Peer Review File

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First Round:

Reviewer A

The authors used a rat model of STZ-induced diabetes to show that inflammatory and metabolic changes in the retina in diabetic rats could be partially inhibited using systemic IMD-0354, a novel IKKB inhibitor. IMD-0354 was reported previously by others to decrease vascular permeability in STZ-induced diabetic mice (ref 26). The current study did not evaluate the retinal vasculopathy associated with DR but showed partial preservation of retinal ganglion cells with IMD-0354 and conclude that NF-κB activation is a critical step in the development of DR. The paper is written relatively well but all the figures do not match the figure legend and citation which made it somewhat difficult to review the manuscript and data presented.

Some specific points to be addressed by authors:

Abstract:
DR is used in abstract without mention of what it stands for.
Abstract conclusion should be reworded since diabetic retinal vasculopathy development was not studied in these Diabetic rats. IMD-0354 effects were limited to ganglion cell density. The abstract conclusion should be worded to be in line with title and purpose.

Reply: We have made appropriate changes according to the reviewer's suggestions.

Change in text: Please see line 53.

Introduction: well written

Methods:
Lines 112-15:
- provide justification or reference for dose and duration of IMD-0354 used in this study
Lines 166-8:

Reply: In ***Amelioration of endotoxin-induced uveitis treated with an IκB kinase β inhibitor in rats*** published on ***Molecular Vision*** in 2012 *[PMID: 23112571]*, the rats were injected intraperitoneally with 3, 10, or 30 mg/ kg of IMD-0354. The results showed that 3 mg/ kg IMD-0354 was not strong enough to make a difference with the control group. Meanwhile, the level of inflammation in the rats' eyes was significantly reduced by 10 and 30 mg/ kg of IMD-0354.

In **IκB kinase-β inhibitor IMD-0354 beneficially suppresses retinal vascular permeability in streptozotocin-induced diabetic mice** published on **Invest Ophthalmol Vis Sci** in 2014 *[PMID: 25205865]*, mice in the diabetic +IMD-0354 group was systemically

administered with IMD-0354 (30 mg/kg) daily for 6 consecutive weeks.

According to the above two studies, we chose to treat the rats in diabetic +IMD-0354 group with IMD-0354 (30 mg/kg) daily for 6 consecutive weeks.

- Method used to measure nuclear NFkB-p65 appears to be missing.
Line 190:

Reply: We are sorry for this inadvertent error. Nuclear NFkB-p65 was measured by WB. Cytoplasmic and nuclear protein of microglia/Müller cells were separated by protein extraction kit (Pierce Biotechnology, Rockford, IL) for the detection of cytoplasmic and nuclear NFκB-p65.

Change in the text: Please see line 230-234.

-provide justification or reference for the dose of IMD-0354 used for in vitro assay

Reply: According to ***IκB kinase-β inhibitor IMD-0354 beneficially suppresses retinal vascular permeability in streptozotocin-induced diabetic mice*** published on ***Invest Ophthalmol Vis Sci*** in 2014 *[PMID: 25205865]*, mice in the diabetic +IMD-0354 group was systemically administered with IMD-0354 (30 mg/kg) daily for 6 consecutive weeks.

Results:
Figures provided do not match the legend and citation
For example Figure 1 image is listed with Figure 7 legend
Figure 2 image is listed with Figure 5 legend
Figure 4 image Is listed with Figure 1 legend
Figure 5 image is listed with Figure 2 legend
Figure 7 image is listed with Figure 6 legend
Figure 6: RGC density appears to have been obtained using retinal flat mount immunostaining. It states that RGC density was decreased in all retina layers with diabetes but RGCs are not normally present in all retinal layers.

Reply: The order error of the figures might probably be occurred during the group upload. The Fig 6. "all layers" description of RGC distribution is a typo instead of "inner layers", we are sorry for this inadvertent error and have it corrected in the updated manuscript.

Reviewer B

The article determines the anti-inflammatory effects of IMD-0354 on glial cells in STZ-induced diabetic retinopathy. The authors indicate that NF-κB activation is a critical step in the development of DR.
Some considerations:
-Line 114: Please, revise the following paragraph regarding the IMD-0354 injection. The article is performed in rats, as animal model, no in mice.

Reply: We have rephrased the sentences according to the reviewer's suggestion.

Change in the text: Please see line 135-140.

-Line 188: Please, include the specific medium used for microglial cell and Müller cell and define if the medium contains any supplementary compound.

Reply: Cell culture media for microglial cell and Müller cell (DMEM F12 supplemented with 10% fetal bovine serum) (Line 180) has been mentioned in the Method part **(Culture of Primary Rat Retinal microglia and Müller Cells)**. No other supplementary compound had been added except for fetal bovine serum.

-Regarding the Nuclear translocation of NFkB-p65 approaches. The authors have shown the total amount of NFkB protein levels, but it is not enough for deciding if there is or not a nuclear translocation, it could be interesting using the nuclear-cytosol isolation protein or using immunofluorescence approach that shows the NFkB nuclear staining.

Reply: We are sorry for this inadvertent error that we ignored to introduce the cytoplasmic -nuclear protein separation part in the Method section. The nuclear NFkB-p65 was measured by WB. Cytoplasmic and nuclear protein of microglia/Müller cells were separated by protein extraction kit (Pierce Biotechnology, Rockford, IL) for the detection of cytoplasmic and nuclear NFκB-p65. The Fig 8. D-F and the Fig 10. D-F exhibited the expression difference between cytoplasmic and nuclear NFκB-p65.

