Granzyme B in Vascular Remodeling and Pathological Angiogenesis

by

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Abstract

Granzyme B (GZMB) is a serine protease that is expressed by a variety of immune cells and is abundant in a large number of chronic inflammatory disorders. GZMB is highly expressed in cytotoxic lymphocytes where it serves as the main effector molecule of the granule exocytosis pathway by which cytotoxic immune cells mediate target cell death through intracellular delivery of GZMB, leading to activation of apoptotic signaling cascades. GZMB can also accumulate extracellularly during inflammation, where it can cleave a range of extracellular matrix (ECM) proteins that may disrupt cell-matrix interactions and modulate the bioavailability of matrix-bound growth factors. In this dissertation I have explored the intracellular and extracellular roles of GZMB in vascular remodeling in disease. By examining human atherosclerotic plaques, I discovered an imbalance between GZMB and its endogenous inhibitor, proteinase inhibitor 9 (PI-9). PI-9 expression by vascular smooth muscle cells (VSMC) in plaques was reduced with increased disease severity. Elevated levels of GZMB in advanced lesions were correlated with reduced PI-9 expression and increased VSMC apoptosis. These findings suggest that VSMC are more susceptible to GZMB-induced apoptosis in advanced lesions due to reduced PI-9 expression. While examining the extracellular activities of GZMB on vascular remodeling, I focused on the role of GZMB-mediated cleavage of fibronectin (FN), a known GZMB substrate. FN has a major role in regulating angiogenesis as it facilitates endothelial cell (EC) migration and capillary formation, as well as binding to angiogenic growth factors in the ECM including vascular endothelial growth factor (VEGF). VEGF is a potent vascular permeabilizing agent that is sequestered in the ECM by binding FN. GZMB-mediated FN cleavage resulted in reduced EC adhesion, migration and capillary tube formation. In addition, GZMB-mediated FN cleavage induced the release of VEGF from the ECM and promoted VEGF-dependent vascular leakage in
vivo. Thus, GZMB may contribute to the progression and/or persistence of chronic inflammation by dysregulating angiogenesis and promoting vascular permeability. Collectively, the results of this work suggest that both intracellular and extracellular GZMB activities contribute to vascular remodeling and pathological angiogenesis.
Preface

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List of Acronyms and Abbreviations

3D: 3- dimensional
α-SMA - α-smooth muscle cell actin
AAA: Abdominal aortic aneurysm
Ab: Antibody
ANOVA: Analysis of variance
AP-1: Activator protein-1
ApoE: Apolipoprotein E
BAL: Bronchoalveolar lavage
bFGF : Basic fibroblast growth factor
CAD: caspase-activated deoxyribonuclease
Caspase: Cysteinyl aspartate specific protease
COPD: Chronic obstructive pulmonary disease
CSVA: cross sectional vascular area
CTL: Cytotoxic T lymphocytes
Ctr: Control
DAB: 3,3′-Diaminobenzidine
DCMY: dilated cardiomyopathy
DNA: Deoxyribonucleic acid
EC: Endothelial cells
ECM: Extracellular matrix
EDA/EDB: extradomain A/B
FAK: Focal adhesion kinase
FN: Fibronectin
GZMA: Granzyme A
GZMB: Granzyme B
Gzmb: Mouse granzyme B
H&E: Hematoxylin and eosin stain
HIF: Hypoxia-inducible transcription factors
HMVEC: Human microvascular endothelial cells
HSP: Heat-shock proteins
HUVEC: Human umbilical vein endothelial cells
ICAD: Inhibitor of caspase-activated deoxyribonuclease
IFN-γ: Interferon-γ
Ig: Immunoglobulin
IL: Interleukin
KO: knock out
LAD: Left anterior descending
LDL: Low-density lipoproteins
LPS: Lipopolysaccharide
MAPK: Mitogen activated protein kinase
MMPs: Matrix metalloproteases
MT1-MMP: Membrane type 1 metalloprotease
NF-κB: Nuclear factor-kappa B
NK: Natural killer
ox-LDL: Oxidized low-density lipoproteins
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate buffered saline
PI-9: Proteinase inhibitor 9/SERPINC9
PlGF: Placental growth factor
RA: Rheumatoid arthritis
RCL: Reactive centre loop
RGD: Arginine-Glycine-Aspartate
SD: Standard deviation
SDS: Sodium dodecyl sulfate
SEM: Standard error of the mean
SMC: Smooth muscle cells
TCR: T cell receptor
TGF: Transforming growth factor
TIMPs: Tissue inhibitors of metalloproteases
TNF-α: Tumor necrosis factor-α
UV: Ultraviolet
VCAM1: Vascular cell adhesion molecule-1
VE-cadherin: Vascular endothelial-cadherin
VEGF: Vascular endothelial growth factor
VEGFR2: Vascular endothelial growth factor receptor 2
VPF: Vascular permeability factor
VSMC: Vascular smooth muscle cells
VVO: Vesiculo–vacuolar organelle
vWF: Von Willebrand Factor
WT: Wild type
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1. Introduction

1.1 Granzymes

Granzymes (Granule secreted enzymes) are a family of serine proteases that are contained within cytosolic granules of both innate and adaptive immune cells [1]. Granzymes were first characterized in cytotoxic T lymphocytes (CTL) and natural killer (NK) cells and are considered the primary effector molecules of the granule exocytosis pathway by which cytotoxic immune cells mediate targeted cell death of virally infected, allogeneic and/or transformed cells [2]. There are five human granzymes with a range of substrate specificities: Granzyme A (GZMA-tryptase), Granzyme B (GZMB-aspase), Granzyme H (GZMH-chymase), Granzyme K (GZMK-tryptase) and Granzyme M (GZMM-metase). In mice this family expands to a total of 11 enzymes which in addition to granzymes A, B, and K also includes granzymes C, D, E, F, G, L and N. The purpose for the multiple gene duplication in mice is unknown, however their expression in CTLs suggests a role in host protection against a wider variety of pathogens in comparison to humans [3]. GZMB is the most abundant and the most studied granzyme whose role in immune-mediated programmed cell death (apoptosis) is well established [4, 5]. GZMA has also been perceived as a major inducer of apoptosis. However, recent findings have challenged this view and suggest that GZMA may instead be primarily involved in immune regulation through the induction of pro-inflammatory cytokine release [5, 6]. Mechanisms leading to cell death by other granzymes (H, K and M, often referred to as orphan granzymes) are ill defined. Nevertheless, the elevated expression of granzymes in a large number of chronic inflammatory disorders have motivated researchers to explore other functions for this family of enzymes, including extracellular activities that may modulate the immune response and contribute to the development and/or progression of chronic diseases.
1.2 Granzyme B

Studies into the mechanisms by which lymphocytes acquire their cytotoxic capabilities have led to the isolation and discovery of GZMB as a potent serine protease that can induce targeted cell death upon release from activated CTLs [7-9]. Also referred to as cytotoxic T lymphocyte-associated serine esterase 1 or granzyme 2, GZMB is a 32-kDa serine protease resembling chymotrypsin with a preference for cleaving after aspartic acid at the P1 position [10, 11]. GZMB is highly basic which renders this enzyme cationic and facilitates its interactions with anionic molecules such as glycosaminoglycan-rich proteoglycans and anionic glycoproteins that are abundant in the extracellular matrix (ECM) [12, 13].

GZMB expression in CTLs and NK cells is mainly upregulated due to activation of antigen receptors and in response to pro-inflammatory cytokines, namely interleukin-2 (IL-2) [14]. Post-translation, the GZMB zymogen is trafficked to cytoplasmic granules where it undergoes proteolytic activation by cathepsin C (dipeptidyl peptidase I) that removes dipeptide (Gly-Gly) residues from the N-terminus [15]. In lymphocytes, GZMB is co-expressed with perforin, a pore forming molecule that facilitates GZMB entry into the target cell after granule exocytosis. Both perforin and GZMB are stored within the cytosolic granules and remain inactive due to their binding to serglycin proteoglycan and the low pH environment within the granule [16]. Upon activation, the lytic granules are polarized towards the targeted cell by the directional organization of the microtubule cytoskeleton [17]. Once delivered to the site of secretion, lytic granules fuse with the plasma membrane and release their contents. The neutral pH outside the granule activates GZMB allowing it to exert its proteolytic activity [16].
1.3 Proteinase inhibitor 9

Proteinase inhibitor 9 (PI-9, SERPINB9) is the only known endogenous inhibitor of human GZMB. PI-9 is a member of the ovalbumin serine proteinase inhibitors (serpins) family, which act as pseudo-substrates to induce an irreversible conformational change of their target proteins [18]. The target specificity of serpin proteinase inhibitors is determined by the reactive centre loop (RCL), which is a sequence of amino acids that project out from the serpin molecule and specifically interact with the protease active site [19]. Once the active site of GZMB binds the RCL of PI-9 and attempts to cleave it, PI-9 undergoes a dramatic conformational change that completely deforms the GZMB active site, resulting in a covalently bound PI-9/GZMB complex and rendering GZMB inactive [20]. PI-9 is only expressed intracellularly as it lacks the N-terminal secretory signal peptide. It is also suggested to be non-functional extracellularly due to the oxidative environment outside the cell [21]. Thus, PI-9 exerts its inhibitory function only on intracellular GZMB and not on extracellular GZMB.

PI-9 is widely expressed in the body, particularly in GZMB expressing immune cells such as CTL and NK cells, where it is thought to protect against self-induced cell death during activation [22]. In this case, GZMB leakage from cytosolic granules and/or accidental uptake by the effector cell itself will be counteracted by PI-9, allowing the survival of the effector immune cell [22]. However, PI-9 is also widely expressed in non-immune cells such as endothelial cells (EC), mesothelial cells [23] and smooth muscle cells (SMC) [24], and is also expressed in immune-privileged sites such as the placenta, testis, ovary and the eye [25]. These observations have led to the hypothesis that constant PI-9 expression serves to protect bystander cells from accidental GZMB-induced apoptosis during inflammation [23]. Thus, it can be argued that any change in PI-9 cellular levels may alter the susceptibility of the cell to GZMB-induced cell death.
Indeed, alteration in PI-9 levels has been suggested to play a role in a number of pathologies. Several studies have observed an increase in PI-9 expression in a number of tumors that was correlated with increased resistance to CTL-mediated GZMB-induced apoptosis, suggesting that elevated PI-9 expression in tumors may serve as means to evade immune-mediated killing through inhibition of GZMB [26-28]. Clinically, higher PI-9 expression is correlated with unfavorable prognosis in patients with melanoma and anaplastic large cell lymphoma [29, 30], further supporting the anti-apoptotic role of PI-9 in tumors. Reduced PI-9 expression by tubular cells is associated with increased apoptosis in the presence of GZMB-positive T cells in biopsies from patients with acute renal graft rejection [29]. Thus, an imbalance between GMZB and its endogenous inhibitor PI-9 may underlie a key mechanism that contributes to disease progression.

PI-9 is regulated by the pro-inflammatory transcription factors nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) [31]. A range of pro-inflammatory cytokines leads to PI-9 upregulation including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) [31, 32]. These observations further support the hypothesis that PI-9 expression is upregulated in situations where cells are more readily exposed to GZMB, such as during an immune response where bystander cells require protection from GZMB insult. Interestingly, estrogen increases PI-9 expression [33], raising the possibility that pre-menopausal women may have increased cytoprotection in diseases where intracellular GZMB activity is likely to play a pathological role such as in atheromatous cardiovascular diseases [34].

1.4 Granzyme B-induced apoptosis

It is now accepted that GZMB is the main effector molecule of the granule exocytosis pathway by which cytotoxic immune cells mediate targeted cell apoptosis [4]. This process is unique to mammalian vertebrate biology where GZMB and perforin-containing granules are
secreted by cytotoxic cells into the immunological synapse [35]. Although a few models have been proposed for the mechanism by which perforin mediates GZMB entry into the target cell cytoplasm, the leading model is one by which GZMB and perforin are endocytosed by the target cell into an endosomal vesicle. Perforin then forms a pore in the endosome membrane allowing the escape of GZMB into the target cell cytoplasm where it cleaves a number of intracellular substrates leading to activation of apoptotic pathways [16] (Figure 1).

Importantly, perforin is required for intracellular delivery of GZMB and the induction of apoptosis as perforin deficiency impairs lymphocyte-mediated cytotoxicity. Moreover, perforin-deficient mice are highly susceptible to viral infection and cancer, further highlighting the role of perforin in mediating targeted cell apoptosis through GZMB delivery [16].

GZMB initiates apoptosis by activating effector caspases either directly or by activating the mitochondrial apoptotic pathway. Once in the cytoplasm, GZMB cleaves and activates a number of key effector caspases, namely caspase 3 [36]. Caspase 3 activates caspase-activated DNase (CAD) by cleaving its inhibitory unit ICAD (inhibitor of CAD), leading to DNA fragmentation and cell apoptosis. GZMB also mediates cytochrome c release from the mitochondria either through direct disruption of mitochondrial transmembrane potential or through the processing of Bid to its truncated form (tBid), which disrupts mitochondrial membrane integrity [37]. Cytochrome c, in concert with caspase 9, facilitates the formation of the apoptosome leading to caspase 3 activation and DNA fragmentation [38] (Figure 1).

Since GZMB induces apoptosis through multiple pathways it is difficult to escape cell death once sufficient GZMB has been delivered into cells. As PI-9 is a potent intracellular inhibitor of GZMB that is able to completely deactivate GZMB on a 1:1 molar ratio, it is
suggested that internal levels of PI-9 regulate cell susceptibility to GZMB-induced apoptosis [21]. Thus, even small amounts of GZMB internalization may be detrimental in cells that express low levels of PI-9.

1.5 Extracellular granzyme B

Although first described as both an intracellular and extracellular protease [39, 40], much of the earlier work on GZMB focused on its intracellular delivery by perforin and the induction of targeted cell apoptosis. Later discoveries revealed that other immune cells including neutrophils, dendritic cells, macrophages, basophiles and mast cells are also capable of expressing and releasing GZMB in the absence of perforin [41]. These findings highlight additional extracellular roles for GZMB during inflammation that are independent of targeted cell recognition and/or entry. Further, under pro-inflammatory conditions GZMB is expressed by non-immune cells that are incapable of forming an immunological synapse including chondrocytes, keratinocytes, type II pneumocytes and syncytial trophoblasts [42-45]. In addition, GZMB accumulates in the ECM of inflamed tissues and is present in several bodily fluids in chronic diseases such atherosclerosis, chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis (RA) [46, 47]. Collectively, these finding indicate the potential extracellular role of GZMB in inflammation, and thus serve as the driving force of current research aiming to explore additional roles of extracellular GZMB in disease.

Mechanisms leading to GZMB release into the extracellular environment are still ill defined but may involve a number of pathways. It was first suggested that granzymes may escape the immunological synapse during CTL degranulation [48]. Indeed, CTLs can constitutively release granzymes after T cell receptor (TCR) activation and prolonged IL-2 exposure, where it was reported that up to a third of GZMB is non-specifically secreted by T
cells following activation [49]. This work was expanded on by Prakash et al, who showed that both active and zymogen forms of GZMB can be constitutively released from stimulated NK cells and CTLs in the absence of target cell engagement, with NK cells primarily releasing the active form via the typical granule pathway and CTLs secreting the inactive zymogen in a granule-independent manner [50]. Furthermore, reports have also documented extracellular granzyme release by lymphocytes in response to cytokine/chemokine stimuli [51], while additional evidence suggests integrin interactions with ECM proteins such as fibronectin and vitronectin may contribute to T cell activation and extracellular granzyme release [52-55].

Several in vitro studies have identified a number of ECM substrates that are cleaved by GZMB, suggesting a number of potential roles for extracellular GZMB activity in disease. These substrates include key proteoglycans and glycoproteins that are essential for maintaining ECM integrity and regulating cell-matrix interactions. GZMB cleavage of aggrecan, a cartilage proteoglycan degraded in RA, has led to several studies investigating the pathological role of GZMB in this disease [42, 56-59]. GZMB proteolysis of fibronectin (FN), vitronectin, laminin and fibrillin-1 results in EC and SMC detachment and death by anoikis as well as reduced cell migration [60, 61]. Purified GZMB from ultraviolet A (UVA) exposed keratinocytes also inhibited migration of keratinocytes cultured on a FN matrix, suggesting a role for GZMB in attenuating skin epithelization [43]. Recently, Boivin et al, demonstrated that GZMB cleavage of decorin, biglycan and betaglycan leads to release of TGF-β1 from the matrix, suggesting that GZMB may indirectly effect normal cell function by altering growth factor bioavailability [62]. In addition to cleaving ECM structural components, GZMB cleaves other extracellular proteins that may influence cell activity including cytokines [63, 64], cell surface receptors [65-67] and proteins involved in hemostasis and angiogenesis such as plasminogen, plasmin, fibrinogen and
von Willebrand Factor (VWF) [68, 69]. Thus, increased expression and release of GZMB by cytotoxic and non-cytotoxic cells during inflammation may lead to considerable alteration in both cellular and extracellular tissue composition with a number of potential consequences that may underlie the pathogenesis of a number of chronic inflammatory diseases (Figure 1).

It is important to note that although GZMB may be inhibited intracellularly by PI-9, to date there is no evidence for the presence of an extracellular inhibitor for GZMB. Indeed, GZMB retains up to 70% of its activity in human plasma [70], suggesting that GZMB is lacking extracellular regulation in contrast to other extracellular protease such as matrix metalloproteases (MMPs) that are tightly regulated by the tissue inhibitors of metalloproteases (TIMPs).
Figure 1. Intracellular and extracellular GZMB activities. During inflammation GZMB is expressed by cytotoxic and non-cytotoxic cells. GZMB is released to the extracellular environment where it can either enter target cell cytoplasm through the activity of the pore forming molecule, perforin, or GZMB can accumulate outside the cell. Intracellularly, GZMB activates the apoptotic pathway by direct activation of effector caspases and via the processing of Bid and release of cytochrome C (Cyt C) from the mitochondria. PI-9 is a potent intracellular inhibitor of GZMB and thus it can rescue the cell for GZMB-induced apoptosis. Extracellularly, GZMB modulates cell activity either indirectly by cleaving ECM proteins leading to cell detachment and impaired cell adhesion and migration as well as releasing matrix bound growth factors. Extracellular GZMB may also have a direct affect on cell function by cleaving cell surface receptors. Finally GZMB may also modulate systemic processes as it cleaves hemostasis regulatory proteins and cytokines.

