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14. ABSTRACT

Proliferative vitreoretinopathy (PVR) is one of the major remaining challenges in retinal surgery. PVR occurs in patients with previous complex retinal surgery and also in patients with penetrating globe injury, of which there are more than 200,000 worldwide per year. PVR is thought to result from proliferation and migration of retinal pigment epithelial (RPE) cells, leading to formation of an epiretinal membrane, retinal detachment, and loss of vision. At present, there are no reliable means of preventing this complication of ocular trauma and retinal surgery. In this project, we have addressed specific aims to [1] analyze the PVR response in wild type versus annexin A2-deficient mice, [2] define the role of A2 in the function of activated macrophages and RPE cells in PVR, and [3] examine the expression pattern of A2 in human PVR. We conclude that A2 plays a fundamental role in the pathogenesis of PVR in the mouse, that its expression is needed in both macrophages and RPE cells, and that A2 is extensively expressed within cells of epiretinal membranes in human PVR. Our data suggest that A2 may represent a "druggable" therapeutic target for the prevention of the PVR response.

15. SUBJECT TERMS
proliferative vitreoretinopathy, annexin A2, retinal pigment epithelial cell, macrophage, ocular injury

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1. Introduction

Proliferative vitreoretinopathy (PVR) is a potentially blinding disorder that occurs in 8-10% of patients with primary retinal detachment, but up to 40-60% of patients with penetrating globe injury, of which there are more than 200,000 worldwide per year[1-3]. In recent studies, up to 43% of civilian and military patients with penetrating eye injuries or intraocular foreign bodies developed PVR[4-6]. In response to a retinal lesion that breaches the blood-retinal barrier, leakage of plasma proteins and circulating blood cells, possibly together with local hypoxia, conspire to stimulate retinal pigment epithelium (RPE) cells to abandon the their attachment to Bruch's membrane, proliferate, migrate, and synthesize collagens and other matrix proteins that lead to the formation of epi- and subretinal membranes with subsequent contraction[7, 8]. The early events that initiate PVR are poorly understood, but the process appears to begin with influx of CD68+ macrophage-like cells that induce delamination and proliferation of RPE cells analogous to epithelial-mesenchymal transition (EMT)[1, 9-12]. There have been few if any major advances in clinical management of PVR since its original description in 1983[13, 14].
Annexin A2 (A2) is a calcium-regulated, phospholipid-binding protein that is expressed on the surface of both macrophages[15] and RPE cells[16]. We demonstrated previously that A2 is required for the full retinal vascular proliferative response in ischemic retinopathy, based upon its ability to bind plasminogen and tissue plasminogen activator, and generate cell surface plasmin activity[17, 18], which degrades fibrin and activates matrix metalloproteinase enzymes[19, 20]. Cell surface plasmin potentiates the migration of cells through fibrin- or laminin-containing tissue matrices.

A2-deficient (*Anxa2*$^{-/-}$), developed in our laboratory, mice have normal development, longevity, and vision, but exhibit increased thrombosis following arterial injury and reduced angiogenesis in a range of postnatal models. A2 forms a high affinity cell surface complex with its binding partner S100A10 (aka p11). Under the auspices of this DoD award, we have established that A2 is required for the full expression of PVR in the standard dispase model in mice (**manuscript in preparation**). These studies revealed four key findings, namely **[1]** that the overall PVR response is greatly attenuated or absent in the *Anxa2*$^{-/-}$ mouse, **[2]** that cells that co-express A2 and the macrophage marker CD68, as well as cells that co-express A2 and the RPE cell marker RPE65, are abundant in human PVR membranes, **[3**] that human RPE cells migrate *in vitro* in response to macrophages only when both cells express A2, and **[4]** that, in response to retinal injury, macrophage inflammatory protein-1α and β (MIP-1α and MIP-1β) are among the macrophage-derived chemokines and cytokines that drive increased expression of annexin A2 on the surface of RPE cells. Our data suggest further that increased cell surface A2 enables the delamination and migration of RPE cells as they undergo fibroblastoid transformation in an EMT-like reaction. Additional data derived from this project suggest that pretreatment of wild type mice with anti-A2 prior to dispase injection largely attenuates the subsequent PVR reaction. Together, these investigations suggest that A2 is central to the pathogenesis of PVR, and that A2 represents a logical and "druggable" therapeutic target for the prevention of the PVR response.

2. Keywords

proliferative vitreoretinopathy
annexin A2
macrophage
retinal pigmented epithelial cell
penetrating ocular injury
epithelial-mesenchymal transition
chronic inflammation

3. Accomplishments

Goals of the Project. At the outset of this project, we proposed three specific aims with the overarching objective of understanding the potential role of the annexin A2 system in the pathogenesis of proliferative vitreoretinopathy (PVR). Under **Aim 1**, our major goal was to analyze the functional role of annexin A2 and related molecules in the classic dispase mouse model of PVR. Under **Aim 2**, we sought to specify PVR-related, annexin A2-dependent interactions between RPE cells and macrophages that might promote or enable RPE cell

migration to the vitreal surface of the retina during the PVR response. **Aim 3** was directed at defining the role of the annexin A2 system in the pathogenesis and progression of human PVR. We have completed all three aims, and, in the process, have identified a molecular pathway that appears to integrate the function of macrophages and RPE cells in the pathogenesis of PVR.

Accomplishments under the Stated Goals

Specific Aim 1. To analyze the functional role of annexin A2 and related molecules in a mouse model of PVR.

Approach: In a standard model of murine PVR, *Anxa2*$^{+/+}$ and *Anxa2*$^{-/-}$ mice were treated with intravitreal dispase (0.3 units/ul; PBS)[21]. The mice (3-4 months old) were first anesthetized with isoflurane by nose cone and administered topical proparacaine HCl (0.5%) to the surface of the eye. The pupil was dilated with phenylephrine (2.5%) and tropicamide (1%), and under microscopic control, a 30-gauge needle fitted to a Hamilton syringe was advanced into the left eye to a depth of 1 mm at the dorsal midline just anterior to the outer canthus. Dispase (3 ul) was injected using a Hamilton syringe. The right eye remained undisturbed.

On days 1, 14, 28, and 42, mice from each group were euthanized by CO_2 inhalation, and the eyes harvested for histologic analysis. The eyes were enucleated, and a nick created in the cornea with a #11 blade prior to incubation in 4% paraformaldehyde. The cornea and lens were removed, and the eye oriented in 30% sucrose/PBS (18h) prior to infiltration with optimal cutting temperature/sucrose (2/1:v/v) and freezing in liquid N2-cooled isopentane. Sections (5-7 um) were arrayed six per slide, and every tenth slide stained with hematoxylin and eosin (H&E) for examination for the presence of vitreous strands, neural retinal folds, epi- and subretinal membranes, and inflammatory cells. The extent of PVR development was scored by masked observers, using a validated scale. In addition, sections were examined by indirect immunofluorescence microscopy, using antibodies directed against markers for macrophages (F4/80), endothelial cells (isolectin B4), pericytes (NG2), glial cells (GFAP), Muller cells (glutamine synthase), neuronal cells (NF160), retinal pigment epithelial cells (RPE 65), and photoreceptor cells (CRALBP). These analyses have allowed us to assess the cellular locations and relative expression levels of A2 and related molecules (S100A10) within the injured eye.

Major Findings:

[1] Dispase-induced PVR is attenuated in the *Anxa2*$^{-/-}$ mouse. Histologic examination at 4 weeks of H&E-stained sections of eyes receiving low-dose dispase revealed migration of RPE cells over the vitreal surface of the retina in *Anxa2*$^{+/+}$ eyes (**Fig. 1**). In *Anxa2*$^{-/-}$ eyes, RPE cells remain posterior to the retina at the injection site. In eyes receiving standard dose dispase, histologic examination at 2, 4, and 6 weeks post injection revealed that RPE cells fail to migrate from their original location in *Anxa2*$^{-/-}$ mice (**Fig. 2**). Whereas RPE cells begin to migrate over the surface of the *Anxa2*$^{+/+}$ retina within 2 weeks of injury, those in *Anxa2*$^{-/-}$ eyes, remained within the RPE cell layer. By six weeks, there was considerable distortion, detachment, and contraction of the retina, which often came to rest posterior to the ocular lens, in *Anxa2*$^{+/+}$, but not *Anxa2*$^{-/-}$, eyes. Large numbers of pigmented RPE cells were seen streaming through the retinal wound, over the vitreal surface of the retina, and within the developing retinal scar in the *Anxa2*$^{+/+}$ eye only. These data indicated that *Anxa2*$^{-/-}$ mice are resistant to dispase-induced PVR, when compared with *Anxa2*$^{+/+}$ mice.

[2] Quantitative histology confirms the importance of A2 in PVR. By examining 7-11 *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ animals, each at 2, 4, and 6 weeks, we quantified the degree of retinal disruption and RPE cell migration using a standardized scoring system (**Fig. 3**). Ten sections at or near the injection site were evaluated by trained, masked observers, and scored according to a standard rubric encompassing the degree of retinal detachment, disorganization of retinal cell layers, and epiretinal membrane formation (**A**). In addition, RPE cell migration was evaluated in a separate scoring system based on the presence of RPE cells within the retina, over the vitreal surface of the retina, or into the extraretinal tissues (**B**). Previously available scoring systems in animal models have been restricted primarily to observations made by direct ophthalmoscopic examination and/or fundus photography[21-24]. To our knowledge, histologic scoring paradigms have not been published for the mouse

dispase system. At all time points examined (2, 4, and 6, weeks), *Anxa2*$^{+/+}$ mice display significantly more extensive retinal detachment, cellular disorganization and epiretinal membrane formation compared with *Anxa2*$^{-/-}$ mice (p values $\leq$ 0.006). These data indicate that the A2-deficient mouse is resistant to dispase-induced PVR.

[3] A2-positive RPE cells migrate over the surface of the retina in murine PVR. In addition, we have used immunofluorescence staining to document the migration of A2-positive cells from the RPE to the surface of the retina in murine PVR (**Fig. 4**). We have detected RPE65-positive cells on the surface of the retina during PVR. This result differs somewhat from data reported by Canto Soler et al., in which RPE65-positive cells were reported to be restricted to subretinal membranes in a dispase (0.2-0.4 u/ul)-induced PVR model in the mouse[21]. In addition, we found extensive collagen deposition within the epiretinal membranes of *Anxa2*$^{+/+}$, dispase-injected eyes, but not *Anxa2*$^{-/-}$ eye (**Fig. 5**). These data indicate that cells migrating over the retinal surface after 2 weeks of PVR are RPE-derived, A2-positive cells.

[4] A2 expression is required for recruitment of bone marrow macrophage precursors to the dispase-injected eye. We conducted bone marrow transplantation studies to determine the origin of macrophages recruited to the eye following dispase-induced PVR (**Fig. 6**). Mice were lethally irradiated and immediately rescued with a transplant of bone marrow from *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ mice, each bearing the LacZ marker gene knocked into the A2 locus (*Anxa2*$^{LacZ/+}$ or *Anxa2*$^{LacZ/-}$ mice). In this experiment, *Anxa2*$^{LacZ/+}$ refers to mice that express LacZ under the A2 promoter, and also have a functioning A2 allele, whereas *Anxa2*$^{LacZ/-}$ mice express LacZ under the A2 promoter, but have no expression of A2 itself. One month after transplant, the mice underwent injection of intravitreal dispase to induce PVR.

