Review

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Natural pattern recognition mechanisms at epithelial barriers and potential use in nanomedicine

Abstract: At epithelial barriers molecular pattern recognition mechanisms act as minesweepers against harmful environmental factors and thereby play a crucial role in the defense against invading bacterial and viral pathogens. However, it became evident that some of the proteins participating in these host defense processes may simultaneously function as regulators of tissue regeneration when in the extracellular matrix, thus coupling defense functions with regulation of stem cells. Although molecular pattern recognition has complex physiological roles and we just begin to understand its various functions, the simplicity of the underlying principles for recognition of specific classes of molecules may generate novel starting points for nanomedical approaches in drug delivery across epithelial barriers. The present article aims to provide an introduction into the biological context, processes, proteins, and general mechanisms of molecular pattern recognition in humans and, by using selected

examples, to identify potential areas in nanomedicine for the exploitation of these mechanisms.

Keywords: DMBT1; drug delivery; epithelial barrier; innate immunity; pattern recognition.

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Introduction

In innate immunity, molecular pattern recognition is a fundamental and strikingly simple mechanism intimately linked to the emergence of multicellular life. So-called scavenger receptor cysteine-rich (SRCR) proteins comprise one of the families involved in non-self recognition by the innate immune system and represent one of the best examined examples of pattern recognition molecules (PRMs). At first associated only with metazoan organisms, with sponges and sea urchins as the most ancient representatives, SRCR proteins were recently also identified by genome analyses in the multicellular and unicellular green algae *Volvox carteri* and *Chlamydomonas reinhardtii*, respectively (1–4). SRCR proteins have been therefore proposed to have originated in early eukaryotes, before the divergence between the plant and animal kingdom, and while they were lost in higher plants, SRCR proteins further evolved in metazoa (1). In algae, sea urchins, and sponges SRCR proteins mediate different functions related to cell differentiation, reproduction, and regeneration, which have as common factor the recognition of “self structures” (2, 5, 6). However, in sea urchins, which lack an antibody-based adaptive immune system, the functions of SRCR proteins have expanded to comprise the primitive immune system (7), indicating an early expansion to a role in the recognition of non-self.

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In humans some SRCR proteins specialized to particular functions, while others may have maintained a broad role in various physiological processes, e.g., participating in defense and regenerative processes. A detailed review of SRCR proteins has been provided elsewhere (8), which describes the role of SRCR proteins in health and disease and their potential use as therapeutic supplements or as drug targets. With regard to nanomedicine, an exciting question is how the simplicity of molecular pattern recognition could be exploited for drug delivery.

The present review will focus on the facets of molecular pattern recognition and its possible use in nanomedicine strategies. We will describe the biological processes PRMs are involved in, key proteins, and underlying known mechanisms of recognition. After a general introduction, the human multifunctional PRM DMBT1 will be discussed *pars pro toto* in more detail, since recent advances in deciphering the molecular basis of its broad ligand-binding specificity may give rise to applications that could be of interest for the field of nanomedicine. This review may serve as inspiration for exploring these mechanisms for nanomedical applications.

Natural pattern recognition mechanisms

Although there are reasons to anticipate the involvement of pattern recognition mechanisms in various biological processes, PRMs have traditionally been analyzed with respect to their role in the immune defense. The immune defense has evolved as a complex network of interactions and crosstalk between cells with different functions to assure a fast, efficient, and effective recognition and clearance of pathogens (9–13). As founding mechanism, immune cells are capable to distinguish between self and non-self based on the chemically and structurally different molecules on pathogen surfaces. Noteworthy, the immune system can also detect altered molecules on malignant cancer cells but, in an opposite direction, it has also been suggested to favor carcinogenesis when a deregulated activation of immune response and subsequent prolonged inflammation is triggered (14, 15).

The very first line of defense against pathogens is a fast activation of the components of the innate immune system. In addition to immune cells such as macrophages, dendritic cells (DCs) and natural killer (NK) cells described since the beginning as components of the

innate immunity, the past decade has provided increasing evidence that also epithelial cells possess an autonomous defense system, which defines the first frontline of defense. This ancient arm of the immune system uses a limited number of cell-bound receptors or soluble molecules to recognize a broad spectrum of microorganisms via common and highly conserved microbial structures, which are referred to as pathogen-associated molecular patterns (PAMPs) (16). Arrays of carbohydrates, lipids, and peptides that are components of microorganism surfaces as well as bacterial and viral RNA and DNA are ligands for membrane-bound pattern recognition receptors or secreted pattern recognition molecules (hereafter collectively referred to as PRMs; Table 1, Figure 1). In general, pattern recognition can be simplified to two modes of interaction: recognition of carbohydrates via lectins and recognition of poly-anionic patterns by non-lectin proteins.

