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Integrating structure and function of 'tandem-repeat' galectins

Maria F. Troncoso[1], Maria T. Elola[1], Diego O. Croci[2], Gabriel A. Rabinovich[2,3]

[1]Institute of Biological Chemistry and Physicochemistry (UBA-CONICET), Department of Biological Chemistry, School of Pharmacy and Biochemistry, University of Buenos Aires, [2]Laboratory of Immunopathology, Institute of Biology and Experimental Medicine (IBYME-CONICET), [3]Department of Biological Chemistry, School of Exact and Natural Sciences, University of Buenos Aires, Buenos Aires, Argentina

TABLE OF CONTENTS

1. ABSTRACT

Galectins (GALs) are evolutionarily-conserved lectins defined by at least one carbohydrate recognition domain (CRD) with affinity for beta-galactosides and conserved sequence motifs. Although the biological roles of some members of this family, including the 'proto-type' GAL-1 and the 'chimera-type' GAL-3 have been widely studied, the functions of 'tandem-repeat' galectins are just emerging. The subgroup of 'tandem-repeat' galectins (GAL-4, -6, -8, -9, and -12) contain two distinct CRDs, connected by a linker peptide. Here we integrated and distilled the available information on 'tandem-repeat' galectins, their specific structures, potential ligands and biological activities in inflammatory and neoplastic diseases. While GAL-4 has been implicated in inflammatory bowel diseases, either as a pro-inflammatory or pro-apoptotic mediator, GAL-8 plays roles in autoimmune diseases such as rheumatoid arthritis and lupus erythematosus and modulates tumor progression. GAL-9 controls allergic inflammation and Th1/Th17-mediated autoimmunity and has prognostic value in certain tumor types. Finally, GAL-12 plays important roles in adipocyte physiology. Although this information is just emerging, further studies are needed to dissect the biological roles of 'tandem-repeat' galectins in health and disease.

2. INTRODUCTION

Galectins are evolutionarily-conserved lectins defined by at least one CRD with affinity for beta-galactosides and conserved sequence motifs (1). Fifteen members have been defined in mammals with subtle differences in glycan-binding specificity and wide tissue distribution. They can be subdivided into three groups: those containing one carbohydrate recognition domain (CRD) that can dimerize ('proto-type' galectins; GAL-1, -2, -5, -7, -10, -11, -13, -14, and -15); those containing two distinct CRDs in tandem, connected by a linker peptide ('tandem-repeat' galectins; GAL-4, -6, -8, -9, and -12); and GAL-3 ('chimera' galectin) which consists of unusual proline- and glycine-rich short stretches fused onto the CRD (2).

Several roles have been proposed for the linker region of 'tandem-repeat' galectins, including protein-protein interactions, membrane insertion and regulation of CRD presentation. By using mutated/truncated forms of GAL-8, it has been demonstrated that isolated CRDs have impaired biological activity. Interestingly, a comparable effect has been shown, in some circumstances, when shortening of the linker peptide ('hinge' region) was

accomplished. These results indicate that 'tandem-repeat' galectins require cooperative interactions between the two CRDs and a properly oriented 'hinge' region for effective function (3).

Numerous studies have demonstrated that 'tandem-repeat' galectins are considerably more potent than other members of the galectin family in inducing the same cellular responses; for example, GAL-4, -8, and -9 are much more potent than GAL-3 and GAL-1 in eliciting signaling on T cells and neutrophils (4-9). Earl and coworkers demonstrated that the insertion of a linker region between two CRDs confers increased signaling potency by allowing intermolecular interaction of these CRDs, thus promoting higher order multimerization. Thus, 'tandem-repeat' galectins are endowed with the ability to form higher-order multivalent multimers and increased lattice formation on the cell surface. The increased potency of 'tandem-repeat' galectins compared with 'proto-type' galectins is likely due to the property of the linker domain to allow for intermolecular CRD interactions, resulting in the formation of multimers with increased valency (10).

Multiple biological functions related to immune and inflammatory responses have been reported for galectins over the past decade including roles in cellular adhesion, migration, cytokine synthesis, and survival (1, 2, 11). In fact, different galectins are emerging as crucial mediators in acute and chronic inflammatory diseases, autoimmunity and cancer, and are being increasingly recognized as molecular targets for innovative drug discovery (12, 13).

Strikingly, GAL-4 and GAL-8 can serve as bactericidal lectins. Recently, Stowell and colleagues reported a new role for GAL-4 and GAL-8 in innate immunity, demonstrating that these lectins can autonomously kill target bacteria which express blood group B carbohydrates on the side chains of surface glycans. The best characterized of such bacteria is *E. coli* O86, which cross-reacts with human anti-blood group B antibodies and induces significant blood group B antibodies in previously unexposed individuals. Importantly, while blood group A or O individuals produce antibodies that kill *E. coli* O86, blood group B individuals do not generate antibodies capable of altering *E. coli* O86 viability, providing a clear example of a restriction in adaptive immunity toward a blood group antigen-bearing pathogen. The binding of GAL-4 and GAL-8, but not GAL-3, to this class of pathogens fills this gap leading to rapid loss of bacterial motility and viability. Notably, the C-terminal CRD is sufficient for both binding and bactericidal activity. Thus, some 'tandem-repeat' galectins have emerged as autonomous bacteria-killing agents; these unexpected findings clearly indicate a major role of these lectins in innate immunity. These studies also change our view of galectins as key mediators of host defense (14, 15).

Galectins are often up- or down-regulated in cancerous cells and cancer-associated stromal cells (16-20). In general, this altered expression correlates with the aggressiveness of the tumors and the acquisition of metastatic phenotype, indicating that galectins may modulate tumor progression and influence disease outcome. In cancer biology, different functions have been identified for galectins, including tumor transformation, apoptosis, and cell growth regulation (21). In addition, galectins are also involved in various steps of tumor metastasis, including tumor cell adhesion (22-24), homotypic cell aggregation (25, 26), invasiveness (27-28), angiogenesis (29-33), and inflammation (2). Galectins also function as soluble mediators employed by tumor cells to evade immune responses (34-36). The role of galectins in tumor growth and metastasis *in vivo* is well documented (34, 37-45). However, most of these functions have been described for GAL-1 and GAL-3 and the roles of 'tandem-repeat' galectins have been largely overlooked.

Here, we aim to analyze, integrate and distil the available information on 'tandem-repeat' galectins, their specific structures, linker regions, isoforms, potential ligands, and differences in functions that might help to delineate their role as novel therapeutic targets in inflammatory and cancer disorders.

3. GALECTIN-4

GAL-4 is a 'tandem-repeat' galectin, which possesses two CRDs and is primarily expressed in epithelial cells along the gastrointestinal tract (46). GAL-4 has been first discovered as a 17-kDa protein in rat intestinal extracts, but after cloning its gene, Oda and co-workers found that the 17-kDa protein was only a proteolytic fragment of a larger 36-kDa protein (47). GAL-4 mRNA has been identified in human, pig, rat and mouse alimentary tract, and in rabbit bladder (46).

GAL-4 has been initially identified as an adherens junction protein in pig tongue epithelium (48, 49) but more recently this lectin has been reported as a major component of lipid rafts in brush border membranes of small intestinal epithelial cells (50). The role of GAL-4 in lipid rafts has been studied by using interference RNA to silence its expression in a human colon adenocarcinoma cell line. Following this silencing strategy, protein markers of the apical membrane domain were trapped intracellularly, suggesting that GAL-4 may play an important role in the apical delivery of proteins (51).

Using real time RT-PCR strategies, two porcine small intestine GAL-4 isoforms have been identified that differ in the length of their linker regions. The larger isoform, GAL-4.1, is nine amino acids longer in its linker region than the smaller isoform, GAL-4.2. Based on nucleotide sequence similarities, the two isoforms are likely splice variants of GAL-4 pre-mRNA. By immunohistochemical staining, GAL-4 was found to be primarily localized within the cytoplasm of absorptive epithelial cells lining intestinal villi, especially at the villous tips, with progressively less intense staining observed along the sides of villi and into the crypts. GAL-4 is also associated with the nuclei in villous tip cells. Interestingly, in intestinal crypts, a specific subset of cells, probably enteroendocrine cells, expresses GAL-4 at high