Change in the text: Please see line 230-234.

-Line 228: The immune-staining for GFAP and Iba1 showed, is it in retinal section or whole retina?

Reply: The staining of GFAP and Iba1 showed in Figure 2 and Figure 3 was in retinal section, a scale bar represents 50μm was added into the figures.

-Line 291: The authors have written: “These findings suggest that NF-kB activation and nuclear translocation are important pathophysiological events in diabetic retinopathy” however, it is not proper due to the previous suggestion.

Reply: We have rephrased the corresponding sentences to make a more appropriate statement.

Change in text: Please see line 343-349.

-Line 307: the authors have written: “IMD-0354 downregulated the level nuclear NF-κB p65, attenuated oxidative injury, decreased expression of inflammatory….”. But the level nuclear NFkB has not been shown.

Reply: We are sorry that we didn’t describe the nuclear-cytosol isolation method during protein extraction. We actually did examine the nuclear and cytoplasmic levels of NF-kB in both microglia and Muller cells and found significant difference between nuclear and cytoplasmic NF-kB in both cell entities. Please see Fig 8. D-F and the Fig 10. D-F.

-Figure 2: Please include a scale bar in panel A. And it is necessary to show the DAPI or hoescht staining in order to define the specific retinal layer if it is a retinal section. In case of the images are from a whole retina, the authors have to include the retinal thickness that they have used to define the retinal layer selected. Related to microglial polarity status, a representative image of

each microglia polarity could be interesting.

Reply: We have included a scale bar which represents the thickness of 50μm in each figure as the reviewer suggested, the Hoechst staining is marked as blue staining layer. As for the second question, we agree with the reviewer's point of view that investigating the microglia polarity could be meaningful and interesting, we have marked the typical forms of microglia by arrows in Figure 6.

-Figure 5: The Diabetic panel of TUNEL positive cells looks like a bit overexposed…. It is interesting that the retinal thickness in this condition (diabetes for 6 weeks after STZ injection) is not affected. Please, could you comment something about that?

Reply: We performed all the exposure of images under the same standard exposure intensity.

According to ***Correlation of Retinal Structure and Visual Function Assessments in Mouse Diabetes Models*** published on ***Invest Ophthalmol Vis Sci*** in 2021*[PMID:34410299]* by Lin etc., the STZ group showed a significant difference in the inner layer at 3 months (0.0021 mm; $P < 0.05$), and differences in the outer layer were observed at 6 months, but not sign of attenuated retinal thickness within the 6-weeks (1.5 month) time window, which is consistent with our findings.

-Figure 6: The density of RGCs in the retina is redundant information. The figure 5 shows that the RGC layer is the most affected retinal layer and the TUNEL positive cells are increased in this retinal layer.

Reply: We appreciated the reviewer's suggestion. The Figure 5 revealed the distribution of TUNEL positive cells overlapping with the RGC layers, suggesting that the RGCs might be the most affected cells during this time window. While Figure 6 is a further complement for that RGCs experienced a decrease in density in a more visualized way.

-Figure 8 and 10: The specific experimental approach for determining the nuclear translocation is needed. Please, include a specific marker for microglial and Müller cells in the western blot section.

Reply: The nuclear NFkB-p65 was measured by WB. Cytoplasmic and nuclear protein of microglia/Müller cells were separated by protein extraction kit (Pierce Biotechnology, Rockford, IL) for the detection of cytoplasmic and nuclear NFκB-p65. The nuclear NFkB-p65 levels were found significantly elevated in microglia (Panel E of Fig 8) and Muller cells (Panel E of Fig 10). We have added the CD68 as the specific marker for microglia in Fig 8 and added the GFAP as the specific marker for Muller cells in Fig 10 respectively.

-Figure 10: The blots in panel C for HDAC1 and NFkB is not fine.

Reply: We have uploaded a revised version of Figure 10 as the reviewer suggested.

-IMD-0354 Injection: Please, describe the time-point of starting with the treatment. How many days have the rats been with high glucose before the administration of IMD-0354? Have the rats received any doses of insulin previous or during the treatment?

Reply: The rats in diabetic +IMD-0354 group were administered with IMD-0354 from week 6 of STZ injection for 6 consecutive weeks (Line 199). No insulin had been given before or during the STZ treatment.

According to ***Role of NF-κB in cytochrome P450 epoxygenases down-regulation during an inflammatory process in astrocytes*** published on ***Neurochemistry International*** in 2019 *[PMID:31271766]***,** NF-κB specific inhibitor IMD-0354 was added to a final concentration of 1 ng/ml. This concentration of IMD-0354 was able to significantly block NF-κB pathway and reverse the epoxygenase activity down-regulation induced by LPS.

Although some references indicated a lower concentration of IMD-0354 on endothelial cells, considering that microglia and Müller cell belong to glia cell (astrocyte belongs to glia cell as well), we chose to follow the concentration of 1 ng/ml.

Change in text: Please see line 226-227.

Second Round:

Reviewer A

The authors have addressed all points stated in the initial review.

One suggestion: Lines 133-8: add reference for the selected dose

Answer: We have added reference as suggested.