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Contribution of the Plasma and Lymph Degradome and Peptidome to the MHC Ligandome

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Abstract

Every biological fluid: blood, interstitial fluid and lymph, urine, saliva, lacrimal fluid, nipple aspirate and spinal fluid contains a peptidome-degradome derived from the cellular secretome along with byproducts of the metabolic/catabolic activities of each parenchymal organ [1-18]. Over the last decade qualitative and quantitative analysis of the biological fluid peptidome and degradome have provided a dynamic measurement of tissue homeostasis as well as the tissue response to pathological damage. Proteomic profiling has mapped several of the proteases and resulting degradation by-products derived from cell cycle progression, organ/tissue remodeling and cellular growth, physiological apoptosis, hemostasis and angiogenesis. Currently, a growing interest lies in the degradome observed during pathological conditions such as cancer, autoimmune diseases and immune responses to pathogens as a way to exploit biological fluids as liquid biopsies for biomarker discovery [12,13,17,,19-48]. In this review we focus on the current knowledge of the degradome/peptidome observed in two main biological fluids (plasma and lymph) during physiological and pathological conditions and its importance for immune surveillance.

Keywords

Lymph; Degradome; Peptidome; MHC molecules

Interstitial Fluid and Lymph

The interstitial fluid is a thin film of fluid that bathes all the cellular layers of each parenchymal organ [49-51]. In humans, 8-12 liters of interstitial fluid are generated every day, which accounts for about one sixth of the body weight[49,50,52]. A fundamental function of the interstitial fluid is to provide nutrients, water, electrolytes, metabolites, and overall biomolecules to parenchymal cells[50,53-55]. Indeed, since the organ's cellular layers are not in direct contact with the blood, nutrients, protein and soluble molecules need to extravasate into the interstitial fluid to be taken up by parenchymal cells. The movement of proteins and biomolecules from the intravascular to the extravascular/interstitial compartment is associated with movement of water and electrolytes, which altogether forms the elementary composition of the interstitial fluid. Using radioactive tracer it was originally shown that all the major categories of plasma proteins, such as albumin and α, β and γ globulins were

present in the interstitial fluid at a relatively smaller concentration (between 20% to 37%, depending on the anatomical district) than in the plasma[50,56,57]. These proteins, which are pivotal for the maintenance of the intravascular oncotic pressure, extravasate from the blood capillaries following the gradient of hydrostatic pressure present between the arterial and venule-end of the capillary bed [58]. The interstitial fluid will then be progressively enriched with the tissue proteome derived from the cellular secretome, products of metabolic cellular activities, extracellular matrix remodeling and physiological apoptosis[2-5,11-13,16,59-61]. Indeed, although plasma albumin and serum globulins constitute the majority of the lymph proteins, tissue-specific proteins are also highly represented in the lymph proteome when compared to the plasma proteome [2-5,11-13,16,59-61]. Tissue specific proteins are present both as soluble proteins as well as in exosomes and extracellular vesicles, released from parenchymal cells and circulating in the lymph [62-65]. Soluble proteins and their degradation products comprise different members of the extracellular matrix proteome, which is processed by the several Matrix Metalloproteinases (MMPs) involved in tissue growth and remodeling. Among these, MMPs generate collagens, laminins, versican and lumican, among many other cleavage fragments, as a way to accommodate cell movement, division, regulate cellular architecture or to generate specific collagen products that will act as integrin signaling [1-5,66-72]. Additionally, MMPs, ADAMs, Sheddase and γ-Secretase products of cleaved growth factors (SDF1, VEGF) or other surface receptors, (TNF, IL-1R, IL-11R, Notch1 IL-32), have also been mapped in the lymph; where soluble peptides are released following extracellular or intramembrane processing upon receptor ectodomain release [2-5,73-79]. By-products from plasminogen activators and thrombin, such as fibrin and PAR-1,3 and 4 cleaved products have also been found in the lymph [2,3]. Degradation products from kallikreins, involved in blood and lymph flow regulation and electrolytes balance have also been mapped in the serum and lymph peptidome [2,3].