Table 1 Pattern recognition molecules and their cognate ligands.

PRM	Ligands
C-type lectins	
MBL	Mannose, glucose, fucose
ficolins	Acetylated ligands
SP-A, SP-D	Mannose, glucose, fucose
DC-SIGN	Mannose, glucose, fucose
mannose receptor	Mannan, high-mannose
Dectin-1	β -1,3 glucans
Dectin-2	High-mannose
Galectins	
galectins 1-15	β -Gal, lipophosphoglycans (LPG), LacNAc
Siglecs	
siglecs 1-13	Sialic acid-containing glycans
TLRs	
TLR3	dsRNA
TLR4	LPS
TLR7	ssRNA, guanine analogs
TLR9	CpG DNA
Defensins	
α/β defensins	Negatively charged phospholipids
Scavenger receptors	
SRA	LPS, LTA, CpG DNA, proteins
MARCO	LPS, proteins
DMBT1	poly-phosphorylated and poly-sulfated ligands
Properdin	DNA, LPS, heparan sulfate
RLRs	
RIG-I	dsRNA, 5' triphosphate dsRNA
MDA5	dsRNA
LGP2	RNA
NRLs	
NOD-1	d-Glutamyl-meso-diaminopimelic acid (iE-DAP)
NOD-2	muramyl dipeptide (MDP)

Figure 1 Schematic overview of pattern recognition molecules and their cognate ligands.

The different examples of PRMs are depicted with their subcellular localization. PRMs can be secreted, cell surface-associated, cytoplasmic or endosomal. They can largely be grouped into PRMs that recognize carbohydrate structures (lectins) and that recognize poly-anionic ligands. According to their location, they act at different stages of defense, i.e., outside the cells to scavenge pathogens and to prevent infection, at the cell surface to trigger innate immune responses against extracellular pathogens, or inside the cells to signal invading pathogens. CRD: carbohydrate recognition domain; LPS: lipopolysaccharide; LTA: lipoteichoic acid; dsDNA: double-stranded DNA; dsRNA: double-stranded RNA; ssRNA: single-stranded RNA.

Carbohydrate-based pattern recognition via lectins

Lectins are soluble or transmembrane carbohydrate-binding proteins characterized by multiple binding domains. Many lectins are organized as oligomers and different members of the family are characterized by a different number and spatial arrangement of the carbohydrate recognition domains (CRDs) (17, 18). The immune system is equipped with lectins able to efficiently detect patterns of pathogen-associated carbohydrates such as N-acetylglucosamine, N-acetylmannosamine, mannan, β -glucans, and sialic acid-containing glycans and to stimulate an appropriate immune response (19, 20). The arrays of oligosaccharides on the surfaces of bacteria, fungi, and viruses can be recognized as non-self because of specific carbohydrate compositions

and their specific spatial arrangement. Indeed, the specificity of PAMP recognition relies on the spatial organization of the CRDs, the high-avidity binding, and the density of the ligands on the pathogen surface (17, 20).

Lectins that act as PRMs include C-type lectins, galectins, and siglecs (20–23). C-type lectins that act as PRMs are both soluble and membrane-bound proteins that recognize carbohydrate moieties in a Ca^{2+} -dependent fashion and upon ligand binding can trigger pro-inflammatory responses, activation of the complement system, macrophage-mediated phagocytosis, and priming and/or modulation of the adaptive immune response. Examples for this class of membrane-associated PRMs are the mannose receptor, DC-SIGN, and Dectin-1 (24). The pulmonary surfactant proteins SP-A and SP-D are soluble C-type lectins involved in pathogen opsonization via recognition of carbohydrates associated with lipopolysaccharide (LPS) and lipoteichoic acid (LTA)

on bacteria, lipoarabinomannan on mycobacteria, and glycoproteins on fungi and viruses (25). Besides recognition of PAMPs, the recognition of arrays of carbohydrates mediated by lectins constitutes a broadly used interaction system. For example, selectins, which constitute another class of C-type lectins, are used by circulating leukocytes in the blood vessels for the initial steps of extravasation to reach the inflammation site, and the lectin-carbohydrate interaction is also used by pathogens to bind to target cells (e.g., the Influenza virus hemagglutinin) (26).

Lectins such as galectin-3 and SP-D are also secreted to the mucus layer of mucosal epithelia, which commonly represents the first site of pathogen contact (27). This is part of an autonomous defense system regulated at the level of the lining epithelial cells. On the other hand, the mucus layer is rich in glycans, which are targets of bacterial lectins for mucoadhesion (27). Accordingly, exploitation of lectin-carbohydrate interactions via natural or synthetic mimics has emerged as field of intense research in nanomedicine for the design of bioadhesive drug delivery systems, as has been reviewed elsewhere (28, 29).

