Restoring microglia homeostasis as a treatment for glaucoma

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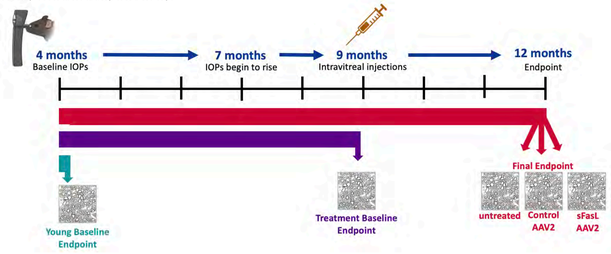
INTRODUCTION

In glaucoma, elevated pressure within the eye (IOP) is a major risk factor for the development of glaucoma and lowering IOP remains the only treatment for this disease. However, continued disease progression in patients with reduced IOP indicates that IOP-independent mechanisms contribute to glaucoma. In the healthy eye, inflammation is tightly regulated to protect the delicate tissues necessary for vision, but in glaucoma inflammation in the eye becomes dysregulated and in animal models of glaucoma, microglia activation and inflammation persist even after the IOP has been successfully lowered. Microglia are considered the resident immune cells of the retina and are responsible for normal maintenance of the retina, as well as the local response to injury. Chronic activation of microglia is thought to contribute to the death of retinal ganglion cells (RGCs) in glaucoma by promoting harmful inflammation and in animal models of glaucoma, the extent of microglia activation correlates with the extent of RGC death and optic nerve degeneration. We previously identified a protein, soluble Fas ligand (sFasL), that is essential in preventing inflammation in the healthy eye. Microglia activation and the development of glaucoma coincides with the loss of this protein. Therefore, we hypothesized that sFasL is essential in preventing retinal microglia activation and with the loss of sFasL in glaucoma, the microglia become chronically activated, promoting neurodestructive inflammation that continues even after the IOP is lowered. Therefore, the question we asked in this project was whether treatment with sFasL after glaucoma injury could "restore" the homeostatic phenotype of activated retinal microglia and prevent the continued progression of disease.

DESIGN & METHODS

***In vivo* studies:** DBA/2J (D2) mice received an intravitreal injection of AAV2.control or AAV2.sFasL at 9 months of age. Untreated DBA/2J mice and mice treated with AAV2.control served as controls. Rebound tonometry was used to monitor IOP. Mice were euthanized at 4, 9, and 12 months of age and an optic nerve grading scale was used to assess axon degeneration (no glaucoma, moderate, severe).



***In vitro* studies:** The BV2 microglia cell line, which is a very well characterized immortalized cell line isolated from murine brain tissue, was used for all *in vitro* studies.

Apoptosis and IL-1β assay: BV2 microglia were labeled with CellTrace Red Dye (APC-A) and incubated with increasing doses of mFasL- or neo-vesicles (no FasL) for 18hrs. Just prior to flow cytometry, all cells were stained with a Live/Dead fixable green stain (FITC). Labeled BV2 cells with media alone served as a negative control and BV2 cells with triton 10x served as maximum apoptosis. Supernatant was collected to assess IL-1β secretion by ELISA and BV2 cells incubated with media alone or neo-vesicles (no FasL) served as negative controls.

qPCR: Quantitative PCR was performed on BV2 microglia following 18hr treatment with sFasL (2ug/ml) or mFasL vesicles (1:100). Relative gene expression was normalized to the mean of housekeeping genes (Ppia, Hsp90ab1, B2m, Actb). Data are expressed as fold changes in gene expression calculated with respect to the media-only group. Each sample is a pool of three individual RNA samples per treatment group.

M1/M2 Flow cytometry assay: BV2 microglia were incubated with media, LPS (1mg/ml), or sFasL (100ug/ml). For samples undergoing both treatments, LPS was added 2 hours before sFasL. After 18hrs, microglia were stained for CD40 (M1 marker) and CD206 (M2 marker). Isotype and media-alone served as negative controls.

RESULTS

Figure 1: Post optic nerve injury treatment with sFasL-AAV2 prevents disease progression. DBA/2J mice were treated with sFasL-AAV2 or control-AAV2 at 9 months of age, when 34% of the mice display moderate to severe optic nerve degeneration. **(A)** sFasL-AAV2 treatment had no effect on IOP. **(B)** An optic nerve grading scale was used to assess disease stage and (**C**) at 12 months of age, a significant attenuation of axon degeneration was observed in mice treated with sFasL-AAV2 as compared to untreated or AAV2-control vector.

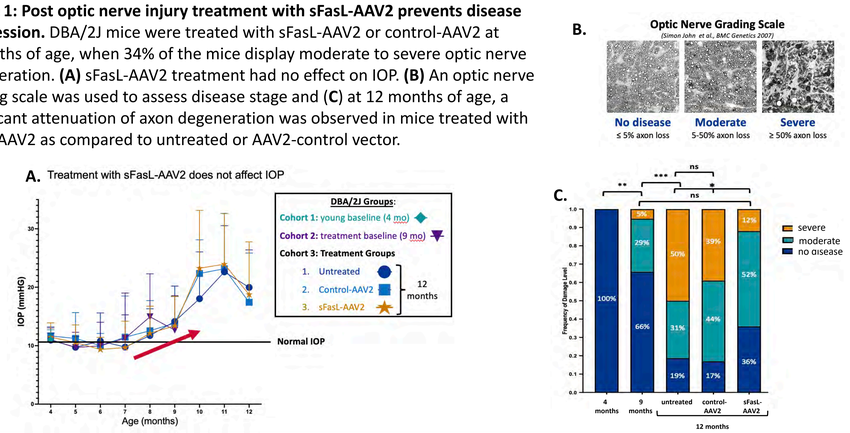


Figure 2: mFasL-induces IL-1β secretion in BV2 microglia. BV2 microglia were incubated with mFasL-vesicles or neo-vesicles (no FasL) at 1:200, 1:100, 1:20, and 1:10 dilutions. Data are presented as mean IL-1β +/- SEM N=3, **P <0.01, ****P<0.0001.

