The impact of the extracellular matrix on inflammation

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Abstract | The advent of *in situ* immunology and intravital analyses of leukocyte movement in tissues has drawn attention to the previously neglected extracellular matrix (ECM) and its role in modulating immune cell behaviour in inflamed tissues. The ECM exists in different biochemical and structural forms; both their individual components and three-dimensional ultrastructure impart specific signals to cells that modulate basic functions that are important for the early steps in inflammation, such as immune cell migration into inflamed tissues and immune cell differentiation. In chronically inflamed tissues, aberrant ECM expression and fragments of the ECM that are derived from tissue-remodelling processes can influence immune cell activation and survival, thereby actively contributing to immune responses at these sites.

Intravital microscopy
A technique that is used for the examination of biological processes, such as leukocyte–endothelial cell interactions, in living tissues. In general, translucent tissues are used, such as the mesentery or cremaster muscle. These tissues can be exposed and mounted for microscopic observation.

Extracellular matrix
(ECM). The secreted products of many cell types that form an organized scaffold for cell support.

Extravasation
The cellular process in which circulating leukocytes bind to and migrate through the endothelium into the underlying tissue.

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There is extensive information on the role of cell adhesion molecules, inflammatory cytokines and chemokines in promoting leukocyte infiltration and functions in inflamed tissues[1–3]. The advent of *in situ* immunology and intravital microscopy for the study of immune responses has shown that the extracellular matrix (ECM) has an important role in influencing immune cell behaviour in inflamed tissues. The ECM exists in several biochemical and structural forms and is secreted and assembled by the cooperative activity of numerous cell types. The individual components of the ECM and its three-dimensional ultrastructure and biophysical properties can signal specific information to cells and modulate essential immune functions, such as immune cell migration into and within inflamed tissues, immune cell activation and proliferation, and cell differentiation processes, such as T cell polarization.

In chronically inflamed tissues, inflammatory cytokines and proteases (in particular, matrix metalloproteinases (MMPs)) that are released by extravasating cells or by activated tissue-resident cells can modify the ECM. Cytokines, in particular, tumour necrosis factor (TNF), interferon-γ (IFNγ) and transforming growth factor-β (TGFβ), modulate the expression of a wide range of ECM molecules. However, MMPs, which were previously considered as broad range ECM degraders, instead fine-tune inflammatory processes by selectively cleaving ECM proteins and their receptors[4–6] among other substrates including chemokines and cytokines. This results in the generation of 'bioactive' fragments of the ECM that influence the activity and/or function of both infiltrating and resident cells. This Review focuses on the ECM of non-lymphoid organs, with emphasis on the direct role of the ECM in controlling immune cell infiltration into inflamed tissues and the potential roles of the ECM in immune cell activation in chronically inflamed tissues during autoimmune disease. As such, this Review is divided into two distinct sections, the first dealing mainly with early extravasation events and the second dealing with tissue remodelling in inflamed tissues.

What is the ECM?

The ECM is voluminous and highly insoluble and typically consists of proteins that are composed of independent structural domains, the sequences and arrangements of which are highly conserved. These domains are glycosylated and frequently contain glycosaminoglycan chains that are sulphated, which leads to negative charges[7,8]. The domain structure of ECM molecules may reflect conserved structure–function relationships, such as the recognition of specific integrin or non-integrin receptors by dystroglycan, oligomerization by collagen domains and calcium binding by C-type lectin domains. However, mosaic assembly of independent domains also results in unique functions, as does the selective cleavage and release of isolated domains. The negative charge of many ECM molecules, in particular the proteoglycans, and the large area that they occupy in tissues provides large potential for interactions with other charged molecules, such as growth factors and chemokines, thus influencing the local concentration or accessibility of these factors[8]. As a highly organized, insoluble suprastructure, the ECM can, in a spatially patterned and regulated manner, integrate and deliver multiple complex signals to leukocytes that affect their behaviour in inflamed tissues.

