GRANZYME B INHIBITS KERATINOCYTE MIGRATION BY DISRUPTING
EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)-MEDIATED SIGNALING

by

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Abstract

Chronic skin ulceration is a common complication and cause of morbidity in the elderly, diabetic, obese and/or immobile populations. Effective therapies that adequately promote efficient closure and remodelling of chronic wounds are lacking. Inflammation and excessive protease accumulation and activity are thought to play key roles in the impairment of normal wound healing. Granzyme B (GzmB) is a serine protease that was, until recently, believed to function exclusively in cytotoxic lymphocyte-mediated apoptosis. However, during dysregulated and/or chronic inflammation, GzmB can accumulate in the extracellular milieu, retain its activity, and cleave a number of important extracellular proteins. Epidermal growth factor receptor (EGFR) is a transmembrane receptor involved in cellular processes such as proliferation and migration. EGFR signaling is integral to the wound healing process. I **hypothesized** that GzmB impairs EGF-induced keratinocyte migration by impairing EGFR signaling. The present study investigated the effects of GzmB on keratinocyte cell migration. Using Electric Cell Substrate Impedance Sensing (ECIS) and other *in vitro* wound healing assays, the present study demonstrates that GzmB inhibits keratinocyte migration by interfering with the EGFR pathway. GzmB limited cell transition into a migratory morphology and was found to reduce ligand-induced EGFR phosphorylation. Inhibition of GzmB reversed the aforementioned effects in HaCaT cells. In summary, data from the present study suggests a key role for GzmB in the pathogenesis of impaired wound healing through the reduction of EGFR signaling and cell migration.

Preface

- All experimentation, analyses and writing was done by Yulia Merkulova in consultation with Dr. Granville.
- In Chapter 1:
 - Figure 2 was obtained and reproduced with permission from “Granzyme B in injury, inflammation and repair” by Hiebert PR and Granville DJ (*Trends Mol Med.* 2012 Dec;18(12):732-41).
 - Figure 4 was obtained and reproduced with permission from “Granzyme B in injury, inflammation and repair” by Hiebert PR and Granville DJ (*Trends Mol Med.* 2012 Dec; 18(12):732-41).
- All chapters of this thesis contain portions of the text from a manuscript entitled “Granzyme B inhibits keratinocyte migration by disrupting epidermal growth factor receptor (EGFR)-mediated signaling” accepted for publication in *Biological Chemistry*.

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List of Acronyms and Abbreviations

AAA	Abdominal Aortic Aneurysm
ApoE	Apolipoprotein E
BAL	Bronchoalveolar Lavage
COPD	Chronic Obstructive Pulmonary Disease
CSF	Cerebrospinal Fluid
CTL	Cytotoxic T Lymphocytes
Dcn	Decorin
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPI	Dipeptidyl Peptidase I
ECIS	Electric Cell-Substrate Impedance Sensing
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Signal-Regulated Kinases
EMT	Epithelial Mesenchymal Transition

FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FN	Fibronectin
GzmA	Granzyme A
GzmB	Granzyme B
GzmH	Granzyme H
GzmK	Granzyme K
GzmM	Granzyme M
HGF	Hepatocyte Growth Factor
HUVEC	Human Umbilical Venous Endothelial Cells
Hz	Hertz
IFN	Interferon
IL	Interleukin
KO	Knockout
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinases

MMP	Matrix metalloproteinase
MS	Multiple Sclerosis
NF- κ B	Nuclear Factor Kappa B
NK	Natural Killer
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
Pfn	Perforin
PG	Proteoglycan
qPCR	Quantitative Polymerase Chain Reaction
RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
Sa3n	Serpin a3n
SDS	Sodium Dodecyl Sulphate
siRNA	small interfering Ribonucleic Acid
SMC	Smooth Muscle Cell

TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline-Tween 20
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitor of Metalloproteinases
TNF α	Tumor Necrosis Factor Alpha
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
vWf	von Willebrand Factor
WT	Wild Type

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Dedication

I would like to thank my family for their tremendous support in everything I do: my mother – Irina Merkulova, my father – Andrey Merkulov, and my grandmother – Vera Kukharensko. Without the support of my family I would never have the ability to advance in my studies as far as I have and accomplish what I have so far. I am grateful for having their support and I dedicate my thesis to my entire family.

Chapter 1: Introduction

1.1 Skin anatomy

Skin is the largest organ in the body serving as a barrier to protect us from pathogens, dehydration, and bleeding. It serves as a thermoregulatory, excretory and sensory organ allowing us to sense and adjust to the environment. Breaking the skin barrier makes the body vulnerable to infections, thus there is a complex mechanism in place to promote wound healing and tissue repair (1).

Skin consists of three layers which are epidermis, dermis, and subcutaneous tissue. The epidermis is the outermost skin layer which protects the deeper layers of the skin (1). The epidermis is made up of 40-50 layers of stacked squamous skin epithelial cells, called keratinocytes, with rapidly dividing stem cells located at the base of the epithelium (stratum basale) and dead cells facing the outside environment (stratum corneum). The layers of epidermis are stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale (1). As keratinocytes divide, they mature and start expressing keratin as they move upward, with the outer dead keratinocytes containing mainly keratin. Another cell type located in the epidermis is melanocytes – cells that produce a dark pigment melanin which absorbs ultraviolet (UV) light to protect skin from sunburns. Less abundant cell types in the epidermis include Langerhans cells and Merkel cells which activate the immune response to pathogens and assist in tactile function respectively. The epidermis does not contain any blood vessels and receives nutrition via diffusion of molecules from the dermis to which it is attached through basement membrane (1).

Dermis – the largest skin layer found under the epidermis is highly vascular and innervated. The dermis consists of papillary dermis composed of loose connective tissue intertwined with the basement membrane of the epidermis, and reticular dermis composed of dense irregular connective tissue containing proteoglycans and glycoproteins. In addition to nerves and blood vessels the dermis contains sebaceous glands, hair follicles, fibroblasts, and immune cells. Dermis provides skin the elasticity and stretch and is the site where inflammation takes place (1).

Subcutaneous tissue, or hypodermis, is connected below the dermis and contains larger blood vessels, subcutaneous adipose tissue, and loose connective tissue. Subcutaneous tissue provides a connection for upper skin layers with the underlying organs and serves as energy storage (1).

1.2 Extracellular matrix of the skin

Once believed to be a metabolically inactive space filler, extracellular matrix (ECM) is now known to be an active substance that not only provides structural support, but also serves as reservoir of growth factors important in a variety of physiological processes. ECM is integral to the proper functioning of skin (2).

One of the main components of ECM are proteoglycans (PGs) - heavily glycosylated proteins possessing an overall negative charge which facilitates binding to cations and attracts water molecules (2, 3). Fibrous proteins such as collagen comprise another major category of ECM proteins. Smaller proteins such as decorin (Dcn) provide additional structural support (3).

Basal lamina – ECM secreted by epithelial cells which is composed of laminin, fibronectin (FN), type IV collagen, and PGs (2, 4). Basal lamina connects integrins located on basal surface of the epithelium to the dense irregular connective tissue underneath (4).

Collagen is the major component of the dermis (2). There are distinct 28 collagen types identified in vertebrates, however type I, III, and IV are the most abundant in skin (2). Dermis is heavily occupied by type I collagen fibers which provide tensile strength to skin (2, 3). Dcn is an arch-shaped proteoglycan that regulates collagen fibrillogenesis, growth factor storage, and cellular growth (5). Dcn rests on top of collagen fibers near the molecular cross-linking sites of individual collagen molecules and regulates fiber spacing and organization (5). Elastin is another major component of the dermis which provides recoil and elasticity to skin (2). Fibrillin is a protein providing stabilization and scaffolding essential for the formation of elastin fibers (2). Fibroblasts are the main sources of the ECM proteins found in the dermis (2, 3).

In addition to structural support ECM acts as a regulator of GF availability. Dcn can bind to, and sequester transforming growth factor beta (TGF- β) while FN can bind and sequester vascular endothelial growth factor (VEGF) (6, 7). Other GFs including insulin-like growth factor (IGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF) also associate with ECM (8). The availability of GFs contained by ECM is regulated by matrix metalloproteinases (MMPs) which cleave many ECM molecules and release bioactive growth factors as well as ECM fragments with pro-inflammatory properties (9).

ECM is an integral component of many tissues and deficiencies or abnormalities in ECM proteins lead to diseases including osteogenesis imperfecta, Marfan syndrome, scleroderma, fibrosis, hypertrophic scarring, and many others (2).

