Shaping the immune landscape in cancer by galectin-driven regulatory pathways

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Abbreviations List

ALCAM: Activated leukocyte cell adhesion molecule

AP-1: Activating protein-1

BCR: B-cell receptor

C2GnT1: Core 2 β-1,6-N-acetylglucosaminyltransferase 1

cHL: Classical Hodgkin lymphoma

CLL: Chronic lymphocytic leukemia

CTL: Cytotoxic T lymphocytes

CTLA-4: Cytotoxic T-lymphocyte antigen 4

CRD: Carbohydrate-recognition domain

DCs: Dendritic cells

EBV: Epstein-Barr virus

EC: Endothelial cell

EMT: Epithelial mesenchymal transition

4-F-GlcNAc: 4-fluoro-glucosamine

GlcNAc: N-acetilglucosamine

HIF-1α: Hypoxia-inducible factor-1α

HMGB1: High mobility group box 1

IDO: Indoleamine 2,3-dioxygenase

IL: Interleukin

KSHV: Kaposi's sarcoma-associated herpes virus

LacNAc: N-acetyllactosamine

LAG-3: Lymphocyte activation gene-3

MDSCs: Myeloid-derived suppressor cells

MHC: Mayor histocompatibility complex

MICA: Major histocompatibility complex class I-related chain A

NF-κB: Nuclear factor-κB

NKT cells: Natural killer T cells

NSCLC: Non-small-cell lung carcinoma

PDAC: Pancreatic ductal adenocarcinoma

PD-1: Programmed cell death protein-1

PD-L1: Programmed death ligand-1

PGE_2: Prostaglandin E_2

PTLD: Posttransplant lymphoproliferative disorders

Siglecs: Sialic acid-binding immunoglobulin-type lectins

STAT-3: Signal transducer and activator of transcription-3

TCR: T-cell receptor

TDG: thiodigalactoside

TF antigen: Thomsen–Friedenreich antigen

TGF-β: Transforming growth factor-β

TIM-3: T-cell immunoglobulin and mucin-domain containing-3

TNF: Tumor necrosis factor

Tregs: T regulatory cells

VEGF: Vascular endothelial growth factor

VEGFR2: Vascular endothelial growth factor receptor-2

Abstract

Along with the discovery of tumor-driven inflammatory pathways, there has been considerable progress over the past 10 years in understanding the mechanisms leading to cancer immunosurveillance and immunoediting. Several regulatory pathways, typically involved in immune cell homeostasis, are co-opted by cancer cells to thwart development of effective antitumor responses. These regulatory circuits include engagement of inhibitory checkpoint pathways (CTLA-4, PD-1/PD-L1, LAG-3 and TIM-3), secretion of immunosuppressive cytokines (TGF-β, IL-10) and expansion and/or recruitment of myeloid or lymphoid regulatory cell populations. Elucidation of these pathways has inspired the design and implementation of novel immunotherapeutic modalities, which have already generated clinical benefits in an important number of cancer patients. Galectins, a family of glycan-binding proteins widely expressed in the tumor microenvironment (TME), have emerged as key players in immune evasion programs that differentially control the fate of effector and regulatory lymphoid and myeloid cell populations. How do galectins translate glycan-containing information into cellular programs that control immune regulatory cancer networks? Here we uncover the selective roles of individual members of the galectin family in cancer-promoting inflammation, immunosuppression and angiogenesis. Moreover, we highlight the relevance of corresponding glycosylated ligands and counter-receptors and the emerging function of these lectins as biological liaisons connecting commensal microbiota, systemic inflammation and distal tumor growth. Understanding the molecular and cellular components of galectin-driven regulatory circuits, the implications of different glycosylation pathways in their functions and their clinical relevance in human cancer might lead to the development of new therapeutic approaches in a broad range of tumor types.

Keywords: Cancer; Galectins; Glycans; Immunotherapy; Tumor Immunity; Inflammation

1. Galectin-glycan regulatory pathways in tumor-associated inflammation and immunity

Inflammation is a hallmark of cancer [1]. Chronic inflammatory conditions such as ulcerative colitis or chronic infections are known to increase the risk of carcinogenesis. In addition, independently of the role of chronic inflammation in tumor initiation, an influx of inflammatory cells is a universal occurrence in the microenvironment of established tumors. Inflammation at tumor beds includes differentiated and immature hematopoietic cells (primarily of the myeloid lineage), cytokines produced by leukocytes, fibroblasts or tumor cells, and complement components. Overall, inflammation promotes, rather than blunting, malignant progression, at multiple levels [2-4]. Firstly, inflammation fuels the proliferation and survival of malignant cells by activating transcription factors such as nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3), which drive proliferative and anti-apoptotic pathways. Secondly, inflammatory cells, and in particular myeloid leukocytes, are required for the generation of new blood vessels that support further tumor growth. In addition, inflammatory cells contribute to the formation of pre-metastatic niches that promote malignant spreading. Inflammatory cells and their products also impair the effectiveness of chemotherapeutic agents, and therefore represent a major target to gain understanding on cancer initiation and malignant progression, as well as for the design of novel therapeutic interventions [1,2].

Along with the discovery of tumor-driven inflammatory pathways, over the past 10 years there has been considerable progress in understanding cancer immunosurveillance and immunoediting based on the protection against development of spontaneous and chemically-induced tumors in animal models and the identification of targets for immune recognition in human cancer [5]. In fact, in the microenvironment of many established solid tumors, T cells can spontaneously exert clinically relevant pressure against malignant progression [5], and dramatically delay the progression of transplantable tumors [5]. Yet, in spite of these advances, a number of hurdles prevent the development of robust and durable antitumor responses. Thus, similar to inflammation, another independent although partially overlapping hallmark of cancer, is the ability of tumor cells to elude or thwart antitumor immunity [6]. The

mechanisms underlying these immune escape strategies involve: a) impairment of antigen presentation; b) activation of negative costimulatory signals- also called immune inhibitory checkpoints, including cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed death-ligand 1 (PD-L1), lymphocyte-activation gene 3 (LAG-3) and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3); and c) elaboration of a myriad of immunosuppressive factors such as transforming growth factor-β (TGF-β), interleukin (IL)-10 and indoleamine 2,3-dioxygenase (IDO). In addition, a number of regulatory cell populations, including $Foxp3^+$ and $Foxp3^-$ regulatory T cells (Tregs), natural killer T (NKT) cells , myeloid-derived suppressor cells (MDSCs) and mature immunosuppressive dendritic cells (DCs), contribute to undermine T-cell mediated tumor immunity [6]. In fact, accumulating evidence highlights the clinical benefit of blocking immune inhibitory checkpoints, either as monotherapies (e.g. anti-CTLA-4 or anti-PD-1 mAb) or in synergism with other immunotherapeutic modalities, to induce durable cancer regression and improve overall survival in patients with various malignancies by overcoming cancer-induced immunosuppression [5].