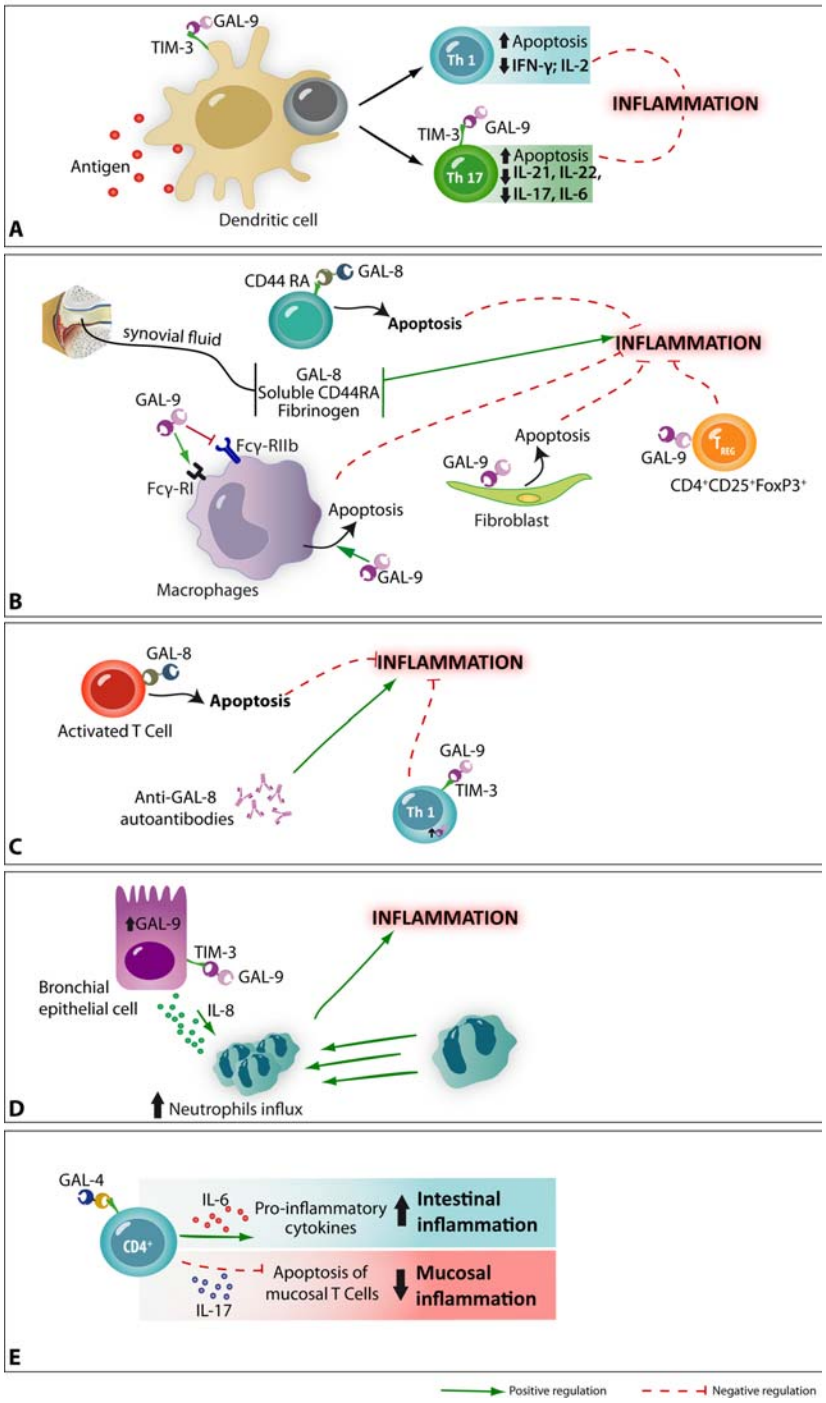

Figure 1. 'Tandem-repeat' galectins in autoimmune inflammation. Rheumatoid arthritis, systemic lupus erythematosus, cystic fibrosis and inflammatory bowel diseases are common autoimmune diseases in which galectins play complex roles. (A) Due to the potent role of GAL-9 in T-cell suppression and its ability to limit Th1- and/or Th17-mediated autoimmune inflammation, it has been proposed to serve as a therapeutic agent in experimental autoimmune encephalomyelitis, rheumatoid arthritis, nephrotoxic serum nephritis model, contact dermatitis, psoriasis, diabetes, graft versus host disease and liver ischemia/reperfusion injury after transplantation. (B) In rheumatoid arthritis, GAL-8 has an anti-inflammatory action and promotes apoptosis of synovial fluid cells by interacting with cell surface CD44vRA. However, in the synovial fluid, fibrinogen forms complexes with GAL-8 and soluble CD44vRA and traps GAL-8, inhibiting its anti-inflammatory effect. GAL-9 suppresses inflammation by inducing apoptosis of fibroblasts-like synoviocytes, inhibiting pro-inflammatory cytokines, enhancing induction of T regulatory cells, preventing the generation of Th17 cells, and modulating FcgammaR expression on macrophages. (C) Sera

from systemic lupus erythematosus patients carry circulating function-blocking anti-GAL-8 autoantibodies, which inhibit the pro-apoptotic activity of GAL-8 on activated T cells, thus fueling inflammation. On the other hand, GAL-9 interacts with TIM-3 and dampens Th1 response. (D) In cystic fibrosis airway, up-regulated GAL-9 induces IL-8 production in bronchial epithelial cells, via TIM-3, leading to neutrophil dominated inflammation. (E) In intestinal inflammation, GAL-4 stimulates $CD4^+$ T cells to produce IL-6 and contributes to the exacerbation of chronic intestinal inflammation. Full green arrows indicate induction of inflammation while dashed red arrows indicate inhibition of inflammation.

levels. Yet, GAL-4 distribution patterns are similar in duodenum, jejunum, and ileum of porcine small intestine (52).

The fine carbohydrate specificity of the two CRDs is different (53-55). GAL-4 binds to sulfated glycosphingolipids carrying 3-*O*-sulfated-galactosides and carcinoembryonic antigen (CEA), but not to glycosphingolipids with 3-*O*-sialylated-galactosides; the C-terminal domain shows a 12-fold higher affinity than the N-terminal domain for $SO_3$3Gal-beta-1-3GalNAc-alpha-1-*O*-benzyl oligosaccharides (53). The N-terminal CRD reacts strongly with human blood group ABH precursor and desialylated porcine salivary glycoproteins, pig gastric mucin, and the linear tetrasaccharide Gal-beta-1-3GlcNAc-beta-1-3Gal-beta-1-4Glc (lacto-*N*-tetraose) (54). Notably, GAL-4 (but not GAL-1, -3, and -8) binds to cholesterol 3-sulfate, although this molecule has no carbohydrate moieties (56). As mentioned above, GAL-4 can also bind carbohydrate structures of the human blood groups A and B, and *E. coli* O86, an enteropathogenic bacteria strain that expresses an antigen essentially identical to human blood group B antigen as the side chain of its surface lipopolysaccharide. Remarkably, both domains of GAL-4 specifically recognize *E. coli* O86 and possess bactericidal effects (14). The fine specificity and natural ligands found on intestinal cells for the two CRDs of GAL-4 deserve further attention. In addition, it is important to further analyze the consequences of shortening the linker region on ligand binding and cross-linking to address the role of this peptide in modulating the biology of these proteins, independently of the CRDs.

3.1. Galectin-4 in inflammation

3.1.1. Galectin-4 in intestinal inflammation

Inflammatory bowel disease is characterized by two forms of intestinal chronic inflammation, Crohn's disease and ulcerative colitis, which show immune imbalance, dysregulated host/microbial interaction, and genetic susceptibility (57, 58). Hokama and coworkers provided the first evidence showing that GAL-4 interacts with the immunological synapse and serves as a stimulator of $CD4^+$ T cells to aggravate intestinal inflammation (59). Interestingly, neutralization of GAL-4 activity *in vivo* by administration of a specific blocking antibody suppressed the progression of chronic colitis that spontaneously developed in immunodeficient mice, whereas pre-treatment with this antibody failed to abolish the development of colitis in these mice. Thus, GAL-4 seems to contribute to exacerbation, rather than initiation, of chronic intestinal inflammation. Because it can be predicted that both acute (induction of inflammation) and healing (recovery from inflammation) processes are simultaneously involved in chronic intestinal inflammation, GAL-4-mediated exacerbation of inflammatory bowel disease may result from a suppression of the healing process. Indeed, treatment with recombinant GAL-4 successfully delayed the recovery from a chemically-induced acute intestinal inflammation, whereas treatment with anti-GAL-4 antibody enhanced the recovery from acute inflammation. Specifically, GAL-4 stimulated $CD4^+$ T cells, but not other immune cells such as B cells or macrophages to produce IL-6, a well-known cytokine involved in the pathogenesis of not only intestinal inflammation, but also colon cancer. Notably, only $CD4^+$ T cells that are present in inflamed, but not non-inflamed, intestine could respond to GAL-4, suggesting differences in their 'glycophenotypes'. Moreover, splenic $CD4^+$ T cells even from diseased mice were unable to respond to GAL-4, suggesting differences in the susceptibility to GAL-4 between local inflamed and systemic compartments (Figure 1) (57).

Recently, Stowell and coworkers have demonstrated that treatment of resting T cell lines (MOLT-4 and HL60) with GAL-4 induces phosphatidylserine exposure independently of cell death, and that this lectin does not induce such an effect on activated T cells; although it does promote phosphatidylserine exposure in activated neutrophils (7). This unique activity was recently coined 'preaparesis', as exposure of phosphatidylserine may favour leukocyte turnover and homeostasis by preparing these cells for phagocytic removal (7). Additionally, GAL-4 specifically binds to the lipid rafts on $CD4^+$ T cells to activate protein kinase C signaling cascade, a fundamental pathway operating in inflammation cascades. Specific ligands for GAL-4 may be expressed on intestinal $CD4^+$ T cells only under inflammatory conditions. Accordingly, some of the most important glycosyltransferases involved in the synthesis of O-glycans are significantly altered in intestinal $CD4^+$ T cells under inflammatory conditions as compared with healthy individuals (57, 58). Supporting a role for GAL-4 in intestinal inflammation, Mathieu and colleagues recently found, in a model of acute colitis in BALB/c mice and in a model of chronic colitis in *Il-10*$^{-/-}$ mice, an increased GAL-4 expression in crypt epithelial cells, at both cytoplasmic and nuclear levels. Thus, GAL-4 seems to contribute to the exacerbation of intestinal inflammation, although different mouse strains show striking differences in galectin expression (60).