1.6 Granzyme B in chronic inflammation

Accumulating evidence from a number of clinical studies has indicated that GZMB levels in several bodily fluids are elevated during chronic inflammation [71]. These observations have
led to numerous studies implicating a role for either intracellular and/or extracellular GZMB in disease pathogenesis.

In COPD, a disease that is characterized by inflammation, alveolar epithelial cell apoptosis, and proteolytic ECM degradation [72, 73], increased GZMB and perforin expressing CD8+ and CD3+ cells are evident in the bronchoalveolar lavage (BAL) and blood [74]. Presence of GZMB positive T cells are correlated with bronchial epithelial cell apoptosis in bronchial and alveolar wall of COPD patients [74] suggesting that intracellular GZMB-induced apoptosis may contribute to epithelial cell damage in this disease. Interestingly, GZMB was also identified in alveolar macrophages and in type II pneumocytes of control and COPD patients in this study, indicating that these non-cytotoxic cells may contribute to extracellular GZMB accumulation which may lead to alveolar wall degradation.

In patients with RA, soluble extracellular GZMB is increased in the plasma and synovial fluid [59]. GZMB is expressed by invading immune cells including CD4+, CD8+, NK cells, macrophages as well as by resident chondrocytes [47, 58, 75, 76]. GZMB expressing cells are localized to the invasive front of the pannus, an area prone to cartilage destruction, suggesting there is an abundant source of GZMB in this area of proteolysis [57]. Extracellularly, GZMB can degrade aggrecan, a key proteoglycan and a major constituent of cartilage, thereby further compromising cartilage integrity [56, 57]. Additional cellular sources of GZMB native to the cartilage are chondrocytes, which can express GZMB and perforin, suggesting that GZMB expression by these cells may not just be involved in matrix degradation, but also play a role in cytotoxicity [42].
Further evidence for the role of GZMB in chronic inflammatory diseases is derived from *in vivo* studies using GZMB deficient mice. In a mouse model of skin aging, GZMB deficiency contributes to reduced skin thinning and improved collagen organization, possibly through a reduction in decorin cleavage [77]. This observation is in line with several studies implicating a role for GZMB in a variety of inflammatory skin disorders (reviewed in [71]). GZMB deficiency as well as GZMB inhibition is also protective against the development of abdominal aortic aneurysm (AAA) [78, 79]. GZMB cleavage of fibrilin-1 and decorin is suggested to contribute to elastic lamella instability and collagen disorganization, respectively, in aneurysm development.

In a mouse model of transplant vasculopathy, GZMB deficiency resulted in reduced intimal thickening and decreased endothelial cell apoptosis, suggesting that GZMB-induced apoptosis plays a role in disease pathogenesis [80, 81]. Further evidence for a role of GZMB in atheromatous diseases, in particular atherosclerosis, is discussed in detail below.

It is important to note that while perforin-deficient mice are compromised in their ability to clear virus infected cells and tumor cells, GZMB-deficient mice develop normally, do not develop spontaneous tumors and are expected to withstand viral infection [2]. GZMB-deficient CTLs retain their cytotoxic activity by employing additional pro-apoptotic signals, mainly through the activation of the Fas receptor on the target cell by the Fas ligand on the CTL membrane. Fas activation leads to subsequent processing of effector caspases, including caspase 3, resulting in target cell apoptosis [16]. In addition, several groups have suggested that other granzymes (GZMA, GZMM, GZMK, GZMH) can promote perforin-dependent cell death, providing redundant mechanisms for inducing apoptosis that are independent of GZMB expression [82-85]. However, recent studies have questioned the pro-apoptotic roles of these granzymes [5]. GZMB-deficient humans have not yet been described.
Apart from the direct effect of GZMB in altering tissue integrity, either through intracellular or extracellular activities, GZMB may also have an indirect role in modulating chronic inflammation. GZMB converts the pro form of IL-18 to its active form, leading to increased IFN-γ release from human keratinocytes [63]. A recent study by Afonina et al. identified IL-1α as a substrate for GZMB, where cleavage by GZMB greatly increases the biological activity of this cytokine both \textit{in vitro} and \textit{in vivo} [64]. As both these cytokines are potent pro-inflammatory mediators it is suggested that GZMB may indirectly promote inflammation through enhancing cytokine activity. Additional indirect roles for GZMB in inflammation may include alteration of angiogenesis and vascular permeability through cleavage of FN and altering vascular endothelial growth factor (VEGF) bioavailability. Evidence for the later is presented in chapter 5 and 6 of this dissertation.

\textit{1.7 Atherosclerosis}

Atherosclerosis is the underlying condition responsible for the vast majority of heart attacks and strokes [86]. The development of an atherosclerotic plaque is initiated by injury and activation of the endothelium followed by accumulation and retention of lipids and low-density lipoproteins (LDL) that are modified and oxidized (ox-LDL) in the subendothelial layer of the artery known as the intima [87]. Lesion progression is characterized by infiltration of macrophages that are transformed into foam cells due to increased accumulation and reduced clearance of modified LDL particles in the cytoplasm [88]. In addition, a large array of pro-inflammatory cytokines are released from macrophages, EC and vascular smooth muscle cells (VSMC) leading to further recruitment of macrophages, lymphocytes and mast cells into the artery. A major contributor to intimal thickening and plaque progression is the migration of VSMC from the media to the intima and their transformation from a contractile to a synthetic
phenotype, leading to increased ECM production by these cells [89]. Both the accumulation of macrophage foam cells and synthetic VSMC in the intima promote plaque growth which may compromise lumen diameter leading to disrupted blood flow within the affected artery [90].

Observations of high immune cell infiltration in atherosclerotic lesions have refocused the research in this field, suggesting that a lipid/cholesterol imbalance may serve as key initiating factors, however it is the inflammatory response that mediates and determines the outcome of this disease [91]. Activated EC increase the expression of vascular cell adhesion molecule-1 (VCAM1) and endothelial-selectins which facilitates monocyte adhesion and recruitment into the intima. Increased expression of chemokines by differentiated macrophages in the intima mediates T cell infiltration that can be activated by responding to antigen presentation of ox-LDL and heat-shock proteins (HSP) [92]. Further immune activation is achieved by the release of pro-inflammatory cytokines that are abundant in the plaque including TNF-α, IL-1β and IFN-γ, leading to increased lymphocyte accumulation particularly within the shoulder region of the plaque [91]. Expression of matrix degrading enzymes such as MMPs by macrophages as well as induction of cell apoptosis by CTLs and NK cells greatly contributes to the development of a necrotic core and thinning of the fibrous cap, further leading to plaque destabilization [93]. An unstable plaque may progress to a point of abrupt rupture of the intimal surface, exposing the pro-thrombotic material of the plaque leading to the formation of an occlusive thrombus that can be fatal if occurring in major blood vessels that perfuse the heart or the brain. Thus, the development of a chronic inflammatory response in atherosclerosis plays a major role in plaque progression and destabilization [91].
1.7.1 Apoptosis and atherosclerotic plaque stability

A large plaque that compromises lumen diameter may result in reduced organ perfusion with detectable clinical manifestation. However, the development of non-stenotic vulnerable plaques, that may progress unnoticed, may result in the abrupt rupture of an unstable plaque and the formation of an occlusive thrombus leading to a heart attack or stroke [94]. Indeed, most vulnerable plaques may not affect lumen diameter as angiographic studies on patients with acute coronary syndromes indicate that more than two-thirds of disrupted plaques are less than 50% occlusive [95]. Plaques may grow outwards in a process referred to as positive remodelling where the plaque grows towards the media layer which may prevent substantial luminal narrowing [95]. Thus, plaque size alone may not be used as a sole indicator of disease severity. For that reason current research is focused on understanding factors that contribute to plaque instability. Apart from plaque size the major determinants of unstable plaques include a thin and inflamed fibrous cap and a large necrotic core that occupies over a quarter of the plaque area [95, 96]. Pathological mechanisms leading to fibrous cap thinning and necrotic core expansion are suggested to increase plaque vulnerability.

Observations of increased vascular cell apoptosis in concert with inflammation in advanced atherosclerotic plaques have indicated that immune-induced apoptosis may serve as a major mechanism leading to reduced plaque cellularity and decreased plaque stability [97-99]. Apoptosis of EC is suggested to contribute to plaque erosion and exposure of the pro-thrombotic content of the plaque [99, 100]. Macrophage apoptosis may be induced by immune-derived cytokines such as TNF-α and IFN-γ, as well as by increased oxidative DNA damage due to ox-LDL uptake [98]. Macrophage death may lead to reduced scavenging capacity and an inability to remove apoptotic bodies of vascular and immune cells within the plaque leading to secondary
necrosis and the release of cellular contents that can further augment the inflammatory response [98].

VSMC apoptosis is also evident in advanced lesions. VSMC are abundant in plaques and contribute to ECM production in the formation of the fibrous cap. Moreover, VSMC are responsible for the production of interstitial collagen fibers that promote fibrous cap integrity and stability [101]. Thus, in advanced lesions, VSMC loss not only contributes to reduced plaque cellularity but also leads to reduced collagen synthesis and fibrous cap thinning. Using the transgenic human diphtheria toxin receptor/ApoE<sup>−/−</sup> mouse model, Clark et al. demonstrated that both acute and chronic induction of VSMC apoptosis resulted in reduced fibrous cap size, increased necrotic core and plaque calcification [102, 103], all of which are common features of rupture-prone plaques in humans [104-106]. However, a direct cause and effect relationship between apoptosis and plaque rupture is currently lacking. This is due to the controversial argument that atherosclerotic plaques in mice do not progress to the stage of rupture such as seen in humans [107, 108]. Nevertheless, evidence of increased apoptosis in all major cell types that constitute the plaque in association with inflammatory infiltrate suggest that immune-induced apoptosis plays a major role in altering plaque composition and stability.

1.7.2 The role of granzyme B in atherosclerosis

Evidence to date suggests that elevated local and systemic GZMB expression in atherosclerosis corresponds to increased disease severity and plaque instability. Previous work from our laboratory has demonstrated that while GZMB is not present in healthy, non-atherosclerotic arteries, increased GZMB expression is evident in macrophage foam cells, CTLs,
VSMC and detected extracellularly in advanced lesions [109]. Several clinical studies support a correlation between elevated plasma GZMB levels and atherosclerotic lesion severity. GZMB plasma levels are significantly higher in patients with unstable carotid plaques compared to patients with stable lesions and corresponds to increased incidences of cerebrovascular events [110]. Peripheral blood mononuclear cells (PBMC) isolated from patients with unstable angina produce and release higher levels of GZMB than PBMC isolated from patients with stable angina [111]. Elevated GZMB plasma levels are also detected in patients with acute coronary syndrome [46]. In the latter study, increased GZMB levels were detected following the acute phase, indicating that GZMB may also be involved in ventricular remodelling after ischemic injury [46]. Taken together, GZMB appears to be an important contributor to atheromatous disease pathogenesis [34, 109].

GZMB may promote plaque instability through the induction of apoptosis in EC, macrophage foam cells, intimal VSMC and/or by cleaving ECM proteins that are imperative to maintaining plaque stability (Figure 2). Incubation of EC with GZMB and perforin results in a marked apoptotic response, suggesting that GZMB induces intracellular apoptotic pathways in EC [80]. Splenocytes from wild type (WT) mice induced higher VSMC death compared to splenocytes from perforin KO mice indicating that VSMCs are sensitive to cell death through the granule exocytosis pathway [61]. Importantly, in the latter study it was demonstrated that GZMB is able to induce VSMC and EC apoptosis in the absence of perforin by cleaving ECM proteins such as FN in vitro, leading to cell detachment and anoikis [60, 61]. The additional extracellular roles of GZMB in modulating the inflammatory response, such as generating pro-inflammatory ECM fragments and cytokine processing, as discussed above, may exacerbate the inflammation, further compromising plaque stability.
1.8 Angiogenesis

The delivery of blood to a target tissue is an absolute requirement for maintaining organ function. For that reason, the ability of the vascular network to expand and respond to local changes within the tissue is imperative, especially during embryonic development and growth.
but also during health and disease in the adult [112]. In the embryo, initial capillary formation occurs through a process called vasculogenesis, where endothelial progenitor cells assemble together to form a primitive network of capillaries. This network further expands with mural cells encapsulating the first layer of endothelium, giving rise to a spectrum of vascular structures from arteries to capillaries and veins [113]. During organ development and growth, the existing vasculature responds to the increased demand in blood supply by a process called angiogenesis, which refers to the sprouting growth of new capillaries from pre-existing blood vessels. In the adult, blood vessels are mostly quiescent and physiological angiogenesis occurs only as part of the menstrual cycle and in the placenta during pregnancy [113]. However in disease and injury, local tissue changes can lead to activation of angiogenesis which may result in an excessive response that can further contribute to disease pathogenesis, such as in cancer and chronic inflammation [114]. In other cases, insufficient angiogenesis and/or malformation of new capillary networks leads to reduced revascularization preventing adequate healing and regeneration such as in ischemic heart disease and impaired wound healing. Indeed, an ever growing list of disorders are characterized by dysregulation of angiogenesis while the continued discovery of key regulatory mechanisms of angiogenesis in disease is serving as a platform for the development of new therapeutics aimed at normalizing this response [114].

Angiogenesis is a complex process that is regulated by multiple factors including the interaction of EC with the surrounding matrix as well as EC response to growth factors and cytokines [113]. Tissue hypoxia is amongst the most potent stimulators of angiogenesis. This is achieved by the activation of hypoxia-inducible transcription factors (HIF) in residing cells, leading to upregulation of a number of angiogenic factors, namely vascular VEGF [115]. Other important stimulators of angiogenesis include placental growth factor (PIGF) and basic fibroblast
growth factor (bFGF) [112]. In response to these growth factors EC are reprogrammed and adopt an invasive and proliferative phenotype that will support their progression into the avascularized interstitium. Initially, activated EC lose cell-cell interactions by transiently dissolving cell junctions and adhesion molecules, as well as breaking down the basal lamina. Cytoskeleton reorganization allows EC to move towards the underlying matrix while membrane type 1 metalloprotease (MT1-MMP) degrade the pericellular matrix [116]. In addition, cell surface integrins, including $\alpha_v\beta_3$, $\alpha_5\beta_3$ and $\alpha_5\beta_1$, interact with matrix proteins allowing EC adhesion and migration. Ultimately, EC are coordinated according to cues from the ECM and form a functional capillary structure. Thus, the effect of growth factors in concert with the close interaction of EC with the ECM is critical for neovessel formation throughout all stages of angiogenesis [116].

1.8.1 The role of fibronectin in angiogenesis

FN is a large glycoprotein consisting of two similar but not identical ~250 kDa monomers linked by a disulfide bond at the C-terminus. It consists of multiple repeats of protein domains with conserved amino acid sequences including 2 type I, 2 type II, 15 -17 type III repeats (Figure 3). Although encoded by a single gene there are over 20 variations of this protein as a result of alternative gene splicing. Two type III domains that are subject to alternative splicing are called EDA (ED for “extradomain” A) and EDB. In addition a variable region that is not homologous to other parts of the FN molecule is present closer to the C-terminus [117]. FN is expressed as a soluble protein by hepatocytes and circulates in the plasma at high levels (~300 µg/ml). FN can also be expressed locally within tissues by a variety of cell types including SMC, EC and fibroblasts. While cellular FN can be expressed with both EDA and/or EDB domains, plasma FN is devoid of both of these domains [118].
Figure 3. Human FN. Schematic diagram of human FN showing the organization of the type I, type II and type III repeats and the location of the binding domains to either matrix molecules at the N-termini, integrin binding domain in type III repeats 9-10, and growth factor binding domain in type III repeats 12-14.

FN has important roles in mediating cell-matrix interactions, providing an essential 'bridge' between the cell cytoskeleton network and the surrounding ECM through interactions with cell surface integrins [119]. Specific regions within the FN molecule have distinct roles that define the function of this protein (Figure 3). The N-terminal region is mainly responsible for the incorporation and assembly of FN into the matrix by interacting with collagen, fibrin and multimerization to other FN molecules in a process that is dependent on specific cell surface sites [120]. The central region, also referred as the cell binding region, is a site for integrin binding. Importantly, this site includes the RGD domain, a sequence of Arg-Gly-Asp amino acids that binds α₅β₁ and αvβ₃ integrins [121]. The RGD binding site is important for cell
adhesion and migration on FN where mutation of this site (RGD to RGE) [122] as well as treatment with synthetic RGD fragments that compete over integrin binding [123] results in defective vascular formation and reduced angiogenesis. The C-terminus of FN contains growth factor and heprin binding domains [124]. Several growth factors were shown to bind to this domain such as VEGF and bFGF [125, 126]. Binding of these growth factors to FN is thought to convey important cell signals that potentiate and normalize angiogenesis through the synergistic interaction of integrin and growth factor receptors on EC membranes [124, 127].

