ROLE OF EXTRACELLULAR GRANZYME B (GZMB) IN THE DISRUPTION OF
THE OUTER BLOOD-RETINA BARRIER (OBRB) AND REMODELING OF BRUCH’S
MEMBRANE (BM)

by

Yuan Tian

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(NEUROSCIENCE)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

April 2020

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis entitled:

Role of extracellular Granzyme B (GzmB) in the disruption of the outer blood-retina barrier (oBRB) and remodeling of Bruch’s membrane (BM)

submitted by Yuan Tian in partial fulfillment of the requirements for the degree of Master of Science in Neuroscience

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Abstract

Age-related macular degeneration (AMD) is a poorly understood chronic inflammatory eye disease, which is characterized by the atrophy of retinal pigment epithelium (RPE) and the breakdown of Bruch’s membrane (BM), often leading to the formation of choroidal neovascularization (CNV), a hallmark of the exudative form of AMD. Currently, the effective treatments are anti-vascular endothelial growth factor (VEGF) therapies targeting CNV growth. Few studies have been conducted to explore the functions of Granzyme B (GzmB) in AMD. GzmB is a serine protease, stored in the granules of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), and also expressed by different immune and non-immune cells. It was initially believed to be involved in immune-targeted cell death, and recently many studies have revealed an additional role and an extracellular function in several chronic inflammatory disorders, such as asthma, multiple sclerosis (MS), and rheumatoid arthritis (RA). Whether extracellular GzmB activity is involved in the pathogenesis of AMD, a chronic inflammatory disease of the eye, is still not known. This present study hypothesized that extracellular GzmB may affect the function of the outer blood-retina barrier (oBRB) and BM remodeling by cleaving potential substrates in the outer retina. In order to test this hypothesis, this study used immunohistochemistry (IHC) to identify increased numbers of GzmB+ choroidal cells in aged and CNV human ocular tissues, most of which were confirmed to be choroidal mast cells. RPE cells were also found to be another source of GzmB in the outer retina. This study then used cell culture methods and immunocytochemistry (ICC) and western blot to show that exogenous GzmB cleaves RPE cell-cell adhesion proteins (ZO-1, JAM-A, occludin) and RPE-derived extracellular matrix (ECM) substrates (fibronectin, laminin), leading to damaged RPE barrier
function and the degradation of ECM components in BM *in vitro*. These results support the hypothesis that extracellular GzmB may play a role in disrupting the oBRB function and BM remodeling. This study is the first to explore such pathophysiological implications of extracellular GzmB activity in AMD.
Lay Summary

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly in North America. In the late stages of the “wet” form of AMD, patients suffer from permanent visual loss due to the growth of abnormal blood vessels in the outer retina. The outer blood-retina barrier (oBRB) is essential for the health of the retina; unknown causes lead to the disruption of oBRB, which consequently allows abnormal blood vessel growth. This study focuses on the activity of a candidate enzyme, granzyme B (GzmB) and its ability to affect the integrity of oBRB. Our findings showed that resident cells in the eye express this enzyme, which induces the oBRB breakdown. This study supports GzmB as a novel potential target for therapeutic intervention in AMD.
Preface

Joanne Matsubara and David Granville conceived and designed the study, obtained funding. All experiments, analyses, and data interpretation in Chapter 2 and Chapter 3 were done by Yuan Tian, under the consultation of Jing Cui. Writing was done by Yuan Tian, under the consultation of Dr. Joanne Matsubara. The work with human tissues reported in Chapters 2 was covered by UBC Ethics Certificate number H00-70411.

In chapter 1:

- Figure 1 was adapted from Molecular Aspects of Medicine, 33(4), Bhutto I, Lutty G, Understanding age-related macular degeneration (AMD): Relationships between the photoreceptor/retinal pigment epithelium/Bruch’s membrane/choriocapillaris complex, 295-317, Copyright (2012), with permission from Elsevier.

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and disease: challenging the dogma, Boivin WA, Cooper DM, Hiebert PR, Granville DJ, [2009], advance online publication, 21 September 2009 (doi: 10.1038/labinvest.2009.91.)

- Figure 5 was adapted from Nita M, Strzałka-Mrozik B, Grzybowski A, Mazurek U, Romaniuk W (2014). Age-related macular degeneration and changes in the extracellular matrix. Med Sci Monit. 2014 Jun 18;20:1003-16. doi: 10.12659/MSM.889887. Published under Creative Common Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) license.

A manuscript of the thesis was published with the title “Retinal Distribution and Extracellular Activity of Granzyme B: A Serine Protease that Degrades Retinal Pigment Epithelial Tight Junctions and Extracellular Matrix Proteins.” Joanne A. Matsubara, **Yuan Tian**, Jing Z. Cui, Matthew Zeglinski, Sho Hiroyasu, Christopher T. Turner and David J. Front Immunol. 07 April 2020; 11:574. doi: 10.3389/fimmu.2020.00574. Author Contributions: Joanne Matsubara and I wrote the manuscript. Joanne Matsubara and David Granville conceived and designed the study, obtained funding, analyzed and interpreted the data, and critically revised the manuscript. I and Jing Cui performed the experiments, analyzed and interpreted the data and generated figures. Matthew Zeglinski, Sho Hiroyasu, and Christopher Turner provided expert opinion and technical advice on GzmB assays and assisted with study design and interpretation. Matthew Zeglinski generated and purified endogenous GzmB from YT cells for use in this study.
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<th>Description</th>
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<tbody>
<tr>
<td>AMD</td>
<td>Age-Related Macular Degeneration</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>BFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BM</td>
<td>Bruch’s Membrane</td>
</tr>
<tr>
<td>CC</td>
<td>Choriocapillaris</td>
</tr>
<tr>
<td>CNV</td>
<td>Choroidal Neovascularization</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GA</td>
<td>Geographic Atrophy</td>
</tr>
<tr>
<td>GzmB</td>
<td>Granzyme B</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional Adhesion Molecule</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>MTT</td>
<td>Methylthiazolyldiphenyl-Tetrazolium Bromide</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>BRB</td>
<td>Blood-Retina Barrier</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>Pfn</td>
<td>Perforin</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal Pigment Epithelium</td>
</tr>
<tr>
<td>TEER</td>
<td>Trans-Epithelial Electrical Resistance</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight Junction</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula Occludens</td>
</tr>
</tbody>
</table>
Acknowledgements

I thank Dr. Joanne Matsubara for enlarging my vision of science and providing endless support to my study during this journey. I give my gratitude to all the past and present lab members in Dr. Joanne Matsubara’s lab, who have provided enduring help and inspired me to continue my work in this field. I owe particular thanks to Dr. Jing Cui, whose penetrating questions taught me to explore more deeply. I would like to thank Matthew Zeglinski, Sho Hiroyasu, and Christopher Turner in Dr. David Granville’s lab, who have provided expert opinion and technical advice for my study. I would like to extend my thanks to all committee members Dr. David Granville, Dr. Deborah Giaschi and Dr. Cal Roskelley for their academic support in my project, offering me precious advice for my questions. I would like to thank my dear boyfriend Stanley Chia for his love, patience, and companionship. Special thanks are owed to my parents, who have supported me throughout my years of education, both morally and financially.
Dedication

I dedicate this thesis to my parents, Quanzhou Tian and Yanqing Shan, who have always been there for me. Without their support at my back, I would not have achieved both success and happiness today. I am always grateful for their endless love throughout my life.
Chapter 1: Introduction

1.1 Age-Related Macular Degeneration

1.1.1 Background

Age-related macular degeneration (AMD) is the leading cause for blindness in the elderly (ages 60 years or older) in North America.¹ Many risk factors contribute to its complex, multifactorial pathogenesis, including smoking, hypertension, gender, family history, inflammation.²⁻⁶ AMD mainly affects the macular retina, which is responsible for the central vision. Typical symptoms include distorted images and blind spots in the center field of view.

In early AMD, drusen are found between the basal surface of retinal pigment epithelium (RPE) and the choroid. As the disease progresses, early AMD may evolve into two late forms: non-exudative or dry AMD (85 - 90 %) is featured by large or numerous drusen, pigment changes, RPE and photoreceptor atrophy; exudative or wet AMD (10 - 15 %) is characterized by choroidal neovascularization (CNV), secondary to the degradation of the RPE layer and Bruch’s membrane (BM) which allows leaky choroidal neo-vessels to invade the outer retina. Patients with non-exudative AMD may slowly develop limited vision, including reduced nocturnal vision, or difficulty in reading. Patients with exudative AMD may suffer a more rapid onset of retinal deterioration leading to permanent central vision loss within an aggressive timeline.⁷

Due to the lack of sufficient knowledge in AMD pathogenesis, current treatments can only slow down the progression of the disease instead of stopping its initial formation.⁸ Common approaches include antioxidant and mineral supplementation for early and intermediate stage non-exudative AMD. Laser ablation and anti-vascular endothelial growth factor (VEGF)
therapies are indicated for the exudative forms of AMD. In the Age-Related Eye Disease Study (AREDS), researchers found that uptake of daily supplements (vitamins C and E, beta carotene, zinc) reduces the risk of developing advanced AMD.\(^9\) Photocoagulation uses focused laser light to locally kill dying retinal cells with minimal damage to surrounding tissue; photodynamic therapy destroys abnormal new vessels through delivering photosensitizing agents in blood circulation prior to laser treatment and activating these agents by laser energy.\(^{10,11}\) Intravitreal injections of anti-angiogenic agents (VEGF inhibitors) inhibit the growth of CNV.\(^{12}\)

### 1.1.2 The Structure of the Outer Retina

The development of the eye happens between the third week and the tenth week during the process of embryogenesis. To differentiate the retina, the developing optic vesicle (neuroectoderm) forms distinct ocular tissues: the neural retina, RPE, and optic stalk which will hold the optic nerve. The mesenchyme (mesoderm) surrounding the optic cup contributes to the pigmented vascular layer, known as the choroid, which is in contact with the RPE.\(^{13}\) BM forms between the RPE and the mesenchyme; the differentiated RPE and RPE-derived production of basic fibroblast growth factor (BFGF) and vascular endothelial growth factor (VEGF) are essential in BM formation and the development of choroidal vasculature.\(^{7,14}\) During the retinal development, proteases (such as calpains and caspases) are needed to selectively eliminate inappropriate neuronal connections.\(^{15,16}\) Proteases are enzymes that catalyze proteolysis and promote the cleavage of proteins involved in many physiological and pathological processes; they also have roles in the retinal degeneration and angiogenesis.\(^{17}\) For example, Lambert et al found that matrix metalloproteinase (MMP)-2 and MMP-9 synergize in the course of experimental CNV and MMP-9 contributes to CNV.\(^{18,19}\) Another group also confirmed that
MMP-2 and MMP-9 cause alterations of the blood-retina barrier, by the proteolytic degradation of the tight junction protein occludin between endothelial cells.\(^\text{20}\)

In healthy human eyes, the RPE monolayer is the outermost layer of the retina and resides on the BM, forming a complex with choriocapillaris (CC), a layer of capillaries which is adjacent to the BM in the choroid (Figure 1-1). This RPE/BM/CC complex offers a supporting environment for photoreceptors (rods and cones) in the process of phototransduction, which switches photon energy into neuronal signals. In the center of the macula (5.5 mm), there is a foveal zone (1.5 mm), which has only cones and responsible for visual acuity; this foveal zone receives oxygen from the blood vessels in the choroid. The RPE cells, situated between photoreceptors and the choroid, provide nutrients to maintain the active metabolism of the overlying photoreceptors, phagocytize photoreceptor debris, form the outer blood-retinal barrier (oBRB), and also produce growth factors, e.g. fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), VEGF.\(^\text{21-27}\)

In addition to versatile RPE cells, the BM also plays an irreplaceable role in maintaining normal visual function. The BM is a connective tissue which is composed five layers: the basement membrane of RPE, the inner collagenous layer, the elastic layer, the outer collagenous layer, and the basement membrane of CC endothelium. RPE cells and CC endothelial cells synthesize protein components and contribute to their basement membrane respectively. Besides, invading fibroblasts can produce protein components including collagen and elastin in the extracellular spaces of BM contributing to the other three layers during the development.\(^\text{7,28,29}\) The main components of the different layers in BM are listed in Table 1-1, including fibronectin, laminin,
elastin and different types of collagen. Among these molecules, collagen IV subunits are specific to specialized basement membranes; besides, specific laminins (e.g., laminin 5) are synthesized by RPE cells to help themselves adhere to the BM interacting with integrin molecules; fibronectin is also an important constituent which is abundant in the innermost layers of BM. The damage of these key structural components would lead to pathological conditions in diseases. As a physical and biochemical barrier between RPE cells and CC, BM not only supports RPE cell adhesion, but also regulates the transport and diffusion of metabolic molecules between RPE cells and CC. Age-related changes of BM could progress into AMD pathological changes, through the uncontrolled complement activation, or the occurrence of CNV. Extracellular matrix (ECM) ligands (e.g., laminin) in BM promotes RPE attachment to its basement membrane through interacting with on integrin molecules its basal side, and these ECM ligands could undergo degeneration or remodeling during AMD progression, therefore inducing RPE detachment; this RPE detachment from ECM in BM may contribute to RPE cell death.
Figure 1-1: The Structure of the Retina around the Center of the Macula.

