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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Proliferative vitreoretinopathy (PVR) occurs in patients with penetrating ocular injury, of which there are more than 200,000 worldwide per year among both military and civilian personnel. It is a major challenge in ophthalmology and retinal surgery. Experts agree that PVR results from proliferation and migration of retinal pigment epithelial (RPE) cells, through a retinal wound and over the vitreal surface of the retina. There, RPE cells secrete collagen and other matrix proteins that form an epiretinal scar-like membrane that exerts tractional forces on the retina, often leading to retinal detachment and loss of vision. At present there are no reliable means of treating or preventing PVR. This program would develop a potential new biologic therapy, based on targeting the annexin A2 cell surface fibrinolytic system and stimulators of the system (macrophage inflammatory protein (MIP) 1-alpha and MIF 1-beta, for early point-of-care prevention of PVR.

15. SUBJECT TERMS

NONE LISTED

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1. Introduction

The primary goal of this research project is to test the hypothesis that blockade of the annexin A2 protease system will prevent the development of dispase-induced PVR in mice by inhibiting the recruitment of circulating innate inflammatory cells into the retina and impeding the delamination of RPE cells and their transition to migratory scar-forming cells within the retina. A secondary goal is to explore the postulate that blockade of the macrophage inflammatory protein system will prevent or curtail the development of PVR by blocking assembly of the A2 protease system on the surface of migrating RPE cells.

2. Keywords

annexin A2, macrophages, macrophage inflammatory protein, proliferative vitreoretinopathy, retinal pigmented epithelial cell

3. Accomplishments

Goals and accomplishments slated for Year 1 are listed according to the Statement of Work for this project.

SPECIFIC AIM 1: DEMONSTRATE THE ABILITY OF ANTI-A2 BLOCKING ANTIBODIES TO PREVENT PROLIFERATIVE VITREORETINOPATHY (PVR) IN TWO STANDARD ANIMAL MODELS

Major Task 1: Preparatory approvals and reagent certification.
[1] In the first 3 months of the project, we were successful in obtaining both Weill Cornell Medicine IACUC approval and ACURO approval for the proposed animal experiments.

[2] In collaboration with the Tri-institutional Therapeutic Discovery Institute (TDI), we conducted Octet/BLI binding studies which verified that antibody 02L21A, like our positive control (1A7) demonstrates strong binding to immobilized annexin A2, thus confirming our earlier ELISA results. In a chromatographic heparin binding assay, furthermore, 02L21A showed low heparin binding, suggesting that non-specific binding to surfaces is unlikely to be a liability for this agent.
On the other hand, 36H22A, as second candidate antibody, demonstrated strong binding to A2 by ELISA, but only negligible binding to A2 in an Octet/BLI assay. Further studies using Octet and surface plasmon resonance (SPR) are ongoing to understand this discrepancy in more detail.

[3] We have identified and procured two lots of dispase. Both induce mild-moderate PVR when given at a concentration of 0.1 U/ml (3 ul/eye). Histologic data indicate that the resulting injury is not so severe as to obliterate the overall ocular anatomy, but severe enough to reliably show RPE cell migration and epiretinal membrane formation. Based on previously funded studies, we have developed and validated an expanded scoring system to quantify overall histologic anatomy (**Figure 1** - Appendix) as well as the degree of RPE cell migration (**Figure 2** - Appendix) in PVR.

Major Task 2: Optimize timing of IgG Intervention
We tested the efficacy of antibody 1A7 injected at 1, 4, and 7 days following dispase treatment in mice. Eyes were harvested at 4 weeks, sections prepared, and tissue stained with hematoxylin and eosin. Because injection at the three time points tested were equally effective in blocking PVR development, we did not test the 2-day time point. In addition, we did not test the -2 day time point (i.e. give. antibody 2 days prior to dispase injection), as this scenario does not reflect the likely use of anti-A2 in the field, and was included in the original plan in case post-dispase injection was ineffective due to proteolysis by dispase. Give the finding of equal efficacy at days 1, 4 and 7, we have chosen to use day 1 after dispase as the time point of antibody injection going forward.