Pattern recognition-based activation of the complement cascade

Activation of the complement system is the basis for severe adverse side effects that can be caused by nanotherapeutics (30). Although such reactions are rather occurring upon intravenous administration, it is worth considering that molecular pattern recognition likely plays an important role in triggering complement activation. Moreover, activation of the complement cascade via pattern recognition is not limited to the circulation system. The complement system is the main humoral component of the innate immunity and it is composed of about 35 soluble and membrane-bound proteins. The complement system can be activated by different molecular patterns via three different mechanisms: the classical, the lectin, and the alternative pathway (Figure 2). All pathways converge in the activation of a C3 convertase, a serine protease that is the initial effector for the release of activated components of the complement, which results in the formation of pro-inflammatory mediators, opsonins and lysis of targeted cells (31, 32).

The classical pathway is activated by the interaction between the soluble molecule C1q and antibodies (IgM and IgG) bound to the surfaces of microorganisms (33, 34). The lectin pathway is activated by carbohydrate-interacting PRMs. The sensor molecules for the lectin pathway are the mannan-binding lectin (MBL) and ficolins, which are

Figure 2 Schematic representation of the main branches of the complement cascade.

Three major pathways for activation of the complement cascade are characterized, of which two include pattern recognition mechanisms. The point of convergence is the C3-activation via C3-convertases with the formation of the membrane attack complex as endpoint, resulting in permeabilization of the pathogens' surfaces. Left: the classical pathway is activated via C1q recognition of antibodies bound to pathogen antigens. This results in formation of the C1 complex (C1). Center: the lectin pathway is based on the recognition of non-self carbohydrate patterns on the surfaces of the pathogens, which in turn results in recruitment and activation of mannan-binding lectin-associated serine proteases (MASPs). Both the classical and the lectin pathway result in the formation of the C4bC2a C3-convertase. Right: the alternative pathway comprises a steady-state surveillance mechanism. In the bloodstream C3 is hydrolyzed at low but constant rate promoting the formation of the C3 convertase C₃_{H₂O}Bb. The subsequent binding of C3b on pathogen surfaces triggers the recruitment of Factor B (fB) and properdin (P), resulting in the assembly and stabilization of the C3bBbP complex that acts as C3-convertase. Via this mode, properdin-mediated pattern recognition confers positive regulation to the complement cascade. Negative regulators (not shown) produced by intact host cells counteract the activation of the complement cascade against self-structures. The two different forms of the C3-convertase, i.e., C4bC2a and C3bBb, subsequently catalyze cleavage of C3 and the formation of the membrane attack complex further downstream.

C-type lectins (Table 1, Figure 1) that recognize patterns of pathogens-associated carbohydrate or acetyl group-containing ligands, respectively (e.g., mannose, glucose, fucose, GlcNAc, GluNAc) (35).

The alternative pathway (AP) represents a constitutive monitoring for detection of microorganisms and the recognition of PAMPs is involved only in subsequent steps for the stabilization and amplification of the activated complement cascade. The C3 effector of the complement system is constitutively activated via spontaneous, but not efficient, hydrolysis, which promotes the formation of an initial C3 convertase ($C3_{H_2O}$ Bb) that in turn generates C3b fragments, which can covalently bind nearby surfaces. The interaction of C3b with PAMPs triggers the formation of the AP C3 convertase (Figure 2). To avoid the assembly of the AP C3 convertase on non-pathogenic targets and consequent risk of autoimmune responses, proper negative and positive control cues have to be in place. Molecular pattern recognition provides regulatory elements for the stabilization of the immune response via the AP. For example, only upon recognition of PAMPs the rapid decay of the AP C3 convertase C3bBb is reduced by the plasma protein properdin (CFP, PFC; Figure 2). Recently it has been shown that properdin, besides stabilizing C3bBb, can mediate recruitment of C3b to the pathogen surface and trigger the formation of the C3 convertase complex. Properdin binds glycosaminoglycans (GAGs) likely by interacting with the sulfate moieties and it recognizes and binds to early apoptotic T cells, late apoptotic and necrotic T cells, *Neisseria gonorrhoeae*, and zymosan (36–38). In general, properdin seems to be a PRM for poly-anionic structures, because it also interacts with DNA and LPS (37, 39). The features of properdin-ligand interactions reveal some striking similarities to DMBT1 as discussed further below.

Collectively, these mechanisms suggest that understanding the rules underlying molecular pattern recognition could aid the rational design of nanotherapeutics, which consequently will lead to a decreased risk of inducing severe reactions due to complement activation.