1.3 Granzymes

Granzymes are a family of serine proteases originally discovered in the granules of cytotoxic T cells (CTLs) and natural killer (NK) cells (10-12). Granzymes are a conserved group of proteases, with 5 members identified in humans (Gzm A, B, H, K and M) and 11 in mice (Gzm A, B, C, D, E, F, G, K, L, M, and N) (10). While considerable amount of research focuses on granzymes A (GzmA) and B (GzmB), other granzymes received less attention as they are less abundant (10). Granzymes are structurally similar and range from 27-65 kDa in size, with GzmA being the only dimer in the family, thus having the largest size (11, 13). Despite structural similarities, granzymes vary in substrate specificities, and depending on catalytic activity are genetically linked to loci encoding groups of functionally similar proteases including tryptases, chymases and metases (10, 11). The catalytic site of granzymes is conserved and contains three residues – histidine, aspartic acid, and serine (11). Once synthesized, granzymes are tagged with mannose-6-phosphate which targets them to cytotoxic granules (14). Granzymes are produced as zymogens, and are cleaved at the time of packaging into cytotoxic granules (11). Dipeptidyl peptidase I (DPPI) cleaves the activation peptide present on the pro-form of granzymes, generating the active form of these proteases (15). Inside the lytic granules granzymes are stored on a serglycin scaffold – a chondroitin sulphate proteoglycan, which in combination with acidic pH keeps granzymes inactive while inside cytotoxic cells (11, 16, 17).

GzmA is a tryptase which cleaves after Lysine and Arginine, and is the only dimer in the granzyme family, with subunits held by disulfide bonding (11). The gene for GzmA is encoded on chromosome 5, with two different GzmA transcripts produced through alternative splicing. GzmA is found in CTLs, NK cells, $\gamma\delta$ T cells, and thymocytes. GzmK, another tryptase encoded on chromosome 5, is a 28 kDa protein with unique substrate specificity distinct from that of GzmA (18, 19). Sources of GzmK include CTLs and NK cells (11). GzmB - an aspartase encoded on chromosome 14, is a 32 kDa protease released by CTLs, NK cells, $\gamma\delta$ T cells, Tregs, and many non-lymphoid and non-immune cell types, and will be further discussed in more detail (6, 11). GzmB has been shown to cleave after Aspartate, with a preference for Arginine-Glycine-Aspartate (RGD) motif (20). GzmH, another 32 kDa protease encoded on chromosome 14, is a chymase which has only been identified in humans (11, 21). GzmH cleaves after Tyrosine or Phenylalanine, and its sources include CTLs and NK cells (11, 22, 23). GzmM - a metase, known as the “orphan enzyme” has not been widely studied until recently (11). The gene for GzmM is encoded on chromosome 19, and the full-length protein is 30 kDa (11, 24). GzmM cleaves after Methionine or Leucine and is expressed by NK cells (11, 24).

While granzymes share substrate specificity to some degree, each granzyme possesses unique array of substrates, suggesting a specific role in cell-mediated immune response for each enzyme as well as other roles extending beyond inducing apoptosis of target cells.

1.3.1 Release of granzymes

Granzymes are released from cytotoxic granules into a cellular synapse formed between effector and target cell (25). When released by immune cells, granzymes are co-secreted with perforin (Pfn) - a pore-forming protein which allows granzymes to enter target cell cytoplasm (25). Interestingly, studies have shown that effector cells can release granzymes in the absence of target cell engagement (25, 26). It is believed that besides non-specific granzyme secretion into the extracellular milieu, they can also leak out of cellular synapses and accumulate extracellularly, causing tissue damage (20, 25).

1.3.2 Granzymes in disease

Originally believed to function exclusively in immune cell-mediated cytotoxic killing of target cells, granzymes are now seen as multifaceted proteases with functions extending beyond cellular cytotoxicity (10, 27). The former dogma that granzymes are purely cytotoxic has been challenged with research done using Pfn knockout mice, which shed light on the extracellular roles of granzymes (10, 27).

Pfn-dependent roles of granzymes entail initiating apoptosis of target cells – a well-established concept (20, 28). Pfn-dependent functions of granzymes contribute to responses to viral infections, tumour clearance, and immunological tolerance (20, 28). In tumour or viral cell elimination granzymes are released by CD8⁺ T cells (29). Previous research shows that Pfn-deficient mice develop spontaneous B cell lymphomas and lack appropriate anti-tumour responses, highlighting the importance of Pfn (30). In immune peripheral tolerance, CD4⁺ Tregs target overactive CTLs for destruction by releasing GzmB with Pfn (31).

Granzymes possess a unique array of substrates both intra- and extracellular, and may play a specific role in immune-mediated target cell killing and tissue injury (20). As granzymes are primarily released by immune cell types, they are highly abundant at sites of chronic inflammation, and have been a subject of interest as potential biomarkers of disease (27). Granzymes have been found in bodily fluids including blood, bronchoalveolar lavage (BAL) fluid, cerebrospinal fluid (CSF), and synovial fluid (27). The presence of granzymes in bodily fluids is associated with specific diseases is summarized in Figure 1.

GzmA has been detected in synovial fluids of rheumatoid arthritis patients and plasma of patients suffering infections (25). Besides pro-apoptotic roles, new pro-inflammatory roles of GzmA have been demonstrated, particularly in cytokine induction and processing. At low concentrations in the absence of Pfn, GzmA is able to induce IL-1 β , TNF- α , and IL-6 expression in monocytic cells and mouse macrophages (32). Interestingly, GzmA is capable of extracellular proteolytic activity and has been shown to cleave FN and collagen IV (33).

GzmK levels are increased in plasma of septic patients, BAL of patients with acute lung inflammation, and plasma of patients with viral infections (25). Similarly to GzmA, its activity leads to pro-inflammatory cytokine expression in lung fibroblasts including, IL-6, and IL-8 through cleavage of protease activated receptor-1 (PAR-1) and IL-1 β expression in macrophages (18, 34, 35). GzmK has been shown to act in immunomodulation executed by a subset of NK cells (CD56^{bright}), a process important in neuroprotection in multiple sclerosis (36).

Granzyme M – an orphan enzyme, has been implicated in responses to lipopolysaccharide (LPS), and seems to contribute to the cytokine storm associated with LPS stimulation. Granzyme M knockout mice survived longer after LPS challenge and produced reduced amounts of IL-1 α , IL-1 β , TNF, and IFN- γ relative to wild type controls (37). GzmH – another orphan enzyme which has received little attention in recent years. GzmH is predominantly expressed by NK cells, and as such, it is believed to be an alternative inducer of cell death, activating pathways distinct from those activated by other granzymes (22). GzmH is capable of effectively killing tumour cells in a Pfn-dependent manner (38). A novel function for GzmH in adenovirus DNA binding protein degradation has been discovered implicating a unique niche for GzmH (39).

Figure 1. Granzyme presence in bodily fluids associated with various diseases.

A line represents the presence of a particular granzyme in bodily fluids of patients with the disease connected to the granzyme.

1.4 Granzyme B – role in apoptosis

Induction of apoptosis or controlled cell death, was the first biologically relevant function discovered for GzmB, summarized in Figure 2 (28). Eliminating cancer cells, or virally infected cells from the body is crucial to prevent spreading of the disease and is accomplished primarily through the release of GzmB by CTLs or NK cells (20). T helper cells have also been shown to release GzmB and target overactive CTLs for apoptosis, an important process in immune tolerance. Co-released with Pfn into the cellular synapse, GzmB enters target cell cytoplasm through Pfn pores and cleaves a number of intracellular substrates (20, 28). The first substrate possessing biological relevance identified for GzmB was a caspase (40). Caspases are potent inducers of apoptosis and must be proteolytically cleaved from the precursor form into the active form to perform their function (28). Once activated, caspases cleave intracellular proteins leading to downstream cascades resulting in apoptosis (20, 28). GzmB has been shown to cleave caspase-3 and caspase-8 *in vitro* and *in vivo* following granule-mediated release and target cell entry (41-43).

GzmB is also capable of caspase-independent apoptosis induction (28). Mitochondria are important players in the apoptotic pathways and upon their disruption release cytochrome c – a pro-apoptotic protein, into the cytoplasm (28). By cleaving BID, another pro-apoptotic protein, GzmB has been shown to cause mitochondrial collapse, resulting in the release of cytochrome-c (44-46). Interestingly, GzmB is capable of acting through another caspase-independent pathway to induce apoptosis, by inducing DNA fragmentation (47).

Figure 2. Pro-apoptotic roles of GzmB.

GzmB is co-released with Pfn from CTLs and NK cells during cytotoxic immune response. Pfn creates pores in the target cell membrane allowing GzmB to exit the endosome and translocate into the cytoplasm of the target cell. Once in the cytoplasm, GzmB cleaves many intracellular proteins resulting in the activation of multiple pathways leading to apoptosis. Direct cleavage of caspase-3 and caspase-8 results in their activation; cleavage of BID leads to mitochondrial disruption; other proteolytic functions of GzmB result in DNA fragmentation – all of these pathways lead to target cell apoptosis as a result of intracellular GzmB.

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1.5 Extracellular roles of GzmB

GzmB can cleave a number of extracellular proteins with many physiological and pathological implications. Table 1 lists known GzmB substrates and their roles in tissue injury and inflammation. While the physiological relevance of some GzmB-mediated proteolysis remains to be determined, *in vivo* experiments demonstrated that cleavage of certain extracellular matrix proteins and cytokines proves to be relevant in the context of many pathologies.