Major Task 3: Select lead IgG candidate
We examined the ability of 4 different antibodies (02L21A, 36H22A, 1A7, and control 1D4) given on day 1 after dispase injury to prevent the development of PVR in mice (**Figures 3 and 4**). Eyes were harvested humanely at 4 weeks after dispase injection, sections cut, and tissue stained with hematoxylin and eosin. Our data indicate that the negative control (1D4) had minimal effect on overall histology, whereas the anti-A2 positive control (1A7) partially blocked histologic change. The strongest effect was seen with our new antibodies (02L21A and 36H22A) which inhibited about 60% of the structural changes in the eye (**Figure 3**). With respect to RPE migration, we found very similar results, observing the strongest inhibition of RPE cell invasion with 02L21A and 36H22A.

Major Task 4: Confirm lead candidate optimal dose
Using the dispase model in mice, we injected lead candidate antibodies 02L21A at 15 ug/ml and 36H22A at 5 ug//ml. Eyes were harvested at 4 weeks, sections prepared, and tissue stained with hematoxylin and eosin. Double blind scoring revealed strong effects at these doses. To determine whether higher doses induce an even stronger effect, we will complete the analysis of additional doses in Year 2.

SPECIFIC AIM 2: CONFIRM THAT BLOCKADE OF MIP-1α/β CAN PREVENT PVR *IN VIVO*

Major Task 1: Effect of anti-MIP IgG on development of PVR in mice

We tested control, anti-MIP-1α and MIP-1β, or both in mice subjected to the dispase model of PVR. Eyes were harvested at 4 weeks, sections prepared, and tissue stained with hematoxylin and eosin. Double blind scoring of histologic sections revealed that overall histology and RPE cell migration were inhibited by 60-70% (**Figures 3 and 4**). We are now in the process of repeated this experiment with additional animals in order to apply statistical analyses.

Major Task 2: Identify the optimal anti-MIP dose in mice
All of the preliminary experiments presented above were conducted at an anti-MIP dose of 30 ug/ml (3 ul per eye). We will be testing additional doses in Year 2.

SPECIFIC AIM 3: DEFINE THE PHARMACOKINETICS AND POTENTIAL TOXICITY OF LEAD CANDIDATES IN MICE

Major Task 5: Assess visual function
Due to the fact, as noted below **(#5 Changes/Problems**), that the Weill Cornell Medicine Visual Function Core facility has closed, we undertook training in retinal optical coherence tomography (OCT) and retinal fundus imaging in the mouse with experts in the Dyson Institute in the WCM Department of Ophthalmology (DoO). We are now able to independently perform OCT analyses and fundus imaging, and plan to engage an OCT expert in the DoO to independently review our results.

Training Impact: This project has provided Dr. Valentina Dallacasagrande with invaluable experience in techniques involving induction and evaluation of PVR in mice. She has mastered our injection protocol, the scoring rubric, statistical analysis, and OCT and fundoscopic procedures involved in the project. In addition, she will be presenting a related project on diabetic retinopathy at an international conference (the Gordon Conference on Plasminogen Activation and Extracellular Proteolysis in Ventura, California) later this month. In addition, she presents her data and experimental plans at our lab meetings on a weekly basis, and meets with Dr. Hajjar approximately once per week.

Dissemination: Nothing to report

Plans for the next reporting period:
According to the original SOW, in Year 2 we will [1] test whether a second dose of anti-A2 offers any benefit over single dose treatment in reducing PVR, [2]identify the optimal dose of anti-MIP using a range of 10, 30, and 90 ug/ml for treatment of murine PVR, [3]evaluate whether a second dose of anti-MIP offers an added benefit, [4] identify the optimal administration of antibody administration in the rabbit PVR model, and determine whether anti-MIPS prevent PVR in the rabbit. We will also repeat selected experiments performed in Year 1 in order to increase the number of animal per group from 3 to 6.

4. Impact

Impact on current discipline: In the previous funding period, we established that development of PVR in the mouse dispase model is critically dependent upon expression of annexin A2 in the host. This work suggested that A2 supports the cell transformation and migration of retinal pigment epithelial cells that underlie the formation of fibrotic membranes on the surface of the retina. These epiretinal membranes can develop into scars that may contract leading to retinal detachment and major vision loss. Given that the current treatment for PVR remains unsatisfactory, we have initiated studies in mice determining whether annexin A2 may be a new potential therapeutic target. Over the past year, we have developed an expanded scoring system to gauge the severity of dispase-induced PVR in mice, using a comprehensive array of assessment tools including overall histology, evaluation of retinal pigment epithelial cell migration, fundoscopy, and optical coherence tomography (OCT). Our preliminary data suggesting that immuno-inhibition of A2 may prevent or significantly reduce the development of epiretinal membranes in the dispase model of murine PVR. In addition, we find that inhibition of macrophage inflammatory protein (MIP) 1-alpha and MIP1-beta also reduces the degree of PVR in mice. These new data provide further evidence that blockade of these agents may have therapeutic value. They also provide additional mechanistic confirmation of the role of the MIP-annexin A2 axis in the development of PVR. This work has not yet been publicly reported.

