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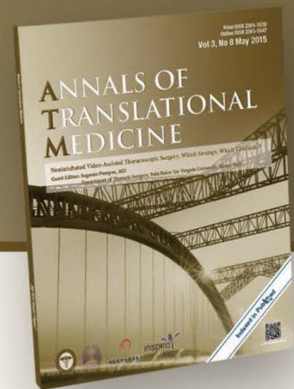

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Peer Review File

Article information: http://dx.doi.org/10.21037/atm-20-4548

Reviewer comments

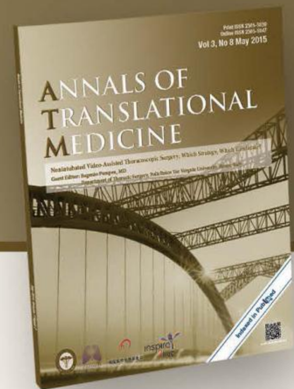
Major revisions:

1. Abstract: Results mention that there was a great similarity between NHP and humans, were these based on homologs, orthologues…it is not clear.

   Reply 1: By gene expression profile analysis, we obtained 38 and 20 overlapped genes in both human and cynomolgus monkey PBMCs with PHA and LPS stimulation respectively. These overlapped DEGs mainly involved in the same biological functional process in two species, such as *MCM complexes (MCM3, MCM4, MCM6, MCM7)* and *PCNA* in 'DNA replication', *PLK1* in 'cell cycle', and *CCL3, CD74, IL1A, IL8* in 'immune response', and *CCL3, IL1A, IL8* in 'inflammatory response'. The evaluation of these genes with their corresponding function conserved between primates could provide a possible explanation of mechanism for a great similarity in immune response between human and monkey. we have modified our text in results of abstract as advised **(see Page2-3, line 44-51).**

2. Overall, the message of this manuscript is not clear. The figures need clarification and presentation improved. The text is too wordy and a lot of the processes and pathways discussion should be summarized in tables so that the reader can read along.

   Reply 2 : In order to make the message clearer in the discussion part of this manuscript, the subheading has been added in discussion, and the important results have been re-discussed according to the following outline:



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- **PBMC proliferation induced by PHA/LPS**

  In contrast to LPS, human PBMCs showed higher proliferative capacity than that of monkeys after PHA stimulation.

- **Cytokine profile induced by PHA/LPS**
  - INFγ: The stronger secretion and up-regulated genes associated with IFNγ in human PBMCs with PHA/LPS stimulation could reflect potential striking different mechanisms in the innate and adaptive immune responses across the primates.
  - IL8: The similar changes in IL8 in different species after LPS stimulation, suggesting that IL8 could be a bridge biomarker to reflect the activation state of innate immune response in mammalian.
  - IL5: It was not detected in monkey PBMCs that received either PHA or LPS. The distinct of memory T cells in human and cynomolgus monkeys, especially in juvenile monkeys, might contribute to this different secretion of IL5.
- **Biologic functional features of DEGs in human and monkey PBMCs with PHA stimulation**
  - A large number of up-regulated genes related to mitosis may be one of the reasons for stronger response to PHA in human.
  - The overlapped DEGs associated with DNA replication and cell cycle reflect the highly conserved signatures of immune responses to PHA stimulation across the primates.
- **Biologic functional features of DEGs in human and monkey PBMCs with LPS stimulation**
  - In contrast to PHA, the count of DEGs in the same BP was similar in the two species under LPS stimulation, suggesting human and cynomolgus monkeys had similar characteristics in immune pathways and response intensity in innate immune response to LPS.

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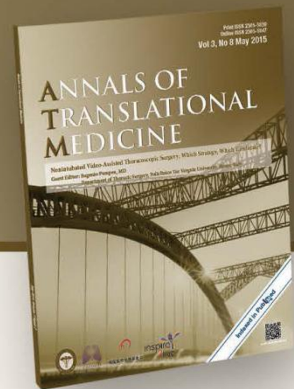

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- The overlapped DEGs data indicated that IL8 was highly conserved among the primates and might play an important role in the innate immune response to LPS.
- Antigen processing and presentation mainly via MHC class Ⅱ other than MHC Class I was found in human after LPS stimulation.

Revised comments in detail:

(1) Results: we modified our text to make the language more concise **(See Page 14-15, line 295-315; Page 16, line 321-325; Page 17, line 357-363; Page 18, line 373-381; Page 19, line 392-395; Page 20, line 412-420).**

(2) Discussion: we added the subheading **(Page 23, line 483, 495; Page 28, line 590-591; Page 31, line 651-652).** We modified our text to make the language more concise **(See Page 24, line 496-500, 506-511; Page 25, line 518-539; Page 26, line 547-555; Page 28, line 594-603; Page 29, line 609-614, 621-622; Page 30, line 640-642, 648).**

(3) Conclusion: We modified our text to make the language more concise **(See Page 33, line 715-716).**

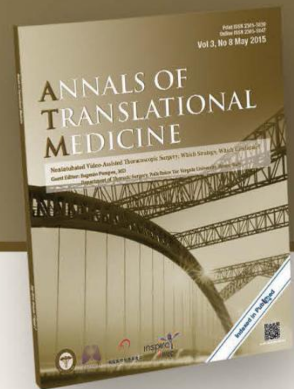
(4) Also, the figure 6 and 7 were replaced by the updated table 3 and 4 for comparing the categories. Other figures (Fig 1-3) were improved as advised.