Apoptotic cells, which have been detected in the lymph [80], have also been shown to release intracellular proteins such as cytosolic enzymes (carboxypeptidase, enolase-3, LDH, GAPDH, different kinases), cytoskeletal proteins (α-actinin-4, filamin-α), chaperones (14-3-3, hsc-70 and hsp-90), mitochondrial and nuclear proteins (histones HMGB1)[81–85]. Degradation products due to caspase activity have also been mapped in the lymphatic fluid [2-5].

During pathological conditions, including acute and chronic inflammation, cancer, autoimmune and degenerative diseases, several proteomic analyses have shown that the interstitial fluid and the lymph are further enriched with by-products of tissue injury, necrosis, apoptosis and overall cellular damage [11,16,29,86-92]. Following trauma changes in the lymph proteome could be observed within the first 30 minutes including triggering of coagulation and pro-inflammatory responses, changes in proteases/antiproteases homeostasis and release of products from cellular damage[11]. Similarly, changes in the lymphatic fluid were observed following acute pancreatitis with release of pancreatic enzymes and a necrosis-related degradome[90]. In patients with rheumatoid arthritis the inflammatory process could be clearly mapped in the lymph draining the affected joints. Finally, lymph analysis in different models of cancer has shown the potential of exploiting interstitial and lymphatic fluid for early detection of metastasis. Indeed, one of the main goals of cancer research is the ability to detect and possibly prevent metastasis formation,

and liquid biopsy is considered the key to monitor disease progression. We recently performed a comparative proteomic analysis between lymph and plasma in several patients with melanoma, as compared to healthy controls. We determined that the lymph was significantly enriched in melanoma biomarkers such as Melan-A, S100B, and S100A8 [93,94], whereas the same proteins were undetectable in blood (manuscript in preparation). Additionally, factors known to strongly correlate with metastatic potential in melanoma, including colony stimulating factor-1 (CSF-1), Galectin-3, and matrix metalloproteinases (MMP)-2 and −9 [95], were also orders of magnitude higher in lymph compared to plasma (manuscript in preparation). When melanoma patients without metastasis were compared with those with metastasis, as well as with healthy patients, a distinct protein signature in the lymph, that was absent in the plasma, and that characterized the lymph of metastatic melanoma patients was found. Highly expressed were proteins related to cytoskeletal rearrangement and adhesion (myosin, actin, tropomyosin, troponin, integrins), matrix remodeling (vimentin, enolases, cathepsins), histone variants and glycolytic enzymes (triosephosphate isomerase). Most of these proteins were previously shown to be differently regulated in tumor progression and metastasis [9697].

Overall during the last twenty years several proteomic analyses performed on bovine, ovine, rodent, and human lymph sampled in physiological and pathological conditions have determined that the lymphatic fluid is not a simple ultrafiltrate of the plasma but collects the “omic signature” of the organ from which it drains [2-5 ,11-13,16 ,59-61].

Blood

The blood peptidome-degradome was the first, among all other biological fluids, to be investigated. Over the years several mass spec analyses have provided a comprehensive mapping of blood peptides in physiological and pathological conditions. Overall over 10,000 peptides deriving from both intracellular and extracellular sources have been identified using bottom up and top down mass spectrometry. Most of these peptides derive from proteolytic processing performed by a variety of peptidases involved in several cellular processes including tissue remodeling, membrane receptor editing, cellular secretion and cellular apoptosis. The amount and complexity of the extracellular degradome/proteome is further increased during inflammatory and degenerative conditions as well as cancer, all conditions associated with increased protease activity. As such the blood peptidome-degradome has been considered a valuable source of biological information, as a liquid biopsy for identifying plasma-based markers of disease. Notably, quantification of insulin peptides in serum is used as a biomarker for diabetes[98], collagen fragments as well as peptides from cartilage proteins, as a marker of osteoporosis and degenerative joint conditions, β-amyloid and TAU peptides for Alzheimer’s disease[99] and angiotensin II for hypertension[100], to name a few. For cancer peptides a comprehensive database (http://crdd.osdd.net/raghava/cancerpdf/), has recently been generated that reports peptides mapped in serum and plasma from ~30 forms of cancers. The reported degradome/peptidome highlights protein processing and degradation in the tissue environment as biomarkers of cancer-associated activities. An automated technology platform has also been developed at Memorial Sloan-Kettering Cancer Center (MSKCC) to extract and map peptides from patients’ serum. Using robotic automation on a MALDI-TOF target plate, around 600 peptides were identified in a

