Non-Cytotoxic Roles of Granzymes in Health and Disease

Katlyn C. Richardson[1], Karen Jung[1], Julian Pardo[2], Christopher T. Turner[3†], David J. Granville[1†#]

[1]International Collaboration on Repair Discoveries (ICORD), British Columbia Professional Firefighters' Wound Healing Group, Vancouver Coastal Health Research Institute; Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada

[2]Fundación Instituto de Investigación Sanitaria Aragón (IIS Aragón), Biomedical Research Centre of Aragon (CIBA); Department of Microbiology, Radiology, Pediatrics and Public Health, University of Zaragoza; CIBER de Enfermedades Infecciosas, Instituto de Salud Carlos III.

[3]Faculty of Pharmacy & Pharmaceutical Sciences, Monash University, Melbourne, VIC; Future Industries Institute, University of South Australia, Adelaide, SA, Australia

†Co-senior authors

#Correspondence

David Granville, ICORD Centre, Rm 4470, 818 West 10th Ave.

Vancouver, BC, V5Z 1M9, Canada. Tel: +1-604-675-8869 Email: dgranville@icord.org

15-20 Word Summary

Granzymes exert cytotoxic and non-cytotoxic roles in health and disease. The present review focuses on novel non-cytotoxic roles of granzymes.

Running Title

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ABSTRACT

Granzymes are serine proteases previously believed to play exclusive and somewhat redundant roles in lymphocyte-mediated target cell death. However, recent studies have challenged this paradigm. Distinct substrate profiles and functions have since emerged for each granzyme while their dysregulated proteolytic activities have been linked to diverse pathologies.

1. – INTRODUCTION

GRANZYMES

Granule-secreted enzymes (granzymes, Gzms) are a family of serine proteases first identified in 1987 (1, 2). The human genome comprises five granzymes (Gzms A, B, H, K, M) that are located on chromosomes 5 (Gzms A, K), 14 (Gzms B, H) and 19 (GzmM), encoding proteases that exhibit distinct substrate specificities (3, 4). Human and mouse GzmA and GzmK (tryptases) cleave after basic residues; GzmB (asp-ase) cleaves after acidic residues; GzmM (met-ase) cleaves after aliphatic residues; and human GzmH/mouse GzmC (chymase) cleave after aromatic residues. The functional characteristics of each granzyme are summarized in Table I. Despite human granzymes sharing approximately 40% structural sequence homology (5), differences in substrate binding clefts dictate unique substrate specificities and downstream consequences in health and disease (6–9). As such, there is an emerging body of work investigating the physiologic and/or pathologic roles for each granzyme.

Historically, granzymes have been viewed as redundant mediators of cytotoxic lymphocyte-mediated target cell death through a process involving the pore-forming protein, perforin, that facilitates granzyme entry into cells. There have been many excellent reviews written on the mechanisms of granzymes and perforin in the induction of cell death (6, 7, 10–13). In recent years, in addition to cytotoxicity, diverse roles of granzymes, particularly GzmB, have been delineated in inflammation, extracellular matrix (ECM) degradation, impaired wound healing, scarring, basement membrane disruption, blistering, loss of epithelial barrier function, vascular permeability and autoimmunity (9, 14–17). GzmA and GzmB are the most widely studied granzymes, with less understood pertaining to the roles of Gzms H, K and M, which are occasionally referred to as the ‘orphan’ granzymes (18). Notably, the roles of Gzms A, K and M in immune cell-mediated killing are currently an area of controversy (19, 20). Thus, as our understanding of granzymes evolves, this may prompt the need to reassess earlier studies, characterizing elevated granzymes in fluids, cells and tissues from diverse human pathologies, through a new, non-cytotoxicity-focused lens. As the functions of granzymes are further delineated with advanced genomics,

proteomics, degradomics and other tools, other pathophysiological roles for granzymes are likely to emerge. The purpose of the present review is to provide insights into the non-cytotoxic functions of granzymes and contextualize the relevant literature within the framework of health and disease.

ORIGINAL CONCEPT: GRANZYMES IN PERFORIN-DEPENDENT, LYMPHOCYTE-MEDIATED CELL DEATH

As granzymes were first observed within the granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, initial research established a role for granzymes in lymphocyte-mediated cell death. Under this paradigm, granzymes cleave intracellular substrates to initiate cell death (apoptosis, pyroptosis, necrosis) of target cells. This field of research gained particular traction in the 1990s — coinciding with the peak of apoptosis research — after a landmark study was published ascertaining synergistic roles for granzymes and the membrane-perforating molecule, perforin (formerly known as cytolysin), in cell death (21). Since then, granzymes and perforin — particularly, GzmB and perforin — have been recognized as major constituents of lytic granules within cytotoxic cells and the main effectors of granule-dependent cell death (7).

Both CTLs and NK cells are capable of synthesizing and storing cytotoxic granules. Within these granules, granzymes are rendered as zymogens which, at least in mice, require N-terminal processing by proteinases, cathepsin C (also dipeptidyl peptidase I) (Gzms A, B, K) or cathepsin H (GzmB), to become fully, proteolytically active (22–24). Upon engagement of a target cell, the lytic granules are rapidly polarized toward the immunological synapse, which allows for transport of activated granzymes, perforin, and other contents towards the plasma membrane along a microtubule cytoskeleton (25). Subsequently, perforin helps to deliver granzymes into a target cell. Although different mechanisms have been proposed to explain how perforin releases granzymes into the cytosol of target cells (26), recent evidence suggests that this process is dependent on the ability of perforin to form pores in the plasma membrane of the target cell (27, 28). The successive delivery of granzymes into the cytoplasm of the target cell rapidly induces cell death through the cleavage of substrates both in the cytosol and nucleus. Apart from the release of granzymes triggered by target cell

recognition, granzymes, (i.e. GzmB), may be constitutively released to the extracellular space, albeit the exact regulation of this mechanism and its relevance is not clear (29).

The role of the GzmB/perforin pathway in cytotoxic lymphocyte-mediated apoptosis is well-documented. Within the target cell, GzmB activates the caspase cascade directly by processing effector caspases 3 and 7 or indirectly through cleavage of pro-apoptotic BH3-interacting domain death agonist (Bid) (30–33). GzmB may also cleave Bid into a truncated form (gtBid) (30–33). Further, GzmB can bypass caspases and mitochondria, cleaving related substrates involved in apoptosis execution directly (34–41). The relative contribution of the different pathways to cell death induced by GzmB may depend on differences between the substrate specificity of human and mouse GzmB (42, 43). In mice, ten granzymes have been identified (Gzms A to G, K, M and N) and are primarily named after their human homologs, apart from GzmC, which is a homolog for the closely related human GzmH. The relevance of these findings, predominantly derived using recombinant proteins in in vitro settings, is still unclear (44). The cytotoxic potential of the remaining granzymes is less clear and differences in the profile of substrates cleaved by human or mouse granzymes may add to the obscurity (43, 45). Still, current investigations indicate that cell death pathways activated by other granzymes are non-apoptotic and caspase-independent, although their relevance and potential implications in disease remain to be confirmed and fully characterized (12). More detailed information on these pathways and their potential relevance can be found in other reviews (6–14, 18–20, 46–48).

Despite the breadth of literature describing the differential role(s) of granzymes in various cell death pathways, there remains significant controversy over the specific contributions of individual granzymes to cell death. As perforin is an essential precursor for granzyme delivery by CTLs, a lack of perforin would result in the loss of granzyme internalization by target cells. Based on this fact and the assumption that granzymes could only elicit a physiological effect intracellularly, perforin knockout mice were at one time used to dismiss the contribution of all granzymes to disease (6, 46, 49). We now know this is not the case, and that granzymes can exhibit perforin-

independent and/or other non-cytotoxic intracellular/extracellular functions as discussed in the following sections.

EMERGING CONCEPTS: NON-CYTOTOXIC ROLES FOR GRANZYMES

Over the past 15 years, diverse roles for granzymes (especially GzmB) have been forwarded. Extracellular roles for granzymes have been identified along with increased granzyme levels observed in the extracellular space and biofluids. Extracellular granzymes can accumulate in the extracellular milieu due to leakage from immunological synapses of CTLs/NK cells, constitutive secretion (29, 50) and/or secretion by other immune and non-immune cell types that do not express perforin and/or form immunological synapses (reviewed in Turner et al. (16) and Boivin et al. (9)). In recent years, the extracellular roles of GzmA and GzmB have been investigated. The role of extracellular GzmB independent of perforin was first demonstrated in an in vivo mouse model of abdominal aortic aneurysm, whereby GzmB deficiency increased overall survival while perforin deficiency showed no improvement (51). In the latter study, a role for extracellular GzmB was proposed and later confirmed by Ang et al. (52) using an extracellular GzmB inhibitor (Serpina3n). In the extracellular milieu, GzmA (53) and GzmB (54–56) can induce perforin-independent cell detachment in anchorage-dependent cells through the cleavage of ECM proteins. In cultured rat small intestine epithelial cells, GzmA mediates collagen type IV and fibronectin degradation, promoting reduced cellular adhesion (53). GzmB cleaves fibronectin, vitronectin and laminin, leading to endothelial cell detachment and anoikis as well as inhibition of tumour cell spreading, migration and invasion (55). Similarly, in smooth muscle cells (54), fibroblasts (56) and endothelial cells (55), the addition of GzmB in the absence of perforin induces anoikis through the cleavage of fibronectin and other ECM proteins. Together, these observations underscore novel functional roles of granzymes outside of cytotoxicity that were previously not considered. As granzymes are observed in abundance in conditions as described below, often in the absence of perforin, it is important to consider non-cytotoxic, perforin-dependent (intracellular) and -independent (extracellular) roles.

Interest in non-cytotoxic roles for granzymes has been fueled by observations suggesting that granzymes can be expressed and secreted by both immune and non-immune cells as well as observations demonstrating granzyme accumulation and retention of proteolytic activity in the extracellular milieu. Granzymes are expressed in diverse populations of immune cells including: CD34+ hematopoietic progenitor cells (GzmB (57)), regulatory CD4+ T cells (GzmB (48)), B cells (GzmB (58–61)), $CD4^+$ T cells (GzmA (62, 63), GzmH (64), GzmK (63)), CD3+, CD56+ and gamma delta T-cells (GzmM (65)), type I innate lymphoid cells (mouse GzmC (66)), intestinal T cells (GzmM (67)), intraepithelial γδ lymphocytes (GzmA (68), GzmB (68)), macrophages (GzmB (69), GzmK (70)), type II pneumocytes and alveolar macrophages (GzmA (71), GzmB (71)), NK cells (GzmA (65, 71, 72), GzmB (64, 71), GzmH (64, 73), GzmK (72), GzmM (65, 73, 74)), mast cells (GzmA (75), GzmB (51, 56, 76–81), GzmH (75, 78)), basophils (GzmB (76, 82)), monocyte-derived dendritic cells (GzmB (83)), plasmacytoid dendritic cells (GzmB (84–91)); as well as non-immune cells including: platelets (GzmA (92), GzmB (93)), keratinocytes (GzmB (94–96)), testicular Sertoli cells and placental syncytial trophoblasts (GzmB (97)), articular chondrocytes (GzmB (98)), visceral adipose tissue (GzmB (99)), photoreceptor cells of the retina (GzmM (100)); and cancer cells: B-chronic lymphocytic leukemia cells (GzmB (59)), breast carcinoma cells (GzmB (101)), urothelial carcinoma cells (GzmB (102)), nasal NK/T-cell, gamma delta T-cell and intestinal T-cell lymphomas (GzmM (67)).