While CTLs and NK cells are the major sources of GzmB, other cells have been shown to secrete it, including activated T helper cells, macrophages, dendritic cells, mast cells, basophils, chondrocytes, keratinocytes, and trophoblasts (10, 20). Interestingly, mast cells, basophils, and dendritic cells as well as non-immune cell types (UV-activated keratinocytes, chondrocytes) release GzmB (20).

By cleaving fibrillin-1 and Dcn, GzmB can promote abdominal aortic aneurysm (AAA), whereby the aorta ruptures due to medial disruption and instability of the adventitia (48, 49). In these studies of GzmB^{-/-}ApoE^{-/-} mice exhibited increased survival and decreased incidence of aortic rupture, and increased fibrillin staining compared to ApoE^{-/-} or ApoE^{-/-}Pfn^{-/-} mice. Additionally, inhibition of GzmB with Sa3n reduced frequency of aortic rupture, reduced Dcn degradation and increased collagen density (48, 49).

GzmB cleavage of Dcn contributes to loss of collagen fibre organization and leads to loss of skin tensile strength. Using ApoE knockout mice, Hiebert et al. demonstrated that GzmB/ApoE double knockout mice exhibited improved wound closure and contraction in comparison to ApoE knockout mice. In a mouse model of UV-induced skin aging, Parkinson

et al. demonstrated that GzmB deficiency prevents wrinkle formation and prevented the loss of collagen density in the dermis. Interestingly, in this study GzmB-generated FN fragments induced the expression of matrixmetalloproteinase-1 (MMP-1), an extracellular enzyme that degrades collagen. Hsu et al. demonstrated that extracellular GzmB inhibition resulted in faster wound closure in a diabetic mouse model of delayed wound healing (50). This study also found that GzmB inhibition resulted in faster wound re-epithelialization and contraction, enhanced granulation tissue maturation characterized by elevated cellular proliferation and differentiation, and improved collagen deposition. The above studies demonstrate that mast cells are a major contributors of extracellular GzmB in skin, with elevated levels of mast cells and GzmB in healed diabetic skin tissue (50).

Previous research demonstrated the involvement of GzmB in cytokine processing. IL-18, a pro-inflammatory cytokine, can be cleaved by GzmB resulting in a shorter, active form of the cytokine (51). Later studies discovered that GzmB can process IL-1 α into a shorter, more potent peptide, thus leading to an amplified immune response (52). Interestingly, IL-1 α fragments similar to those generated by GzmB were identified in BAL from patients with pulmonary diseases including COPD, cystic fibrosis, and bronchiectasis.

GzmB-mediated ECM proteolysis plays a role in regulating the inflammatory response. FN fragments produced by GzmB show chemotactic properties and are able to recruit monocytes and fibroblasts to the site of inflammation (53-55). Additionally, FN, Dcn, and biglycan fragments can serve as danger signals and stimulate Toll-like receptors 2 and 4 (55-57).

By cleaving Dcn, biglycan, betaglycan, and FN, GzmB can act as a modulator of growth factor availability in tissues. Hendel et al. have shown that GzmB-mediated FN cleavage releases bioactive vascular endothelial growth factor (VEGF) and promotes vascular permeability. In this study, GzmB promoted vascular leakage, however its effect was abolished in the presence of anti-VEGF neutralizing antibody. Additionally, GzmB^{-/-} mice demonstrated reduced inflammation-mediated vascular leakage compared to WT mice (7). While there hasn't been *in vivo* evidence demonstrating GzmB-mediated TGF- β release, *in vitro* data suggests GzmB may play a role in regulation of TGF- β bioavailability by cleaving Dcn, biglycan, and betaglycan, releasing active TGF- β capable of eliciting cell responses in tissue culture (6).

Recent evidence supports the role of GzmB in cardiac fibrosis, where GzmB and not Pfn deficiency served a protective role in Angiotensin-II induced cardiac fibrosis (58). This study demonstrated a new mechanism of vascular permeability induction by GzmB – through cleavage of VE-cadherin, an endothelial junctional protein.

GzmB-mediated ECM degradation has been shown to result in anoikis in certain cell types. Choy et al. observed cell detachment and death in response to GzmB treatment of smooth muscle cells (59). Later studies by Buzza et al. demonstrated the same effect in breast carcinoma and endothelial cells (60). This effect has been attributed to GzmB-mediated degradation of FN, vitronectin, and laminin.

While ECM degradation by GzmB is a detrimental part of many pathological processes, it is physiologically important for immune cell extravasation (61). GzmB null T cells and NK cells demonstrated the ability to adhere to blood vessel wall *in vivo*, however

lacked the ability to extend lamellipodia and transmigrate into the tissues (61). The study also showed that lymphocytes release GzmB to locally degrade ECM and facilitate movement driven by chemokines.

Table 1: List of Granzyme B substrates and their physiological functions.

Substrate	Biological Function
Decorin	Collagen fibrillogenesis and organization TGF- β binding and sequestration (6, 62-64)
Fibronectin	Comprises basement membrane Regulates cell migration and proliferation, and cutaneous wound closure (59, 60, 65)
Laminin	Comprises basal lamina, facilitates cell migration (60, 66)
Vitronectin	Binds integrins and growth factors, facilitates cell migration and proliferation (60, 67)
Biglycan	TGF- β sequestration and collagen fibre spacing (6)
Betaglycan	TGF- β sequestration and collagen fibre spacing (6)
Aggrecan	Proteoglycan, essential component of cartilage and the function of joints (68)
Von Willebrand Factor	Platelet adhesion and clotting (69, 70)
Plasminogen	Dissolves fibrin blood clots, degrades ECM (71, 72)
Fibrinogen	Essential blood clotting enzyme (20, 73)
Fibrillin-1	ECM protein, sequesters inactive TGF- β , supports ECM framework (6, 74)
Neuronal Glutamate Receptor	Neuronal plasticity modulation, essential in memory and learning (6, 75)
Il-1α	Pro-inflammatory cytokine, promotes fever and sepsis (6, 20)
VE-cadherin	Endothelial junctional protein (58)

1.6 Inhibitors of GzmB

In order to prevent apoptosis of the effector cells during production and storage of GzmB, in humans there are intrinsic inhibitory molecules that bind to GzmB to block its activity (10). Protease Inhibitor-9 (PI-9) is the only known endogenous inhibitor of GzmB expressed by cytotoxic T cells, NK cells, DCs, epithelial cells, vascular SMCs, and hepatocytes to serve as protection against GzmB-mediated apoptosis (76, 77).

Serpin a3n – a molecule isolated from mouse Sertoli cells, possesses inhibitory activity against human and mouse GzmB, however it can inhibit other proteases as well (50, 78). Serglycin scaffolding within secretory vesicles combined with low pH provide additional reduction of GzmB activity to prevent unwanted proteolysis (10).

Developing extracellular inhibitors of GzmB to inactivate it extracellularly is currently an area of interest focused on developing therapeutic agents to prevent or reverse tissue injury associated with chronic inflammatory environments.

1.7 Acute wound healing

Normal wound healing consists of a series of tightly regulated and overlapping stages including hemostasis, inflammation, granulation tissue formation and re-epithelialization, and tissue remodelling (20). The time line of wound healing response and relative abundance of inflammatory cells is summarized in Figure 3.

Immediately after tissue injury, inflammatory mediators are released by damaged cells resulting in rapid vasodilation and platelet aggregation (79). At the injured site platelets release a variety of molecules including fibrinogen, Von Willebrand factor, FN, thrombospondin, epidermal growth factor (EGF), TGF- β and other molecules (79). Platelet

activation initiates clotting cascades, and results in the formation of the fibrin plug which stops the bleeding and initially fills up the wound space. This completes the hemostasis stage (79).

The inflammatory stage proceeds next which involves the recruitment of leukocytes via diapedesis through the activated endothelium of dilated blood vessels and chemokine gradient-mediated migration through ECM (80-82). Resident macrophages respond to inflammatory signals and bacterial molecules and release pro-inflammatory cytokines such as interleukin-8 (IL-8) which is a leukocyte chemotactic factor (80, 83, 84). Neutrophils are the first subset of leukocytes to migrate to the wound site where they engulf and destroy bacteria, release reactive oxygen species, and proteases such as neutrophil elastase and cathepsin G, which can further promote tissue damage (80, 85). Followed by neutrophils, monocytes are recruited to the wound site 48-96 hours after injury to continue phagocytosis (80, 84, 86). Resident and recruited macrophages release cytokines including tumor necrosis factor alpha (TNF- α), TGF- β , VEGF, FGF, platelet-derived growth factor (PDGF), promoting vascular permeability and inflammation (80, 83, 87, 88). T cells and B cells are recruited 5-7 days later in the immune response (80).

In addition to releasing pro-inflammatory cytokines, macrophages synthesize ECM molecules including FN, vitronectin, thrombospondin, and PGs that culminates with the deposition of granulation tissue (84). Fibroblasts actively synthesize type III collagen which is the main component of granulation tissue cells use as a scaffold for migration (89). Keratinocytes at the edges of the wound commence proliferation and migration, which peaks 1-2 days post wounding (90). During migration basal keratinocytes at the edges of the wound

flatten, elongate, rearrange actin cytoskeleton to form lamellipodia and filopodia and lose their cell-cell and cell-ECM contacts. Simultaneously myofibroblasts contract to reduce the wound size (79, 81, 91-95).