In contrast to these findings, Paclik and coworkers showed in a model of experimental colitis that administration of recombinant GAL-4 results in lower secretion of pro-inflammatory cytokines including IL-17, increased apoptosis of mucosal T cells, and lower mucosal inflammation (61). The authors found that GAL-4 induces apoptosis of activated peripheral and mucosal lamina propria T cells via calpain-, but not caspase-, dependent pathways. GAL-4 binds to activated peripheral and

Table 1. 'Tandem-repeat' galectins in human tumors

Galectin	Tumor	Expression levels			Clinical Implications	Reference
		Protein levels	RNA levels	Micro-array		
Circulating GAL-4	Colon carcinoma	+ serum			Tumor screening/ diagnostic value (CEA/CA19-9/GAL-4)	68
Circulating anti-GAL-4 autoantibodies	Colon carcinoma	+ serum				66
GAL-4	Colon carcinoma	↓	↓	↓ DNA/Tissue	Prognostic value	46, 63-65, 67
	Hepatocellular carcinoma		↑			69
	Ileal carcinoids tumors	↑		↑ Tissue		71
	Gastroenteropancreatic neuroendocrine tumors	↑	↑	↑		72
	Barrett's esophagus	↑	↑			73
	Sinonasal adenocarcinoma	↑	↑	↑ DNA	Diagnostic marker?	75
	Breast carcinoma	↑ DCIS[1]				46
	Mucinous epithelial ovarian tumors	↑	↑	↑ DNA	Diagnostic marker	76, 77
GAL-8	Prostate carcinoma	↑	↑		Diagnostic marker	83
	Lung squamous cell carcinoma	↑	↑			85, 86, 89, 108
	Colon carcinoma	↓			Prognostic value	65, 110, 114
	Astrocytic tumors	High level but unchanged with malignancy				112
	Laryngeal squamous cell carcinoma	↑			Prognostic value	113
	Hepatocarcinoma	↓				114
	Endothelial cells in colon carcinoma	↓				32
GAL-9	Melanoma	↓			Prognostic factor	168
	Oral squamous cell carcinoma	↓	↓		Prognostic factor	169
	Cervical squamous cell carcinoma	↓			Differentiation biomarker	170
	Breast cancer	↓ (in metastasis)			Prognostic factor, antimetastatic potencial	171, 172

Abbreviations: DCIS[1]: ductal carcinoma *in situ*

mucosal lamina propria T cells mainly in CD3-bearing glyco-epitopes. Thus, by ameliorating experimental colitis via induction of mucosal T-cell apoptosis and inhibition of pro-inflammatory cytokine release, mucosal GAL-4 has been postulated as a potential novel candidate to treat mucosal inflammation (61). Although these findings are apparently contrasting, it is possible that $CD4^+$ T-cell activation and apoptosis would represent two interrelated events that occur early or late during the course of immune responses and would be modulated by GAL-4. Moreover, Paclik´s findings showing that GAL-4 binds only to activated, but not resting T cells, might reinforce those described by Hokama and colleagues showing that the reactivity of $CD4^+$ T cells to GAL-4 is only elicited under inflammatory conditions in which most T cells are highly activated (57, 58).

The CD3 receptor complex is required for cell surface expression of T-cell receptor (TCR) ensuring T-cell signaling and activation. Thus, the binding of extracellular GAL-4 to this complex or a tightly associated glycoprotein as major targets implies that GAL-4 is capable of modulating central T-cell functions. The carbohydrate-dependent binding of GAL-4 located within a CD3 glyco-epitope is fully functional and inhibits T-cell activation, cycling, and expansion. Further supporting its role in regulating T-cell function, GAL-4 blockade using antisense oligonucleotides inhibits T-cell death induced by a tumor necrosis factor (TNF)-alpha inhibitor (61).

Additionally to its role in T-cell physiology, GAL-4 significantly enhances *in vitro* migration of intestinal epithelial cells, suggesting its potential role in wound healing. This process, which is termed epithelial restitution, does not require cell proliferation (62). GAL-4 also increases cyclin B1 expression and consequently cell cycle progression, while GAL-1 inhibits cell cycle progression. No induction of apoptosis on epithelial cells by GAL-4 has been detected. In conclusion, GAL-4 binds to intestinal epithelial cells and promotes tissue regeneration, which may be relevant in the context of inflammatory diseases characterized by severe mucosal injury (62).

Taken together, these findings support a central, albeit controversial role of GAL-4 in intestinal inflammation. Importantly, given that chronic inflammation is a direct cause of colitis-associated cancer, understanding the role of GAL-4 in intestinal inflammatory tissues may provide some clues in developing a new class of therapeutic agents in the near future.

3.2. Galectin-4 in cancer

Several studies have identified the expression of GAL-4 in tumor cells both at the transcript and protein levels (Table 1). In colon adenocarcinoma, GAL-4 is significantly down-regulated, although controversial data

Table 2. Animal models used to show the involvement of 'tandem-repeat' galectins in cancer

Galectin	Murine model	Tumor type	*In vivo* effects	Reference
GAL-4	Human gastric cancer cell lines grafted onto nude mice	Metastatic cirrhous gastric carcinoma	High GAL-4 expression in peritoneal metastasis	70
GAL-8	*In vivo* treatment with anti-GAL-8 antibody on nude mice carrying DU-145 tumors	Prostate carcinoma	Tumor size regression	83
	In vivo treatment with anti-GAL-8 antibody + doxorubicin on nude mice grafted with human lung squamous carcinoma cells	Lung squamous cell carcinoma	Tumor size regression	109
	Human HCT-15, LoVo, CoLo201, DLD-1 colon cancer cells grafted onto nude mice	Colon carcinoma	GAL-8 expression is inversely related to tumor growth rate	110
	HER-2/*neu* transgenic mice	Estrogen receptor-negative breast cancer	GAL-8 is identified as an up-regulated tumor antigen	111
GAL-9	Administration of recombinant GAL-9 in tumor-bearing mice	Meth-A sarcoma	Enhanced $CD8^+$ T cell-mediated antitumor immunity and prolonged survival	165
		Melanoma		166
	C57BL/6 mice injected with highly metastatic B16F10 melanoma cells, transfected with a secreted form of Gal-9	Melanoma	Suppression of pulmonary metastasis	173
	Daily recombinant Gal-9 injection after inoculation of B16F10 melanoma cells (C57BL/6 mice) or Colon26 colon adenocarcinoma cells (BALB/c mice)	Melanoma and colon adenocarcinoma		

has also been published. Rechreche and coworkers demonstrated that in most cases of colon carcinoma GAL-4 mRNA is 1.5–50 times lower in tumor tissue as compared with adjacent normal colon tissue. However, these authors reported no significant correlation between decreased GAL-4 expression and degree of tumor differentiation or Dukes stage (63). Similarly, 21 genes have shown continuous down-regulation in the normal-adenoma-carcinoma colon sequence, including GAL-4 (64). By inmunohistochemistry, Huflejt and colleagues found GAL-4 in the normal colonic mucosa, in dense supra-nuclear formations in crypt cells, and with a cytosolic distribution in some cells closer to the lumen. In adenomatous polyps proximal to adenocarcinomas, the supra-nuclear formations of GAL-4 are still present, but increased diffuse cytosolic expression was also apparent. In adenocarcinomas, the supra-nuclear formation of GAL-4 is absent and instead there was a diffuse cytosolic expression. Thus, during malignant transformation, a progressive loss of the dense supra-nuclear GAL-4 aggregates is evident, especially for the normal crypt and upper crypt epithelial cells, as well as a diffuse cytosolic GAL-4 distribution (46). By immunohistochemical galectin fingerprinting, GAL-4 was found to be associated with a significant prognostic value in Duke B colon tumors; GAL-1, -3, and -4 may be involved in the early stages of human colon carcinoma development, whereas GAL-8 is involved in the later stages. Remarkably, the prognostic values associated with expression of GAL-1 and -4 were found to be independent of the Dukes stages for Dukes A and B tumors (65). Interestingly, although their significance still remains in veil, autoantibodies against GAL-4 have been identified in sera from patients with colon cancer (66).

More recently, Satelli and coworkers described the function of GAL-4 as a tumor suppressor in human colorectal cancer, demonstrating that loss of GAL-4 is a common and specific event in these types of tumors. GAL-4 expression is significantly down-regulated in adenomas and is essentially absent in invasive carcinomas as assessed by immunohistochemistry. Knocking down GAL-4 resulted in increased cell proliferation, migration, and motility. Moreover, GAL-4 was found to be associated with Wnt signaling pathway, and its expression led to down-regulation of Wnt signaling target genes. Therefore, GAL-4 may represent a novel target in the control of Wnt signaling proteins (67).

In colorectal carcinoma, Watanabe *et al.* have recently showed that circulating GAL-4 may serve as a tumor prognostic biomarker for patient follow-up. Serum levels of GAL-1 and GAL-4 significantly decreased after surgery, and the levels of GAL-4 were below the cut-off value in most patients. The levels of circulating GAL-4 significantly increased as the tumor stage progresses, whereas those for GAL-1 were relatively high from an early stage. The combined use of GAL-4 and CEA and/or cancer antigen 19-9 (CA19-9) considerably expanded the proportion of colorectal cancer patients who were positive for tumor markers. Thus, it was concluded that GAL-4 may serve as a tumor marker for patient follow-up when combined with CEA/CA19-9, while GAL-1 might be used for tumor screening (68).

In contrast to the results found in colorectal cancer, GAL-4 expression levels were higher in hepatocellular carcinomas (69) and in gastric cancer cells with increased metastatic potential (Table 2) (70), as compared to the corresponding normal tissues. Rumilla and colleagues have examined the expression of GAL-4 in primary and metastatic human ileal carcinoid tumors, as well as in carcinoid tumors of the stomach, lung, and rectum. Gene expression profiles revealed that GAL-4 is highly expressed in ileal carcinoids, especially in primary ileal carcinoids compared to metastatic tumors. Gastric carcinoids also express GAL-4, but very few pulmonary or rectal carcinoids do. Hence, distribution of GAL-4 in carcinoid tumors appears to be specific along the gastrointestinal tract and the lungs (71). Notably, GAL-4 is one of the most highly represented genes in human

gastroenteropancreatic neuroendocrine tumors (72), and in Barrett's esophagus with squamous epithelium (73).

