



Galectin-mediated immune recognition: Opsonic roles with contrasting outcomes in selected shrimp and bivalve mollusk species

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ABSTRACT

Galectins are a structurally conserved family of β -galactoside-binding lectins characterized by a unique sequence motif in the carbohydrate recognition domain, and of wide taxonomic distribution, from fungi to mammals. Their biological functions, initially described as key to embryogenesis and early development via recognition of endogenous ("self") carbohydrate moieties, are currently understood as also encompassing tissue repair, cancer metastasis, angiogenesis, adipogenesis, and regulation of immune homeostasis. More recently, however, numerous studies have contributed to establish a new paradigm by revealing that galectins can also bind to exogenous ("non-self") glycans on the surface of potentially pathogenic virus, bacteria, and eukaryotic parasites, and function both as pathogen recognition receptors (PRRs) and effector factors in innate immunity. Our studies on a galectin from the kuruma shrimp *Marsupenaeus japonicus* (MjGal), revealed that it functions as a typical PRR. Expression of MjGal is upregulated by infectious challenge, and can recognize both Gram (+) and Gram (–) bacteria. MjGal also recognizes carbohydrates on the shrimp hemocyte surface, and can cross-link microbial pathogens to the hemocytes, promoting their phagocytosis and clearance from circulation. Therefore, MjGal contributes to the shrimp's immune defense against infectious challenge both as a PRR and effector factor. Our studies on galectins from the bivalve mollusks, however, have shown that although they can function in immune defense as MjGal, protistan parasites take advantage of the recognition roles of the host galectins, for successful attachment and host infection. We identified in the eastern oyster *Crassostrea virginica* two galectins (CvGal1 and CvGal2) that not only recognize a large variety of bacterial species, but also the protozoan parasite *Perkinsus marinus*. Like the shrimp MjGal, both oyster galectins function as opsonins, and promote parasite adhesion and phagocytosis. However, *P. marinus* survives intrahemocytic oxidative killing and proliferates, eventually causing systemic infection and death of the oyster host. In the softshell clam *Mya arenaria* we identified a galectin (MaGal1) that displays carbohydrate specificity and recognition properties for sympatric *Perkinsus* species (*P. marinus* and *P. chesapeaki*), that are different from CvGal1 and CvGal2. Our results suggest that although galectins from bivalves can function as PRRs, *Perkinsus* parasites have co-evolved with their hosts to subvert the galectins' immune functions for host infection by acquisition of carbohydrate-based mimicry.

1. Introduction

A critical step for the host's successful immune response against infectious challenge is the immediate detection of foreign surface structures or soluble products from the potential pathogens and parasites by pattern recognition receptors (PRRs) that can induce multiple downstream effector functions aimed at immobilizing, killing, and clearing the pathogen or parasite from the host's internal milieu (Janeway and Medzhitov, 2002). For example, carbohydrate moieties on bacterial lipopolysaccharides and exopolysaccharides, viral envelope glycoproteins, and various surface or soluble glycans from eukaryotic parasites can be recognized by one or more carbohydrate-binding proteins (lectins) from the host (Laine, 1997; Vasta and Ahmed, 2008). Lectins can form soluble or membrane-associated oligomeric assemblies of two or more polypeptide subunits, resulting in multivalent carbohydrate-binding proteins that enable cross-linking of target ligands

(Dam and Brewer, 2008, 2010). Thus, lectin-mediated recognition can lead to agglutination and immobilization, opsonization, phagocytosis, and intracellular oxidative killing of the pathogens, as well as activation of complement or prophenoloxidase cascades that can promote their extracellular killing, followed by clearance from the host. Therefore, in both invertebrates and vertebrates, lectins are critical components of innate immune responses to infection (Vasta et al., 2007; Wang and Wang, 2013a).

While lacking the typical adaptive immune response of vertebrates characterized by immunoglobulins, and B and T cells, invertebrates share with vertebrates very rapid and effective innate immune mechanisms against infection (Loker et al., 2004). These mechanisms include both cell-associated receptors and soluble recognition molecules (Janeway and Medzhitov, 2002; Lemaitre and Hoffmann, 2007; Wang and Wang, 2013a) among which lectins play a key role (Vasta and Ahmed, 2008; Wang and Wang, 2013b). The presence of unique amino

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acid sequence motifs and conserved structural fold of the carbohydrate recognition domains (CRDs) have enabled the classification of lectins into several major families, such as galectins (formerly S-type lectins), C-, F-, L-, P-, X-, and I-types, heparin-binding, and others. Although lectins from several families are exclusively involved in intracellular functions, C-, F- and X-type lectins have been extensively studied as PRRs (Vasta and Ahmed, 2008; Zhang et al., 2014; Zhang et al., 2018; Xu et al., 2014a, b, Vasta et al., 2017).

2. Galectins: A structurally conserved and functionally diversified lectin family

A. Structural Aspects Among the lectin families described so far, galectins are characterized by their binding affinity for β -galactosides, a unique conserved sequence motif in their CRD, wide taxonomic distribution from fungi to invertebrates and vertebrates (Leffler et al., 2004; Vasta and Ahmed, 2008; Cummings et al., 2015–2017), and striking functional diversification (Leffler et al., 2004; Vasta, 2009; Rabinovich and Croci, 2012). Based on structural features, galectins from vertebrates have been classified into the “proto”, “chimera”, and “tandem-repeat” types (Hirabayashi and Kasai, 1993). The first (proto-type) is characterized by single CRD subunits that usually form non-covalently-linked homodimers. The proto-type galectin-13 (“pregnancy protein 13”) is an exception, since in solution the dimer is established by a disulfide bridge between Cys¹³⁶ and Cys¹³⁸ (Than et al., 2004). The chimera-type galectin subunits display a C-terminal CRD, and an N-terminal domain rich in proline and glycine that can promote oligomerization into trimers or pentamers. In the tandem-repeat type, two CRDs are joined by a linker peptide, are mostly monomeric. Galectin-related proteins that do not follow the canonical structure of the three major galectin types include the eye lens protein GRIFIN (Galectin related inter-fiber protein), the galectin-related protein GRP (previously HSPC159; hematopoietic stem cell precursor), and the Charcot-Leyden crystals (Galectin 10) (Su et al., 2018; Zhou et al., 2018; Ogden et al., 1998). Galectins from invertebrates can display structural features and domain organizations that do not fit within the three mammalian galectin types describe above, with some molecular species from bivalve mollusks housing four CRDs (Vasta et al., 2015), or constituting hybrid structures, such as the chimeric galectin-related protein (GREP) in the snail *B. glabrata* (Dheilly et al., 2015).

The structure of mammalian galectin-1 (Gal1) in complex with N-acetyl-lactosamine (LacNAc) enabled the identification of the specific amino acid residues of the CRD that interact with the carbohydrate ligand (Liao et al., 1994). The 135 amino acids-long polypeptide subunit of Gal1 folds into a β -sandwich that comprises two antiparallel β -sheets of five and six strands each (S1–S6 and F1–F5). The primary carbohydrate binding site is formed by three strands (S4–S6) in which H⁴⁴, B⁴⁶, R⁴⁸, H⁵², B⁶¹, W⁶⁸, E⁷¹, and R⁷³ establish direct interactions with LacNAc (Liao et al., 1994). Further, W⁶⁸ establishes a hydrophobic interaction with the non-reducing terminal galactose ring, while additional water-mediated interactions between H⁵², D⁵⁴, and R⁷³ and the nitrogen of the N-acetyl group determine the higher affinity for LacNAc over lactose (Lac). In this regard, the dissociation constants of Gal1 for Lac, LacNAc, and thiodigalactoside (TDG) are in the range of 10^{-5} M, with two binding sites per Gal1 dimer (Schwarz et al., 1998). In addition to the primary binding site in the galectin CRD, additional carbohydrate-binding features (“extended binding site”) can enhance interactions with more complex glycans. For example, the galectin-3 (Gal3) carbohydrate-binding site is shaped as a cleft open at both ends, resulting in increased affinity for polylactosamines and ABH blood group oligosaccharides (Seetharaman et al., 1998). For galectins from invertebrates, such as the *Caenorhabditis elegans* 16-kDa galectin and the oyster (*Crassostrea virginica*) galectins CvGal1 and CvGal2, their binding specificity for blood group oligosaccharides is determined by a shorter loop 4 in the primary binding cleft in the galectin CRD (Ahmed et al., 2002; Feng et al., 2013; Vasta et al., 2015).

