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Seeing strangers or announcing "danger": Galectin-3 in two models of innate immunity

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Recent investigations on the molecular mechanisms by which our immune system recognizes infections and initiates defense against those infections have led to the proposition of two models explaining the way our innate immunity system functions; the self-nonself model and the Danger model. In this review, the roles of galectin-3 in innate immunity against infections—host-pathogen interactions—will be discussed. We will shed light on the potential contribution of a β-galactoside binding mammalian lectin, galectin-3 as a molecule implicated in innate immunity from the angle of both the self-nonself model and the Danger model.
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Abbreviations: **CRD, carbohydrate recognition domain; IL, interleukin; bFGF, basic fibroblast growth factor; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; TLR, Toll-like receptor; LPS, lipopolysaccharide.**

Innate immune system and galectin-3

Immunity—our protection system against infectious diseases—is composed of two broad components: innate immunity and adaptive immunity. The innate immune system protects the body from infections through mechanisms that are not specific to the particular antigens of a pathogen, whereas the adaptive immune system utilizes its machinery with a high degree of specificity towards the antigens presented by pathogens and is endowed with the ability to specifically 'remember' these pathogens in subsequent infections. In general, the innate immune response clears most invading pathogens within a few days. That the adaptive immune response to the invading pathogen is not induced in the early stages of infection (within approximately one week) suggests that the innate immune system can efficiently get rid of the pathogenic entity without the aid of the adaptive immunity in most cases of infection.

Attempts to decipher how the innate immune system recognizes the invasion of pathogens in the absence of adaptive immunity has led to the elaboration of two models; the "self-nonself" model proposed by Janeway [1,2] and the "Danger" model proposed by Matzinger [3,4]. Those models are not mutually exclusive, but there are some definite differences between them. The self-nonself model emphasises the recognition by innate immunity of "nonself", or the degree of 'foreignness' of the pathogenic entity [2,5]. In contrast, in the Danger model, the most critical aspect of the recognition of invading pathogen is the determination of the foreign entity capacity to stress or injure tissues [4,6–8]. In this review, the potential roles played by galectin-3 in innate immunity will be discussed from those two models, the self-nonself model and the Danger model, points of view. In this regard, the biochemical features of galectin-3 will be first introduced, then the immunomodulative activity of galectin-3 will be discussed as it would fit in the self-nonself model and the Danger model.

Characteristics of galectin-3

Galectin-3 (previously known as Mac-2 antigen, εBP, L-29 or CBP30/35) is a $\sim$30kDa soluble β-galactoside binding protein

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and belongs to the galectin family as defined by conserved peptide sequence elements involved in carbohydrate binding activity [9–14]. The number of galectin members in mammalians has increased rapidly and more than 10 galectins have been identified to date [11–19]. Certain galectins contain one carbohydrate recognition domain (CRD), and exist as dimers (for example, galectin-1, 2) (prototype), while other galectins, such as galectin-4, 8, and 9 contain two CRDs connected by a short linker region (tandem repeat) [20]. The divalent characteristics enable these galectins to cross-link their ligands expressed on cell surfaces. In contrast to the prototype and tandem repeat type galectins, galectin-3 is quite unique, as it usually exists as a monomer and is composed of only one *C*-terminal CRD combined with a regulatory domain containing multiple repeats of a sequence rich in glycine, proline, and tyrosine at the N-terminal end (Figure 1 insert box, chimera type) [11–14]. Upon binding to its glycoconjugate ligands at the cell surface, the conformation of galectin-3 appears to change in a concentration and time dependent manner, and galectin-3 begins to oligomerize through the self-assembly of the *N*-terminal regulatory domain of the galectin-3 molecules (Figure 1) [21–24]. The oligomerization results in the formation of a galectin-3 molecule that possesses multivalent CRD. This dynamism between monomer and oligomer as a part of regulation of "lectin activity"—activity to cross-link the ligands—is a distinctive feature of galectin-3.

