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Review Article

Reprogramming the tumor metastasis cascade by targeting galectin-driven networks

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A sequence of interconnected events known as the metastatic cascade promotes tumor progression by regulating cellular and molecular interactions between tumor, stromal, endothelial, and immune cells both locally and systemically. Recently, a new concept has emerged to better describe this process by defining four attributes that metastatic cells should undergo. Every individual hallmark represents a unique trait of a metastatic cell that impacts directly in the outcome of the metastasis process. These critical features, known as the hallmarks of metastasis, include motility and invasion, modulation of the microenvironment, cell plasticity and colonization. They are hierarchically regulated at different levels by several factors, including galectins, a highly conserved family of β-galactoside-binding proteins abundantly expressed in tumor microenvironments and sites of metastasis. In this review, we discuss the role of galectins in modulating each hallmark of metastasis, highlighting novel therapeutic opportunities for treating the metastatic disease.

Introduction

Along with the improvement of surgical techniques and the implementation of neoadjuvant therapies, the mortality rate of many primary tumors has dramatically decreased [1], being metastatic spread the most lethal attribute of neoplastic cells [2]. To describe the process that epithelial carcinomas should undergo to achieve a higher grade of malignancy, a model of interconnected events known as 'the Hallmarks of Metastasis' has been proposed. This new concept, based on the Hallmarks of Cancer described by Hanahan and Weinberg [1,3], introduced four well-defined attributes shared by all metastatic cells [2]. Every hallmark represents a trait of the metastatic cell which directly impacts in the success of the 'metastatic cascade'. To proceed with the metastatic process, tumor cells must acquire these specific hallmarks, namely: (a) *motility and invasion* which enables tumor cells to leave primary sites and spread towards the pre-metastatic niche; (b) *modulation of the microenvironment*, which allows interactions with the stroma to promote a tissue architecture endowed with the pro-metastatic configuration; (c) *plasticity* to survive in the hostile conditions of the new environment; and (d) *colonization* to usurp the pre-metastatic niche and to establish a supportive neighborhood for seeding and outgrowth [2]. This categorization, based on distinctive and complementary capabilities of the metastatic cell, intends to associate each hallmark with genotypic and phenotypic attributes that are crucial for the metastatic outgrowth. A better understanding of these features would lead to the identification of novel biomarkers, to improve early diagnosis and to boost therapeutic strategies.

In the past decade, galectins, a family of highly conserved β-galactoside-binding proteins, emerged as key regulators of tumor progression, not only by influencing primary tumor growth but also by shaping the metastatic process [4]. Galectins are glycan-binding proteins that control a wide range of biological processes including cell invasion, migration, immune evasion, and angiogenesis. At the molecular level, galectins fine-tune signaling of different cell surface receptors by establishing multivalent interactions with specific glycosylated ligands [5,6]. Different tumors exhibit differential galectin

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landscapes that hierarchically serve as 'on–off' switchers that control different Hallmarks of Cancer including invasion and metastasis. Blockade of galectin–glycan interactions thus represents a novel strategy to halt tumor progression [7]. In this review, we will discuss the involvement of galectins in different hallmarks of metastasis and the molecular pathways that govern their function in the context of tumor progression.

The hallmarks of metastasis

The metastatic process consists of sequential events that must be accomplished for successful secondary organ colonization. This multistage process, known as the metastatic cascade, is endowed with defined features characterized as the 'Hallmarks of Metastasis' including the acquisition of motility and migration, dynamic modification of the metastatic milieu and the plasticity to overcome hostile environments in order to colonize the pre-metastatic niche in target organs (Figure 1). As a first step, tumor cells must leave the primary tumor site and invade local tissue surroundings by degrading the basal membrane (*invasion*); then they foster the formation of new blood vessels (*neo-angiogenesis*), leak into the bloodstream (*intravasation*) and travel in the circulation (*dissemination*) until anchoring in the distant stroma (*extravasation*), where it finally settles in the target organ (*colonization*) [8]. Cells that have already left the primary tumor, either at an early or late stage of the disease, are known as disseminated tumor cells (DTC) [9]. DTCs experience a reprogrammed phenotype which endows these cells with the capacity to circumvent unfavorable events and to adapt to environmental changes [10]. Once the secondary organ is reached, DTC can either proliferate and give rise to macrometastasis or remain dormant for years or decades [11,12]. This hibernation state is reversible, and many factors have been proposed to awake the dormant cell. This 'awakening' process is in general triggered by microenvironmental stimuli but could also be driven by internal signals and activation of certain transcription factors [13].

Galectins: key players in the control of the metastatic cascade

Galectins are a family of glycan-binding proteins that recognize *N*-acetyl-lactosamine (Galβ1-4NAcGlc; LacNAc) residues in complex branched *N*-glycans and extended core 2-*O*-glycans. Galectins display both, intracellular and extracellular functions that contribute to tumor progression. Members of this family can be classified, based on their architecture, in three major subgroups called: (a): 'proto-type' galectins (Gal1, Gal2, Gal5, Gal7, Gal10, Gal11, Gal13, Gal14, and Gal15) consisting of subunits with a single carbohydrate-recognition domain (CRD) and the ability to self-associate as dimers via non-covalent bonds; 'tandem-repeat' galectins (Gal4, Gal6, Gal8, Gal9, and Gal12) sharing two homologous CRDs connected by a linker peptide and the 'chimera-type' galectins, being Gal3 the unique member of this group, which may exist as monomer, but can

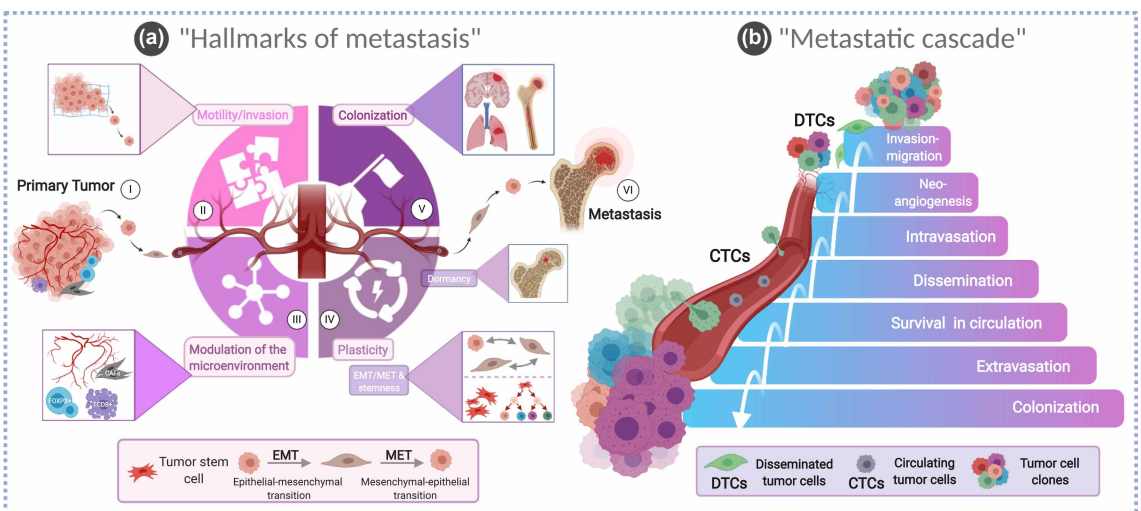

Figure 1. The Hallmarks of Metastasis.

(**a**) Welch and Hurst proposed a subcategorization of the Hallmarks of Cancer named as the Hallmarks of metastasis to describe the four traits that successful metastatic tumor cells share [2]. (**b**) Every feature has a direct impact on the progression of the metastatic cascade leading to colonization and formation of macrometastasis. Created with BioRender.com.

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also dimerize or multimerize (particularly in pentamers). The N-terminal domain as well as the beginning of the a C-terminal carbohydrate-binding domain (CBD) of Gal3, are involved in dimerization and multimerization processes, making higher-order complexes in the presence of higher-order sugars [14–17] (Figure 2). Through binding to different glycosylated receptors (glycoproteins or glycolipids) on the surface of different cell types, galectins can trigger activation of signaling pathways that co-operate to reprogram several key processes including antitumor immunity, angiogenesis, genomic instability, and neoplastic transformation [4].

Accumulating evidence indicates that galectin expression is altered in tumors and in the tumor-associated stroma, playing active roles during tumor progression. Recently, we reviewed mechanisms, signaling, and pathways through which galectins may influence different 'Hallmarks of Cancer' [7]. In this review, we will dissect the impact of galectins in the so-called 'Hallmarks of Metastasis' focusing on how these endogenous lectins influence different events of the tumor metastasis cascade.