Importantly, it is now established that activation of granzyme-positive immune cells can leak or secrete granzymes into the extracellular milieu. Previous studies into the accumulation of extracellular granzymes in response to tissue damage and inflammation have elucidated novel roles for granzymes in disease pathogenesis, with pathologic roles for granzymes under active investigation (7, 103). Within the extracellular milieu, GzmB in particular can cleave and activate various substrates including cell junction proteins, cell surface receptors, extracellular matrix proteins, cytokines/growth factors, and plasma proteins, as will be discussed in the next sections of this review (6, 16, 104–106). Granzymes have also been implicated in mechanisms underlying viral clearance by inactivating diverse viral proteins independently of their ability to kill the host cell (reviewed in

Jong et al. (107); however, the scope of this review is limited to granzyme function as it relates to cellular physiology.

Collectively, studies characterizing the expression of granzymes in cells other than CTLs/NK cells, cells expressing granzymes that lack perforin, accumulation of granzymes in the extracellular space and biofluids, and retention of proteolytic activity in biofluids, suggest that granzymes could play consequential, non-cytotoxic roles in various pathologies.

2. – GRANZYMES: NON-CYTOTOXIC MECHANISMS

The steady discovery of non-cytotoxic roles for granzymes has identified novel mechanisms and key roles in health and disease. In this section, substrates for each of the granzymes are discussed and classified based on their subcellular localization and primary functional role. This information is also summarized in Table II. As indicated above for cell death, it is important to note that the substrate specificities of human and mouse granzyme homologues are different and this may influence their pathogenic mechanisms and resulting biological functions (43).

CELL JUNCTION PROTEINS

To date, there is little in vitro or in vivo evidence to suggest that Gzms A, H, K or M disrupt cell adhesion via the cleavage of desmosomal or hemidesmosomal proteins. However, in recent years, GzmB-mediated cleavage of both desmosomal and hemidesmosomal proteins has been observed in a number of in vivo models, suggesting GzmB plays an important role in the disruption of epithelial barrier function, vascular permeability and/or disruption of the basement membrane zone (68, 76, 108–111). While much of this work has focused on skin, lessons learned are beginning to be transferred to other epithelial tissues (reviewed in Jung et al. (112)).

Several studies have emerged recently suggesting a role for GzmB on epithelial dysfunction in different epithelial pathologies/tissues including the skin (cleavage of cell-cell junction proteins, filaggrin cleavage and loss of epithelial barrier function in dermatitis) (111), colon (intraepithelial γδ lymphocyte release of GzmB

inducing cell epithelial shedding in Crohn's disease) (68), eye (disruption of tight junctions of the retinal pigment epithelium, ECM remodelling of the Bruch's membrane and disruption of the blood-retina barrier in macular degeneration) (110), and airways (NK-derived extracellular GzmB-mediated epithelial protease-activated receptor (PAR)-2 activation, IL-25 production and Th2 response in asthma) (108). While much of the data pertaining to GzmB and epithelial dysfunction is at its infancy, increasing evidence suggests a number of key junctional proteins are susceptible to GzmB-mediated proteolysis, including desmoglein-1 and desmoglein-3 (111), epithelial (E)-cadherin (111), filaggrin (111), junctional adhesion molecule (JAM)-A (56, 110), zonula occludens (ZO)-1 (56, 110, 111), and occludin (110). Another mechanism for GzmB-mediated epithelial barrier dysfunction involves the production of soluble E-cadherin fragments (~80 kDa) (111). Soluble E-cadherin fragments are elevated in multiple conditions (113), including those with demonstrated GzmB activity and junctional protein dysfunction, such as dermatitis. In atopic dermatitis, soluble E-cadherin fragments correlate with disease severity and may disrupt cell-cell junctions important in epithelial barrier function maintenance (114). Indeed, attenuation of GzmB activity, achieved through genetic deletion or pharmacological inhibition, reduces the loss of barrier function in atopic dermatitis by inhibiting E-cadherin and filaggrin cleavage as demonstrated using murine and ex vivo human skin models (111).

GzmB also promotes endothelial barrier disruption in blood vessels. GzmB cleavage of vascular endothelial cadherin (VE-cadherin) (80), platelet endothelial cell adhesion molecule (PECAM)-1, JAM-A and ZO-1 (56) may result in multiple pathologic consequences within the vasculature ultimately leading to increased vascular permeability and inflammation. In the context of macular degeneration, GzmB-mediated cleavage of occludin was proposed to contribute to pathologic angiogenesis and microvasculature permeability (110).

In the basement membrane zone, GzmB mediates disruption through cleavage of α6 and β4 integrins (109), collagen VII (109), and collagen XVII (BP180) (76, 109). Cleavage of the hemidesmosomal proteins by GzmB is proposed to contribute to the onset and progression of subepidermal blistering (pemphigoid) diseases (bullous pemphigoid, dermatitis herpetiformis, epidermolysis bullosa acquisita), whereby GzmB-mediated cleavage of

these desmosomal proteins results in the separation of the epidermis from the dermis. In a recent study by Hiroyasu et al. (76), further proof of concept and target validation was demonstrated using a combination of both GzmB knockout mice and topical GzmB inhibitor approaches in conjunction with three models of autoimmune sub-epidermal blistering. Elevated GzmB was observed in human blister fluid from patients with bullous pemphigoid and inhibition of GzmB in murine models resulted in a significant reduction in blistering that coincided with the inhibition of hemidesmosomal protein cleavage (76).

CELL SURFACE RECEPTORS

Cell surface receptors are key signalling mediators between extracellular and intracellular environments and are highly susceptible to extracellular protease-mediated degradation.

GzmA cleaves numerous PARs, a family of G protein-coupled receptors activated by cleavage of their extracellular domain, exposing de novo N termini which function as self-activating tethered ligands to promote transmembrane signalling (115, 116). In the blood, GzmA cleaves the thrombin (PAR-1) receptor on platelets, desensitizing their response to thrombin-induced aggregation (15, 117). However, the relevance of GzmA/PAR-1 in coagulation is not well understood. In an in vivo model of sepsis, GzmA knockout mice show reduced coagulatory damage, suggesting GzmA/PAR-1 inhibition of thrombin-mediated aggregation in platelets is likely not a key mechanism during sepsis (118). Even so, GzmA may be able to mediate the effects of other ligands that interact with PAR-1, like endotoxin, which is associated with the development of sepsis (103). Differences in the cytokine profiles elicited from monocytes exposed to GzmA versus thrombin suggests that GzmA activates monocytes via a different receptor (119). Further investigation will be required to establish the conditions and cell types where GzmA/PAR-1 activation is favoured. GzmA-mediated PAR-1 cleavage has also been proposed to supress tumour progression by promoting JAK2/STAT1 signal activation-induced apoptosis (120). In hepatocellular carcinoma patients, the loss of GzmA-mediated PAR-1 cleavage is observed and this may contribute to tumor progression (120). Hence, it may be of interest to examine the levels of GzmA/PAR-1 activity in other cancers and its correlation with disease severity. GzmA-mediated thrombin receptor cleavage

elicits morphological changes in neural cells, as demonstrated by detection of weakened calcium ion (Ca^{2+}) signals (117). GzmA also cleaves the thrombin-like receptor on neurites, leading to neurite retraction and reversed stellation of astrocytes (15, 121). Hence, GzmA may play an important role in the development of nervous system impairments (121). GzmA-mediated activation of PAR-2 has been proposed; however, it has not been conclusively demonstrated (122).

GzmB is capable of cleaving both PAR-1 and PAR-2 (108, 123). GzmB-mediated PAR-1 activation in neurons and was found to induce neuronal cell death/atrophy associated with multiple sclerosis (123). Conversely, in the context of asthma, extracellular GzmB was not toxic, but rather activated PAR-2 in the epithelium, resulting in IL-25 expression and secretion (108). IL-25 production was augmented by IL-13, provoking a type II immune response (108). Thereafter, both pulmonary group 2 innate lymphoid cells (ILC2s) and T helper 2 (Th2) cells were activated, leading to subsequent eosinophilic recruitment and allergic airway disease (108). GzmB may also cleave other cell surface receptors pertaining to autoantigen generation, including the acetylcholine receptor in myasthenia gravis (124), neuronal glutamate receptor 3 in Rasmussen's encephalitis (125), as well as fibroblast growth factor receptor 1 (FGFR1, CD331) and Notch Homolog 1 (Notch1) in prostate cancer (126). Although there is a diverse range of autoantigens predicted to be cleaved by GzmB, few have been validated. Literature pertaining to GzmB-mediated autoantigen generation has been reviewed previously (127).

GzmK may also cleave PAR-1, promoting pro-inflammatory cytokine and chemokine release in cultured lung fibroblasts (IL-6, IL-8 and MCP-1) (128), endothelial cells (IL-6 and MCP-1) (129), keratinocytes (IL-6) and pro-inflammatory M1 macrophages and peritoneal macrophages (IL-1β) (70, 130). GzmK-mediated PAR-1 activation also induces cell proliferation and endothelial activation (70, 128, 129). The expression of pro-inflammatory cytokines IL-1β and IL-6 and chemokines IL-8 and MCP-1 are implicated in a variety of inflammation-driven processes and can contribute to local tissue inflammation (131–134). In fibroblasts and endothelial cells, GzmK-dependent production of these cytokines/chemokines requires mitogen-activated protein kinases (MAPK), extracellular-signal regulated kinase (ERK)1/2 and p38 phosphorylation (128, 129).

EXTRACELLULAR MATRIX (ECM) PROTEINS

The ECM is a vital component of all tissues, providing scaffolding for cell adherence, but also plays a key role in regulating cellular behaviour and processes such as migration, proliferation, inflammation, differentiation and homeostasis. Consequently, proteolytic processing of the ECM is tightly regulated. While much attention has been focused on the ECM cleavage capacity of matrix metalloproteinases (MMPs), their activities are tightly regulated by Tissue Inhibitors of Metalloproteinases (TIMPS) (135). Further, MMPs are critical regulators of many physiologic processes and broad MMP inhibition can exacerbate inflammation by suppressing MMP-mediated chemokine processing (136). Of note, it is estimated that of the twenty-four human MMPs, up to ten may exert anti-inflammatory or anti-tumorigenic roles. As such, they have been referred to as 'anti-targets', whereby their function should perhaps be promoted rather than inhibited (137). Conversely, there are no known endogenous extracellular inhibitors of GzmB; thus, accumulation and proteolytic activity associated with inflammation remains unregulated. While there is increasing evidence for extracellular GzmB in pathogenesis, our understanding of other granzymes in ECM cleavage, including their activity retention in the extracellular milieu and/or ECM substrates is poorly understood.

As described in a review article by Butler and Overall (138) on MMPs and their respective TIMPS, the protease web is tightly regulated. Thus, it could be postulated that dysregulated proteases in the ECM could lead to disruptions of other proteases in the protease web, resulting in proteolytic amplification and/or other pathological consequences. In the context of granzymes, investigations into elevated and unimpeded granzyme activity in the extracellular space and its implications on other proteases are emerging. GzmB retains its activity in plasma (139) and none of the anti-proteases in the lung inhibit GzmB activity (140). As a consequence of this, studies by Parkinson et al. (81) suggest that aberrant GzmB activity can impact other proteases. GzmB-generated fibronectin fragments were found to induce MMP1 and MMP3 expression in dermal fibroblasts, suggesting that GzmB may disrupt the protease web by indirectly inducing other proteases (81). Moreover, Geng et al. (141) have shown that decorin binds to the surface of collagen fibrils to impede access and proteolytic cleavage by

MMP1, while Parkinson et al. (81) reported GzmB-mediated decorin cleavage rendered collagen I susceptible to MMP1-mediated cleavage. In another example of how GzmB may influence other proteases, Hiroyasu et al. (76) demonstrated that GzmB-induced macrophage inflammatory protein (MIP)-2 (mouse homolog of IL-8) expression, promoted neutrophil recruitment and neutrophil elastase expression in models of autoimmune blistering. Discussed later in this review, GzmB-mediated release of ECM-sequestered growth factors (VEGF, TGF-β) may also influence the activities of other proteases.