2. Glycans and galectins in the tumor microenvironment (TME)

Although the complex regulatory pathways leading to tumor inflammation and immunosuppression have been largely studied at the gene, mRNA and protein levels, the contribution of the glycome (the complete repertoire of cellular glycans) to these processes is poorly understood. Because of the non-template nature of carbohydrate synthesis, the macro- and microheterogeneity of glycosylation patterns of cell surface receptors and the dynamic regulation of glycan structures in different physiologic and pathologic processes, deciphering the information encoded by the cellular glycome has proven a difficult task [7,8]. However, in spite of these limitations, endogenous glycan-binding proteins or lectins have been demonstrated to efficiently translate glycan-containing information into functional cellular responses including cell cycle progression, chemotaxis, differentiation, cytokine synthesis and apoptosis by interacting with a discrete number of glycan structures [9,10]. In fact, lectins contribute to tumor growth and metastasis by influencing signaling thresholds of glycosylated receptors or by modulating cell-cell interactions in the TME, leading to

alterations of tumor cell migration, angiogenesis, inflammation and immune escape [11-13]. However, In this regard, changes in protein glycosylation profiles have been largely observed not only in cancer cells themselves, but also in tumor-associated fibroblasts, endothelial cells and immune cells [13,14]. Although the biological relevance of these changes is far from being completely understood, it has been demonstrated that inflammatory cytokines typically up-regulated in cancer, and in particular IL-6 and IL-1β, can change the glycosylation pattern of pancreatic and hepatocellular carcinoma cells, which contributes to accelerate malignant progression [15-17]. In addition, *O*-glycan branching also regulates the trafficking and effector activity of T cells, which are the major drivers of spontaneous anti-tumor immunity against established tumors, as well as memory T-cell differentiation [18, 19]. The activity of enzymes that drive the elongation of branched structures on *O*-glycans is also influenced in T lymphocytes by a variety of cytokines such as IL-2, IL-4 or IL-15, with dissimilar activities [19].

Yet, what are the most prominent changes in glycosylation observed in the TME? One of the most notable hallmarks observed during tumor progression is the increased frequency of β1–6 branching of complex *N*-glycans, resulting from enhanced expression of N-acetylglucosaminyltransferase 5 (GnT5; encoded by MGAT5) [20], as well as augmented expression of the bisecting GlcNAc branch generated by the N-acetylglucosaminyltransferase 3 (GnT3; encoded by MGAT3) in malignant compared to healthy tissues [21]. Moreover, incomplete glycosylation has been reported to be a common feature of cancer-associated mucins, including expression of the T antigen (Galβ1–3GalNAc-α1-O-Ser/Thr), also called Thomsen–Friedenreich (TF) antigen or expression of the Tn (GalNAc-α1-O-Ser/Thr) or sialyl-Tn antigens [22]. Furthermore, cancer cells may also display altered sialylation, as demonstrated by augmented α2,6-linked sialic acid attached to external N-acetyllactosamine (Gal-β1-4GlcNAc) units [23]. These aberrantly expressed glycosylated structures can be specifically recognized by endogenous lectins, forming multivalent lectin-glycan complexes that positively or negatively influence malignant progression [8,24]. Although lectins can recognize complex glycan determinants with relatively high affinity in the submicromolar range [25], it has been demonstrated that the structure, number and density of glycan

epitopes in multivalent glycoproteins, as well as the density of glycosylated receptors expressed on the cell surface and the multivalent nature of some lectins, all together determine the avidity of lectin-glycan interactions and their signaling potency [24]. Modeling of these interactions revealed the formation of two- and three-dimensional arrangements of multivalent lectins and glycans, often termed 'lattices' [26]. Although these high-ordered supramolecular complexes need further characterization at the cellular and molecular levels, lectin-glycan interactions have been proposed to serve as scaffolds for organizing plasma membrane domains and modulating the signaling threshold of relevant surface glycoproteins including the T cell receptor (TCR), B cell receptor (BCR), vascular endothelial growth factor receptor-2 (VEGFR2) and cytokine receptors [8, 27].

Whereas several lectins, including C-type lectins and sialic acid-binding immunoglobulin-type lectins (siglecs) may regulate signaling processes and control regulatory programs in the TME, we focus here on galectins, an evolutionarily conserved family of soluble glycan-binding proteins [12]. In spite of the wealth of information reporting the expression of individual members of the galectin family during tumor progression [28], there are only few attempts to dissect the complete galectin signature of individual cancers [28-34]. In general, galectin-1 overexpression was almost universally associated with poor outcome, while expression of galectins-3 and -9 appeared to be tumor type-dependent [28]. Interestingly, galectins may function extracellularly by interacting with a myriad of glycosylated receptors on the cell surface and extracellular matrix and regulating several processes including homotypic and heterotypic tumor cell adhesion, migration, epithelial mesenchymal transition (EMT), angiogenesis and tumor-immune escape [28, 35-38]. On the other hand, galectins can control critical intracellular events including oncogenic (e.g. K-ras; H-ras) signaling, splicing and autophagy [39-41]. However, in contrast to their extracellular functions, most of the intracellular events mediated by galectins take place through glycan-independent pathways [39]. From a structural viewpoint, galectins are classified into three different families: a) ‘proto-type’ galectins (galectin-1, 2, 5, 7, 10, 11, 13, 14 and 15) which display one carbohydrate recognition domain (CRD) and can dimerize; b) ‘tandem-repeat’ galectins (galectin-4, 6, 8, 9 and 12) which

contain two homologous CRDs in tandem in a single polypeptide chain; and c) the chimera-type galectin-3 which displays a CRD connected to a non-lectin N-terminal region responsible for oligomerization [42]. Although galectins lack the typical signal sequence required for the classical secretory pathway, most of them are externalized through a non-classical mechanism that is still not clearly understood [43].