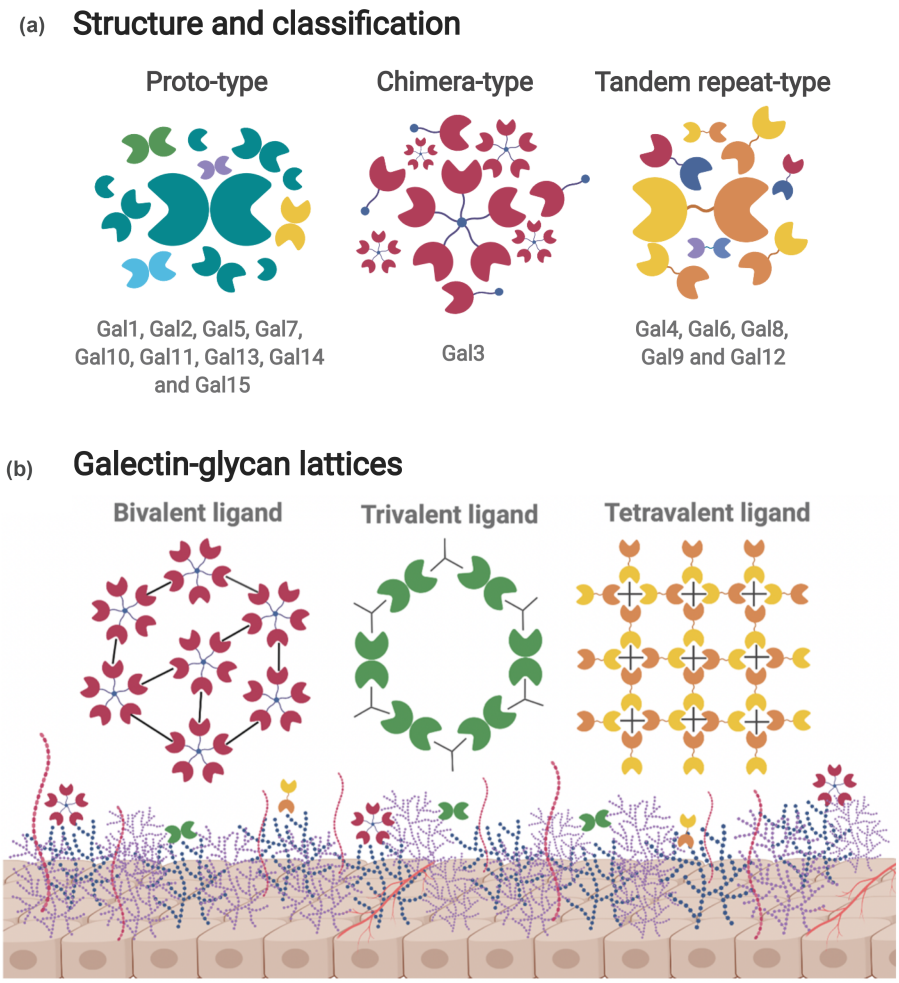

Figure 2. Galectins: structural and biochemical features.

(**a**) Structural representation of different galectin family members. Galectins are subdivided into three groups: (i) 'Prototype' (Gal1, Gal2, Gal5, Gal7, Gal10, Gal11, Gal13, Gal14, and Gal15) with one CRD and ability to form homodimers; (ii) 'Tandem-repeat' galectins (Gal4, Gal6, Gal8, Gal9, and Gal12), with two different CRDs connected by a linker peptide; and (iii) the 'Chimera type' galectin, being Gal3 the only member of this family, which can exist as monomer but can also dimerize or multimerize (particularly in pentamers). (**b**) Scheme of distinct types of lattices, that could be potentially assembled through interactions between multivalent galectins and glycans. Bivalent, trivalent and tetravalent ligands associated with multivalent galectins can give rise to the formation of distinct three-dimensional configurations and arrays that trigger or re-wire signaling pathways. Created with BioRender.com.

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Motility and invasion

Extracellular and intracellular signals govern tumor cell fate promoting the invasion of the tumor surrounding area as a first hallmark of the metastatic process. Tumor cells may co-opt galectin-driven pathways that are used during normal development, for invasion and dissemination. For example, normal mammary epithelial cells proliferate and migrate, within their surrounding stroma, during the branching stage of mammary gland morphogenesis. This process may be controlled by the dynamics of Gal1 subcellular localization [18]. Interestingly, at the invading edge of the normal mouse mammary gland, Gal1 was found to be abundant in the nucleus of proliferating epithelial cells of the end buds, where it is involved in pre-mRNA splicing and coexists in a complex with Gemin-4 [18]. Thus, in spite of its nuclear localization and function in physiologic settings, this lectin impacts significantly on the migration and invasion of the transformed mammary cell. Accordingly, Gal1 is forced to translocate to the nucleus of malignant breast cancer cells due to an increased α2,6-sialylation (α2,6 SA) of membrane glycoconjugates, that are restrictive for its binding. Thus, Gal1 serves as a major driving force for tumor cell invasion during breast tumor progression [18].

Reinforcing the idea that galectins play a role in normal invasive programs, Gal1 and Gal3 have been described as active members of the human trophoblast invasion machinery. Blocking both galectins in HTR-8/SVneo cells derived from human placenta, led to a significant reduction in cell migration and invasion [19,20]. Moreover, Gal7 has also been proposed as a key regulator of epithelial cell homeostasis since it modulates cell growth, differentiation, and apoptosis. Likewise, in epithelial wound healing, a non-transformed scenario of cell invasion, Gal7 controls keratinocytes migration in mouse corneas and epidermis [21,22].

As generally occurs in physiological events involving cell migration and invasion, most tumor cells invade surrounding tissues either as clusters in a process named collective migration or as single cells depending on the microenvironmental conditions. Collective migration relies in the interactions of tumor cells with their neighborhood, where cell–cell junctions as desmosomal proteins, gap junctions, and tight junctions are key players, forming a cohesive group of cells that proceed with migration and invasion by degrading extracellular matrix (ECM) components [23]. In this regard, cell–cell junctions are modulated by several signaling pathways such as phosphatidylinositol 3-kinase (PI3K)/AKT, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), focal adhesion kinase (FAK), and Rho GTPases [23] that sense the environmental conditions and modulate homotypic and heterotypic cell interactions. On the contrary, single cells can invade the surrounding area following two distinct mechanisms, a mesenchymal invasion or a Rho/ROCK-dependent ameboid process. In this regard, Gal1 can favor both types of migration by favoring mesenchymal transformation and increasing the number and length of filopodia on tumor cells via the Rho-dependent signaling pathway [24].

Interestingly, DTC have the ability of modulating the microenvironment and at the same time, are endowed with remarkable plasticity mainly through a mesenchymal and/or stem cell phenotype. The strong plasticity of a DTC has direct impact on its motility and invasion capacities, suggesting that the four Hallmarks of Metastasis are deeply interconnected, and all are functionally related. Here, we will dissect the role of galectins in regulating motility and invasion of tumor cells by remodeling the extracellular matrix, orchestrating the stromal cells cross-talk, and favoring development of pro-metastatic niches.

Modulation of the microenvironment

Extracellular matrix remodeling

Several studies showed that galectins can affect the cross-talk between tumor cells and the ECM [4]. Galectins bind to a wide array of glycoproteins and glycolipids on the cell surface and the ECM, delivering signals and mediating cell–cell and cell–ECM adhesion. Accordingly, galectins can interact with both *N*- and *O*-glycans present on glycoproteins of the ECM such as fibronectin, elastin, hensin, and laminin [4]. In particular, Gal1 promotes attachment of melanoma cell lines (A375 and A2058) and ovarian cell lines (AZ224, SK-OV-3, AZ382 OVCAR-3, and AZ224) to laminin-1 and fibronectin in a dose-dependent manner [25,26]. In addition, Gal3 interacts with both soluble and insoluble elastin, regulating the adhesive interactions between breast carcinoma cells (MDA-MB-231 and BT-474) with the ECM [27]. Moreover, in melanoma cells, Gal3 oligomers controlled the interactions of elastin and αvβ3 integrin modulating their invasive capacity [28] (Figure 3).