FN plays a major role in regulating angiogenesis both during development and disease. FN null mice are embryonic lethal due to severe vascular defects, while a similar phenotype was demonstrated in α₅β₁ null mice and in RGD/RGE mutant mice suggesting a fundamental role for FN-EC interactions during vascular development and angiogenesis. During inflammation, plasma FN accumulates in the affected area due to leakage of hyperpermeable capillaries. Once incorporated in the ECM, FN undergoes a conformational change, exposing matricryptic RGD binding domains that facilitate cell adhesion and migration as part of provisional matrix formation during inflammation, further supporting EC sprouting and capillary formation [117, 128] (Figure 4). In vitro studies using three dimensional (3D) culture matrices demonstrated the requirement of FN for neovessel formation [129, 130] while human EC cultured in collagen gels required FN in order to successfully integrate with the vascular network after implantation in mice [131]. Moreover, fragmentation of FN is suggested to alter angiogenesis as several FN fragments can induce and/or inhibit EC migration and proliferation [132-135]. FN fragmentation may particularly influence angiogenesis during inflammation as FN fragments are evident in several bodily fluids in a number of chronic inflammatory diseases [136-138]. Indeed, FN is susceptible for proteolysis by proteases that are highly expressed during inflammation including
mast cell chymase, cathepsin G [139], MMPs [140], elastase [141] and GZMB [60, 61]. Interestingly, several FN fragments are reported to have distinct biological activities such as monocytes chemotaxis [142] and the induction of MMPs expression in chondrocytes [143]. Thus, alteration of FN may have important consequences on vascular morphogenesis and angiogenesis especially during inflammation.

Figure 4. The role of FN in angiogenesis. FN is circulating in the plasma in a closed, globular form. Vascular leakage during inflammation promotes FN extravasation to the interstitium where it undergoes a conformational change that opens the molecule and exposes cell binding domains (RGD). FN can also be expressed locally by fibroblasts, EC and SMC. FN binds and sequesters VEGF in the ECM. During angiogenesis, EC break the basal lamina and migrate towards the interstitium. FN supports EC migration and correct spatial alignment by interaction of RGD with integrin receptor on EC surface. Presentation of VEGF to EC by FN further stimulates directional migration and provides spatial cues that support capillary formation. Inflammatory cells serves as a source for GZMB accumulation in the ECM in disease. GZMB may cleave FN ad thus disrupt the angiogenic process.
1.8.2 Angiogenesis in chronic inflammation

The alteration of vascular architecture is a prominent characteristic of inflammation. Increased vascular permeability and expression of adhesion molecules by activated EC facilitates immune cell recruitment to the affected area [144]. During chronic inflammation a large array of inflammatory mediators can modulate the vascular response and induce angiogenesis. Increased angiogenesis during chronic inflammation facilitates the delivery of oxygen and nutrients to the inflamed site. However, the formation of leaky and unstable neovessels can further promote the inflammatory response by providing additional access points for immune cell infiltration and may delay the resolution of inflammation [145]. This particular phenotype of inflammatory angiogenesis including leaky, incomplete and pericyte devoid endothelium is observed in a large number of chronic inflammatory diseases including RA, psoriasis, atherosclerosis, AAA, ocular diseases and cancer [145]. Moreover, inability to form stable and functional neovessels may retard tissue regeneration and healing through expression of immune derived proteases and anti-angiogenic factors that delay the angiogenic response. The latter may have a particular impact during ischemic diseases as well as chronic wound healing often characterized by reduced granulation tissue formation and impaired angiogenesis [146, 147]. It is not surprising therefore that manipulating angiogenesis during disease is perceived as a major treatment strategy that may impact a variety of chronic inflammatory disorders [148]. While classical views are focused on developing agents that can either promote or inhibit angiogenesis, more recent approaches are shifting the focus towards exploring targets that will normalize aberrant vasculature, which may offer a better prospect for treating inflammatory diseases characterized by uncontrolled neovascularization.
Proteolysis of the ECM during inflammation has a significant impact on neovessel formation and stability. Immune cells, such as macrophages, neutrophils and mast cells contribute to ECM remodelling via the release of extracellular proteases such as MMPs, cathepsins, elastases and serine proteases [149]. Proteolytic breakdown of the ECM by these enzymes has substantial implications for the angiogenic process [150]. Cleavage of basal lamina proteins, such as laminin, is implicated in increased EC sprouting and migration [151] while laminin synthesis is important for neovessel stability and pericyte recruitment. ECM cleavage products that contain important integrin binding domains, most importantly the RGD motif, can regulate EC migration and proliferation by interacting with integrin receptors on the EC surface [152]. In addition, pericellular proteins within the ECM, such as decorin, fibrinogen and FN can bind to and sequester pro-angiogenic growth factors. The presentation of growth factors by the ECM is suggested to induce synergistic signals from both growth factor receptors and integrin receptors that promote stable neovessel formation [153] (Figure 4). Proteolytic degradation of the ECM during inflammation may alter growth factor bioavailability giving rise to leaky and irregular vascular beds [124, 151, 152, 154]. Thus, immune-mediated tissue remodelling during inflammation regulates angiogenesis and may have considerable impact on disease progression.

1.9 Vascular endothelial growth factor

VEGF is a designation that was given to the first member of the VEGF family, VEGFA, which was originally described as vascular permeability factor (VPF) [155]. This family consists of 5 members: VEGFA, VEGFB, VEGFC, VEGFD and PIGF. All family members are structurally related, however they differ by their receptor binding affinity and biological activities [156]. Often referred to as a 'master' regulator of vascular development and
angiogenesis, VEGFA has an imperative role in regulating EC migration proliferation and survival, while deletion of only a single allele results in severe vascular abnormalities which are embryonic lethal [157]. VEGF is expressed by most paranchymal cells and may act in a paracrine manner on adjacent EC. In addition, the autocrine affect of VEGF when expressed by EC is essential to maintain EC survival and may not be sufficiently compensated by paracrine VEGF [158].

VEGF signals predominantly through binding to VEGF receptor 2 (VEGFR2), a tyrosine kinase cell membrane receptor that is highly expressed in EC particularly during embryonic vasculogenesis [159] and in pathological conditions associated with aberrant angiogenesis such as in cancer and chronic inflammation [160]. Upon ligation VEGFR2 dimerizes and undergoes trans/autophosphorylation in a number of intracellular tyrosine residues. Key phosphorylated residues include Y951, Y1054, Y1059, Y1175 and Y1214. Signal transduction is conducted by a series of adaptor binding proteins that consecutively phosphorylate target proteins leading to a range of biological activities [156]. Induction of cell proliferation is mediated by activation of the RAS/RAF/ERK/MAPK pathway [161]. Activation of focal adhesion kinase (FAK) leads to rearrangement of the EC cytoskeleton and serves to regulate cell migration [162, 163]. VEGF-induced endothelium permeability is achieved through a number of pathways (discussed below). One of which includes the phosphorylation of the adaptor protein SRC which in turn phosphorylates the junctional protein vascular endothelial-cadherin (VE-cadherin), leading to endocytosis of the latter and causing increased paracellular fluid movement [164, 165]. Finally, termination of the signal is achieved by rapid internalization of VEGFR2 from the cell surface, or alternatively dephosphorylation by phosphotyrosine phosphatases [156].
1.9.1 Vascular endothelial growth factor interaction with the extracellular matrix

Since VEGF was first isolated as a heparin-binding secreted factor, it was suggested that ECM proteoglycans that binds heparin may also bind to VEGF [166]. Further investigations revealed that the VEGFA gene encodes a number of splice variant VEGF isoforms that differ in their preference to bind the ECM [167]. VEGF 165 is the most abundant isoform and has a cluster of basic residues within its C-terminus which allows it to interact with the anionic heparin-containing proteoglycans in the ECM. VEGF 121 lacks the basic residues in its C-terminus, making this isoform more acidic and less likely to interact with the ECM allowing it to diffuse freely upon secretion. On the other hand, isoforms VEGF 189 and 206 are longer and contain additional basic residues compared to VEGF 165, allowing them to bind the ECM more tightly [168]. The expression of different VEGF isoforms is suited to create a biochemical gradient that supports the angiogenic response. While more diffused isoforms will act on distant EC to promote migration and proliferation, ECM bound isoforms will fine tune the spatial arrangement of the growing neovessels [169].

Further evidence for the pathophysiological effects of VEGF-ECM binding on angiogenesis and vascular morphogenesis arises from studies using proteases that liberate VEGF from the ECM. Plasmin cleaves VEGF intramolecularly and produces a diffusible and active VEGF fragment that induces EC proliferation in vitro and increased vascular leakage in vivo [168]. MMP-3 cleaves VEGF, yielding a non-ECM binding fragment that gives rise to enlarged, leaky neovessels, while matrix bound VEGF gives rise to thinner, more branched and less leaky capillaries [170]. This study indicates that the release of VEGF from the ECM modulates the angiogenic response, resulting in the formation of irregular capillary networks that resemble the features of pathological angiogenesis observed in cancer, impaired wound healing and chronic
inflammation [170]. Interestingly, mice expressing only the diffused isoform, VEGF 120 (mice VEGF isoforms are shorter by one amino acid compared to human VEGF), die perinatally and exhibit thin and disorganized branches [171] while mice expressing only VEGF 164 are normal [172]. In addition, MMP-2 and -9 induce VEGF-dependent and inflammatory-dependent angiogenesis in vivo potentially through release of VEGF from ECM stores [173], further supporting previous observations pertaining to a role for MMP-9 in promoting angiogenesis and tumor growth through release of VEGF [174]. Thus, VEGF bioavailability is regulated by the ECM and has important implications on angiogenesis and vascular patterning.

Mechanisms that convey specific signals to EC by matrix bound VEGF are a result of synergistic interactions between integrins and VEGF receptor. VEGF promotes complex formation between VEGFR2 and \( \alpha_v \beta_3 \) integrin [175]. This interaction enhances VEGFR2 activation which further induces p38MAPK and FAK activation leading to increased EC proliferation and migration [175]. ECM-anchored VEGF 165, but not diffused VEGF 121, leads to prolonged phosphorylation of Y1214 of VEGFR2 which also results in increased p38MAPK activation, as well as association of VEGFR2 with \( \beta_1 \) integrin and focal adhesion proteins [176]. Interestingly, the intrinsic structure of FN allows this protein to facilitate this co-stimulatory signal as it contains both a central integrin binding domain and an N-terminal VEGF binding domain (Figure 3). Wijelath et al. demonstrated that FN-bound VEGF enhances EC migration and proliferation, a response that is significantly higher than when treated with either FN or VEGF alone, and it is specifically mediated by \( \alpha_5 \beta_1 \) integrin [125, 177]. Importantly, treatment of engineered FN fragments containing both the cell binding domain and VEGF binding domain enhances VEGF-mediated chronic wound healing in diabetic mice to a greater extent than either FN or VEGF alone through increased granulation tissue formation and an adequate angiogenic
response [178]. These findings further highlight the importance of VEGF-FN interaction in normalizing neovascularization and supporting tissue regeneration.

1.9.2 Vascular endothelial growth factor induced permeability and pathological angiogenesis

Although considerable debate still exists on the precise mechanisms by which VEGF induces vascular permeability one consensus remains; VEGF is among the most potent vascular permeabilizing agents currently known. With activity of less than 1 nM, VEGF is about 50,000 times more potent than histamine on a molar basis and it can induce permeability within minutes [179]. First described as VPF, its permeability inducing effect was demonstrated in vivo using the Miles assay in guinea pigs [155]. In this assay Evans blue dye that binds albumin in the circulation is injected intravenously prior to localized injection of a test permeabilizing agent, most commonly in the skin. Dye extravasation to the tissue can be clearly visualized and serve as a direct indicator of vascular leakiness [180]. Detailed quantification is achieved by dye extraction followed by absorbance measurement [181].

Currently, mechanisms for VEGF-induced vascular permeability are described by three general models. VEGF promotes intercellular fluid escape by disassembling the adheres junction, VE-cadherin, in a process that involves sequential phosphorylation of the SRC family of adaptor proteins leading to endocytosis of VE-cadherin [164, 182]. Alternatively, a body of evidence exists that supports a transcellular pathway for capillary leakiness. Transmission electron microscope studies on EC led to the discovery of numerous, grape-like clusters of cytoplasmic vesicles that are referred to as the vesiculo–vacuolar organelle (VVO) [183]. These vesicles span from the apical to the basal side of EC and are linked by stromata that are normally closed by a thin diaphragm. It is suggested that fusion of VVO with the plasma membrane creates a transendothelial channel that facilitates fluid movement across the endothelium [184].
Although the mechanisms leading to VVO fusion are ill defined, it was observed that VEGF treatment results in the passage of large molecular tracers such as ferritin through interconnected VVO in EC [185]. Finally, the induction of fenestrae in VEGF-treated EC has been suggested as an additional model for VEGF-mediated transendothelial fluid escape. Fenestrae are 50–150 nm areas of extreme endothelial cell thinning that are generally closed by diaphragms [186, 187]. Fenestrae exist in normal microvessels of several tissues where increased extravasation is required such as in the kidneys, islets of Langerhans and the adrenal cortex. VEGF is suggested to mediate the opening of fenestrae, however the molecular mechanisms leading to this event has not been clarified [188, 189].

Vascular hypermeability is a common feature of pathological angiogenesis for which VEGF is suggested to be responsible for in a range of pathologies including cancer, healing wounds and chronic inflammatory diseases [190-195]. Pathological angiogenesis is characterized by leaky, incomplete, tortuous, pericyte-devoid endothelium that forms functionally impaired vascular networks [179]. Several *in vivo* studies highlighted the role of increased VEGF bioavailability in inducing pathological angiogenesis. High VEGF expression by transduced myoblasts implanted in mice muscle resulted in the formation of aberrant, leaky neovessels where low VEGF expressing myoblasts gave rise to stable, pericyte-coated capillaries [196]. This study demonstrates that the precise microenvironmental levels of VEGF is a key determinant to achieve effective angiogenesis while an increase in VEGF bioavailability leads to pathological angiogenesis [196]. Mechanisms leading to VEGF-mediated pathological angiogenesis include a series of structural alterations to the existing vasculature starting with the formation of dilated capillaries with degraded basement membrane and reduced pericyte support. These vessels also referred to as mother vessels, further split through cell invasion both into the
stroma and towards the lumen to form intraluminal bridges. This process may give rise to small, poorly organized, leaky capillaries that resemble renal glomeruli [179]. It is suggested that angiogenic inducing therapies using high doses of VEGF may be limited by the growth of abnormal blood vessels [196]. On the other hand, administration of low dose, matrix-bound VEGF promotes the formation of stable capillaries and improves wound healing [178]. Thus, alteration in the microenvironmental levels of VEGF and its interaction with the matrix may induce pathological angiogenesis further supporting chronic inflammation and retard tissue regeneration.
2. Rational, Hypothesis and Aims

The past decade was an exciting time for granzyme research that brought with it a dramatic change in our understanding of the biological roles of these proteases in health and disease. Since its discovery over 3 decades ago it has been firmly established that GZMB has a major role in cellular immunity as a potent initiator of target cell apoptosis when delivered intracellularly. This has served as the basis for an elaborative body of research that demonstrated the role of GZMB in eliminating virus infected cells and tumors. However observations of elevated GZMB levels in a variety of chronic inflammatory diseases has raised further questions about the function of this enzyme in the broader context of chronic inflammation.

Earlier work from our laboratory provided evidence that GZMB-mediated apoptosis plays a role in TVD [80] and atherosclerosis [109]. Reduced TVD development in GZMB deficient mice corresponded with the ability of GZMB to induce EC apoptosis, which was suggested as a key mechanism that contributes to TVD onset and progression [80]. Studies in human atherosclerotic plaques demonstrated an association of apoptotic VSMC with increased GZMB levels, raising the possibility that GZMB-mediated apoptosis may play a role in promoting VSMC cell death as a mechanism that promotes plaque instability [109]. These studies highlighted a potential pathological function for GZMB in modulating the cellular composition of effected arteries by inducing either EC or VSMC cell death. However, both EC and VSMC express PI-9, the endogenous intracellular inhibitor of GZMB, which is suggested to protect host cells from apoptosis due to misdirected GZMB release [23]. This fact introduced a challenge to explain why vascular cells are not protected from GZMB-mediated apoptosis, especially in the context of atherosclerosis where plaques are composed of host cells. One possibility was that PI-
9 expression in vascular cells is reduced during atherogenesis leading to increased susceptibility to GZMB-mediated apoptosis in this disease.

Apart from its intracellular role, it is now acknowledged that GZMB can act extracellularly and cleave an ever-growing list of ECM proteins [71]. Constitutive release of GZMB by stimulated NK cells and CTLs without target cell engagement as well as the expression of this enzyme by other immune and non-immune cells during chronic inflammation (most often in the absence of perforin) provide several potential sources of extracellular GZMB in disease. Several changes in cellular biology due to GZMB cleavage of ECM proteins has been proposed including cell detachment, impact on cell migration and altering cell signaling by either cytokine processing and/or release of biologically active molecules from the matrix [71]. However, mechanistic evidence relating extracellular GZMB activity to specific pathological processes that contribute to disease progression remains to be fully understood. Interestingly, GZMB cleaves a number of ECM proteins that have a key role in regulating angiogenesis such as FN [60]. A dysregulated angiogenic response underlies a range of chronic inflammatory disorders that are associated with extracellular GZMB accumulation and FN fragmentation. Furthermore, VEGF, a 'master' regulator of angiogenesis and a potent inducer of vascular permeability is sequestered in the ECM, in part by binding to FN [177]. Thus, GZMB processing of the ECM may have both direct and indirect effects on microvascular architecture by cleaving FN and altering VEGF bioavailability providing a mechanism that contributes to pathological angiogenesis in chronic inflammation.

In light of the above I hypothesize that GZMB contributes to changes in vascular integrity in disease through both intracellular and extracellular mechanisms. To better address
this overarching hypothesis I derived the following sub-hypotheses within the subsequent specific aims:

1. Increased GZMB and downregulation of the GZMB inhibitor PI-9 contributes to atherosclerotic plaque progression through increased VSMC susceptibility to GZMB induced apoptosis.

