EXTRACELLULAR GRANZYME K MEDIATES ENDOTHELIAL INFLAMMATION
THROUGH THE CLEAVAGE OF PROTEASE ACTIVATED RECEPTOR-1

by

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Abstract

Granzymes are a family of serine proteases that were once thought to function exclusively as mediators of cytotoxic lymphocyte-induced target cell death. However, non-lethal roles for granzymes, including Granzyme K (GzK), have been recently proposed. As recent studies have observed elevated levels of GzK in plasma of patients diagnosed with sepsis, we hypothesized that extracellular GzK induces a pro-inflammatory response in endothelial cells. In the present study, extracellular GzK proteolytically activated Protease Activated Receptor-1 (PAR-1) leading to increased IL-6 and MCP-1 production in Human Umbilical Venous Endothelial Cells (HUVEC). Enhanced expression of ICAM-1 along with an increased capacity for adherence of THP-1 cells was also observed. Characterization of downstream pathways implicated the MAPK p38 pathway for ICAM-1 expression, and both the p38 and the ERK1/2 pathways in cytokine production. GzK also increased TNFα–induced inflammatory adhesion molecule expression. Furthermore, the physiological inhibitor of GzK, IαIp, significantly inhibited GzK activity in vitro. In summary, extracellular GzK is not cytotoxic but promotes a pro-inflammatory response in endothelial cells.
Preface

- All experimentation, analyses and writing was done by Mehul Sharma in consultation with Dr. Granville.

- In chapter 1:
  - Table 2 was obtained with permission and modified from Zhao P, Metcalf M, Bunnett NW. Biased signaling of protease-activated receptors. Front Endocrinol (Lausanne). 2014;5:67.
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<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>Activated Protein C</td>
</tr>
<tr>
<td>Ape1</td>
<td>Apurinic Apyrimidinic Endonuclease</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>CLIP</td>
<td>Cross-linking Immunoprecipitation</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocytes</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EBM</td>
<td>Endothelial Basal Media</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein - Barr virus</td>
</tr>
<tr>
<td>ECIS</td>
<td>Electric Cell-substrate Impedance Sensing</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGM</td>
<td>Endothelial Growth Media</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EPCR</td>
<td>Endothelial Protein C Receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GzA</td>
<td>Granzyme A</td>
</tr>
<tr>
<td>GzB</td>
<td>Granzyme B</td>
</tr>
<tr>
<td>GzH</td>
<td>Granzyme H</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered Saline-Tween 20</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptors</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>TVD</td>
<td>Transplant Vascular Disease</td>
</tr>
<tr>
<td>VCP</td>
<td>Vasolin Coated Protein</td>
</tr>
<tr>
<td>vWf</td>
<td>von Willebrand factor</td>
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</tbody>
</table>
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Dedication

Dedicated to thinkers.
Chapter 1: Introduction

1.1 Immune System

The first line of defence in our body against any infection is physical barriers, such as the skin, which prevents entry of foreign pathogens. If the pathogen breaches these barriers, the next line of defence, the immune system, is activated. Innate and Adaptive immunity make up the two components of the immune system and upon entry of the pathogen the innate immune system is initially activated. The response generated can be largely non-specific and short-lived. The subsequent adaptive immune response is more specific, requires antigen presentation to the immune cells, thereby creating a long lasting memory against the pathogen (1).

Complement system and leukocytes of the innate immune system mediate the initial effects against a pathogen. Prostaglandins and cytokines produced by infected cells at the site of infection result in increased blood flow and recruitment of white blood cells, facilitating an inflammatory response to fight off the infection and initiate healing. The second arm of the innate immune system is mediated by innate immune cells – neutrophils, macrophages, dendritic cells, mast cells, eosinophils, basophils and natural killer (NK) cells. Mast cells, basophils, eosinophil are involved responding to allergens. Neutrophils and macrophages largely act as phagocytes in clearing the infection and dendritic cells and macrophages can act as antigen presenting cells. The presentation of antigens to T and B cells of the adaptive immune system usually takes place in lymph nodes resulting in activation of the next line of defence, the adaptive immune system (1).
B-cells, of the adaptive immune system, mainly produce antibodies specific to the antigen. These antibodies can target pathogens in circulation and activate the complement cascade to clear/kill them. The functions of T-cells vary considerably and they can be generally classified as T-regulatory, CD4+ T-helper cells, CD8+ cytotoxic T cells (CTLs) and γδ T cells. In general, T-helper cells, through cytokine release and cell-to-cell interactions, regulate immune responses by helping to activate different immune cells against different pathogens. CTLs recognize stressed and infected cells and induce apoptosis upon target cell recognition (2, 3).

Both CTLs and NK cells, can initiate target cell death through death receptor or the granule exocytosis pathways. The latter is the more common mechanism of cytotoxicity administered by these cells. Granules packed with serine proteases, granzymes, along with the cell membrane perforating protein, perforin, are released in the immune-synaptic cleft and taken up by target cells.

Recent studies have demonstrated that perforin might not be forming a pore on the cell membrane but that perforin and granzymes are endocytosed in clathrin coated vesicles and perforin facilitates granzymes entry into the target cell by forming pores in the endosome. In either case, perforin is thought to be essential in the delivery of granzymes into target cell (4). Granzyme B is the primary mediator of apoptosis in allogenic, and/or virally-infected cells (5).

1.2 Granzymes

Granzymes (granule-secreted enzymes) are a family of serine proteases, that were initially discovered in the granules of CTLs and NK cells (6–10). Five types of granzymes are characterized in humans: Granzyme A, B, H, K and M. They are a family of structurally similar
enzymes, with a triad of residues - histidine, aspartic acid and serine - conserved at the catalytic site (11). However, these serine proteases, expressed on three different gene clusters on different human chromosomes, widely differ in their substrate specificities (12, 13).

GzA and GzK are tryptases and are both found on chromosome 5 (12). GzA is a dimer and has a highly cationic surface. GzK is similar in residue composition on its surface but is a monomer, and therefore it is suspected that GzA arose following GzK gene duplication in the evolutionary process (14). Although both their predicted substrate cleavage sites are after basic amino acids, Lysine or Arginine, studies have shown that GzK will cleave at sites distinct from GzA and that they target different intracellular substrates (15, 16).

The genes for GzB and GzH are located on chromosome 14 (12). GzB is an aspartase, cleaving after aspartic acid residues. Caspase-3 and caspase-7 are some well-established intracellular substrates of GzB upon whose cleavage the cell undergoes apoptosis (17). GzH, although most closely related to GzB, has been shown to induce programmed cell death in host cells without displaying cleavage of caspase-3 or cleavage of Bid, pathways that are vastly associated with GzB (18). GzH is described as a chymase and is mainly thought to have predicted cleavage sites after hydrophobic amino acid groups (11).

The final human granzyme, GzM, is a metase. It is highly expressed in all subsets of NK cells, its genes are located on chromosome 19 and it cleaves after the amino acid Methionine or Leucine (12). Along with GzH and GzK, it was initially thought to be an orphan enzyme as functional roles of these three enzymes were assumed to overlap those of GzA and GzB. However, studies have now shown that GzM, similar to GzK and GzH, can exert distinctive roles in inducing tumour cell apoptosis that are independent of pathways characterized for GzA and
GzB (19, 20). Furthermore, the unique reported cleavage capability for GzH, GzK and GzM, as mentioned above, suggest that each granzyme might have a distinct role in the fighting off infections and tumor cells that evade host recognition and escape the mechanisms mediated by other granzymes (21).