Lastly, tissue remodelling occurs during which fibroblasts replace type III collagen with type I collagen – a much stronger fiber, restoring tissue tensile strength nearly to the original degree (2, 94, 95). TGF- β signaling plays a key role in stimulating fibroblast function during this process. In later stages of wound healing immune cells undergo apoptosis (80).

Figure 3. Timeline of recruitment and relative abundance of inflammatory cells during acute wound healing response.

Information obtained from Park JE and Barbul A (86).

1.8 Chronic wound healing

Chronic wounds are clinically defined as defects in the skin barrier that failed to begin healing in the period of three months or have not fully healed in twelve months (96, 97). In the chronic wound microenvironment one or more of the healing pathways may be impaired resulting in failure of the skin to heal. Depending on the etiology of the wound and the pre-existing health problems of patients, chronic wounds can be classified into diabetic, pressure, and decubitus skin ulcers (98). Figure 4 highlights the differences between acute and chronic wound environments.

Chronic wound healing is not caused by a single factor, but rather a combination of processes that have gone awry resulting in a vicious cycle of inflammation and tissue injury. The pathological characteristics associated with chronic wounds include accumulation of inflammatory infiltrates, elevated levels of proteases and reactive oxygen species, ECM degradation, infection, impaired re-epithelialization and zones of necrotic tissue at the site of inflammation (80, 99).

Entrapment in the inflammatory stage is one of the hallmarks of chronic wounds. Studies have shown increased recruitment of proinflammatory cellular infiltrates including neutrophils, macrophages, and T cells to the site of chronic inflammation (100, 101). In comparison to acute wounds, chronic wounds have higher levels of CTLs compared to T helper cells, and macrophage populations are shifted to M1 (killer) type reducing the healing response (101). As a result, levels of proinflammatory cytokines including $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and $\text{IFN-}\gamma$ are elevated in chronic wounds (102, 103). Levels of GzmB are also elevated, as described earlier (20). Additionally the functionality of neutrophils is compromised. In

particular, neutrophils in chronic wounds produce greater amounts of reactive oxygen species including hydroxyl radicals, superoxide ions, and hydrogen peroxide leading to a highly oxidative microenvironment which further damages the surrounding the tissue (104, 105).

Elevated levels of cytokines lead to elevated levels of MMPs secreted by inflammatory cells leading to excessive proteolytic activity in the chronic wound environment. Previous studies report elevated levels of MMP-1 and MMP-8 (collagenases), MMP-2 and MMP-9 (gelatinases), MMP-3, MMP-10, and MMP-11 (stromelysins), neutrophil elastase, cathepsin G and other proteases in chronic wounds (106-110). Interestingly, studies report an inverse correlation between proteases and their inhibitors, with reduced levels of tissue inhibitor of matrix metalloproteinases (TIMPs) observed in chronic wounds (109, 111). Consequently, this leads to ECM degradation, which in turn promotes further inflammation through immune cell recruitment by generated ECM fragments (112). While many MMP functions are proinflammatory, recent discoveries following failed cancer clinical trials where MMPs were targeted with inhibitors showed unexpected roles of MMPs in suppressing inflammation (113). For instance, MMP-12 has a role in terminating the recruitment of neutrophils and macrophages to the site of inflammation, MMP-14 is involved in the upregulation of anti-inflammatory cytokine expression, MMP-25 functions in the clearance of apoptotic neutrophils (114-116). These anti-inflammatory roles are crucial for wound healing progression and anti-inflammatory MMPs are now looked at as drug antitargets (115). Instead, the research is now shifting towards the understanding the unique proteolytic signatures of MMPs and the factors involved in regulating the balance between their activities which would greatly benefit the understanding of the chronic wound environment.

Other abnormalities in the wound healing response have been documented in chronic wounds. Insufficient angiogenesis leads nutrient starvation, hypoxia and tissue necrosis (80, 117). Previous studies report an increase in antiangiogenic proteins such as myeloperoxidase and a decrease in pro-angiogenic factors such as superoxide dismutase in chronic wound samples (118). In parallel with these findings, elevated levels of apoptotic marker annexin V were observed in diabetic wound fluids (118). Fibroblast senescence has previously been reported, rendering fibroblasts dysfunctional (119). Senescent fibroblasts have been shown to secrete MMP-2, MMP-3, and MMP-9 (120). Reduced keratinocyte migration is another important characteristic observed (80, 110).

The complex interplay of cell-cell and cell-ECM interactions poses many challenges in delineating a comprehensive pathway network involved in driving chronic wound healing pathogenesis. While much is known about the chronic wound environment, there is still a need to broaden the understanding of how separate factors come together to promote pathogenesis of chronic wounds.

Figure 4. Schematic comparison of acute (left) and chronic (right) wound environment with the emphasis on roles of GzmB in chronic wound pathogenesis.

During acute wound healing all stages progress to termination to restore tissue functionality. In a chronic inflammatory environment, the progression of wound healing stages is impaired due to elevated levels of proteases which degrade ECM, activate proinflammatory cytokines, and lead to recruitment of more inflammatory cells, overall resulting in prolonged inflammation and tissue injury.

The figure was obtained and reproduced with permission from “Granzyme B in injury, inflammation and repair” by Hiebert PR and Granville DJ (*Trends Mol Med.* 2012 Dec; 18(12):732-41).

1.9 Epidemiology of chronic wounds

Chronic non-healing skin wounds (or skin ulcers) are a heavy burden on the economy and a serious cause of morbidity in our society. In the U.S. chronic wounds affect approximately 6.5 million patients with an estimated annual healthcare cost of over US\$25 billion (121, 122). The healthcare cost of chronic wound treatment is expected to rise with the increasing global incidence diabetes and obesity as well as increasing medical care costs.

The morbidity associated with chronic wounds is a serious impact on the quality of life of affected individuals. These individuals suffer from pain, impaired sleep, impaired mobility, restricted work capacity. Patients suffering from chronic wounds also suffer socially, due to fear of injury and negative body image (122).

Overall, chronic non-healing skin wounds are associated with high morbidity, healthcare costs, reduced productivity, and reduced quality of life and as such present a significant health problem.

1.10 Current treatments

Many therapies are available for patients suffering chronic wounds such as various types dressings, decellularized skin, cellular dermal constructs, bilayered constructs, with many new therapies under development (121).

Passive or occlusive dressings are used to prevent infection and provide a medium for keratinocytes to migrate on. While providing pain relief and faster healing in acute wounds, passive dressings lack effectiveness in chronic wounds (121). It has been shown that chronic wound fluid under passive dressings is inhibitory to cell proliferation (123). Active dressings such as those containing collagen and anti-bacterial agents were a promising idea in theory,

but did not demonstrate improvements in wound closure of diabetic foot ulcers and pressure ulcers (124, 125).

Many growth factors have been considered as topical therapeutic agents for chronic wounds, however currently only PDGF holds Food and Drug Administration (FDA) approval (125). Despite many topical single growth factor treatments attempted, PDGF is the only one which showed success in clinical trials (126-128). Epidermal Growth Factor (EGF) therapies have been attempted, however clinical trials produced marginal improvements with no significant differences between treatment and placebo groups (129). The main challenge with developing growth factor therapies is ensuring the bioavailability of the growth factor in the chronic protease-rich wound environment (96).

Engineered skin such as cellular dermal constructs and bilayered constructs provide hope for chronic wound treatment. These constructs contain fibroblasts and ECM proteins and have had some success in previous studies (130-133).

Despite numerous clinical trials, large scale studies are lacking to provide quality evaluations of current therapies. Most treatments provide marginal or no improvements and new more effective therapies are needed to address the problem of chronic wounds.

1.11 Epidermal growth factor receptor

Epidermal Growth Factor Receptor (EGFR) belongs to the ErbB family of tyrosine kinase receptors which includes ErbB1 (EGFR), ErbB2, ErbB3, and ErbB4 (134). EGFR is a transmembrane receptor with a single membrane spanning domain. The N-terminus of the receptor contains 4 domains which comprise the extracellular portion of the receptor (135). The C-terminus of the receptor faces the cytoplasm and contains many tyrosine residues

which get phosphorylated after receptor engagement (135, 136). Epidermal Growth Factor (EGF), an EGFR-specific ligand, binds the N-terminal domain of a EGFR monomer, causing a change in receptor conformation and subsequent receptor dimerization (135). It is believed that ligand binding transforms the receptor from an inactive monomeric form into an active dimer form (135). While EGF can only bind EGFR, EGFR can recognize other ligands including amphiregulin, transforming growth factor alpha, betacellulin, heparin-binding EGF-like growth factor, epiregulin, and epigen (137, 138). Upon dimerization, the C-terminal tyrosine kinases autophosphorylate the C-termi transducing the signal from phospho-tyrosines to a multitude of signaling molecules such as Akt, ERK1/2, PI3K, etc (135, 136, 139). Once the signal has been transduced, the receptor is internalized and targeted for degradation, with partial receptor recycling back to the cell membrane (140).