In spite of a well-studied role of GAL-4 in tumor growth, less information is available on how this lectin contributes to the metastatic phenotype. During peritoneal dissemination of gastric cancers, the 'runt-related transcription factor 3' (RUNX3) participates in gastric carcinogenesis. Gene expression profiles revealed substantial down-regulation of GAL-4 in RUNX3-stable transfectants of gastric cancer cells. Whether GAL-4 is actually regulated by RUNX3 or is involved in peritoneal dissemination still remains to be investigated (74).

In sinonasal adenocarcinoma -which usually develops in the ethmoid sinuses-, a microarray analysis validated by quantitative RT-PCR and immunohistochemistry has demonstrated that GAL-4 is differentially expressed in tumors compared to normal tissues: the lectin is overexpressed in all ethmoid tumors except the high-grade non-intestinal type adenocarcinoma tumors which are poorly differentiated. Its expression seemed to correlate with histological type and differentiation status of the adenocarcinoma, but further evaluation in precancerous stages and low grade tumors is necessary to evaluate the value of GAL-4 as a diagnostic marker (75).

During breast malignant transformation, the immunohistochemical patterns of GAL-4 expression are well-defined: normal tissues surrounding the malignant component show minimal or no GAL-4 expression, while weak induction of this lectin was associated with epithelial proliferation. Accordingly, the highest levels of GAL-4 expression were found in ductal carcinoma *in situ* and in a subset of infiltrating ductal carcinomas (46).

GAL-4 is also highly overexpressed in mucinous epithelial ovarian cancers compared to the other ovarian carcinoma subtypes and normal ovarian surface epithelium. It is expressed at high levels in mucinous borderline tumors and in benign mucinous cysts, consistent with activation of GAL-4 expression early in mucinous epithelial ovarian cancer development. Hence, GAL-4 expression may be useful as an early diagnostic marker of mucinous epithelial ovarian cancers, either alone or in combination with other markers (76). Accordingly, another study has reported GAL-4 among other 46 genes that are overexpressed in mucinous epithelial ovarian cancers compared to the other histological subtypes of ovarian carcinoma and to normal ovarian surface epithelium (77).

4. GALECTIN-6

'Recently'(~2 million years ago) after mouse and rat diverged, *LGALS6* gene, which encodes GAL-6 protein, appeared by a tandem duplication of the *LGALS4* gene in the mouse genome (78-80). The sequence of *LGALS6* gene has evolved under the influence of strong positive selection, probably leading to the gain of a new function. Unexpectedly, despite this selection pressure, the *LGALS6* gene is present in some mouse species, but not all. Furthermore, even within the species and populations where it is present, it appears that the *LGALS6* gene is never fixed (78). It has been hypothesized that a balanced selection and a neutral retention of ancient polymorphism may explain this paradox.

GAL-6 is a 33-kDa-beta-galactoside binding lectin which has been isolated in the course of cloning GAL-4 cDNA from mouse colon (79, 80). The major difference between GAL-4 and GAL-6 is that the linker region that connects the two CRDs is shorter in GAL-6. It is found in small intestine, colon, and stomach (79) but RT-PCR analysis revealed that *LGALS6* mRNA is also expressed in the liver, kidney, spleen, and heart (81). The functional role of GAL-6 has not yet been defined; however, a biological function is expected within the gastrointestinal tract.

5. GALECTIN-8

GAL-8 is another member of the 'tandem-repeat'-type family of galectins, which possesses two CRDs and thus behaves as a bivalent molecule. This lectin has been initially cloned from a rat liver cDNA library; the isolated clone contains an open reading frame that encodes 316 amino acids, which compose a protein of about 35 kDa (82). In humans, a prostate carcinoma tumor antigen-1 (PCTA-1), which is selectively expressed in prostate carcinoma cells, but not in normal prostate or benign prostate hypertrophy, has been identified as GAL-8 (83). Similarly, Po66, a mouse IgG1 monoclonal antibody produced by immunization with squamous cancer cells, recognizes a carbohydrate-binding protein (Po66-CBP) which has also been identified as GAL-8 (84, 85). GAL-8 exhibits some particular features, for example, its linker peptide length may vary giving rise to different isoforms (86). In fact, the GAL-8 gene (*LGALS8*) encodes numerous mRNAs (probably seven) generated through alternative splicing, mostly on intron VIII (i.e.: 3 'tandem-repeat' type lectins called GAL-8a, -8b, and -8c, or 4 'proto-type' ones named GAL-8d, -8e, -8f, and -8g harboring only one CRD) in normal and tumor cell lines (87-89). Because the N-terminal domain of GAL-8 intrinsically dimerizes (8), cleavage of the linker region between GAL-8N and GAL-8C may allow the possibility to dissect potential signaling pathways initiated by each separate domain. Moreover, GAL-8 linker region displays sensitivity to cleavage by thrombin (90), providing at least one possible regulatory pathway capable of separating the potential functional consequences downstream of these two domains.

With respect to the carbohydrate binding specificities, the N-terminal CRD of GAL-8 has a particularly strong affinity for 3-*O*-sialylated and 3-*O*-sulfated galactosides, not shared by the C-terminal CRD (91). For example, the N-terminal CRD of GAL-8, but not the C-terminal CRD, allows the former, but not the latter, to bind alpha-2-3-sialylated or 3-*O*-sulfated galactosides (91-93). The first crystal structures of GAL-8N in complexes with lactose, 3'sialyl-lactose and 3'sulfo-lactose have recently revealed that the residues Arg45, Gln47, and Arg59 are indispensable and coordinately contribute to

GAL-8N binding affinity to sialylated/sulfated oligosaccharides (92). GAL-8 can tightly bind human blood group A and B carbohydrates; some bacteria such as those belonging to the enteropathogenic *E. coli* O86 strain express blood group B residues on the side chains of the surface lipopolysaccharides, and therefore, these bacteria are recognized and killed by this galectin (14).

Depending on the cellular context and the mode of presentation (soluble or immobilized), GAL-8 can regulate cell adhesion, spreading, growth, and apoptosis (94-98). Its role has been mostly studied in relation to tumor malignancy (86). However, there is some evidence of functions of GAL-8 in the context of autoimmunity, T-cell homeostasis, and inflammatory disorders.

5.1. Galectin-8 in inflammation

5.1.1. Galectin-8 in autoimmune inflammation

A role for GAL-8 in rheumatoid arthritis has been proposed by Eshkar Sebban *et al.* The authors found, in an autoimmune model of joint inflammation, that synovial fluid and synovial cells from rheumatoid arthritis patients contain substantial amounts of GAL-8 and CD44, particularly the CD44vRA isoform which acts as a GAL-8 high-affinity ligand and is a selective variant of CD44 preferentially localized within the arthritic synovia. In the arthritis model, GAL-8 displayed anti-inflammatory activity by promoting apoptosis of synovial fluid cells. Thus, binding of GAL-8 to cell surface CD44vRA induces apoptosis of joint inflammatory cells resulting in the resolution of inflammation. In the synovial fluid, fibrinogen, which forms a complex with GAL-8 and CD44vRA, usually accumulates in the inflamed joint, and traps GAL-8 and soluble CD44vRA. Following this process, more inflammatory cells survive resulting in aggravated arthritis. Thus, the balance between cell surface and soluble glycans present in CD44vRA determine the severity of joint inflammation (Figure 1) (99).

In systemic lupus erythematosus (SLE), a prototypic autoimmune disease, function-blocking autoantibodies recognizing GAL-8 are found. Thirty percent of the SLE patients versus 7% of control patients show autoantibodies. Although the precise role of these autoantibodies still remains uncertain, they may act by controlling pathogenic alterations of the immune system through selectively blocking GAL-8 functions (Figure 1) (100). Autoantibodies against galectins have been described in a variety of autoimmune diseases (101), although their pathogenic roles and their function-blocking activities remain unknown.

5.1.2. GAL-8 in the control of T-cell function and homeostasis

GAL-8 shares with other galectins the capacity of modulating the viability of effector T cells, thus contributing to T-cell homeostasis. This lectin induces apoptosis in Jurkat T cells through mechanisms involving activation of the complex phospholipase-D/phosphatidic acid signaling pathway, which results in activation of the extracellular signal regulated kinases 1 and 2 (ERK1/2) and type 4 phosphodiesterases (102). GAL-8 induces apoptosis in more than 90% of the cells showing higher killing activity compared to GAL-1 and GAL-3. This mechanism involves caspase-3 activation and nuclear condensation in nearly 30% of the cells. Thiodigalactoside, a well-known beta-galactoside which competes with galectin CRDs, inhibits GAL-8 apoptotic effects, demonstrating dependence on galectin-carbohydrate interactions. Affinity-purified anti-GAL-8 autoantibodies from patients with SLE significantly blocked the apoptotic effects of GAL-8. These results implicate GAL-8 as a novel T-cell suppressive factor, which might be counterbalanced by function-blocking autoantibodies during the development of autoimmune inflammation. Thus, in spite of certain similarities with other members of the family, the pro-apoptotic function of GAL-8 seems to be unique in its susceptibility to inhibition by anti-GAL-8 autoantibodies and the molecular mechanism underlying execution of death signals. Phosphatidic acid produced by phospholipase-D promotes ERK1/2 activation by recruiting Raf-1 to the plasma membrane or by phosphatidic acid phosphohydrolase-mediated conversion into diacylglycerol, which then recruits Ras. The contribution of the diacylglycerol-dependent pathway was assessed by inhibiting phosphatidic acid phosphohydrolase activity with propranolol, which inhibits phosphatidic acid conversion into diacylglycerol as described in Jurkat T cells. Propranolol decreases ERK1/2 activation induced by GAL-8, suggesting that diacylglycerol effectively contributes to ERK1/2 activation by the phospholipase-D/phosphatidic acid pathway. ERK1/2 activation subsequently leads to expression of the death factor Fas ligand that triggers apoptosis. GAL-8 also induces apoptosis in human peripheral mononuclear cells activated by anti-CD3 and anti-CD28 stimulating antibodies. Thus, GAL-8 appears to be a potent inducer of Fas ligand expression that may contribute to eliminate activated T cells. This immunosuppressive role seems to be counterbalanced by function-blocking autoantibodies in autoimmune disorders (102).