Oligomerization of galectin subunits (proto and chimera types) or the presence of multiple distinct CRDs in TR galectins results in binding multivalency and increased avidity (Dam and Brewer, 2008, 2010), that is critical to galectin function. Interactions of multivalent galectins with multivalent glycans on the cell surface can lead to their cross-linking and formation of clusters, microdomains, and lattices that are critical for signaling or receptor endocytosis, as well as cross-linking of two or more cells, and adhesion of cells to glycosylated surfaces (Rabinovich et al., 2007; Garner and Baum, 2008; Kutzner et al., 2019) (Fig. 2A). Further, while in proto and chimera galectins, the multivalent oligomers display CRDs with identical specificity and affinity, for the vertebrate TR galectins and the multi-CRD galectins from invertebrates the carbohydrate specificity of the CRDs present in the polypeptide may be similar but not identical, enabling the protein to crosslink different glycans or cells (Houzelstein et al., 2004; Krejcirikova et al., 2011; Vasta et al., 2015). In addition, galectins that are bound to carbohydrate ligand are less susceptible to proteolytic cleavage or oxidative inactivation (Liao et al., 1994; Stowell et al., 2009).

B. Functional Aspects: Galectins can mediate key biological functions in both the intracellular (cytosolic and nuclear) and extracellular compartments. By binding to endogenous (“self”) glycans, galectins can mediate developmental processes, and tissue repair, as well as regulation of immune homeostasis, cancer metastasis, angiogenesis, and adipogenesis, among others (Leffler et al., 2004; Vasta and Ahmed, 2008; Cummings et al., 2015–2017). Multivalent interactions of extracellular galectins with cell surface glycans can modulate cell function by inducing their reorganization or association in lattices, regulating turnover of endocytic receptors, and modulating signaling pathways (Garner and Baum, 2008; Rabinovich et al., 2007; Dam and Brewer, 2010). Furthermore, differences in carbohydrate specificity, affinity, and avidity of the various galectin types, subtypes, and isoforms, endow the galectin repertoire in any given species with substantial diversity in recognition properties, that together with their unique tissue-specific expression, distribution and local concentrations, results in extensive functional diversification (Cooper, 2002; Vasta and Ahmed, 2008; Vasta, 2009; Takeuchi, 2018).

Soon after the discovery of galectins, the observation that those present in chicken muscle were developmentally regulated and preferentially recognized polylactosamines present on the myoblast surface and the extracellular matrix (ECM), suggested a function in embryogenesis and early development (Reviewed in Cummings et al., 2015–2017). Later research on Gal1 and galectin-3 (Gal3) revealed their roles in development of notochord, skeletal muscle and central nervous system (Craig et al., 2010; Colnot et al., 1997; Colnot et al., 2001; Georgiadis et al., 2007). The increasing availability of null mice for selected galectins enabled the analyses of developmental phenotypes and the elucidation of their unique functions. More recently, genetically-tractable models such as *C. elegans*, *Drosophila*, and zebrafish (*Danio rerio*) have become alternative useful systems for addressing the diverse functions of galectins (Pace et al., 2002; Vasta et al., 2004; Ahmed et al., 2002; Nemoto-Sasaki et al., 2008; Craig et al., 2010; Shi et al., 2014; Feng et al., 2015; Eastlake et al., 2017; Takeuchi, 2018).

Galectins are expressed and distributed in most mammalian tissues, including cells of the innate and adaptive immune system (Stowell et al., 2008; Rabinovich et al., 2012). Galectins are regulators of innate immune homeostasis (DiLella et al., 2011), and can function as pro- or anti-inflammatory factors (Hirashima et al., 2002; Stowell et al., 2008; Liu et al., 2012; Toledo et al., 2014). Galectins are also modulators of lymphocyte development and adaptive immune responses (Rabinovich et al., 2012; Liu et al., 2012). Gal1-mediated interactions of lymphocyte precursors with stromal cells in bone marrow and thymus are a key step for their further development, selection, and migration to the periphery (Rossi et al., 2006). Further, galectins can also either promote or attenuate apoptosis or proliferation, modulate cytokine synthesis and secretion by T cells, and promote feto-maternal tolerance (Rabinovich et al., 2012; Liu et al., 2012; Blois et al., 2007, 2019). Thus, it has

become firmly established that galectins can modulate immune homeostasis with either beneficial or detrimental effects on pathological conditions that result from depressed or exacerbated immune function, such as cancer, inflammation, allergy, and autoimmune disorders (Rabinovich et al., 2012; Liu et al., 2012; Hsu and Liu, 2008; Martínez Allo et al., 2020).

The multiple roles of galectins in cancer has been addressed with increasing interest over the past two decades (Reviewed in Méndez-Huergo et al., 2017). Expression profiles of selected galectins in melanoma, prostate and ovarian cancer can be associated with malignancy stage or metastatic potential (Blidner et al., 2015; Hill et al., 2010), and increased angiogenesis (Crocini et al., 2014). In early stages of prostate adenocarcinoma, Gal3 expression is silenced by promoter methylation (Ahmed et al., 2009; Ahmed and AlSadek, 2015), but is expressed in later stages and enhances tumor angiogenesis, tumor-endothelial cell adhesion, metastasis, and evasion of immune surveillance (Guha et al., 2013). In recent years, the adipogenic roles of Gal 12 and Gal3 and their associations with type 2 diabetes, obesity, and inflammation have been characterized in detail (Yang et al., 2011a,b; Hsu et al., 2018; Pejnovic et al., 2013).

During the past decade, evidence has mounted in support of new paradigm by revealing that galectins can also bind to exogenous (“non-self”) glycans on the surface of potentially pathogenic virus, bacteria, and eukaryotic parasites and function as PRRs. Furthermore, downstream of pathogen recognition, galectins also function as effector factors in innate immunity, as they can inhibit pathogen adhesion and/or entry into the host cell, or either directly kill certain pathogens by disrupting its surface structures, or promote their agglutination, phagocytosis or encapsulation, and clearance from circulation (Vasta, 2009; Vasta et al., 2012; Shi et al., 2014).

3. Galectins as PRRs in defense against infection: recognition of exogenous (“non-self”) glycans and effector functions

The recent finding that galectins can recognize glycans on the surface of viruses, bacteria, protistan parasites, and fungi, has supported their proposed roles in innate immunity as PRRs (Reviewed in Vasta, 2009) (Fig. 2B). As discussed above, the considerable diversity in fine carbohydrate specificity of the galectin repertoire(s) in each species, suggest a broad capacity for recognition of potential pathogens and parasites. Furthermore, the capacity for multivalent binding of all three galectin types enables the direct recognition of pathogens and parasites, their crosslinking to the host's phagocytic cells, and the activation of signaling pathways and downstream processes that lead to modulation of immune responses (Di Lella et al., 2011). Additionally, as multiple innate immune receptors such as TLRs and C-type lectins are present in any single species, cooperative defense functions of galectins with other receptors have been described (Esteban et al., 2011; Jouault et al., 2006). As will be discussed in the following sections, the binding of host galectins to the pathogen or parasite surface glycans, and their crosslinking to host cell receptors can lead to various outcomes beneficial to the host, including direct killing of the pathogen, blocking of pathogen attachment to the host cell surface, or opsonization, phagocytosis, encapsulation, and clearance of the pathogen. However, this is not always the case, and recognition of infectious agent by galectins can also lead to detrimental outcomes for the host, by facilitating attachment, entry and infection by pathogens and parasites. This subversion of the defense role(s) of the host galectins by the pathogens and parasites is likely the product of the host-pathogen co-evolutionary process (Vasta, 2009).