Specific Aim:

1.1 To characterize the relationship between GZMB and PI-9 during the development of atherosclerotic lesions in humans. Earlier observations suggested that PI-9 expression is reduced in atherosclerotic plaques [24]. However, no correlation was made to GZMB-mediated VSMC apoptosis in these studies. Moreover, no evidence was provided to determine whether the observed reduction in PI-9 is due to reduced plaque cellularity or reduced expression of this protein by VSMC. Thus, the goal of this aim was to determine the expression pattern of PI-9 by VSMC across different stages of disease severity and correlate these with GZMB expression and evidence of VSMC apoptosis.

2. Extracellular GZMB promotes pathological angiogenesis by cleaving the ECM leading to disrupted EC-matrix interaction and increased VEGF bioavailability.

Specific Aims:

2.1 To determine the direct effect of GZMB-mediated FN cleavage on EC angiogenic potential. FN regulates EC adhesion, migration and capillary tube formation through the interaction of cell binding domains in FN with integrin receptors on EC. Several studies indicated that FN proteolysis may promote angiogenesis by exposing cell binding
domains further enhancing EC adhesion and migration, while others indicated that FN fragmentation attenuates EC angiogenic response [152, 197]. The goal of this aim is to explore the direct consequences of FN proteolysis by GZMB on EC adhesion migration and capillary tube formation.

2.2 To determine the effect of GZMB-mediated FN cleavage on VEGF bioavailability and its impact on vascular permeability. VEGF is sequestered in the ECM by binding FN. Since VEGF is a potent vascular permeabilizing agent and an inducer of pathological angiogenesis, we sought to explore whether GZMB cleavage of FN results in the release of VEGF from the ECM and what impact this process might have on vascular permeability \textit{in vivo}. 

3. Materials and methods

3.1 Cases and tissue processing

Serial sections of human left anterior descending (LAD) coronary arteries from 22 male heart transplant patients exhibiting different stages of atherosclerotic lesion development were obtained from the BioBank registry at St. Paul’s Hospital. All samples were processed for immunohistochemical analysis, as described previously [198]. Briefly, following removal during transplant surgery, intact LAD coronary arteries were isolated and removed from explanted hearts. Only the proximal segments of the LAD were used for this study. Proximal LAD segments (~1.5 cm) were further processed by cutting the segment to 4 pieces. Each piece (~ 3 mm) was then preserved in either 10% formalin or flash-frozen in liquid nitrogen. Flash-frozen sections that were adjacent to sections preserved by formalin (used for immunohistochemical examination) were utilized for western blot analysis. Formalin fixed samples were embedded in paraffin and sectioned transversely at 5 mm intervals. Sections were stained with Hematoxylin and eosin (H&E) (3 sections per case), examined by a cardiac pathologist and categorized according to the widely established revised AHA classification [106] as diffuse intimal thickening (n=7) (intimal thickening ranged from 0.15-0.55mm), pathological intimal thickening (n=6) (defined as an establish intimal lesion that includes SMC and foam cells accumulation without a necrotic core), and fibrous cap atheroma (n=9) lesions (defined as a well-formed atheromatous plaque with a necrotic core and an overlying fibrous cap) (Table 1). H&E slides were digitally scanned by the Aperio ScanScope system and analyzed using ImageScope software (version 10.2.2.2) (Aperio Technologies, Inc., Vista, CA). Detailed measurements of lesion morphological characteristics
and patient age distribution is presented in Table 1. The study protocol was approved by the Providence Health Care Research Ethics Board, University of British Columbia.

Table 1. Patient information and morphological characteristics of left anterior descending coronary artery lesions.

<table>
<thead>
<tr>
<th>Pathological diagnosis</th>
<th>Intimal thickening (mm)</th>
<th>Plaque size (% of CSVA)</th>
<th>Necrotic core area (% of plaque size)</th>
<th>Age</th>
<th>Pathological diagnosis (macroscopic examination)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffuse intimal thickening</td>
<td>0.33(0.17)</td>
<td>-</td>
<td>-</td>
<td>53 (8.41)</td>
<td>DCMY (n=6)</td>
</tr>
<tr>
<td>Pathological intimal thickening</td>
<td>1.14(0.17)</td>
<td>68(10)%</td>
<td>-</td>
<td>58 (5.2)</td>
<td>Coronary atherosclerosis (n=5)</td>
</tr>
<tr>
<td>Fibrous cap atheroma</td>
<td>1.66(0.58)</td>
<td>70(15)%</td>
<td>30.62(22)%</td>
<td>52 (7.3)</td>
<td>Coronary atherosclerosis (n=9)</td>
</tr>
</tbody>
</table>

CSVA, cross sectional vascular area; DCMY, dilated cardiomyopathy. Values presented as mean (SD)

3.2 Immunohistochemistry

Formalin-fixed, paraffin-embedded sections of LAD coronary arteries were serially sectioned (5 µm; total of 10 sections per case). Sections were deparaffinised and rehydrated in xylene and decreasing concentrations of ethanol. Antigen retrieval was performed by boiling slides in citrate buffer (pH= 6) (Invitrogen, Carlsbad, CA) for 15 min followed by a 30 min cooling at room temperature. Endogenous peroxides were quenched by incubating sections in
3% H₂O₂ for 15 min. Sections were blocked with either 10% normal horse serum (for slides probed with PI-9, GZMB, and CD8 antibodies (Abs) or 10% goat serum (for slides probed with cleaved caspase-3 Ab) for 30 min and incubated over night at 4°C with either of the following primary antibodies: PI-9 (Alexis Biochemicals, San Diego, CA) (1:50 dilution), mAb α-SMA (Abcam, Cambridge, MA) (1:100 dilution), CD8 (RDI, Flanders, NJ) (1:100 dilution), cleaved caspase-3 (Cell Signaling Technology, Danvers, MA) (1:100 dilution) and mAb GZMB (generously supplied by Dr. Joseph Trapani (Peter MacCallum Cancer Center, Melbourne, Australia) (1:100 dilution). Sections were incubated with 1:350 dilution of biotin horse anti-mouse or biotin goat anti-rabbit for 30 minutes followed by signal amplification using Vectastain® ABC system (Vector Laboratories Inc., Burlingame, CA), according to manufacturer’s instructions. Staining was visualized using DAB or Nova Red (Vector Laboratories Inc., Burlingame, CA). For double fluorescence immunolabelling, sections were initially probed with anti-cleaved caspase-3 antibody followed by incubation with goat anti-rabbit secondary antibody and using the ABC system as described above. Sections were then blocked using the Avidin/Biotin blocking system (Vector Laboratories Inc., Burlingame, CA) according to manufacturer’s instructions. Slides were blocked with 10% normal horse serum and probed with α-SMA antibody followed by secondary horse anti-mouse incubation and application of the ABC system, as described above. Slides were visualized using TSA™ Plus Fluorescence Systems (PerkinElmer Life and Analytical Sciences, Inc., Shelton, CT) substrate as follows: Fluorescein was used to detect cleaved caspase-3; Cyanin 3 was used to detect α-SMA. Hematoxylin was used as a nuclear counter stain in sections that were used for bright field imaging. Sections stained without primary antibody were used as negative controls. Tissue
sections taken from tonsil tissue were used as positive control for PI-9, GZMB and cleaved caspase-3 Abs.

3.2 Image analysis and staining quantification

Bright field images were captured using Nikon Eclipse E600 microscope (Nikon). 4-5 images per section were captured (10x magnification) in conserved areas relative to the lumen in all examined cases. ImagePro Plus™ (Media Cybernetics, Silver Spring, MD) was used to quantify positive immunostaining as reported previously [199]. Threshold between positive and negative staining was defined by using positive and negative control sections and applied by a color cube algorithm in ImagePro Plus™ with manual selection of positive stained colors. The same threshold for brown color for sections stained for PI-9 and threshold for red color for sections stained for α-SMA were applied to all examined cases (diffuse intimal thickening, pathological intimal thickening and fibrous cap atheroma). Manual tracing of the media and intima layers using ImagePro Plus™ was used to determine the measurable area in each image, where the intima was defined as the area between the lumen and the internal elastic lamina (IEL), and the media was defined as the area between IEL to the external elastic lamina (EEL). Values for positive staining divided by the measurable area were used to calculate the staining fraction. Staining fraction is defined as the area of positive staining divided by total measurable tissue area. Positive staining from all captured images per case was averaged to give a value to be used for analysis.

3.3 Confocal microscopy, image acquisition and processing

As been described previously [200], confocal images of fluorescently labeled tissue sections were acquired with a Leica AOBS SP2 laser scanning inverted confocal microscope (Leica, Heidelberg, Germany) using a high resolution Leica Plan-Apochromat oil immersion
objectives. The acquisition software was Leica Confocal Software TCS SP2. The laser lines for specific excitations included laser line 488 (for Fluorescein), and 543 nm (for Cyanin 3). The width of the slits in front of each PMT could be software adjusted so that each PMT could detect spectral regions spanning from a 5-nm bandwidth up to the overall spectral capacity of the system (400–800 nm). Using this unique option, spectral scanning was performed on each fluorescent dye to confirm their specificity. Because of the broad nature of emission spectra of Fluorescein and Cyanin 3, the fluorescent signals were collected only from peak emission regions. The AOBS based emission ranges were 500-535 nm and 555-615 nm for Fluorescein and Cyanin 3 emissions, respectively. Further, the images from these dual stained samples were acquired sequentially to eliminate cross-talk between the emission signals. Acquired images were overlaid using Volocity software (Improvisions, UK).

3.4 Recombinant PI-9 expression

PI-9 expression plasmid (pCMV/PI-9) was a kind gift from Dr. Phillip Bird (Monash University, Melbourne, Australia). COS cells were grown to 70% confluency in a 6-well plate in DMEM growth media (DMEM, 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin). Transfection media was generated by mixing 800µl/well of Opti-Mem media (Invitrogen Carlsbad, CA) with 12 µl of Lipofectamine (Invitrogen Carlsbad, CA) in a microcentrifuge tube. In a separate tube, 2 µg of the above plasmid was added to 800 µl of Opti-Mem media. Tubes were incubated for 5 min at room temperature. To generate a transfection complex, Lipofectamine and plasmid containing media were mixed and incubated for 30 min at room temperature. After the removal of growth media, transfection media was added to the COS cells and incubated for 8 hours in 37°C (5% CO₂). Transfection media was then replaced with DMEM growth media for an additional 48 hours. Cells were then washed twice with DPBS and lysed
using CellLytic M (Sigma St. Louis, MO) lysis buffer with 1% proteinase inhibitor cocktail (Sigma St. Louis, MO).

3.5 Western blotting

Non-fixed, flash frozen coronary arteries (LAD) whole tissue samples collected from the same patients screened by immunohistochemistry (n=3 from each category) were analyzed for PI-9 expression by western blot analysis. Samples were homogenized in lysis buffer (50 mM Tris-Base pH=8, 150 mM NaCl, 1% Triton-x, 0.1% SDS, proteinase inhibitor cocktail (1:100) followed by 3 freeze thaw cycles, centrifuged at 12,000 rpm for 10 min and the isolated supernatant was spun for an additional 10 min and collected. Total protein concentration was quantified using BioRad Bradford protein assay (BioRad Laboratories), and 45µg of total protein was run on a 10% SDS-PAGE polyacrylamide gel and transferred onto a PVDF membrane pre-activated by methanol. Membrane was blocked using 2.5% skim milk powder in PBS+ 1% Tween-20 for 30 min and incubated over night at 4°C with 1:700 dilution of PI-9 mAb in blocking solution. After repeated washes, membrane was incubated with goat anti-mouse IRDye™ 800 antibody at 1:2000 dilution for 1 hour at room temperature and fluorescent signal was imaged using the Li-COR Odyssey Infrared Imaging System (Li-COR Biosciences). Densitometry was used to quantify PI-9 expression. Membrane was stripped by incubating the membrane with a stripping buffer (to 10ml of 10% SDS, 0.5M Tris pH=6.8, added 35µl of β-mercaptoethanol) for 10 min in a 60°C water bath, followed by repeated washes and re-probed with mAb β-actin for loading control.

3.6 FN cleavage assay

For GZMB-mediated plasma FN cleavage, 48 well plates were coated with human purified plasma FN (20 µg/ml) (Millipore, Billerica, MA) in Dulbecco’s Phosphate-Buffered
Saline (DPBS) (150 µl/well) for 1 h at 37°C. Wells were then blocked with 1% bovine serum albumin (BSA) in DPBS for 30 min at 37°C. GZMB treatments were prepared by diluting human purified GZMB (Axxora, San Diego, CA) to various concentrations using 50 mM Tris buffer, pH 7.4. GZMB specific inhibitor, Compound 20 (UBC Centre for Drug Research and Development, Vancouver, BC) [201], was added to a final concentration of 50 µM in the GZMB 100 nM + inhibitor treatment group. All treatment groups were pre-incubated at RT for 30 min and then added to the FN coated wells for 24 h at RT. Supernatants were carefully removed and separated by 10% SDS-PAGE gel. Transferred membranes were blocked in 2.5% skim milk solution in PBS+ 0.1% Tween 20 for 30 min and then immunoblotted with monoclonal mouse anti-human FN Ab (R&D systems, Minneapolis, MN) at 1:1000 dilution in blocking solution, and incubated over night at 4°C. Detection was carried by incubating the membranes with IRDye 800 anti-mouse Ab (LI-COR Biosciences, Lincoln, NE) at 1:10,000 dilution in blocking solution for 1 h at RT and fluorescent signal was imaged using the Li-COR Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE).

3.7 Human microvascular endothelial cell and SMC-derived FN cleavage assay

Human microvascular endothelial cell (HMVEC) or human coronary artery SMC (Clonetics/Lonza, Walkersville, MD) were seeded on a 6 well plate and grown to confluence in complete growth media (EGM2+2%FBS for HMVEC or SmGM + 5%FBS for SMC; Clonetics/Lonza, Walkersville, MD). Cells were maintained in serum-reduced (0.2% FBS) media for 7 d with changing media every 2 d. To remove the cells while leaving the ECM intact, cells were washed 3 times with DPBS and 200 µl/well of 0.25 M ammonium hydroxide was added and incubated for 20 min at RT. Wells were washed 3 times with dH₂O and cell removal was confirmed by microscopical examination of the wells. ECM was then blocked with 1% BSA for
30 min at 37°C. 300 µl/ well of 50 nM GZMB in 50mM Tris buffer, pH 7.4, was added to the wells and incubated for 24 h at RT. Supernatants were carefully removed and separated by 10% SDS-PAGE gel. FN fragments were detected by western blot.

3.8 Adhesion assay

Forty-eight wells culture plates were coated with 150 µl/ well of 20 µg/ml FN in DPBS and incubated for 1 h at 37°C. Wells coated with 5% BSA were used as negative controls. Wells were washed once with DPBS and blocked with 1% BSA for 30 min at 37°C. GZMB treatments were prepared by diluting GZMB to various concentrations to a volume of 150 µl/ well in adhesion media (M199/EBSS medium + 1Xglutamax; HyClone, Logan, UT). Compound 20 (50 µM), was added to the GZMB 100 nM group as indicated. All treatment groups were pre-incubated at RT for 30 min and then added to the FN coated wells for 24 h at RT. To stop the reaction after the incubation period, Compound 20 was added to a final concentration of 50 µM to both the controls and the remaining GZMB treatment groups and incubated for 30 min at RT prior to the addition of cells to the wells. HMVEC, passage 4-7, were grown to confluence on 30 µg/ml purified bovine collagen I (BD Biosciences, Bedford, MA) pre-coated flasks in endothelial growth media (EGM2+2%FBS). Cells were harvested with 0.125% Trypsin EDTA and washed first with DPBS+5%FBS, centrifuged down and washed again with only DPBS to remove any remaining FBS. Cells were centrifuged again, and cell pellet was reconstituted in adhesion media. 2x10⁴ cells in 50 µl/ well were then seeded on the wells that were pretreated with GZMB, which was inactivated by Compound 20 as described above. Cells were then incubated for 30 min at 37°C to allow for adhesion. Non-adherent cells were removed by pipetting out the media and washing the wells 3 times with DPBS. Adherent cells were fixed
with 200 µl/well of 1:1 ice cold methanol: acetone followed by placing the plate in -20°C for 10 min. Wells were washed 3 times with DPBS and adherent cells were stained with hematoxylin.

To maximize the area to be examined for the adherent cell quantification, wells were photographed at 3 conserved areas using a Nikon Eclipse TE300 microscope (X4 magnification). Triplicate images per well were then merged using the automatic photomerge function in Photoshop® (Adobe Systems Incorporated, San Jose, CA). Images were adjusted with ImagePro Plus® (Media Cybernetics, Silver Spring, MD) using a consistent macro that was applied to all images and cells were counted by the software. Experiments were repeated at least 3 times with 3 triplicates per condition.

3.9 Migration assay

For haptotaxis assays, 8 µm pore size transwell membrane inserts (BD Biosciences, Bedford, MA) were coated on the underside with either FN (10 µg/ml) or collagen I (10 µg/ml) in DPBS for 1 h at 37°C in a 24-well plate. Inserts were washed with DPBS and blocked with 1% BSA for 30 min at 37°C. Membranes were then treated with either 50 nM GZMB or 50 nM GZMB+ 50µM Compound 20 in 400 µl/well in migration media (M199/EBSS medium + 1Xglutamax+1%BSA). Control groups included treatments with migration media and vehicle alone (1:1000 diluted DMSO), and migration media with Compound 20 (50 µM). Membranes were incubated with the above treatments for 24 h at RT. HMVEC were grown to confluence as described above, and growth media was replaced by serum reduced media (M199/EBSS medium + 1Xglutamax+1%FBS) for 2 h prior to cell harvest. 4.5×10^4 cells/well in 200 µl were seeded on the top chamber where additional 200 µl of migration media was added to the lower chamber for a final volume of 600 µl. Cells were allowed to migrate towards FN for 6 h at 37°C. Cells were then fixed with 4% paraformaldehyde and cells that adhered to the top part of the membrane
were removed. Inserts were stained with hematoxylin and membranes were mounted on microscope slides. Four fields were photographed per membrane at X10 magnification. Images per membrane were merged and analyzed using Photoshop® and ImagePro Plus® as described above. Experiments were repeated at least 3 times with 3 triplicates per condition.