Toll-, NOD-, and RIG-I-like receptor-mediated pattern recognition

A broad spectrum of PAMPs is recognized by the Toll-like receptor (TLR) family, which constitutes a powerful functional bridge between the innate and adaptive immune response (10, 40). TLRs are expressed by immune cells such as dendritic cells (DCs), neutrophils, macrophages, natural killer (NK) cells, mast cells, B and T cells, but also by epithelial cells, which need to provide the first line of defense against pathogens. TLRs can be divided into two groups based on their cellular localization, which matches the topological sites where the recognized PAMPs can be

found (Table 1, Figure 1). TLRs associated to the cellular membrane are activated by conserved constituents of bacterial and fungal walls or bacteria-specific structures (e.g., LPS, LTA, peptidoglycan, flagellin), while intracellular TLRs are localized in the endosomal compartment, where they can bind to foreign DNA and both single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) (41). Notably, the localization of TLRs in the endosomal compartment minimizes the probability to interact with endogenous nucleic acids, while it enhances the likelihood to detect bacterial and viral nucleic acids that are engulfed and targeted to the lysosomes by phagocytosis, autophagy, or macropinocytosis (41).

NOD-like receptors (NLRs) and the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) represent two other well-characterized families of cytosolic PRMs. NOD-1 (nucleotide-binding oligomerization domain-1) and NOD-2 are members of the NLRs and recognize intracellular dipeptides derived from bacterial peptidoglycan (42). RIG-I and MDA5 are cytoplasmic RNA helicases of the RLR family that recognize dsRNA and trigger antiviral immune responses (43).

Thus, in addition to properdin, also RLRs and many TLRs show specificity for poly-anionic ligands.

Defensin- and scavenger receptor-based pattern recognition

Activated leukocytes but also epithelial cells, especially within mucosal epithelia that provide the first line of defense in response to pathogenic stimuli, secrete antimicrobial peptides called defensins (44). Defensins are cationic peptides synthesized as pro-peptides and mediate pathogen neutralization by binding the negatively charged surface of bacteria and yeast cells via electrostatic interactions. Elimination of the microorganisms is achieved by permeabilization of the target membrane (45–47). Accordingly, positively charged arginine residues were found to be critical for defensin activity (48). This is in line with defensins representing another group of PRMs that recognize poly-anionic patterns.

Scavenger receptors, first discovered as a class of receptors that mediate internalization of modified LDL by macrophages (49), are now known to constitute a family of structurally unrelated oligomeric glycoproteins that function as PRMs (50, 51). Despite the differences in domain compositions, scavenger receptors are characterized by highly conserved shape and charge distribution at the ligand binding surface, which is thought to be responsible

for their affinity for poly-anionic structures (52). Scavenger receptors, alone or in conjunction with co-receptors, mediate a broad range of functions and in immune cells are known to be involved in functions such as adhesion, endocytosis, and phagocytosis. Many members of the family act as PRMs and bind to Gram-positive and Gram-negative bacteria. SR-AI and MARCO were reported to recognize unmethylated bacterial CpG DNA, Lipid A – the core element of the endotoxin LPS – and LTA (53). Sequence comparisons identified a particular protein domain, referred to as scavenger receptor cysteine-rich (SRCR) domain, that is common to many but not all scavenger receptors and further occurring in proteins that had not been implicated in pattern recognition before (54). Back in 1997, the human Deleted in Malignant Brain Tumors 1 (DMBT1; Figure 3) was identified as a new member of the SRCR superfamily (55).

The pattern recognition molecule DMBT1

The gene *DMBT1* was first cloned as tumor suppressor gene in brain tumors, but soon afterwards was demonstrated to

also code for glycoprotein-340 (gp-340), thought to function as receptor for SP-D opsonized bacteria, and salivary agglutinin (SAG), a protein present in saliva with binding and agglutination activity for cariogenic and numerous other pathogenic bacteria, including *Helicobacter pylori* (55–60). These studies established that the main, but not exclusive, sites of DMBT1 expression are epithelial cells at mucosal and non-mucosal epithelia and associated glands. However, in certain epithelia and in tumors DMBT1 is frequently found in the extracellular matrix (ECM).

Orthologs of DMBT1 are mouse *crp-ductin* (now referred to as *Dmbt1*), formerly described as protein with specific expression in the crypts of the intestinal tract (61), and rat *ebnerin* (now referred to as *dmbt1*), which has been linked to activation of stem/progenitor cells upon liver damage (62). Several lines of evidence, including studies on *hensin*, the rabbit ortholog of human DMBT1, strongly indicate that that DMBT1 as ECM protein may also participate in processes related to cell polarization and differentiation (58, 63–74). General support for functions related to regenerative processes was lent by concerted up-regulation of DMBT1 together with trefoil factors (TFFs), which function in wound healing, and by studies demonstrating

Figure 3 DMBT1 structure and ligand recognition.

