At present manuscript submitted by Lee et al., is highlighted on sulfated CXCR3 peptide trap as a promising therapeutic approach for age-related macular degeneration. It is very interesting for the age-related macular degeneration which has not enough therapeutic strategy in clinic. However, the following question should be conceived.

1. The abstract should be supplemented in materials and methods, in fact, the constructed CXCR3 should be introduced in the presented abstract.

- In response to the inquiry from reviewerd2, we incorporated the following statements into the abstract (lines 22-24). We generated expression vectors for mCXCR3-S2 and hCXCR3-S2 with GST domains and their respective cDNA sequences. Following overexpression in E. coli BL21 (DE3), we purified the fusion proteins from cell lysates using affinity chromatography.

2. Introduction: there is not enough data or information for the scientific hypothesis. Please supplemented the data or information in logic.

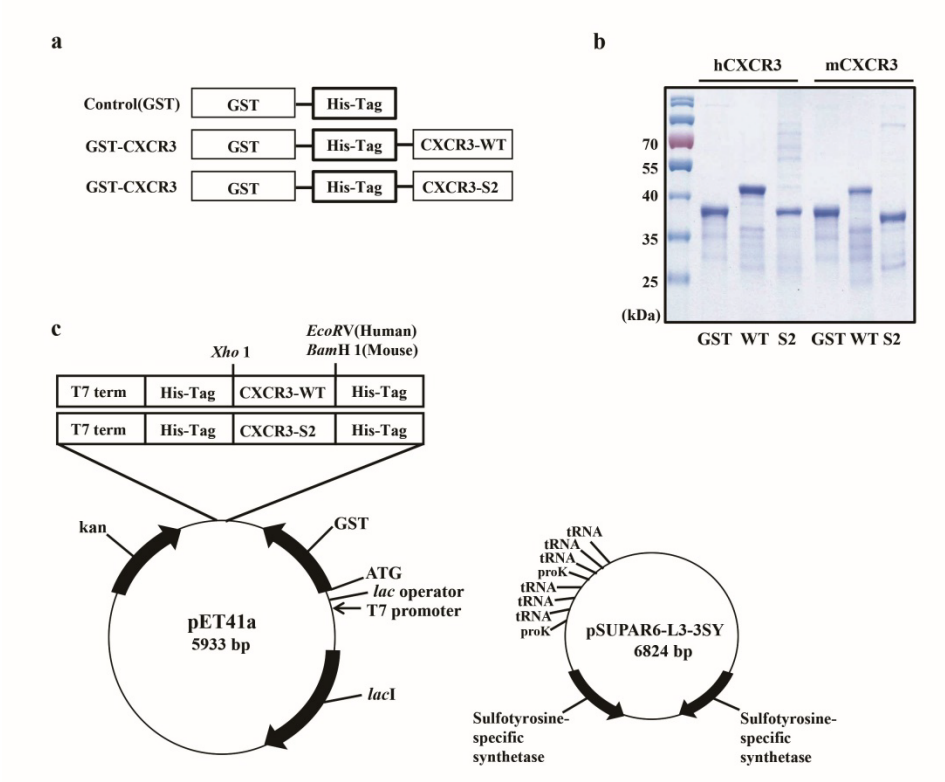
- In addressing reviewer 2's query, we revised the introduction section (lines 55-60, lines 74-83, lines 87-90). Please refer to the highlighted portions in our revised manuscript for specific changes.

Age-related macular degeneration (AMD), a condition marked by the progressive decline of the retinal pigment epithelium (RPE), retina, and choriocapillaris, stands as the leading cause of vision impairment among the elderly worldwide [1,2]. The stages of AMD are categorized into early, intermediate, or advanced. Extracellular deposits, such as drusen and subretinal drusenoid deposits, and focal hypopigmentation/hyperpigmentation are typical characteristics of early and intermediate AMD. Advanced/late AMD with choroidal neovascularization (CNV) or geographic atrophy is characterized as neovascular AMD (nAMD). As the vascular endothelial growth factor (VEGF) is a key pathogenic factor in the development of nAMD [3], intravitreal injection of anti-VEGF agents is currently the only established treatment for nAMD. However, anti-VEGF treatment has several limitations [4]. The exact pathogenic mechanism of AMD remains unclear; however, a genome-wide association study revealed that immunological disturbances, including complement abnormalities, are partially responsible for the development and progression of AMD [5]. Chemokines are chemotactic agents classified into four main subgroups: CC, CXC, CX3C, and C [6]. Chemokines have various biological functions; some perform proinflammatory activities, whereas others regulate cellular homeostasis and cell migration; accordingly, they have potential roles in the development or progression of neuroinflammatory diseases [7]. In AMD, CC chemokines, particularly CCL2 has been extensively studied. It recruits monocytes and macrophages, and elevated levels of CCL2 are associated with AMD progression [8]. Although CXC chemokines play multiple roles, including chemotaxis of immune cells as well as regulation of angiogenesis and epithelial–mesenchymal transition [9], the pathogenic roles of CXC chemokines in AMD are still not fully understood. Among the CXC chemokines, CXCR3 is a transmembrane receptor associated with G proteins. Predominantly located on immune cells, especially activated T lymphocytes and natural

