Author's response to reviews

Title: Antagonism of cannabinoid receptor 2 pathway suppresses IL-6-induced immunoglobulin IgM secretion

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Author's response to reviews: see over
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David Farb, PhD
Editor Office
BMC Pharmacology and Toxicology

Dear Dr. David Farb,

On behalf of the authors, I am sending you our revised manuscript titled as “Antagonism of cannabinoid receptor 2 pathway suppresses IL-6-induced immunoglobulin IgM secretion”, which has been reviewed by the experts and needs revision before acceptance for publish in BMC Pharmacology and Toxicology. We have followed the reviewers’ comments in the revised manuscript and given a point-by-point response to their good comments. Meantime, as the native English speakers, Professor Christine Milcarek and Dr. Patrick Bartlow have read and corrected language errors. This is to respond the 2nd Reviewer’s suggestion on the Quality of written English.

Regarding the authorship update, Dr. Patrick Bartlow has been removed from the author list and mentioned in the Acknowledgements section for his technical assistance and editing and journal submission.

Should you have any other problems or questions regarding this manuscript, please don't hesitate to contact me.

Sincerely,

Dr. Xiang-Qun (Sean) Xie, PhD, Professor
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Point-by-point response to Reviewer’s Comments

Reviewer: Santhi Gorantla

“Xie et al showed that cannabinoid 2 receptor (CB2R) antagonist or inverse agonist blocks the IgM secretion in SKW 6.4 B cell line induced by IL-6, and this blockade can be stopped by CB2R agonist confirming that antagonist induced blocking of IgM secretion is via CB2R. They also showed that transcription factors including Bcl6, PAX5, XBP-1, NF-κB and STAT were involved in the regulation of IL-6 induced IgN expression by CB2R antagonist.”

“B-lymphocytes express highest levels of CB2R mRNA among all the leukocytes. Very little is known about effects of CB2R modulation in B-lymphocytes on differentiation and function. The authors tried to investigate the modulatory activity of CB2R antagonist and inverse agonist on a human B cell line.”

Major concerns of the present study are:

(1). the purpose and rationale of the study is not explained well.”

Response: We thank the reviewer’s suggestion and have added description to address the purpose and rationale of the study in the background section.

(2) why the antagonist or inverse agonist were employed to investigate the CB2R modulation in B cell line? Is there any therapeutic implication to the particular study?

Response: Since the CB2 agonists such as Hu-308 failed to show distinct effects on IgM production, we worked on several antagonists as chemical probes to see whether they show any potential modulation on antibody secretion. We do have the expectation to use them for the possible treatment of some immunoglobulin-overproduced conditions, e.g., hyperimmunoglobulinemia, multiple myeloma.

(3). What is the effect of CB2R agonist on the B cell line?

Response: These are legitimate concern and it still remain controversial. CB2 agonist may inhibit the forskolin-stimulated cAMP. The references show that cannabinoids have the immunosuppressive effects. The effects of agonists on B cell migration are also concentration-dependent, transient and reversible (Biol Pharm Bull. 2011; 34(7):1090-3.). Delta-9-tetrahydrocannabinol, a partial agonist for CB1 and CB2, inhibits mouse plaque-forming cell assays for antibody formation. Cannabinoid receptor 2 (CB2) mediates immunoglobulin class switching from IgM to IgE in murine B cells (J Neuroimmune Pharmacol. 2008; 3(1):35-42). Agonist CP55,940 can enhance the proliferation of B cells (Blood.
Our unpublished data show that CB2 antagonist but not agonist could inhibit the proliferation of myeloma cells, which are malignancy plasma B cells.

In Figure 2 it is claimed that CB2R agonist reverses the inhibitory effect of the inverse agonist, but the inhibitory effect does not look very significant. There are no statistical analyses included.

**Response:** We have added the analyses as described in the Figure 2 legend: “*P<0.05 compared with the control (IL-6 treated alone).” Meantime, we also re-wrote the results of “CB2 agonists reverse the inhibitory effect of SR144528 on IL-6-induced IgM secretion”.

**Reviewer: Barbara L. F Kaplan**

“Feng and colleagues prepared the manuscript entitled “Antagonism of cannabinoid receptor 2 pathway suppresses IL-6-induced immunoglobulin IgM secretion” to be considered for publication in BMC Pharmacology and Toxicology. While the data have potential significance and address important effects of cannabinoid modulation of IgM production in a human B cell line via CB2, the authors should address several issues prior to publication.”

1. There is no information provided on statistical analyses performed. In fact, statistics are only provided in Figure 3. For instance, what does “slightly disturbed” mean? Statistical analysis is especially critical for the data presented in Figure 2 in which the authors conclude that the inhibitory effects of SR144528 are reversed by HU308. It is not clear why the authors did not perform a concentration response of reversing SR144528 with HU308, especially since HU308 at concentrations up to 20 μM (seemingly) did not produce any effects on IgM or viability. In addition, it was difficult to determine how many times experiments were replicated in many figures, especially since some data were reported without any error bars.