The top line provides a schematical presentation of the domain organization of a DMBT1 monomer with the symbols depicted in the legend. The second line zooms into the amino acid sequence of one of the 13 tandem repeated SRCR domains of DMBT1. The sequence GRVEVLYRGSW was shown to represent the minimal peptide that recognizes bacteria. This sequence binds to molecules with a poly-anionic structure, such as heparan sulfate (HS), chondroitin sulfate (C), dextran sulfate sodium (DSS), and carrageenan (poly-sulfated molecules), LTA of Gram-positive bacteria, LPS of Gram-negative bacteria, DNA, dNTPs (poly-phosphorylated molecules) via recognition of the structurally related phosphate and sulfate groups. Amino acid residues identified as critical for ligand interactions are depicted in red. SID: SRCR-interpersed domain.

a pro-angiogenic role for DMBT1 in vitro and in vivo (75, 76). Moreover, the work of Müller et al. established that endothelia represent a further site of DMBT1 expression, that human DMBT1 in the ECM interacts with the lectin galectin-3, and that DMBT1 modulates Notch signaling, a key pathway in stem cells (76).

Initially, the broad spectrum of DMBT1 functions has been sought to be explained by interactions with various protein ligands. *DMBT1* codes for a secreted protein and its core region is composed of SRCR domains (Figure 3), which previously had been exclusively connected to the mediation of various protein-protein interactions. After an N-terminal signal peptide, up to 13 SRCR domains follow (55). The number of these domains can vary based on interindividual genetic polymorphisms (77, 78). Towards the carboxy-terminal end a fourteenth SRCR domain flanked by two complement C1r/C1s-Uegf-Bmp1 (CUB) domains and a final zona pellucida (ZP) domain follow (55, 77, 78). The ZP domain is known to mediate oligomerization in other proteins (79) and it is likely to cause the formation of high molecular weight oligomers of secreted DMBT1 (56, 80).

A number of host and pathogen proteins were identified as interaction partners of DMBT1. DMBT1 interacts with viruses such as influenza A virus (81) and HIV-1 (82), the latter through its interaction with viral glycoprotein gp120 (83, 84). A baseline protective function of DMBT1 is suggested by studies showing that it prevents bacterial invasion in vitro, intestinal inflammation in vivo, and that *DMBT1* polymorphisms giving rise to a shortened protein variant may predispose to Crohn's disease (85–88). Moreover, DMBT1 interacts with several components of the innate immunity, which include SP-D, SP-A, IgA, lactoferrin, mucin 5B (MUC5B), and C1q (56, 60, 81, 89–97), and the lectin galectin-3 and TFF2 as accessory proteins in the mediation of differentiation and regeneration (76, 98–101). Additionally, DMBT1 stimulates recruitment of alveolar macrophages and interacts also with the bacterial proteins antigen I/II, SspA and SspB (102–105). Despite the abundance of evidence for DMBT1-mediated recognition of numerous proteins, the interaction partners lack common features, suggesting that a more generalized binding mechanism is alternatively or at least additively active.

DMBT1-mediated pattern recognition

Via a systematic approach, the bacterial binding activity of DMBT1 could first be mapped to the protein region

containing the highly homologous 13 SRCR domains and subsequently it was refined to a 16-amino acid motif (QGRVEVLYRGSWGTVVC), which is present with slight variations in each of these SRCR domains (106). The minimal sequence for bacterial binding was finally mapped to an 11-amino acid motif (GRVEVLYRGSW) referred to as DMBT1 pathogen-binding site 1 (DMBT1pbs1) (107). Remarkably, this 11-amino acid motif retains not only the broad bacterial binding activity but also the property to aggregate bacteria upon binding. An alanine substitution scan indicated that the amino acid sequence VEVLXXXXW is critical for bacterial binding (107). Binding and competitive aggregation assays demonstrated that DMBT1 has affinity for poly-sulfated ligands. Using synthetic peptides containing the DMBT1pbs1 sequence, DMBT1 purified from saliva, and human recombinant DMBT1 it was shown that molecules such as heparin sulfate, chondroitin sulfate, dextran sulfate sodium (DSS), and carrageenan are able to bind to DMBT1 and to inhibit bacterial binding and aggregation via the synthetic peptides and DMBT1. By contrast, corresponding agents lacking sulfate groups did not exert inhibitory effects (108, 109). Likewise, poly-phosphorylated ligands such as LTA of Gram-positive bacteria and LPS of Gram-negative bacteria, DNA, but also dNTPs and phosphate anions in solution exerted inhibitory effects on DMBT1 binding properties. Binding studies with bacteria synthesizing different LPS variants confirmed that negatively charged phosphate groups are the structure recognized by DMBT1pbs1 (109). Taken together, these data indicated that DMBT1 functions as PRM for poly-anionic ligands, in particular for those that expose phosphate and the structurally related sulfate groups.