Galectins of all three major types have been experimentally shown to recognize a variety of glycans on the surface of virus, bacteria, protozoan parasites and yeasts (Reviewed in Vasta, 2009). Upon recognition of the pathogen or parasite by a galectin, several different outcomes have been reported. Some galectins can not only bind to, but also directly kill the pathogens. The tandem repeat galectins Gal4 and

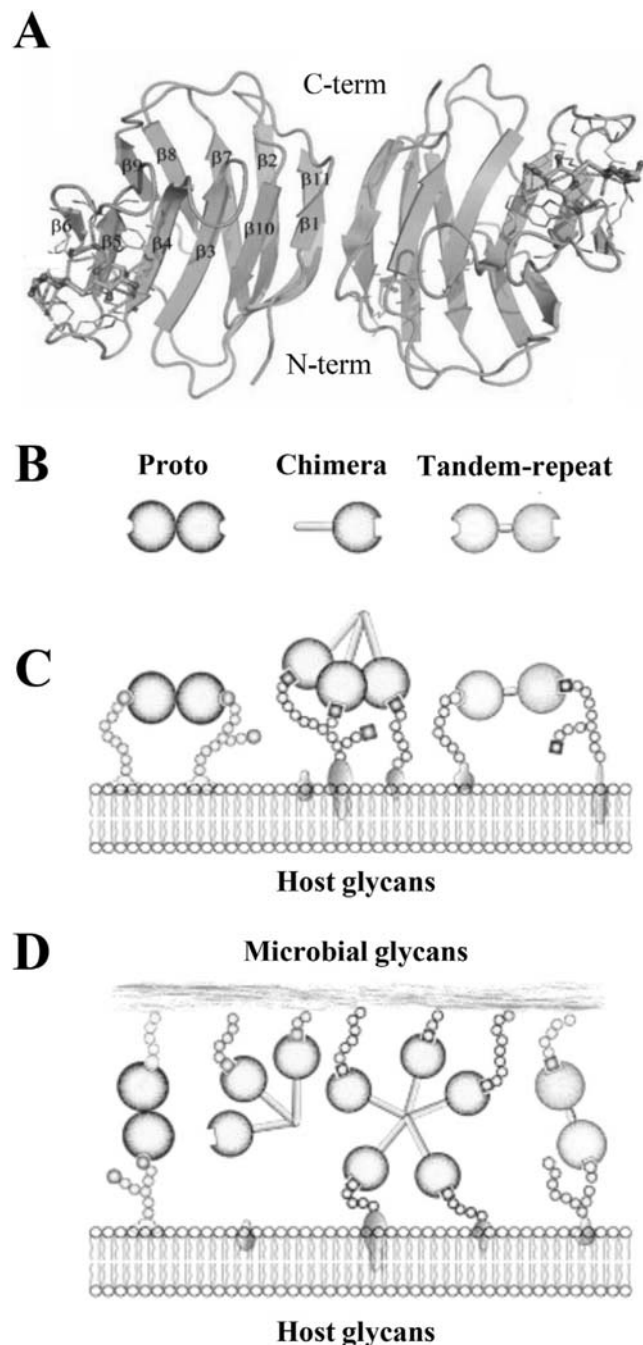


Fig. 1. Galectin structure, types, and functions in “self”/“non-self” recognition. A. Crystal structure of galectin-1 dimer in complex with LacNAc. B. Galectins are classified into three main types: proto, chimera and tandem-repeat types. Proto-type galectins contain one carbohydrate-recognition domain (CRD) per subunit and are usually homodimers of non-covalently linked subunits (Galectins 1, 2, 5, 7, 10, 11, 13, 14 and 15). In contrast, chimera-type galectins are monomeric with a carboxy-terminal CRD, joined to an amino-terminal peptide that contains a collagen-like sequence rich in proline and glycine, and can oligomerize as trimers (Galectin 3). In the tandem-repeat galectins, two CRDs are covalently joined by a linker peptide (Galectins 4, 6, 8, 9 and 12). C. Recognition of “self” glycans by galectins: In the extracellular space, galectins form multivalent oligomers that cross-link cell surface glycoproteins and glycolipids, form microdomains and lattices, and activate signaling pathways. D. Recognition of “non-self” glycans by galectins: Proto, chimera, and tandem-repeat galectins can function as pattern recognition receptors (PRRs) and establish trans-interactions with the host cell surface and microbial glycans.

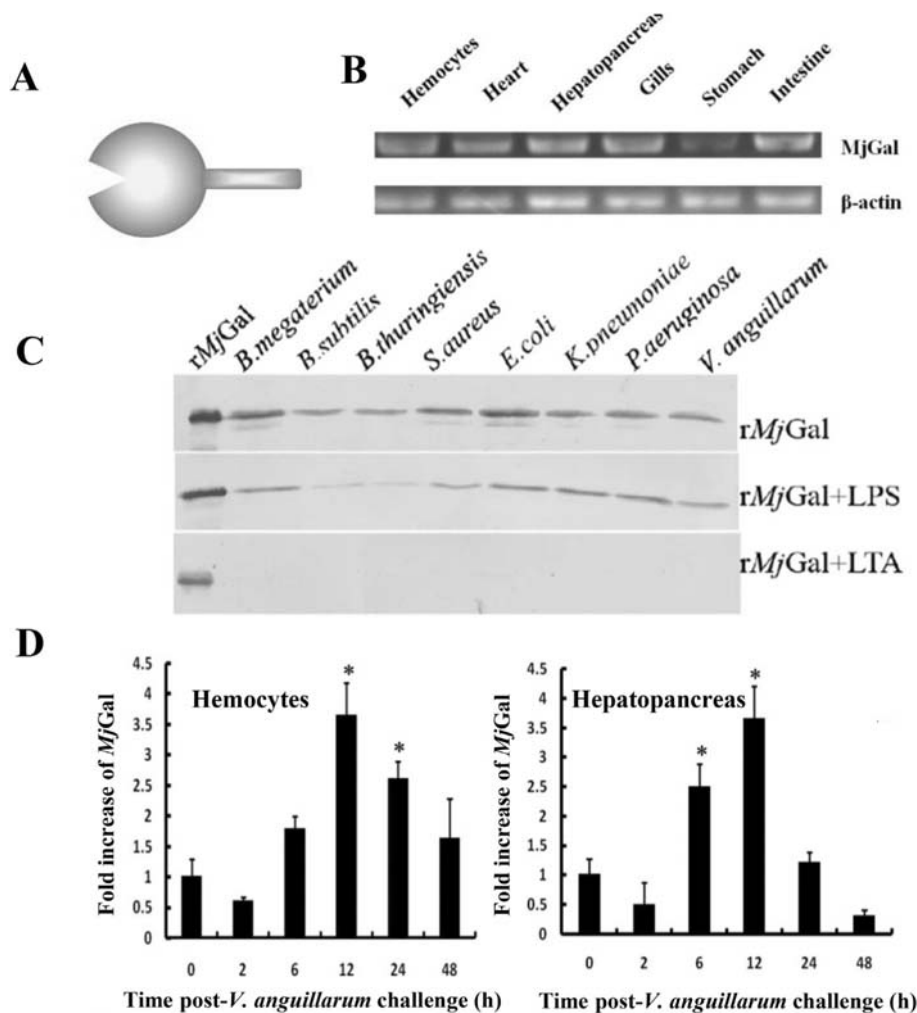


Fig. 2. The shrimp galectin MjGal: Domain organization, tissue-specific expression, recognition of bacteria, and upregulation of expression upon infectious challenge: A. Domain organization of MjGal: the cartoon illustrates the N-terminal CRD, followed by the peptide extension at the C-terminal end; B. Expression of MjGal in different tissues of naïve animals as assessed by RT-PCR, with β -actin serving as the reference gene. C. Binding of recombinant MjGal (rMjGal) to bacteria by Western blot, and inhibition by LPS and LTA: Purified, His-tagged rMjGal (500 μ g/ml) was pre-incubated with TBS alone or excess LPS or LTA for 1 h, after which bacterial suspensions were added and incubated at room temperature for 1 h. The bacteria were centrifuged, washed four times with TBS, and treated with 7% SDS for 1 min. The eluate was collected by centrifugation, subjected to SDS-PAGE, and the eluted rMjGal detected by Western blot using anti-His antibody. D. The time-course expression of MjGal was upon *V. anguillarum* challenge was analyzed by qRT-PCR in hemocytes (Left panel) and hepatopancreas (Right panel), with β -actin serving as the reference gene. The histograms show the statistical analysis of the quantitative real-time PCR results. The asterisks indicate significant differences (** $P < 0.01$, *** $P < 0.001$) between the *V. anguillarum*-challenged and PBS-injected group. Error bars represent $\pm$ SD of three independent PCR amplifications and quantifications (Adapted from Shi et al., 2014).