For chemotaxis assays, FN fragments were generated as described for the plasma FN cleavage assay above in 24-well plates at a volume of 300 µl with 50 nM GZMB in migration media with two additional control groups having migration media alone. Wells were incubated for 24 h at RT. To stop GZMB activity 50 µM of Compound 20 was added to both the GZMB treatment groups and to one of the control groups. The second control group was supplemented with vehicle alone (1:1000 DMSO). Transwell inserts were coated on both sides of the membranes with collagen I (10 µg/ml) in DPBS for 1 h 37°C and blocked with 1%BSA. Supernatants from the above cleavage assays were added to the bottom chamber and supplemented with 300 µl of migration media for a final volume of 600 µl. HMVEC were seeded at 4.5x10⁴ cells/well in 200 µl on the top membrane and allowed to migrate for 6 h at 37°C. Membranes were processed and analyzed as described above. Migration media containing VEGF (50 ng/ml) (R&D systems, Minneapolis, MN) was used as positive control.

3.10 3D collagen gel tube formation assay

Collagen gels were prepared by mixing 4 ml of purified bovine collagen I (Advanced BioMatrix, Poway, CA) with 500µl 10XRPMI media (Life Technologies Inc. Burlington, ON), 400 µl NaHCO₃ (2.5%)/NaOH (0.5N) solution, 100 µl of 1 M HEPES buffer (Lonza, Walkersville, MD) to a final collagen I concentration of 2.5 mg/ml. 25 µl of 6 M HCL was added to adjust pH to 7.4 followed by addition of laminin (0.6 µg/ml) (Sigma, St. Louise, MO), and FN (10 µg/ml). 200 µl/well of collagen gel solution was added to 48 well plates and allowed to
solidify for 1 h at 37°C. HUVEC were seeded on top of the collagen gel at 4x10^4 cells/well in 200 µl serum reduced growth media (EGM-2 +1%FBS) and incubated for 10 h at 37°C to form a monolayer. Media was removed and gels were washed once with warm DPBS to remove non-adherent cells followed by addition of a second layer of collagen gel solution (100 µl) on top of the cell monolayer. Plates were incubated for an additional 1 h at 37°C to allow for gel solidification. Treatment media including 50 nM GZMB, 50 nM GZMB + 50µM Compound 20, Compound 20 alone, and vehicle alone in serum reduced growth media at 200µl/well were added on top of the gels and incubated for additional 12 h at 37°C. Gels were fixed with 3% formalin overnight and photographed using Motic AE31 inverted microscope at X4 magnification in order to capture the entire well in a single image. Images were processed by ImagePro Plus® and quantified by tracing total tube length per gel. Branching quantification was performed by counting points where two or more tubes converged. Experiments were repeated 3 times with 3 triplicates per condition.

Fluorescence staining was performed by incubating gels with 0.1% Triton X for 30 min at RT and blocking with 1%BSA. Gels were then incubated over night with 1:100 dilution of Alexa Fluor® 594 phalloidin (Life Technologies Inc. Burlington, ON) in DPBS at 4°C. Gels were washed with PBS and imaged using Leica multiphoton microscope system setup as described [200] at X20 magnification. Z-section images were scanned and compiled and the 3-dimensional image restoration was performed using Volocity software (Improvisions, Inc. Waltham, MA). Supernatants collected at the end of the tube formation assay were analyzed by western blot to detect FN fragments as described for the FN cleavage assay above.
3.11 VEGF release assay

For GZMB-mediated VEGF release from FN, 48 well plates were coated with human purified plasma FN (20 µg/ml) in DPBS for 1 h at 37°C. Wells were then blocked with 1% BSA in DPBS for 30 min at 37°C. VEGF (50ng/ml) was added to the FN coated wells and incubated for 2 hrs at 37°C followed by extensive washing to remove unbound VEGF. H purified GZMB (50 nM) in Tris buffer with either vehicle control (1/100 DMSO) or GZMB inhibitor (Compound 20) were added to the well for additional 2 hrs at 37°C. Supernatants were analyzed by VEGF ELISA (R&D systems, Minneapolis, MN) according to the manufacturer instruction. For VEGF release from HUVEC matrix, HUVEC were cultured in a 6 well plate and grown to confluence in complete growth media (EGM2+2%FBS). Cells were maintained in serum-reduced (0.2% FBS) media for 9 d with changing media every 2 d. Cell removal was performed as described above. Remaining ECM was then blocked with 1% BSA for 30 min at 37°C, followed by addition of VEGF (50 ng/ml) in 1%BSA for 2 hrs at 37°C. Unbound VEGF was removed by washing the wells with DPBS and GZMB (50 nM) in Tris buffer with either vehicle control or Compound 20 were added to the well for additional 2 hrs at 37°C. Supernatants were analyzed by VEGF ELISA.

3.12 VEGFR2 phosphorylation by GZMB-mediated VEGF release from FN

HUVEC were grown to confluence in 6 well plate in complete growth media. Media was changed to EGM no VEGF +1%FBS media for 2 days prior to treating cells with VEGF release supernatants. VEGF release from FN coated wells was performed as described above, however this time treatments were prepared in M199 media to allow the use of the supernatants as a treatment media for HUVEC culture. VEGF release supernatants were added to HUVEC culture for 7 min followed by rapid cell lysis with lysis buffer that included phosphatases inhibitor
cocktail (1:100) and proteinase inhibitor cocktail (1:100). Cell lysates were resolved by 10% SDS PAGE and immunoblotted using pVEGFR2y1214, pVEGFR2y1175, total VEGFR2 (Cell Signaling Technology, Danvers, MA) (1:700 dilution in 2.5% milk). β- tubulin immunoblotting was used as a loading control. Densitometry was used to quantify pVEGFR2 and values were divided by the expression levels of total VEGFR2 and normalized to loading control (n=3 with three replicates per treatment).

3.13 In vivo permeability assay

8-10 weeks old CD1 female mice were injected with 200µl Evan’s Blue dye (0.5% in injectable saline)(Sigma St. Louis, MO) via the tail vein. The following treatments were prepared in injectable saline to a final volume of 10µl (n=5 for each treatment group): Recombinant mouse granzyme B (Gzmb) (100ng)(Sigma St. Louis, MO), Gzmb (100ng) + anti mouse VEGF antibody (1.5µg), Gzmb (100ng) + goat IgG control (1.5µg). Treatments were injected locally to the ear. After 30 min mice were euthanized and perfused with saline to clear the circulation from any remaining dye. Ear tissues were excised using a 7 mm punch biopsy and dried in 60°C incubator overnight. Tissues were weighed and dye was extracted by incubating tissues with 300µl formamide (Sigma St. Louis, MO) in 60°C incubator for 24 hrs. Absorbance of the extracted dye was measured at 610 nm and values were normalized to tissue weight. Studies were approved by of the University of British Columbia Animal Care Committee.

3.14 Statistics

A one way analysis of variance (ANOVA) with Bonferroni's multiple comparison post test was performed to determine statistical differences between multiple groups. A paired t test was performed to determine statistical difference between two matching groups. A value of P<0.05 was considered significant.
4. The relationship between GZMB and PI-9 in human atherosclerotic plaque development

4.1 Introduction

Chronic inflammation in atherosclerotic lesions is a major contributor to plaque destabilization [202]. Infiltrating lymphocytes, macrophages and mast cells, orchestrate the inflammatory response through the production of an array of cytokines and chemokines that further augment immune cell recruitment to the developing atheroma [88]. Plaque weakening is attributed in part to immune-mediated apoptosis that is executed by CTLs and NK cells as the presence of these cells is associated with a high degree of intimal apoptosis [97]. Apoptosis of intimal VSMC leads not only to a reduction in plaque cellularity but also results in a significant decrease in extracellular matrix production that is vital for maintaining plaque integrity [98]. In concert with the secretion of matrix-degrading enzymes and the induction of apoptotic events, the immune response plays an active role in promoting plaque destabilization and rupture [202].

Accumulating evidence suggests a role for GZMB in the induction of apoptosis and ECM remodelling that is observed in advanced atherosclerotic lesions [34, 41, 109]. Previous work from our laboratory has demonstrated that increased GZMB is present in advanced atherosclerotic plaques and localizes to macrophage foam cells, CTLs and vascular VSMC, and is associated with macrophage and intimal VSMC apoptosis in the shoulder regions of advanced plaques [109]. Increased plasma levels of GZMB are also detected in patients with carotid atherosclerotic lesions in correlation with increased cerebrovascular events, unstable versus stable angina pectoris, and in patients following acute myocardial infarction [46, 110, 203]. Hence, the aforementioned findings indicate the potential involvement of GZMB in plaque
instability, and that GZMB inhibition might serve as a useful strategy to promote plaque stability.

The only known endogenous inhibitor of human GZMB is PI-9. PI-9 is highly expressed in immune cells, including CTLs and NK cells, and in immune-privileged sites where it protects cells from misdirected GZMB release and accidental cell death [25]. Interestingly, both EC and VSMC express PI-9 in the normal vasculature, suggesting they may be protected against GZMB insult under normal conditions [23, 24]. Thus, PI-9 may serve as an endogenous regulator within the vasculature that can potentially attenuate plaque weakening in the presence of elevated levels of GZMB.

Since PI-9 is thought to confer protection against misdirected GZMB in many tissue types [25], it is important to assess whether an imbalance between PI-9 and GZMB expression during atherogenesis corresponds to increased disease severity and apoptotic activity within the plaque. Hence, the purpose of this study was to assess the expression of PI-9 in human coronary arteries in relation to GZMB expression and apoptotic activity during atherosclerotic lesion progression. Reduced PI-9 expression by VSMC during lesion formation would provide a potential mechanism for increased plaque susceptibility to immune-mediated GZMB-induced apoptosis.

4.2 Results

4.2.1 PI-9 expression by VSMC is reduced with increased atherosclerotic lesion development

PI-9 expression in whole tissue samples of human LAD coronary arteries exhibiting diffuse intimal thickening, pathological intimal thickening and fibrous cap atheroma lesions (Table 1), was determined by western blot analysis using total protein extracted from frozen
samples (Figure 5 A). Recombinant PI-9 was used as a positive control. In agreement with previous studies [24], total PI-9 expression levels were significantly lower in fibrous cap atheromas relative to diffuse intimal thickening arteries (*P<0.01). No significant difference was noted in PI-9 protein levels between diffuse and pathological intimal thickening lesions using this method (Figure 5 B).

Figure 5. Reduced PI-9 expression in human coronary arteries with increasing lesion severity. A. Lysates from tissues samples collected from the same patients screened by immunohistochemistry (Figure 6), were separated on 10% SDS-PAGE gels and immunoblotted using anti-PI-9 Ab. β-actin was used as a loading control. Recombinant PI-9 expressed by COS cells was used as positive control (right panel) for PI-9 Ab. B. Densitometry quantification of the above blot shows a significant reduction in PI-9 expression in fibrous cap atheroma lesions (n=3 for each lesion type)(*P < 0.01).
Since total protein extracts analyzed by western blot cannot be linked to variation in protein expression by any specific cell type, it was essential to determine the expression pattern of PI-9 by VSMC in different stages of lesion development. As medial VSMC and intimal VSMC are known to hold diverse phenotype characteristics [89], we also examined the expression pattern of PI-9 between medial and intimal VSMC. Immunohistochemistry of serial sections of human LAD coronary arteries were stained with anti-PI-9 Ab (Figure 6 A-D) and α-SMA Ab (Figure 6 E-H). In diffuse intimal thickening arteries, PI-9 staining was abundant in the media and was highly associated with medial VSMC. Within the intima of these arteries, PI-9 staining was punctate and localized to α-SMA staining (Figure 6 A, E). In pathological intimal thickening lesions, PI-9 staining was faint and scattered, while α-SMA staining was abundant in the intima (Figure 6 B, F). Within the media of these lesions, PI-9 staining was diffuse and less intense in some vessels (Figure 6 C). α-SMA staining in the media was similar to that observed in diffuse intimal thickening arteries, although in some arteries the medial layer was thinner (Figure 6 G). In the intima of fibrous cap atheromas, weak to no PI-9 staining was observed in the presence of notable α-SMA staining, especially in the shoulder region of the fibrous cap (Figure 6 D, H). Medial staining for PI-9 in these lesions was faint and diffuse, whereas α-SMA staining was prominent (Figure 6 H).
Figure 6. PI-9 and α-SMA expression in intima and media of human coronary arteries with varying stages of atherosclerosis. PI-9 expression was detected by immunohistochemistry (DAB, brown color) in diffuse intimal thickening arteries (intima and media) (A); in pathological intimal thickening lesions in the intima (B) and in the media (C); and in fibrous cap atheroma lesions (intima and media) (D). Images E-H are 5μm serial sections of the corresponding images (A-D) stained for α-SMA (Nova red, red color) indicating the presence of VSMC. H&E insets (x 4 for A-C, x 2 for D) represent the location of the magnified image (x 10) within the artery. Scale bar= 100μm.
To evaluate whether the observed reduction in PI-9 staining with increased lesion severity is due to reduced cellularity or reduced expression by VSMC, staining of PI-9 and α-SMA in serial sections were quantified using ImagePro Plus™. PI-9 staining was significantly lower in both media and intima of pathological intimal thickening (\(^*\)P<0.05) and fibrous cap atheroma lesions (\(^\dagger\)P<0.01) when compared to diffuse intimal arteries (Figure 7 A). No significant difference between the different groups was observed in α-SMA staining in the media and the intima (Figure 7 B). Values for PI-9 staining fraction were then normalized to the corresponding α-SMA staining values in the same area within the matching serial sections to give the expression ratio of PI-9 in VSMC. PI-9 expression ratios were significantly lower in both the media and intima of pathological intimal thickening and fibrous cap atheroma lesions relative to that observed in diffuse intimal thickening arteries (\(^*\)P<0.05) (Figure 7 C). Interestingly, there was a significant lower PI-9 staining ratio between the media and the intima within diffuse and pathological intimal thickening lesions (\(^\#\)P<0.05) (Figure 7 C).
4.2.2 GZMB localizes to VSMC, foam cells and CD8+ cells in human atherosclerotic lesions

Tissue sections were analyzed for GZMB expression. As was observed in previous studies [109], moderate GZMB staining was evident in medial and intimal VSMC in human LAD coronary arteries (Figure 8 A). In the adventitia of several pathological intimal thickening lesions, infiltrating lymphocytes exhibited intense GZMB staining (Figure 8 A, middle panel). Serial sections stained with anti-CD8 Ab revealed that GZMB is localized to CD8 positive cells.
Figure 8. GZMB associates with VSMC, CD8+ and foam cells in coronary arteries with atherosclerotic lesions. A. GZMB expression was detected by immunohistochemistry (DAB, brown color) in arteries with varying lesion stages (top row). Corresponding serial sections were stained for α-SMA (Nova red, red color) indicating the presence of VSMC (bottom row) (x 20) that are associated with GZMB staining (arrows). Lower magnification photomicrograph (x 2) of fibrous cap atheroma lesion, stained for α-SMA is shown in the lower right image. * denotes location of necrotic core. Adventitial infiltrating lymphocytes are indicative of positive control staining for GZMB Ab as depicted in the pathological intimal thickening lesion micrograph (top middle panel). Inset in this image is a representative negative control for GZMB Ab. B. GZMB staining (brown color) is co-localized to CD8+ cells (red color; arrows) in serial sections of fibrous cap atheroma lesion in proximity to the developing necrotic core (upper panel, x 2 magnification; middle and lower panel, x 40 magnification). C. GZMB is localized to foam cells in lipid rich areas within the shoulder region of pathological intimal thickening and fibrous cap atheroma lesions.Insets are lower power (x 20 for intimal thickening; x 2 for fibrous cap atheroma) micrographs representing the location of the magnified images (x 40). * denotes location of necrotic core. Scale bar= 100μm.
4.2.3 **PI-9 expression by VSMC in coronary arteries with increase lesion severity exhibits inverse association to cleaved caspase-3 in the presence of GZMB**

To correlate changes in PI-9 expression levels with incidence of apoptotic events in VSMC, serial sections were immunostained with anti-PI-9 Ab (DAB, brown) and anti- GZMB Ab (DAB, brown), and fluorescently immunostained using anti- α-SMA (Cyanin 3, red) and anti-cleaved-caspase-3 (fluorescein, green) Abs. The latter was used as both an early indicator of cellular apoptosis and as a downstream product of GZMB activity [29, 204, 205]. In diffuse intimal thickening arteries, PI-9 staining was prominent in the media as well as in the intima (Figure 9 A). No cleaved caspase-3 was observed in the confocal images generated from the same field of view (Figure 9 C), although some diffuse GZMB staining was identified (Figure 9 B). In the intima of pathological intimal thickening lesions, areas of low PI-9 staining and positive GZMB staining were associated with positive cleaved-caspase-3 labeling that was partly co-localized to α-SMA, evident as the yellow color in the overlaid confocal image, indicating that these intimal VSMC are undergoing apoptosis (Figure 9 D-F). In fibrous cap atheroma lesions, areas of low to no PI-9 staining and intense GZMB staining within the intima were associated with prominent cleaved-caspase 3 and α-SMA co-localization, indicating a high degree of VSMC apoptosis in these lesions. (Figure 9 G-I).
Figure 9. Reduced PI-9 expression is corresponded to increased cleaved caspase 3 in intimal VSMC and associates with elevated GZMB in varying stages of atherosclerotic lesions. Serial sections were immunostained for PI-9, GZMB (DAB, brown color) using bright field imaging and fluorescently immunolabeled for α-SMA (cyanin 3, red color) and cleaved caspase-3 (fluorescein, green color) using multi-channel confocal imaging. Panels A-C, D-E, G-I represent diffuse intimal thickening, pathological intimal thickening and fibrous cap atheroma lesions respectively. Images C, F, and I are the overlaid confocal images. Co-localization of cleaved caspase-3 with α-SMA is observed as yellow color. Inset in image G is a lower magnification photomicrograph (x 2). * denotes location of necrotic core. Scale bar= 100µm for bright field images. For images C and F scale bar=150 µm. Image I scale bar= 75µm.
4.3 Discussion

In this study, reduced PI-9 expression in human VSMC was associated with atherosclerotic lesion progression and inversely related to the extent of apoptosis within the intima of atherosclerotic plaques. Since VSMC are the most abundant cell type in the arteries and have been previously shown to express PI-9 [24], it was essential to determine whether the observed reduction in PI-9 expression was due to reduced VSMC presence within the growing atheroma, or due to reduced expression of PI-9 by these cells. This study indicates that the observed decrease in PI-9 is due to reduced expression by VSMC and not due to lack of cellularity within the plaque. In agreement with previous observations [109], GZMB was observed in atherosclerotic lesions with levels increasing in arteries with advanced lesions. Importantly, detection of cleaved caspase-3 in intimal VSMC of advanced lesions was correlated with reduced PI-9 expression in the presence of high GZMB levels.