Biochemical studies with purified galectin-3 have revealed that the reversibility of galectin-3 activity can be mediated by certain proteolytic enzymes, such as bacterial collagenase IV and the matrix metalloproteases-2 and 9 (Figure 1D) [23–26]. Those enzymes are able to cleave galectin-3, resulting in truncated galectin-3 lacking the N-terminal regulatory domain, which mediates the oligomerization (Figure 1D). As this truncated form of galectin-3 can still bind to its ligands, but can not exert most of its typical activities [11–16], truncated galectin-3 could be considered a 'dominant negative' form of galectin-3 [27]. Recent reports have indicated that phosphorylation of

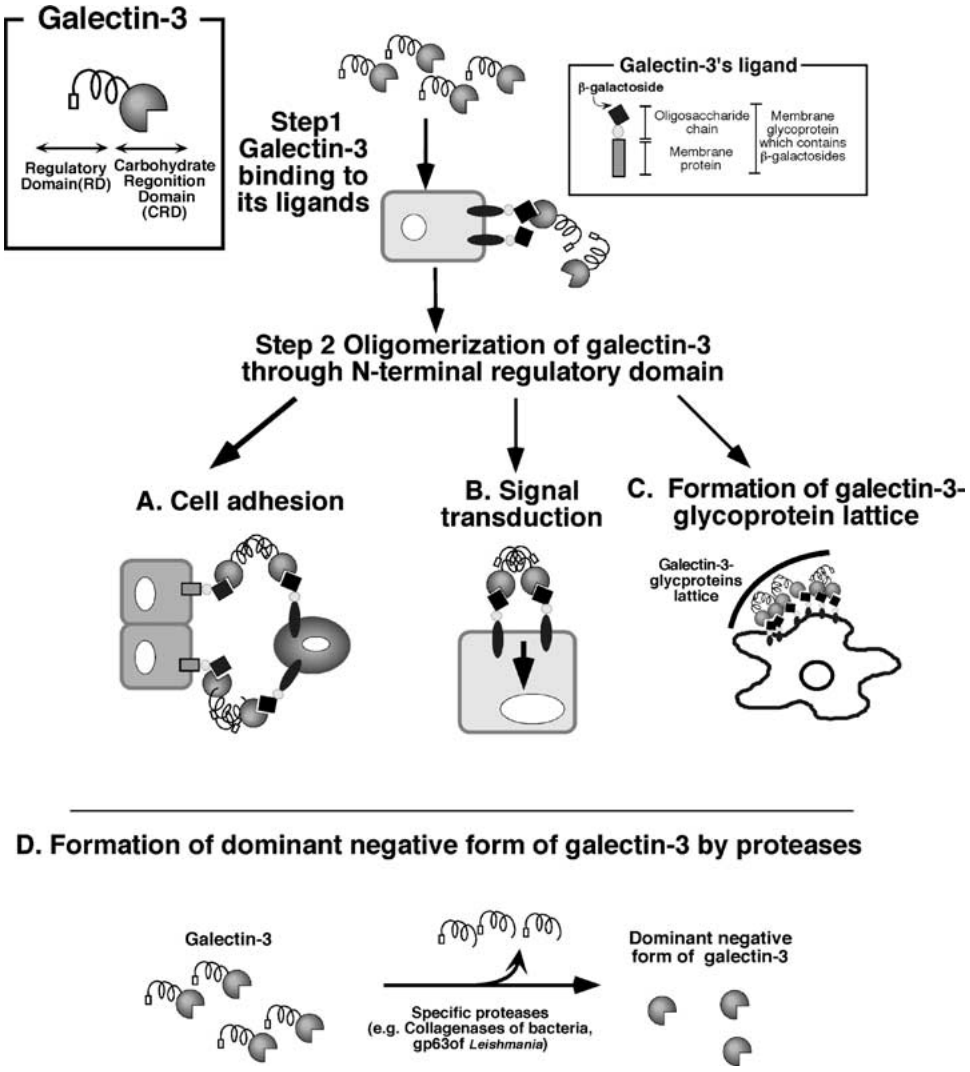

Figure 1. Oligomerization of galectin-3 results in cross-linking of receptors on cells.

the N-terminal regulatory domain of galectin-3 significantly reduces its affinity for the ligands. It has been speculated that the phosphorylation of the regulatory domain alters its ability to participate in multivalent interactions, which stabilize the interaction between galectin-3 and its ligands [28].

