GRANZYME B DISRUPTS CELL-CELL ADHESION AND EPITHELIAL BARRIER FUNCTION

by

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Abstract

The skin is comprised of multiple layers of keratinocytes which together form a barrier to the external environment, regulating temperature, water loss, and pathogen exposure. As such the skin barrier is vital for health as well as disease prevention. Disruption of the epithelial barrier can result in infection, allergen exposure, and inflammation, culminating into severe conditions. Many autoimmune conditions, such as pemphigus, involve a dysregulation and accumulation of immune cells, this results in a disruption in skin barrier causing a loss of function. Granzyme B (GzmB) is a serine protease that is expressed and secreted by a variety of immune and non-immune cells. It can accumulate in the extracellular milieu and retain its proteolytic functions resulting in chronic inflammation and impaired tissue repair due to extracellular matrix (ECM) remodeling. As such, I hypothesized that GzmB disrupts epithelial barrier function through the proteolytic cleavage of cell junction proteins. The present study investigated the impact of GzmB on epithelial barrier dysfunction using Electric Cell-substrate Impedance Sensing (ECIS) and western blot analyses of intercellular junction cleavage fragments. Human formalin fixed, paraffin embedded blistered skin tissue was assessed for the presence of GzmB. GzmB treatment resulted in a loss of E-cadherin staining on the cell membrane which was supported by western blot analysis of the cell supernatants. Additionally, we observed a dose-dependent increase in E-cadherin fragmentation in GzmB-treated cells compared to controls. HaCaT cells exhibited a significant decrease in barrier function when treated with GzmB while cells treated with GzmB in the presence of a specific GzmB inhibitor remained unaffected. While absent in normal skin, GzmB was observed in abundance within the intra-epidermal blister in addition to the surrounding epithelium. In summary, GzmB contributes to a decline in epithelial barrier function in part through the proteolytic cleavage of cell-cell junctions.

Lay Summary

Skin blistering occurs when different layers of skin cells lose their ability to ‘stick’ to one another. Skin blisters are often thought of as those caused by friction or burns. However, there are also rare skin diseases, referred to as autoimmune blistering skin diseases in which the body’s own immune cells target and eliminate skin cell connections resulting in cells separating. As these connections are necessary to form a barrier that maintains fluid balance, temperature, and ward off bacterial infection, when this barrier is compromised, the body’s equilibrium is lost. Specifically, my work focussed on a protein-degrading enzyme known as Granzyme B, that is released from immune cells. My research indicates that Granzyme B accumulates in areas of high inflammation and areas where blistering is occurring. Furthermore, Granzyme B was found to break down the barrier function by specifically cleaving important adhesion proteins that hold skin cells together.

Preface

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List of Acronyms and Abbreviations

A.A.	Amino acid
ADAM	A Disintegrin And Metalloproteinase
BAL	Bronchoalveolar Lavage
BP	Bullous Pemphigoid
C20	Compound 20 Inhibitor
Cldn	Claudin
COPD	Chronic Obstructive Pulmonary Disease
CTL	Cytotoxic T Lymphocytes
DMEM	Dulbecco's Modified Eagle Medium
Dsc	Desmocollin
Dsg	Desmoglein
E-cad	E-cadherin
ECIS	Electric Cell-substrate Impedance Sensing
ECM	Extracellular Matrix
EMT	Epithelial Mesenchymal Transition
FBS	Fetal Bovine Serum

GzmA	Granzyme A
GzmB	Granzyme B
GzmH	Granzyme H
GzmK	Granzyme K
GzmM	Granzyme M
IFN	Interferon
IL	Interleukin
JAM	Junctional Adhesion Molecule
JMD	Juxtamembrane Domain
MMP	Matrix Metalloproteinase
NK	Natural Killer
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline – Tween 20
PECAM	Platelet Endothelial Cell Adhesion Molecule
PF	Pemphigus Foliaceus
PI-9	Protease Inhibitor 9

PV	Pemphigus Vulgaris
SDS	Sodium Dodecyl Sulphate
sE-cad	Soluble E-cadherin
TAILS	Terminal Amine Isotopic Labeling of Substrates
tBid	Truncated Bid
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline – Tween 20
TEER	Trans-Epithelial Electrical Resistance
TEWL	Trans-Epidermal Water Loss
UV	Ultraviolet
ZO	Zonula Occludens

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Dedication

I dedicate my thesis to my parents, Patricia and Javier Santa Cruz, who have gone through so much and, despite all that, have always been there for me. Without their constant support at my back I wouldn't have had the success and skill I do today. I have been, and always will be, grateful for their endless support and day to day encouragement throughout my life. From the bottom of my heart, Thank You!

Chapter 1: Introduction

1.1 Skin Structure and Function

Human skin, unlike other organs, is constantly and directly exposed to the external environment. It is the largest organ in the body and its purpose is to provide protection against infections, mechanical and chemical insults, water loss, and ultraviolet radiation.[1] The skin forms an intricate structure composed of two layers that include *i*) the epidermis, forming the outermost layer, and *ii*) the dermis whose connective tissue provides the epidermis with nutrition.[2] The dermis is primarily composed of connective tissue with approximately 90% being collagen (I & III), the remainder being elastic fibres, blood and lymph vessels, muscle fibres, and sweat glands.[3]

All mammals are equipped with physical barriers that separate the internal from the external. The major barriers include, but are not limited to, the respiratory, gastrointestinal tracts, and the cutaneous barrier. The skin, is the first line of defence from the external environment and must protect and minimize possible damage from external environmental insults. This barrier is formed by specialized epithelial cells called keratinocytes that link together to form a dynamic and continually renewing stratified epithelium.[4]

The basal layer of cells, known as the stratum basale, contain the only dividing cells in the epidermis. It is composed of undifferentiated and mitotically active keratinocytes which are positioned immediately above the basement membrane.[3,4] The basal layer proliferates to move cells into the more superficial layers; the next layer being the stratum spinosum. Due to the intercellular junction desmosomes, it creates a spine-like appearance. After which the spinous layer begins to differentiate into the stratum granulosum where it becomes recognizable due to the

multiple densely packed granules. The outermost layer is the stratum corneum which is the outermost layer made up of corneocytes, which are enucleated, protein rich, and terminally differentiated keratinocytes. Corneocytes are surrounded by an extracellular lipid matrix composed of cholesterol, long-chain fatty acids, and ceramides.[1–4] The stratum corneum is a critical component of the skin barrier function as it prevents water loss and over hydration.[3] The structure and arrangement of the different layers of the epidermis is illustrated below (Figure 1).

Keratinocytes make up roughly 80% of the cells in the epidermis. Other cell types include melanocytes, Langerhans cells, dendritic cells, monocytes, macrophages, mast cells, and leukocytes, all of which span the into the dermis and epidermis.[3,4] The web of epithelial and immune cells collectively form a defensive barrier to the external environment.[4]

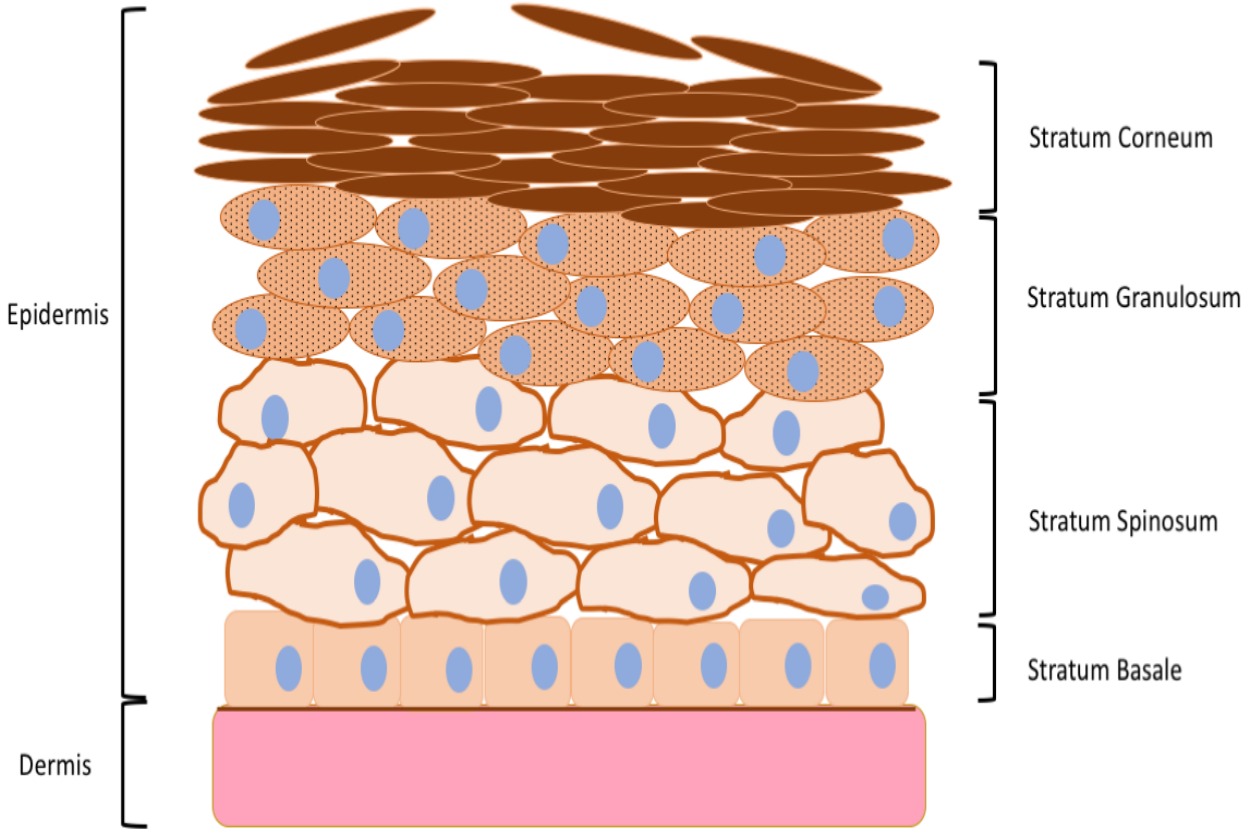


Figure 1: Structure of the Epidermis. The first layer of cells immediately above the dermis is the mitotically active stratum basale, followed by the stratum spinosum. The stratum granulosum is last layer of nucleated keratinocytes and is full of lamellar granules. Finally, the stratum corneum is a layer of enucleate corneocytes that get sloughed off with regular turnover.

1.2 Barrier Function and Intercellular Junctions

Although the stratum corneum provides much of the physical barrier from the external environment, the lower layers of the epidermis also contribute substantially to barrier function. This is highlighted by the fact that there is only a moderate increase in trans-epithelial water loss (TEWL) when the stratum corneum is removed through tape stripping.[1] However, complete loss of the epidermis, as seen in suction blisters, will lead to a large destruction in barrier maintenance through the loss of the cell-cell connections known as intercellular junctions.[1] Therefore, it is imperative to consider how the epidermis maintains a barrier and the intercellular junctions that support it.

Intercellular junctions are comprised of tight junctions, adherens junctions, and desmosomes/ hemidesmosomes (Figure 2). The epidermis is highly stratified with each layer expressing a unique combination of intercellular junctions. Hemidesmosomes, are present exclusively in the stratum basale as they link the basal layer to the basement membrane, whereas desmogleins and desmocollins appear to have a varying specificity within the different layers of the epidermis. Adherens junctions, typically containing E-cadherin, are present in all viable layers, whereas tight junctions tend to localize in the stratum granulosum. Tight junctions are also much more varied in protein composition compared to adherens junctions, with the protein claudins (cldn) and occludins being the most abundant.[4,5]

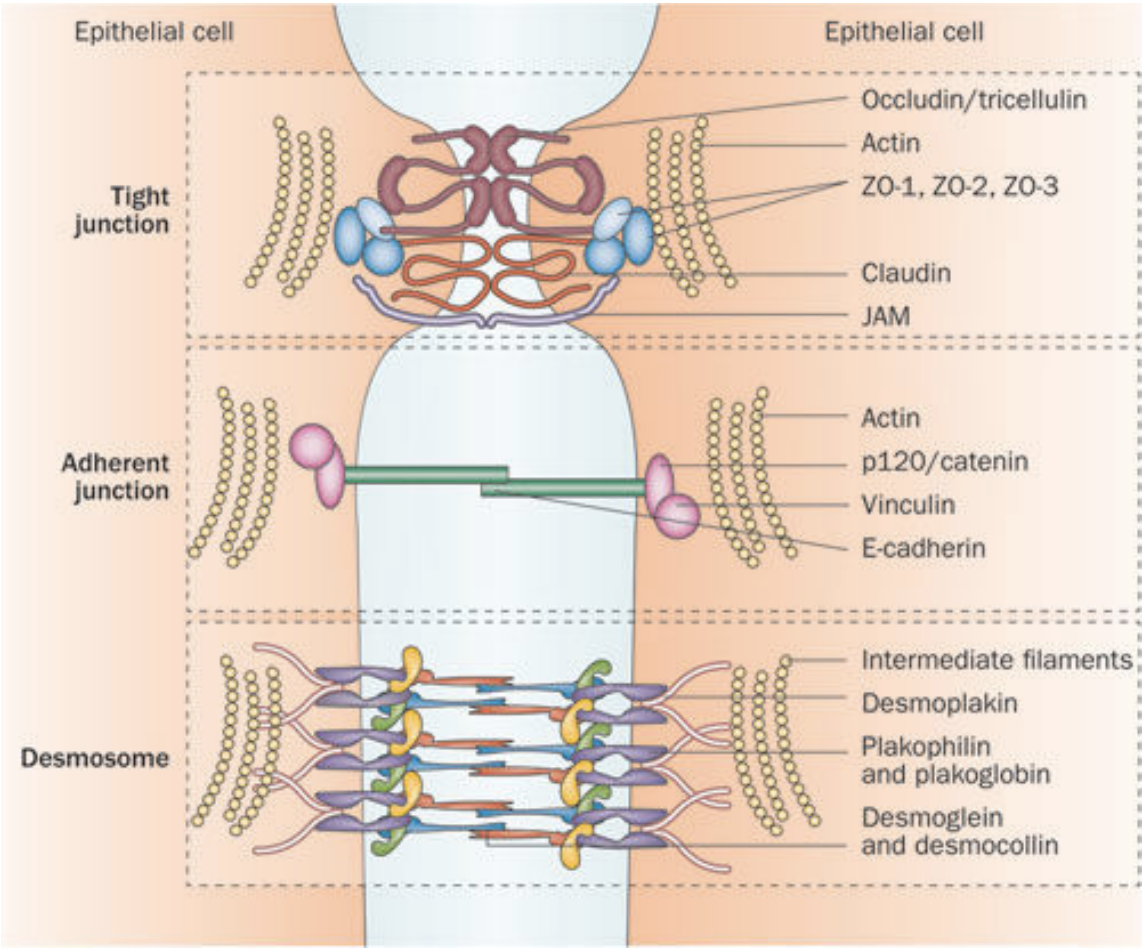


Figure 2: Structure of Intercellular Junctions. Tight junctions, Adherens junctions, and desmosomes are the primary intercellular junctions that connect adjacent epithelial cells. Tight junctions lie in the upper-most (apical) region of the cell, thus providing polarity. They seal the intercellular space, regulating paracellular movement and barrier formation. Adherens junctions and desmosomes form a connection between cells providing cell-cell adhesion and mechanical strength against external stress aiding in barrier maintenance.

1.2.1 Tight Junctions

Tight junctions form the apical domain of the cell providing polarity and function as a gate-keeper controlling the paracellular movement of ions, water, and solutes. In the skin, tight junctions are relatively impermeable compared to the small intestine or renal tubules.[6] Tight junctions seal the intercellular spaces between keratinocytes and are under constant dynamic regulation. Previously, epidermal barrier functions were thought to be exclusively maintained by the intercellular lipids in the stratum corneum, however other studies have shown an important role of tight junctions in barrier function in the epidermis.[4,7] In fact, a study by Furuse *et al*. (2002) showed that claudin-1 knockout mice displayed a wrinkled morphology with severe trans-epidermal water loss (TEWL) and died within a day of birth, while no changes in lipid structures in the stratum corneum were noted.[8]

Various proteins that form the molecular components of tight junctions include claudins, occludins, junctional adhesion molecules (JAM), and tight junction plaque proteins.[4,8–12] Occludin is a 65kDa transmembrane protein which crosses the cell membrane four times with two extracellular loops and its localization to tight junctions is controlled by phosphorylation. If non-phosphorylated, occludin is contained within cytoplasmic vesicles or along the basolateral membrane.[11] Initially suggested to participate solely in homophilic binding, occludin can interact with claudin and junction adhesion molecule A (JAM-A) through its second extracellular loop.[11,13]

The claudins consist of 24 subtypes ranging in size from 20-27 kDa and, like occludin, are transmembrane proteins that span the membrane four times with two extracellular loops. However, this similarity is not due to amino acid (a.a.) sequence homology. Claudins regulate paracellular tight junction channels due to their similarity to ion channels (acting as the gate), specifically ion charge selectivity, and ion concentration dependent movement.[11]

Junction adhesion molecules (JAM) are composed of three types: JAM-A, JAM-B, & JAM-C and belong to the immunoglobulin super-family. All JAMs contain a transmembrane domain, and an intracellular tail. Originally characterized as a tight junction protein in endothelial and epithelial cells, more recent investigations have found a role for JAM proteins in regulating cell polarity.[10]

Zonula occludens proteins (ZO) are the intracellular plaque proteins that anchor the tight junctions and are also composed of three types: ZO-1, ZO-2, ZO-3 and bind to adherens junctions and tight junctions. ZO-1 has been shown to be a scaffolding protein linking transmembrane and cytoplasmic proteins and possibly link adherens and tight junctions. Adhesion junction development through E-cadherin is correlated with the localization of tight junctions, namely ZO-1.[11] This suggests that E-cadherin in required for the proper formation of tight junctions.

In the epidermis, Cldns-1,-4,-6,-7,-11,-12,-18, occludins, and zonula occludens (ZO-1 and ZO-2) are the most abundant, particularly within the stratum granulosum. ZO-1, ZO-2, cldns-4,-6,-18 are seen within the spinous layer and cldn-1-7-12, JAM-A have been observed in all viable layers.[4]

1.2.2 Adherens Junctions

Adherens junctions play critical roles in the initiation and stabilization of cell-cell adhesion, intracellular regulation of gene transcription, and actin cytoskeleton organization. Anchoring junctions are composed of transmembrane adhesion proteins which have a cytoplasmic tail that binds to one or more anchoring proteins and an extracellular domain that links to either the extracellular matrix or the extracellular portion of another extracellular adhesion protein.[14] The core of adhesion junctions are primarily made up of cadherins, namely E-cadherin, and the catenin family. Together they control the maintenance and function of adhesion junctions.[11] In basal cells,

adhesion junctions are found along the lateral and apical membranes while in suprabasal cells they are located all over the cell surface.[15]

Adherens are cadherin-based junctions that consist of Ca^{2+} dependent adhesion proteins, with E-cadherin being the most prominent transmembrane cadherin within the epidermis.[11,15] Cadherin proteins have five extracellular cadherin repeat domains which form the basis behind trans-cadherin cell-cell adhesion between adjacent cells. Binding of Ca^{2+} to each extracellular cadherin domain is essential for correct protein conformational organization.[11] The Ca^{2+} ions are situated between cadherin repeats, and in the absence of Ca^{2+} ions, the extracellular portion of the cadherin becomes floppy and susceptible to proteolytic cleavage.[16] Additionally, adhesion junctions appear to be required for the proper formation of tight junctions as blocking antibodies directed toward adhesion junctions also block tight junctions.[14]

E-cadherin is the most abundant cadherin expressed in the epidermis, the extracellular portion participates in homophilic binding with adjacent E-cadherins.[11,16] The cytoplasmic domain binds to anchoring proteins such as α- and β-catenin that regulate intracellular signalling pathways, gene transcription, and control of actin cytoskeleton organization. Cadherin mediated cell-cell adhesion facilitates cell reorganization and dispersal.[11] When E-cadherin is down-regulated, as in epithelial-to-mesenchymal transition (EMT), cells lose their polarity and adhesive properties. This is commonly seen in carcinogenesis, where E-cadherin expression is ablated leading to increased proliferation and invasiveness.[11] During extracellular cleavage of E-cadherin, the intracellular domain is acted upon by intracellular proteases that generate fragments that promote intracellular signaling and cell proliferation. However, it is uncertain which fragments are responsible for which processes. Nevertheless, it is known that extracellular proteases can cleave the ectodomain of E-cadherin, generating a fragment known as soluble E-cadherin (sE-cad), which can propagate

autocrine or paracrine signaling and/or disrupt other intercellular junctions (Discussed further in section 1.2.2.1).[17]

Cadherins are transmembrane adhesion proteins that indirectly link to the actin cytoskeleton *via* a highly conserved cytoplasmic tail that links to a series of anchoring proteins called catenins.[16] The catenin family is made up of p120-catenin, β-catenin, and α-catenin.[11,18–20] P120-catenin directly binds E-cadherin at a highly-conserved sequence within the juxtamembrane domain (JMD).[19] It is believed that this association helps stabilize E-cadherin to the plasma membrane during the formation of cell-cell contacts. Without this connection, the adhesive properties of E-cadherin are diminished.[11,19] Additionally, p120-catenin may prevent the internalization and degradation of E-cadherin.[11] β-catenin binds the cytoplasmic C-terminal domain of E-cadherin and is regulated by phosphorylation. β-catenin is tightly regulated through phosphorylation, in part, because β-catenin can bind to transcription factors and regulate the transcription of genes involved in cell proliferation.[11,21] β-catenin directly links to the next anchoring protein, α-catenin. α-Catenin directly binds to actin filaments and other proteins such as vinculin, ZO-1, and α-actinin and participates in force-dependent conformational changes.[18]