GzmB mediates disruption of cellular interactions within the basement membrane zone through cleavage of collagens IV and VII (109, 142, 143). GzmB proteolysis of collagen IV also has implications in lymphocyte transmigration (142, 143). The contribution of collagen VII to pathomechanisms of subepidermal blistering is well-established, with therapeutic efficacy of a topical GzmB inhibitor observed in more than three different blistering disease murine models to date (144).

Increasing evidence suggests proteoglycans are key proteins targeted by GzmB in aging and wound healing pathologies. Within cartilage tissue, GzmB has been shown to cleave cartilage proteoglycans including aggrecan (145). Aggrecan was also found to be cleaved by GzmA, and later, it was shown that GzmA knockout mice were less susceptible to collagen-induced arthritis than wild-type mice (146). Here, it was shown that GzmA contributed to arthritis by promoting osteoclastogenesis by the induction of tumour necrosis factor (TNF)-α release in precursor cells (146). GzmB can also cleave ECM proteins fibronectin (147), laminins-332,-511 (55, 110) and vitronectin (55) as stated previously in this review. Further, GzmB cleavage of fibronectin can induce release of fibronectin-sequestered VEGF (148). Given the important pathologic role for VEGF in macular degeneration, it is exciting to speculate whether GzmB, which is elevated in aging and diseased eyes (110), contributes to the increase in VEGF that is observed in macular degeneration.

The small leucine rich proteoglycan decorin is abundant in the skin and other tissues. While it is associated primarily with the collagen-matrix, decorin also interacts with and governs the activities of diverse proteins,

including fibronectin, thrombospondin-1, WNT-inducible signaling pathway protein 1, toll-like receptors (TLR) 2/4 and several receptor tyrosine kinases (EGFR, HER2, MET, and VEGFR2) (149). Further, decorin plays a key role in collagen organization and fibrillogenesis. Reduced decorin is associated with increased scarring and fibrosis; thus, it follows that decorin has been investigated as an anti-fibrotic agent in vivo (150, 151). Notably, decorin is perhaps the most well-studied extracellular GzmB substrate. GzmB-mediated decorin cleavage has been observed in several in vivo models of skin conditions (age-impaired wound healing, diabetic wound healing, accelerated skin aging, photoaging, pressure injury in aged skin) and aneurysm (52, 81, 111, 152–155), the pathologies and phenotypes of which will be described in detail in a later section of this review. GzmB cleavage of decorin, biglycan and β-glycan has also been observed to sequester TGF-β1 (156).

In addition to their roles in aging and wound healing, decorin and fibronectin are known to affect tumour cell survival and metastasis, prompting Arias et al. (8) to hypothesize that GzmB cleavage of these ECM substrates may also be relevant in cancer. However, this hypothesis has not been investigated experimentally. Rather, these suggestions were made on the basis that decorin and fibronectin functions underlie critical processes related to tumor progression and these ECM components are observed at reduced levels in a variety of human cancers. Decorin can facilitate cell cycle arrest, cell death, anti-angiogenic and anti-metastatic programs (157). Further, TGF-β is a known effector cytokine underlying epithelial-mesenchymal transition (EMT) and cancer progression (158). Hence, GzmB-mediated decorin cleavage may promote tumour survival signaling and metastasis. Within the tumour microenvironment, fibronectin has important functions in proliferation, angiogenesis, invasion and metastasis (159). Expression of pro-inflammatory fibronectin fragments is increased in human oral cancer and regulates cancer cell spreading, migration and invasion (160). Hence, GzmB cleavage of fibronectin may also affect tumour development. Moreover, GzmB cleavage of fibronectin releases VEGF which can enhance angiogenesis (148, 161). It is possible that GzmB-mediated release of fibronectin-sequestered VEGF could contribute to tumour angiogenesis; however, further elucidation is required. All these hypotheses and preliminary results regarding GzmB-mediated ECM remodelling in cancer need to be further experimentally

confirmed in biologically relevant in vivo models. Investigation into the relevance of additional GzmB ECM substrates to this mechanism is also warranted. In urothelial carcinoma, GzmB is expressed in the absence of perforin, retains proteolytic activity and cleaves cell-matrix substrate vitronectin, suggesting that GzmB degradation of other ECM components may contribute to oncogenesis (102).

GzmB can also cleave ECM forms of fibrinogen and Von Willebrand Factor (VWF), leading to impaired platelet aggregation (162). Moreover, GzmB delays ristocetin-induced platelet aggregation and inhibited platelet adhesion and spreading (162). GzmB cleavage of (VWF) is dependent on conformation; thus, it may not be observed in all pathophysiological settings (162). Nonetheless, while not demonstrated in vivo, GzmB has a potential role in coagulation, warranting further investigation.

CYTOKINE PROCESSING & INDIRECT CYTOKINE, GROWTH FACTOR RELEASE

A small number of cytokines are processed intracellularly within the cytoplasm by GzmA and GzmB into their active state for release.

GzmA processes pro-IL-1β to IL-1β in macrophages; however, the mechanism remains to be confirmed, with speculation it occurs through either direct or indirect activation of caspase-1/the inflammasome (105, 163–165). Initially, GzmA was believed to cleave pro-IL-1β directly (164). Metkar et al. (165) observed GzmA to stimulate IL-1β in vitro that was then reversed by a caspase-1 inhibitor, suggesting a role for the inflammasome in this process. Further, genetic deletion of GzmA in mice decreased lipopolysaccharide (LPS)-induced toxicity, confirming the potential relevance of GzmA-mediated IL-1β release in LPS-induced shock (165). A follow up study by Hildebrand et al. (163) demonstrated that GzmA secretion mediated by the bacterial *Pasteurella multocida* toxin (PMT) was able to process pro-IL-1β without inducing cell death via caspase-1 and inflammasome activation. In both instances, the resulting mature IL-1β (17 kDa) is bioactive, but the functional consequences have yet to be explored. GzmA indirectly elicits the release of pro-inflammatory cytokines through the activation of TLRs which play an important role in the innate immune response. In monocytes and

macrophages, TLR signaling is required for GzmA-mediated cytokine release including IL-6 and TNF-α (92, 166–168). LPS-pre-sensitized macrophages elicit GzmA cleavage of TLRs 2, 4 and 9, and release of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α (165, 169). GzmA can also induce the release of cytokines from cultured human peripheral blood mononuclear cells (IL-6, IL-8 and TNF-α or IL-1β, IL-6, IL-8 and TNF-α) (119, 165), purified monocytes (IL-6, IL-8 and TNF-α or IL-8 and MCP-1) (92, 119), macrophages (IL-1β) (165), plasmacytoid dendritic cells (type I interferons) (168), fibroblasts and epithelial cells (IL-6, IL-8) (170), albeit the mechanisms involved have not been fully elucidated. Current evidence supports that GzmA-mediated pro-inflammatory cytokine processing and production promotes the development of colorectal cancer (171). GzmA has been investigated in patients with ulcerative colitis, a chronic inflammatory condition closely linked to colorectal cancer, as a biomarker for response to anti-inflammatory immunotherapy as discussed in a later section (172).

Several reports have described GzmB in the direct cleavage and indirect induction of pro-inflammatory cytokines. GzmB cleaves IL-1α (17 kDa) resulting in the generation of a more pro-inflammatory form of IL-1α than its precursor (173). IL-1α proteolysis by GzmB is likely involved in the activation and link between the innate and adaptive immune response (173). GzmB processes pro-IL-18 resulting in activation and subsequent release of IL-18 (111, 174, 175). IL-18 promotes T cell activation and expansion and is a critical inducer of the inflammatory cytokine IFN-γ. GzmB can indirectly promote the release of cytokines IL-8 (from keratinocytes) (76) and IL-25 (from lung epithelial cells) (108) in addition to growth factors (156), which are outlined briefly here. In a murine model of blistering disease, GzmB impeded secretion of the neutrophil chemoattractant MIP-2 (mouse homolog of IL-8) (76). Correspondingly, GzmB induced IL-8 secretion from human primary keratinocytes in a dose-dependent manner in vitro (76). In a model of asthma, GzmB induced IL-25 secretion from the epithelium through activation of PAR-2 (108). GzmB is also capable of releasing ECM-sequestered growth factors, VEGF (148) and TGF-β (156). GzmB triggers the release of VEGF through fibronectin cleavage (148). Further, VEGF release leads to VEGFR2 activation (161). GzmB-mediated decorin/biglycan/β-glycan

cleavage triggers the release of active TGF-β1 (156). While the precise functional roles of GzmB-mediated ECM-sequestered growth factors require further elucidation in vivo, the implications are potentially extensive.

There is also evidence supporting a role for GzmK in cytokine induction. GzmK indirectly promotes cytokine release through a PAR-1-dependent mechanism in cultured lung fibroblasts (IL-6, IL-8 and MCP-1) (128), endothelial cells (IL-6 and MCP-1) (129), skin fibroblasts and keratinocytes (IL-6) (70), and pro-inflammatory M1 macrophages and peritoneal macrophages (IL-1β) (70, 130). GzmK also indirectly elicits the release of pro-inflammatory cytokines via TLR activation. In contrast to GzmA, GzmK supports LPS-CD14 complex formation, which binds to TLR4 (169). In vitro, GzmK can enhance LPS-induced cytokine release from human primary monocytes (TNF-α) (176) and mouse peritoneal macrophages (IL-1β) (130). Similarly, GzmK can enhance TNF-α-induced cytokine release from endothelial cells (IL-6, MCP-1) (129). Also in endothelial cells, GzmK can promote the expression and secretion of soluble VEGFR1, which sequesters VEGF-A and impairs subsequent pro-angiogenic signalling (177). The underlying mechanisms remain unknown but do not appear to involve PAR-1 activation (177). In support of these findings, GzmK was observed to positively correlate with sVEGFR1 protein levels and negatively correlate with T4 intratumoural angiogenesis and tumour size in human colorectal cancer (177).

GzmM is also capable of indirectly eliciting a pro-inflammatory response. In a mouse model of LPS-induced endotoxicosis, GzmM knockout mice were resistant to LPS-induced toxicity which corresponded with reduced levels of serum IL-1α, IL-1β, TNF and IFN-γ (178). This GzmM pro-inflammatory response was reasoned to operate downstream of LPS-TLR4 signaling, which may have implications is sepsis/endotoxicosis and other diseases (178).

PLASMA PROTEINS

The proteolytic activity of circulating GzmA has been investigated. GzmA cleaves pro-urokinase plasminogen activator (uPA) which converts single-chain human pro-urokinase into active two-chain enzyme and plays a putative role in plasmin generation (15, 179).

GzmB-mediates cleavage of clotting factors plasmin (180) and plasminogen (180). In systemic sclerosis, GzmB cleaves plasminogen which limit the pro-angiogenic function of plasmin and increased levels of antiangiogenic angiostatin (180). A potential role for GzmB in C3 and C5 processing to C3a and C5a, respectively has also been proposed (181); however, more research must be done to confirm a pathologic role for GzmB in hemostasis.

GzmM cleaves both denatured and soluble plasma-derived platelet aggregation plasma protein VWF (182). This proteolysis prevents binding of VWF to coagulation factor VIII (182), affecting the VWF/coagulation factor VIII ratio which is important in the clinical management of blood coagulation.

OTHER/UNDEFINED

The breadth of research that granzymes have impacted is further showcased in this section as more research groups are investigating the consequences of granzyme activity.

GzmA-mediated myelin basic protein (MBP) degradation results in myelin destruction and is implicated in the pathogenesis of multiple sclerosis (15, 183).

GzmB is also considered an important contributor to axonal injury and neuronal death in multiple sclerosis (184). GzmB inhibition using serine protease inhibitor a3n (Serpina3n) prevents loss of myelin and overall disease severity in experimental autoimmune encephalomyelitis (EAE) and is under investigation as a potential novel therapeutic approach (184). Though not fully understood, observations that GzmB is expressed by regulatory T and B cells, both with and without perforin, in tumour microenvironments highlight potential roles for GzmB in tumour progression that are independent of ECM remodelling (reviewed in Arias et al. (8)) as discussed in a later section.