cohort of 27 patients with metastatic thyroid cancer and 32 controls. A statistically significant diverse peptidome could be mapped between the two groups [46]. Similar analyses have mapped degradome/peptidomes in patients with oral, breast, ovarian, colon-rectal cancer as well as metastasis from different malignancies[24-26,28,31,32,36,37,40,43,101-103]. All these analyses point to the importance of the peptidome/degradome, present in biological fluid, as an important tool for biomarker discovery[37].

Lymph and Blood Circulation in Spleen and Lymph Nodes

In the paragraphs below the anatomical structures of spleen and lymph nodes are described. Our goal is to illustrate how lymph and blood flows through these secondary lymphatic organs and how the anatomical structure facilitates the process of clearance and sampling of the blood and lymph proteome/peptidome for immunosurveillance. The variety, complexity and functionality of the different immune cell populations present in these organs are beyond the goal of this review.

Blood Clearance in the Spleen

Anatomically the spleen is divided into the red and white pulp[104]. Since a major role of the spleen is to clear/filtrate the incoming blood the splenic circulation differs from the classical pattern of arterioles, capillaries, and venules, as present in all the other parenchymal organs[104]. The red pulp is divided into anatomical regions by connective tissue called the “cords of Billroth". The cords are formed by a meshwork of fibroblast-like reticular cells supported by extracellular matrix and reticulin fibers without endothelial lining [104]. The anatomical cavernous spaces of the cords directly receive arterial blood from terminal arterioles and arterial capillaries[104]. Several macrophages and dendritic cells are present within the cords attached to the extracellular matrix components, whose job it is to filtrate pathogens, abnormal/old/damaged red blood cells from the blood and to sample the overall circulating proteome [104-106]. From the cords the blood flows into the venous sinuses, which represent a second system of filters, before entering the venule-end of the splenic circulatory system[104]. The venous sinusoidal system consists of a network of contractile reticular fibers, composed of actin and myosin-like filaments, known as stress fibers, which run both circumferentially and longitudinally forming a filtration lattice. Elongated endothelial cells, on top of this fenestrated basal membrane, as well as a population of macrophages known as metallophilic macrophages, form this additional filter to ensure no pathogens or damaged red blood cells re-enter the general circulation [104,106].

The spleen white pulp contains around 30% of the total lymphocyte population which are structured around a central arteriole as “periarteriolar lymphocyte sheaths” (PALS). Most of the T cells are in the inner part of the PALS whereas B cells and plasma cells are in the outer PALS. In the periarteriolar areas T cells interact with dendritic cells and B cells. Next to the PALS bona fide B cell follicles are present where clonal expansion of B cells, isotype switching and somatic hypermutation occurs[104]. Anatomical integrity of these areas is controlled by a complex of chemokine gradients (CXCL13, CCL19, CCL21) and their respective receptors which guide T and B cell trafficking and localization [104]. Altogether PALS and B cell follicles are surrounded by a corona-like structure, called the marginal

zone, which also contains B cells, the marginal zone macrophages and different dendritic cell subsets[104]. The marginal zone is located between the white and red pulp and is an important transit area for cells entering the PALS from the blood stream.

PALS and B cell follicles resemble the anatomical structures present in the lymph node albeit an important difference is that the spleen is void of afferent lymphatics. As such the lymph does not circulate in the splenic parenchyma and blood filtration is the primary immunological function of the spleen. On the other hand, structures similar to efferent lymphatics have been described in the spleen as an exit way for T, B and dendritic cells to leave the white pulp and re-circulate into the lymphatic system [49]. These lymphatic vessels, present in the T and B areas of the white pulp, have been described flowing from the outer to the inner PALS, running in parallel to the arteriole and draining into the pancreatic-splenic lymph nodes[49]. From there the lymph flows into the intestinal lymph trunk, which then enters the cisterna chyli and finally drains into the thoracic duct.