1.2.2.1 Soluble E-cadherin

As discussed above, sE-cad is the ectodomain of E-cadherin that has been extracellularly cleaved by proteases. These proteases include metalloproteinases (MMP-3,-7,-9), a disintegrin and metalloproteinases (ADAM-10,-15), plasmin, and kallikrein.[22,23] These proteases convert the whole 120 kDa E-cadherin protein into an extracellular N-terminus fragment (~80 kDa) and an intracellular C-terminal fragment (~38 kDa). Formation of sE-cad is primarily discussed in the context of cancer, specifically its contribution of tumor development. First identified as ~80 kDa peptides released from MCF-7 human carcinoma cells,[24] these fragments retain their adhesive

properties and can disrupt cell-cell contacts.[22,23,25] Additionally, the released extracellular fragment can act locally or systemically to act as an autocrine/paracrine signaling molecule.[22] The first identified function of sE-cad was the disruption of cell-cell contacts and three potential mechanisms have been proposed: *i*) the reduction of adhesion-competent E-cadherin on the cell surface, *ii*) sE-cad retains its adhesive properties and can disrupt E-cadherin homodimers between adjacent cells, *iii*) sE-cad has chemotactic properties and can travel through the blood stream and become embedded in the extracellular matrix (ECM), this would serve as an anchoring point for whole E-cadherin on migrating cells to adhere, or *iv*) the proteolytic formation of sE-cad contributes to a positive feedback loop by upregulating major MMPs and ADAMs to further degrade basement membrane proteins (Figure 3).[22]

In addition to the involvement in cancer proliferation and migration, sE-cad is found to be elevated in a variety of skin diseases including bullous pemphigoid, psoriasis, pemphigus vulgaris, and other inflammatory diseases.[26] The levels of sE-cad are elevated in autoimmune blistering diseases with twice as much in the blister fluid of bullous pemphigoid than in that observed in serum. These sE-cad levels tend to decrease following therapeutic intervention.[27] In psoriasis, there is a weak yet significant correlation between levels of sE-cad and PASI score (Psoriasis Area and Severity Index) which measures the severity and extent of disease.[26] Thus, sE-cad is a useful marker for characterizing the severity of many diseases, and elevated levels of sE-cad can inhibit cell-cell adhesion which promote the pathologies seen in cancer and blister formation.[25,26]

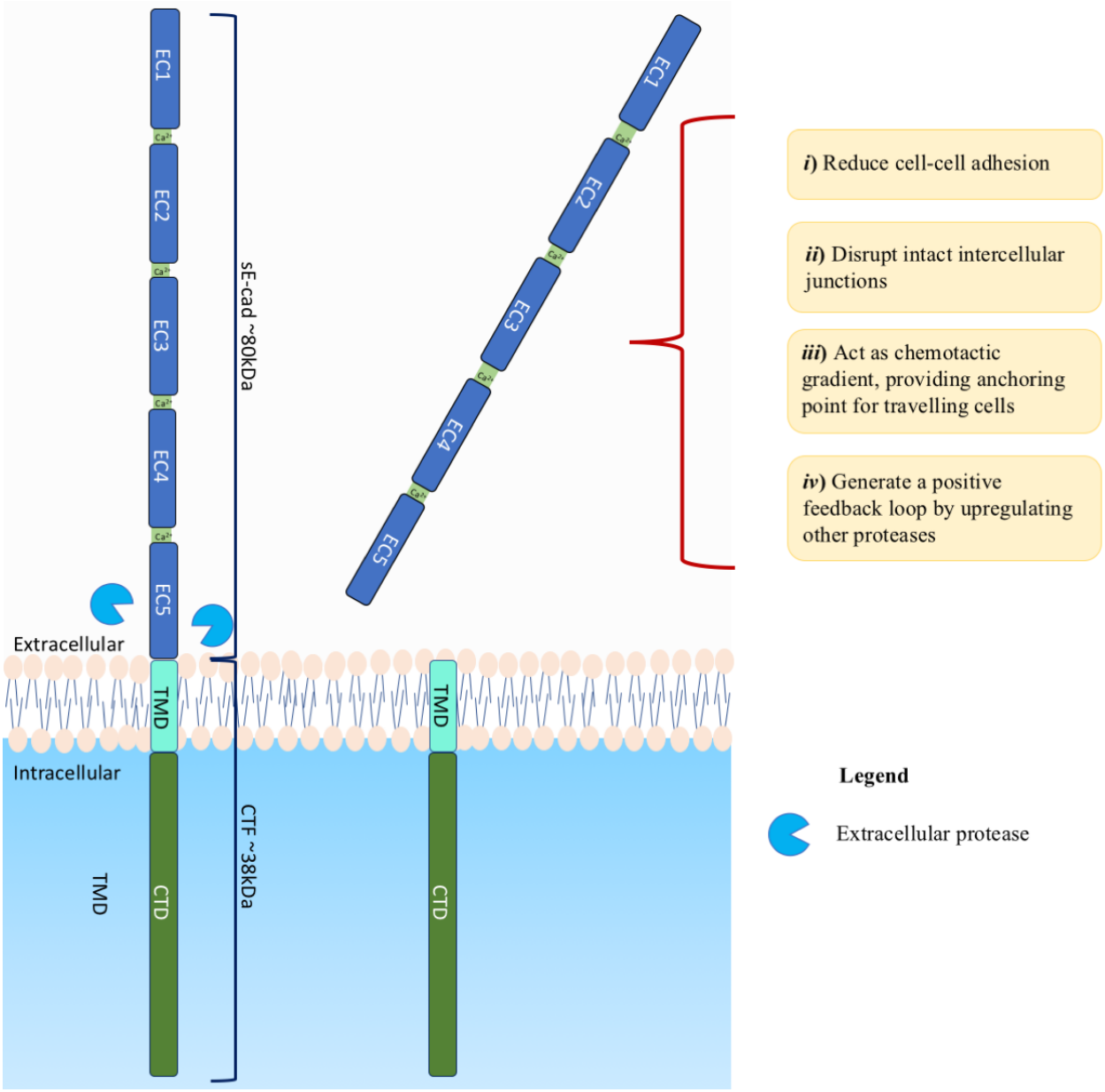


Figure 3: Structure of E-cadherin. E-cadherin is the main adherens junction involved in adhesion in epithelial cells. It contains 5 extracellular repeats (EC1-5), a transmembrane domain (TMD), and an intracellular C-terminal domain (CTD). Cleavage of the extracellular domain releases an 80kDa fragment known as soluble E-cadherin (sE-cad) which can be released into the extracellular space. This fragment can then participate in multiple downstream effects such as reducing cell adhesion, disrupting intact cell-cell junctions, providing an anchoring point for travelling cells, and upregulating other proteases thereby increasing sE-cad production. Meanwhile a 38kDa fragment remains attached to the cell membrane and can undergo further proteolytic cleavage where it will be freed from the membrane and act as a downstream messenger.

1.2.3 Desmosomes

Desmosomes are part of the cadherin superfamily and form around other transmembrane cadherins and mediate calcium-dependent cell-cell adhesion. There are two sub-types of desmosomes which include *i*) desmogleins (Dsg) and *ii*) desmocollins (Dsc). There are four isoforms of Dsg and three of Dsc, each of which is expressed in a cell specific manner.[28] With respect to the skin, Dsg3 is more abundantly present in the lower epidermis and virtually absent in the upper layer, while Dsg1 expression becomes more abundant upward towards the granular layer.[29] Desmosomes are linked to intermediate filaments thus providing much of the tensile strength and resistance to mechanical stress.[15] Therefore, desmosomes bolt cells together through extracellular adhesive structures and link to the cytoskeletal filaments to disperse forces.[28]

The extracellular portion of the desmosomes is structurally similar to E-cadherin in that it contains five cadherin repeats which form Ig-like globular domains with Ca^{2+} separating each repeat. The cytoplasmic domains of both Dsg and Dsc contain a conserved intracellular anchor and a cadherin-like sequence. The major intracellular anchoring proteins that link the desmosomes to intermediate filaments are plakoglobin, plakophilin, and desmoplakin.[28]

Plakoglobin is a member of the armadillo family proteins and is closely related to β-catenin.[11,28] Plakoglobin binds to the cadherin-like sequence domain of all desmosomes and bridges the connection between the cytoplasmic tail and the intermediate filament binding proteins. Plakophilin is a member of the p120-catenin subfamily, humans express three subtypes that are differentially expressed depending on cell type. Desmoplakin is a member of the plakin family and connects intermediate filaments to plakoglobin and plakophilins. Collectively these structures are known as desmosomal plaques.[28] Humans have one desmoplakin gene that is alternatively spliced

to produce desmoplakin I and II. Desmoplakin II has been identified to be more important for desmosome-mediated adhesion in HaCaT cells compared to desmoplakin I.[28,30]

The list of intercellular junctions is, not surprisingly, much longer than what is discussed here, however, these are among the most abundant and therefore most studied. Tight junctions are the main junctions involved in barrier formation and a loss of structural integrity of any of these proteins could lead to increased TEWL.[8,10,11,14,31] E-cadherin and desmosomes both belong the cadherin superfamily meaning that they are Ca^{2+} dependent and inhabit the basal layer of the cell. The main functions of cadherins are adhesion and resistance to mechanical stress.[15]

Table 1: Summary of Major Intercellular Junctions and Their Anchoring Proteins

	JUNCTION	LOCATION AND FUNCTION
TIGHT JUNCTIONS	OCCLUDIN[32,33]	• Epithelial and endothelial cells. • Anchoring proteins ZO-1, ZO-2, ZO-3. • Tetraspanning transmembrane protein. • Regulates paracellular permeability through its phosphorylation status.
	CLAUDIN 1-24[32,33]	• Epithelial and endothelial cells. • Anchoring proteins ZO-1, ZO-2, ZO-3. • Tetraspanning transmembrane protein. • Forms the backbone of tight junctions by forming. homophilic and heterophilic binding through extracellular loops. • Major barrier forming component.
	JAM-A/B/C[9,32,33]	• Epithelial and endothelial cells. • Anchoring proteins ZO-1, ZO-2, ZO-3. • Member of Ig superfamily. • JAM-A directly involved in tight junction assembly and maintenance, cell polarity, and regulation of barrier function.
ADHERENS	E-CADHERIN[32]	• Epithelial cells. • Anchoring proteins α- and β-catenins, plakoglobin, p120. • Forms homophilic binding. • Controls cell-cell contact, barrier integrity, and cytoskeletal tension.
DESMOSOMES	DESMOGLEIN 1-4[28,34]	• Epithelial and endothelial cells. • Plakoglobin, plakophilin, and desmoplakin. • Intracellular sequences including intracellular anchor, and cadherin like sequence, proline rich linker region (IPL), repeat domain unit (RUD), and desmoglein terminal domain (DTD). • Mediate cell-cell adhesion and resists mechanical stress.
	DESMOCOLLIN 1-3[28,34]	• Refer to desmoglein 1-4. • Lacks IPL, RUD, and DTD. • Function in strong cell adhesion and sensing/ responding to mechanical stress.

1.3 Pemphigus

Pemphigus refers to a group of autoimmune skin blistering diseases characterized by blisters forming within the epidermis. There are a variety of pemphigus disorders with an incidence ranging between 0.76-32 cases per million.[35] Globally, the incidence of pemphigus varies depending on geographical region, with pemphigus vulgaris being more prevalent in Europe and USA, while pemphigus foliaceus is more common in Africa with other underdeveloped nations reaching an incidence of up to 3% (discussed further in sections 1.3.1 and 1.3.2 respectively).[36] Pemphigus has a slight female predominance (1.5:1), and occurs between the ages of 40-50 years.[37] Autoantibodies are directed against the desmosome adhesion molecules within the epidermis causing the separation of keratinocytes, a process known as intraepidermal acantholysis.[37,38] Pemphigus blisters are characterized by flaccid blisters or erosions on the skin and mucous membranes. Mucosal blisters, such as oral and genital blisters, typically precede full blown pemphigus symptoms by three to four months.[37] Pemphigus is a very serious disease because antibodies directed against desmosomal adhesion molecules cause a loss of barrier function which leads to increased TEWL and pathogen exposure causing secondary infection. If left untreated, this disease is often fatal.[36]

All forms of pemphigus are characterized by the presence of skin fixed and circulating autoantibodies (collectively known as intercellular antibodies) against desmosomes. Circulating autoantibodies are present in roughly 80% of patients, and titres correlate with severity of disease.[36] Desmoglein 1 and 3 are the most important and best studied antigens in pemphigus; however, there is increasing evidence for autoantibodies directed at other desmosomal proteins such as Dsc and desmoplakin.[37] Diagnosis of pemphigus subtypes are based on clinical features

(such as appearance, texture, and localization within the body), histology, and immunological tests.[36]

Due to the potential life-threatening nature of pemphigus, a more intensive therapeutic regimen is required compared to other autoimmune skin blistering conditions. The severity of skin lesions, age, current medication, and the impact of co-morbidities will direct the time-point and extent of local or systemic immunosuppressive therapy.[37] The use of systemic corticosteroids is often combined with adjuvant immunosuppressive/modulatory drugs (for example azathioprine, mycophenolate mofetil, rituximab, and cyclophosphamide) in severe cases.[35,39,40] Case studies on intravenous immunoglobulins and immunoadsorption in combination with rituximab have also be published.[35–37] In some variants of pemphigus there little to no immune infiltrate however, T-cell responses could regulate the formation of pathogenic autoantibodies.[36] The most well-known and common forms of pemphigus will be discussed further below.

1.3.1 Pemphigus Vulgaris

Pemphigus vulgaris (PV) is the most common variant of the disease, with an incidence ranging from 0.1-3.2/100 000 per year.[41] In PV, intercellular antibodies are directed against Dsg3 and to a lesser extent Dsg1.[35,36,42] As Dsg3 is situated in the lower epidermis, blisters form in this area just above the stratum basale and are present in the skin and mucous membranes. Oral blisters are common in patients with PV, known as limited oral pemphigus vulgaris, as the oral epithelium consists predominantly of Dsg 3.[36] Blisters are common on the torso, face, axillae, esophagus, conjunctiva, nasal mucosa, and genitalia.[42] Intact blisters are rare due to their fragility and thus rupture easily. The erosions that form after a ruptured blister is often painful and bleed easily.

Rupture leads to the loss of epidermal barrier function which can lead to secondary infections.[36,38,42]

Another form of pemphigus is pemphigus vegetans which is a variant of PV and is much rarer. Pathology of the disease is associated with vegetating proliferation of the epidermis. Lesions typically appear in the axillae, groin, and inframammary area. Pemphigus vulgaris is thought to arise when therapy has been successful in preventing new blisters but are unable to heal old blisters.[36]

1.3.2 Pemphigus Foliaceus

Pemphigus foliacues (PF) is a less common form of the disease with an incidence about a fifth to a tenth that of PV.[36] In PF, intercellular antibodies are directed against Dsg1, and thus blister formation is more superficial and scaly in appearance compared to PV. Pemphigus foliaceus onset is often subtle with a few crusted lesions scattered throughout the body, and no apparent mucous membrane involvement.[42] The pathology of PF is so superficial that there is not enough epithelial tissue to trap fluid and thus, it is unable to form a blister. Lesions usually begin as multiple coin-sized patches on the upper torso, face and scalp. Crusts can be removed easily leaving superficial erosions that if left untreated will not heal and increase in number over the span of weeks and months. However, the prognosis of untreated PF is better than that of PV, most likely due to the lower risk of barrier dysfunction, and therefore lower risk of infection and fluid loss.[36] Another form of PF is fogo selvagem, an endemic form that is concentrated in rural areas along creeks and streams. Primarily concentrated in Brazil and neighboring countries including Colombia, Venezuela, Ecuador, and Peru.[43] The most affected areas are the face, scalp, neck, and upper trunk.

Ultraviolet (UV) exposure augments and triggers skin lesions leading to a rapid disease progression of weeks to months.[43]

1.3.3 Hailey-Hailey Disease

Hailey-Hailey disease, also known as benign chronic familial pemphigus, was first described in 1939 by the Hailey brothers. It is an autosomal dominant condition with most patients having no symptoms until the third or fourth decade of the disease. Exacerbating factors include heat, friction, and stress with symptoms worsening in the summer months.[44] Blisters typically appear in the genital area, neck, and axillae, and often rupture leaving an eroded base. The gene responsible for the defect is ATP2C1 which encodes the secretory pathway calcium/manganese-ATPase (SPCA1) which is a calcium and manganese pump.[45] Ca^{2+} is needed for a variety of cellular processes including proper assembly of desmosomes. Epidermal Ca^{2+} gradient shows substantially lower levels in the superficial layers of the skin with unchanged concentrations in the basal layer.[45,46]

1.3.4 IgA Pemphigus

IgA pemphigus is very rare and does not discriminate between males and females or age (1 month to 85 years old).[38] It is characterized by neutrophil infiltration, acantholysis, and tissue bound/circulating IgA antibodies.[47] There are two different types of IgA pemphigus: subcorneal pustular dermatosis (SPD) and intraepidermal neutrophilic (IEN). In SPD, IgA deposition is limited to the upper epidermis, while in IEN, IgA deposition is either limited to the lower epidermis or throughout the entire epidermis.[48] Blisters commonly form in the axillary and groin areas, trunk, and proximal extremities, with very rare mucous membrane involvement.[38]

1.3.5 Paraneoplastic Pemphigus

Paraneoplastic pemphigus is clinically presented with lesions affecting both the mucosal and cutaneous tissues.[38] It has recently been described in patients with a history of neoplasms including, lymphomas, bronchogenic carcinoma, and chronic lymphocytic leukemia.[38,49] Mucosal lesions typically present as severe erosions particularly as uncontrollable stomatitis/ inflammation especially in the oral cavity. Skin blisters are polymorphic and typically are present on the trunk, palms, and soles and morphologically resemble that of PV.[38,48,49] Severe progressive paraneoplastic pemphigus can lead to complications in the respiratory system including pulmonary infections.[49] The cause of death in patients with paraneoplastic pemphigus is most often due to respiratory complications and septicemia.[49]

Histological analysis of paraneoplastic pemphigus includes intraepidermal acantholysis, and keratinocyte necrosis.[49] Immunoprecipitation reveals desmoplakin I & II, envoplakin, bullous pemphigoid antigen 1, and periplakin as the typical antigens present in the sera of patients with paraneoplastic pemphigus.[49] Summary of each pemphigus disease is discussed in Table 2.

Table 2: List of Pemphigus Variants and Targeted Antigens

Subtype	Pathology
Pemphigus Vulgaris[35–37,42]	• Autoantibodies against Dsg-3 and sometimes Dsg-1 • Blisters form in the suprabasal region of the epidermis • Blisters typically begin in and around the oral mucosa, and then spread to the epidermis • Affected areas include torso, face, axillae, esophagus, conjunctiva, nasal mucosa, and genitalia • Variations of the disease include limited oral pemphigus vulgaris and pemphigus vegetans
Pemphigus Foliaceus[35–37,42,48]	• Autoantibodies against Dsg-1 • Lesions form in the superficial layers of the epidermis • Due to the location of the lesions, blisters are not typically formed as there are not enough layers of epithelial cells to form a blister • Affected areas include the face, scalp, neck, and upper trunk
Hailey-Hailey Disease[44–46]	• Also known as benign chronic familial pemphigus • Affected gene is ATP2C1, altering Ca^{2+} movement and localization in the epidermis • Lack of available Ca^{2+} results in improperly developed desmosomes • Affected areas include the genital area, neck, and axillae • Blisters often rupture leaving an eroded base
IGA Pemphigus[38,47,48]	• IgA antibodies target desmosomal and non-desmosomal cell surface components • Two different types of IgA pemphigus: subcorneal pustular dermatosis (SPD) and intraepidermal neutrophilic (IEN) • Affected areas include the axillary and groin areas, trunk, and proximal extremities
Paraneoplastic Pemphigus[38,48,49]	• Typical antigens include desmoplakin I & II, envoplakin, bullous pemphigoid antigen 1, and periplakin • Affected areas include the oral cavity, mucosa, trunk, palms, and soles • Blisters resemble that of pemphigus vulgaris • Complications typically arise via respiratory complications

1.4 Granzymes

Granzymes (**gran**ule secreted en**zymes**) are a family of serine proteases that were primarily known to be stored in the granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells and be released upon immune mediated targeted cell apoptosis. In humans, there are five granzymes: Granzyme A (GzmA), Granzyme B (GzmB), Granzyme H (GzmH), Granzyme K (GzmK), and Granzyme M (GzmM), with GzmA and GzmB being the most abundant and best characterized.[50–52] In mice, there are ten functional granzymes (GzmA-G, K, M and N).[50,53–55] In combination with the pore-forming protein, perforin, granzymes are thought to be directly involved in the targeted cell death of infected or foreign cells.[50,56–58] Granzymes are organized into three chromosomal clusters, GzmA cluster, GzmB cluster, and GzmM cluster. GzmA and GzmK are grouped on chromosome 5 (GzmA cluster), GzmB and GzmH are grouped on chromosome 14 (GzmB cluster), and GzmM in on chromosome 19 (GzmM cluster).[51,54,55] Together, their DNA sequence homology is between 57- 61%.[51,55]

Granzymes are first synthesized as zymogens (or proenzymes) which will be cleaved at the time of packaging into cytolytic granules. Cleavage of the zymogen leaves two amino acids which will later be removed by cathepsin C (also known as dipeptidyl peptidase I) which is constitutively expressed in lysosomes.[52,59] Cleavage of the proenzyme's remaining dipeptide activates the granzyme.[50,52] In the granule, once the pro-enzyme has been activated by cathepsin C, granzymes are then stored on a scaffold consisting of serglycin (a chondroitin sulfate proteoglycan).[50,60] The combination of storage on serglycin and the acidic pH inside the granule serves to maintain the low enzymatic activity of granzymes inside secretory vesicles.[50,60] Granzymes are maximally active at a pH of 7.5 and so are catalytically active upon release from the secretory vesicles into the cytoplasm.[52]