EGFR is present on apical and basolateral surfaces of polarized epithelial cells, however its abundance on cell surface varies depending on cell type (138). EGF can be delivered to cells through platelets, macrophages, as well as from epithelial cells themselves through cleavage by sheddase TACE/ADAM17 - a process known as ectodomain shedding (141, 142). During early stages of wound healing platelets and macrophages serve as sources of EGF, and during later stages, including migration and proliferation ectodomain shedding from epithelial cells is the major source. A schematic representation of EGF release and EGFR signaling is depicted in Figure 5.

EGFR signaling generates a very potent response, and while essential in wound healing, can be detrimental as overactive EGFR will initiate epithelial mesenchymal

transition (EMT) potentially leading to malignancies. Constitutive EGFR signaling is a hallmark of many cancers including breast, lung, glioblastoma, and others (143-145).

Figure 5. EGFR release and mechanism of action.

(A) Schematic representation of EGF release at a wound site. Platelets, macrophages, and keratinocytes serve as sources of EGF which binds EGFR on the surface of keratinocytes. Platelets and macrophages release stored EGF via exocytosis. Keratinocytes expressed a tethered form of EGF localized to cell membrane. During wound healing EGF on the surface of keratinocytes is released as a result of proteolytic cleavage by a sheddase. (B) Upon EGF binding, EGFR molecules dimerize and phosphorylate C-terminal tyrosines which transduce the signal to induce migration, proliferation, and survival.

1.12 Role of EGFR in wound healing

Following injury, a transient elevation of EGFR and its ligand levels occurs in tissues (83). EGF acts on EGFR in autocrine, paracrine, endocrine, and intercrine manner to stimulate wound healing (146, 147). Studies performed using EGFR-null skin grafts showed delayed healing, decreased re-epithelialization, and decreased keratinocyte proliferation and migration in EGFR-null skin compared to WT control (148). Other studies demonstrated the requirement of specific phosphorylation sites on the C-terminus of EGFR to initiate migration. Boucher et al. demonstrated that cells mutated at sites encoding tyrosines 845, 1068, or 1086 failed to migrate at the same rate as control, with doubled wound closure time relative to WT controls (149). Other studies demonstrated the potent ability of EGFR to induce migration (142, 150).

1.13 Rationale and hypothesis

Tissue damage due to elevated protease activity is a hallmark of chronic wound pathogenesis. GzmB may exhibit multiple pathogenic roles that impair the wound healing process. As discussed earlier, a causative role for GzmB in chronic wound healing has been demonstrated in several *in vivo* delayed wound healing models (50, 151-153). These studies showed that GzmB KO or inhibition resulted in improved re-epithelialization, wound contraction, granulation tissue formation and collagen remodelling. The abundance of extracellular substrates of GzmB as indicated in Table 1 suggests that it could play a role in all stages of wound healing, with the precise role, and many potential pathogenic mechanisms remaining to be elucidated.

In the highly proteolytic chronic wound environment the balance between growth factor release and their corresponding receptor signaling is lost due to growth factor or receptor degradation (146, 154, 155). Chronic wounds are known to contain reduced levels of EGF, TGF- β , and PDGF (156). Additionally, growth factors that are present in the wound are likely trapped by proteolytically-generated ECM fragments, nullifying the bioavailability of these growth factors (96, 157). For these reasons many growth factor therapies, including topical EGF therapy have not been very effective in patients with chronic non-healing wounds (96).

EGFR – a vital receptor for initiation of cell migration during wound healing can be extracellularly cleaved by an MMP (141). Using GraBCas V1.0 substrate predictor software, I was able to identify a potential GzmB cleavage site on the N-terminus of EGFR which is the extracellular domain of the receptor.

I *hypothesize* that GzmB impairs EGF-induced keratinocyte migration by impairing EGFR signaling.

Specific aims:

1. To investigate the effect of GzmB on EGFR-dependent keratinocyte migration
2. To investigate the effect of GzmB on EGFR signaling
3. To determine whether EGFR is a substrate of GzmB

Chapter 2: Materials and Methods

2.1 Cells and tissue culture

The HaCaT human keratinocyte cell line (spontaneously immortalized cell line at high calcium levels and temperature conditions) was obtained from AddexBio (San Diego, CA). The HeLa cervical carcinoma cell line was obtained from ATCC (Manassas, VA). MCF7 caspase-3-deficient breast cancer cell line was obtained from Kevin Bennewith (BC Cancer Research Center, Vancouver, BC). All cell lines were grown in DMEM supplemented with 10% FBS and 1% Penicillin and Streptomycin at 37°C in 5% CO₂ incubator, unless indicated otherwise.

2.2 Reagents and antibodies

Antibodies for P-EGFR (Y1068), total EGFR, and GapDH were obtained from Cell Signaling (Danvers, MA). Antibody for GzmB was obtained from BD Biosciences. Antibody for p53 was obtained from Santa Cruz Biotechnology (Dallas, TX). EGF and EGFR N-terminal domain peptides were obtained from LifeTechnologies. EGFR N-terminus antibody obtained from Abcam. Secondary antibody used in western immunoblotting, Goat anti-Rabbit-AlexaFluor 680, was obtained from LifeTechnologies. In immunofluorescence experiments Goat anti-Rabbit-AlexaFluor 488 (LifeTechnologies) and Donkey anti-Mouse-AlexaFluor 532 (LifeTechnologies) were used for detection. GzmB was obtained from EmeraldBio. Serpin a3n was obtained from Dr. R. Chris Bleackley, University of Alberta, Edmonton, AB.

2.3 Electric cell-substrate impedance sensing

HaCaT cell migration was measured using Electric Cell-Substrate Impedance Sensing (ECIS) method. Cells were seeded on L-cysteine pre-coated 8W1E PET ECIS arrays (Applied Biophysics) at 2×10^5 cells/chamber in 400 μ l of DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin. Cells were incubated for 1 h at RT to allow attachment, and afterwards incubated overnight at 37°C, 5% CO₂ tissue culture incubator on ECIS ZTheta Platform (Applied Biophysics) with electric fence on. Electric fence settings were set to default and measurements were taken at multiple frequencies. On the next day cells were washed with PBS and incubated with serum free DMEM $\pm$ 100 nM GzmB $\pm$ 300 nM Sa3n for 6 h at 37°C. At the end of the 6 h incubation fresh medium $\pm$ GzmB $\pm$ Sa3n was added to cells and 100 ng/ml EGF was added to specific wells. Cells were put back on the ECIS platform and electric fence was turned off to initiate migration. The time point when electric fence was turned off is referred to as 0 h. Migration was measured by an increase in resistance values at the frequency of 4 kHz in real time as cells covered the electrode. Every assay contained a well without cells and a well without electric fence as endogenous controls. PBS was used as control treatment.

2.4 Scratch migration assay

Cell migration was alternatively assayed using a traditional scratch assay method. HaCaT cells were seeded on 12 well tissue culture plates at 5×10^5 cells/well. The next morning cells were pre-treated with 100 nM GzmB for 6 h in serum free medium. Cells were then scratched with a p200 pipette tip, washed with PBS, and serum free medium containing fresh GzmB $\pm$ 100 ng/ml EGF was added. Immediately cells were imaged at 20x magnification using a light microscope. A scratch mark was made with a razor blade on the

bottom of each well to serve as reference point for imaging. Cells were also imaged at 18 h after the addition of EGF. ImageJ was used to trace scratches and measure their area in pixels. Percent original area at 18 h was calculated relative to the area at the time of scratching.

2.5 Immunofluorescence

HaCaT cells were grown to confluency on 8 well Lab Tek II chamber slide system (Nunc, Roskilde, Denmark) and serum starved overnight. In the morning, cells were washed with PBS, scratched with p200 pipet tip, and treated with 100 nM GzmB for 6 h. Next 100 ng/ml EGF was added to cell medium for 2 h. Cells were then washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X-100, blocked with 1% BSA and stained for EGFR and GzmB. F-actin staining was performed using Phalloidin-AlexaFluor488 (LifeTechnologies) according to manufacturer's protocol. Confocal imaging was performed on Leica AOBS SP2 laser scanning confocal microscope (Leica, Heidelberg, Germany) and Leica Confocal Software TCS SP2. Images were analyzed using Volocity 3D Image Analysis Software (PerkinElmer, Waltham, MA).

