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Lymph formation, composition and circulation: a proteomics perspective

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REVIEW

Abstract

During the last 20 years a deeper understanding of the lymphatic circulatory system, lymph formation and composition has emerged. This review will examine the current knowledge on the organization of the lymphatic vascular tree, the formation of lymph from the extracellular fluid, lymph circulation and the lymph proteomic composition during physiological and pathological conditions. Formation of the lymph fluid is dependent on pressure gradients in the capillary beds and the composition of the endothelial cell glycocalyx, which acts as a molecular sieve. Fluid propulsion toward the draining node is dependent on the intrinsic pumping mechanism of the lymphangions and their unidirectional valves. The lymph 'omics' composition is dependent on the ultrafiltration of plasma proteins as well as proteins and molecules derived from the metabolic and catabolic activities of each parenchymal organ from which the lymph drains. Altogether, these new insights have brought about a new awareness of the importance of the lymphatic system in human physiology and pathology.

Keywords: antigen processing and presentation, inflammation, peptidomic, tissue proteomic

Introduction

The lymph is a biological fluid produced as the affluent of interstitial fluid from most parenchymal organs; it collects products of tissue metabolism and catabolism as well as circulating immune cells and transports them to the regional lymph nodes. As such the lymph has a pivotal role in every immunological process, including maintenance of immunological tolerance, autoimmunity, inflammation, cancer metastasis, and cardiovascular and metabolic disorders (1).

Until recently the detailed composition of the lymphatic fluid was virtually unknown, primarily due to the technical difficulties of cannulating lymphatic collectors, the scarcity of the collected lymph, which precluded many biological assays, and the low sensitivity of mass spectrometry (MS) instruments for proteome mapping. As many of these issues have been overcome, lymph biology has progressively received more attention. During the last 10 years important progress has been made toward understanding the mechanisms of lymph formation, circulation and composition. In this review we will summarize emerging concepts on the mechanisms of lymph formation and circulation and the development of proteomic analysis to map the lymph proteome in physiological and pathological conditions.

Lymph formation

Protein extravasation from the microcirculation into the interstitial fluid

The interstitial fluid, which is the precursor of the prenodal lymph, is formed as an ultrafiltrate of capillary microcirculation; as such many of the proteins found in the blood are present in the lymph as well. According to the Starling principle, the microvascular ultrafiltration process is determined by the net balance between hydrostatic and osmotic pressures across the microvascular endothelium (2, 3). The principle states that the pressure gradient between the arterial and venous halves of the capillary beds drive the fluid filtering from the arterial end into the interstitial space and reabsorption into the venous system. However, this concept, which has been accepted for over 100 years, has recently been revised. Indeed, more-sophisticated measurements of fluid exchange in the capillary beds indicate that in most tissues the steady-state pressures (hydrostatic and osmotic)

provide a low level of fluid filtration from the microcirculation, at both arterial and venous ends, toward the interstitial fluid (4, 5).

Fluids, proteins and small molecules filter across the walls of the microvessels into the interstitium. The exchange occurs through the glycocalyx, a matrix of glycoproteins and glycosaminoglycans with a thickness between 50 and 500 nm that is present on the luminal surface of the endothelial cells, which extends over the intercellular clefts (6, 7). The glycocalyx structure and organization has been shown to be similar in both fenestrated and continuous endothelial cells (6, 7). Thus, fluids, small molecules and proteins, with the exception of large macromolecules, are filtered through the glycocalyx, which acts as a molecular sieve of various porosity, to influence the filtration rate from the capillary lumen to the interstitium (8, 9). Through this mechanism, plasma proteins contribute to the lymph proteome (Fig. 1).

The contribution of parenchymal organs to lymph formation

Until 10 years ago it was thought that the qualitative protein composition of the lymph would totally overlap with that of