Interestingly, Gal1 induces up-regulation of matrix metalloproteinases MMP-2 and MMP-9 and stimulates actin cytoskeleton reorganization in oral cancer and in lung adenocarcinoma (Figure 3). Also, Gal7, another prototype galectin that is highly expressed in oral squamous cell carcinoma (OSCC), promoted invasiveness

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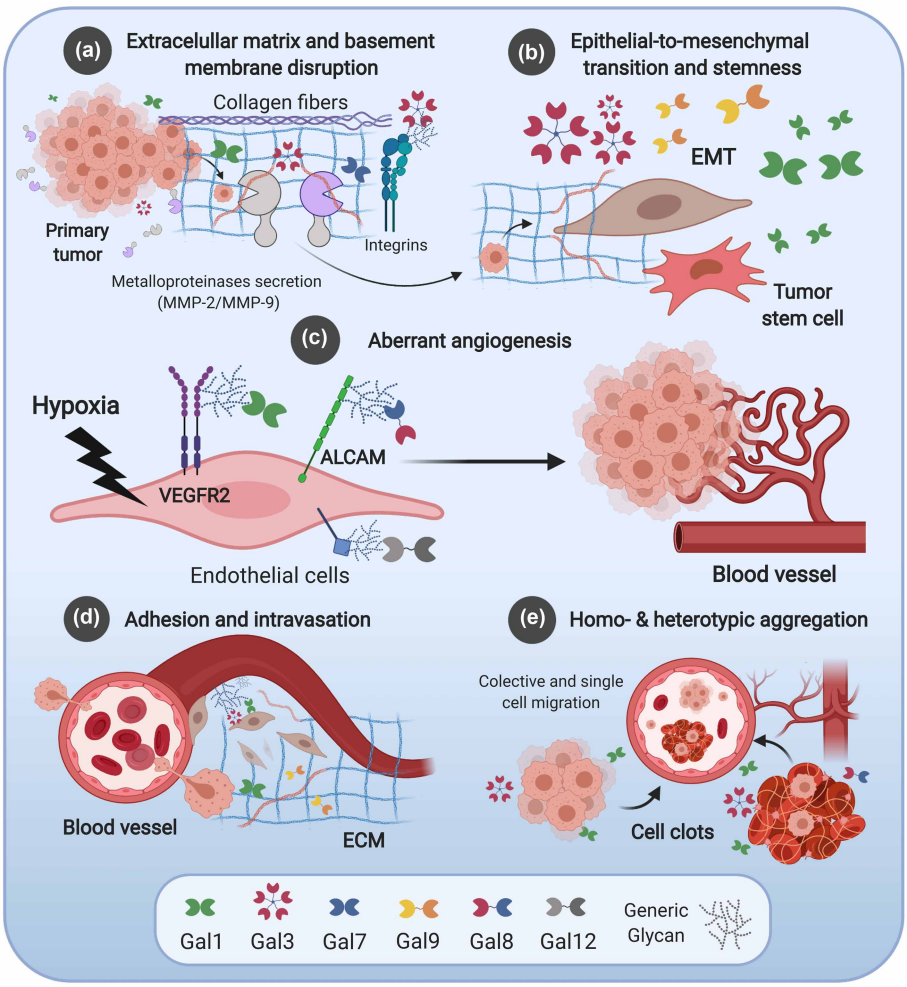

Figure 3. Galectins control tumor cell migration, invasion, and intravasation processes.

(**a**) Tumor cells may disrupt the basement membrane to invade the stroma. Gal1, 3 and 7 enhance metalloproteinases (MMP-2 and MMP-9) secretion and integrin/collagen disruption. (**b**) Gal1, Gal3 and Gal9 influence cell plasticity promoting epithelial-to-mesenchymal transition (EMT) and stemness (Wnt/β-catenin) having a direct impact on motility, migration, and invasion. (**c**) Galectins promote neoangiogenesis through binding to VEGFR2 (Gal1), by stimulating the proangiogenic capacity of tumor-associated macrophages (TAMs) or through binding to αvβ3 integrin (Gal3), through association with ALCAM/CD166 (Gal8), or by recognizing 3′ fucosylated residues (Gal12). (**d**) Gal3 interacts with Thomsen–Friedenreic (TF) antigen enhancing adhesion and intravasation and Gal9 promotes ECM adhesion. (**e**) Cells can migrate either as single cells or in clusters. Galectins promote homotypic and heterotypic aggregation of tumor cells. Gal1, Gal3, and Gal9 enhance the aggregation of platelets with tumor cells aiding survival in the circulation. Interaction between Gal3 and MUC1 favor emboli formation and heterotypic aggregation. Created with BioRender.com.

through ERK and JNK signaling and facilitated migration via the expression of MMP-2 and MMP-9 [29] (Figure 3). Similarly, Gal3 increased the activities of the same metalloproteinases, promoting cell invasion in pancreatic and tongue cancer cells through PI3K/AKT and β-catenin signaling pathways [30–32] (Figure 3). Furthermore, Gal3 binding to complex *N*-glycans on mammary epithelial tumor cells, modulated fibronectin-dependent matrix remodeling, promoted α5β1 integrin recruitment and FAK activation, thus increasing tumor cell motility [33]. Therefore, different members of the galectin family foster tumor cell adhesion and migration not only by stimulating interactions with glycoconjugates present in the ECM, but also by promoting remodeling of its composition and structure.

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Tumor–stroma cross-talk

Mammary ductal carcinoma *in situ* (DCIS) is a proliferation of neoplastic luminal cells that are confined to the duct-lobular system of the breast. If DCIS progresses to invasive ductal carcinoma (IDC), tumor cells infiltrate the parenchyma after basement membrane disruption [34]. Consistent with the effect of immune regulatory mediators in shaping tumor evolution toward an invasive phenotype, a switch toward a tolerogenic immune environment during the transition from DCIS to IDC was recently demonstrated. At early stages of the dissemination process, dysregulation in the balance of pro- and anti-metastatic signals dictates the outcome the tumor progression. Accordingly, the galectin gene expression signature of the breast tumor stroma was found to be associated with positive or negative clinical outcomes. In this context, triple-negative (TN) and HER2+ breast cancer patients showed a stromal signature expression characterized by the prevalence of Gal1, Gal3, and Gal9 that was associated with poor prognosis [35]. Within the TME, cancer-associated fibroblasts (CAFs) play a major role during tumor progression [36], through the secretion of growth factors and cytokines that activate receptors present on tumor cells. Several studies revealed that Gal1 released by human pancreatic stellate cells (which function as CAF in the pancreatic TME) induces progression of preneoplastic pancreatic lesions and promotes the release of stromal cell-derived factor-1 (SDF-1) which increases migration and invasion of pancreatic cancer cells [37,38]. Importantly, in a mouse model of pancreatic ductal adenocarcinoma (PDAC), tumoral Gal1 induces activation of CAF, thus contributing to TME remodeling [39]. In line with these findings, in human cells, knocking down Gal1 inhibited CAF-induced tumor cell migration and invasion by reducing the production of monocyte chemoattractant protein-1 (MCP-1/CCL2) [40]. Furthermore, in gastric carcinoma cells, CAF-derived Gal1-induced epithelial-mesenchymal transition (EMT) via hedgehog pathway activation [41]. Thus, galectins govern tumor–stroma interactions through mechanisms involving immune and inflammatory components, further contributing to immune escape (Figure 4).

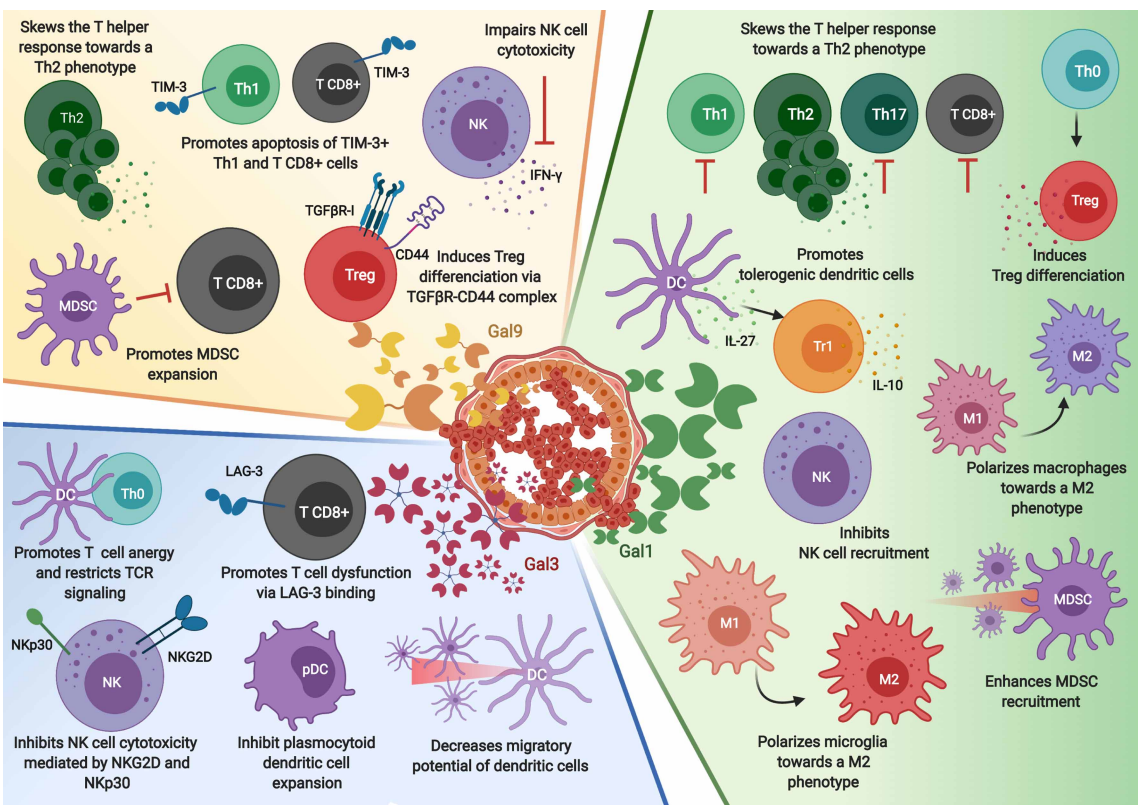

Figure 4. Galectins reprogram antitumor immune response by shaping myeloid and lymphoid cell compartments.
Effects of Gal1, Gal3, and Gal9 on different innate and adaptive immune cell types and the consequence of these interactions in immune evasion mechanisms and metastatic events. Created with BioRender.com.