Inside the thymus, GAL-8 induces apoptosis in $CD4^{high}CD8^{high}$ thymocytes through activation of caspases. The intrathymic expression and the pro-apoptotic effect of GAL-8 suggest a pivotal role for GAL-8 in shaping the T-cell repertoire (103). Conversely, in the peripheral T cell compartment, GAL-8 induces an antigen-independent proliferation of $CD4^+$ T cells that may contribute to fuel adaptive immunity and inflammatory responses. It has been demonstrated that GAL-8 can also synergize with TCR signaling to enhance specific T-cell responses in the presence of low doses of an ovalbumin (OVA) peptide (which comprises 323-339 amino acids of cognate OVA) in DO11.10 mice transgenic for TCR_{OVA}. Importantly, GAL-8 also costimulates T cells even at low concentrations; this activity may be functional not only under normal conditions but also during the induction of autoimmunity where the threshold of T-cell activation is lower (104).

Stowell and coworkers have demonstrated that dimeric GAL-8 signals phosphatidylserine exposure in HL60 human promyelocytic cells. The authors postulated that GAL-8 exits in a dimeric state expressing four CRDs,

which enables bivalency in its functions. Neither domain alone induces phosphatidylserine exposure and the N-terminal domain -but not the C-terminal domain- is critical for dimerization, suggesting that cross-linking of functional cell surface receptors must rely on recognition by a functionally bivalent C-terminal domain. Phosphatidylserine exposure occurs independently of apoptosis in HL60 cells, implying that there was no DNA fragmentation and cell viability was completely unaltered following GAL-8 treatment. Finally, GAL-8 dimerization may not only provide functional bivalency with two C-terminal domains but may also enhance the affinity of binding to poly-N-acetyllactosamine-containing glycans to successfully induce phosphatidylserine exposure (8, 105).

GAL-8 may also modulate the adhesive properties of T cells through specific binding to $alpha_4$ integrins. Thus, GAL-8 induces Jurkat T-cell adhesion to culture plates, whereas single-CRD GAL-1 inhibits adhesion of T cells to extracellular matrix glycoproteins. GAL-8 also induces adhesion of peripheral blood leucocytes to human umbilical vein endothelial cells (HUVEC). These results suggest that the di- or multivalent structure of GAL-8 is essential for its pro-adhesive properties. GAL-8-induced cell adhesion was accompanied by stress fibre formation, which suggests that intracellular signaling is required. In fact, inhibition of $alpha_4$ integrin function through antibody-mediated blockade reduced sensitivity to GAL-8 treatment. Furthermore, phosphorylation of proline-rich tyrosine kinase-2 (PYK) and ERK1/2, which occurs upon integrin-mediated signaling, was up-regulated following GAL-8 treatment (106).

5.1.3. Galectin-8 and neutrophils: key mediators of acute inflammation

Neutrophils play a central role during the acute phase of inflammation, particularly as a result of bacterial infection, environmental exposure, and some types of tumors. The recruitment of these cells is a hallmark of acute inflammation, being the first cells that migrate toward sites of inflammation. Recruitment of circulating neutrophils to an inflamed tissue proceeds through several defined steps, namely, attachment, rolling, firm adhesion to endothelial cells and transendothelial migration. GAL-8 plays an important role in this process by inducing firm and reversible adhesion of peripheral blood neutrophils *in vitro* (107), suggesting that this lectin may be a novel modulator of neutrophil function. In fact, when added to serum-containing medium this lectin induced firm neutrophil attachment. Immobilized GAL-8 also supported neutrophil adhesion, but the number of neutrophils adhering was less than 40% compared to that recorded when GAL-8 was used as a soluble ligand. Blocking anti-$alpha_M$, but not anti-$alpha_L$ integrin antibodies, strongly inhibited GAL-8-mediated neutrophil adhesion. Moreover, GAL-8 binds to pro-matrix metalloproteinase-9 (pro-MMP-9) and accelerates its processing to MMP-9, probably through modulation of its capacity to cleave structural proteins such as collagens during inflammation. GAL-8 also regulates neutrophil function: it is as effective as formyl-Met-Leu-Phe (a potent chemotactic mediator) in inducing superoxide production by peripheral blood neutrophils, which is a key factor in their microbicidal activity (107).

5.2. Galectin-8 in cancer

Due to the wide expression of GAL-8 in tumor tissues (brain, breast, large intestine, germ cells, head and neck, kidney, muscles, ovary, pancreas, thyroid gland, placenta, prostate, uterus, lung, stomach, and esophagus) (89, 95), different studies have attempted to validate an association between expression of this lectin and neoplastic transformation (86). As mentioned above, GAL-8 has been identified as the prostate carcinoma tumor antigen-1, or PCTA-1, selectively expressed in prostate carcinoma, but not in normal prostate or benign prostate hypertrophy (Table 1). The protein sequence of PCTA-1 displays 81% sequence homology with rat GAL-8. Reverse transcription PCR experiments have demonstrated selective expression of PCTA-1 in prostate carcinomas versus normal prostate and benign prostatic hypertrophy. Similarly, frozen sections from patient-derived prostate cancer specimens react strongly with anti-GAL-8 antibodies in prostate carcinoma cells, but not in adjacent benign glands or in tissue sections containing normal prostate or benign prostatic epithelium. Moreover, *in vivo* treatment with the anti-GAL-8 antibody of athymic nude mice carrying established DU-145 tumors significantly reduced tumor size while animals receiving an irrelevant antibody isotype developed actively growing tumors. In summary, GAL-8 may contribute to establish differential diagnosis and staging and may serve as a novel therapeutic target in prostate cancer (Table 2) (83).

The expression of GAL-8 isoforms has also been carefully studied in normal and tumor prostate cell lines using RT-PCR by Ahmed and coworkers. Out of seven isoforms of GAL-8, the 'proto-type' isoforms GAL-8e and -8g were found to be up-regulated in malignant LNCaP compared to normal PrEC prostate cells, whereas the two 'tandem-repeat' isoforms GAL-8a and -8b were equally expressed in these cells. Little or no expression of all isoforms was detected in benign prostate epithelial cells BPH-1 (87).

Po66, a mouse IgG1 monoclonal antibody generated by immunizing Balb/c mice with human lung squamous carcinoma cells (84), recognizes a carbohydrate-binding protein (Po66-CBP) which has also been identified as GAL-8 (Po66-CBP displays 82% nucleotide and 98.7% amino acid sequence identity with PCTA-1). DNA cloning and gene library analysis have provided clear evidence that all the isoforms derive from a unique gene by alternative splicing. Tissue-specific expression studies have been carried out by database analysis using Po66-CBP cDNA sequence, which revealed that Po66-CBP transcripts are found in tumoral and embryonic lung, while their expression is absent in healthy lung (89). This observation confirms the results described by Hadari *et al.* (95). An immunohistochemical study has also been performed on primary and secondary malignant lung tumors of various histological origins, in which GAL-8 was found to be abundant in squamous cell carcinoma, weakly expressed in adenocarcinoma and undetectable in small cell carcinoma (85, 86). Moreover, a correlation between GAL-8

expression and the degree of differentiation of squamous cell carcinomas and neuroendocrine tumors was detected (85, 108). Studies conducted in nude mice grafted with human squamous cell carcinoma cells have shown that injection of ^{131}I-radiolabeled Po66 antibody and doxorubicin causes tumor size regression (Table 2) (109), further supporting the potential role of GAL-8 in neoplastic transformation and tumor growth (86).

In contrast, in human colon cancer, immunohistochemical staining revealed a significant decrease of GAL-8 expression according to tumor malignancy. The more aggressive the tumor was, the less GAL-8 protein it expressed. In fact, there was less GAL-8 in colon tumors than in normal or benign colon tissues. Accordingly, grafting four experimental human colon cancer models onto nude mice has enabled the observation that GAL-8 expression inversely correlates with tumor growth rate. Further *in vitro* studies revealed that this lectin reduced tumor migration rate only in those human experimental models that exhibit the lowest growth rate *in vivo* (Table 2) (110).

In search for potential tumor antigens in *Neu* (HER-2/neu, human epidermal growth factor receptor 2) transgenic mice, a model for estrogen receptor–negative breast cancer with significant similarity to human premenopausal breast cancer, Lu and colleagues identified GAL-8 as one of the five most frequently expressed tumor antigens. In this case, a serological analysis of recombinant cDNA expression libraries was conducted, and serum samples were collected from animals bearing spontaneous tumors as well as control tumor-free mice. Results revealed that the majority of the identified antigens (including GAL-8) were associated with immunogenic human homologues, which may be exploited as potential targets for therapeutic use (i.e., tumor rejection antigens) (Table 2) (111).