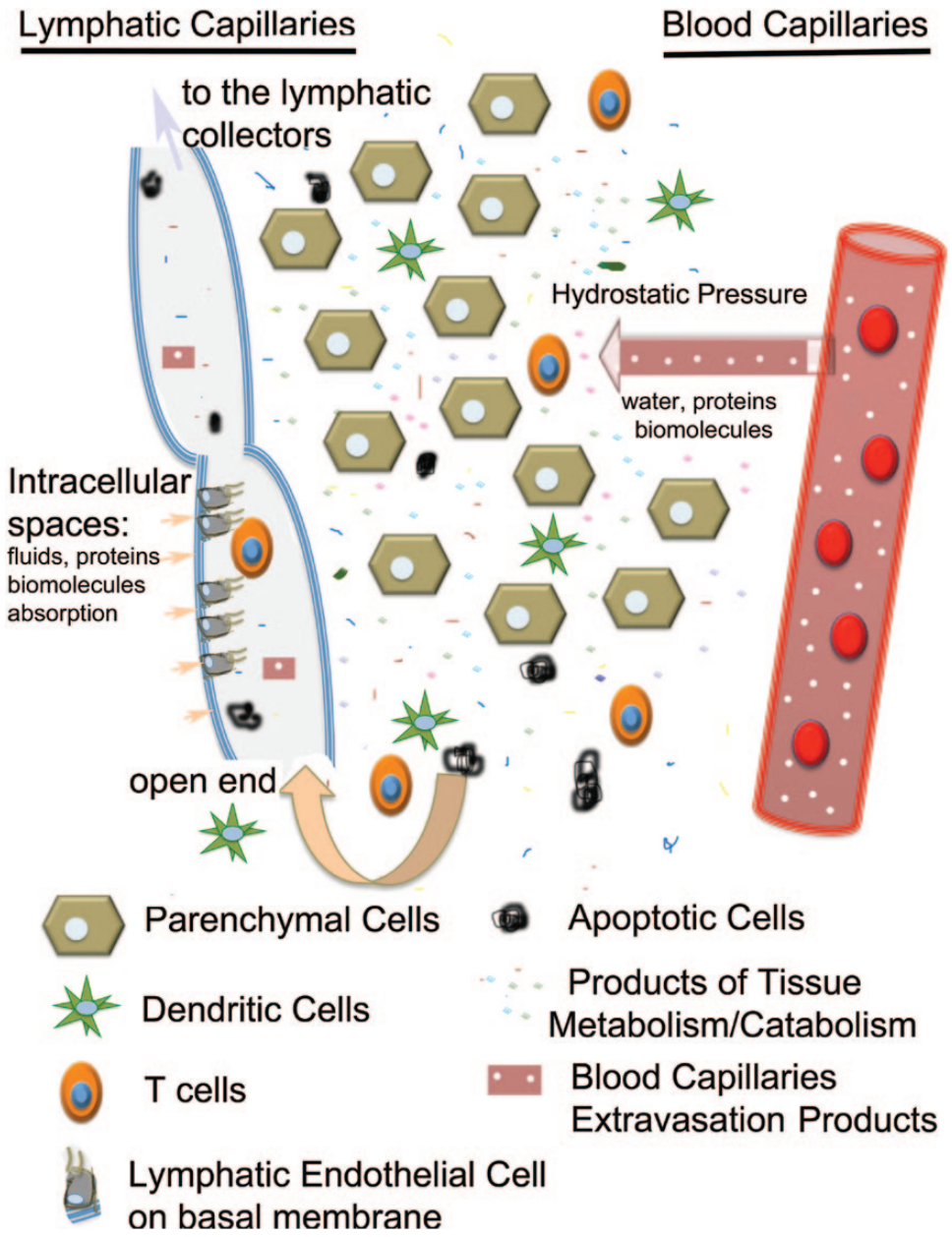

Fig. 1. Schematic of lymph formation. In every parenchymal organ the interstitial fluid is formed by products of cellular secretions, metabolism/catabolism as well as solute, molecules and proteins that extravasate from blood capillaries. Lymphatic capillaries have one open blunt-end, which facilitates drainage of the interstitial fluid and the movement of immune cells and apoptotic cells into the lymphatic circulation. The other end will coalesce into a lymphatic collector. Additionally gaps between lymphatic endothelial cells, which function as a one-way valve, further facilitate cell and solute collection into the lymphatic capillaries.

the plasma (10). However, this notion has been challenged by several proteomic analyses performed on lymph fluid (11, 12), collected in physiological and pathological conditions, which indicated how the metabolic/catabolic activities of parenchymal organs contribute to the lymph proteome (Fig. 1). Analyses performed on human (11–13), rodent (14–17), ovine (18) and bovine (19) lymph samples have revealed qualitative and quantitative differences between plasma and lymph. Altogether two major differences can be pointed out: first, proteins deriving from extracellular matrix (ECM) processing, tissue growth and remodeling, cellular metabolic/catabolic activities and cell death are enriched in the lymph as compared with the plasma; second, tissue-specific proteins have been mapped in lymph draining from different organs, indicating a region-specific 'omic' signature (11, 12). Tissue-derived proteins will eventually be phagocytosed by tissue-resident and nodal antigen-presenting cells (APC) and only a small fraction will reach the venous circulation.