A cross section from a Macaque monkey shows the structure of the retina. To the left and right of the center of macula, the retina includes 10 layers from the inner most to the outer most: (1) the inner limiting membrane (ILM); (2) the retinal nerve fiber layer (RNFL); (3) the ganglion cell layer (GCL); (4) the inner plexiform layer (IPL); (5) the inner nuclear layer (INL); (6) the outer plexiform layer (OPL); (7) the outer nuclear layer (ONL); (8) the outer limiting membrane (OLM); (9) the photoreceptor layer (PR; IS, photoreceptor inner segments; OS, photoreceptor outer segments), and (10) the retinal pigmented epithelium (RPE) monolayer. ILM, RNFL, OLM are not discernible at this magnification. Bruch’s membrane (BM) is labeled by the red line. RPE cells reside on BM and form a complex with choriocapillaris (CC) in the choroid. The health of this micro-environment is essential for the physiology of the macula. [Adapted from Molecular Aspects of Medicine, 33(4), Bhutto I, Lutty G, Understanding age-related macular degeneration (AMD): Relationships between the photoreceptor/retinal pigment epithelium/Bruch’s membrane/choriocapillaris complex, 295-317, Copyright (2012), with permission from Elsevier.]
<table>
<thead>
<tr>
<th>Layer</th>
<th>Component</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>The basement membrane of RPE</td>
<td>Types IV and V collagen, laminin, heparan sulfate, chondroitin sulfate</td>
<td>29, 30, 31, 33, 34</td>
</tr>
<tr>
<td>The inner collagenous layer</td>
<td>Types I, III, and V collagen, fibronectin, chondroitin sulfate, dermatan sulfate</td>
<td>29, 30</td>
</tr>
<tr>
<td>The elastic layer</td>
<td>Type VI collagen, Elastin, fibronectin</td>
<td>7, 30</td>
</tr>
<tr>
<td>The outer collagenous layer</td>
<td>Types I, III, and V collagen, fibronectin, chondroitin sulfate, dermatan sulfate</td>
<td>29, 30</td>
</tr>
<tr>
<td>The basement membrane of choriocapillaries</td>
<td>Types IV, V, and VI collagen, laminin, heparan sulfate, chondroitin sulfate</td>
<td>29, 30, 31</td>
</tr>
</tbody>
</table>
1.1.3 Pathophysiology

Dry AMD

A hallmark of the early stage of dry AMD is the presence of drusen, yellowish deposits between the RPE and Bruch’s membrane (Figure 1-2). A few, small drusen may not affect vision, yet as the number and size increase, patients may experience impaired vision and those with large drusen are more likely to progress to late stages of AMD. Studies show that these pathological drusen are formed by proteins and lipids. Mullins et al identified different types of serum proteins including immunoglobulins, complement proteins (C5 and C5b-9 complex) in drusen, which may come from the choroidal circulation. Johnson et al immunohistochemically examined inflammatory elements related to complement activation in drusen and adjacent RPE cell cytoplasm, suggesting chronic inflammatory process may be involved in drusen biogenesis and AMD. The Alzheimer’s amyloid beta (A beta) peptide has been found associated with complement activation in pathologic deposits. Also, a proteomic study revealed a wide variety of protein components, showing that oxidative injury may contribute to the pathogenesis of AMD.

Along with drusen, another hallmark for dry AMD is abnormal pigmentation in RPE cells in the macular area. As the course of the disease advances towards geographic atrophy (GA), an advanced form of dry AMD, the loss of RPE cells appears and photoreceptors also undergo atrophy in the macula, leading to blind spots in central vision. Many studies have been focused on the imbalance of lipid metabolism and oxidative stress, which is a possible reason for RPE degeneration. Pigment changes, reduced melanosomes, and increased lipofuscin granules are observed in aged RPE cells. Toxic substances accumulate in RPE under oxidative stress, when
excess oxidized waste products are generated in abnormal lipid metabolism.\textsuperscript{21,42} Unfortunately, RPE cells and photoreceptors do not regenerate after they die, and presently there is no effective cure for GA.\textsuperscript{47} Preclinical and clinical studies on stem cell-based therapies showed that induced pluripotent stem cell (iPSC)-derived stem cells might be able to rescue degenerated RPE cells, thus the iPSC-derived RPE transplantation may provide potential therapeutics for AMD in the future.\textsuperscript{48,49}

**Wet AMD**

In wet AMD, abnormal choroidal blood vessels grow from the CC into the subretinal space underneath the macula. Briefly, the damaged integrity of the oBRB, composed of RPE tight junctions (TJ), fails at preventing nonspecific exchange of material between the choroid and the retina, and allows the formation of CNV (Figure 1-2). These abnormal blood vessels finally lead to hemorrhage in the retina, resulting in diminished vision or permanent loss of central vision. The pathogenetic mechanism underlying CNV is not well understood, but some investigators observed an imbalance between angiogenic factors and inhibitors regulating the angiogenesis process: either increased production of angiogenic factors or decreased production of angiogenic inhibitors may promote CC endothelial cells (EC) to proliferate towards the retina.\textsuperscript{50,51} RPE cells produce and secrete angiogenic factors, and particularly VEGF, which is the most important contributor to ocular angiogenesis.\textsuperscript{52} Increased VEGF levels have been observed in both the vitreous and CNV specimens from patients with wet AMD.\textsuperscript{53-55} Anti-VEGF therapies effectively inhibit the growth of CNV in experimental models and human AMD subjects.\textsuperscript{56,57} CNV development may also require matrix metalloproteases (MMPs), generated by vascular endothelium and immune cells such as macrophages, or other enzymes to break down the
surrounding ECM, permitting EC migration into the retina through incomplete BM.\textsuperscript{58,59}

Considering that current opinions point towards a chronic inflammatory component in AMD pathogenesis,\textsuperscript{60,61} it is reasonable to predict the possibility that inflammatory cells may induce breaks in BM via degrading ECM by proteases, such as MMPs, or other enzymes such as granzyme B.
Figure 1-2: The Histopathological Changes of the Outer Retina and the Choroid in AMD.

(a) An electron micrograph of mouse outer retina. From the top to the bottom, there are the choroid (CH), Bruch’s membrane (BM), retinal pigment epithelium (RPE), photoreceptor outer segments (OS). (b) Normal outer retina. (c) Lipofuscin accumulation in RPE cells. (d) Drusen between RPE cells and BM. (e) Atrophy of RPE cells and overlying photoreceptors (PR). (f) Choroidal neovascularization (CNV). IS, photoreceptor inner segments.

1.2 **Granzyme B (GzmB)**

Granzymes ("granule-secreted enzyme") are a family of conserved serine proteases, stored in the granules of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs). CTLs were first discovered to contain granzymes whose functions are involved in immune-targeted cell death.\(^{62}\) There are 5 types of granzymes (A, B, H, K, M) found in humans, and 11 types (A, B, C, D, E, F, G, K, L, M, and N) in mice.\(^{63,64}\) Granzyme B (GzmB) is the most abundant and well-studied member in this family. As mentioned before, proteases are enzymes with the ability to catalyze proteolysis, which means they can promote the cleavage of proteins under different physiological and pathological conditions.\(^{17}\) GzmB is a serine protease in which serine serves as the nucleophilic amino acid at its active site, and has a preference for cleaving the peptides adjacent to aspartate (Asp) residues.\(^{65,66}\) Many studies have revealed GzmB activity in different diseases.\(^{67,68}\) This enzyme is expressed not only by CTLs and NK cells, but also by other immune cells and non-immune cells, such as macrophages, mast cells, basophils, dendritic cells, epidermal keratinocytes, etc.\(^{65}\) Its gene maps to chromosome 14 on the human genome, containing five exons and four introns; the encoding product is a 32-kDa serine protease (GzmB), with two 6-stranded $\beta$ sheets and three trans-domain segments.\(^{64}\)

GzmB has an aspartic acid preference for cleaving peptides, due to its active site with an adjacent arginine (Arg) residue.\(^{69}\) To date, protease inhibitor-9 (PI-9) is the only known endogenous inhibitor of GzmB in humans, expressed by immune privileged tissues, CTLs, dendritic cells, mast cells, endothelial and mesothelial cells, liver cells, etc. PI-9 protects cells from GzmB-mediated cytotoxicity during immune responses.\(^{70-74}\) Serpina3N is a GzmB inhibitor found in mouse Sertoli cells; it was shown to inhibit both mouse and human GzmB *in vitro*.\(^{75}\)
Originally, GzmB was discovered to mediate perforin-dependent apoptosis in chronic inflammatory disorders, while recent studies have focused on its extracellular activity in various diseases, exploring its effects on ECM modification, cytokine processing, etc.65,76

1.2.1 GzmB-Mediated Apoptosis

The intracellular role of GzmB is immune-targeted cell death. To induce cell death, GzmB is facilitated by perforin (a Ca\(^{2+}\) dependent pore forming cytolytic protein) entering the cytoplasm of targeted cells.77 In a perforin-deficient mouse model, cytotoxicity mediated by T cells and NK cells is greatly impaired; in human subjects, cytotoxic lymphocyte dysfunction is associated with perforin deficiencies in genetic disorders.78,79 After GzmB enters targeted cells (internalization), it can activate multiple apoptotic pathways through targeting different substrates in the cytoplasm and the nucleus (Figure 1-3). GzmB cleaves the BH3 interacting-domain death agonist (Bid), a pro-apoptotic Bcl-2 protein, into a truncated form (gtBid) which recruits the pro-apoptotic protein Bax/Bak to the mitochondria to disrupt its membrane integrity, leading to the release of the apoptogenic factor, mitochondrial cytochrome c. Cytochrome c promotes the formation of apoptosome, which results in caspase activation.80–82

GzmB can also process pro-caspases in mitochondria-independent pathway.83–85 In addition, other intracellular substrates of GzmB include the inhibitor of caspase-activated deoxyribonuclease (ICAD), nuclear envelope intermediate filament protein (lamin B), poly (ADP ribose) polymerase (PARP), etc.86–88 Although there are unsolved questions about this complicated signaling network, GzmB-mediated apoptosis plays an important role in elimination of virus-infected cells and tumor cells.
Figure 1-3: The Intracellular Roles of GzmB.
GzmB enters targeted cells under the facilitation of perforin. Upon internalization, GzmB can initiate apoptosis through the mitochondrial pathway, or directly cleave caspases and/or caspase substrates, leading to caspase activation followed by cell death. (Reprinted by permission from [2009, Springer Nature]: [Springer Nature] [Laboratory Investigation] [Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma, Boivin WA, Cooper DM, Hiebert PR, Granville DJ], [2009], advance online publication, 21 September 2009 (doi: 10.1038/labinvest.2009.91).)
1.2.2 GzmB-Mediated Extracellular Cleavage