Galectins were originally identified by their capacity to bind glycoconjugates bearing the N-acetyllactosamine [Galβ(1–4)-GlcNAc; LacNAc] disaccharide; however compelling evidence indicates substantial differences in glycan-binding specificities of individual members of the galectin family, which might explain differences in their biological activitites [44-47]. For instance, galectin-1 can bind to α2,3-sialylated and non-sialylated complex *N*-glycans containing poly-N-LacNAc residues, but does not bind to α2,6-sialylated glycans, whereas galectin-3 recognizes both α2,3- and α2,6-linked sialic acid [48, 49]. Furthermore, galectin-2 exhibits reduced binding to all sialylated carbohydrates [50] and galectin-8 shows higher affinity for 3'-O-sulfated or 3'-O-sialylated glycans and Lewis X-containing glycans than for neutral complex *N*-glycans [51]. Surprisingly, galectin-10 recognizes mannose-containing instead of β-galactoside-related saccharides [52]. Although these carbohydrate residues are widely distributed among different cells and tissues and are shared by a number of glycoproteins and glycolipids, individual members of the galectin family may co-opt a selected repertoire of glycosylated receptors on different cell types, suggesting that additional mechanisms including protein-protein interactions, conformational determinants and/or orientation of glycan motifs, may also determine galectin-binding preferences. To illustrate this concept, galectin-1 binds to CD45, CD43 and CD7 glycoproteins as well as to the GM1 glycolipid, while galectin-3 preferentially cross-links CD45, CD71 and LAG-3 on the surface of T cells [53-55]. On the other hand, galectin-8 interacts mainly with CD44 and β_1 integrin [56, 57], whereas galectin-9 binds to TIM-3 and CD44 on effector T cells and Tregs respectively [58, 59]. Within the myeloid compartment, galectin-1 binds and cross-links CD43 on DCs [60-62] and CD45 in macrophages/microglial cells [63]. Furthermore, at different stages of endothelial cell (EC) activation, VEGFR2, neuropilin-1 and CD146 have been proposed as candidate receptors for galectin-1 and galectin-3 [37, 64-66], whereas activated leukocyte cell

adhesion molecule (ALCAM/CD166) has been shown to serve as a preferential counter-receptor for galectin-8 [67]. Thus, individual members of the galectin family may co-opt a different set of glycosylated receptors on the surface of individual cell types.

Interestingly, while some galectins are widely expressed, either constitutively or in an inducible fashion in immune cells and tissues, others have a more limited cellular localization. For example, galectin-1 is considerably up-regulated in inflammatory macrophages and immunosuppressive DCs [61, 68, 69], activated T and B cells [70, 71], $CD4^+CD25^+$ Tregs and uterine NK cells [72, 73], whereas galectin-10 expression appears to be restricted to eosinophils and $CD4^+CD25^+$ Tregs [74, 75]. However, galectin expression is markedly deregulated in the TME including tumor cells themselves and tumor-associated stromal, endothelial and immune cells through mechanisms involving gene duplications and/or transcriptional or epigenetic regulation [28, 76]. How do galectins translate glycan-containing information into cellular programs that control immune regulatory networks in the TME? In the next sections we describe pioneering work and new findings that facilitate our understanding of the role of galectin-glycan interactions in tumor immunity.

3. Galectins shape tumor immunity through different mechanisms

Galectins can influence a broad spectrum of immune cell processes including maturation, activation, differentiation, polarization, trafficking, cytokine synthesis and viability [47, 77]. (**Figure 1**). These immunoregulatory effects and the marked up-regulation of galectins in human and mouse tumors [76] prompted the investigation of their roles in tumor immunity and inflammation. Studies in a melanoma model initially demonstrated the role of galectin-1 in tumor-immune escape through modulation of T cell viability and cytokine production. Targeted inhibition of galectin-1 gene expression in melanoma cells unleashed otherwise repressed $CD4^+$ and $CD8^+$ T cell responses, resulting in inhibition of tumor growth in syngeneic mice [78]. Supporting these findings, Cedeno-Laurent and colleagues demonstrated that disruption of galectin-1 ligands using peracetylated 4-fluoro-glucosamine (4-F-GlcNAc), a metabolic inhibitor of N-acetyllactosamine biosynthesis, decreased tumor growth in melanoma by boosting

antitumor immunity [79], suggesting that a dynamic galectin-1-glycan axis controls immune responses in the TME. Mechanistic studies revealed that this lectin acts by selectively deleting Th1 and Th17 cells through glycosylation-dependent mechanisms; these cell subsets share the repertoire of glycans (particularly high frequency of core 2-*O*-glycans and low amounts of α2,6-linked sialic acid) that are important for galectin-1 binding and apoptosis [48]. Furthermore, galectin-1 can also act by triggering the differentiation of human and mouse tolerogenic DCs [61] and M2-type pro-resolving macrophages [63, 80] and is a potent inhibitor of T-cell adhesion and transendothelial migration [81, 82], suggesting that this protein functions as a pleiotropic immunosuppressive factor in the TME.

Further studies in classical Hodgkin lymphoma (cHL), demonstrated that galectin-1 conferred immune privilege to Reed Sternberg cells by promoting a non-productive inflammatory infiltrate and skewing the balance toward Th2 and Treg cell responses [83, 84]. Interestingly, expression of galectin-1 was found to be up-regulated via an enhancer dependent on the AP-1 transcription factor both in cHL and in posttransplant lymphoproliferative disorders (PTLD), Epstein Barr virus (EBV)-driven B-cell malignancies [83, 85], suggesting that oncogenic viruses may usurp the galectin-1 pathway to promote tumor growth and immune escape. Similarly, Kaposi's sarcoma-associated herpes virus (KSHV) up-regulates galectin-1 to promote tumorigenesis in both human and mouse Kaposi's sarcoma cells [36]. These findings were further substantiated in models of lung cancer and neuroblastoma indicating that tumor-derived galectin-1 contributes to tumor-immune escape by shaping T cell and DC compartments [86-88]. Expanding these findings to other tumor types, studies in the 4T1 breast cancer model showed that galectin-1 also contributes to immunosuppression and metastasis by enhancing the number and function of Tregs [89]. Accordingly, intraperitoneal injection of thiodigalactoside (TDG), a galectin-binding saccharide, raised the levels of $CD8^+$ T cells in 4T1-bearing Balb/c mice [90]. Moreover, genetic ablation of galectin-1 in a model of pancreatic ductal adenocarcinoma (PDAC; *Ela-myc* mice) dampened tumor progression by inhibiting proliferation, desmoplastic reaction and by stimulating a tumor-associated T-cell response, yielding a 20% increase in relative mice survival [91]. Accordingly, in co-