GAL-8 expression was also analyzed in tumors of the central nervous system by means of immunohistochemistry and RT-PCR. The levels of GAL-8 expression were found to be very high in human pilocytic astrocytomas, astrocytomas, anaplastic astrocytomas, and glioblastomas, and remain unchanged during the progression of malignancy. Functional studies revealed that GAL-8 can stimulate glioblastoma cell migration *in vitro*, suggesting that GAL-8 may be involved in cancer cell invasion of the brain parenchyma *in vivo* (112).

By immunohistochemical analysis of laryngeal squamous cell carcinomas, a strong positive expression of GAL-8 was associated with T-stages, nodal stages, and clinical stages of the disease. However, histopathologic grades were not correlated with GAL-8 expression. These results suggested that expression of GAL-8 could be used as a clinical prognostic factor for squamous cell carcinoma of the larynx (113).

In an extensive study, Danguy and coworkers have compared the expression of GAL-8 in a variety of different tumors versus the corresponding normal tissues. In the large intestine, pancreas, liver, skin, and larynx, immunohistochemical analysis revealed decreased GAL-8 expression in cancerous versus normal tissues. The expression of GAL-8 was augmented in cancer versus normal tissues from lung, bladder, kidney, prostate and stomach, suggesting tissue-specific regulation of GAL-8 expression in cancer (114).

Angiogenesis is crucial for the growth and survival of tumor cells, which also increases the likelihood that tumor cells may enter the circulation and generate metastasis. Thijssen and coworkers have reported the expression of GAL-1, -3, -8, and -9 in the human endothelium. In normal tissues, the expression of GAL-8 was more diffuse while in tumor tissues it was restricted to the nuclei. Moreover, different endothelial cells such as HUVEC, HMEC-1, RF24, and $EVLC_2$ expressed GAL-8 (32). GAL-8 was also highly expressed in primary human dermal lymphatic endothelial cells, and was involved in promoting their adhesion and migration, mainly by interacting with podoplanin (a sialomucin-like membrane protein highly expressed in lymphatic but not in blood vascular cells) (115). We have recently described a critical role for GAL-8 in the promotion of *in vitro* and *in vivo* angiogenesis. Our results show that *in vitro* capillary-tube formation and endothelial cell migration are stimulated by GAL-8 through interaction with CD166 on BAEC cells. Interestingly, when endothelial cells were transfected with a GAL-8-specific small interference RNA, a significant reduction of tube length and endothelial cell migration was observed as compared to non-transfected cells. GAL-8-induced tubulogenesis and migration were significantly prevented by specific disaccharide inhibitors. Likewise, matrigel supplemented with GAL-8 and injected into mice resulted in induction of *in vivo* angiogenesis (33). Taken together, these data imply a key function for endothelial GAL-8 (either vascular or lymphatic) in angiogenesis, which may be exploited for cancer therapy. Interestingly, another aspect of tumor progression and metastasis involves the interactions of platelets with tumor cells. Recently, Romaniuk and colleagues found that GAL-8 binds to specific glycans on the platelet surface and triggers spreading, calcium mobilization and fibrinogen binding. It also promotes aggregation, thromboxane generation, P-selectin expression and granule secretion. Notably, glycoproteins $alpha_{IIb}$ and Ib-V were identified as putative Gal-8 counter-receptors (116). These effects, which were considerably more potent than those triggered by GAL-1 (117), may certainly contribute to heterotypic interactions between platelets, tumor cells and the endothelium to critically influence metastatic spreading.

This heterogeneous scenario where GAL-8 is up- or down-regulated in many tumor types and plays a variety of functions, prompts a final discussion related to the complexity of the study of GAL-8 within tumor microenvironments. GAL-8 has probably a more complex gene regulation than other members of the galectin family, giving rise to numerous messenger RNAs and seven isoforms; yet it still represents an interesting tool to understand the biology of different types of cancer and a potential target for therapy in selected tumor types.

6. GALECTIN-9

This 'tandem-repeat' galectin has been originally isolated from mouse embryonic kidney cells (118). Its expression is developmentally regulated (119) and widely distributed in rat and mouse tissues (120). Human GAL-9 has been first identified as a putative autoantigen in patients with Hodgkin's disease and has been suggested to play an important role in the regulation of immune system (121). Initially, it was named 'ecalectin' due to its potent eosinophil chemoattractant activity (122). In humans, it is expressed in peripheral blood leukocytes and lymphatic tissues (121). Also, GAL-9 is produced by endothelial cells stimulated with IFN-gamma and is up-regulated in the vasculature of human inflammatory lesions (123). Furthermore, an altered expression of this lectin has been reported in cystic fibrosis-derived bronchial epithelial cells (124).

Mouse GAL-9 has been cloned and characterized by Wada and Kanwar (120). Sequence analysis of its cDNA revealed the presence of two different domains linked by a stretch of about 30 amino acid residues, with 39% of sequence homology. The N-terminal domain shows merely moderate homologies with other known galectins and the C-terminal lectin domain is highly homologous to rat GAL-5 (120, 121). Both N- and C-terminal CRDs exhibit high affinity for branched complex-type sugar chains, especially for tri- and tetraantennary N-linked glycans bearing multiple N-acetyllactosamine units (125). More recently, the crystal structure of mouse GAL-9 N-terminal CRD has revealed the basic mechanism of carbohydrate recognition, confirming the binding of N-acetyllactosamine and also T-antigen (Gal-beta1-3GalNAc) (126). However, the structural analysis of human GAL-9 N-terminal CRD has revealed properties that completely differ from those observed in the mouse orthologue (127, 128). In fact, human GAL-9 exhibits higher affinity for the Forssman pentasaccharide and two putative recognition modes for poly-N-acetyllactosamine binding.

Similarly to GAL-8, alternative splicing leads to the formation of three splice variants, which have been clearly detected in Jurkat T cells. The 35.9-kDa medium-sized isoform (GAL-9M) corresponds to authentic GAL-9 whereas the long and small-sized isoforms (GAL-9L and S) have a 32-amino acid insertion and a 12-amino acid deletion, respectively in the linker peptide (129). GAL-9 is known to have a variety of cellular functions, including cell differentiation, adhesion, aggregation, and cell death (130). Through modulation of cellular activities, this lectin can regulate multiple physiological and pathological processes such as immunity, inflammation, and cancer.

6.1. Galectin-9 in inflammation

Although T helper cell-mediated response is involved in host immunity against pathogens and tumors, uncontrolled T helper activity leads to tissue injury in many inflammatory and autoimmune diseases. Recombinant GAL-9 induces apoptosis in thymocytes, Jurkat T cells and activated $CD4^+$ and $CD8^+$ T cells through the calcium-calpain and caspase-1 pathway (119, 131). GAL-9 has been identified as a ligand for the 'T-cell immunoglobulin mucin domain 3' (TIM-3) and by virtue of this interaction, this lectin selectively induces apoptosis in activated Th1 but not Th2 cells (132-134). Due to its potent roles in T-cell suppression and tolerance induction, GAL-9 has been considered as a therapeutic candidate for autoimmune and inflammatory diseases (135, 136).

By activating the TIM-3 signaling pathway, GAL-9 also plays a role in innate immunity by favoring a pro-inflammatory response (137, 138). It has been demonstrated that GAL-9 induces maturation of human monocyte-derived dendritic cells. In fact, dendritic cells treated with recombinant GAL-9 secrete IL-12 but not IL-10, which in turn elicit the production of Th1 cytokines by alogeneic $CD4^+$ T cells (138). Furthermore, intracellular GAL-9 activates gene promoters of IL-1alpha, IL-1beta and IFN-gamma through physical interaction with the nuclear factor NF-IL6 (C/EBPbeta) in THP-1 monocytic cells, demonstrating its role in the regulation of inflammatory cytokines (139).

These results suggest that the GAL-9/TIM-3 signaling pathway play a dual role either by promoting innate immunity or by terminating adaptive immune responses. Recently, it has been demonstrated that the two CRDs in the N- and C-terminal regions of GAL-9 contribute differently to its multiple functions. While the N-terminal extreme is responsible for the effect of GAL-9 on the innate immune response, the C-terminal region is involved in inducing T-cell death (140). Interestingly, it has been demonstrated that GAL-9 promotes apoptosis in T helper cells and stimulates the production of pro-inflammatory cytokines independently of TIM-3 (141), suggesting multiple mechanisms and binding partners through which GAL-9 can exert immunomodulatory effects.

6.1.1. Galectin-9 in allergic inflammation

GAL-9 has been initially described as a potent eosinophil chemoattractant produced by T lymphocytes and thus, implicated in allergic responses (122, 142). It has been shown to inhibit apoptosis in eosinophils from eosinophilic patients but it enhances apoptosis in those from healthy subjects (143). Remarkably, eosinophils have been found to adhere to IFN-gamma-stimulated fibroblasts in a lactose- and GAL-9-dependent manner (144).

Given these pioneer findings, the role of GAL-9 has been further investigated in a murine model of experimental allergic conjunctivitis. This is a Th2-mediated disease that is induced by active immunization with ragweed (RW) followed by RW challenge in eye drops. Treatment with an anti-GAL-9 neutralizing antibody did not affect the severity of the disease. However, when the splenocytes from RW-primed mice treated with anti-GAL-9 antibodies during the induction phase were stimulated *in vitro* and adoptively transferred into naive recipients, they were found to induce severe conjunctivitis. In contrast, when RW-primed splenocytes were re-stimulated with RW in the presence of anti-GAL-9 antibodies, they induced a less severe disease and produced significantly less IL-5 and

IL-13, and more IFN-gamma, as compared to IgG-treated control splenocytes (145).