Using single cell RNA and antigen receptor sequencing, a recent study has identified a GzmK-expressing population of CD8+ T-cells as key contributors to inflammaging in humans and mice, although no GzmK substrates were directly implicated in this study (185). Termed "age-associated T-cells", human and mouse GzmK+CD8+ T-cells shared transcriptomic and epigenetic signatures, and displayed similarities to terminal, exhausted T cells isolated from mice with chronic infection (185). The circulating GzmK+CD8+ T-cell population clonally expanded with age, was detected in all organs with age, was the primary source of GzmK detected in the aging mice, and correlated with increased levels of pro-inflammatory cytokines IL-6, IL-8, and TNF-α (185). Findings derived from immune cells in young and old mice showed that GzmK, with and without IFN-γ, enhanced the senescence-associated secretory phenotype (SASP) in fibroblasts (185). While it remains to be seen if deletion of GzmK would attenuate the observed inflammaging phenotypes in vivo, the study findings suggest that GzmK could be a key mediator in inflammaging.

3. – GRANZYMES IN RELEVANT PATHOLOGIES

Tissue injury, inflammation and repair are key elements underlying the pathophysiology of many conditions, and elevated protease activity is thought to be a key contributor. While granzymes are not the only proteases involved in these processes, granzyme substrates are key mediators and granzymes have been observed in diverse pathologies in multiple body systems. Putative role(s) of granzymes in disease pathology is context-dependent – dependent upon the cell source, degree and site of protease accumulation, and protease access to substrates/tissues. The current understanding of the best known granzymes (Gzms A, B, K) and their established roles in various pathologies is summarized in Figures 1-4 and discussed below.

GZMA IN SEPSIS

Elevated extracellular GzmA is observed in plasma, serum, synovial fluid and bronchoalveolar lavage fluid in patients with inflammatory conditions, ranging from rheumatoid arthritis (186–188) gut disease (189) to sepsis (190). Granzyme release can be stimulated in NK cells by bacterial products in the absence of target cells, which could contribute to extracellular GzmA expression (191). Several detailed studies have delved into the

pathogenic role of GzmA in sepsis (103). Extracellular GzmA levels are significantly increased in severe sepsis, septic shock, and endotoxemia (190, 192). In fact, increased serum GzmA levels (relative to healthy donors) precedes sepsis onset in people with peritonitis, one of the leading disease cofounders (103). Further, GzmA was positively correlated with sequential organ failure assessment (SOFA) score, a clinical predictor of patient mortality (103). In human subjects injected with LPS, there was a transient increase in GzmA expression in plasma, corresponding to similar elevations observed in bacteremic melioidosis patients (192). Notably, GzmA release appears to be part of a general response to bacterial infection rather than being pathogen specific (192).

While the non-cytotoxic mechanisms of extracellular GzmA in disease remains to be fully characterized, in vitro studies using purified, recombinant GzmA have elucidated potential pathologic roles in sepsis. Exposure to purified GzmA triggered pro-inflammatory cytokine release in cultured fibroblasts (IL-6 and IL-8), epithelial cells (IL-8), human peripheral blood mononuclear cells (IL-6, IL-8 and TNF-α), monocytes (in conjunction with LPS, IL-6, IL-8 and TNF-α) and macrophages (IL-1β, IL-6 and TNF-α) (15, 103). The inflammasome may be required for pro-inflammatory IL-1β cytokine expression as caspase-1 depletion ameliorates secretion (163), although a separate study showed GzmA activates IL-1β directly by cleaving the precursor form (164).

Studies involving a mouse model of sepsis have identified an influx of GzmA-positive cells. In vivo, GzmA is predominantly expressed by NK cells, which mediates macrophage expression of IL-6 and TNF-α through a TLR4-dependent mechanism (103, 118). A recent study by Hu et al. (68) identified GzmA-expressing intraepithelial γδ lymphocytes in Crohn's disease which is characterized by an enteric bacteria invasion similar to sepsis (68). After a fatal challenge with mouse pathogen *Brucella microti*, GzmA knockout mice displayed increased survival, which correlated with reduced expression of IL-1α, IL-1β and IL-6 (118). In a model of *E. coli*-induced sepsis, there was increased survival in GzmA knockout mice, along with a lower sepsis score and reduced expression of IL1-α, IL-β and IL-6 (193). In a cecal ligation and puncture model, both GzmA knockout mice and wild-type mice treated with an extracellular GzmA inhibitor exhibited increased survival compared to untreated wild-type mice (166). Notably, the loss of GzmA activity in these mice ameliorated infection-related

pathology (inflammation) but not bacterial clearance, suggesting the protease may be a therapeutic target for the prevention of bacterial sepsis without affecting immune control of the pathogen (103, 118, 193). Interestingly, some studies suggest that the contribution of GzmA to sepsis might depend on the type of bacterial infection. Bronchoalveolar lavage fluid from patients with pneumococcal pneumonia presented increased levels of GzmA and GzmA knockout mice showed increased resistance to pneumosepsis induced by *Streptococcus pneumonia* infection (194). In contrast, GzmA deficiency did not affect the susceptibility to *Klebsiella pneumoniae*-induced sepsis (195). Again, in both cases, the immune control of the pathogen was unaffected in the absence of GzmA. Collectively, GzmA is an emerging therapeutic target for inflammation in bacteria-mediated sepsis with potential application of GzmA as a biomarker of peritoneal sepsis development and severity (103).

GZMA IN ULCERATIVE COLITIS & COLORECTAL CANCER

The development of colorectal cancer is strongly linked to chronic inflammation observed in ulcerative colitis (196), and the current literature suggests GzmA could be a key mediator of inflammation underlying both conditions. High *GZMA* expression has been detected in tumour samples from human colorectal cancer patients co-expressed with genes encoding inflammatory markers IFN-γ, TNF-α, and IL-2 (171), as well as from tissue obtained from the intestinal mucosa of patients with active Crohn's disease or ulcerative colitis (197). Mechanistically, extracellular GzmA was reported to induce IL-6 in macrophages through the NFκB pathway, and in turn activate oncogenic STAT3 signaling in colon cancer cells (171). Genetic ablation of GzmA or pharmacological inhibition with GzmA inhibitor Serpinb6b in mouse models attenuated severity of colitis, inflammatory cytokine levels, as well as colorectal cancer development (171), suggesting that GzmA could be a key therapeutic target for both inflammatory bowel disease and colorectal cancer. Furthermore, a study on ulcerative colitis patients revealed GzmA to be a robust marker of treatment response with novel, efficacious therapeutic, etrolizumab, supporting the utility of GzmA as a potential predictive biomarker (172).

GZMB IN CARDIOVASCULAR INJURY

A role for extracellular GzmB in disease, independent of perforin, was first observed in a mouse model of abdominal aortic aneurysm using both GzmB and perforin knockout mice (51). Here, GzmB was abundant in both mouse and human disease (51). Further, GzmB deficiency decreased aortic aneurysm, reduced rupture, and increased overall survival, perforin-deficient mice exhibited no improvement in survival compared to controls, suggesting a perforin-independent role for GzmB in aortic aneurysm (51). In this initial study, fibrillin-1 was identified as a substrate that was cleaved by GzmB in the medial layer, leading to medial disruption (51). GzmB deficiency reduced fibrillin-1 cleavage, medial disruption, aortic rupture and mortality (51). In a follow-up study by Ang et al. (52), decorin was identified as a key GzmB substrate that was cleaved in the adventitia. Decorin plays an important role in collagen organization and fibrillogenesis; hence, the loss of decorin was predicted to reduce overall circumferential strength of the aorta. Indeed, GzmB-mediated cleavage of decorin led to reduced collagen organization, aneurysm and rupture, which most likely was attributed to a loss of sustained circumferential tensile strength in the adventitia (52). Intravenous injection of Serpina3n, a potent, irreversible, non-specific, systemic inhibitor of GzmB, prevented the loss of decorin, resulting in increased collagen organization, aneurysmal rupture and survival in a dose-dependent manner (52). As such, current evidence suggests an important role for GzmB-mediated decorin cleavage in models of impaired vascular wound healing.

The contributions of GzmB activity to degradation of ECM substrates have also been linked to microvascular damage. In addition to the cleavage of cell-cell adhesion proteins, it was demonstrated that GzmB disrupts endothelial adhesion, migration, and capillary tube formation through degradation of fibronectin (148, 161). GzmB has also been shown to promote vascular permeability through the proteolytic release of fibronectin-sequestered VEGF (161). In studies performed by Hendel et al, GzmB-mediated fibronectin cleavage triggered the release of ECM-sequestered pro-angiogenic VEGF (198), leading to VEGFR2 activation (161). While the link between GzmB and VEGF in vivo requires further elucidation, anti-VEGF treatment was able to attenuate GzmB-induced microvascular permeability in a murine model of oxazolone-induced dermatitis (111). More

recently, using prematurely aged mice, GzmB reduced levels of fibronectin, increased VEGF and enhanced microvascular hemorrhage in a murine model of pressure injury (199).

The GzmB/perforin pathway was originally investigated in the context of allograft vasculopathy, an accelerated form of arteriosclerosis and major cause of chronic solid organ rejection (54, 200). Reduced luminal narrowing was observed in both murine GzmB and perforin knockout models, supporting a role for the GzmB/perforin-apoptosis pathway in this accelerated form of transplant arteriosclerosis (54, 200). Of note, albeit separate studies, greater protection was observed in the perforin knockout mice, suggesting that other granzymes could also be involved. Subsequently, as elevated circulating GzmB was observed in patients with unstable plaques and increased cerebrovascular events (201) as well as following acute myocardial infarction (202), the role of GzmB and perforin in native atherosclerosis was investigated in an apolipoprotein E (ApoE)-knockout model (152). In this study, perforin and GzmB knockout mice exhibited distinct roles in atherogenesis. ApoE/perforin-deficient mice exhibited greater protection versus ApoE/GzmB-deficient mice, suggesting a role for other granzymes in atherogenesis (155). However, ApoE/GzmB-deficient mice exhibited reduced decorin and increased collagen in plaques, suggesting a potential role for GzmB in plaque instability and rupture (155).

A role for GzmB in cardiac fibrosis has also been proposed. GzmB was elevated in fibrotic human heart sections as well as fibrotic murine hearts isolated from an angiotensin II-induced model of cardiac fibrosis (80). In vivo, independent of perforin, GzmB deficiency or Serpina3n administration led to reduced angiotensin II-induced cardiac hypertrophy and fibrosis, microhemorrhage, inflammation as well as fibroblast recruitment (80). These observations were hypothesized to be dependent on GzmB cleavage of VE-cadherin, resulting in subsequent vessel wall permeability, inflammation and fibroblast activation (80). Of note, GzmB-mediated decorin cleavage did not appear to be involved in this purported mechanism of action.

GZMB IN INFLAMMATORY SKIN CONDITIONS

Elevated GzmB is documented in multiple inflammatory dermatological conditions and skin injury, including atopic dermatitis (111, 203), autoimmune blistering disease bullous pemphigoid (76, 109), Stevens-Johnson syndrome/toxic epidermal necrolysis (204, 205), diabetic wounds (147, 153), pressure injuries (199), and aged skin (152, 154, 199), with dysregulated GzmB contributing to pathogenic roles through proteolytic degradation of substrates within the epidermis, dermal-epidermal junction (DEJ), and dermis. It is important to emphasize that the impact of extracellular GzmB on skin pathology is determined by the cell source, area of accumulation (e.g., epidermis, DEJ, dermis, etc.), and substrates/cleavage site exposure to GzmB, which appears to vary between these skin conditions.