Innate antitumor immunity

Galectins have the ability to shape the innate immune compartment, thus influencing early antitumor immunity and promoting a predominant immunosuppressive or tolerogenic profile (Figure 4). In a B16 melanoma model, Gal1 fostered the differentiation of tolerogenic dendritic cells (DCs) [42] and inhibited transendothelial migration of inflammatory DCs [43]. This lectin facilitated the recruitment of monocytic and granulocytic myeloid-derived suppressor cells (MDSCs) and TAMs ($CD11b^{high}F4/80^{+}$) into the TEM in a mouse glioma model (GL261 cell line) [44]. In addition, Gal1 polarized macrophages and microglia into an M2 anti-inflammatory profile [45,46]. In this regard, Gal1 expression has been considered a mechanism of evasion of innate immunity in another glioma model (GL26). Gal1 knockdown in GL26 cells resulted in heightened inflammatory responses leading to rapid recruitment of Gr-1(+)/CD11b(+) myeloid cells and NK cells into the brain TME, thus influencing tumor immunoediting [47]. Additionally, tumor-derived Gal3 impacts the cytotoxic potential of NK cell activating-receptors such as NKG2D and NKp30. In particular, in bladder tumor cells, Gal3 binding to core 2 *O*-glycans present on MHC class I-related chain A molecule (MICA), dismantled the interactions between this ligand and NKG2D in NK cells, thus blunting NK-mediated killing potential [48]. Moreover, in human cervical cancer and breast cancer cell lines, Gal3 acts as a soluble inhibitory ligand for the NKp30 receptor [49]. In a mouse tumor model of melanoma, Gal3 also enhanced polarization of macrophages toward a M2 phenotype [50], thus tilting the balance toward an immunosuppressive antitumor response. Recently, it was shown that Gal9 binds to the mannose receptor CD206 present on M2 macrophages and stimulates the release of fibroblast growth factor (FGF)-2 and MCP-1, thus supporting tumor growth in metastatic melanoma patients [51]. Of note, Gal9 also favors expansion of granulocytic (CD11b + Ly6G+) MDSCs promoting tumor growth in EL4 thymoma cells and 4T1 mouse breast cancer cells [52]. On the other hand, in colon cancer, Gal9 modulates NK cells by promoting F-actin polarization of NK cells via Rho/ROCK1 activation [53]. Thus, galectins may serve as critical mediators of tumor-promoting inflammation and regulators of innate immunity acting at early stages of tumor development and preventing activation of antitumor adaptive responses.

Adaptive antitumor immunity

Galectins may influence the progression of the metastatic cascade by blunting the antitumor adaptive responses. In particular, Gal1 expression thwarts antitumor immunity through several mechanisms, including the promotion of T-cell apoptosis, suppression of T-cell activation, induction of anti-inflammatory Th2 responses and expansion of regulatory T cells (Tregs) [54–56]. Mechanistically, Gal1 induces selective death of Th1 and Th17 cells due to differential glycosylation of these cells as evidenced by low frequency of α2,6-linked sialic acid (α2,6SA) and higher elongation of core 2 *O*-glycans. On the contrary, Th2 cells are protected from Gal1-induced cell death through α2,6 sialylation of cell surface glycoproteins. As a result, Th2 cells are protected from Gal1-induced cell death, leading to a relative increase in the frequency of this T-cell subpopulation [57]. Taken together, these properties license Gal1 as part of an immune evasive program confirmed in several tumor types including melanoma, classical Hodgkin lymphoma, HNSC, neuroblastoma, glioblastoma as well as ovary, pancreatic, prostate and breast adenocarcinoma, among others. In addition, Gal1 released by human PSCs promotes a Th2-skewed cytokine profile along with activation of caspases-9 and 3 and triggering of the mitochondrial apoptotic pathway [58]. Deletion of Gal1 in a Kras-driven model of PDAC (*Ela-KrasG12Vp53*$^{-/-}$) promoted mice survival and increased T-cell infiltration in established tumors [59]. This profile was also evident in classical Hodgkin lymphoma which expressed Gal1 via an enhancer of the activating protein 1 (AP1) transcription factor [60].

T-cell exclusion thwarts antitumor responses by limiting access of T cells to tumor antigens. In HNSC, Gal1 expression prevented T-cell infiltration into the tumor bed and increased resistance to anti-PD-1 immune checkpoint blockers by inducing Gal9 expression in endothelial cells (ECs) [61]. In breast cancer, invasion of tumor-draining lymph nodes (TDLN) by metastatic cells correlated with poor prognosis and was associated with local immunosuppression, which was partly mediated by the expansion of Tregs. Luminal tumors are in general poorly infiltrated or are eventually infiltrated with Tregs [62]. However, TN and HER2-positive subtypes have a pronounced immune infiltration, mostly composed of $CD8^{+}$ tumor-infiltrating T lymphocytes (TILs). Whether galectins influence the composition and the quality of the immune infiltration in metastasis and in breast cancer tumors has been poorly explored. Expression of Gal1 correlated with tumor grade in human breast cancer particularly in a TN breast cancer model. Silencing tumor-derived Gal1 reduced tumor

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burden and diminished lung metastasis through a mechanism involving neutralization of local and systemic immunosuppression [54]. Importantly, the frequency of Tregs in the TME, TDLN, spleen, and metastatic lungs was significantly reduced in Gal1 knockdown tumors. Furthermore, silencing tumor-derived Gal1 induced down-regulation of the immunoregulatory adaptor LAT in Tregs [54], thus counteracting the immunosuppressive capacity of these cells.

Likewise, Gal3 confers immunosuppression to tumors by modulating T cell signaling and anergy. Importantly, through its oligomeric structure, Gal3 is able to form lattices that promote distancing of critical immune receptor molecules, thus modulating signaling thresholds of T cells in the TME. When Gal3 binds to cytotoxic T cells, it promotes distancing of the TCR from CD8 molecules, thus impairing IFN-γ secretion and fostering T-cell anergy in TILs. Treatment with LacNac, a disaccharide that dissociates Gal3–glycan interactions, restores TCR-CD8 colocalization and effector function of $CD8^+$ T cells isolated from several sources including breast carcinoma metastasis, tumor ascites or blood [63]. In addition, Gal3 promotes CD8 T cell dysfunction by acting as a non-canonical ligand of the inhibitory checkpoint LAG-3 in a breast cancer HER-2/neu transgenic mouse model [64]. Likewise, Gal3 traps the immune checkpoint molecule cytotoxic T lymphocyte antigen-4 (CTLA-4) on the surface of T cells, thus sustaining inhibitory signals and contributing to disarm antitumor T-cell responses [65]. In this context, the presence of anti-Gal3 autoantibodies has been reported in the plasma from patients with metastatic melanoma after partial or complete response to anti-CTLA-4 plus anti-VEGF mAb therapies, suggesting that Gal3 may potentially have prognostic and predictive value for immunotherapy response [66]. Interestingly, in immunologically desert tumors, Gal3 binds to glycosylated IFN-γ and other chemokines in the TME, thus preventing IFN-γ diffusion and avoiding the formation of the IFN-γ-induced chemokine gradient required for tumor T-cell infiltration [67] (Figure 4).

Like Gal-1, Gal-9 has been defined as a negative regulator of Th1 responses, by selectively deleting TIM-3+ Th1 cells. In fact, systemic Gal9 was associated with Th2 polarization and reduced overall survival in patients with metastatic melanoma [68]. *In vitro*, the addition of Gal9 impairs the proliferation of healthy PBMCs, leading to Th1 cell apoptosis, and promoting Th2 cell phenotypes [68]. In addition, Gal9 enhanced the stability and function of inducible Tregs by forming a trimolecular complex with CD44 and transforming growth factor (TGF-β) receptor I (TGF-βRI) that promotes SMAD3 activation. Exogenous Gal9 co-operates with TGF-β1 to reinforce iTreg cell differentiation and maintenance. In fact, it was demonstrated, that Gal9 acts directly through the non-coding conserved sequence CNS1 region of the Foxp3 locus, thereby modulating iTreg cell induction [69]. Interestingly, Gal8, a tandem-repeat type galectin with the ability to recognize α2,3-sialylated glycans, promotes Treg conversion by activating TGF-β and IL-2R signaling [70].