All members of the galectin family contain one or two CRD(s) and have affinities for many β-galactoside-containing glycoconjugates (except galectin-10), but specific β-galactoside glycan structures are necessary for tight binding [11–16]. It has been well established that galectin-3 has a strong preference for the polylactosamine structure (Galβ1-4/3GlcNAc)$_n$, which is often located at the peripherally exposed position (non-reducing terminus) of glycoconjugates. The affinity of galectin-3 for this polylactosamine increases in proportion to the number of lactosamine units present on the glycan chain [29,30]. Substitution of the non-reducing terminal galactose residue with blood group H (Fucα1-2), A (GalNAcα1-3(Fucα1-2)) or B (Galα1-3(Fucα1-2)) increases the affinity of the lactosamine repeats to galectin-3 [29,30]. Interestingly, galectin-1 does not show such a preference, suggesting that galectin-3 has a different carbohydrate binding pocket in its CRD [29–32]. Recent structure analyses of galectin-1 and -3 have also revealed that galectin-3 indeed has an extended carbohydrate binding pocket when compared to galectin-1 [33–36]. Thus, this extended binding pocket contributes to the binding specificity of galectin-3 for its specific ligands.

Subcellular localization of galectin-3

In eukaryotes, soluble extracellular proteins are generally secreted through the "classical" secretory pathway (endoplasmic reticulum-Golgi apparatus). Nascent polypeptides, which contain either signal sequences or potential transmembrane domains, are sorted to the secretory pathway, wherein glycoconjugates are attached to soluble secretory proteins or membrane proteins as they progress through the secretory apparatus. Although galectin-3 was first identified as a cell surface component on inflammatory macrophages, this lectin is not a conventional membrane constituent. Galectin-3 does not contain any of the sequences that are necessary to be sorted to the secretory pathway [11–16].[1] Instead, galectin-3 is synthesized and accumulated in the cytoplasm of various cells including phagocytic leukocytes (macrophages, monocytes, dendritic cells, mast cells, neutrophils),[2] epithelial cells, and keratinocytes [11–16]. Since it is known that galectin-3 is found in the extracellular space and at the cell surface, galectin molecules have to be exported from those cells [39]. As galectin-3's ligands are found either in the lumina of the secretory pathway or the extracellular surfaces, it is only possible for galectin-3 to exert many of its activities after it is extracellularly released.

There could be at least two ways for the cytosolic galectin-3 to be released: (1) release caused by cell necrosis (passive release) and (2) active secretion from live cells through a unique pathway called the "leaderless (alternative)" secretory pathway [39]. Passive release from damaged cells certainly is the easiest way for many cytoplasmic proteins to encounter their extracellular ligands. However, it has been also well established that significant amounts of intracellular galectin-3 or other cytoplasmic proteins, such as interleukin (IL)-1 and basic fibroblast growth factor (bFGF) are actively secreted from live cells through the "leaderless" secretory pathway without any major disruption of membrane integrity [40–46]. This active secretion of galectin-3 is tightly regulated in a differentiation-specific manner [39,47–51]. For example, peritoneal inflammatory macrophages exudated by thioglycollate injection secrete ~50% of their galectin-3 within 3 h, while immature or resident peritoneal macrophages or neutrophils[3] are unable to secrete galectin-3 and retain it cytoplasmically [48]. The tight regulation of galectin-3 secretion implies the strict control on the compartmentalisation of galectin-3 in the cytosol, away from its ligands, which are present on cell surface and in the Golgi apparatus. In fact, the importance of the segregation of cytosolic ligand from its receptor to regulate the activity has been demonstrated for the other cytoplamic protein, bFGF [52]. bFGF is a potent growth factor and angiogenic factor. Like galectin-3, bFGF does not contain any sequence for the secretory pathway but is secreted through the "leaderless" pathway. Expression of bFGF variant created by the fusion of bFGF with a secretory-signal sequence results in autocrine transformation of the cells, suggesting that the interaction of this bFGF variant with FGF receptor inside cells induces the transformation. We recently proposed that extracellular release of galectin-3 triggers a unique neutrophil emigration into the lungs during infection with *Streptococcus pneumoniae* [38]. Together, the regulation of the release of intracellular galectin-3 could control various galectin-3 activities, especially its immunomodulative activities (see the section on Galectin-3 and the Danger model).