When either *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ were reconstituted with *Anxa2*$^{LacZ/+}$ bone marrow, and then subjected to dispase injection, cells doubly positive for both LacZ (as indicated by positive Xgal staining) and F4/80, a macrophage marker, were present in the injured retina at 48 hours after receiving intravitreal dispase. In fact, approximately 90% of F4/80-positive cells were Xgal-positive. At the same time, Ly6G-positive cells (neutrophils) were also present, but did not co-expressed Xgal; this result was expected since neutrophils express significant amounts of A2[15]. Notably, when irradiated *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ mice were reconstituted with *Anxa2*$^{LacZ/-}$ bone marrow, fewer F4/80-positive cells were present in and around the injured retina, and none expressed Xgal. Ly6G-positive cells did infiltrate the retina, but none expressed Xgal. Together, these data strongly suggest, first, that macrophages recruited to the injured retina came from the bone marrow, and, second, that A2 is required for recruitment of macrophage precursors to the injured eye.

Based upon these data, we concluded that dispase injection results in a dramatic, chronic inflammatory response in the normal mouse eye. This response appears to be characterized by A2-dependent recruitment of bone marrow-derived macrophage precursors into the injected eye. Recruitment of macrophages is followed by migration of A2-positive cells through the retinal wound and over the vitreal surface of the retina. Ultimately, there is extensive deposition of collagen, contraction of the epiretinal membrane, and retinal detachment, leading to severe disorganization and destruction of the normal retina and adjacent structures. We show for the first time, that the absence of annexin A2 is associated with reduced recruitment of bone marrow-derived macrophages and a reduced PVR response. Together, these data suggested that A2 may play a dual role by **[1]** promoting recruitment of macrophages to the injured eye, and **[2]** enabling migration of transformed, fibroblast-like RPE cells to the surface of the retina , giving rise to the formation of epiretinal membranes.

[5] Antibody blockade studies. We carried out preliminary experiments to examine the possibility that blockade of A2 might prevent PVR in wild type mice (**Fig. 7**). Mice were pretreated with an anterior chamber injection of 1A7 (5 ug/ml, 3 ul), 2E6 (15 ug/ml, 3 ul) or 1D4 (15 ug/ml, 3 ul) 2 days prior to induction of PVR by intravitreal dispase. Mice were sacrificed and eyes harvested 2 or 4 weeks later. Whole eyes were fixed, embedded, and sectioned. Inspection of H&E-stained sections suggested that two anti-A2 IgGs (1A7 and 2E6) blocked the development of PVR at 2 weeks (**Fig. 7A**). On the other hand, the eye injected with antibody 1D4, a control that does not recognize A2 and does not block plasmin generation, showed a robust PVR reaction, with detachment of the entire retina. At four weeks, the eye injected with 1A7 showed intact cell layers along

with a few retinal folds, whereas the non-injected eye showed severe PVR (**Fig. 7B**). Quantitative evaluation of PVR and RPE histologic scoring of non-injected versus anti-A26-injected eyes revealed scores of 3-5 for non-injected or 1D4-injected eyes versus 1-2 for eyes receiving 1A7 or 2E6 (**Fig. 7C and 7D**). Mice receiving these antibodies showed no obvious untoward effects. These data indicate that inhibition of A2 profibrinolytic activity may be a useful and safe approach to the prevention of PVR.

Specific Aim 2: To specify PVR-related, annexin A2-dependent interactions between RPE cells and macrophages.

Approach: We first established methods for isolating and propagating mouse RPE cells. We next designed a murine cell migration system for analysis of macrophage-induced RPE cell migration. *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ murine RPE cells were seeded on laminin-coated, 2-micron pore filters within the upper chambers of transwell assemblies. Lower wells contained a variety of test substances, including RPE cell medium, macrophage medium, macrophage-conditioned medium, or *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ bone marrow-derived macrophages. After 18 hours, we enumerated RPE cells that had migrated through the transwell barrier to the under surface of the filter; we first removed residual cells on the upper side of the filter, and then staining the underside with crystal violet. Stained cells were enumerated using NIH Elements software, or by spectrophotometric analysis of extracted crystal violet (A540 nm) and quantitation using a standard curve. We also developed an analogous system for examining human macrophage-induced RPE cell migration, using human RPE and macrophage cell lines, ARPE-19 and THP1, respectively. Finally, we conducted cytokine/chemokine profiling of mouse retinas from mice undergoing PVR and control mice using a commercial kit.

Major Findings:

[1] In macrophage-induced RPE cell migration studies *in vitro*, A2 expression is required in both cell types. We observed significant migration of RPE cells across the filter only when both the RPE cells and macrophages were of the *Anxa2*$^{+/+}$ genotype (**Fig. 8A**). We demonstrated, further, that IgG directed against A2 can block 60% of RPE cell migration, and that a global fibrinolytic inhibitor, tranexamic acid (TXA), has a similar effect (**Fig. 8B**). These experiments indicate that A2 is essential for macrophage-induced RPE migration, must be expressed in both cell types, and works most likely via is pro-fibrinolytic activity.

[2] In PVR, macrophage inflammatory proteins (MIPs) are elevated in the *Anxa2*$^{+/+}$, but not *Anxa2*$^{-/-}$, retina. To further understand the mechanism by which macrophages might recruit RPE cells, we conducted cytokine profiling experiments on pooled retinal homogenates from *Anxa2*$^{+/+}$ and *Anxa2*$^{-/-}$ PVR or non-injected eyes (**Fig. 9A and 9B**). At 2 weeks, we found no change in a series of immunomodulatory molecules, including proinflammatory cytokines (IL-1β, IL-6, TNFα, RANTES), an immunostimulatory cytokine (INFγ), or an anti-inflammatory cytokine (IL-10) in either *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ dispase injected eyes. At 2 days, however, we noted a 6-fold increase over non-injected controls in the level of chemokine CCL3 (aka macrophage inflammatory protein-1α, MIP-1α) in *Anxa2*$^{+/+}$ retinas. We also noted a doubling in CCL4 (aka MIP-1β) at the same time point. *Anxa2*$^{-/-}$ retinas, in contrast, showed no increase at all in either CCL3 or CCL4.

CCL3 is a member of the "CC" chemokine, or "chemotactic cytokine," family of molecules that attract mononuclear cells to sites of chronic inflammation[25, 26]. CCL3 is produced by inflamed tissue and interacts with G-protein coupled receptors, CCR1 and CCR5, on monocytes and macrophages that express CCR1 or CCR5. In the subretinal fluid and vitreous humor of human eyes exhibiting PVR, CCL3 levels, along with those of other cytokines and chemokines, have been reported to be significantly higher than in control eyes or those with rhegmatogenous retinal detachment without PVR[27, 28]. Exactly how A2 might contribute to the elevated CCL3 in wild type retinal lysates, however, is unclear. One possibility is that production of CCLs reflects the number of macrophages infiltrating the retina, and is higher in the *Anxa2*$^{+/+}$ mouse due to more efficient recruitment of macrophages. Another possibility is that A2 plays a direct role in the production or secretion of these chemokines by macrophages.

[3] MIPs enable macrophage induced RPE cell migration *in vitro* by increasing RPE cell surface A2 expression. As noted above, we have found that MIP-1α and MIP-1β, alone or in combination, induced migration of human RPE cells (ARPE-19) across laminin-coated filters in a transwell system. For MIP-1α and MIP-1β, this effect appeared to be additive, and possibly synergistic (**Fig. 8C**). Furthermore, IgG directed against human MIP-1α and MIP-1β blocked RPE cell migration by 40-50% in the transwell system (**Fig. 8D**). Thus, anti-MIPs, like anti-A2 can specifically impair macrophage-induced RPE migration.

To elucidate the mechanism by which MIP-1α and MIP-1β induce RPE cell migration, we examined expression of total A2, as well as cell surface A2, in cultured human RPE cells (**Fig. 9A and B**). After exposure of RPE cells to medium conditioned by RPE cells and macrophages, or to MIP-1α and MIP-1β, for 18 hours, cell surface proteins were labeled with non-cell permeable biotin. After extensive washing, the cells were lysed, and labeled proteins captured with immobilized streptavidin. Captured proteins were eluted, resolved on SDS polyacrylamide gels, and probed with antibody to A2. The results indicate no change in total annexin A2, but a significant increase in surface A2 on RPE cells exposed to MIP-1α and MIP-1β. Several agents, such as heat shock and thrombin, are known to induce phosphorylation of A2 at tyrosine 24, and it is known that this phosphorylation event triggers translocation of the $A2_2$-$S100A10_2$ complex to the cell surface[29, 30]. We have also verified that MIP-1α and MIP-1β are produced by macrophages, but not by glial cells or by RPE cells themselves (**not shown**). Together, the data presented above indicate that MIP-1α and MIP-1β increase cell surface A2 and plasmin activation, thereby promoting RPE cell delamination and migration in the development of PVR.

Specific Aim 3: To define the role of the annexin A2 system in the pathogenesis of human PVR.

Approach: Human epiretinal membrane samples were collected at surgery by Dr. Szilard Kiss, placed on sterile MF-Millipore filters, and retrieved within 5 minutes by a member of Dr. Hajjar's research group. PVR membrane-containing filters were placed in tissue culture dishes, and the membrane fixed with 2% paraformaldehyde (10 min, 21°C). The membrane was then rinsed three times with PBS containing Ca^{++} and Mg^{++} and stored at 4°C for up to 22 days. For immunofluorescence staining, autofluorescence was quenched with NH_4Cl, and sections then blocked with normal donkey serum. For A2 staining, incubation with primary and secondary antibodies was done at 4°C overnight and 30 minutes at 21°C, respectively, using rabbit anti-annexin A2, followed by Cy3-labeled donkey anti-rabbit. Sections were counterstained with DAPI to visualized cell nuclei, and were imaged using Nikon 80i microscope at 200x power.

Major Findings:

[1] Human epiretinal membranes contain abundant cells that are doubly positive for A2 and RPE65. To address the potential role of A2 in human PVR, we have been fortunate to be able to collect PVR membranes from 11 subjects undergoing retinal surgery, under an IRB-approved protocol and in collaboration with Dr. Szilard Kiss (Director, Retina Service, Department of Ophthalmology, Weill Cornell Medicine). In addition, we have vitreous fluid samples from 7 subjects, and plasma and peripheral blood mononuclear cells from 5 subjects. Immunostaining of epiretinal membrane samples removed from surgical subjects revealed them to be highly cellular. A2-expressing cells were easily identified in every samples tested. In addition, cells that stained positive for both RPE65, an RPE cell marker, and A2 were readily detected (**Fig. 11**).

[2] Human epiretinal membranes contain abundant cells that are doubly positive for A2 and CD68. Immunostaining also revealed abundant epiretinal membrane cells that are positive for both A2 and CD68, a macrophage cell marker (**Fig. 12**).