Neo-angiogenesis and intravasation

Once the tumor cell has detached from the primary site and has invaded and migrated through the peritumoral surrounding area, it may get into the bloodstream through a process called intravasation. This critical step is profoundly influenced by the architecture and properties of tumor-associated vessels, both from blood and lymphatic origin [71]. Tumor-associated vasculature differs substantially from normal vasculature as it is fenestrated, aberrant and involves weak interactions of adjacent ECs due to the absence of pericyte coverage, which together with the ability to penetrate endothelial barriers, facilitates the tumor intravasation process. Tumor angiogenesis refers to the formation of new blood vessels within a tumor, being hypoxia, the major driver of the angiogenic response. This process is crucial for metastatic spread and is governed by different growth factors present in the TME including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and FGF, which triggers EC activation. In addition, lymphangiogenesis significantly contributes to the metastatic cascade, by regulating immune cell influx and tissue fluid homeostasis [71]. Based on these features, it is not surprising that galectins are also involved in the formation of vascular networks and promotion of angiogenesis and lymphangiogenesis, thus nourishing tumors and providing novel escape routes.

Expression of Gal-1 is regulated by hypoxia through mechanisms that may depend on either hypoxia-inducible factor 1α (HIF-1α) or nuclear factor κB (NF-κB activation [72–74]. Importantly, this lectin reprograms EC biology promoting VEGFR-2 phosphorylation [73,75] and angiogenesis [73,76–78]. Interestingly, hypoxia also remodels the glycan profile of ECs, thus promoting a Gal1 permissive phenotype which controls paracrine EC signaling. In this context, Gal1 present in the TME, binds to complex *N*-glycans on VEGFR2 and promotes neovascularization [73]. Moreover, a decrease in α(2,6)-SA and an increase in complex branched *N*-glycan structures, as well as elongation of poly-LacNAc residues facilitate Gal1 binding to VEGFR2 on ECs, thereby promoting aberrant angiogenesis and conferring resistance to anti-VEGF mAb

therapy [79] (Figure 5). Furthermore, ECs can take up Gal1 and stimulate their proliferation and migration through the activation of the H-Ras signaling [80]. Interestingly, in cervical cancer cells, receptor of activated C-kinase 1 (RACK1) facilitated tumor cell invasion and promoted lymphangiogenesis and LN metastasis through a mechanism involving Gal1 expression and secretion. Interestingly, this lectin feeds a positive loop increasing RACK1 levels via activation of the β1 integrin signaling, further reinforcing the pro-metastatic effect [81].

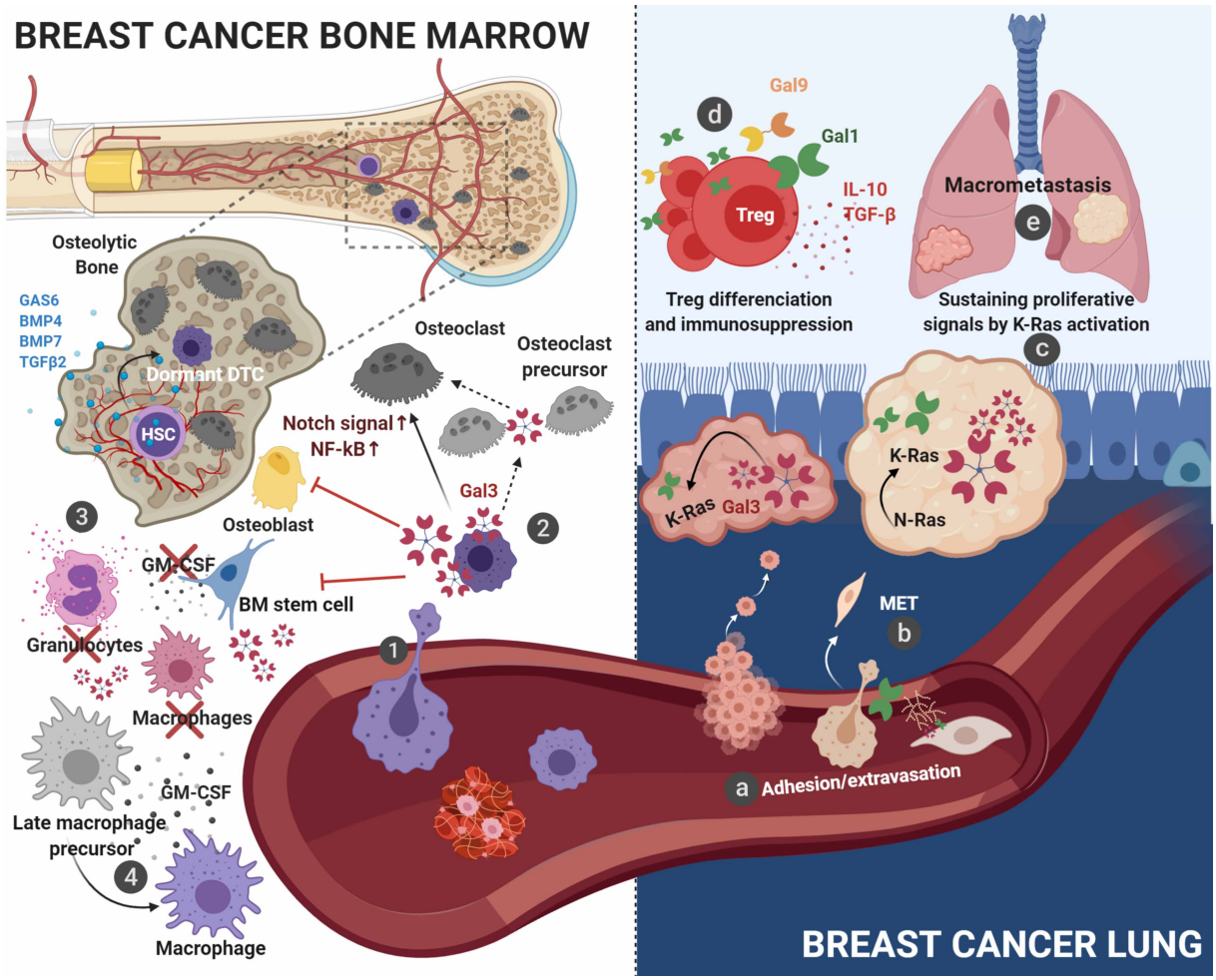

Figure 5. Extravasation and colonization: Schematic representation of bone marrow and lung metastasis in breast cancer models.
(**a**) Adhesion and extravasation: (1) Tumor cells may adhere to the endothelium by means of galectin-glycan interactions. Gal1 interacts with cell surface glycosylated receptors including CD44 or CD326, thus influencing extravasation and cancer cell attachment to the endothelium. Gal3 triggers the exposure of CD44 and E-selectin and promotes adhesion and extravasation through the Thomsen-Friedenreic (TF) antigen. *Bone marrow* (*BM*): (2) Gal3 promotes osteoclast differentiation in the presence of RANKL, triggering Notch and NF-κB signaling. Osteoclasts play key roles in remodeling the pre-metastatic niche. (3) Gal3 inhibits BM stem cell differentiation and GM-CFS secretion; however, in the presence of late macrophage precursors it enhances GM-CFS production and macrophage recruitment and polarization. Hematopoietic stem cells can secret factors such as BMP and TGF-β_2 favoring bone remodeling and sustaining dormant phenotypes. *Lung*: (**b**) Cells undergoing EMT must turn back into an epithelial phenotype in order to form macrometastasis. (**c**) Gal3 promotes the proliferation of tumor cells upon activation of RAS signaling, favoring a molecular switch from NRAS to KRAS, and the formation of macrometastasis. Also, Gal1 promotes tumor cell proliferation via KRAS interaction. (**d**) Since Gal1 may promote Treg cell polarization and increase Treg cell frequency in the lungs, this lectin may contribute to create an immunosuppressive microenvironment for those DTCs in the lung. In this figure, we represent in colors different tumor cell clones with distinct tropism (violet-bone marrow/pink and strong grey-lung). Created with BioRender.com.

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Gal1 is far from being the only galectin associated with the angiogenic process. Gal3 is a key player in tumor vascularization as it stimulates the proangiogenic capacity of tumor-associated macrophages (TAMs) through mechanisms involving a TGF-β_1 and VEGF cross-talk [82]. Moreover, Gal3 also modulates VEGF- and bFGF-mediated neovascularization through binding to N-glycans on integrin αvβ3 in ECs [83]. Importantly, Gal3 is also expressed in ECs and interacts with cancer-associated Thomsen–Friedenreic (TF) antigen, a glycan biomarker exposed in many metastatic human carcinoma cells, [84,85], initiating DTC adhesion to the endothelium [86]. Moreover, in breast and prostate cancer cells, Gal3 also increases adhesion to the endothelium of well-differentiated microvessels [87]. Furthermore, Gal3 released into the bloodstream of colorectal and melanoma cancer patients, promotes hematogenous dissemination via its interaction with TF-expressing MUC1 on the cancer cell surface [88]. These data suggest that Gal3 not only promotes angiogenesis but may also act as an endothelium activator affecting the first steps of the DTC intravasation process.