Lymph circulation

All parenchymal organs, with the exception of the brain, contain a network of open-ended lymphatic capillaries, which collect the interstitial fluid (1). The capillaries are formed by a single layer of lymphatic endothelial cells supported by a sparse basement membrane (20). The endothelial cells are connected by specialized intercellular junctions, containing platelet endothelial cell adhesion molecule 1 (PECAM1) and vascular endothelial cadherin (VE cadherin), which function as one-way valves to facilitate entry of proteins, fluids, macromolecules, small molecules and immune cells (21–25). The lymphatic capillaries coalesce into progressively larger lymphatic collectors. The collectors are formed by one layer of lymphatic endothelial cells supported by a more organized basal membrane containing lymphatic muscle cells, connective tissue and fibroblasts (20). The muscle cells, which share characteristics of both smooth and striatal muscles, are important for maintaining a basic vessel tone and spontaneous contraction to propel the lymph toward the draining lymph node (26, 27) (Fig. 2).

The directional flow of lymph is also maintained through a series of unidirectional valves, positioned along the collectors, which open and close in synchrony with the vessel contraction. The valves are bicuspid, formed by connective tissue overlaid by lymphatic endothelial cells (28, 29). The segment of lymphatic collectors positioned between the two sets of valves is called a lymphangion (Fig. 2). Contraction from the more distal lymphangion toward the one closer to the lymph

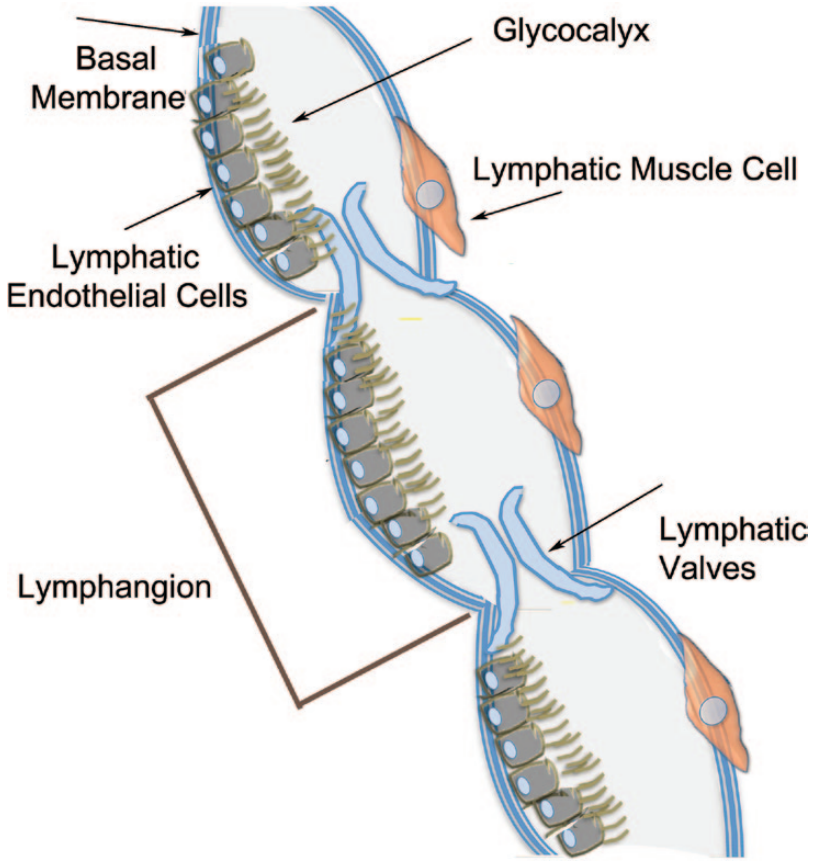

Fig. 2. Schematic of a lymphatic collector. A monolayer of lymphatic endothelial cells pave the lumen of the lymphatic collector. The endothelial cells are supported by a basal membrane formed by ECM proteins such as collagens, laminins, fibronectin, vitronectin, thrombospondins and proteoglycans. Intercalated with the ECM are lymphatic muscle cells which, upon contraction, propel the lymph forward. A series of valves, which rhythmically open and close, prevent lymph backflow. A segment of the lymphatic collector positioned between two series of valve is called a lymphangion.

node, in synchrony with directional valve closure, favors unidirectional lymph transport and prevents backflow, altogether enabling the collectors to work as pumps (26, 27, 30).

