Review

Galectin-9: From cell biology to complex disease dynamics

Sebastian John and Rashmi Mishra*

Department of Neurobiology and Genetics, Division of Disease Biology, Rajiv Gandhi Centre for Biotechnology, Poojappura, Thiruvananthapuram 695014, India

**Corresponding author (Email, rashmimishra@rgcb.res.in; r.mishra2007@gmail.com)*

Both authors have contributed equally to the manuscript.

Galectins is a family of non-classically secreted, β-galactoside-binding proteins that has recently received considerable attention in the spatio-temporal regulation of surface 'signal lattice' organization, membrane dynamics, cell-adhesion and disease therapeutics. Galectin-9 is a unique member of this family, with two non-homologous carbohydrate recognition domains joined by a linker peptide sequence of variable lengths, generating isoforms with distinct properties and functions in both physiological and pathological settings, such as during development, immune reaction, neoplastic transformations and metastasis. In this review, we summarize the latest knowledge on the structure, receptors, cellular targets, trafficking pathways and functional properties of galectin-9 and discuss how galectin-9-mediated signalling cascades can be exploited in cancers and immunotherapies.

[John S and Mishra R 2016 Galectin-9: From cell biology to complex disease dynamics. *J. Biosci.* **41** 507–534]

1. Introduction

1.1 *The 'Sugar code' and galectins*

A novel mode of unambiguous biological information transfer and storage has recently emerged in the form of 'sugar code', which is far more abundant, complex and diverse than the genetic code itself and is defined by the specific sugar moieties resident on the head groups of lipids and proteins. These 'sugar codes' store the information for the execution of various spatio-temporal cellular events such as cell division, differentiation, adhesion, migration, metabolism and signal transduction (Villalobo and Gabius 1998; Gabius 2000; Gabius *et al.* 2004; Murphy *et al.* 2013). Parallel to the evolution of these sugar codes, a unique family of secreted proteins that recognizes and interprets these codes has emerged, and is termed as galectins due to its binding affinity to β-galactoside-containing oligosaccharides and a display of lectin-like clustering activity (Kasai and Hirabayashi 1996).

Galectins are evolutionary expressed from nematodes to humans (Nagae *et al.* 2008; Yang *et al.* 2008), and all mammalian galectins have an evolutionary conserved carbohydrate recognition domain (CRD) of about 130 amino acids which bind to oligosaccharides via the recognition of the β-galactoside units. To date, 16 mammalian galectin protein members have been identified and all of them lack a recognizable signal sequence for their transport into the classical ER-Golgi cargo trafficking machinery, and hence they are proposed to be secreted out into the extracellular environment directly from the cytoplasm. The identified galectins can be further classified into three types on the basis of number of CRDs, namely proto, chimera and tandem-repeat types. Prototype galectins (gal-1, -2, -5, -7, -10, -11, -13, -14, -15, and -16) are characterized by one CRD, while the chimera-type gal-3 has one C-terminal CRD and a long N-terminal tail composed mostly of collagen-like repeats that terminate in a short non-collagenous terminal peptide sequence. The tandem-repeat type galectins (gal -4,-6,-8,-9,-12) possess two CRDs connected by a linker domain of variable length that governs several distinct biophysical properties and functions of these proteins (Heusschen *et al.* 2013).

Galectins are reported to have multifarious roles in the nucleus, cytoplasm, different organelles, cell surfaces and in the extracellular matrices. Besides, the secretory pathway of

Keywords. Cancer; carbohydrate recognition domain; galectin-9; immune-regulation; linker peptide

Supplementary materials pertaining to this article are available on the Journal of Biosciences Website.

DOI: 10.1007/s12038-016-9616-y

Published online: 16 July 2016

galectins is highly intriguing. Just prior to secretion galectins give an appearance of accumulated free polysomes in the cytoplasm and are secreted out as soluble non-glycosylated galectins through the ER/Golgi-independent pathway. Whether there exists a convergence and co-ordination of the secretory pathway of galectins with that of other non-classically secreted growth factors, cytokines (e.g. fibroblast growth factors FGF-1 and FGF-2 and interleukin IL-1β) and intravesicles is an interesting possibility that still needs to be explored (Hughes 1999).

1.2 *Focus on galectin-9*

Galectin-9 is a 34–39 kDa tandem repeat type protein (LGALS9 in human, lgals9 in mouse) with two non-homologous N- and C-terminal CRDs joined by a short polypeptide called the linker domain. Variable lengths of the linker domain generate Gal-9 isoforms that bind to different glycan units with differential avidities and affinities. This CRD binding event is found to be intriguingly pH sensitive, implicating a crucial regulatory role of acidification on the assembly and disassembly of galectin-glycan lattices for spatio-temporal control of signal transduction (Mishra *et al.* 2010). Further, Gal-9 oligomeric properties cluster homo and hetero protein–lipid modules that may cumulatively act as a functional signalling unit in diverse locations such as on the surface, in the endocytic-exocytic vesicles for cargo trafficking, at the chromatin as a histone modifier and transcription factor, at the junction of two or more cells for cell–cell communications and in the exosomes for paracrine signal dispersal in tissue morphogenesis. Hence, co-existence of several of these events can collectively trigger a specific function. Indeed, with these multivalent properties, Gal-9 finds its functions in cell adhesion, cell surface recognitions, migration, chemoattraction and as a modulator of important signalling between growth and apoptosis (Heusschen *et al.* 2013). Therefore, a detailed study on Gal-9 as a decoder of glycome or sugar diversities, which enables its diverse genetic and epigenetic phenomena, will certainly illuminate its potential as a key diagnostic and therapeutic agent. Hence, we find it timely to put forward this review that summarizes the latest information on the structural, biochemical and extracellular–intracellular functional properties of Gal-9 and further discusses the recent findings on its use in various biomedical applications.

2. Structure and biochemial characteristics of Gal-9

Gal-9 was initially described by three independent groups as (i) an auto-antigen derived from the tissue involved in Hodgkin's lymphoma (Tureci *et al.* 1997), (ii) as an ecalectin, a 36 kDa eosinophil chemoattractant expressed on T lymphocytes (Matsumoto 1998) and (iii) as a urate transporter, a transmembrane protein cloned from the rat kidney (Tao 1997). Later these three proteins were recognized as the variants of Gal-9 with minute differences in the amino acids that overall influenced their functional and structural characteristics with one of them (urate transporter) being transmembranous and others being either membranous or secretory in nature (Matsumoto 1998; Miyanishi *et al.* 2007).

With the two-CRDs, Gal-9 exhibits multivalency even as a monomer and hence can act as a linking bridge between specific carbohydrates. The homo and hetero oligomerization of Gal-9 forms ordered arrays of complexes upon binding to multivalent glycoconjugates, akin to the lattices formed by antibodies and multivalent antigens. Such supramolecular lattice organization, in two and three dimensions with saccharide sequences that are displayed as *N*- or *O*-glycans on the cell surface accounts for a specific biological function. Therefore, Gal-9 lattices can potentially cluster several multivalent glycoconjugates between the two cells of the same or different type and simultaneously trigger the cellular interaction with the extracellular matrix proteins (Nonaka *et al.* 2013).

Recognition of the β-galactoside residue by the S4 strand is the most conserved feature of galectin-binding activity (Nagae *et al.* 2006, 2008). The four –OH groups of the β-galactoside residue forms the critical hydrogen bonds with highly conserved residues within the galectin CRD (Nagae *et al.* 2006, 2008). Even though the individual N- and C-terminal of Gal-9 CRDs have different oligosaccharide-binding affinities and in general mark a substantially lower activity than full-length wild-type Gal-9 protein, the two CRDs have the potential to exert their own individual biologically important activities based on the linker polypeptide forms. However, the anti-proliferative and pro-apoptotic activity of Gal-9 CCRD is shown to be greater than that of G9NCRD and may be due to the difference between the activities of G9CCRD and G9NCRD. These findings suggest that each CRD has the potential to form oligomers to exert its activity.

The recombinant chimeric proteins consisting of two NCRDs or two CCRDs joined by a linker display the same eosinophil chemoattractant activity as wild-type galectin-9; however, the cleavage of the linker peptide is documented to bring about a decreased physiological activity of Gal-9 as an eosinophil chemoattractant (ECA) (Hughes 1999; Asakura *et al.* 2002; Sato *et al.* 2002; Nishi *et al.* 2006) suggesting the indispensability of linker domain in computation of the strength of interaction *vs.* the heterogeneity of the N- and C-terminal domains in at least some biological processes.

The structures of the NCRD (Nagae *et al.* 2006, 2008; Solís *et al.* 2010) and CCRD (Yoshida *et al.* 2010) were solved, by X-ray crystallography. In addition to this, a study (Nonaka *et al.* 2013) used nuclear magnetic resonance

(NMR) experiments to investigate Gal-9 CCRD. Even though the complete structural analysis of full-length Gal-9 has never been successful due to the low solubility of Gal-9 (Nonaka *et al.* 2013), details on biochemical and functional aspects of Gal-9 has emerged from the N- and C-terminal crystal structures and have strongly influenced the interpretations of experimental observations. Of note is that the two CRDs of Gal-9 display conserved amino acids that are involved in β-galactoside binding but overall they have only 38% similarity with each other (Tureci *et al.* 1997) .

2.1 *N-terminal carbohydrate recognition domain (NCRD)*

The NCRD of Gal-9 has 124–147 amino acids with six (S1-S6)- and five (F1-F5)-stranded β-sheets. They are closely packed to resemble a sandwich (Nagae *et al.* 2006) where the β-galactoside binding moiety lies in the S4-S6 strands, with Tryptophan (W), Histidine (H) and Asparagine (N) as the key Gal-9 and β-gal interaction residues. Among these residues, H (60th position in mouse Gal-9 and 61st in human) and W (81^{M}/ 82^{H}) (81st position in mouse Gal-9 and 82th in human) facilitate the alignment of CRD with the glycan epitope, while W forms the CH-π interaction with the galactose ring. A crucial hydrogen bond is formed by N and H with O4 of galactose and an additional hydrogen bond between O6 of galactose and N completes the interaction. The other crucial residues that take part in the glucose recognition are the two residues R (arginine) at 64^{Mouse}/65^{Human} and 86^{Mouse}/87^{Human} and of E (glutamic acid) at 84^{Mouse}/85^{Human}

Apart from other lectin-carbohydrate-binding interactions, the overall structure of NCRD does not change for energy minimization during ligand binding due to a basic amino acid patch near NCRD in contrast to prototype galectins that bears a negative patch of amino acids in the same region. These variations of Gal-9 NCRD may account for the differences in ligand affinities as compared to prototype galectins (Nagae *et al.* 2006, 2008). Evidences suggest that the net structure of Gal-9 NCRD is similar to the CRD of Gal-3 and Gal-7 with differences only in the carbonyl backbone of S6 strand of oxygen molecules (Nagae *et al.* 2006).

The mouse Gal-9 (mGal-9) NCRD shows only 67% identity with human Gal-9 (hGal-9) NCRD, unlike other galectins that show more than 75% similarity with their mouse counterparts (Nagae *et al.* 2008). This difference is projected in the differential ligand affinities and dimerization abilities of the two species. The binding specificity of human Gal-9 NCRD differs significantly from that of the mouse protein. hGal-9NCRD showed higher affinity for galactosyl glycans, glycolipid-type glycans and sialylated glycans than the mouse counterpart. For example, affinity for Forssman pentasaccharide by hGal-9 NCRD is 93 times higher than for mGal-9, the reason being the presence of A136 and N137 (A=alanine, N=asparagine) in human NCRD. In addition to this, the crystal structure of mGal-9 shows dimer formation, whereas purified hGal-9 NCRD is suggested to exist in equilibrium between the monomer and dimer. In mouse, dimer formation between NCRDs is achieved through the interactions between R68, E84 and M88 (R=arginine, E=glutamic acid, M=Methionine) of S6 strands in mouse. Another study demonstrates that due to the mutation of M88 to T89 (M=Methionine, T=Tyrosine), self-dimerization is not seen for hGal-9 NCRD (Nagae *et al.* 2006, 2008). However, surface plasmon resonance based studies have indicated that hGal-9 NCRD forms a dimer with relatively very weak interaction, but with an unknown activity. Interestingly, the crystal structures of the human and mouse Gal-9 NCRDs has elucidated the molecular basis of this difference and shows that hGal-9 NCRD has no hydrophobic cluster exposed to the solvent on the F3 and F4 strands (Nonaka *et al.* 2013), and hence may not require self-dimerization to mask the hydrophobic regions. Whether the difference in self-dimerization in human and mouse Gal-9 NCRDs is also responsible for differences in anti-proliferative activities needs further elucidation. Also, Asn137 in human Gal-9 NCRD is replaced by serine in mouse Gal-9 NCRD. Thus, Asn137 would be an important residue for the tight interaction with poly-*N*-acetyllactosamine in human galectin-9 NCRD (Nagae *et al.* 2009). These observations suggest that the differences in the physiological properties of galectin-9 isoforms from different species may be driven due to amino acid substitutions on the protein surface (Nagae *et al.* 2008).

2.2 *C-terminal carbohydrate recognition domain (CCRD)*

The Gal-9 CCRD consists of 138 amino acids forming a β-sandwich structure with six (S1-S6)- and five (F1-F5)-stranded β-sheets stacked one over the other with F5 and S2 joined by an additional short α-helix. Although several histidine residues (8 His residues/146 amino acids) are found in Gal-9CCRD (Nonaka *et al.* 2013), carbohydrate binding region is formed by S3-S6 strands, with R and E as the key residues participating in carbohydrate recognition as that also seen in NCRD. Interaction with carbohydrate binding region is achieved through W (255), R (239), N (237), E (258) and H (235), forming the stacking interaction with galactose ring, while R, N and H build a hydrogen bond with the axial O4 of galactose. The rest of the amino acids involved in the interaction with galactose through water-mediated hydrogen bond networks are H (235), N (237) and W (255). However, despite the similarity between the ligand-bound and ligand-free structure of CCRD, small structural variations at the loop regions are observed (Yoshida *et al.* 2010).

Again, despite the same carbohydrate recognition key residues of Gal-9 NCRD and CCRD, there exists a structural

difference in the loop region connecting the strands. This structural change in loop region is brought about by the amino acids (added, removed or substituted) in either of the two CRDs. This difference changes the positioning of the glycan units on the CRD, leading to differences in affinity for even the same glycan unit (Yoshida *et al.* 2010). The hGal-9 CCRD is fairly homologous to NCRD (amino acid sequence identity of 37%) but exhibits 3.3 to 5.7 times lowered affinities for N-acetyllactosamine oligomers Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (LN2), Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (LN3) and Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (LN5) than NCRD (Hirabayashi *et al.* 2002).

Curiously, the two residues Tyr298 and Asn303, located at the F5-H1 loop behind the S4 and S5 strands, were not considered to be involved in the carbohydrate binding, but recently, NMR spectroscopy results have shown that significant changes can be brought about by the carbohydrate binding in the structural dynamics of the S4 and S5 strands that can be transmitted to the F5-H1 loop via the hydrophobic core that can enable ligand binding (Nonaka *et al.* 2013).

It has been recently shown that Gal-9CCRD is a basic protein with pI 8.54 and can easily aggregate at a higher salt concentration or at neutral pH (Nonaka *et al.* 2013). Interestingly, Gal-9 CCRD has 78% amino acid similarity with that of Gal-5 CRD. Therefore, the oligomerization property of Gal-5 CRD can give additional clues about the oligomer formation by Gal-9 CCRD. However, trimerization of CCRDs has also been observed under *in vitro* condition in the presence of a bivalent Ni ion. Recently, it has been shown that at higher concentrations, CCRDs have the ability to self-associate through F3-F4 strands. Disrupting this association is found to decrease the anti-proliferative activity of CCRD. Also in physiological conditions and at higher concentrations, oligomerization of full-length Gal-9 protein can be achieved through the C-terminal domain, suggesting that Gal-9 can perform different physiological functions at different concentrations/states of multimerizations. Hence, the differences between the Gal-9 NCRD and CCRD may also be predominantly due to the tendency to self-associate, rather than just towards ligand-binding ability, which may crucially influence the individual roles of both of the CRDs (Nonaka *et al.* 2013).

2.3 *The linker domain*

Both the CRDs of Gal-9 are connected by a 14- to 56 amino-acid-long polypeptide chain, called the linker domain. Based on the shortest, medium and longest length of the linker peptide, i.e. Gal-9 with linker length of 14, 26 and 56 amino acids (Sato *et al.* 2002), three isoforms of Gal-9 are described as small (S), medium (M) and long (L) (constituting of 311, 323 and 355 amino acids, respectively). These differences, as well as conservations in the linker regions, confers Gal-9 isoforms with different functional properties. In contrast to the earlier unclear functions of the linker domain, recent evidences speculate the role of linker region in protein–protein interactions, membrane insertions and regulation of CRD presentations. Furthermore, these diverse linker domains are also involved in strengthening the signalling potency by intermolecular interaction of the CRDs and through multimerization, thereby leading to the formation of multivariant multimers that increases the lattice formation on the cell surface. Thus the linker peptides can give multivalent property to Gal-9 that leads to several important physiological functions at a greater potency as compared to the proto-type galectins.

2.4 *Significance of Gal-9 protease cleavage sites in the linker domain of galectin-9*

Gal-9L has a metalloproteinases (MMP3/ stromelysin), elastases and thrombin cleavage sites on its linker domain (Nishi *et al.* 2005, 2006). MMPs are metal-dependent enzymes capable of degrading various extracellular matrix proteins and are involved in cleavage of cell surface receptors, release of apoptotic ligands, chemokine/cytokine activation and processing of bioactive molecules through which they can regulate various cellular activities such as proliferation, migration, adhesion, differentiation, angiogenesis, apoptosis and host defence mechanisms. Gal-9 contains an MMP-3 cleavage site and is shown to aid its secretion into the extracellular milieu. In Jurkat cells, unknown MMPs are involved to facilitate Gal-9 secretion through the protein-kinase-C-dependent pathway (Chabot *et al.* 2002). Elastases, on the other hand, are the protease that break down elastin, an elastic fiber that together with collagen determines the mechanical properties of the connective tissues, extracellular matrices and stretch prone proteins. In lung cystic fibrosis, Gal-9 and its surface receptor TIM-3 (T-cell immunoglobulin mucin domain-3) are demonstrated to undergo rapid proteolytic degradation primarily because of neutrophil elastase and proteinase-3 contributing to neurophil-driven inflammation (Vega-Carrascal *et al.* 2011). Further, thrombin is a serine protease implicated in blood clotting and inflammation. Its recognition site in the linker region of hGal9L is –PRPRGRRQ– (cleavage of peptide bond at the C-terminal side of R175) and that in mGal9L is –QFPRTPKGRK– (cleavage of peptide bond possibly at the C-terminal side of R170). Since this insertion portion is absent in medium and short isoforms, these Gal-9 forms are resistant to thrombin cleavage. However, in Gal-9L, such protease cleavage may generate free N- and C-CRDs, which can homo-oligomerize and bind to different

glycoconjugates to effect different outcomes of a cellular event; for instance, Gal-9 N-terminal is efficient in activating dendritic cells (DC), whereas C-terminal oligomerization contributes to T-cell death.

Interestingly, in the case of thrombin cleavage, physiological activity of Gal-9 is decreased as an eosinophil chemoattractant (ECA) (Nishi *et al.* 2006) due to the fact that independent N- and CCRDs of Gal-9 after thrombin cleavage can efficiently bind to various closely related glycoconjugate ligands on the eosinophil membrane and crosslink them to remodel them for downstream signalling (Asakura *et al.* 2002). The isolated domains probably bind to different glycoconjugates than the full-length Gal-9, contributing to decreased eosinophil chemoattractant (ECA) activity. Hence, thrombin cleavage site serves as regulatory switch on the activity of Gal-9 protein. Therefore, this process of thrombin cleavage is associated with regulation of movement, adhesion and tissue extravasations of eosinophils to the site of inflammation and is also required during bronchial asthma, helminthic infection, atopic dermatitis, rhinitis and in certain types of malignant neoplasm, e.g. Hodgkin's lymphoma (Matsumoto 1998). Suggestively, the suppression of this thrombin cleavage site in Gal-9 may be also advantageous in certain pathological events. For instance, activated CD4+ T-cells in the case of HIV-1 infection express chemoattractant receptors, similar to those on eosinophils, such as CCR5, CXCR4 and α4β7 [figure 1a(5)]. Recombinant Gal-9 NC (human–*E. coli* recombinant Gal-9 bearing N- and C-terminals with modified thrombin cleavage site in the linker domain, rendered to be protease resistant) is shown to significantly reduce the T-cell chemoattractant surface receptor expression probably via clustering, endocytosis and subsequent degradation. Hence, when the thrombin cleavage site is suppressed in full-length Gal-9, it may affect membrane retention of chemoattractant receptors on T cells, suppressing T-cell proliferation and activation, suggesting a selective advantage in inhibition of T-cell HIV invasion (Elahi *et al.* 2012).