Granzymes are characterized by the His-Asp-Ser catalytic triad.[61,62] GzmA & K are tryptases, cleaving after basic amino acids, GzmB cleaves after aspartic acid residues dependent on the presence of an arginine in the binding pocket, GzmH is a chymase, cleaving after hydrophobic residues, and GzmM is a metase preferentially cleaving methionine or leucine residues.[51,54] Some granzymes share substrate targets nevertheless, their degradomes are different suggesting only a partial overlap of substrate specificities.[54] A possible explanation for granzymes with different substrate specificities and distinct pro-apoptotic functions may be due to their ability to either combat pathogens and/or prevent tumor evasion. However, there is no consensus in the literature and is still highly debated.[55]

Granzyme A is the only granzyme that exists as a dimer with a total molecular weight of ~52 kDa and preferentially cleaves after Arginine and Lysine. In addition to its pro-apoptotic role, GzmA can induce the maturation and release of the inflammatory cytokines TNFα, IL-1β, and IL-6 in macrophages, in the context of chronic inflammation.[55,63] Additionally, the ECM proteins proteoglycan, fibronectin, and collagen IV are also proteolytic targets for GzmA, suggesting a more prominent role for GzmA in inflammation.[55,64]

Similar to GzmA, GzmK cleaves after Arginine and Lysine and has a similar cytotoxic potential. GzmK has been shown to be a good prognostic marker in cancer as elevated levels of GzmK in patients with high risk acute lymphoblastic leukemia do not relapse after chemotherapy.[65] However, elevated levels of GzmK have also been seen in inflammatory diseases including, but not limited to, atherosclerosis, chronic obstructive pulmonary disease, and sepsis.[50,55,66]

GzmH is a human specific granzyme that is expressed in NK cells and preferentially cleaves after phenylalanine and tyrosine. The cytotoxic potential of GzmH is very low, as the lethal dose 50 (LD50) of cells is more than 170-fold greater than GzmB.[55,67] Alternatively, GzmH may possess antiviral activity as GzmH can cleave the adenoviral protein 100K. 100K is an adenovirus derived GzmB inhibitor and an essential assembly protein. The direct cleavage of this protein by GzmH allows the recovery of GzmB activity providing a mechanism that overcomes viral defenses.[68]

GzmM, is involved in immune cell-mediated apoptosis. However, GzmM-mediated cell death does not induce DNA fragmentation, caspase activation, or mitochondrial death pathways, suggesting a novel form of perforin dependent targeted cell death.[69] However, GzmM has been implicated in STAT3 activation and induction of epithelial-mesenchymal transition in cancer cells. Therefore, GzmM appears to play a significant role in cancer invasion, metastasis, and chemoresistance.[58]

1.5 Physiological Role of Granzyme B

GzmB is one of the most abundant and best characterized members of the granzyme family from both a structurally and functionally perspective.[55] The principle function of GzmB is the perforin-dependent targeted cell death of virus-infected and other potentially harmful cells. Perforin-deficient mice show a complete loss of granzyme-mediated cell death and are much more susceptible to viral and other intracellular pathogens.[57] GzmB has the greatest apoptotic potential out of all other granzymes due to its ability to cleave substrates at key aspartic acid residues.[52] A variety of caspases are known to be directly or indirectly activated by GzmB.[51,52,70] This led to the model where the activation of caspase 3 by GzmB was responsible for the induction of CTL-mediated cell death via the caspase-dependent pathway.[51,70]

Additionally, GzmB can activate a caspase-independent form of cell death known as the mitochondrial pathway. Cleavage of the pro-apoptotic protein Bid into a granzyme-truncated Bid (gtBid) results in its translocation to the outer mitochondrial membrane where it induces the release of other pro-apoptotic proteins including cytochrome c.[52] The release of cytochrome c stimulates the accumulation of dATP, apaf-1, and procaspase-9 (collectively known as the apoptosome) which subsequently leads to autocatalytic maturation and complete activation of caspase 9 and 3 (Figure 4).[50,71,72]

The only known endogenous inhibitor of GzmB in human cells is protease inhibitor-9 (PI-9) also known as serpinB9. Immune cells express PI-9 as a defence mechanism against accidental cytosolic GzmB activity.[50,73] Additionally, endothelial cells, vascular smooth muscle cells, and hepatocytes have been shown to express PI-9 to block GzmB mediated cell death.[50,74]

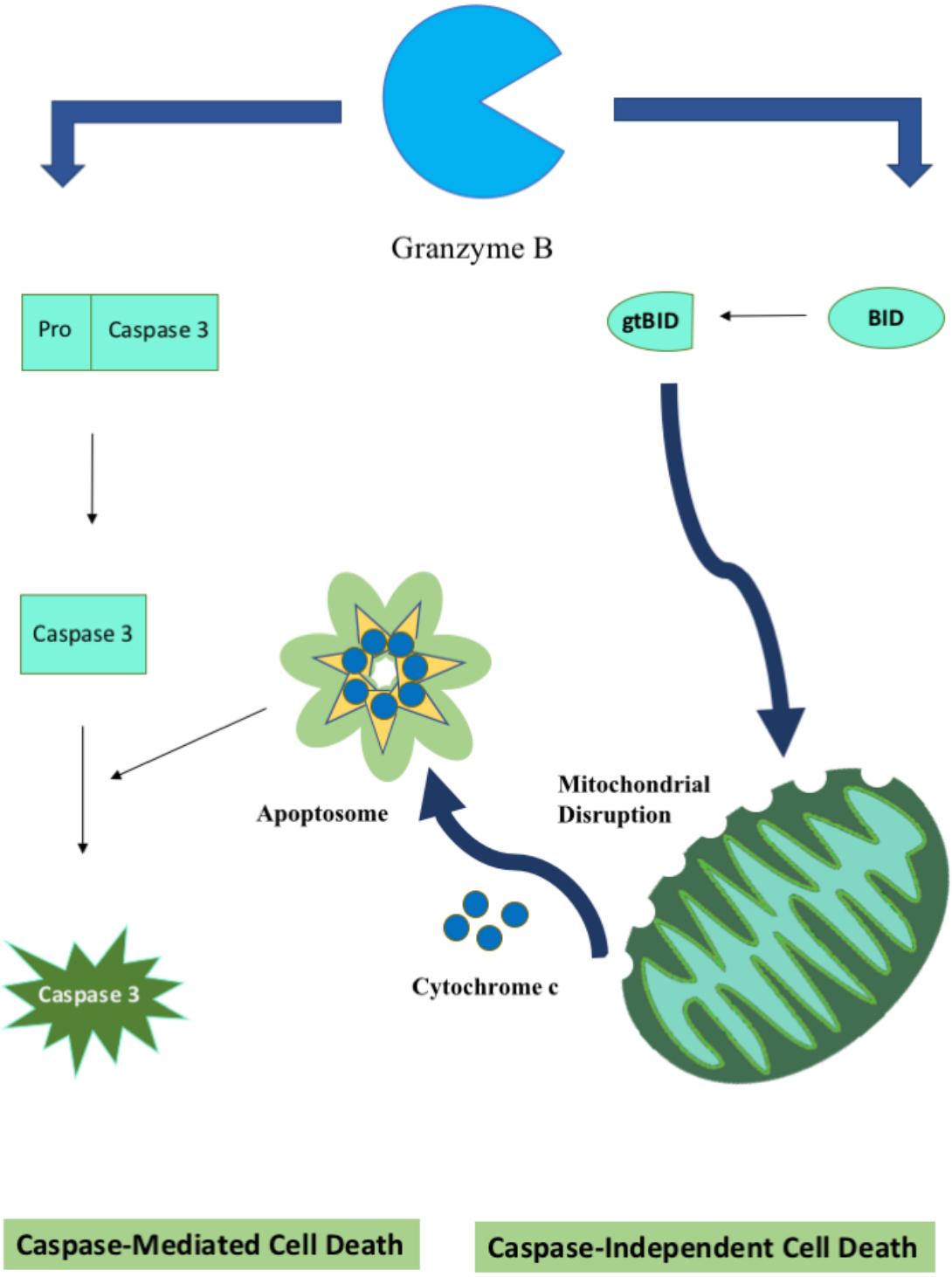


Figure 4: Granzyme B in Apoptotic Cell Death. GzmB is internalized into the cell via perforin. Once inside the cell, GzmB initiates apoptosis via cleavage of Bid into a granyme-truncated form (gtBid) which induces mitochondrial disruption and release of cytochrome c. This leads to the formation of the apoptosome. GzmB can also cleave procaspase-3 into a partially activated caspase-3 however, the apoptosome is required to develop the fully activated caspase-3.

1.6 Extracellular Role of Granzyme B

Although GzmB was solely thought to be released by CTLs and NK cells, recent work has proven that a wide variety of immune and non-immune cells can express GzmB.[50,75,76] During pro-inflammatory conditions, T-helper cells, mast cells, activated macrophages, neutrophils, and basophils can express GzmB. Additionally, non-immune cells, such as keratinocytes and chondrocytes, have recently been added to the list of cells expressing GzmB under chronic inflammation/stress.[50]

GzmB is present in the ECM of tissue and in bodily fluids (plasma, cerebrospinal fluid, synovial fluid, bronchoalveolar lavage) in conditions associated with chronic inflammation.[53,55,77] It is now recognized that non-immune cells (eg. keratinocytes, chondrocytes, pneumocytes) and a wide variety of other immune cells (mast cells, basophils and plasmacytoid dendritic cells, B cells) can express GzmB. As these cells do not express perforin and/or do not form an immunologic synapse, an extracellular role for GzmB is favoured.[50] GzmB can be released into the extracellular space and retains its proteolytic function. Cleavage of ECM proteins can lead to ECM remodeling, cell death, and inflammation. Perforin-independent cell death of adherent cells or anoikis can occur when cells detach from the ECM and die.[51,53,77–79] Alternatively, GzmB may target cell adhesion proteins promoting cell detachment and migration, in addition to cleaving a variety of receptors to influence cell growth and survival. GzmB may also contribute to the production of autoantigens and onset of autoimmune diseases.[51]

Elevated levels of GzmB in the extracellular milieu may contribute to delayed wound healing, in part, because GzmB cleaves a variety of ECM proteins essential for wound closure and remodelling.[53] For example, fibronectin, vitronectin, and laminin were all identified targets for

GzmB, each of which plays a critical role in keratinocyte attachment and migration.[78] Additionally, GzmB cleavage fragments can act as a chemotactic gradient and/or as ligands for cell-signaling (Figure 5).[50,61] Chronic low-dose UV irradiation results in an increased accumulation of GzmB in the skin which ultimately leads to an increase in fibronectin fragments and decorin degradation. The increase in fibronectin fragments increases MMP-1 expression by fibroblasts, while loss of decorin leaves the collagen fibrils susceptible to MMP-1 degradation, thus leading to a disordered dermis and wrinkle formation.[80]

A study by Pardo et al. showed that bone marrow derived mast cells can express GzmB but not GzmA or perforin *in vivo*. Immunohistochemical staining showed an elevated GzmB expression in skin but not lung associated mast cells, suggesting different environmental stimuli can alter mast cell phenotype and distribution.[81] The GzmB released from these mast cells caused a disorganization of endothelial cell-cell contacts including vascular-endothelial cadherin (VE-cadherin), PECAM, JAM-A, and ZO-1 *in vitro*.[81]

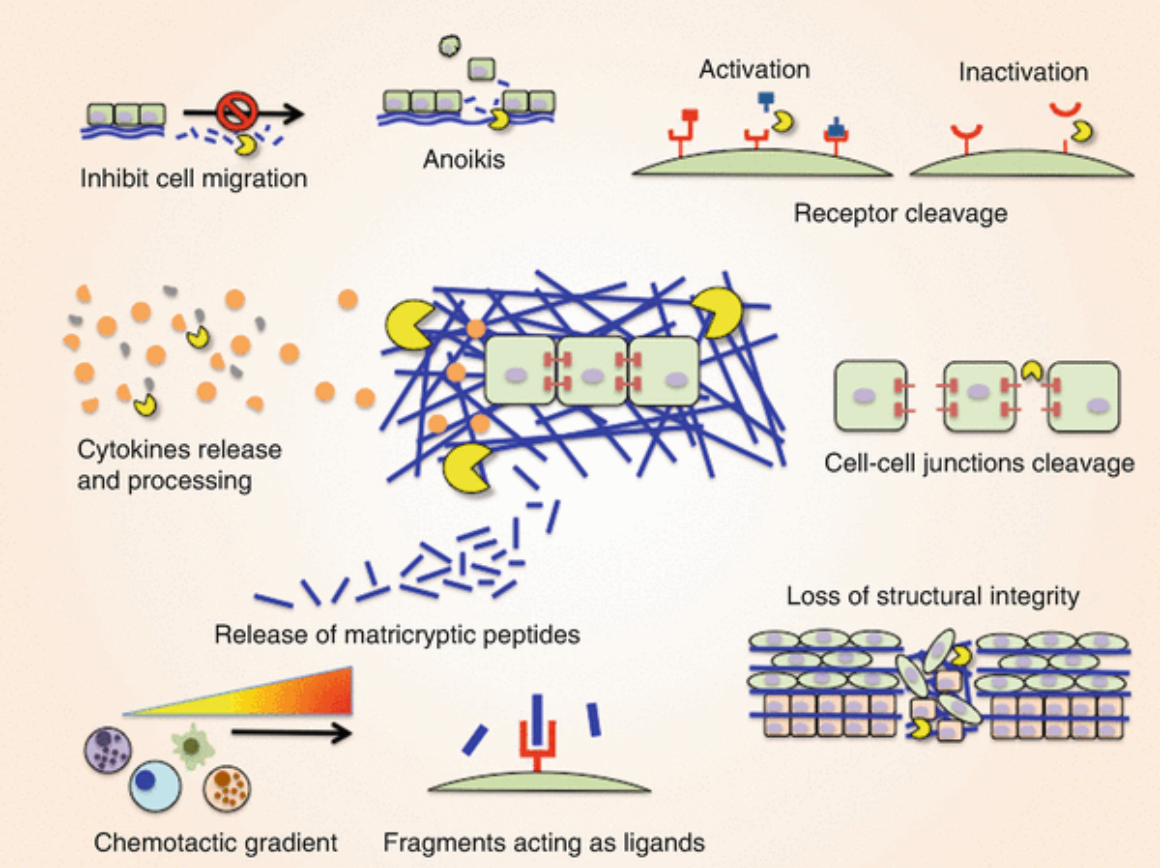


Figure 5: Putative Extracellular Roles for Granzyme B. Release of GzmB is elevated during chronic inflammation and retains its proteolytic function, suggesting that extracellular inhibitors of GzmB are limited. Once released, GzmB is capable of cleaving cell surface receptors, ECM proteins, and intercellular junctions. This results in receptor inactivation/deactivation, cell detachment, inhibition of cell migration in wound healing, and disorganized structural integrity. Additionally, GzmB can induce cytokine production and activation, thus providing the opportunity for increased inflammation.

Encyclopedia of Signaling Molecules, Granzyme B, 2nd Edition, 2017, page1 -7, Christopher T. Turner, Valerio Russo, Stephanie Santacruz, Cameron Oram, David J. Granville, (original copyright notice as given in the publication Turner CT, Russo V, Santacruz S, Oram C, Granville DJ. Granzyme B. In: *Encyclopedia of Signaling Molecules*. Cham: Springer International Publishing; 2018:2244-2250. doi:10.1007/978-3-319-67199-4_101961.) With permission of Springer.

1.6.1 Granzyme B in Inflammation and Disease

GzmB is elevated in a variety of different inflammatory diseases in different organs including: lung, vascular, skin, and autoimmune diseases, as well as neurological disorders. Chronic obstructive pulmonary disease (COPD) has increased CTLs and NK cells expressing GzmB in the blood and bronchoalveolar lavage (BAL) of both smoking and non-smoking patients with COPD.[50,56] Asthma patients also show an increase in lymphocytes expressing GzmB in the BAL of patients suffering an allergen induced asthma.[77,82]

Neurological disorders such as multiple sclerosis have displayed an elevated CTL infiltrate. Multiple sclerosis relapses have shown an elevated GzmB mediated neurotoxicity in the cerebrospinal fluid.[83] Neuronal cell death is induced through the PAR-1 activation via a perforin independent pathway.[83,84]

Atherosclerotic lesions exhibit elevated GzmB levels with increasing disease severity and lipid rich regions.[50,61,78] Increased levels of GzmB in unstable plaques showed an association with increased frequency of strokes. Furthermore, perforin deficiency in LDLR-KO mice had no effect on disease progression, further supporting an active role for extracellular GzmB.[85] Similarly, GzmB-mediated apoptosis is shown to be increased due to recruitment of inflammatory cells during acute transplant rejection.[50] Additionally, serpina3n, a potent GzmB inhibitor, injected into APOE-KO mice prior to angiotensin II pump implantation had a significantly lower abdominal aortic aneurysm rupture than mice treated with saline alone.[86]

As mentioned in the previous section, UV photoaging can lead to increased expression of GzmB in the skin. Induction of GzmB by UVA and UVB is seen in HaCaT keratinocytes, as well as in human skin with GzmB localization almost exclusively to the epidermis.[79] Keratinocytes will

also express GzmB in response to confluence.[87] In atopic dermatitis and psoriasis, there is a significantly higher concentration of GzmB compared to healthy controls. Plasma concentrations of GzmB showed a correlation with the severity of the disease in atopic dermatitis.[88]

Autoimmune diseases such as systemic lupus erythematosus (SLE), scleroderma, and type I diabetes show an elevated GzmB response. GzmB-mediated apoptosis of β-cells in type I diabetes correlates with loss of insulin secretion by islet cells.[89] Although caspase-8 and GzmB share substrate homology, GzmB shows distinct cleavage sites that are unique to GzmB. For example, autoantigens topoisomerase I, Ku-70, XRCC4, and RNA polymerase II large subunit were cleaved by GzmB but not caspase-8.[90,91] This suggests that GzmB plays a role in autoantigen production which could lead to the formation of autoantibodies and subsequent provocation of autoimmunity.

1.7 Rationale and Hypothesis

The epidermis is made up of intercellular junctions that collectively form the skin's barrier. A loss of this barrier would result in an increase in TEWL, secondary infections, and temperature dysregulation.[1,2,4] GzmB is expressed and/or secreted by a variety of cells and retains its proteolytic activity in the extracellular milieu.[50,79,81] GzmB is capable of cleaving ECM proteins including: fibronectin, vitronectin, laminin, decorin, fibrillin-1, Vascular Endothelial cadherin (VE-cadherin), JAM-A, and ZO-1.[50,78,81] This suggests that GzmB can cleave a variety of intercellular junctions that help maintain barrier integrity.

Pemphigus is an autoimmune skin blistering disease that develops due to the presence of autoantibodies directed against desmosomes.[36] Granzyme B has been shown to cleave proteins at highly conserved sites that are not common in other proteases, suggesting novel autoantigen

production by GzmB.[91] Therefore, GzmB may play a role in autoantigen production that could ultimately lead to autoimmune disease.

Another possibility that could progress the pathology of pemphigus and other blistering skin diseases is extracellular proteolysis. A study by Schiltz *et al*. demonstrated that a non-IgG pemphigus acantholysis factor could induce acantholysis in healthy skin explants and speculated that this factor was a proteolytic enzyme originating from the skin.[92] Therefore, it is possible that proteolytic cleavage of cell-cell junctions, namely E-cadherin, occurs after disease onset. sE-cad is elevated in several skin diseases including, PV, BP, and psoriasis, with levels decreasing in response to therapy.[25,26] The role and origin of sE-cad are still unknown; however, elevated levels of sE-cad would favour bullous formation by inhibiting cell-cell adhesion. The PV antibody produced an extra band of E-cadherin in western blot, suggesting that this antibody induces proteolytic degradation of E-cadherin.[25]

I ***hypothesize*** that GzmB disrupts epithelial barrier function through the proteolytic cleavage of intercellular junctions and that GzmB activity could contribute to intraepidermal blistering.

My specific aims are:

1. To assess the impact of GzmB on skin barrier function
2. To identify intercellular junctions that are susceptible to GzmB-mediated cleavage.
3. To assess whether GzmB-mediated intercellular junction cleavage is observed in pemphigus blistering diseases.

Chapter 2: Materials and Methods

2.1 Cell Culture

HaCaT (**H**uman **A**dult low **Ca**lcium high **T**emperature) human keratinocytes were grown and maintained in DMEM (Gibco) supplemented with 10% (*vol/vol*) FBS and 1% (*vol/vol*) Penicillin/Streptomycin (P/S) (Sigma) and kept in a humidified chamber with 5% CO_2 at 37°C. For all experiments, cells were serum starved for 4 h in DMEM and 1% P/S, unless otherwise indicated.

2.2 MTS Cell Viability Assay

HaCaT cells were seeded at $2x10^4$ cells in 96-well tissue culture plates (Greiner) and grown overnight. The next day cells were washed in PBS and serum starved for 3 h. After 3 h, cells were treated with 0 nM, 50 nM, 100 nM, 150 nM, or 200 nM GzmB (Emerald Bio) ± a GzmB inhibitor; 50 μM C20 inhibitor (CDRD) for 72 h. After 72 h, 20 μL of MTS assay solution (Promega) was added to each well and placed back in 37°C incubator for 2 h. Absorbance was read at 490 nm using TECAN Infinite M1000 Pro plate reader.

2.3 Trans-Epithelial Electrical Resistance

HaCaT barrier function was measured using the Electric Cell-substrate Impedance Sensing (ECIS) Zθ system (Applied Biophysics). ECIS arrays (8W10E+ PET) were pre-coated in 0.2 M cysteine solution and cells seeded at a density of $4x10^5$ cells/well in complete growth medium. Cells were incubated for 1 h at room temperature to allow for cell attachment. Cells were then placed in a humidified 37°C and 5% CO_2 incubator for 24 h on the ECIS Zθ platform with measurements taken at multiple frequencies. After 24 h, cells were washed once with PBS and

incubated with serum free DMEM for 4 h. Cells were then treated with either 0 nM, 50 nM, 100 nM, 150 nM, and 200 nM GzmB ± 50 μM C20 inhibitor. Cells were returned to the incubator and incubated for another 70 h on the ECIS platform. The time at which cells were treated with GzmB is referred to as 0 h. Barrier function was measured by changes in resistance at a frequency of 4 kHz in real time. A well with a no cells (400 μL DMEM) was used as an endogenous control. All resistance readings were normalized to the control at 0 h (when cells were treated with GzmB).