Many investigators proposed an extracellular function of GzmB in disease pathogenesis.\textsuperscript{89,90} The process of GzmB release into extracellular spaces has not been fully understood, but it had been proposed that GzmB may leak into ECM from “immunological synapses” during immune-targeted cell death: upon target cell engagement, GzmB molecules may be released through granule-mediated pathway or constitutive secretory pathway without granules, and then gradually accumulate in ECM spaces in autoimmune or chronic inflammatory conditions.\textsuperscript{91} Prakash et al recently found that GzmB can also be constitutively released from CTLs and NK cells in the absence of target cell engagement.\textsuperscript{92} GzmB becomes active once reaching the neutral pH of ECM milieu, or there may be an extracellular activator of inactive GzmB zymogen.\textsuperscript{92} Extracellular GzmB molecules do not degrade because of the lack of an endogenous extracellular GzmB inhibitor, which may be one factor related to extracellular GzmB activity in diseased tissues.\textsuperscript{65}

GzmB expression was discovered in non-cytotoxic cells like keratinocytes and chondrocytes.\textsuperscript{93,94} Wagner and colleagues found GzmB exists within the cytotoxic arsenal of neutrophils.\textsuperscript{95} Pardo and coworkers confirmed GzmB expression in activated mouse mast cells, possibly contributing to delayed cell death, increased vascular permeability, leukocyte extravasation and subsequent inflammatory processes in affected tissues.\textsuperscript{96} Other researchers discovered that human mast cells produce and release GzmB upon activation.\textsuperscript{97} More intriguingly, mast cell-derived GzmB decreased the efficacy of anti-angiogenic therapy by affecting pro-angiogenic factors, implying that inhibition of mast cell-derived GzmB may improve the efficacy of anti-angiogenic therapy.\textsuperscript{98} In addition, GzmB has also been reported in bodily fluids such as synovial fluid (SF) and
cerebrospinal fluid (CSF). The physiological level of GzmB is 20–40 pg/ml in the plasma of healthy individuals, yet extracellular GzmB concentration can be 1 – 1000 times higher than plasma levels under some pathological conditions, including inflammation, infections, arthritis, etc.

Several extracellular substrates of GzmB have been identified (Table 1-2). Figure 1-4 illustrates pathological consequences in different tissues caused by GzmB cleavage. Extracellular GzmB can cleave vitronectin, fibronectin, and laminin, leading to ECM remodeling and affecting the associated network of cells in many diseases. The loss of interaction between resident cells and adhesive ECM proteins caused by GzmB, can induce anoikis (cell death) in different tissues, including smooth muscle cells, endothelial cells, fibroblasts. GzmB-mediated ECM fibronectin fragments are chemotactic for immune cells, such as monocytes and neutrophils, and reinforce their recruitment during inflammatory processes. Moreover, the cleavage of the ECM by GzmB releases growth factors stored in the ECM; GzmB also promotes the maturation of secretion of cytokines. Apart from ECM proteolysis, GzmB also degrades cell receptors and produces auto-antigenic fragments or abnormal signaling. Gahring et al proved the cleavage of a neuronal glutamate receptor subunit 3 (GluR3) by GzmB generates the GluR3B auto-antigenic peptide; in addition, acetylcholine receptors (AchRs) at neuromuscular junctions are also cleaved by GzmB, generating auto-antigenic fragments that may trigger autoreactive responses. Loeb et al revealed fibroblast growth factor receptor-1 (FGFR1) and Notch1 to be substrates of GzmB which are important to cell proliferation and survival. In spite of the fact that extracellular activity of GzmB is an emerging topic in chronic inflammatory diseases, it is worth noting that there is no study that explores how GzmB behaves...
in the domain of the ocular tissues, and particularly in the development of AMD, a chronic inflammatory eye disease with several features known to be induced by GzmB in other diseases, such as the compromise of the blood barrier function and ECM remodeling. It is possible for GzmB to similarly function and cleave its substrates in AMD eyes, leading to ECM changes in BM, the degraded integrity of RPE monolayer and the increased release of cytokines, which may abnormally enhance the signaling along the angiogenesis pathway.
Figure 1-4: The Extracellular Roles of GzmB.
In chronic inflammatory tissues, extracellular GzmB can cleave substrates in extracellular matrix (ECM) such as fibronectin, leading to the loss of structural integrity and detachment-mediated cell death (anoikis). GzmB-mediated ECM fragments have chemotactic properties and can induce the recruitment of immune cells. The cleavage of GzmB can release growth factors and cytokines from ECM. GzmB can also degrade cell surface receptors and produce auto-antigenic fragments. {Reprinted by permission from [2009, Springer Nature]: [Springer Nature] [Laboratory Investigation] [Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma, Boivin WA, Cooper DM, Hiebert PR, Granville DJ], [2009], advance online publication, 21 September 2009 (doi: 10.1038/labinvest.2009.91.)}
### Table 1-2: Extracellular Substrates of GzmB.

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<td>Aggrecan; Cartilage proteoglycans; Decorin; Biglycan; Betaglycan; Fibromodulin; Brevican; Syndecan</td>
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<td>Cell receptors</td>
<td>Neuronal glutamate receptor (GluR); Acetylcholine receptors (AchRs); Fibroblast growth factor receptor-1 (FGFR1)</td>
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1.3 Rationale and Hypothesis

The defined functions of granzymes are still under diverse investigation, and the best studied ones among this family are GzmB and more recently GzmA. They are serine proteases with the ability to catalyze proteolysis, and their cleavage of proteins could promote pathological changes in different tissues.⁶⁵,⁹³,¹⁰⁴,¹¹⁹ Although GzmB and GzmA share some similar extracellular substrates, GzmB seems superior to be the focus of this thesis study, because the effects of its cleavage have ever been observed in the skin which also has a barrier function like the eye: GzmB is secreted by keratinocytes and immune cells in the dermis, cleaving its substrates and inducing a damaged skin barrier function and ECM remodeling.⁹³,¹⁰⁴,¹²⁰ Thus, it is possible that GzmB may play a similar role in the outer retina. Figure 1-5 shows the potential substrates of GzmB in the outer retina. In healthy eyes, the retina, RPE layer, BM and choroid are intact. As the outermost layer of the retina, the RPE monolayer resides on the BM and separates the retina from the choroidal blood vasculature. RPE cells connect to each other through TJ-associated proteins which consist of transmembrane proteins including occludin, junctional adhesion molecules (JAMs), adaptors proteins such as zonula occludens (ZOis), signaling proteins, and transcriptional and posttranscriptional regulators.¹²¹ The BM is composed of fibronectins, laminins, and several types of collagens, as shown by Table 1-1.³⁰–³⁵ It has been shown that GzmB can cleave some of these ECM components in other tissues, such as fibronectin and laminin.¹⁰⁶–¹⁰⁸ Also, Pardo et al reported that GzmB can induce a disorganization of endothelial cell-cell contacts, including JAM-A and ZO-1.⁹⁶ Except for GzmB, there are other proteases known to cleave ECM substrates and cell-cell junctions, which play essential roles in CNV development.¹⁷ As an example, MMPs are main enzymes responsible for ECM remodeling in diverse tissues, and abnormal activities of MMPs can lead to pro-angiogenic environment
favorable for CNV growth.\textsuperscript{122–125} MMPs can also proteolytically degrade the tight junction protein occludin between endothelial cells, and cause alterations of the blood-retina barrier.\textsuperscript{20} Based on these observational evidence, it is reasonable to predict that GzmB, which possesses a similarly extracellular function of cleavage like MMPs, may also be involved in ECM degeneration and the oBRB breakdown in CNV development. During the aging and AMD progression, extracellular GzmB may gradually act on these substrates, interrupting the RPE monolayer and BM. This cleavage of GzmB in the outer retina may not only disrupt the oBRB function, but also release potential inflammatory or angiogenetic factors (e.g. VEGF), which are normally restrained in ECM. Considering that the eye is an immune-privileged organ, with a physical blood-retina barrier, thus it might be more likely that resident cells in the eye, rather than infiltrating cells (e.g., neutrophils, eosinophils, and basophils), are the sources of GzmB in ocular tissues at the early stages of diseases. Yet, it is also not clear whether the infiltration of inflammatory cells from the systemic circulation might contribute to GzmB level in the outer retina after the disruption of blood-retina barrier and BM destruction.

This thesis study will address several questions:

\textbf{Question 1:} What are the cell sources of GzmB in the outer retina and the choroid?

\textbf{Question 2:} Does extracellular GzmB cleave its potential substrates in the outer retina?

\textbf{Question 3:} How does GzmB affect the oBRB and BM?

Question 1 will be discussed in Chapter 2. Questions 2 and 3 will be discussed in Chapter 3.

\textbf{Hypothesis:} GzmB is produced by resident cells in the outer retina and the choroid, and extracellular GzmB may cleave RPE tight junctions (TJ)-associated proteins and extracellular matrix (ECM) substrates in Bruch’s membrane (BM), and thereby disrupt the outer blood-retina barrier (oBRB) and promote abnormal BM remodeling.
**Aim 1**: To identify cellular sources of GzmB in the outer retina and the choroid.

**Aim 2**: To test whether exogenous GzmB could affect the oBRB function and BM remodeling through cleaving RPE TJ-associated proteins and BM components.

**Significance**: This study is the first to explore pathophysiological implications of extracellular GzmB activity in AMD. By cleaving RPE TJ proteins and BM components in the outer retina, GzmB has the potential to disrupt the oBRB function and induce BM remodeling *in vivo*. The role of GzmB in the outer retina and the choroid will add new knowledge to this yet unexplored area of study.

**Outline of Experiments**:

In Chapter 2, *ex vivo* experiments were designed to visualize GzmB expression in human outer retina and choroid by different staining methods, including the immunohistochemistry (IHC) and the immunofluorescence (IF). GzmB expression was first compared among healthy young and aged eyes, and diseased (AMD) eyes; toluidine blue was included to identify resident mast cells; anti-mast cell tryptase antibody was used to examine whether tryptase is co-localized with GzmB in mast cells; CD68 was chosen to identify resident macrophages.

In Chapter 3, RPE cell cultures were used to assess if exogenous GzmB could cleave RPE TJ-associated proteins in the outer retina by using the immunocytochemistry (ICC). At the same time, the barrier function of RPE cells was measured by both the trans-epithelial electrical resistance (TEER) and the FITC-dextran permeability assay, to confirm whether exogenous GzmB could disrupt the oBRB. Western Blot assay was conducted to examine whether exogenous GzmB could cleave ECM components in BM and induce BM remodeling.
Figure 1-5: The Potential Substrates of GzmB in the Outer Retina.