Focused exploration on the exact functional significance of having such a thrombin cleavage site can further provide us with the key to Gal-9 functional maneuverings that maybe exceedingly important for therapeutic applications. For instance, the plasma-membrane-associated, G-protein-coupled, Proteinase-Activated Receptors (PARs) are direct substrate of thrombin that is located on eosinophils, platelets and endothelial cells (Cirino *et al.* 2000). PAR1 is a peptide receptor that carries its own ligand which remains silent until it is cleaved by thrombin at R41-S42 site of the N-terminal extracellular domain. The newly exposed N-terminus then serves as a tethered ligand for self-activation and transmembrane signalling with pleuripotent effects such as platelet activation, migration, clotting, NO and prostaglandin release (Strukova 2001). In endothelial cells, thrombin-activated PAR1 increases vascular permeability, partly by transactivation of SIP3 (sphingosine 1 phosphate receptor 3) with PAR1-SIP3 coupling in DCs, leading to the systemic inflammation in the late stages of severe sepsis (Obinata and Hla 2012). Similarly, thrombin cleavage may also self-activate Gal-9 into homo NNCRD and CCRD oligomers with different outcomes as described for PAR1.

2.5 *Sugar-binding specificities, raft association and binding partners of Gal- 9*

Gal-9 with different carbohydrate binding specificities exhibits bi/multivalent binding properties to cluster multiple multivalent glycoconjugates or oligosaccharides. Furthermore, clustering events finally exert a functional output through transmembrane signalling cascades. These signalling events involve interactions between similar or different types of cells with that of extracellular matrix proteins. Different intermolecular interactive techniques such as Frontal Affinity Chromatography have been applied to elucidate these sugar–protein binding specificities of Gal-9 NCRD and CCRD. Results show that Gal-9 involves a specific protein–protein as well as protein–lipid interactions, i.e. with specific inter- and intramolecular galectin bindings (with Gal-9, Gal-3 and -8 but not with Gal-1) (Miyanishi *et al.* 2007) as well as with distinct carbohydrate interactions by NCRD and CCRDs of Gal-9 (Nagae *et al.* 2006; Yoshida *et al.* 2010). The hGal-9 showed a higher affinity for branched *N*-glycan-type oligosaccharides than linear structured oligolactosamines (1–3-linked poly-*N*-acetyllactosamines).

Interestingly, Gal-9 CCRD and NCRDs exhibit different specificities for oligolactosamine and glycolipid-type glycans, the Forssman pentasaccharides and A-hexasaccharides. Independently, CCRD and NCRD show similar affinities towards branched oligosaccharides (Yoshida *et al.* 2010) and other glycolipid-type glycans such as for GA1, GM1, GD1a, GD1b and Gb4 and FGL (Ohtsubo *et al.* 2005; Nagae *et al.* 2008).

Only a few membrane receptors for Gal-9 have been so far identified even though a large number of glycoconjugates with potential β-galactoside binding sites are expressed on the membrane. Limited recognition of these binding sites could be a result of limited availability of binding sites due to conformational changes resulting from the differential environmental and cellular conditions. Exogenously introduced Gal-9 binds to CD44 and forms a CD44/ BMP receptor complex that leads to phosphorylation of smad 1, 5, 8 and results in osteoblast proliferation (Tanikawa *et al.* 2010) [figure 1a(C)]. Although the specific glycan ligands required for Gal-9-induced T-cell death are not yet known, complex *N*-glycans are found to be important for Gal-9-induced T-cell death. CD44 is found to be one of the target glycoproteins of

Gal-9 which acts as an important adhesion molecule for migrating lymphocytes and eosinophils. Gal-9 interaction with CD44 prevents CD44 from binding to hyaluronic acid, which is a principal ligand for CD44 and for providing a foothold for migrating cells; hence, this process attenuates accumulation of activated lymphocytes and eosinophils in the inflamed lesion (Niki *et al.* 2009).

In general, Gal-9 may bind to several cell surface receptors (Heusschen *et al.* 2013) and might influence intracellular molecules as well. These multipotent binding properties of Gal-9 have been assigned to its two N- and C- terminal carbohydrate recognition domains (CRD) (Nagae *et al.* 2006). The involvement of Gal-9 in several functions depends on the ligand availability on the cell surface which can change with different type of cells or with the (activation) status of the same cell type. Hence, Gal-9 can execute different functions depending on the cell type.

To date, receptors or surface binding partners that have been reported for Gal-9 includes glucose transporter 2 (GLUT- 2) (Ohtsubo *et al.* 2005), protein disulphide isomerase (PDI) (Bi *et al.* 2011) [figure 1a(I)], Forssman glycosphingolipid (FGL) (Mishra *et al.* 2010) [figure 1a(G)], T-cell immunoglobulin mucin-3 (TIM-3) (Zhu *et al.* 2005), immunoglobulin E (IgE) (Niki *et al.* 2009) [figure 1a(C)] and Glucagon receptor (Gcgr) (Johswich *et al.* 2014). Various other molecules are known to interact with Gal-9 with functional consequences, including the Epstein-Barr virus latent membrane protein-1 (Mengshol *et al.* 2010).

Furthermore, the multivalency of Gal-9 is shown to cluster membrane lipid rafts on osteoblasts, with an activity more potent than other galectins. Although very few receptors on lipid rafts have been identified, Gal-9 is shown to mediate important effects via binding to glycoconjugates present in these sphingolipid-cholesterol-enriched microdomains (Clayton *et al.* 2014).

Gal-9 is also found to be a resident membrane raft protein in nasopharyngeal carcinoma and is a partner of another membrane-associated protein LMP1 which is observed to have role in malignant phenotype of nasopharyngeal carcinoma (Pioche-Durieu *et al.* 2005). Gal-9 is also observed to cluster membrane rafts in human osteoblast and bring about phosphorylation of c-src on Tyr^{281} residue and ERK, which further leads to osteoblast proliferation [figure 1a(C)].

In Madin-Darby canine kidney epithelial cells type II (MDCKII), it has been shown that knockdown of Gal-9 can cause severe depletion of its glycosphingolipid receptor (FGL) suggestive of the negative feedback loop and coupling of lipid–protein biosynthetic pathways (Mishra *et al.* 2010). Here, FGL-Gal-9 interaction is reported for cell polarization. FGL glycan chain acts as the specific membrane-associated receptor. FGL receptor cycles Gal-9 to the Golgi apparatus from which the protein is recycled back to the apical surface (Mishra *et al.* 2010), thereby maintaining polarization of cells [figure 1a(G)].

However, normal human cells lack the ability to produce FGL, but a subset of human population has been reported to express Forssman glycoconjugates in colon, cervical and gastric cancers, thus implying an active relationship between Forssman antigen expression and cancers (Nagae *et al.* 2008). Also crystallization studies show that the NCRD of hGal-9 exhibit a striking affinity for the complex glycoconjugate Forssman pentasaccharide than the hGal-9 CCRD (Nagae *et al.* 2008). These results emphasize the need to determine the physiological as well as the pathological meaning of interaction between Gal-9 and Forssman pentasaccharide/antigen.

Another interesting example comes from hepatic Gcgr, a class-B G-protein-coupled glucagon receptor involved in glucagon action and development of hyperglycaemia/insulin resistance. Gal-9 is shown to promote crosslinking and dimerization of Gcgr, thereby slowing down the surface receptor mobility to organize a lattice, wherein the other glycoprotein receptors are also recruited to increase the Gcgr ligand sensitivity and this promotes hyperglycaemia, attributed to the diabetic condition (Johswich *et al.* 2014). Hence, in such cases, glucagon receptor binding sites for galectin must be inhibited by small molecule drugs or synthetic sugars that can effectively compete with Gal-9 binding.

Galectin-9 interaction with the cell surface glycoproteins serves to regulate the formation and maintenance of plasma membrane domains (Bi *et al.* 2011). The glucose transporter GLUT-2 involved in glucose-stimulated insulin secretion acts as an important Gal-9 ligand, thereby gaining an indirect control over the blood glucose homeostasis in response to the dietary intake. Gal-9 recognizes carbohydrate moiety of GLUT-2-expressed extracellularly on the pancreatic β-cell surface by binding to the N-glycan branch of GLUT-2. This facilitates the plasma membrane retention of GLUT-2 and prevents the GLUT-2 endocytosis into the cytoplasm and thereby sustains the primary glucose-stimulated insulin secretion response. Hence, Gal-9 can also de-promote the development of diabetes, unlike the case in which it interacts with Gcgr (Ohtsubo *et al.* 2005).

Gal-9 also enables a similar plasma membrane retention phenomenon with the soluble oxidoreductase family member, protein disulphide isomerases (PDI). By modifying the thiols on the cell surface proteins, PDI can alter the redox status of the plasma membrane, thereby influencing many of the T-cell events. PDI is the unique Th2-cell receptor and Gal-9 binds at the *O*-glycans of PDIs [PDI (P4HB), PDIA3 (ERP57, GRP58), and PDIA6 (ERP5)]. This binding increases the endogenous activity of PDI thereby enhancing the β3 integrins or CD61-mediated extracellular matrix migration of Th2 cells (Bi *et al.* 2011) which is brought about by the redox status of cell surface glycoproteins that remains associated with PDI. PDI can form complex with HIV gp120, CD4 and CXCR4 on the surface of T cells

[figure 1a(1)]. Contrastingly, Gal-9 binding to PDI on T cells potentiates infection with HIV.

Galectin-9 recognizes and interacts with carbohydrate(s) covalently attached to the surface of TIM-3 (T-cell immunoglobulin mucin-3) by binding to the *N*-glycan on TIM-3 (Yoshida *et al.* 2010). TIM-3 is one of the best studied natural ligand of Gal-9 and is a type 1 membrane protein with a structurally conserved immunoglobulin variable (IgV) domain and a mucin stalk that anchors to an intracellular tail with SH2 phosphorylation domain (Sakuishi *et al.* 2010). This glycoprotein was initially identified on activated Th1 cells and is found to be involved in T-cell apoptosis and migration, DC differentiation, phagocytosis of apopototic cells and anti-microbial immunity, etc. When the amino acids, crucial for carbohydrate recognition, R64 and R239, were mutated to A in Gal-9, TIM-3 binding was completely abolished (Zhu *et al.* 2005). This suggests the participation of both CRDs in TIM-3 binding and T-cell death. TIM-3 can be found within CD8+ T-cell lipid rafts at the immunological synapses where its ligand Gal-9 also recruits other receptors such as CD45 and CD148 (Clayton *et al.* 2014). TIM-3 is a crucial interaction partner of Gal-9 on immunological synapses and is dynamically partitioned into the lipid rafts wherein this couple recruits other glycoproteins such as CD44 (Zhu *et al.* 2005; Wu *et al.* 2014) and integrin to enable TCR signalling (Bi *et al.* 2011). Thus, the structural analysis of the specific carbohydrate recognition properties of galectin-9 CRD should provide insights into the physiologic function of galectin-9 as the biological activity of galectin-9 depend on the ligand specificity of each CRD and the subsequent multivalent binding conferred by two CRDs.

Gal-9 also exhibits prominent binding to IgEs, a highly glycosylated protein from human (myeloid), mouse (Spe7) and rat (IR162). The IgE protein binding to Gal-9 was stronger than the interaction of Gal-9 with Gal-1, -3, -4, and -7. After binding to IgE, Gal-9 was shown to suppress the interaction between IgE and antigen in a concentration-dependent manner (Niki *et al.* 2009).

2.6 *pH as a determinant of galectin-9 glycoconjugate binding affinities*

The binding of Gal-9 to the cell surface receptor FGL is found to be pH sensitive (Mishra *et al*. 2010). Glycoconjugate-Gal-9 binding interactions for cell polarization and protein recycling in cells can be well discussed in connection with the activity exerted by pH on Gal-9 protein. In detail, galectin-9 binding to surface glycosphingolipid results in clustering of membranes at the normal pH in a concentration-dependent manner. The clustering of Gal-9-FGL couple beyond the line tension causes negative membrane curvature, resulting in endocytosis (this physical principle is demonstrated in supplementary figure 1A). Since the pH of the endosome is far below the physiological pH (4.9–5.3), some dissociation of Gal-9 from its binding partner (e.g. FGL) is expected to occur. This would further result in the delivery of free Gal-9 by the endosomes to the Golgi network with simultaneous lysosomal degradation of the dissociated glycosphingolipid receptors. Thus, trans Golgi network (TGN)-delivered (at pH 6) Gal-9 can interact with fresh glyco-conjugates, such as FGL, in the Golgi lumen to form a vectorial cargo recycling circuit, facilitating the maintenance of apico-basal compartments in a polarized cell [figure 1a(G)]. The TGN to plasma membrane cargo trafficking in this case seems to represent a mirror process which was encountered at the plasma membrane. In this reverse mirror process, post-Golgi vesicular cargo vectorially fuses to apical membrane and rejuvenates its composition and architecture that is imperative for its defined function (supplementary figure 1B). This is the first demonstration of the role of pH in galectin–receptor coupling and membrane targeting which further finds its proof in similar studies conducted in the perspective of Gal-3 (Straube *et al.* 2013; von Mach *et al.* 2014). These current findings hence provide a novel insight into merging of the non-classical mode of protein secretion into the classical biosynthetic pathway and generates a mechanistic link between the two evolved pathways of membrane trafficking.

3. Gal-9 gene and its regulation

The tandem-repeat-type gene encoding Gal-9 is located on chromosome 17, in its longer arm at 11.2 loci (q11.2). The gene has undergone duplication, with two Gal-9-coding gene segments in the shorter arm at 11.2 loci (p11.2). In humans, genes encoded by longer arm is termed as LGALS9 and those encoded by shorter arm are termed LGALS9B and LGALS9C. Initially, LGALS9B and C were considered pseudogenes, but due to good exon-intron structure, they are now considered as true genes. The presence of gene/gene like sequence of iNOS in close vicinity of Gal-9 gene (both in p11.2 and q11.2) is a highly intriguing feature of this gene. The significance of this co-existence may be highly relevant in immune response where both the genes have functional significance in vascular permeability and inflammatory cascades (Leffler *et al*. 2004).

Apart from the full-length variant, which consists of all 11 exons (Gal-9FL or Gal-9L), two other LGALS9 splice variants are frequently described in the literature, i.e. Gal-9Δ5 (or Gal-9M, lacking exon 5) and Gal-9Δ5/6 (or Gal-9S, lacking exons 5 and 6) (Heusschen *et al*. 2013). Endothelial cells were found to express five LGALS9 splice variants, two of which have not been reported before. Splicing was found to be confined to exons 5, 6 and 10.

Deletion of exon10 shifts the frame of translation placing a stop codon before the second CRD is translated. Hence, isomer with exon10 deletion results in loss of second CRD. This isomer is identified only in human but not in mouse. The isomer with exon 5 and 10 deletions seems to have importance in pregnancy, since a decrease in its expression was seen during spontaneous abortion. Variants of membrane-associated Gal-9 (Ecalectin, hUAT) is also seen, which differs only in few amino acids (Heusschen *et al.* 2013). Further, splicing of the linker encoding exons has been shown to influence galectin-9 valency and function (Heusschen *et al.* 2013).

Analysis of proximal promoter region of rat and mouse Gal-9 revealed an identity of 76% between them. The promoters of Gal-9 of two species have common transcription factor (TF) binding sequences (eg: E2 box, CF1 and AP3) and distinct TF binding sequences (e.g. C/EBP, Insulin II enhancer, AP1 for rat and AP2, TCF2α, GATA 1/2/3 for mouse). hGal-9 (LGALS9) shows TF binding sequence for Kid3, WT1, p300, SREBP, TCFII, STAT, etc., most of which are involved in cell growth, differentiation, lipid biosynthesis and uptake and cancers (analysis via TRANSFAC: transcription factor analysis software). Gal-9 is expressed during most of the events involving these transcription factors. For example, SREBP regulates lipid homeostasis by transcribing enzymes that are required for phospholipid, cholesterol and fatty acid synthesis. In MDCK cells, Gal-9 is involved in FGL trafficking from apical surface to Golgi apparatus (Mishra *et al.* 2010). There is a high probability of Gal-9 getting transcribed by SREBP transcription factor, along with the enzymes involved in lipid biosynthesis for recycling lipids. But, experimental evidences are required to confirm the co-relation of these transcription factors in transcription of Gal-9 and Gal-9 involvement with their function.

The stimulation of Gal-9 expression is cell specific and is seen under the activation of IFN-γ, IFN-β, IL 1β, IL-1α, Phorbol 12-myriastate 13-acetate (PMA), LPS, Poly IC (Polyinosinic Polycytidylic acid, a synthetic double-stranded RNA that mimics viral nucleic acid) and TLR-3. Galectin-9 expression can be induced by IFN-γ in various tissues such as the endothelial cells (via HDAC3/PI3K/IRF3) (Alam *et al.* 2011), fibroblasts (via MAPK, PI3K and JAK/STAT) (Park *et al.* 2011) and astrocytes TCR (TNF/TNFR1/JNK/c-Jun pathway) (Zhu *et al.* 2005; Steelman *et al.* 2013) [figure 1a(8), a(Hi)]. Chabot *et al.* (2002) and Kashio *et al.* (2003) found that the release of Gal-9 from Jurkat cells is induced by PMA stimulation (Kashio *et al.* 2003). In periodontal ligament derived cells, LPS stimulation increased Gal-9 expression (Chabot *et al.* 2002); however, in the endothelial cells, Gal-9 is induced by the Poly IC but not by the LPS (Imaizumi *et al.* 2007) [figure 1a(8)]. Primary neuron cultures treated with IFN-γ upregulated the transcription of Gal-9 mediated by STAT1. Membrane PI3K and IRF-3 are also involved in expression of Gal-9 during TLR-3 activation (Bi *et al.* 2011); matrix metalloproteinase and protein kinase C are involved in the release of galectin-9 from Jurkat cells (Chabot *et al.* 2002). All these evidences thereby strengthen the fact that Gal-9 expression is cell specific.

Other TFs of importance that can increase the Gal-9 expression are KLF2 and SOX11(see QIAGEN's Ingenuity Target Explorer, QIAGEN's, *https://targetexplorer.ingenuity.com/gene/EG/3965*. KLF2 is a flow-responsive transcription factor in endothelial cells and SOX11 is a transcription factor upregulated in fetal brain that plays an important role in the embryonic development (Dekker *et al.* 2006). It is also highly expressed in many cancers such as glioblastomas and lymphomas (Weigle *et al.* 2005; Gustavsson *et al.* 2010). These TFs involvement in upregulation of Gal-9 may be indirect and the reason for upregulation is not clear. However, it is interesting that biophysical-force-responsive gene or mechanosensitive genes such as KLF2 forms a regulatory axis of Gal-9, thereby raising a need to further elucidate the role of Gal-9 in mechanotranduction [figure 1a(Hii, Hiii)]. In the context of mechanosensitivity, a novel model for Gal-9 extracellular release has been suggested, wherein a change in the colloidal osmotic pressure by serum deprivation in the cultured tumour cells creates a plasma membrane-associated nonlethal oncotic pores for galectin transport. However, the gene machinery involved in this pore formation and Gal-9 release remains to be elucidated. In a similar context, Gal-3 is already reported to be secreted and recycled into the biosynthetic machinery via shear flow stress. This secreted form further clusters the integrin receptors to mediate mechanotransduction associated with vasculogenic mimicry in tumours, angiogenesis from endothelial cells and migration (Baptiste *et al.* 2007).

Although the factors involved in Gal-9 expression have been reported, the mechanisms involved in Gal-9 gene regulation are not yet clear. Possible transcription of Gal-9 by important TFs may be correlated with its spatial or temporal expression in correspondence with the relevant TF expression. Finding the accessory factors that drive the TFs to transcribe this gene will further give insights into the regulation of this gene. Since main function of Gal-9 involves carbohydrate recognition, it is possible that these glycan units may be involved in the regulation of this gene product.