1.3 Extracellular Granzymes and Diseases

Induction of cytotoxicity is not the sole physiological function of granzymes (22). Various studies have identified granzymes in extracellular space/fluids as possible biomarkers and/or therapeutic targets in diseases as elevated levels of extracellular granzymes are found in circulation of patients with various chronic inflammatory conditions and/or autoimmune disease (22, 23). Listed in Table 1 are some of the diseases associated with elevated extracellular granzymes in mice and humans.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Elevated Extracellular Granzymes Implicated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid arthritis</td>
<td>GzA, GzB – Plasma, Synovial fluid</td>
<td>(24, 25)</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disorder (COPD)</td>
<td>GzB - Bronchoalveolar lavage (BAL) of patients with COPD and in asymptomatic smokers</td>
<td>(26)</td>
</tr>
<tr>
<td>Asthma</td>
<td>GzB – BAL of allergen challenged patients with asthma</td>
<td>(27, 28)</td>
</tr>
<tr>
<td>Hypersensitivity Pneumonitis</td>
<td>GzA, GzB – Increased levels in BAL fluid of patients</td>
<td>(29)</td>
</tr>
<tr>
<td>UV photoaging and skin aging</td>
<td>GzB – Increased levels observed by immunohistochemistry in skin samples of UV-treated mice, and human skin punch biopsies. GzB KO rescues skin aging in ApoE KO mice.</td>
<td>(30, 31)</td>
</tr>
<tr>
<td>Multiple Sclerosis (MS)</td>
<td>GzB – Cerebral spinal fluid of patients with relapsing-remitting MS</td>
<td>(32)</td>
</tr>
<tr>
<td>Myasthenia Gravis</td>
<td>GzB – Thymus glands of patients, measured by immuno-blotting of homogenates.</td>
<td>(33)</td>
</tr>
<tr>
<td>Delayed Chronic Wound Healing</td>
<td>GzB – Elevated levels in granulation tissue of diabetic mice; GzB activity suppression rescued delayed wound closure in ApoE KO and diabetic mice.</td>
<td>(34, 35)</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>GzB – Highly present in atherosclerotic lesions. Higher in blood of patients with unstable atherosclerotic plaques</td>
<td>(36–39)</td>
</tr>
<tr>
<td>Aortic Aneurysm</td>
<td>GzB – Vessel walls of mice following aortic rupture</td>
<td>(40)</td>
</tr>
<tr>
<td>Viral infections</td>
<td>GzA, GzB – Plasma of patients with Epstein-Barr Virus (EBV) or HIV GzK – Bronco-alveolar lavage of patients with viral pneumonitis GzM – Plasma of patients with Human Cytomegalovirus (HCMV) infection</td>
<td>(41–44)</td>
</tr>
<tr>
<td>Plasmodium falciparum infections.</td>
<td>GzA, GzB – Plasma of malaria patients</td>
<td>(45)</td>
</tr>
<tr>
<td>Experimental endotoxemia or sepsis</td>
<td>GzA, GzB – Plasma of patients with melioidosis caused by the gram-negative bacterium <em>Burkholderia pseudomallei</em> GzM – Plasma of meningococcal sepsis patients GzK – Plasma of patients in hospital with clinical sepsis</td>
<td>(46–49)</td>
</tr>
<tr>
<td>Acute airway inflammation</td>
<td>GzK – BAL of patients with acute asthma after an allergen challenge</td>
<td>(50)</td>
</tr>
</tbody>
</table>
Because granzymes are largely thought to be transported via the immune-synapse and perforin channels into target cells, the finding of elevated granzyme levels in extracellular tissue has sparked research into possible mechanisms that can account for this. Leakage of granzymes out of the immunological synapse during a CTL and NK cell response is one possibility (23). Alternatively, granzymes can also be actively secreted during inflammation. GzA and GzB have both been shown to be released upon incubation of blood with LPS or bacteria, and injection of LPS in healthy individuals (49). This finding suggests that degranulation of immune cells as a possible mechanism even in the absence of cytotoxicity. Lastly, granzymes can be directly secreted out from cytotoxic lymphocytes even in the absence of any stimulation antigenic cells as granzymes are found in circulation of healthy patients (51).

The observations of elevated extracellular granzymes levels prompted investigations into the possible functions granzymes can have in the extracellular milieu. Granzymes have now been shown to act on various components in the extracellular matrix (ECM). GzB has been shown to cleave fibronectin, decorin, fibrillin-1, and vitronectin \textit{in vitro} and \textit{in vivo}. Cleavage of these ECM proteins in mice via extracellular GzB has been shown to contribute to abdominal aortic aneurysm, skin aging and delayed wound closure (52–54). GzA has also been shown to degrade fibronectin and collagen type IV; however the physiological relevance of this has not been properly evaluated (55, 56).

Apart from ECM degradation, GzA, GzB, GzK and GzM have all been shown to partake in the production, release or processing of proinflammatory cytokines (23). Direct cytokine release from cell lines by GzB is not seen but GzB has been shown to cleave IL-1α precursor (57) and pro-IL-18 (58, 59), activating both these proinflammatory cytokines. Furthermore, GzB
synergistically enhances LPS-induced TNF-α release from monocytes in vitro (60). This potentiation of LPS-induced cytokine response has also been shown to be consistent with GzK and GzA, and does not require the proteolytic activity of these enzymes (60). Extracellular GzA has also been shown induce IL-6 and IL-8 from fibroblast and epithelial cell lines (61) and can also activate monocytes to release TNF-α, IL-6, and IL-8 (62), all requiring the catalytic activity of GzA. Intracellularly, GzA cleaves pro-IL-1β into IL-1β, while displaying a lack of cytotoxicity, in monocytes (63, 64). GzK, which shares similar catalytic activity as GzA, also displays significant extracellular and proinflammatory capacity and will be discussed in more detail in the following section.

1.4 GzK in Inflammatory Disease

To understand the role that GzK plays in disease states, the proteolytic capability of GzK has been examined. Initially GzK was shown to cleave a myriad of intracellular substrates leading to apoptosis, summarized in Figure 1.
Figure 1: Intracellular pathways activated by GzK. GzK can be released into the immunological synapse by cytotoxic lymphocytes and internalized into the target cell with perforin. In the target cell, GzK can cleave Bid and Ape1, both can disrupt mitochondrial functionality leading to apoptosis. It can also induce apoptosis through DNA fragmentation through release of DNases after cleavage of the SET complex or its proteolytic activity can induce ER stress mediated apoptosis.
Upon uptake of GzK in target cells with perforin, GzK can cleave bid causing cytochrome C release from mitochondria and resulting in apoptotic cell death (65). GzK can also cleave apurinic apyrimidinic endonuclease (Ape1) – a redox/repair enzyme – leading to disrupted inner mitochondrial membrane potential and increased oxidative stress causing apoptosis (66). Cleavage of other intracellular substrates – SET complexes are cleaved to release DNases which cause DNA fragmentation in the nucleus, and vasolin coated protein (VCP) are cleaved increasing endoplasmic reticulum stress – also initiate apoptosis in the target cell (67, 68). Following these findings GzK was discovered to target the importin α/β, a nuclear transport protein, and a physiological target whose degradation inhibits the replication of influenza virus in host cells (69, 70).

The ability of GzK to fight off other viral infections was evaluated by studying the role of GzK in fighting off lymphocytic choriomeningitis virus (LCMV) infection (71). Subsequently, they found that GzK, or GzK expressing CTLs were not cytotoxic in an LCMV infection. Furthermore, they showed that uptake of GzK can induce IL-1β release from macrophages, and that fighting LCMV infection was likely through the activation of the NLRP3 inflammasome rather than apoptosis of target cells (71). The pro-inflammatory capacity of GzK was further built upon by the study of Cooper et al. (2011) which showed that extracellular GzK can activate lung fibroblasts to induce IL-6, IL-8 and MCP-1 inflammatory cytokine production (72).

Found in its active form at 26 kDa, physiological evidence for elevated extracellular GzK levels has been observed in the bronchoalveolar lavage of patients with acute pneumonia and in asthmatic patients that were exposed to an allergen challenge (41). Elevated levels of the active form of GzK are also found in the blood of patients suffering from sepsis, where GzKs most
likely physiological inhibitor, Inter-alpha-inhibitor protein (IαIp), was down-regulated. The up-regulation of GzK in sepsis has now been demonstrated in multiple studies (47, 60).

In a study by Wensink et al. (59) it has been shown that GzK has the capacity to bind to disaggregated lipopolysaccharide (LPS) from micelles and can augment the LPS-CD14 complex formation. This formation can signal through TLR4 on the surface of macrophages and immune cells, potentiating the LPS inflammatory response from these cells. This finding, along with the finding that GzK can induce inflammatory activation of lung fibroblasts, suggests that elevated levels of GzK may contribute to disease progression.

1.5 GzK Inhibitor, Inter Alpha Inhibitor Protein (IαIp)

In the study done by Rucevic et al. (46) regarding sepsis, GzK was found at a high molecular weight in plasma of health controls, as determined by blotting with an anti-GzK antibody. In patient groups which are clinically diagnosed with sepsis, a band at 26 kDa was detected on an SDS-gel when blotted with the same antibody. This band is suspected to be the active form of GzK. It is therefore suggested that in healthy patients GzK might be complexed with inhibitors found in the blood or glycoproteins that can inhibit the proteolytic action of the enzyme. In fact, GzK shares a similar C-terminal helix with that of α-thrombin that can bind to heparin, a highly sulfated glycosaminoglycan (14). However, the exact mechanism of action that inhibits GzK activity in healthy patients still remains unclear.

Wilharm et al. (73) initially showed that GzK activity was observed to be inhibited with increasing concentrations of plasma. Upon addition of heparin, further inhibition of GzK activity
was noted. This suggested that heparin-accelerated serine protease inhibitors of human plasma could be mediating GzK inhibition. After looking at a few of these serine protease inhibitors present in plasma – anti-thrombin III, heparin co-factor II, α1-proteinase inhibitor and IαIp – it was determined that IαIp was the most efficient inhibitor of GzK found in plasma (74).

Produced and secreted by the liver into human plasma, IαIp are found at really high concentrations in circulation, between 500-800mg/L. They are composed of three or four heavy chains and one light chain subunit, which is covalently linked with a glycosaminoglycan chain. Release of active proteases during inflammation, especially neutrophil elastase, has been implicated in cleaving the glycosaminoglycan chain (75). This frees up the light chain peptide of IαIp, referred to as Bikunin, which is thought to exert the inhibitory effects of IαIp (76). Bikunin is inactive when linked to the heavy chains but once freed has been shown to inhibit a range of plasma proteases present during inflammation, including elastase, plasmin and Cathespin G (77). Bikunin is then readily cleared via glomerular filtration and receptor mediated uptake (78).