Once secreted, galectin-3 binds to β-galactoside-containing glycoconjugates on cell surfaces (Figure 1, step 1). Upon binding to their glycoprotein ligands at the cell surface, galectin-3 begins to oligomerize (Figure 1, Step 2, oligomerization). This oligomerization leads to the cross-linking of surface glycoproteins, thus inducing various activities such as cell-cell adhesion (Figure 1A), signal transduction through receptor clustering (Figure 1B) or the formation of a multivalent galectin-3-glycoprotein lattice on the cell surface (Figure 1C) [11–16,53]. Table 1 lists some of the proposed roles played by galectin-3 in immune responses.

Galectin-3 and the model of self-nonself: Pathogen recognition by galectin-3

According to the self-nonself model proposed by Janeway, the innate immune system uses two distinct strategies for recognizing invading pathogens: the recognition of nonself entity and the recognition of missing self [5]. To detect nonself, innate immunity possess the machinery to recognize pathogen-associated molecular patterns (PAMPs), which are distributed broadly among pathogens but are not produced by the host

Table 1. Proposed functions of galectin-3 in immune responses

Function	*Reference*
Cell-cell adhesion or pathogen-cell interaction (Figure 1A)	
Promotes neutrophil adhesion to laminin	[55]
Mediates neutrophil adhesion to vascular endothelial cells	[38]
Promotes *P. aeuruginosa* to corneal epithelial cells	[56]
Signal transduction through receptor clustering (Figure 1B)	
Induces Ca^{2+} influx in T lymphocytes and monocytes	[54,57]
Triggers oxidative burst in macrophages and neutrophils	[51,58,59]
Augments IL-1 production in monocytes	[60],
Initiates degranulation in mast cells	[61]
Down-regulates IL-5 expression in leukocytes	[62,63]
Is chemoattractant for monocytes and macrophages	[54]
Is chemoattractant for endothelial cells	[64]
Stimulates capillary tube formation and induce angiogenesis	[64]
Induces leukocyte recruitment *in vivo*	[54][a]
Immunoregulation through surface multivalent galectin-3lattice (Figure 1C)	
Increases thresholds for T cell activation	[65]
Inhibits granulocyte-macrophage colony stimulating factor-induced bone marrow cell proliferation	[66]

[a]Pelletier I, Nieminen J, Sato S, unpublished observation.

[2]. PAMPs are recognized by receptors called pattern recognition receptors (PRRs) [2]. Toll-like receptors (TLRs), which are comprised in the PRRs, are expressed on cell surface, and can activate signal transduction that induces anti-microbial effector responses and inflammation upon recognition of PAMPs [5]. For example, an integral component of the outer membranes of gram-negative bacteria, lipopolysaccharides (LPS), which can provoke strong inflammatory responses, is recognized by the complex of TLR4 and MD-2, possibly with cross-talk with CD14, resulting in activation of the signalling pathways involved in innate immunity [67]. Some of those PAMPs are also recognized by secreted PRRs, binding of which marks pathogens for destruction either by the complement system or by phagocytosis. Examples of these secreted PRRs are collectins, which can distinguish 'nonself' glycans on the pathogens from 'self' glycans found on our cells [68]. It is suggested that collectin can recognize the macropattern that are the repeating 45 Å spans of C3-OH and C4-OH groups of GlcNAc, Glu, Fuc, or Man residues [68]. This macropattern is present on microbial cell walls but absent from complex self glycans.