(A) The 5 layers of BM and major ECM proteins are shown in blue text. The PR and RPE sit above the BM on the RPE basement membrane. The choriocapillaris sits below BM, on its basement membrane. (B) Changes in the outer retina and the choroid associated with AMD pathology. RPE undergo atrophy and cell death, soft drusen deposits accumulate below RPE. Laminin, elastin and fibronectin undergo cleavage resulting in overall ECM fragility; altered forms of collagen increase, causing thickening of BM. Choriocapillaris undergoes atrophy, with closure of some vessels, identified as ghost vessels. (C) Enlargement of box in (A) depicting tight junctional and cell adhesion proteins on RPE cleaved by GzmB in blue text. Abbreviations: PR- photoreceptors; RPE- retinal pigment epithelium; RPE Bm- basement membrane of RPE; ICL- inner collagenous layer; EL- elastin layer; OCL- outer collagenous layer; ChC Bm- basement membrane of the choriocapillaris; COL- collagen; LAM- laminin; FN- fibronectin; Ghost Ves- closed lumen of vessel with dead endothelial cells; JAM- junctional adhesion molecules; ZO- zonula occludens. [Adapted from Nita M, Strzałka-Mrozik B, Grzybowski A, Mazurek U, Romaniuk W (2014). Age-related macular degeneration and changes in the extracellular matrix. Med Sci Monit. 2014 Jun 18;20:1003-16. doi: 10.12659/MSM.889887. Published under Creative Common Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) license.]
Chapter 2: Cell Sources of GzmB in Human Outer Retina and Choroid

2.1 Introduction

Although GzmB is expressed by a variety of cells, there are no studies reporting what cells, if any, are responsible for GzmB expression in the eye. Parkinson et al described that GzmB is colocalized to keratinocytes and to an increased mast cell population in ultraviolet light (UV)-induced skin photoaging in mouse model. In their work, the authors illustrated that keratinocytes and increased mast cells express GzmB and GzmB deficiency protects against wrinkle formation. They also demonstrated that GzmB can cleave ECM components such as fibronectin, leading to enhanced ECM degradation and a phenotype of aged skin. In a similar manner, as the retina ages or AMD develops, the complement system can be activated to produce excessive inflammatory mediators, and redundant oxidative species can also be generated from oxidative stress, triggered by an interaction of multiple risk factors including age, smoking and sunlight exposure. These changes of the immune system and inflammation responses might stimulate relevant cells to produce GzmB in the eye. I proposed that GzmB may also be expressed by potential resident cells in the outer retina and the choroid during the process of aging or AMD, to cleave potential substrates in extracellular milieu and cause AMD phenotypes. Therefore, Chapter 2 addressed Question 1: What are the cell sources of GzmB in the outer retina and the choroid? In this chapter, ex vivo experiments were designed to visualize GzmB expression in human outer retina and choroid by immunostaining methods. Specific cell-markers were also included to identify or compare cell types of GzmB⁺ cells. Next, GzmB expression was compared among healthy young and aged eyes, and diseased (AMD) eyes.
2.2 Methods

2.2.1 Donor Tissue Preparation

Donor tissues with the following pathologies were excluded in this study: local or systemic infection, progressive brain pathologies, systemic diseases of unknown origin, lymphoproliferative or myeloproliferative disorders or any intrinsic eye disease. All eyes were consented for research in this study.

Sixteen pairs of non-diseased donor eyes were obtained from the Eye Bank of British Columbia (Vancouver, British Columbia, Canada). Eyes were collected within 12 hours (hrs) from death and immersed in 10 % buffered formalin.

Eyes with geographic atrophy (GA), choroidal neovascularisation (CNV) or soft drusen deposits were obtained from the Department of Pathology, UBC. Tissue sections from the macular area were prepared as above, stained by hematoxylin and eosin, and then assessed by ophthalmic pathologists to identify and screen for ocular pathologies. In total, eight eyes with GA (from AMD), six eyes with CNV (from AMD), nine eyes with numerous soft drusen (from non-AMD eyes) were included in this study. To compare more than two groups, One-way ANOVA test and Tukey’s multiple comparisons post-hoc test were used, to compare the average ages of all groups (Table 2-1; Figure 2-1). Data are presented as Mean ± SEM. Statistical significance level was set at $p < 0.05$. No significant differences were found between the average age of old donors and the average ages of diseased donors, therefore old donor eyes can be used as age-matched non-diseased controls for diseased groups.
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Figure 2-1: The Comparison of Ages of Human Donors.
Analysis of ages of young donors (≤ 55 years, n = 8), old donors (≥ 70 years, n = 8), GA donors (n = 8), CNV donors (n = 6) and soft drusen donors (n = 9). One-way ANOVA Test and Tukey’s multiple comparisons post-hoc test (mean ± SED; **** p < 0.0001).
2.2.2 Immunohistochemistry (IHC)

Eight older donor (≥ 70 yrs) eyes, eight younger donor (≤ 55 yrs) eyes, eight eyes with GA (from AMD), six eyes with CNV (from AMD), nine eyes with numerous soft drusen (from non-AMD eyes) were used to measure the level of GzmB immunoreactivity in human outer retina and choroid. Eyes were fixed in 10 % formalin and embedded in paraffin. Tissue sections (6 µm thickness) were prepared by using a rotary microtome (Leica RM2255). The immunohistochemical procedures and analysis followed those previously described. After deparaffinization, sections were incubated with Proteinase K (1:20 in Tris-EDTA buffer) for 20 minutes (mins) for antigen retrieval and incubated with 3 % H₂O₂ in a humidified chamber to block endogenous peroxidase activities at room temperature (RT) for 10 mins. Tissues were then treated with 10 % normal goat serum (Vector Laboratories, S-1000) to block non-specific proteins for 20 mins and then probed with a primary antibody against GzmB (Abcam, ab4059) at RT for 1 hr before overnight incubation at 4° C. On the next day, biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, BA-1000) was used to incubate tissues at RT for 45 mins in dark room. ABC–AEC development system was prepared using VECTASTAIN® Elite® ABC HRP Kit (Vector Laboratories, PK-6100) and AEC Substrate Kit (Vector Laboratories, SK-4200). Slides were incubated with the prepared ABC reagent for 30 mins and then the AEC substrate working solution at RT for 8 - 13 mins. Mayer’s Hematoxylin solution (Sigma-Aldrich, MHS32) was used to counterstain tissues at RT for 30 seconds in humid chamber. Slides were mounted and covered with 1mm coverslips. In this experiment, human tonsil tissues from healthy donors were fixed in 10 % formalin and embedded in paraffin; tissue sections (6 µm thickness) were prepared by using the same rotary microtome and used as
positive controls, as recommended by the manufacturer of the antibody. The primary antibody was omitted and replaced with non-specific IgG in negative control tissue samples.

2.2.3 Toluidine Blue Staining

As a basic thiazine metachromatic dye with high affinity for acidic tissue components, toluidine blue is used to highlight mast cell granules, rich in acid mucopolysaccharides and glycoaminoglycans, in tissue sections.\textsuperscript{127,128} Healthy donor eyes were prepared to test whether mast cells are the source of GzmB immunoreactivity. Tissues were prepared as the method described in section 2.2.1, and then paraffin sections with intact retina/RPE/choroid were put in 60° C incubator to be deparaffinized for 20 mins, followed by hydration into distilled water. Sections were stained with 0.1 \% [weight (g)/volume (ml)] toluidine blue working solution (Sigma-Aldrich, T3260) for 2 - 3 mins, and then washed three times in distilled water. Sections were dehydrated quickly through 95 \% and 2 changes of 100 \% alcohol (10 dips each since toluidine blue stain fades quickly in alcohol), and then washed in 2 changes of xylene for 3 mins each. Afterwards, sections were coverslipped with resinous mounting medium (Permount, Fisher Chemical\textsuperscript{™}, SP15-500). After having been imaged and photographed by brightfield microscopy, slides were washed and measured for GzmB immunoreactivity in the outer retina and the choroid following the same method in section 2.2.2. Photographs taken of GzmB\textsuperscript{+} cells were then obtained and images were compared to toluidine blue images to determine single and double labeled cells.
2.2.4 Immunofluorescence (IF)

Healthy donor eyes were prepared following the method described in section 2.2.1. Paraffin sections were deparaffinized at 60°C for 20 mins. After deparaffinization, sections were incubated with Proteinase K (1:20 in Tris-EDTA buffer) for 20 mins for antigen retrieval and incubated with 3 % H₂O₂ in a humid chamber to block endogenous peroxidase activities at room temperature (RT) for 10 mins. Tissues were then treated with 10 % normal goat serum (Vector Laboratories, S-1000-20) to block non-specific proteins for 20 mins. To conduct GzmB and tryptase or GzmB and CD68 double labeling, a mixture of a primary antibody against GzmB (Abcam, ab4059) and a primary antibody against tryptase (Abcam, ab2378), or a mixture of a primary antibody against GzmB (Abcam, ab4059) and a primary antibody against human CD68 (Dako, M0876) were used to incubate slides at RT for 1 hr before leaving overnight at 4°C. On the following day, slides were washed with PBS, and then probed with a mixture of Alexa 488 conjugated goat anti-rabbit secondary antibody (Thermo Fisher Scientific, A-11070) and Alexa 546 conjugated goat anti-mouse secondary antibody (Thermo Fisher Scientific, A-21133) at RT for 1 hr in the dark room. After being washed with PBS, slides were mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, H-1200), and then covered with 1.5 mm coverslips. The Zeiss LSM 800 confocal laser scanning microscope was used to image samples at the wavelengths of 405 nm, 488 nm, and 543 nm.

2.2.5 Statistical Analysis

The GzmB level was visualized by immunohistochemistry. The GzmB⁺ cells in the choroidal layers (not including GzmB⁺ cells in choroidal vessels) and immunoreactive RPE cells were counted and normalized to 1050 μm length units of BM. The immunoreactivity was compared
among older donors \((n = 8)\) and younger donors \((n = 8)\), and between donors with GA, CNV or soft drusen \((n = 6\) for CNV, \(n = 8\) for GA, \(n = 9\) for soft drusen). All experiments were repeated in triplicate or quadruplicate. Statistical analysis was performed using GraphPad Prism Ver7. To compare more than two groups, one-way ANOVA test and Tukey’s multiple comparisons post-hoc test were used. Data are presented as Mean ± SEM. Statistical significance level was set at \(p < 0.05\).
2.3 Results

2.3.1 GzmB Choroidal Immunoreactivity in Older and CNV Donor Eyes

First, more GzmB⁺ choroidal cells were found in the outer retina and the choroid of postmortem eyes from older (≥ 70 yrs) than younger (≤ 55 yrs) donors (Figure 2-2 A, B, G). Next, donor eyes with choroidal neovascularization (CNV, wet AMD) contained greater numbers of GzmB⁺ choroidal cells compared to donor eyes with geographic atrophy (GA, dry AMD) or soft drusen (SD, an early precursor to AMD pathology) (Figure 2-2 C, D, E, F, H). The statistics showed that the number of GzmB⁺ choroidal cells was significantly higher in CNV eyes compared to those of all other groups, while the number of GzmB⁺ choroidal cells in older donor eyes was significantly higher than the number of younger donor eyes (Figure 2-2 I).

2.3.2 GzmB Immunoreactivity in RPE Cells in Eyes with Soft Drusen or CNV

RPE were also immunoreactive for GzmB, demonstrating significant labeling in the basal cytoplasmic compartment (Figure 2-2 C, D, E, H). RPE cells displayed weaker GzmB immunoreactivities in older, younger healthy eyes and GA eyes, compared to CNV eyes (Figure 2-2 A, B, E, F, G, H, J). Next, CNV eyes had fewer RPE cells containing GzmB compared to eyes with soft drusen, with one example shown in E near drusen site (black arrowhead) (Figure 2-2 C, D, E, F). One-way ANOVA Test and Tukey’s multiple comparisons post-hoc test showed that the number of GzmB⁺ RPE cells was significantly higher in soft drusen eyes compared to all other groups, while the number of GzmB⁺ RPE cells in CNV eyes was significantly higher than younger, older and GA eyes (Figure 2-2 J). These data in Figure 2-2 suggest that increased GzmB is associated with aging and some forms of AMD (CNV, soft drusen) in human eyes.
Figure 2-2: GzmB Immunoreactivity in Healthy and Diseased Human Donor Eyes.