Rucevic et al. (79) noted that along with elevated levels of active GzK in sepsis patients, a decrease in plasma concentrations of IαIp was also noted. Extensive secretion of proteases during inflammation may cause activation and eventual exhausting of IαIp. In fact, decreased levels of IαIp are observed in multiple sepsis studies of adults and neonates (80–82). Since GzK activity is shown to be inhibited by IαIp, the decrease in IαIp clinically could suggest that there is an increased systemic activity of GzK in these patients. The finding that administration of exogenous IαIp in models of adult and neonatal sepsis as well as models of endotoxic shock demonstrates improved morbidity and mortality (83–85) brings up the question of GzK activity in sepsis.
1.6 Sepsis: A Condition Resulting from a Dysregulated Immune Response

Sepsis is a wide-spread inflammatory reaction in response to an infection. It is a complex heterogeneous disease in terms of its inciting cause, initially presented severity and conditions that drive its course and progression. A common symptom in all sepsis patients is a dysregulated and destructive response to the invading microorganism (86). Progression of sepsis is often a cause of the immune reaction initiated by this pathogen. In fact bacterial products themselves are only detected in blood of about 30% of patients (87). Microbial factors are readily cleared from the blood but the resulting immune response drives a pro-inflammatory cytokine storm in the host, referred as the early phase of sepsis. This is then followed with a prolonged inhibited immune function phase. Either of these phases can prove to be fatal (86).

The exact mechanism behind sepsis progression is complex and still requires significant research but most are characterized by an initial microbial invasion. This activates the innate immune system. Bacterial virulence factors can activate different pattern recognition receptors (PRRs) – namely toll like receptors (TLRs) and NOD-like receptors – within the host immune cells and activate pro-inflammatory signals through various intracellular pathways (88). The response by the host towards lipopolysaccharide, released by gram negative bacteria, is another major factor in activating the immune system, initiating a subsequent pro-inflammatory response from the host (89, 90). Key cytokines produced as a result of these microbial factors involved in initiating and maintaining a cytokine storm are TNFα, IL-1, IL-6, IL-12 and IFNγ. These cytokines can further recruit more macrophages/monocytes, neutrophils, NK cells and cytotoxic...
T-cells, which can produce more cytokines, and thereby make the situation fatal for the patient (91).

Excess inflammatory signals can be damaging to the host, but an inhibited immune system can cause growth of bacteria which have evaded clearance. TGF-β, IL-10 and IL-4 are the main cytokines involved in this stage; each serving the purpose to contain the intense initial immune response (91). The excess inflammation also damages the endothelium and causes production of pro-coagulant factors in blood which can form blood clots in the microvasculature and subsequently lead to multiple organ failure, an end stage of sepsis leading to death (92).

Endothelial dysfunction is a hallmark of sepsis and combating endothelial dysfunction as a therapy for sepsis has gained exposure over the past decade (93). After numerous studies revealed that sepsis pathophysiology involved functional interactions between inflammatory signals and coagulation (93, 94), recombinant human Activated Protein C (rhAPC) became the first biological drug approved by the Food and Drug Administration (FDA) for sepsis treatment (95, 96). Although recently discontinued for sepsis treatment, APC may combat both the inflammatory and coagulant actions in sepsis (97, 98). Mechanistically, APC can convey its protective effects through upregulation of anti-apoptotic genes (99). Studies have shown that APC has barrier protective and cell proliferative properties in endothelial cells and is also protective towards circulating mononuclear cells (100, 101).

As an anti-coagulant, APC cleaves factors Va and VIIIa of the coagulation cascade and inactivates them (102). Consequently there is a decrease in thrombin production, ultimately leading to a decrease in thrombin induced fibrin deposition. By lowering thrombin production APC also reduces the inflammatory and coagulant properties that thrombin activates on
endothelial cells leading to disease progression. Thrombin interaction with Protease Activated Receptor-1 (PAR-1) on endothelial cells causes rapid endothelial barrier permeability, causing exposure of underlying smooth muscle cells and collagen leading to platelets enhancing platelet-aggregation. Thrombin-PAR-1 pathway also induces nitric oxide production – dilating blood vessels and reducing blood pressure – and inflammatory cytokine, as well as adhesion molecule expression from endothelial cells, each contributing to sepsis progression (102, 103).

1.7 Protease Activated Receptors (PAR)

Active cysteine and serine proteases, like GzK, in the blood usually exert their effects by acting upon cell surface receptors on endothelial cells, platelets and leukocytes, particularly the family of PARs (104). Elevated GzK levels in sepsis and the implications of PARs in diseases which harbour endothelial dysfunction, like sepsis (104, 105), dictate the need for a more thorough understanding of PAR functionality.

PARs are a family of seven-transmembrane G-protein coupled receptors that mediate the cellular effects of proteases. Four PARs (1-4) have been identified in humans and they are found in different combinations on different cell types. Although they are vastly expressed on platelets and endothelial cells, PARs can also exert their effects to a lower extent on a variety of other cell types including vascular smooth muscle cells, circulating monocytes, fibroblasts and neuronal cells (104, 106, 107).

Understanding PAR mediated physiology is complex and requires an understating of the unique PAR mechanisms. First, PAR activation is unusual: there is a two-step mechanism in
their activation. A protease cleaves the extracellular domain of the receptor; subsequently the exposed tethered domain binds to the receptor itself causing an intramolecular rearrangement of the receptor inducing a downstream pathway. This activation is irreversible, restricting their reactivation. Second, PARs could be coupled to different G-proteins that signal through multiple different intracellular signalling pathways. Third, different proteases can trigger different activation of PARs. For example: a protease which cleaves at Site 1 in Figure 2 leaves a large portion of the extracellular motif of the receptor and does not allow the receptor to bind to itself, these proteases are called disabling proteases. A protease cleaving at Site 2 allows for intramolecular binding of the receptor and is therefore labelled as an activating protease. Different activating proteases can also cleave at distinct sites and activate distinct signals within the cell, a property of PAR known as biased agonism. Fourth, cofactor receptors can aid in facilitating the activation of PARs and can cause a change in the downstream activation of the cell (104, 108).

![Figure 2: Canonical and non-canonical mechanisms in the cleavage of the PAR-1 receptor. Activating proteases cleave at a site closer to the N-terminus than where deactivating, disarming, proteases cleave. Cleavage site of the activating protease could activate distinct pathways within the cell as long as the cleavage site allows for intra-molecular binding of the receptor.](image-url)
The four PARs (PAR1–4) identified in humans belong to a rhodopsin-like GPCR subfamily. PAR1, the first member, was identified as a receptor for thrombin, a serine protease coagulation factor (109). PAR2 was next discovered accidentally while screening a mouse genomic library, it was identified to have an extracellular amino terminus containing a trypsin cleavage site (110). The discovery of PAR2 prompted a search for other PARs as there was suspicion that a thrombin response could be facilitated by other PARs after the finding that PAR-1 deficient mice still partially respond to a thrombin effect. Subsequently PAR3, another receptor for thrombin, and PAR4, a receptor for both thrombin and trypsin, were discovered (111, 112). PARs, originally thought to be receptors for thrombin and trypsin have now been shown to be cleaved by many different proteases. This has made it difficult to define PARs with their functionality, as different proteases can trigger the activation of different PARs with different physiological outcomes on different cell types (108). Previously GzA, GzB and GzK have all been shown to cleave PAR-1, GzA and GzB in neuronal cells and GzK in lung fibroblasts (108, 113, 114).

1.7.1 Protease Activated Receptor-1 (PAR-1)

Thrombin, a prominent plasma serine protease, plays an important role in coagulation and hemostasis. Even though PAR-1, PAR-3 and PAR-4 are all responsive towards thrombin, PAR-1 carries out most of the cellular actions of thrombin (115). As such, PAR-1 is an important receptor in the regulation of endothelial physiology. In blood, thrombin is readily produced at the site of vascular injury and different cells carry out different actions of thrombin. Thrombin-
mediated PAR-1 cleavage on platelets allows for platelet aggregation and clotting, PAR-1 cleavage on endothelial cells causes a pro-inflammatory response and disrupts the endothelial barrier and cleavage on vascular smooth muscle cell causes proliferation (104, 115).

However these properties of PAR-1 activation are specific to thrombin activation and the biased agonism of PAR-1 becomes important when considering other proteases that might be activating this receptor. As mentioned previously, cleavage at distinct sites can induce a differential response. Some known protease activities on PAR-1 functionality based on cleavage site are presented in Table 2. Of importance is APC, which we have encountered before as a protease with potentially beneficial effects in sepsis. APC cleaves PAR-1 at a site separate from that of thrombin (116). On endothelial cells, cleavage of PAR-1 with APC conveys anti-inflammatory and endothelial barrier protective properties (117). Another intriguing example is of the protease Factor Xa, which cleaves PAR-1 at the same site as thrombin but requires the Endothelial Protein C Receptor (EPCR) as a co-factor (118). The physiological response generated by this is quite distinct from that of thrombin or APC as shown in Table 2.