In the case of galectin-3, studies of carbohydrate binding specificity of galectin-3 as well as of galectin-3 ligands suggest that galectin-3 is designed to recognize 'self' glycans on various host cells rather than 'nonself' glycans on pathogens, thus it is very unlikely that galectin-3 can serve as a general PRR. However, a number of pathogenic entities including gram-negative and gram-positive bacteria, can (or have managed to evolve to) synthesize glycoconjugates that contain β-galactosides similar to those present in host cells [69]. For example, strains of gram-negative mucosal pathogens, such as *Neisseria meningitidis, N. gonorrhoeae* and *Haemophilus influenzae* express the polylactosamine structure (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) on their lipooligosaccharides and LPS. *S. pneumoniae*, a gram-positive bacteria contains the serotype-specific capsular polysaccharides, and certain serotypes, such as type XIV also contain the polylactosamine structure. It has been proposed that galectin-3, which is present in macrophages or mucosal epithelial cell layers, could recognize those polylactosamine containing glycoconjugates on pathogens [69].

Elegant work by Mey *et al*. suggests that galectin-3 indeed interacts with LPSs of *Klebsiella pneumoniae, Salmonella minnesota*, *S. typhimurium*, and *Escherichia coli* not only through binding to β-galactoside residues of LPS, but also through binding to a non-glycan structure, Lipid A/inner core region of LPS [70]. Thus, galectin-3 binds to polygalactoside (α1–3 and β1–3) containing LPS of *K. pneumoniae* through the *C*-terminal CRD in a carbohydrate biding activity dependent manner. It has been revealed that the lipid A/inner core oligosaccharide region of LPS from various pathogens can also bind to the N-terminal regulatory domain of galectin-3 and the interaction is independent of lectin activity. Those data raise the possibility that galectin-3 acts as a PRR, although the biological significance of the recognition of LPS by galectin-3 requires further investigation. However, such recognition of LPS by galectin-3 may mediate the interaction between pathogen and host cells since galectin-3 molecules could oligomerize upon binding to their glycoconjugate ligands at the cell surface, thereby cross-linking surface glycoproteins and causing cell-cell adhesion (Figure 1A). For example, Gupta *et al*. suggest the implication of galectin-3 in the binding of *Pseudomonas aeruginosa* to corneal epithelial cells [56].

Recent works using CD14-deficient and TLR 4-deficient mice reveal that innate immunity can control infection

efficiently in the absence of major PRRs, CD14 and TLR-4 [71,72]. Early and intense neutrophil emigration following the infection that leads to accelerated clearance of *E. coli* (10 times more efficient than control) is induced in CD14-or TLR-4 deficient mice. Interestingly, these efficient responses against infection take place despite the fact that the mice do not exhibit typical proinflammatory response to LPS. The authors also have found that this novel pathway functions in a manner independent of TLR-2 as well as the complement and coagulation cascades, suggesting that innate immunity employs alternative PRRs. The lipid A domain of LPS, which itself is a weak proinflammatory component, can induce early neutrophil emigration not only in CD14 deficient and TLR-4 deficient mice, but also in normal mice. Thus, it was concluded that Lipid A of LPS stimulates a unique and critical innate immune response that is CD14 and TLR-4-independent, resulting in early neutrophil emigration and enhanced bacterial clearance [71]. As lipid A can be recognized by galectin-3 *N*-terminal domain [70], it is tempting to propose that galectin-3 acts as a PRR, which is implicated in this CD14 and TLR independent pathway. Thus, potential and biological significance of galectin-3 as LPS-binding protein certainly requires further investigation.

Galectin-3 and the self-nonself model: Roles of the surface multivalent galectin-3-glycan lattices on leukocytes in immunosuppressive regulation

The second strategy of innate immune recognition is based on the recognition of self-specific molecules that are expressed on the surface of uninfected cells but are absent on pathogens [5]. For example, natural killer (NK) cells express at least two kinds of receptors: receptors containing *C*-type lectin-like motifs,[4] which induce a killing signal by NK cells and the receptors that recognize 'self molecule' MHC class I molecule which suppress the killing signal induced by the lectin-like receptors. When NK cells recognize "missing self", only the *C*-type lectin-like receptors for killing are engaged, resulting in cell-killing. Thus, based on this strategy, the master switch of our innate immune system remains 'OFF' as long as the system keeps recognising self-specific molecules [74,75].