Gal 8 are expressed in the human intestinal tract, and can specifically recognize and kill *Escherichia coli* strains that display B-blood group oligosaccharides; other *E. coli* strains or bacterial species are not affected (Stowell et al., 2010). Recognition is carried out by the C-CRD, whereas the N-CRD would be responsible for the bactericidal activity by disrupting the target cell surface integrity (Stowell et al., 2010). Similarly, Gal3 displays recognition, agglutination and bactericidal effect against *Helicobacter pylori* and displays a potent, associated with drastic changes in the bacterial morphology (Park et al., 2016). Galectin-3 has also been reported to bind to and directly kill fungal pathogens such as *C. albicans* and *C. neoformans*, although the detailed mechanism(s) involved in the fungicidal activity has not been fully elucidated (Kohatsu et al., 2006; Almeida et al., 2017).

Galectins can bind to the pathogen or parasite surface and while not killing or inactivating it, they can hinder their attachment to the host cell surface and prevent infection. Gal1 and Gal 9 can bind to the envelope glycoprotein of influenza A virus and reduce infection severity (Yang et al., 2011a,b; Machala et al., 2019; Hattori et al., 2013; Katoh et al., 2014). Gal1 can also recognize Dengue virus inhibiting viral adhesion and internalization into host cells *in vitro* (Toledo et al., 2014). Direct interaction of Gal1 with the envelope glycoproteins of the Nipah virus Gal1 can reduce cell-cell fusion and attenuate the pathophysiological effects of infection (Levroney et al. (2005); Garner et al. (2015). Similarly, the zebrafish Gal1 and Gal3 can bind to the infectious hematopoietic necrosis virus and hinder adhesion to the fish epithelial cell surface (Nita-Lazar et al., 2016; Ghosh et al., 2019). A similar mechanism has been proposed for Gal4, which hinders attachment of

Bordetella pertussis and *Helicobacter pylori* by binding to their gut epithelial cell surface receptors (Danielsen et al., 2006; Ideo et al., 2005).

Galectins can also function as opsonins by binding to and promoting phagocytosis of pathogens and parasites, which are killed by intracellular oxidative burst, and cleared from the internal milieu. If the infectious particle is too large to be phagocytosed by a single cell, it can be encapsulated by deposition multiple layers of cells, and killed either by oxidative stress or melanization (Vazquez et al., 2009; Nakamura et al., 2012; Xia et al., 2018).

In the following sections we discuss two examples of the role(s) of galectins from invertebrates in recognition, opsonization, and phagocytosis of pathogens and parasites, with contrasting outcomes. In the first example, we illustrate the successful opsonic role of an innate immune defense in an invertebrate with the galectin MjGal from the kuruma shrimp, *Marsupenaeus japonicus*. In the second example, the subversion of the opsonic role of galectins from bivalve mollusks by the parasite *Perkinsus* sp. is discussed.

4. A shrimp galectin is a successful opsonin for bacterial pathogens

Our studies on a galectin (MjGal) from the kuruma shrimp *Marsupenaeus japonicus*, revealed that it functions as an inducible typical PRR and effector factor that is effective in the recognition and clearance of bacterial pathogens (Shi et al., 2014). MjGal was identified in our *M. japonicus* EST database as a 33.5 kDa galectin that displays a single 127-residue long CRD on the N-terminal region, followed by a C-

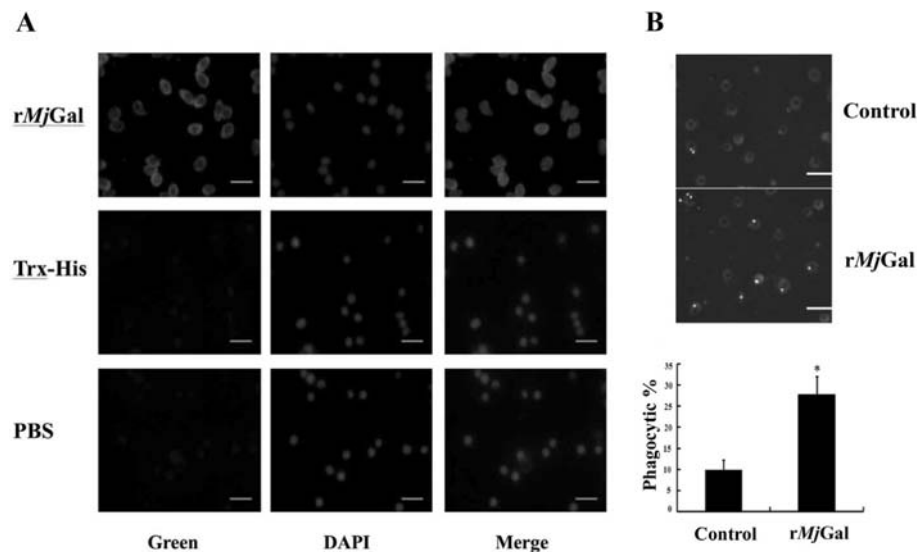
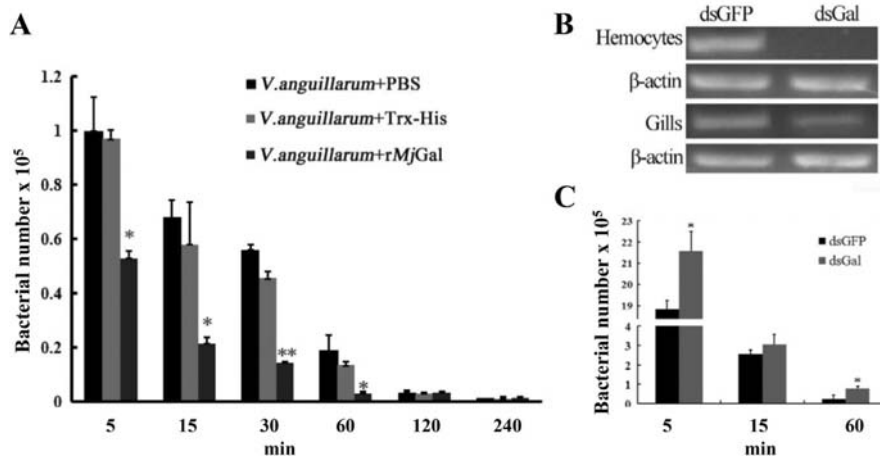


Fig. 3. *In vivo* binding of rMjGal to shrimp hemocytes, and opsonization of bacteria. **A.** rMjGal, Trx-His, or PBS were injected into shrimp and the hemocytes were collected and analyzed by immunohistochemistry. The left panel indicated the binding signal detected by the anti-Histidine antibody for the recombinant protein (Green), the middle panel showed the hemocyte nucleus location (Blue), and the right panel showed the merge of previous two panels. Bar = 20 mm. **B.** Bacteria phagocytosis assay: fluorescently labeled *V. anguillarum* (5×10^7 cells) were coated with either Trx-His (Top panel, control) or rMjGal (Middle panel) and injected into shrimp. The hemocytes were collected after 30 min and placed onto the glass slides. Subsequently, trypan blue solution (2 mg/ml PBS) (Amresco) was added to quench the fluorescence of non-phagocytosed bacteria. The phagocytosis by hemocytes was observed at $400\times$ magnification, and phagocytosed bacteria counted at $200\times$ magnification under fluorescence microscope. The phagocytic percentages of two groups ($*P < 0.05$) are shown in the lower panel. Bar = 50 mm (Adapted from Shi et al., 2014). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



was extracted from hemocytes and gills of two groups, and tested by RT-PCR to confirm silencing of MjGal expression. **C.** The bacterial counts in hemolymph samples collected at 5, 15 and 60 min from shrimp from the dsMjGal and dsGFP groups (Adapted from Shi et al., 2014).