Strong labeling with amino-ethyl carbazole chromogen (red) indicates GzmB$^+$ cells in the choroid (black arrows) and as well in the basal compartment of the RPE cell (black arrowheads). Blue arrows point to GzmB staining in the extracellular spaces in the choroid. (A-B) GzmB$^+$ cells in older healthy eyes. (C-D) GzmB$^+$ cells in eyes with soft drusen. (E-F) GzmB$^+$ cells in CNV eyes. (G) An example of GzmB$^+$ choroidal cell in a younger healthy eye (black arrow). (H) An example of GzmB$^+$ choroidal cell in a diseased, GA eye (black arrow). (I) Analysis of GzmB immunoreactivity of choroidal cells in older (≥ 70 years, n = 8) and younger (≤ 55 years, n = 8) healthy donor eyes, CNV eyes (n = 6), GA eyes (n = 8) and eyes with soft drusen (n = 9). One-way ANOVA Test and Tukey’s multiple comparisons post-hoc test (* p < 0.05; ** p < 0.01). (J) Analysis of GzmB immunoreactivity of RPE cells in older (≥ 70 years, n = 8) and younger (≤ 55 years, n = 8) healthy donor eyes, CNV eyes (n = 6), GA eyes (n = 8) and eyes with soft drusen (n = 9). One-way ANOVA Test and Tukey’s multiple comparisons post-hoc test. (* p < 0.05). Scale bar: 20 µm.
2.3.3 Verification of Mast Cells in Human Donor Eyes

To explore GzmB⁺ cell types in the choroid, we first measured GzmB immunoreactivity in healthy donor eyes \((n = 5)\), and then used toluidine blue to identify those mast cells that were immunoreactive for GzmB. Toluidine blue has a high affinity for acidic tissue components, and is commonly used to highlight or visualize the granules of mast cells. Results found many GzmB⁺ cells (red) were also positive for toluidine blue (purple), which confirms that choroidal mast cells are a cellular source of GzmB in human choroid (Figure 2-3).

![Figure 2-3](image)

Figure 2-3: Verification of Mast Cell Origin by Toluidine Blue Staining and Double Labeling with GzmB Immunoreactivity in Healthy Donor Choroids.

\((A)\). GzmB AEC immunoreactivity reveals a GzmB⁺ cell in choroid (black arrow). \((B)\). Sequential staining with toluidine blue on the same section demonstrates that the GzmB⁺ cell is also positive for toluidine blue, a marker of mast cells. Higher powers of the boxed region in \((A)\) and \((B)\) are shown in \((C)\) and \((D)\) respectively. Scale bar = 100 µm in \((A), (B)\) and 50 µm in \((C), (D)\).
2.3.4 Different Populations of Mast Cells in Human Donor Eyes

Next, we assessed if GzmB+ mast cells in healthy human choroids could also express tryptase, which has been reported to contribute to pathological changes in AMD. Lutty et al reported that the increased degranulation of mast cells was observed in all AMD choroids, compared to healthy controls. During the degranulation process, choroidal mast cells release proteolytic enzymes or other molecules from granules (secretory vesicles). Lutty et al also reported that mast cell-derived tryptase binds to BM in GA, suggesting it may cause ECM modifications which contribute to RPE atrophy and choroidal thinning in GA.\textsuperscript{129,130} Interestingly, another study demonstrated that tryptase does not alter the expression of ZO-1 and JAM-A in RPE cells.\textsuperscript{131} Thus, it was questioned whether both tryptase and GzmB could independently contribute to changes during aging and pathological changes during AMD development. To test whether GzmB exists in the same population of mast cells that contain tryptase, we performed double immunolabeling of GzmB and tryptase in healthy donor eyes ($n = 3$). This result showed that GzmB+ mast cells do not overlap with tryptase+ mast cells in healthy donor choroids, suggesting two subpopulations of mast cells: GzmB+ or tryptase+ populations of choroidal mast cells (Figure 2-4). This is a novel finding which might suggest that GzmB and tryptase could induce different changes in the process of aging or in the development of AMD.
Figure 2-4: GzmB and Tryptase Immunofluorescence in Healthy Donor Choroids.

(A) An example of a double-labeled choroidal tissue section depicting GzmB (green) and tryptase (red) immunofluorescence in separate mast cells. DAPI (405 nm) labeling of nuclei is shown in blue. The yellow dashed line indicates the approximate border between RPE layer and choroid. (B) Green channel image shows the single choroid cell is GzmB positive (green). (C) Red channel image shows the single choroid cell is tryptase positive (red). (D) Blue channel image shows DAPI (405 nm) labeling of nuclei (blue). Scale bar = 20 μm.
2.3.5 Verification of Macrophages in Human Donor Eyes

To further explore GzmB$^+$ cell types in the choroid, we also performed the double labeling using antibodies against GzmB and CD68, a marker of macrophages, in healthy donor eyes ($n = 3$). Result showed a few choroidal cells were CD68$^+$ macrophages (red), which were also immunoreactive for GzmB (green), suggesting that macrophages contain GzmB in the choroid of human outer retina. (Figure 2-5).

![Figure 2-5: Verification of Macrophages by GzmB and CD68 Double Labeling in Healthy Donor Choroids.](image)

(A) An example of a double-labeled choroidal tissue section depicting GzmB (green) and CD68 (red) immunofluorescence in macrophages (the white arrow points to one yellow cell double stained for GzmB and CD68; the arrowhead points to a single GzmB-labeled cell in the choroid). DAPI (405 nm) labeling of nuclei is shown in blue. Autofluorescence was seen along the apical side of RPE layer. (B) Green channel image shows two choroidal cells are GzmB positive (green). (C) Red channel image shows one choroidal cell is CD68 positive (red). (D) Blue channel image shows DAPI (405 nm) labeling of nuclei (blue). Scale bar = 20 µm
Chapter 3: GzmB-Mediated Extracellular Cleavage in the Outer Blood-Retinal Barrier (oBRB) Disruption and Bruch’s Membrane (BM) Remodeling

3.1 Introduction

Chapter 3 focused on answering Question 2: Does extracellular GzmB cleave its potential substrates in the outer retina, and Question 3: How does GzmB affect the oBRB and BM? In this chapter, RPE cells were cultured and they formed a viable monolayer, mimicking the morphology and function similar to those of RPE cells in vivo. This model provides a convenient way to study the GzmB cleavage function in the outer retina. As these cultured RPE cells grow and contact each other, they form intracellular tight junctions (TJs) and adherens junctions (AJs). TJs between RPE cells were studied in the GzmB cleavage assay, because they form the outer blood-retina barrier (oBRB) which is disrupted in CNV. TJs consist of transmembrane proteins including occludin, junctional adhesion molecules (JAMs), adaptors proteins such as zonula occludens (ZO)s, signaling proteins, and transcriptional and posttranscriptional regulators. Research showed that GzmB can induce a disorganization of endothelial cell-cell contacts, including ZO-1 and JAM-A. Therefore, in the GzmB cleavage assay, the immunocytochemistry was used to visualize the expression of ZO-1, JAM-A, and also another main transmembrane protein occludin (Table 3-1). In addition, the barrier function of cultured RPE cells was measured through the trans-epithelial electrical resistance (TEER) and the permeability across RPE monolayer, to examine whether exogenous GzmB could cleave TJs, and therefore potentially disrupt the oBRB.

Previous studies have demonstrated important ECM components existed in the BM (Table 1-1), which are responsible for its normal functions; some of these proteins have been confirmed to be
substrates of GzmB in other tissues, including fibronectin and laminin. As described previously, fibronectin is abundant in three innermost layers of the BM, laminin-5 molecules interact with integrin molecules and help RPE cells adhere to the BM, and collagen IV exists specifically in two basement membranes of the BM, therefore these candidates were chosen to be tested in the GzmB cleavage assay (Table 3-1). Briefly, cultured RPE cells were used to produce and secrete these ECM protein molecules in vitro, and western blot was undertaken to detect cleaved bands of ECM proteins released into the culture medium after exogenous application of GzmB.
Table 3-1: List of Extracellular Matrix (ECM) and RPE Tight Junctional (TJ)-Associated Proteins Tested in GzmB Cleavage Assays in Chapter 3.

<table>
<thead>
<tr>
<th>Components</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPE Tight junction (TJ)</td>
<td>Zonula Occludens-1 (ZO-1)</td>
</tr>
<tr>
<td>RPE Tight junction (TJ)</td>
<td>Junctional Adhesion Molecule-A (JAM-A)</td>
</tr>
<tr>
<td>RPE Tight junction (TJ)</td>
<td>Occludin</td>
</tr>
<tr>
<td>Extracellular Matrix (ECM)</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>Extracellular Matrix (ECM)</td>
<td>Laminin-5 (Laminin-332)</td>
</tr>
<tr>
<td>Extracellular Matrix (ECM)</td>
<td>Collagen-IV</td>
</tr>
</tbody>
</table>
3.2 Methods

3.2.1 Cell Culture
Passage 3 - 5 primary fetal RPE cells and passage 5 - 7 ARPE-19 cells (a human RPE cell line, ATCC) were grown and maintained in Dulbecco’s Modified Eagle Medium/F12 medium (DMEM, Life Technologies) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin and kept in a humidified chamber with 5% CO2 at 37°C. Primary RPE were isolated from human fetal donor eyes, with no known pathology, that were consented for research. Exogenous GzmB used in this study was isolated from YT cells, a NK cell line devoid of GzmA and GzmK activity. Procedures of GzmB extraction and purification were modified from Shi et al.135

3.2.2 Methylthiazolyldiphenyl-Tetrazolium Bromide (MTT) Cell Viability Assay
Fifty mg methylthiazolyldiphenyl-tetrazolium bromide (MTT) powder (Sigma, M5655) was dissolved in 10 ml sterilized PBS, filtered through a 0.2 µm filter and stored at 4°C as MTT stock buffer (5 mg/ml). ARPE-19 cells were grown to confluence on 96-well plates. Cells were washed with PBS and starved for 3 hrs in serum-free DMEM before GzmB stimulation with increasing doses (0 nM, 10 nM, 20 nM, 50 nM, 100 nM), and then the 96-well plate was cultured in 37°C incubator for 24 hrs. After 24 hrs, culture medium was removed, 250 µl of 0.5 mg/ml MTT buffer (1:10 dilution in DMEM) was added to each well, and the plate was incubated at 37°C for 2 hrs. After that, MTT solution was removed, 250 µl DMSO was added to each well and the 96-well plate was re-incubated at 37°C for 15 - 20 mins. In the end, the absorbance at 570 nm wavelength was read using the Hybrid Multi-Mode Reader (BioTek Synergy H1).
3.2.3 Immunocytochemistry (ICC)

Tight junctional (TJ) proteins were visualized by immunofluorescence on ARPE19 cells grown and stimulated by GzmB in chamber slides. Approximately $1.6 \times 10^5 / \text{cm}^2$ ARPE-19 cells were seeded on 8-well chamber slides (LabTek II) in DMEM containing 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin and kept in a humidified chamber with 5 % CO₂ at 37°C. When ARPE-19 cells reached confluence, they were washed with PBS, and starved for 2 hrs in serum-free DMEM before GzmB stimulation. Next, DMEM medium with GzmB (0 nM, 1 nM, 10 nM, 50 nM, 100 nM) was added to culture slides at 37°C for 6 hrs. After incubation, culture medium was removed and cells were fixed in situ with 100 % methanol on ice for 15 mins. Cells were then washed with PBS, and blocked in 2 % (wt/vol) normal goat serum (Vector Laboratories, S-1000) at RT for 30 mins, followed by incubation with ZO-1 polyclonal antibody (ThermoFisher, 61-7300), JAM-A (CD321) monoclonal antibody (ThermoFisher, 14-3219-82), or Occludin monoclonal antibody (E-5) (Santa Cruz Biotechnology, sc-133256) in PBS with 0.1%(vol/vol) TX-100 for at RT 1 hr before overnight incubation at 4°C. Primary antibodies were omitted for negative controls. Next, cells were washed with PBS, and then incubated with Alexa 488 conjugated goat anti-rabbit secondary antibody (ThermoFisher, A-11070) or Alexa 488 conjugated goat anti-mouse secondary antibody (ThermoFisher, A-11001) at RT for 1 hr in the dark room. After being washed with PBS, a blade was used to remove chambers and silicon tapes, and marker pen was used to label the locations of different chambers on slides. Slides were mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, H-1200) and then covered with 1.5 mm coverslips. A Zeiss LSM 800 confocal laser scanning microscope was used to image samples at 405 nm and 488 nm wavelengths: a control chamber, which was not stimulated by GzmB, was first observed to find a layer clearly showing RPE nuclei and a
strongest signal for the targeted TJ-associated protein by manually adjusting the focus. All software settings were identified with the control section and used throughout for all samples. Next, for each consecutive sample in the experiment the previously identified control software settings were used, and the focus was manually adjusted to identify the focal plane with the strongest signals for targeted TJ-associated proteins. Images were obtained at 40x resolution, and exported as tiff files. The immunofluorescence was quantified using Adobe Photoshop (Adobe Inc., San Jose, California): the “color range” tool was used to select a range of colors for the immunostaining of the targeted protein on an image taken from the control chamber, and then the number of pixels of all selected colors was measured by using the “histogram” tool; the same range of colors for the immunostaining was saved and applied to all images to get the numbers of pixels; all pixels were used for statistical analysis afterwards.