killer cells, CXCR3 plays a crucial role in guiding effector cells to infection sites and assisting in the clearance of pathogens, contributing significantly to immunological functions [10]. The human CXCR3 gene encodes a 45.65 kDa (415 aa) protein, and its partial cDNA was cloned from a CD4 + T lymphocyte cDNA library in 1996 [11]. In a study utilizing rapid amplification of cDNA ends (RACE) [12], Lasagni et al identified a splice variant of human CXCR3, subsequently named CXCR3B. The original transcript was renamed CXCR3A, although it is still commonly referred to as CXCR3. Post-translational modifications of CXCR3 are crucial for its interactions with ligands. In a study employing site-directed mutagenesis [13], the impact of sulfation on CXCR3 binding and activation was identified. Specifically, sulfation of Tyr 27 and Tyr 29 residues is necessary for CXCR3 activation, along with the crucial role of the first 16 residues of the N-terminal region for optimal binding of CXCL10 and CXCL11. CXCR3 binds to ligands such as CXCL9, CXCL10, and CXCL11 [9]. Increased levels of aqueous CXCL10 have been reported in patients with AMD [14,15]. Furthermore, elevated levels of CXCR3 and one of its ligands, CXCL10, have been observed in eyes affected by CNV compared to control eyes [16,17]. Through its interaction with the CXCR3 receptor, CXCL10 triggers chemotaxis, apoptosis, cell growth, and angiostasis. While CXCL10's increased expression is noted in various inflammatory conditions, including infectious diseases, immune dysfunctions, and tumor development [18], its specific role in nAMD remains unclear. Considering the intimate connection between inflammation and neovascularization, we proposed that targeting CXCL10 could be a therapeutic strategy for treating CNV. Tyrosine residues in the N-terminal of chemokine receptors are post-translationally sulfated. These sulfated tyrosine residues modulate the binding to chemokine ligands that induce intracellular signaling pathways. Studies have also shown that the sulfated peptides of chemokine receptors increase the binding affinity of peptides to their cognate ligands [19,20]. In this study, we aimed to develop a sulfated-CXCR3 trap designed to target CXCR3 ligands like CXCL10. The goal was to demonstrate its efficacy in the murine laser-induced CNV model, specifically addressing its impact on CNV.

3. The experimental designed should be completed.
(1) The authors should be supplemented the data of CXCR3 identification. At present, the authors just provided the coomassie brilliant blue staining on 10% SDS-PAGE. Please provided the MS data, etc.
- In response to Reviewer 2's inquiry, conducting MS analysis of the purified protein is currently not feasible. Moreover, conducting MS analysis would require an additional few months, which exceeds our current revision deadline. However, we would like to note that this aspect has been well established by Liu et al., providing a robust reference for our study's context [Liu et al .Chang C Liu, Susan E Cellitti, Bernhard H Geierstanger and Peter G Schultz, Efficient expression of tyrosine-sulfated proteins in *E. coli* using an expanded genetic code, Nature protocols. 4 (2009) 1784-1789.]

We incorporated the cloning maps for CXCR3 peptide trap genes into Figure 1.



(2) The wound healing assay and invasion assay should be presented the picture.
- In response to reviewer 2's inquiry, we incorporated the images of the wound healing assay and invasion assay into Figure 2.

(3) Please supplemented the pharmacodynamics of the animal model. At present, the data is too little to confirm the pharmacological effect.
- We believe that conducting pharmacodynamic analysis on animals falls outside the scope of our current manuscript. Moreover, undertaking such a study would require an extension of several months. We hope the reviewer will understand the practical constraints we are facing.

4. The discussion should be widely revised with the results.
- In addressing Reviewer 2's query, we have extensively revised the Discussion section (lines 323-361). Additionally, we updated the Conclusions section (lines 380-384) to succinctly summarize our findings. Please refer to the highlighted portions in our revised manuscript for specific changes.