Together, these data indicate that expression of A2 in both RPE- and macrophage-like cells is characteristic of human PVR.

Opportunities for Training

Dr. Nadia Hedhli, a postdoctoral fellow, worked on Aims 1 and 2 in this project and gained extensive experience in the dispase model of PVR, ocular histology in the mouse, isolation of murine bone marrow derived and peritoneal macrophages, and isolation of primary mouse RE cells. She is now a professor of biology at Hudson Community College in Jersey City, New Jersey.

Dissemination

[1] An abstract describing these findings was submitted this year to the 2018 Military Health System Research Symposium:

Hajjar, KA, N Hedhli, D Almeida, MP Gupta, S Kiss. "The Phospholipid-Binding Protein Annexin A2 Mediates Development of Proliferative Vitreoretinopathy in a Murine Model of Penetrating Ocular Injury," Vision-Ocular Trauma Casualty Management Grand Rounds, 2018 Military Health System Research Symposium; https://mhsrs.amedd.army.mil/Conference/Lists/AbstractsForm/Abstracts2018.aspx, Abstract #MHRS-18-1290.

[2] A manuscript describing these findings is in the final stages of preparation:

Luo, M, D Almeida, N Hedhli, MP Gupta, S Kiss, **KA Hajjar**. "Annexin A2 Enables Retinal Pigment Epithelial Cell Transformation in Proliferative Vitreoretinopathy."

Next reporting period goals

Because this is the final report for this project, there are no associated goals to report.

4. Impact

[1] Upon publication, this study will have significant impact on the field of annexin A2 biology. Although there are several reports describing the roles of the various human annexins in the control of inflammation and host responses, our work shows for the first time that A2 expression governs the wound healing response in the retina. To our knowledge, no other reports have touched on the role of A2 in PVR. In addition, our data propose new paradigms whereby expression of annexin A2 may influence chemokine secretion, and whereby cell surface A2 may direct cell migration in EMT-like responses to injury in the eye and beyond.

[2] We feel that our work will also have a significant impact on the field of ophthalmology. At present there is no effective treatment or preventive modality for PVR. Our data strongly suggest that blockade of A2 may prevent PVR if given early following penetrating ocular injury.

[3] Regarding impact on technology transfer, this work has led us to conduct preliminary tests on the effectiveness of anti-annexin A2 blocking antibodies in PVR. We recently filed a provisional patent on the antibodies and their use in this and other applications. A final patent application is in preparation.

[4] With respect to the impact on society beyond science and technology, this work has laid the groundwork for development of a new preventive treatment for PVR. While lacking at present, development of such treatment could offer the prospect of preserved vision to post-surgical and post-trauma patients with PVR. For example, we envision point-of-care prophylactic treatment with intra-ocular anti-A2 at the time of, or shortly after, ocular injury for the development of PVR.

5. Changes/Problems

We previously reported that our ability to procure human epiretinal membrane samples was temporarily limited. We have now overcome this issue, as competing projects have ended, and as we have developed more

robust consenting and procurement procedures. In the last year, we have doubled our collection of human epiretinal membranes to the point where subject recruitment no linger limits progress in this aim. We have now collected 11 samples, and stained 7. We feel that these numbers are now sufficient to draw preliminary conclusions about the degree and extent of A2 expression in epiretinal membrane cells.

6. Products, Inventions, Patent Applications, and/or Licenses

[1] An abstract describing these findings was submitted this year to the 2018 Military Health System Research Symposium.
[2] A manuscript describing these findings is in final preparation.
[3] A provisional patent (62/576,518) has been filed on the use of our recently developed anti-annexin A2 antibodies for treatment of PVR.

7. Participants & Other Collaborating Organizatoins

Name	Katherine A. Hajjar, MD
Project role	PI
Researcher identifier	
Nearest person month	2
Contribution	Oversight of all aspects of the project; review of data.
Funding support	This grant

Name	Szilard Kiss, MD
Project role	Co-PI
Researcher identifier	
Nearest person month	1
Contribution	Human sample procurement; review of data.
Funding support	This grant

Name	Dena Almeida
Project role	Technician
Researcher identifier	
Nearest person month	4
Contribution	Execution of dispase model; development of cell based models; all tissue processing, staining, and documentation
Funding support	This grant

8. Special Reporting Requirement - Quad Chart

Please see attached.

9. Appendices

Figures
Quad chart

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Figure 1

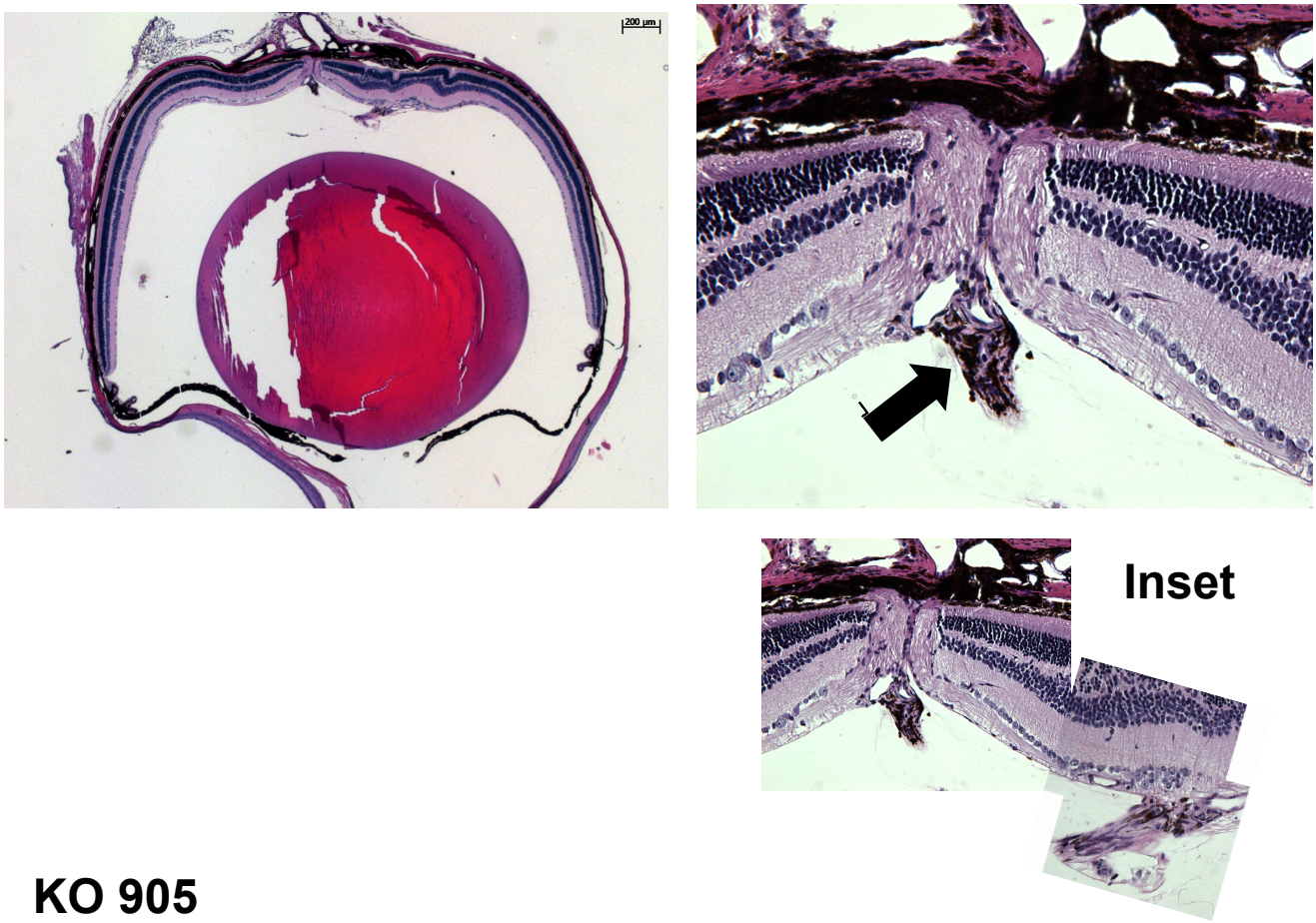


Figure 1. C57Bl/6 mice, either *Anxa2*$^{+/+}$ (WT) or *Anxa2*$^{-/-}$ (KO) received intravitreal injections of 0.003 units/ul of dispase in the left eye. At 4 weeks, representative H & E-stained sections through the WT eye show early migration of pigmented cells from the RPE layer through the retinal wound and over the vitreal surface of the retina (arrow). In the KO eye, RPE cells remain behind the retina. Original magnification 25x left and 200x right.