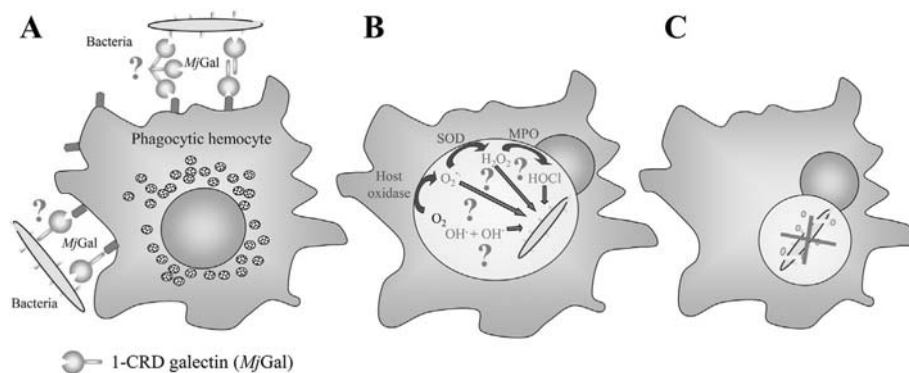


Fig. 5. Schematic model of the proposed role of the shrimp galectin MjGal in the uptake and clearance of bacteria by hemocytes. **A.** The galectin MjGal from the kuruma shrimp *M. japonicus* can opsonize and cross-link the circulating bacteria to carbohydrate moieties on the hemocyte surface. It remains unknown if MjGal forms multivalent oligomers (dimers or trimers) that may contribute to the cross-linking of bacterial and hemocyte glycans. **B.** MjGal-mediated recognition and opsonization of bacteria promotes their phagocytosis. The phagocytosed bacteria are most likely killed by the typical oxidative burst. **C.** The phagocytosed bacteria are cleared from the shrimp internal milieu.

terminal polypeptide extension (Fig. 2A). In this regard it resembles the chimera-type galectins described in mammals (Fig. 1B), but with the CRD located on the opposite end of the polypeptide.

In the naïve shrimp MjGal was expressed in all tissues examined (Fig. 2B), and can bind to both Gram-positive and Gram-negative

bacteria (Fig. 2C). Interestingly, the binding to bacteria could not be inhibited by lactose or related sugars that are typical inhibitors for most vertebrate and invertebrate galectins (Shi et al., 2014). In contrast, recognition of some bacterial species by MjGal could be partially inhibited by preincubation of the galectin with lipopolysaccharide (LPS),

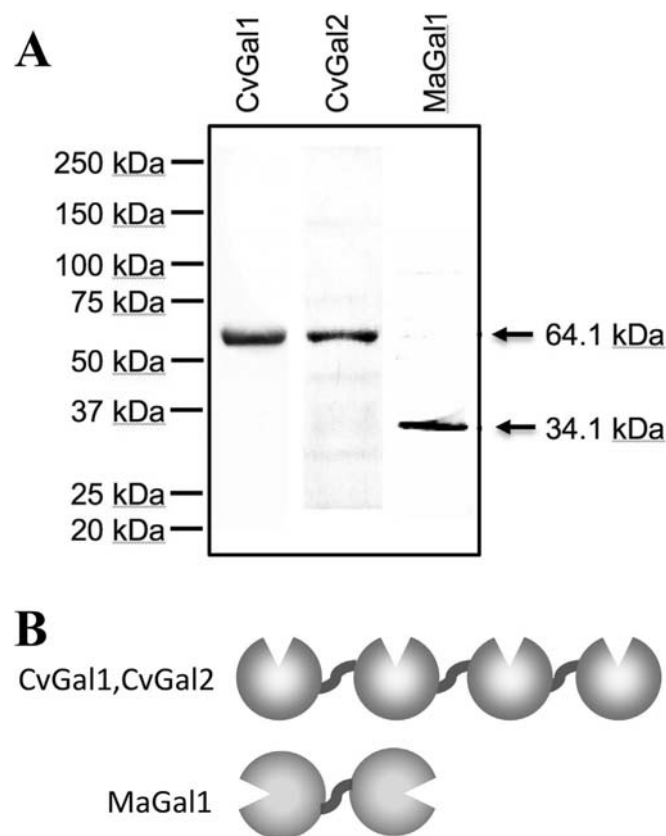


Fig. 6. Galectins CvGal1 and CvGal2 from the eastern oyster, and MaGal1 from the softshell clam. **A.** Coomassie (CBB) stain of recombinant CvGal1, CvGal2, and MaGal1 containing either four or two CRDs. Arrows indicate the expected molecular weight of the proteins. **B.** Schematic illustration of 4 CRDs of CvGal1 and CvGal2 and 2 CRDs of MaGal1 [Adapted from Tasumi and Vasta, 2007, with permission from the American Association of Immunologists].

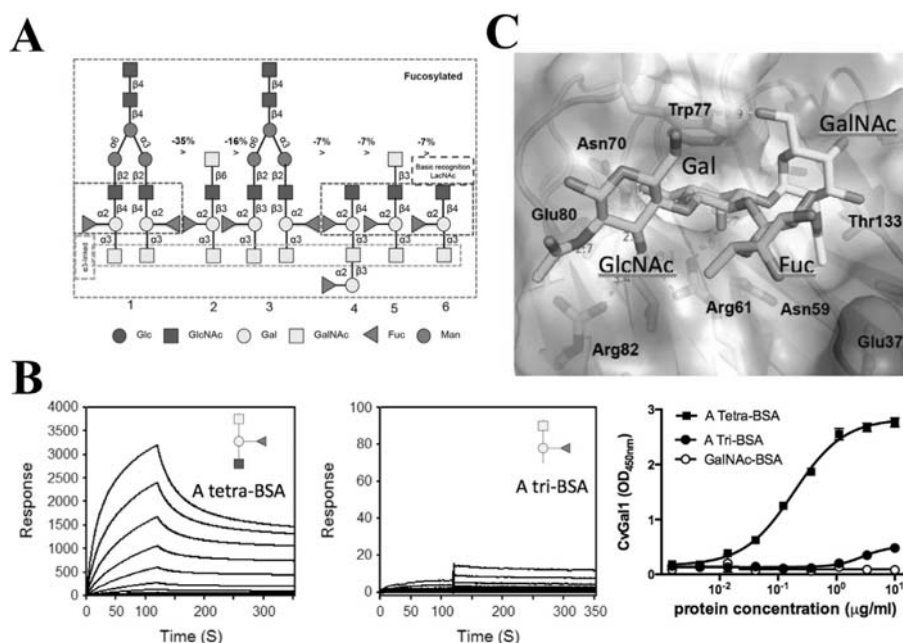


Fig. 7. Analysis of the carbohydrate specificity of rCvGal1 by glycan array and surface plasmon resonance analyses, and homology modeling of the interactions with the ligand. **A.** Glycan array analysis: The six best glycans ranked by their affinity for rCvGal1 in glycan array analysis. The negative percentages are the evaluation of the % change in the fluorescent signal ($(F_i - F_0)/F_0 \times 100\%$). **B.** Surface plasmon resonance: Blood group A tetrasaccharide-BSA (A Tetra-BSA; left panel) or blood group A trisaccharide-BSA (A Tri-BSA; middle panel) were immobilized up to 1000 response units on CM5 chips, and the binding of rCvGal1 was assessed by SPR with rCvGal1 being injected as analyte. The SPR sensorgrams were recorded with 2-fold serial dilutions of the analyte starting from 100 µg/ml. Solid phase analysis: A Tetra-BSA, A Tri-BSA, or N-acetylgalactosamine-BSA (GalNAc-BSA) were added at the concentrations indicated (serial dilution starting from 10 µg/ml, 100 µl/well) into 96-well plates and the binding of 0.2 µg/ml of rCvGal1 was assessed by ELISA. Data show optical density at 450 nm (OD_{450nm}) in triplicates with standard error (SEM). **C.** Homology modeling of the interactions of CvGal1 with blood group A tetrasaccharide: A2 blood tetrasaccharide docked at the binding pocket of the CvGal1 model of the first CRD. CvGal1-binding site is shown as semi-transparent solvent-accessible surface colored by its vacuum electrostatic potential (positive in blue to negative in red). The schematic model of the protein is visible across the surface showing the interacting residues in a stick representation. H-bonds recognizing hydroxyl groups of the A2 oligosaccharide are displayed as dashed lines with their distances (in Å) between heavy atoms indicated. [Adapted from Feng et al., 2013, with permission from the American Society for Biochemistry & Molecular Biology]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

while lipoteichoic acid (LTA) behaved as a more effective inhibitor (Fig. 2C). These results suggest that MjGal binds to bacteria through the recognition of either LTA or LPS, and that the binding is strong enough that simple sugars cannot outcompete these interactions. The alternative explanation that bacterial recognition could take place via a non-CRD region of MjGal was ruled out, as the mutant rMjGal^{Δ102–106} that lacks the two cysteine residues and three additional residues in the CRD showed weaker recognition for the tested bacteria, or the isolated LPS and LTA (Shi et al., 2014).

