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RE: Manuscript by Faulks *et al.* Number ID: 02738864; ESPS manuscript NO: 28904

Dear Prof. Ji Fang-Fang,

We are grateful to you and both peer-reviewers for the positive responses to our invited article entitled "P2X7 receptor activation causes phosphatidylserine exposure in canine erythrocytes". Please find uploaded a revised version of our manuscript for your further consideration. In addition, please find below our responses to both peer-reviewers. We hope you find this revised version suitable for publication in in the *World Journal of Hematology*.

Kind regards,

Ronald Sluyter

Response to Peer-reviewer 1

The authors do not tell whether the 24h incubation with ATP induces lysis of erythrocytes or changes in their morphology (comparing treated cells with controls).

Due to the editorial request to submit the revision in no more than 2 weeks and the *ad hoc* availability of dog samples we are unable to perform any additional experiments. However, we added text to the Results (page 6, paragraph 1) and Discussion (page 8, paragraph 1) addressing these points as follows, respectively:

ATP incubation also caused visible hemolysis compared to cells incubated in the absence of ATP (results not shown), but neither this nor other changes in erythrocyte morphology were investigated further.

In the current study, ATP caused visible hemolysis of canine erythrocytes, however this was not formally investigated. We have previously shown that 24 hour ATP incubation induces a small but significant amount hemolysis of erythrocytes from English springer spaniels compared to those incubated in the absence of ATP (16% vs 1%, respectively)[12]. Future studies are required to explore if this ATP-induced hemolysis is mediated by P2X7 or other purinergic receptors, such as P2X1 or P2Y1, which can also mediate hemolysis[20,21]. Also, it remains unknown if 24 hour ATP incubation causes other changes in erythrocyte morphology. Five minute with 1 mM ATP of beagle erythrocytes increases cell viscosity as assessed by filterability of packed cells, but not changes in cell shape as observed by light microscopy[26]. Therefore, further studies could explore if activation of P2X7 or other purinergic receptors alters canine erythrocyte morphology.

Deeper analyses of the role of the P2X7 receptor system in erythrocytes in healthy functions and disease, or the function of the splice variants, or on other cells are not presented or discussed.

The current manuscript aimed to explore P2X7-mediated PS exposure in canine erythrocytes; not to provide a deep analysis of the role of the P2X7 receptor system in erythrocytes, which was discussed in depth in our 2015 review [9]. Thus, we fell it is unnecessary to reiterate this in the current manuscript. However, we added discussion of splice variants in erythrocytes to the Discussion (page 7, paragraph 3) as follows:

It remains unknown why the relative amounts of P2X7 differ between canine and human erythrocytes, but we have previously speculated[12] that this difference may be due to variations in the proteolytic systems responsible for maturation-associated degradation in reticulocytes between these two species. Differences in erythrocyte P2X7 activity between these two species are unlikely to be due to altered expression of splice variants. Previous immunoblotting studies using an antibody to the extracellular loop of P2X7, which is predicted to bind all known splice variants of canine P2X7 (URL: http://www.ncbi.nlm.nih.gov/gene/448778) and human P2X7[23,24], demonstrated only the full-length receptor in erythrocytes from both species[12]. Notably, the lifespans of canine and human erythrocytes are similar (approximately 115 days)[25] suggesting that P2X7-induced PS exposure in erythrocytes is unlikely to influence the removal of senescent cells.

Response to Peer-reviewer 2

Data are indicative of increased expression and activity of P2X7 in canine erythrocytes compared to human erythrocytes. Please discuss biological explanation for this and its physiological importance, if any.

The physiological importance in the increased P2X7 expression and activity in canine erythrocytes compared to human erythrocytes remains unknown. This was not discussed in detail as the current manuscript offers no direct comparison between the two species and this topic has been subject to previous speculation in our previous papers [12, 18]. Nevertheless we have briefly extended this discussion along with splice variants in the revised manuscript (page 7, paragraph 3) (see response 2 to peer-reviewer 1).