Activation of PAR-1 via different proteases could also render the receptor non-responsive to thrombin activation and can interfere with the physiological properties of thrombin. Cleavage of PAR-1 by deactivating peptides, like plasmin or Cathespin G, can render the receptor non-responsive to further cleavage by any protease (119). Similarly PAR-1 cleavage by an agonist can leave it inaccessible to further cleavage by other proteases. Furthermore, the desensitization of the receptor can also be carried out through the phosphorylation of the receptor after activation and subsequent internalization from the cell membrane (104, 108). This mechanism is currently under controversy as it was recently noticed that it is possible for different proteases to
create a different profile of PAR-1 receptors on the cell membrane. Distinct trafficking of PAR-1 was seen when both APC and thrombin are co-incubated with endothelial cells. PAR-1 cleaved by APC remained on the surface for longer times before being internalized and therefore are selectively activated for longer times when compared to thrombin activation of PAR-1 (120).

The above references are only a short summary towards the complexity associated with PAR-1 activation. This has made it very difficult for the implementation of a PAR-1 inhibitor in a disease model. In fact, the first inhibitor of PAR-1, SCH 530348, was approved only this year as an anti-coagulant for prevention of cardiovascular events in patients with peripheral arterial disease or history of myocardial infarction (121). In a disease like sepsis, however, where many proteases can act on PAR-1, there has been no implications of PAR-1 inhibitors. In fact, PAR-1 is shown to have contrasting roles in different stages of sepsis and likewise PAR-1 knockout models show no difference in disease severity after LPS injection (122, 123).
Table 2: PAR-1 activation by different proteases lead to different pathway activation

<table>
<thead>
<tr>
<th>Protease</th>
<th>Cleavage site</th>
<th>Signaling pathways</th>
<th>Physiological response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>38LDPR↓SFLL45</td>
<td>ERK1/2, p38 MAPK, JNK, NF-κB, Rho-GTP, Ca2+, Gα12/13-Rho</td>
<td>Platelet aggregation, endothelial barrier disruption, cytokine secretion, vascular smooth muscle cells proliferation</td>
<td>(124, 125)</td>
</tr>
<tr>
<td>Factor Xa</td>
<td>35LDPR↓SFLL45</td>
<td>Gαq/Ca2+, Gα12/13-Rho, β-arrestin/ ERK</td>
<td>Pro-inflammation, endothelial barrier protection, inhibition of cancer cell migration, fibroblast proliferation</td>
<td>(118, 126, 127)</td>
</tr>
<tr>
<td>Plasmin</td>
<td>36ATLD↓PRSF43</td>
<td>Gα12/13-Rho, MAPK</td>
<td>Platelet thrombogenesis and clot retraction, disruption of barrier function, inflammation matrix remodeling, vascular angiogenesis</td>
<td>(129, 130)</td>
</tr>
<tr>
<td>MMP1</td>
<td>39DPRS↓FLLR46</td>
<td>Gαq/Ca2+, ERK1/2</td>
<td>Participate in β-adrenergic receptor over activation-dependent cardiac dysfunction in cardiac cells</td>
<td>(129, 131)</td>
</tr>
<tr>
<td>MMP13</td>
<td>39DPRS↓FLLR46</td>
<td>Gαq/Ca2+, ERK1/2</td>
<td>Participate in β-adrenergic receptor over activation-dependent cardiac dysfunction in cardiac cells</td>
<td>(129, 131)</td>
</tr>
<tr>
<td>Elastase</td>
<td>42SFLL↓RNPN49</td>
<td>Gαi/MAPK</td>
<td>Stress fiber formation and endothelial barrier permeability</td>
<td>(132)</td>
</tr>
<tr>
<td>APC</td>
<td>43FLLR↓NPND50</td>
<td>β-arrestin/ Rac1, Akt</td>
<td>Cytoprotective, anti-inflammatory, endothelial barrier protection</td>
<td>(116, 117)</td>
</tr>
<tr>
<td>GzA</td>
<td>Unknown</td>
<td>Neutrite retraction and reversal in the stellation of astrocytes</td>
<td></td>
<td>(133)</td>
</tr>
<tr>
<td>GzK</td>
<td>Unknown</td>
<td>ERK1/2, p38 MAPK</td>
<td>Cytokine secretion and fibroblast proliferation</td>
<td>(72)</td>
</tr>
</tbody>
</table>
1.7.1.1 Predicted PAR-1 Cleavage Site by GzK

It is important to know the GzK-PAR-1 outcome on endothelial cells to determine if GzK can exacerbate endothelial dysfunction. Even though the PAR-1 extracellular domain is susceptible to cleavage by GzK, the site of cleavage is still unknown. Predicting the site of cleavage by GzK could provide some insight into possible signalling pathways that GzK might be activating and whether GzK interferes with other proteases acting upon the same receptor. Figure 3 identifies some possible hypothesized cleavage sites of the extracellular domain of PAR-1 by GzK.

As mentioned previously, GzK prefers Arg or Lys at the P1 position for cleavage. While GzB requires a tripeptide synthetic substrate for cleavage, there is no consensus motif around the P1 cleavage site in the peptides for GzK (134). In accordance with this, the P2-P4 substrate specificities appear broad for both GzK and GzA, which is consistent with known cleavage sites reported in B-tubulin by these enzymes (15). Although there is a lack of clear consensus around the P1 site of Arg or Lys, the substrate specificity of GzK and GzA are clear and distinct from each other. This suggests that exosite-exosite interaction between either of the two granzymes and their substrates might be important in determining cleavage sites. Both GzA and GzK are highly charged molecules on the surface; these positively charged loops could provide docking sites for the selection of substrates to the active site. In this context, the tertiary structure of the granzymes around the active site can play a role in determining substrate specificity and differences between GzA and GzK substrates can be accounted for, as GzK is a monomer but GzA is a dimer with an extended active side cleft (15, 16). However this does not give us enough
specific information to pinpoint the location of PAR-1 cleavage by GzK and the effects of GzK cleavage in endothelial cells will need to be determined experimentally.

Figure 3: Predicted cleavage sites of GzK on PAR-1 extracellular domain as represented by the black arrows. The red arrow is the thrombin cleavage site of PAR-1. Based off of reported biochemical properties of GzK by Bovenschen et al. (135) and Plasman et al. (136)
1.8 Rationale and Hypothesis

The five known granzymes in humans have all been identified to have different substrate specificities. Along with this, different granzymes are preferentially expressed in different subsets of immune cells implying different functionality. GzK, one of the tryptases in the granzyme family, is preferentially expressed in CD56\textsuperscript{bright} NK cells, γδ T-cells, expressed in a set differentiation phase of memory CD8\textsuperscript{+} T-cells, and CD3\textsuperscript{+}CD56\textsuperscript{+}NKT cells (137, 138). In disease states activation of these immune cells can leak granzymes into the extracellular milieu. GzK has been found to be up-regulated in the plasma of patients admitted for severe sepsis. Its most likely physiological inhibitor, IαIp, is also found down-regulated in sepsis. GzK has the capability to cleave PAR-1 in lung fibroblasts, a G-protein coupled receptor highly expressed in endothelial cells. Since PAR-1 activation can have detrimental/inflammatory effects on the endothelium, if GzK also cleaves PAR-1 on endothelial cells, it is possible that GzK can be furthering disease conditions like sepsis by inducing an inflammatory response from the endothelium. A summary of the potential pathways contributing to inflammation and dysfunction of the endothelium that GzK could activate is shown in the Figure 4.

I hypothesize that GzK cleavage of PAR-1 in the endothelium causes pro-inflammatory cytokine production and endothelial barrier disruption.

Specific aims:

1. To assess the role of extracellular GzK-mediated PAR-1 cleavage on endothelium-derived pro-inflammatory cytokine production.
2. To delineate the downstream pathways that mediate GzmB-induced endothelial inflammation.
Figure 4: Putative downstream pathways activated by GzK in endothelial cells by PAR-1.

Inflammatory mediators production, angiogenesis
Chapter 2: Methods and Materials

2.1 Cell Culture

Human Umbilical Vein Endothelial Cells (HUVEC) were (Lonza, Walkersville, MD) grown and maintained in EGM-2 (EBM-2 supplemented with human Epidermal Growth Factor (hEGF), Vascular Endothelial Growth Factor (VEGF), R3-Insulin-like Growth Factor-1 (R3-IGF-1), Ascorbic Acid, Hydrocortisone, human Fibroblast Growth Factor-Beta (hFGF-β), Heparin, Gentamicin/Amphotericin-B) supplemented with 5% (vol/vol) FBS and kept in a humidified atmosphere with 5% CO$_2$ at 37ºC. For all experiments, cells were serum-starved for 2h in EBM-2 with 0.1% FBS before treatments. During treatments, cells were maintained in EBM-2 with 1% FBS unless otherwise mentioned.

THP-1 monocyte cells (Lonza) were cultured in RPMI 1640 containing 10% FBS (vol/vol) FBS, 10mM HEPES, 2mM glutamine, 100U/ml penicillin and 100ug/ml streptomycin and maintained in a humidified atmosphere under 5% CO$_2$ at 37ºC.

2.2 5-Bromodeoxyuridine Incorporation Assay to Determine Cell Proliferation

We modified the BrdU method as mentioned by Suda et al. (100) to study cell proliferation. Briefly, HUVEC were seeded in 96-well plates at 80% confluence and incubated in EBM-2 with 2% FBS for 24 h. HUVEC were then treated with increasing doses of GzK (Enzo Life Sciences, Farmingdale, NY), and after 16 h, 5-Bromodeoxyuridine (BrdU; 10 ug/mL) was added to each well. Two hours later, the culture media were removed, and the BrdU
incorporation was estimated using a BrdU mouse antibody and subsequently an anti-mouse IgG HRP-linked antibody. Incorporation was measured according to manufacturer’s instructions (Cell Signalling, Beverly, MA) at an absorbance wavelength of 450nm.