As discussed before, galectin-3 preferentially binds to 'self' glycans expressed on the cell surface. In general, those 'self' glycans on surface glycoproteins contain more than two lactosamine residues, thus galectin-3 ligands are intrinsically multivalent. Cross-linking of bivalent galectin-3 and its multivalent ligands leads to the formation of two- and three-dimensional cross-linked complexes called galectin-3 lattices (Figure 1C) [76,77]. Galectin-3 has been found to be stably associated with the cell surfaces of leukocytes including macrophages, dendritic cells, and T lymphocytes lattices [22,48,65,78–80], suggesting that such two- or three-dimensional galectin-3 lattices are present on those cells. It has been proposed that the galectin-3-glycoproteins lattices, which keep recognizing 'self' glycans, could robustly restrict the lateral mobility of certain receptors, likely raising the threshold for ligand-dependent receptor clustering and signal transduction [77]. Indeed, the biological importance of the galectin-3 glycoprotein lattice has been demonstrated recently by a study with mice deficient in β1,6 *N*-acetlyglucosaminyltransferase V (Mgat5), which is critically involved in the biosynthesis of the oligosaccharide ligands for galectin-3 [65]. T cells from Mgat5-deficient mice lack galectin-3 lattice, which can be found on the T cell from normal mice. Their data suggest that T cells purified from mice deficient in Mgat5 or T cells from normal mice pretreated with lactose are hypersensitive for activation. *In vivo*, it appears that the absence of the multivalent galectin-3 lattices induces increased susceptibility to experimental autoimmune diseases and enhanced delayed-type hypersensitivity (Th1 response) *in vivo*. Thus, the recognition of 'self' glycans by galectin-3 could maintain the baseline of immunity in the 'OFF' state through formation of surface galectin-3-glycoprotein lattices.

Recently, our laboratory has found that truncated galectin-3 which lacks N-terminal regulatory domain (Figure 1D) is generated by a protozoa, parasite *Leishmania*'s protease, gp63, during early infection with *Leishmania major* but not with *L. donovani* [27]. Galectin-3 first recognizes *L. major*-specific polygalactosyl repeats $(Gal\beta 1\text{–}3)_n$ expressed on the major surface glycoconjugates, lipophosphoglycans. The association of galectin-3 with *L. major* parasites results in the formation of the truncated galectin-3. Because the truncated galectin-3 can not oligomerize, the multivalent galectin-3 lattices on leukocytes, such as macrophages, may be depleted by the infections with *L. major* but not by the infections with *L. donovani*. It is also possible that excess truncated galectin-3 molecules compete with remaining intact galectin-3 (a dominant negative effect) as has been shown in other systems [54]. Thus, our data raise the possibility that *L. major* infections could induce enhanced immune reactions through this species-specific depletion of the surface galectin-3 lattices. In fact, leishmaniasis manifests itself quite differently, depending on the *Leishmania* species: Infection with *L. major* develops in dermal tissues as cutaneous lesions (often non-lethal) with the induction of strong local inflammation at the site of infection, while *L. donovani* infections result in a visceral form of the disease (lethal if untreated) and a very limited inflammatory response at the primary site of infection [81,82]. As the presence of surface galectin-3 lattices on leukocytes suppresses the development of strong immune responses, we propose that by disrupting the surface galectin-3 lattices, *L. major* infections (but not *L. donovani* infections) decrease the threshold for the initiation of signal transduction pathways in leukocytes, biasing the immune response towards a Th1 type profile and/or strong local inflammatory responses, which are observed in *L. major* infections. Thus, together, the robust immunosuppresion by the multivalent galectin-3-glycoprotein lattice could be released, thereby innate immunity could be 'ON' if galectin-3 is cleaved by the pathogenic entity, such as *L. major* (through gp63), *E. coli* (through collagase IV) [23,24], or

Pseudomonas aeruginosa (through collagenase IV) [83]. That the recognition of 'self' by the multivalent galectin-3 lattice could be a new type of self-nonself regulation in immunity is indeed intriguing.