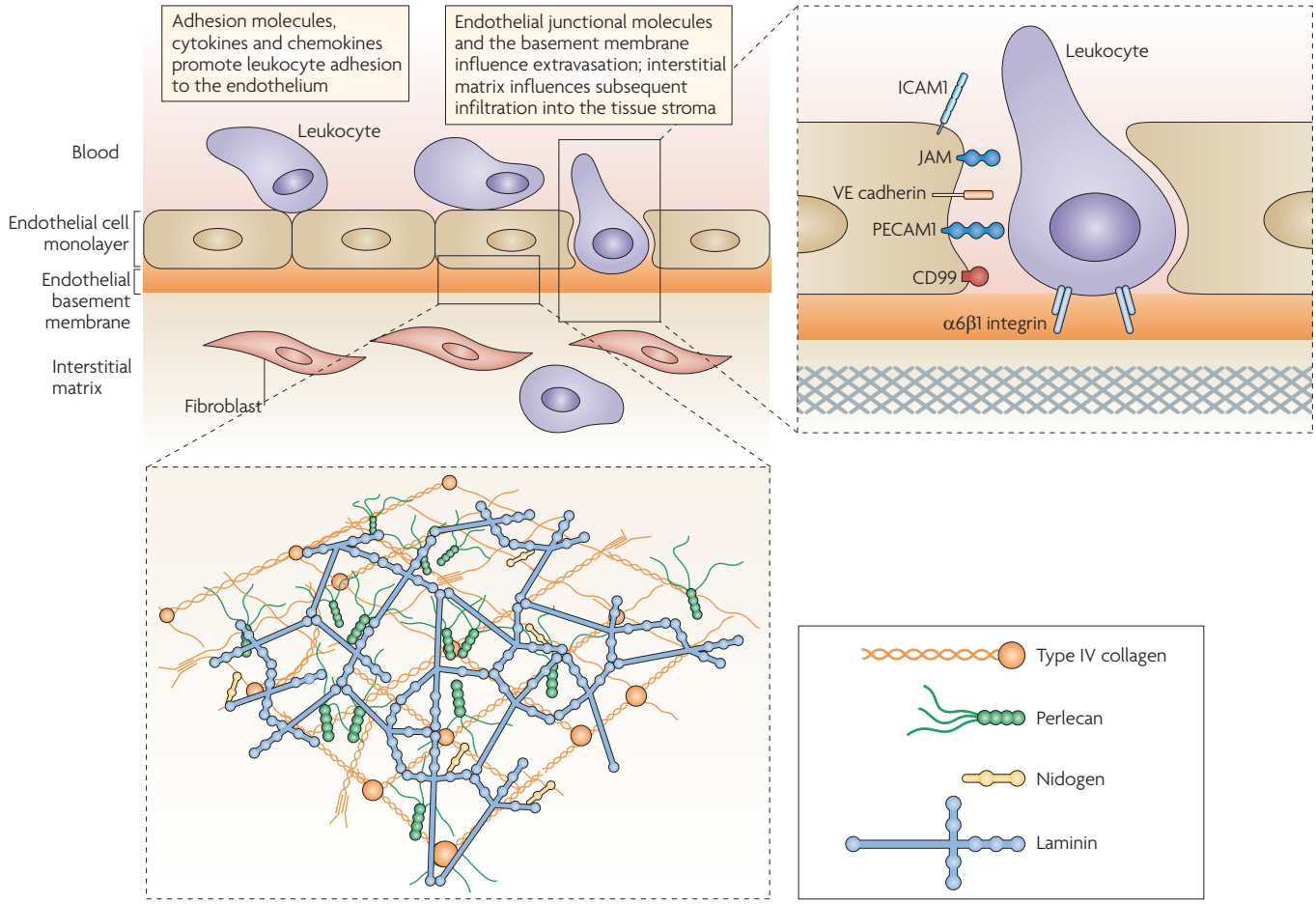


Figure 1 | **Leukocyte infiltration into inflamed tissues.** Infiltration of leukocytes into inflamed tissues requires interactions between leukocyte- and endothelial cell-expressed adhesion molecules, chemokines and cytokines; the collective action of these molecules leads to firm adhesion. In addition, cell infiltration is controlled by endothelial junctional molecules, the basement membrane and the interstitial matrix. The basement membrane and the underlying interstitial matrix are biochemically and structurally distinct extracellular matrix (ECM) suprastructures. The interstitial matrix is mainly composed of fibrillar type I collagen and has varying amounts of type III and type V collagens (depending on the tissue type), non-collagenous glycoproteins (such as tenascins, fibronectin, vitronectin) and proteoglycans carrying glycosaminoglycan chains (such as chondroitin-, dermatan- and keratan-sulphate proteoglycans). By contrast, basement membranes are thin, tightly crosslinked networks (50–100 nm in thickness) that consist of 1–2 laminin isoforms, normally a singular type IV collagen isoform, nidogen 1 and/or nidogen 2, the heparan sulphate proteoglycan perlecan and several minor glycoproteins[35]. During diapedesis, infiltrating leukocytes disrupt the interactions of several junctional adhesion molecules (JAMs). Interruption of some of these adhesion complexes, including those that involve platelet/endothelial cell adhesion molecule 1 (PECAM1), CD99 (in the case of neutrophils) or CD99 antigen-like-2 (CD99L2) (in the case of lymphocytes), have been implicated in the subsequent penetration of the endothelial basement membrane and in the upregulation of α6β1 integrin, which is a receptor for several laminin isofoms[2]. ICAM1, intercellular adhesion molecule 1; VE cadherin, vascular endothelial cadherin.

The two basic forms of the ECM are basement membranes, which are thin networks of highly crosslinked glycoproteins, and the loose fibril-like interstitial matrix (FIG. 1). In addition, specialized ECM structures, which combine features of both the basement membrane and the interstitial matrix, form the reticular fibre network of secondary lymphoid organs[9,10] and share characteristics with the provisional matrix that forms at sites of injury. This provisional matrix can be well-populated by immune cells during wound healing[11].

Barriers encountered by leukocytes

The initial steps in leukocyte extravasation are well-characterized; these steps involve selectin-mediated rolling on the vascular endothelial cell surface, chemokine-dependent activation of α4β1 and β2 integrins and subsequent firm adhesion to the endothelial cell surface, which is followed by penetration of the endothelial cell monolayer[1,2] and the underlying endothelial cell basement membrane (FIG. 1). Transcellular migration may also occur[12] but is probably a rare event compared with paracellular migration and therefore will not be considered here.

Electron microscopy studies originally suggested that the transmigration of basement membranes was a rate-limiting step in leukocyte extravasation, as leukocytes were frequently found to be localized between the endothelium and the underlying basement membrane[13–15]. These early observations have been substantiated by more recent intravital microscopy studies[16–19]. In particular, studies of T cell extravasation have shown that although T cells only require 9–10 minutes to penetrate the endothelium, they are retained for up to 30 minutes at the outer vessel surface[19]. Although these studies do not directly visualize the endothelial basement membrane, they imply its involvement in this retention and highlight transmigration of the endothelial basement membrane as a rate-limiting step in the extravasation process.

This hypothesis is supported by confocal fluorescent and electron microscopy analyses of interleukin-1β (IL-1β)-induced neutrophil extravasation across cremaster muscle post-capillary venules. These analyses have shown that genetic or antibody-mediated blockade of the homophilic interactions between the cell adhesion molecules CD99, CD99L2 and platelet/endothelial cell adhesion molecule 1 (PECAM1), which are expressed both at the junctions between adjacent endothelial cells and on the surface of several leukocyte types[2,20], significantly reduces neutrophil infiltration and leads to arrest of the infiltrating cells between the endothelial cell and the underlying basement membrane[21–26]. PECAM1-mediated interactions between infiltrating neutrophils and vascular endothelial cells have been proposed to upregulate α6β1 integrin expression on the infiltrating neutrophils (in an IL-1β-dependent but TNF-independent manner). As α6β1 integrin is a receptor for laminins, which are a specific class of basement membrane components, upregulation of α6β1 integrin by extravasating neutrophils has been suggested to facilitate the subsequent transmigration of the endothelial basement membrane[26]. Indeed, blocking of α6β1 integrin also results in reduced neutrophil infiltration and the trapping of neutrophils between the endothelium and the basement membrane in the cremaster muscle model[26]. How these homophilic interactions between infiltrating neutrophils and the endothelium facilitate penetration of the basement membrane and whether they are relevant for other immune cell types remain to be investigated.

Structure and functions of the ECM

All basement membranes consist of four major components: type IV collagen, laminins, nidogen and the heparan sulphate proteoglycan perlecan. Of these, only type IV collagen and laminin can self-assemble to form networks[27] (FIG. 1). According to genetic data, laminins are indispensable for the formation and long-term integrity of the basement membrane[27–30]. The type IV collagen network forms independently of the laminin network and the two networks are interconnected by nidogens and perlecan[27]. However, each of these four main basement membrane components represents a family of several isoforms; for example, there are six different type IV collagen isoforms, two nidogen isoforms, two major heparan sulphate proteoglycans (agrin and perlecan) and 16 different laminin isoforms. These isoforms can differentially combine to form basement membranes that are biochemically and functionally distinct. In addition, more than 50 other glycoproteins, including netrin 4 (REF. 31), agrin, fibulin 1, fibulin 2 (REF. 32), secreted protein acidic and rich in cysteine (SPARC; also known as BM40 and osteonectin)[33] and collagen (types VII, VIII, XV and XVIII[34]), are minor components of some basement membranes and have distinct functions. Even in the vascular system, the endothelial cell basement membrane composition varies with vessel type, and laminin isoforms show the greatest heterogeneity[35].

In most tissues, except the lungs[36], leukocyte extravasation occurs at the post-capillary venule, the vessel wall of which is composed of an endothelial cell monolayer and an underlying basement membrane that is embedded with pericytes. This basement membrane contains laminin 411 (which is composed of laminin subunits α4, β1 and γ1) and laminin 511 (which is composed of laminin subunits α5, β1 and γ1)[37–39], as well as type IV collagen (which is composed of two α1 chains and one α2 chain)[28,40], perlecan[41], nidogen 1 and nidogen 2 (REF. 42). Although endothelial cells can produce all basement membrane components, their secretion is dynamic and is altered by the growth or physiological state of the endothelium[39,43] and by subjacent cells. In particular, pericytes, which are encased in the endothelial cell basement membrane, probably contribute to the composition of the basement membrane either directly through the secretion of basement membrane molecules[44,45] or indirectly by secreting factors that influence endothelial cell secretion of basement membrane molecules[46].