Lymph Clearance in the Lymph Nodes

During the past few years new research has elucidated how the interstitial fluid enters the initial lymphatics to form lymph. It was originally proposed, and recently experimentally proven, that tissue movement transiently increases interstitial pressure, generating a suction force that facilitates drainage into the open-ended lymphatics[49]. These initial lymphatics do not contract whereas the downstream collecting lymphatics propel the lymph forward through a Ca^{++}-initiated contraction of smooth muscle present in the vessel wall. Additionally, the presence of a one-way valve separating anatomical segments of the vessels at equal distance (each segment is called a lymphangion) synergizes with the vessel pumping activity favoring lymph movement towards the lymph node[107].

Several studies have indicated that subcutaneously injected fluorochrome- labeled proteins or particles reach the draining node within minutes after injection, signifying the efficiency of the lymphatic circulation in transporting soluble and particulate material to the regional lymph nodes[107]. Additionally, subsets of dendritic cells that patrol peripheral organs also enter the lymphatics, following a CCR7- CCL21 gradient into the lymph node [108]. These afferent lymphatic vessels, draining well-defined anatomical regions, enter through the lymph nodal capsule into the sub-capsular sinuses, which are pool-like areas where the lymph flows and is filtrated by the macrophages and dendritic cells lining the walls of the sinuses[4]. $CD11b^{+}.CD169^{+}.MHCII^{+}$ macrophages and dendritic cells that have been characterized on the floor of the sub-capsular and medullary sinuses, that act as “flypaper” to capture incoming pathogens for T and B cell presentation. These cells function as pivotal gatekeepers, which prevent pathogen dissemination[109]. From the sinuses the lymph travels along two possible routes; particulate material and proteins with a molecular weight above 80 kDa flow from the sub-cortical and medullary sinuses into the efferent lymphatic to the next lymph node, whereas proteins and small molecules below 80 kDa enter the conduit system. The conduit system comprises a series of 100-200 nm diameter channels, within the nodal T cell areas, formed by a central pillar of collagens I and V and peripheral walls formed by perlecan, fibronectin and laminins [110].-[114]. These channels physically connect the sub-cortical and para-cortical regions of the node with the medullary spaces and the high

endothelial venule. Low molecular weight proteins and small molecules passing through the conduit will encounter dendritic cells, scattered throughout the T cell areas, whose dendrites directly penetrate the conduit system[110-114].

Through this highly regulated size exclusion mechanism two fundamentally important functions of the lymph node are achieved: fluid homeostasis and pathogen immunosurveillance. Indeed, the vast majority of water/fluid from the interstitial fluid and pre-nodal lymph will pass through the conduit and flow directly into the high endothelial venule as a mechanism to control body fluid homeostasis[115]. On the other hand particulate materials and pathogens are excluded from the conduit as a safeguard mechanism to avoid bacteria/viruses entering the bloodstream. Pathogens that will not be phagocytized by the dendritic cells and macrophages present in the sub-cortical and medullary sinuses will exit through the efferent lymphatic into another lymph node[4]. Indeed in humans the 600-800 lymph nodes present throughout the body are organized in chains of 6-8 nodes to ensure proper pathogen clearance. As such the lymph nodes act as a sieve that uses size exclusion as a mechanism to capture the self and non-self proteome for immunosurveillance.