Upon experimental infectious challenge with *V. anguillarum*, one of the bacterial species strongly recognized by MjGal (Fig. 2C), galectin expression was upregulated in circulating phagocytic cells (hemocytes) and hepatopancreas (Fig. 2D). These results suggested that MjGal functions in innate immune defense as a PRR that is inducible by microbial infection. In addition to binding to the surface of a variety of G^+ and G^- bacteria, MjGal also binds to the shrimp hemocyte surface (Fig. 3A). It is noteworthy, however, that unlike the recognition of bacteria MjGal binds to the hemocyte in a lactose dependent-manner (Shi et al., 2014), as typically described for most galectins. The binding to bacteria, presumably by binding to LTA or LPS, and the binding to the hemocyte surface carbohydrate moieties enable MjGal to function as an opsonin, cross-linking the potentially pathogenic bacteria to the hemocyte surface and promoting their phagocytosis (Fig. 3B).

In vivo experimental infections in mature shrimp with *V. anguillarum* that had been pre-exposed to either rMjGal, Trx-His, or PBS alone showed that MjGal significantly contributes to the clearance of the injected bacteria from circulation over the baseline levels (Fig. 4A). This was confirmed by an *in vivo* loss-of-function approach, through silencing expression of MjGal by RNAi. The injection of MjGal dsRNA into mature shrimp could significantly inhibit levels of the MjGal transcript in hemocytes (Fig. 4B). As a result, the MjGal dsRNA-injected animals were less effective in the clearance of *V. anguillarum* than the GFP dsRNA-injected controls (Shi et al., 2014) (Fig. 4C). Therefore, MjGal plays a key role in the ability of the shrimp to recognize and clear a bacterial infectious challenge (Fig. 5). The detailed mechanisms involved in the opsonization activity remain to be unraveled, but in order

CvGal1 model of the first CRD. CvGal1-binding site is shown as semi-transparent solvent-accessible surface colored by its vacuum electrostatic potential (positive in blue to negative in red). The schematic model of the protein is visible across the surface showing the interacting residues in a stick representation. H-bonds recognizing hydroxyl groups of the A2 oligosaccharide are displayed as dashed lines with their distances (in Å) between heavy atoms indicated. [Adapted from Feng et al., 2013, with permission from the American Society for Biochemistry & Molecular Biology]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

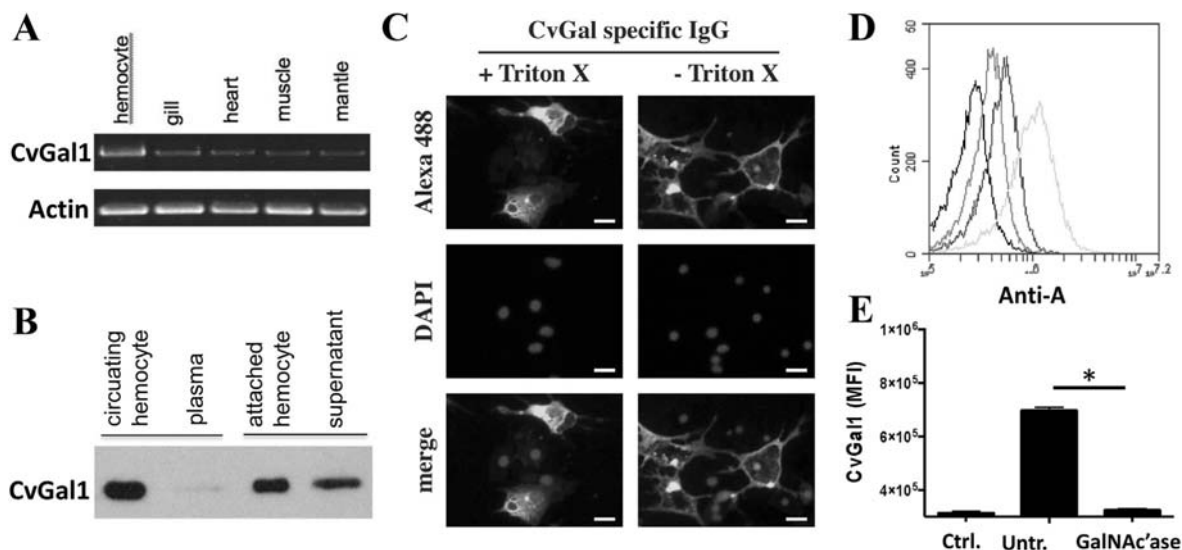


Fig. 8. The oyster galectin CvGal1: Tissue-specific expression, secretion, and binding to the hemocyte surface blood group A moieties: **A.** Expression of CvGal1 different tissues of eastern oysters as assessed by RT-PCR, with β -actin serving as the reference gene. **B.** Upon hemocyte attachment and spreading, CvGal1 is secreted. Western blotting of unattached hemocytes, plasma, attached-spread hemocytes, and supernatant. **C.** Immunofluorescence staining with anti-CvGal1 and DAPI staining of attached-spread hemocytes with (+) or without (-) Triton X treatment, showing the presence of CvGal1 in the cytoplasm, and on the external surface of the hemocyte plasma membrane, respectively. Scale bar, 10 μ m. **D.** Fixed hemocytes were stained with dilutions of anti-blood group A antibody (red, 1:2000; blue, 1:500; yellow, 1:100) or buffer only (black) in flow cytometry analysis. **E.** Binding of rCvGal1 to unattached hemocytes with α -N-acetyl-galactosaminidase treatment (GalNAc'ase) or no treatment (Untreated) were measured by flow cytometry, whereby a sample without rCvGal1 was the control (Ctrl). Data show mean fluorescence intensity (MFI) $\pm$ S.E. of each sample. * indicates significant difference ($p < 0.05$) between samples from One-Way ANOVA analysis. [Adapted from Tasumi and Vasta, 2007, with permission from the American Association of Immunologists and the American Society for Biochemistry & Molecular Biology]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

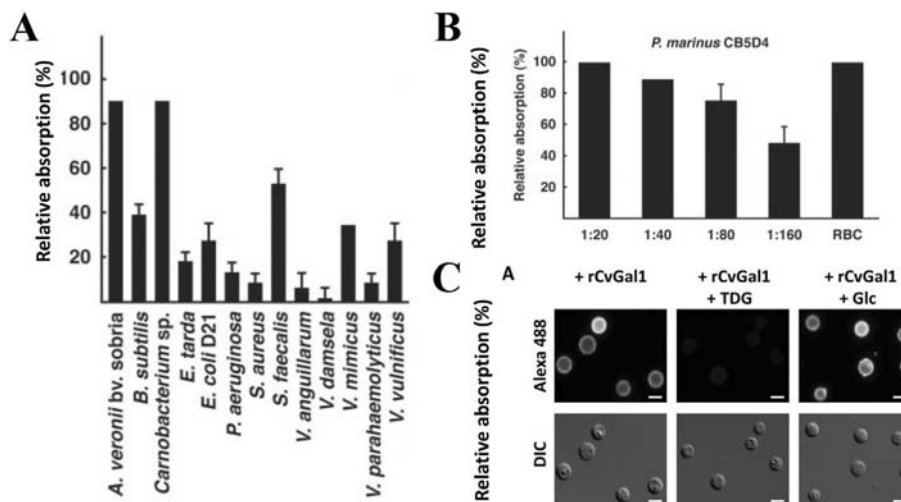


Fig. 9. The oyster galectin CvGal1 binds to a variety of environmental bacteria and to the parasite *Perkinsus marinus*. **A.** CvGal1 binds to a variety of environmental bacteria: Absorption test of rCvGal1 with selected microorganisms. Results are shown as relative absorption. Bars show SDs of quadruplicate experiments. **B.** Absorption test of rCvGal1 *P. marinus* trophozoites at various cell pellet-to-CvGal1 solution ratios. Bars show SDs of quadruplicate experiments. **C.** Binding of rCvGal1 to *P. marinus* trophozoites: Carbohydrate-specific binding of rCvGal1 to *P. marinus* trophozoites in the presence and absence of thiodigalactose (inhibits rCvGal1 binding) or glucose (does not inhibit rCvGal1 binding) was analyzed by immunofluorescence staining. Scale bar, 10 μ m [Adapted from Tasumi and Vasta (2007), with permission from the American Association of Immunologists and the American Society for Biochemistry & Molecular Biology.

to explain the cross-linking of bacterial surface carbohydrates (LPS or LTA) to the hemocyte surface glycans both interactions taking place via the *MjGal* CRD, it is possible that *MjGal* can form oligomers, dimers or trimers, as illustrated in the cartoon (Fig. 5A). Furthermore, although the bacterial numbers in circulation are reduced by the opsonic activity of *MjGal*, it remains to be elucidated whether the phagocytosed bacteria are killed by the typical hemocyte oxidative burst and digested by lysosomal activity (Fig. 5B and C), as observed in other invertebrate models (Vazquez et al., 2009; Nakamura et al., 2012; Xia et al., 2018).