Further, miRNA-TF factor networks play a crucial regulatory role on the functions of any protein. MicroRNAs (miRNAs) are short RNA molecules of 20–25 nucleotides in length that negatively regulate gene expression in animals and plants primarily by targeting 3′ untranslated regions of mRNAs. Many miRNA are predicted to regulate Gal-9; however, only few are confirmed so far (Yang *et al.* 2015). A detailed investigation into this network along with mechanisms of Gal-9 mRNA regulation via alternative

polyadenylation will provide new insights into the mechanism of quiescence/activation of Gal-9 transcripts and isoforms in various physiological and pathological conditions in different cell types in which Gal-9 is evidenced to be expressed.

4. Cellular /tissue distribution functional properties of galectin-9

Galectin-9 is found to participate in a number of intracellular and extracellular biological processes including glycoprotein trafficking, protein folding, cell–cell or cell–ECM interactions, signal transduction, fertilization and development (Vasta *et al.* 2012). When first described, it was noted to be abundantly expressed in fetal and adult murine liver (Wada *et al.* 1997). It was also found to be heavily expressed in the thymus, where it was proposed to play a role in deleting developing thymocytes (Nebbia *et al.* 2012). Thus Gal-9 is distributed in a wide variety of mammalian cell types with some tissue specificities because the microenvironment and specificity of cells greatly influenced the gal-9 gene expressions (refer to *http://www.proteinatlas.org/ENSG00000168961-LGALS9/tissue*). These microenvironments comprise both internal and external factors such as certain physiological and pathological factors responsible for growth, differentiation, development and protection (Hirashima *et al.* 2004). While the Gal-9 specificity is tightly modulated by the controlled and uncontrolled cellular divisions and immune (tolerative, suppressive and defence) mechanisms, etc., interestingly, distinctive cellular phases may account for distinct isoforms of Gal-9 corresponding to the distinct tissue metabolisms which can be seen in the altered expression of galectins in tumour cells compared with their normal counterparts (Yang *et al.* 2008).

Indeed, significantly wide distribution and functions of Gal-9 can be seen in the skeletal system (Tanikawa *et al.* 2008; Wiersma *et al.* 2013), muscular system (Wada *et al.* 1997; Leal-Pinto *et al.* 1997; Spitzenberger *et al.* 2001), respiratory system (Leal-Pinto *et al.* 1997;Wada *et al.* 1997; Tureci *et al.* 1997; Matsumoto 1998; Spitzenberger *et al.* 2001), digestive system (stomach and epithelial cells lining the crypts of small intestine, intestinal mucosa, colon, liver, gall bladder) (Wada *et al.* 1997; Matsumoto 1998; Spitzenberger *et al.* 2001; Thijssen *et al.* 2008), urinary system (renal cortex, arterioles, inter tubular capillaries of medulla and cortex, glomerular mesangium of kidneys) (Wada *et al.* 1997; Leal-Pinto *et al.* 1997; Spitzenberger *et al.* 2001), reproductive system (ovary, uterus, mammary gland, testis) (Leal-Pinto *et al.* 1997; Bauersachs *et al.* 2006; Shimizu *et al.* 2008), cardiovascular system (heart and aorta) (Wada *et al.* 1997; Leal-Pinto *et al.* 1997; Spitzenberger *et al.* 2001), lymphatic system (spleen, lymph node, thymus, reticulum network outlining dendritic processes of stromal/epithelial cells of thymus) (Leal-Pinto *et al.* 1997;Wada *et al.* 1997; Matsumoto 1998), nervous system (brain, corneal and conjuctival epithelia, stromal cells of conjunctiva, iris, ciliary body, sclera, retinal blood vessels, retinal pigment epithelium, choroid and keratinocytes) (Leal-Pinto *et al.* 1997; Schlotzer-Schrehardt *et al.* 2012), integumentary system (Igawa *et al.* 2006), myeloid (Tureci *et al.* 1997; Matsumoto 1998) and lymphoid immune systems (Tureci *et al.* 1997).

Galectin-9 has both endogeneous and exogenous cellular distribution (Heusschen *et al.* 2013). On the extracellular surface, Gal-9 selectively binds to galactose-containing oligosaccharides and glycoproteins (such as laminin, fibronectin, vitronectin) in the extracellular matrices and to the cell-surface glycoconjugates *via* either bivalent or multivalent crosslinking (Nobumoto *et al.* 2008). This mechanism of galectins enables homo- or heterotypic aggregation and bridges the cells with extracellular matrix proteins. Intracellular functional translocation between the nucleus and cytoplasm enables galectins to undergo both protein–protein or lectin–carbohydrate interactions, to mediate fundamental development, growth, immunological and death mechanisms (Yang *et al.* 2008). Extracellular, secreted form of Gal-9 can mediate cell adhesion, chemoattraction, receptor endocytosis, lipid recycling, raft clustering, etc. via binding to glycan ligand (Matsushita *et al.* 2000; Hughes 2001; Asakura *et al.* 2002; Kasamatsu *et al.* 2005b; Nobumoto *et al.* 2008; Mishra *et al.* 2010).

Cytoplasmic Gal-9 induces cancer cell aggregation leading to inhibition of metastasis (Irie *et al.* 2005). This phenomenon was observed in breast cancer cells and hepatocellular carcinoma, in which cytoplasmic Gal-9 was observed to be associated with aggregation of cells which inhibits the cells from invasion, detachment from tumour and attachment to vascular endothelium. The expression of Gal-9 in this case was neither seen in the nucleus nor on the surface of the cells. The aggregation was surprisingly not induced by exogenous Gal-9 (Irie *et al.* 2005). These results clearly suggest the involvement of cytoplasmic Gal-9 in cell aggregation which could probably be through Gal-9 mediated interaction and stabilization of cell–cell adhesion junction machinery such as desmoplakin-3/JUP and beta-catenin (Irie *et al.* 2005; Zhang *et al.* 2012; Ewing *et al.* 2007) (also see *https://targetexplorer.ingenuity.com/gene/EG/3965*) [figure 1a(A)]. In metastatic melanoma cells, nuclear expression of Gal-9 is seen unlike in the breast cancers and hepatocellular carcinomas (Kageshita *et al.* 2002). Also, aggregation of melanoma cells was seen with Gal-9 added exogenously (Kageshita *et al.* 2002). This suggests that differential localization of Gal-9 is associated with its different functions.

Nuclear localization of Gal-9 has been observed in placental, liver, colon endothelial cells, monocytes and melanoma cells (Thijssen *et al.* 2008; Barjon *et al.* 2012; Matsuura *et al.* 2009; Ma *et al.* 2013; Kageshita *et al.* 2002). But not

much about the nuclear functions of Gal-9 is known. Transient transfection of Gal-9 could increase the expression of IL-1α, IL-1β in THP-1 (monocyte) cells (Matsuura *et al.* 2009). The promoter activity of IL-1A gene was increased by all the three isoforms, but the small isoform could increase the promoter activity of IL-1β and IFN-γ (Matsuura *et al.* 2009). Expression of these genes however results due to co-involvement of several transcription factors such as AP-1, NF-IL6 and NF-kB, etc. (Matsuura *et al.* 2009). Gal-9 is implicated in increasing the activity of AP-1 and NF-IL6. Physical interaction of Gal-9 and NF-IL6 is also observed, suggesting the increase of activity linked to their physical association (Matsuura *et al.* 2009). LPS-stimulated monocytes have been reported to show nuclear translocation of Gal-9-NF-IL6 that activates IL-1α, IL-1β and IFN-γ. NF-IL6 is found to further activate IL-8, IL-1 and IL-β production [figure 1a(11)] (Matsuura *et al.* 2009). Physical interaction of Gal-9 and β-catenin is shown by immunoprecipitation (Ewing *et al.* 2007). Another galectin member, Gal-3, also binds to β-catenin and forms a complex with TCF4 that drives expression of c-myc and cyclinD1 genes that are involved in cell cycle and growth (Dumic *et al.* 2006). Gal-9 also forms a complex with β-catenin and may drive similar transcription events.

4.1 *Galectin-9 in cell adhesion and cell–cell communication*

The participation of Gal-9 in cell adhesion is mainly based on its multivalent properties and the ability to bind to cell surface and glycosylated components of ECM. Gal-9 seems to have integrin-mediated strong adhesion to laminin III, but the initial contacts are carbohydrate mediated and largely depend on Gal-9 and Gal-3. The interaction with collagen I, however, is mediated through integrins, even at the initial stages. Gal-9 is involved in cell matrix interaction and appears to regulate adhesion at multiple levels, both directly and indirectly. Gal-9 on the surface of eosinophils can mediate its adhesion to fibroblast allowing them to perform their effector functions in tissues for prolonged time. Gal-9 can promote host-pathogen interactions (e.g. *Leishmania major* interaction with macrophages) and the involvement of Gal-9 in either increasing or decreasing the adhesion also depends on the cell origin (Pelletier *et al.* 2003). Exogenously introduced Gal-9 can reduce the interaction between a melanoma and ECM, making it to aggregate and leading to anti-metastasis. The same phenomenon was seen in malignant breast cancer cells when Gal-9 was overexpressed (Kageshita *et al.* 2002). One of the major mechanisms of suppression of metastasis and invasion by Gal-9 seems to be through the inhibition of the adhesion molecules from coupling to each other because Gal-9 binds to one of the adhesion partner and blocks its interaction domain. In B16F10 and COLON26 tumour cells, Gal-9 blocks the interaction between CD44-hyaluronic acid and VLA-4 and VCAM-1, which is critical for adhesion of tumour cells to endothelium (Nobumoto *et al.* 2008) [figure 1a(9)]. Interestingly, while Gal-3 is reported to contribute to tumour invasion and metastasis, Gal-9 suppresses detachment of tumour cells from primary sites and its adhesion to ECM plays a major role in cancer metastasis regulation through the property of adhesion.

5. Galectin-9 in immunoregulation

Distinct glycan signatures on pathogens are read by lectin molecules in the immune cells, distinguishing the non-self-entities from self. This assists in eliciting a series of changes to recruit more immune cells to the site of infection that will ultimately kill the pathogen. Gal-9 is involved actively at various stages of immune response, for normal functioning. Every function is associated with specific concentration change of Gal-9, which is important in bringing out a definite physiological effect [figure 1(b)].

A dendritic cell (DC) involved in immune response, captures foreign molecules through lectin–glycan interaction and undergoes series of changes that leads to its maturation. Maturation of dendritic cell is important for initiating immune response. Gal-9 added exogenously can induce maturation of dendritic cell (indicated by upregulated expression of co-stimulatory molecules like CD40, CD54, CD80, CD83 etc., and HLA-DR). The various other factors that contribute to DC maturation are IL-1β and IFN-γ which can also increase the expression of Gal-9. In response to identification of a pathogenic attack, these molecules may possibly increase the expression of Gal-9 in DC that can help in its maturation. The antigen engulfed DC migrates to lymph nodes – a high expression of Gal-9 can also help in DC maturation. The matured DC induces proliferation of naïve T-cells to activated T-cells (Dai *et al.* 2005).

The effect of Gal-9 on activated T-cells is concentration dependent. At higher concentrations, Gal-9 induces apoptosis of activated T-cells (CD8+ and CD4+), but at lower concentrations it increases the cytokine production by activated T-cells. At higher concentrations, bivalent Gal-9 can oligomerize, and hence may bring together more number of signalling proteins or induce binding with more number of ligands, which may be important for apoptotic signalling. One of the ligands responsible for inducing apoptosis in activated T-cells by Gal-9 is TIM-3. TIM-3 is a glycoprotein expressed on activated T-cells under prolonged viral infection or upon repeated polarization. It contains an N-glycan chain, to which Gal-9 binds and triggers a series of events like calcium mobilization, calpain and caspase-1 activation that ultimately drives the T-cells to apoptosis (Kashio *et al.* 2003). Apoptosis by TIM-3-Gal-9 may be a mechanism

through which exhausted T-cells are eliminated [figure 1a(10)]. High concentration of Gal-9 in the absence of TIM-3 can also lead to apoptosis, indicating the presence of other ligands of Gal-9 on activated T-cells. In another subset of T-cells involved in type I diabetics, CD40+CD4+T cells, higher concentration of Gal-9 has been shown to drive necrotic cell death through binding of Gal-9 with CD40 (Kanzaki *et al.* 2012) [figure 1a(12)].

Time- and dose-dependent response of Gal-9 show surprising effects on T-cells and these effects were reported to be TIM-3 independent in the study conducted by Gooden and team. Here, high Gal-9 concentration (of up to 1000 nM) with short incubation periods accounted for the T-cell apoptosis whereas the non-lethal doses, i.e. low concentration (15–30 nM approx.) had a stimulatory effects on T-cells. Although high-dose Gal-9 exhibited rapid T-cell death, notable T-cell expansion was seen after a major wave of the cell death at lower doses. Here, Gal-9 activated and strongly expanded the surviving T-cells in a TIM-3-independent manner by a shift from naïve towards a central memory and IFN-γ-producing TH1 phenotype. On the other hand, in the presence of T-cell activating signals (anti-CD3/IL-2), Gal-9 did not trigger expansion of T-cells, but shifted the normal CD8/CD4 balance towards a predominant CD4+ phenotype. These experimental evidences disclose the fact that Gal-9 has diverse immunomodulatory effects depending on the concentration, receptors and skewing signals available for the interactions (Gooden *et al.* 2013).

Gal-9 apoptosis is also seen with the B-cells (BALL-1), monocytes (THP-1), and myelocytes (HL-60) other than the T-cells (Kashio *et al.* 2003). But this apoptotic property of Gal-9 is absent in TIM-3-negative Th2 or in Treg cells (Wiersma *et al.* 2012, 2013). Th2-cells are proposed to be resistant to Gal-9 apoptosis due to the lack of cell surface receptor TIM-3 (Bi *et al.* 2011). But Gal-9 could induce the migration of Th2-cells through PDI receptor on the Th2-cells. Gal-9 binding of N-glycan receptors is necessary for inducing the T-cell death. But in the Th2 cells, where a significant amounts of α-2,6-linked sialic acid is added to Nglycan receptors (rendered non-permissive to Gal-9 binding), Th2 cell counts are uninhibited by Gal-9 (Wiersma *et al.* 2013; Gooden *et al.* 2013).

During activation of T-cells, a rapid decline of Gal-9 concentration at the lymph node is observed. At lower concentration of Gal-9, Th1 and Th17 cells produced IFN-γ and TNFα. The cytokine producing active T-cells recruit more immune cells towards the defense area for eliminating the pathogen. Also, at lower concentration, the CD40+CD4+T subset of T-cells transform to conventional CD4+T-cells that decreases the number of aggressive CD40+CD4+T-cells. These cells are correlated with insulitis in type I diabetics. Decrease in this subset of T-cells would help in combating type I diabetes, indicating application of Gal-9 in treating type I diabetes (Kanzaki *et al.* 2012).

Gal-9 plays a crucial role in cellular differentiation and maintenance of Th17 and Tregs. When CD4-naive T-cells are stimulated with TGF-β1 and IL-6, Gal-9 can almost completely suppress IL-17 mRNA expression and induction of Th17 cells in mice. Apart from this, Gal-9-CD44 complex synergizes with TGF-β1 to activate and translocate Smad3 to the nucleus that further promotes Gal-9 and FOXP3 transcription that generates stable iTregs (Treg differentiation) cells which is crucial in regulating immune homeostasis, autoimmunity, responses to infections, cancers and inflammation (Ji *et al.* 2013) [figure 1a(2)]. Thus by mediating the immune suppression and differentiation, Gal-9 participates in Th17/Treg immune regulatory functions.

Cytokines generated by the activated T-cells recruit other innate immune cells like neutrophils and eosinophils to the site of infection. Function of Gal-9 as an eosinophil chemoattractant helps in recruitment of eosinophil by T-lymphocytes. Gal-9 can trigger two major events in eosinophil activation, which are aggregation of eosinophil and superoxide production by eosinophil. Aggregation of eosinophil occurs at high concentration and superoxide production is a dose-dependent event, which increases with an increase in the dose of Gal-9. Another interesting effect of Gal-9 on eosinophil is that it increases the survivability of eosinophil isolated from allergic patients but not of those eosinophils from normal patients. This indicates changes happening on glycome of eosinophil during a disease condition. Understanding this change is important for management of allergic diseases driven by eosinophil (Matsumoto *et al.* 2002).

Overall participation of Gal-9 in the immune system depends on its ability to recognize and bind the glycan units. In all the functions of Gal-9 mentioned above, except for DC maturation, not only do CRDs have a major role, the bivalency of Gal-9 is also important. Most of the functions of Gal-9 are concentration dependent. Different concentrations lying at two extreme ends can bring about different physiological functions [figure 1(b)]. At lower concentrations, Gal-9 may bind to only a few receptors that may activate a small and transient signal module resulting in production of chemokine. However, at higher concentrations, lot more surface domains are brought together not only by bivalent interactions but also by oligomerization, resulting in a mega function like apoptosis. However, CRDs independent role in DC maturation indicates the presence of protein–protein interaction of Gal-9 that adds on to multivalent functions of Gal-9.

6. Gal-9 in diseases and its associated therapeutic potential/biomedical applications

The involvement of Gal-9 has been observed in infections, antimicrobial immunity, autoimmune disorders, allergic

responses, cancers and degenerative diseases. In many of these disease cases, expression of Gal-9 is reported to vary from the normal levels, which indicates a possible involvement of Gal-9 in the progression or regression of the disease [figure 1(a,b,c)]. For example, the elevation of serum Gal-9 in the patients with type 2 diabetes and chronic kidney disease is closely and negatively linked to the estimated glomerular filtration rate (eGFR) which may be due to the alteration of the immune response and inflammation (Gal-9 is high in patients detected with CKD). Hence, the altered expression levels of Gal-9 (e.g. Gal-9 in serum and blood plasma) itself can act as the diagnostic tool for several diseases. Also, for instance, in CNS, under normal condition, expression of Gal-9 is at low levels. The levels are seen to rise during experimental autoimmune encephalomyelitis (EAE) (at peak of T-cell infiltration), which modulate the T-cell population, resists pathogenic Th1 and controls inflammation.

Hence, Gal-9 is essentially anti-inflammatory, as suggested by studies in several disease animal models including collagen-induced arthritis (CIA) (Sakai *et al.* 2011), asthma (Katoh *et al*. 2007), nephrotoxic serum nephritis (Tsuchiyama *et al.* 2000), diabetic nephropathy (Baba *et al.* 2005) lipopolysaccharide (LPS)-induced inflammation (Kasamatsu *et al.* 2005a), experimental allergic conjunctivitis (EAC) (Fukushima *et al.* 2007), experimental allergic encephalitis (Zhu *et al.* 2005), coxsackievirus B3 (CVB3)-induced myocarditis (Zhang *et al.* 2014b), autoimmune hepatitis (AIH) (Lv *et al.* 2012) and non-alcoholic fatty liver disease (NAFLD) (Tang *et al.* 2013). Hence, Gal-9 crucial roles in diseases and its therapeutic implication are discussed as follows:

6.1 *In autoimmune diseases*

1. Systemic lupus erythematosus (SLE):

Systemic lupus erythematosus is a polymorphic and multigenic inflammatory autoimmune disease that predominantly affects women. This inflammatory disease is characterized with serum anti-double-stranded DNA marker antibodies along with nephropathy inducing chronic renal failure (Moritoki *et al.* 2013). Here, adjustment of T-cell balance and a decrease in anti-dsDNA antibodies are effected by Gal-9. Gal-9 induces a decrease in TIM-3+Th1 and Th17 as Th17 and Tregs are critical for SLE pathogenesis. Furthermore, Gal-9 treatment increases CD19+ cells in MRL/lpr lupus-prone mice and it reduces CD19−CD138+ plasma cells but not CD19+CD138+ plasmablasts. This plasma cell reduction may result in the suppression of anti-dsDNA antibody production, ameliorating clinical severity in SLE pathogenesis (Moritoki *et al.* 2013).

2. Nephrotoxic serum nephritis:

Nephrotoxic serum nephritis is characterized by kidney glomerular crescent formation (defined as the presence of two or more layers of cells within Bowman's space) and glomerular influx of CD8+ cells and macrophages into the glomerular capillaries, which results in renal failure in the absence of immunosuppressive therapies. Gal-9 selectively induces apoptosis of CD8+ cells and reduces the infiltration of CD8+ cells and macrophages, leading to the inhibition of crescent formations (Tsuchiyama *et al.* 2000).