2.4 Dextran Permeability

HaCaTs were seeded on 24-well transwell inserts (Corning) at $5x10^5$ cells per insert. Cells were grown overnight to reach confluence. Confluence was measured using ECIS, when resistance had reached a plateau signal. Cells were then serum starved for 4 h before addition of 0-200 nM GzmB. Cells were treated for 72 h prior to the addition of the 70 kDa Rhodamine B dextran (Sigma) to the top chamber. Cells were placed back in the incubator for and additional 5 h. Fluorescence intensity was read from the bottom chamber using the Tecan Infinite M1000 Pro plate reader, excitation: 520 nm, emission: 590 nm.

2.5 Terminal Amine Isotopic Labeling of Substrates

HaCaT cells were grown to confluence and then serum starved for 4 h. Cells were treated with 100 nM GzmB or left untreated for 24 h. Afterward, the supernatant was collected and protein samples were denatured in 3M guanidinium hydrochloride (GuHCl), reduced with DTT and free cysteines alkylated with iodoacetic acid (IAA) before blocking primary amines by reductive amination with formaldehyde. After acetone precipitation, resuspended samples were digested with Trypsin (1:100 *w/w*) and N-terminal peptides originating from proteolysis were enriched by removal of unblocked internal peptides with hyperbranched polyglycerol-aldehydes (HPG-ALD)

polymer. Samples were desalted and concentrated by C18 stagetip before loading on a 75 μm ID capillary column packed with 1.9 μm C18 particles. Samples were analyzed on an Easy nanoLC hyphenated to a Bruker Impact II Q-TOF mass spectrometer operated in data-dependent acquisition mode. Data was searched against a Uniprot human proteome database using Mascot, with a peptide level false discovery rate (FDR) <1%.

2.6 Western Blotting

Cells grown to confluence were serum starved for 4 h and treated with 0-200 nM GzmB ± 50 μM C20 inhibitor in serum free medium for 24 h. Supernatants were collected and cells were lysed immediately using Cell Lytic M Solution (Sigma) supplemented with protease inhibitor cocktail (Sigma). Protein lysates were quantified using the Bio-Rad Protein assay system. Supernatants were concentrated in four volumes of ice cold acetone and 20 mM DL-Dithiothreitol (DTT) (Sigma) to one volume of protein sample for 1 h. Sample was then centrifuged at 10 000 x*g* for 15 min at 4°C. Supernatant was discarded and pellet was air dried for 30 min. The pellet was dissolved in 8 M Urea and used in SDS-PAGE. Samples were heat denatured, and separated on a 10% polyacrylamide gel and transferred to PVDF membrane. Membranes were blocked in a 5% skim milk TBST solution and probed for E-cadherin (R&D), JAM-A (Abcam), ZO-1 (Invitrogen), Dsg1 (Abcam), and Dsg3 (Santa Cruz) overnight at 4°C with gentle shaking. Blots were washed in TBST and primary antibodies were detected using Alexa Fluor 790 donkey anti-rabbit (Life Technologies) and Alexa Fluor 680 donkey anti-mouse (Invitrogen). Blots were imaged and quantified using the LiCor Odyssey Fc system.

2.7 Biochemical Cleavage Assay

JAM-A, ZO-1, E-cad, Dsg1, Dsg3 were incubated with 50 nM or 100 nM GzmB ± C20 inhibitor. GzmB was pre-incubated with 50 uM C20 inhibitor at 37°C for 1 h prior to addition of substrates. Cleavage reactions were incubated at 37°C for 2 h, After which, 6x denaturing sample buffer was added to stop the reaction. Proteins were then separated by SDS-PAGE and the gel stained with Coommassie Brilliant Blue R Concentrate (Sigma) as per manufacturer's protocol. Gels were imaged on the LiCor Odyssey or LiCor Odyssey Fc systems under the 700 nm channel.

2.8 Immunocytochemistry

HaCaT cells were grown to confluency on glass cover slips (Fisher Scientific) in 24-well tissue culture plates and serum starved for 4 h. Cells were then washed with PBS and treated with 0-200 nM GzmB for 8 h. Cells were then washed with PBS, fixed with 10% formalin, permeabilized with 0.1% Triton-X-100, blocked with 10% donkey or horse serum and incubated with E-cadherin (Abcam), JAM-A (Abcam), ZO-1 (Invitrogen), Dsg1 (Abcam), and Dsg3 (Santa Cruz) at 4°C overnight. Slides were washed three times with PBST and incubated with Alexa Fluor 488 secondary antibody for 2 h at RT. Confocal imaging was performed using the Zeiss AxioObserver Z.1 laser scanning confocal microscope and images captured using the Zen Software.

2.9 Immunohistochemistry

Formalin fixed, paraffin embedded human skin tissue blocks were sectioned at 5 um, de-waxed in xylene and hydrated in 100%-70% EtOH before haematoxylin and eosin (H&E), or immunohistochemical staining. Slides were then boiled for 15 min in 1x citrate buffer and cooled to RT. After which slides were washed in TBS three times and incubated in either goat, or horse protein serum block solution for 1 h at RT depending on secondary antibody. Sections were

incubated with GzmB (Abcam) and E-cadherin (Abcam) at 4°C overnight. The next day, sections were washed three times with TBS and incubated with horse anti-mouse and goat anti-rabbit biotinylated secondary antibody for 30 min. Slides were washed and then stained with 3,3'-diaminobenzidine and then counterstained with haematoxylin. Tissue sections were scanned using the Aperio CS2 slide scanner (Leica, Heidelberg, Germany)

2.10 Immunofluorescence Double Staining

Immunofluorescence was performed on the same skin tissue sections mentioned in section 2.9. Blocks were sectioned at 5 um, dewaxed, and hydrated as per the protocol in section 2.9. Sections were rinsed in TBS then blocked 10% donkey serum. Sections were then incubated in the mixture of GzmB (Abcam) and E-cadherin (R&D) primary antibodies in 10% donkey serum at 4°C overnight. Slides were rinsed in TBS and sections incubated in the mixture of two Alexa Fluor 488 and Alexa Fluor 594 secondary antibodies in TBS for 1 h at RT. Rinsed with TBS and counterstained with DAPI (1:1000) for 5 min at room temperature. Finally, sections were rinsed, mounted and visualized using confocal imaging on Zeiss AxioObserver Z.1 laser scanning confocal microscope and images were captured using the Zen Software.

2.11 Statistical Analysis

All data represent results from at least 3 independent experiments. ECIS and Dextran permeability data was analyzed with one-way ANOVA followed by Tukey's multiple comparisons post-hoc test. Data is represented as mean ± standard deviation (SD). *P*-values are represented as follows: $p < 0.05$ is denoted by *, $p < 0.01$ by **, $p < 0.001$ by ***, and $p < 0.0001$ by ****. $p < 0.05$ was considered significant in all experiments. All statistical analyses were performed in GraphPad Prism version 6.0 (GraphPad Software, San Diego, California).

Chapter 3: Results

3.1 Extracellular Granzyme B Does Not Reduce Viability

Due to GzmB's physiological role in targeted apoptosis, the first step was to determine if low levels of extracellular GzmB altered cell viability. In the absence of perforin, HaCaT viability was evaluated using the MTS incorporation assay. Granzyme B was administered to a confluent HaCaT monolayer after starving the cells for 4 h. No significant change in cell viability was seen up to 200 nM GzmB at 72 h (Figure 6).

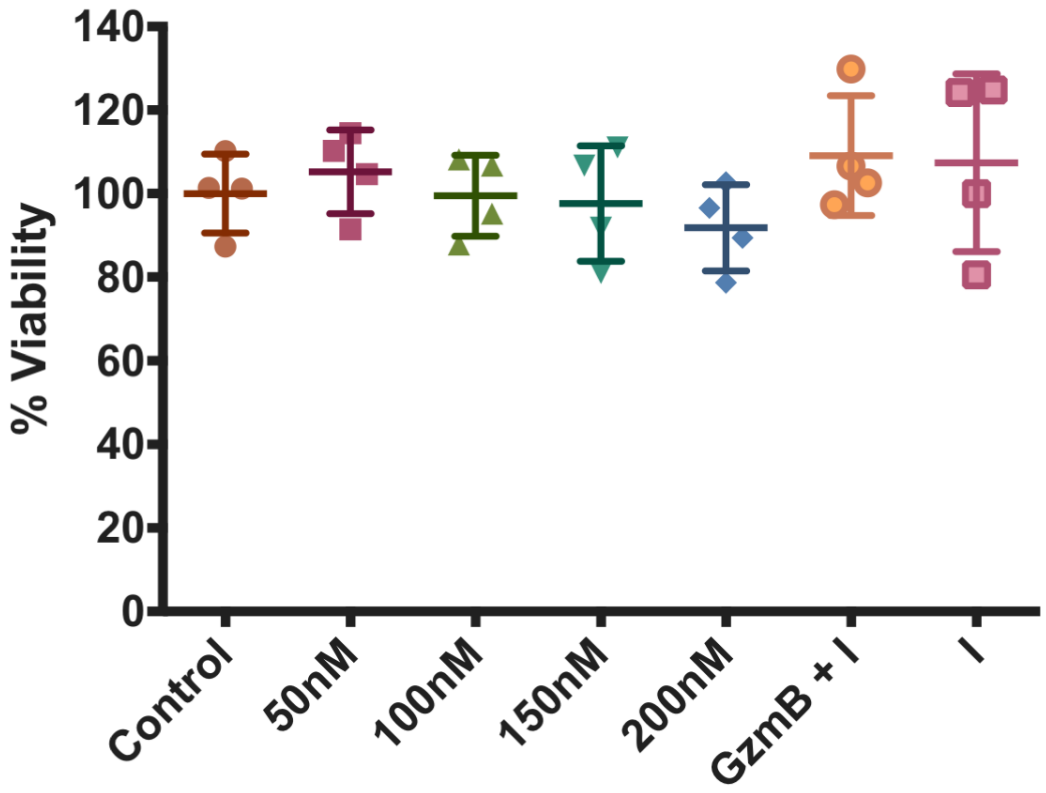


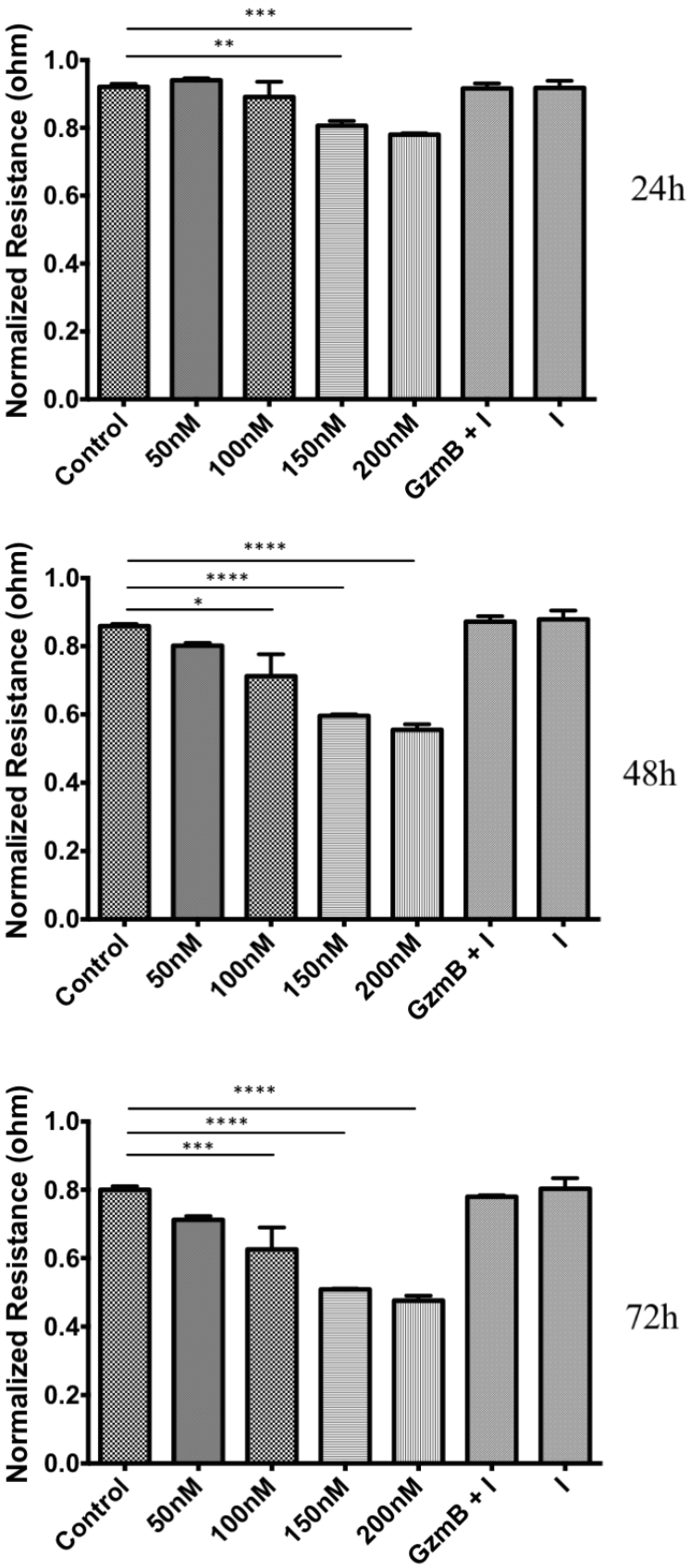
Figure 6. Impact of Granzyme B on HaCaT viability. HaCaTs were treated with increasing doses of GzmB for 72h, after which, MTS solution was added to each well and the absorbance was read at 490 nm. Data was collected from four independent replicates. Data was analyzed using one-way ANOVA followed by Tukey's multiple comparisons post-hoc test, values represent mean ± SD.

3.2 Granzyme B Decreases Epithelial Barrier Function

A major symptom of pemphigus disorders is the loss of epithelial barrier function due to blister formation within the epidermis. GzmB can inhibit cell migration and cleave extracellular matrix proteins (Figure 5), which may play a critical role in epithelial cell reorganization and wound healing. Therefore, we used ECIS to determine whether extracellular GzmB has any effect on epithelial permeability. HaCaTs showed a dose-dependent decrease in barrier function with increasing concentration of GzmB over time (Figure 7A).

A decrease in barrier function in the skin has been correlated with an increase in TEWL,[1,7,8] suggesting that increased permeability is a symptom of the disorder. In order to confirm loss of barrier function, we next looked at dextran paracellular flow through using transwell inserts. 70kDa Rhodamine B dextrans were added to the top chamber following 72 h of GzmB treatment and flow through was read from the bottom well using a fluorescent plate reader. A dose dependent increase is fluorescent signal was observed in the bottom wells of the GzmB treated cells with significance seen in the 200nM lane (Figure 7B). There was a significant ($p < 0.001$) increase in fluorescent signal in the bottom wells of cells treated with 200 nM GzmB as compared to untreated controls. There was no significant difference in the fluorescent signal amongst all other treatment groups however, there appears to be an increasing dose-dependent trend.

A


***
**
Normalized Resistance (ohm)
1.0
0.8
0.6
0.4
0.2
0.0
Control
50nM
100nM
150nM
200nM
GzmB + I
I
24h
****
****
*
48h
****
****
***
72h

B

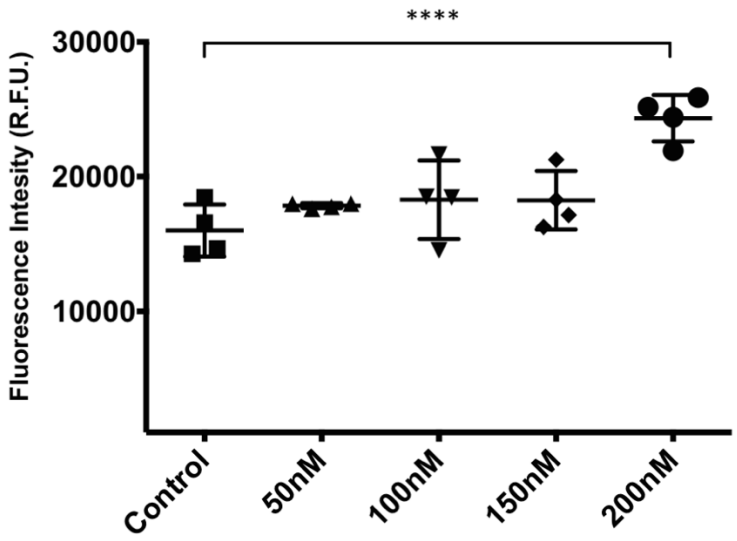


Figure 7: Granzyme B decreases epithelial barrier function. (A) Trans-epithelial electrical resistance of HaCaT keratinocytes measured using ECIS. GzmB was added to a confluent monolayer and resistance was measured continuously thereafter. Results display resistance at 24 h, 48 h, and 72 h post treatment. Values represent mean ± SD. (B) 70 kDa Rhodamine B dextran flow through measured using the Tecan plate reader. Ex: 520 nm Em: 590 nm. In both experiments, data was analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Values represent mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$.

3.3 TAILS Analysis of HaCaT Supernatant

Terminal amine isotopic labelling of substrates (TAILS) is a method of identifying novel N-termini fragments produced by proteolysis compared to the native/natural N-termini. HaCaT cells were grown to confluence and incubated in serum starved media ± 100 nM GzmB for 24 h. The supernatant was collected for TAILS analysis to identify GzmB cleavage substrates. Addition of GzmB showed a long list of potential substrates that are cleaved which included both intracellular and extracellular targets (data not shown). Among those listed were several intercellular junction proteins (Table 3). Table 3 lists identified adhesion junctions, tight junctions, and desmosomes, with their identified target sequences. Three adhesion junctions identified with 3 cleavage sites for

E-cadherin, 4 cleavage sites for protocadherin (FAT) 2, and 1 cleavage site for protocadherin gamma C3. One tight junction was identified with 1 cleavage site identified in JAM-A. Of the desmosomes listed there are 4 identified desmogleins targeted: 1 cleavage site for Dsg-1, 2 cleavage sites for Dsg-2, 1 cleavage site for Dsg-3, and 3 cleavage sites for Dsg-4. Of the desmocollins: 2 cleavage sites were identified for Dsc-2, and 4 cleavage sites for Dsc-3. Of this list of junction proteins, we selected several targets for further analysis. Dsg-1 and Dsg-3 were chosen specifically because they are the two most common autoantigens seen in pemphigus skin blistering disease. E-cadherin was selected for further analysis it is the most prominent and best studied adherens junction, and literature has revealed that cleavage products are implicated in a variety of diseases. JAM-A was chosen as it was the only tight junction identified in the TAILS analysis. To identify where in the protein GzmB cleaves, we mapped the cleavage sites on intercellular junction protein schematics (Figure 8). Specific locations and domains of cleavage sites was determined using the Uniprot website. (http://www.uniprot.org/)[93]

Protein Name	P10 to P1	Sequence	P1' Position	Gene Names	PEP	Ratio GrzB/Control normalized
Adherens Junctions						
Cadherin-1;E-Cad/CTF1/CTF2/CTF3	EQKITYRIWR	DTANWLEINPDTGAISTR	528	CDH1	4.49E-21	1.7519
Cadherin-1;E-Cad/CTF1/CTF2/CTF3	NFEDCTGRQR	TAYFSLDTR	66	CDH1	4.74E-05	1.3372
Cadherin-1;E-Cad/CTF1/CTF2/CTF3	PPVGVFIIER	ETGWLKVTEPLDR	210	CDH1	0.012572	0.49765
Protocadherin Fat 2	SPVSPGPVYR	LVASDLDEGLNGR	1266	FAT2	0.023499	1.7398
Protocadherin Fat 2	SLKFEKAVYR	VQLSEFSPPGSR	369	FAT2	0.053358	1.7072
Protocadherin Fat 2	KLFNVRLPER	LSPVSPGPVYR	1255	FAT2	1.51E-17	1.3106
Protocadherin Fat 2	ASDWGSPFRR	EKEVSIFLQLR	545	FAT2	0.031377	0.79425
Protocadherin gamma-C3	LLLLGALNKA	STVIHYEIPEER	30	PCDHGC3	0.013769	1.0617
Tight Junctions						
Junctional adhesion molecule A	AILLCSLALG	SVTVHSSEPEVR	28	F11R	2.07E-21	0.90215
Desmosomes						
Desmoglein-1	REVTPFFIIY	CRALNSMGQDLERPLELR	127	DSG1	0.028243	1.2067
Desmoglein-2	KHPHLVRQKR	AWITAPVALR	50	DSG2	7.39E-34	0.80633
Desmoglein-2	LLTGYALDAR	GNNVEKPLELR	136	DSG2	0.0172	0.62216
Desmoglein-3	FHQSVISRYR	VQSTPVTIQVINVR	365	DSG3	0.026505	1.3673
Desmoglein-4	YNLVVRGSDR	DGAADGLSSECDCR	243	DSG4	0.0061079	2.1331
Desmoglein-4	LIYCRALNSR	GEDLERPLELR	134	DSG4	0.017557	1.6054
Desmoglein-4	SLLNYVLGTY	TAIDLDTGNPATDVR	412	DSG4	0.018281	0.98662
Desmocollin-2	ENTVDVEILR	VTVEDKDLVNTANWR	376	DSC2	0.027121	1.9507
Desmocollin-2	LKAINDTAAR	LSYQNDPPFGSYVVPITVR	635	DSC2	0.031357	1.1958
Desmocollin-3	EYDVFDLIAY	ASTADGYSADLPLPLPIR	215	DSC3	0.00030894	1.5929
Desmocollin-3	VKPLNYEENR	QVNLEIGVNNEAPFAR	430	DSC3	0.0054584	1.5214
Desmocollin-3	RDEPDTMHTR	LKYSILQQTPR	280	DSC3	0.0032082	0.59823
Desmocollin-3	ENAFNVEILR	IPIEDKDLINTANWR	376	DSC3	0.0043228	0.59105

Table 3: List of Intercellular Junction Targets for Granzyme B. Listed are protein names, a.a. sequence before (P10-P1) and after cleavage site, P1' position, gene names, posterior error probability (PEP), and ratios. The PEP is defined as the ratio between the decoy database and the true database; a low value indicates a low probability of error. The GzmB/control normalized ratio is how frequently the given fragment appears in the GzmB treated supernatant compared to the control supernatant.