In addition to Gal1 and Gal3, Gal8 also induces migration and morphogenesis of vascular ECs *in vitro* and promotes angiogenesis *in vivo*, in an experimental model of breast cancer. The mechanisms underlying this effect involve glycosylation-dependent interactions between Gal8 and activated leukocyte cell adhesion molecule (ALCAM)/CD166) [89].

It has been well-established that certain subpopulations of macrophages significantly contribute to metastasis, by either promoting angiogenesis or facilitating tumor spreading. Particularly, in breast cancer, the association of podoplanin-expressing macrophages (PoEMs) with tumor-associated lymphatic vessels correlates with metastasis in LN and in distant organs. In this setting, it has been demonstrated that lymphatic vessels synthesize Gal8, which in turn promotes adhesion of PoEMs. Once in the lymphatic vessel neighborhood, PoEMs induce lymphangiogenesis and LN invasion in a glycan-dependent fashion via matrix remodeling, growth factor release and activation of pro-migratory integrin β_1 [90].

Remarkably, alternative splicing has been shown to regulate the length of the linker region between both CRD of Gal9, affecting its valency and function. This molecular effect is particularly relevant during vascular development since different Gal9 isoforms selectively control vascularization, exhibiting controversial effects depending on their concentration and environmental context. The Gal9Δ5 isoform induces *in vitro* sprouting and migration of human umbilical vein endothelial cell (HUVEC), but paradoxically, triggers inhibitory effects on angiogenesis *in vivo* [91]. In addition, we recently found that adipose tissue-derived Gal12 promotes *in vitro* angiogenesis through binding to 3′-fucosylated glycans on ECs (Figure 3). Moreover, analysis of *in vivo* adipose tissue vasculature showed reduced vascular networks in Gal12-deficient ($Lgals12^{-/-}$) compared with wild-type mice, thus emphasizing the role of galectins in the cross-talk between vascular and adipose tissue [92]. Thus, Gal1, Gal3, Gal8, Gal9, and Gal12 emerge as novel regulators of EC biology and angiogenesis in the TME. Whether these endogenous galectins selectively control different stages of the vascularization process in a spatio-temporal manner remains to be elucidated.

Cell plasticity

Metastatic cells have the intrinsic ability to move as individual cells or as a community cluster. The cell migration process is prevented by E-cadherin junctions that tend to preserve epithelial cells integrity, further counteracting cell dissociation or association with other substrates. To overcome this hurdle, tumor cells may hijack EMT. Interestingly, EMT involves the expression of transcription factors such as Slug, Snail, Twist, ZEB1, and ZEB2 that orchestrate the transition from an epithelial towards a mesenchymal phenotype, negatively modulating the expression of E-cadherin, a trademark of epithelial identity. Several galectins have been linked to EMT programs in several types of cancers. Gal1 induces EMT in human ovarian cancer cells (SKOV3-ip and SKOV3) via activation of the JNK/p38 signaling pathway [93,94]. Moreover, this lectin also induces EMT in other tumor types such as hepatocellular carcinoma [95,96], gastric cancer [41,97,98], PDAC, [99], and colon cancer [100] (Figure 3). Interestingly, Gal3 expression also promotes EMT via a Wnt/β-catenin-dependent activation in oral tongue squamous cell carcinoma [32] and fosters the expression of EMT-related markers in colon carcinoma cells [101] (Figure 3). Furthermore, in prostate cancer patients, Gal4-driven EMT was associated with poor survival [102]. Interestingly, evidence from experimental models and clinical data indicates that EMT programs share characteristics with stemness programs. Cancer stem cells (CSCs), stem-like cells within tumors, have been proposed to play central roles in the metastatic process by virtue of their self-renewal capacity and resistance to different anticancer treatments including chemotherapy. For example, in breast cancer cells, EMT induction drives the generation of cells with stem-like properties characterized by a

$CD44^{high}/CD24^{low}$ surface phenotype [103]. Additionally, in numerous tumor types, including several forms of brain cancer, prostate cancer, colon cancer, lung cancer, hepatocellular carcinoma, and ovarian cancer, the marker CD133 has been proposed to identify a cell population with CSC properties [104]. In this context, Gal1 plays a pivotal role in tumorigenesis and invasiveness of $CD133^{+}$ CSCs in lung adenocarcinoma [105]. Even more, Gal3 promotes lung cancer stemness properties by activating the EGFR/c-Myc/Sox2 axis [106] and the αvβ3 integrin/KRAS/NFkB pathway [107]. Gal3 also supports stemness in A2780 and OVCAR3 ovarian cancer cells by activating the intracellular domain of Notch1 [108]. Finally, it was proposed that the association of Gal9 and TIM-3 expressed on the surface of leukemic stem cells, activates both NF-κB and β-catenin pathways, promoting stemness and self-renewal in human acute myeloid leukemia (AML) [109]. Thus, galectins have a broad impact in the control of cell plasticity, an essential feature that shapes migratory, invasive and metastatic properties of tumor cells through activation of EMT and stem cell programs.

Colonization

Survival in the circulation and extravasation

Once the tumor cell penetrates the basement membrane, it gets into the blood or lymphatic vessels and traverses through the bloodstream until reaching the colonization site. During this journey, circulating tumor cells (CTCs) need to overcome the unfavorable conditions of the bloodstream and survive. For that purpose, tumor cells form clusters, which protect them from the shear forces and allow their survival in the circulation until they reach a distant organ for seeding.

The maintenance of cancer cell clusters is dependent on the ability of tumor cells to form heterotypic and homotypic adhesive interactions, that are critical for the establishment of secondary lesions in distant tissues [110,111]. Several studies showed that Gal1 promotes both homotypic and heterotypic aggregation of tumor cells [112,113] by cross-linking several glycosylated ligands presents on the tumor cell surface as well as in ECM glycoproteins including laminin and fibronectin [26] (Figure 3). Gal1 mediates homotypic cell aggregation in melanoma cells by interacting with the glycoprotein 90K/Mac-2BP (a Gal3-binding protein) [113]. In addition, Gal1 interacts with adhesion molecules such as CD44 or CD326 affecting extravasation and cancer cell attachment to the endothelium in breast cancer [114].

Interestingly, Gal3 in its pentameric form, binds to core 1 antigen (TF; Galβ1,3GalNAcα-1-0-Ser/Thr) and induces heterotypic and homotypic aggregation. Elevated levels of Gal3 in cancer patient's plasma augmented cancer cell adhesion to ECs under both static and flow conditions, further increasing transendothelial invasion. The interaction of Gal3 with the TF antigen present in ECs permits the polarization of cancer-associated MUC1 protein and exposes surface adhesion molecules such as CD44 and E-selectin, thus enhancing cancer-endothelium adhesion in colorectal and melanoma cancer cells [88]. Interaction between Gal3 and MUC1 promotes emboli formation and survival in the circulation of colon and breast cancer CTCs, leading to profound consequences in metastasis formation [115]. In addition, serum levels of Gal2, Gal4, and Gal8 significantly increased in plasma from patients with metastasis, enhancing the interactions between the CE and the TF disaccharide present on cancer-associated MUC1 [116].

As mentioned above, the role that Gal9 plays in metastasis is less clear and even controversial. Evidence indicates that certain Gal9 isoforms may promote cell adhesion and cluster formation, while others inhibit these pro-metastatic processes. Whereas Gal9L (or gal-9FL) isoform decreases E-selectin expression, Gal9M (or gal-9Δ5) and S (or gal-9Δ5/6) increase E-selectin levels in LoVo colon carcinoma cells. Overexpression of these three isoforms in LoVo cells increased attachment to ECM proteins, while Gal9M and S overexpression fosters adhesion to HUVEC *in vitro* [117]. Furthermore, in luminal breast cancer cells (MCF7 cell line) Gal9 induced cluster formation, while Gal9M and S variants inhibited the adhesion to collagen, fibronectin, vitronectin and laminin [118]. Moreover, in OSCCs, Gal9 increased adhesion to laminin and fibronectin [119] (Figure 3).

As mentioned above, platelets may influence the metastatic potential of CTC by contributing to clog formation and/or increasing the adhesion to the endothelium and transmigration via P-selectin [120]. Through the formation of heterotypic aggregates, platelets may protect tumor cells against immune attack and circulation shear forces [120], while secreting pro-inflammatory mediators that sustain tumor growth and stroma formation [121]. A growing body of experimental evidence indicates that Gal1 controls platelet homeostasis with potential implications in metastasis. Gal1 induces platelet and leukocyte aggregation in a carbohydrate-dependent manner, synergizing with ADP or thrombin to form platelet clusters and ATP release. At the molecular level, Gal1 induces conformational changes in the integrin complex GPIIb/IIIa, a pre-requisite for

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platelet cross-linking, and favor conformational changes in platelets, including F-actin polymerization and lamellipodia as well as filopodia extension [122]. Also, Gal8 promotes the transition of αIIbβ3 integrin towards an activate state favoring platelet aggregation and clustering [122,123]. Mechanistically, both lectins promote thromboxane generation, P-selectin expression, and granule secretion that favors the aggregation phenomenon [123,124]. Thus, by interacting with glycosylated ligands present on ECs or platelets, galectins mediate clog formation and platelet-induced metastasis. Once CTCs have surmounted the challenge of moving through the circulatory tract and have already extravasated, they are prepared to colonize the pre-metastatic niche, further generating dormant or active metastasis.