culture systems, human pancreatic stellate cells enhanced apoptosis and anergy of T cells through galectin-1-dependent mechanisms [92], sustaining the role of galectin-1 in tumor-immune escape. Recently, this immune evasive program was elegantly demonstrated in models of glioblastoma, although in this particular tumor type, modulation of the functionality of macrophages [93] and NK cells [94] was observed. Thus, galectin-1 may de-activate both innate and adaptive components of antitumor immunity, leading to increased tumor progression. However, although galectin-1 is typically up-regulated in cancer cells, in some tumor types immune and/or stromal cells appear to be the main galectin-1 source. Particularly, in ovarian cancer models γδ-T lymphocytes were found to be major galectin-1 producers that contribute to systemic immunosuppression [95]. Also, in human chronic lymphocytic leukemia (CLL), galectin-1 was found to be predominantly secreted by accompanying myeloid cells (nurse-like cells, macrophages and DCs) and contributed to establish the appropriate microenvironmental conditions required for leukemic progression [96]. In contrast, in patients with advanced-stage cutaneous T-cell lymphoma, malignant cells were the predominant supply of this lectin which contributed to immunosuppression by blunting T cell proliferation and skewing the balance toward a Th2 profile [97]. Notably, lack of CD7 rendered leukemic cells themselves resistant to galectin-1-induced cell death, particularly in mycosis fungoides and Sezary syndrome [98, 99]. Most recently, in the context of hematological malignancies, Lykken and colleagues reported a key role of galectin-1 as a mechanism of resistance to anti-CD20 (Rituximab) therapy in non-Hodgkin lymphoma [100]. These results emphasize the common role of galectin-1 in conferring immune privilege to both solid and hematologic tumors.

However, in spite of the wealth of information linking galectin-1 and tumor-immune escape, other members of the galectin family also play important roles in tumor immunity. Demotte and colleagues found that galectin-3 plays a key role in distancing the TCR from CD8 in effector T lymphocytes, thus providing an alternative explanation for T-cell anergy occurring after several rounds of antigen stimulation [101]. The authors further demonstrated that GCS-100, a polysaccharide currently in clinical development, can detach galectin-3 from tumor-infiltrating lymphocytes and

boost cytotoxicity and secretion of antitumor cytokines [102]. These results were further substantiated by other findings showing inhibitory effects of tumor-derived galectin-3 on T-cell activation and survival [103, 104]. Furthermore, in two elegant studies tumor-released galectin-3 has been shown to dampen tumor immunity through modulation of NK cell biology. First, Tsuboi and colleagues showed that galectin-3 can interfere with the binding of tumor-associated major histocompatibility complex class I-related chain A (MICA) and NKG2D through mechanisms involving extension of core 2-O-glycans by the core 2 β-1,6-N-acetylglucosaminyltransferase 1 (C2GnT1) [105]. In a subsequent study, Wang *et al* showed that galectin-3 can serve as an inhibitory ligand for human NKp30, another cytotoxic NK cell receptor, thus impairing NK-cell mediated immunity [106]. More recent studies demonstrated that galectin-3 can also influence tumor immunity through binding to LAG-3, a negative regulatory checkpoint, on activated antigen-committed $CD8^+$ T cells, leading to suppression of cytotoxic T lymphocyte (CTL) effector function; this effect was accompanied by inhibition of plasmacytoid DC expansion [55]. Thus, galectin-3 may impair antitumor responses through multiple mechanisms involving potentiation of T-cell anergy, activation of inhibitory receptors such as LAG-3 and inhibition of NK cytotoxic receptors including NKG2D and NKp30.

Finally, galectin-9 can also influence the course of antitumor responses through activation of regulatory pathways. Galectin-9 became more popular after its identification as a candidate ligand for TIM-3, an inhibitory receptor and T-cell exhaustion marker [58]. Disruption of galectin-9-TIM-3 interactions *in vivo* resulted in abrogation of T-cell tolerance in several models of autoimmunity, infection, transplantation and cancer [107]. Accordingly, a number of studies showed that inhibition of the galectin-9-TIM-3 axis decreased apoptosis of CTLs and attenuated tumor growth [107-109]. Interestingly, these interactions also promoted expansion of myeloid regulatory cells which in turn suppressed CTL responses [110] (more details are provided in *Section 4*). In contrast, other studies revealed that galectin-9 can instead potentiate antitumor immunity via TIM-3-dependent interactions between DCs and $CD8^+$ T cells [111]. To reconcile these findings, further studies are warranted to dissect the inhibitory or stimulatory effects of the TIM-3-galectin-9 pathway in tumor

immunity. In this regard, recent studies revealed TIM-3-independent effects of galectin-9 and galectin-9-independent TIM-3 activities in several pathologic contexts [112, 113]. Supporting this notion, tumor-associated DCs in tumor models and cancer patients showed high expression of TIM-3 which suppressed innate immune responses through recognition of nucleic acids by Toll-like receptors and cytosolic sensors via a galectin-9-independent mechanism. In this setting, TIM-3 interacted with the alarmin high mobility group box 1 (HMGB1), but not with galectin-9 to inhibit recruitment of nucleic acids into DC endosomes [114]. Thus, TIM-3 may play distinct roles in innate and adaptive tumor immunity through galectin-9-dependent or -independent mechanisms. Interestingly, galectin-9 may also trigger tolerogenic responses through TIM-3-independent pathways as binding of this lectin to CD44, in the presence of TGF-β, induced Treg cell conversion and maintained their function and stability [59]. In addition, recent studies indicated that galectin-9 can also act as an agonist of 4-1BB (CD137), a member of the tumor necrosis factor (TNF) receptor superfamily by directly binding to a site different from 4-1BBL, promoting 4-1BB aggregation and signaling [115]. Collectively, these data suggest essential, although partially overlapping functions of individual members of the galectin family, in regulating antitumor immunity (**Figure 1**).