In addition, administration of GAL-9 in a murine model of asthma has been found to reduce Th2-associated airway inflammation and airway hyper-responsiveness. Interestingly, binding of GAL-9 to CD44 inhibits the interaction between CD44 and hyaluronan, which is known to mediate the migration of T cells to the lung (146). In a model of asthma in guinea pigs, GAL-9 suppressed degranulation of mast cells stimulated by IgE and the specific antigen. Moreover, this lectin strongly and specifically bound IgE preventing IgE-antigen complex formation (147). Thus, GAL-9 appears to suppress allergic inflammation through multiple mechanisms including alteration of Th1/Th2 cytokine balance, modulation of T-cell migration and suppression of mast cell degranulation.

Conversely, in another mouse model of allergic asthma induced by ovoalbumin, an increase in GAL-9 expression has been detected in the lungs (148). In this model, it was found that GAL-9 may contribute to the development of allergic airway inflammation due to the control of eosinophil recruitment and promotion of Th2 responses. These controversial results, which may reflect different experimental strategies used, emphasize the need of new studies to validate the therapeutic potential of this galectin in the management of allergic processes.

6.1.2. Galectin-9 in autoimmunity and other chronic inflammatory disorders

Nephrotoxic serum nephritis model, which is characterized by glomerular influx of $CD8^+$ T cells into glomerular capillaries, is induced in Wistar Kyoto rats by injecting rabbit anti-glomerular basement membrane serum. In this model, GAL-9 has been shown to induce apoptosis of activated $CD8^+$ T cells (149). Later on, the role of GAL-9 in controlling Th1 response has been confirmed *in vivo* by treatment of immunized mice with this galectin during the induction phase of experimental autoimmune encephalomyelitis, a model of multiple sclerosis. In such a model, a selective loss of IFN-gamma-producing cells and suppression of Th1 autoimmunity has been observed (Figure 1) (133).

More recently, the potential use of recombinant GAL-9 in the treatment of rheumatoid arthritis has been proposed. This galectin induced apoptosis in fibroblast-like synoviocytes from rheumatoid arthritis patients and in rheumatoid synovial tissue implanted into severe combined immune deficient mice (150). Additionally, GAL-9 reduced the severity and incidence of the disease in a collagen-induced arthritis model by suppressing the generation of Th17 pathogenic cells and favoring the induction of regulatory T cells (151). Moreover, in GAL-9-treated mice, the severity of immune complexes-induced arthritis and the levels of pro-inflammatory cytokines were markedly reduced. This suppressive effect was, at least in part, a result of the regulation of Fc-gammaR expression on macrophages (Figure 1) (152).

In addition, non-obese diabetic (NOD) mice in which GAL-9 was overexpressed were significantly protected from type 1 diabetes, a Th1-dominant autoimmune disease that specifically destroys insulin-producing beta cells. These findings demonstrated that the Th1 cell population was markedly decreased in the spleen, pancreatic lymph node and pancreas following treatment with a GAL-9-encoding plasmid, indicating a negative regulatory role of GAL-9 in the development of Th1-mediated diseases. Thus, GAL-9/TIM-3 interaction may serve as a therapeutic target to ameliorate autoimmune diabetes (Figure 1) (135). It has been hypothesized that dysregulation of the GAL-9/TIM-3 pathway can trigger chronic autoimmune diseases, such as multiple sclerosis, whereas low levels of TIM-3 might allow Th1 cells to escape GAL-9-induced cell death (136, 153).

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease. The expression of TIM-3 on peripheral blood mononuclear cells from SLE patients remains normal; however, an increased expression of GAL-9 was found on lymphocytes from these patients (154). These results suggest that the GAL-9/TIM-3 pathway may play a role in the pathogenesis of SLE and it could be used as a therapeutic target for this disease (Figure 1) (155).

Murine contact hypersensitivity is a widely used model of contact dermatitis, which is a common skin disease. It is generally thought that the Th1-type response is responsible for this reaction. Psoriasis vulgaris is another common skin disease that has a chronic clinical course and is resistant to different treatments. IFN-gamma-producing Th1 cells are thought to be key players in the pathogenesis of psoriasis. A reduction in the Th1- and/or Th17-mediated skin inflammation has been observed when the therapeutic effects of GAL-9 were assessed in inflammatory reactions associated to contact hypersensitivity and psoriasis (Figure 1) (156).

Cystic fibrosis is a chronic severe disease characterized by persistent pulmonary infections accompanied by a dysregulation of pro-inflammatory cytokines and inflammation dominated by polymorphonuclear neutrophils. Recently, it has been demonstrated that TIM-3 and GAL-9 are substantially up-regulated in cystic fibrosis-derived bronchial epithelial cells (124). Moreover, GAL-9 induced the production of IL-8 in a lactose- and TIM-3-dependent manner. Also, TIM-3 and GAL-9 undergo rapid proteolytic degradation in the cystic fibrosis lung. These results led to the conclusion that TIM-3, through binding to its ligand, GAL-9, promotes neutrophil influx, even in the absence of microbial invasion. This effect could be potentiated by lipopolysaccharides after infection (124). Thus, the TIM-3/GAL-9 interaction may possess therapeutic value in cystic fibrosis (Figure 1).

The TIM-3/GAL-9 axis has also been validated in the context of liver homeostasis. Blockade of this pathway by using TIM-3- or GAL-9-specific neutralizing antibodies, led to increased hepatocellular damage, local neutrophil infiltration, T-cell and macrophage accumulation and liver cell apoptosis in a mouse model of liver ischemia/reperfusion injury (157). These findings provide important information about the crucial role of GAL-9 in local inflammation, including organ ischemia-reperfusion injury, which occurs frequently after major hepatic resection or liver transplantation.

Recently, recombinant GAL-9 has been proposed as an attractive candidate for the treatment of the acute graft versus host disease (GvHD), a serious complication of allogeneic transplantation. A search for potential mechanisms underlying this effect revealed the ability of GAL-9 to ameliorate the progression of the disease in a murine transplantation model by inducing T-cell death (158). In conclusion, GAL-9 promotes the loss of IFN-gamma-producing Th1 cells, and controls a variety of Th1-mediated autoimmune and inflammatory disorders, supporting its role as a promising and selective anti-inflammatory agent (Figure 1).

6.1.3. Galectin-9 in infection-associated inflammation

Numerous studies have described the role of galectins in host-pathogen interactions and infection (2). It has been demonstrated that GAL-9 has suppressive effects on bacterial infection-induced inflammation. Mice treated with recombinant GAL-9 are less sensitive to the Shwartzman reaction. Accordingly, GAL-9-deficient mice are more susceptible to infection-induced inflammation (159). The anti-inflammatory activity of GAL-9 on lipopolysaccharide-induced vasculitis involved chemoattraction of prostaglandin E2-producing polymorphonuclear neutrophils (159).

It has also been demonstrated that GAL-9 suppresses lung inflammation in an experimental model of pneumonitis induced by *Trichosporon asahii*, as it reduces the amounts of IL-1, IL-6, IL-17, and IFN-gamma (160). Interestingly, Jayaraman and colleagues recently showed that TIM-3 expressed on Th1 cells interacts with GAL-9, which is expressed by *Mycobacterium tuberculosis*-infected macrophages (161). This interaction inhibits intracellular bacterial growth by inducing caspase-1-dependent IL-1beta secretion. Thus, TIM-3/GAL-9 interaction might have evolved to restrict growth of pathogens and to avoid further tissue inflammation by reducing Th1 responses.

The roles of GAL-9 in viral infections have also been reported. GAL-9 has been found at very high levels in the serum of patients with chronic hepatitis C infection and its expression is significantly up-regulated in Kupffer cells (162). Furthermore, IFN-gamma stimulates the production of GAL-9 by macrophages. GAL-9, in turns, induces cytokine production, expansion of regulatory T cells and apoptosis in virus-specific cytotoxic T lymphocytes. In this regard, it has been demonstrated that chronic inflammatory reactions provoked by herpes simplex virus infection in the eye are controlled by GAL-9/TIM-3 interactions. Administration of GAL-9 diminishes the severity of eye lesions, by inducing apoptosis of T cells and facilitating the expansion of regulatory T cells (163). Accordingly, $CD8^+$ T-cell responses to herpes simplex virus are substantially enhanced in GAL-9-deficient mice (164). These results reveal a central role of GAL-9 in the control of immunity against viral infection.

6.2. Galectin-9 in cancer

The role of GAL-9 in different steps of tumor progression is relatively well-established. Administration of recombinant GAL-9 prolongs the survival of Meth-A sarcoma tumor-bearing mice, being this effect a consequence of T cell-mediated immune response. Although GAL-9 induces apoptosis in $CD4^+$ Th1 cells, this lectin appears to increase both the number of IFN-gamma-producing TIM-3^+ $CD8^+$ T cells and TIM-3^+ $CD86^+$ dendritic cells. Thus, GAL-9 potentiates $CD8^+$ T cell-mediated anti-tumor immunity through modulation of the interactions between $CD8^+$ T cells and dendritic cells via TIM-3 (Table 2) (165). Interestingly, administration of GAL-9 also prolongs the survival of mice bearing B16 melanoma by increasing expansion of NK cells, $CD8^+$ T cells and macrophages. However, GAL-9 may also promote NK cell-mediated anti-tumor activity by expanding macrophages showing a plasmacytoid-like phenotype (Table 2) (166). Additionally, many enzymes involved in protein glycosylation and also some lectins, including GAL-9, are up-regulated in the liver of mice bearing the Engelbreth-Holm Swarm sarcoma, a specific model that involves systemic inflammation and repression of multiple hepatic drug metabolizing enzymes and transporters (167). These results imply an important role of GAL-9 in cancer-induced inflammation.