Figure 2

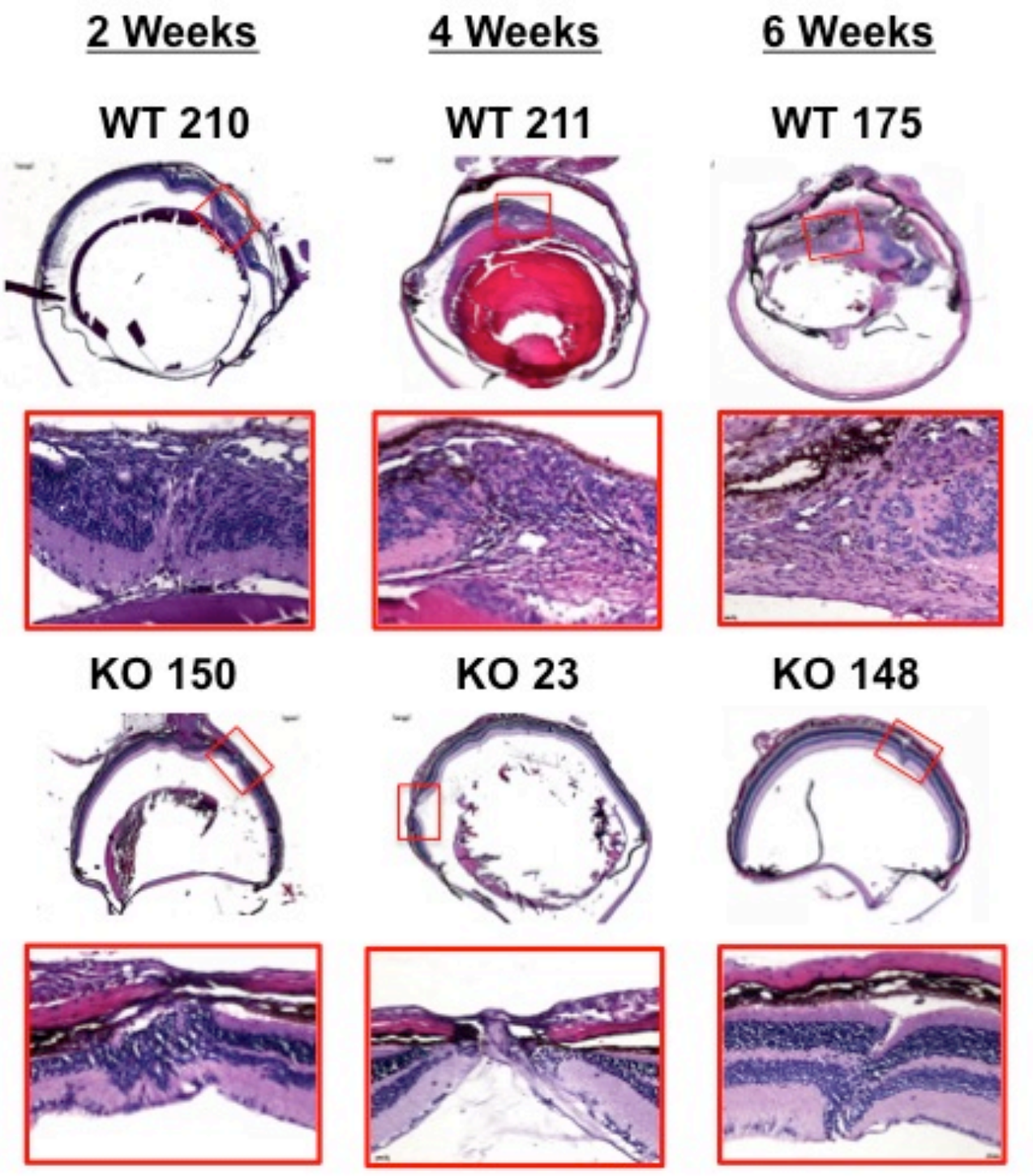


Figure 2. C57Bl/6 mice, either *Anxa2*$^{+/+}$ (WT) or *Anxa2*$^{-/-}$ (KO) received intravitreal injections of 0.3 units/ul of dispase in the left eye. At 2, 4, and 6 weeks, representative H & E-stained sections through the WT eye shows extensive migration of pigmented cells from the RPE layer through the retinal wound and onto the vitreal surface of the retina in apposition to the lens. In the KO eye, migrating pigmented cells are not detected and no epiretinal membrane has formed. Original magnification 25x left and 200x right.

Figure 3

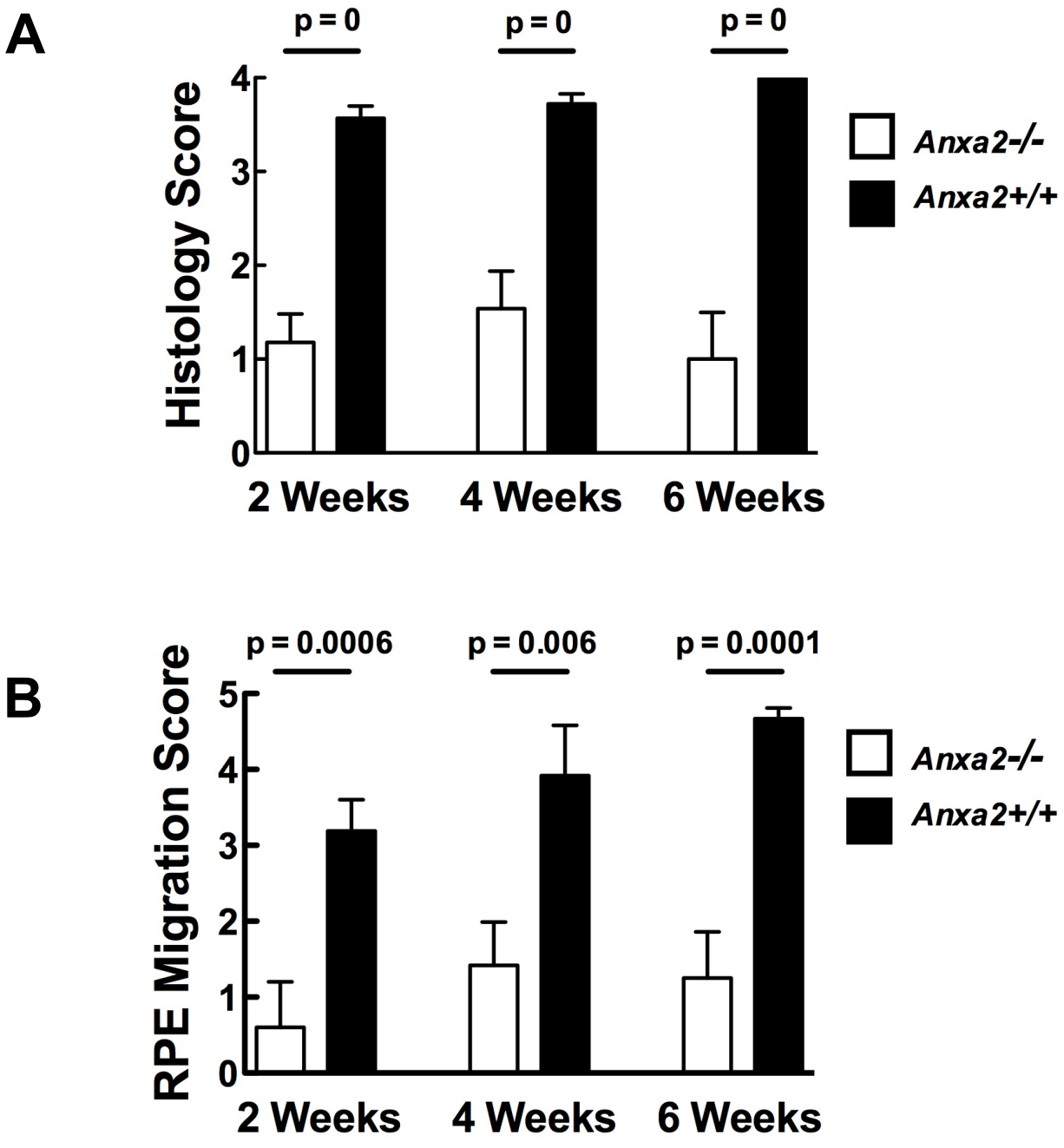


Figure 3. Hematoxylin- and eosin-stained sections (10 section per dispase-injected eye) from a total of 53 mice and were analyzed by 3 trained observers in a double-blind fashion. Sections were from six groups of mice (n=7-11 mice per group) representing *Anxa2*$^{-/-}$ or *Anxa2*$^{+/+}$ mice at 2, 4, or 6 weeks and were scored using a standard algorithm. Inter-observer variability for the 6 groups averaged 10.4 + 3.1%. The results indicate a highly significant difference in severity of PVR in *Anxa2*$^{+/+}$ *vs. Anxa2*$^{-/-}$ mice, as judged by overall histology score (**A**) and the extent of RPE cell migration (**B**).

Figure 4

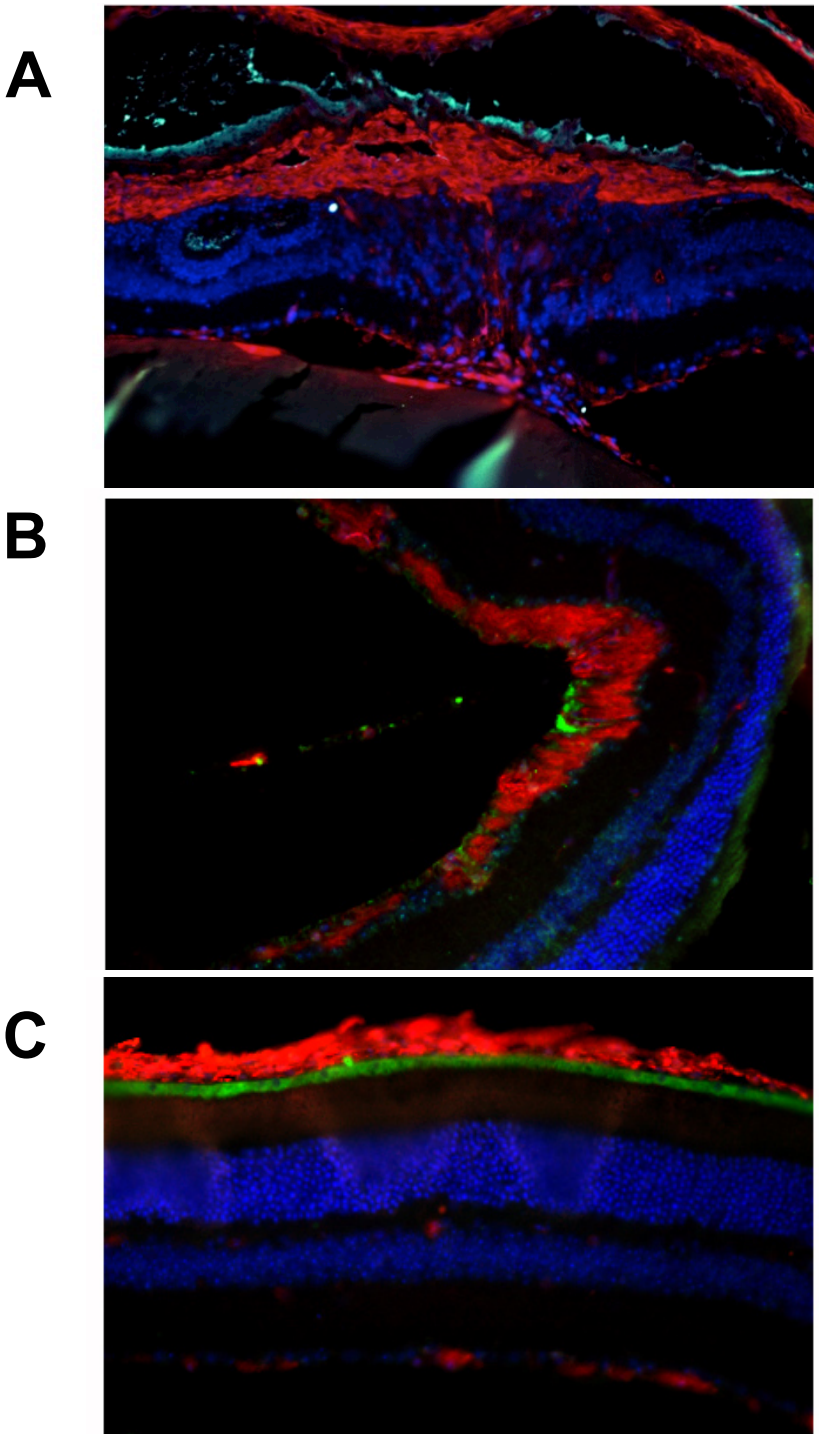
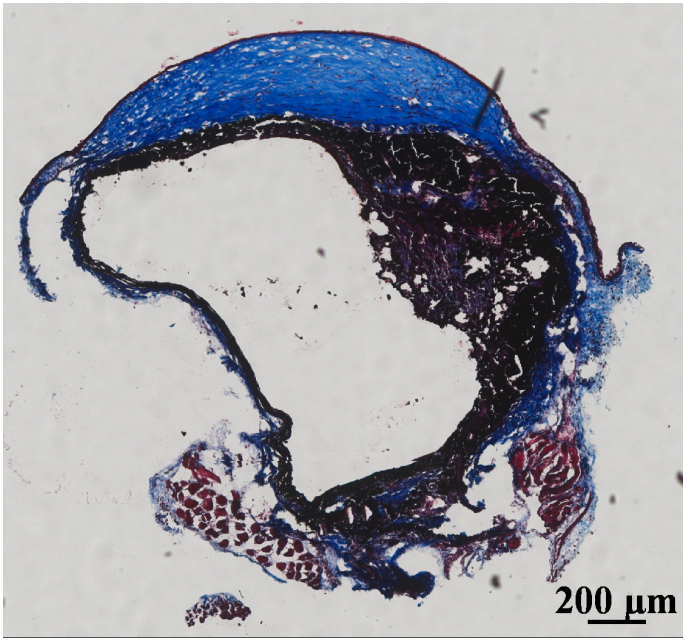


Figure 4. Immunofluorescence staining of sections of *Anxa2*$^{+/+}$ dispase-injected eyes harvested at 2 weeks. **A**. At the injection site, cells migrating from the scleral side of the retina through the retinal wound and over the vitreal surface of the retina are strongly positive for A2 (red). **B.** Cells within the developing epiretinal membrane are positive for both A2 and RPE65, indicating RPE cell lineage. **C.** In control, PBS-injected retinas, A2 staining is limited to choroid and retinal blood vessels, and RPE65 staining remains within the undisturbed RPE cell layer. DAPI nuclear staining is shown in blue. Original magnification 200x.