5. The opsonic activity of galectins from bivalve mollusks can promote parasite infection

During our initial study on the mechanism(s) for uptake of the protozoan parasite *Perkinsus marinus* by the eastern oyster (*Crassostrea*

virginica) hemocytes we identified in EST databases two galectins (CvGal1 and CvGal2) of unique CRD organization (Tasumi and Vasta, 2007; Feng et al., 2013; Feng et al., 2015). A similar search on our RNAseq database from the softshell clam *Mya arenaria* we identified one galectin transcript (MaGal1) that was about half the length of the oyster galectins, this observation supported by the electrophoretic mobilities of the recombinant proteins (Fig. 6A) (Vasta et al., 2020). Based on sequence analysis, CvGal1 and CvGal2 have 4 tandemly arrayed CRDs, whereas the clam galectin (MaGal1) has 2 CRDs in each polypeptide (Fig. 6B) Vasta et al. (2020). Within each polypeptide, the 4 CRDs are structurally similar but not identical, suggesting that they differ in their fine specificity. As follows below, our biochemical, structural and functional studies on these galectins are illustrated with CvGal1. Our *in vitro* binding-inhibition studies showed that the recombinant CvGal1 prefers GalNAc to Gal, and strongly binds to

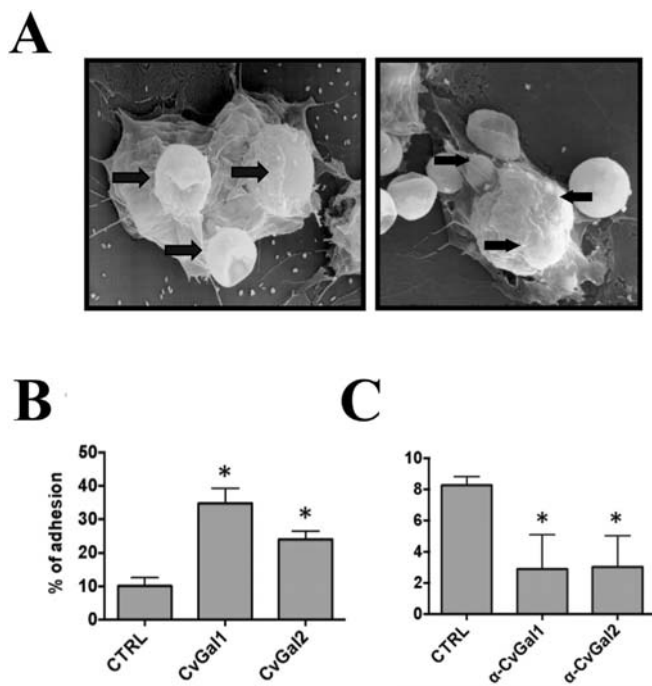


Fig. 10. The oyster galectin CvGal1 promotes adhesion of *Perkinsus marinus* trophozoites to the hemocyte surface. **A.** SEM of attached oyster hemocytes with *Perkinsus marinus* trophozoites attached to the cell surface and in the process of being phagocytosed. **B.** Pre-incubation of *P. marinus* trophozoites with rCvGal1 or rCvGal2 for 1 h significantly enhances their adhesion to the hemocyte cell surface. **C.** Pre-incubation of oyster hemocytes with anti-CvGal1 or anti-CvGal2 purified Igs for 1 h significantly reduces the adhesion of *P. marinus* trophozoites to the hemocyte cell surface. * indicates significant difference ($p < 0.05$) from control sample [Adapted from Tasumi and Vasta, 2007, with permission from the American Association of Immunologists and the American Society for Biochemistry & Molecular Biology].

glycoproteins that are rich in blood group A oligosaccharides such as the porcine stomach mucin (PSM) (Tasumi and Vasta, 2007; Feng et al., 2013; Feng et al., 2015).

Analysis of CvGal1 specificity on a glycan microarray confirmed these results (Fig. 7A) and showed that oligosaccharides that display nonreducing terminal GalNAc linked in α 1–3 to a subterminal Gal, such as the complex bi-antennary GalNAc α 1–3(Fuca1–2)-Gal β 1–4GlcNAc β 1–2Man α 1–6 [GalNAc α 1–3(Fuca1–2)-Gal β 1–4GlcNAc β 1–2Man α 1–3]Man β 1–4GlcNAc β 1–4GlcNAc β and GalNAc α 1–3(Fuca1–2)-Gal β 1–3GlcNAc β 1–2Man α 1–6 [GalNAc α 1–3(Fuca1–2)-Gal β 1–3GlcNAc β 1–2Man α 1–3]Man β 1–4GlcNAc β 1–4(Fuca1–6)GlcNAc β were the strongest recognized ligands. These structures

further suggested that the anomeric linkage linked in β 1–4 or β 1–3 of the subterminal Gal to the GlcNAc affects the binding and that CvGal1 prefers type 2 backbone structures Gal β 1–4 GlcNAc in N-acetylglucosamine, typical of most galectins described to date, and shows weaker binding to type 1 backbones Gal β 1–3GlcNAc. In addition, a Fuca1–2 bound to the subterminal Gal, as typical of blood group A oligosaccharides, further strengthens the binding. CvGal2 also preferentially recognizes ABH oligosaccharides, but here are significant differences in specificity with CvGal1 and MaGal1. The assessment of the binding affinity of CvGal1 by surface plasmon resonance (SPR) and solid phase binding studies revealed that this oyster galectin prefers the blood group A tetrasaccharide over the trisaccharide (Fig. 7B). A model of the CvGal1 binding site and docking of the blood group A tetrasaccharide enabled the rationalization of the structural basis for the CvGal1 preference for the blood group A tetrasaccharide (Fig. 7C) (Feng et al., 2013).

CvGal1 is expressed in most tissues tested but the transcripts are most abundant in hemocytes suggesting that the signals observed in other tissues may originate in infiltrating hemocytes (Fig. 8A). In circulating hemocytes most of the CvGal1 protein remains as an intracellular protein, but when hemocytes attach and spread on a foreign surface, the CvGal1 is secreted to the extracellular space (Fig. 8B). The secreted CvGal1 binds to the external surface of the attached and spread hemocytes, as shown by immunofluorescence on non-permeabilized hemocytes (-TritonX₁₀₀) (Fig. 8C). Based on the results from the specificity analysis of CvGal1, we tested the hemocytes for the potential presence of blood group A surface moieties with an anti-A monoclonal antibody and obtained strong staining as assessed by FACS (Fig. 8D). Pretreatment of the hemocytes with GalNAc α ase, a glycolytic enzyme that cleaves non-reducing terminal GalNAc, reduced the anti-A monoclonal antibody to the baseline (Fig. 8E). As GalNAc is the key sugar that characterizes blood group A oligosaccharides and determines the specificity of anti-A antibodies and lectins, these results led to the conclusion that CvGal1 recognizes blood group A moieties on the hemocyte surface (Tasumi and Vasta, 2007; Feng et al., 2013; Feng et al., 2015). This was rigorously confirmed by a glycoproteomic study by MS on the oyster hemocyte isolated glycoproteins (α integrins and dominin) that are recognized by CvGal1 (Feng et al., 2013; Kurz et al., 2013).