A recent quantitative analysis of the nodal clearance capacity, which utilized state-of-the-art, label-free quantitative (LFQ) proteomics complemented by tandem mass tag isotope labeling, was used to identify proteomic changes in the pre- and post-nodal mesenteric lymph [116]. The proteomic data were supplemented with analysis of lymphatic transport of fluorochrome-labelled proteins, bacteria and beads, by direct cannulation of pre-nodal lymphatics followed by post-nodal collection and quantification. The picture that emerged is that lymph nodes act as very efficient filtration organs, with concentration-dependent filtration efficiency across molecular sizes [116]. Indeed, by direct lymphatic cannulation with injection of titrated amounts of fluorophore-labeled proteins, at a physiological flow pressure, it was determined that for protein concentrations up to 5 μg/ml, corresponding to the concentrations of all tissue-specific antigens, the efficiency of protein clearance upon nodal passage was up to 80-90%. For much higher protein concentrations, corresponding to the ones normally observed for proteins involved in the maintenance of oncotic pressure, efficiency of protein clearance upon nodal passage was lower[116]. Similarly, the lymph node could efficiently clear a pathogen load up to a million bacteria, but when ten million bacteria were injected into the pre-nodal lymph, around 30% of the pathogen load could be observed in the post-nodal lymph [116].

Proteases generating the blood and lymph peptidome

Protease genes comprise 1.7% of the human expressed genome but despite their relevance in a variety of biological activities many of the proteases, their substrates, activators and inhibitors are yet to be fully characterized. Proteases can be divided into five catalytic classes including metallo, serine, cysteine, threonine and aspartic proteases. Each class is further divided into different families, mostly based on primary amino acid sequence and three-dimensional structure (MEROPS database). During the last few years there has been a keen interest in the analysis of the proteases, their inhibitors and degradation substrates in serum and lymph. The overall goal of these liquid biopsies is to map changes during physiological and pathological conditions, which can help identify early stage of disease and

monitor its progression. Indeed, albeit a degradome can be mapped in both fluids under physiological conditions, its composition changes both qualitatively and quantitatively in pathological conditions generating degradomic maps and peptide hot-spots as potential disease biomarkers. In the paragraphs below we review current literature on the known family of proteases generating the degradome/peptidome in the blood and lymph in physiological and pathological conditions. Although all the below mentioned enzymes are involved in several pathological processes our analysis will only report on enzymatic processing in relationship to the blood and lymph peptidome.

MMPs and ADAMs

During physiological conditions the lymphatic fluid degradome is highly represented in byproducts derived from the action of collagenolytic matrix metalloproteinase (MMPs) and disintegrin metalloproteinases (ADAMs), which are involved in extracellular matrix remodeling in parenchymal organs.

There are over 20 MMPs and over 40 ADAMs family members in the human genome. MMP proteolysis forms space for cell migration by cleaving intercellular junctions or the basement membrane, regulate tissue morphology through proteolysis of cell-cell junctions, and by cleavage can activate/deactivate signaling molecules[117]. For example, MMP9 cleavage of collagen IV α3 chain has been shown to generate an anti-angiogenic peptide which binds to the αvβ3 integrin[118]. As such the majority of the generated peptidome/degradome consists of collagens, laminins, elastins, fbronectins, proteoglycans and surface molecules, such as cadherins and integrins. Indeed, cleavage products from all these proteins have been extensively mapped in the lymph and plasma[2-5,38,116,119].

In pathological conditions MMPs and ADAMs have been closely associated with cancer growth, invasion and metastasis. Advancement in activity-based profiling of protease function have enabled tracking MMPs and ADAMs tissue proteolytic activities in different stages of cancer[120]. Additionally, transgenic mice harboring gene deletion of individual MMPs have shown how these proteases facilitate neoplastic progression by degrading the extracellular matrix structure, processing cell-cell and cell-matrix adhesion molecules. Indeed, MMP2, MMP13 and 14 have all been implicated in pericellular tumor proteolysis[121]. Collagens, laminins, growth factors and cytokine processing have all been linked to MMP3, 7, 8, and 11 in different cancer models spanning ovarian, liver cancer, intestinal adenomas, and squamous cell carcinoma[121,122]. Besides aiding tumor invasion, MMPs also contribute to tumor growth by VEGF cleavage that alter tissue neo-angiogenesis[121].