Changes in PI-9 levels are associated with a number of pathologies. Elevated PI-9 expression in tumors is believed to provide a protective mechanism that allows tumor cells to evade immune-mediated insult through inhibition of GZMB-induced apoptosis [26-28]. Clinically, higher PI-9 expression is correlated with unfavorable prognosis in patients with melanoma and anaplastic large cell lymphoma [29, 30], further supporting the anti-apoptotic and pro-survival roles of PI-9 in tumors. Interestingly, reduced PI-9 expression by tubular cells was associated with increased apoptosis in the presence of GZMB-positive T cells in biopsies from patients with acute renal graft rejection [29]. Our findings indicate that alterations in PI-9 expression may play a role in the pathogenesis of atherosclerotic lesion progression. In agreement with previous studies [24], we found a decrease in PI-9 protein levels in advanced lesions relative to earlier lesion stages. Importantly, we were able to determine that the observed
reduction in PI-9 levels was due to reduced expression by medial and intimal VSMC and not due to reduced cellularity. By normalizing PI-9 staining to α-SMA, we observed a significant reduction in PI-9 expression by intimal and medial VSMC in pathological intimal thickening and fibrous cap atheroma lesions relative to diffuse intimal thickening arteries, thus indicating reduced PI-9 expression by VSMC with increased lesion severity. Although the results from the western blot analysis indicated that there was no difference in PI-9 levels between diffuse and pathological intimal thickening lesions, it is important to note that it was not possible to differentiate between specific areas within the artery using whole tissue lysate as total protein levels were derived from all areas of the artery and the surrounding tissue including the adventitia. In several cases, a localized lymphoid nodule was observed in the adventitia of pathological intimal thickening lesions (Figure 8 A, middle panel). Lymphocytes express high levels of PI-9 [25], thus while examining whole tissue lysates by western blot, other cellular sources of PI-9 might have influenced the total amount of protein, resulting in an insignificant difference between these groups. Based on all the experimental data collected in this study, we can conclude that PI-9 expression is reduced in intimal and medial VSMC with the progression of atherosclerotic lesion development.

In addition to differences in PI-9 expression between lesion stages, we also observed variations in PI-9 expression between medial and intimal VSMC in both diffuse and pathological intimal thickening lesions. Alterations in protein expression in intimal VSMC have been suggested to play a central role in the pathogenesis of atherosclerotic lesion development [206]. Migration of medial VSMC into the intima has been proposed as one of the main mechanisms involved in the progression from early lesion to a more severe, stenotic-type lesion [207], although it is now apparent that the origin of intimal VSMC may be derived from diverse
sources, including adventitial fibroblasts and circulating bone marrow-derived stem cells [208, 209]. Once in the intima, VSMC switch from the contractile phenotype to the synthetic phenotype [89]. *In vitro* experiments on animal-derived VSMC revealed several features attributed to the synthetic phenotype, including enhanced proliferative activity, increased extracellular matrix production, changes in lipoprotein interactions and increased proteolytic activity [206]. Interestingly, intimal VSMC from rat arteries exhibit higher sensitivity to apoptotic stimuli, such as reactive oxygen species and retinoic acid [210, 211]. Conversely, medial VSMC isolated from human arteries were heterogeneous in their resistance to Fas-mediated apoptosis, where Fas-sensitive cells exhibited lower expression of proteins that inhibit the Fas-induced apoptotic pathway, including cFLIP and c-IAP-1 [212]. Our observations indicate lower PI-9 levels in intimal VSMC compared to medial VSMC in earlier lesions. Although not directly tested in this study, the observed changes in PI-9 expression between medial and intimal VSMC could be attributed to the phenotypic modulation of intimal VSMC, and may imply an additional mechanism enhancing intimal VSMC sensitivity to apoptosis.

Apoptosis of VSMC plays a key role in determining plaque vulnerability and can influence plaque progression [101]. Using the transgenic human diphtheria toxin receptor/ApoE KO mouse model, Clark *et al.* demonstrated that both acute and chronic induction of VSMC apoptosis resulted in reduced fibrous cap size, increased necrotic core and induced focal cap accumulation of macrophages and cellular debris [102, 103], all of which are common features of rupture-prone plaques in humans [104-106]. However, the physiological mechanisms that promote VSMC apoptosis during atherogenesis are not completely understood. Since PI-9 is a key cellular regulator of GZMB-induced apoptosis it was important to examine whether the observed reduction in PI-9 expression by VSMC is correlated to increased apoptotic activity in
the presence of GZMB. Our observations revealed high levels of PI-9 expression in VSMC of diffuse intimal thickening arteries with no evidence of activated caspase-3 in these cells in the presence GZMB. On the other hand, in pathological intimal thickening lesions, and more significantly in fibrous cap atheroma lesions, intimal VSMC showed reduced to no PI-9 expression and higher incidence of cleaved caspase-3 that was associated with elevated levels of GZMB within these lesions. Although our results suggest that reduced PI-9 expression in VSMC may increase susceptibility to GZMB -induced apoptosis, additional experimental approaches should be undertaken to establish a direct cause and effect relationship between changes in PI-9 levels and induction of VSMC apoptosis by GZMB in the context of atherogenesis. A limitation of this study is the relative small sample size, especially of the fibroatheroma cases, where future studies on larger patient cohorts will assist in clarifying this potential imbalance between GZMB and PI-9 in atherosclerosis disease progression. Moreover, it is important to note that the observed VSMC apoptosis could also be attributed to other pro-apoptotic mechanisms such as receptor-mediated cell death, oxidative stress and mechanical stimuli [100]. As reduction in PI-9 expression in other cell types, including immune cells, tumor cells, liver cells and tubular cells has been found to increase susceptibility to GZMB -induced apoptosis [22, 29, 213, 214], it is likely that reduced PI-9 expression in intimal VSMC may predispose these cells to GZMB -induced apoptosis in atherosclerotic lesions.

In summary, we have demonstrated changes in the expression levels of GZMB and its endogenous inhibitor, PI-9, with the progression of atherosclerotic lesion development. The reduction in PI-9 expression in intimal VSMC was inversely correlated with active caspase-3, an indicator of apoptosis within the intima. Decreased expression levels of PI-9 in the presence of GZMB in advanced lesions may represent a new mechanism that can increase VSMC
susceptibility to GZMB-induced apoptosis. Thus, this work highlights the imbalance that exists between GZMB and PI-9 as an underlying mechanism that may contribute to atherosclerotic lesion progression.
5. Extracellular GZMB cleavage of FN disrupts endothelial cell adhesion, migration and capillary tube formation

5.1 Introduction

Angiogenesis, the formation of new capillaries from pre-existing vessels, is a tightly regulated process that is critical for normal development, ensuring adequate blood supply to the target tissue [113]. However, dysregulated angiogenesis, which refers to the formation of unstable, incomplete and immature neovessels, plays a pivotal role in several pathological conditions including cancer and other chronic inflammatory diseases [112, 144]. The formation of immature, unstable capillaries promotes inflammation by enabling immune cell infiltration across the endothelium. Conversely, formation of a stable capillary network is required for adequate resolution of inflammation and initiation of repair processes [215]. As such, properly orchestrated angiogenesis during inflammation is considered a key determinant for the progression of numerous chronic inflammatory disorders [145].

The previous chapter provided evidence for an imbalance between PI-9 and GZMB in atherosclerotic plaques, which may promote VSMC apoptosis. As PI-9 is an intracellular inhibitor of GZMB it may not inhibit extracellular GZMB activities. GZMB accumulates in the ECM in a number of chronic inflammatory diseases that are highly associated with dysregulated angiogenesis [41, 71]. While emerging evidence suggests that extracellular GZMB activity can impair tissue integrity, the direct consequences of GZMB-mediated ECM cleavage on the angiogenic process has yet to be explored.

The interaction of EC with the surrounding ECM, in concert with tightly regulated pericellular proteolysis, are critical for neovessel formation [130, 151, 197]. Initial proteolysis of
basal lamina proteins and controlled breakdown of surrounding ECM is required to liberate EC, allowing capillary sprouting into the interstitium [113, 116]. Subsequent interaction between ECM proteins that contain cell binding domains, namely RGD domains, with integrin receptors on EC cell surfaces facilitate EC adhesion and migration to support the correct alignment of EC to form functional capillaries [216].

FN is an important ECM protein that has a central role in regulating vascular morphogenesis [217]. During inflammation, FN accumulates in the affected area due to leakage from capillaries. Once incorporated in the ECM, FN undergoes a conformational change, exposing matricryptic RGD binding domains that facilitate cell adhesion and migration as part of the provisional matrix formation [117, 128]. Previous studies suggest that GZMB cleaves FN at the RGD binding domain, implying that GZMB-mediated FN proteolysis may alter cell-matrix interactions [60]. Indeed GZMB induces detachment and anoikis in EC, SMC and fibroblasts [60, 61, 218]. However, during the angiogenic process, detachment of EC from a quiescent monolayer and destabilization of the basal lamina is required in order to initiate EC sprouting and migration [113, 116, 153]. Moreover, proteolytic alteration of the pericellular matrix may expose cryptic domains in FN that further support or inhibit EC angiogenesis [152, 197]. Since immune-derived proteases are believed to regulate the angiogenic response during inflammation through ECM processing [150], it is important to explore the effects of GZMB-mediated FN cleavage on EC angiogenic behavior. The current study demonstrates that GZMB cleavage of FN impairs EC adhesion, migration and capillary tube formation.
5.2 Results

5.2.1 GZMB cleaves plasma and endogenous FN

To test whether GZMB cleaves plasma FN in its matrix form, plates were coated with human plasma FN and treated with increasing concentrations of GZMB. Western blot analysis of the supernatants identified a number of cleavage fragments that appear at greater intensity with increasing GZMB concentration, indicating that GZMB-mediated FN cleavage is dose-dependent (Figure 10 A). Pre-incubation of GZMB with Compound 20 [201] reduced the appearance of the FN cleavage fragments, confirming that GZMB is responsible for generating the observed FN fragments.

FN can be produced locally within the tissue by a large number of cells including SMC, fibroblasts and EC [128, 219, 220]. However, cells may produce a different splice variant of FN that differs from plasma FN [221]. Moreover, FN multimerization within the matrix is dependent on interaction with cells and other components of the ECM [129, 222]. Thus, it was important to examine whether GZMB can cleave endogenous FN from cellular-derived ECM. As indicated in Figure 10 B and C, supernatants from GZMB treated ECM exhibited FN fragments that were absent in control wells, indicating that GZMB cleaves and releases FN fragments from both EC and SMC-derived ECM.
Figure 10. GZMB cleaves plasma FN and cell-derived FN. A. Culture wells were coated with 20 µg/ml human plasma FN. Increasing concentrations of GZMB were added to FN coated wells, with or without GZMB inhibitor (Compound 20) and incubated for 24 h at RT. Supernatants were removed from the wells and FN fragments in the supernatants were detected by western blotting. B. HMVEC and C. Human SMC, were grown to confluence and maintained in serum-reduced media for 7 d. Cells were removed from the culture wells by adding NH4OH followed by extensive washing. ECM was then treated with GZMB for 24 h at RT. Supernatants were removed from the wells and FN in the supernatants was detected by western blotting.
5.2.2 GZMB-mediated cleavage of FN reduces endothelial cell adhesion

To explore the effect of GZMB-mediated cleavage of FN on EC adhesion, FN coated wells were treated with increasing concentrations of GZMB for 24 h. After inhibiting GZMB through the addition of Compound 20 to all experimental groups, EC were added to the wells and were allowed to adhere. GZMB treatment resulted in a dose dependent decrease in EC adherence to FN-coated wells (Figure 11). This decrease reached significance (P<0.05) when GZMB was used at 50 nM and 100 nM concentrations. Pre-incubation of 100 nM GZMB with Compound 20 restored EC adhesion to FN-coated wells, while treating the wells with the inhibitor alone resulted in no difference in cell adhesion compared to controls. Non-specific adherence of cells to the wells was negligible in these experimental conditions as only low number of cells adhered to wells that were coated with 5% BSA (Figure 11).
Figure 11. GZMB-mediated FN cleavage reduces endothelial cell adhesion. A. Representative images (x 4) of adherent cells on FN-coated (20 µg/ml) wells pre-treated with increasing concentrations of GZMB with or without Compound 20 (Inhibitor) (50µM) and with Compound 20 alone. B. Quantification of adherent cell number. Results are presented as mean± SEM of n=3 with three triplicates per condition (*=P<0.05). Pre-coated wells with 5% BSA were used as a negative control.

5.2.3 GZMB-mediated cleavage of FN reduce endothelial cell migration

The role of FN cleavage by GZMB on EC migration was determined using a haptotaxis migration assay to assess the directional movement of the cells according to a gradient of surface bound matrix molecule [223]. To achieve this gradient, transwell membranes were coated with FN only on the underside and then treated with 50 nM GZMB for 24 h at RT. Although FN cleavage occurs at lower concentrations of GZMB (Figure 12), 50 nM GZMB was used as it was the lowest concentration that resulted in a significant difference in the cell adhesion experiments.
(Figure 11). GZMB pre-treatment of FN-coated membranes resulted in a significant reduction (P<0.05) in EC migration to the underside of the membrane (Figure 12 A,B). Inhibiting GZMB restored EC migration while the inhibitor alone had no effect on migration compared to controls (Figure 12 B). No cell migration occurred when the transwell membranes were coated with 5% BSA (data not shown).

Since FN fragments may directly influence cell migration [224], we tested whether GZMB-generated FN fragments possess chemotactic properties. No difference in EC migration was observed between control wells and wells that were treated with FN fragments generated by GZMB. As expected, more cells migrated toward wells treated with VEGF (Figure 12 C). GZMB inhibitor alone had no affect on EC migration.

Although GZMB concentration was diluted in the lower chamber prior to the addition of EC to the top chamber, there was a possibility that GZMB could act directly on the cells to prevent cell migration. For this reason, we performed an additional experiment where the underside of the transwell membranes were coated with either FN or collagen I. Since collagen I is not cleaved by GZMB [13, 60, 61], any change in EC migration in the collagen I coated wells would indicate a direct effect of GZMB on the cells. While FN-coated membranes treated with GZMB resulted in a significant reduction in EC migration, no difference was observed between control and GZMB treated membranes coated with collagen I (Figure 12 D). This observation indicates that the reduction in EC migration in the GZMB treated groups is substrate-dependent and it is not due to the direct effect of GZMB on EC.
5.2.4 *GZMB disrupts capillary tube formation in 3D collagen gels*

To further understand the effect of GZMB-mediated cleavage of FN on EC formation of 3-dimensional capillary structures, we employed a 3-dimensional collagen gel tube formation assay. EC were cultured between two layers of collagen gels containing FN and treated with...
GZMB, GZMB + Compound 20 or the inhibitor alone. After 12 h, tubular structures were evident in the gels. To confirm the 3-dimensional characteristic of the observed tubular structures, gels were incubated with fluorescently labeled phalloidin that binds F-actin within EC and imaged with multiphoton microscopy. This technique avoided any sectioning of the gels and allowed in situ imaging to be performed, due to the high resolution and scanning penetration of up to 400 µm into the gels. The resulting 3D images confirmed the three-dimensional nature of the observed tubular structures (Figure 13 A). Gels that were treated with GZMB had tubules that were shorter with reduced connections compared to the other treatment groups (Figure 13 A). To quantify this effect, lower magnification (x 4) phase contrast microscopy images were used to measure total tube length and branching points throughout the entire gel (Figure 13 B). Gels that were treated with GZMB had significantly shorter (P<0.01) total tube length and reduced branching (P<0.05) compared to controls, whereas incubation of GZMB with the inhibitor resulted in no difference in tube length and branching compared to controls (Figure 13 C,D). Incubating gels with the inhibitor alone had no effect on tube formation. To further confirm that the reduced tube formation in the GZMB treated gels was related to GZMB-mediated FN cleavage, supernatants from the gel culture were analyzed by western blot to detect FN fragments. Supernatant samples from FN-coated culture plates that were treated with or without GZMB were used as a positive control. Although several bands of FN fragments were detected in the gel culture supernatants from all treatment groups, only GZMB treated gels exhibited a lower molecular weight (approximately 60 kDa) band similar to the band that was produced in the FN-coated wells treated with GZMB (Figure 13 E). Overall, these data show that GZMB cleaves FN within the collagen gel leading to reduced EC tube formation.
Figure 13. GZMB disrupts endothelial cell tube formation. A. 3D reconstruction of capillary tube structures in collagen gels stained with fluorescently labeled phalloidin and imaged by multiphoton microscopy (x 20). B. Representative low magnification (x 4) phase contrast images of tubules formed in 3D collagen I gels treated with either GZMB (50 nM), GZMB+ Compound 20 (Inhibitor) (50 µM) and GZMB inhibitor alone. C. Quantification of total tube length and D. branch points, per each treatment group. Results are presented as mean+ SEM of n=3 with three triplicates per condition (**=P<0.01; *=P<0.05). E. Western blot of supernatants from the tube formation assay immunoblotted for FN. Supernatants from FN coated wells treated with either culture media or media with GZMB (50 nM) were used as positive control.
5.3 Discussion

FN plays an important role in regulating new blood vessel formation [217]. However during inflammation, FN proteolysis can alter this role thereby altering angiogenesis [150, 197]. In the present study we demonstrated that GZMB cleaves both plasma-derived and cellular-derived FN. Cleavage of FN resulted in reduced EC adhesion, migration and capillary tube formation, which was partially restored with a specific GZMB inhibitor. These observations suggest that GZMB disrupts EC neovessel formation through altering the ECM microenvironment.