Subjacent to the endothelial cell basement membrane is the interstitial matrix of the tissue stroma, which loosely interconnects mesenchymal cells or fibroblasts and is responsible for the tensile strength and elasticity of tissues (FIG. 1). In most tissues, with the notable exception of the central nervous system (CNS), the interstitial matrix is mainly composed of fibrils that contain type I, type III, type V and/or type XI collagen interspersed with other suprastructural elements. These elements include non-collagenous glycoproteins, such as tenascins, fibronectin, vitronectin and chondroitin-, dermatan- or keratan-sulphate proteoglycans and the non-glycosylated protein elastin. Although the fibrils convey tensile strength, the non-collagenous glycoproteins, in particular proteoglycans, resist compressive forces and control collagen fibril diameter and density[47].

Role of the ECM in initial leukocyte migration

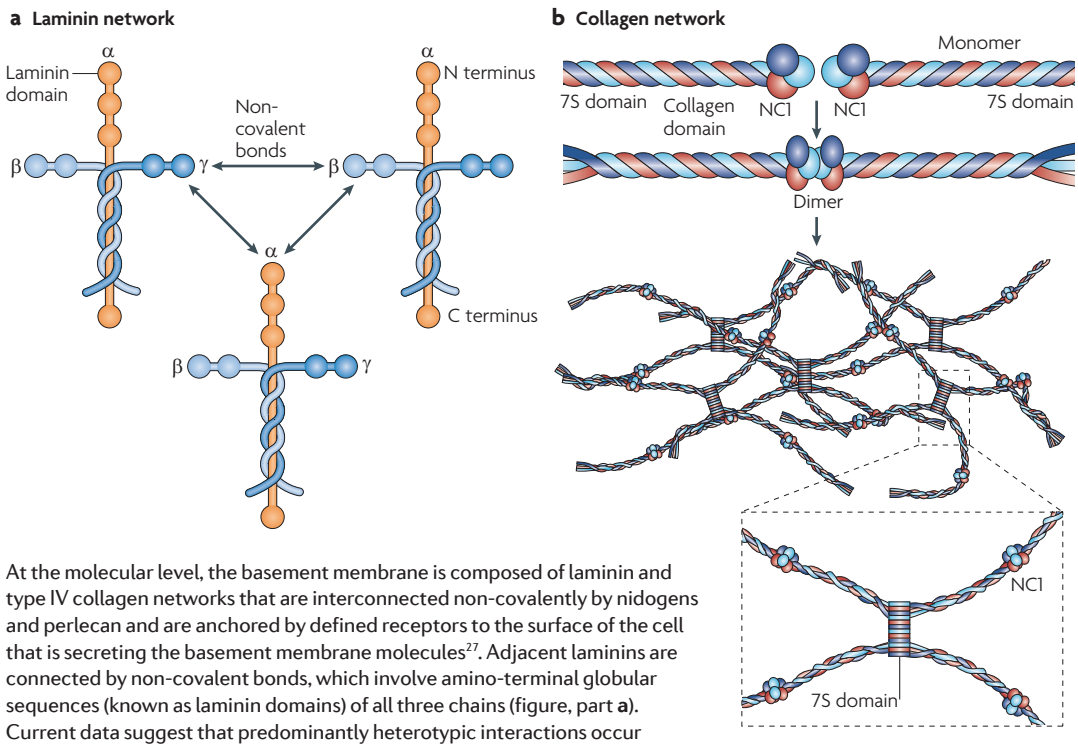
Few studies have addressed how immune cells infiltrate the endothelial cell basement membrane and how this process may influence subsequent immune cell behaviour in the inflamed tissue. The reason for this is the difficulty in reconstituting basement membranes *in vitro* owing to their complexity and tissue-specific characteristics. Although no commonly accepted model for immune cell transmigration of endothelial basement membranes exists, several mechanisms have been proposed, including migration through preformed holes or slits, proteolytic digestion of basement membrane components and non-proteolytic force-dependent mechanisms[48].

Cell adhesion molecules
Proteins (for example, CD99, CD99 antigen-like-2 (CD99L2) and platelet/endothelial cell adhesion molecule 1 (PECAM1)) that are located between adjacent endothelial cells but that are also expressed by neutrophils, monocytes and lymphocytes and are known to have important roles in leukocyte transmigration of the endothelium in inflammation. CD99 (also known as MIC2) is a small highly glycosylated glycoprotein with no known similarity to any other protein family. CD99L2 is distantly related to CD99 and has 32% sequence identity; PECAM1 is a member of the immunoglobulin supergene family.

Pericytes
Cells that are embedded in the vascular basement membrane of microvessels and that are thought to be derived from the vascular smooth muscle cell lineage. They make close cellular contact with endothelial cells and this interaction is essential for the maintenance of vessel function, as well as for the regulation of angiogenesis and vascular remodelling.

Tensile strength
The maximum load that a material can support during stretching without irreversible disruption and is expressed per unit area. When stresses less than the tensile strength are removed, a material returns either completely or partially to its original shape and size.

Box 1 | **Intermolecular interactions in basement membranes**



At the molecular level, the basement membrane is composed of laminin and type IV collagen networks that are interconnected non-covalently by nidogens and perlecan and are anchored by defined receptors to the surface of the cell that is secreting the basement membrane molecules[27]. Adjacent laminins are connected by non-covalent bonds, which involve amino-terminal globular sequences (known as laminin domains) of all three chains (figure, part **a**). Current data suggest that predominantly heterotypic interactions occur between the laminin domains of the α-, β- and γ-chains[30]; this suggests that although laminin isoforms with severe N-terminal truncation of the α-chain (such as those that contain laminin α4) can polymerize, they are likely to form less dense networks than laminins with three full length N-terminal domains (such as laminin α5), owing to fewer inter-laminin connections. Therefore, it may be that the more loose and therefore more pliable laminin 411 networks allow the passage of a cell, given sufficient force. By contrast, the more interconnected and therefore tighter network of laminin 511 presents more resistance to infiltrating cells. This could account for the preferred transmigration of endothelial basement membrane sites that are devoid of laminin α5 (but contain laminin α4). Type IV collagen network assembly requires covalent bonding between the carboxy-terminal non-collagen domain (NC1) and the N-terminal 7S domains of adjacent monomers[40] (figure, part **b**). However, there is also evidence that supports non-covalent interactions between type IV collagen monomers[111] and differences in the number of NC1 dimers that are covalently crosslinked between basement membranes[111]. Differences in the extent of crosslinking may reflect areas that are more readily penetrated by leukocytes, given sufficient force.