Increased levels of active MMP9 and MMP2 have also been found in the CSF and sera of patients with multiple sclerosis (MS) prior to a relapse albeit their specific substrates in the disease have not yet been characterized [123]. Similarly, active ADAMs such as ADAM-17 and its processing substrates (TNF-α and CX3CL1) have been found in the serum of patients with relapsing MS [123].

Cleavage products following regulated-proteolysis of membrane receptors have also been found released in the interstitial fluid/lymph[1-5,116], including products derived from IL-6R

and IL-11R proteolysis by ADAM10 and ADAM17, which releases peptide fragments of around 20 amino acids [124], similarly an 11 amino acid fragment is released from proteolysis of the TNF-R [125], or fragments released following proteolysis by α–β and γ secretases of the IL-1R and IL-2R, EGFR, TLR-superfamily and other chemokines or cytokines[79,126-128].

Serine Proteases

Serine proteases are a large family of proteases whose most notable members include chymotrypsin, trypsin, elastase, clotting factors (Xa, XI, thrombin, plasmin, plasminogen activators), kallikreins, granzymes, cathepsin G, and factors from the complement cascade (C1r, C1s and C3 convertases). Although the degradome of some of these proteases is only present in biological fluids in pathological conditions, other enzymes of this family are active in a wide variety of physiological functions. For example, a degradome derived from the activity of clotting factors is physiologically present in both serum and lymph [1-3,87,119,129,130]. Similarly, kallikrein-related degradomes, due to their involvement in fluid and electrolytes regulation, have also been mapped in the lymph[2].

On the other hand serine proteases, which are essential mediators of gastrointestinal physiology, serving digestive purposes as well as mucosal tissue homeostasis,[131] are only found in serum and plasma during pathological conditions. Increased plasma serine protease activity has been observed in inflammatory bowel disease, Ulcerative Colitis and Crohn's disease[131]. The pathological relevance of these enzymes is highlighted by the finding that re-establishment of proteolytic homeostasis, using protease inhibitors, reduces the severity of these conditions[131]. Degradation products from trypsin, chymotrypsin, elastase and other serine proteases have been mapped in the lymph collected from patients or mice models of bowel disease[11,13,132].

Three main pathways characterize the complement system: classical, lectin, and alternative. Although the classical and lectin pathways generally are activated following recognition of exogenous/pathogenic materials, the alternative pathway is constitutively active at low levels in physiological conditions[133]. This is often referred to as the *tickover mechanism* and allows the system to stay primed for rapid activation[133]. Indeed a degradome for the alternative pathway has been mapped in lymph and plasma collected from healthy individuals [1-3,87,119,129,130]. On the other hand the activity of the complement cascade is highly increased in acute and chronic inflammatory conditions as well as degenerative pathologies in every parenchymal organ[24-26,31,32,36,37,101,121].

The serine proteases subfamily of elastase, tryptase, proteinase 3, and Cathepsin G are mostly active during inflammation when they are released from mast cells and granulocytes and rapidly degrade connective tissue proteins[134].

Cysteine Proteases

Cysteine proteases include some of the Cathepsin family members, Caspases and Calpains. Cathepsins (F,K,L,O,S,V,X and W) are endopeptidases, whereas cathepsins B, H, X and C possess exopeptidase activity. They are mostly found in endolysosomal compartments and at least some members are secreted extracellularly. Under physiological conditions, an extracellular degradome has been associated with the role of cathepsins L in skin and hair

follicle morphogenesis[135] and cardiac remodeling[136] and a cathepsin K role in bone resorption[137]. Cathepsins are up-regulated and secreted in almost every form of cancer, where they are generally associated with poor prognosis. The degradome associated with their functional activity relates to neoangiogenesis (laminin, collagen IV, fibronectin proteolysis, processing of angiogenic inhibitors or proangiogenic factors), cell proliferation and invasiveness (ECM degradation, activation of MMPs, processing of cell-cell adhesion)[122,138-141].Additionally, during acute and chronic inflammatory conditions macrophages, dendritic cells and also non-immune cells secrete active Cathepsins, which contribute to the tissue degradome[142-146].