3. Diabetic nephropathy:

The injection of Gal-9 into *diabetic/diabetic* mice was shown to significantly inhibit the glomerular hypertrophy, mesangial matrix expansion and urinary albumin excretion. Gal-9 was observed to reverse the high glucose–mediated upregulation of p27Kip1 and p21Cip1, by reducing the glomerular expression of TGF-β1 and thereby inducing the G1-phase-cell-cycle arrested p27Kip1 and p21Cip1-positive glomerular cells to progress from G1 to G2 phase of the cell cycle, inhibiting the cell-cycle-dependent hypertrophy. Gal-9, hence, can be developed into a therapeutic drug towards diabetic nephropathy (Baba *et al.* 2005).

Type 1 diabetes (T1D) immune suppression: Type 1 diabetes (T1D) is a T-cell-mediated autoimmune disease that selectively destroys the insulin-producing β-cells in the pancreas. Non-obese diabetic (NOD)/severe combined immunodeficient SCID mouse is an animal model to study the pathogenesis of T1D. Decrease in the numbers of aggressive CD40+CD4+T-cells (Th1) immune cells by Gal-9 inhibits the development of autoimmune diabetes in NOD mice, and therefore the overexpression of Gal-9 or recombinant Gal-9 can be a useful therapeutic strategy against T1D, which can be further used for prolonging the survival of islet grafts or other transplants during pancreatic regeneration (Kanzaki *et al.* 2012; Chou *et al.* 2013).

Type 2 diabetes (T2D) suppression: Gal-9 is found to have an indirect control over type 2 diabetes in maintaining the blood glucose homeostasis. The GLUT-2 surface receptor which is involved in glucose-stimulated insulin secretion, and acts as an important Gal-9 ligand; thereby Gal-9 gains an indirect control over the blood glucose homeostasis in response to the dietary intake. Gal-9 recognizes and retains the glucose transporter GLUT-2 on the pancreatic β-cell surface, inhibiting Glut-2 endocytosis and thereby sustaining the primary glucose-stimulated insulin secretion response (Ohtsubo *et al.* 2005). In addition, a report shows that the elevated serum Gal-9 levels in the patients with type 2 diabetes and chronic kidney disease is negatively linked to the estimated glomerular filtration rate, which in turn may be related to the alteration of the immune response and inflammation in the patients type 2 diabetes and CKD (Kurose *et al.* 2013).

4. Anti-glomerular basement membrane glomerulonephritis (anti-GBM GN):

Anti-glomerular basement membrane crescentic glomerulonephritis, the most aggressive form of GN, is a Th1- and

Th17-predominant autoimmune disease characterized with the greatest probability of renal insufficiency and the highest frequency of crescent formation at the time of diagnosis. The protective role of Gal-9 in anti-GBM GN is associated with the inhibition of Th1- and Th17-cell-mediated immune responses that shifts immune response from Th1 to Th2 and finally enhances the Th2 immunity in the kidney of anti-GBM GN (Zhang *et al.* 2014a).

5. Autoimmune hepatitis (AIH):

AIH is a disease of the liver that occurs when the body' immune system attacks cells of the liver. When the effector cells express low TIM-3, a Gal-9 ligand, they become less susceptible to Treg-immune cell-mediated suppression. Reduced signalling of the TIM-3/galectin-9 axis contributes to impaired Treg control during AIH (Liberal *et al.* 2012). Hence, Gal-9-mediated T-cell activation plays a crucial role in AIH as also demonstrated in the murine model of liver injury, in Con A-induced hepatitis, wherein a single injection of Gal-9 in mice was protective against Con A-induced hepatitis.

6. Liver ischaemic reperfusion injury (IRI):

Liver IRI is an exogenous antigen-independent inflammatory pathology wherein CD4+ T-cells acts as a key mediator. Liver IRI occurs frequently after major hepatic resection or liver transplantation. The blockade of TIM-3/galectin-9 pathway was shown to exacerbate the local inflammation and liver damage due to TNF and IL-6 and amplified T-cell activity of Kupffer cells. These results suggest the importance of TIM-3/galectin-9 signalling in the maintenance of liver homeostasis and in controlling dysregulated liver immune response (Liberal *et al.* 2012; Hirao *et al.* 2015).

7. Non-alcoholic fatty liver disease (NAFLD):

Immune modulation of NK (natural killer) T-cells has a significant therapeutic impact in fighting the ever-growing trends of obesity and fatty liver diseases. Upon endo/exogenous activation, NKT cells secrete many cytokines, such as IFN-γ and IL-4, and upregulates the expression of TIM-3. IFN-γ induces the production of Gal-9 by Kupffer cells and leads to TIM-3+NKT-cell apoptosis, which limits the inflammatory response and inhibits the destructive immunity. Gal-9 also interacts with TIM-3 expressed on Kupffer cells to produce IL-15, which induces the proliferation of NKT cells. This eventually leads to the homeostasis of NKT cells and balance of the local immune microenvironment. Importantly, exogenous Gal-9 can also significantly improve the diet-induced steatosis and obesity in an NKT-cell-dependent manner through TIM-3/Gal-9 signalling pathway, thus stressing out a significant therapeutic impact in fighting the ever-growing trend of obesity and fatty liver diseases (Tang *et al.* 2013).

8. Immune thrombocytopenia (ITP):

Immune thrombocytopenia is an autoimmune bleeding disorder in which T- and B-cells recognize platelet antigens and initiate a pathogenic reaction. The patients with ITP possess activated platelet-autoreactive T-cells with increased pro-inflammatory cytokine secretion, due to loss of peripheral tolerance in patients with ITP. The complex dysregulation of cellular immunity of ITP includes a significant shift towards T helper (Th1) and Th17 pro-inflammatory immune responses, and a loss of tolerance resulting from a decreased number and defective functioning of CD4+CD25+ regulatory T-cells (Tregs). There is also platelet destruction by cytotoxic T-lymphocytes (CTLs) and defects in antigen-presenting cells (APCs), which can potentially lead to abnormal processing and/or presentation of self-antigens and stimulate anti-platelet autoimmunity. Recently, a link between ITP and the TIM-3 pathway has been suggested wherein Gal-9 can therapeutically influence the pathways, as the immune modulatory roles of Gal-9 is already well established (Zhang and Shan 2014).

9. Experimental autoimmune encephalomyelitis or experimental allergic encephalomyelitis:

EAE is an inflammatory and demyelinating autoimmune disease of the central nervous system. EAE is shown to be limited by exogenous administration of Gal-9 in experimental allergic encephalitis as the TIM-3/galectin-9 pathway may have evolved to control the population expansion and tolerance of Th1 cells in the immune compartment and to prevent prolonged inflammation in target tissues (Zhu *et al.* 2005).

10. Gal-9 in multiple sclerosis:

Multiple sclerosis (MS) is a chronic progressive degenerative disorder of the central nervous system, characterized by inflammation, demyelination, ultimate failure of remyelination and axonal loss (Stancic *et al.* 2011). MS is Th1-dependent autoimmune disease and is characterized by high amounts of IFN-γ. Gal-9 was found in nuclei of microglia in active lesions of this disease, whereas it is found in cytoplasm in the inactive lesions (Stancic *et al.* 2011). This suggests that Gal-9 may be involved in promoter regulation of genes involved in MS-associated inflammation. Hence, this observation need urgent further investigation as this may shed light on the mechanisms of Gal-9-mediated activation/quiescence of MS lesions. Further, cerebrospinal fluid of secondary progressive multiple sclerosis patients is also reported with higher levels of Gal-9 than healthy controls (Burman and Svenningsson 2016).

11. Rheumatoid arthritis (RA):

Rodent models of immune-mediated arthritis (RMIA) are commonly used to evaluate the mechanisms of inflammatory joint disease as well as to test the efficacy of anti-arthritic compounds. Collagen-induced arthritis (CIA), adjuvant-induced arthritis (AIA) and collagen antibody-induced arthritis all come under the animal models of rheumatoid arthritis (RA) that is widely used to address questions of disease pathogenesis and to validate therapeutic targets. RA is found to be both Th1 and Th17-driven autoimmune

inflammation, and in this context, Gal-9 negatively regulates arthritis by inducing differentiation of naïve T-cells to Tregs (T suppressor cells) and thereby induces apoptosis of TIM-3+ Th1 and pro-inflammatory Th17 cells (Seki *et al.* 2007). Gal-9 also induces apoptosis of hyper-proliferating synoviocytes in RA joints, which consequently decreases the levels of pro-inflammatory, IL-6, thereby enabling effective RA treatment. Supporting data also suggests that in experimental autoimmune arthritis, incubation with recombinant Gal-9 decreases the proinflammatory cytokines such as IL-1β, IL-6, TNF-α, MCP-1, MIP-2, IL-12 and IL-17 [figure 1a(13)].

6.2 *In allergic disorders*

In a murine model of asthma, administration of Gal-9 is shown to reduce Th2-associated airway inflammation and airway hyper-responsiveness. Mechanistically, Gal-9 binds to CD44, inhibiting the interaction of CD44 with hyaluronan, which would otherwise mediate the migration of T-cells to lungs, aiding allergic responses (Katoh *et al.* 2007). Gal-9 administration has been shown to suppress the immediate-phase asthmatic reaction in guinea pigs, by forming a complex with IgE and hence preventing the degranulation of mast cells, which would otherwise release several proinflammatory mediators (Niki *et al.* 2009) [figure 1a(3)]. Together with the previously reported functions of Gal-9 in suppressing CD44-dependent migration of inflammatory cells to the lesions, the current studies add more value to Gal-9 as a potential candidate for the development of a novel protein drug for the treatment of asthma and allergic disorders. Evidently, when the sputum samples of asthma-infected patients were evaluated for airway inflammation analysis, the macrophages were observed to express low levels of Gal-1 and Gal-9. These defective expressions of Gal-1 and Gal-9 in asthma patients may interpose inflammatory responses associated with this disease (Sanchez-Cuellar *et al.* 2012). Galectin-9 may also be involved in the modulation of the threshold activation of splenocytes by producing significantly less cytokines and thus reducing the severity of Th2-mediated allergic conjunctivitis in the mouse experimental allergic conjunctivitis (Fukushima *et al.* 2007; Yang *et al.* 2008)

6.3 *In infectious diseases*

Involvement of Gal-9 is seen in infections caused by *Mycobacterium tuberculosis, Leishmania major, Trichosporon asahii* and viral infections of HCV and HSV. Previous studies have revealed the elevated plasma levels of Gal-9 in acute HIV-1 and dengue-virus-infected individuals (Saitoh *et al.* 2012; Chagan-Yasutan *et al.* 2013) but no changes are observed in Gal-9 protein levels in experimental pneumococcal meningitis (Bellac *et al.* 2007). Hence, Gal-9 serves as a biomarker in acute viral infections like dengue and HIV and the levels of expression directly indicates the activated immunological reactions, like release of inflammatory cytokines, chemokines, immunological complexes and other immune modulators. These findings also corroborate with the observation that when anti-influenza virus drugs were administered to the patients, Gal-9 levels were seen to decline. It was also demonstrated that Poly IC (which mimics viral nucleic-acid-mediated immune reaction) but not LPS induces Gal-9 production *in vitro*. In the case of Dengue hemorrhagic fever, increased Gal-9 expression was observed in patients with severity of dengue viral infection and the expression level gradually decreased in the late recovery stages (Chagan-Yasutan *et al.* 2013).

Gal-9 is further involved in the reactivation of HSV (*Herpes simplex virus*) which was seen to be delayed and reduced when Gal-9 was knocked out. After HSV infection, the CD8+T-cells are accumulated in the trigeminal ganglion and maintained latency of HSV. But intermittent latency and reactivation was brought about by TIM-3/Gal-9 interaction. It was shown that trigeminal ganglions with virus (replicating/latent) have upregulated Gal-9, which interacts with TIM-3 of CD8+T-cells and reduces the effector function of T-cells (Reddy *et al.* 2011). In ocular infection with herpes simplex virus 1 (stromal keratitis), which results in human blindness, severity of the disease was demonstrated by the absence or suppression of Treg cell responses and it could be treated by expanding or activating Tregs and reducing the effector cells responsible for tissue damage. A novel therapeutic strategy was stated to control this viral immunopathology through a combined treatment with monoclonal antibody to TNFRSF25 (tumour necrosis factor receptor superfamily member 25) and Gal-9 wherein the former expands and activates Tregs specifically and the latter causes apoptosis in TIM-3 expressing effector cells and pro-inflammatory cells. This combined treatment was also shown to aid in the reduction of pro-inflammatory molecules and simultaneous upregulation of anti-inflammatory molecules (Reddy *et al.* 2012).

Upregulation of TIM-3 and accumulation of Foxp3+ Treg_cells are characteristics of HCV infection, which suppresses effector T-cell response that is essential for viral clearance. HCV-infected hepatocytes express higher levels of Gal-9 and TGF-β and upregulate TIM-3 expression and regulatory cytokines TGF-β/IL-10 by co-cultured CD4+ T-cells driving conventional CD4+T-cells into CD25+Foxp3+ Treg-cells. Recombinant Gal-9 also acts in synergy with TGF-β, transforming TCR-activated CD4+T-cells into Foxp3+Treg-cells in a dose-dependent manner (Ji *et al.* 2013). Similarly, Gal-9 causes apoptosis in the Th17-cells by interaction with TIM-3 leading to the reduced expression of IL-17, which is responsible for

the anti-microbial immunity, bacterial clearance and survival rates in the case of *Klebsiella pneumoniae* infection (Wang *et al.* 2011). As mentioned earlier, Gal-9 seems to ameliorate Con-A lectin-induced hepatitis and its absence seems to increase the disease severity. Con-A induces CD4+ T-cell activation plays a crucial role in the liver injury. Gal-9 increases the T_{reg} to T_{eff} ratio, which has been demonstrated to cause selective apoptosis of ConA activated CD4+T cells that further prevents the release of TNFα, IL-6 and IFN-γ. These pro-inflammatory cytokines are thought to cause cytotoxic effects on hepatocytes *in vitro*, in a direct and synergistic manner, contributing to the development of hepatitis. Inhibition of pro-inflammatory cytokines represents an alternative inhibitory mechanism for explaining suppressive properties of Gal-9 on T-cell mediated diseases. Hence, administration of Gal-9 could serve as a therapeutic tool for the treatment of hepatitis (Lv *et al.* 2012).

On contrary, surface-retained PDI, a ligand for Gal-9, forms a complex with HIV gp120 and catalyses the disulphide bond arrangement on HIV envelope, thereby promoting its entry into the cell, augmenting HIV infection of T-cells. In this context, Gal-9 aids in pathology of HIV infection. Similarly, reducing local expression of galectin-9 in genital mucosa is suggested to reduce infection of T cells with HIV (Bi *et al.* 2011). Further, in *Hepatitis B virus* (HBV)-related liver inflammation, increased expression of Gal-9 is observed in Kupffer cells, which has also been reported in the hepatocellular carcinoma pathology (Nebbia *et al.* 2012). Gal-9/TIM-3 interaction leads to the reduction in the immunological efficacy in some acute infectious states like influenza attack. Upon infection, virus-specific CD4 and CD8 cells expresses TIM-3 at higher levels, leading to apoptosis of these cells and results in lack of immunity against the virus. Also in T-cell responses, antibody responses and disease control against this virus has been seen to be reduced in the presence of Gal-9. Hence, it is suggested that inhibiting Gal-9/TIM-3 interaction would serve to enhance the immunity against influenza A viral infections (Sharma *et al.* 2011).

Interestingly, Gal-9 exhibited the therapeutic effects on Cecal ligation and puncture-induced (CLP) polymicrobial sepsis in mice, potentially by expanding NKT cells, plasmacytoid (p)DC-like macrophages, and by modulating the production of early and late proinflammatory cytokines by decreasing the frequency of TIM-3+CD4 T-cells. Adversely, delayed Gal-9 treatment prolonged the survival of polymicrobial sepsis (Kadowaki *et al.* 2013). Similarly, Gal-9 administration was found to be beneficial in *Mycobacterium tuberculosis* (Sada-Ovalle *et al.* 2012; Jayaraman *et al.* 2013), *Leishmania major* (Pelletier *et al.* 2003), *Pseudomonas aeruginosa* (Vega-Carrascal *et al.* 2014) infections, in pneumonitis by *Trichosporon asahii* (Arikawa *et al.* 2010) and in bacterial damage of periodontal tissue (Kasamatsu *et al.* 2005a) and *Coxsackie virus B3*-mediated lethal heart injury (Zhang *et al.* 2014b).

7. Gal-9 and its effect on cancer

A major portion of the galectin-oriented cancer research has focused on the membrane-anchored cell surface receptors binding via CRDs. To date, a series of experimental and clinical evidences have been reported to support a correlation between galectin expressions and neoplastic transformation due to receptor crosslinking, lattice formation and transmembrane signalling cascade events leading to cellular transformations. A better understanding of the extra- and intracellular functions of galectins in cancer cells which is necessary to create an immunosuppressive tumour microenvironment and tumour heterogeneity is much needed to explore the new anticancer therapies (Vladoiu *et al.* 2014).

Galectin-9 exhibits pro/antitumoural characteristics depending on the concentration, type of tumour and its progression status. These characteristics are found to be further dependent on the large diversity of binding partners, compartmentalization and its expression pattern contingent to the cell type. Intracellular localization of galectin-9 differs according to the cell type and tumour progression stage and impacts the biological functions with regards to cancer progression. The expression of galectin-9 is elevated with neoplastic progression in certain malignancies, and therefore, Gal-9 can play an important role as potential biomarkers that may help to identify the disease progression and may also serve as a therapeutic agent as described in the following sections:

1. Cell transformation:

Although the mechanisms involved for galectin-9-induced malignant transformation is still not completely clear, a negative correlation between the expression of galectin-9 and malignant transformation has been established. In cervical squamous cell carcinoma, decreased expression of Gal-9 may be associated with the de-differentiation of normal epithelial cells to cervical intra-epithelial neoplasia. Gal-9 loss is associated with the loss of E-cadherin in epithelial cells (Mishra *et al.* 2010). The decreased expression of E-cadherin in cervical cancers might therefore be involved in the severity of lesions and malignant transformation (Liang *et al.* 2008).

2. Apoptosis:

Apoptosis regulation by galectin-9 is one of the most studied intracellular functions of Gal-9, relevant to tumour progression. Although the underlying mechanisms of galectin-9 regulation on apoptosis and proliferation are not fully understood, binding partners, compartmentalization, dose-time responses, and cell types, all are found to influence the differential effects of Gal-9 on proliferation *vs.* apoptosis of cancer cells. For example, in endothelial cells,

IFN-γ induces both a cell cycle arrest and cell proliferation. Here the increased expression of galectin-9 exhibits an inverse relation between galectin-9 and proliferation. Numerous studies are on to explore the relationship between increased proliferative potential and apoptotic resistance with consequent loss of galectin-9, in many malignant cells. The observation that galectin-9 induces apoptosis of many blood cancer cells (Kobayashi *et al.* 2010; Kuroda *et al.* 2010; Pena *et al.* 2014) appears to contrast with the reports that these cells have an increased galectin-9 expression (Lahm *et al.* 2001).