Original studies by Smith *et al.* (31) indicated a flow rate of 1–5 ml h^{-1} in both pre-nodal and post-nodal lymph under physiological conditions. However, lymph flux to the draining lymph nodes can change notably under pathological conditions, which can be associated with increased lymphangiogenesis (the formation of new lymphatic vessels from preexisting ones), increased cellular trafficking, increased lymph volume and flux and increased pro-inflammatory mediators, which affect lymphatic contractility (32–34).

Increased lymphangiogenesis has been observed in several pathological conditions, including primary and secondary lymphedema, acute and chronic inflammation, and cancer (35–38). In all these conditions the increased lymphangiogenesis relates to an increased production of different vascular endothelial growth factors (VEGF-A, VEGF-C, VEGF-D and VEGFR-3) released by immune and stromal cells (39–42). Additionally, nuclear factor-κB up-regulates the transcription factor Prox1 that promotes lymphatic endothelial cell proliferation (41, 43, 44). At the moment it is still unclear whether the increased lymphangiogenesis observed under inflammatory conditions serves a beneficial role. While it decreases tissue edema and clears inflammatory cells and cytokines/chemokines it also expands the dissemination of pro-inflammatory cells and mediators. Less controversial is the role of lymphangiogenesis in cancer where the expansion of lymphatic vessels facilitates metastasis and is a strong negative prognostic factor (35, 36).

Lymph proteomics

During the past decade, proteomic analysis of lymphatic fluid has exponentially expanded with the ultimate goal of finding protein biomarkers in different diseases and from different lymphatic districts (10). Owing to its intimacy with the immune and circulatory systems, the lymph proteome is an attractive source of protein markers and immune mediators. Because lymph has been long considered a product of a capillary filtration process, lymph composition was originally deemed to overlap with that of the plasma.

Although early studies confirmed that the lymph proteome was actually enriched with plasma and blood cell components (45), 10 years of proteomics investigations on lymph from humans or animal models have shed light on the complexity and distinctive traits of the proteome in comparison with plasma. Herein a comprehensive overview of the lymph proteome, based on published findings, is reported (Supplementary Table 1, available at *International Immunology* Online). Briefly, a total of 1063 lymph proteins have been documented from different species, including humans (630 proteins), rats (356), mice (60), bovines (9), ovines (3) and other mammals such as *Sus scrofa* and *Equus caballus*.

Lymph is a compositionally unique biofluid

The first proteomics report on lymph dates back to 2004, when Leak *et al.* (45) provided a comparison between ovine lymph and plasma proteomes using a 2D PAGE—MS/MS workflow (where proteins are separated by 2D PAGE, then subjected to multiple steps of MS). The compositional unicity of the lymph proteome was reported for the first time, with a subset of protein spots being uniquely identified or found to be higher in abundance in lymph compared with plasma samples, including glial fibrillary acidic protein, neutrophil cytosol factor-1, fibrinogen α- and β-chains, IgG, to name a few.

In 2008, Mittal *et al.* (16, 46) performed a quantitative analysis of the rat mesenteric lymph proteome in response to alterations in the dietary regimen (rats fed a normal diet versus fasting). A total of 150 proteins were measured using a relative quantification approach in lymph from both the fasted and fed states. A proteome significantly enriched in protease inhibitors and proteins related to innate immunity was observed.

Human lymph proteome coverage has recently expanded as well (11, 13), confirming findings from animal models. Analyses of matched plasma and lymph from trauma patients resulted in the identification of 548 proteins in total, including 232 shared entries.

Similar findings have recently been reported and expanded by Clement *et al.* (12, 47, 48). Here 144 common proteins could be found between plasma and lymph. Most of the shared proteins were represented by soluble secreted, interstitial and extracellular proteins; the rest derived from intracellular organelles (nucleus, cytosol and plasma membrane). The complement system, transporters, metabolism regulators and protease inhibitors covered half of the proteome shared by the two fluids, which is suggestive of a core of conserved functional features between plasma and lymph.