Galectin-3 and the Danger model

In contrast to the self-nonself model which proposes that immune response is initiated upon recognition of non-self, the Danger model propose that what really matters for innate immunity against infections is whether the pathogenic entity is harmful or not [3,6]. If not, the cells at the infection site are healthy or the cells die through apoptosis and are then scavenged; no immune response is initiated. However, if cells die through necrosis, or become stressed or damaged, innate immunity is triggered. Thus, the ultimate controlling signals, the Danger signals, are endogenous, not exogenous. In other words, it is the intracellular components or molecules released passively from damaged, stressed or necrotic cells that trigger the initial innate immune response to infection is [3,6]. Innate immunity has the ability (and receptors) to recognize intracellular molecules and to respond to those molecules. Indeed, growing is the list of such intracellular molecules, which can potentially initiate immune response: examples are DNA [84], RNA [6], cytoplasmic heat shock proteins [85], fibroblast growth factors [44,86], thioredoxine [87], S100 family proteins [88], and IL-1 [89]. As discussed above, galectin-3 is also an intracellular molecule and possesses various immunomodulative activities (Table 1) [11–15]. Thus, we have recently proposed that galectin-3 should also be added to the growing list of molecules acting as 'Danger signals' in the Danger model [53]. Some of those activities, that are closely associated with initial innate immune responses, appear to be the results of either signal transduction through galectin-3 mediated receptor clustering or cell-cell adhesion through cross-linking of two different types of cells by galectin-3 (Table 1). Among those activities, it is noteworthy that galectin-3 is implicated in neutrophil emigration to infected or inflammatory sites [38, 93,94] and that injection of galectins-3 *in vivo* could induce leukocyte emigration [54][5] since one of the most important responses in the initial innate immunity against infections is recruitment of phagocytic leukocytes, especially neutrophils and macrophages, to the infected site. The exudated leukocytes could control infections by phagocytozing pathogens and by releasing various bactericidal factors, such as reactive oxygen/nitrogen intermediates, production of which could be triggered in neutrophils and macrophages by extracellularly released galectin-3 [51,58,59].

The Danger model states that the alarm signals are of two types: pre-packaged and inducible [6]. Galectin-3 is found in various cells present at the site of infection including neutrophils,[6] macrophages, dendritic cells, endothelial cells, fibroblasts, and keratinocytes [11–16]. As discussed above, those cells, with the notable exception of inflammatory macrophages, can not secrete galectin-3 actively [39]. Thus, when cells at the infection site are damaged or stressed, 'pre-packaged' galectin-3 could be released passively from those damaged cells and therefore could send Danger signals to the immune system. Interestingly, inflammatory macrophages secrete galectin-3 actively from live cells, therefore in this case, at first glance, active secretion of galectin-3 from inflammatory macrophages without any cellular damage may not seem to fit very well with the Danger model. However, when we take timing of the recruitment of inflammatory macrophages into consideration, this notable exception can be considered as a Danger signal sent by the system through active secretion of galectin-3 from inflammatory macrophages. In general, neutrophils are the first leukocyte to be recruited to the infection site. If the infection is persistent and exudated neutrophils have not controlled the infection, then inflammatory macrophages are recruited to the sites [90–93]. Thus, the emigration of inflammatory macrophages itself is one of the situations where the primary defense response is to send the 'Danger signals' to the general immune system for further protection. As discussed before, galectin-3 is actively secreted by the inflammatory macrophages but not by the resident macrophages. Thus, such strict control of galectin-3 secretion from macrophage could also be a clear indication that galectin-3 is an 'inducible' molecule representing 'Danger signals' for the innate immunity [53].

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Notes

1. A recent report suggests that alternative spliced forms of galectin-3 which contains transmembrane domain are detected in chicken osteoblast-like cells and intestine [37].
2. Human neutrophils express galectin-3 intracellularly while mouse neutrophils do not appear to contain significant amount of galectin-3 [38].
3. Julie Nieminen and Sachiko Sato, unpublished observation.
4. The majority of *C*-type lectin like motifs of NK cell receptors do not appear to contain the aminoacid residues critical for their carbohydrate binding domains, thus the binding should be mediated by the mechanism independent of the carbohydrate binding activity [73].
5. Pelletier I, Nieminen J, Sato S, unpublished observation.
6. Human neutrophils express galectin-3 intracellularly but can not secrete it (Julie Nieminen and Sachiko Sato, unpublished observation).

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