In addition to binding to the endogenous (“self”) hemocyte surface glycans, the oyster CvGal1 can also recognize and bind to a wide variety of glycans of bacteria, both G+ and G-, with variable degrees of preference for specific species and strains (Fig. 9A), and also several species of microalgae that are components of the phytoplankton on which the oyster feeds (Tasumi and Vasta, 2007; Vasta, 2009; Vasta et al., 2015, 2020). Most importantly, it can also recognize the parasite *Perkinsus marinus* trophozoites with the highest affinity (Fig. 9B), and this binding is specific for galactosyl moieties, as it can be completely inhibited by thiodigalactoside (TDG) but not by glucose (Glc) (Fig. 9C) (Tasumi and

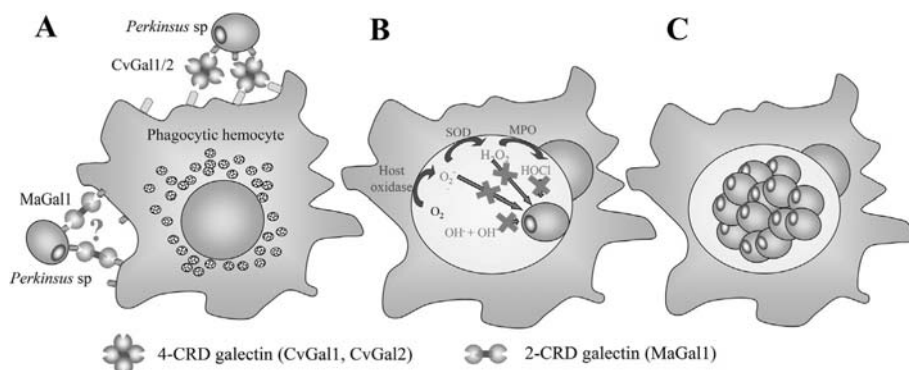


Fig. 11. Schematic model of the proposed roles of an oyster galectin (CvGal1 or CvGal2) in the uptake of *Perkinsus marinus* trophozoites by hemocytes. **A.** The 4-CRD galectin (CvGal1 or CvGal2) from the eastern oyster (*C. virginica*) is secreted by the hemocytes and can cross-link the blood group oligosaccharide moieties on the hemocyte surface to glycans of similar topology on the surface of the parasite *P. marinus*. **B.** CvGal1-mediated recognition and opsonization promotes adhesion of the trophozoites and leads to enhanced phagocytosis. The *P. marinus* trophozoites escape oxidative killing by expression of anti-oxidative enzymes (Superoxide dismutases, ascorbate-dependent peroxidases, etc). **C.** Surviving phagocytosed *P. marinus* trophozoites proliferate within the hemocyte, eventually causing systemic infection and death of the oyster host.

Vasta, 2007).

The strong recognition of the parasite *P. marinus* trophozoites by oyster hemocytes leads to their adhesion to the hemocyte cell surface as the first step to their phagocytosis (Fig. 10A). Pre-exposure of the parasite *P. marinus* trophozoites to CvGal1 or CvGal2 significantly enhances their adhesion of the *P. marinus* trophozoites surface (Fig. 10B), whereas pre-incubation of the oyster hemocytes with anti-CvGal1 or anti-CvGal2 antibodies significantly hinders trophozoite adhesion to the hemocyte surface (Fig. 10C). These results strongly suggest that function as active opsonins for *P. marinus* trophozoites, promoting their adhesion to the phagocytic cell surface. Taken together, these results strongly suggested that both extracellular oyster galectins CvGal1 or CvGal2 can function as opsonins that recognize *P. marinus* trophozoites and promote their adhesion to the phagocytic cell surface. This opsonic activity contributes to phagocytosis of the *P. marinus* trophozoites (Fig. 11A). The galectin MaGal1 also effectively recognizes *P. marinus* trophozoites, but the details of its potential role as opsonin for the parasite remain to be elucidated (Fig. 11A). The *P. marinus* trophozoites are endowed of a very powerful anti-oxidative enzyme repertoire that includes two SODs and two APXs, and the phagocytosed parasites survive intracellular killing by inhibiting or degrading the ROS produced by the oyster hemocytes (Fig. 11B) (Schott et al., 2003a,b, 2019; Fernández-Robledo et al., 2008). Therefore, the trophozoites take advantage of the intra-hemocytic environment for nutrition, they multiply intracellularly, and eventually cause a systemic infection and kill the oyster host (Fig. 11C) (Vasta, 2009).

6. Summary and conclusions

As discussed throughout this review, while galectins can participate in multiple regulatory functions by recognizing endogenous (self) cell surface, ECM, and soluble glycans, evidence has accumulated in the past few years to support that galectins can also recognize exogenous (non-self) glycans present on the surface of viruses, bacteria, and eukaryotic parasites. Therefore, they can be considered *bona fide* pattern recognition receptors (PRRs). An illustrative example of the recognition of structural topologies ("patterns") on distinct glycans by a galectin is the binding of the proto-type galectin from the toad *Bufo arenarum* to different carbohydrates with similar affinity (Bianchet et al., 2000). The crystal structures of the toad galectin in complex with LacNAc and TDG revealed that the protein establishes identical interactions with the non-reducing terminal Gal that is shared by both disaccharides, but different contacts with the sub-terminal sugar unit (GlcNAc in LacNAc, and Gal in TDG). The number and quality of H-bonds, however, is similar in both complexes, revealing the structural plasticity of the galectin CRD to establish compensatory protein/sugar interactions for the two chemically distinct disaccharides that result in similar binding affinity (Bianchet et al., 2000). Galectins, however, do not rigorously fit the definition of PRRs as described by Janeway and Medzhitov (2002). PRRs recognize pathogens *via* widely distributed, highly conserved microbial molecules (pathogen- or microbe-associated molecular patterns, PAMPs or MAMPs) such as LPS or peptidoglycan, which are absent in the host. In contrast, galectins can recognize similar carbohydrate ligands that are displayed on both the host and the pathogen cell surface. This apparent paradox reveals our limited knowledge about the actual diversity in recognition of the host galectin repertoire, and as well as the structural and biophysical aspects of multivalent ligand binding (Vasta, 2009; Dam and Brewer, 2008, 2010).

As discussed above, the recognition of glycans on the surface of bacteria and parasites, and on the surface of the invertebrate phagocytic cells (hemocytes) enables soluble galectins to function as effective opsonins. Thus, the host galectins promote adhesion of the pathogen to the hemocyte and contribute to the phagocytosis of the infectious agent. In the shrimp, the opsonic activity of MjGal leads to enhanced clearance of the circulating bacteria, supporting their effective role in innate immune response to infection. In the oyster, however, although

galectins are equally capable of recognizing multiple environmental bacteria, the parasite *Perkinsus marinus* appears to subvert galectin function by taking advantage of this recognition system to enter the host hemocytes, block intracellular oxidative killing, and proliferate to produce a systemic infection. Thus, the opsonic activity of invertebrate galectins, can lead to various and sometimes opposite outcomes, that benefit either the host or the pathogen. The key roles played by galectins in development, tissue repair, and immune homeostasis via recognition of endogenous glycans, has most likely imposed severe functional constraints against evolutionary structural changes, resulting in the striking conservation of this lectin family in both structure and specificity. Therefore, it should be not surprising that pathogens would have co-evolved with their hosts by adapting their surface glycomes to "hijack" the recognition and opsonic properties of host galectins for attachment and entry into the host cells in a "Trojan horse" model (Vasta, 2009).

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List of abbreviations

MjGal	Marsupenaeus japonicus galectin
MjGal ^{Δ102–106}	deletion mutant of MjGal
CvGal1	Crassostrea virginica galectin 1
CvGal2	Crassostrea virginica galectin 2
MaGal1	Mya arenaria galectin 1
BaGal1	Bufo arenarum galectin-1
hGal-1	human galectin-1
CGL2	Coprinus cinereus galectin-2
CRD	carbohydrate recognition domain
GalNAc	N-acetyl-D-galactosamine
GlcNAc	N-acetyl-D-glucosamine
LacNAc	N-acetyl-lactosamine
LTA	lipoteichoic acid from Staphylococcus aureus
LPS	lipopolysaccharide from Escherichia coli
PGN	peptidoglycan from Micrococcus luteus
TDG	thiodigalactose
BSM	bovine submaxillary mucin
OSM	ovine submaxillary mucin
PSM	porcine stomach mucin
Trx-His	recombinant thioredoxin carrying a His tag
RNAi	RNA interference
dsRNA	double stranded RNA
SPR	surface plasmon resonance
PRR	pattern recognition receptors

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