Impact on other disciplines: Once complete, findings from this study could provide new treatment avenues and a better mechanistic understanding of other "scarring" disorders, such as renal, pulmonary, or hepatic fibrosis.

Impact on technology transfer: Nothing yet to report.

Impact on society: Nothing yet to report.

5. Changes/Problems

Changes in approach: Nothing to report.

Problems or delays: From the outset of the project on October 1, 2021, we were negatively impacted by Covid-19. First, we received needles for antibody injection from World Precision Instruments (WPI), which turned out to be defective; the needles were excessively blunt and not properly sharpened. When we tested the dull needles, we noted an inordinate degree of ocular injury, which obscured the effect of dispase. Upon ordering a second batch from WPI, we noted that they were also inappropriately dull. We ordered a third batch in March from the only other vendor, Hamilton Syringe, but these were on back order for 3.5 month due to "supply line" issues. This caused a delay in initiating the planned experiments, which are now moving forward.

While waiting for needles to arrive, we initiated experiments originally scheduled for year 3 (Specific Aim 3, Major Task 5). During the period of Covid lockdown, our Visual Function Core was shut down, and then bever reopened due to personnel issue. Therefore, we identified collaborators in the Department of Ophthalmology's Dyson Institute at Weill Cornell Medicine. We received training in OCT, fluorescein angiography, and fundus photography, and started initial analyses using these techniques on dispase-injected, antibody-treated mice. This work is now moving forward.

Changes significantly impacting expenditures: Nothing to report.

6. Products

Nothing to report.

7. Participants & Other Collaborating Organizations

Not applicable

8. Special Reporting Requirements

Not applicable.

9. Appendices

Figure 1: Scoring System for Evaluation of General Histology. Standard 5-um, fixed, paraffin-embedded retinal sections were evaluated based upon the extent of RPE cell migration. Ocular sections were evaluated according to the degree of retinal detachment and the degree of disorganization of retinal cell layers. Scoring was validated by two trained observers in a double-blind manner, and interobserver variation was less than 10% per eye.

Score	Retinal Detachment	Disorganization of Retinal Layers
0	Absent	Absent
1	Focal (<25%)	Absent
2	Extensive (≧ 50%)	Absent
3	Absent	Focal (<25%)
4	Focal (<25%)	Focal (<25%)
5	Extensive (≧ 50%)	Focal or Partial (<50%)
6	Absent	Extensive (≧ 50%)
7	Focal (<25%)	Extensive (≧ 50%)
8	Extensive (≧ 50%)	Extensive (≧ 50%)

Figure 2: Scoring System for Evaluation of RPE Cell Migration. Standard 5-um, fixed, paraffin-embedded retinal sections were evaluated based upon the extent of RPE cell migration. Scoring was validated by two trained observers in a double-blind manner, and interobserver variation was less than 10% per eye.

Score	Description
0	NO RPE migration
1	RPE migration above PRL
2	RPE migration limited to the retina by injury site
3	RPE migration and cell layer disorganization limited to the retina by injury site
4	RPE migration within retina and on retinal surface. Retinal layers remain intact.
5	RPE migration within retina. Retinal layers at injection site are disorganized.
6	RPE migration within retina and on retinal surface. Retinal layers at injection site are disorganized.
7	RPE migration beyond retinal surface and into extraretinal tissue
8	Complete destruction

Figure 3: Effect of Anti-A2 and Anti-MIP Antibodies on Overall Ocular Histology During Development of Dispase-Induced PVR in Mice. 5-um, fixed, paraffin-embedded Each dot represents the average of two scores made by two masked observers and describing histologic changes for a single standard section through the injection point within in a single eye.