A Caspase-generated degradome/peptidome is also observed following physiological tissue apoptosis and presence of apoptotic cells in the lymph[80]. Apoptosis occurs physiologically, as a homeostatic mechanism to maintain cell populations in tissues and it is estimated that around 10 billion cells are made each day to balance the ones dying by apoptosis[147]. Peptides derived from Caspase 3 processing have been mapped to several intracellular proteins as detailed in the paragraph above[2,3]. Both inflammatory (Caspases 1-4-5 in humans and 1-11 and 12 in mice) and apoptotic Caspases (initiators 2-8-9-10 and executioners 3-6-7) are critically active in inflammatory responses where they participate in maturing pro-inflammatory cytokines (IL-1β and IL-18), and as the major executioners of cell death. As such a Caspase degradome has been mapped in the plasma during acute and chronic inflammatory conditions such as myocardial infarction, chronic hepatitis C and nonalcoholic Fatty liver disease, COPD and hemorrhage [148149-151].

Calpains are non-lysosomal cytosolic proteases mostly involved in cleavage of intracellular proteins involved in cellular motility and trafficking. Calpains can also be secreted by immune cells and are present on the plasma membrane where EGF activate calpain 2 via ERK/mitogen-activated protein kinases to facilitate productive locomotion of adherent cells[152-154]. As such Calpain degradation products have been mapped in the lymph and eluted from DC MHC II[2]. Notably, inflammatory conditions increase Calpain secretion from activated macrophages and T cells and tissue specific antigens, such as myelin proteins [123], have been shown to be processed by Calpains.

Aspartic Proteases

Cathepsin D and E are endo-lysosomal aspartic proteases. A cathepsin D degradome has been previously reported in the lymph; likely released from apoptotic cells or during exosomal exit following fusion of the multivesicular late endosomes with the plasma membrane[2,98]. Elevated plasma levels of Cathepsin D have been reported in several inflammatory and neoplastic conditions. In non-alcoholic steatohepatitis plasma levels of Cathepsin D directly correlate with disease development and regression[155]. Plasma Cathepsin D has also been found elevated following myocardial infarction, hepatic carcinoma, breast cancer and inflammatory joint disease associated with cartilage degradation[156-159].

Threonine Proteases

The Proteasome is the enzyme complex harboring the threonine class of proteases and its role in MHC I and MHC II-cross presentation has been very well characterized. In physiological conditions low levels of circulating proteasomes have been observed in normal human blood [160]. Also few proteasome-cleaved peptides have been mapped in the lymph. Likely these peptides are either secreted from parenchymal or immune cells [98]. or released from the cytosol of apoptotic cells[80]. On the other hand, high levels of proteasomal activity have been mapped in the plasma of patients with solid tumors and hemopoietic malignancies, metastatic melanoma, different kinds of trauma, rheumatoid arthritis and SLE [160-164].

Lymph Peptidome and Degradome contribution to the MHC I and MHC II immunopeptidome

Research in the last 30 years has clearly established that MHC I and MHC II molecules sample both exogenous and endogenous antigens. The former paradigm that MHC I would only bind cytosolic antigens and MHC II would sample phagocytized proteins is now rectified through the discovery of cross-presentation and autophagy. It is now apparent that both MHC molecules intersect intracellular and extracellular pathways[165-168]. As such many more non-canonical pathways have been added to the canonical MHC-I proteasome and MHC-II- endosomes pathway. Additionally, from early analysis in which only a few MHC peptides could be reliably fragmented and analyzed, several groups have now reported very large sets of naturally processed peptides[2,23,169-181]. The increased number of mapped epitopes associated with the availability of incrementally sophisticated software programs that allow mapping of the processing proteases (MEROPS, BRENDA, CutDB) or the analysis of post-translational modifications has allowed the field to move from a merely compilative analysis of the MHC-eluted immunopeptidome to a more mechanistic analysis of how differential MHC-immunopeptidomes contribute to immunosurveillance. We recently sequenced over 3000 HLA-DR1-eluted peptides derived from *ex vivo* purified dendritic cells [2]. The eluted immunopeptidome was analyzed using a series of databases comprising experimentally determined peptide cleavage sites. The analysis underscored the wide variety of enzymes and processing pathways that contribute to the MHC II immunopeptidome; besides the endosomal processing pathway that generates Cathepsins-cleaved peptides, tissue-specific proteases, including MMPs, ADAMs, Caspases, Granzymes among many others, were shown to add to the MHC II self peptidome. Some of the eluted peptides overlapped with those found in the lymph and did not derive from cathepsin processing[2]. Overall this ensures that dendritic cells present a broad self-peptidome which includes epitopes generated by a multiplicity of proteases with the ultimate goal to present the sampled environment to patrolling T cells[2].