### 3.2.4 Western Blot

To detect the cleavage products in primary RPE cell after GzmB stimulation, confluent primary RPE cells, grown in 6-well plates, were stimulated with GzmB (0 nM or 100 nM) at 37°C for 5 hrs. After 5 hrs, supernatants were collected, centrifuged at 13,000 rpm at 4°C for 10 mins and aliquoted without disturbing sediments at the bottom of the vial. Adherent RPE cells were rinsed with PBS twice on ice, and treated with 200 µl of lysis buffer (10 ml RIPA lysis buffer with proteinase inhibitor cocktail (Roche, 5892791001)). Next, the lysates were collected from each well, centrifuged at 13,000 rpm at 4°C for 10 mins, and aliquoted without disturbing the genomic DNA cluster at the bottom of the vial. Cell lysate samples were quantified by Pierce TM BCA Protein Assay Kit (Thermo Fisher Scientific, 23225) for total protein concentration, read at 562 mm wavelength by using Synergy H1 Hybrid Multi-Mode Microplate Reader.
(BioTek). The Gen5 Software generated data for the protein concentrations of all protein samples.

Next, 10 µl of Fisher BioReagents™ EZ-Run™ Prestained Rec Protein Ladder (Fisher BioReagents™, BP3603500) and each sample with loading buffer (2:1) were loaded on 1.5 mm gels, and run on the gels using the Bio-Rad PowerPac 300 Electrophoresis Power Supply under reducing conditions at 120 V for 7 - 8 hrs. Total proteins were transferred from the gels to the membranes (Immobilon® Transfer membrane, Millipore, ISEQ00010, pore size 0.2 µm) at 100 V for 120 mins. When the transfer was complete, membranes were incubated with 5 % [weight (g)/volume (ml)] skimmed milk in 0.2 % Tween-1X PBS at room temperature for 60 mins. Next, membranes were incubated with primary antibodies anti-fibronectin (Abcam, ab2413), anti-laminin 5 (Abcam, ab14509) or anti-collagen-IV (Santa Cruz, sc-398655) in blocking buffer (1:1000) at 4° C overnight; vinculin (Abcam, ab129002) was used as internal protein loading control. Secondary antibodies including goat anti-rabbit IgG HRP affinity purified PAb (R&D, HAF008) and donkey anti-mouse IgG HRP affinity purified PAb (R&D, HAF018) in washing buffer (1:1000) were used to incubate membranes in a shallow tray at room temperature for 2 hrs. Targeted proteins on membranes were detected with Pierce™ ECL Western Blotting Substrate Kit (ThermoFisher, 32106). The protein band intensity was measured using Image J (NIH, Bethesda, MD).

3.2.5 Trans-Epithelial Electrical Resistance (TEER)

The barrier function of primary RPE cells was measured using the Electric Cell-substrate Impedance Sensing (ECIS) ZΘ system (Applied Biophysics). Briefly, approximately $1.6 \times 10^5$
primary RPE cells were seeded in complete growth medium (DMEM containing 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin) on laminin-coated transwell inserts (0.4 µm pore size, 12 mm diameter, Fisher Scientific) and kept in a humidified incubator with 5 % CO₂ at 37°C. After overnight incubation, medium was changed to 1 % FBS. After cells reached confluence on transwell inserts in 24-well plates, the inserts were transferred into an 8-well ECIS trans-filter adapter (Applied Biophysics), connecting to the ECIS ZΩ platform. Cells were then washed with PBS and starved for 2 hrs in serum-free DMEM before GzmB stimulation. Next, DMEM medium with 0 nM (controls) or 100 nM GzmB was placed in the upper inserts of the transwell systems, and cells were returned to the incubator and incubated for 24 hrs on the ECIS ZΩ platform. Experiments were conducted after the electrical resistance reached a steady state. The time at which GzmB stimulation began was referred to as 0 hr, and the trans-epithelial electrical resistance (TEER) across the RPE monolayer was recorded over 24 hrs. An empty well without cells was used as an endogenous control.

3.2.6 FITC Dextran Permeability Assay
Approximately 1.6×10⁵/cm² primary RPE or ARPE-19 cells were seeded in complete growth medium (DMEM containing 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin) on laminin-coated transwell inserts (0.4 µm pore size, 12 mm diameter, Fisher Scientific) and kept in a humidified chamber with 5 % CO₂ at 37°C. After overnight incubation, medium was changed to 1 % FBS. When cells reached confluence on transwell inserts in 24-well plates, they were washed with PBS and starved for 2 hrs in serum-free DMEM before GzmB stimulation. DMEM medium with 0 nM (controls) or 100 nM GzmB was placed in the upper inserts of the transwell systems at 37°C for 5 hrs. After 5 hrs, culture medium was removed without
disturbing cells, and DMEM culture medium with 1 mg/ml FITC-labeled dextran (70 kDa) was added into all inserts for another 5 hrs. Next, all inserts were removed and the fluorescence intensity at 490 nm (excitation)/520 nm (emission) wavelengths was read using the Hybrid Multi-Mode Reader (BioTek Synergy H1). Next, membranes at the bottom of transwell inserts were cut out and adherent ARPE-19 cells underwent immunocytochemistry with anti-ZO-1 antibody following the method described in section 3.2.3. An LSM 800 laser-scanning microscope was used to obtain images at 405 nm and 488 nm wavelengths, as described previously. The pixel of immunofluorescence was measured using Adobe Photoshop (Adobe Inc., San Jose, California).

### 3.2.7 Statistical Analysis

All experiments were repeated in triplicate or quadruplicate. Statistical analysis was performed using GraphPad Prism Ver7. To compare two groups, independent sample T-test was used for ICC of ARPE-19 cell samples, western blot and FITC-dextran permeability assay of primary RPE cell samples. To compare more than two groups, one-way ANOVA test and Tukey’s multiple comparisons post-hoc test was used for MTT cell viability assay and ICC of ARPE-19 cell samples. Data are presented as Mean ± SEM. Statistical significance level was set at $p < 0.05$. 
3.3 Results

3.3.1 Extracellular GzmB Does Not Affect the Viability of RPE Cells

We used MTT cell viability assay to test whether GzmB stimulation could affect RPE cell viability. The results showed that ARPE-19 cells stimulated with exogenous GzmB at concentrations from 10 nM - 100 nM for 24 hrs demonstrated no changes in cell viability compared to controls (GzmB at 0 nM), suggesting GzmB stimulation in vitro at tested dosages does not affect RPE cell viability (Figure 3-1). Thus, these in vitro cultured RPE cells were used for the following morphological and functional studies of GzmB cleavage assays.
Figure 3-1: The Effect of Exogenous GzmB on the Viability of ARPE-19 Cells.
ARPE-19 cells were stimulated with exogenous GzmB (10 nM, 20 nM, 50 nM, 100 nM) for 24 hrs, and compared to controls (GzmB = 0 nM). No significant changes were observed in the viability at any of the GzmB concentrations. Data were analyzed using one-way ANOVA test and Tukey's multiple comparisons post-hoc test (mean ± SEM, n = 5 per group).
3.3.2 Extracellular GzmB Cleaves Tight Junctional (TJ) Proteins on RPE-19 cells

To explore the effects of exogenous GzmB, ARPE-19 cells were grown and stimulated with GzmB at 0, 1, 10, 50 and 100 nM for 6 hrs in chamber slides. Zonula occludens-1 (ZO-1), a TJ protein, was visualized by immunofluorescence. Cell boundaries marked by ZO-1 were clearly shown in control group and most areas of ARPE-19 cells stimulated by 1 nM and 10 nM GzmB. However, the fluorescence signal was much weaker in the group with 50 nM GzmB, and the loss of the fluorescence signal associated with ZO-1 immunoreactivity implied a significant degradation of ZO-1 by almost 90% in cells stimulated by 100 nM GzmB (Figure 3-2).

Therefore, we chose 100 nM GzmB to stimulate cells in following in vitro assays. It is of note that a cellular internalization effect was seen in Figure 3-2 (A, B, C, D)\textsuperscript{136}, which may potentially mean that RPE ZO-1 (peripheral membrane protein) was endocytosed in the cytoplasm during the permeabilization step (Triton X-100) in the ICC.

*In vitro* studies were undertaken to assess the impact of exogenous GzmB on two additional RPE TJ proteins. The immunoreactivities demonstrated TJ contacts (green, 488 nm) on ARPE-19 cells in control groups (0 nM GzmB), while exogenous GzmB stimulation (100 nM) degraded JAM-A and occludin on ARPE-19, as shown by the loss of fluorescence signal (Figure 3-3, Figure 3-4). Both differences between control and GzmB-stimulated groups were statistically significant. Similarly, the internalization effect was also observed in Figure 3-3 (A) and Figure 3-4 (A). RPE JAM-A and occludin are transmembrane proteins, thus it suggests that the permeabilization step (Triton X-100) could be omitted to avoid the internalization and improve the quality of images in future experiments.
Figure 3-2: The Reduced Immunoreactivity of Zonula Occludens-1 (ZO-1) in ARPE-19 Cells Stimulated by Exogenous GzmB.

(A-E) ARPE-19 cells were stimulated with exogenous GzmB (0 nM, 1 nM, 10 nM, 50 nM, 100 nM) for 6 hrs. The immunoreactivity of ZO-1 tight junctional contacts (green, 488 nm) between cells (white arrowheads) was reduced as the concentration increased. (F) Omission of primary antibody demonstrates a lack of green ZO-1 immunolabeling. DAPI (blue, 405 nm) labeling of nuclei is shown in blue. Scale Bar = 20 μm. (G) Significant differences were observed between controls (0 nM GzmB) and stimulated groups with 50 nM and 100 nM GzmB. A one-way ANOVA and Tukey’s multiple comparisons post-hoc test (n = 6 per group) were used to compare the control group with GzmB-stimulated groups: mean ± SEM; * p < 0.05; *** p < 0.001.
Figure 3-3: The Reduced Immunoreactivity of Junctional Adhesion Molecule-A (JAM-A) in ARPE-19 Cells Stimulated by Exogenous GzmB.