Epidermis

Recent discoveries uncovering the role of extracellular GzmB in skin afflicted with atopic dermatitis have revealed novel mechanisms underlying the pathogenesis of the disease. GzmB is elevated in atopic dermatitis lesional skin compared with healthy and non-lesional tissue, and is detected both within the epidermis and dermis (111, 206). GzmB detection in the plasma of atopic dermatitis patients is correlated with pruritus (itchiness) and disease severity (203). Extracellular GzmB, predominantly secreted from mast cells, was demonstrated to cleave the epidermal barrier proteins filaggrin, E-cadherin, desmoglein-1 and desmoglein-3 (111). GzmB further disrupts cell junctions through the cleavage of cell junction proteins leading to a loss of barrier function in vitro. ZO-1 and JAM-A were also identified as GzmB substrates in the skin in vitro (56, 111). Using an in vivo model of hapten-induced dermatitis, GzmB knockout mice exhibited reduced inflammation, epidermal thickness, lesion formation, epithelial barrier dysfunction, erosions (an indicator of scratching and indirect measure of pruritus) as well as overall disease severity (111). Furthermore, topical administration of a potent, small molecule inhibitor of GzmB (VTI-1002, viDA Therapeutics, Vancouver, Canada) also reduced dermatitis severity compared to controls, providing further target validation (111).

Xerosis and pruritus are common features of atopic dermatitis as well as aging skin and some preliminary investigations have been performed in the context of GzmB (111, 203). As GzmB directly cleaves structural

proteins key to epidermal barrier function which could contribute to the development of xerosis and pruritus, further investigations into GzmB-mediated xerosis and pruritus are warranted. In the aging population, xerosis and pruritus are among the most common skin health concerns due to aging-related declines in functions of the epidermal barrier, immune system and nervous system (207, 208). Particularly, the epidermal barrier composition is altered with age, and the capacity for barrier repair is reduced (207, 209). In support of this, GzmB has been reported to be elevated in aged skin (81, 199). Strikingly, in a murine model of accelerated aging and skin aging, ApoE/GzmB double knockout mice exhibited significantly decreased erosions compared to the control ApoE-/- mice (152). Whether reduced erosions were due to reduced pruritus requires further elucidation. Taking into account that GzmB is elevated in skin aging (81, 199), correlated with increased pruritus severity (203) and inhibition reduces transepidermal water loss in a murine dermatitis model (111), there is evidence to support a role for GzmB in age-related xerosis and pruritus (112).

Beyond the skin, extracellular GzmB activity has been implicated in barrier dysfunction in other tissues. Tight junctional proteins, JAM-A and occludin, as well as fibronectin, laminin-332, and collagen IV have been identified as substrates of GzmB in retinal pigment epithelial cells, with implications for age-related macular degeneration (110). Furthermore, as dysregulated extracellular GzmB activity has been noted to play key roles in pathological inflammation of the airway epithelium (108) and the gut epithelium (68), the role of GzmB in promoting epithelial barrier dysfunction in other tissues could be speculated.

Dermal-epidermal junction (DEJ)

A study by Russo et al. (109), identified GzmB to be elevated at the DEJ in multiple autoimmune blistering diseases: human bullous pemphigoid, dermatitis herpetiformis, and epidermolysis bullosa acquisita. GzmB, but not perforin, is abundantly expressed along the DEJ in SJS/TEN (210). GzmB cleaves key basement membrane substrates present in the DEJ including collagen XVII, collagen VII, and α6β4 integrins (109). Laminin-511 (previously known as laminin-10), highly expressed in the basement membrane, is also identified as a GzmB

substrate (55), but its cleavage in the context of GzmB and blistering has not been reported. GzmB knockout mice displayed reduced disease severity in two models of epidermolysis bullosa acquisita and a bullous pemphigoid model (76). In this study, GzmB was found to contribute to skin blistering through the cleavage of collagen XVII and α6-integrin (76). Similarly, topical application of the GzmB inhibitor VTI-1002 reduced degradation of anchoring proteins collagen XVII and α6-integrin, neutrophil infiltration, and histological blistering score (76). While these studies provide evidence and focus on the disruption of the DEJ/basement membrane zone in skin, lessons from these findings could be applied to other tissues where GzmB levels may be elevated in the basement membrane zone.

Dermis

GzmB accumulation in skin has been observed in conditions impacted by aging, chronic inflammation and/or impaired wound healing. Similar to our observations in vessel wall injury and repair, decorin degradation has also been observed in several skin pathologies. Decorin is a key proteoglycan that associates with collagen in the skin, providing tensile strength, binding to growth factors such as TGF-β and protecting collagen from cleavage by MMPs and other proteases. Decorin is the best characterized GzmB substrate in the dermis. Elevated GzmB and decorin degradation has been detected primarily in the dermis of human and/or murine skin exhibiting accelerated aging (152, 154), pressure injuries in aged skin (199), ultraviolet (UV) light exposure (81), as well as impaired wound healing from pressure injuries (199) and diabetic wounds (153). In a mouse model of accelerated skin aging, GzmB deficiency ameliorated decorin degradation, loss of dermal collagen density, collagen disorganization and skin thinning (152). As approximately 80-90% of premature skin aging can be attributed to sun/UV radiation exposure, referred to as photoaging, the role of GzmB in photoaging was investigated in mice using a 20-week, chronic model in which mice were exposed every other day to low level (1 MED) UVA/UVB radiation (81). In this model, mast cells were identified as a major source of GzmB and the absence of GzmB prevented cleavage of decorin, loss of collagen integrity, and wrinkle formation (81). In an in vivo mouse model of impaired diabetic burn wound healing and scarring, inhibition of GzmB activity using

topical GzmB inhibitor VTI-1002 prevented the loss of decorin, augmented collagen organization, and improved overall wound quality and tensile strength (153). In support of these observations, fibrotic scars from diverse tissues exhibit reduced decorin levels (211, 212), whilst mouse studies indicate decorin administration to wounds reduces fibrosis (213, 214). Most recently, GzmB was also shown to be elevated in the dermis of pressure injury wounds in humans and mice (199). In the latter study, decorin levels and tensile strength were significantly increased in an aging mouse model of pressure injury when GzmB was absent (199). As wounds typically heal with reduced tensile strength, especially in the elderly or diabetic populations, previous exposure to pressure injuries is a predictive risk factor for subsequent pressure injuries. As such, this work shows promise as a potential therapeutic option to reduce the risk of future pressure injuries by increasing tensile strength.

The mechanisms of GzmB in the pathogenesis and exacerbation of inflammatory skin conditions are rapidly emerging and better understanding of the consequences of its uninhibited, dysregulated proteolytic activity in the skin will shed light on its efficacy as a novel therapeutic target as well as other therapeutic opportunities.

GZMB IN NEUROINFLAMMATION

Multiple sclerosis (MS) is a chronic neuroinflammatory disease characterized by demyelination of the central nervous system and axonal damage caused by infiltrating immune cells, including T cells (215). While intracellular GzmB accumulates in the neural soma (216) and studies have suggested that it induces cytotoxicity through the classical, perforin-dependent mechanism (217, 218), accumulating evidence also indicates that GzmB contributes to neuronal damage independent of perforin. In the absence of perforin, recombinant GzmB induces toxicity in neurons cleaving caspase-3 and α-tubulin in vitro (216, 219), gaining entry through mannose-6-phosphate receptor (216). GzmB is also reported to cleave intracellular substrate transaldolase, the loss of which is found in myelinating cells oligodendrocytes at sites of demyelination, along with loss of myelin basic protein (220).

Predominantly released by activated CD8+ T cells, GzmB is expressed at high levels in active lesions (216) and cerebrospinal fluid of patients with MS (123). This extracellular GzmB induces neurotoxicity through cleavage of cell surface receptor, PAR-1 (221), whereby inhibition of PAR-1 prevented GzmB-mediated toxicity (123). In further support of a GzmB-PAR-1-mediated mechanism, using a murine model of late/chronic EAE, siRNA specifically targeting GzmB significantly reduced the cumulative EAE disease severity scores compared to controls (222). In the same study, the proposed mechanism involved Eomes+ CD4+ T cell-mediated secretion of GzmB which facilitated neurotoxicity through a process that could be attenuated using a PAR-1 antagonist (222).

More recently, a role for GzmB in a non-apoptotic mechanism that may underlie the pathogenesis of multiple sclerosis has been uncovered. As CD4+ T cells derived from MS patients are resistant to suppression by regulatory T cells (223), the authors questioned whether extracellular GzmB, which has been linked to autoimmunity, may play a role (223). Extracellular GzmB was shown to inhibit suppression of non-regulatory, responder T cells by regulatory T cells without decreasing viability of regulatory T cells (223). Importantly, extracellular GzmB inhibitor Serpina3n has shown efficacy in reducing axonal and neuronal injury in a mouse model of EAE (184). In the latter study, Serpina3n-treated mice exhibited a significant reduction in myelin loss and cumulative EAE scores (184). Given that extracellular GzmB is a key contributor in mediating inflammation in other tissues, further investigation may reveal other non-cytotoxic roles for extracellular GzmB in neuroinflammatory disease.

GZMB IN CANCER PROGRESSION

The role for the GzmB/perforin pathway in cytotoxic lymphocyte-mediated tumour cell apoptosis is well-documented and described elsewhere. However, it is recognized that immune cells possess multiple mechanisms in their arsenal with respect to tumour cell killing (eg. Fas/CD95/FasL/CD95L, TRAIL, other granzymes, etc.). As such, loss of GzmB alone does not augment tumorigenesis (224). In light of the emerging non-cytotoxic mechanisms of GzmB, such as ECM remodelling and manipulating immune homeostasis/tumour escape

programs, GzmB is now also appreciated for its roles in promoting tumour progression (reviewed in Arias et al. (8) and Tibbs and Cao (225)).

Elevated GzmB has been observed in various cancers, where GzmB may contribute to pathology through cleavage of substrates within the surrounding tumour microenvironment. Emergent discoveries on the role of ECM remodeling/degradation in cancer have underscored a putative role for extracellular GzmB in urothelial cancer promotion. A study by D'Eliseo et al. (102) identified GzmB, in the absence of perforin, to be elevated in neoplastic urothelial cancer cells undergoing EMT at the cancer invasion front. In vitro, GzmB expressed by tumour cell lines cleaved vitronectin which is a vital component of the ECM and GzmB inhibition suppressed bladder cancer cell invasion (102). In urothelial cancer, GzmB expression was detected in T cells, with negligible levels of perforin (226), supporting potential roles for GzmB in invasion and metastasis. It is likely that GzmB cleavage of other known ECM substrates, such as decorin and fibronectin, is also relevant to the progression of other solid tumours (8).

Another mechanism by which tumours promote survival and invasion is through manipulating immune homeostasis and escape mechanisms. In healthy individuals, regulatory cells employ various mechanisms involved in downregulating the pro-inflammatory activities of T cells, known as immune checkpoints (227). During cancer development, tumour cells can activate these host mechanisms, establishing an immunosuppressive microenvironment which dampens anti-tumour T cell responses to promote tumour survival and invasion (228). GzmB has been found within pro-tumourigenic regulatory cells, mainly $CD4^+$ Treg and IL21-dependent Breg cells (8). Here it was found in mice in vivo cancer models that GzmB-positive $CD4^+$ Treg cells favour tumour development by mediating elimination of effector anti-tumoural NK and $CD8^+$ T cells by a mechanism dependent on perforin (229). Few studies to date have examined GzmB expression and activity in regulatory cells infiltrating solid carcinomas in vivo (230–232) and more research is required to validate the role of GzmB in cancer immunosurveillance. Finally, though the key roles of GzmB in mediating inflammatory responses from diverse immune and non-immune cells has been demonstrated in recent years as described

elsewhere in this review, its relevance in the context of tumour progression is also unclear and warrants further investigation. Our understanding of granzymes and their contributions to tumour development is at its infancy. As current findings support both pro-tumour and anti-tumour effects of granzymes in tumour environments, not unlike other pathologies, the roles of granzymes appear to be influenced by their cell source, sub-cellular location, microenvironment, as well as access to substrates. As such, the relative tumourigenicity of cells transplanted into GzmB-deficient mice compared to wild-type mice is unresolved and may be dependent on cell type (reviewed in Arias et al. (8))

GZMK IN SKIN INFLAMMATION

Expressed at negligible levels in healthy skin, GzmK is elevated in response to tissue injury and inflammation, localizing with the inflammatory cell infiltrate predominantly in the dermis. In acute burns, GzmK expression was demonstrated to be predominantly expressed by pro-inflammatory M1 macrophages (70).