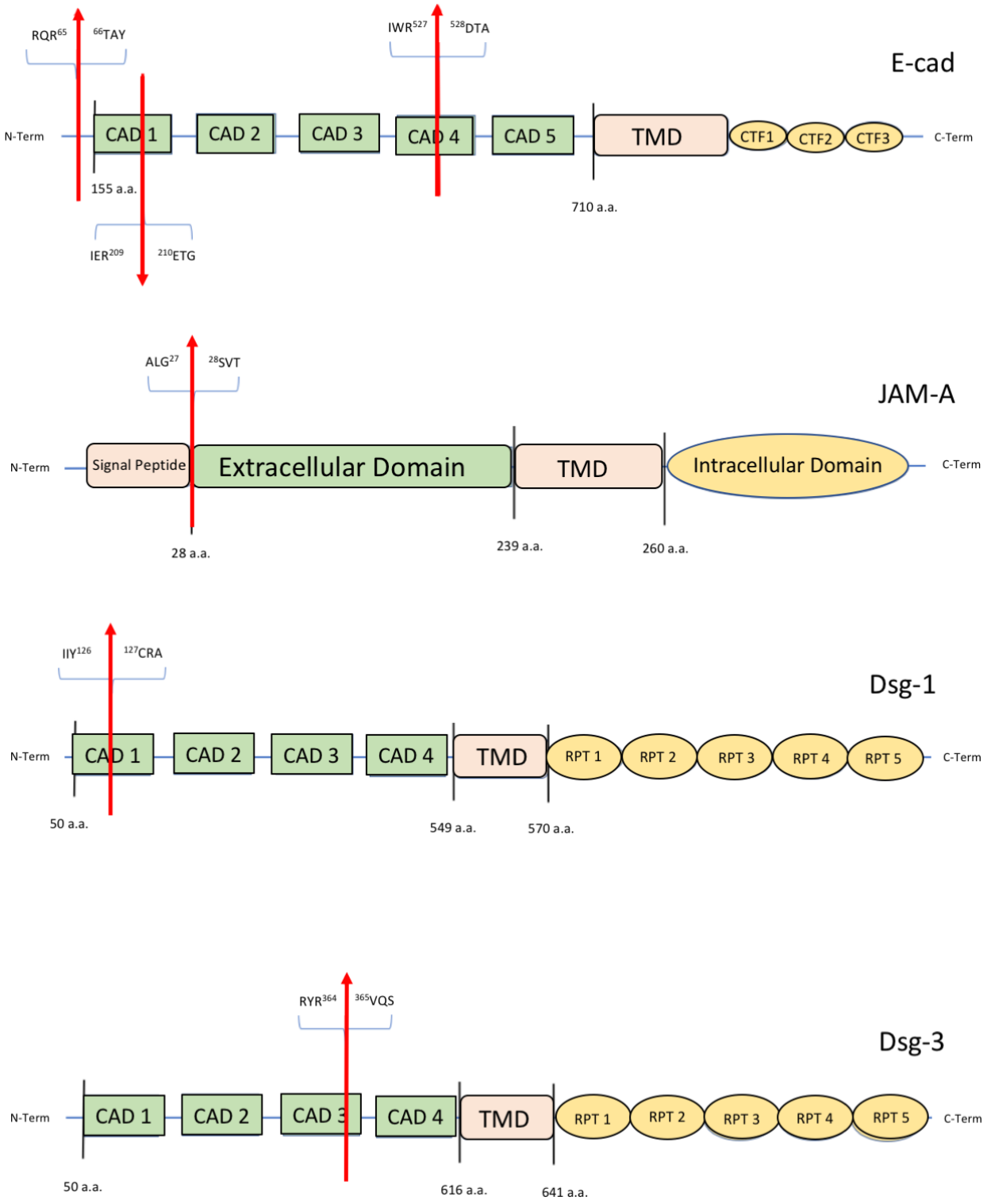


Figure 8: Schematic of granzyme B cleavage sites. Intercellular junction domain schematics with detected GzmB cleavage sites (red arrows) in E-cad, JAM-A, Dsg-1, and Dsg-3. TMD = Transmembrane Domain, Cad = Cadherin, RPT = Intracellular Cadherin Repeats, CTF = C-Terminal Fragment.

3.4 Granzyme B Cleaves Intercellular Junctions

With the loss of epithelial barrier function following GzmB treatment (Figure 6), our next step was to determine whether this was accomplished via proteolytic cleavage of cell-cell adhesion proteins. From the results obtained in section 3.3 (Table 3), we selected several targets for *in vitro* confirmation of proteolytic cleavage (Figure 8). The tight junction protein, JAM-A, the adherens junction protein, E-cadherin, and the desmosomes, Dsg-1 and Dsg-3 were selected for further analysis. ZO-1 was also included in the analysis based on recent findings by Pardo's group, which found that GzmB could cleave intercellular junctions in endothelial cells including, platelet endothelial cell adhesion molecule (PECAM), JAM-A, and ZO-1.[81]

First, we performed a biochemical cleavage assay of the selected intercellular junctions listed above. Substrates were incubated with GzmB for 2 h prior to being separated via SDS-PAGE to identify cleavage fragments. All five proteins showed a reduction of whole protein and an increase in fragmentation when incubated with GzmB versus protein alone or GzmB + C20 (Figure 9). E-cadherin and Dsg-1 showed an almost complete loss of whole protein with 100 nM GzmB, whereas JAM-A, ZO-1, and Dsg-3 showed less cleavage as whole protein was still detectable after GzmB treatment.

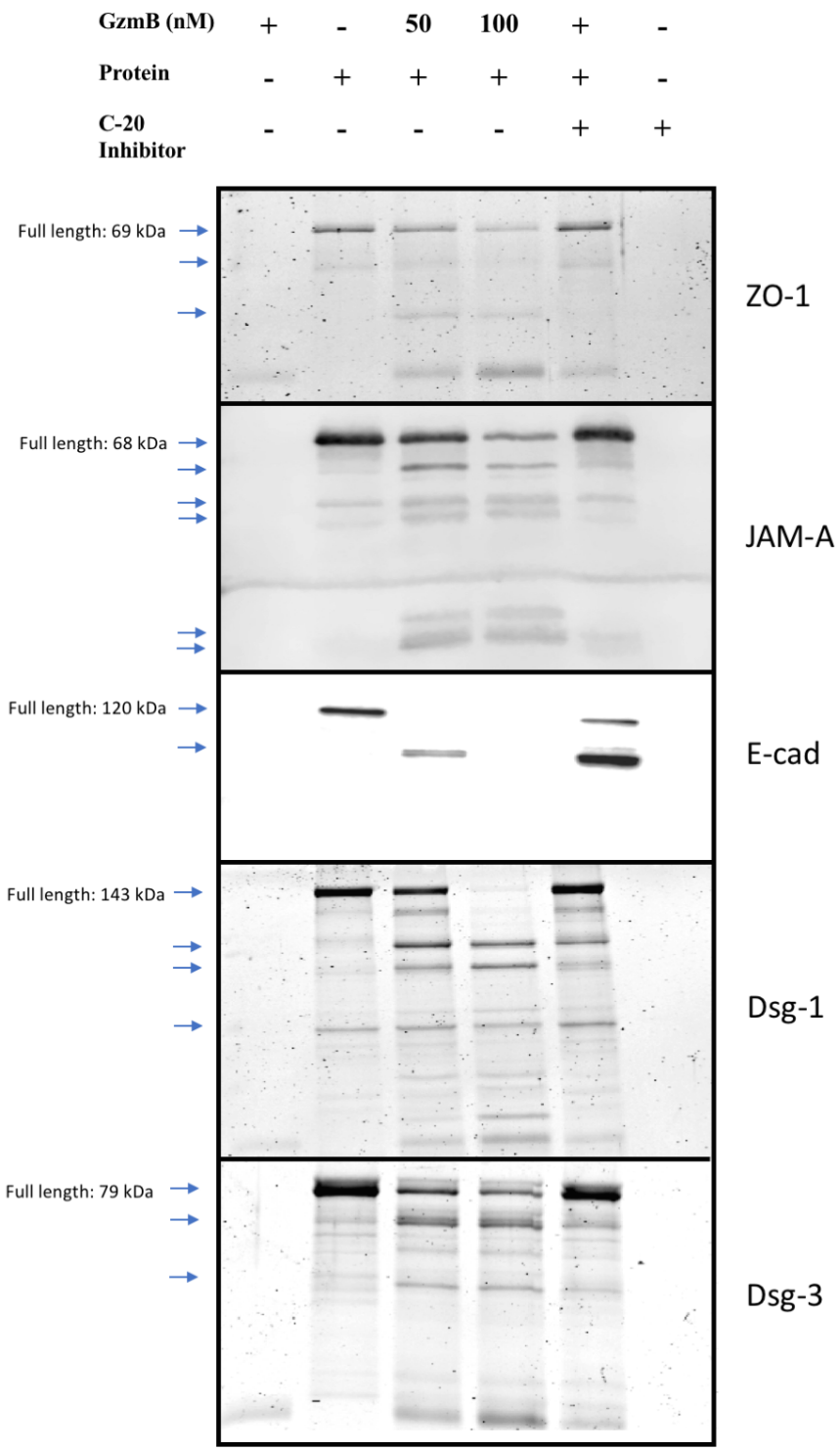
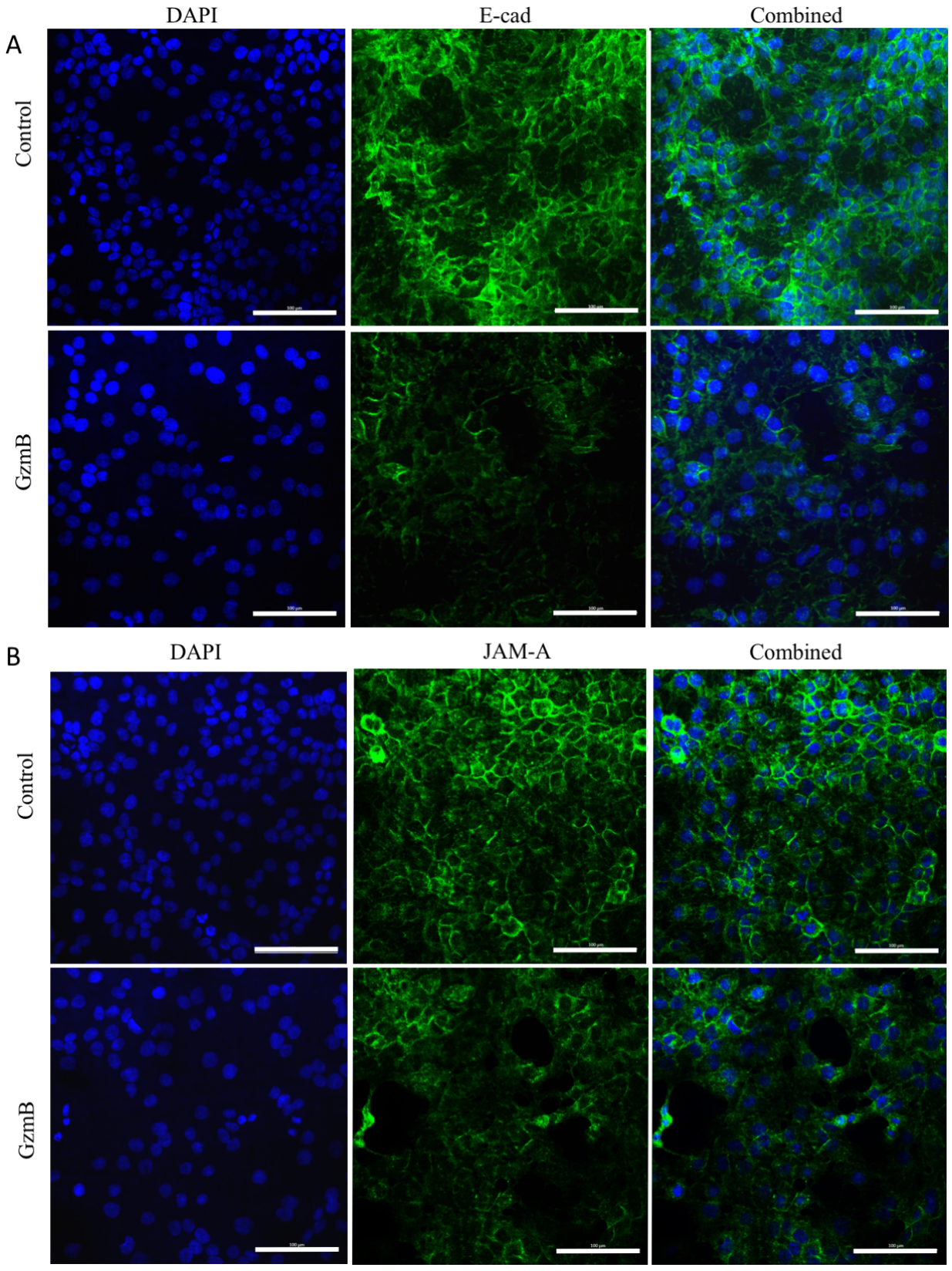
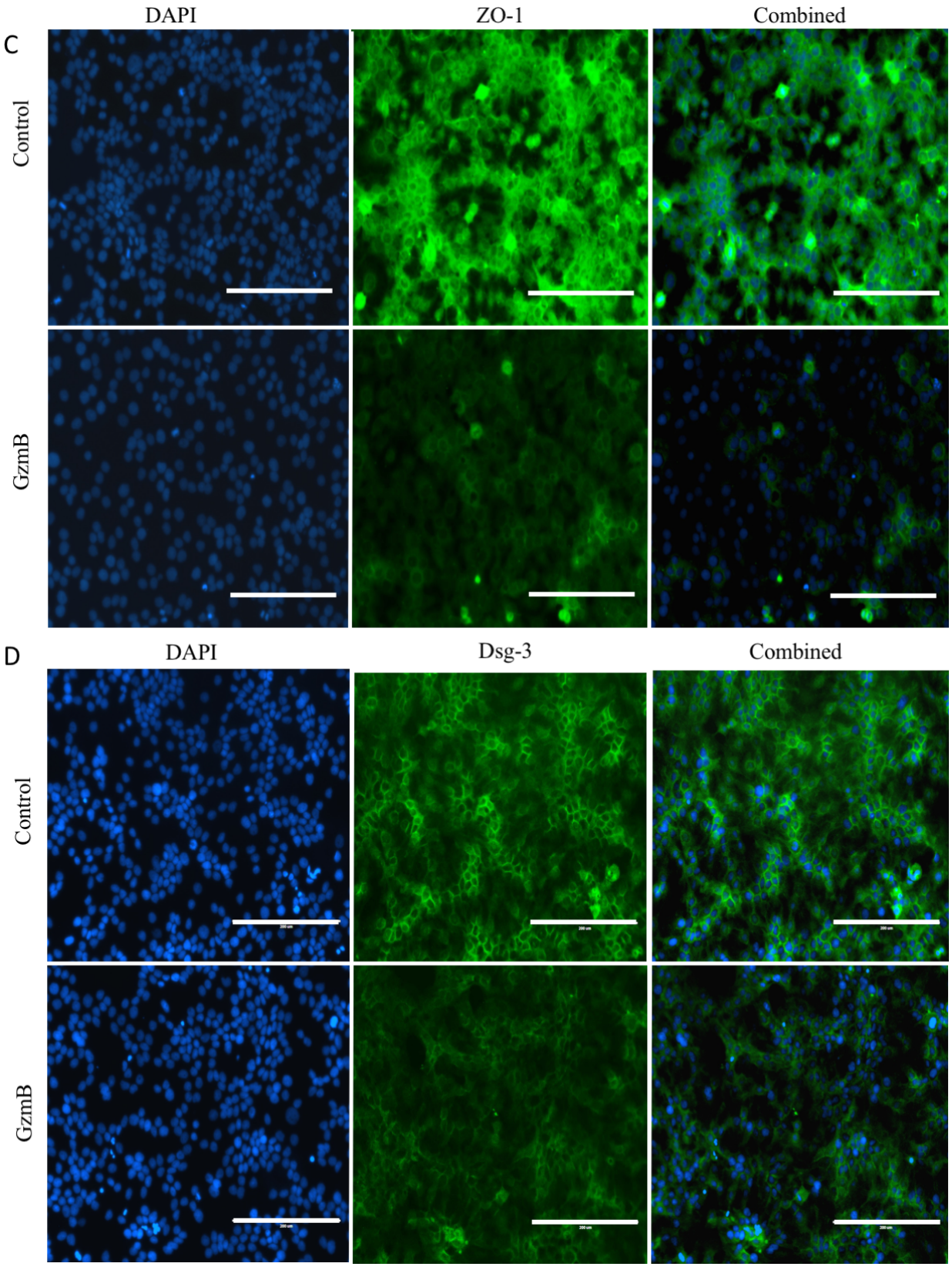


Figure 9: Biochemical cleavage of intercellular junctions. Whole protein was incubated with GzmB ± C20 inhibitor for 2h. Protein was run on SDS PAGE gel and analyzed by western blotting (JAM-A, E-cad) or by Coomassie staining (ZO-1, Dsg-1, Dsg-3). Blue arrows indicate full length protein (labelled at top) and subsequent fragments underneath.

To assess the morphological changes of intercellular junctions *in vitro*, in the presence of GzmB, HaCaT cells were serum starved and incubated with 100 nM GzmB or left untreated for 8h. Cells were then fixed in formalin and imaged by immunofluorescence for intercellular junction morphology. Untreated cells showed a linear pattern at cell junctions, while GzmB treatment showed a more disperse and irregular pattern when stained with E-cadherin (Figure 10A). JAM-A showed a weaker, more diffuse signal in the GzmB treated *vs*. untreated cells (Figure 10B). ZO-1 displayed a strong signal along closely clustered cells in the control, while in the GzmB treated images there is a large drop in fluorescence with ZO-1 only appearing in dividing and densely clustered cells (Figure 10C). Dsg-3 and Dsg-1 Figure 10D & 10E respectively) both show a drop in fluorescence with Dsg-1 displaying a scattered and irregular appearance in cell morphology.


A
DAPI
E-cad
Combined
Control
GzmB
B
DAPI
JAM-A
Combined
Control
GzmB


C
DAPI
ZO-1
Combined
Control
GzmB
D
DAPI
Dsg-3
Combined
Control
GzmB

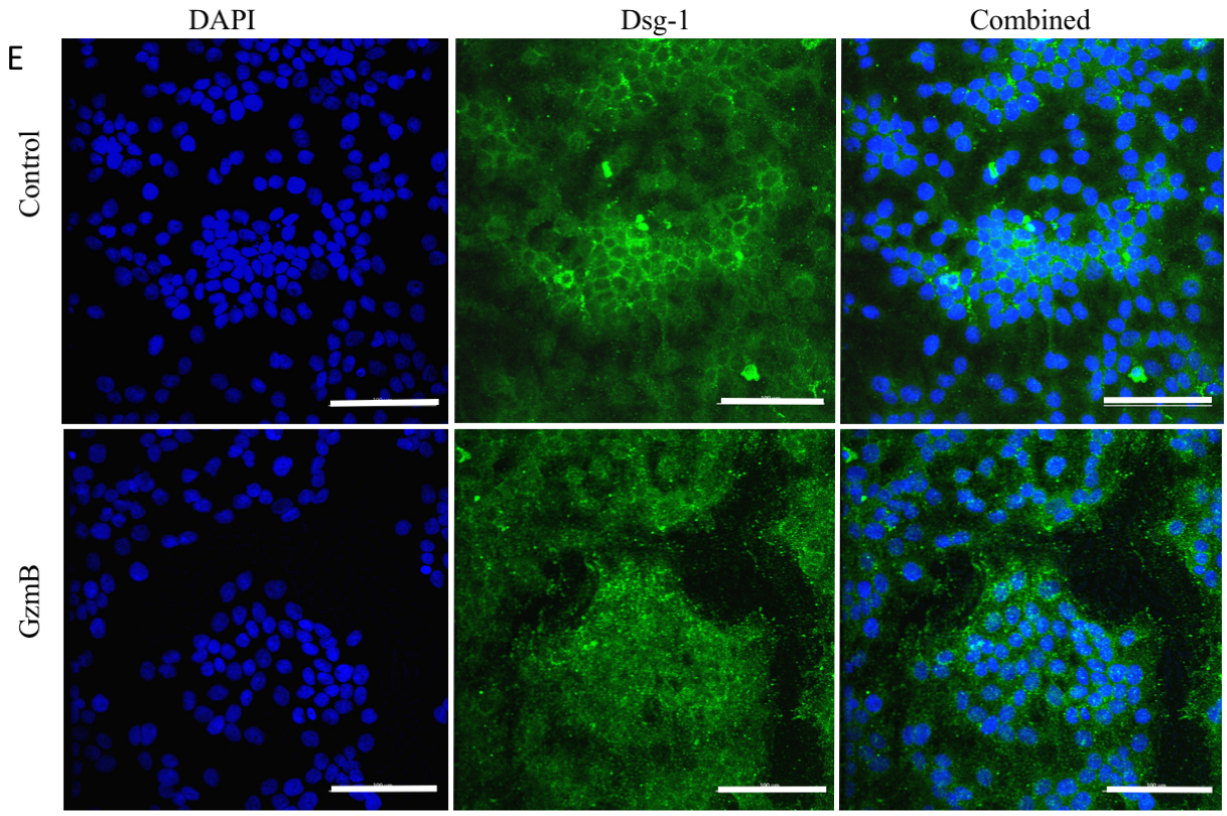


Figure 10: Immunocytochemistry of cell-cell junctions. Confocal images showing immunofluorescence staining of HaCaT cells for (A) E-cadherin, (B) JAM-A, (C) ZO-1, (D) Dsg-3, and (E) Dsg-1 after treatment with 100 nM GzmB or untreated for 8h. DAPI was used as a nuclear stain. Scale bars = 100 μm.