4. Galectin-driven regulatory pathways in 'emergency' myelopoiesis, inflammation and DC-mediated immunosuppression

Tumor-induced secretion of inflammatory cytokines such as IL-1β, IL-6, and prostaglandin E_2 (PGE_2) elicits pathological myelopoiesis via signaling on bone marrow myeloid precursors [116-118]. Sterile expanded (‘emergency’) myelopiesis is a mechanism co-opted by tumors that takes place to fight pathogens [119]. Pathological myelopoiesis leads to the accumulation of myeloid cells retained at an immature stage of differentiation into the blood, lymph nodes, spleen, bone marrow and tumor sites in cancer-bearing hosts [120, 121]. Unlike other infectious inflammatory conditions, however, this heterogeneous assortment of myeloid cells at different stages of differentiation acquires the capacity to suppress anti-tumor T cell responses in cancer

through poorly investigated mechanisms, and therefore turn into a cell type termed MDSCs. Two main subsets of MDSCs therefore represent precursors of granulocytic *vs.* monocytic cells that become immunosuppressive upon the influence of cancer-driven factors. Among other tumor microenvironmental cell types, both subtypes of MDSCs produce galectin-1, which contributes to their immunosuppressive activities, at least in ovarian cancer [95]. In addition, granulocytic MDSCs, which outnumber monocytic MDSCs by a ratio of 3:1 [122], are more active at generating immunosuppressive adenosine. This is critical as adenosine signaling is sufficient to drive the up-regulation of galectin-1 in naïve γδ-T cells [123]. These effects can be recapitulated by incubation of naïve γδ-T cells with adenosine agonists, but not with other factors such as IL-6, PGE_2, or TGF-β. Interestingly, γδ-T cells represent the major producers of galectin-1 on a per cell basis in the microenvironment of human ovarian carcinomas, while galectin-1 production turns them into highly immunosuppressive cells in a variety of autochthonous and transplantable tumor models [95, 123, 124]. Therefore, MDSCs suppress anti-tumor immunity directly, through multiple mechanisms that include the secretion of galectin-1, and indirectly by rendering γδ-T cells into regulatory lymphocytes that produce even higher levels of galectin-1. Besides galectin-1, production of immunosuppressive galectin-3 has been also identified in MDSCs, primarily of the monocytic lineage [125]. Furthermore, TIM-3-galectin-9 interactions have been associated with MDSCs proliferation in preclinical models [110]. Finally, both galectin-1 and galectin-3 have been found to be produced by activated M2-like macrophages [126], the predominant phenotype in the microenvironment of multiple tumors. However, only the secretion of galectin-3 was significantly up-regulated in macrophages differentiated *in vitro* under M2-skewing conditions, compared to classically activated M1-type macrophages [126]. Thus, galectins are actively involved in pathological myelopoiesis associated to cancer inflammation.

Multiple tumor-derived factors progressively abrogate the capacity of DCs to activate T cell-mediated anti-tumor immunity in advanced tumors [69, 127, 128]. Interestingly, while macrophages represent the most abundant hematopoietic cell type in the microenvironment of most malignancies, *bona fide* (but inflammatory) DCs are prominent in ovarian cancer masses, but not in human tumor ascites [69, 128].

Because a continuum of differentiation of myeloid precursors receiving conflicting signals in tumor-bearing hosts complicates the categorization of myeloid populations in the TME, differentiating macrophages from DCs in solid tumors is more complex than categorizing leukocyte subsets in the steady-state. However, $CD1c^{+}MHC\text{-}II^{+}CD19^{-}$ $CD11c^{+}$ leukocytes expressing *ZBTB46* at significantly higher levels than splenic macrophages outnumber $CD11c^{+}CD1c^{-}$ macrophages in dissociated ovarian tumor masses, while *Zbtb46*$^{+}$Dngr1/$Clec9a^{+}CD11c^{+}MHC\text{-}II^{+}CCR7^{+}Fc\gamma RI^{+}$ (inflammatory) DCs represent the most abundant hematopoietic cell in different autochthonous and transplantable ovarian cancer models [69].

Remarkably, at least in ovarian cancer, DCs not only become ineffective antigen presenting cells; they are also transformed into accomplices in tumor growth and immune evasion through the acquisition of immunosuppressive activities. Further investigation of the mechanisms whereby DCs inhibit anti-tumor immunity demonstrated that these regulatory functions rely on the secretion of galectin-1 by DCs at tumor beds [69]. Galectin-1 production is driven by unremitting expression of the genomic organizer Satb1 in tumor-associated DCs, which occurs in response to inflammatory microenvironmental signals such as overexpressed S100 proteins. Accordingly, *in vivo* silencing of Satb1 in tumor-associated DCs boosted protective immunity by decreasing galectin-1 production, leading to significant immunological impairment of malignant progression [69]. The relevance of Satb1-dependent up-regulation of galectin-1 specifically in TME DCs was further supported by additional experiments ectopically overexpressing Satb1 in wild-type *vs.* galectin-1-deficient DCs, which were co-administered with tumor cells into different cohorts of naïve mice. These studies clearly showed that tumors progress significantly faster in the presence of Satb1-overexpressing wild-type DCs, compared to mock-transduced control DCs. However, Satb1-dependent acceleration of tumor growth was abrogated when tumor-associated DCs lacked the capacity to synthesize galectin-1. Therefore, sustained overexpression of Satb1 in DCs, elicited by tumor cells, drives tumor-promoting activities in tumor-associated DCs through up-regulation of galectin-1 [69]. These results are in agreement with previous studies demonstrating that galectin-1-expressing DCs contribute to the resolution of inflammation during the course of autoimmune diseases [61].

5. Galectins link commensal microbiota, cancer-promoting inflammation and immunosuppression

Humans are colonized by trillions of bacteria, viruses, fungi and protozoan that populate the intestine, skin, respiratory and genitourinary tracts in a symbiotic relationship. Although recent studies have questioned the ratio of bacteria *vs.* human cells in a typical human being, it is still clear that commensal microorganisms dramatically outnumber human nucleated cells. The normal flora of humans is accordingly quite complex, with up to 1,000 commensal bacterial species only in the intestine [129]. Commensal microorganisms maintain homeostasis at mucosal surfaces, and influence metabolic processes, including obesity. Most importantly, colonization by commensal microorganisms is required for the development of a robust immune system and a broad repertoire of T cell receptors [130-135].

Despite the importance of the microbiota on broad protective immune responses, however, the relevance of commensal bacteria in anti-tumor immunity has only emerged in the last two years, with independent studies demonstrating that the effectiveness of different immunotherapeutic approaches requires the presence of commensal bacteria [136, 137]. This work has been followed by additional studies showing that individual bacterial species (rather than the overall microbiome) can be used to enhance the effectiveness of both CTLA-4 and PD-L1 blockade [138, 139].