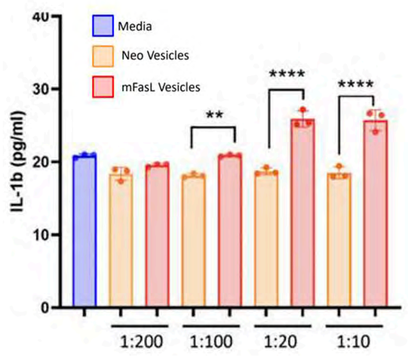


Figure 3: BV2 Microglia are resistant to mFasL-induced apoptosis. Labeled BV2 microglia (APC) were incubated with increasing doses of mFasL- or neo-vesicles and a Live/Dead fixable green stain (FITC) was used to detect apoptosis.

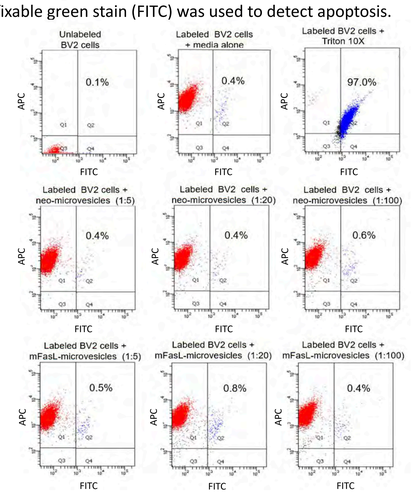
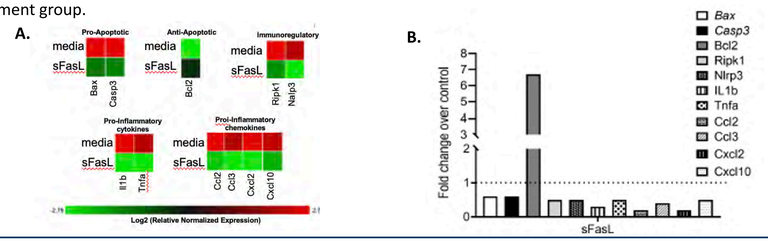
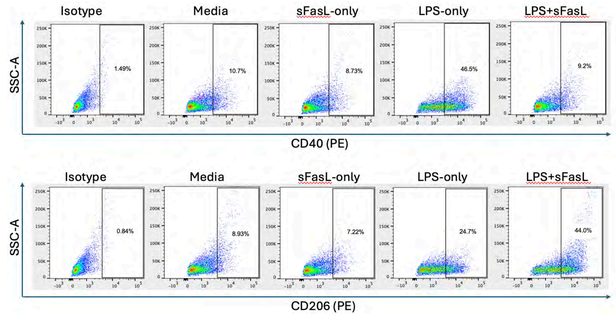


Figure 4: sFasL inhibits pro-inflammatory and proapopotic genes in activated BV2 microglia. (A) heatmap showing relative gene expression normalized to the mean of housekeeping genes (Ppia, Hsp90ab1, B2m, Actb). (B) Fold change in gene expression calculated with respect to the media-only group. Each sample is a pool of three individual RNA samples per treatment group.



RESULTS

Figure 5. Treatment with sFasL promotes M2 microglia. BV2 microglia were treated with media, sFasL, LPS, or LPS (2hrs) followed by sFasL. The flow cytometry plots demonstrate that treatment with LPS triggers M1 microglia with high CD40 (M1 marker, 46.5%) and low CD206 (M2 marker, 24.7%). By contrast, subsequent treatment with sFasL (LPS+sFasL) reverts microglia back to a more M2 phenotype with low CD40 (M1 marker, 9.9%) and high CD206 (M2 marker, 44%). These results are representative of three independent experiments.



CONCLUSIONS

- sFasL-AAV2 gene therapy is neuroprotective and prevents disease progression, even when administered after evidence of axon loss.
- Microglia are resistant to mFasL-induced apoptosis and instead become activated, upregulating inflammatory genes and secreting the proinflammatory cytokine, IL-1β.
- sFasL on its own is non-apoptotic and non-inflammatory.
- Treatment with sFasL reverses the phenotype of LPS-activated microglia from a pro-inflammatory, neurodestructive M1 phenotype to a neuroprotective M2 phenotype.

NEXT STEPS

- Demonstrate effectiveness of the sFasL-AAV2 gene therapy in a large animal preclinical model of glaucoma.
- Complete retinal microglia RNAseq analysis from DBA/2J mice treated with sFasL-AAV2 to identify the genes and signaling pathways that are turned off/on following treatment with sFasL, helping us to better understand the mechanism by which sFasL resets retinal microglia and identify new gene-based targets for the treatment of glaucoma patients.

ACKNOWLEDGEMENTS

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