3.5 Granzyme B Produces sE-cad Fragments

Soluble E-cadherin is the extracellular portion of whole E-cadherin that has been proteolytically cleaved. As discussed in section 1.2.2.1, sE-cad is implicated in many different diseases ranging from cancer to pemphigus.[23,25,26] The TAILS analysis (Table 3 & Figure 8) identified three separate cleavage sites, with the predicted fragment at position 528 showing the most promise for the formation of sE-cad. Therefore, our next step was to identify whether GzmB is capable of producing sE-cad *in vitro* by analyzing the supernatant of HaCaT cells exposed to GzmB (Figure 11A). HaCaTs were treated with 50 nM or 100 nM of GzmB or left untreated for 24 h. Supernatants were collected, concentrated and separated *via* SDS-PAGE. Antibodies directed toward the N-terminal region of E-cadherin showed a band of increasing intensity with increasing concentrations of GzmB (Figure 11A). Western blot analysis of whole cell lysates demonstrates that there is no difference in intracellular E-cad levels for either the N- or C-terminus. This suggests that intracellular levels of E-cad remain relatively constant (Figure 11B).

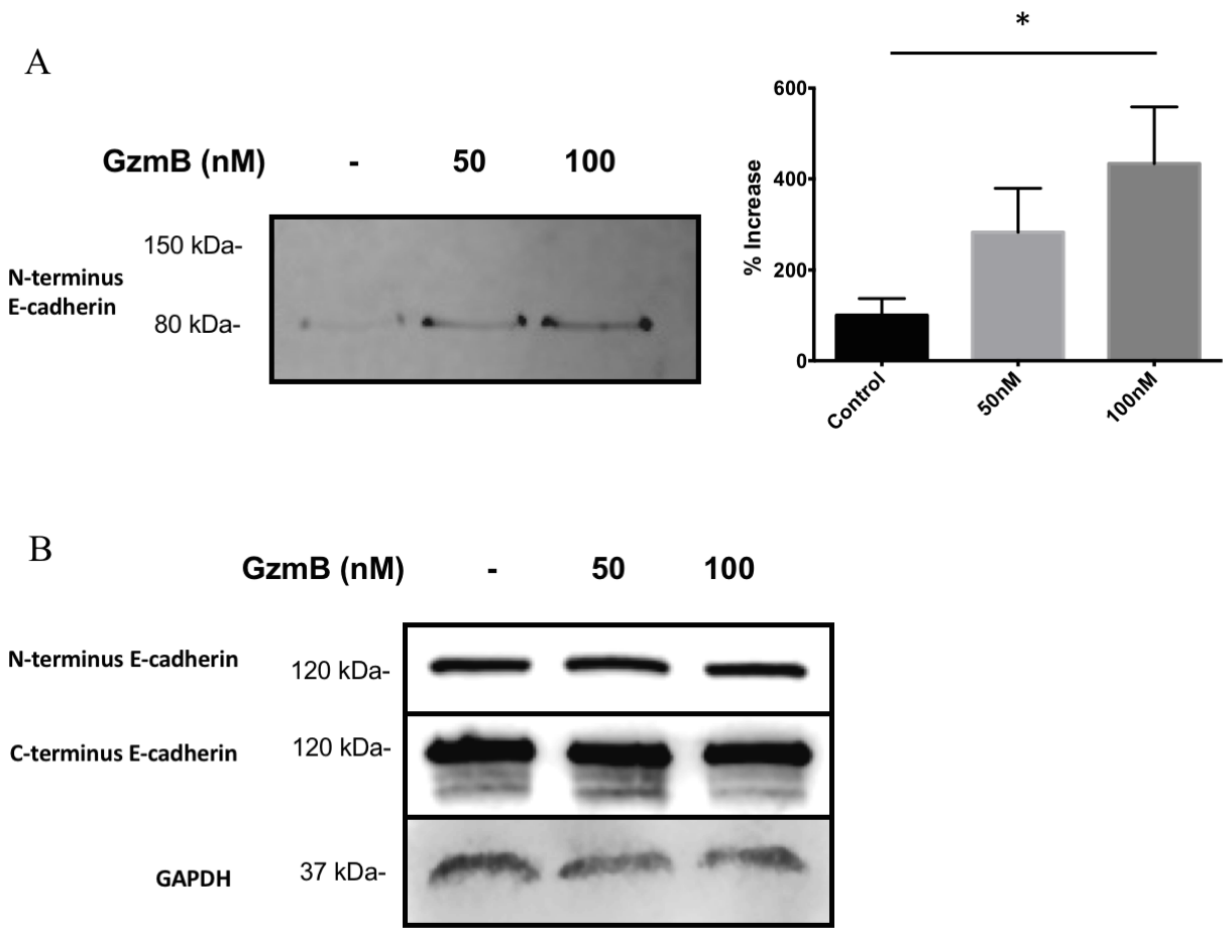
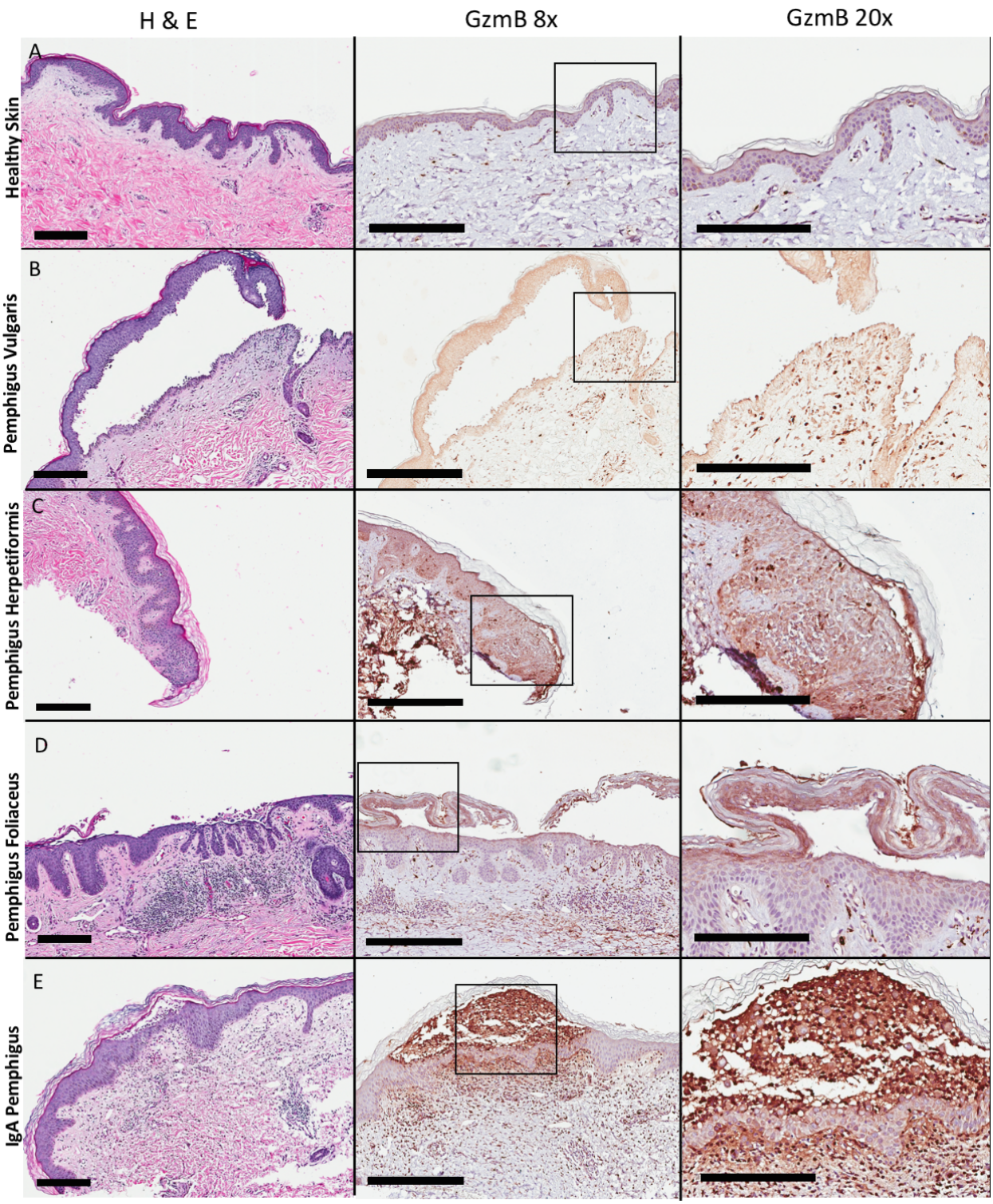


Figure 11: Granzyme B Produces sE-cad Fragments. (A) Western blots of concentrated cell supernatants collected after 24 h of GzmB exposure. HaCaTs were treated with 50 nM or 100 nM GzmB, or left untreated. Bar graph shows levels of sE-cad released in the supernatant as percentages compared to control. Values are expressed as mean ± SD (B) HaCaT cell lysates were used for Western blotting of E-cadherin. Monoclonal antibodies for the N- and C-terminus of E-cad were used. GAPDH was used as an internal control. Data was analyzed using one-way ANOVA followed by Tukey's multiple comparisons post-hoc test. Values represent mean ± SD, *$p < 0.05$.

3.6 Elevated Levels of Granzyme B in Pemphigus Tissues

A major characteristic of pemphigus diseases is a loss of epithelial barrier function. This is due, in part, to the loss of cell-cell adhesion mediated by the loss of functional desmosomes. Therefore, we looked at *ex vivo* sections of human skin blisters from patients with different pemphigus subtypes in order to establish whether GzmB is present in the epidermis in or around the lesion. Formalin fixed, paraffin embedded pemphigus blister tissues were analyzed by hematoxylin & eosin (H&E) staining and immunostaining for GzmB (Figure 12). Compared with healthy skin (Figure 12A), diffuse GzmB staining within the epidermis and sites of acantholysis are noted in pemphigus herpetiformis (Figure 12C), pemphigus foliaceus (Figure 12D), and pemphigus vegetans (Figure 12I). The largest increase in GzmB was observed within the blister and blister fluid, especially in IgA pemphigus (Figure 12E), Hailey-Hailey disease (Figure 12F), and limited oral pemphigus vulgaris (Figure 12H). While pemphigus vulgaris (Figure 12B) and paraneoplastic pemphigus (Figure 12G) exhibit lower levels of GzmB however, there is still evidence of GzmB accumulation within the dermis.


H & E
GzmB 8x
GzmB 20x
A
Healthy Skin
B
Pemphigus Vulgaris
C
Pemphigus Herpetiformis
D
Pemphigus Foliaceus
E
IgA Pemphigus

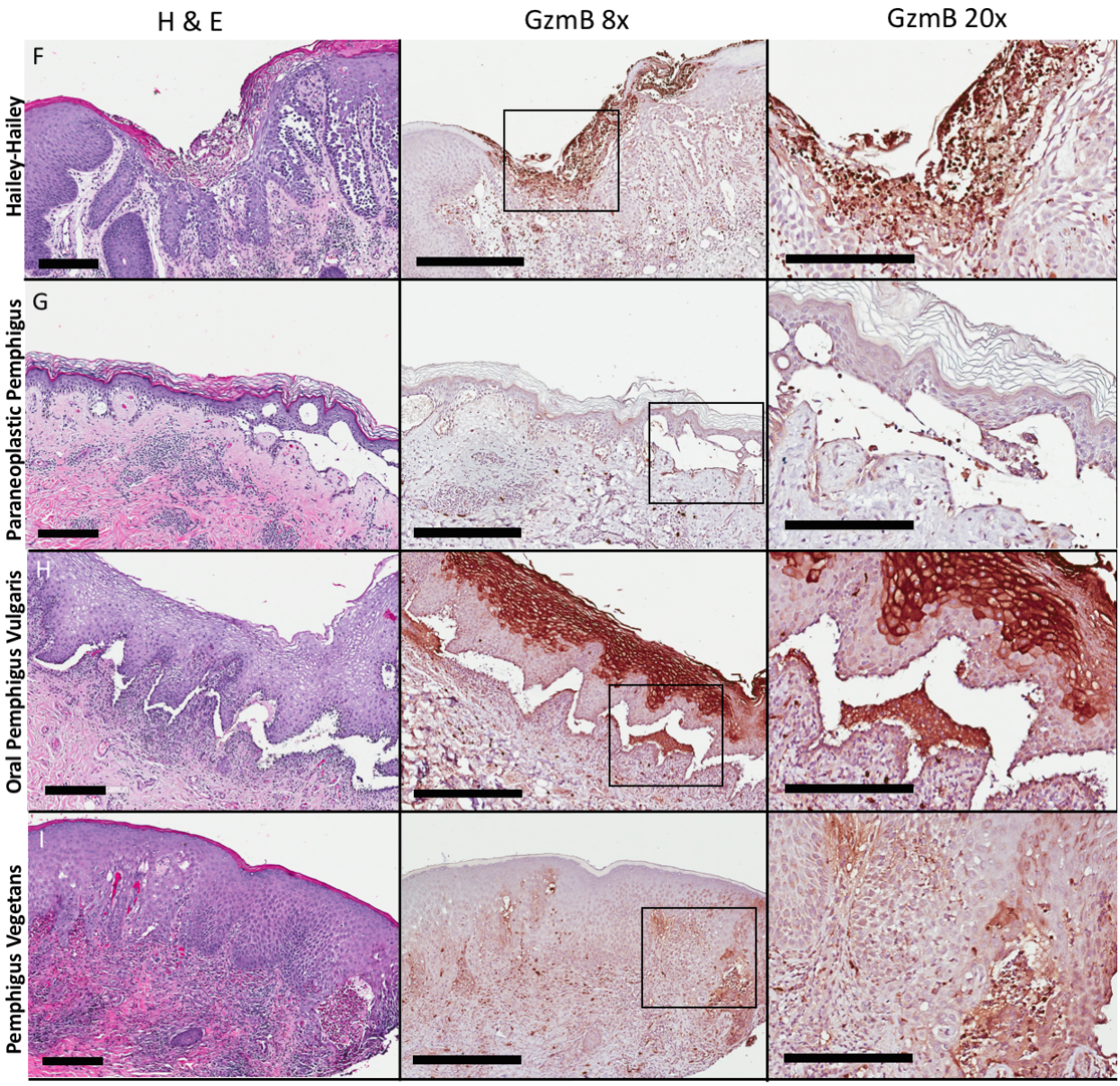
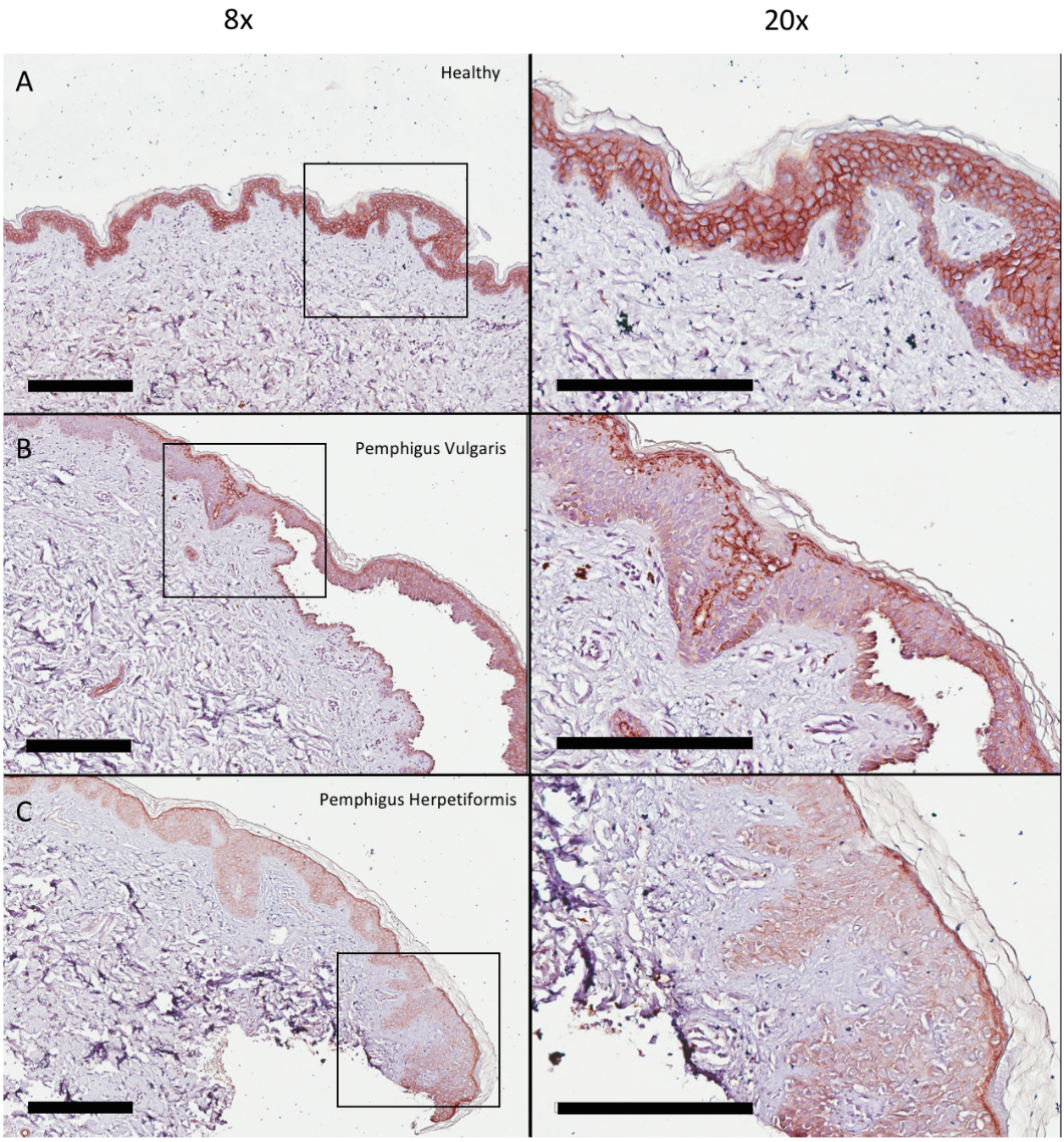
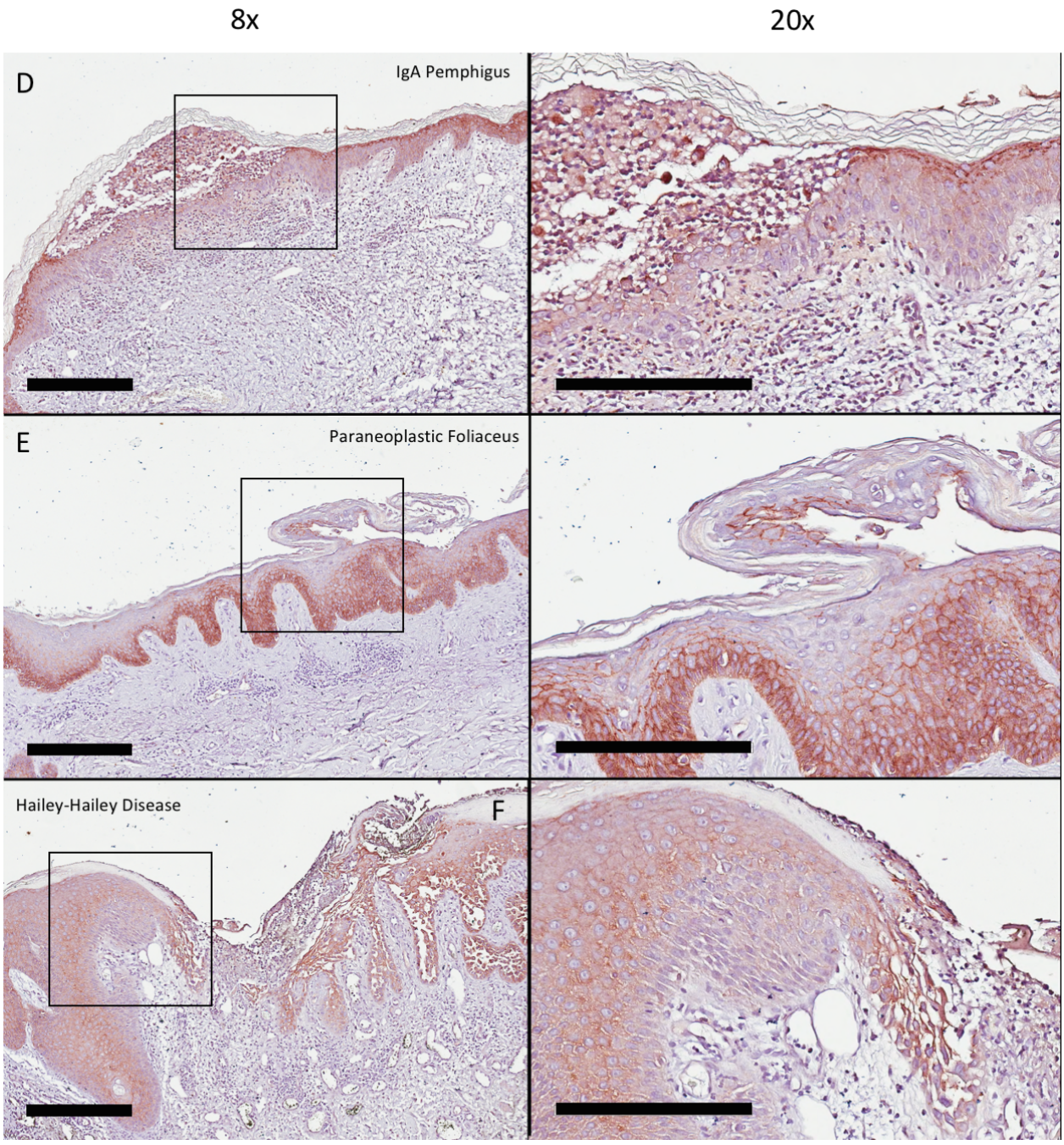


Figure 12: Elevated Levels of GzmB in Pemphigus Tissues. The left column are representative images of H&E staining and in the middle (8x) and right (20x) columns are GzmB immunostaining of healthy or blister skin biopsies. Abundant GzmB is observed within the epidermis and in the blister, as well as within the dermis. (A) Healthy skin, (B) pemphigus vulgaris, (C) pemphigus herpetiformis, (D) pemphigus foliaceus, (E) IgA pemphigus, (F) Hailey-Hailey disease, (G) paraneoplastic pemphigus, (H) limited oral pemphigus vulgaris, and (I) pemphigus vegetans. Black box indicates area of magnification. Left and right column: black bars = 200 μm, middle column: black bars = 300 μm.