A role for proteases. As only type IV collagen monomers are covalently linked in the basement membrane (BOX 1) and as different leukocyte types, in particular neutrophils, secrete proteases that can digest ECM molecules *in vitro*, early work proposed that leukocyte transmigration of basement membranes involved the digestion of basement membrane components. There are reports of proteolytic cleavage of basement membrane molecules, including the endothelial basement membrane components type IV collagen, laminin α5 and nidogen 1. However, although this cleavage can result in bioactive fragments that can attract neutrophils and macrophages *in vitro*[49–53], all of these studies involve *in vitro*-generated ECM fragments and cannot be verified *in vivo*. In addition, several facts argue against degradation of the endothelial basement membrane. For example, normal immune surveillance of tissues *in vivo* requires the continuous trafficking of large numbers of T cells and dendritic cells (DCs) across endothelial basement membranes which, in contrast to the remodelling events that are associated with embryonic development or pathological conditions, occurs without detectable perforations or alterations[15,36,54,55]. Although diapedesis is associated with disruption of the retention properties of the basement membrane, there are no overt morphological changes to the basement membrane and the effects on it are transient, suggesting that they are reversible[36,56–58]. Current data suggest that the role of proteases in inflammation is strictly controlled and that, rather than being important in the early extravasation steps, they instead act to fine-tune the nature of the immune response or to remodel the interstitial matrix during chronic inflammation[5,59] (BOX 2).

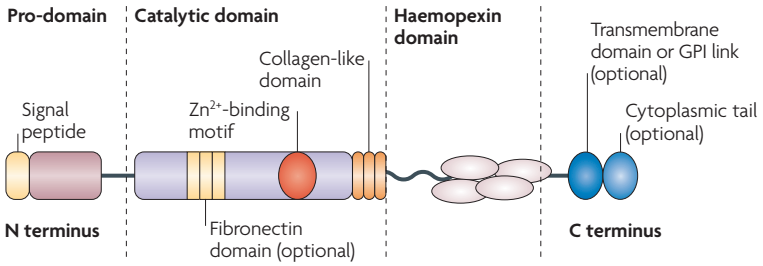
Preformed slits in the endothelial basement membrane. Preformed slits have been proposed to occur in the endothelial basement membrane of lung alveolar capillaries, which is the site of leukocyte extravasation in this tissue. Transmission electron microscopy has revealed slits of up to 1 μm in length through which neutrophils can pass[36,60]. Although such extreme cellular compression may be tolerated by neutrophils, owing

Diapedesis
The migration of leukocytes across the endothelium. This migration generally occurs by squeezing through the junctions between adjacent endothelial cells, although in some settings leukocytes have been shown to pass through transiently formed gaps in the cytoplasm of endothelial cells. It is the last step in the leukocyte–endothelial cell adhesion cascade, which includes tethering, triggering, tight adhesion and transmigration.

Box 2 | **MMPs: what they can do and what they actually do *in vivo***

Matrix metalloproteinases (MMPs) are endopeptidases that are characterized by a conserved Zn^{2+} binding motif in the catalytic domain and several conserved protein domains (figure). At present, they comprise a family of 25 different members in mice and 24 different members in humans[112]. MMP expression is transcriptionally regulated by factors, including pro-inflammatory cytokines[113–115], growth factors and hormones. However, they are produced and secreted as neutral pro-enzymes, or zymogens, that require activation in the tissue by removal of the amino-terminal pro-domain[112]. Activation can be mediated by other MMPs or proteases and some are activated intracellularly by furins. In addition, chemicals, such as organomercurials, urea, some detergents and reactive oxygen species, can act as activators of MMPs. Furthermore, MMP activity in tissues is regulated by tissue inhibitors of MMPs (TIMP1–TIMP4) that bind to and inactivate most MMPs. The major endogenous MMP inhibitor in serum is α2-macroglobulin, which binds MMPs and leads to their clearance by endocytosis. Hence, MMP mRNA expression or even protein expression does not provide information on the *in vivo* localization of MMP activity, which is assessed by *in situ* zymography methods[55] and antibody-capture activity assays. The MMPs are classified according to the substrates that they can cleave that are identified by *in vitro* experiments — the reader is referred to (REF. 116) for a description of how MMPs bind their substrates and exert their catalytic functions.

In vitro assays that use high protease to substrate ratios have shown that MMPs can cleave a wide range of ECM molecules. This has led to the widely-held concept that MMP-mediated cleavage of ECM molecules is the means by which infiltrating cells, including leukocytes and transformed cells, penetrate ECM barriers and that MMPs are essential for all remodelling processes in developing or regenerating tissues. However, the advent of mouse transgenic technology and the elimination of genes for one or more MMPs has resulted in surprisingly few spontaneous phenotypes, with the notable exception of membrane type 1 MMP[115], leading to the growing realization that the proteolytic function of these molecules is more subtle than originally considered and that the traditional methods that are used to identify MMP substrates are not relevant in the complex cellular or tissue context. The development of new protease 'degradomic' techniques[4] — which is the application of proteomic and genomic approaches to characterize proteases, their substrates and inhibitors on a system-wide scale in an organism — provide a more accurate assessment of the role of proteolytic processes *in vivo* and suggest a role for MMPs as 'processing' rather than 'degrading' enzymes. The best example of this is the selective cleavage of several chemokines by MMPs that results in either their inactivation[117,118] or potentiation of their effects[119] (TABLE 1). The process also results in control of extracellular pools of inflammatory cytokines[120–122] and the selective cleavage of ECM domains, which results in bioactive fragments that have functions distinct from the parent molecule[78]. In addition, there is recent evidence that supports a non-catalytic function for MMPs that is mediated by the haemopexin domain of the latent form of some MMPs, in particular pro-MMP2 and pro-MMP9, in cell migration[123].



to their fragmented nuclei, it is unlikely to be feasible for lymphocytes, which are 7–8 μm in diameter, or for DCs and monocytes, which can be up to 20 μm in diameter and have a nuclear diameter of 3–5 μm. Ultrastructural analyses of different basement membranes are limited; however, atomic-force microscopy combined with high-resolution scanning electron microscopy of specific basement membranes has revealed a complex topography of tightly interlaced networks with pores of 40–70 nm that cover up to 15% of the total surface[61–63]. These pores are too small to allow the passage of an immune cell but could be sites of preferred extravasation that can be stretched by the transmigrating cells (BOX 1).

Variability in basement membrane composition. Recent *in vivo* studies have suggested that the biochemical composition of the endothelial basement membrane defines the preferred sites of T cell[37,38] and neutrophil extravasation[18]. The endothelial basement membrane composition also determines the extent of T cell[38], monocyte[64] and neutrophil[18,64] extravasation into inflamed tissues, and the laminin family has a decisive role.

Perivascular cuff
The immune cell infiltrate that is immediately adjacent to the outer surface of the post-capillary venule wall and, in the case of central nervous system vessels, is bordered by the inner endothelial cell basement membrane and the outer parenchymal basement membrane.

