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SUBJECT: Review of Mikołaj Kocikowski thesis for Ph.D. degree

Dear Committee

I have read with great interest and enjoyment the Ph.D. thesis of Mr Kocikowski entitled "Of Dogs and Men. Tracing Immune Checkpoint Signatures Across Cancers and Unleashing the Potential of Canine PD-1 Antibodies". I find it acceptable in large part to satisfy an award of Ph.D. However, while the thesis is generally well written and documented appropriately with copious detail on the methods used to analyze data, I have the following questions and comments laid out according to chapters.

INTRODUCTION

Generally from the **Introduction: Chapter 1** I can understand that murine models are poor for use in humans because humans are mostly outbred and live in an immunologically dirty environment. Dogs also live in an immunologically stimulating environment like humans. However, given the ease with which humans can breed dogs for desirable traits then it must be true that dogs are more likely to be inbred than humans.

How might such inbreeding affect the use of dogs as a model? For example, I can see some texts state that 1 in 3 dogs develop cancer versus 1 in 5 humans. Is this higher rate in dogs related to dogs being more likely to be inbred to maintain a given set of traits than in humans? While even Plato suggested that humans might be bred to enhance beneficial phenotypes, such eugenics are generally frowned upon. Not true with dogs where this is the norm. Given that certain breeds may also carry genetic risks for certain types of cancers, as do human families, how is their genomic heritage considered if at all in use as a model for human cancers? For example, terriers are at higher risk for bladder cancer than other breeds.

Cancers can certainly run in human families as well. In addition, large dog breeds also tend to have higher rates of osteosarcoma, or bone cancer, than smaller breeds. Given that taller people have a higher risk of cancer – due to their growth rate that made them taller – is there a correlation with the same process in large dogs?

I understand the use of canine cancer as a model is justifiable but to what degree are their hot and cold tumors related to those in humans? Are there direct parallels such that a cold tumor in humans will also be a cold tumor in dogs? If not then there might be some key to how to improve IC use.

Chapter 2. IMMUNE CHECKPOINT LANDSCAPES OF HUMAN AND CANINE CANCERS

From Limitations (2.3.12) in Chapter 2 please explain further the functional ramifications toward developing therapies when you write "One caveat is the possibility of cancer cells themselves expressing functional IC receptors, a hypothesis lacking consistent evidence. We followed the common assumption that comparatively high IC expression indicates a promising target."

Chapter 3. COMPARATIVE CHARACTERIZATION OF TWO NOVEL ANTIBODIES AGAINST CANINE PD-1

On page 76 there is a bit of sloppiness in sentence construction “Maekawa et al. identified rat anti-bovine PD-L1 antibodies that recognized canine PD-L1 and also blocked its interaction with PD-1 [180]. The antibody was recombined to create a canine-rat chimera and subsequently tested in seven dogs with cancers. In this study, exploratory in nature, responses were observed in two of the dogs [183].” Is the underlined sentence intended to mean that two dogs were cured and the other five were not? Or is it just that there was some response? While I could guess your meaning, it is better to clean this up explicitly stating what the response was for these two dogs.

On page 77 you state “We used murine hybridoma technology to generate monoclonal antibodies (mAbs) against the canine PD-1 protein.”, but I can’t find there or in the methods how you made the protein. Once made, how did you know it was folded properly? I can see in Figure 1 that your antibodies are detecting a protein of the right molecular weight but how did it come to be folded properly after expressed? How was it purified for phage display? On page 97 I read “Briefly, Balb/c mice were immunized with a recombinant protein fusing the complete canine PD-1 sequence and a his-tag (Sino Biological).”but how did you know the protein folded properly? Is all of this in REF 186? And who did all the preparative work – Borek’s group at Moravian? It should be made clear early on that you leveraged the work prior work in Brno. Otherwise, the reader goes along not be certain what you did or didn’t do until closer to the end.

On page 80 “Both PD1-1.1 and PD1-2.1 exhibited a shift in signal peaks for cells transfected with the PD-1 as compared to the control cells, indicating specific binding of PD-1.” The use of with word “peaks” needs an adjective to better describe it than signal. After all a “peak” is usually a signal and the word peak is simply common jargon that we all use in speaking but in writing please try to be more explicit. So this text is a bit sloppy in writing. Would be better to write what you see in the plots which is “…a shift [in cell count as a function of fluorescence intensity] for cells…” or something like this but more descriptive. I would try not to write peak unless it has a descriptive adjective prior to it.

Chapter 4. CANINIZED ANTIBODIES: STRATEGIES FOR PROTEIN DEIMMUNIZATION

The introduction to the problem appears, even though it’s not my expertise, to be well written. I would consider publishing it as a small review.

It wasn’t until page 143 that I understood “*As this project aimed to build a resource of highly reliable sequences that could be used to construct functional antibodies and train artificial intelligence models, the limiting yet stringent approach was appropriate.*” Prior to that I was thinking you were trying to make an actual caninized mAb. This should be pointed out early in the chapter. I would start each chapter with a clearly stated GOAL to reminder the reader and avoid them going back to the introduction to look for the reasoning. For example, this chapter’s goal might be: Here we compared the efficacy of different in silico strategies to predict best approaches to caninize mAbs.

In the end it was clear you were developing in silico methos but these statements are a biut sloppy “*In the project's culmination, we generated caninized antibody variants and identified those that best retained the key structural features. The top-scoring antibodies will undergo further validation in laboratory assays. ”* because you didn’t’ generate antibody variants, which to me implies you made them physically, but rather you identified sequence variants that are promising. Is this correct? It just means to me that you need to write more carefully. For example, don’t say you generated antibody variants when you mean you predicted antibody variants.

Page 151 Chapter 4

4.3.2 Future work – this section is interesting to point out but it isn't your thesis work but rather just your thoughts on the pitfalls of actually making the antibodies. I'm not sure it should appear before the next section **4.3.3 Limitations and improvements**. This section 4.3.3. follows directly from your dissertation. Logically I would place it prior to 4.3.2. Notably, the above future work section 4.3.2. might be better named. Perhaps Limitations of antibody production. 4.3.3 though falls directly from your work and I would have put it before 4.3.2

Chapter 5.

This statement "*Canine antibodies are not identical to human ones, but at the moment have to be modeled based on human templates, as no canine antibodies feature in the PDB database. Crystallographic data of any canine antibodies could lead to better results."* appears to be the single most limiting factor in what you have tried to accomplish. Perhaps an opinion piece should be published extolling the virtues of having these structures???

Chapter 6.

Interesting discussion of peptides as ICs. What are ways to avoid their proteolysis which is the most obvious problem with their use? Would it be feasible to design a peptide mimetic with amide like bonds that cannot be hydrolyzed? Since you go into the field of bacteria, please explain the contribution of William Coley in the early 20th century who injected patients with live and heat-killed bacteria. What components of the bacteria do you think might have been responsible for the positive outcomes he found? Why was his work neglected for him only to one hundred years later now be referred to as the Father of cancer immunotherapy?

In summary, overall the thesis is well written and sufficiently referenced with apparently sufficient detail to reproduce the work. The challenges - presented and addressed as best possible - by the student are also of great current interest generally across the biological sciences community. I therefore find the document sufficient for the purpose of defending a Ph.D. degree.

Sincerely,

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