In 2013, Clement *et al.* (12, 47) reported a comparative proteomics analysis of matched human plasma versus lymph, highlighting the presence of 72 lymph-enriched proteins, involved in apoptosis, cell catabolism or ECM remodeling (collagens, cartilage and other ECM proteins). Gene ontology annotation of their lymph proteome results showed enrichment of intracellular proteins (functionally classified as 25% extracellular, 32% from cytoplasm, 21% from nucleus and 11% from plasma membrane) compared with plasma. Consistently with this, Dzieciatkowska *et al.* recently reported that, in matched lymph versus plasma samples from individuals experiencing major trauma, the former fluid was characterized by 105 unique identifications, most of which involved cell lysis products, and mediators of pro-inflammatory responses and immune system activation. Of note, lymph was specifically enriched with mediators of vascular hypoactivity/neoangiogenesis and energy/redox metabolism—two key markers of metabolic adaptations to trauma—as well as ECM components and lymph-specific immunomodulators (14, 15).

As technical strides in proteomic technologies enable the detection of increasingly lower abundance proteins in complex biological fluids, most of the proteins previously identified as lymph-specific have been progressively included in the non-redundant list of the plasma proteome, as part of the Human Plasma Proteome Project. However, it should be noted that quantitative differences between the two fluids have consistently been observed including in more recent studies (11). Targeted quantitative proteomics approaches that utilize standards labeled with heavy isotopes for absolute quantification will help us revise our current understanding of the specific composition of the fluids.

Proteins from intracellular sources

Compositional analysis of the human lymph proteome has hitherto focused on mesenteric and peripheral pre-nodal lymph, and results are hereby merged in Supplementary Table 1, available at *International Immunology* Online. Proteins from different lymph proteomics studies are reported in Supplementary Table 1, available at *International Immunology* Online, together with an indication of the study and the organism on which the study was performed. In this view, it is worth noting that protein hits reported in these studies may sometimes have resulted from searches on proteomics databases of orthogonal species (e.g. such as in the case of *Bos Taurus* hits in an *Ovis aries* experimental dataset, Goldfinch *et al.* (18)). However, it should be noted that the expression profile of lymph is likely to be uniquely defined by the anatomical region from which it was derived, since the lymph is in direct contact with each cellular layer of the parenchymal organ during its formation, from the parenchymal interstitial space, to its collection into the lymphatics (12, 46).

Indeed, when compared with plasma, lymph is enriched with proteins of cellular origin released from the parenchyma. The list includes proteins of nuclear origin, such as histones, and splicing and transcription factors, as well as proteins involved in the translation and protein synthesis pathways (12). Proteins derived from the cytosol and organelles have also been mapped in the lymph. These proteins are released following cellular apoptosis, during physiological conditions, or during cellular necrosis during pathological conditions such as acute and chronic inflammation and trauma/shock.

Beside mitochondrial, ribosomal and endosomal proteins, proteomics reports have identified cytosolic enzymes as well as membrane and cytoskeletal components. Interestingly, intracellular enzymes such as glycolytic enzymes (e.g. glyceraldehyde 3-phosphate dehydrogenase, aldolase, triosephosphate isomerase and lactate dehydrogenase) might also have additional distinct functions when found in the extracellular milieu. These potential 'moonlighting proteins' are relevant when considering inflammation and immune modulation such as in the role of mesenteric lymph in mediating multiple organ failure and metabolic derangement subsequent to trauma/hemorrhagic shock (49).

Although all of these cellular components, or fragments thereof, are likely to be present in plasma at some level (50, 51), the increase in relative abundance reported under physiological (12) or pathological (11) conditions highlights the role of lymph as a conduit for the tissue proteome (47, 52).

Proteins from the ECM

Enrichment of ECM proteins in lymph has been extensively reported (11, 12, 14). ECM proteins identified in lymph include collagens, laminins, additional glycoproteins and proteoglycans. ECM proteins constitute the structural component of each tissue and play a key role in maintaining tissue morphological integrity, regulation of cell growth, cellular division and migration, and provide a reservoir of cytokines, chemokines and growth factors (48, 53).

The ECM undergoes physiological turnover and proteolytic processing. The low level of matrix turnover that occurs in the skin and all parenchymal organs is further accelerated in response to various cellular stimuli, which range from dynamic homeostasis to full-blown tissue remodeling, as occurs during inflammation, wound healing and cancer (53–56). ECM protein turnover is controlled by the activity of specific matrix proteases, including matrix metalloproteinases (MMPs), a disintegrin and MMPs (ADAMs), ADAMs with thrombospondin motifs (ADAMTs, Shiomi *et al.* (56)), and cathepsins. The level and activity of these proteins in lymph increases under stress/disease conditions including, but not limited to, trauma/hemorrhagic shock (14).