The microvasculature of the CNS is ideal for defining the site of extravasation across the vessel wall, as it has a specialized double basement membrane structure that is designed to limit solute and cellular movement and allows precise localization of sites of immune cell infiltration (FIG. 2). The CNS post-capillary venule consists of endothelial cells, which are joined by complex tight junctions, and an underlying endothelial basement membrane; these are further ensheathed in a second parenchymal basement membrane and a layer of astrocytes[65] (FIG. 2). During inflammation in the CNS, infiltrating leukocytes must first penetrate the endothelial monolayer and its basement membrane. The infiltrating cells subsequently accumulate in the perivascular space between the inner endothelial and outer parenchymal basement membranes, forming a perivascular cuff and allowing precise localization of the sites of extravasation[37,38,55]. One of the best studied models of CNS inflammation is experimental autoimmune encephalomyelitis (EAE) induced with myelin oligodendrocyte glycoprotein (MOG)-peptide in C57BL/6 mice; this is a model of $CD4^+$ T cell-mediated autoimmune disease with similarities to the human

Table 1 | **Examples of factors that can modulate the ECM in inflamed tissues**

Factor	Effects on ECM	Refs
Cytokines		
TNF	Upregulation of osteopontin, MMP9 and the vascular laminins α4 and α5; downregulation of most other ECM molecules	37,113, 124,125
IL-17	Upregulation of MMP9	114
TGFβ	Upregulation of most ECM molecules	125
IFNg	Downregulation of most ECM molecules; downregulation of MMP1, MMP2, MMP3, MMP7, MMP9 and MMP10	125,126
IL-1β	Upregulation of laminin α4; upregulation of MMP1, MMP3, MMP7 and MMP9;	37,39,121
Proteases		
MMP2, MMP9	Cleavage of cell–matrix receptors (for example, dystroglycan)	55
MMP2, MMP9	Inactivation of chemokines (for example, CXCL12)	127
MMP9	Activation of chemokines (for example, CXCL8, CXCL6 and CXCL5); surface release of TNF, which alters local concentrations	119,120
MMP2, MT1–MMP	Production of CXCR3 receptor antagonists (for example, CCL7); degradation of IL-1β, which alters local concentrations of this cytokine	118,121, 122
MMP1, MMP3	Production of CXCR3 receptor antagonists (for example, CCL2, CCL8 and CCL13)	128
MMP7	Cleavage of syndecan 1 and syndecan 4; cleaved syndecans bind to chemokines, which alters their local availability	129

CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; CXCR, CXC-chemokine receptor; ECM, extracellular matrix; IFNγ, interferon-γ; IL, interleukin; MMP, matrix metalloproteinases; TGFβ, transforming growth factor-β; TNF, tumour necrosis factor.

disease multiple sclerosis. Although other EAE models exist and are characterized by distinct inflammatory infiltrates[66], the MOG-induced EAE model is the most extensively studied because it can be used to assess disease in transgenic mice that are often on a C57BL/6 genetic background. In CNS post-capillary venules, laminin α4 is ubiquitously localized in the endothelial basement membranes, whereas laminin α5 has a patchy distribution (FIG. 2). In MOG-induced EAE, $CD4^+$ T cell transmigration occurs at regions of the endothelial cell basement membrane where laminin α4 is present but laminin α5 is absent, owing to an inhibitory effect of laminin α5 on T cell transmigration[38]. For T cells to distinguish between laminin α4- and laminin α5-containing areas, they require surface expression of α6β1 integrin; antibody-mediated inhibition or genetic ablation of this integrin on leukocytes selectively inhibits T cell transmigration across the endothelium[38].

In laminin α4-deficient mice, laminin α5 shows a compensatory ubiquitous localization in all endothelial basement membranes[38,67]. Studies of these mice in the MOG-induced EAE model, but also in models of acute skin inflammation, delayed-type hypersensitivity (DTH), thioglycollate-induced peritonitis and subcutaneous air pouch models[64], have shown that laminin α4-deficient mice have significantly decreased immune cell infiltration to sites of inflammation. Intravital microscopy of neutrophil extravasation across cremaster muscle post-capillary venules in laminin α4-deficient mice showed normal neutrophil adhesion to the vessel wall and migration in the interstitial matrix of the stroma, but reduced diapedesis across the vessel wall; this suggests a common inhibitory effect of laminin α5 on the extravasation of $CD4^+$ and $CD8^+$ T cells, neutrophils and monocytes.

The existence of such a 'pathway of least resistance' that is defined by the absence of laminin α5 is also supported by studies of IL-1β-induced neutrophil extravasation across cremaster muscle post-capillary venules. In these studies, intravital microscopy was used to identify sites of neutrophil extravasation and confocal microscopy of excised tissue was then used to characterize these sites[18]. Neutrophil extravasation across post-capillary venules occurs preferentially at sites where expression of laminin α5 and type IV collagen (but not perlecan) is lower; these are termed low-expression regions (LERs)[18]. LERs are associated with endothelial cell junctions and the gaps between pericytes and are transiently increased in size but not in number in the presence of IL-1β, coincident with neutrophil extravasation. In contrast to findings in the MOG-induced EAE model, laminin α5 was reported to be detectable on the surface of 15–20% of neutrophils during IL-1β-induced extravasation. As isolated neutrophils do not express mRNA that encodes laminin α5 and an elastase inhibitor reduced both neutrophil extravasation and the transient increase in LER size during IL-1β treatment, it was proposed that the laminin α5-containing network is cleaved by neutrophil elastase during the extravasation process[18]; this suggests a fundamental difference between neutrophil extravasation in these models and $CD4^+$ T cell extravasation in the MOG-induced EAE model.

According to current theories of basement membrane formation and integrity, and in view of the low turnover of basement membrane components in mature tissues[37,43], degradation of an integral component, such as laminin α5, is inconsistent with the temporally restricted changes in vessel permeability that are observed at sites

Delayed-type hypersensitivity
(DTH). A cellular immune response to antigen that develops over a period of ~24–72 hours. The response is characterized by the infiltration of T cells and monocytes and depends on the production of T_H1-type cytokines.

of extravasation. Therefore, it remains to be shown whether neutrophil elastase can cleave basement membrane proteins in this process, rather than acting on other factors (possibly in the interstitium) to generate chemoattractants.

Owing to the double basement membrane structure of CNS vessels, the EAE model provides a unique opportunity to examine how $CD4^+$ T cells penetrate the parenchymal basement membrane, which is a non-endothelial basement membrane that is biochemically distinct from the endothelial basement membrane and contains laminin α1 and α2 (REF. 37). $CD4^+$ T cell penetration of this basement membrane in the MOG-induced EAE model is not laminin dependent, but instead requires focal MMP2 and MMP9 (REF. 55), which selectively cleave the dystroglycan receptor that anchors astrocyte end-feet to parenchymal basement membrane components. However, parenchymal basement membrane components or other ECM-binding receptors on astrocyte end-feet remain unaffected, indicating a specific regulatory role for these proteases[55]. Because basement membrane anchorage is essential for its formation and integrity[27], loss of β-dystroglycan-mediated anchorage might compromise basement membrane stability (as suggested by the astrocyte-specific deletion of the entire dystroglycan complex in mice[68]), leading to distortion of intermolecular interactions in the basement membrane and thereby facilitating leukocyte transmigration.

Although comprehensive studies that compare different T cell or macrophage subpopulations or other immune cell types, such as DCs, have not been carried out, the data described above suggest not only that there are common cues that are followed by leukocytes that define sites of preferred extravasation across the post-capillary wall (for example, low expression of laminin α5), but also that the same leukocyte type can use different mechanisms depending on the biochemical composition of the basement membrane. However, possibilities to test this further are very limited owing to the need to use *in vivo* models for basement membrane studies and the absence of models in which immune cells can be tracked.