Besides the role of the microbiome on immunotherapeutic effectiveness, our studies demonstrated that commensal bacteria spontaneously modulate the progression of non-mucosal tumors at places that are distal from locations of bacterial colonization [95]. Specifically, the microbiota regulates systemic tumor-promoting inflammation in cancer-bearing hosts: In tumors dominated by systemic up-regulation of IL-6 (e.g., ovarian cancer), commensal microorganisms, in the absence of measurable bacterial translocation, spontaneously accelerate malignant progression by driving higher levels of serum IL-6. Accordingly, depletion of commensal bacteria with a cocktail of antibiotics results in systemic down-regulation of IL-6 and, subsequently delayed tumor growth, in the absence of any additional therapeutic intervention or any effect on the tumor cell cycle. In contrast, in tumors where IL-6 is

only mildly up-regulated (e.g., luminal breast cancer), IL-17 dominates tumor-promoting inflammation through interactions with the microbiota, and depletion of commensal bacteria accelerates, rather than delaying, malignant progression [95].

Remarkably, these studies demonstrate that IL-6 up-regulation driven by dysbiosis (changes in the equilibrium between commensal microbial communities) in cancer-bearing hosts leads to over-production of immunosuppressive galectins by multiple cell types in the microenvironment of various tumors, including ovarian cancer [95]. Mechanistically, IL-6 up-regulation drives MDSC mobilization in cancer-bearing individuals. As aforementioned, MDSCs themselves, along with myeloid cells differentiated from them (macrophages and DCs) produce galectin-1, both at lymphatic (antigen priming) locations and tumor beds. Most interestingly, granulocytic MDSCs induce γδ-T cells to produce galectin-1, thus rendering them into immunosuppressive cells that abrogate protective anti-tumor immunity (**Figure 2**). Although under-investigated, γδ-T cells outnumber Tregs in microenvironments of human tumors such as ovarian and breast cancer and, as major producers of galectin-1, could represent major therapeutic targets to restore the protective activity of tumor-reactive γδ-T cells. Overall, these studies underscore the potential relevance of the microbiota in the glycobiology of cancer microenvironments and the effectiveness of anti-cancer interventions, which could differ in individual tumor types. Understanding how the repertoire of commensal microbes can be specifically manipulated to boost the benefit of immunotherapies and how galectin-glycan regulatory pathways could mediate these processes could open new avenues for more effective combinatorial interventions. Although translating the success of preclinical models to humans with less homogeneous repertoires of commensal bacteria, larger volumes of dilution and heterogeneous diets, ages and genetic backgrounds could bring major challenges, several lines of evidence suggest that mouse models could reflect the biology of human tumors with regards to the effects of the microbiota. Firstly, higher expression of galectin-1 is also obvious in tumors from TLR5-competent ovarian cancer patients, which exhibit stronger tumor-promoting inflammation, compared to ~7% of patients carrying a heterozygous polymorphism that abrogates TLR5 signaling and dampens IL-6-driven tumor-promoting inflammation [95]. Secondly, elegant studies by Vetizou and colleagues [139] demonstrated that reconstitution of

germ-free mice with the particular repertoire of commensal bacteria generated in melanoma patients upon the administration of CTLA-4 inhibitors was sufficient to enhance therapeutic effectiveness after tumor challenge. Future studies on the role of the microbiome in forging spontaneous or immunotherapeutically driven anti-tumor immunity should provide further insight into the role of galectins and glycans in both mucosal and distal tumors.

6. Galectin-glycan regulatory pathways at the interface of tumor immunity and angiogenesis

Notwithstanding this article focuses on the role of galectin-glycan pathways in tumor immunity, the effects of these regulatory lectins in EC biology are relevant in terms of the intimate connections between immunosuppression and angiogenesis and the importance of blood and lymphatic vessels in immune and tumor cell dissemination. In this regard, distinct members of the galectin family, including galectins-1-, -3-, -8 and -9 have been shown to play key roles in tumor vascularization by engaging a distinct repertoire of EC receptors, activating EC signaling pathways and/or regulating different events in the angiogenic process [13, 140-143]. We demonstrated that galectin-1 interactions with complex *N*-glycans couple tumor hypoxia to angiogenesis in models of Kaposi's sarcoma, melanoma, lung adenocarcinoma and T-cell lymphoma [36, 37]. Exposure to hypoxic microenvironments up-regulated galectin-1 expression in different tumor types through hypoxia-inducible factor-1α (HIF-1α)-dependent [144, 145] or NF-κB-dependent [36] mechanisms. Moreover, hypoxia also favored exposure of galectin-1-specific ligands, as it increased the amounts of β1-6GlcNAc-branched *N*-glycans and poly-LacNAc structures and reduced the levels of α2,6 sialylation on ECs [37]. Targeting galectin-1 suppressed vascularization in several tumor types including melanoma [35, 37, 146], Kaposi's sarcoma [36], prostate carcinoma [32], lung adenocarcinoma [37], T-cell lymphoma [37], pancreatic adenocarcinoma [91] and glioblastoma [93]. Moreover, analysis of human tumor biopsies revealed a marked correlation between the number of blood vessels and galectin-1 expression in prostate carcinoma [32], non-small-cell lung carcinoma (NSCLC) [147] and Kaposi's sarcoma [36]. More recently, we identified a glycosylation-based mechanism mediated by

galectin-1-*N*-glycan interactions that serves to preserve angiogenesis in vascular endothelial growth factor (VEGF)-targeted therapies [37]. We found that galectin-1 bound directly to non-sialylated *N*-glycans on VEGFR2, promoted segregation and retention of this glycosylated receptor on the surface of ECs and recapitulated VEGF-like signaling, including VEGFR2, Erk1/2 and Akt phosphorylation. Remarkably, tumors that were refractory to VEGF blockade produced high amounts of galectin-1 in response to hypoxia or anti-VEGF treatment and their associated vasculature displayed glycosylation patterns that facilitated galectin-1 binding and signaling. Targeting galectin-1 eliminated resistance to anti-VEGF treatment, suppressed the formation of aberrant tumor vascular networks and augmented anti-tumor immune responses in several tumor models [37]. Interestingly, antibody-mediated galectin-1 blockade promoted transient normalization of tumor-associated vessels, which facilitated access of effector T cells to TME [37], thus emphasizing the critical role of galectin-glycan regulatory pathways in the control of tumor vascularization and immunity.