GzmK knockout mouse models and mechanistic in vitro studies have further delineated GzmK-specific roles, particularly involving inflammation of the skin. Exposure to purified GzmK induces pro-inflammatory cytokine secretion: IL-6 from keratinocytes (70), IL-6 and IL-8 from skin fibroblasts (70, 128) and IL-1β from pro-inflammatory M1 macrophages and peritoneal macrophages (70, 130). GzmK also increases expression of MCP-1 in fibroblasts and endothelial cells as well as VCAM-1 and ICAM-1 in endothelial cells, adhesion molecules that facilitate immune cell recruitment (70, 129). In the presence of GzmK, THP-1 monocyte attachment to endothelial cells in culture was elevated (129), which further supports a role for GzmK in promoting immune cell infiltration. Using a murine model of thermal skin injury, GzmK contributed to a prolonged pro-inflammatory stage of wound repair (70). Moreover, GzmK knockout mice with thermal injury showed reduced expression of IL-1β, IL-6, MCP-1, ICAM-1 and VCAM-1, corresponding to decreased detection of macrophages (70). GzmK knockout mice also exhibited improved keratinocyte migration, re-epithelialization, matrix organization and wound closure (70).

GZMK IN SEPSIS

A role for GzmK in sepsis is also a current area of investigation. Plasma GzmK levels are elevated in patients with putative diagnoses of sepsis compared to healthy individuals while physiological inhibitors of GzmK, inter-alpha inhibitor proteins, are significantly decreased in patients with sepsis (233). Using in vivo models of bacterial sepsis, a key role for GzmK in exacerbation of sepsis was implicated, with GzmK-deficient mice displaying lower sepsis scores than wild-type mice (193).

4. – CURRENT KNOWN GRANZYME INHIBITORS & FUTURE THERAPEUTIC OPPORTUNITIES

It has been estimated that approximately five to ten percent of all pursued drug targets are proteases (234). The unique, non-cytotoxic, pathologic roles that granzymes exert make this family of proteases suitable drug targets. In particular, the identification of granzymes in the extracellular space and the emergence of their roles in disease in recent years have significantly increased their potential as druggable targets. This could explain why few granzyme inhibitors were developed when granzyme function was solely believed to be linked to perforin and intracellular functions. Perhaps the most attractive and best studied target at present is GzmB, with many studies supported by in vitro, ex vivo and in vivo studies validating extracellular GzmB as a target for certain cardiovascular, neurologic and cutaneous conditions. Recent studies using a combination of knockout, Serpin, siRNA and small molecule approaches to validate granzymes (GzmB in particular) as a target have demonstrated proof-of-concept and support further advancement towards the clinic. To our knowledge, viDA Therapeutics (Vancouver, Canada) is the only industrial group that is actively developing pharmacologic inhibitors against granzymes, with a focus on GzmB and GzmK. The development of inhibitors of other granzymes is still in its infancy. Known naturally occurring and synthetic inhibitors of granzymes are listed in Table I.

GzmA is detectable in circulation with activity tightly regulated by extracellular inhibitors aprotinin, anti-thrombin III (ATIII)/Serpinc1, α2-macroglobulin and CI esterase inhibitor (235–237). GzmA inhibition involves the formation of a stable covalent ester linked complex through the active site of the serine protease, blocking the active site from substrate-binding. These non-specific inhibitors are known to modulate systemic

inflammation associated with multiple cardiac events (238). However, inhibitor levels are reduced in sepsis patients, which may explain why increased active GzmA levels are observed in the blood and correlates with disease severity (166). Serpin family inhibitors Serpine2/Protease Nexin-1 and Serpinb12 are slow binding inhibitors of GzmA and hepsin found in the blood and tissues (239, 240). Serpinb6b has also been identified as an inhibitor of mouse GzmA but not human GzmA (241). Administration of Serpinb6b by intraperitoneal injection to mice induced with a model of bacterial sepsis improved survival and reduced serum IL-6 levels (193).

Serpinb9, also known as protease inhibitor 9, PI-9, is an intracellular inhibitor of human GzmB that exists only in the cytoplasm (242, 243). Thought to serve as a layer of protection for cytotoxic lymphocytes against GzmB leakage from granules (244), Serpinb9 is the only known endogenous inhibitor of human GzmB (242). There is currently no known endogenous inhibitor of extracellular human GzmB. Serpina3n is a naturally occurring, non-specific extracellular inhibitor of GzmB that is only found in mice (245, 246). Serpina3 is a family of thirteen related inhibitors from the same gene locus in mice that are all orthologues of human antichymotrypsin (ACT) (247) though Serpina3n is the only orthologue that inhibits GzmB and human ACT is not a GzmB inhibitor (9). Though Serpina3n is not a specific inhibitor of GzmB and can inhibit other proteases, in vivo administration has been used as a means of inhibiting extracellular GzmB activity in vivo to demonstrate proof-of-concept. Serpina3n has demonstrated efficacy in murine models of abdominal aortic aneurysm, whereby Serpina3n attenuated decorin cleavage, prevented rupture and increased survival (52). Serpina3n has also been assessed in a murine EAE model whereby Serpina3n was found to attenuate GzmB-mediated axonal and neuronal injury compared to the vehicle-treated controls. Further, Serpina3n also prevented the loss of myelin and reduced disease severity (184). VTI-1002 (viDA Therapeutics, Vancouver, Canada) is a potent and highly specific extracellular GzmB inhibitor that has demonstrated efficacy in a topical formulation for scarring (153), atopic dermatitis (111), and autoimmune blistering disease (76).

Identification of inhibitors against the orphan granzymes have trailed behind those targeting GzmA and GzmB, though some studies have emerged. To date, Serpinb1 is the only intracellular inhibitor for human GzmH that has been identified (248). Inter-alpha-inhibitor protein (IαIP) is a natural physiological inhibitor of human and mouse GzmK found in plasma, mediated by the second Kunitz-type domain of its bikunin subunit (249). Levels of IαIP are inversely correlated with levels of free extracellular GzmK (26 kDa) in the blood and disease severity in human patients with sepsis (233), suggesting that in addition to inducing inflammation, elevated levels of GzmK may contribute to the onset and/or progression of sepsis. However, to our knowledge, no pharmacologic studies using GzmK inhibitors have been performed in any in vivo models to validate GzmK as a target. An irreversible GzmM-specific tetrapeptide chloromethylketone inhibitor has been designed against the catalytic cleft of human GzmM (250). Serpinb4 is also an intracellular inhibitor of human GzmM, in addition to GzmB (251). In vitro, Serpinb4 inhibited GzmM cleavage of substrates α-tubulin and nucleophosmin while overexpression of Serpinb4 in human HeLa tumour cells inhibited recombinant GzmM and NK cell-mediated cell death (251).

Apart from GzmB, most of the granzyme inhibitors identified to date are non-specific, large protein molecules, rendering them less than ideal for pharmacologic development due to synthesis and manufacturing costs among other challenges. With respect to GzmB, topically applied VTI-1002 has demonstrated efficacy in preclinical models of scarring, dermatitis, and autoimmune blistering. Given the recent explosion in studies demonstrating novel mechanistic roles for granzymes in different pathologies, further therapeutic developments in this area are inevitable.

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DISCLOSURES

D.J.G. serves as a co-Founder and Chief Scientific Officer of viDA Therapeutics. No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.C.R. prepared figures; K.C.R., K.J., and C.T.T. drafted the manuscript; K.C.R., K.J., J.P., C.T.T., and D.J.G. edited and revised the manuscript; K.C.R., K.J., J.P., C.T.T., and D.J.G. approved the final version of the manuscript.

Table I: Human Granzyme Family Substrate Specificity, Biological Functions & Inhibitors

	Chromosome	Type	Cleavage Specificity (Amino Acid Abbreviation)	Original Concept	Emerging Concepts	Inhibitors
GzmA	5q11-12	Tryptase	Basic residues (Arg, Lys)	Caspase-independent cell death	· Carcinogenesis · ECM Degradation · PAR Activation · Pro-inflammatory Cytokine Release · Osteoclastogenesis	Extracellular · Antithrombin III · Aprotinin · α2-macroglobulin · CI esterase inhibitor · Nexin 1 · Serpinb12 Intracellular · Serpinb6b (mice only)
GzmB	14q11.2	Asp-ase	Acidic residues (Asp, Glu)	Apoptosis	· Antibacterial · Autoimmunity · Barrier Dysfunction · Basement membrane Disruption · ECM Degradation · Impaired Remodelling · PAR Activation	Intracellular · Serpinb9/PI-9 · Compound 20 Extracellular · Serpina3n (mice only) · VTI-1002
GzmH	14q11.2	Chymase	Aromatic residues (Phe, Trp, Tyr)	Cell Death	· Antiviral	Intracellular · Serpinb1
GzmK	5q11-12	Tryptase	Basic residues (Arg, Lys)	Necrosis	· Endothelial Activation/Dysfunction · PAR Activation · Pro-inflammatory Cytokine Release · SASP	Extracellular · Inter-alpha inhibitor proteins · Bikunin
GzmM	19p13.3	Met-ase	Aliphatic residues (Leu, Met)	Unknown	· Innate Immunity	Intracellular · Serpinb4 Extracellular · Tetrapeptide chloromethylketone

ECM, extracellular matrix; PAR, protease-activated receptor; SASP, senescence-associated secretory phenotype

Table II: Non-Cytotoxic Human Granzyme Substrates, Arranged by Biological Significance