2.6 Western immunoblotting

Cells grown to approximately 80% confluency were serum starved overnight and treated with 25-300 nM GzmB $\pm$ Sa3n in serum free medium for 6 h and stimulated with 100 ng/ml EGF for 10 min. Cycloheximide (Sigma-Aldrich, 10 μ g/ml) was used to block protein synthesis prior to GzmB treatment, and p53 protein levels were used as controls as p53 possesses a 20 min half-life. Supernatants were collected and cells were lysed immediately using Cell Lytic M Solution (Sigma) supplemented with Protease Inhibitor Cocktail (Sigma)

and Phosphatase Inhibitor Cocktail (Sigma). Protein amount in lysates was quantified using Bio-Rad Protein assay and equal amounts of protein were used. Supernatants were concentrated in 10K spin columns (Millipore) and the entire sample was used in SDS-PAGE. Samples were denatured, ran on 10% polyacrylamide gel, transferred to PVDF membrane, immunoblotted, and scanned using Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, New England). Antibodies were diluted in 5% skim milk solution in TBST. All membrane washes were done in TBST, except the wash immediately prior to imaging, which was done using TBS solution. ImageJ was used to perform semi-quantitative band intensity analysis.

2.7 Biochemical cleavage assays

EGF (6 ug) or extracellular domain of EGFR (500 ng) were incubated with 100 nM GzmB in PBS, with total reaction volume of 30 μ l. GzmB was pre-incubated with 300 nM Serpin a3n for 1 h at 37°C prior to addition to substrates. Cleavage reactions were incubated at 37°C for 24 h. Denaturing sample buffer was added to stop reactions. Samples were run on SDS-PAGE and were used in western immunoblotting or gel staining with SimplyBlue Stain (Invitrogen) following manufacturer's protocol.

2.8 Flow cytometry

Cells were treated with GzmB for 6 h, collected using TrypLE (Gibco), washed with cold PBS, and stained with AnnexinV AlexaFluor 488 (LifeTechnologies) following manufacturer's protocol. Cells were analyzed on Gallios Flow Cytometer (Beckman Coulter) and Kaluza Analysis Software version 1.3 (Beckman Coulter) was used for data analysis.

2.9 Calcein viability assay

HaCaT cells were seeded at 2×10^4 cells/well on black 96 well tissue culture plates (Greiner). Cells were serum starved the next day and then treated with 0.1-200 nM GzmB for 6 and 24 h. Dead cells controls were incubated with 70% Methanol for 30 min. At the end of each treatment cells were washed with PBS, and incubated with 4 μ M Calcein-AM (LifeTechnologies) for 10 min at RT. Cells were then washed and incubated with 100 μ l PBS. Fluorescent measurements were taken using Tecan Infinite M1000 Pro plate reader.

2.10 Statistical analyses

All data represent results from at least 3 independent experiments. ECIS and Scratch assay data was analyzed with ANOVA followed by Tukey's multiple comparisons test. Data is represented as mean $\pm$ standard error. Mann-Whitney Test (two-tailed, unpaired) was used to make two-group comparisons. P-values are represented as follows: $p < 0.05$ is denoted by *, $p < 0.001$ is denoted by **, and $p < 0.0001$ is denoted by ***, and $p < 0.05$ was considered significant in all experiments. All statistical analyses were performed in GraphPad Prism version 5.04 (GraphPad Software, San Diego, California).

Chapter 3: Results

3.1 Granzyme B impairs keratinocyte migration through an EGFR-dependent mechanism.

HaCaT keratinocyte migration was assessed *in vitro* by ECIS and scratch assay. GzmB untreated/EGF stimulated cells migrated at a faster rate than untreated control cells, while GzmB treated/EGF unstimulated cells did not migrate (Figure 6A). Interestingly, the migration of GzmB treated/EGF stimulated cells was delayed by 8 h compared to GzmB untreated/EGF stimulated cells. The quantitative analysis further showed a 2-fold migration time delay in GzmB treated/EGF stimulated cells as compared to GzmB untreated/EGF stimulated cells. GzmB inhibition with Serpin a3n restored the migration phenotype to that of GzmB untreated/EGF stimulated cells (Figure 6A). Alternatively, migration was assessed by scratch assay (Figure 6B). In agreement with the ECIS results, GzmB reduced HaCaT migration in GzmB treated/EGF stimulated cells approximately 3-fold in comparison to GzmB untreated/EGF stimulated cells. Similarly, GzmB alone reduced cell migration in comparison to control treated cells.

Figure 6. GzmB impairs keratinocyte migration.

(A) ECIS migration assay on HaCaT cells treated with 100 nM GzmB for 6 h while grown with electric fence turned on. After 6 h, cells were washed with PBS, and incubated with fresh GzmB $\pm$ 100 ng/mL EGF with electric fence turned off. Fold change in resistance was calculated relative to the values obtained at the start of incubation without electric fence (0 h time point). Right panel shows time to 100% resistance for selected samples. Data was obtained from 5 independent experiments. (B) Scratch assay showing the effect of GzmB on keratinocyte migration. HaCaT cells treated with 100 nM GzmB for 6 h were scratched, washed with PBS, then incubated for 18 h with fresh GzmB treated medium $\pm$ 100 ng/mL EGF. Cells were imaged at the time of scratching (0 h) and 18 h after scratching. Scratch areas from at least 4 independent experiments were quantified and expressed as a percentage relative to the scratch area at 0 h. For both experiments data was by one-way ANOVA followed by Tukey's multiple comparisons test and is represented as mean values $\pm$ SD ; $p < 0.05$ denoted by *, $p < 0.001$ denoted by **.

3.2 GzmB impairs transition to migratory cell morphology

HaCaT cell morphology was examined at the wound edges of scratches made through the cell monolayers. GzmB treatment significantly reduced the number of cells that displayed a migratory phenotype (Figure 7A and B). In GzmB untreated/EGF stimulated group, more cells exhibited cell spreading with defined leading edges, cell polarity, filopodia and lamellipodia, which are characteristics of migrating cells (92, 158).

Figure 7. GzmB alters migratory cell morphology.

(A) Confocal images of immunofluorescent staining of HaCaT cells for EGFR after creating a scratch with a pipette tip followed by a 6 h treatment with control or 100 nM GzmB and a 2 h stimulation with 100 ng/ml EGF. DAPI used as nuclear stain. Scale bars = 20 μ m. White arrows point toward cells with migratory morphology. Representative images picked from 4 independent experiments. (B) Number of edge cells with migratory morphology. Values represent the average of 4 independent experiments. Data analyzed by a two-tailed, unpaired Mann-Whitney test and represents mean $\pm$ SD, $p < 0.05$ denoted by *.

3.3 GzmB-treated HaCaT cells lack actin rearrangements required for migration

To further examine the migratory cell phenotype lacking in cells following GzmB treatment, actin cytoskeleton architecture was examined in HaCaT cells using immunofluorescent staining and confocal microscopy. In a confluent cell layer, filamentous actin (F-actin) is evenly distributed across the cell, maintaining its symmetrical shape. During migration, F-actin undergoes coordinated polymerization to extend cellular processes (filopodia and lamellipodia) towards the acellular space (159). Filopodia are thin finger like projections filled with tight parallel strands of F-actin, while lamellipodia are filled with interlocking branches of F-actin (160). During migration, actin filament rearrangements push and extend the cell membrane forward in the direction of migration.

In the absence of GzmB, the majority of cells at the leading edge displayed clear actin spikes protruding towards the scratch zone creating a clear phenotype difference between edge cells and cell within the monolayer (Figure 8A and B). Closer examination of the actin spikes reveals a thin parallel arrangement characteristic of filopodia (Figure 8B). With GzmB treatment, leading edge cells lose the actin spikes, with majority of cells displaying straight edges and looking very similar to cells within the monolayer (Figure 8C and D). Upon closer examination, little or no filopodia are observed at the leading edge cells (Figure 8D).

Figure 8. GzmB interferes with filopodia formation in HaCaT cells.

Confocal images of immunofluorescent staining of HaCaT cells for F-actin after creating a scratch with a pipette tip followed by a 6 h treatment with control (A and B) or 100 nM GzmB (C and D) and a 2 h stimulation with 100 ng/ml EGF. DAPI used as nuclear stain. Scale bars individually defined. White arrows point toward cells with evident filopodia. White boxes define areas represented in (B) and (D). Representative images picked from 3 independent experiments.

3.4 GzmB reduces ligand-induced EGFR phosphorylation

To investigate whether the functional changes in HaCaT cells were a direct result of EGFR signaling, EGFR phosphorylation levels were assessed by western immunoblotting after treating cells with GzmB. GzmB significantly reduced ligand-dependent EGFR phosphorylation in a dose dependent manner (Figure 9A). Inhibiting GzmB with Sa3n restored P-EGFR level to that observed in controls. The same effect on P-EGFR was observed in HeLa cells (Figure 9B). The presence of cycloheximide, a protein synthesis blocker, did not affect the ability of GzmB to reduce P-EGFR levels (Figure 9C). Interestingly, treatment of HaCaT cells with GzmK, another member of GzmB family, following EGF stimulation did not lead to a reduction in P-EGFR levels (Figure 9D).

Figure 9. GzmB reduces EGFR phosphorylation.