The apoptotic activity on cancerous cell by Gal-9 is like the two faces of a coin, resulting in positive or negative regulation in various cancerous cell types. In detail, the stable dimeric structure of Gal-9 was found to be important in inducing apoptosis in multiple myeloma cells, through activation of JNK and p38-MAPK signalling and through independent activation of caspase-8, -9 and -3 (Kobayashi *et al.* 2010). Alternatively, these kinases may phosphorylate H2AX, leading to DNA fragmentation and apoptosis [figure 1a(10)]. Due to this, hGal9 can be used as a new therapeutic target for multiple myeloma as it bears anti-proliferative effect and enough potential to overcome resistance to conventional chemotherapy. Also, in chronic myeloid leukaemia, human Gal-9 is found to undergo ATF3-Noxa apoptotic pathway, wherein the loss of mitochondrial membrane potential and induction of endoplasmic reticulum stress is followed by activation of caspase-3/caspase-8/caspase-9 and caspase-8/caspase-4 cascades (Kikushige *et al.* 2015) [figure 1a(10)]. In contrast to this, high amounts of Gal-9 carrying exosomes released from the *Epstein–Barr virus*-associated nasopharyngeal carcinoma (NPC) induced the apoptosis of matured Th1-lymphocyte through the interaction between Gal-9 of NPC exosomes and TIM-3 receptors on the T-cells, leading to the suppression of anti-tumoural T-cell responses. This Th1 suppressive effect by NPC exosomes promotes pro- tumoral responses by Gal-9 in NPC. Thus, to improve the therapeutic efficacy for NPC (Klibi *et al.* 2009), TIM-3/Gal-9 interaction should be effectively blocked [figure 1a(6)]. Similar TIM-3/ Gal-9 pro-tumoral responses are seen in patients who are chronically infected with HCV or HBV. Here, galectin-9 induces TIM-3-mediated apoptosis of effector T-cells and favours the expansion of Tregs and attenuates adaptive immune responses to induce failure of the immune response facilitating viral persistence. Hence, Gal-9 shows tumoural effect by mediating T-cell dysfunction in NPC and HCC, predicts poor prognosis in patients with HBV-associated HCC (Bacigalupo *et al.* 2013), and shows anti-proliferative effects in multiple myeloma.

Gal-9 with its apoptotic ability, can effectively eliminate chronic myeloid leukaemia (CML) (Kuroda *et al.* 2010), Burkitt's and Hodgkin's lymphoma cells (Tureci *et al.* 1997). Gal-9 can directly trigger cell death in sarcoma via association of Gal-9 with TIM-3+DCs and TIM-3+CD8+Tcells that further generates granzymeB, perforin and IFN-γ for apoptotic elimination (Wiersma *et al.* 2013; Gooden *et al.* 2013) [figure 1a(10)]. Also Gal-9 is shown to significantly retard tumour growth of myeloma xenografts in mice. But the inhibitory effect of Gal-9 on solid cancers (breast, colon, oral, gastric etc.) seems to rely on interference with various metastatic features of cancer cells rather than the immediate cytotoxicity. Although B-cells do not express TIM-3, B-cell lymphoma cell lines are found to be sensitive to the induction of cell death by recombinant Gal-9 (Bacigalupo *et al.* 2013). Thus, Gal-9 induces apoptotic cell death in many cancer types like melanoma (Wiersma *et al.* 2012), breast cancer, hepatocellular carcinoma, adult T-cell leukaemia (Kashio *et al.* 2003), multiple myeloma (Kobayashi *et al.* 2010), chronic myeloid leukaemia (Kuroda *et al.* 2010), Burkitt's lymphoma, Hodgkin's lymphoma, sarcoma (Nagahara *et al.* 2008), gall bladder carcinoma (Tadokoro *et al.* 2016), gastric cancers (Takano *et al.* 2016), cholangiocarcinoma (Kobayashi *et al.* 2015), etc. However, TIM-3/Gal-9 ligations in myeloid leukaemia activates ERK1/2 and AKT pathways that enables β-catenin and NFkB to co-operatively drive the pan myeloid autocrine loop to develop malignant stem cells (Elahi *et al.* 2012) [figure 1a(4)].

3. Adhesion, migration and invasion:

Detachment of cancer cells from its primary site by loosening cell–cell contacts, invasion into the extracellular matrix by cell–cell and cell–matrix interactions, attachment of cancerous cells to vascular endothelium, invasion of cancer cells through vascular endothelium, migration to distant sites, establishing tumour growth with neo-vascularization are the essential steps in the malignant tumour metastasis (Leber and Efferth 2009; Martin *et al.* 2013).

In cancer studies, numerous findings evidently support the indispensability of Gal-9 (Lahm *et al.* 2000, 2001; Danguy *et al.* 2002; Kageshita *et al.* 2002; van den Brule *et al.* 2004; Kasamatsu *et al.* 2005b; Irie *et al.* 2005; Liang *et al.* 2008; Zhang *et al.* 2009, 2012; Heusschen *et al.* 2013;). Gal-9 plays an important role in the cell-aggregate formation (Kageshita *et al.* 2002; Irie *et al.* 2005), cell-matrix (Nobumoto *et al.* 2008; Zhang *et al.* 2009) and cell-endothelial inhibitory interactions (Zhang *et al.* 2009) and prevention of cancer cell invasions (Kageshita *et al.* 2002; Irie *et al.* 2005; Liang *et al.* 2008; Zhang *et al.* 2012), etc., wherein the overall effects varies with different cell types, receptors, compartmentalization as well as with the Gal-9 concentrations [figure 1b]. The process of cell aggregation such as in MCF7 breast cancer cell lines can be carried out by the secreted Gal-9 in a dose-dependent manner. The cellular aggregation occurs at increased levels of Gal-9 while

it shows a decreased tendency at reduced concentrations (Irie *et al.* 2005; Heusschen *et al.* 2013).

While Gal-9 promotes cell aggregation, it prevents the cellular adhesion of cancer cells with the vascular endothelial cells by blocking the interaction between vascular cell adhesion molecule-1 and very late antigen-4 (VCAM1-VLA4) [figure 1a(9)] (Nobumoto *et al.* 2008). Both the endogenous form and exogenously secreted Gal-9 can inhibit the cell-free contact (Nobumoto *et al.* 2008; Zhang *et al.* 2009). This anti-metastatic role of high levels of Gal-9 is well observed in the murine models of melanoma and colon carcinoma, where Gal-9 prevents the tumour cell adhesion to endothelial cells, resulting in the metastasis inhibition (Nobumoto *et al.* 2008). Hence, Gal-9 can trigger cancer cell aggregation (Kageshita *et al.* 2002; Irie *et al*. 2005), can impair cell detachment (Nobumoto *et al.* 2008), and may prevent escape of cancer cells from the primary tumour (Irie *et al.* 2005; Kageshita *et al.* 2002; Liang *et al.* 2008; Zhang *et al.* 2012).

Gal-9 can also interfere with the invasion of cancer cells into the extracellular matrix through blocking of adhesion of the cells to ECM components; for example, in some cancer cell types such as breast, colon, and melanoma, Gal-9 can inhibit the metastatic process through blocking of the cellular adhesion to ECM components, such as with collagen, laminin, and fibronectin (Nobumoto *et al.* 2008). But, low galectin-9-expressing oral squamous carcinoma cells (OSCC) showed increased adhesion to collagen and fibronectin (Kasamatsu *et al.* 2005b). Other than this, Gal-9 was shown to also inhibit the CD44-hyaluronic acid interaction and thereby blocked the metastasis formation preferentially by inhibiting the binding of cell-surface-expressed CD44 to hyaluronic acid. Indeed, this metastatic spread could be inhibited with antagonistic anti-CD44 antibodies (Katoh *et al.* 2007).

Reportedly, TIM-3 expressed by endothelial cells facilitates metastasis by melanoma cells and in such cases recombinant Gal-9 can be used to reduce cancer cell adhesion/metastasis by competitively blocking TIM-3 on the endothelial cells.

Interestingly, the expression of Gal-9 itself reveals the status of tumour progression and metastasis formation in various types of cancers. Gal-9 is highly expressed in melanocytic lesions of low malignancy, whereas metastatic lesions are characterized by low or no expression of Gal-9. In HCC, the Gal-9 downregulation resulted in lymph node metastasis, vascular invasion and intrahepatic metastasis, raising a significant risk for patient survival, while Gal-9 expression resulted in anti-metastatic ability by enhancing aggregation, inhibiting cell-endothelial adhesion and blocking endothelial invasion of the cancer cells (Zhang *et al.* 2012; Bacigalupo *et al.* 2013). Similarly, primary breast carcinoma tumours were characterized by high levels of endogenous Gal-9, whereas all distant metastasis lacked expression of Gal-9 (Irie *et al.* 2005). Also, normal epithelium and low-grade cervical carcinoma had high levels of Gal-9, whereas high-grade cervix carcinoma lesions had very low levels of Gal-9 (Liang *et al.* 2008). Therefore, the loss of Gal-9 is a negative prognostic factor in these cancer types. In line with these clinical observations, Gal-9 transfected cancer cells showed less metastasis than parental Gal-9-negative cancer cells in mouse models of melanoma and colon cancer. Furthermore, intravenous administration of recombinant Gal-9 significantly reduced the formation of metastasis in mice injected with wild-type melanoma and colon cancer cells (Kageshita *et al.* 2002). Taken together, these data indicate that endogenously expressed Gal-9 as well as recombinant Gal-9 inhibits the formation of metastasis in certain types of cancers.

Altogether, galectin-9 may positively or negatively modulate the invasive behaviour of cancer cells such that its complete mechanisms and outcomes remain largely unknown with varying cancer types. For example, galectin-9 is found to increase the adhesion of melanoma (Kageshita *et al.* 2002), oral (Kasamatsu *et al.* 2005a, b) and colon cancer cells (Zhang *et al.* 2009), but reduces adhesion of breast cancer cells and (Irie *et al.* 2005). One of the reasons for this observed disparity maybe due to the variants and isoforms of Gal-9 expressed in different tumours. The isoforms of Gal-9 can differentially regulate the tumour progression depending on the cell types. Probably, the adhesion depends on the relative abundance of individual galectin-9 splice variants, for example, increased Gal-9FL and Gal-9Δ5/6 inhibited the adhesion of MCF7 breast cancer cells to different extracellular matrix components like collagen, fibronectin, vitronectin and laminin (Irie *et al.* 2005), while high abundance of Gal-9Δ5 probably promoted it. It is stated that S- and L-type Gal-9 inhibits the adhesion of tumour cells onto fibronectin, vitronectin and laminin of extracellular matrix molecules and collagen type IV of endothelial cells, hence preventing metastasis. Interestingly, exogenous Gal-9 5 appears to have a bimodal effect on endothelial cell migration in the *in vivo* CAM (chick chorioallantoic membrane) model (Heusschen *et al.* 2014), wherein at low concentrations, migration is stimulated, while at higher concentrations, it is hampered. Also, various Gal-9 isoforms may differently regulate E-selectin expression in certain colorectal cancer cells. E-selectin expression is normally observed in tumour endothelium (HUVEC) and E-selectin expression on endothelial cells is a negative prognostic factor in some malignancies including in colorectal carcinoma. Zhang *et al.* (2009) showed that increased expression of galectin-9 modulates E-selectin levels in LoVo colon carcinoma cells. Interestingly, specific galectin-9 splice variants showed diverging effects. Gal-9FL overexpression induced downregulation of E-selectin expression in these cells while the Gal-9Δ5 and Gal-9Δ5/6 overexpression resulted in the

upregulation of E-selectin, with concomitantly increased adhesion of these cells to the endothelial cells. In addition, while Gal-9FL overexpression did not result in an increased adhesion of LoVo cells to endothelial cells, it did result in an increased adhesion of these cells to matrigel and this explains the ligand-dependent adhesion effect of Gal-9 (Chabot *et al.* 2002; Asakura *et al.* 2002). Further, work needs to be done in the perspective of Gal-9 isoforms and splice-variant-driven adhesion, and metastasis biology; further efforts needs to be directed towards the precise role of the linker domains in these processes as linker domains can be manipulated to switch the metastasis phenotype into non-oncogenic niches.

4. Immune escape:

Galectin-9 has been extensively studied in the context of immunity and inflammation as it can act as a cytokine and can modulate the activity and function of numerous types of immune cells; however, its role in tumour immune escape remains largely unexplored. In immune context, galectin-9 is well characterized as an eosinophil chemoattractant (Matsumoto 1998; Sato *et al.* 2002), with Gal-9FL being the most potent splice variant for this effect (Sato *et al.* 2002). Eosinophils are predominantly associated with anti-tumour activity and serve as good prognosis markers (Thijssen *et al.* 2015). Interestingly, eosinophilia is often observed in haematological and colon tumours (Samoszuk 1997; Munitz and Levi-Schaffer 2004; Wedemeyer and Vosskuhl 2008), and as discussed previously, these tumours frequently display increased galectin-9 expression compared to normal tissue. It remains to be determined whether tumour-derived galectin-9 indeed mediates infiltration of eosinophils into the tumour micro-environment and whether the loss of galectin-9, as observed in several solid tumour types, indeed allows tumours to escape from an eosinophil-mediated anti-tumour response.

The ability of galectin-9 to modulate the differentiation, expansion and migration of other immune cells (e.g. Th17 differentiation, Th1 apoptosis and Th2-cell migration) supports the immunosuppressive activity of galectin-9 in tumour immune escape. This immunosuppressive effect of galectin-9 was indeed proposed as the mechanism by which *Epstein–Bar virus*-infected nasopharyngeal carcinoma cells escape immune surveillance (Keryer-Bibens *et al.* 2006; Klibi *et al.* 2009). A similar role of immune escape is also seen with HBV-associated hepatocellular carcinoma through galectin-9/TIM-3 axis (Bacigalupo *et al.* 2013; Nebbia *et al.* 2012). When PBMCs are exposed to galectin-9, apoptosis is potently induced in CD4+ and CD8+ cells, but not in CD4+CD25+ cells, suggesting that endothelial galectin-9 might indeed confer a tolerant environment around the tumour vasculature.

On the other hand, galectin-9 administration was also shown to induce the expansion of dendritic cells resulting in the potentiation of CD8+ or natural killer (NK) cell-mediated anti-tumour immunity in sarcoma and melanoma models respectively (Clayton *et al.* 2014). Furthermore, the observation that galectin-9 predominantly suppresses immune function seems hard to reconcile with the poor outcome in patients with low galectin-9 expression. Hence, most probably, galectin-9 expression is lost during the course of tumorigenesis, enabling tumour cells to metastasize more easily as soon as multiple other modes of immune escape have developed. However, a recent report shows a positive co-relation in gastrointestinal stromal cancers and WHO grade of glioma tumor tissue with Gal-9 levels and TIM-3 expression on the tumor infiltrating T cells. Therefore a link between Gal-9 and overall clinical manifestation in tumors progression needs special attention to ascertain it as a potential target to inhibit immunoevasion (Komita *et al.* 2015; Liu *et al.* 2016).

5. Tumour angiogenesis:

Tumour angiogenesis is initiated when tumour cells grow beyond a mass that can be supplied with oxygen and nutrients by diffusion. Such a tumoural mass becomes hypoxic and triggers production and secretion of growth factors that are sensed by endothelial cells in the nearby vessels. Vessel dilation and matrix degradation occurs and the endothelial cells start to migrate and proliferate into the growth factor gradient. Finally, the growing vessels mature, providing the growing tumour mass with an irregular but functional blood supply. This angiogenesis process is essential for tumour progression. The vascular cells i.e. the endothelial cell, responsible for angiogenic process (i.e. growth of new blood vessels out of existing capillaries) express different galectin family members (Heusschen *et al.* 2013). Galectin-9 splice variants are one among them with unclear mechanisms in tumour angiogenesis. Gal-9 is reported to attract eosinophils and expands dendritic cells, which will further release angiogenic growth factors like VEGF. In addition, altered galectin-9 levels on the endothelial cell layer might affect infiltration of anti-tumour immune effector cells, e.g. Th1 and Tc-cells, as well as immune-suppressive cells, e.g. eosinophils and Tregs, thereby interfering with a proper anti-tumour immune response. However, at the higher concentrations of exogenous galectin-9, *in vivo* angiogenesis in the CAM (chick chorioallantoic membrane) model resulted in an inhibition of angiogenesis, leading to a dramatically reduced endothelial cell proliferation and a decrease in total vessel length, branch points and end points [figure 1a(7)] (Heusschen *et al.* 2014). At a lower concentration exogenous Gal-9Δ5 did not interfere with an efficient angiogenic response; indeed, it showed a trend towards an increased vessel length, with suggested Gal-9Δ5 role in modulation of endothelial cell migration. Hence, probably endothelial galectin-9 is mainly involved in tumour immune escape rather than tumour angiogenesis (Heusschen *et al.* 2013).

7.1 *Therapeutic potential and other biomedical applications*

1. Gal- 9 as a graft rejection marker:

Recently, Qiao *et al.* (2014) highlighted a novel therapeutic strategy comprising Gal-9 and TIM-3 for the prolonged survival of allografts. Although a number of independent Gal-9 and TIM-3 studies have been conducted in the perspective of skin and cardiac allografts, islet grafts in autoimmune diabetes, corneal allografts, mesenchymal stem cell grafts in cartilage damage, kidney allografts, human renal transplants and liver allografts (Wang *et al.* 2007; Arikawa *et al.* 2010; Wiersma *et al.* 2013; Chou *et al.* 2013; Shimmura-Tomita *et al.* 2013; Ungerer *et al.* 2014; Fukata *et al.* 2014); Qiao *et al.* made robust attempts to describe the enhanced role of TIM-3 and its ligand galectin-9 in allografts and the important roles played by these molecular couples in the pathogenesis of rat lung transplant rejection, wherein TIM-3/Gal-9 were evaluated as sensitive valuable markers for monitoring the pulmonary acute rejection responses in the early stages of lung transplantation (Qiao *et al.* 2014). Hence, the ability of Gal-9 to suppress pathogenic T-cell responses in autoimmune disease models and experimental allograft transplantation is now well documented. In this context, a new galectin-9/collagen matrix using collagen-binding galectin-9 fusion proteins were made which could cause local, contact-dependent immune suppression during transplantation. Hence, Gal-9-modified collagen matrices could serve as a valuable resource for the demand of new immunosuppressive agents in organ transplantation (Arikawa *et al.* 2010; Fukata *et al.* 2014). Further, recent success of cardiac allografts has been demonstrated with a combined administration of Gal-9 and EX-527, a Sirtuin-1-specific inhibitor (Tao *et al.* 2015).

2. Gal-9 and pregnancy:

The expression of galectins at maternal-fetal interface may reduce the danger of maternal immune attacks on the fetal semiallograft, hence providing immune tolerance mechanisms, which can help in sustaining the embryo implant for longer gestation period (Than *et al.* 2009; Li *et al.* 2016; Hao *et al.* 2015).

Increased amount of Gal-9 is found in the sera of pregnant women and is suggested to be one of the supporting factors for safe progression of pregnancy. The immunotolerance level attained to regulate and control the pro-inflammatory mechanisms during pregnancy is suggested to be under the influence of the physiologically increased Gal-9. These regulatory mechanisms brought about by the Gal-9 expressing regulatory T-cells, TIM-3+ cytotoxic T-cells and NK-cells are found to be depended on cellular receptor expression and activation, inflammatory stimuli, and stages of pregnancy (Than *et al.* 2009; Sun *et al.* 2016) [figure 1a(B)].