Gzm	Substrate	Perceived Consequence of Cleavage	Reference(s)
Cell Junction Proteins			
GzmB	α6β4 integrin	Loss of dermal-epidermal adhesion; implicated in BP and EBA pathogenesis	(109)
	Collagen XVII (BP180)	Loss of dermal-epidermal adhesion, implicated in BP and EBA pathogenesis; production of collagen XVII fragments (~97 kDa) may be autoantigenic with possible implication in linear IgA bullous disease	(76, 109)
	Desmoglein-1	Epidermal barrier dysfunction; implicated in atopic dermatitis pathogenesis	(111)
	Desmoglein-3	Epidermal barrier dysfunction; implicated in atopic dermatitis pathogenesis	(111)
	E-cadherin	Epidermal barrier dysfunction; implicated in atopic dermatitis pathogenesis; production of sE-cadherin fragments (~80 kDa) impairs epithelial barrier function	(111)
	Filaggrin	Epidermal barrier dysfunction, implicated in atopic dermatitis pathogenesis	(111)
	JAM-A	Loss of cell-to-cell contact integrity leading to reduced endothelial and retinal barrier function and an increase in vascular permeability and leukocyte extravasation, leading to inflammation and fibrosis	(56, 110)
	Occludin	Loss of cell-to-cell contact integrity leading to reduced retinal barrier function	(110)
	PECAM-1	Loss of endothelial cell-to-cell contact integrity leading to reduced endothelial barrier function and an increase in vascular permeability and leukocyte extravasation, leading to inflammation and fibrosis	(56)
	RPE-derived tight junctions	Loss of cell-to-cell contact integrity leading to reduced retinal barrier function	(110)
	VE-Cadherin (CD144)	Loss of endothelial cell-to-cell contact integrity leading to reduced endothelial barrier function and an increase in vascular permeability and leukocyte extravasation	(56, 80)
	ZO-1	Loss of cell-to-cell contact integrity leading to reduced epidermal and retinal barrier function	(56, 110, 111)
Cell Surface Receptors			
GzmA	PAR-1, thrombin and thrombin-like receptor	Competitively interacts with PAR-1 against thrombin; desensitizes response to thrombin-induced aggregation by platelets; in hepatocellular carcinoma, low expression of GzmA and PAR-1 in tumour tissues is correlated with aggressive clinicopathological characteristics and poor prognosis; mechanistically, GzmA activates PAR-1 on tumor cells to induce tumor suppression and cell death via the activation of the JAK2/STAT1 pathway; elicits morphological changes in neural cells, as demonstrated by detection of weakened Ca2+ signals; leads to neurite retraction and reversed stellation of astrocytes; may be implicated in nervous system impairments	(117, 120, 121, 170, 252)
GzmB	Acetylcholine Receptor	May be autoantigenic; implicated in myasthenia gravis	(124)
	FGFR1 (CD331)	May be autoantigenic; implicated in prostate cancer	(126)
	Neuronal Glutamate Receptor 3	May be autoantigenic; implicated in Rasmussen's encephalitis (severe form of pediatric epilepsy)	(125)
	Notch1	May be autoantigenic; implicated in prostate cancer	(126)
	PAR-1	Neuronal death	(123)
	PAR-2	IL-25 release (with IL-13) in epithelial cells and promotes type II immune response	(108)
GzmK	PAR-1	Endothelial dysfunction; releases IL-6, MCP-1	(128, 129)
Extracellular Matrix Proteins			
Collagen Fibres			
GzmA	Collagen IV	Lymphocyte transmigration through basement membrane remodeling	(53, 253)
GzmB	Collagen IV	Lymphocyte transmigration	(142, 143)
	Collagen VII	Loss of dermal-epidermal adhesion; implicated in sub-epidermal, autoimmune blistering, EBA pathogenesis	(109)
Proteoglycans			
GzmA	Aggrecan	Major constituent of cartilage; implicated in arthritis pathogenesis	(254)
GzmB	Aggrecan	Cartilage degradation; implicated in arthritis pathogenesis	(145)

	β-glycan (soluble)	Sequesters TGF-β1; implicated in cardiovascular disease pathogenesis	(156)
	Biglycan	Sequesters TGF-β1; implicated in cardiovascular disease pathogenesis	(156)
	Decorin	ECM remodelling; decreases structural integrity and strength; disrupts collagen fibrillogenesis/collagen organization/thick bundle formation; sequesters TGF-β1; implicated in atopic dermatitis, age-impaired wound healing, diabetic wound healing, ApoE aging, photoaging, aneurysm and cardiovascular disease pathogenesis	(52, 81, 111, 152–156)
Other			
GzmB	Fibrillin-1	Loss of elastic lamellae, medial degeneration, vessel wall instability; implicated in Marfan syndrome and abdominal aortic aneurysm pathogenesis	(51)
	Fibrinogen (matrix form)	Impairs platelet integrin to mediate platelet adhesion; forms platelet-platelet bridges; contributes to thrombus growth; putative role in local coagulation during inflammation	(162)
	Fibronectin	Impairs integrin-mediated cell-matrix adhesion/signaling leading to cell detachment and death, vasomotor dysfunction, increased inflammation; implicated in vascular disease pathogenesis and diabetic wounds; production of fibronectin fragments (various ~80-230 kDa) increases vascular permeability and induces MMP-1/3 expression in fibroblasts; implicated in photoaging	(54, 55, 81, 110, 147, 148)
	Laminin-332 (previously Laminin-5), Laminin-511 (previously Laminin-10)	Impairs integrin-mediated cell-matrix adhesion/signaling leading to cell detachment and death	(55, 110)
	Vitronectin	Impairs integrin-mediated cell-matrix adhesion/signaling leading to cell detachment and death	(55)
	VWF (matrix form)	Interferes with VWF-platelet interaction (delays ristocetin-induced platelet aggregation and inhibits platelet adhesion and spreading); putative role in local coagulation during inflammation	(162)
Cytokine Processing			
GzmA	pIL-1β	Produces cytokine IL-1β; Dysregulated inflammation	(163, 164)
GzmB	IL-1α	Enhances IL-1α activity; Dysregulated inflammation	(173)
	pIL-18	Produces cytokine IL-18; Dysregulated inflammation	(111, 174, 175)
Plasma Proteins			
GzmA	uPA	Generates plasmin during T-cell mediated processes	(179)
GzmB	C3	Produces anaphylatoxin C3a; activates the complement system	(181)
	C5	Produces anaphylatoxin C5a; activates the complement system and neutrophil chemoattractant	(181)
	Plasmin	Produces angiostatin fragments; antiangiogenic activity	(180)
	Plasminogen	Produces angiostatin fragments; antiangiogenic activity	(180)
GzmM	VWF	Inhibits platelet aggregation and destabilizes coagulation factor VIII in plasma; putative role in local coagulation during inflammation	(182)
Other/Undefined			
GzmA	MBP	Damages myelin; implicated in neurodegenerative disease pathogenesis (e.g., multiple sclerosis)	(183)
GzmK	Unidentified	Senescence-associated inflammation (SASP: IL-6, CCL2, CXCL1)	(185)
GzmM	Ezrin	Inhibits activation of AKT and MAPK survival pathways; putative role in cell death; inhibits tumor metastatic progression	(255)
	PAK2	Unknown	(256)

BP, bullous pemphigoid; CCL, CC chemokine ligand; CXCL, chemokine (C-X-C motif) ligand; EBA, epidermolysis bullosa acquisita; E-cadherin, epithelial cadherin; FGFR1, fibroblast growth factor receptor 1; IL, interleukin; JAK/STAT, janus kinase/signal transducer and activator of transcription; JAM-A, junctional adhesion molecule A; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MMP, matrix metalloproteinases; Notch1, notch homolog 1; PAR, protease-activated receptor; PECAM-1, platelet endothelial cell adhesion molecule 1; RPE, retinal pigment epithelium; SASP, senescence-associated secretory phenotype;

TGF-β, transforming growth factor beta; PAK2, P21 activated kinase; uPA, pro-urokinase plasminogen activator; VE-cadherin, vascular endothelial cadherin, VWF, von Willebrand factor; ZO-1, zonula occludens

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FIGURE LEGENDS

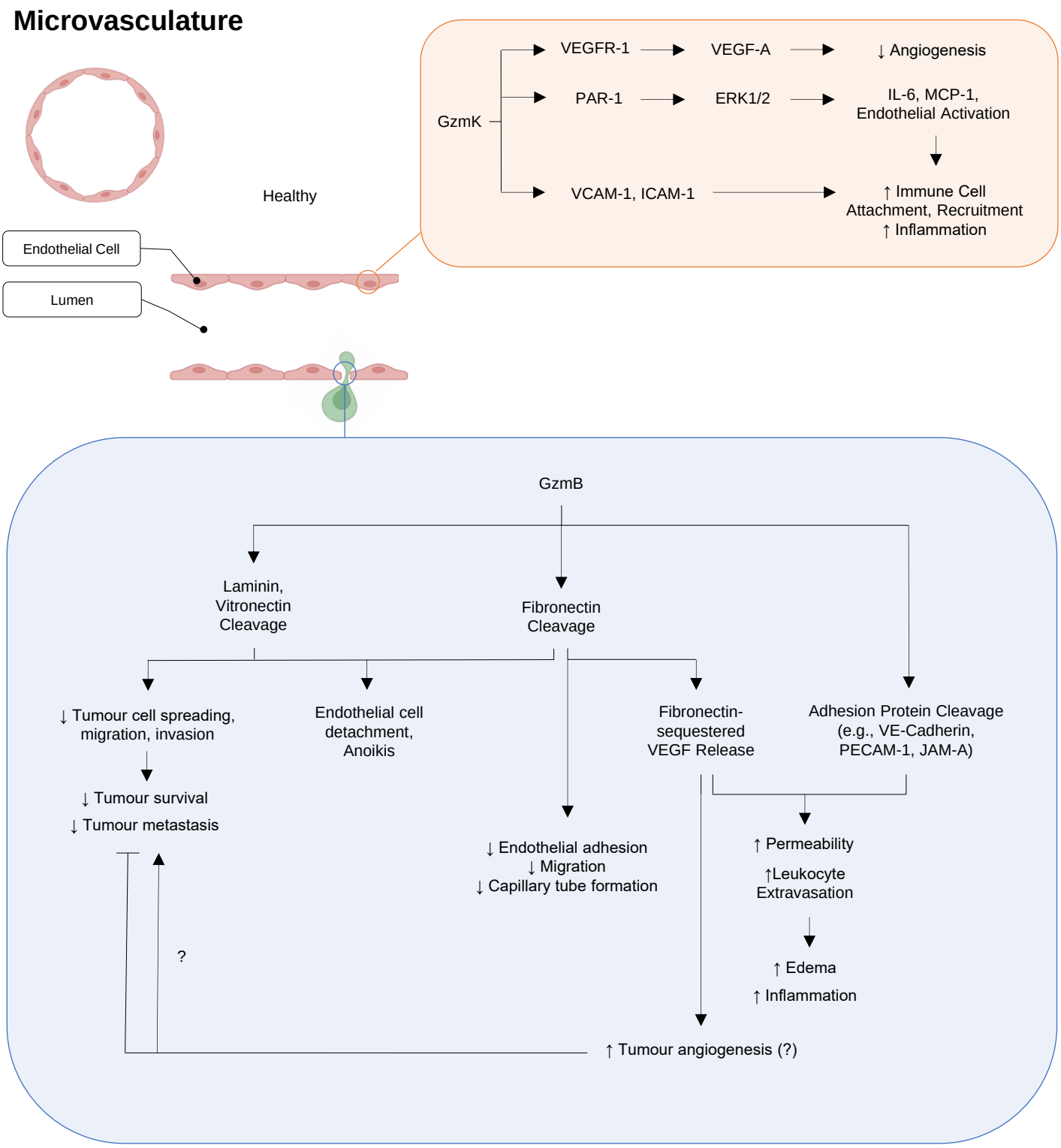
Figure 1: GzmB and GzmK in endothelial dysfunction. GzmB contributes to endothelial permeability through the release of fibronectin-sequestered VEGF, which may promote endothelial permeability. Secondly, GzmB cleaves key cell adhesion proteins (e.g., VE-cadherin) resulting in reduced cell-cell adhesion. GzmB cleavage of fibronectin disrupts endothelial adhesion, migration and capillary tube formation. GzmB contributes to anoikis through cleavage of fibronectin, laminin and vitronectin. In a tumour microenvironment, cleavage of these matrix proteins may discourage tumour cell survival and metastasis, which may be enhanced or impeded by VEGF-mediated pro-angiogenic signaling. GzmK may promote endothelial dysfunction through a process involving PAR-1 activation leading to pro-inflammatory cytokine release as shown.

Figure 2: GzmB in atherosclerosis. GzmB accumulates with increased atherosclerotic severity. GzmB may contribute to plaque instability and rupture via the cleavage of decorin in the atherosclerotic cap region resulting in reduced collagen stability and rupture. GzmB may also contribute to smooth muscle cell death via apoptosis/anoikis.

Figure 3: GzmB in abdominal aortic aneurysm. GzmB elevation has been observed in lymphocytes in the intraluminal thrombus, deep intima, media and adventitia. GzmB may contribute to medial disruption through the cleavage of fibrillin-1, a key component of microfibrils, and further disruption to the elastic lamellae. GzmB accumulation in the adventitia is proposed to contribute to the cleavage of decorin, an important mediator of collagen fibrillogenesis and organization. Loss of decorin contributes to impaired adventitial collagen remodeling leading to reduced circumferential strength resulting in dilatation and rupture. Decorin also binds to, and retains TGF-β. At present, while GzmB has been shown to release TGF-β from decorin and biglycan in vitro, the significance of these findings in vivo is unknown.