7. Conclusions and Perspectives

In the present article, we highlight the multifunctional roles of galectins (particularly galectin-1, -3, and -9) in the TME with particularly emphasis on their ability to govern the immunological landscape of different tumors, their involvement in vascularization programs and their regulatory roles in linking commensal microbiota, systemic inflammation and cancer immunosuppression. Because of their immune inhibitory and pro-angiogenic activities, targeting galectin-glycan interactions has emerged as a promising therapeutic approach, either alone or in combination with other treatment modalities, to restore anti-tumor immunity and to attenuate abnormal vascularization of established tumors. In this regard, a number of galectin blocking strategies with different degrees of selectivity for individual members of the family have been considered, including glycan-based inhibitors [102, 148-152], allosteric antagonists or peptidomimetics such as anginex, 6DBF7, 0118 or OTX008 [153-156], natural or modified polysaccharides such as citrus pectin [157] and anti-galectin specific neutralizing antibodies [36, 37, 85]. Some of these inhibitory agents have recently

been designed or purified, whereas others are being tested in preclinical models or are already undergoing clinical trials. However, before galectin-based therapeutic agents can be embraced in clinical settings, a more thorough understanding of the mechanisms involved in galectins functions is required. In this regard, a number of questions remain to be addressed: a) To what extent is there functional redundancy and specificity of action within the galectin family in the TME?; b) Are there therapeutic advantages in using less selective glycan inhibitors that simultaneously target several members of the galectin family or is it preferable to use specific antagonist of individual galectins?; c) Do galectins interact with other immune evasion programs or do they play hierarchical independent roles in tumor-immune escape? Further studies are warranted to further understand the role of galectin-glycan regulatory pathways in the TME and their relevance in cancer immunity and immunotherapy.

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Legends to Figures

Figure 1. Galectin-driven regulatory pathways in the TME. Galectins-1, -3- and -9 fuel immune evasive programs in different tumor types through activation of multiple tolerogenic mechanisms. Galectin-1 tilts the balance of the immune response toward a Th2 profile by selectively deleting Th1, Th17 and $CD8^+$ T cells. Moreover, it drives the differentiation of T regulatory cells (Tregs), endows dendritic cells (DCs) with tolerogenic potential, polarizes macrophages toward an anti-inflammatory M2-type profile, inhibits NK cell recruitment and limits transendothelial T-cell migration. In addition, this lectin is a key player of a regulatory circuit that links commensal microbiota, systemic inflammation and tumor growth through mechanisms involving expansion of myeloid-derived suppressor cells (MDSCs) and $\gamma\delta$-T cells. Interestingly, galectin-1-glycan interactions can also couple tumor hypoxia to vascularization and preserve angiogenesis in tumors refractory to anti-vascular endothelial growth factor (VEGF) treatment. On the other hand, galectin-3 acts by restricting T cell receptor (TCR)-mediated signaling and promoting T-cell anergy and exhaustion by distancing the TCR from CD8 and engaging LAG-3 on the surface of $CD8^+$ T cells. In addition, this lectin impairs the antitumor activity of NK cells by inhibiting NKp30-mediated cytotoxicity and interrupting NKG2D-MICA interactions. In addition, galectin-3 may also control the expansion of tumor-associated plasmacytoid DCs. Finally, galectin-9 confers immune privilege to tumor cells through TIM-3-dependent or -independent mechanisms. While it selectively kills terminally-differentiated TIM-3^+ Th1 and $CD8^+$ T cells, it also binds to CD44 and cooperates with TGF-β_1 to promote Treg cell differentiation and favors expansion of immunosuppressive MDSCs. Galectin-1 (green) is indicated as a non-covalent homodimer each containing one carbohydrate-recognition domain (CRD), galectin-3 (light blue) is indicated in its pentameric structure and galectin-9 (purple and blue) is depicted as two CRDs in tandem connected by a linker peptide.

Figure 2. Galectin-1 links commensal microbiota, tumor-promoting inflammation and immunosuppression. In the presence of a tumor, TLR5-dependent crosstalk between commensal bacteria (purple and brown) and hematopoietic cells at mucosal surfaces

boosts the up-regulation of IL-6 in the blood stream. Systemic up-regulation of IL-6 promotes pathological myelopiesis, leading to the mobilization of galectin-1-producing myeloid-derived suppressor cells (MDSCs). Granulocytic MDSCs, to a greater degree than their monocytic counterparts, render γδ-T cells with anti-tumor potential into galectin-1-secreting immunosuppressive players, representing ~6% of total T cells in the TME. By producing galectin-1, γδ-T cells dramatically impair the anti-tumor activity of effector T cells at tumor beds, thus fueling malignant progression in a microbiota-dependent manner.