Figure 5

WT 12



KO 15

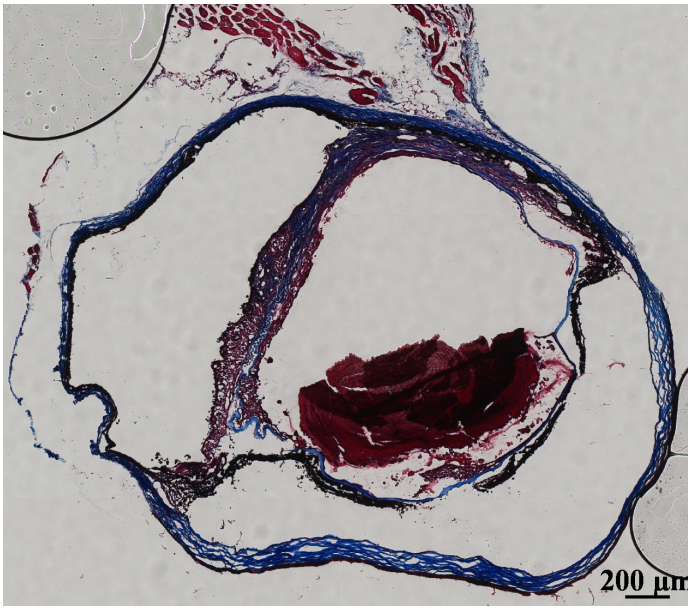


Figure 5. Masson's trichrome stain of dispase-injected *Anxa2*$^{+/+}$ (WT) and *Anxa2*$^{-/-}$ (KO) eyes. Note extensive collagen deposition (blue) in WT eye, compared with the KO eye. Original magnification 25x.

Figure 6

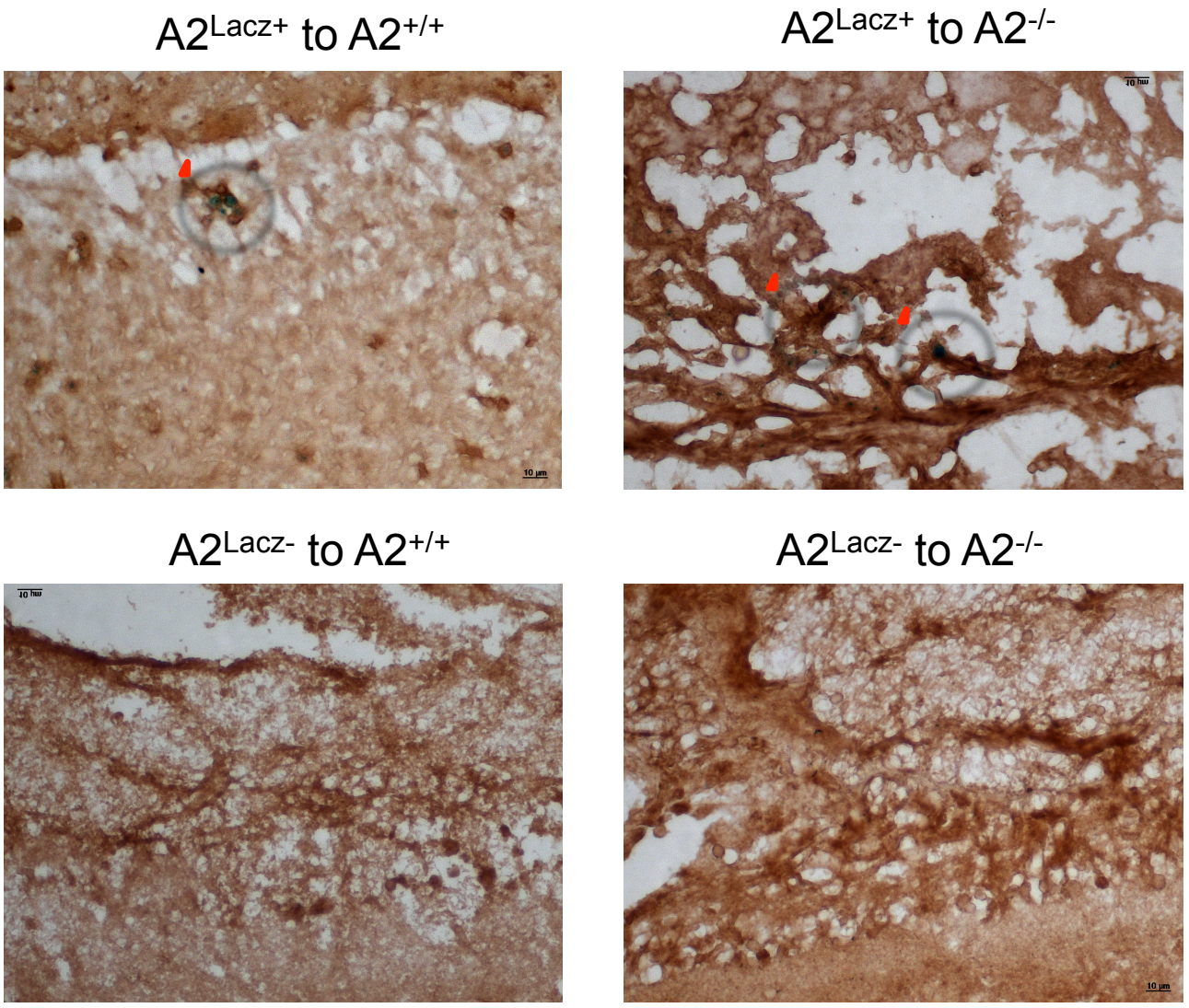


Figure 6. Xgal (LacZ) (blue) and F4/80 (dark brown) staining at 48 hours after intravitreal dispase injection in *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ mice transplanted with bone marrow from either *Anxa2*$^{LacZ/+}$ or *Anxa2*$^{LacZ/-}$ mice. Mice were lethally irradiated prior to bone marrow reconstitution. Note Xgal and F4/80 co-staining in some cells in retinas of mice receiving *Anxa2* $^{LacZ/+}$, but not *Anxa2*$^{LacZ/-}$, bone marrow.

Figure 7

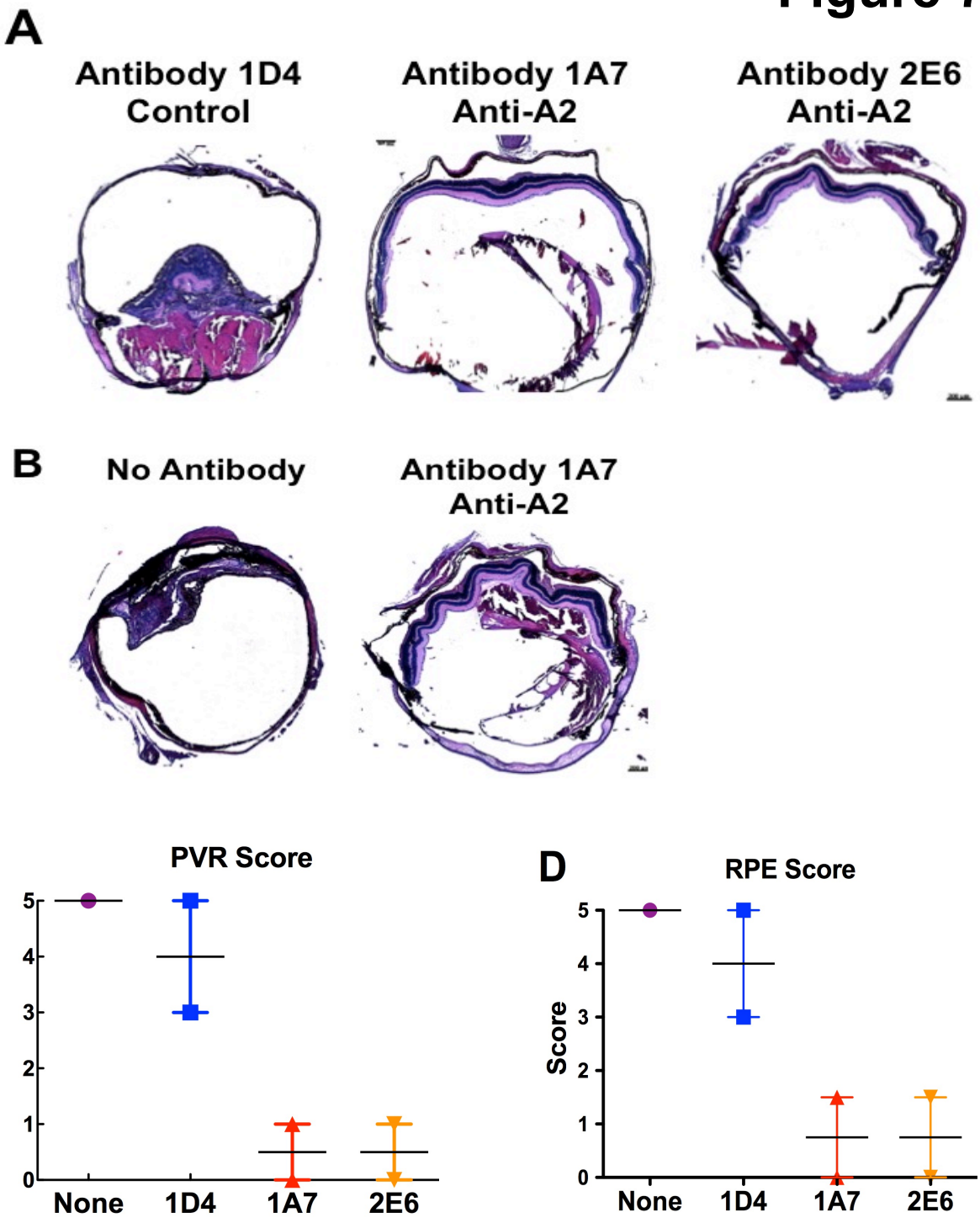
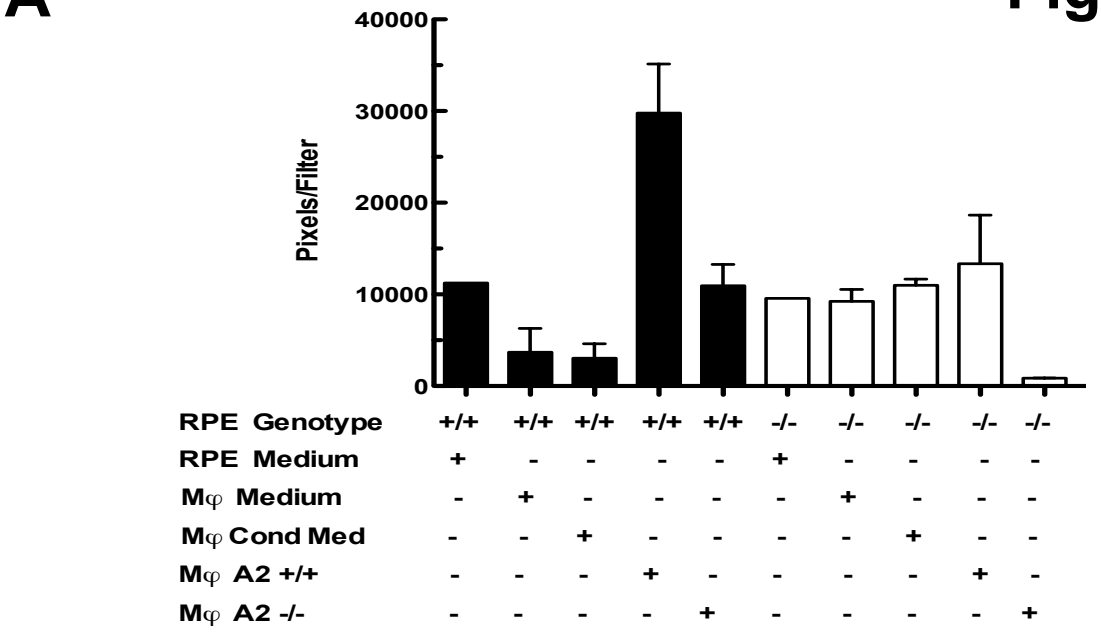