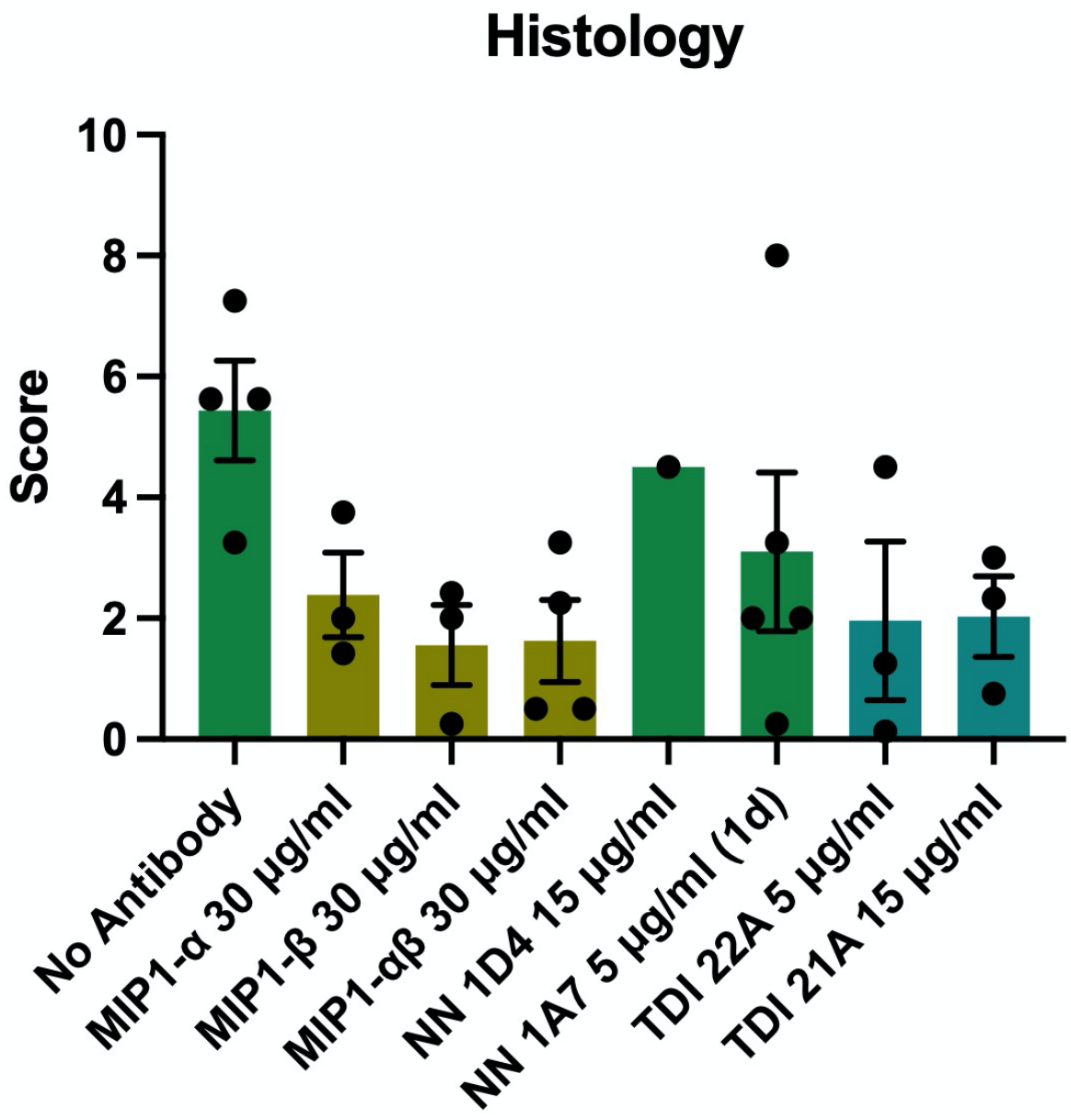

Figure 4: Effect of Anti-A2 and Anti-MIP Antibodies on RPE Cell Migration During Development of Dispase-Induced PVR in Mice. Each dot represents the average of two scores made by two masked observers and describing changes in RPE cell migration for a single standard section through the injection point within in a single eye.