Colonization of the pre-metastatic niche

Once the vasculature is left behind, the tumor cell needs to adapt to the new microenvironment in order to colonize and form metastasis. DTCs establish the pre-metastatic niche through a complex cross-talk between the primary tumor and stromal cells, by secreting growth factors and chemokines that systemically stimulate the bone marrow (BM) to produce 'pro-niche' molecules. For example, breast cancer DTCs secrete IL-11 and express Jagged1, which modulate osteoclast function, thus favoring bone metastasis through the release of survival factors from the bone matrix. However, the survival of the DTCs in the pre-metastatic niche does not guarantee the formation of large macroscopic metastasis. In fact, DTCs that reach the metastatic organ can remain in a dormant state for years and even decades. For example, in BM, hematopoietic stem cells (HSCs) secrete pro-dormancy factors such as GAS6, BMP4, BMP7, and TGF-β_2 [11]. As galectins play important roles in shaping stroma–tumor cell interactions, it is likely that they could influence the establishment of a pre-metastatic niche. In fact, Gal3 restructures the BM microenvironment which in turn regulates its cleavage status during osteoclastogenesis. At a mechanistic level, intact Gal3 interacts with myosin-2A enhancing osteoclastogenesis in the presence of RANKL, while its cleavage suppresses osteoblast differentiation via Notch signaling, thus contributing to pro-metastatic osteolysis in breast and prostate cancer [125–128]. Gal3 also affects the differentiation of BM-derived precursors [129], thus influencing commitment toward osteoclast, osteoblast, lymphoid and myeloid cell compartments and enhancing colonization and metastasis in breast and prostate cancer (Figure 5).

A dormant cell may remain in a latent state in the target organ for years. However, a perturbation in the homeostatic state of the environmental conditions may occur, inducing awakeness of dormant cells. Signals may arise from inside the cells and may include activation of proliferative signals or may involve the release of inhibitory brakes that control cell cycle progression. Both Gal1 and Gal3 can interact with oncogenic RAS proteins, inducing RAS membrane anchorage and activation, opening the gates towards proliferation and macrometastasis [130,131]. Moreover, Gal1 promotes tumor cell proliferation through activation of ERK1/2 upon Ras activation in lung cancer models [132]. Also, Gal3 activates KRAS, promoting a molecular switch from NRAS to KRAS in breast cancer patients [133] (Figure 5). Although further studies are awaited, these growing body of experimental evidence, even when they are indirect, may suggest a potential role of galectins in dormancy.

The roles of galectins in modulating EMT and stemness, in regulating metalloproteases that remodel the microenvironment, in generating local and systemic immunosuppression and in inducing angiogenesis have been discussed here. Altogether, these reports suggest that galectin–glycan interactions may have a broad impact in the colonization of the metastatic organ and will largely contribute to the metastatic burden.

Galectin-targeted strategies

As galectins modulate different Hallmarks of Metastasis, targeting galectin–glycan interactions may represent a promising therapeutic strategy to treat or reprogram metastatic disease. Several inhibitors have been developed, including small-molecule inhibitors, multivalent saccharide ligands, antagonistic peptides, and blocking antibodies and some of them are currently under evaluation in clinical trials.

Carbohydrate-derived small-molecule inhibitors

This approach is mainly based on the use of chemically modified natural galectin ligands, such as the disaccharides lactose (Lac) or *N*-acetyllactosamine (LacNAc) to target the CRD of galectins. As the development of these inhibitors involves a full understanding of the biochemistry of galectin–glycan interactions, efforts are being made to generate galectin inhibitors that target individual members of the family with higher affinity and selectivity (particularly Gal1, Gal3, and Gal7). The disadvantages of these inhibitors for use in clinical

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Table 1. Clinical trials that target galectins and their respective ligands (Adaptation from 156) Part 1 of 2

Sponsor	Compound	Proposed target	Origin	Indication	Phase	Title of the study	NTC Number	Status
La Jolla pharmaceuticals	GSC-100	Gal3	Plant-based	Chronic kidney disease	1	Safety study of GCS-100 to treat chronic kidney disease	NCT01717248	Completed ($n = 29$)
				Chronic kidney disease	2	A phase 2a study of weekly doses of GCS-100 in patients with chronic kidney disease	NCT01843790	Completed ($n = 120$)
				Chronic lymphocityc leukemia	2	Study of the safety of GCS-100 in subjects with chronic lymphocytic leukemia (PR-CS008)	NCT00514696	Completed ($n = 24$)
				Multiple myeloma	1	Safety and efficacy study of GCS-100LE in the treatment of multiple myeloma	NCT00609817	Terminated (due to lack of funding)
				Diffuse large B-cell lymphoma	2	GCS-100LE in combination with etoposide and dexamethasone in relapsed or refractory diffuse large B-cell lymphoma (GCS-100LE)	NCT00776802	Withdrawn (due to lack of funding)
Galectin therapeutics	DAVANAT (GM-CT-01)	Gal1 and Gal3	Plant-based	Advanced solid tumors	1	Safety of GM-CT-01 with and without 5-fluorouracil in patients with solid tumors	NCT00054977	Completed ($n = 40$)
				Melanoma	2	Peptide vaccinations plus GM-CT-01 in melanoma	NCT01723813	Terminated (due to end of validity of peptide vaccine)
				Colorectal cancer	2	A new agent GM-CT-01 in combination with 5-FU, avastin and leucovorin in subjects with colorectal cancer	NCT00388700	Withdrawn (due to financing and re-organization)
				Gallbladder cancer	2	Study to test the benefit and safety of GM-CT-01 in combination with 5-FU to treat bile duct and gall bladder cancer	NCT00386516	Withdrawn (due to financing and re-organization)
				Colorectal cancer	2	GM-CT-01 plus 5-fluorouracil as third- or fourth-line therapy for metastatic colorectal cancer	NCT00110721	Terminated (study protocol amended to a new treatment regimen: study DAVFU-006)
Galectin therapeutics	GR-MD-02	Gal3	Plant-based	Metastatic melanoma	1	Galectin inhibitor (GR-MD-02) and ipilimumab in patients with metastatic melanoma	NCT02117362	Completed ($n = 8$)
				NASH advanced fibrosis	1	Phase 1 study to evaluate safety of GR-MD-02 in subjects with non-alcoholic steatohepatitis (NASH) and advanced fibrosis	NCT01899859	Completed ($n = 31$)

Continued

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Table 1. Clinical trials that target galectins and their respective ligands (Adaptation from 156) Part 2 of 2

Sponsor	Compound	Proposed target	Origin	Indication	Phase	Title of the study	NTC Number	Status
				Melanoma, lung and head and neck cancer	1	GR-MD-02 plus pembrolizumab in melanoma, non-small cell lung cancer, and squamous cell head and neck cancer patients	NCT02575404	Recruiting ($n = 22$)
				NASH cirrhosis	2		NCT02462967	Completed ($n = 162$)
				NASH advanced fibrosis	2	Clinical trial to evaluate efficacy of GR-MD-02 for treatment of liver fibrosis in patients with NASH with advanced fibrosis (NASH-FX)	NCT02421094	Completed ($n = 30$)
				Psoriasis	2	An open-label, phase 2a study to evaluate safety and efficacy of GR-MD-02 for treatment of psoriasis	NCT02407041	Completed ($n = 10$)
Massachusetts general hospital	Modified citrus pectin	Gal3	Plant-based	Osteoarthritis	3	Blocking extracellular galectin-3 in patients with osteoarthritis	NCT02800629	Unknown
				High blood pressure	3	Galectin-3 blockade in patients with high blood pressure	NCT01960946	Active, not recruiting ($n = 59$)
EcoNugenics	PectaSol-C	Gal3	Plant-based	Prostatic neoplasms	3	Effect of modified citrus pectin on PSA kinetics in biochemical relapsed PC with serial increases in PSA	NCT01681823	Completed ($n = 60$)
GalectoBio	TD139	Gal3	Synthetic chemistry	Idiopathic pulmonary fibrosis	2	RCT (randomized control trial) of TD139 vs placebo in HV's (human volunteers) and IPF patients	NCT02257177	Completed ($n = 60$)
OncoethixGmBH	OTX008	Gal1	Synthetic chemistry	Advanced solid tumors	1	A phase I, first-in-man study of OTX008 given subcutaneously as a single agent to patients with advanced solid tumors	NCT01724320	Unknown

settings include low *in vivo* bioavailability, susceptibility to glycosidase hydrolysis, and fast clearance. Although many alternatives have emerged over the years, the poor selectivity still remains a hurdle for this approach [14,134].