3.7 Granzyme B and E-cadherin in Pemphigus Tissue

As seen in section 3.6 and Figure 10, GzmB can cleave the extracellular portion of E-cad known as sE-cad. Additionally, as mentioned in a review by Furukawa et al., western blotting for sE-cad is observed in blister fluids.[25] Using the same pemphigus tissue blocks mentioned above, we next looked at the staining pattern of E-cad. Antibodies directed toward the N-terminus region of E-cad was used to visualize the extracellular E-cad (Figure 13). Healthy skin displays an intact E-cad staining, lining the edges of the cells (Figure 13A). Pemphigus herpetiformis (Figure 13C) and pemphigus vegetans (Figure 13I) show an overall diffuse signal throughout the epidermis. E-cadherin staining appears very diffuse in the lower epidermis of pemphigus vulgaris (Figure 13B) and around the blister in IgA pemphigus (Figure 13D). E-cadherin staining is concentrated in the lower epidermis of pemphigus foliaceus with almost absent signal in the upper layers (Figure 13E). Hailey-Hailey disease (Figure 13F) and limited oral pemphigus vulgaris (Figure 13H) show irregular staining patterns throughout the epidermis, while paraneoplastic pemphigus (Figure 13G) shows a staining pattern very similar to healthy skin. However, in the case of Hailey-Hailey disease, this diffuse pattern in E-cad staining is mostly likely due to the inability to transport Ca^{2+}, and thus cadherin formation is incomplete.[45]


8x
20x
A
Healthy
B
Pemphigus Vulgaris
C
Pemphigus Herpetiformis


8x
20x
D
IgA Pemphigus
E
Paraneoplastic Foliaceus
Hailey-Hailey Disease
F

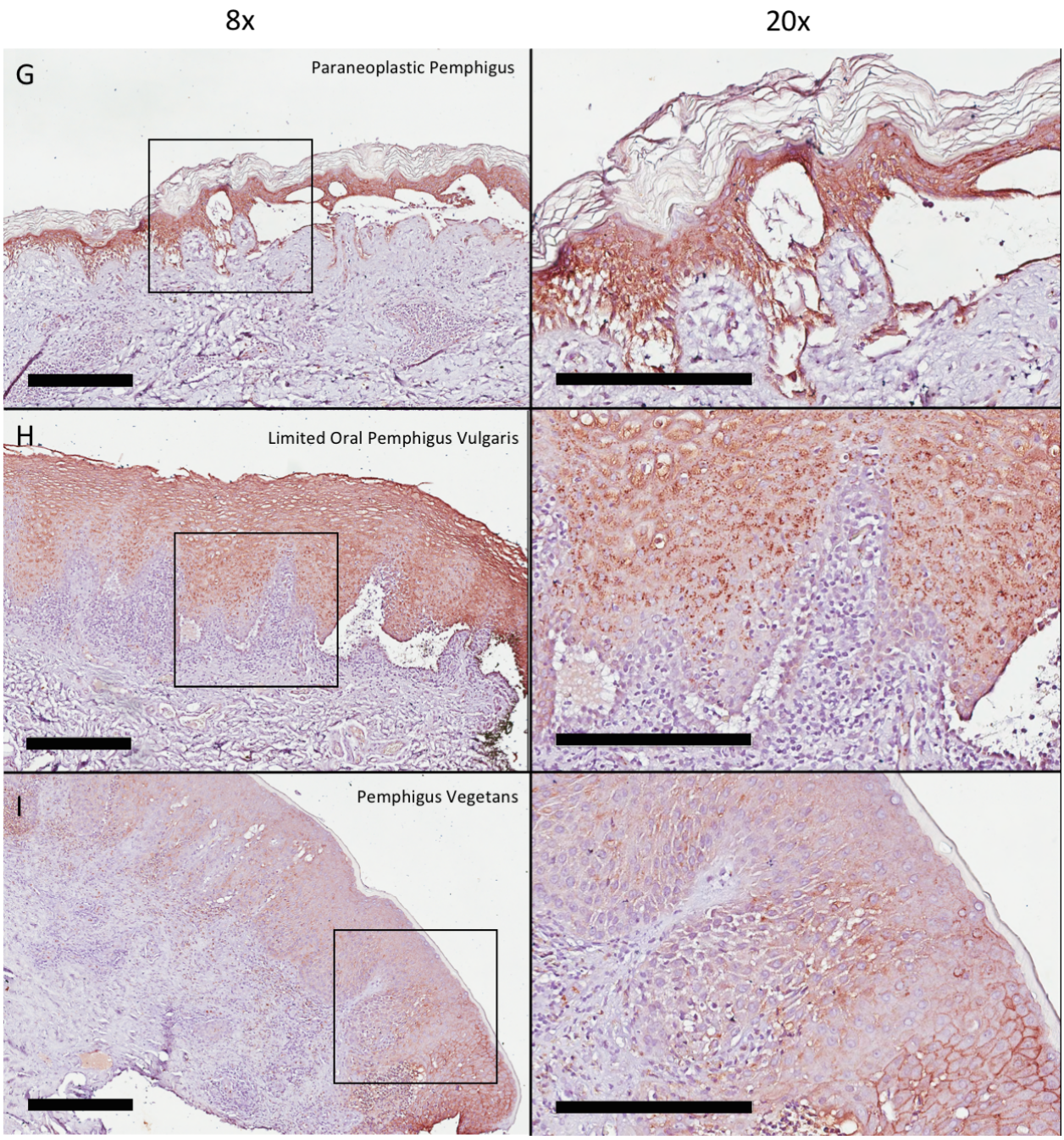
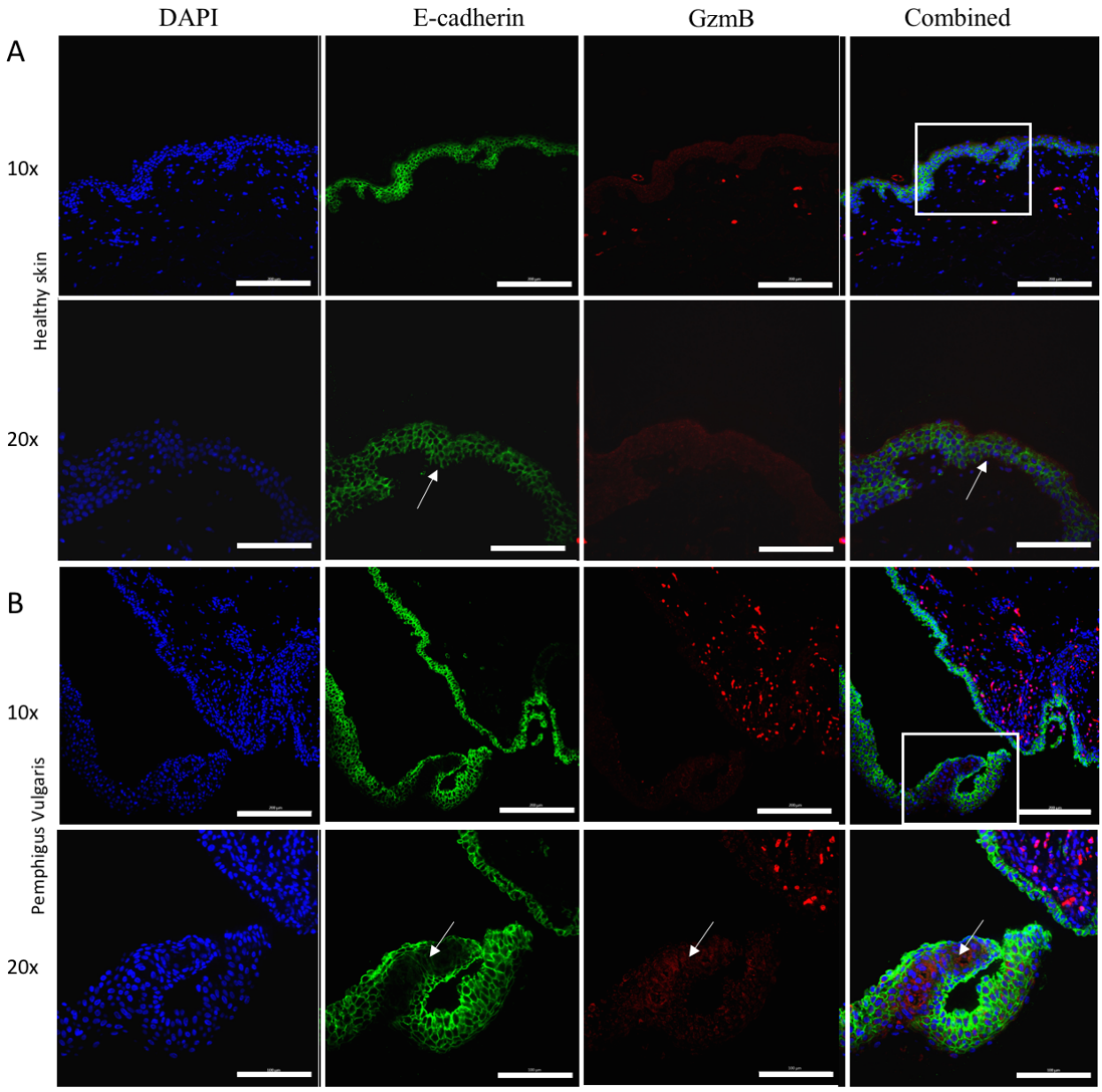
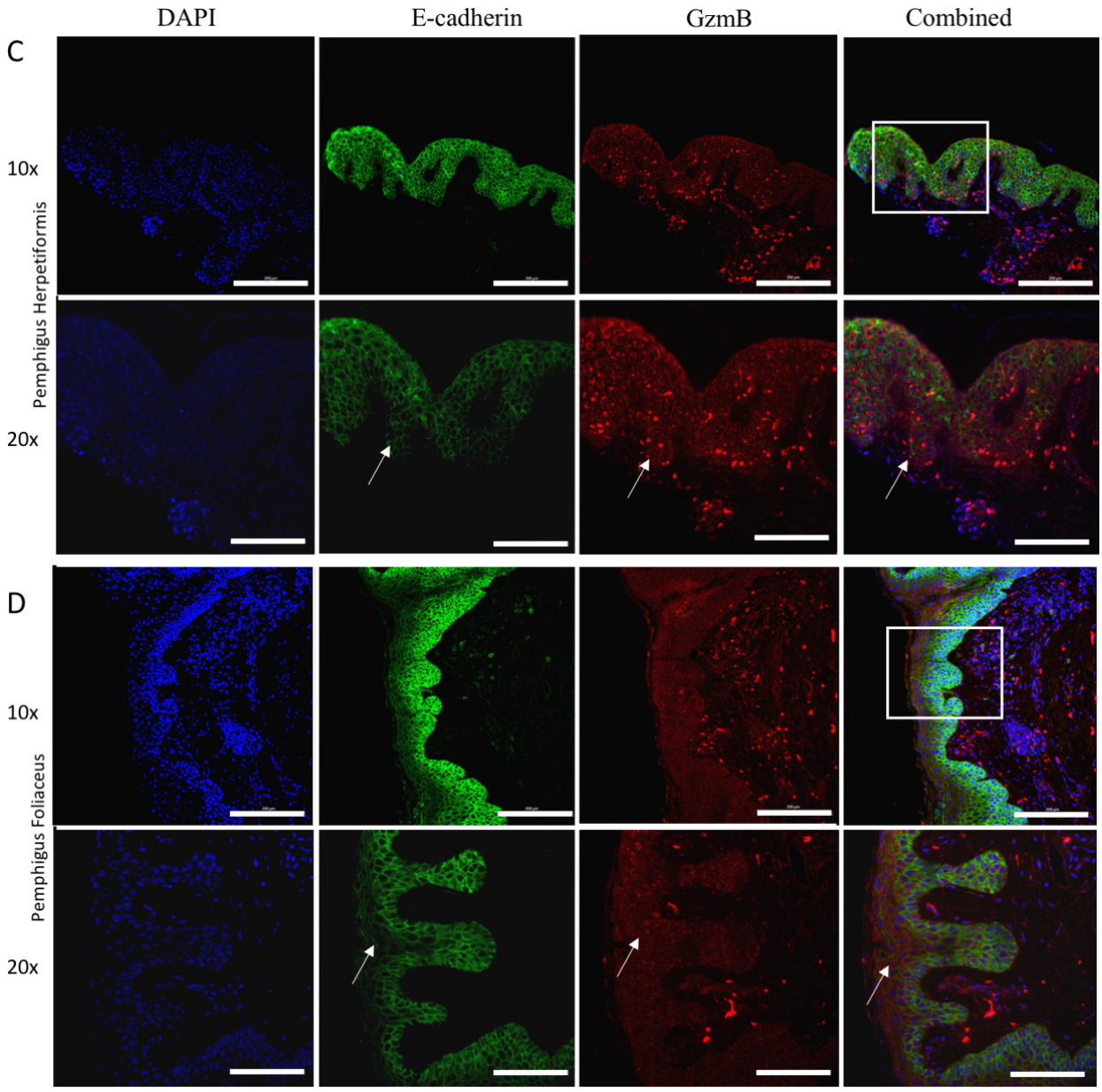
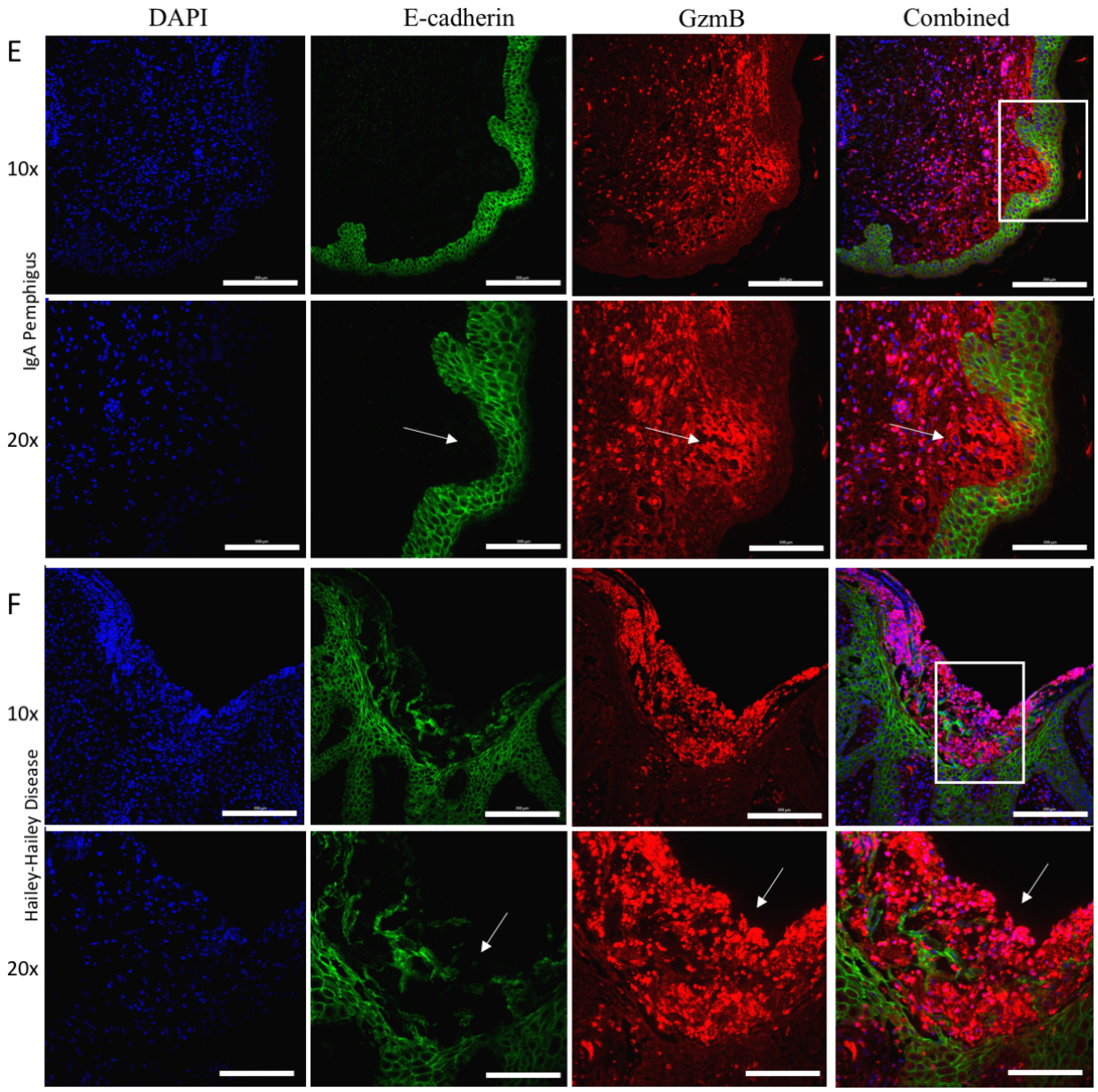


Figure 13: E-cadherin Immunohistochemistry. E-cadherin immunostaining of the indicated healthy or pemphigus biopsies. Extracellular immunostaining of E-cadherin appears intact within the healthy tissue but diffuse and faded in many pemphigus samples. (A) Healthy skin, (B) pemphigus vulgaris, (C) pemphigus herpetiformis, (D) pemphigus foliaceus, (E) IgA pemphigus, (F) Hailey-Hailey disease, (G) paraneoplastic pemphigus, (H) limited oral pemphigus vulgaris, and (I) pemphigus vegetans. 8x magnification in left column (black box indicates magnified area), 20x magnification in right column. Left column black bars = 300 μm, right column black bars = 200 μm.

The same pemphigus tissue blocks mentioned above were co-stained by immunofluorescence for DAPI, GzmB, and E-cad in order to determine if presence of GzmB correlated with a loss of E-cad staining (Figure 14). E-cadherin staining in healthy skin shows a clear fluorescence along the borders with very little GzmB staining (Figure 14A). IgA pemphigus and Hailey-Hailey disease show highly elevated levels of GzmB in the blister but with unperturbed E-cad (Figure 14E & F respectively). Pemphigus foliaceus, paraneoplastic pemphigus (Figure 14G) and limited oral pemphigus vulgaris show similar E-cad and GzmB staining patterns as seen in figures 12 and 13. Pemphigus foliaceus has a drop in E-cad fluorescence along the upper epidermis, and limited oral pemphigus vulgaris shows lower E-cadherin staining where GzmB is elevated (Figure 14D & H respectively). Pemphigus vulgaris and pemphigus herpetiformis have elevated levels of GzmB; however, E-cadherin shows minimal changes in fluorescence (Figure 14B & C respectively). The E-cadherin antibody used for immunofluorescence is a polyclonal antibody and therefore, the staining pattern appears different compared to Figure 13, suggesting a possible loss in N-terminal E-cadherin with an intact C-terminus.