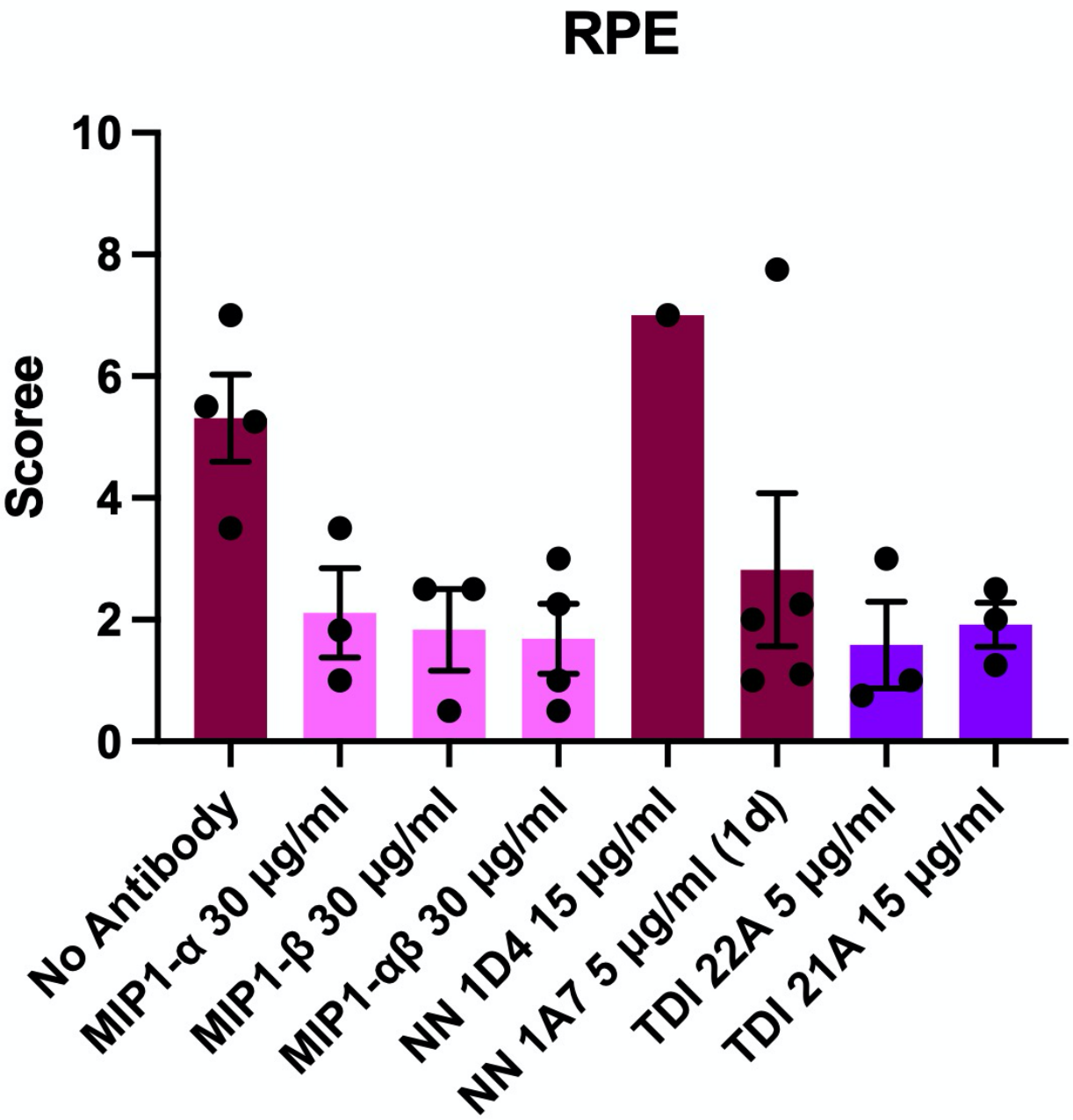

Figure 5. Optical Coherence Tomography (OCT) and Fundus Imaging in Normal and PVR Mice. Top Left: OCT image displaying normal retinal architecture and cells layers from choroid (bottom) to vitreal surface (top). **Top Right:** Fundoscopic image of retina in same eye showing the optic disc and regular array of blood vessels radiating across the retina. **Bottom Left:** OCT image acquired at 4 weeks in mouse with dispase-induced PVR demonstrating intraretinal detachment. **Bottom right:** Fundoscopic view of same PVR eye at 4 weeks after diapase injection demonstrating intraretinal detachment.