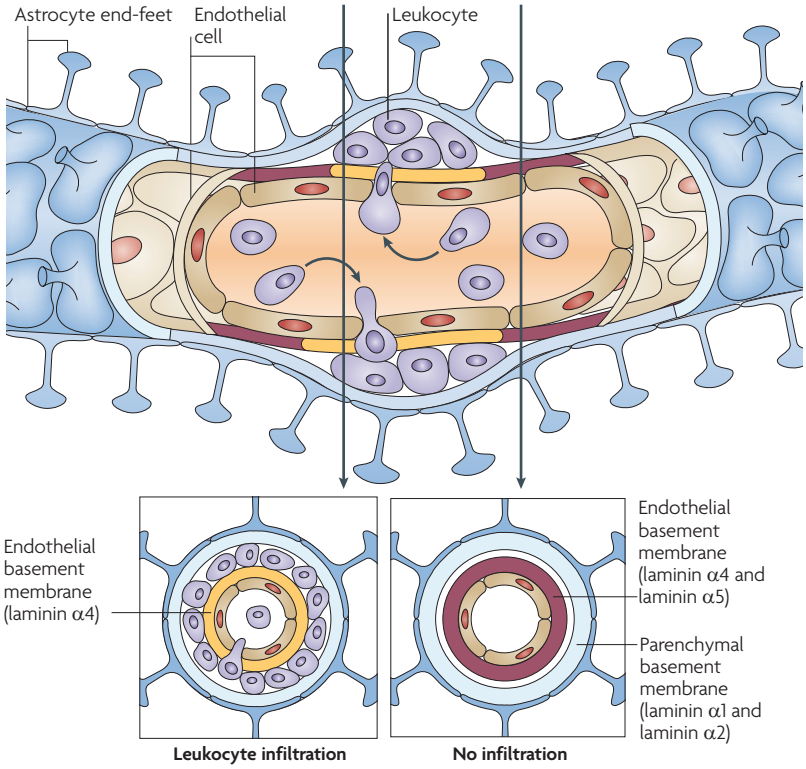


Figure 2 | **Cellular and ECM layers that are encountered by leukocytes infiltrating CNS post-capillary venules.** Leukocyte extravasation occurs only where laminin α4 alone, but not laminin α5, is localized in the endothelial cell basement membrane[38]. Following transmigration of the endothelial monolayer and underlying basement membrane, leukocytes in the central nervous system (CNS) still face a second biochemically distinct basement membrane, the parenchymal basement membrane (containing laminin α1 and laminin α2 chains). In contrast to penetration of the endothelial cell basement membrane, penetration of the parenchymal basement membrane by infiltrating T cells is associated with focal matrix metalloproteinase 2 (MMP2) and MMP9 activity[55]. Osteopontin, which is upregulated in the perivascular cuff, promotes the differentiation of T helper 1 (T_H1) and T_H17 cells and their prolonged survival, thereby contributing to the transition from the relapsing and remitting forms of experimental autoimmune encephalomyelitis (EAE) to the more severe progressive type of EAE without phases of remission[89,91].

Migration through interstitial matrices. In most tissues other than the CNS, after leukocytes have penetrated the endothelial monolayer and its basement membrane they encounter the loose fibrillar network of the interstitial matrix, which affects their subsequent migration to the site of inflammation. The most comprehensive studies on immune cell migration through the interstitial matrix involve DCs, and describe an amoeboid mode of migration for these cells along the collagen fibril scaffold that is independent of β1, β7, αv and β2 integrins[69]. Similarly, $CD4^+$ and $CD8^+$ T cell blasts and monocytic cell lines have also been shown to migrate through fibrillar collagen gels *in vitro* in a protease- and β1 integrin-independent manner[70]. Data suggest that external resistance, which is defined by collagen fibril size and density, together with high internal resistance due to the size and rigidity of the nucleus, determines whether the migrating DCs[69] use only the force of the actin network for forward probing of the leading edge or whether myosin II-dependent contraction of the trailing end is required to squeeze the nucleus forward. This 'flowing and squeezing' migration provides a model for leukocyte migration through the interstitial matrix without degradation or permanent remodelling and only transient deformation of the pericellular matrix. It is noteworthy that this process differs from the protease- and integrin-dependent mode of migration of mesenchymally derived cells[71] and tumour cells[72,73].

Force as a general mechanism for penetration of the ECM. It is possible that sensing of external forces by transmigrating leukocytes may also influence penetration of the endothelial basement membrane. It has been shown that neutrophils that migrate on the surface of the endothelium under laminar shear stress can sense the compliance of the underlying substrate,

which controls the degree of mechanical force that is generated by the migrating neutrophil[74,75]. The maximum force that is exerted by an actively migrating neutrophil is substantially greater than the force that is generated by an adherent cell that does not transmigrate. Furthermore, substrate rigidity that underlies the endothelial monolayer further modifies mechanical forces that are induced by the transmigrating neutrophil[74,75]. Such force generation requires engagement of integrins, which connect the extracellular environment to the cytoskeleton, for greater force generation. This mechanical force can disrupt cell–cell contacts[74] but may also have a role in the subsequent penetration of the basement membrane. Consistent with this force detection model, recent studies have shown that T cells[76], neutrophils and monocytes[77] that crawl across endothelial cell surfaces express invasive filopodia, which have been proposed to probe for sites that are permissive to transendothelial cell migration and, theoretically, could also test the compliance of the endothelial basement membrane.

Role of the ECM in inflamed tissues

Immune cells as modulators of the ECM during chronic inflammation. In inflamed tissues, both ECM turnover and protease secretion by tissue-resident cells are affected by cytokines, such as TGFβ, TNF and IFNγ (TABLE 1), that are released by the infiltrating cells. There is increasing evidence that suggests that such aberrantly expressed ECM molecules can influence immune cell activation, differentiation and survival, and that some of these molecules are selectively cleaved by proteases, in particular by the MMPs (BOX 2), resulting in bioactive peptides that can act as chemoattractants or can alter immune cell activity. Therefore, the remodelled ECM of inflamed tissues affects the propagation of the inflammatory response and the development of chronicity. Although this is an interesting aspect of ECM research in inflammation, data remain limited and fragmentary; hence, selected examples of how the altered ECM in inflamed tissues may influence immune cell behaviour are discussed below.

ECM fragments as chemoattractants. The best evidence so far for the existence of *in vivo* bioactive ECM fragments that are generated by selective cleavage events is provided by a chemotactic fragment of type I collagen. An acetylated tripeptide, Pro-Gly-Pro (acetyl-PGP), that results from the cleavage of type I collagen by MMP8 or MMP9 has recently been shown to mimic the chemotactic effects of CXC-chemokine ligand 8 (CXCL8) on neutrophils in a lung inflammation model[78]. During inflammation, MMP8 and MMP9 are released from activated neutrophils and cleave type I collagen into fragments of 30–100 amino acids in size. These fragments are further processed by prolyl endopeptidase and are acetylated by acetylases that are present at sites of inflammation. These acetyl-PGP fragments show structural homology to chemokines and chemotactic formyl peptides of bacterial origin that act on G-protein-coupled receptors (such as CXC-chemokine receptor 1 (CXCR1) and CXCR2) to promote neutrophil recruitment to sites of inflammation[79]. Acetyl-PGP is detectable in bronchoalveolar lavage samples from individuals with obstructive pulmonary diseases and may therefore represent a biomarker or therapeutic target for neutrophilic inflammatory diseases[80].