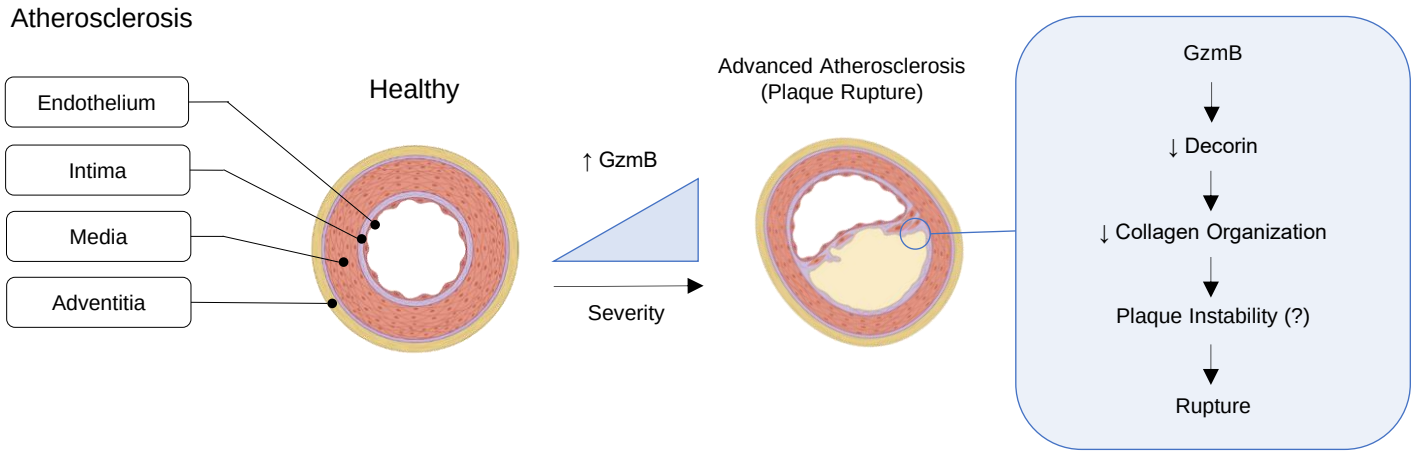
Figure 4: GzmB and GzmK in aging and/or inflammatory skin conditions. The role of GzmB in skin is context-dependent based on the location, cell source, and substrates exposed to proteolysis. In the epidermis, GzmB contributes to reduced epithelial barrier function through the cleavage of cell-cell junction proteins. GzmB also induces IL-8 release from keratinocytes resulting in neutrophil recruitment and may augment neutrophil elastase activity, as demonstrated in autoimmune blistering. The impact of GzmB on epithelial dysfunction is an area of active study in other epithelial tissues where GzmB may augment a Th2 immune response (asthma), cleave desmosomal proteins in the retinal pigment epithelium (macular degeneration) or promote epithelial shedding (Crohn's disease). In the basement membrane (dermal-epidermal junction), GzmB accumulation results in cleavage of hemidesmosomal proteins (collagen VII and XVII as well as a6b4 integrin) leading to separation and sub-epidermal blistering in bullous pemphigoid, dermatitis herpetiformis and epidermolysis bullosa acquisita. While GzmB-mediated laminin cleavage has been observed in vitro, to date, its cleavage has not been investigated in vivo. GzmB accumulation in the dermis has been observed in extrinsic skin aging (e.g., photoaging) and chronic wound healing (e.g., diabetic, age-impaired) and scarring (thermal injury). In the dermis, GzmB-mediated cleavage of decorin contributes to impaired collagen remodeling and reduced tensile strength. Decorin can also impede MMP-1-mediated collagen cleavage while GzmB-generated fibronectin fragments promote dermal fibroblast MMP-1/3 expression. GzmB-mediated decorin cleavage also increases TGF-β release and scarring. Ultraviolet light induces GzmB expression in keratinocytes and increases GzmB+ mast cells in the dermis. GzmK appears to act on PAR-1 in the epithelial cells and possibly the dermal

microvasculature via PAR-1 to induce proinflammatory cytokine production. GzmK also impedes re-epithelialization of keratinocytes.



Note: Figures are to be redone by APS professional artist as stated in review invitation

Macrovasculature



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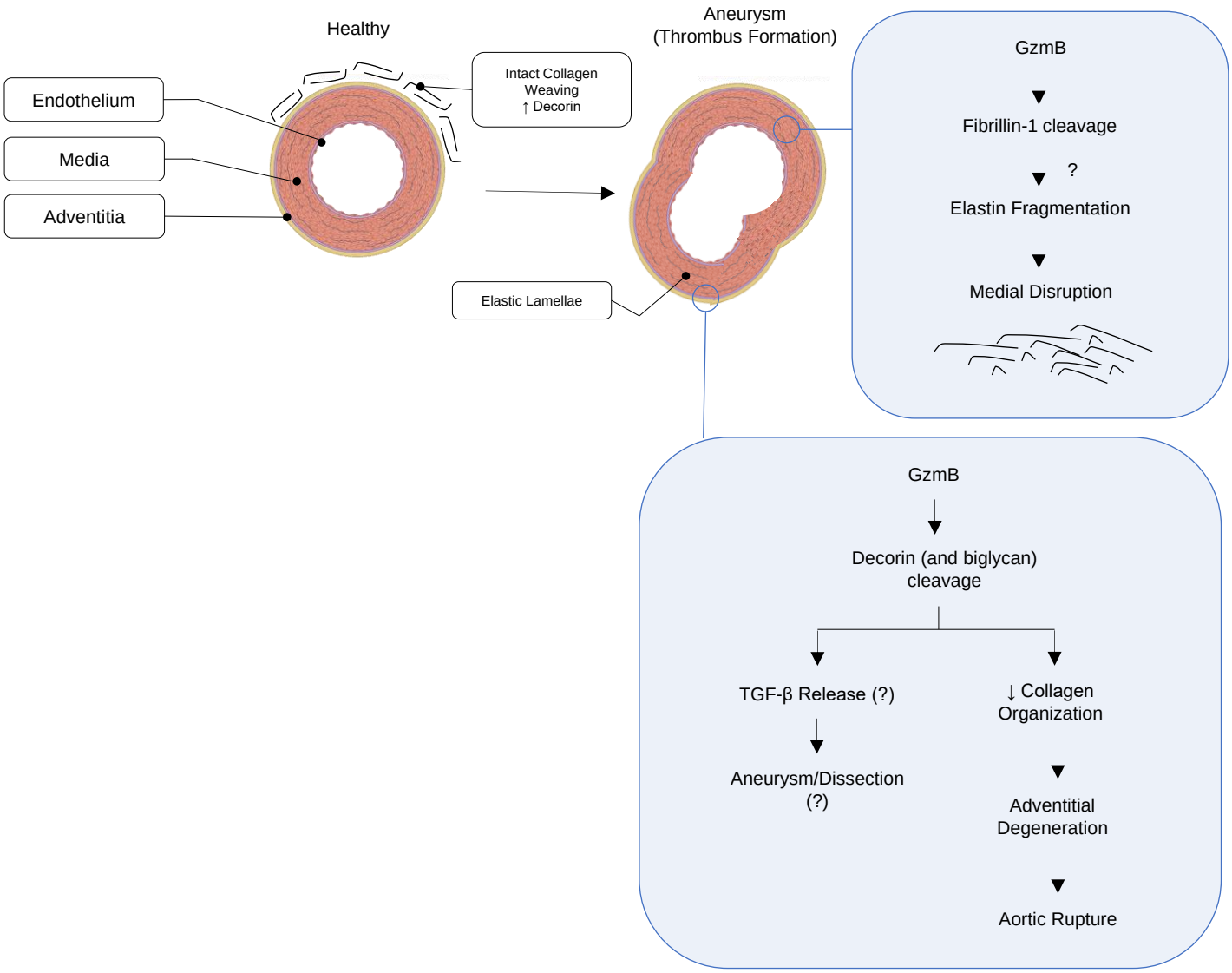
Macrovasculature

Abdominal Aortic Aneurysm



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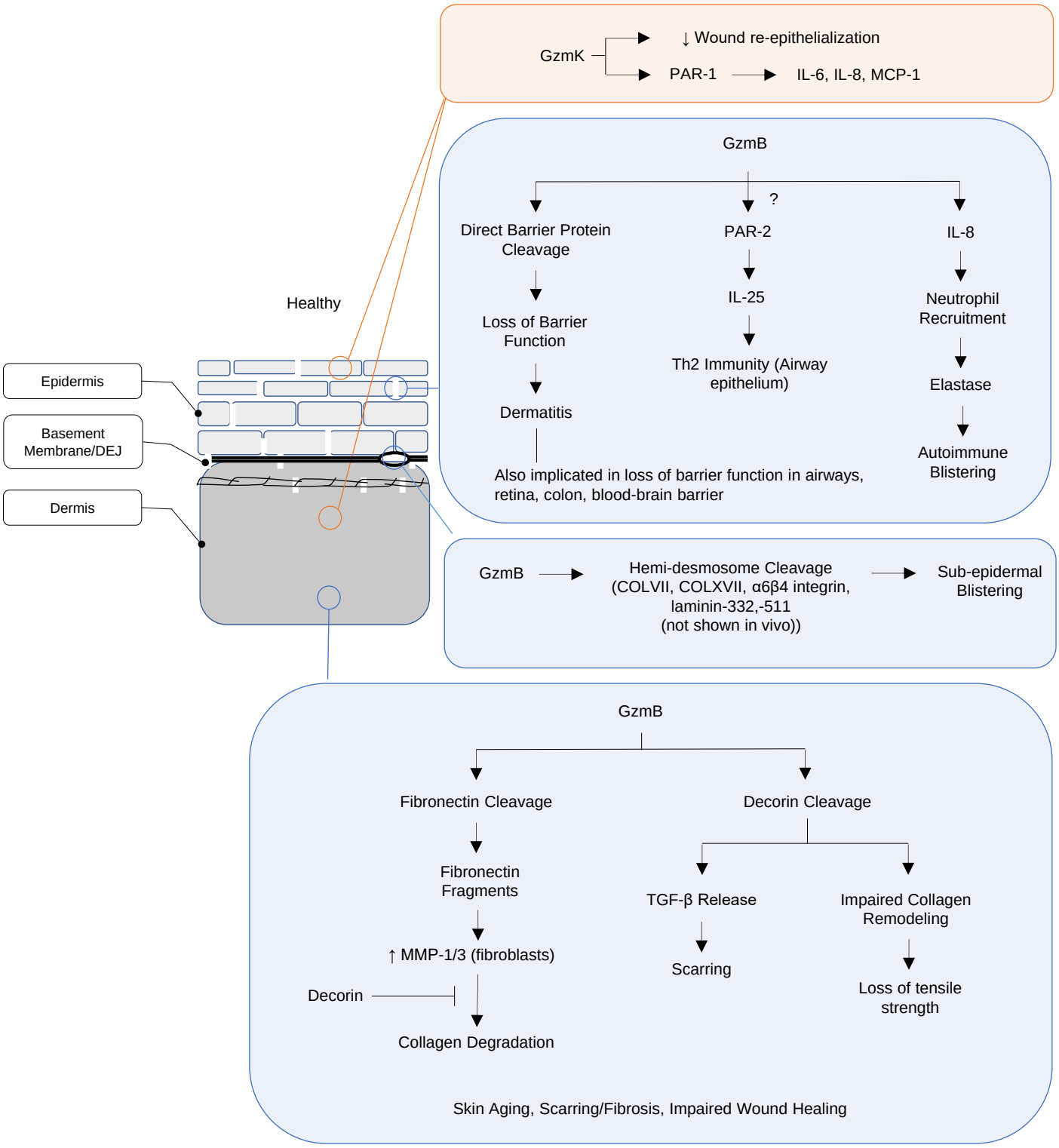
Skin/Epithelium



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