2.3 Detection of IL-6 and MCP-1

IL-6 and MCP-1 levels were measured using IL-6 and MCP-1 ELISA kits according to manufacturer’s protocol (R&D Technologies, North Kingstown, RI). Supernatants of HUVEC treated with GzK (0.1-50 nM) without any delivery/cell permeabilizing agent were collected after 24 h and analysed via ELISA. For inhibitor assays: HUVEC were pretreated with the PAR-1 antibody (ATAP-2; 5μg/ml; Invitrogen, Grand Island, NY), p38 MAPK inhibitor (SB202190; 10μM; Sigma, St. Louis, MO) or ERK inhibitor (U0126; 10μM; Sigma) for 30 min prior to treatment with GzK (50 nM). HUVEC were treated with 0.1% DMSO as vehicle control to obtain baseline readings.

For inhibitor assays using IαIp (Athens Research & Technology, Athens, Georgia), GzK was pre-incubated with IαIp (1μg of IαIp and 1μg of GzK) for 30 min in 10μL of EBM-2 at 37°C. HUVEC were then treated with the above compounds for 4 hours and supernatants were collected and analysed via ELISA for IL-6 and MCP-1.

2.4 Western Blot Analysis of MAPK Proteins, IκBα and ICAM-1

After pre-treatment with the PAR-1 antibody (ATAP-2; 5μg/ml), p38 MAPK inhibitor (SB202190; 10μM) or ERK inhibitor (U0126, 10μM; or PD98059, 10 μM) for 30 min and subsequent treatment with GzK (50 nM), HUVEC were lysed in mammalian cell lysis buffer
containing protease inhibitor (Sigma) cocktail and phosphatase-inhibitor cocktail (Sigma), followed by cell-scraping. Cell debris was removed by centrifugation (12000xg for 15 min).

Samples were separated on 10% SDS gels, transferred to nitrocellulose membranes and probed for phosphor-p44/42 (9106s), phospho-p38 (9216), IκBα (9242S) or ICAM-1 (4915) (Cell Signalling) Antibody detection was achieved using the Li-COR Odyssey Infrared imaging system (Li-COR biosciences, Lincoln, NE). Membranes were stripped and re-probed with primary antibodies for total p44/42 (cat no: 9102), total p38 (cat no: 9212s) and GAPDH (cat no: 2118) and once again analyzed via Li-COR. Densitometry was used to quantify all bands and relative ratios are presented.

### 2.5 THP-1 Adhesion Assay on HUVEC

The THP-1 adhesion assay was carried out as described by Fountain et al. (139) and modified slightly. Briefly, HUVEC were seeded into 96-well plates and allowed to form confluent monolayers. Cells were then pretreated with p38 MAPK inhibitor or PAR-1 neutralizing antibody for 30 min prior to treatment with GzK (50 nM) for 8 h at 37°C. Separately, THP-1 (1x10⁶ cells/ml) were loaded with 5 μM Calcein-AM in RPMI-1640 with 10% FBS for 30 min. Following loading, THP-1 cells were re-suspended to 1x10⁶ cells/ml in PBS buffer and added to PBS washed HUVEC monolayers, allowing cells to adhere for 15 min at 37°C, 5% CO₂. Non-adhered cells were aspirated and adhered cells were washed twice with PBS before the fluorescence intensity (496 nm excitation; 516 nm emission) was recorded using a Tecan plate reader (Switzerland).
2.6 Measuring Endothelial Barrier Function through Electric Cell-substrate Impedance Sensing (ECIS)

ECIS allows determination of changes in the impedance to current flow across an electrode. Impedance was used as a measure of endothelial barrier disruption in real time. Barrier disruption assay with ECIS was carried out as mentioned by Fox et al. (140) and modified slightly. Briefly, wells of sterile 8W10E+ electrode arrays were reduced with 10 mM l-cysteine for 10 minutes. HUVEC were then plated and grown to confluence in EGM-2 in these wells.

All experiments allowed for 30 minutes of monolayer serum starvation in EBM-2, and were conducted in EBM-2 with 2% FBS. Impedance measurements were collected at 16,000 Hz on the ECIS machine (Applied Biophysics, Troy, NY) and normalized for presentation. Thrombin (Enzyme Research Laboratories, South Bend, IN) at 2 U/ml, a known molecule which compromises barrier integrity was initially used as a positive control, and wells with only EGM-2 served as a negative control. 50 nM of GzK was utilized in all experiments, unless otherwise indicated, and 5mg/ml of ATAP-2 was used as a PAR-1 neutralizing antibody. All experiments were carried out at 37°C with 5% carbon dioxide.

2.7 Cleavage Assay and SimpleBlue Coomassie G-250 Blue Stain of SDS-Gel

GzK (50nM) and Thrombin (2U/ml) were incubated in 50μL of EBM-2 media for 4 h at 37°C with 5% carbon dioxide. GzK (50nM) itself in 50μL and Thrombin (2U/ml) itself in 50μL were the two controls. The samples were ran and separated on a 10% SDS-gel. The gel was washed 3 times in deionized water for 5 minutes each and then stained in SimpleBlue stain (Life Technologies, Burlington, ON) for 1 hour. The gel was then washed once for 30 minutes before
being left for a wash overnight in deionized water. It was subsequently visualised and appropriate bands were identified.

2.8 Real Time PCR Analysis for Adhesion Molecule Expression

HUVEC treated with GzK were washed with PBS, and RNA was extracted using RNeasy kit (QIAGEN, Valencia, CA). After DNase I digestion, cDNA was generated from normalized RNA using Superscript II reverse transcriptase. Samples were assayed by quantitative real time PCR for levels using TaqMan Fast Mastermix (Applied Biosystems, Waltham, Massachusetts) and TaqMan primer-probes (Life Technologies) for ICAM-1, VCAM-1, E-selectin and housekeeping gene GAPDH on a ViiA 7 System (Applied Biosystems). All gene amplifications were performed in duplicates System (Applied Biosystems). All gene amplifications were performed in duplicates. Gene expression levels were evaluated using the ΔΔCT method as recommended by Livak et al (141). For each sample the gene of interest was normalized to that of GAPDH. This value was then expressed as a fold change over either the no treatment control or the TNFα only control.

2.9 Statistical Analysis

Quantified western blots are expressed as a mean +/- SEM for three separate experiments.

ELISA data is expressed as mean +/- SEM for three separate experiments each with three biological replicates. Statistical analysis was performed using One-Way ANOVA followed by Dunnets post hoc analysis for multiple group comparisons. Differences were considered significant at p<0.05. qPCR analysis is expressed as +/- SEM for three separate experiments each
with two biological replicates. Statistical analysis was performed using the Mann–Whitney U test. Differences were considered significant at p<0.05.

Impedance measurements on ECIS, an indicator of barrier integrity, are presented with errors bars representing one standard deviation. All ECIS experiments were carried out in triplicates and were carried out at two different time points.
Chapter 3: Results

3.1 Extracellular GzK is Not Cytotoxic to Endothelial Cells

The impact of GzK on HUVEC viability was evaluated using a BrdU incorporation assay. GzK was administered without any delivery agent to a HUVEC monolayer and no change was seen in cell proliferation or cell death up to 50 nM GzK (Figure 5). These results were verified using cell counts (data not shown).
Figure 5: Extracellular GzK is not cytotoxic to HUVEC. HUVEC were serum starved for 2 h and then incubated with growth media in the presence or absence of GzK (1nM, 10nM, 50nM). After 24 h the BrdU assay was conducted to quantify cell growth (n=6) and incorporation was measured in a plate reader at absorbance wavelength of 450nm.