The susceptibility of FN to proteolysis [117, 139] as well as the identification of FN fragments in bodily fluids during chronic inflammatory diseases [136-138] suggests that FN proteolysis during inflammation serves as either a regulatory or a disruptive event that can impact vascular morphogenesis [152]. Accumulation of extracellular GZMB in chronic inflammatory disorders that are associated with disruptive neovascularization, including atherosclerosis, abdominal aortic aneurysm and rheumatoid arthritis [41, 71], has led to the present investigations into the specific effects of GZMB-mediated cleavage of FN on EC angiogenic potential.

Similar to previous studies [60], the present study found that plasma FN is cleaved by GZMB in a dose-dependent manner resulting in the generation of dissociated FN fragments in the supernatant. Since FN is also produced locally by resident cells, in different isoforms containing the EDA and/or EDB domains [128, 219, 220], we tested whether GZMB cleaves FN from endogenously produced FN. GZMB cleaved both SMC- and EC-derived FN suggesting that compensatory production of FN by resident cells is susceptible to GZMB degradation. An earlier study by Buzza et al. [60] suggested that the FN fragments generated by GZMB are
cleaved after AspEE149EE, located at the RGD cell-binding domain. This domain is essential for integrin receptor binding, namely α5β1 and αvβ3, facilitating EC adhesion and migration [225-227]. In our study we observed bands at 120 kDa and 100 kDa that correspond to FN fragments that contain the cell binding domain [119, 132], further supporting the previous suggestion by Buzza et al. However, the appearance of lower molecular weight fragments including a 60 kDa band suggests that there are multiple GZMB cleavage sites on FN. Further sequencing is required to determine the specific GZMB cleavage sites in FN.

Previous studies indicated that FN fragmentation may either support [132, 133] or prevent [134, 135] EC migration and proliferation. Other investigations have focused on the role of specific FN fragments on cell behavior showing distinct biological activities that differ from the intact FN protein [224, 228, 229]. In this study, we explored the net effect of FN fragmentation by GZMB on EC angiogenic behavior. We included all cleavage products and/or any intact protein that remained after GZMB processing of FN. In this way we were able to establish a direct cause and effect relationship between GZMB activity on FN and its overall effect on EC interaction with FN; an environment that would be more similar to that which occurs in vivo. GZMB-mediated cleavage of FN resulted in a dose dependent reduction in EC adherence to FN. Previous studies showed that GZMB induces EC, SMC and fibroblast cell detachment and anoikis through cleavage of the ECM, including cleavage of FN [60, 61, 218]. It is important to note that GZMB treatment in these studies was applied to an established cell monolayer. In the context of angiogenesis, EC detachment from the basement membrane is an initiating factor that is required to allow EC sprouting and migration and is dependent on the ability of detached EC to adhere to the surrounding matrix [113, 116, 153]. Further, FN proteolysis exposes biologically active cryptic domains that promote cell adhesion and migration
[140, 152]. Therefore, we examined whether GZMB-mediated FN cleavage alters the interaction of detached EC to FN. Our results expand on previous studies indicating that GZMB cleaves FN to an extent that free EC are not able to adhere to FN. Thus, GZMB may have a role in both disrupting existing vessels by promoting cell detachment as well as a role in modulating the ECM in a way that prevents EC adhesion.

Incorporation of FN in the provisional matrix supports EC haptotactic migration. This form of migration describes cell movement down a gradient of immobilized ECM protein, a process that is key for EC sprouting [223]. GZMB cleavage of FN exhibited a significant reduction in EC haptotactic migration. It has been speculated that GZMB-mediated ECM cleavage may result in the generation of bioactive fragments with chemotactic properties [71]. For that reason, we assessed whether FN fragments generated by GZMB could induce EC chemotaxis. No chemotactic effect was observed in collagen I coated membranes in comparison to VEGF treatment, used as positive control. Collagen I coating was chosen as opposed to FN coating in this experiment as it allowed any FN receptors on EC to be free and available to interact with FN fragments in the media. These results suggest that GZMB-mediated FN cleavage disrupts EC migration over the intact FN protein while the resultant FN fragments do not contribute to this process. However, whether GZMB mediated-FN fragments are chemotactic for other cell types such as inflammatory cells cannot be ruled out.

In order to form functional capillary structures, EC must coordinate in a 3D configuration and establish specific cell-cell interactions. The ECM has a major role in regulating this process by providing key molecular and mechanical signals [130] and FN is essential for EC morphogenesis and formation of capillary tubes [129]. Interestingly, capillary tubes formed by human EC cultured in collagen I and FN 3D matrices were able to functionally integrate with the
mouse microvasculature after implantation [131]. While 3D in vitro cultures lack the inherent complexity of in vivo angiogenic models, they do allow to explore specific molecular events that regulate capillary morphogenesis, in particular the examination of EC-matrix interactions that may not be directly analyzed in vivo [130]. Using multiphoton microscopy we were able to confirm the 3D characteristic of the capillary tube structures formed by EC cultured in 3D collagen I and FN gels. GZMB treatment impaired tube formation giving rise to shorter, less branched tubes, while inhibiting GZMB activity prevented this impairment, suggesting that GZMB processing of the ECM disrupted EC tube formation. To further support the role of GZMB-mediated FN cleavage in disrupting EC tube formation, we examined the supernatants from these cultures for evidence of FN fragmentation. Although all cultures presented evidence of FN fragmentation, only GZMB treated cultures contained a distinct ~60 kDa FN cleavage fragment. The latter fragment corresponded to the FN fragment generated by GZMB treatment of FN coated wells. FN fragmentation in the other samples is likely be due to the activity of other proteases that contribute to EC tube formation [153]. Nevertheless, the correlation between the appearance of a distinct ~60kDa FN fragment and the reduced tube formation after GZMB treatment suggests that GZMB cleavage of FN impairs EC tube formation in this assay.

Increased extracellular GZMB accumulation in the interstitium and in bodily fluids has been observed in a large number of chronic inflammatory diseases. This has promoted a change in focus regarding our understanding of the pathological role of this enzyme in disease; from its pro-apoptotic intracellular role to its role in modulating the extracellular environment in disease [13, 41, 71, 230]. The present study provides evidence for a novel role for GZMB in regulating EC angiogenic potential through degradation of FN, a process that may impair neovascularization. We propose that the inability of EC to effectively interact with the matrix
due to GZMB activity will result in the formation of incomplete, unstable, less branched and leaky neovessels, which are hallmarks of pathological angiogenesis [144, 150]. Interestingly, GZMB is abundant in a number of chronic inflammatory disorders, including RA, atherosclerosis, AAA, cancer and skin lesions, in which aberrant neovascularization is perceived as a mechanism that contributes to the persistent chronic inflammatory state [145]. Moreover, GZMB expression in proximity to microvessels in both skin lesions [231] and AAA [78], as well as the high affinity of GZMB to the anionic, glycosaminoglycan-rich ECM [13], positions this enzyme in an ideal location to disrupt vascular morphogenesis and neovascularization. As interactions between EC and the ECM may be disrupted by GZMB activity, elevated GZMB activity could exacerbate inflammation by promoting the formation of unstable and immature capillaries. Importantly, the discovery of GZMB expression and release from mast cells within the skin and the effect of GZMB on disrupting EC junctions [218] further supports a role for this enzyme in regulating microvascular response in concert with other vascular regulators that are released by mast cells during inflammation.
6. The effect of GZMB on VEGF bioavailability and its impact on vascular permeability

6.1 Introduction

Increased vascular permeability is one of the earliest manifestations of inflammation resulting in extravasation of protein-rich plasma into the effected tissue. Acute vascular permeability allows the deposition of circulating plasma matrix proteins including fibrin and FN which facilitate cell migration in the inflamed area. This process also provides an access point for immune cells and immunoglobulins to enter the tissue and help fight foreign antigens [232]. In contrast, chronic vascular hyperpermeability is suggested to sustain the inflammatory response and retard resolution, further promoting the development of chronic inflammation [145, 232]. This type of vascular hyperpermeability underlies the pathogenesis of a large number of chronic disorders including RA, psoriasis, ocular disease, cancer and chronic wounds [145, 232].

VEGF is a potent vascular permeabilizing agent that is highly expressed during chronic inflammation [179]. VEGF induces vascular leakage by activating VEGFR2 in EC leading to the opening of intercellular and/or intracellular pathways that facilitate plasma extravasation [156]. Cellular sources for VEGF during inflammation may include macrophages and mast cells however it may also be expressed by EC and may act in a paracrine and autocrine fashion [158].

The ECM has a major role in regulating VEGF bioavailability. VEGF contains a cluster of basic residues that facilitate the interaction with anionic ECM proteins [168]. VEGF interaction with the ECM greatly determines its bioavailability as the majority of VEGF is retained in the ECM after cell secretion [168]. Several proteases are suggested to alter VEGF interaction with the ECM including plasmin [168] and MMPs [170, 173], giving rise to increased
microvessel leakage and formation of aberrant neovasculature that is characteristic of pathological angiogenesis [170]. Thus, VEGF that is sequestered in the ECM can be liberated by proteases and this process has significant implications on vascular integrity. Importantly, VEGF binds the growth factor binding domain in FN (type III 12-14; Figure 3) [125, 126, 177]. Binding of VEGF to FN enhances EC migration and proliferation, and has been shown to promote angiogenesis [125, 177, 178]. However, the consequences of VEGF release from FN by a specific protease and its impact on vascular integrity has yet to be explored.

In the previous chapter we demonstrated that GZMB can effectively cleave FN to multiple fragments leading to reduced EC migration and impaired capillary tube formation. Given that FN binds VEGF, we hypothesized that GZMB-mediated FN cleavage will release VEGF from the ECM and promote vascular leakage. Thus, the purpose of this study is to examine the role of GZMB in altering VEGF bioavailability and explore its impact on vascular permeability in vivo.

6.2 Results

6.2.1 GZMB releases VEGF from plasma FN

To examine the ability of GZMB to release VEGF from FN, culture plates were coated with human plasma FN followed by addition of VEGF. VEGF was effectively bound to FN as only small amount of VEGF spontaneously dissociated to the supernatant in the control group (37.72 pg/ml ± 23.95) (Figure 14). However, GZMB treatment elicited significant release of VEGF to the supernatant (350 pg/ml ± 61.35; P<0.001). The release of VEGF was dependent on GZMB activity as co-treatment with GZMB inhibitor (Compound 20) significantly reduced
VEGF release (147 pg/ml ± 8.3; P<0.05). Thus, GZMB promotes the release of VEGF from plasma FN.

![Figure 14](image)

Figure 14. GZMB releases VEGF from FN. VEGF (50 ng/ml) was added to FN coated wells and incubated for 2 hrs at 37°C. Unbound VEGF was removed by washing with PBS. GZMB (50 nM) with vehicle control (DMSO) or GZMB with inhibitor (Compound 20, 50 µM) were added to the wells and incubated for 2 hrs at 37°C. Supernatants were removed and analyzed for VEGF by ELISA. n=3 with three replicates per treatment (*P<0.05; ** P<0.01).

6.2.2 GZMB releases VEGF from HUVEC matrix

In the previous chapter we demonstrated that GZMB can cleave both plasma and cell derived FN. Since the endogenous cellular matrix is more complex and may contain other pericellular matrix proteins that can bind VEGF [124] it was essential to explore whether GZMB could mediate VEGF release from endogenous matrix. VEGF was added to HUVEC-derived matrix and unbound VEGF was removed by washing. Similar to experiments done on plasma FN, GZMB treatment significantly increased VEGF release from HUVEC matrix compared to
control (control 135pg/ml ± 33.66; GZMB 562pg/ml ± 39.86; P<0.01) (Figure 15). Inhibition of GZMB effectively reduced GZMB-mediated VEGF release (275pg/ml ± 63.8; P<0.05). Thus, GZMB promotes VEGF release from both plasma FN and from endogenously produced EC matrix.

Figure 15. GZMB releases VEGF from HUVEC matrix. HUVEC were grown to confluence and maintained in serum-reduced media for 9 d. Cells were removed by adding NH4OH followed by extensive washing. Remaining ECM was incubated with VEGF (50 ng/ml) for 2 hrs at 37°C. Unbound VEGF was removed by washing with PBS. GZMB (50 nM) or GZMB with inhibitor (Compound 20, 50 µM) were added and incubated for additional 2 hrs at 37°C. Supernatants were removed and analyzed for VEGF by ELISA. n=3 with three replicates per treatment (*P<0.05; ** P<0.01).

6.2.3 GZMB-mediated VEGF release from FN activates VEGFR2

VEGFR2 dimerizes and undergoes rapid transphosphorylation in a number of tyrosine residues upon ligation with VEGF [156]. We were determined to examine whether VEGF that is released from FN by GZMB retains its activity. HUVEC were treated with VEGF release
supernatants, generated as in Figure 14 and VEGFR2 activation was examined by immunoblotting for the phosphorylation of Y1214 and Y1175. Even small amounts of VEGF that spontaneously dissociate from FN activate VEGFR2 as control supernatants show distinct signal for both Y1214 and Y1175 phosphorylation, while HUVEC treated with media containing no VEGF (-ve control) show no signal. However, VEGF release supernatants that were generated due to GZMB activity caused a significant increase in VEGFR2 phosphorylation in both Y1214 and Y1175 (Figure 16). VEGF released from FN treated with GZMB + inhibitor leads to attenuated VEGFR2 activations. Thus, VEGF that is released from FN by GZMB is active and it can effectively lead to VEGFR2 phosphorylation.
Figure 16. GZMB-mediated VEGF release from FN activates VEGFR2. FN coated wells were incubated with VEGF and treated with GZMB or GZMB with inhibitor as in Figure 14. Supernatants were removed and added to HUVEC monolayer culture for 7 min. Cell lysates were analyzed by western blotting using A. phospho Y1214 VEGFR2 Ab (pVEGFR2 Y1214) and B. phospho Y1175 VEGFR2 Ab (pVEGFR2 Y1175). Total VEGFR2 and β-tubulin Abs were used as loading controls. Quantification is presented as the densitometry ratio of pVEGFR2 to total VEGFR2 normalized to β-tubulin. n=3 with three replicates per treatment (*P<0.05).

6.2.4 GZMB -mediates VEGF dependent vascular permeability in vivo

To test the impact of GZMB-mediated VEGF release on vascular leakage we used the Miles assay by injecting mouse GZMB (Gzmb) to the ear and measured Evan's blue extravasation as described in the Material and Methods. To minimize the background of Evan's blue dye that is present in the circulation and did not extravasate we ensured to perfuse the animal with large volume of saline (10ml) prior to analyzing the tissue. Vascular leakage at the tip of the ear was due to holding the ear with forceps to stabilize the ear during injections. Only the injection area at the base of the ear was excised with 7 mm punch biopsy for subsequent dye extraction. Absorbance of the extracted dye was normalized to tissue weight. Gzmb injection resulted in increased vascular leakage compared to saline injections (n=5) (Figure 17 A). To further establish whether the increase in vascular leakage is dependent on VEGF activity we co-injected Gzmb with anti-mouse VEGF Ab. Vascular leakage was significantly reduced when Gzmb was injected with VEGF Ab compared to Gzmb + IgG control injections (n=5) (Figure 17 B). Thus, Gzmb leads to VEGF dependent increase in vascular leakage in vivo.
Figure 17. Gzmb induces a VEGF-dependent increase in vascular permeability in vivo. Evan’s blue (0.5%) was injected via the tail vein prior to ear injections of A. mouse granzyme B (Gzmb) (100 ng) or saline control or B. Gzmb with anti-mouse VEGF Ab (1.5 µg) or Gzmb and IgG control Ab (1.5 µg). Ear tissue was excised as indicated using a 7 mm punch biopsy (areas in circle). Evan’s blue extraction was performed by drying the tissue and immersing it in formamide at 55oC for 24 hrs. Results are presented as absorbance (610nm) normalized to tissue weight (mg). (n=5 for each experimental group; *P<0.05).

6.3 Discussion

The ECM has a major role in regulating growth factors bioavailability by sequestering them and limiting their release [124]. VEGF is a potent vascular permeability agent that is retained in the ECM by binding to FN [125, 126, 177]. In this study we have demonstrated that GZMB increases VEGF bioavailability by releasing it from both FN and endogenous EC matrix.
GZMB-mediated VEGF release leads to activation of VEGFR2 in HUVEC. Importantly, we demonstrated that GZMB mediates vascular leakage \textit{in vivo} in a VEGF dependent way as co-treatment of GZMB and anti-VEGF Ab reduced vascular leakage. Thus, GZMB regulates VEGF bioavailability by cleaving FN and promoting vascular leakage \textit{in vivo}.