3. Gal-9 in mesenchymal stem cell (MSC) therapies and chondrocyte differentiation:

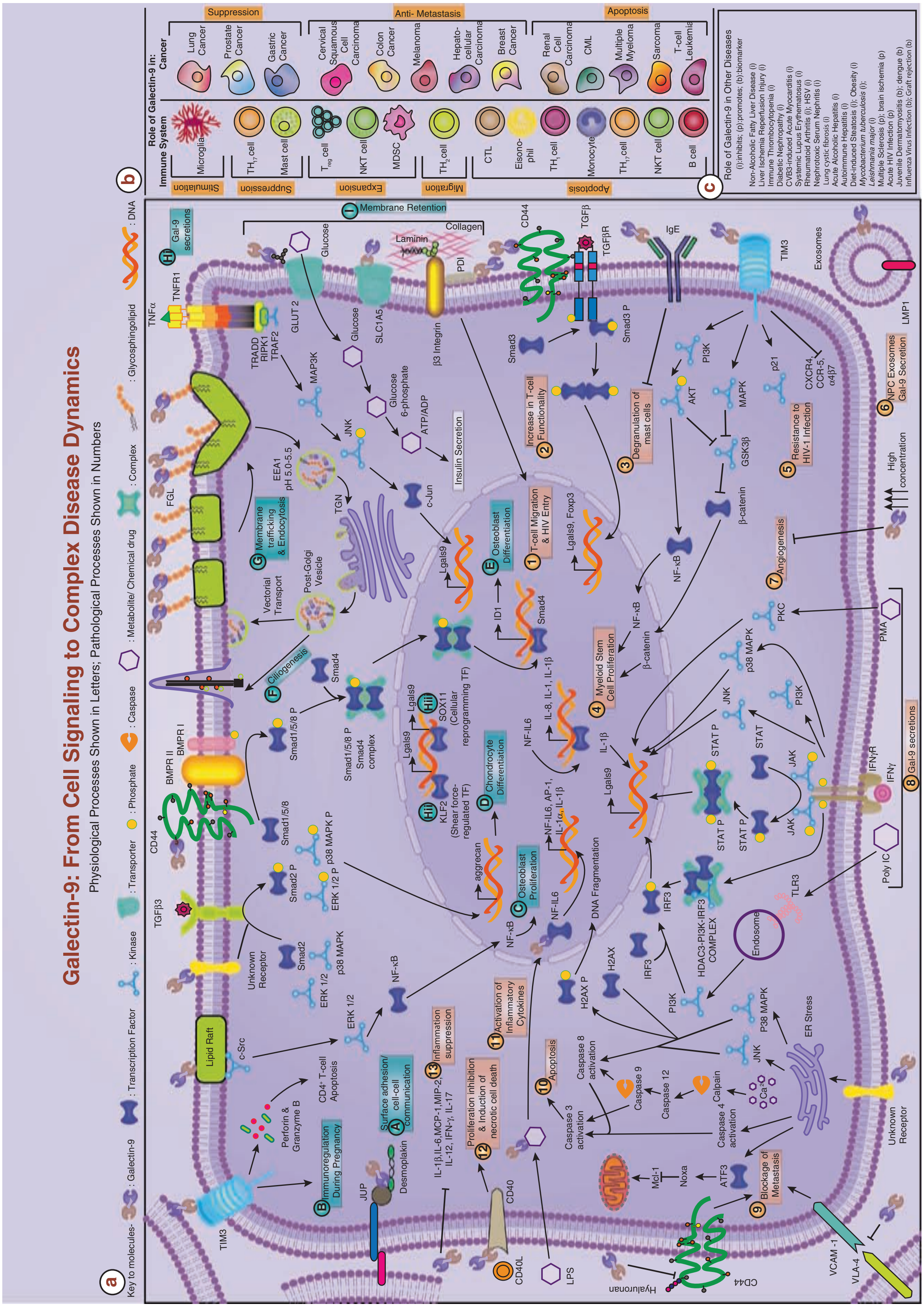
Gal-9 acts as a predictive indicator for clinical MSC therapy as it can serve as a beneficial biomarker in therapeutic applications wherever MSCs are employed for regenerative medicine, transplantation, and in treatment of autoimmune diseases. Here, Gal-9 helps in the prediction of higher or lower immune modulatory potentials of single cell (MSC) preparations, and thereby helps in distinguishing the potencies of MSCs derived from different donors. Further, TGFβ3 and Gal-9 has been shown to synergistically amplify TGFβ3 signalling mediators such as p38 MAPK, ERK1/2, and Smad2, leading to chondrocyte differentiation of human mesenchymal stem cells as ascertained by upregulation of collagen type X, aggrecan, SOX9 and other chondrocyte differentiation markers (Arikawa *et al.* 2010).

4. Gal-9 in osteoblast differentiation and proliferation:

Endogenous Gal-9 clusters inner plasma membrane lipid rafts in human osteoblasts to induce sc-Src-ERK-NFkB-mediated osteoblast proliferation (Tanikawa *et al.* 2008; Wiersma *et al.* 2013) [figure 1a(C)]. Conversely, in the absence of bone morphogeneic proteins (BMPs), exogenous galectin-9 has been shown to stimulate CD44 surface glycoproteins to form complex with BMP receptor type II leading to the phosphorylation of Smad1, Smad5, and Smad8, which causes the translocation of complexes with Smad4 to the nucleus for activation of Id1 promoter in a dose-dependent manner. Id1, inhibitor of differentiation 1 protein then promotes the differentiation of osteoblastic cells, and hence Gal-9 induces osteoblast differentiation through the Smads signalling pathway (Tanikawa *et al.* 2010) [figure 1a(D)].

5. Gal-9 in ciliogenesis:

Evidently, Gal-9 enables the biogenesis of primary cilium in MDCK kidney epithelial cells. Interestingly, Gal-9 knockout in this study resulted in a total loss of cilia with concomittent induction of epithelial-to-mesenchymal-like transition. At the same time, when Gal-9 recombinant protein was administered extracellularly, the cilium was totally regenerated (Mishra *et al.* 2010). This suggests that Gal-9 may be associated with biogenesis and trafficking of ciliary cargo [figure 1a(F)]. Being a mechanosensitive organelle of cell, cilia enables cell to monitor its extracellular microenvironment and generate responses through which the cells can constantly adapt themselves to various states. As defects in ciliogenesis are associated well with various diseases ranging from mental retardation, and kidney failure to tumorigenesis, the current finding identify Gal-9 as a promising target for diagnostic and therapeutic applications in ciliopathies.


Galectin-9: From Cell Signaling to Complex Disease Dynamics
Physiological Processes Shown in Letters; Pathological Processes Shown in Numbers
Key to molecules- : Galectin-9 : Transcription Factor : Kinase : Transporter : Phosphate : Caspase : Metabolite/ Chemical drug : Complex : Glycosphingolipid : DNA
Role of Galectin-9 in:
Immune System
Cancer
Stimulation
Suppression
Expansion
Migration
Apoptosis
Anti- Metastasis
Microglia
Mast cell
NKT cell
MDSC
CTL
Monocyte
B cell
Lung Cancer
Prostate Cancer
Gastric Cancer
Cervical Squamous Cell Carcinoma
Colon Cancer
Melanoma
Hepato-cellular Carcinoma
Breast Cancer
Renal Cell Carcinoma
CML
Multiple Myeloma
Sarcoma
T-cell Leukemia
Role of Galectin-9 in Other Diseases
(i):inhibits; (p):promotes; (b):biomarker
Non-Alcoholic Fatty Liver Disease (i)
Liver Ischemia Reperfusion Injury (i)
Immune Thrombocytopenia (i)
Diabetic Nephropathy (i)
CVB3-induced Acute Myocarditis (i)
Systemic Lupus Erythematosus (i)
Rheumatoid Arthritis (i); HSV (i)
Nephrotoxic Serum Nephritis (i)
Lung cystic fibrosis (i)
Acute Alcoholic Hepatitis (i)
Autoimmune Hepatitis (i)
Diet-induced Steatosis (i); Obesity (i)
Mycobacterium tuberculosis (i);
Leishmania major (i)
Multiple Sclerosis (p); brain ischemia (p)
Acute HIV Infection (p)
Juvenile Dermatomyositis (b); dengue (b)
Influenza Virus Infection (b); Graft rejection (b)
A Surface adhesion/ cell-cell communication
B Immunoregulation During Pregnancy
C Osteoblast Proliferation
D Chondrocyte Differentiation
E Osteoblast Differentiation
F Ciliogenesis
G Membrane trafficking & Endocytosis
H Gal-9 secretions
I Membrane Retention
1 T-cell Migration & HIV Entry
2 Increase in T-cell Functionality
3 Degranulation of mast cells
4 Myeloid Stem Cell Proliferation
5 Resistance to HIV-1 Infection
6 NPC Exosomes Gal-9 Secretion
7 Angiogenesis
8 Gal-9 secretions
9 Blockage of Metastasis
10 Apoptosis
11 Activation of Inflammatory Cytokines
12 Proliferation inhibition & Induction of necrotic cell death
13 Inflammation suppression
TIM3
CD44
TGFβ3
BMPR II
BMPR I
FGL
TNFα
TNFR1
TRADD RIPK1 TRAF2
GLUT 2
SLC1A5
β3 Integrin
PDI
Laminin
Collagen
TGFβR
IgE
Exosomes
LMP1
Lipid Raft
c-Src
Perforin & Granzyme B
CD4+ T-cell Apoptosis
JUP
Desmoplakin
CD40
CD40L
LPS
Hyaluronan
VCAM -1
VLA-4
Unknown Receptor
Smad2
Smad2 P
Smad1/5/8
Smad1/5/8 P
Smad4
ERK 1/2
p38 MAPK
NF-κB
MAP3K
JNK
c-Jun
EEA1 pH 5.0-5.5
TGN
Post-Golgi Vesicle
Vectorial Transport
Glucose
Glucose 6-phosphate
ATP/ADP
Insulin Secretion
Smad3
Smad3 P
PI3K
AKT
MAPK
GSK3β
β-catenin
p21
CXCR4, CCR-5, α4β7
High concentration
PKC
PMA
IFNγR
IFNγ
JAK
STAT
STAT P
Poly IC
TLR3
Endosome
IRF3
HDAC3-PI3K-IRF3 COMPLEX
H2AX
H2AX P
DNA Fragmentation
ER Stress
Calpain
Caspase 12
Caspase 9
Caspase 8 activation
Caspase 3 activation
Caspase 4 activation
ATF3
Noxa
Mcl-1
IL-1β,IL-6,MCP-1,MIP-2, IL-12, IFN-γ, IL-17
aggrecan
KLF2 (Shear force-regulated TF)
SOX11 (Cellular reprogramming TF)
Lgals9
ID1
Lgals9, Foxp3
NF-IL6
IL-8, IL-1, IL-1β
NF-IL6, AP-1, IL-1α, IL-1β
IL-1β

◀ **Figure 1.** Galectin-9-associated signalling pathways and diseases: (**a**) This section shows Galectin-9 associated 'physiological' processes labelled in alphabets and 'pathological' processes labelled in numbers. Briefly, galectin-9 physiological roles are as follows: (A) Surface adhesion and cell–cell communication, (B) immunoregulation during pregnancy, (C) osteoblast proliferation, (D) chondrocyte differentiation, (E) osteoblast differentiation, (F) ciliogenesis, (G) membrane trafficking and endocytosis, (H) Gal-9 transcription and secretion and (I) membrane retention of surface receptors. Galectin-9 roles in diseases/pathology are in: (1) T-cell migration and HIV entry, (2) increase in T-cell functionality, (3) degranulation of mast cells, (4) myeloid stem cell proliferation, (5) resistance to HIV infection, (6) Gal-9 secretion on NPC exosomes, (7) inhibition of angiogenesis, (8) Gal-9 transcription and secretion, (9) blockage of metastasis, (10) apoptosis, (11) activation of inflammatory cytokines, (12) proliferation inhibition and induction of necrotic cell death and (13) inflammation suppression. (**b**) This section shows effects of Galectin-9 on immune and cancer cells. (**c**) This section highlights some major diseases associated with galectin-9 wherein it has been shown to act as an inhibitor or promoter of disease or can act as a biomarker for disease diagnosis. (See supplementary material for a quick guide to the detailed legend of this figure.). Abbreviations used in the figure: α4β7: Integrin alpha 4 beta 7; AGC: Aggrecan; AKT: Protein Kinase B; AP-1: Activator Protein-1; ATF3: Activating Transcription Factor 3; BMPR I: Bone Morphogenetic Protein Receptor Type I; BMPR II: Bone Morphogenetic Protein Receptor Type II; CCR-5: C-C Chemokine Receptor Type 5; CD44: Cluster Of Differentiation 44; CML: Chronic Myeloid Leukaemia; CTL: Cytotoxic T Lymphocyte; CXCR4: C-X-C Chemokine Receptor 4; EEA1: Early Endosome Antigen 1; ERK 1/2: Extracellular Signal Regulated Kinase 1/2; FGL: Forssman Glycosphingolipid; FOXP3: Forkhead Box P3; GLUT 2: Glucose Transporter 2; GSK3β: Glycogen Synthase Kinase 3 Beta; H2AX: H2A Histone Family Member X; HDAC3: Histone Deacetylase 3; ID-1: DNA-Binding Protein Inhibitor of Differentiation-1; IFN-γ-: Interferon Gamma; IFN-γR: Interferon Gamma Receptor; IgE: Immunoglobulin E; IkB: Inhibitor of Kappa B; IkK: Inhibitor Of Kappa B Kinase; IL-12: Interleukin 12; IL-17: Interleukin 17; IL-1α: Interleukin 1 alpha; IL-6: Interleukin 6; IL-8: Interleukin 8; IL-1β: Interleukin 1 beta; IRF3: Interferon Regulatory Factor 3; JAK: Janus Kinase; JNK: C-Jun N-Terminal Kinase; JUP: Junction Plakoglobin; KLF2: Kruppel Like Factor 2; Lgals9: Galectin 9; LMP1: Latent Membrane Protein 1; LPS: Lipopolysaccharide; MAP3K: Mitogen Activated Protein Kinase Kinase Kinase; MAPK: Mitogen Activated Protein Kinase; Mcl-1: Induced Myeloid Leukaemia Cell Differentiation Protein; MCP-1: Monocyte Chemoattractant Protein-1; MIP-2: Macrophage Inflammatory Protein 2; NF-IL6: Nuclear Factor For Interleukin 6; NFkB: Nulcear Factor Kappa B; NKT Cell: Natural Killer T Cell; P38-MAPK: P38 Mitogen Activated Protein Kinase; PDI: Protein Disulphide Isomerase; PI3K: Phosphoinositide 3 Kinase; PKC: Protein Kinase C; PMA: Phorbol 12-Myristate 13-Acetate; Poly IC: Polyinosinic: Polycytidylic Acid; RIPK1: Receptor-Interacting Serine/Threonine Protein Kinase 1; SLC1A5: Solute Carrier Family 1 (Neutral Amino Acid Transporter), Member 5; SOX11: Sex Determining Region Y-Box 11; STAT: Signal Transducers And Activators Of Transcription; TF: Transcription Factor; TGFβ: Transforming Growth Factor Beta; TGFβ3: Transforming Growth Factor Beta 3; TGFβR: Transforming Growth Factor Beta Receptor; TGN: Trans Golgi Network; TIM-3: T-Cell Immunoglobulin Domain And Mucin Domain 3; TLR3: Toll-Like Receptor 3; TNF A: Tumour Necrosis Factor alpha; TNFR1: Tumour Necrosis Factor Receptor 1; TRADD: Tumour Necrosis Factor Receptor 1-Associated Death Domain Protein; TRAF2: Tumour Necrosis Factor Associated Factor 2; VCAM-1: Vascular Cell Adhesion Molecule 1; VLA-4: Integrin α4β1 (Very Late Antigen 4).

8. Conclusions and future perspectives

It is evident that Gal-9 is a multifunctional protein with central participation in many crucial cellular processes. However, how Gal-9 and its isoforms/splice variants are regulated at the expression, polyadenylation, post-translational modification and secretory levels is not yet clear. Also, even though Gal-9 can bind to diverse glycans with varying affinities and avidities, there is only scattered knowledge on the actual binding partners of Gal-9 in different cell types and in different physiological and pathological conditions. The lack of this knowledge hampers the most effective manoeuvrings of Gal-9 and its receptors. If this is elucidated, then, on the one hand, competitive synthetic carbohydrate vaccines and drugs can be designed to inhibit Gal-9 binding to its receptors in relevant cases and, on the other, potent Gal-9 stimulators like IFN-γ and probiotics can be administered to enhance positive functions of Gal-9 in a context-dependent manner. For instance, in cow milk allergy, Gal-9 expression plays a preventive role, where supplementation of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides along with *Bifidobacterium breve* M-16V (GF/Bb) can induce Gal-9M production by intestinal epithelial cells which can successfully suppress mast cell degranulation and this reduces the acute hypersensitivity responses (AHR) (de Kivit *et al.* 2012). This report interestingly also suggests the role of gut microbiota in natural regulation of Gal-9 isoforms for disease therapeutics. In this direction, since high levels of Gal-9 prevent distant metastasis, screening and development of probiotics that can enhance Gal-9 expression is much required. Indeed, supporting data also exists that both age and diet have a strong regulatory role on the expression of Gal-9 isoforms (Rhodes *et al.* 2013), and hence robust studies on Gal-9 regulation via nutritional biochemistry may bring safe and effective cure to several diseases and lifestyle disorders

Gal-9 has also emerged as a promising candidate in human system with therapeutic potential in various diseases ranging from cancers to degeneration and in the regenerative medicine as it is involved in biogenesis of cilium (Mishra *et al.* 2010), osteoblast (Tanikawa *et al.* 2010) and chondrocyte differentiation (Arikawa *et al.* 2009) and in epithelial

cell polarization (Mishra *et al.* 2010). Again, 4-1BB, a member of TNFR superfamily which holds immense potential to promote immune responses against autoimmune disorders, inflammations, viral infections and cancers, is found to be a direct binding partner of Gal-9. Gal-9 binds to 4-1BB at a site distinct from the binding site of antibodies and the natural ligand of 4-1BB. Gal-9 aids 4-1BB aggregation, signalling and operational activity in T-cells, dendritic cells and natural killer cells. The action of anti-4-1BB antibodies in suppressing autoimmune and allergic inflammation is found to be entirely reliant on Gal-9 that controls the therapeutic activity of 4-1BB-targeting antibodies (Madireddi *et al.* 2014).

Further work in cell biology and clinical medicine will allow us to manoeuvre Gal-9 for homeostatic regulation via activation/inhibition regimes, given the fact that Gal-9 does act as a hub molecule in major cellular signalling interactomes. Also, Gal-9 has a potential for being used for 'subcellular targeting' of drugs for specific inhibition of localized cellular targets. This strategy will reduce the concentration at which drug needs to be administered, overcoming toxicity issues on one side with enhanced specificity on the other. Similar subcellular targeting principles can be used for Gal-9 to deliver agents into cells and tissues for biomedical imaging. Recently, Shiga toxin B subunit is being effectively tried for cellular targeting wherein StxB subunit interacts with Gb3/CD77 on cancer cells, leading to the CTL responses along with Th1 polarization (Johannes and Romer 2010). When StxB interacts with DCs, it induces CTL responses to viral infections and tumours that are long lasting and more efficient. Since Gal-9 also binds Gb3 glycosphigolipid-like StxB (Nagae *et al.* 2008), emerging concepts on biomedical applications of StxB for tumour and viral drug targeting/vaccination can be very well applied to Gal-9, which is preferable as Gal-9 is a natural mammalian protein (Johannes and Romer 2010; Rajendran *et al.* 2010).

The diverse effects of Gal-9 on cells and the reciprocal cellular responses in different stages in the normal metabolic conditions or during the various diseases and disorders are found to be specifically dependent and greatly influenced by various modulating factors that constitute the extra/intracellular signals and foreign pathogenic components. Hence Gal-9 can both promote and inhibit several diseases (figure 1c).

In summary, the latest knowledge on Gal-9, presented in this review, directly reflects its potential in biomedical applications such as in tissue engineering/organ morphogenesis/stem cell proliferation, angiogenesis, immune system functions (autoimmune diseases, allergic infections and immune-tolerance), inflammation, ischaemia, cancers, diabetes, obesity as well as in viral, bacterial and pathogenic infections. Gal-9 can be developed as a PCR/immunohistology/ELISA-based potential biomarker for several diseases such as dengue fever and nasopharyngeal carcinoma, and can also act as a potential predictor of graft rejection and safe progression of pregnancy. Gal-9, in a concentration-dependent manner, may provide a novel cure for ciliopathies and its endocytic pathways can be further exploited for subcellular delivery of drugs and biomedical imaging probes. Hence, there is a wide scope of harnessing galectin-9-associated biology for therapeutic purposes in the form of diagnostic and prognostic biomarkers, as a part of drug delivery system and as a direct drug target.

Acknowledgements

We thank the Department of Biotechnology, India, for award of Ramalingaswami Fellowship Grant (BT/RLF/Re-entry/16/2011) to RM for this work.

