Dear reviewer,

We sincerely thank you for thorough evaluation of the manuscript. Bellow we respond point-by-point. We hope that we have responded satisfactorily to the comments and we are prepared to make further changes at the request of the Editor and the Reviewers. We thank you for considering our manuscript.

Sincerely,

Špela Knez, Mojca Narat, Jernej Ogorevc

I have revised the manuscript entitled "Differential Gene Expression Induced by Different TLR Agonists in A549 Lung Epithelial Cells Is Modulated by CRISPR Activation Of TLR10" (biomolecules-1940708). This is an interesting paper aimed to review the function of TLR10 in A549 lung epithelial cells, and the differential expression of immune related to genes in the challenged A549 cells. But this idea was not clearly developed. The research is quite limited— some other minor problems were also found.

The list of specific comments that should be addressed are defined as follows:

1). symbols for genes should be italicized.

We italicized gene symbols.

2). Line 45: Delete extra spaces.

Extra space removed (line 45).

3.) Some abbreviations are repeated. (e.g. " RT -qPCR " " CRISPRa ", " CRISPRi"). Please check and correct.

Repeated abbreviation (RT-qPCR) deleted.

4). Once the abbreviation has been announced, the corresponding molecule should no longer be written by its full name. (e.g. " dCas9 ").

Instead of pCMV-dCas9: NLS: VPR abbreviation dCas9-VPR was used (lines 124 and168).

5).it is: "TBK1 (TANK-binding kinase 1)", it should be: "TANK-binding kinase 1 (TBK1)".

All the abbreviations were corrected according to the presented format.

6). Line89: There is a problem with the title serial number.

Title numbers corrected, line 89.

7). Some abbreviations must be clearly indicated in Figure 1 (e.g. "ORF", "C").

Abbreviations in Figure 1 explained in the legend, line 227.

8). Figure 1. C needs to be remade with high resolution.

Response: Western Blot was repeated and new image with higher resolution was included.

9). In Figure 1, the authors stated that the combination of sgRNA2 and sgRNA3 showed the strongest upregulation of endogenous TLR10 and was assayed at the protein level, but specific statistical analysis of the variability was lacking.

Response: Specific statistical analysis of variability added (lines 194-199, 224-227, 259-264, 278-279, 312-317). Values are expressed as mean ± standard deviation (SD). Statistical analysis was performed using Student's t-test for paired samples. A P-value of ≤ 0.05 was considered statistically significant.

10). What is the efficiency of transduction using different sgRNAs vectors and dCas9-VPR plasmids in A549 cells? Is it stably expressed?

Response: We performed transfection of plasmids (not viral vectors). The transfection was transient – the plasmid wasn't integrated into the genome of the transfected cells. According to our experiments with GFP and the producer's data the expected transfection efficiency is approximately 60-70 %.

11). The authors mentioned that no changes in cell viability, growth rate or morphology were observed between cells transfected with TLR10 overexpressing cells and cells transfected with the empty vector. However, no specific experimental results were seen in the article or in the supplementary. Whether TLR10 overexpression has any effect on the status and proliferation of A549 lung epithelial cells needs further experiments.

Response: In our preliminary experiments we monitored cell viability and proliferation by counting and staining the cells using Trypan blue. After the transfection with either of the plasmids (empty or TLR10OE) the we didn't observe any difference in growth rate or viability between the cells overexpressing TLR10 and the cells transfected with empty vector. We added explanation in *Results section* (lines 211-214).

12). Whether TLR10 overexpression in A549 cells has effects on the expression of other TLRs?

Response: In our preliminary experiments we already checked for expression of other TLRs, the data is now presented in Supplementary figure 2 and explained in the main text (lines 210-211). We observed no statistically significant differential expression for TLR2 subfamily or TLR3 and TLR4. We also investigated expression differences of specific TLRs and TLR10 when treated with ligands, data was added to Supplementary figure 5 and explained in the text (lines 238-239).

13). All abbreviations used in Fig. 4 should be explained in figure legend.

Response: Abbreviations were added to the figure legend (Figure 5) (lines 372-378).

14). TLR10 overexpression in the cells leads to reduced proinflammatory cytokines expression and increased the production of anti-inflammatory cytokine, which needs to be verified by ELISA or Western blotting at protein levels.

Response: To confirm biological relevance at the protein level, ELISA was performed for IL1β, CXCL10, TNFα, CCL20, IL8, IL10 and IFNβ using commercial kits as indicated in *Material and methods* section 2.6. Results from ELISA are presented in figure 4A and in the text (lines 313-317).

Results from ELISA showed inhibitory effect of TLR10 also at the protein level; secretion of IL1β, TNFα, CXCL10, IFNβ, CCL10 and IL8 was downregulated in challenged A549-TLR10 OE cells compared to challenged control cells. Contrary, IL10 secretion was higher in challenged A549-TLR10 OE compared to challenged control cells.

15). The authors used only cDNA samples for gene expression profiling, so does TLR10 have an inflammatory inhibitory effect *in vivo*?

Response: In this study we were interested in potential immunomodulatory function of TLR10 in lung epithelial cells. Cell models are an established alternative to *in vivo* experiments, therefore we believe

the use of a relevant cell model is relevant, especially in case of TLR10 where *in vivo* studies on mouse model are limited, because mice lack functional TLR10. Based on our results, it is reasonable to believe TLR10 would also show *in vivo* effects in lung epithelial cells. In fact, that has already been demonstrated by for example Jiang et al., 2016, who showed reduced TLR-induced response in leukocytes of transgenic mice with inserted hTLR10 (lines 38-42). However, we agree further that further studies on relevant cell and animal models are required for better functional annotation of TLR10 in lung and other cell types/tissues. Discussion added (lines 420-423).