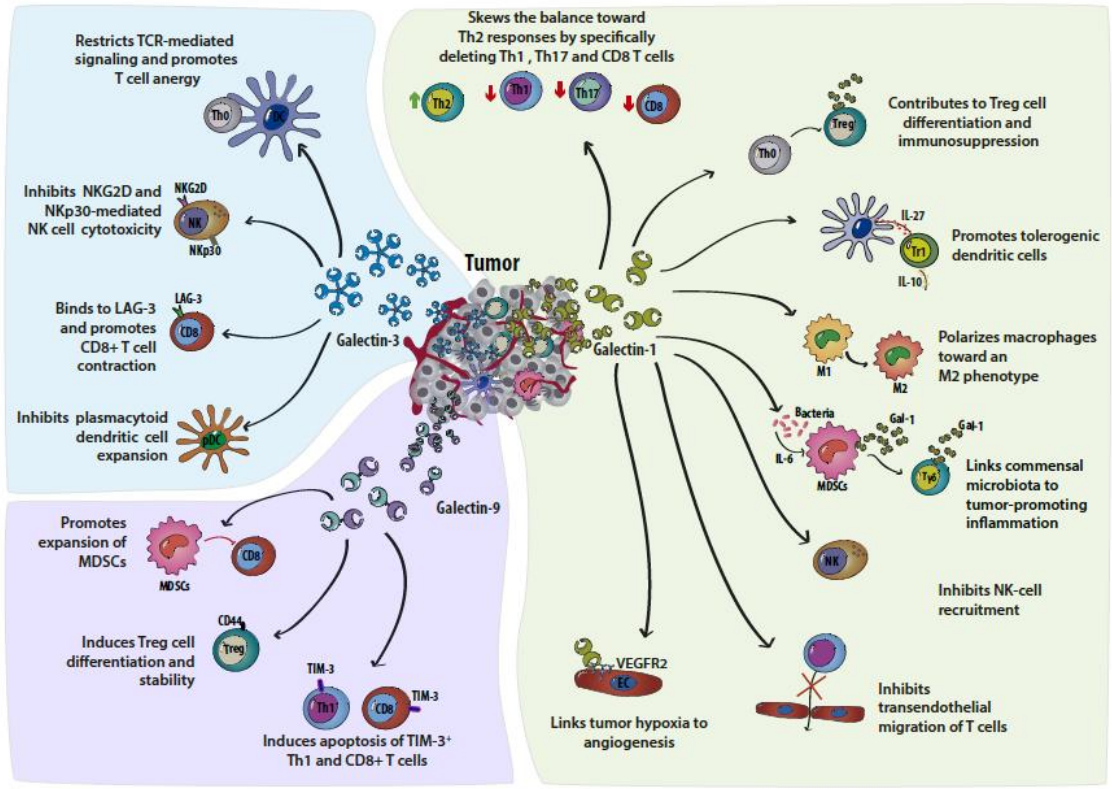


Figure 1

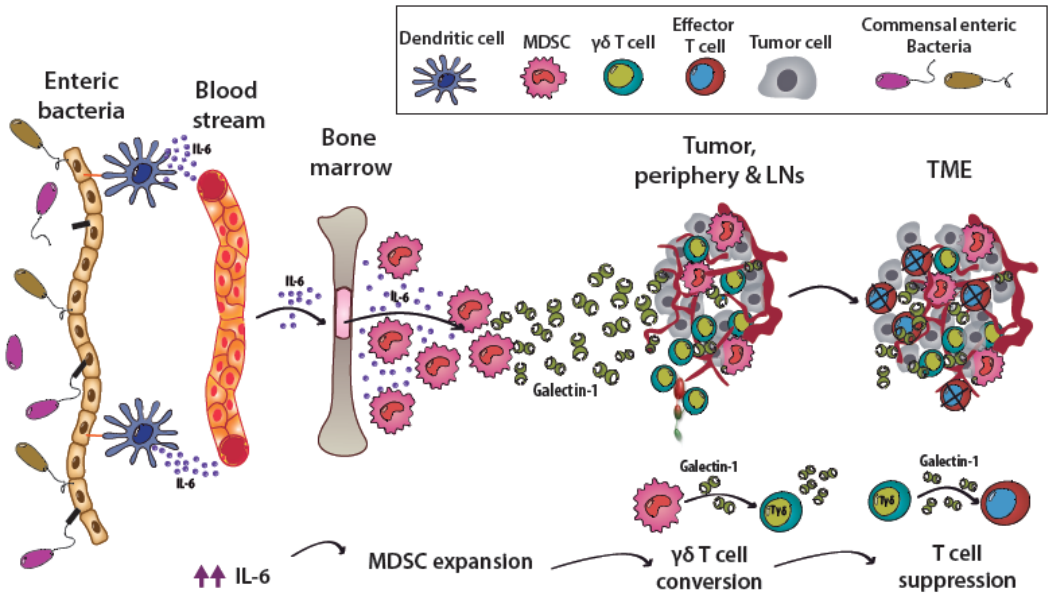
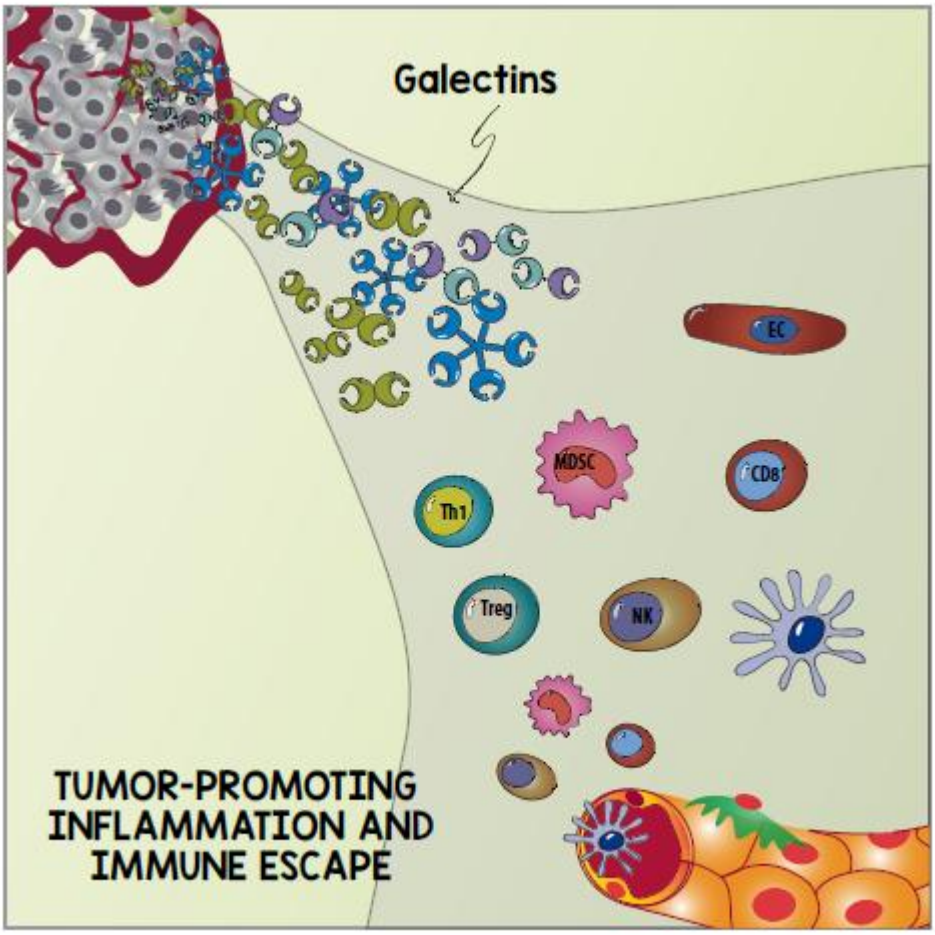


Figure 2



Graphical abstract

Highlights

*Galectins are key players in immune evasion programs in cancer

*Galectins-glycan interactions control immune and endothelial cell compartments

*Galectin-1 links commensal microbiota, systemic inflammation and tumor-immune escape

*Targeting galectin-glycan interactions may contribute to unleashing antitumor immunity