated the log-rank statistics from 0.07 to 0.13 of IL-6 relative expression by 0.001. Please see Figure S3.

Recognizing that we have no validation population to test the generalizability of the IL-6 cutoff value for predicting the incidence of CLAD and BOS, we have modified the limitation section. Please see Page 15 of the Manuscript Body.

We have calculated cumulative incidence of CLAD, BOS and RAS according to quartiles of IL-6 relative expression to illustrate the relationship between IL-6 and CLAD, BOS and RAS incidence. Please see Page 11 of the Manuscript Body and Figure 2.

There seemed to be no significant difference in IL-6 expression levels between early-onset BOS(≤3 years) and late-onset BOS(>3 years)(p=0.637, data not shown in the Manuscript).

Reviewer: 4

Comments to the Author
This study is a very interesting observation that pre-implant donor levels of IL-6 and to a lesser degree IL-1b are strongly associated with poorer long term lung allograft function. While IL-6 measurements in the donor lung probably reflect a general overall inflammatory status in the lung, it may either be a good biomarker or direct therapies targeted to the level of inflammation in the donor lung prior to implantation. While this is a small overall cohort, these observations are potentially important and can be considered hypothesis generating.

There are some limitations in the current analysis. BOS, rejection and death are time dependent variables. The modeling does not take this into account.

In response to the reviewer's comment, we have conducted a time-dependent Cox regression analysis with treating Grade A2-4 acute rejection as a time-dependent variable. Please see Pages 9 and 11 of the Manuscript Body and Table 3.

The advantage of the overall long follow up is the high event rate. However, the importance of early allograft dysfunction is more important than late dysfunction. From this perspective, the authors refer to differences in early BOS and CLAD. How are they defining early BOS or CLAD? What is the time interval? Examining the curves, there appears that the relationship to developing allograft dysfunction persists over time.

In response to the reviewer's comment, we have clarified and defined early-onset BOS as BOS of which onset date is within 3 years after lung transplantation as previously described (Sato et al, J Heart Lung Transplant 2013).

We have also conducted a survival analysis and a time-dependent Cox regression analysis on early-onset BOS. Please see Figure 1C, Table 3 and Table S2.

As the reviewer expected, the effect of relatively higher IL-6 expression seemed persistent over time as it was associated with both early-onset BOS and all BOS. Please see Table 3 and Table S2.

Lack of correlation between acute rejection and development of BOS is interesting given that this has been the most common variable correlated to BOS. The very low incidence of acute rejection in their study cohort suggests that biopsies were not routinely done as part of surveillance but for clinical indications only.

In response to the reviewer's comment, we have clarified the description of our center practice regarding transbronchial lung biopsies (TBBxs) which were performed as routine surveillance as well as for clinical indication in Materials and methods section. Please see Page 7 of the Manuscript Body.

We have also reported the number of TBBxs and the percentage of patients with each grade of acute rejection according to the timing of TBBxs. Please see Figure S1.

We have also expanded the discussion to include possible factors related to the low incidence of Grade A2-4 acute rejection. The low incidence of Grade A2-4 acute rejection might be due to our study population inclusion criteria, inadequate sampling and inter-observer variability in the pathologic grading. Please see Page 14 of the Manuscript Body.

There are no data provided regarding HLA sensitization which has been linked to earlier development of CLAD and BOS.

In response to the reviewer's comment, we have added to the limitation section a comment to acknowledge that we were unable to take into account HLA sensitization due to insufficiency of the clinical data of the study cohort. Please see Page 15 of the Manuscript Body.

The use of this current data set should be treated as the training set and not the testing set. By using the current data set to determine the optimal statistical differentiating point, the investigators have separated the curves optimally as would be expected. Without a follow up test cohort, there is limited confidence that this is the appropriate value. A sensitivity curve demonstrating the incidence of BOS or CLAD at a time interval for different quartile/quintiles of IL-6 would be more illustrative.

In response to the reviewer's comment, we have calculated the cumulative incidence of CLAD, BOS and RAS according to quartiles of IL-6 relative expression to illustrate the relationship between IL-6 and CLAD, BOS and RAS incidence. Please see Page 11 of the Manuscript Body and revised Figure 2.

We have also developed the limitation section to clarify that we have no testing set to validate the IL-6 cutoff value for predicting the incidence of CLAD and BOS. Please see Page 15 of the Manuscript Body.

Finally, the reporting of lung allograft dysfunction is important. However, patient survival is a much more important end-point. Survival outcomes need to be reported.

In response to the reviewer's comment, we have reported the survival outcome of the study cohort. Please see Page 9 of the Manuscript Body, Figure 1B and 1C.