3.2 GzK Induced IL-6 and MCP-1 Production in HUVEC via PAR-1

GzK administration resulted in a dose dependent increase in IL-6 and MCP-1 production in HUVEC (Figure 6 A-B). The production of MCP-1 and IL-6 was ablated when HUVEC were pre-treated with a PAR-1 neutralizing antibody, ATAP-2 (Figure 6 C-D). As such, GzK induction of cytokines from HUVEC is mediated though PAR-1 activation. No significant increase in TNFα production was observed (data not shown).
Figure 6: GzK induces the release of IL-6 and MCP-1 in a dose dependent manner from HUVEC via PAR1. HUVEC were exposed to GzK (0.1-50nM) for 24hrs. Supernatants from cell cultures were analyzed for (A) IL-6, (B) MCP-1 secretion by ELISA. HUVEC were pre-incubated with PAR-1 neutralizing antibody (ATAP-2; 5ug/ml) for 30min and then treated with 10nM GzK for 24 h. Supernatants were collected and screened for C) MCP-1 and D) IL-6 production via ELISA. * represent statistically differences compared to media controls (p<0.05), ** (p<0.01), *** (p<0.005)
3.3 Extracellular GzK Activates the p38 MAPK and ERK1/2 Pathway in HUVEC

To assess PAR-1 signalling, HUVEC were grown to confluence in 6-well plates, starved and treated with GzK (50 nM) and had their lysates analyzed via western blot. Upon 50 nM GzK treatment of HUVEC there was downstream phosphorylation of p38 MAPK and ERK1/2 (Figure 7 A-D). The phosphorylation was apparent after 5 min, and by the end of one hour the phosphorylation levels had returned to near baseline. NF-κB activation, as detected through IκBα degradation, was not observed via western blotting (Figure 7 E-F).
Figure 7. GzK induces the phosphorylation of the p38 and ERK1/2 pathway in HUVEC. HUVEC were incubated with GzK (50nM) for 0 – 50min. Lysates were collected and screened via western blots for A) ERK1/2 phosphorylation, C) p38 MAPK phosphorylation and E) IκBα degradation. Relative levels of phosphorylation over control are expressed as a ratio of B) p-ERK1/2 to total ERK1/2, D) ratio of p-p38 to total p38 and F) ratio of IκBα to GAPDH.
3.4 GzK Regulates Cytokine Production in HUVEC via the ERK1/2 and p38 MAPK Pathways

ERK1/2 phosphorylation was completely inhibited when cells were pre-treated with ATAP-2 (5 mg/ml) or the ERK1/2 inhibitor U0126 for 30 min (Figure 8 A). Phosphorylation of p38 was also reduced after pre-treatment with the p38 inhibitor, SB202190, and ATAP-2 (Figure 8 B). JNK phosphorylation and IκBα degradation were not observed (data not shown). Pre-treatment of cells with U0126 or SB202190 reduced levels of IL-6 and MCP-1 (Figure 8 C-D).

![Figure 8: Inhibitors of ERK1/2 and p38 MAPK abolish GzK induced cytokine production in HUVEC. HUVEC were pre-incubated with ERK1/2 inhibitor U0126 (10uM) or p38 inhibitor SB202190 (10uM) for 10 min, then treated with GzK (50nM) for 5 min. Lysates were collected and screened via western for A) ERK1/2 phosphorylation and B) p38 phosphorylation. The effects of the inhibitors on IL-6 and MCP-1 production are presented. HUVEC were pre-incubated with U0126 or SB202190 for 30min, treated with GzK (50nM) and then supernatants were collected for determination of C) IL-6 and D) MCP-1 production via ELISA. Data are expressed as % change over vehicle control (0.1% DMSO in EBM-2) of (mean +/- SEM from triplicates). * represent statistically differences compared to GzK treatment alone (p<0.05).
3.5 GzK Induces ICAM-1 Expression through the p38 MAPK Pathway

At the site of inflammation the leukocytes need to adhere to the endothelium to facilitate trans-endothelial migration and induce site-specific inflammation (142). GzK treatment of HUVEC resulted in an increase in ICAM-1 expression. This effect was abrogated via pre-treatment with ATAP-2 or SB202190 (Figure 9 A, B). mRNA levels for adhesion molecule genes (ICAM-1, VCAM-1 and E-selectin) in HUVEC were significantly up-regulated after 4 h of GzK treatment as compared to control (Figure 9 C). This effect was abolished when HUVEC were pre-treated with the PAR-1 neutralizing antibody or the p38 inhibitor (SB202190). There was no decrease in ICAM-1 expression in GzK activated HUVEC which were pre-treated with ERK1/2 inhibitor, U0126 or ERK1/2 inhibitor PD98059; nor did these inhibitors cause a decrease in expression of adhesion molecules at an mRNA level (data not shown).
Figure 9. GzK induces adhesion molecule expression in HUVEC via PAR-1 cleavage and subsequent MAPK p38 activation. HUVEC were incubated with GzK (50nM) for 8 h. Cell lysates were collected and analyzed via western blot for A) ICAM-1 expression (n=3). Relative level of B) ICAM-1 expression compared to GAPDH were measured via densitometry. HUVEC were pre-treated with PAR-1 neutralizing antibody (ATAP-2; 5ug/ml) or p38MAPK inhibitor (SB202190; 10uM) for 30 min and subsequently treated with GzK for 4 h. Cells were lysed and the mRNA expression of C) ICAM-1, VCAM-1 and E-selectin was measured via qPCR and expressed as fold change relative to GAPDH over expression in controls (n=6). * represent statistically differences (p<0.05).
3.6 The GzK-PAR1 Mediated Adhesion of THP-1 Monocytes to HUVEC via p38 MAPK Activation

A dose dependent increase in THP-1 cell adherence after 8hrs was seen in the GzK activated HUVEC monolayer (Figure 10A). The almost 2 fold increase in adherence was not present when the GzK activated HUVEC were pre-treated with the PAR-1 neutralizing antibody, ATAP-2, (Figure 10B), and the p38 MAPK inhibitor (Figure 10C). TNFα was used as a positive control as HUVEC activated with TNFα displayed an almost 5 fold increase in adherence (data not shown). HUVEC incubated with 0.1% DMSO in media was used as a negative control.
Figure 10: Undifferentiated THP-1 cells are more likely to adhere to GzK activated HUVEC via PAR-1 and subsequent p38 MAPK activation. HUVEC monolayers treated with increasing dose of GzK for 8 h were followed by a wash and incubation with THP-1 cells for 15 min and presented as A) % Adhered THP-1 cells. B) Fold change in THP-1 adherence when GzK activated HUVEC were pre-treated with PAR-1 neutralizing antibody, p38 inhibitor or 0.1% DMSO over control adherence. * represent statistically differences (p<0.05), ** (p<0.01), *** (p<0.005).
3.7 GzK Increased the Inflammatory Effect when Administered on HUVEC Primed with TNFα

The nature of the extracellular milieu often determines the activity of endothelial cells (143). It has been proposed that thrombin potentiates the inflammatory capability of TNFα treated endothelial cells, specifically adhesion molecule expression (144). Conversely, APC, down-regulates adhesion molecule expression following TNFα challenge in endothelial cells (145). The effect of GzK on HUVEC that were pre-treated with TNFα was therefore investigated. GzK exhibited similar effects to 2U/ml thrombin, amplifying the mRNA expression of adhesion molecules (Figure 11A). In addition, GzK administered in combination with TNFα displayed enhanced production of IL-6 and MCP-1 (Figure 11B-C). This increase in cytokine production does not seem to be an amplification of a TNFα response, rather an additive response of both TNFα and GzK. We also show that GzK administered upon TNFα activated HUVEC do not induce the activation of NF-κB as observed by IκBα degradation (Figure 11D).
Figure 11: GzK exacerbates a TNFα response in HUVEC. HUVEC monolayers were pre-treated with TNFα (10ng/ml) for 2 h and then incubated with GzK (50 nM) or Thrombin (2U/ml) or APC (50nM) for 4 h. Cell were then lysed and A) the mRNA expression of ICAM-1, VCAM-1 and E-selectin was measured via qPCR and expressed as fold change relative to GAPDH levels over expression in controls (n=6). Pre-treated HUVEC with TNFα (10ng/ml) for 2 h and then incubated with GzK (50nM) or Thrombin (2U/ml) or APC (50nM) for 24 h had their supernatant collected and screened for B) IL-6 and C) MCP-1 expression via ELISA (n=9) and significance determined as change relative to only TNFα treatment. NF-κB activation was studied in D) as a possible mechanism for GzK induced potentiation of the TNFα response. Lysates were collected at different time points before and after GzK administration and detected via western blot for IκBα degradation. * represent statistically differences (p<0.05), ** (p<0.01).
3.8 Inter-alpha Inhibitor Protein Efficiently Inhibits Extracellular GzK Activity

Pre-incubation of GzK with IαIp inhibited the extracellular GzK activity in vitro (Figure 12). GzK-induced adhesion molecule expression (ICAM-1, VCAM-1 and E-selectin) and MCP-1 and IL-6 production were diminished when IαIp was pre-incubated with GzK (Figure 12 A and Figure 12 B – C respectively).
Figure 12. IαIp efficiently inhibits GzK induced HUVEC activation. 50 nM GzK was pre-incubated with IαIp (1:1) for 30 min, or incubated alone in EBM-2. HUVEC were treated with the above and 0.1% DMSO control for 4 h. Cells were lysed and A) the mRNA expression of ICAM-1, VCAM-1 and E-selectin was measured via qPCR and expressed as fold change relative to GAPDH over expression in controls (n=6). HUVEC in the above condition were also left for 24 h and had their supernatant collected and screened for B) IL-6 and C) MCP-1 expression via ELISA (n=9). * represent statistically differences (p<0.05).

3.9 GzK Does Not Cause Endothelial Barrier Disruption

It has been established that other proteases besides thrombin can induce endothelial barrier permeability via the PAR-1 receptor (Table 2). We have shown that GzK can interact with the
PAR-1 receptor in endothelial cells. Therefore we used the Electric Cell-substrate Impedence Sensing (ECIS) to determine endothelial permeability. Increasing concentration of GzK had no effect on endothelial barrier functionality as measured by impedance at 16000Hz (Figure 13), whereas 2U/ml of thrombin caused barrier disruption within 10 minutes.