(A) HaCaT (B) HeLa cells were treated with increasing concentrations of GzmB for 6 h followed by a 10 min stimulation with 100 ng/mL EGF. Western blotting was performed using antibodies for P-EGFR (Y1068), EGFR, and GAPDH. Bar graph shows EGFR phosphorylation levels expressed as percentages relative to total EGFR levels as determined by semi-quantitative band intensity analysis. Serpin α_3 n was used to inhibit GzmB activity in (A). Data in (A) was analyzed by a two-tailed, unpaired Mann-Whitney test and is represented as mean values $\pm$ SD. (C) GzmB-induced P-EGFR response analyzed in HaCaT cells pre-treated with 10 μ g/ μ l cycloheximide (using p53 as a short half-life control) for 24 h prior to the start of experiment done following the procedure in (A). (D) Western immunoblotting of HaCaT cell lysates following a 6 h treatment with 100 nM GzmK and a 10 min stimulation with 100 ng/ml EGF. Western blots shown represent results of at least 3 independent experiments.

3.5 GzmB does not cleave EGF or the extracellular domain of EGFR

To determine whether the cleavage of EGFR or its ligand by GzmB was responsible for EGF-induced P-EGFR reduction, biochemical cleavage assays were performed. GzmB did not cleave EGF as determined by protein gel staining where no EGF band intensity changes were observed between control and GzmB containing reactions (Figure 10A). GzmB did not cleave the extracellular domain of EGFR as determined by western immunoblotting (Figure 10B) and protein gel staining (Figure 10C).

Figure 10. GzmB does not cleave EGFR or EGF.

(A) EGF cleavage assay with 500 nM GzmB incubated overnight analyzed by SimplyBlue stained protein gel. (B) EGFR N-terminus cleavage assay using 100 nM GzmB±Sa3n analyzed by Western Immunoblotting for EGFR. (C) EGFR N-terminus cleavage assay run in duplicates with 100 nM GzmB±Sa3n analyzed by SimplyBlue stained protein gel. Images for panels B, C, and D represent data obtained from 3 independent experiments.

3.6 GzmB does not reduce HaCaT cell viability

GzmB has previously been shown to induce anoikis in breast adenocarcinoma, endothelial and smooth muscle cells (59, 60). A downstream effector of anoikis, Caspase-3 has been shown to cleave the C-terminus of EGFR and reduce its activity (161-163). We investigated whether induction of anoikis and caspase-3 were involved in GzmB-dependent reduction in P-EGFR levels. Cell viability after GzmB treatment was assessed by AnnexinV/PI staining and flow cytometry. GzmB did not induce apoptosis in HaCaT cells over the period 6 and 24 h (Figure 11A). Additionally, GzmB did not reduce cell viability as determined by calcein viability assay after 6 and 24 h (Figure 12). To further rule out the potential involvement of caspase-3 in reducing P-EGFR levels, a caspase-3 deficient MCF-7 epithelial cell line was treated with GzmB and stimulated with EGF which still resulted in the reduction of P-EGFR levels (Figure 11B). Additionally, cleaved caspase-3 was not detected by western immunoblotting in HaCaT cells after 6 and 24 h of 100 nM GzmB treatments (data not shown).

Figure 11. GzmB does not affect HaCaT cell viability.

(A) Flow cytometry analysis of Annexin V/PI stained HaCaT cells following a 6 and 24 h treatment with 100 nM GzmB. Bar graphs represent % of Annexin V⁺ and Annexin V⁺PI⁺ cells. Data combined from 3 independent replicates. Mann-Whitney test was used for statistical comparisons, data represented as mean ± SD. (B) Western immunoblotting for P-EGFR (Y1068) of MCF-7 cells following a 6h treatment with 100 nM GzmB and a 10 min stimulation with 100 ng/mL EGF. Western blot is a representative image from 3 independent experiments.

Figure 12. Calcein viability assay.

HaCaT cells treated with 0.1-200 nM GzmB were incubated with 4 μ M calcein-AM for 10 min, followed by fluorescence measurements. Methanol (70%) treated cells used as dead cells control. Data combined from 3 independent replicates. Mann-Whitney test was used for statistical comparisons. Data represented as mean $\pm$ SD.

3.7 GzmB is internalized by HaCaT cells over time.

To address the migration rescue effect after 8 h of incubation with GzmB, its depletion in the medium was assessed by western immunoblotting. GzmB abundance in cell medium decreased over time (Figure 13A). In parallel, the internalization of GzmB by HaCaT cells was evaluated by immunocytochemistry and confocal microscopy. After 6 h of incubation with 100 nM GzmB, HaCaT cells contained cellular compartments positive for GzmB staining (Figure 13B).

Figure 13. GzmB internalization by HaCaT cells.

(A) Western immunoblotting of concentrated cell supernatants initially containing 100 nM GzmB collected at different time points, blotted with anti-GzmB antibody. Equal volumes of supernatants were added to cells and collected for concentration. (B) Confocal images showing immunofluorescence staining of HaCaT cells for GzmB and EGFR after a 6 h treatment with 100 nM GzmB (top panel) and untreated cells with the same staining (bottom panel). DAPI used as nuclear stain. Scale bars = 6 μ m. Representative images picked from 3 independent experiments.

Chapter 4: Discussion

Cell migration is essential in many physiological and pathological processes including embryogenesis, wound healing, vascular repair and metastasis (164-166). In the context of cutaneous wound healing, epithelial cell migration is an essential step in tissue repair (167). Due to impairment of wound healing processes, non-healing wounds are characterized by chronic inflammation, leading to accumulation of a variety of proteases at the wound site (20, 110). These proteases include matrix metalloproteinases, cathepsin G, neutrophil elastase and others (110, 112), which collectively degrade many extracellular matrix proteins such as FN, Dcn, and vitronectin – all vital for proper wound healing. GzmB is also abundant at sites of chronic inflammation, including non-healing wounds, which cleaves a wide array of extracellular substrates, including the aforementioned proteins (20). Previous studies have highlighted the negative roles of GzmB in mouse models of chronic inflammation and impaired wound healing. Using ApoE knockout mice, Hiebert et al. demonstrated that GzmB/ApoE double knockout mice exhibited improved wound closure and contraction in comparison to ApoE knockout mice. GzmB inhibition by Serpin a3n resulted in faster wound closure, improved re-epithelialization, and granulation tissue formation in a diabetic mouse model of impaired wound healing (50). While inflammatory infiltrates are a large source of GzmB (20), through different mouse models, it has also been established that mast cells also serve as a significant source of GzmB (50, 58, 153).

Wound healing is a complex interplay of cellular and extracellular matrix interactions, as many processes overlap and are interdependent on each other. This study investigated the effect of GzmB on epithelial cell migration – the core process involved in the re-epithelialization stage, with HaCaT keratinocytes used as an *in vitro* model for skin

epithelium. While cell migration is a process driven by many factors including extracellular matrix-derived granulation tissue, growth factor stimulation, and cell-to-cell contact rearrangement, this study focused on the growth factor dependent migration process. EGFR is a potent inducer of cell migration and proliferation (138, 150). During physiological wound healing, platelets and macrophages serve as initial sources of EGF during hemostasis and inflammatory stage, respectively (142). In later stages, EGF is released from the surface of keratinocytes by ectodomain shedding in order to stimulate cell migration in an autocrine manner for induction of a local effect (138). Due to the presence of protease generated extracellular matrix fragments, proteases, and necrotic cell debris, the bioavailability of EGF at the wound site is believed to be compromised (96). For this reason clinical trials using topical EGF therapy have been attempted, many of which showed no or marginal improvements with EGF treatment, and while few show promising results, there is still a long term concern of inducing skin malignancies as well as making the therapies financially viable (96). Increasing the bioavailability of EGF that is already in the wound and preventing EGFR signaling impairment may be a promising therapy for non-healing wounds.

The present study demonstrates that GzmB inhibits migration in HaCaT keratinocytes through an EGFR-dependent pathway. GzmB treatment alone and, interestingly, followed by EGF stimulation, impaired keratinocyte migration significantly. While GzmB treatment alone fully prevented migration measured by ECIS, it significantly delayed migration induced by EGF. While EGFR is the only receptor that binds EGF, it is unlikely that other ErbB receptors are activated by EGF stimulation in the present study (134). To further support the claim that EGFR-dependent pathway is affected, additional work can be done using siRNAs to target the remainder of ErbB family receptors (ErbB-2, ErbB-3, and ErbB-

4), expecting the same response to EGF and GzmB+EGF treatment as observed in Figure 1. Additionally, knocking down EGFR with siRNAs or targeting it with a specific inhibitor, such as AG1478, which selectively inhibits EGFR tyrosine kinase activity would provide further support to the interpretation of the aforementioned results (168). Performing experiments with EGFR knockdown or inhibition will change the migration pattern of EGF treated cells to that of untreated cells, and GzmB+EGF treated to that of GzmB treated, as seen in Figure 1, which would further support the claim that the effect observed in Figure 1 is EGFR-dependent. Cell migration rescue after 8 h may be due to GzmB internalization by cells, thus reducing the concentration of enzyme in the medium. Extracellular GzmB concentration declined in a time-dependent manner after addition to HaCaT cells, and intracellular compartments positive for GzmB staining were observed inside the cells after 6 h (Figure 13). These findings are consistent with studies where GzmB was found within cellular compartments and without the addition of Pfn, did not display cytotoxic capabilities (169). To investigate the effect of GzmB on EGFR signaling, tyrosine 1068 was chosen as an indicator of P-EGFR state as it was previously shown to be one of the essential phosphotyrosines involved in migratory signaling pathways (149). GzmB treatment induced a dose-dependent reduction in P-EGFR levels, in both HaCaT and HeLa cells, with Serpin a3n leading to restoration of normal P-EGFR levels in HaCaT cells. Interestingly, GzmK, another member of the granzyme family, did not reduce P-EGFR levels. This data suggests that reduced P-EGFR is a GzmB-specific effect and may be applicable to different epithelial cell types.