Figure 7. Anti-A2 antibodies attenuate dispase-induced PVR. Mice received anterior chamber injections of either 1A7 (15 ng), 2E6 (45 ng) or control 1D4 (45 ng) 2 days prior to induction of PVR with intravitreal dispase. Inspection of H&E-stained sections of eyes harvested at 2 (**A**) and 4 (**B**) weeks suggests that 1A7 and 2E6 were effective in blocking the development of PVR (note intact retina), whereas 1D4, with no anti-A2 reactivity, had no effect (note detached, contracted retina). PVR and RPE histologic scores are shown in **C** and **D**, where 0 represents normal appearing retina, and 5 indicates extensive retinal disruption, detachment, and contraction with the presence of RPE cells within the retinal scar and within extraretinal tissues.

Figure 8



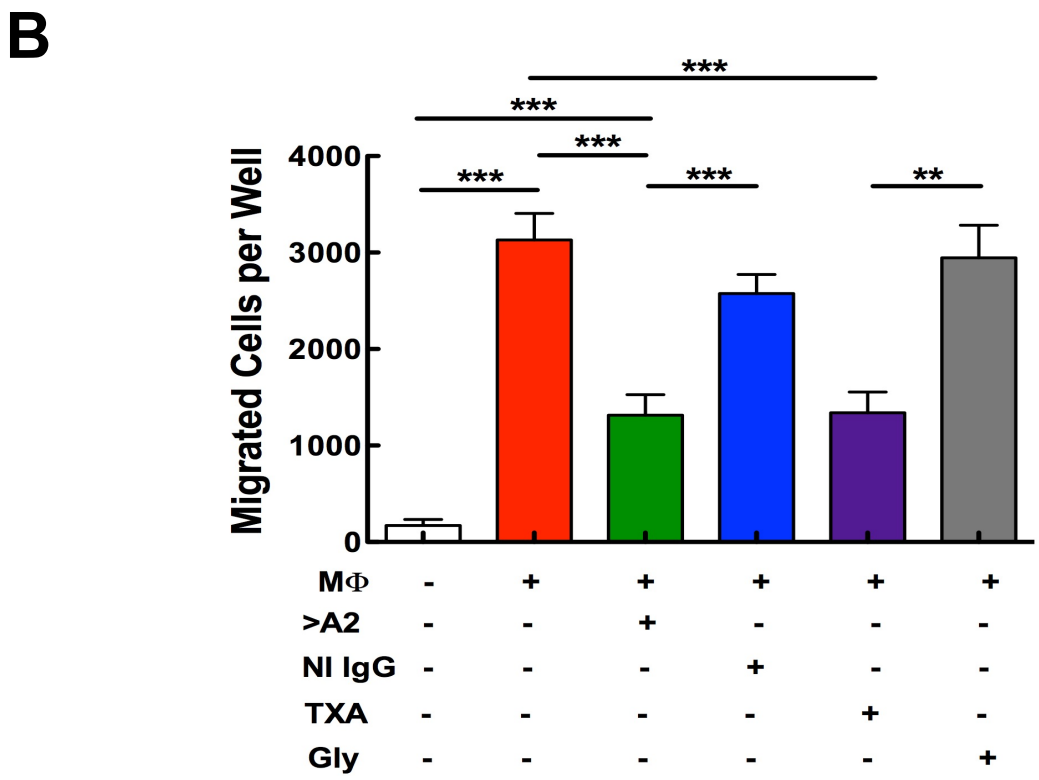


Figure 8. RPE migration assay. **A.** *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ RPE cells were seeded on laminin-coated, 3-micron pore, transwell filters positioned above a lower well containing either RPE medium, macrophage medium, macrophage conditioned medium, or *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ bone marrow-derived macrophages, as indicated. After 24 hours, cells migrated to the underside of the filter were stained with crystal violet, and enumerated using NIH Elements image software. **B.** Human RPE and macrophages were arrayed as described under A, and migration in the presence of anti-A2, control IgG, tranexamic acid (TXA), or glycine (Gly, control) assessed using crystal violet elution and quantitative absorption at 540 nm.

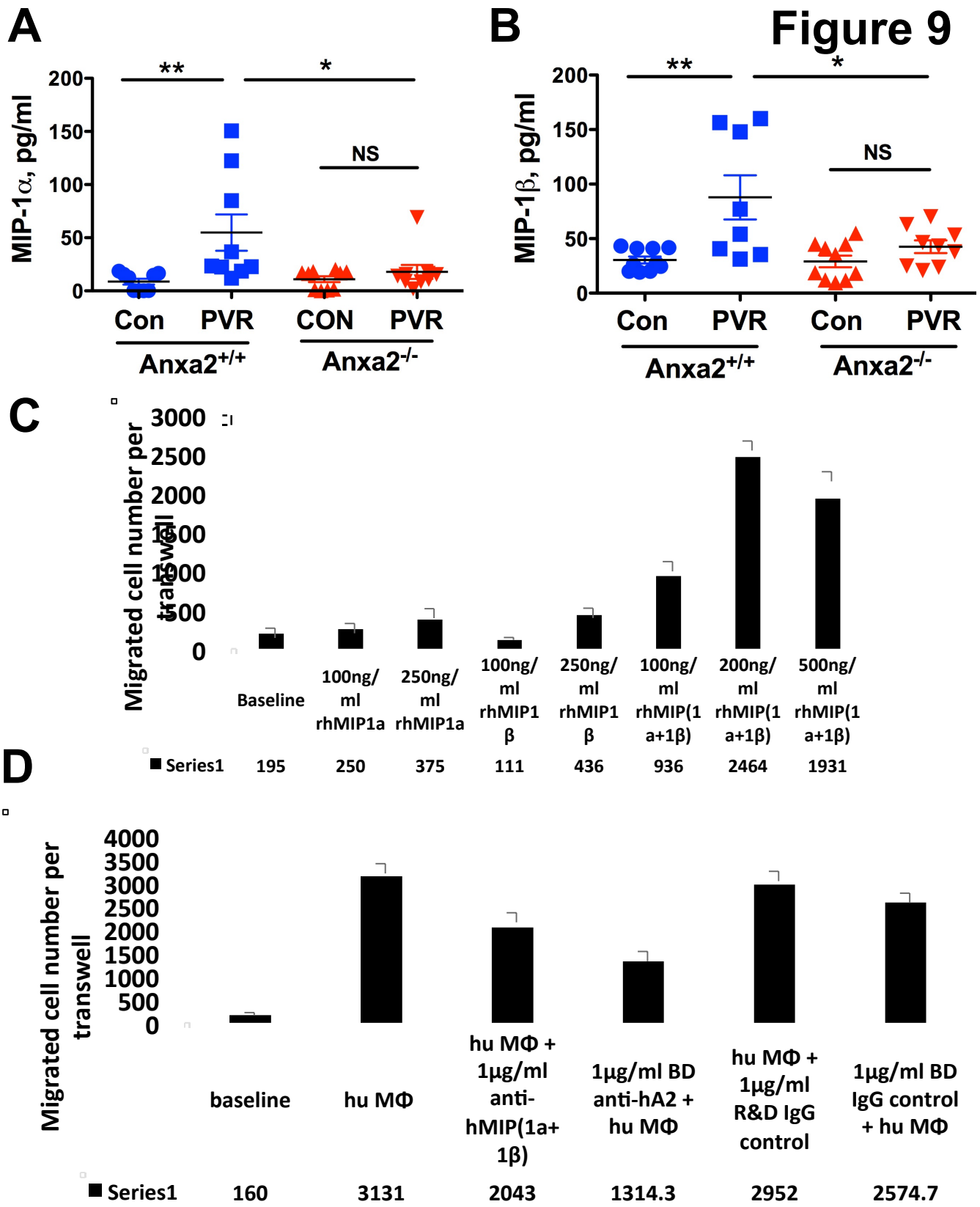
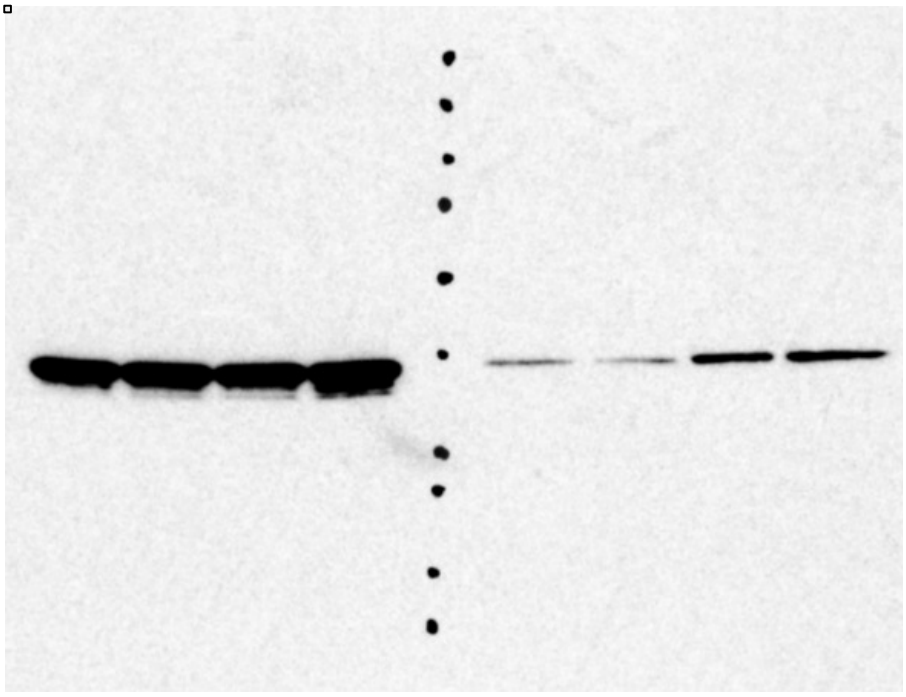


Figure 9. MIPs and human RPE cell migration. **A, B**. Elevated concentrations of MIPs are present in PVR retinas from *Anxa2*$^{+/+}$, but not *Anxa2*$^{-/-}$ mice. **C.** MIP-1α and MIP-1β induce migration of RPE cells *in vitro* in an additive manner. **D.** Anti-MIPs and anti-A2 block macrophage-induced RPE cell migration.