**Figure 13:** GzK does not cause endothelial barrier disruption. HUVEC grown to monolayer on an 8W10E+ plates on the ECIS were subjected to increasing concentrations of GzK (10, 50, 100nM) and barrier dysfunction was determined via impedance readings across the membrane at 16000Hz. Impedance readings on the graph are normalized to the time right before treatment. Thrombin (2U/ml) was used as a positive control. Data is represented as an average of triplicate data points and was also conducted 3 separate times. One standard deviation error bars included.

### 3.10 GzK Interferes with Thrombin Induced Permeability

The nature of PAR-1 allows it to become desensitized either by making the receptor inaccessible to cleavage by other proteases due to the location of the cleavage site. It can also be desensitized to proteases after the receptor is activated, phosphorylated and internalized (104, 108). These
findings raise the question as to whether GzK activity on endothelial cells desensitizes thrombin mediated PAR-1 response. Incubation of endothelial cells with GzK (50nM) for 30min and then washing it away did not alter the response of thrombin (2U/ml) mediated endothelial permeability (Figure 14.1). However, co-incubation of GzK with thrombin lowered permeability caused by thrombin in endothelial cells (Figure 14.2). PAR-1 neutralizing antibody, ATAP-2, also significantly lowered the effects of thrombin induced permeability. Heat-inactivated GzK does not convey the same effects as active GzK on thrombin induced barrier dysfunction as determined by impedance readings (Figure 14.3) The proteolytic effects of GzK towards thrombin itself was considered as a possible mechanism of action towards a decrease in thrombin activity and no proteolysis of thrombin was noticed when GzK (50 nM) with thrombin (2U/ml) for 1 hour (Figure 14.4). Figure 14.5 showed that continuous presence of thrombin (2U/ml) maintains endothelial permeability, whereas when thrombin is washed away then barrier recovers. This suggests that GzK could interfere with thrombin signalling via directly competing with PAR-1, thereby restricting a continual activity of thrombin.
Figure 14: GzK interferes with thrombin induced endothelial barrier dysfunction. HUVEC grown to monolayer on an 8W10E+ plates on the ECIS machine and barrier dysfunction was determined via impedance readings across the membrane at 16000Hz. Impedance readings on the graph are normalized to the time right before treatment. The readings in A) are representative of 30 min of GzK incubation before 3x wash with PBS and subsequent incubation with thrombin (2U/ml). Thrombin effect measured after 30 min incubation with GzK and no wash, along with the effect of pre-incubation of HUVEC with ATAP-2 (mg/ml) on thrombin effect are measured and presented in B). Incubation with heat-inactivated and active GzK for 30 min and subsequent effects of thrombin are presented in C). The catalytic activity of GzK with thrombin are presented in D) on an SDS-gel stained with Coomassie Blue. Impedance readings of washing thrombin away after incubation for 30 min on HUVEC monolayer is presented in E). All impedance data is represented as an average of triplicate data points. One standard deviation error bars are included.
Chapter 4: Discussion

These findings support a role for GzK in promoting inflammation. Of particular relevance to the present study are the reports of elevated levels of GzK in sepsis patients. As endothelial dysfunction is a hallmark of sepsis, GzK could be contributing to the promotion of inflammation through its effects on endothelial functionality (65, 67, 68, 146). Other studies suggest that mouse GzK is not cytotoxic but, rather, promotes inflammation through a process that involves IL-1β production (71). It has been recommended that similar studies need to be done with human GzK to determine its cytotoxicity, however the characterization of human and mouse GzK suggests functional equivalence between the two (147, 148). Furthermore, elevated levels of GzK are reported in circulation of patients with inflammatory conditions like viral pneumonia and sepsis. And extracellular GzK induces PAR1-dependent pro-inflammatory cytokine production in pulmonary fibroblasts, and also induces an immune response by binding and potentiating an LPS response from human monocytes. These findings support a role for GzK in promoting inflammation.

PAR-1 activation in endothelial cells has been implicated in various patho-physiological responses such as vascular injury, angiogenesis, inflammatory cytokine production and clotting cascade initiation, resulting in progression of cardiovascular and inflammatory diseases (104, 105, 149). However, PAR-1 response varies depending on the cell type in which it is expressed, the protease that cleaves it, and/or the co-receptor – Endothelial Protein C Receptor (EPCR) or other PARs – that might be necessary for activation. Various proteases have been shown to cleave PAR-1, including MMP1, MMP13, elastase, APC and Factor Xa, each with a distinct physiological effect (Table 2). Understanding the particular effect that GzK-mediated PAR-1
activation has on HUVEC will provide insight into the possible roles that GzK can play in endothelial inflammatory conditions.

Given the immense body of literature regarding the cytotoxic role of intracellular granzymes (147), research showing the role that extracellular granzymes can induce cell death via induction of anoikis (150), and that GzB activation of PAR-1 can cause neuronal cell death, (114) it was first important to consider whether extracellular GzK is cytotoxic to endothelial cells. We found that extracellular GzK is not cytotoxic to endothelial cells, and extracellular GzK indeed participated in endothelial cell mediated inflammatory production through PAR-1.

GzK-PAR1 interaction caused IL-6 and MCP-1 production from HUVEC. The productions of these cytokines were dependant on MAPK p38 and ERK1/2 phosphorylation and independent of NF-κB activation, as studied by IκBα degradation. Both MCP-1 and IL-6 are important for the recruitment of innate immune cells to the site of inflammation (142, 151). IL-6 has been shown to have significant effects in activation/differentiation of adaptive immune cells (151). IL-6 has also been implicated in disease severity by enhancing the LPS-mediated coagulation cascade initiated in chimpanzees and inducing expression of Tissue Factor (TF) in monocytes (152, 153). TF is the main initiator of blood coagulation and its increased expression on platelets, leukocytes, fibroblasts and endothelial cells is associated with thrombotic complications and mortality in conditions such as sepsis, disseminated intravascular coagulation and coronary artery disease (154, 155).

Although IL-6 has not been shown to induce definite sepsis like state, characteristic of cytokines like TNFα and IL-1, IL-6 does function to induce fever, and mediate an acute phase response (151, 156). These general systemic responses makes IL-6 a key cytokine in determining
disease severity. Elevated serum IL-6 levels correlate with disease severity indicators such as stress after surgery and trauma, rheumatoid arthritis, Crohn’s disease, multiple organ failure and septic shock, and general overall mortality (91). As such, the increase in production of IL-6 could contribute to the ongoing inflammatory response in diseases like sepsis, and could also be involved in the initiation of dysregulated coagulation, thereby aiding in the progression of disease which are co-related with increased plasma levels of GzK.

The MAPK p38 phosphorylation also triggered expression of adhesion molecules on the cell surface leading to an enhanced adhesion of THP-1 cells (undifferentiated monocytes) to HUVEC (Figure 9 and Figure 10). Adherence of leukocytes to the endothelium is a critical step in initiating and maintaining site specific inflammation as it facilitates transendothelial migration for leukocytes (157). There was a lack of signalling through ERK1/2 in the expression of adhesion molecules, unlike p38 involvement. The lack of involvement of ERK1/2 in adhesion molecule expression has also been shown in thrombin-mediated PAR-1 activation (158).

Extracellular GzK activity differs from thrombin most distinctly in inducing endothelial permeability. Thrombin, at higher than normal physiological levels plays a role in cell contraction inducing endothelial permeability through the PAR-1 receptor (159). This response was absent from the GzK-PAR1 interaction suggesting that an intracellular pathway profile different from thrombin is activated when GzK cleaves PAR-1. The lack of GzK mediated endothelial permeability suggests that GzK might not have any direct role in initiating the coagulation cascade. Endothelial permeability exposes the underlying tissue, composed of collagen and smooth muscle cells, to blood allowing for platelet binding and aggregation through glycoprotein receptors (160). Thrombin also induces the production of Tissue Factor (TF) on endothelial cells via PAR-1, creating yet another positive feedback loop for the extrinsic
coagulation pathway. This expression of TF in endothelial cells is regulated by p38 MAPK activation (158). Since GzK activated p38 MAPK, and coagulation is important in late stage disease models of sepsis, GzK induced TF expression in endothelial cells was studied. GzK administration on HUVEC did not show any upregulation of TF mRNA expression in HUVEC (data not shown). These findings suggest that GzK does not have any direct significant role in the initiation of the coagulation cascade through endothelial cells. Since the coagulation cascade involves a series of serine proteases present in circulation and GzM has been shown to have a role in cleavage of coagulation factors, vWF and FVII, thereby interfering with the coagulation cascade (46), studying the role that GzK could have as a protease in the coagulation cascade in future studies could be insightful.

GzK did not desensitize the PAR-1 receptor towards a thrombin mediated endothelial effect (Figure 14A). It is possible that this observation was a consequence of the cleavage site of GzK being closer to the N-terminus of PAR-1, thereby allowing further activation of PAR-1 via thrombin. However, when GzK was not washed away, co-incubated with thrombin, (Figure 14B) the effects of thrombin mediated permeability were reduced, although slightly but significantly. Since PAR-1 partly conveys the permeability effects of thrombin, and GzK does not have any proteolytic activity towards thrombin (Figure 14D), it is possible that GzK influences the thrombin effect by being directly involved with the PAR-1 receptor but without desensitizing the receptor to the thrombin effect. The slight decrease in permeability upon co-incubation of high concentration of GzK administration (50nM) could be due to the robust effects that small amounts of thrombin (10nM) can have in the activation of the Rho-GTP pathway leading to barrier dysfunction/endothelial permeability (161). Working with
concentrations of thrombin lower than 2U/ml, ~10nM, might show a clearer GzK co-incubation effect on endothelial permeability.