As a result of the activity of a wide variety of these and other proteases, a vast array of lymph-circulating peptides have been documented. For example, the activity of MMPs (especially MMP2, MMP8, MMP9 and MMP13) on ECM proteins has been recently associated with increased neutrophil activation, a phenomenon mediated by the MMP-dependent release of bioactive peptides (such as acetyl-PGP originating from collagens), which are recognized by CXC receptors to promote chemoattraction but not adhesion (57, 58). ECM-derived peptides from collagens (over 70 different peptides have been mapped) or laminins (including α1, 4, 5 and γ1, 2 and 3 isoforms) are among the most abundant components of ECM processing (48). Less represented were peptides derived from glycoprotein and proteoglycan catabolism, examples being fibrosin-1, aggrecan, mucins and fibronectin.

The lymph proteome changes with disease status

Lymph proteomes are not only defined by the anatomical region from which lymph was derived, but also mirror disease processes and underlie systemic adaptation to pathological conditions, such as in the case of sepsis (18, 59–62), inflammation (46, 61, 62) (e.g. pancreatitis or asthma) or traumatic events (such as trauma/hemorrhagic shock (15, 17, 63)).

Animal models of sepsis induced by cecal ligation and puncture resulted in the differential detection of 158 distinct proteins identified in lymph samples from the sepsis group (64). In particular, the levels of five proteins involved in lipid metabolism—apolipoprotein E (ApoE), annexin A1 (Anxa1), neutrophil gelatinase-associated lipocalin (NGAL), S100a8 and S100a9—were proportional to the gravity of sepsis.

Sheep infected with the parasitic nematode *Teladorsagia circumcincta* showed alterations of the ovine gastric lymph, as gleaned through 2D PAGE separation followed by Matrix-assisted laser desorption/ionization time of fly and MS/MS analysis. Goldfinch *et al.* (18) determined a significant increase of gelsolin, α-1 β-glycoprotein and hemopexin in lymph collected from infected animals as compared with healthy controls, providing potential biomarkers for determining extent of infection (18).

Other examples of lymph proteomics studies using animal models with potential translational value have been authored by Popova *et al.* (60), whereby whole proteome analysis of mouse lymph nodes was documented in response to cutaneous exposure to anthrax. More than 380 proteins were detected in the normal intranodal lymph, whereas the infectious process resulted in profound changes in the semi-quantitative abundance of proteins and the appearance of 297 proteins. The lymph proteome is also affected by systemic inflammation (61), as induced in rats through LPS exposure,

resulting in increases in the levels of tumor necrosis factor-α, IL-1β, IL-6, IL-10 and ADAMST1, the latter detected only in response to LPS treatment. Analogously, the lymph composition was altered during taurocholate-induced acute pancreatitis in rats (46), or in response to asthma-inducing diisocyanates (62).

An appreciation for the role of soluble components from lymph, but not plasma, in mediating multiple organ failure secondary to trauma followed by hemorrhagic shock (49) has fostered a series of proteomics investigations on mesenteric lymph in rat/canine models and humans. Significant contributions in this field include differential proteome analyses of pre-shock versus post-shock lymph samples, in the presence or absence of sham controls to discriminate between the effect of trauma and hemorrhagic shock in rat models (15, 17). Follow-up papers have focused on documenting time-course changes to the lymph proteome in rats following hemorrhage (14). Altogether, these studies documented that, after trauma and hemorrhagic shock, a progressive impairment of the ratio of serine proteases to antiproteases (SERPINs) involved in clotting responses occurred; this dynamic early decrease in the levels of SERPINs was followed by a late progressive increase in their levels. Decreased levels of serine proteases are accompanied by progressive increases in the levels and activity of MMPs (as determined through zymography assays) and ECM proteins, suggesting a role for the protease/anti-protease balance in mediating post-hemorrhage responses.

Alternatively, canine models of trauma and hemorrhagic shock have highlighted the release of damage-associated molecular patterns from host tissue after the trauma and hemorrhagic shock. These events are coupled to the activation of polymorphonuclear cells, leading to acute lung injury and systemic inflammatory response syndrome (63).