Thiodigalactoside (TDG), a non-selective inhibitor of Gal1, is an alternative disaccharide to overcome the poor bioavailability of lactose derivatives. Ito and colleagues observed that following TDG administration, tumor progression and metastasis in murine models of breast and colon adenocarcinoma decreased. Interestingly, TDG prevents binding of Gal1 to CD44 and CD326 receptors on the surface of CSCs [114,135,136]. Symmetrical modifications of TDG at O3 and O3′ positions increased galectin-binding affinities providing the potent small-molecule inhibitors of Gal3 [137]. This includes the C2-symmetric TD139 (from Galecto Biotech) or its asymmetrical derivative TAZTDG [138]. Phase Ib/IIa clinical trials of TD139 as a dry powder for inhalation revealed early promising results in patients with idiopathic pulmonary fibrosis (Table 1). Further studies should be conducted to generate more selective and potent small-molecules inhibitors targeting individual galectins.

Carbohydrate-derived polysaccharide inhibitors

Natural polysaccharides have emerged as high-affinity galectin inhibitors. Modified citrus pectin (MCP) and Davanat (GM-CT-01) are the best-studied galectin inhibitors derived from natural sources. Pectins are a family of complex polysaccharides, which are found in high amounts in the plant primary cell wall [134]. MCP, a soluble, orally ingested dietary carbohydrate fiber, has been shown to inhibit Gal3 function and suppress tumor growth *in vivo* [134,139,140]. Different commercial forms of MCP have been incorporated into clinical trials. Among these compounds, Pectasol and its most recent version Pectasol-C (EcoNugenics), GCS-100, and GBC-590 (Safescience) can be mentioned. GM-CT-01 (DAVANAT) is a β-D-(1 → 4)-galactomannan-based compounds isolated from the seeds of *Cyamopsis tetragonoloba* (Guar gum). At present, there is an ongoing phase II clinical trial using a GM-CT-01 vaccine in patients undergoing diffuse melanoma. GR-MD-02 is a pectin-derived galactoarabino-rhamnogalacturonan polysaccharide and a modified version of DAVANAT® [141]. Both DAVANAT and GR-MD-02 display comparable inhibition of Gal1 and Gal3. There is early encouraging data from a phase 1b clinical trial of GR-MD-02 (Galectin Therapeutics) in combination with the anti-PD-1 mAb Pembrolizumab, (Merck) demonstrating its effectiveness against advanced melanoma with 5 of 8 responders (2 complete responders and 3 partial responders). In addition, a clinical trial of GR-MD-O2 in combination with the anti-CTLA-4 mAb Ipilimumab for metastatic melanoma treatment is currently active. However, in spite of considerable progress, more work remains to be done to link the therapeutic activity of these compounds with mechanistic determinants of galectin binding and function.

Peptide-derived inhibitors

This strategy is based on the use of peptides or peptidomimetics to target galectins at their CRD or/at distant sites. G3-C12 is an oligopeptide (ANTPCGPYTHDCPVKR) that binds Gal3 CRD (K_d = 72 nM) [142]. This peptide prevented the metastasis of breast cancer cells to the lung in mouse models. Despite the need of structural studies describing Gal-3/G3-C12 peptide interaction, preliminary results showed encouraging data to be used as a proof-of-evidence to target Gal3-overexpressing cancers such as melanoma [143]. Anginex is an anti-angiogenic synthetic peptide that binds to the β-sheet configuration of Gal1 [144]. Anginex binds to Gal1 and inhibits neoplastic proliferation, migration, and angiogenesis, thereby inhibiting tumor growth [76]. Anginex prevents Gal1 uptake by ECs preventing translocation of H-Ras-GTP and signaling via the Raf/MEK/ERK kinase cascade. Anginex may also recognize other galectins such as Gal2, 7, 8 (N-terminal), and 9 (N-terminal), although with lower affinities [145]. The results of several clinical studies using anginex in combination with radiotherapy and/or chemotherapy showed that this peptide sensitizes tumor-associated ECs to radiotherapy, thus enhancing clinical success [146,147]. OTX008 is a caliraxene-based compound and a non-peptidic mimetic of anginex. It down-regulates cancer cell proliferation, invasion, and tumor angiogenesis in a variety of tumor cells [148] and has undergone a phase I clinical trial administered subcutaneously to patients with advanced solid tumors (Table 1) [149]. Further studies are awaited to design more selective peptide inhibitors targeting individual galectins and to elucidate the effect of glycosylated ligands on its effects.

Neutralizing antibodies

To develop galectin inhibitors, an important obstacle to overcome is the high similarity in the CRD structures among different members of the galectin family. The differential activities of individual galectins in normal and pathological processes [150,151] explain the urgent need of developing potent and selective inhibitors such as blocking antibodies. A neutralizing anti-Gal1 (F8.G7) mAb inhibited tumor growth and angiogenesis in mice with Kaposi's sarcoma by targeting Gal1 binding to VEGFR2 and its subsequent phosphorylation [73,79]. Recently, a novel anti-human Gal1 mAb (Gal1-MAb3) with both angioregulatory and immunostimulatory capacities has been developed [152]. Interestingly, several trials are currently recruiting patients to evaluate the efficacy of blocking antibodies against TIM-3, a glycosylated receptor recognized by Gal9, for the treatment of advanced solid tumors and hematologic malignancies (NCT02817633, Eli Lilly and Co. and NCT03066648 Novartis).

Small interfering RNA (siRNA) and aptamers

Small interfering RNA (siRNA) has also been proposed as an alternative approach for galectin inhibition. Park et al. demonstrated that siRNA-mediated silencing of Gal3 in a human osteosarcoma cell line resulted in decreased tumor cell migration and invasiveness [153]. Interestingly, these nucleic acid-based antagonists have

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become an alternative approach to overcome drug delivery through the blood–brain barrier. Van Woensel et al. used Gal1 siRNA (siGal1) loaded on a chitosan nanoparticle delivered intranasally to mice in an experimental model of glioblastoma multiforme. The siGal1 administration reduced Gal1 expression in the TME, inhibited tumor cell proliferation and migration, and increased $CD5^+$ B cell- and $CD8^+$ T-cell activation [154]. More recently, *in vivo* delivery of a Gal1-specific aptamer showed promising results suppressing tumor growth *in vivo* and restoring T cell-mediated immunity by blocking interactions between Gal1 and CD45 [155]. A better understanding and definition of the therapeutic window of application of these treatments during early dissemination and dormancy will be crucial for better therapy design. Moreover, combination with current therapies, such as immunotherapies, may enhance therapeutic outcomes and increase overall survival and progression-free survival in a larger number of patients.

Conclusions and future perspectives

Galectins have emerged as master regulators of the metastatic cascade providing tumor cells with a wide range of abilities considered to be 'Hallmarks of Metastasis'. By co-opting or subverting physiologic cellular circuits, galectins hierarchically shape the dissemination of malignant cells, rising as novel biomarkers and therapeutic targets. Particularly, a molecular signature, dominated by Gal1, Gal3 and Gal9, is associated with poor prognosis in many cancer types and directly or indirectly linked to modulation of early or late metastatic events. Targeting of galectins or their specific glycosylated ligands either alone or in combination with current therapies (chemotherapy, immunotherapies, anti-angiogenic therapy, targeted therapies, radiotherapy) [156,157] may represent the sleeping-beauty of the next generation of anticancer treatments. Notwithstanding several research groups are developing galectins inhibitors, it is still unclear whether the intra- or extracellular activities should be targeted in order to restrain tumor progression. Future studies should be focused on elucidating the role of galectins in early dissemination and dormancy programs to more precisely establish the therapeutic window required for better clinical responses. By adjusting spatio-temporal variables, dissecting their precise roles in the metastatic cascade, and designing more selective and rational inhibitors, anti-galectin therapy is becoming a novel and promising therapeutic approach for treating metastatic disease in several human cancers.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

BM, bone marrow; CAFs, cancer-associated fibroblasts; CTCs, circulating tumor cells; CTLA-4, cytotoxic T lymphocyte antigen-4; DCIS, ductal carcinoma *in situ*; DCs, dendritic cells; DTC, disseminated tumor cells; ECM, extracellular matrix; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FGF, fibroblast growth factor; IDC, invasive ductal carcinoma; JNK, c-Jun N-terminal kinase; MDSCs, myeloid-derived suppressor cells; OSCC, oral squamous cell carcinoma; PDAC, pancreatic ductal adenocarcinoma; PoEMs, podoplanin-expressing macrophages; SDF-1, stromal cell-derived factor-1; TAMs, tumor-associated macrophages; TDG, thiodigalactoside; TDLN, tumor-draining lymph nodes; TF, Thomsen–Friedenreic; TILs, tumor-infiltrating T lymphocytes; TN, triple-negative; VEGF, vascular endothelial growth factor.

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