Elastase and MMP12 digests of elastin have also been shown to be chemotactic for monocytes in chronically inflamed lungs[81–83]. So far, these processes have been characterized in lung inflammation models, in which type I collagen is upregulated and elastin is a major interstitial matrix component, but they are likely to be relevant to other tissues in which these molecules predominate or are upregulated during inflammation, such as the joint synovium or the dermis of the skin.

The ECM as an activator of immune cells

Several recent publications have suggested that ECM fragments or ECM molecules that are upregulated during inflammation can also activate immune cells, thereby perpetuating the inflammatory response. *In vivo* data supporting this hypothesis are limited, but selected studies that are mainly relevant to chronic inflammatory conditions exist and are discussed here. In many cases, with the notable exception of osteopontin, these effects are mediated by the Toll-like receptor family, members of which recognize defined molecular patterns that are associated with pathogens or tissue damage. Activation of TLRs initiates innate immune responses that subsequently influence the nature of the adaptive immune response. Existing data suggest that endogenous TLR ligands that are associated with tissue damage are variable and are probably related to the pathological situation[84].

Osteopontin and T cell differentiation in multiple sclerosis. Osteopontin is a glycoprotein that is secreted by many fibroblast-like cells and also by activated T cells and other immune cells. It was originally identified as a constituent of the ECM of bone and is thought to recruit osteoblast precursors and bind them to the mineralized matrix of the bone[85]; hence its name and classification as an ECM molecule. However, data suggest that osteopontin has several distinct functions that are probably related to the differential glycosylation or phosphorylation state of osteopontin and whether or not it is crosslinked to other ECM molecules. Expression of osteopontin is upregulated during several autoimmune diseases, including systemic lupus erythematosus[86], rheumatoid arthritis[87] and inflammatory bowel diseases[88], and it has been implicated in the pathogenesis of these diseases.

The involvement of osteopontin in autoimmune diseases has been emphasized by studies of EAE and multiple sclerosis. It was first identified as a factor in multiple sclerosis by large-scale sequencing of cDNA libraries derived from perivascular cuffs that were dissected from the brains of patients with multiple sclerosis[89]. Osteopontin is expressed in the perivascular cuff of inflamed vessels and is upregulated in the brain and serum in both rodent models of EAE and in patients

Filopodia
Slender cytoplasmic projections that extend from the leading edge of migrating cells.

Osteopontin
An extracellular matrix protein that supports the adhesion and migration of inflammatory cells. It has recently been recognized as an immunoregulatory T_H1-type cytokine.

Toll-like receptor family
(TLR family). A family of receptors that are unique to microorganisms that recognize conserved products (such as lipopolysaccharide) known as pathogen-associated molecular patterns (PAMPs), as well as damage-associated molecular patterns (DAMPs). TLRs signal to the host that a microbial pathogen is present or that tissue damage has occurred.

with multiple sclerosis during relapses of the disease[90]. It has been implicated in the transition from relapsing and remitting forms of multiple sclerosis to the more severe progressive type of disease without phases of remission[89,91].

Osteopontin acts in different ways; it exacerbates EAE by promoting the differentiation of IFNγ-producing T helper 1 (T_H1) cells and IL-17-producing T_H17 cells that are associated with disease progression[92]. In addition, osteopontin stimulates IL-2 secretion, which is required for T cell survival, and simultaneously inhibits apoptosis of effector T cells, thereby enhancing the survival of autoreactive T_H1 and T_H17 cells in the CNS[91,93,94]. It has been proposed that apoptotic elimination of an already activated autoreactive T cell population after the initial autoimmune response might be a mechanism for the deletion of autoreactive T cells that escape elimination in the thymus during development. Indeed, T cell death in the CNS during EAE normally occurs concurrently with spontaneous disease remission[95,96]; hence, the concentration of osteopontin in the perivascular cuff and its pro-survival effects on autoreactive T cell populations suggests that this molecule might modulate disease progression.

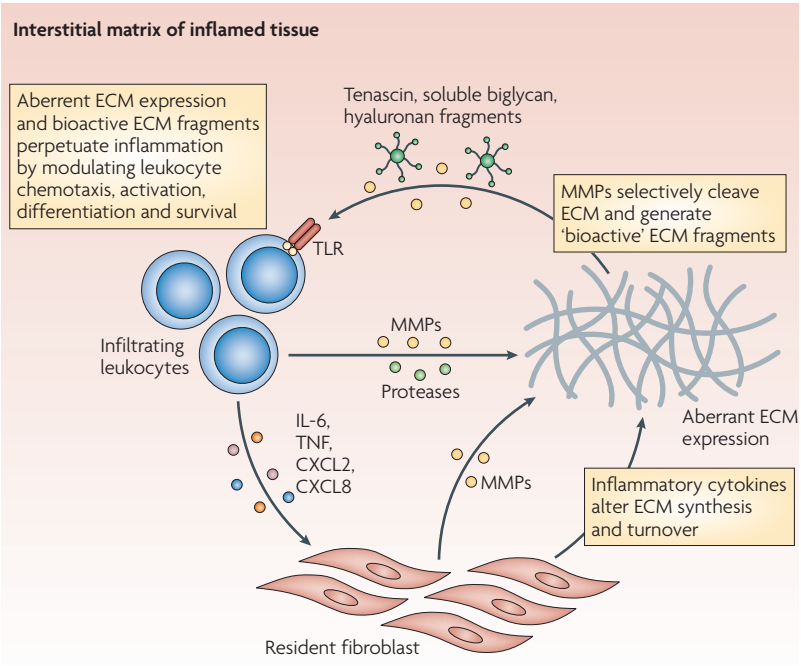


Figure 3 | **Potential modes of ECM-mediated activation of immune cells.** Infiltrating immune cells secrete cytokines and proteases, including matrix metalloproteinases (MMPs), which activate resident cells in the tissue interstitium, altering extracellular matrix (ECM) synthesis and/or the selective cleavage of ECM domains. Both aberrant ECM expression and 'bioactive' ECM fragments can influence the inflammatory response by modulating immune cell chemotaxis, activation, differentiation or survival, thereby contributing to the perpetuation of the inflammatory response, in some cases through the activation of Toll-like receptor 2 (TLR2) and/or TLR4. These events typically occur in the interstitium of tissues during chronic inflammation, for example, in patients with rheumatoid arthritis, chronic lung diseases or skin inflammatory diseases. CXCL, CXC-chemokine ligand; IL-6, interleukin-6; TNF, tumour necrosis factor.

TLR activation by tenascin, biglycan and hyaluronan. Recent data implicate constituents or fragments of the ECM as activators of TLRs in particular TLR4 but also TLR2. In all cases, the molecules that are involved are upregulated or proteolytically processed in inflamed tissues and are typically interstitial matrix molecules, such as the tenascin C isoform, the small leucine-rich proteoglycan biglycan[97] and fibronectin[98], as well as heparan sulphate[100,101] and the glycosaminoglycan hyaluronan[99]. Because TLR2 and TLR4 are typically activated by bacterial antigens (such as lipopolysaccharide (LPS)), one concern with these studies is the use of recombinant ECM proteins or fragments that are generated in bacterial systems; this raises the possibility that even small amounts of residual LPS activity may account for the observed results. In addition, the mechanisms of how the diverse ECM components interact with these two TLRs remain unclear, as they do not share similar structural domains.