Washing thrombin away results in recovery of the endothelial barrier (Figure 14 E) suggesting that continuous thrombin activation of the PAR receptors – PAR-1 and PAR-3 are both involved in mediating thrombin induced endothelial permeability – might be necessary to maintain barrier permeability. Previously PAR-1 agonist proteases have been shown to activate the receptor by first docking on the receptor, then cleaving the extracellular domain (162). It is possible, based on our findings, that GzK binds to PAR-1 and competitively inhibits thrombin binding and therefore reduces endothelial permeability. Further studies with cross linking immuno-precipitation (CLIP) need to be done to give us insight into whether GzK binds and competes with thrombin for the PAR-1 active site, thereby verifying or rejecting our proposal. It is also possible, as suggested by Schuepbach et al. (163) with regards to APC-PAR-1 signalling, that GzK is creating a distinct set of PAR-1 receptors on endothelial cell surface which are retained on the cell surface for long times and do not respond to thrombin. However this mechanism is unlikely as GzK incubation for 30 min and subsequent washing does not display any reduced permeability after thrombin treatment (Figure 14 A), suggesting a lack of GzK specific PAR-1 receptors in the first 30 min of GzK incubation. Since PAR-1 is a major regulator of endothelial physiology and pathology, studying the effects that other proteases can have in mediating PAR-1 functionality, especially with regards to barrier functionality, is important.

Thrombin, the natural ligand for PAR-1, has the most pronounced effects and is the most efficient activator of this receptor. It was previously suggested that APC could be anti-inflammatory in endothelial cells due to low pro-inflammatory chemokine production in response to APC-mediated PAR-1 cleavage as compared to thrombin, which is ~10,000 times
more potent (164). It was noticed that GzK also does not display thrombin-like inflammatory potency upon GzK mediated PAR-1 activation. Although low levels of GzK (1nM) were enough to provoke inflammatory cytokine production; 50 nM GzK was required for ICAM-1, E-selectin and VCAM-1 gene expression. 50 nM GzK was also needed to induce a robust inflammatory response as noted by THP-1 cell adhesion, ICAM-1 expression and cytokine expression.

As discussed, APC and thrombin have opposing effects regarding inflammatory adhesion molecule gene expression in TNFα pre-treated cells. Specifically, APC down-regulates ICAM-1, VCAM-1 and E-selectin expression whereas thrombin up-regulates the expression (99, 165). The effects of APC and thrombin on TNFα treated cells were confirmed by our data (Figure 9C). GzK (50 nM) exacerbated the effects of TNFα similar to thrombin. TNFα does not increase the expression of PAR-1 on endothelial cells, but does increase the expression of PAR-2 and PAR-4 (166). Therefore the potentiation of the TNFα effect is likely not mediated by the GzK-PAR1 nexus but by some other extracellular receptor or through some intracellular pathway. The cytokine production response after GzK administration on TNFα pre-treated cells was additive of only TNFα treatment and only GzK treatment; whereas adhesion molecule expression appeared to be synergistic. Thrombin and TNFα have some overlapping and some separate intracellular activation profiles in endothelial cells (158). It is possible that GzK, in a similar way to thrombin, is working through two separate intracellular pathways, one that overlaps with the TNFα pathway, thereby potentiating the TNFα adhesion molecule expression response, and one which is separate from the TNFα response. It is interesting to note that neither of these pathways is dependent upon NF-κB activation, as IκBα degradation was noted after TNFα administration to cells, but not after GzK administration (Figure 11D). As TNFα is a common inflammatory
stimulus found in many diseases, including sepsis (167), elevated GzK may be acting to enhance TNF-mediated inflammation in such conditions.

As PAR-1 can elicit both a protective and deleterious response in disease states, the use of PAR-1 antagonists can be challenging for clinical applications (168). Furthermore PAR-1 deficient models do not affect survival in mouse models of endotoxemia, possibly because PAR-1 can switch between pro and anti-inflammatory roles during different stages of sepsis in mice (123). Although efforts are still underway for optimization of an ideal antagonist of PAR-1 these efforts largely focus on the coagulation pathway initiated by PAR-1 in platelets.

The early phase of sepsis consists of an intense immune reaction which feedbacks upon itself to initiate a cytokine storm setting up a vastly dysregulated immune system (91). As such, inhibition of proteases that facilitate inflammatory signals might be considered an effective therapeutic strategy in diseases like sepsis (91). The use of IαIp in treatment of sepsis has been considered previously in murine models. Neonatal sepsis models of mice mortality was reduced when the mice were treated with exogenous IαIp (169). Previously, lower levels of IαIp were observed in plasma of human patients with sepsis along with corresponding higher levels of unbound ~26 kDa GzK (47). Our data suggests that an elevation in GzK, as observed in the aforementioned study, would promote endothelial dysfunction and inflammation in septic patients. More recently, ulinastatin, the active chain of IαIp, has been shown to reduce sepsis mortality in humans (170). IαIp has been demonstrated to act against many proteases that might be released during an inflammatory response, including neutrophil elastase and cathepsin G (171–173). We provide evidence that among other proteases, IαIp, can also act to inhibit GzK-mediated pro-inflammatory activity.
The inflammatory potential of GzK could also provide insight into the mechanism behind the functionality of CD56\(^{\text{bright}}\) NK cells. GzK is significantly expressed in CD56\(^{\text{bright}}\) NK cells (137), and this specific subset of NK cells are implicated in inflammatory diseases such as vasculitis, chronic respiratory infections, bronchiectasis and deep skin ulcers (174). They are largely considered the inflammatory subset of NK cells (175, 176), although some unexpected cytotoxicity mediated by these cells have been implicated in Multiple Sclerosis (177). Furthermore, this subset of NK\(^{\text{bright}}\) cells are known to express lower levels of perforin, an indication that released granzymes could have larger extracellular roles. Combined with the present data demonstrating a role for extracellular GzK in endothelial dysfunction, this could provide new clues as to how CD56\(^{\text{bright}}\) NK cells contribute to inflammation.
Chapter 5: Conclusion and Future Directions

In summary, extracellular GzK can have pro-inflammatory effects in endothelial cells via PAR-1, and these effects are largely carried out by the p38 MAPK and ERK1/2 pathways. GzK can enhance TNF-mediated inflammatory responses in HUVEC. IαIp, the hypothesized physiological inhibitor of GzK, inhibits GzK activity in an in vitro setting. GzK-PAR-1 activation is distinct compared to that of PAR-1 activation which is observed by its natural ligand, thrombin. These findings are summarized in Figure 15 below.
Figure 15: Downstream outcomes of GzK mediated PAR-1 activation in HUVEC. GzK activation of PAR-1 leads to p38 and ERK1/2 phosphorylation. Downstream transcription factors activated lead to increased cytokine production, increased adhesion molecule gene expression, and adherence to THP-1 cells. GzK-PAR-1 activation lacks endothelial permeability phenotype in HUVEC. GzK also potentiates a TNFα response in HUVEC and extracellular GzK can be inhibited by IαIp. The dotted arrows represent hypothesized pathways, whereas the straight arrows represent results from this study.

Advancing from this study the pro-inflammatory effects of GzK need to be further scrutinized. It is essential to study the in vivo role of GzK. LPS administration has been tried on both GzA and GzB knockout mice models. The survival challenge with LPS in GzB knockout mice is conflicting and needs more research (64, 178). However, GzA deficiency has been shown to play a survival role in LPS challenge, as GzA deficient mice were more likely to survive the inflammatory response induced via LPS (64). GzK knockout mice can reveal whether
GzK also plays a role in LPS mediated pathology. The finding that GzK levels are elevated in sepsis also prompts us to consider the role that innate immune cells might have in the production of GzK. GzK has been shown to be expressed in certain subsets of NK cells and CD8$^+$ T-cells, but no study regarding their expression in neutrophils has been published. Neutrophils are the first line of defence of the innate immune system and are vastly present in circulation during sepsis (179). Even though initially thought to not express granzymes (180), GzA has been recently shown to be expressed in polymorphonuclear neutrophils (181).

In our research we have also delineated the pathway utilized by GzK to induce an inflammatory response from the endothelium. If GzK is indeed shown to be pro-inflammatory in a diseased in vivo model, then it might be useful to target one of these pathways as a therapeutic tool to combat GzK mediated inflammation. The use of IαIp to combat inflammation, although inhibitory against GzK, can also be inhibitory against other proteases in circulation like trypsin, elastase and other neutrophil proteases (77). And as discussed in Table 2, elastase has barrier protective effects via PAR-1. The role of GzK in activating PAR-2, or PAR-3 and PAR-4, was not addressed in this study. PAR-2 activation, via neutrophil serine proteinase 3, can lead to barrier protective properties, possibly as a mechanism to shut the endothelial barrier after the passage of infiltrating leukocytes to the site of infection (182). GzK role in activating other PARs can provide knowledge for understanding completely the activity of GzK in endothelial biology.
References