As cells transition into a migratory state, they undergo changes in morphology. Upon receiving an external migration-inducing signal cells undergo polarization, membrane

spreading and extension, and protrusion in the direction of movement (91, 170). In a migrating monolayer of cells, only edge cells display migratory morphology, while the rest retract in support of collective cell movement (91). While some cell types will form strong attachments to the substrate during migration, others, such as keratinocytes, do not form noticeable attachments but, rather, glide over the surface (91). In migrating cells the nucleus gets displaced in the direction opposite from that of migration (91, 170). The present study showed that GzmB treatment impairs the induction of EGF-induced migratory cell phenotype further indicating that GzmB interferes with cellular migration physiology. Further actin cytoskeleton staining revealed a reduction in filopodia formation in GzmB-treated cells while untreated cell exhibited an abundance of filopodia along the leading edge cell membranes. (Figure 8). This difference in cytoskeletal architecture presents an interesting avenue for future investigations.

Previous studies indicate that GzmB can induce cell death through anoikis in the absence of Pfn (59, 60). With keratinocytes, however, we found no indication of cell viability loss or cell death induction after 24 h of treatment with 100 nM GzmB. There was, however, a slight increase in the number of dead cells as indicated by AnnexinV/PI staining at 24 h in GzmB treated cells (Figures 11 and 12), however the increase was small and insignificant. Previous studies showed that caspase-3 is able to cleave EGFR intracellularly, which would lead to P-EGFR reduction (161, 162). Demonstrating the P-EGFR reduction in response to GzmB in caspase-3 deficient MCF-7 cells further ruled out cell death-mediated P-EGFR reduction. While the data in HaCaT cells contradicts previous reports in other cell types, it can be attributed to a higher degree of adherence to substratum and neighboring cells by

keratinocytes and the overall high degree of resilience to external stimuli compared to previously investigated cell types.

GzmB has the ability to cleave many extracellular substrates and has overlapping roles with matrix metalloproteinases extracellularly, and caspases intracellularly (20, 60). EGFR has previously been reported to be a matrix metalloproteinase substrate (141), and while we originally hypothesized a GzmB cleavage site on EGFR, we did not identify EGFR as a substrate of GzmB. Though the mechanism of action of GzmB on EGFR-dependent migration still remains elusive, this study demonstrates a novel role of GzmB on cell migration. Elucidating how GzmB affects the EGFR signaling pathway would shed further light with respect to negative roles of GzmB in the wound healing processes.

It is well established that despite being taken up by cells over time, GzmB remains internalized in vesicles in the absence of Pfn (165). Blocking protein synthesis with cycloheximide in this study still resulted in the reduction of EGFR phosphorylation after GzmB treatment, suggesting that the effect of GzmB is unlikely due to the production of new protein and is likely extracellular. While not being able to cleave EGFR, GzmB may still interact with the receptor preventing or interfering with its dimerization, hindering EGF binding, and resulting in reduced P-EGFR levels. Preliminary experiments aimed at investigating EGFR dimerization in response to GzmB treatment were initiated in this study, however the protocols are in need of extensive troubleshooting and the results remain to be determined.

Previous studies demonstrate the ability of ECM proteins to induce EGFR internalization and degradation, thus resulting in the overall reduction in EGFR signaling.

Santra et al. found that Dcn core protein can bind to the ligand-binding domain of EGFR (171). Studies from the same group later showed that Dcn binding leads to EGFR internalization and degradation (172). This ability of Dcn to reduce EGFR cell surface availability was shown to have physiological relevance in cancer, with Dcn treated mice exhibiting reduced tumour growth (173-175). As discussed earlier, GzmB is known to cleave Dcn, thus it would be interesting to investigate whether GzmB-generated Dcn fragments elicit the same effect on EGFR in keratinocytes leading to receptor internalization and downstream degradation.

E-cadherin is an epithelial calcium-regulated junctional protein present abundantly on basolateral surface of cells and primarily responsible for cell-cell adhesion (176). EGFR and E-cadherin colocalize on epithelial cell membranes and their functions are interconnected by direct cross talk through cytoplasmic signaling pathways including β -catenin signaling (177, 178). If one protein is taken up by the cell, the other follows, due to their tight colocalization. Preliminary data in our lab has shown that GzmB cleaves the extracellular domain of E-cadherin (unpublished). It is possible that by cleaving E-cadherin leading to its internalization and downstream degradation GzmB may cause a secondary effect on EGFR leading to non-specific receptor uptake and reduction of cell surface receptor availability.

While providing novel insight into the roles of GzmB in the context of wound healing, the main limitation of this study is *in vitro* nature of all experiments. HaCaTs, while widely used in skin research, may contain differences from keratinocytes observed in the human body. The cell line was developed under high calcium (1.4 mM Ca^{2+}) and high temperature conditions (38.5°C) which deviate from physiological environment (179).

The results of this study provide compelling evidence for GzmB in the impairment of keratinocyte migration and EGFR signaling and are paralleled in HeLa cells. These results may be relevant in other tissues such as the vasculature as EGFR is present on endothelial cells and during angiogenesis endothelial cells migrate to form new blood vessels (180, 181). It would be interesting to investigate the effects of GzmB on endothelial migration as during chronic inflammation angiogenesis is dysregulated and new vessels are dysfunctional.

During extravasation immune cells release GzmB to locally degrade the ECM of vascular basement membranes (62). In GzmB null mice NK cells adhere to the endothelium but fail to transmigrate into the tissue (62). Certain cancer cells, such as the urothelial cancer cells have been shown to produce GzmB (182). It is believed that GzmB contributes to urothelial cancer pathogenesis by promoting tumour invasiveness via ECM degradation. In the study by D'Eliseo et al., GzmB was expressed at the edges of urothelial tumours in cell undergoing epithelial mesenchymal transition (182). Interestingly, Pfn was not expressed in these cells (182). This study demonstrated that inhibition of GzmB reduced tumour cell invasiveness *in vitro*. The same group later published that colorectal cancer cells are also capable of GzmB expression and its inhibition inhibited epithelial mesenchymal transition and tumour invasion (183).

Chapter 5: Conclusion and Future Directions

In conclusion, my thesis studies revealed a novel function of GzmB in the context of cutaneous wound healing. GzmB significantly delayed keratinocyte migration through EGFR-dependent pathway as measured by two distinct techniques and inhibited cell transition to migratory morphology with reduction in actin cytoskeleton rearrangements to form filopodia. GzmB impaired ligand-induced EGFR phosphorylation in HaCaT and HeLa cells. GzmB did not cleave EGFR or EGF and did not cause a secondary effect on EGFR by inducing anoikis and caspase-3 induction and sustained its effect on EGFR in caspase-3 deficient MCF7 cells. The summary of this study is schematically represented in Figure 14.

These studies provide groundwork for future investigations into the mechanism of action of GzmB on EGFR. Using primary human keratinocytes as a more physiologically relevant skin cell model would provide more insight into the involvement of GzmB in cell migratory pathways. As discussed earlier, multiple avenues can be investigated to delineate the exact mechanism of action of GzmB on EGFR including potential inhibitory effects of ECM fragments, inhibition of EGFR dimerization, and non-specific EGFR internalization during E-cadherin degradation. Upon the identification of the mechanism behind GzmB-induced EGFR phosphorylation reduction, performing mouse chronic wound healing studies would determine the extent of the impact of GzmB interference with EGFR pathway on the speed and quality of healing of chronic wounds. Using established mouse models of chronic wound healing P-EGFR levels can be profiled over the course of wound closure and compared to normal levels to determine if the *in vitro* data obtained in this study translates to detectable phenotypes *in vivo*. Lastly, it would be interesting to investigate the effect of

GzmB on other members of ErbB receptors to determine if similar effect on receptor phosphorylation is observed.

The functional diversity of GzmB makes it an interesting target of scientific investigation. While being a required mediator of apoptosis in cytotoxic immune response and immune cell transmigration, when dysregulated, GzmB inflicts substantial damage to tissue structure via ECM degradation. This study broadens the understanding of harmful effects of GzmB on keratinocyte migration – a vital component of wound healing. While much remains to be investigated, this study provides novel insight into the negative impacts of GzmB in EGFR-mediated cell migration.

Figure 14. Summary of the current investigation and findings.

GzmB reduced keratinocyte migration by reducing ligand-dependent EGFR phosphorylation. The upstream mechanisms were investigated however the mechanism of action of GzmB on EGFR remains elusive. Proposed mechanisms behind the observed results are included and present avenues for future investigations.

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