Minor revisions:

1. Abstract, starts with mention of TGN1412, which needs to be introduced first. Other details such as the Genus and species and strain of NHP should be elaborated at first mention and also in methods.

   Reply1: we added some information about TGN1412 as advised **(see Page 2, line 26-28).** Also, we added the species and strain of NHP in the Abstract **(see Page 2, line 33-35).**

2. Mention Institutional protocol number for the use of NHPs.



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Reply 2: In the part of ethical statement, our institution protocol number for the use of NHPs was mentioned **(see Page 35, line 739).**

3. The English in this paper needs to be revised in general…. for e.g. In the abstract, results, the language usage should be "a large number" not "a large amount" and so on. Also, general conventions are not always followed here, please check on these. Also spellings such as 'upregularted' should be carefully checked.

   Reply 3: we modified these English spelling and convention mistakes as advised **(see Page 2, line 36-37; see Page 4, line 78; see Page 8, line 160; Page 22, line 470; Page 23, line 477).**

4. Heparin (used as a coagulant here) may have an effect on gene expression in blood and also effect further perturbations of the transcriptome. Please explain how this potential confounder was ruled out in this case.

   Reply 4: First of all, the heparinized blood sample undergoes the process of dilution, centrifugation, cell separation, washing, and re-culturing before PHA or LPS treatment, which may reduce the effect of heparin on PBMCs. Secondly, heparin binds to enzyme inhibitor anti-thrombin Ⅲ, causing a conformational change that results in its activation. The activated AT then inactivates thrombin, factor Xa and other proteases. PBMC cells in the blood are mainly lymphocytes, so heparin has little effect on the function and phenotypic characteristics of lymphocytes. Finally, during the gene expression profile analysis in this study, the blood sample of the control group were also heparinized. In the DEGs analysis, the PHA/LPS treatment groups were compared with the corresponding control group. In addition, the results

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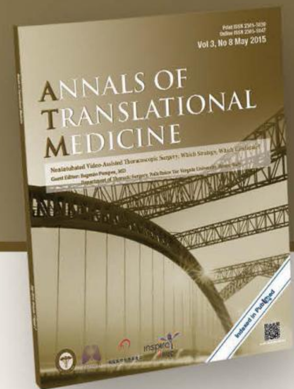

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of GO analysis indicated that the DEGs were associated with immune response, DNA replication and cell proliferation. Therefore, this potential confounder by heparin was ruled out in this study.

5. 3 biological replicates were used in this study. This is a low number for whole transcriptome analyses in general, can the authors justify the use of this low number and cite reasons why the dataset is robust and reproducible from this small number of replicates.

   Reply5: The aim of this comparative study is to permit better extrapolation of animal toxicity data in preclinical drug safety studies using non-human primates. The three biological samples can cover the genetic polymorphisms, and it is also the smallest number of animals selected in each group when performing conventional toxicology studies using non-human primates. This also has certain economic considerations. In this study, the results of gene expression profile were consistent with the cell proliferation and cytokine secretion, which suggesting the data was robust and reproducible. Moreover, the qRT-PCR validation showed that the microarray measurements were robust.

6. What RIN value was used as a cutoff for the hybridizations? In general, the methods need more specific information, such as chip information, labeling kits used, pre-amplification kits and reagents. Basically, according to MIAME (PMID: 11726920) and ARRIVE recommendations, other researchers should be able to re-create the wet lab methodology of all –omics publications and this section should be revised.

   Reply 6: We added more specific information for gene expression profile as advised (**See Page 9, line178-184**).

7. In the Methods, how were the 10 genes chosen for qRT-PCR analyses?

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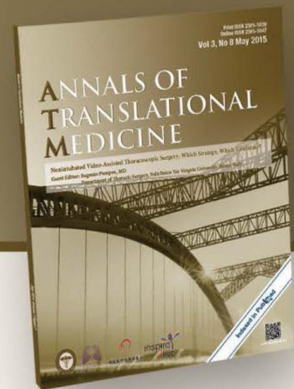

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Reply 7: In order to establish bridging biomarkers shared by human and monkey, the overlapped 38 and 20 genes under PHA and LPS stimulation respectively were obtained in both species. By correlating the same genes in the overlapped biological functional groups (the same GO terms), with PHA stimulation, it was found that the genes (CCL2, IL8) were related to 'immune response' and 'inflammatory response', the genes (MCM6, MCM3, MCM7, MCM4, PCNA) were related to 'DNA replication' and 'DNA-dependent DNA replication initiation', the genes (PLK1, CDKN2C) were related to 'mitosis' and 'cell division' and 'G1/S transition of mitotic cell cycle'. Similarly, with LPS stimulation, it was found that the overlapped genes (CCL3, IL1A, IL8) were related to 'immune response' and 'chemotaxis' and 'inflammatory response', the gene CD74 was related to "cytokine-mediated signaling pathway" and 'immune response'. Therefore, these overlapped genes with similar biological function might reflect the highly conserved signatures of immune response to PHA/LPS stimulation across the primates.