DAPI
E-cadherin
GzmB
Combined
A
10x
Healthy skin
20x
B
10x
Pemphigus Vulgaris
20x


DAPI
E-cadherin
GzmB
Combined
C
10x
Pemphigus Herpetiformis
20x
D
10x
Pemphigus Foliaceus
20x


DAPI
E-cadherin
GzmB
Combined
E
10x
IgA Pemphigus
20x
F
10x
Hailey-Hailey Disease
20x

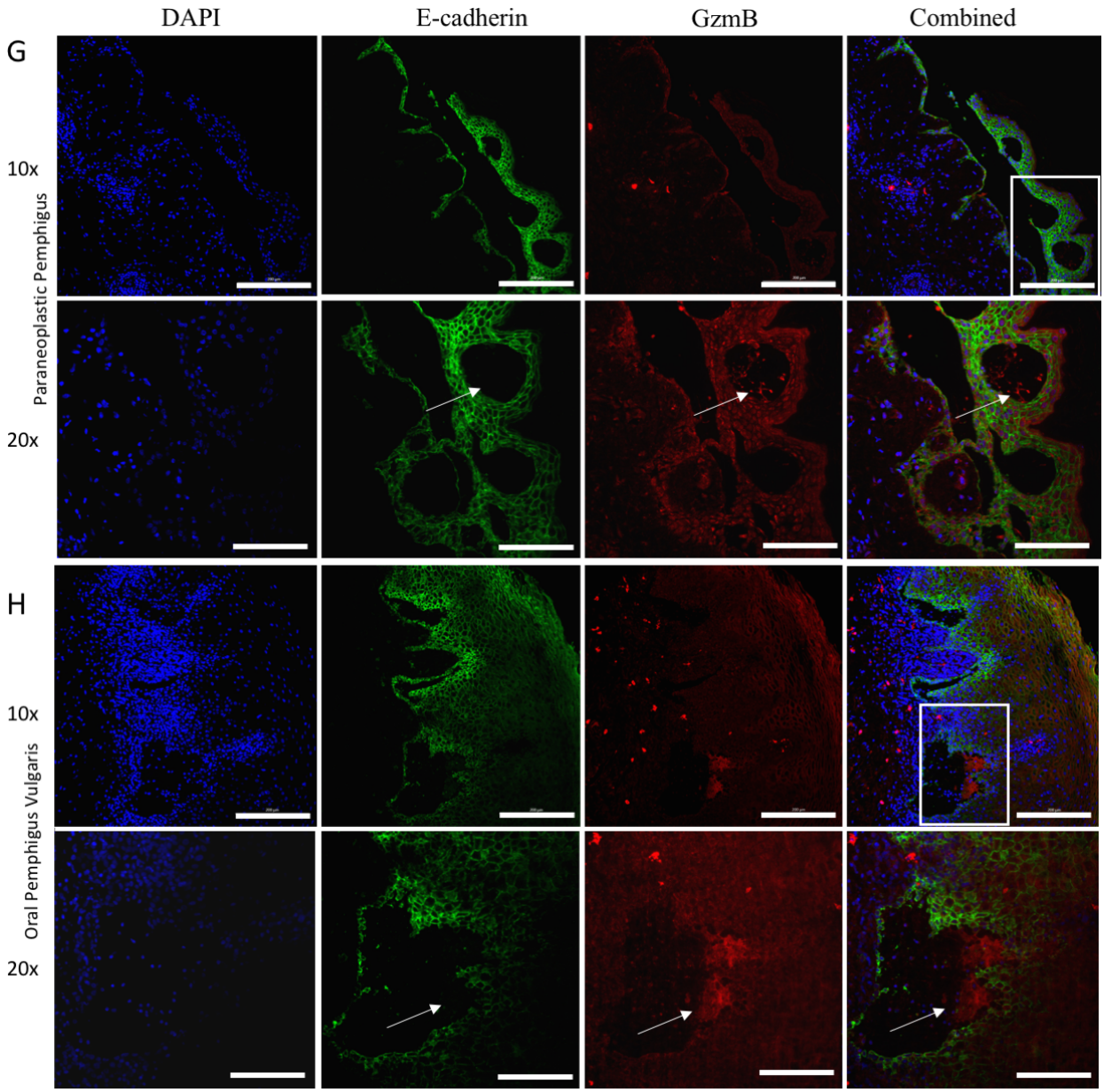


Figure 14: Granzyme B and E-cadherin Co-staining. Confocal images of fluorescent staining pattern of DAPI (blue), E-cadherin (green) and GzmB (red). (A) Healthy skin, (B) pemphigus vulgaris, (C) pemphigus herpetiformis, (D) pemphigus foliaceus, (E) IgA pemphigus, (F) Hailey-Hailey disease, (G) paraneoplastic pemphigus, and (H) limited oral pemphigus vulgaris. Top row is 10x magnification (Scale bars = 200 μm), bottom row is the 20x magnification (Scale bars = 100 μm) of the same image as indicated by the white box. White box indicates area of magnification, white arrows indicate areas of interest.

Chapter 4: Discussion

The epithelial barrier is essential for maintenance of internal homeostasis. The skin, as a whole, provides a protective layering as both the stratum corneum and the keratinocytes in the lower layers form the two major epidermal barriers. In fact, only a partial increase in TEWL is seen after tape stripping removes the stratum corneum,[1] whereas removing a significant portion of the epidermis through suction blisters will cause a severe disturbance in barrier function characterized by an extreme loss in TEWL.[1] Therefore, cell-cell connections are imperative in the maintenance of a healthy epithelial barrier. Intercellular junctions are the backbone of cell adhesion, and when extracellular proteases, cleave these adhesion proteins, the extracellular fragments can have multiple downstream effects. GzmB cleaves many ECM proteins in the skin including laminin, fibronectin, vitronectin, and decorin.[78,80] Thus, the presence of GzmB within the skin suggests a possible pathologic role within the epidermis in diseased skin.

The epithelial barrier exerts critical roles throughout the body including skin, mucosa, gastrointestinal and respiratory tracts, and the choroid plexus in the blood-cerebrospinal fluid barrier.[32,94] Epithelial barrier dysfunctions is observed in conditions such as allergic airway inflammation, asthma, inflammatory bowel disease, multiple sclerosis, atopic dermatitis, and pemphigus.[4,5,12,32,94] Compared to other epithelial barriers, only the skin has an extra external barrier known as the stratum corneum. The intestinal and respiratory tracts, and the choroid plexus are almost entirely dependent on cell-cell junctions for a regulated gateway and barrier formation. Many publications have studied the role of MMPs and ADAMs in the role of barrier dysfunction, while other studies have noted a beneficial role of serine proteases in barrier maintenance.[95,96] However, the role of GzmB-mediated proteolysis in epithelial barrier function had yet to be investigated. My studies demonstrated that GzmB significantly impairs barrier function by

decreasing TEER and increasing paracellular permeability. Thus, GzmB appears to decrease resistance by increasing intercellular spaces and ultimately creating a more permeable monolayer.

As mentioned above, there is a significant decline in barrier function in cells treated with GzmB, thus, the next step was to determine if this was due to cell death. GzmB has previously been implicated in cell death, either through apoptosis and/or anoikis.[50] Therefore, we tested whether exogenous GzmB could reduce keratinocyte cell viability. Our findings indicated no significant difference in viability between untreated and GzmB over 72 h (Figure 6). Any cell death noted is likely due to maintaining cells in serum starved media for a prolonged period, however, this is equal throughout all conditions.

Epidermal barrier function is maintained by the stratum corneum and intercellular junctions. When studying barrier function, tight junctions are at the forefront of investigations. Of the tight junction proteins, claudins are by far the most studied, however, JAMs, ZOs, and E-cad are all gaining popularity as their importance in the formation and maintenance of barrier function becomes known.[9,97] Pardo *et al.* has shown the extracellular GzmB cleavage of intercellular junctions in endothelial cells[81], however no one has looked at the conditioned media of keratinocytes with GzmB. Therefore, we wanted to determine if GzmB was capable of cleaving intercellular junction proteins in an *in vitro* setting. To determine extracellular targets for GzmB, the culture media of GzmB treated HaCaT cells were analyzed by TAILS. Among the long list of targets one tight junction, four adherens junctions, and six desmosomes were identified as potential targets for GzmB, many of which had multiple cleavage products (Table 3 & Figure 8). Additionally, several intracellular anchoring proteins were discovered including junction plakoglobin, plectin, desmoplakin, alpha catenin, vinculin, and periplakin (data not shown). These proteins are important in cell-cell adhesion as they are involved in linking the junction protein to

the intracellular cytoskeleton and providing stability. This suggests that GzmB can act extracellularly and intracellularly without the addition of perforin, to disrupt cell-cell junctions and decrease barrier integrity.

One of the most well studied extracellularly cleaved intercellular junction protein is E-cadherin. Cleavage of E-cad produces a biologically active fragment called soluble E-cadherin (sE-cad) that is ~75-80 kDa.[22,23,25–27] soluble E-cadherin production has been studied in a variety of protease models including ADAMs, cysteine cathepsins, kallikreins, plasmin and MMPs. Furthermore, some studies have identified the presence of sE-cad but fail to identify the protease responsible.[27] With the evidence presented, GzmB may be responsible for the generation of sE-cad. Cancers of the prostate, bladder, colorectal, ovarian, gastric, and skin are highly associated with elevated levels of sE-cad.[22,23,27] soluble E-cadherin has been mainly studied as a biomarker in cancer, however many non-cancerous conditions show elevated levels of sE-cad including dermatitis, psoriasis, acute pancreatitis, diabetes, bullous pemphigoid (BP), and pemphigus vulgaris (PV).[26,27] A study by Matsuyoshi *et al*. demonstrated significantly elevated levels of sE-cad in the serum of patients with BP and PV, with psoriasis and other inflammatory diseases showing a minor but significant increase.[26] In cancer, sE-cad appears to be correlated with invasion and malignancy, while in a non-cancer setting, sE-cad is correlated with disease severity and therefore, a marker of inflammation.[27,98]

Typical consequences of sE-cad, are disruptions of cell-cell connections, migration and invasion (in cancer), cell signaling, and proliferation/survival.[27] A study by Symowicz *et al*. demonstrated an elevated level of sE-cad in ovarian carcinoma cells.[99] They tested the E-cad ectodomain shedding via an MMP-9 dependent mechanism, however with addition of a broad spectrum MMP inhibitor (GM6001) there was still some sE-cad present, suggesting that not all

the MMP enzyme was inhibited or sE-cad production could also be produced by other extracellular proteases. Additionally, when ovarian cancer cells were incubated with a recombinant form of the human 80 kDa sE-cad ectodomain, an increase in cell dispersion was seen compared to control cells. This suggests that sE-cad alone can disrupt preformed adherens junctions.[99] soluble E-cadherin has been shown to disrupt cell-cell junctions and therefore could ultimately decrease barrier function.[23,27,99] As we have shown GzmB to elevate sE-cad significantly compared to control (Figure 11), it is possible that GzmB-mediated cleavage of cell junctions could create a downstream effect where the cleavage fragments themselves could go on to continue disrupting cell-cell adhesion; specifically, that of E-cad.

Pemphigus is a rare autoimmune skin blistering disease with the hallmark symptom of impaired barrier function due to targeted autoantibodies against desmosomes within the epidermis.[36,49] The present study has established that GzmB is capable of impairing barrier function (Figure 7) and can cleave multiple intercellular junctions (Table 3 and Figures 8, 9, & 10), especially E-cad (Figure 11A). Thus, the next step was to analyze whether GzmB is present in autoimmune skin blistering conditions. To determine whether GzmB was present in pemphigus skin, immunohistochemistry was performed on *ex-vivo* skin for patients with different subtypes of pemphigus disease. Elevated levels of GzmB were present within and around the blister with the highest amounts seen in Hailey-Hailey disease, IgA pemphigus, and limited oral pemphigus vulgaris, with sporadic GzmB staining in areas of acantholysis and visible cell-cell separation (Figure 12).

E-cadherin staining within pemphigus tissue samples show a major reduction when staining for N-terminal E-cad (Figure 13), but very little difference when co-staining for E-cadherin and GzmB (Figure 14). This is due to, in part, from the difference in antibodies used; the antibody used

in the pemphigus histology was a monoclonal antibody specific for the N-terminus of E-cad, while the antibody used for the immunofluorescence is a polyclonal antibody with several epitopes. This result is also mirrored in the Western blot analysis (Figure 11B) as there appears to be an increase in sE-cad released into the supernatant but no difference is seen in E-cad probing within the cell lysate for either the N- or C-terminus. This phenomenon is also seen in ovarian carcinoma cells, where integrin aggregation induces MMP and Src kinase dependent sE-cad production results in an increase in sE-cad in supernatant but no difference in E-cadherin expression within the lysates.[99]

Autoantigen production occurs when peptides are captured and presented by antigen presenting cells to other immune cells. If the peptide is not tolerated (ie. not recognized as self-proteins), it will drive an autoimmune response.[50] A paper by Sekiguchi *et al*. tested the autoantibodies collected from the sera of 43 PF and 40 PV patients and studied the epitope recognition patterns.[100] The dominant epitopes for PF and PV autoantibodies appear to recognize the N-terminus of Dsg-1 and Dsg-3, however, there are autoantibodies directed against the middle portions of Dsg-1 and -3 and show no significant clinical differences in the patients whose sera was collected.[100] There is a large heterogeneity in the specificity of these autoantibodies suggesting that there may be a process in which GzmB and other proapoptotic proteases uncover cryptic epitopes that were not present during immune cell development. This would cause immune cells to identify the peptide as foreign and therefore, become autoantigenic.[50] A study by Casciola-Rosen *et al*. demonstrated that GzmB can efficiently cleave numerous autoantigens targeted in systemic lupus erythematosus, diffuse and limited scleroderma, Sjogren syndrome, and autoimmune myositis. Many of the autoantigens were directly cleaved by GzmB into unique fragments compared to caspase-3.[91] This study has found that GzmB is capable of cleaving not only Dsg-1 and Dsg-3 (the two most common autoantigens seen in pemphigus) but also Dsg2, Dsg4, Dsc2, and Dsc3 (some of which are also

found as targets in pemphigus), as shown in the TAILS analysis (Table 3). Therefore, it is possible for GzmB to contribute to autoantigen production during the early stages of pemphigus development, however, this is still an unknown area and remains to be investigated.

Conversely, GzmB may be acting as a secondary effector, seen later on in the progression of the disease. Not all forms of pemphigus show a major increase in GzmB, such as paraneoplastic pemphigus and PV, suggesting some forms of pemphigus may produce some inflammatory response as opposed to others which display little to no inflammation. In such cases, GzmB may be introduced as an inflammatory response mediated by keratinocytes, neutrophils, and other immune and non-immune cells within and around the blister. Moreover, multiple studies have revealed that keratinocytes can express GzmB under chronic stress such as exposure to UVA and UVB, and in high confluence *in vitro*.[50,79] In fact, HaCaT cells irradiated at 100kJ/m^2 UVA showed an elevated expression of GzmB and MMP-1, whereas MMP-2 and MMP-9 (known to produce sE-cad fragments) were unchanged. Granzyme B protein was found diffusely throughout the epidermis.[79] This suggests that GzmB plays a unique and specific role that differs from that of various MMPs, and under chronic stress, GzmB may be expressed by keratinocytes, leading to further barrier disruption and acantholysis.

The role of GzmB in the pathogenesis of pemphigus requires further elucidation, however it is evident that GzmB is present in and around the blister (Figure 12). It is also evident from my in vitro work that GzmB is capable of separating the layers of epidermis to initiate blistering. GzmB's role in disease progression is functionally very diverse which makes it an interesting target for further investigation. In addition to GzmB's role in targeted apoptosis, when uninhibited, GzmB can cause harmful destruction to ECM proteins and, as seen above, intercellular junctions. These proteolytic fragments have the ability to block cell-cell junctions[99], inhibit cell migration[78], and

can act as ligands implicating them in further downstream cell signaling.[101] Therefore, GzmB could be contributing to the pathology of pemphigus in more than one way and inhibition of GzmB could result in improvement of epidermal barrier function and possibly prevent recurring blister formation.

Chapter 5: Conclusion and Future Directions

Extracellular GzmB retains its proteolytic role and decreases epithelial barrier function. These effects are carried out, in part, throughout the cleavage of cell-cell junctions. Loss of cell monolayer resistance and increased paracellular movement show a decrease in cell-cell contacts. Additionally, GzmB can cleave a variety of intercellular junctions from tight junctions, adherens junctions, and desmosomes, resulting in a loss of cell-cell adhesion. Pemphigus is an autoimmune skin blistering disease characterized by loss of cell adhesion and barrier function therefore, we considered whether GzmB is present. Figures 12 & 14 show that GzmB appears to be present both intracellularly, within immune cells and keratinocytes, and extracellularly, within the blister fluid. Whether GzmB is playing a secondary role, accumulating as a consequence of elevated inflammation, plays a role in the initiation with autoantigen production, or both, remains an area that requires further elucidation.

The work presented here provide the foundation for future work to look into a more extensive mechanism of action that GzmB has on barrier function. The degradomics data identify many potential intercellular targets that many be cleaved by GzmB, however there was also a surplus of other targets identified that remain to be investigated. Additionally, the targets mentioned above only pertain to the extracellularly cleaved/secreted substrates as only the supernatant was analyzed therefore, a cell lysate from GzmB treated keratinocytes may provide a more comprehensive list. It is also important to note that not all of the extracellular targets were picked up by the TAILS analysis, as ZO-1, which was identified by Pardo *et al*., is also cleaved by GzmB.[81]

HaCaT keratinocytes provide an ideal initial starting place for experimentation as they are relatively easy to maintain and grow. However, to provide a more physiologically relevant model,

healthy and diseased primary cells may offer a better understanding of how GzmB affects human skin and barrier function. Alternatively, an animal model may give a more in depth study of *in vivo* processes that occur during the course of pemphigus development and delineate whether GzmB is present in the beginning phases of the disease. A pemphigus model combined with a GzmB knockout model could provide insight into whether loss of GzmB ameliorates the symptoms and epithelial physiology. However, obtaining more human samples of pemphigus would be the best course of action. Unfortunately, due to how rare such conditions are, tissues are limited so we were forced to obtain tissues from many different cases based on availability. Furthermore, blister fluid samples for each blister would provide a lot of information on levels of GzmB present and whether the levels of GzmB in the blister fluid could induce barrier dysfunction in healthy skin cells. Unfortunately, these samples are even harder to come by, only a few variations of pemphigus produce blister fluid, and those that do provide very low levels.

These findings have identified a novel role for GzmB in the loss of epidermal barrier function through the cleavage of vital cell-cell junctions. GzmB is a highly active and destructive protease in the extracellular milieu that is capable of cleaving ECM proteins required for maintaining epithelial structure and integrity. Future work should consider expanding the role of GzmB in intra-epidermal blistering pathogenesis as well as other conditions characterized by a loss of barrier function. A summary of my key thesis findings is highlighted in the figure below (Figure 15).

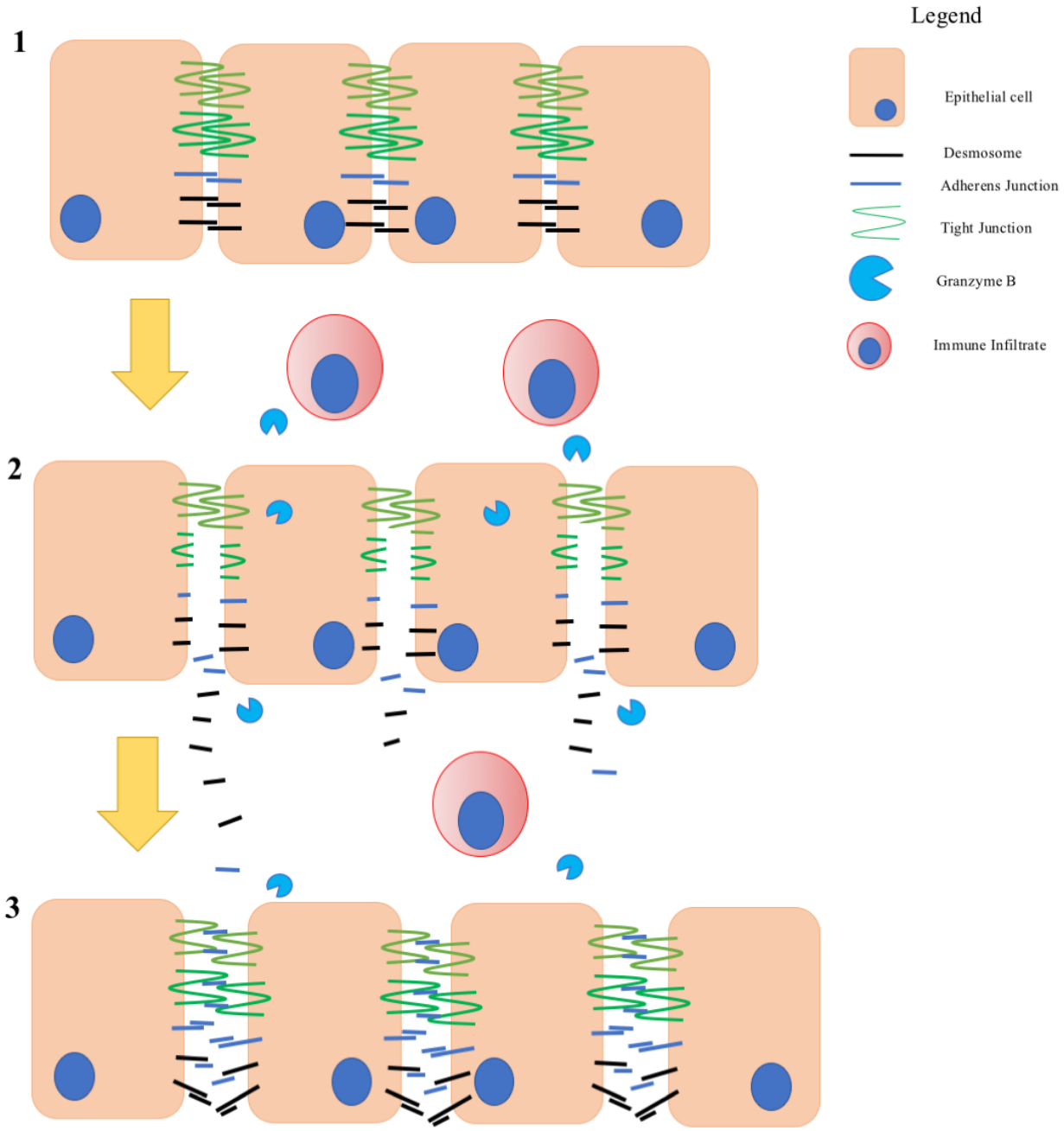


Figure 15: Summary of Findings. (1) A healthy epithelium consists of intact cell-cell connections. (2) In certain autoimmune diseases, GzmB is produced and secreted by surrounding immune cells and/or epithelial cells. GzmB accumulation in the intercellular junction results in the cleavage of key adherens and/or tight junctional proteins. In addition, bioactive, cleaved fragments may be released into the extracellular milieu. (3) As GzmB-mediated junctional destruction and protein cleavage ensues, the resulting fragments may retain their adhesive properties and further disrupt other intact junctions leading to a further drop in barrier function and loss of cell adhesion. Based on the results presented here, it appears that GzmB is coming from neutrophils and even the keratinocytes themselves, however this has yet to be proven.

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