Functions of DMBT1 at biological barriers

DMBT1 is expressed and secreted from glands and epithelial cells of the respiratory and gastrointestinal tract and is an effector of the innate immune system (58, 59, 69, 110, 111), which is in accordance with its predominant expression at sites immediately exposed to infectious threats. As detailed above, DMBT1 has been shown to interact with components of the immune system, as well as with various ligands at viral and bacterial surfaces (97, 112). At mucosal surfaces DMBT1 interacts also with lactoferrin, MUC5B, TFF2 and TFF3, which are endogenous ligands and components of mucosal secretions (93, 95, 101, 113, 114). Lactoferrin is known to confer direct cytostatic effects to bacteria via iron sequestration (115). Mucins

in general play a role in neutralization and clearance of microorganisms on epithelial surfaces through their entrapment in the viscous mucus layer (116). However, to date the role of the interactions of these various proteins with DMBT1 is not yet fully understood. A conceptual possibility is that endogenous ligands, like mucins and other innate immune components, compete with PAMPs for the respective binding sites within DMBT1. Accordingly, in the absence of challenge by pathogen ligands DMBT1 could be mucoadhesive via interaction with sulfated carbohydrate side chains of mucins and/or via interaction with other proteins in the mucus layer. Poly-anionic surfaces of pathogens could compete for these interactions, resulting in major re-assembly in the mucus layer, DMBT1-mediated binding and aggregation of pathogens, release of the formerly bound defense factors, and, potentially, to signal the perturbation of the mucus layer integrity to the underlying cells, which may elicit downstream responses, including the up-regulation of innate defense components. As initial support of this hypothesis, it has been shown that bovine lactoferrin competes with *S. mutans* for binding to DMBT1 and that the two ligands have the same binding site, locating in DMBT1pbs1 (93, 96). However, the mechanisms might be more sophisticated, because it has also been observed that DMBT1 adhesion to hydroxyapatite, which resembles the tooth surface, may change its binding to bacteria and promote their adhesion rather than their agglutination (117–120). This would indicate that some pathogens have evolved mechanisms that allow them to utilize molecular pattern recognition at their advantage. Accordingly, it has remained an open question as to whether DMBT1 plays a role as pro- or anti-carcinogenic factor. A similar scenario seems to apply for the consequences of DMBT1 interaction with HIV-1. Already in the late 1980s it was observed that saliva can inhibit HIV infection and salivary agglutinin (DMBT1) was later on identified as a possible mediator of this protective effect (121, 122). DMBT1 has been shown to interact with HIV type 1 virus by binding the envelope glycoprotein gp120, which is the viral protein responsible for binding to the CD4 receptor on T cells and dendritic cells and for initiating viral entry. Initial studies suggested that DMBT1 interaction with HIV-1 suppresses infection of epithelial cells (123). Of particular note, DMBT1-HIV-1 interaction may be for the major part based on a protein-protein interaction, involving the SRCR1 domain of DMBT1 and the N-terminal stem of the V3 loop of gp120 (82, 83, 123, 124). Thus, the site in gp120 recognized by DMBT1 overlaps with the gp120 binding site for the virus co-receptor CCR5 (83, 125). Consecutive studies revealed that DMBT1 promoted HIV-infection of PHA-stimulated peripheral blood mononuclear

cells via epithelial cells derived from the human genital tract (126). Cannon and colleagues showed that epithelial cells of the genital tract express DMBT1, which is cell surface-associated, resulting in enhanced HIV-1 binding (60, 126). Moreover, using monocyte-derived macrophages shown to have surface-associated DMBT1, it was demonstrated that HIV-1 binding and fusion with target cells can be promoted (84). It is thought that DMBT1 might promote HIV-1 infection by increasing the local concentration of virus on the cell surface and facilitate transcytosis of the viral particles via the mucosal epithelium of the female genital tract (127). This supports the view that DMBT1 may represent a double-edged sword, which has important defense functions, but is actively engaged by some pathogens for productive infection.