A number of proteases were previously shown to alter VEGF-matrix interaction with significant consequences to vascular morphogenesis. Houck \textit{et al.} were the first to demonstrate that VEGF expressing cells treated with plasmin gave rise to a VEGF fragment that dissociates to the supernatant and is capable of inducing vascular leakage in guinea pig skin [168]. MMP-3 generates an unbound VEGF fragment that promotes the formation of aberrant, dilated and leaky neovessels [170]. On the other hand, MMP-resistant, matrix bound VEGF promoted smaller diameter and highly branched neovessels [170]. These studies highlight the fact that VEGF activity is regulated by its interaction with the ECM, while proteolytic processing of VEGF and its dissociation from the matrix promote vascular permeability and disrupt normal neovessel formation [169]. Although our study clearly demonstrates that GZMB can mediate VEGF release from the ECM, it is yet to be determined whether GZMB cleaves VEGF intramolecularly, giving rise to active VEGF fragments. Alternatively, GZMB may promote VEGF release by direct cleavage of FN, liberating the VEGF binding domain on FN from the rest of the molecule. Relying on our finding that GZMB cleavage of FN results in the release of multiple FN fragments [233] we speculate that VEGF release from the ECM is dependent on FN processing by GZMB, however this assumption requires further validation. Nevertheless, VEGF that is released from the ECM by GZMB retains its activity as signified by the increased phosphorylation of VEGFR2.
Changes in the microenvironmental levels of VEGF due to ECM processing may serve as a key mechanism for the induction of pathological angiogenesis in disease. In an elegant study by Ozawa et al. it was demonstrated that local low levels of VEGF expression is critical for the formation of stable, non-leaky neovessels, where local high levels of VEGF expression results in enlarged, unstable, leaky capillaries that are characteristic of pathological angiogenesis [196]. MMP-9 and MMP-2 induce tumor angiogenesis [174] as well as promote inflammatory angiogenesis by releasing VEGF [173]. Moreover, these proteases were shown to promote ovarian ascites formation due to increased VEGF-dependent vascular leakage [234]. However, the role of MMPs in inducing angiogenesis has been challenged by observations suggesting that MMPs may be involved in neovessel regression due to the formation of anti-angiogenic agents via ECM proteolysis and by destabilizing EC-matrix interaction through ECM processing [235-238]. Thus, it has been suggested that MMPs serve as local modulators rather than directly induce or suppress angiogenesis [170]. Similarly, GZMB processing of the ECM disrupt EC-matrix interaction which may pertain to its anti-angiogenic role [233]. The current study may suggest that increased VEGF release by GZMB may potentially serve to induce angiogenesis. Although the direct effect of GZMB-mediated VEGF release on angiogenesis has not been examined in this study we suspect that similar to MMPs GZMB may serve as a modulator of the angiogenic response. However, in contrast to MMPs, which play a major role in normal tissue remodeling and homeostasis, elevated GZMB levels are only evident in chronic inflammatory disorders, most of which are highly associated with pathological angiogenesis, including RA, atherosclerosis, AAA and skin pathologies [41, 71]. Given that GZMB increases VEGF bioavailability and induces VEGF-dependent vascular permeability, a hallmark of pathological angiogenesis, we postulated that GZMB may contribute to the formation of aberrant, unstable
and leaky neovessels through ECM proteolysis and by altering microenvironmental levels of VEGF.

Cumulative evidence supports the role of extracellular GZMB in altering tissue integrity and promoting inflammation through a number of mechanisms. Extracellular GZMB potentiates IL-1α activity by cleaving it and generating an IL-1α fragment that enhances inflammation [64]. We have previously demonstrated that cleavage of fibrillin-1 and decorin by GZMB contributes to AAA rupture in mice [78, 79], while decorin degradation in the skin contributes to skin thinning and frailty [77]. Recently we have demonstrated that GZMB may also have an indirect effect on tissue remodelling as GZMB promotes the release of active TGF-β from the ECM by cleaving a number of proteoglycans [62]. The current study further corroborates the role of extracellular GZMB in altering growth factors and cytokines bioavailability. Importantly, we discover a novel role for GZMB in promoting vascular leakage through VEGF release, a process that may support the development of inflammation. VEGF is a proinflammatory cytokine that was shown to promote increased T cell infiltration and epidermal hyperplasia during the development of psoriatic-like lesions in mice [239], while up regulation of VEGF in RA synovial tissue induces leukocyte recruitment and promotes inflammation [240]. Since GZMB is highly evident in both of these pathologies [71], it is likely that GZMB may contribute to chronic inflammation by increasing VEGF bioavailability. However, further work will be required to examine the role of GZMB-mediated VEGF release on immune cell recruitment.

Interestingly, previous studies from our laboratory and others suggest that mast cells are a major source of GZMB in the early stages of inflammation [77, 218]. Extracellular release of GZMB from mast cells contributes to cell detachment and disorganizes EC intercellular adherens junctions as a mechanism that may induce vascular leakage [218], however evidence for GZMB-
mediated vascular leakage has not been provided in the later study. Although we cannot rule out the possibility that GZMB may contribute to vascular leakage by its direct effect on EC in our *in vivo* experiments, the reduction in vascular leakage in mice that were treated with GZMB + anti VEGF Ab indicates that GZMB-mediates vascular permeability predominantly through altering VEGF bioavailability.

In summary, extracellular GZMB releases VEGF from the ECM by cleaving FN and promotes vascular permeability *in vivo*. This process may serve as a novel mechanism that promotes chronic inflammation in diseases where GZMB is evident while strategies aiming to inhibit GZMB activity may attenuate vascular leakage and reduce the inflammatory response.
7. Future directions

The journey of discovery begins with a question. Although there are no guarantees for the type of answers one will find, this process ultimately gives birth to further questions that serve as a platform for future research in this field. As such, I have outlined a number of questions that arise from this work and proposed potential experimental approaches that may be used to answer them.

What are the mechanisms that regulate PI-9 expression in atherosclerotic plaques?

Findings from the work presented in chapter 4 suggest that PI-9 expression in VSMC is reduced during atherogenesis [241]. Reduction in PI-9 may increase VSMC susceptibility to GZMB-induced apoptosis. For that reason it will be essential to understand the mechanisms that contribute to the observed reduction in PI-9 expression in atherosclerotic plaques. Modifying these mechanisms may be used to increase VSMC survival by increasing PI-9 expression as a strategy to promote plaque stability.

PI-9 is regulated by NF-κB and AP-1transcription factors [31], both of which are activated during inflammation [242, 243]. Moreover, PI-9 expression is increased in response to pro-inflammatory cytokines including IL-1β, TNF-α and IFN-γ [31, 32]. These observations generate an interesting paradox; since the atherosclerotic plaque is an inflamed tissue with elevated levels of pro-inflammatory agents, the expression of PI-9 should have been increased and not decreased as our study indicates. To address this issue we refocused the question and asked; what atherosclerotic specific factors may alter PI-9 regulatory pathways in this disease? We hypothesized that ox-LDL particles, which are abundant in the intima during atherosclerosis, may either suppress PI-9 expression and/or may interfere with the regulatory pathways that
promote PI-9 expression. This hypothesis relies on a number of studies showing a role for ox-LDL in modulating AP-1 and NF-κB signaling pathways. ox-LDL increases activation of AP-1 but suppresses NF-κB activation due to a pro-inflammatory stimuli in VSMC [244]. In EC, treatment with pro-inflammatory mediators, including LPS and TNF-α, induced the expression of the anti-apoptotic gene, A20, through activation of the NF-κB pathways [245]. Treatment with ox-LDL suppressed the activation of NF-κB resulting in reduced A20 expression and inducing EC apoptosis [245]. These studies pertain to the role of ox-LDL in interfering with NF-κB pathway, which may prevent PI-9 expression. We have conducted a number of experiments in order to examine this hypothesis. Ox-LDL did not change the expression pattern of PI-9 in either VSMC or EC. Moreover, combination treatments of ox-LDL and pro-inflammatory cytokines that regulate PI-9 expression, including TNF-α, IL-1β and IFN-γ has also resulted in no change in PI-9 expression. Surprisingly, even treatments with the above cytokines alone did not induce higher expression of PI-9 in VSMC or EC in our experimental settings (data not shown). Thus, we believe that exploration into the regulatory mechanisms that control PI-9 expression in primary cells is worth revisiting as we were unable to validate the proposed regulatory pathways suggested by others.

What is the net effect of GZMB on angiogenesis in disease?

Angiogenesis is a complex process that comprises multiple regulatory mechanisms involving specific interactions between cells, matrix and growth factors [127]. Aberrant neovascularization is characteristic of a large number of chronic inflammatory disorders, and may play a role in exacerbating the inflammatory response by facilitating immune cell infiltrations and delaying resolution of the inflammatory response [145]. Evidence presented in chapter 5 and 6 demonstrates that FN proteolysis by GZMB impairs EC-matrix interactions and
disrupts the formation of newly formed capillaries in vitro [233], while the same cleavage event also leads to increased VEGF release from the matrix resulting in increased permeability of existing vessels in vivo. These observations produce an additional paradox in the context of the angiogenic process; on one hand GZMB may be inhibiting angiogenesis by proteolysis of FN, while on the other hand this process may promote angiogenesis due to increased VEGF bioavailability. Thus, it will be essential to explore the overall effect of GZMB on angiogenesis in experimental settings that involves combination of matrix proteins and growth factors.

It is now recognized that binding of growth factors to the matrix facilitates synergistic signals to EC through both growth factor receptors and integrin receptors, further supporting capillary formation and stabilization [127]. Specifically, FN-VEGF interaction enhances EC migration and proliferation; a response that is greater than treatment with either FN or VEGF alone [125, 177]. Moreover, lower microenvironmental levels of VEGF potentially achieved by binding of VEGF to the matrix promote stable, non-leaky capillary formation while high levels of unbound-VEGF induces pathological angiogenesis [170, 196]. On the basis of these observations we can hypothesize that GZMB cleavage of FN and liberation of VEGF will promote pathological angiogenesis giving rise to unstable, incomplete, leaky neovessels that may further exacerbate the inflammatory response. Employing ex-vivo angiogenic models, such as the mouse/rat aortic ring model in FN+VEGF matrix will provide mechanistic understanding of the role of GZMB in modulating matrix-growth factor interactions and its impact on angiogenesis. Moreover, conducting studies in animal models of disease where GZMB is playing a pathological role, such as in RA, AAA and skin pathologies [71], using either GMZB KO or GZMB inhibition treatment approaches will provide further insights to the effect of GZMB in promoting pathological angiogenesis in disease.
Is there a link between intracellular and extracellular GZMB activities and its role in vascular remodeling in disease?

The work that is presented in this dissertation provides evidence for the role of GZMB in promoting vascular remodeling in disease through seemingly two separate mechanisms. The first involves intracellular GZMB-mediated apoptosis of VSMC in atherosclerosis, while the second relates to an extracellular GZMB function whereby GZMB leads to alteration of microvessel formation through FN proteolysis and induction of vascular leakage through liberation of VEGF from the matrix. Can both mechanisms act in parallel to promote disease pathogenesis? This could be the case in chronic inflammatory vascular diseases in which both vascular cell apoptosis is evident in concert with aberrant, leaky neovasculature. For example, in atherosclerosis, intraplaque neovessel leakage and hemorrhage is a major contributor to increased plaque instability [246, 247]. Degradation of red blood cells within the plaque is a major source for cholesterol (derived from red blood cells membrane) which may further potentate plaque expansion [247]. Moreover, increased neovessel leakage within the plaque and the formation of intraplaque thrombus may further augment the inflammatory response and promote plaque instability. Thus, elevated levels of GZMB in atherosclerosis may promote plaque instability through both intracellular GZMB-mediated VSMC apoptosis and also through extracellular ECM proteolysis, promoting intraplaque neovessel leakiness and hemorrhaging. In TVD, GZMB may promote disease pathogenesis through meditating EC apoptosis, as described [80], but it may also contribute to the development of chronic inflammation and increased incidence of graft rejection through disrupting microvasculature architecture and promoting immune infiltration mediated by VEGF release and increased neovessel permeability. Thus, future studies
would benefit from examination of both intracellular and extracellular GZMB activities as mechanisms that can act in concert to promote vascular disease pathogenesis.
8. Conclusions

GZMB is an immune derived protease that is highly evident in a large number of chronic inflammatory diseases. The intracellular, pro-apoptotic role of GZMB is suggested to contribute to increased tissue damage during inflammation, which may lead to detrimental outcomes such as in the case of atherosclerotic plaque destabilization. Although a direct cause and effect relationship between intracellular GZMB activity and the development of an unstable plaque is still lacking, there is considerable evidence derived from our work and others, in concert with clinical findings that implicate GZMB as a causative factor for immune-mediated apoptosis in advanced atherosclerotic lesions. However, targeting intracellular GZMB as a strategy to reduce apoptosis in the plaque might be highly challenging as intracellular drug delivery is complex, especially in the case of large inhibitory proteins such as monoclonal Abs. Moreover, since the 'killing' efficiency of GZMB is substantial, employing a variety of pathways to induce cell death, a small molecule competitive inhibitor might not be sufficient to rescue from GZMB-mediated apoptosis as it may take only a few molecules of GZMB to initiate the apoptotic cascade. For that reason different approaches for inhibiting GZMB-mediated apoptosis should be considered.

Vascular cells, including VSMC express the only known human endogenous inhibitor for GZMB, PI-9. Thus, these cells are equipped with a protective mechanism that can prevent GZMB-induced apoptosis. Importantly, PI-9 is highly effective in inhibiting GZMB activity as it is a 'suicide' inhibitor with an inhibitory ratio of 1:1 (ie. it takes one molecule of PI-9 to completely deactivate one molecule of GZMB) [21]. As GZMB-PI-9 engagement also deactivates PI-9, the inhibitory capacity of PI-9 is directly proportional to its level of expression. Thus, a reduction in PI-9 expression is expected to increase cell susceptibility to GZMB-induced apoptosis. In this dissertation we showed that PI-9 expression in VSMC is reduced with
increased atherosclerotic disease severity in human. The reduction in PI-9 expression is correlated with increased intimal VSMC apoptosis and elevated levels of GZMB. These observations suggest that intimal VSMC are highly susceptible to GZMB-mediated apoptosis in advanced lesions. Thus, we propose a new mechanism by which impairment of PI-9 expression by intimal VSMC contributes to increased cell death in the plaque, and may contribute to increased plaque instability. These findings highlight the potential role of PI-9 in attenuating VSMC apoptosis and open the door to further explorations aiming to promote PI-9 expression in VSMC as a novel strategy to reduce GZMB-induced apoptosis and promote plaque stability.

The accumulation of GZMB in the ECM during chronic inflammation and its ability to cleave a large number of ECM proteins provides an additional mechanism by which GZMB may contribute to tissue damage in disease. Moreover, recent findings allude to a potential pro-inflammatory role for extracellular GZMB in disease as this protease enhances pro-inflammatory cytokine activities. Interestingly, many ECM proteins that are cleaved by GZMB have an important role in regulating vascular morphogenesis, notably FN. Aberrant neovascularization is a common characteristic of a range of chronic inflammatory disease, many of which present increased extracellular GZMB accumulation. Dysregulated angiogenesis and the formation of incomplete, leaky neovessels may exacerbate the inflammatory response by providing further access for immune cell infiltration and may retard healing.

In our studies we demonstrated that GZMB cleavage of FN has a significant impact on EC angiogenic potential. FN proteolysis by GZMB impairs EC adhesion, migration, and capillary tube formation, all of which are key components of the angiogenic process. Moreover, we discovered that GZMB-mediated FN cleavage releases VEGF from its extracellular stores, effectively altering VEGF bioavailability. This cleavage event has a significant impact on
vascular permeability as GZMB induces VEGF dependent vascular leakage *in vivo*. Thus, we propose a new mechanism whereby extracellular GZMB contributes to chronic inflammation by dysregulating the angiogenic response and contributing to the formation of unstable, leaky neovessels. These findings may pave the way for the development of treatment approaches that aim to normalize neovessels in inflammatory diseases by inhibiting extracellular GZMB activity.

While inhibition of intracellular GZMB activity may be challenging, extracellular GZMB inhibition will be more achievable. Since GZMB-deficient mice develop normally without susceptibility to tumor development and/or virus infection, little to no adverse effects are expected due to the administration of specific GZMB inhibitor. Moreover, current treatment strategies aiming to improve the angiogenic response during wound healing utilize FN-VEGF matrix constructs to stimulate effective angiogenesis. Our studies indicate that elevated levels of GZMB will release VEGF from FN, which interferes with the proposed beneficial outcome of these matrix constructs, and in turn may promote pathological angiogenesis and increased neovessel leakage as more VEGF is liberated from the matrix. Thus, combination therapy which includes FN-VEGF matrices together with GZMB inhibition may prove highly effective in promoting therapeutic angiogenic response that can improve tissue regeneration and healing.

In summary, both intracellular and extracellular GZMB activities have a major impact on vascular integrity and play an important role in regulating neovessel formation through ECM processing and the altering of VEGF bioavailability. The expression of GZMB inhibitor, PI-9, is reduced in VSMC during atherogenesis, while GZMB levels are increased in advanced lesions. This imbalance may result in increased VSMC apoptosis and promote plaque instability. Extracellular GZMB activity disrupts EC-matrix interaction as cleavage of FN by GZMB impairs EC adhesion, migration and capillary tube formation. Further, GZMB alters VEGF
bioavailability through ECM proteolysis, leading to increased vascular permeability \textit{in vivo}. These events may contribute to the persistence of inflammation leading to tissue impairment in the large range of chronic inflammatory disorders wherein GZMB is highly evident.
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