Nevertheless, novel epitopes can be generated during pathological conditions through different mechanisms including: (i) up-regulation and down-regulation of various tissue proteases which contribute to changes in protein processing and sequence of processed epitopes, (ii) changes in the redox microenvironment which favors protein post-translational modifications, such as carbonylation, glycation and nitrosylation and (iii) changes in the

expression of proteins of the antigen processing machinery, such as HLA-DM and HLA-DO, which contribute to peptide selection mechanisms[182].

Proteomic analysis of blood and lymph under physiological or pathological conditions has clearly underscored how the degradome signature can change; the next challenge is to determine whether distinctive degradomic signatures can be specifically associated to disease states and used as liquid biopsy for diagnostic and therapeutic purposes. Additionally, how changes in tissue-specific proteins and proteases affect epitope processing, formation of neo-epitopes and epitope copy number presented by MHC I and MHC II molecules are still questions to be addressed.

An additional aspect arising from these studies is the influence of drugs on the MHC ligandome. For example, gemcitabine, a cytostatic drug used in cancer chemotherapy, not only increases the density of MHC I molecules on the cell surface but also alters immunoproteasome composition and the MHC ligandome[183]. For another example, carfilzomib alters the HLA-presented peptidome of myeloma cells and impairs presentation of peptides with aromatic C-termini [184].

Similarly, tissue redox changes, associated with acute and chronic inflammatory states, degenerative conditions and cancer have been extensively mapped. During these conditions the cellular and extracellular proteome is qualitatively modified by oxidation (carbonylation, nytrosilation, formylation), glycation (carboxymethylysine or carboxymethylarginine) and lipoxidation (4-hydroxynonenal (HNE), malonaldehyde), to name a few of the over four-hundred possible modifications. Both MHC classes are found to present phosphorylated peptides[185], and for MHC class I ligands glycosylations have also been reported[186].

Although most amino acids are susceptible to oxidation by these reactive species, lysine, arginine, methionine and cysteine are the most susceptible due to the presence of free amino and sulfhydryl groups on their side chains. For example, in our extensive database of HLA ligands from human tumor and normal tissues, around 8% of HLA class II and 6% of HLA class I ligand entries are oxidized at one or more amino acids [187,188]. Other notable modifications are arginine citrullination, which has been linked to autoimmune diseases such as rheumatoid arthritis [189], peptide oxidative cleavage for generation of neo-epitopes and autoimmunity, [190] and peptide glycation in diabetes and metabolic syndrome [191,192]. The full effects of protein modifications on the MHC I and MHC II antigen processing machinery, peptide processing loading affinity and presentation need to be further evaluated. Additionally, how peptides and specific PTM can be exploited diagnostically and therapeutically, in cancer, autoimmune and degenerative diseases is also an important open question.

Conclusions

Overall the literature from the last decade has solidified the notion that plasma and lymph provides a rich degradome/peptidome that could facilitate the discovery of novel biomarkers that reflect disease status[4,12,17,20,37,87,129,130]. In contrast to organ biopsies, which are limited to a small number of cells, liquid biopsy can provide a more accurate fingerprint of

the overall organ physiological or pathological state. In particular, unlike the blood, liquid biopsy of lymphatic fluid can provide a specific signature of the organ from which it drains [107]. How to diagnostically use the blood and lymphatic fluid peptidome/degradome and how to therapeutically exploit it for immunotherapy design are important challenges for the future.

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