After 6 hrs with GzmB stimulation (100 nM), JAM-A immunoreactivity (green, 488 nm) is reduced on ARPE-19, compared to controls (0 nM GzmB). DAPI (blue, 405 nm) labeling of nuclei is shown in blue. Omission of primary antibody demonstrates no immunoreactivity in negative control. Significant differences were found using an independent-sample T-Test ($n = 6$ per group) between control and GzmB-stimulated group: mean ± SEM; ** $p < 0.01$. Scale bar = 20 µm.
Figure 3-4: The Reduced Immunoreactivity of Occludin in ARPE-19 Cells Stimulated by Exogenous GzmB.
After 6 hrs with GzmB stimulation (100 nM), occludin immunoreactivity (green, 488 nm) is reduced on ARPE-19, compared to controls (0 nM GzmB). DAPI (blue, 405 nm) labeling of nuclei is shown in blue. Omission of primary antibody demonstrates no immunoreactivity in negative control. Significant differences were found using an independent-sample T-Test (n = 6 per group) between control and GzmB-stimulated group: mean ± SEM; ** p < 0.01. Scale bar = 20 µm.
3.3.3 Extracellular GzmB Decreases the Barrier Function of RPE Cells

The barrier function of primary RPE cells was tested using the trans-epithelial electrical resistance (TEER) and the FITC-labeled dextran flux assay in confluent primary RPE cells grown on transwell inserts. In Figure 3-5 (A), TEER measurements of resting RPE cells ranged from 160 - 180 ohms. After initiation of GzmB stimulation (time = 0 hr), the resistance of RPE cultures decreased to 125 - 135 ohms (time = 10 hrs) as shown by yellow and grey traces. The control cells maintained resistance at 160 - 165 ohms (time = 10 hrs) as shown by the blue and orange traces. Furthermore, the fluorescence flux associated with FITC-labeled dextrans (70 kDa) from the upper inserts to the lower compartments was measured after exogenous GzmB (100 nM) stimulation for 5 hrs. The fluorescence flux through primary RPE monolayer was read using synergy H1 microplate reader (excitation: 490 nm; emission: 520 nm). In Figure 3-5 (B), the fluorescent intensity was higher in lower wells of RPE cells stimulated by GzmB compared to that in controls ($p < 0.01$), suggesting GzmB stimulation can affect the permeability of RPE monolayer in vitro. Subsequent ZO-1 immunocytochemistry on the same RPE cells attached to membranes of transwell inserts demonstrated a loss of ZO-1 immunofluorescence ($p < 0.0001$), consistent with the increased permeability observed by the FITC-dextran increase in the lower compartments (Figure 3-5 C, D, E).
Figure 3-5: The Damaged Primary RPE Barrier Function after GzmB Stimulation.
To test the primary RPE barrier function, cells were grown on transwell inserts until confluent, and stimulated with GzmB (100 nM) for 5 hrs. (A) Trans-epithelial resistance measurements on resting cells range from 160-180 ohms. After initiation of GzmB stimulation (time = 0), the resistance of RPE cultures decreases to 135-125 ohms (time = 10 hrs) as shown by yellow and grey traces. The control cells maintained resistance at 160-165 ohms (time = 10 hrs) as shown by the blue and orange traces. (B) FITC-dextran flux assay demonstrated increased FITC-dextran fluorescence intensity measured in the lower compartment of GzmB stimulated wells compared to control wells. Independent-sample T-Test (n = 6) was used to measure the fluorescence intensity of FITC-dextran flux between control and GzmB-stimulated group: mean ± SEM; ** p < 0.01. (C-E) After the flux assay, transwell insert membranes on which cells were attached were cut out and subjected to immunocytochemistry. The ZO-1 immunoreactivity on primary RPE cells was decreased in control wells (0 nM GzmB) shown in (C), compared to that in experimental wells (100 nM GzmB) shown in (D). DAPI (405 nm) labeling of nuclei is shown in blue. (E) The ZO-1 level was quantified between controls and GzmB-stimulated wells using an independent-sample T-Test (n = 6): mean ± SEM; **** p < 0.0001.
3.3.4 Extracellular GzmB Degrades RPE-derived Extracellular Matrix (ECM) Proteins

The effect of exogenous GzmB on RPE-derived fibronectin, laminin-5 and collagen-IV was studied in vitro. Primary RPE cultures were grown to confluence and stimulated with exogenous GzmB (0 nM, controls vs 100 nM GzmB) for 5 hrs. These cultured RPE cells could produce and secrete ECM protein molecules in vitro, which may adhere to RPE basal and lateral sides, and be degraded after GzmB stimulation. GzmB cleaved bands may then be released into the culture medium, thus supernatant samples containing these cleaved bands were probed with primary antibodies against fibronectin, laminin-5 and collagen-IV by western blot. For fibronectin and laminin-5, several cleaved bands (red arrows) were revealed in those samples stimulated with 100 nM GzmB compared to controls (0 nM GzmB). Meanwhile, quantitative analysis demonstrated that differences in cleaved band intensity for fibronectin and laminin-5 reached significance between control and 100 nM GzmB groups (p < 0.05 and p < 0.001 respectively) (Figure 3-6 A, B, D, E). For collagen-IV, cleaved bands were found in supernatant samples (red arrow), yet they did not reach statistical significance (p > 0.05) (Figure 3-6 C, F). These results supported that GzmB can degrade some but not all of the RPE cell-derived ECM proteins.
Figure 3-6: The Cleavage of Primary RPE-Derived Extracellular Matrix (ECM) Proteins by Exogenous GzmB.

Primary RPE cells were stimulated with exogenous GzmB (100 nM) or GzmB (0 nM, controls) for 5 hrs, and supernatant samples were processed with primary antibodies against fibronectin, laminin-5 (now known as laminin-332) and collagen-IV in western blot. Vinculin (124 kDa, housekeeping gene) antibody was used for quality control and demonstrates supernatant samples did not contain cell lysates. (A) Cleavage bands (red arrows) at lower molecular weight were found in GzmB-stimulated RPE cells (lanes 5-7), but not in controls (lanes 2-4). The densitometric analysis of the band with asterisk (*) is graphed in (D) and demonstrates significant differences between control and 100 nM GzmB groups. (B) A cleavage band (red arrows) was found in samples from GzmB stimulation group (lanes 5-7), but not in controls (lanes 2-4). The densitometric analysis of the band with asterisk (*) is graphed in (E) and demonstrates significant differences between control and 100 nM GzmB groups. (C) A cleavage band, identified by red arrow, was in samples from both GzmB stimulation group (lanes 5-7) and controls (lanes 2-4). The densitometric analysis of the band with asterisk (*) is graphed in (F) and demonstrates no significance between control and 100 nM GzmB groups. (D-F) Independent-sample T-Test (n = 3 per group) was used between controls and GzmB-stimulated groups: mean ± SEM; * p < 0.05, *** p < 0.001.
Chapter 4: Discussion

Age-Related GzmB Expression

This is the first study to have assessed the spatial localization of GzmB in the healthy and diseased (AMD) eyes. I hypothesized that GzmB is produced by potential resident cells in the outer retina and the choroid. My work found a significant increase in the number of GzmB\(^+\) choroidal cells in aged human donor eyes (Figure 2-2). Given that aging is a major risk factor for AMD, our observed increase in GzmB\(^+\) cells in the choroid is consistent with the premise that GzmB may play a role in the development of AMD. In human donor eyes, GzmB expression was not only found inside cells (choroidal and RPE cells), but also detected in the extracellular spaces in one choroid of soft drusen group (Figure 2-2 C), suggesting a secretory process of GzmB into extracellular spaces might have been involved as the disease evolved. It is possible that the fixation and antigen retrieval methods in human tissues emphasized GzmB labeling in the intracellular compartments, but were not optimal to preserve GzmB in the extracellular space. Therefore, effective methods will be needed for more evidence about GzmB staining in ECM. Development of novel immunohistochemistry/immunofluorescence methods for precise measurements of extracellular GzmB molecules would allow researchers to have a direct observation of spatial localization of GzmB expression on formalin-fixed, paraffin-embedded human tissues, and explore more questions related to GzmB activity in extracellular spaces in diseases.

GzmB\(^+\) Choroidal Cells and CNV

Human CNV eyes were found to have increased number of GzmB\(^+\) choroidal cells, compared to GA eyes, soft drusen eyes and non-diseased groups (Figure 2-2). A number of previous studies
disclosed GzmB upregulation in other diseases, such as asthma, atherosclerosis, acne, etc.\textsuperscript{137–140} The present study found that GzmB is also upregulated in AMD, a multifactorial and chronic inflammatory eye disease, and thus AMD can be added to the list of human diseases associated with GzmB. AMD is a complex disease with many forms and stages, including early AMD with drusen, non-exudative or dry AMD with various stages including the late stage geographic atrophy (GA), and exudative or wet AMD with various stages including the late stage of choroidal neovascularization (CNV). The increase of GzmB in the CNV form may indicate that certain stimuli during CNV formation enhance GzmB expression or activity, which in turn accelerates CNV formation. A remarkable feature of CNV, compared to other forms of AMD, is the angiogenesis process, regulated by an imbalance between angiogenic factors and inhibitors. For example, many studies report that increased MMPs promoted CNV in rodent models.\textsuperscript{19,58} It is likely that the observed increase in GzmB may also contribute to CNV. Future work may focus on investigating potential pathological stimuli, which may initiate or regulate GzmB overexpression in CNV in rodent models.

**Mast Cells, Tryptase vs GzmB**

Many GzmB\textsuperscript{+} choroidal cells were further identified to be mast cells in this study (Figure 2-3), consistent with the earlier finding that the total number of mast cell and the number of degranulated mast cells were increased in all types of AMD choroids from studies by Lutty et al.\textsuperscript{129} They also suggested the degranulation of these mast cells released tryptase, another serine protease, which leads to ECM modifications and pathological changes in GA.\textsuperscript{130} There are two main discoveries to be noted here in this study compared to Lutty et al’s work. One disparity is that, their data suggested tryptase is associated with GA eyes, while our results shown here
revealed that GzmB is more significant in CNV eyes, but rarely present in GA eyes (Figure 2-2 E, F, H, I). Moreover, we confirmed that GzmB⁺ choroidal cells are in a population of mast cells distinguished from tryptase⁺ choroidal cells in human donor eyes (Figure 2-4). It is not known whether both mast cells mediators could play a separate or combined role in the development of AMD. Interestingly, our data showed GzmB can cleave TJ-associated proteins including ZO-1 and JAM-A in vitro, yet another study demonstrated that tryptase does not alter the expression of ZO-1 and JAM-A in RPE cells.¹³¹ Thus, it might be possible to speculate that different pathological stimuli initiate tryptase expression in GA, and GzmB expression in CNV, contributing to distinct directions in the pathogenesis of AMD. Future work could study the distribution of GzmB⁺ mast cells in different AMD groups, and illustrate how GzmB and tryptase might contribute to different directions of AMD progression by using in vivo models.

**Macrophages and CD 68 immunohistochemistry**

In addition to mast cells, a small number of macrophages were occasionally seen in human donor eyes (Figure 2-5). This result indicates that macrophages may also be one origin of GzmB in the eye. There are two subtypes of activated macrophages: classically activated macrophages (M1 phenotype) and alternatively activated macrophages (M2 phenotype). CD68 is a total marker of all macrophages, while both types of macrophages have their specific markers, such as CD80 and CD86 for M1 macrophages, and CD163 and CD 206 for M2 macrophages.¹⁴¹,¹⁴² More robust markers need to be included in future work to verify which subtypes of macrophages may be colocalized with GzmB expression in aging and diseased AMD eyes. Classically activated M1 macrophages have proinflammatory functions in classical pathway, while M2 macrophages are capable of generating VEGF and promoting a VEGF-dependent angiogenic response.⁷,¹⁴³–¹⁴⁵
Therefore, M2 macrophages seem to be better target to be studied in future work, which might be associated with GzmB expression and promote angiogenesis in CNV eyes.

**GzmB⁺ RPE Cells and Soft Drusen**

It is of note that RPE cells also appear to be one of the sources of GzmB in human outer retinas (Figure 2-2). Eyes with soft drusen were more likely to have GzmB⁺ RPE cells than the other groups studied, followed by CNV eyes which had a significantly higher number of GzmB⁺ RPE cells compared to normal human donor and GA eyes. This is the first study to describe that human RPE cells can produce GzmB. GzmB immunoreactivity was labeled in the basal cytoplasmic compartment of RPE cells. One limitation of this experiment is that GzmB staining may be obscured by RPE pigmentation, thus not all information about the distribution of GzmB staining in RPE cells can be obtained. Therefore, effective melanin depigmentation methods should be adapted to prepare de-pigmented RPE sections in future experiments. Considering that soft drusen eyes have the highest level of GzmB expression in RPE cells which were often spatially located near drusen deposits, this might hint at a relevance between GzmB activity and inflammatory components in drusen. Thus, it is reasonable to predict that GzmB expression might be upregulated by pathological drusen compositions. Curcio et al summarized a catalog of fifteen phenotypes of RPE cell morphology in advanced AMD, illustrating different fates of RPE cells during the progression of GA. After comparison with their work, GzmB⁺ RPE cells in this study appeared to have the phenotype of “non-uniform”. As described by Curcio et al, “non-uniform” RPE cells have slightly non-uniform morphology and pigmentation, and there are numerous lipoprotein particles (basal laminar deposit, which is the precursor to soft drusen) in the underlying BM. This RPE phenotype could exist in normal aged retinas or at the margin of
atrophy ("relatively healthier zone"), indicating that RPE cells are still at an early stage in the transition to atrophic (dying) RPE in GA. Thus, GzmB+ RPE cells found in soft drusen eyes in this study may suggest that GzmB activity is involved in the early stage of AMD.