While the afore-mentioned data established a role for DMBT1 at the luminal side of epithelia in innate defense, the protein seems to be involved in other remarkable functions when secreted to the ECM. One of these functions in the ECM is an involvement in triggering cell differentiation (58, 61, 72, 73). Much of this knowledge originated from the characterization of the rabbit ortholog of DMBT1 (hensin), which is involved in establishing cell polarity in renal intercalated cells (100, 128). Hensin was identified as the ECM-associated factor responsible for the conversion of β -intercalated cells to α -intercalated cells. This phenotype conversion involves relocation of H^+ -ATPase and Cl^- : HCO_3^- exchanger to the apical and basal membrane, respectively, change of cell shape, acquisition of apical microvilli through actin reorganization and induction of villin and cytokeratin 19 expression, which collectively are signs of terminal epithelial differentiation (74). This process includes polymerization of rabbit DMBT1 in the ECM to fibrillar structures. Activation of $\alpha v \beta 1$ integrin at the basolateral membrane triggers deposition of rabbit DMBT1 in the ECM, concurrent secretion of galectin-3, which functions as interaction partner, and action of the cis-trans prolyl isomerase cypA, which mediates DMBT1 polymerization (70, 71, 129–131). Possibly, interaction of DMBT1 with integrin $\alpha 6$ plays a role in downstream signaling processes (129).

Recently, DMBT1 secretion to the ECM has also been reported for endothelial cells. At this site, DMBT1 has been shown in vivo to have a functional role in promoting angiogenesis and vascular repair (76). In vitro experiments showed that human recombinant DMBT1 (hrDMBT1) binds to the angiogenic growth factors VEGF and EGF and to the Notch ligand Dll4. DMBT1 involvement in vascular development through a crosstalk with the Notch signaling cascade was also shown in vivo (76). These findings supported earlier links between DMBT1 and regenerative

processes and signaling pathways relevant for the regulation of stem/progenitor cells in rat model systems (132). An interesting question in this context is as to whether DMBT1 may interact with sulfated glycosaminoglycans in the ECM, which would be supported by its capability to bind heparan sulfate and chondroitin sulfate, and whether similar competitive mechanisms as hypothesized for the mucus layer would contribute to signaling.

Utilization of DMBT1-mediated pattern recognition at biological barriers

Collectively, there are indications for DMBT1 participating in the defense against invading bacteria and viruses at the first frontline of defense, suppressing inflammation and, potentially, regulating the transition to regeneration as ECM protein. In addition to the interaction with various host and pathogen proteins, DMBT1's role in molecular pattern recognition may underlie its various functions. As mentioned above, after luminal secretion by epithelial cells at mucosal barriers, DMBT1 may associate with mucins and other components via pattern recognition of sulfated glycans. To this end, an interaction with MUC5B has been confirmed (95), although the exact binding mode remains to be elucidated. Conceptually, however, understanding these mechanisms could provide novel starting points for

mucoadhesive nanomedicines, which either mimic DMBT1 binding to the mucus or utilize DMBT1 as adhesive component (Figure 4A). In a certain sense, this would imitate the mode by which certain pathogens abuse innate defense components for the purpose of productive infection. In carries research, it is still an open question whether DMBT1 (SAG) is protective or promoting bacterial colonization. On one hand soluble DMBT1 binds and aggregates cariogenic bacteria, thus hindering their adhesion (57). On the other hand, it was shown that DMBT1 associated to hydroxyapatite, which mimics the tooth surface, facilitating adhesion of bacteria (118–120). Accordingly, it may be critical for the exploitation of DMBT1 properties as PRM in mucoadhesive nanomedicines to understand the differences between these two opposite scenarios and how to translate DMBT1 interactions to a rational design. That said, however, the exploitation of DMBT1 in nanomedicine needs to consider as to whether such enterprise would compromise a delicate immune balance and/or induce severe inflammation. Via pattern recognition-mediated binding of LPS on the bacterial surface, DMBT1 not only prevented bacterial infection and invasion, but also suppressed activation of downstream inflammatory pathways by inhibiting LPS interaction with the epithelial cell surface pattern recognition receptor TLR4 (86). The fact that *Dmbt1* knockout mice were viable and without severe symptoms of disease (85) could indicate that, in the absence of pathogenic challenges, no severe side effects of using endogenous DMBT1 as a drug-carrier or drug-adhesive would be expected. However, because the knockout was constitutive and not

Figure 4 Potential applications of DMBT1-mediated pattern recognition mechanisms in nanomedical drug delivery.