Figure 10

A



B

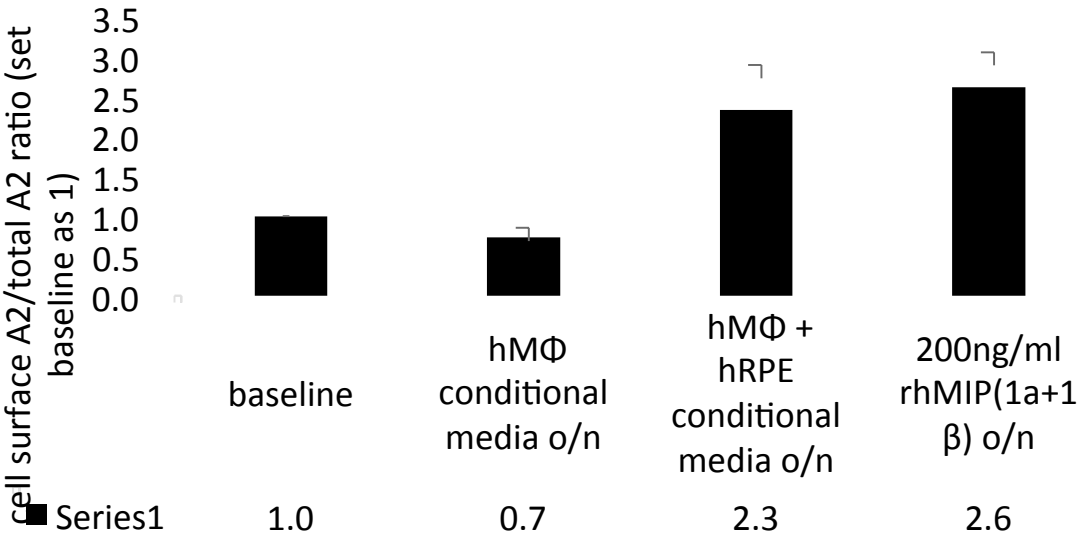


Figure 10. Media conditioned by human THP-1 macrophages with or without recombinant human MIP1a and MIP1β (200 ng/μl) was added to human ARPE-19 cells. After 18 hours, cell surface proteins were biotinylated, isolated from whole cell lysates with immobilized streptavidin, and A2 visualized by immunoblot analysis. **A.** Representative original blot. **B.** Average densitometric values from scanned immunoblots.

Figure 11

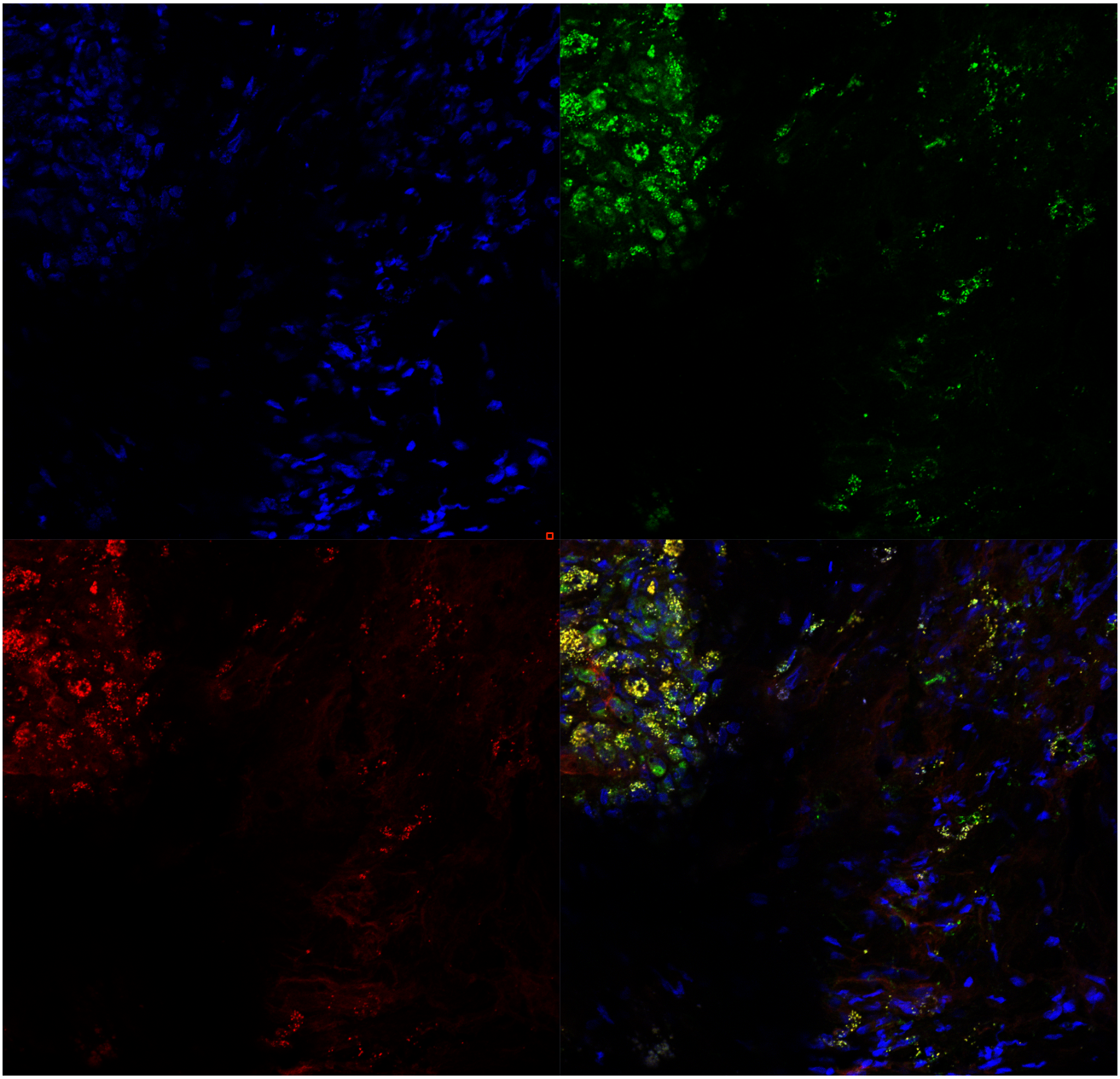


Figure 11. Immunofluorescence staining of sections through a human epiretinal membrane obtained at surgery for membrane removal. Confocal images show nuclear staining (DAPI, blue), staining for macrophages (CD68, green), and staining for annexin A2 (red). Lower right panel shows co-localization of red and green signals in some groups of cells, indicating the presence of A2-expressing monocytes, macrophages, or microglia. Original magnification 250x.
Sample HS685.

Figure 12

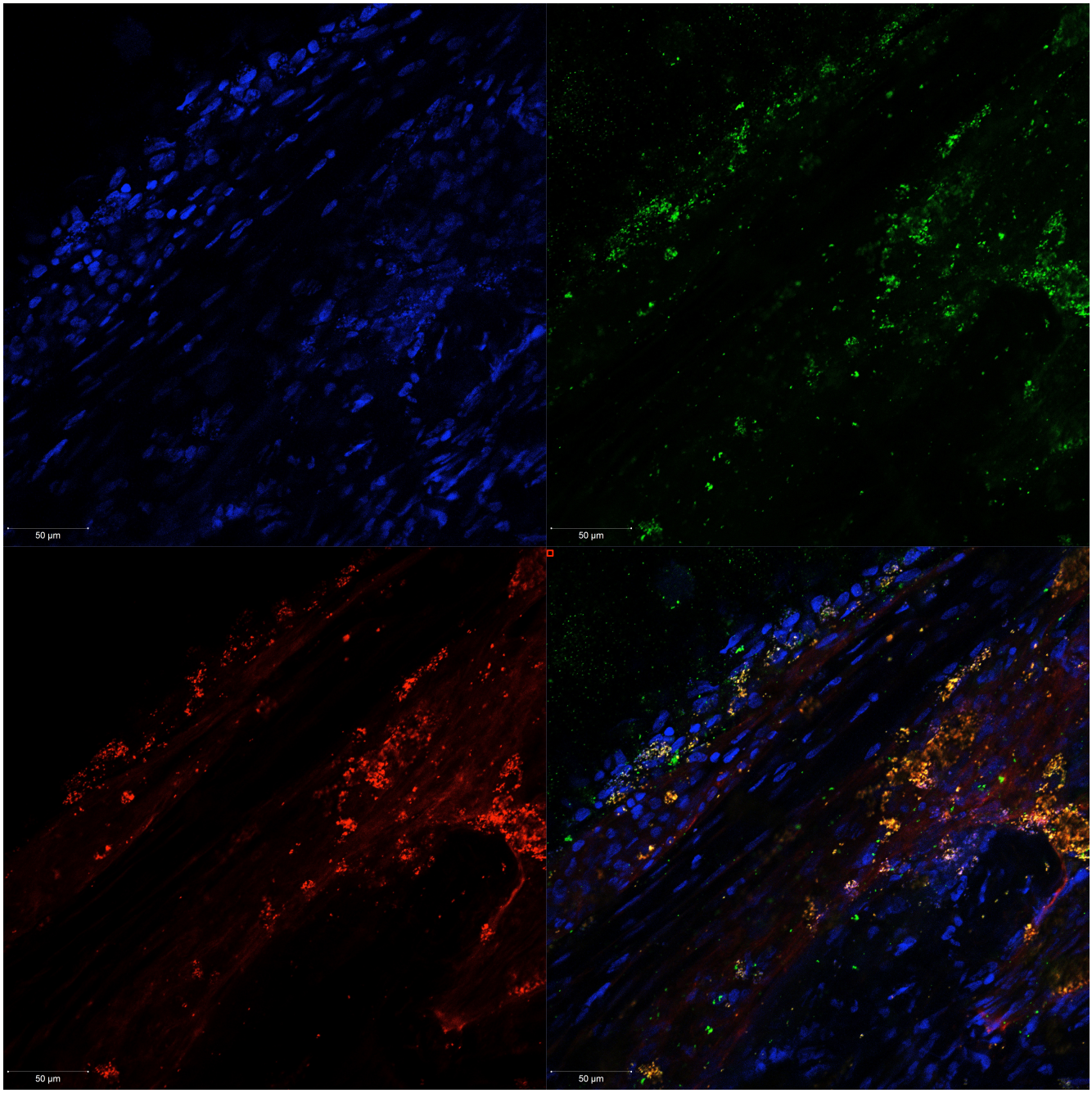


Figure 12. Immunofluorescence staining of sections through a human epiretinal membrane obtained at surgery. Confocal images show nuclear staining (DAPI, blue), staining for RPE cells (RPE65, green), and staining for annexin A2 (red). Lower right panel shows co-localization of red and green signals in some groups of cells, indicating the presence of A2-expressing cells of the RPE lineage. Original magnification 250x.. HS685.