References

Alam S, Li H, Margariti A, Martin D, Zampetaki A, Habi O, Cockerill G, Hu Y, *et al*. 2011 Galectin-9 protein expression in endothelial cells is positively regulated by histone deacetylase 3. *J. Biol. Chem.* **286** 44211–44217

Arikawa T, Matsukawa A, Watanabe K, Sakata K-m, Seki M, Nagayama M, Takeshita K, Ito K, *et al*. 2009 Galectin-9 accelerates transforming growth factor β3-induced differentiation of human mesenchymal stem cells to chondrocytes. *Bone.* **44** 849–857

Arikawa T, Saita N, Oomizu S, Ueno M, Matsukawa A, Katoh S, Kojima K, Nagahara K, *et al*. 2010 Galectin-9 expands immunosuppressive macrophages to ameliorate T-cell-mediated lung inflammation. *Eur. J. Immunol.* **40** 548–558

Asakura H, Kashio Y, Nakamura K, Seki M, Dai S, Shirato Y, Abedin MJ, Yoshida N, *et al*. 2002 Selective eosinophil adhesion to fibroblast via IFN- -induced galectin-9. *J. Immunol.* **169** 5912–5918

Baba M, Wada J, Eguchi J, Hashimoto I, Okada T, Yasuhara A, Shikata K, Kanwar YS, *et al*. 2005 Galectin-9 inhibits glomerular hypertrophy in db/db diabetic mice via cell-cycle-dependent mechanisms. *J. Am. Soc. Nephrol.* **16** 3222–3234

Bacigalupo ML, Manzi M, Rabinovich GA and Troncoso MF 2013 Hierarchical and selective roles of galectins in hepatocarcinogenesis, liver fibrosis and inflammation of hepatocellular carcinoma. *World J. Gastroenterol.* **19** 8831–8849

Baptiste TA, James A, Saria M and Ochieng J 2007 Mechanotransduction mediated secretion and uptake of galectin-3 in breast carcinoma cells: implications in the extracellular functions of the lectin. *Exp. Cell Res.* **313** 652–664

Barjon C, Niki T, Verillaud B, Opolon P, Bedossa P, Hirashima M, Blanchin S, Wassef M, *et al*. 2012 A novel monoclonal antibody for detection of galectin-9 in tissue sections: application to human tissues infected by oncogenic viruses. *Infect Agent Cancer* **7** 16

Bauersachs S, Ulbrich SE, Gross K, Schmidt SE, Meyer HH, Wenigerkind H, Vermehren M, Sinowatz F, *et al*. 2006 Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. *Reproduction* **132** 319–331

Bellac CL, Coimbra RS, Simon F, Imboden H and Leib SL 2007 Gene and protein expression of galectin-3 and galectin-9 in experimental pneumococcal meningitis. *Neurobiol. Dis.* **28** 175–183

Bi S, Hong PW, Lee B and Baum LG 2011 Galectin-9 binding to cell surface protein disulfide isomerase regulates the redox environment to enhance T-cell migration and HIV entry. *Proc. Natl. Acad. Sci. USA* **108** 10650–10655

Burman J and Svenningsson A 2016 Cerebrospinal fluid concentration of Galectin-9 is increased in secondary progressive multiple sclerosis. *J. Neuroimmunol.* **292** 40–44

Chabot S, Kashio Y, Seki M, Shirato Y, Nakamura K, Nishi N, Nakamura T, Matsumoto R, *et al*. 2002 Regulation of galectin-9 expression and release in Jurkat T cell line cells. *Glycobiology* **12** 111–118

Chagan-Yasutan H, Ndhlovu LC, Lacuesta TL, Kubo T, Leano PS, Niki T, Oguma S, Morita K, *et al*. 2013 Galectin-9 plasma levels reflect adverse hematological and immunological features in acute dengue virus infection. *J. Clin. Virol.* **58** 635–640

Chou FC, Kuo CC, Wang YL, Lin MH, Linju Yen B, Chang DM and Sytwu HK 2013 Overexpression of galectin-9 in islets prolongs grafts survival via downregulation of Th1 responses. *Cell Transplant* **22** 2135–2145

Cirino G, Napoli C, Bucci M and Cicala C 2000 Inflammation-coagulation network: are serine protease receptors the knot? *Trends Pharmacol. Sci.* **21** 170–172

Clayton KL, Haaland MS, Douglas-Vail MB, Mujib S, Chew GM, Ndhlovu LC and Ostrowski MA 2014 T cell Ig and mucin domain-containing protein 3 is recruited to the immune synapse, disrupts stable synapse formation, and associates with receptor phosphatases. *J. Immunol.* **192** 782–791

Dai SY, Nakagawa R, Itoh A, Murakami H, Kashio Y, Abe H, Katoh S, Kontani K, *et al*. 2005 Galectin-9 induces maturation of human monocyte-derived dendritic cells. *J. Immunol.* **175** 2974–2981

Danguy A, Camby I and Kiss R 2002 Galectins and cancer. *Biochim. Biophys. Acta* **1572** 285–293

de Kivit S, Saeland E, Kraneveld AD, van de Kant HJ, Schouten B, van Esch BC, Knol J, Sprikkelman AB, *et al*. 2012 Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. *Allergy* **67** 343–352

Dekker RJ, Boon RA, Rondaij MG, Kragt A, Volger OL, Elderkamp YW, Meijers JC, Voorberg J, *et al*. 2006 KLF2 provokes a gene expression pattern that establishes functional quiescent differentiation of the endothelium. *Blood* **107** 4354–4363

Dumic J, Dabelic S and Flogel M 2006 Galectin-3: an open-ended story. *Biochim. Biophys. Acta* **1760** 616–635

Elahi S, Niki T, Hirashima M and Horton H 2012 Galectin-9 binding to Tim-3 renders activated human CD4+ T cells less susceptible to HIV-1 infection. *Blood.* **119** 4192–4204

Ewing RM, Chu P, Elisma F, Li H, Taylor P, Climie S, McBroom-Cerajewski L, Robinson MD, *et al*. 2007 Large-scale mapping of human protein–protein interactions by mass spectrometry. *Mol. Syst. Biol.* **3** 89

Fukata Y, Itoh A, Nonaka Y, Ogawa T, Nakamura T, Matsushita O and Nishi N 2014 Direct cytocidal effect of galectin-9 localized on collagen matrices on human immune cell lines. *Biochim. Biophys. Acta* **1840** 1892–1901

Fukushima A, Sumi T, Fukuda K, Kumagai N, Nishida T, Akiba H, Okumura K, Yagita H, *et al*. 2007 Antibodies to T-cell Ig and mucin domain-containing proteins (Tim)-1 and -3 suppress the induction and progression of murine allergic conjunctivitis. *Biochem. Biophys. Res. Commun.* **353** 211–216

Gabius HJ 2000 Biological information transfer beyond the genetic code: the sugar code. *Naturwissenschaften* **87** 108–121

Gabius HJ, Siebert HC, Andre S, Jimenez-Barbero J and Rudiger H 2004 Chemical biology of the sugar code. *Chembiochem.* **5** 740–764

Gooden MJ, Wiersma VR, Samplonius DF, Gerssen J, van Ginkel RJ, Nijman HW, Hirashima M, Niki T, *et al*. 2013 Galectin-9 activates and expands human T-helper 1 cells. *PLoS One* **8** e65616

Gustavsson E, Sernbo S, Andersson E, Brennan DJ, Dictor M, Jerkeman M, Borrebaeck CAK and Ek S 2010 SOX11 expression correlates to promoter methylation and regulates tumor growth in hematopoietic malignancies. *Mol. Cancer* **9** 187

Hao H, He M, Li J, Zhou Y, Dang J, Li F, Yang M and Deng D 2015 Upregulation of the Tim-3/Gal-9 pathway and correlation with the development of preeclampsia. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **194** 85–91

Heusschen R, Griffioen AW and Thijssen VL 2013 Galectin-9 in tumor biology: a jack of multiple trades. *Biochim. Biophys. Acta* **1836** 177–185

Heusschen R, Schulkens IA, van Beijnum J, Griffioen AW and Thijssen VL 2014 Endothelial LGALS9 splice variant expression in endothelial cell biology and angiogenesis. *Biochim. Biophys. Acta* **1842** 284–292

Hirabayashi J, Hashidate T, Arata Y, Nishi N, Nakamura T, Hirashima M, Urashima T, Oka T, *et al*. 2002 Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. *Biochim. Biophys. Acta Gen. Subj.* **1572** 232–254

Hirao H, Uchida Y, Kadono K, Tanaka H, Niki T, Yamauchi A, Hata K, Watanabe T, *et al*. 2015 The protective function of galectin-9 in liver ischemia and reperfusion injury in mice. *Liver Transpl.* **21** 969–981

Hirashima M, Kashio Y, Nishi N, Yamauchi A, Imaizumi T-A, Kageshita T, Saita N and Nakamura T 2004 Galectin-9 in physiological and pathological conditions. *Glycoconj. J.* **19** 593–600

Hughes RC 2001 Galectins as modulators of cell adhesion. *Biochimie* **83** 667–676

Hughes RC 1999 Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim. Biophys. Acta* **1473** 172–185

Igawa K, Satoh T, Hirashima M and Yokozeki H 2006 Regulatory mechanisms of galectin-9 and eotaxin-3 synthesis in epidermal keratinocytes: possible involvement of galectin-9 in dermal eosinophilia of Th1-polarized skin inflammation. *Allergy* **61** 1385–1391

Imaizumi T, Yoshida H, Nishi N, Sashinami H, Nakamura T, Hirashima M, Ohyama C, Itoh K, *et al*. 2007 Double-stranded RNA

induces galectin-9 in vascular endothelial cells: involvement of TLR3, PI3K, and IRF3 pathway. *Glycobiology* **17** 12C–15C

Irie A, Yamauchi A, Kontani K, Kihara M, Liu D, Shirato Y, Seki M, Nishi N, *et al*. 2005 Galectin-9 as a prognostic factor with antimetastatic potential in breast cancer. *Clin. Cancer Res.* **11** 2962–2968

Jayaraman P, Sada-Ovalle I, Nishimura T, Anderson AC, Kuchroo VK, Remold HG and Behar SM 2013 IL-1beta promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. *J. Immunol.* **190** 4196–4204

Ji XJ, Ma CJ, Wang JM, Wu XY, Niki T, Hirashima M, Moorman JP and Yao ZQ 2013 HCV-infected hepatocytes drive CD4+ CD25+ Foxp3+ regulatory T-cell development through the Tim-3/Gal-9 pathway. *Eur. J. Immunol.* **43** 458–467

Johannes L and Romer W 2010 Shiga toxins–from cell biology to biomedical applications. *Nat. Rev. Microbiol.* **8** 105–116

Johswich A, Longuet C, Pawling J, Rahman AA, Ryczko M, Drucker DJ and Dennis JW 2014 N-glycan remodeling on glucagon receptor is an effector of nutrient sensing by the hexosamine biosynthesis pathway. *J. Biol. Chem.* **289** 15927–15941

Kadowaki T, Morishita A, Niki T, Hara J, Sato M, Tani J, Miyoshi H, Yoneyama H, *et al*. 2013 Galectin-9 prolongs the survival of septic mice by expanding Tim-3-expressing natural killer T cells and PDCA-1+ CD11c+ macrophages. *Crit. Care* **17** R284

Kageshita T, Kashio Y, Yamauchi A, Seki M, Abedin MJ, Nishi N, Shoji H, Nakamura T, *et al*. 2002 Possible role of galectin-9 in cell aggregation and apoptosis of human melanoma cell lines and its clinical significance. *Int. J. Cancer* **99** 809–816

Kanzaki M, Wada J, Sugiyama K, Nakatsuka A, Teshigawara S, Murakami K, Inoue K, Terami T, *et al*. 2012 Galectin-9 and T cell immunoglobulin mucin-3 pathway is a therapeutic target for type 1 diabetes. *Endocrinology* **153** 612–620

Kasai K and Hirabayashi J 1996 Galectins: a family of animal lectins that decipher glycocodes. *J. Biochem.* **119** 1–8

Kasamatsu A, Uzawa K, Shimada K, Shiiba M, Otsuka Y, Seki N, Abiko Y and Tanzawa H 2005a Elevation of galectin-9 as an inflammatory response in the periodontal ligament cells exposed to Porphylomonas gingivalis lipopolysaccharide in vitro and in vivo. *Int. J. Biochem. Cell Biol.* **37** 397–408

Kasamatsu A, Uzawa K, Nakashima D, Koike H, Shiiba M, Bukawa H, Yokoe H and Tanzawa H 2005b Galectin-9 as a regulator of cellular adhesion in human oral squamous cell carcinoma cell lines. *Int. J. Mol. Med.* **16** 269–273

Kashio Y, Nakamura K, Abedin MJ, Seki M, Nishi N, Yoshida N, Nakamura T and Hirashima M 2003 Galectin-9 induces apoptosis through the calcium-calpain-caspase-1 pathway. *J. Immunol.* **170** 3631–3636

Katoh S, Ishii N, Nobumoto A, Takeshita K, Dai SY, Shinonaga R, Niki T, Nishi N, *et al*. 2007 Galectin-9 inhibits CD44-hyaluronan interaction and suppresses a murine model of allergic asthma. *Am. J. Respir. Crit. Care Med.* **176** 27–35

Keryer-Bibens C, Pioche-Durieu C, Villemant C, Souquere S, Nishi N, Hirashima M, Middeldorp J and Busson P 2006 Exosomes released by EBV-infected nasopharyngeal carcinoma cells convey the viral latent membrane protein 1 and the immunomodulatory protein galectin 9. *BMC Cancer* **6** 283

Kikushige Y, Miyamoto T, Yuda J, Jabbarzadeh-Tabrizi S, Shima T, Takayanagi S-i, Niiro H, Yurino A, *et al*. 2015 A TIM-3/Gal-9 autocrine stimulatory loop drives self-renewal of human myeloid leukemia stem cells and leukemic progression. *Cell Stem Cell* **17** 341–352

Klibi J, Niki T, Riedel A, Pioche-Durieu C, Souquere S, Rubinstein E, Le Moulec S, Guigay J, *et al*. 2009 Blood diffusion and Th1-suppressive effects of galectin-9-containing exosomes released by Epstein-Barr virus-infected nasopharyngeal carcinoma cells. *Blood.* **113** 1957–1966

Kobayashi K, Morishita A, Iwama H, Fujita K, Okura R, Fujihara S, Yamashita T, Fujimori T, *et al*. 2015 Galectin-9 suppresses cholangiocarcinoma cell proliferation by inducing apoptosis but not cell cycle arrest. *Oncol. Rep.* **34** 1761–1770

Kobayashi T, Kuroda J, Ashihara E, Oomizu S, Terui Y, Taniyama A, Adachi S, Takagi T, *et al*. 2010 Galectin-9 exhibits anti-myeloma activity through JNK and p38 MAP kinase pathways. *Leukemia* **24** 843–850

Komita H, Koido S, Hayashi K, Kan S, Ito M, Kamata Y, Suzuki M and Homma S 2015 Expression of immune checkpoint molecules of T cell immunoglobulin and mucin protein 3/galectin-9 for NK cell suppression in human gastrointestinal stromal tumors. *Oncol. Rep.* **34** 2099–2105

Kuroda J, Yamamoto M, Nagoshi H, Kobayashi T, Sasaki N, Shimura Y, Horiike S, Kimura S, *et al*. 2010 Targeting activating transcription factor 3 by Galectin-9 induces apoptosis and overcomes various types of treatment resistance in chronic myelogenous leukemia. *Mol. Cancer Res.* **8** 994–1001

Kurose Y, Wada J, Kanzaki M, Teshigawara S, Nakatsuka A, Murakami K, Inoue K, Terami T, *et al*. 2013 Serum galectin-9 levels are elevated in the patients with type 2 diabetes and chronic kidney disease. *BMC Nephrol.* **14** 23

Lahm H, Andre S, Hoeflich A, Fischer JR, Sordat B, Kaltner H, Wolf E and Gabius HJ 2001 Comprehensive galectin fingerprinting in a panel of 61 human tumor cell lines by RT-PCR and its implications for diagnostic and therapeutic procedures. *J. Cancer Res. Clin. Oncol.* **127** 375–386

Lahm H, Hoeflich A, Andre S, Sordat B, Kaltner H, Wolf E and Gabius HJ 2000 Gene expression of galectin-9/ecalectin, a potent eosinophil chemoattractant, and/or the insertional isoform in human colorectal carcinoma cell lines and detection of frameshift mutations for protein sequence truncations in the second functional lectin domain. *Int. J. Oncol.* **17** 519–524

Leal-Pinto E, Tao W, Rappaport J, Richardson M, Knorr BA and Abramson RG 1997 Molecular cloning and functional reconstitution of a urate transporter/channel. *J. Biol. Chem.* **272** 617–625

Leber MF and Efferth T 2009 Molecular principles of cancer invasion and metastasis (review). *Int. J. Oncol.* **34** 881–895

Leffler H, Carlsson S, Hedlund M, Qian Y and Poirier F 2004 Introduction to galectins. *Glycoconj. J.* **19** 433–440

Li YH, Zhou WH, Tao Y, Wang SC, Jiang YL, Zhang D, Piao HL, Fu Q, *et al*. 2016 The Galectin-9/Tim-3 pathway is involved in the regulation of NK cell function at the maternal-fetal interface in early pregnancy. *Cell. Mol. Immunol.* **13** 73–81

Liang M, Ueno M, Oomizu S, Arikawa T, Shinonaga R, Zhang S, Yamauchi A and Hirashima M 2008 Galectin-9 expression links to malignant potential of cervical squamous cell carcinoma. *J. Cancer Res. Clin. Oncol.* **134** 899–907

Liberal R, Grant CR, Holder BS, Ma Y, Mieli-Vergani G, Vergani D and Longhi MS 2012 The impaired immune regulation of

autoimmune hepatitis is linked to a defective galectin-9/tim-3 pathway. *Hepatology* **56** 677–686

Liu Z, Han H, He X, Li S, Wu C, Yu C and Wang S 2016 Expression of the galectin-9-Tim-3 pathway in glioma tissues is associated with the clinical manifestations of glioma. *Oncol. Lett.* **11** 1829–1834

Lv K, Zhang Y, Zhang M, Zhong M and Suo Q 2012 Galectin-9 ameliorates Con A-induced hepatitis by inducing CD4(+)CD25(low/int) effector T-Cell apoptosis and increasing regulatory T cell number. *PLoS One* **7** e48379

Ma CJ, Li GY, Cheng YQ, Wang JM, Ying RS, Shi L, Wu XY, Niki T, *et al*. 2013 Cis association of galectin-9 with Tim-3 differentially regulates IL-12/IL-23 expressions in monocytes via TLR signaling. *PLoS One* **8** e72488

Madireddi S, Eun SY, Lee SW, Nemcovicova I, Mehta AK, Zajonc DM, Nishi N, Niki T, *et al*. 2014 Galectin-9 controls the therapeutic activity of 4-1BB-targeting antibodies. *J. Exp. Med.* **211** 1433–1448

Matsumoto R 1998 Human ecalectin, a variant of human galectin-9, is a novel eosinophil chemoattractant produced by T lymphocytes. *J. Biol. Chem.* **273** 16976–16984

Matsumoto R, Hirashima M, Kita H and Gleich GJ 2002 Biological activities of ecalectin: a novel eosinophil-activating factor. *J. Immunol.* **168** 1961–1967

Matsushita N, Nishi N, Seki M, Matsumoto R, Kuwabara I, Liu FT, Hata Y, Nakamura T, *et al*. 2000 Requirement of divalent galactoside-binding activity of ecalectin/galectin-9 for eosinophil chemoattraction. *J. Biol. Chem.* **275** 8355–8360

Matsuura A, Tsukada J, Mizobe T, Higashi T, Mouri F, Tanikawa R, Yamauchi A, Hirashima M, *et al*. 2009 Intracellular galectin-9 activates inflammatory cytokines in monocytes. *Genes Cells* **14** 511–521

Mengshol JA, Golden-Mason L, Arikawa T, Smith M, Niki T, Mcwilliams R, Randall JA, Mcmahan R, *et al*. 2010 A crucial role for Kupffer cell-derived galectin-9 in regulation of T cell immunity in hepatitis C infection. *PLoS One* **5** e9504

Mishra R, Grzybek M, Niki T, Hirashima M and Simons K 2010 Galectin-9 trafficking regulates apical-basal polarity in Madin-Darby canine kidney epithelial cells. *Proc. Natl. Acad. Sci. USA* **107** 17633–17638

Miyanishi N, Nishi N, Abe H, Kashio Y, Shinonaga R, Nakakita S, Sumiyoshi W, Yamauchi A, *et al*. 2007 Carbohydrate-recognition domains of galectin-9 are involved in intermolecular interaction with galectin-9 itself and other members of the galectin family. *Glycobiology* **17** 423–432

Moritoki M, Kadowaki T, Niki T, Nakano D, Soma G, Mori H, Kobara H, Masaki T, *et al*. 2013 Galectin-9 ameliorates clinical severity of MRL/lpr lupus-prone mice by inducing plasma cell apoptosis independently of Tim-3. *PLoS One.* **8**, e60807

Munitz A and Levi-Schaffer F 2004 Eosinophils: 'new' roles for 'old' cells. *Allergy* **59** 268–275

Murphy PV, Andre S and Gabius HJ 2013 The third dimension of reading the sugar code by lectins: design of glycoclusters with cyclic scaffolds as tools with the aim to define correlations between spatial presentation and activity. *Molecules* **18** 4026–4053