(A) Two general possibilities emerge from the mucus association of DMBT1. Sulfated (or phosphorylated) nanocarriers could be used to mediate interaction with endogenous DMBT1 in the mucus (top) or linking drugs to synthetic DMBT1 peptides (bottom) could be exploited to achieve mucus adhesion. These strategies, however, need to take into consideration to not elicit danger signals in the epithelial cells below the mucus layer, which can occur upon perturbation of mucus integrity. (B) A Trojan-horse-like strategy would exploit the mechanism that enables HIV-1 to sequester DMBT1 for transcytosis through the epithelial cell layer, which might enable to deliver drugs to deeper cell layers via transcytosis. (C) The ability of DMBT1 and synthetic peptides to interact with nucleic acids could be useful for the assembly of nanocomplexes, which may protect nucleic acid-based drugs from degradation and/or assist their delivery to target cells.

conditional, adaptive processes may have compensated for the absence of the pattern recognition activity of Dmbt1 in these mice, so that this interpretation may have some flaws. Finally, utilization of DMBT1 for mucoadhesive drug delivery may be subjected to the general limitation that the mucus is subjected to turnover, so that delivery strategies need to go beyond the simple entrapment of drugs in the mucus layer, e.g., by ascertaining delivery to or transmission by the underlying epithelial cells.

Another interesting option is offered by studies on the role of DMBT1 in HIV infection in the genital tract. Here, the role of pattern recognition is uncertain because so far there is only evidence for a protein-protein interaction between DMBT1 and the viral protein gp120 (82, 83, 123, 124). However, while DMBT1 inhibits infection of the lining epithelium, it promotes transcytosis of the virus, which consecutively infects target cells in deeper tissue layers (84, 126, 127). This indicates that under certain circumstances host DMBT1 might be used as Trojan horse for trans-epithelial drug delivery (Figure 4B). Opposing effects could be the activation of the complement cascade via DMBT1, which so far has been suggested by studies *in vitro* (94, 133, 134), and potential perturbation of ECM signaling processes in which DMBT1 may participate, so that it might be relevant to investigate where the different functionalities in the protein reside. Moreover, the Trojan-horse-like mechanism presumably is complex as it is anticipated to include multiple steps, i.e., mucoadhesion and -penetration, cytoadhesion to and transcytosis by epithelial cells, and cytoadhesion to and entry into target cells. To date, these mechanisms and the components playing a role are poorly understood.

Given the interaction of DMBT1 with poly-sulfated and poly-phosphorylated molecules, another interesting question is whether synthetic DMBT1 peptides could be used to formulate nanoparticles for delivery of poly-anionic molecules such as nucleic acids (Figure 4C). Initial studies pointed to a direct interaction with DNA of both the protein and synthetic peptides (109, 135). However, DMBT1 and synthetic peptides exerted an inhibitory effect on cationic lipid-mediated DNA transfection of cells by interfering with the interaction between DNA and the cationic lipids (135). Although the possibility to use synthetic DMBT1 peptides for nucleic acid transfer has not yet directly been addressed, it seems likely that modifications of such peptides would be necessary to achieve efficient cell delivery. Moreover, variation of the peptide sequences could be advantageous for modulating size and geometry of such nanoparticles. On the other hand, the inherent ability of DMBT1 peptides to bind to nucleic acids could aid the assembly of nano-complexes. Finally, it remains to

be elucidated if nucleic acids in such complexes display increased resistance to nucleases, which would represent another beneficial property.

Conclusions

Molecular pattern recognition can be conceptually divided into the recognition of carbohydrate residues via lectins and the recognition of poly-anionic ligands via diverse membrane-bound or secreted PRMs. Traditionally, PRMs act in pathogen defense and regulation of inflammation via various mechanisms, including activation of the complement cascade at epithelial barriers. However, for some PRMs, like DMBT1, initial evidences have accumulated suggesting the existence of additional functions in the transition from the defense/inflammatory response to regeneration.

The pattern recognition activity of DMBT1 has been mapped in detail to an 11-amino acid motif present in each of its 13 amino-terminal SRCR domains and the ligands recognized by this motif comprise agents with arrayed phosphate groups as well as with structurally similar sulfate groups. The understanding of such molecular pattern recognition mechanisms within the human organism is of general relevance for nanomedicine, as it is commonly desirable to avoid opsonization and/or activation of the complement cascade. Elucidation of the mechanisms of molecular pattern recognition will provide important *a priori* considerations for nanoparticle design. Although DMBT1 seems to participate in a complex variety of processes, its mode of action and its exploitation by some pathogens to achieve a productive infection raise some interesting opportunities for nanomedical applications, which deserve further investigation. It is conceivable to use the mechanisms underlying DMBT1 action or endogenous DMBT1 itself for the design of mucoadhesive nanodrugs or of Trojan horse-like strategies for trans-epithelial delivery. Synthetic peptides have been derived from DMBT1, which retain pattern recognition activity for poly-sulfated and poly-phosphorylated ligands, including nucleic acids, and the ability to aggregate ligands. This raises the interesting question in how far these properties can be utilized to assemble nucleic acid-peptide nano-complexes and whether this can be exploited to modulate the pharmacological properties of nucleic acids and/or for nucleic acid delivery to target cells.

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