**Cleavage of TJ Proteins and oBRB Function**

Earlier studies reported that GzmB cleaves ECM in non-ocular systems, implicating extracellular GzmB activity in pathological chronic inflammation, delayed wound healing, skin injuries, and cardiopulmonary disease, etc. I hypothesized that extracellular GzmB plays a role in the disruption of the oBRB and BM remodeling, two significant events in the development of CNV. This is supported by my results from *in vitro* studies demonstrating the cleavage of RPE TJ-associated proteins (ZO-1, JAM-A, occludin), as well as RPE-derived ECM proteins (fibronectin, laminin-5), induced by exogenous GzmB stimulation.

A MTT assay was conducted to decide the best concentration of exogenous GzmB stimulation for cell studies. In the MTT assay, a range of concentrations of the exogenous GzmB (10 - 100 nM) did not affect RPE cell viability (*Figure 3-1*), suggesting the range of 10 - 100 nM GzmB is safe for *in vitro* RPE cell cultures. In the immunocytochemistry, both 50 nM and 100 nM GzmB induced a significant loss of fluorescence signal associated with ZO-1 immunoreactivity in stimulated RPE cells, compared to other lower concentrations; the loss of fluorescence signal implied a dramatically degradation of ZO-1 by almost 90% in cells stimulated by 100 nM GzmB (*Figure 3-2*). We chose 100 nM GzmB to be used in following *in vitro* assays. As described previously, the physiological range of GzmB is approximately around 20-40 pg/ml in the plasma of healthy individuals, but the extracellular GzmB concentration can be 1 - 1000 times higher in
diseased tissues or bodily fluids.\textsuperscript{65,89,99,101–103} The concentration of 100 nM GzmB, which is 423 - 846 times the physiological concentration in healthy individuals, is therefore within the pathological range of extracellular GzmB concentration under diseased conditions. For future work, 50 nM GzmB also seems to be an ideal concentration.

\textit{In vitro} RPE cell studies were then undertaken to assess the role of exogenous GzmB (100 nM) on the oBRB function and BM remodeling. Earlier non-ocular studies showed that TJ proteins are substrates of GzmB.\textsuperscript{150} My findings validated that RPE-derived TJ and cell adhesion proteins, ZO-1, JAM-A and occludin, were degraded by exogenous GzmB \textit{in vitro} based on ICC (\textbf{Figure 3-2, Figure 3-3, Figure 3-4}). As these RPE TJ and adhesion proteins are important components of the oBRB in the eye, their disruption can contribute to AMD-associated pathological changes in the outer retina.\textsuperscript{151} It is likely to speculate that RPE TJ proteins may be exposed to increased GzmB and undergo degradation gradually during the aging or AMD process.

The oBRB function of the outer retina was measured in two functional tests through using \textit{in vitro} RPE cells stimulated by exogenous GzmB. The resistance of GzmB-stimulated RPE cultures was decreased in TEER test, while the permeability of GzmB-stimulated RPE monolayer was increased in FITC-dextran flux assay (\textbf{Figure 3-5}). The decreased resistance is consistent with the increased permeability, and both together suggest that the integrity of RPE TJs is damaged under GzmB stimulation. These \textit{in vitro} data strongly support my hypothesis that GzmB could disrupt the oBRB function through cleaving RPE TJ-associated proteins.
**Cleavage of BM Components and BM Remodeling**

Together with RPE TJ and adhesion proteins, some RPE-derived ECM proteins were also demonstrated to be substrates of GzmB, including fibronectin and laminin-5, which are main elements of BM, synthesized and secreted by RPE cells (*Figure 3-6 A, B, D, E*). However, GzmB cleavage of collagen-IV did not reach significance, but there were clear cleaved bands of lower weights in the experimental group (*Figure 3-6 C, F*). This may be tackled by improving technical skills towards cleaner western blot images in future experiments. Another possibility is that collagen IV may be a substrate of Gzranzyme A (GzmA), instead of GzmB. These results strongly support my hypothesis that GzmB could induce BM remodeling through cleaving ECM components in BM.

**GzmB in VEGF Release and CNV Growth**

Considering that GzmB has been shown to release ECM-sequestered VEGF from human umbilical vein endothelial cells (HUVEC) ECM *in vitro* and increase VEGF-dependent vascular permeability in a mouse model, it is possible that ocular GzmB may promote abnormal CNV growth by releasing sequestered VEGF from BM. VEGF plays a key role in regulating CNV formation; although anti-VEGF therapy is now commonly used in wet AMD, there is a decline in its long-term efficacy and some patients are non-responders to anti-VEGF drugs. Thus, there should be complex mechanisms involved in exacerbating angiogenic milieu in the outer retina. In 2017, Wroblewski et al found mast cell-derived GzmB decreases the efficacy of anti-angiogenic therapy for tumor angiogenesis by liberating sequestered pro-angiogenic factors, and the inhibition of mast cell degranulation increased the efficacy. This evidence suggests that future studies could focus on VEGF release facilitated by mast cell-derived GzmB in the outer retina.
**Ex vivo** BM-choroidal explant model may help assess the release of angiogenic factor VEGF from the BM and choroid, into the culture supernatants after GzmB stimulation. Furthermore, *in vivo* aging and AMD mouse models would allow us to better understand the mechanism by which GzmB activity gives rise to the cleavage of its substrates and the dysfunction of the oBRB in the outer retina, contributing to a favorable environment for the development of CNV in AMD.

**Strengths and Limitations**

There are both strengths and limitations of my study. As aging is a risk factor of AMD, one strength is that this study utilized healthy young and old donor eyes to analyze age-dependent GzmB expression. Also, human donor eyes from different AMD disease groups (soft drusen, GA, CNV) make it an ideal *ex vivo* model to observe similarities and differences of GzmB immunoreactivity among three stages of AMD disease. *In vitro* RPE cultures offer a convenient method to observe cleaved TJ proteins after GzmB stimulation by using confocal microscopy for high-resolution images. *In vitro* RPE monolayers also make it possible to measure the oBRB resistance and permeability of cultured RPE cells, and provide two separate methods to test whether results on the function of RPE monolayers after exogenous GzmB are consistent and reliable.

There are also some limitations of this study. One limitation is that postmortem eye studies cannot reflect dynamic changes or sequence of events during the development of AMD *in vivo*, so a timeline of GzmB activity in AMD development cannot be clearly described; also, the impact of extracellular GzmB on the oBRB was assessed by only using cultured RPE cells, but
*in vitro* models cannot exactly mimic the sophisticated and dynamic environment in living organisms. Therefore, more questions have to be answered through future *in vivo* studies in animal models, which are needed to further clarify the timeline of GzmB activity in the outer retina and choroid, and the mechanism of its activity in the oBRB disruption and BM remodeling during the formation of CNV in AMD development. Another limitation is that there were no quantifications about the comparison of choroidal mast cells and macrophages among healthy and diseased human donor eyes, due to the limited sources of postmortem eyes; more experiments will be needed to solve more questions about these two cells sources of GzmB in the future.

There are also some technical issues which could be improved in future experiments: (1) in the ICC, the permeabilization step (Triton X-100) may have caused the internalization effect in RPE cells, and the omission of it would improve the quality of JAM-A and occludin (transmembrane proteins) images; (2) in the ICC, the Z-stack images of RPE monolayer was not captured. In future, Z-stack images from confocal microscopy could help visualize TJ-associated proteins more accurately; (3) ICC cannot directly visualize the cleaved products of TJ proteins by GzmB, so diverse methods would help gather more solid evidence. The western blot could be used to test the cleavage of recombinant TJ proteins cleaved by GzmB, and the mass spectrometry could be used to analyze and compare the proteomic profiles of control RPE and GzmB-stimulated RPE cultures; (4) it is not clear if ECM proteins made by RPE were deposited on the bottom of the plastic culture plate. De-cellularization methods could extract RPE cells from ECM matrices\textsuperscript{154,155}, allowing us to obtain the protein lysate of these ECM matrices and analyze the
components in a western blot test. This would help confirm that the cleavage bands were from soluble ECM proteins released in the supernatant after GzmB stimulation.
Chapter 5: Conclusion

Summary
In this study, I hypothesized that GzmB is produced by resident cells in the outer retina and the choroid, and extracellular GzmB cleaves relevant substrates leading to the disruption of the oBRB and BM remodeling. Human ex vivo experiments demonstrated that choroidal mast cells, choroidal macrophages and RPE cells are sources of GzmB in the outer retina and the choroid. Greater numbers of GzmB+ choroidal cells are associated with aged and CNV eyes, and greater numbers of GzmB+ RPE cells are associated with soft drusen eyes. Results from in vitro work showed that exogenous GzmB cleaves RPE cell-cell adhesions (ZO-1, JAM-A, occludin), leading to a damaged oBRB function, and also cleaves RPE-derived ECM substrates (fibronectin, laminin), inducing BM remodeling. These data have answered my questions in this study and support the hypothesis that extracellular GzmB has the potential ability to disrupt the oBRB function and induce BM remodeling.

Significance
As a serine protease, GzmB has been most studied in autoimmune and chronic inflammatory diseases. However, no current studies have investigated its roles in ocular diseases. In addition to its well-established intracellular functions, GzmB also has extracellular functions, which are main focuses of this research. This is the first study to have explored GzmB’s extracellular activity in the outer retina and the choroid towards understanding its role in the development of AMD. During this study, it was discovered that GzmB is expressed by choroidal mast cells, an important resident immune cell, and RPE cells, a novel cell source of GzmB. It also verified that extracellular GzmB is able to degrade RPE TJ-associated proteins and BM components in the
outer retina, promoting the disruption of the oBRB and BM remodeling. My findings have supported the relevance of GzmB activity to AMD pathogenesis and have elicited more questions for future research in this field.

**Future Directions**

Further investigations into the evidence of pathological conditions that promote GzmB expression in mast cells or RPE cells would be beneficial to unraveling the detailed mechanism of GzmB activity in AMD. It would also be important to elaborate the process by which GzmB is secreted into extracellular spaces. Moreover, the recombinant human protease inhibitor-9 (PI-9) could be experimentally used to observe the effects of the inhibition of extracellular GzmB activity on the cleavage of targeted substrates through *in vitro* RPE cell culture. Animal models would help explore many questions *in vivo* in the future: for example, an apolipoprotein-E (ApoE) Knockout (KO) mouse model which has been shown to display accelerated aging and some pathological changes in AMD, could be used to demonstrate if GzmB contributes to angiogenesis in CNV formation *in vivo*. After ApoE KO mice are exposed to potential stimulants (e.g., oxidative stress, sunlight exposure), the extracellular GzmB activity could be detected in this mouse model’s outer retinas and choroids, and histopathological changes of the mouse tissues could be examined to determine the CNV formation. The GzmB KO mouse model would allow researchers to further confirm whether the absence of GzmB reduces CNV formation (e.g., through inhibiting GzmB activity *in vivo*) which may prevent those aging and AMD pathological changes, including CNV development. In the future, novel therapies targeting GzmB activity might be developed to ameliorate CNV formation. In conclusion, this study identified cell sources of GzmB in the outer retina and the choroid, and found that GzmB activity
may play an important role in the pathogenesis of AMD. This study sets the stage for suppressing extracellular GzmB activity towards a novel treatment strategy for AMD.
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Appendices

Appendix A

Table A- 1: List of Antibodies.

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