Previous studies found that GAL-9 induces both cell aggregation and apoptosis of melanoma cells (Table 1) (168). Surprisingly, this protein is strongly expressed in melanocytic nevi, but down-regulated in melanoma cells, especially in metastatic lesions. This study suggested that high GAL-9 expression is inversely correlated with the progression of melanoma. In addition, GAL-9 was found to be down-regulated in oral squamous cell carcinoma cell lines with respect to normal oral keratinocytes (169). Overexpression of GAL-9 increased cell adhesion *in vitro* to fibronectin and collagen type-1, indicating that expression of this lectin may negatively correlate with oral cancer cell-matrix interactions and metastasis. In agreement with these results, the decrease of GAL-9 expression has been associated with the malignant potential of cervical squamous cell carcinoma (Table 1) (170).

In breast cancer, GAL-9 has been proposed to serve as a prognostic factor. Interestingly, 50% of the tumors analyzed in breast cancer patients were found to be positive for GAL-9 while most patients having distant metastasis lacked GAL-9 expression. Also, the cumulative disease-free survival ratio for GAL-9-positive patients was more favorable than that corresponding to the GAL-9-negative group (Table 1) (171, 172). These results suggest an anti-metastatic potential of GAL-9 in breast cancer.

Accordingly, the ability of GAL-9 to inhibit lung metastasis in experimental murine models has been evaluated using the highly metastatic B16/F10 melanoma and Colon26 colon cancer cells. Melanoma cells transfected with a secreted form of GAL-9 evidenced lack of metastatic potential. In addition, intravenous GAL-9 administration reduced the number of metastatic foci in both B16/F10 and Colon26 cells in the lung, indicating that secreted GAL-9 suppresses metastasis (173). It has been proposed that GAL-9 inhibits tumor cell adhesion to the extracellular matrix and blocks invasion to vascular

endothelium through interfering with cell adhesion molecules (Table 2) (173). Noteworthy, overexpression of the three isoforms of GAL-9 in LoVo colon carcinoma cells (which do not express endogenous GAL-9) increases cell adhesion to extracellular matrix proteins, whereas overexpression of isoforms M or S promotes LoVo cell adhesion to human umbilical vein endothelial cells (174).

In addition, the role of GAL-9 has been studied in malignant B cells. It has been found that a protease-resistant GAL-9 inhibits Burkitt and Hodgkin lymphoma cell growth and induces apoptosis (175), suggesting that this modified version of GAL-9 is a potentially useful agent in the treatment of B-cell-related malignancies.

As reported in previous sections, GAL-9 has a potent pro-apoptotic activity toward activated T lymphocytes (131, 176). Interestingly, activating transcription factor-3 mediates chronic myelogenous leukemia cell death induced by GAL-9 and this effect is additive with Imatinib, a therapeutic agent currently used for treating this disease (177). These findings suggest that GAL-9 is an attractive agent to target for overcoming resistance to chronic myelogenous leukemia. Finally, the protease-resistant GAL-9 form inhibits cell proliferation of myeloma cells, including a therapeutic-resistant (Bortezomib) cell line through inhibition of Jun N-terminal kinase (JNK) or p38 mitogen-activated protein kinase (MAPK) pathways (178). GAL-9 could restrict the growth of human myeloma cells xenografted in nude mice, underscoring an alternative therapeutic target in this hematological disease.

7. GALECTIN-12

GAL-12 cDNA has been first cloned from the human Jurkat T cell line. The N-terminal domain has significant homologies with other galectins, particularly it is highly homologous to GAL-8; however, the C-terminal domain displays greater divergence from other members. By Northern blot analysis, GAL-12 mRNA was found to be nearly undetectable in many tissues. Nevertheless, by using RT-PCR, GAL-12 mRNA was detected in the heart, pancreas, spleen, thymus, and peripheral blood leukocytes. It is also present at lower levels in the lung, skeletal muscle, kidney, prostate, testis, ovary and colon but undetectable in the brain, placenta and liver (179).

Interestingly, GAL-12 was found to be preferentially expressed in a human adipose tissue cDNA library. Its mRNA was detected in differentiated 3T3-L1 adipocytes. Whereas GAL-12 mRNA expression did not differ between obese and diabetic animals, caloric restriction and treatment with troglitazone (an anti-diabetic drug that induces apoptosis of large adipocytes and improves insulin resistance) increased GAL-12 mRNA and decreased adipose tissue cell size (180). These studies also suggested that GAL-12 may regulate adipocyte physiology through regulation of cell cycle progression and/or apoptosis (179, 180). In addition, it has been demonstrated that isoproterenol, insulin, tumor necrosis factor-alpha and dexamethasone potently inhibit GAL-12 mRNA expression in 3T3-L1 adipocytes (181). The fact that this galectin was down-regulated by these hormones, suggested that GAL-12 might contribute to insulin resistance. Taken together, these results and those obtained by Hotta *et al.* (180) suggest that GAL-12 down-regulation promotes a reduction of insulin-resistant fat-cells via apoptosis which, in turn, contributes to insulin resistance. These findings underscore a potential role for GAL-12 in the pathogenesis of type 2 diabetes, characterized by insulin resistance of peripheral tissues. In addition, more recent studies have found that GAL-12 plays a role in adipocyte differentiation (182), suggesting that this galectin may serve as a regulator of adipose tissue development and a potential target for the treatment of obesity-associated inflammatory and metabolic diseases.

7.1. Galectin-12 in cancer

Although few studies have been reported on the expression and function of GAL-12 in cancerous tissues, this lectin has been found to be up-regulated in cells synchronized at the G_1 phase or the G_1/S boundary of the cell cycle. Ectopic expression of GAL-12 in cancer cells causes cell cycle arrest at the G1 phase and cell growth suppression, suggesting that, intracellularly, this lectin may act as a regulator of cell cycle. Interestingly, the GAL-12 gene (*LGALS12*) is localized to a region frequently altered in many human cancers. Interestingly, the gene encoding cyclin D1, which is known to be abnormally expressed in some types of leukemia, also maps within this region. Although more work is still needed, these findings suggest a potential role for GAL-12 during the tumorigenesis process (179).

8. SUMMARY AND PERSPECTIVE

Although less studied than 'proto-type' and 'chimera-type' members, 'tandem-repeat' galectins have emerged as central regulators of immune, inflammatory, and neoplastic processes. These lectins exert a variety of function by providing stimulatory or inhibitory signals which act in concert with other cytokines, chemokines or mediators to regulate physiological and pathological processes. For example, the spatio-temporal expression of galectins during initiation or resolution of inflammatory processes is likely to control the magnitude and final outcome of these responses. Moreover, similar to cytokines and growth factors, the same galectin may also display divergent effects depending on cellular compartmentalization, concentration in the local milieu and differentiation status of target cells (183). Furthermore, cell- or tissue-specific profiles of glycosyltransferases leading to the creation or masking of different galectin ligands may also regulate the biological effects of these glycan-binding proteins (184). Accordingly, each 'tandem-repeat' galectin can act on different cell subsets, suggesting lineage-specific recognition. Hence, changes on cell surface glycosylation may have dramatic effects on lectin binding and function (i.e.: immunomodulation, angiogenesis, differentiation, apoptosis, etc).

In future studies, it will be important to examine in detail the domain or domains responsible for dimerization, the linker region length and sensitivity to

proteases cleavage, the oligomeric nature of glycan recognition and signaling as well as the different sugar-binding capacities of both N-terminal and C-terminal CRDs of 'tandem-repeat' galectins.

Taken together, the studies summarized here show a clear association between the structure and function of 'tandem-repeat' galectins. These include spliced variants of these lectins, quaternary structures, cell surface lattice formation and signaling through each CRD that may reflect common mechanisms or different regulatory circuits initiated by each separate domain. Understanding the physiological relevance and tissue distribution of splice variants as well as the different ligands and signaling pathways triggered by these lectins will provide insights for therapeutic interventions in neoplastic and inflammatory diseases. In cancer, 'tandem-repeat' galectins can negatively or positively regulate tumor cell survival and adhesion to the endothelium or extracellular matrix, as well as tumor vascularization, immune-inflammatory reactions and other processes that are crucial for metastatic spread.

However, we are still far from completely understanding the role of 'tandem-repeat' galectins in health and disease. Further work is required to shed light to the biological significance of these complex endogenous lectins in the context of inflammatory responses and tumor progression (184-186), especially using transgenic conditional mice and small interfering RNA that will allow detailed analysis at both the molecular and cellular levels.

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Abbreviations: CA19-9: cancer antigen 19-9, CD: human leukocyte cluster of differentiation, CEA: carcinoembryonic antigen, CRD: carbohydrate recognition domain, ERK1/2: extracellular signal-regulated kinases 1 and 2, GAL: galectin, Gal: galactose, Glc: glucose, IFN-gamma: interferon gamma, IL: interleukin, JNK: Jun N-terminal kinase, *LGALS:* galectin gene, MAPK: mitogen-activated protein kinase, MMP: matrix metalloproteinase, PCTA-1: prostate carcinoma tumor antigen-1, Po66-CBP: Po66 carbohydrate binding protein, RUNX3: transcription

factor RUNX3, SLE: systemic lupus erythematosus, TCR: T-cell receptor, Th1: T helper-1, TIM-3: T-cell immunoglobulin mucin domain 3

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Send correspondence to: Gabriel A. Rabinovich, Laboratorio de Inmunopatologia, Instituto de Biologia y Medicina Experimental, IBYME-CONICET, Vuelta de Obligado 2490, C1428ADN, Buenos Aires, Argentina, Tel: 54 11 4783-2869 (ext. 266), Fax: 54 11 4786-2564, E-mail: gabriel.r@ibyme.conicet.gov.ar

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