However, there are examples in which *in vivo* verification of ECM activation of TLRs has been possible. For example, tenascin C, which is normally expressed at low levels in healthy synovia but is upregulated in inflamed synovia, synovial fluid and cartilage from individuals with rheumatoid arthritis and corresponding mouse models, interacts with TLR4 on macrophages and synovial fibroblasts to induce pro-inflammatory cytokine production that is distinct from that which is induced by bacterial LPS[102]. Mice that lack tenascin C are protected from the synovitis that is induced by zymosan (a TLR2 agonist) and although joint inflammation is induced in an experimental model of rheumatoid arthritis in the absence of tenascin C, the mice show considerably faster recovery and no persistent inflammation[102]. By stimulating the production of pro-inflammatory cytokines, such as IL-6, TNF and CXCL8, by resident fibroblasts and macrophages, tenascin C is proposed to maintain inflammation in the joint and to locally propagate the inflammatory response.

Similar pro-inflammatory effects have been described during renal inflammation for soluble, non-ECM-bound biglycan, which binds to TLR2 and TLR4 on macrophages. This leads to the upregulation of CXCL2 and TNF expression by macrophages and creates a positive feed-forward mechanism that promotes macrophage infiltration and perpetuation of the inflammatory response[103]. By contrast, ECM-bound biglycan is associated with sequestration of cytokines. In particular, ECM-bound biglycan sequesters TGFβ, thereby controlling local concentrations of this anti-inflammatory cytokine. How biglycan is released from the ECM is not clear; proteolytic cleavage has been suggested but has not yet been proven, and it has also been proposed that activated macrophages can secrete the soluble form of the protein[104].

In addition to these glycoproteins, the glycosaminoglycan hyaluronan has been implicated in the activation of TLR2 and TLR4 (FIG. 3). Hyaluronan is a linear heteropolysaccharide that is composed of repeating units of *N*-acetyl-glucosamine and glucuronic acid and normally exists as large molecular weight polymers

(1,000–2,000 kDa). Unlike other glycosaminoglycans, hyaluronan is not sulphated or bound to a core protein, but is synthesized at the inner side of the plasma membrane under the control of hyaluronan synthetases (rather than in the Golgi apparatus) and secreted into the extracellular space[99]. Hyaluronan is produced by several cell types, including epithelial cells, endothelial cells and fibroblast-like cells, and can form a pericellular matrix when it is bound to its main cellular receptor CD44. As part of a pericellular matrix, it can interact with glycosaminoglycan chains of glycoproteins in the interstitial matrix[99]. Owing to its high affinity for water, hyaluronan is considered a filling material in the tissue interstitium or in secretions. It has an exceptionally high turnover and is quickly degraded by free oxygen radicals, by mechanical force and by hyaluronidases and is cleared from tissues by the lymph or receptor-mediated endocytosis. *In vitro* experiments have shown that the enzymatic degradation of hyaluronan results in the generation of pro-inflammatory low-molecular-weight hyaluronan fragments (<250 kDa). However, *in vivo* evidence of such fragments is scarce and only recently have such fragments been identified in the bronchial lavage fluid of individuals with acute lung injury[105]. Such low-molecular-weight hyaluronan fragments have been proposed to be increased in inflamed tissues[106]. In such tissues, hyaluronan fragments interact with TLR2 and TLR4 on resident immune cells, stimulating the expression of pro-inflammatory cytokines and chemokines[105,107] and even promoting interactions between antigen-presenting cells and T cells[108,109], thereby perpetuating the inflammatory response. By contrast, high-molecular-weight hyaluronan has been associated with anti-inflammatory effects and has been shown to promote the survival of epithelial cells in a model of acute lung injury[99].

These extracellular proteins and sugars are postulated to be endogenous danger signals in inflamed tissues and are thought to activate resident immune cells, even in the absence of pathogens (FIG. 3). Therefore, they provide a mechanism for injury recognition that distinguishes damage that is caused by traumatic mechanical tissue destruction from that which is associated with microbial invasion and which is accompanied by the presence of pathogen-associated molecular patterns, such as LPS. However, despite new and exciting research in this field, there are still many unanswered questions. For example, how is the synthesis and subsequent modification of molecules, such as hyaluronan, regulated? What factors induce their production and how is the synthesis of biologically active sugar sequences or ECM fragments controlled? Owing to current technical limitations in the detection of such bioactive protein fragments or sugars *in situ,* these questions remain difficult to answer.

Conclusions and perspectives

This Review draws attention to the complex *in situ* situation at sites of inflammation and highlights the fact that it is not only endothelial and immune cells that are involved in shaping responses at these sites but also the ECM, which encompasses the basement membrane that underlies the endothelium and the interstitial matrix of the tissue stroma. The effects of the ECM in inflammation are varied: the notion that the ECM can have a structural function by acting as a barrier or scaffold for cells that infiltrate inflamed tissues is easy to conceive, even for a non-ECM specialist. However, new data suggest that it does much more; both the biophysical properties of the ECM and its biochemical composition provide specific signals to immune cells that determine their sites of exit from post-capillary venules, their mechanism of migration through endothelial basement membranes and the interstitium to the site of inflammation and, once at the site of inflammation, their ability to promote the inflammatory response. Although the data are currently limited, they suggest that the laminin composition of the endothelial basement membrane defines sites of preferred extravasation for all leukocytes and that immune cells can use different mechanisms for the penetration of different basement membranes. How cells sense biochemically distinct basement membranes remains unanswered — whether it is a matter of force alone or whether it requires specific signal-transduction mechanisms (or a combination of the two) will be an important topic for future investigations. *In vitro* assays may help to distinguish between these two possibilities and to identify the intracellular signal cascades that are triggered by different basement membrane components, but only *in vivo* models can verify their true physiological relevance.

In chronically inflamed tissues, such as those that occur in many autoimmune diseases, the ECM is altered by cytokines and proteases that are produced by infiltrating immune cells. There is strong evidence to suggest that several cytokines, such as TNF or IFNγ, that are upregulated at sites of inflammation can alter both the synthesis and turnover of various ECM molecules, typically those of the interstitial matrix, as well as the secretion and/or activation of proteases in particular the MMPs. There is increasing evidence to suggest that aberrant expression of ECM molecules at sites of inflammation can influence immune cell activation, differentiation and survival and that some of these molecules are selectively cleaved by proteases, resulting in bioactive peptides that can act as chemoattractants or alter immune cell activity. However, the data on the involvement of proteases and bioactive ECM fragments in inflammation are limited and fragmentary owing to the current limitations in the *in vivo* detection of proteases and ECM fragments, which, in general, are short-lived and are present focally at low concentrations. Future 'degradomic' techniques that combine proteomic and genomic approaches to characterize proteases, their substrates and inhibitors on a system-wide scale are likely to provide an overview of the proteolytic processes that are active in inflamed tissues[110]. The ability to mimic or inhibit some of the ECM functions or proteolytic events that are discussed above would provide novel means to manipulate the development of inflammatory responses.

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Competing interests statement
The author declares no competing financial interests.

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