MRC Translational Immune Discovery Unit

UNIVERSITY OF OXFORD

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Assessment of the PhD thesis entitled "5' terminal nucleotide modulates the immunogenicity of RNA" submitted by Jacek Nikodem Szymański

The candidate investigated how RNAs produced by *in vitro* transcription (IVT RNAs) using T7 RNA polymerase activate innate immune responses. This is not only an interesting topic for discovery research. IVT RNAs have growing use as medicines, e.g. mRNA vaccines are made by IVT. As such, the thesis is in an important and timely area. Overall, the research is conducted to high standard, and the data strongly support the conclusions drawn. I not only commend the candidate on the quality of the work, but also on the amount of data obtained. The key result – namely that IVT RNAs with a 5'A activate the innate immune sensor RIG-I more strongly than otherwise identical IVT RNAs with a 5'G – is important not only to the immediate field of innate immune RNA sensing but also to drug development.

Comments on the introduction

This thesis contains a concise and well-written introduction. However, parts of the introduction would have benefitted from enhanced proof-reading, e.g., the section on RLRs contains numerous grammatical errors. The introduction also contains some factual errors (e.g. that NOD2 is the only nucleic acid binding NLR protein) and fails to cite some important papers (e.g. Pichlmair et at., Science 2006). The introduction covers the wider field of innate immune RNA sensors very selectively, e.g. PKR is introduced but OAS proteins and ZBP1 are not.

Comments on manuscript 1 – published in NAR

The key observation of the manuscript is that IVT RNAs with a 5'A induce innate immune responses more potently than IVT RNAs with a 5'G. The candidate and colleagues provide a strong dataset with some advanced experiments including SHAPE analysis in Fig 1. The candidate and colleagues also provide a convincing explanation, namely that 5'A IVT RNA preparations contain more dsRNA. This is elegantly shown by a semi-synthetic RNA produced by splint ligation that does not induce innate immunity unless supplemented with an antisense strand. The manuscript also provides important starting points for future research. Firstly, it will be interesting to determine if the increased abundance of dsRNA in IVT RNAs with a 5'A is due to the IVT reaction and properties of T7 RNA polymerase, or is related to selectivity in RNA purification by PAGE. The data in Figure 7A-B indicate that the latter may be possible. Secondly, future testing of capped, base-modified and polyadenylated RNAs will be important as mRNA therapeutics typically comprise these features.

Comments on manuscript 2 – preprint posted on bioRxiv

This pre-print further intvestigates the role of the 5' nucleobase in RIG-I RNA agonists. Using biochemical (EMSA, ATPase assay) and cell-based (HEK293 and BMDM transfections) experiments, the candidate and colleagues show that RIG-I is more stongly

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activated by RNAs with a 5'A, in comparison with 5'G. The candidate and colleagues further propose a mechanism involving competition of GTP binding proteins with RIG-I for the 5'-end of 5'G RNAs, explaining reduced innate immune activation. This involves a proteomic approach, comparing proteins bound by RNAs with 5'G and a 5'A. Intriguingly, supplementation of cells with guanosine – assumed to elevate intracellular GTP levels – increases RIG-I activation by 5'G RNAs to the levels seen with 5'A RNAs. The candidate and colleagues interpret these data to reflect saturation of GTP binding proteins. These exciting results provide a basis for much future research. For example, it will be interesting to determine if specific GTP binding proteins (such as NUDT16, RAN and RANBP1) compete with RIG-I for 5'G RNAs, or whether there is redundancy between many GTP binding proteins.

Final Conclusion

I, the undersigned, hereby state that the doctoral dissertation of Jacek Nikodem Szymański meets the requirements specified in Article 187 of the Act of July 20, 2018 – Law on Higher Education and Science (c.t., Journal of Laws of 2024, item 1571, as amended). I hereby recommend to the Doctoral Committee of the International Institute of Molecular and Cell Biology in Warsaw to admit Jacek Nikodem Szymański to the subsequent stages of the procedure for the conferment of the doctoral degree in the field of natural sciences, in the discipline of biological sciences.

Moreover, I consider that **this doctoral dissertation should be distinguished**: the candidate demonstrated exceptional productivity with significant new fundamental knowledge and important translational insights gained.

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