Studies on human mesenteric lymph in response to trauma and hemorrhagic shock (11, 13) have confirmed observations on animal models, expanding on the qualitative uniqueness of the lymph proteome in comparison with matched plasma.

The lymph peptidome

Like any biological fluid, including plasma, saliva and urine, the lymph contains a rich peptidome (47, 65–68). The mapped peptides derive from intracellular organelles (nuclei, mitochondria, ribosomes and endosomes) as well as the endoplasmic reticulum, Golgi apparatus and the cytosol. Peptide fragments from cleaved surface receptors, cytokines, chemokines, proteins involved in the innate immune response, coagulation factors and ECM proteins have also been mapped (47).

Analysis of the catabolic pathways involved in the generation of the lymph-bound peptides identified a variety of enzymes including MMPs, cathepsins, caspases, enzymes involved in the innate immune responses such as angiotensin-converting enzyme, complement factor I, granzymes and enzymes of the coagulation cascade including thrombin, plasmin and kallikreins. Altogether these processing pathways underlay the several metabolic/catabolic activities ongoing in each parenchymal organ including ECM degradation, surface receptor cleavage/editing, endosomal processing and cellular apoptosis (48, 52).

The concentration of a few of the lymph-derived peptides has been estimated and it ranges from nanomolar to low micromolar for peptides derived from the most abundant proteins, such as collagen (48). Additionally, it has recently been reported that many of the peptides found in biological fluids are bound to chaperones including albumin, lipoproteins, Igss and transthyretin (69) and exist in equilibrium between their free and bound forms with half-lives estimated to be ~1–20 days (69).

Immunological relevance of the lymph proteome and peptidome

Lymph-carried proteins and peptides are readily available to different APC including tissue-resident dendritic cells (DC) and macrophages, lymph-bound migratory DC, circulating monocytes and all the different APC from the B and monocyte-macrophages-DC lineages that are resident in the lymph node. These APC can acquire the lymph-carried self-antigens through receptor-mediated and fluid phase phagocytosis as well as pre-processed peptides through direct MHC loading (70–75).

Whereas antigens acquired through phagocytosis will generate an MHC II peptidome processed by endosomal enzymes, directly loaded peptides derive from many more different processing pathways, including MMPs, calpains, caspases and granzymes (48, 76–78). Altogether endosomal and non-endosomal processing pathways could potentially generate different sets of peptides from the same protein, qualitatively and quantitatively expanding the number of overall presented epitopes. More importantly these peptides, derived from different catabolic pathways, could be involved in the maintenance of central tolerance, when transported in the thymus by migratory DC, as well as peripheral tolerance (79–89). Indeed, the role of migratory DC in transporting exogenously administered peptides to the thymus to mediate negative selection and to lymph nodes to mediate peripheral T-cell anergy and Treg differentiation has been extensively reported (79, 90–92). However, the lymph-carried peptidome/degradome could also be implicated in autoimmunity (88, 89, 93). Tilting the balance from tolerance to autoimmunity is determined by different factors including peptide MHC II binding affinity/stability, peptide generation by *de novo* processing pathways, copy number of presented epitopes and nature of the presenting APC.

Proteomic analyses have demonstrated how the proteome composition changes under physiological and pathological conditions and how the lymph peptidome/degradome also changes, qualitatively and quantitatively, due to the up-regulation and down-regulation of various tissue proteases, as observed in different pathologies. Hence how the local concentration of tissue-specific proteins and proteases influences epitope processing and the copy number of epitopes presented by MHC I and MHC II molecules, and how *de novo* enzymatic processing during pathological conditions generates novel epitopes, are all important immunological questions yet to be addressed.

Conclusion

Advancements in analytical technologies have allowed for more complete mapping of the lymph fluid proteome and peptidome. These findings have opened new questions

regarding the role of tissue-specific proteins and peptides that are drained to peripheral lymph nodes in homeostasis and disease.

Although a more detailed immunological role of the lymph-carried proteome and peptidome is outside the scope of this review, it is important to note how the lymph proteome, peptidome and degradome has brought new attention to the role of 'self recognition' as a dynamic interaction between APC and the metabolic/catabolic activities ongoing in every parenchymal organ in physiological and pathological conditions.

Supplementary data

Supplementary data are available at *International Immunology* Online.

Conflict of interest statement: The authors declared no conflict of interests.

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