Nagae M, Nishi N, Murata T, Usui T, Nakamura T, Wakatsuki S and Kato R 2006 Crystal structure of the galectin-9 N-terminal carbohydrate recognition domain from Mus musculus reveals the basic mechanism of carbohydrate recognition. *J. Biol. Chem.* **281** 35884–35893

Nagae M, Nishi N, Murata T, Usui T, Nakamura T, Wakatsuki S and Kato R 2009 Structural analysis of the recognition mechanism of poly-N-acetyllactosamine by the human galectin-9 N-terminal carbohydrate recognition domain. *Glycobiology* **19** 112–117

Nagae M, Nishi N, Nakamura-Tsuruta S, Hirabayashi J, Wakatsuki S and Kato R 2008 Structural analysis of the human galectin-9 N-terminal carbohydrate recognition domain reveals unexpected properties that differ from the mouse orthologue. *J. Mol. Biol.* **375** 119–135

Nagahara K, Arikawa T, Oomizu S, Kontani K, Nobumoto A, Tateno H, Watanabe K, Niki T, *et al*. 2008 Galectin-9 increases Tim-3+ dendritic cells and CD8+ T cells and enhances antitumor immunity via galectin-9-Tim-3 interactions. *J. Immunol.* **181** 7660–7669

Nebbia G, Peppa D, Schurich A, Khanna P, Singh HD, Cheng Y, Rosenberg W, Dusheiko G, *et al*. 2012 Upregulation of the Tim-3/galectin-9 pathway of T cell exhaustion in chronic hepatitis B virus infection. *PLoS One* **7** e47648

Niki T, Tsutsui S, Hirose S, Aradono S, Sugimoto Y, Takeshita K, Nishi N and Hirashima M 2009 Galectin-9 is a high affinity IgE-binding lectin with anti-allergic effect by blocking IgE-antigen complex formation. *J. Biol. Chem.* **284** 32344–32352

Nishi N, Itoh A, Fujiyama A, Yoshida N, Araya S, Hirashima M, Shoji H and Nakamura T 2005 Development of highly stable galectins: truncation of the linker peptide confers protease-resistance on tandem-repeat type galectins. *FEBS Lett.* **579** 2058–2064

Nishi N, Itoh A, Shoji H, Miyanaka H and Nakamura T 2006 Galectin-8 and galectin-9 are novel substrates for thrombin. *Glycobiology* **16** 15C–20C

Nobumoto A, Nagahara K, Oomizu S, Katoh S, Nishi N, Takeshita K, Niki T, Tominaga A, *et al*. 2008 Galectin-9 suppresses tumor metastasis by blocking adhesion to endothelium and extracellular matrices. *Glycobiology* **18** 735–744

Nonaka Y, Ogawa T, Oomizu S, Nakakita S, Nishi N, Kamitori S, Hirashima M and Nakamura T 2013 Self-association of the galectin-9 C-terminal domain via the opposite surface of the sugar-binding site. *J. Biochem.* **153** 463–471

Obinata H and Hla T 2012 Sphingosine 1-phosphate in coagulation and inflammation. *Semin. Immunopathol.* **34** 73–91

Ohtsubo K, Takamatsu S, Minowa MT, Yoshida A, Takeuchi M and Marth JD 2005 Dietary and genetic control of glucose transporter 2 glycosylation promotes insulin secretion in suppressing diabetes. *Cell* **123** 1307–1321

Park WS, Jung W-K, Park SK, Heo KW, Kang M-S, Choi YH, Kim G-Y, Park S-G, *et al*. 2011 Expression of galectin-9 by IFN-γ stimulated human nasal polyp fibroblasts through MAPK, PI3K, and JAK/STAT signaling pathways. *Biochem. Biophys. Res. Commun.* **411** 259–264

Reddy PBJ, Schreiber TH, Rajasagi NK, Suryawanshi A, Mulik S, Veiga-Parga T, Niki T, Hirashima M, *et al*. 2012 TNFRSF25 agonistic antibody and galectin-9 combination therapy controls herpes simplex virus-induced immunoinflammatory lesions. *J. Virol.* **86** 10606–10620

Pelletier I, Hashidate T, Urashima T, Nishi N, Nakamura T, Futai M, Arata Y, Kasai K, *et al*. 2003 Specific recognition of Leishmania major poly-beta-galactosyl epitopes by galectin-9: possible implication of galectin-9 in interaction between L. major and host cells. *J. Biol. Chem.* **278** 22223–22230

Pena C, Mirandola L, Figueroa JA, Hosiriluck N, Suvorava N, Trotter K, Reidy A, Rakhshanda R, *et al*. 2014 Galectins as therapeutic targets for hematological malignancies: a hopeful sweetness. *Ann. Transl. Med.* **2** 87

Pioche-Durieu C, Keryer C, Souquere S, Bosq J, Faigle W, Loew D, Hirashima M, Nishi N, *et al*. 2005 In nasopharyngeal carcinoma cells, Epstein-Barr virus LMP1 interacts with galectin 9 in membrane raft elements resistant to simvastatin. *J. Virol.* **79** 13326–13337

Qiao X, Jiang K, Nie J, Fan K, Zheng Z, Wang J and Li J 2014 Increased expression of Tim-3 and its ligand Galectin-9 in rat allografts during acute rejection episodes. *Biochem. Biophys. Res. Commun.* **445** 542–548

Rajendran L, Knolker HJ and Simons K 2010 Subcellular targeting strategies for drug design and delivery. *Nat. Rev. Drug Discov.* **9** 29–42

Reddy PB, Sehrawat S, Suryawanshi A, Rajasagi NK, Mulik S, Hirashima M and Rouse BT 2011 Influence of galectin-9/Tim-3 interaction on herpes simplex virus-1 latency. *J. Immunol.* **187** 5745–5755

Rhodes DH, Pini M, Castellanos KJ, Montero-Melendez T, Cooper D, Perretti M and Fantuzzi G 2013 Adipose tissue-specific modulation of galectin expression in lean and obese mice: evidence for regulatory function. *Obesity (Silver Spring)* **21** 310–319

Sada-Ovalle I, Chavez-Galan L, Torre-Bouscoulet L, Nava-Gamino L, Barrera L, Jayaraman P, Torres-Rojas M, Salazar-Lezama MA, *et al*. 2012 The Tim3-galectin 9 pathway induces antibacterial activity in human macrophages infected with Mycobacterium tuberculosis. *J. Immunol.* **189** 5896–5902

Saitoh H, Ashino Y, Chagan-Yasutan H, Niki T, Hirashima M and Hattori T 2012 Rapid decrease of plasma galectin-9 levels in patients with acute HIV infection after therapy. *Tohoku J. Exp. Med.* **228** 157–161

Sakai K, Kawata E, Ashihara E, Nakagawa Y, Yamauchi A, Yao H, Nagao R, Tanaka R, *et al*. 2011 Galectin-9 ameliorates acute GVH disease through the induction of T-cell apoptosis. *Eur. J. Immunol.* **41** 67–75

Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK and Anderson AC 2010 Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J. Exp. Med.* **207** 2187–2194

Samoszuk M 1997 Eosinophils and human cancer. *Histol Histopathol* **12**(3) 807–812

Sanchez-Cuellar S, de la Fuente H, Cruz-Adalia A, Lamana A, Cibrian D, Giron RM, Vara A, Sanchez-Madrid F, *et al*. 2012 Reduced expression of galectin-1 and galectin-9 by leucocytes in asthma patients. *Clin. Exp. Immunol.* **170** 365–374

Sato M, Nishi N, Shoji H, Seki M, Hashidate T, Hirabayashi J, Kasai K-i, Hata Y, *et al*. 2002 Functional analysis of the carbohydrate recognition domains and a linker peptide of galectin-9 as to eosinophil chemoattractant activity. *Glycobiology* **12** 191–197

Schlotzer-Schrehardt U, Andre S, Janko C, Kaltner H, Kopitz J, Gabius HJ and Herrmann M 2012 Adhesion/growth-regulatory galectins in the human eye: localization profiles and tissue reactivities as a standard to detect disease-associated alterations. *Graefes Arch. Clin. Exp. Ophthalmol.* **250** 1169–1180

Seki M, Sakata KM, Oomizu S, Arikawa T, Sakata A, Ueno M, Nobumoto A, Niki T, *et al*. 2007 Beneficial effect of galectin 9 on rheumatoid arthritis by induction of apoptosis of synovial fibroblasts. *Arthritis Rheum.* **56** 3968–3976

Sharma S, Sundararajan A, Suryawanshi A, Kumar N, Veiga-Parga T, Kuchroo VK, Thomas PG, Sangster MY, *et al*. 2011 T cell immunoglobulin and mucin protein-3 (Tim-3)/Galectin-9 interaction regulates influenza A virus-specific humoral and CD8 T-cell responses. *Proc. Natl. Acad. Sci. USA* **108** 19001–19006

Shimizu Y, Kabir-Salmani M, Azadbakht M, Sugihara K, Sakai K and Iwashita M 2008 Expression and localization of galectin-9 in the human uterodome. *Endocr. J.* **55** 879–887

Shimmura-Tomita M, Wang M, Taniguchi H, Akiba H, Yagita H and Hori J 2013 Galectin-9-mediated protection from allospecific T cells as a mechanism of immune privilege of corneal allografts. *PLoS One* **8** e63620

Solís D, Maté MJ, Lohr M, Ribeiro JP, López-Merino L, André S, Buzamet E, Javier Cañada F, *et al*. 2010 N-domain of human adhesion/growth-regulatory galectin-9: preference for distinct conformers and non-sialylated N-glycans and detection of ligand-induced structural changes in crystal and solution. *Int. J. Biochem. Cell Biol.* **42** 1019–1029

Spitzenberger F, Graessler J and Schroeder HE 2001 Molecular and functional characterization of galectin 9 mRNA isoforms in porcine and human cells and tissues. *Biochimie.* **83** 851–862

Stancic M, van Horssen J, Thijssen VL, Gabius HJ, van der Valk P, Hoekstra D and Baron W 2011 Increased expression of distinct galectins in multiple sclerosis lesions. *Neuropathol. Appl. Neurobiol.* **37** 654–671

Steelman AJ, Smith R, Welsh CJ and Li J 2013 Galectin-9 protein is up-regulated in astrocytes by tumor necrosis factor and promotes encephalitogenic T-cell apoptosis. *J. Biol. Chem.* **288** 23776–23787

Straube T, von Mach T, Honig E, Greb C, Schneider D and Jacob R 2013 pH-dependent recycling of galectin-3 at the apical membrane of epithelial cells. *Traffic* **14** 1014–1027

Strukova SM 2001 Thrombin as a regulator of inflammation and reparative processes in tissues. *Biochemistry (Mosc).* **66** 8–18

Sun J, Yang M, Ban Y, Gao W, Song B, Wang Y, Zhang Y, Shao Q, *et al*. 2016 Tim-3 is upregulated in NK cells during early pregnancy and inhibits NK cytotoxicity toward trophoblast in galectin-9 dependent pathway. *PLoS One* **11** e0147186

Tadokoro T, Morishita A, Fujihara S, Iwama H, Niki T, Fujita K, Akashi E, Mimura S, *et al*. 2016 Galectin-9: an anticancer molecule for gallbladder carcinoma. *Int. J. Oncol.* **48** 1165–1174

Takano J, Morishita A, Fujihara S, Iwama H, Kokado F, Fujikawa K, Fujita K, Chiyo T, *et al*. 2016 Galectin-9 suppresses the proliferation of gastric cancer cells in vitro. *Oncol. Rep.* **35** 851–860

Tang ZH, Liang S, Potter J, Jiang X, Mao HQ and Li Z 2013 Tim-3/galectin-9 regulate the homeostasis of hepatic NKT cells in a

murine model of nonalcoholic fatty liver disease. *J. Immunol.* **190** 1788–1796

Tanikawa R, Tanikawa T, Hirashima M, Yamauchi A and Tanaka Y 2010 Galectin-9 induces osteoblast differentiation through the CD44/Smad signaling pathway. *Biochem. Biophys. Res. Commun.* **394** 317–322

Tanikawa R, Tanikawa T, Okada Y, Nakano K, Hirashima M, Yamauchi A, Hosokawa R and Tanaka Y 2008 Interaction of galectin-9 with lipid rafts induces osteoblast proliferation through the c-Src/ERK signaling pathway. *J. Bone Miner. Res.* **23** 278–286

Tao W 1997 Molecular cloning and functional reconstitution of a urate transporter/channel. *J. Biol. Chem.* **272** 617–625

Tao YF, Lin F, Yan XY, Gao XG, Teng F, Fu ZR and Wang ZX 2015 Galectin-9 in combination with EX-527 prolongs the survival of cardiac allografts in mice after cardiac transplantation. *Transplant. Proc.* **47** 2003–2009

Than NG, Romero R, Goodman M, Weckle A, Xing J, Dong Z, Xu Y, Tarquini F, *et al*. 2009 A primate subfamily of galectins expressed at the maternal–fetal interface that promote immune cell death. *Proc. Natl. Acad. Sci.* **106** 9731–9736

Thijssen VL, Heusschen R, Caers J and Griffioen AW 2015 Galectin expression in cancer diagnosis and prognosis: a systematic review. *Biochim. Biophys. Acta.* **1855** 235–247

Thijssen VL, Hulsmans S and Griffioen AW 2008 The galectin profile of the endothelium: altered expression and localization in activated and tumor endothelial cells. *Am. J. Pathol.* **172** 545–553

Martin TA, Ye L, Sanders AJ, Lane J and Jiang WG 2013 Cancer invasion and metastasis: molecular and cellular perspective. In: *Madame Curie Bioscience database [Internet]* (Austin (TX): Landes Bioscience)

Tsuchiyama Y, Wada J, Zhang H, Morita Y, Hiragushi K, Hida K, Shikata K, Yamamura M, *et al*. 2000 Efficacy of galectins in the amelioration of nephrotoxic serum nephritis in Wistar Kyoto rats. *Kidney Int.* **58** 1941–1952

Tureci O, Schmitt H, Fadle N, Pfreundschuh M and Sahin U 1997 Molecular definition of a novel human galectin which is immunogenic in patients with Hodgkin's disease. *J. Biol. Chem.* **272** 6416–6422

Ungerer C, Quade-Lyssy P, Radeke HH, Henschler R, Konigs C, Kohl U, Seifried E and Schuttrumpf J 2014 Galectin-9 is a suppressor of T and B cells and predicts the immune modulatory potential of mesenchymal stromal cell preparations. *Stem Cells Dev.* **23** 755–766

van den Brule F, Califice S and Castronovo V 2004 Expression of galectins in cancer: a critical review. *Glycoconj. J.* **19** 537–542

Vasta GR, Ahmed H, Nita-Lazar M, Banerjee A, Pasek M, Shridhar S, Guha P and Fernández-Robledo JA 2012 Galectins as self/non-self recognition receptors in innate and adaptive immunity: an unresolved paradox. *Front. Immunol.* **3** 199

Vega-Carrascal I, Bergin DA, Mcelvaney OJ, McCarthy C, Banville N, Pohl K, Hirashima M, Kuchroo VK, *et al*. 2014 Galectin-9 signaling through TIM-3 is involved in neutrophil-mediated Gram-negative bacterial killing: an effect abrogated within the cystic fibrosis lung. *J. Immunol.* **192** 2418–2431

Vega-Carrascal I, Reeves EP, Niki T, Arikawa T, Mcnally P, O'Neill SJ, Hirashima M and Mcelvaney NG 2011 Dysregulation of TIM-3-galectin-9 pathway in the cystic fibrosis airways. *J. Immunol.* **186** 2897–2909

Villalobo A and Gabius H 1998 Signaling pathways for transduction of the initial message of the glycocode into cellular responses. *Acta Anat. (Basel).* **161** 110–129

Vladoiu MC, Labrie M and St-Pierre Y 2014 Intracellular galectins in cancer cells: potential new targets for therapy (Review). *Int. J. Oncol.* **44** 1001–1014

von Mach T, Carlsson MC, Straube T, Nilsson U, Leffler H and Jacob R 2014 Ligand binding and complex formation of galectin-3 is modulated by pH variations. *Biochem. J.* **457** 107–115

Wada J, Ota K, Kumar A, Wallner EI and Kanwar YS 1997 Developmental regulation, expression, and apoptotic potential of galectin-9, a beta-galactoside binding lectin. *J. Clin. Investig.* **99** 2452–2461

Wang F, He W, Zhou H, Yuan J, Wu K, Xu L and Chen ZK 2007 The Tim-3 ligand galectin-9 negatively regulates CD8+ alloreactive T cell and prolongs survival of skin graft. *Cell. Immunol.* **250** 68–74

Wang F, Xu J, Liao Y, Wang Y, Liu C, Zhu X, Chen ZK and Sun Z 2011 Tim-3 ligand galectin-9 reduces IL-17 level and accelerates Klebsiella pneumoniae infection. *Cell. Immunol.* **269** 22–28

Wedemeyer J and Vosskuhl K 2008 Role of gastrointestinal eosinophils in inflammatory bowel disease and intestinal tumours. *Best Pract. Res. Clin. Gastroenterol.* **22** 537–549

Weigle B, Ebner R, Temme A, Schwind S, Schmitz M, Kiessling A, Rieger MA, Schackert G, *et al*. 2005 Highly specific overexpression of the transcription factor SOX11 in human malignant gliomas. *Oncol. Rep.* **13** 139–144

Wiersma VR, de Bruyn M, Helfrich W and Bremer E 2013 Therapeutic potential of Galectin-9 in human disease. *Med. Res. Rev.* **33** E102–E126

Wiersma VR, de Bruyn M, van Ginkel RJ, Sigar E, Hirashima M, Niki T, Nishi N, Samplonius DF, *et al*. 2012 The glycan-binding protein galectin-9 has direct apoptotic activity toward melanoma cells. *J. Invest. Dermatol.* **132** 2302–2305

Wu C, Thalhamer T, Franca RF, Xiao S, Wang C, Hotta C, Zhu C, Hirashima M, *et al*. 2014 Galectin-9-CD44 interaction enhances stability and function of adaptive regulatory T cells. *Immunity.* **41** 270–282

Yang Q, Jiang W, Zhuang C, Geng Z, Hou C, Huang D, Hu L and Wang X 2015 microRNA-22 downregulation of galectin-9 influences lymphocyte apoptosis and tumor cell proliferation in liver cancer. *Oncol. Rep.* **34** 1771–1778

Yang RY, Rabinovich GA and Liu FT 2008 Galectins: structure, function and therapeutic potential. *Expert Rev. Mol. Med.* **10** e17

Yoshida H, Teraoka M, Nishi N, Nakakita S, Nakamura T, Hirashima M and Kamitori S 2010 X-ray structures of human galectin-9 C-terminal domain in complexes with a biantennary oligosaccharide and sialyllactose. *J. Biol. Chem.* **285** 36969–36976

Zhang F, Zheng M, Qu Y, Li J, Ji J, Feng B, Lu A, Li J, *et al*. 2009 Different roles of galectin-9 isoforms in modulating E-selectin expression and adhesion function in LoVo colon carcinoma cells. *Mol. Biol. Rep.* **36** 823–830

Zhang Q, Luan H, Wang L, He F, Zhou H, Xu X, Li X, Xu Q, *et al*. 2014a Galectin-9 ameliorates anti-GBM glomerulonephritis by inhibiting Th1 and Th17 immune responses in mice. *Am. J. Physiol. Renal Physiol.* **306** F822–F832

Zhang XM and Shan NN 2014 The role of T cell immunoglobulin and mucin domain-3 in immune thrombocytopenia. *Scand. J. Immunol.* **79** 231–236

Zhang Y, Jiang L, Zhang M and Lv K 2014b Galectin-9 induced myeloid suppressor cells expand regulatory T cells in an IL-10-dependent manner in CVB3-induced acute myocarditis. *Int. J. Mol. Sci.* **15** 3356–3372

Zhang ZY, Dong JH, Chen YW, Wang XQ, Li CH, Wang J, Wang GQ, Li HL, *et al*. 2012 Galectin-9 acts as a prognostic factor with antimetastatic potential in hepatocellular carcinoma. *Asian Pac. J. Cancer Prev.* **13** 2503–2509

Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, Zheng XX, Strom TB, *et al*. 2005 The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat. Immunol.* **6** 1245–1252

MS received 27 February 2016; accepted 11 May 2016

Corresponding editor: Veena K Parnaik