8. Figure 1, where is the negative control data? It should be included or a line drawn to indicate where background levels are.

   Reply 8: We modified Figure 1 as advised, and added some comments for lymphocyte proliferation ratio (**See Page 8, line 151-152**).

9. Figures 2 and 3, the axes and labeling fonts are very small, suggest using a sans serif font to make reading it easier. Also, is it possible to have consistency of bar colors in all figures. In some they are grey and dark grey, in others orange and blue. Then in Figure 4 we are back again to grey and dark grey.
   Reply 9: We modified the figure 2 and 3 as advised. **(See Fig 2 and Fig3).**

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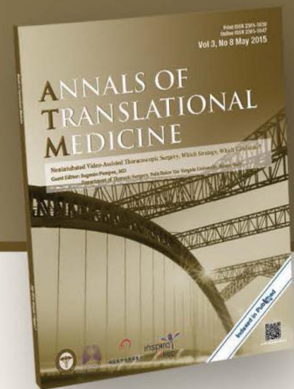

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10. Figure 5 was poorly presented and it is not clear what the message is here? There are labels from the original heatmap and then overlaid with inserted text. Also, there are only 4 groups, the controls should be included and data for all 30 hybridized samples shown. Why are only averages shown. Also the scale of the colors is not sufficient to see any actual differences. I suggest omitting this figure.

    Reply 10: The resulting microarray platform induced 11914 probes in human and 10273 probes in monkey after reducing the probes with intensities < 400. According to the Ration value of 'treatment/control', the DEGs were identified by SAM software. Here, the DEGs in PHA/LPS treatment groups of different concentration clustered together, and more differential expression genes were in high dose groups comparing with those in the low groups as seen in Figure 5. Of course, the results in this figure have been well represented as histogram in Figure 4 (updated). Thus, I would like accept the reviewer's advice to omit this figure and modified the text in manuscript (**See Page 13, line 263-268**).

11. In Fig 6, what is the y-axis? It is not clear. What does the sentence on line 283 mean, increase of 21 times? Also between Figs 6 and 7, I suggest that the authors revise these to show the best way of comparing the categories. Perhaps a table with side by side significance numbers might clear up the message.

    Reply 11: Thanks for this suggestion, and **the updated table 3 and 4 were used to replace Figure 6 and 7.** It showed that the number of DEGs involved in top 10 biological functional groups obviously increased with increasing PHA dose in human PBMCs. For example, the count of genes in 'DNA strand elongation involved in DNA replication' was from 1 in 2μg/ml PHA groups to 21 in 10μg/ml PHA groups, and the count of genes in 'cytokine-mediated signaling pathway' was from 2 in 2μg/ml PHA groups to 43 in 10μg/ml PHA groups in human. As suggested by the

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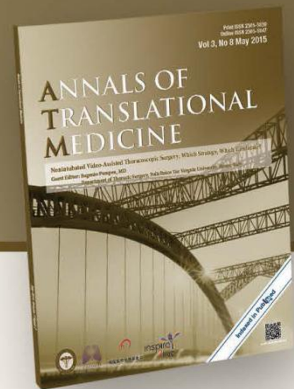

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reviewer, the expression in the manuscript was not clear enough, and it was revised (**See Page 14-15, line 295-307**).

12. Table 3: KIAA0101 is actually a named gene: PCNA Clamp Associated Factor, according to GeneCards, I suggest the authors check their data for such gene names and annotations, especially for tables and figures presented within the article. Also, I suggest, FC are represented to the first decimal place, 3rd decimals are useless information in this context.

    Reply 12: Thanks for the reviewer's comment, the gene symbol, gene name and annotations of human and cynomolgus monkey were checked through GeneCards and PUBMED Gene respectively. The FC value in manuscript and table 3-8 has been modified as advised (**see Page 15-16, line 328, 332, 340, 346, 349; Page 17, line 364; Page 19, line 397, 402-403; Page 20, line 420; Page 21, line 431; Page 29, line 615**).

13. The qRT-PCR validation showed that the microarray measurements were robust, however it was not clear if these were different samples/biological replicates from the ones used for hybridization.

    Reply 13: The samples used for hybridization were exactly the same as those tested by QT-PCR (**See Page 10, line 193**).