Annexin A2 and Proliferative Vitreoretinopathy

Log No. MR130194

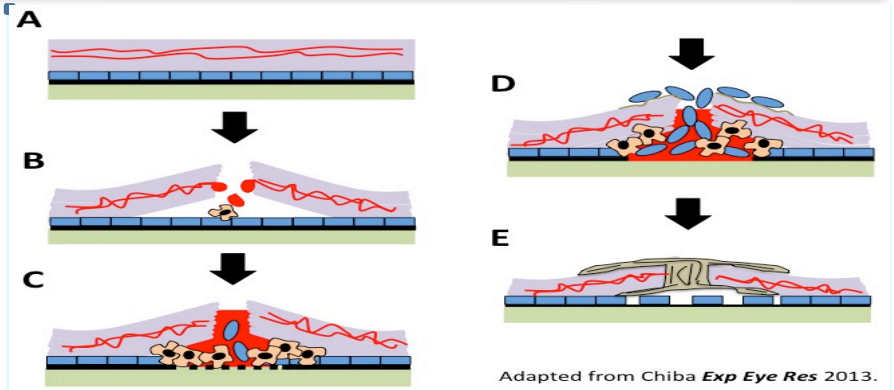
PI Katherine A. Hajjar, MD **Org:** Weill Cornell Medical College **Award Amount: $1,000,000**

Study/Product Aim(s)

- **To analyze the functional role of annexin A2 and related molecules in a mouse model of proliferative vitreoretinopathy (PVR).**
- **To specify PVR-related, annexin A2-dependent interactions between RPE cells and macrophages.**
- **To define the role of the annexin A2 system in the pathogenesis and progression of human PVR.**

Approach

This project will address the hypothesis that, in PVR, early recruitment and activity of macrophages to sites of retinal injury depends upon their expression of annexin A2. We postulate that macrophages produce proteases, growth factors, and signaling molecules that transform quiescent RPE cells into motile, fibrogenic cells that engender pre- and epiretinal scar formation, leading to further retinal damage and loss of vision.



Hypothesis: Upon retinal injury, macrophage (orange) expression of annexin A2 leads to RPE cell (blue) activation, migration, and epi/preretinal membrane scars.

We have recently established that macrophage recruitment to the hypoxic mouse retina is greatly reduced in the annexin A2-deficient mouse.

Timeline and Cost

Activities	CY 14	15	16	17-18
Prepare and submit application				
Aim1: Mouse model completed				
Aim 2: Co-culture studies underway				
Aim 3: Human subject studies underway				
Estimated Budget ($K)	**$000**	**$333**	**$333**	**$334**

Updated: 12/19/18

Goals/Milestones (Example)

CY14 Goal – Submit pre-application

☑ Completed

CY15 Goals – Submit full application and initiate project

☑ Establish PVR model in AnxA2$^{-/-}$, S100A10$^{-/-}$ and S100A4$^{-/-}$ mice

☑ Establish macrophage-RPE co-culture systems

☑ Initiate collection of human PVR samples

CY16 Goals – Continue experiments related to Aims 1-3

☑ Study PVR macrophage-specific A2 knockouts

☑ Continue macrophage and RPE signaling experiment

☑ Initiate human RPE cell-macrophage experiments

CY17-18 Goals – Complete experiments and submit manuscripts

☑ Complete bone marrow transplantation experiments

☑ Complete cytokine profiling

☑ Complete human epiretinal membrane profiling

Comments/Challenges/Issues/Concerns

Budget Expenditure to Date

Projected Expenditure: $1,000,000

The Phospholipid-Binding Protein Annexin A2 Mediates Development of Proliferative Vitreoretinopathy in a Murine Model of Penetrating Ocular Injury

Katherine A. Hajjar, Nadia Hedhli, Dena Almeida, Mrinali Patel Gupta, Szilard Kiss

Background: Proliferative vitreoretinopathy (PVR) is one of the final frontiers in retinal surgery. PVR occurs in 8-10% of patients with primary retinal detachment, but up to 40-60% of patients with penetrating globe injury, of which there are more than 200,000 worldwide per year. In recent studies, up to 43% of civilian and military patients with penetrating eye injuries or intraocular foreign bodies developed PVR, which has increased in frequency as a consequence of modern combat. Although the exact pathogenesis of PVR is poorly understood, it is thought that monocyte-derived macrophages remodel matrix and instruct retinal pigment epithelial (RPE) cells to abandon their attachment to Bruch's membrane, proliferate, migrate, and synthesize collagens and other matrix proteins leading to the formation of epi- and subretinal membranes. Epiretinal membranes can contract causing retinal detachment and severe loss of vision. At present, there is no reliable means of preventing PVR following ocular trauma or retinal surgery.

In the current project, we examined the role of the phospholipid-binding protein annexin A2 (A2) in a murine model of PVR. On the surface of both macrophages and RPE cells, A2 and its partner protein, S100A10, form a complex that binds two pro-fibrinolytic proteins, plasminogen and tissue plasminogen activator. Assembly of these proteins accelerates the activation of the serine protease plasmin, thereby allowing migration of cells across fibrin- or laminin-containing tissue matrices. We hypothesized that A2 potentiates PVR development after ocular injury, and that A2 may constitute a useful drug target for the prevention of PVR following penetrating ocular injury.

Methods: In a classic model of PVR, 3-4 month-old *Anxa2*$^{+/+}$ and *Anxa2*$^{-/-}$ C57BL/6 mice, received a single, 3-ul intravitreal injection of dispase (0.3 unit/ul) according to an IACUC- and ACURO-approved protocol. Mice were sacrificed humanely at intervals thereafter, and whole eyes or retinal tissue harvested for histologic grading using light and immunofluorescence microscopy, as well as cytokine expression studies using commercially available ELISA kits. In addition, RPE cells and bone marrow-derived macrophages were isolated from mice of both genotypes for use in co-culture migration experiments. In some experiments, a cell-based model involving intravitreal injection of 50,000 ARPE-19 or bone marrow derived macrophages was employed. Under an IRB- and HRPO-approved protocol, epiretinal membrane tissue removed at surgery from consenting human subjects, was analyzed histologically for the presence of A2-expressing macrophages and RPE-like cells.

Results: Following challenge with intravitreal dispase, retinas from *Anxa2*$^{+/+}$ mice displayed delamination of RPE cells from Bruch's membrane, and migration of RPE cells through the injection site retinal wound and over the vitreal surface of the retina within 24 hours. By 2 weeks, epiretinal membranes were evident, and within 4 weeks extensive disruption of the normal retinal architecture with invasion of pigmented cells into the retina itself was evident. By 6 weeks, there was retinal detachment and extensive scar formation. Upon dispase injection into *Anxa2*$^{-/-}$ mice, on the other hand, migration of RPE cells into or beyond the retina was not observed at any time point, and normal retinal architecture was preserved at all time points examined. Scoring by three trained, masked observers of hematoxylin and eosin stained sections through the injection site according to a standard algorithm showed reproducibly marked retinal detachment, disorganization, membrane formation, and RPE cell migration in *Anxa2*$^{+/+}$, but not *Anxa2*$^{-/-}$, mice ($p \leq 0.006$, n=4 mice per group, 4 sections per mouse; ANOVA; inter-observer variability 10.4 + 3.1%). Immunofluorescence staining of mouse eyes undergoing PVR revealed that migrating cells specifically expressed A2. In addition, examination of epiretinal membranes removed at surgery from human subjects revealed the presence of cells doubly positive for either A2 and the RPE cell marker RPE65, or A2 and the macrophage marker CD68. *In vitro* co-culture studies revealed that both peritoneal and bone marrow-derived macrophages stimulated migration of primary RPE cells across laminin-coated filters, but only when both cell types were of the *Anxa2*$^{+/+}$ genotype. Examination of retinas from dispase-treated eyes from lethally-irradiated *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ mice

rescued by transplantation of LacZ-marked bone marrow from either *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ mice revealed the presence of intraretinal bone marrow-derived macrophages only when bone marrow was from an *Anxa2*$^{+/+}$ donor. Cytokine ELISAs of whole *Anxa2*$^{+/+}$ and *Anxa2*$^{-/-}$ retinal lysates indicated no difference in production of 5 inflammatory cytokines, including interleukin-1β (IL-1β), IL-6, IL-10, interferon γ, and tumor necrosis factor α, or the chemokine ligand 5 (RANTES) at 24 hours, but did reveal significant induction of macrophage inflammatory protein-1α (MIP-1α, aka chemokine C-C motif ligand, CCL3) and MIP-1β (aka CCL4) in *Anxa2*$^{+/+}$, but not *Anxa2*$^{-/-}$, mice ($p<0.05$, ANOVA, n=4 mice). In two additional models, we observed early PVR in *Anxa2*$^{+/+}$, but not *Anxa2*$^{-/-}$, mice after injection of either ARPE-19 cells or bone marrow derived *Anxa2*$^{+/+}$ macrophages; in both cases, host RPE cells migrated through the retinal wound to the vitreal surface of the retina at 4 weeks in *Anxa2*$^{+/+}$ eyes, but not in *Anxa2*$^{-/-}$ eyes. Further preliminary data indicate that treatment with intravitreal anti-A2 antibody, but not a control antibody, can prevent the PVR response in *Anxa2*$^{+/+}$ mice in the ARPE-19 cell model.

Conclusions: These findings indicate that the phospholipid-binding, pro-fibrinolytic protein annexin A2 is critical for the development of dispase-induced PVR in a mouse model. The results suggest further that recruitment of bone marrow-derived macrophages to the site of injury requires their expression of A2, and that the ability of macrophages to induce RPE cell migration also requires A2, which may enable expression of the chemotactic chemokines MIP-1α and MIP-1β. Finally, these data identify A2 as a possible new therapeutic target in PVR.