4.Discussion

In this study, we explored the impact of the tyrosine-sulfated CXCR3-S2 peptide trap on malignant tumor cell behavior in comparison to the wild-type CXCR3 peptide trap, using MDA-MB-231 and SKOV3 cells. Through a wound healing assay and transwell cell invasion assay, we demonstrated that the sulfated CXCR3-S2 peptide trap significantly reduced CXCL10-induced cancer cell migration and invasion(Figs 1 and 2). CXCR3 has been

identified in numerous malignant cell lines and is linked to the prognosis of individuals with melanoma, colon cancer, and breast cancers [30-32]. Notably, elevated CXCR3 expression in melanoma, colon, and breast cancers is indicative of more malignant and aggressive tumor characteristics. Various studies have highlighted that tyrosine sulfation of different chemokine receptors enhances the binding affinity to their respective chemokines [33–37]. For instance, the sulfation of tyrosine residues on the N-terminus of CXCR3 has been shown to modulate the interaction between CXCL9, CXCL10, CXCL11, and CXCR3, activating CXCL9, CXCL10, CXCL11-CXCR3 signaling [35]. Additionally, Gao et al. demonstrated that sulfation of tyrosine 27 and 29 in the N-terminal region of human CXCR3 increases the binding affinity for CXCL10, indicating that CXCR3 sulfation plays a crucial role in the interaction of CXCL10-CXCR3 [39]. These findings are consistent with our results, indicating that sulfation of the CXCR3 peptide trap reduces CXCL10-induced cancer cell migration and invasion by binding to its ligand, CXCL10.

Intravitreal administration of the fusion proteins significantly reduced CNV volume in a laser-induced CNV mouse model compared to wild-type CXCR3 and GST control, further confirming the significant role of the CXCR3 signaling axis in CNV formation (Fig. 3; Figs S1 and S2). Previously, Fujimura et al. reported increased CXCR3 and IP-10 mRNA in laser-induced models, noting larger CNV in CXCR3-deficient mice and worsening CNV with anti-CXCR3/anti-IP-10 antibodies [40], suggesting a protective role for CXCR3 in CNV. This contrasts with our findings, where recombinant CXCR3-S2 peptide traps significantly reduced CNV and macrophage recruitment compared to wild-type CXCR3 proteins. Discrepancies may arise from several factors: increased macrophage recruitment and elevated CCL2 levels in CXCR3-deficient mice potentially leading to larger CNV, minimal angiostatic effects of recombinant IP-10 on CNV in wild-type mice, and the possibility that anti-CXCR3/anti-IP-10 antibodies used were not fully validated as neutralizing agents, thereby affecting interpretation. While our study demonstrated the efficacy of recombinant CXCR3-S2 peptide traps in reducing CNV and macrophage recruitment, Denoyer et al. showed that a small molecule CXCR3 antagonist in a rat model of ocular hypertension decreases intraocular pressure, prevents retinal neurodegeneration, and preserves visual function by restoring trabecular function [41]. While it remains debatable, given the membrane structure of CXCR3—a G protein-coupled receptor with transmembrane domains—a CXCR3 trap peptide might be a more effective strategy compared to small molecule inhibitors.

Importantly, the mCXCR3-S2 peptide trap specifically targets CXCR3 receptor ligands on cells. This trap peptide directly binds to CXCR3 ligands like CXCL10 found near inflammation sites, mimicking the CXCR3 receptor binding site. Consequently, the CXCR3-S2 peptide trap operates by eliminating ligands for the CXCR3 receptor on cells, thereby preventing the recruitment of inflammatory cells, including macrophages. These results were consistent with those of previous studies, which reported that sulfation of chemokine receptors plays a critical role in their signaling [42-44]. Several research findings indicate that the recruitment of macrophages plays a significant role in the development of CNV in AMD

[1,45,46]. In our study, diminished recruitment of F4/80$^+$ cells was observed in sulfated CXCR3 antibody-injected mice. Whether macrophages play a protective or aggravating role in CNV remains unclear; however, macrophages trigger VEGF secretion in the angiogenic environment of the choroid [47,48]. We speculated that a sulfated CXCR3 peptide could capture CXCL10 before the latter could induce the chemotaxis of M1 macrophages [49] and therefore, could exert a protective effect against CNV. It should be noted that the incorporation of sulfotyrosine, an unnatural amino acid, has some drawbacks, such as low yield, low permeability, and leaky suppression [15]. Further studies are warranted to overcome these limitations.

5. Conclusions

Intravitreal application of recombinant CXCR3-S2 peptide traps reduced CNV and macrophage recruitment in a mouse CNV model, indicating CXCL10-CXCR3 axis targeting as a potential nAMD therapy. Further studies on CXCR3-S2 peptide traps for AMD and CXCR3-related cancers are encouraged to explore broader therapeutic applications.