**Response:** We sincerely thank reviewer’s constructive suggestions. We have labeled the bars with * where appropriate apply, and added “*P<0.05 compared with IL-6 treatment alone” in the Figure 1 and Figure 2 legends. Some data were reported without error bars (Fig. 1A and 1E, Fig. 3A) due to the kit unavailability. Meanwhile, we think the data are already distinct, thus there are no needs to repeat again. We did perform a concentration response of reversing SR144528 with HU308 (5 uM and 10 uM), the results, however, were not linear relationship. Therefore, we didn’t show the 10 uM data to avoid confuse the readers. To follow the reviewer’s comment, we have added the analyses as described in the Figure 2 legend: “*P<0.05 compared with the control (IL-6 treated alone).” Meantime, we also re-wrote the results of “CB2 agonists reverse the inhibitory effect of SR144528 on IL-6-induced IgM secretion”.

2. Although the authors reported in the Materials and Methods that vehicle controls were performed, there was only one figure (1E) in which a vehicle control was identified. Are the other figures and legends missing the description of this control? In particular in the studies in which combinations of drugs were used, the vehicle controls are critical to maintain consistent DMSO concentrations across combination treatments.

**Response:** we have followed the reviewer’s comments and added the vehicle control. In addition, in the Methods, we added “For all the cell cultures with CB2 ligands, the final concentrations of DMSO were always equal or less than 0.05%.” This criterion is consistent with the general requirement of cellular pharmacology. For instance, the final DMSO in the combination wells of CB2 ligands in Figure 2 is only 0.03%, DMSO’s influence on the results is negligible. LPS and TPA were not prepared by DMSO.

3. It was not clear why the concentration of IL-6 was different between experiments. In some figures and/or legends, the concentration of IL-6 was not provided. The authors should clarify this.

**Response:** We have followed reviewer’s suggestion and provided the IL-6 concentration in the figure legends. Since IL-6-induced IgM production needs very long 4 days, whereas treatment time in the western blot assay and RT-PCR test is respectively only 45 min and shorter period, we thus used higher concentration of IL-6 (300 U/ml) to treat the cells to get clear results. We also explained the reasons to use higher concentration of IL-6 in Figure 3B-3D in the text.

4. While SR144528 and Bay11-7085 act similarly, this is not evidence that SR144528 inhibits NF-kB. This should be directly measured, especially since several studies have demonstrated that NF-kB is a target of suppression by cannabinoid agonists. Also, can the authors quantify the inhibition of IkB-a and p-STAT3 in Figure 3B?

**Response:** We thank the reviewer’s comment very much. After carefully checked our data and quantify the inhibition of IkB-a and p-STAT3 in Figure 3B, we found that NF-kB pathway might not play important role in the inhibition of IL-6-induced IgM production by CB2 antagonist. We have re-written the sentences and discussed these in the new version of this manuscript.

**Minor Essential Revisions**

5. “Concentration” instead of “dose” should be used throughout.

**Response:** We have changed it throughout the text.
6. “Immunomodulative” should be “immunomodulatory” (Background).

**Response:** We have corrected it.

7. “Hu308” should be “HU308” throughout.

**Response:** We have changed it throughout the text.

8. Use of only SR144528 to determine the effect of “CB2 ligands” isn’t a valid statement since only one ligand was used (Results describing Fig 1E).

**Response:** We have changed “CB2 ligands” to “CB2 ligand SR144528”.

9. The data that SR141716A did not suppress IL-6-induced IgM production are sound, but they do not rule out the CB1 pathway entirely. The authors should alter this wording.

**Response:** We agree with reviewer’s suggestion and have tuned down the blunt tone conclusion in the revised version. “This suggests that CB2, but not CB1 antagonism, may be mainly involved in the inhibition of IL-6-induced IgM secretion in the present cellular system. Together with the demonstration that human B cells displayed large amount of CB2 receptor mRNAs, the above results led us to assume that the inhibition activity observed on plasma cells could be mediated mainly through the CB2 receptor.”

10. In Figure 3A, a line indicating that IL-6 was present in all groups should be included.

**Response:** We have added a line to show that IL-6 was present in all groups as in Figure 3A.

11. In the Methods, the symbol for μl is incorrect in the “Reagents and cell culture” section.

**Response:** We thank the reviewer. We have corrected it.

12. In the Results, “lipopolysaccharides” should be “lipopolysaccharide”.

**Response:** We have corrected it.

13. There should be a citation after this sentence in the Results: “CB2 is primarily expressed in B plasma cells.”

**Response:** We did it and cited reference 3.

14. In Figure legend 1, the authors indicate that the “Cell DNA synthesis was determined with 3H-thymidine assay as in the Materials and Methods.” All assays are reported in the Materials and Methods so the latter part of this sentence is not
necessary.

**Response:** We deleted the latter part of this sentence.

Discretionary Revisions

15. In the regulation of IgM by Bcl-6 and Pax5, Blimp-1 is also an important regulator of this pathway. The authors should provide data on Blimp-1 or state why they only focused on 2 of the 3 proteins in this pathway.

**Response:** We have performed the PCR for Blimp-1 but the amplification data look not very good, we thought the primers might be an important issue for Blimp-1 PCR. Meantime, we have described clearly the relationship between Bcl-6 and Blimp-1 and wrote in the Results and Discussion: “Transactivation of Bcl-6 would be expected to continue to suppress plasma cell differentiation through negatively targeting the Blimp-1 gene, thus impeding differentiation to secretion.”