Gabriel A. Rabinovich
Juan M. Ilarregui

Conveying glycan information into T-cell homeostatic programs: a challenging role for galectin-1 in inflammatory and tumor microenvironments

Authors' addresses
Gabriel A. Rabinovich[1,2], *Juan M. Ilarregui*[1]
[1]Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas, Ciudad de Buenos Aires, Argentina.
[2]Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad de Buenos Aires, Argentina.

Correspondence to:
Gabriel A. Rabinovich
Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental
Consejo Nacional de Investigaciones Científicas y Técnicas
Vuelta de Obligado 2490
C1428 Ciudad de Buenos Aires, Argentina
Tel.: +54 11 4783 2869 ext. 236
Fax: +54 11 4786 2564
e-mail: gabyrabi@ciudad.com.ar

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Summary: The immune system has evolved sophisticated mechanisms composed of several checkpoints and fail-safe processes that enable it to orchestrate innate and adaptive immunity, while at the same time limiting aberrant or unfaithful T-cell function. These multiple regulatory pathways take place during the entire life-span of T cells including T-cell development, homing, activation, and differentiation. Galectin-1, an endogenous glycan-binding protein widely expressed at sites of inflammation and tumor growth, controls a diversity of immune cell processes, acting either extracellularly through specific binding to cell surface glycan structures or intracellularly through modulation of pathways that remain largely unexplored. In this review, we highlight the discoveries that have led to our current understanding of the role of galectin-1 in distinct immune cell process, particularly those associated with T-cell homeostasis. Also, we emphasize findings emerging from the study of experimental models of autoimmunity, chronic inflammation, fetomaternal tolerance, and tumor growth, which have provided fundamental insights into the critical role of galectin-1 and its specific saccharide ligands in immunoregulation. Challenges for the future will embrace the rational manipulation of galectin-1-glycan interactions both towards attenuating immune responses in autoimmune diseases, graft rejection, and recurrent fetal loss, while at the same overcoming immune tolerance in chronic infections and cancer.

Keywords: *galectin-1, glycosylation, autoimmunity, tumor-immune escape, tolerance, inflammation*

Galectin–glycan interactions as novel regulatory checkpoints in immune-cell homeostasis

Complex strategies have evolved in mammals that serve to orchestrate immune responses to respond to pathogenic infections and to prevent tumor growth. A failure to mount a protective response can result in increased susceptibility to microbial invasion or neoplastic transformation, while the inability to shutdown exuberant inflammatory responses can lead to devastating pathological conditions (1–3). Several interwoven mechanisms have been proposed to control the magnitude of immune responses, to limit the extent of tissue damage, and to restore peripheral tolerance. These include a

number of receptors, cytokines, and inhibitory pathways, which may act in concert during the lifespan of immune cells to achieve homeostasis (1–3). Remarkably, dysfunction of these regulatory pathways may result in a diversity of inflammatory and autoimmune conditions. Conversely, aberrant activation of these counter-regulatory mechanisms may represent a significant hurdle for the generation of specific immunity to malignant tumors and chronic infectious pathogens (3).

Galectins, a conserved family of glycan-binding proteins, have emerged as pleiotropic regulators of innate and adaptive immune responses (4, 5). Members of this family share a consensus amino acid sequence and a carbohydrate-recognition domain (CRD) that is responsible for their β-galactoside-binding activity (4, 6). To date, 15 galectins have been identified in mammals, which have been classified on a structural basis into three different subfamilies: (i) the 'proto-type' galectins (galectin-1, -2, -5, -7, -10, -11, -13, -14, and -15) that have one CRD and can dimerize; (ii) 'tandem repeat-type' galectins (galectin-4, -6, -8, -9, and -12) that contain two different CRDs separated by a linker of up to 70 amino acids, and (iii) the 'chimera-type' galectin-3 that contains a CRD connected to a non-lectin N-terminal region (4, 7). While some members of the galectin family such as galectins-1 and -3 are distributed in a wide variety of tissues, others such as galectins-10 and -12 have a more restricted localization (4). Some members of the galectin family, particularly those secreted in a soluble form, can signal immune cells through multivalent recognition of cell surface carbohydrate structures, either by forming ordered arrays termed 'lattices' or through direct ligand–receptor interactions (7–10). A typical galectin CRD commonly recognizes poly-N-acetyllactosamine [-3Galβ1-4GlcNAcβ1-]n (polyLacNAc) sequences on cell surface glycoconjugates, although considerable variations have recently been described among the glycan specificities of different members of the galectin family (9, 11, 12). These critical differences, which are chiefly associated with the multiplicity of LacNAc residues, the extent of N-glycan branching, and/or the modification of terminal saccharides (i.e. sialylation or fucosylation) (9, 11, 12), might offer a possible explanation for the different and some times contrasting functions of these glycan-binding proteins during an inflammatory response.

Galectins do not have the signal sequence required for the classical secretion pathway involving transport through the endoplasmic reticulum and Golgi apparatus; yet these proteins are released in high quantities into the extracellular milieu (6, 13). Although the mechanisms underlying this secretory pathway are still uncertain, mutant cell lines which are deficient in the biosynthesis of galectin-1 saccharide ligands have impaired secretion of galectin-1 (14). Interestingly, it has been speculated that galectin-1 counter receptors may act either at the intracellular level by recruiting cytoplasmic galectins to the non-classical export pathway or at the extracellular level by exerting a pulling force to promote directional transport of galectins across the plasma membrane (15). Despite this complex scenario, it is still uncertain why galectins are preferentially found in the extracellular compartment, in spite of the fact that their structure suggests that they were designed to be intracellular proteins. From an evolutionary standpoint, it was speculated that galectins were designed to play intracellular roles but then acquired extracellular activity with the appearance of multicellular organisms (16, 17).

While other endogenous lectins, including C-type lectins and Siglecs control immune cell homeostasis through cell–cell contact-dependent interactions (18–21), some members of the galectin family, including galectin-1, function as soluble mediators which act in an autocrine or paracrine fashion to convey glycan-containing information into distinct transmembrane signaling events (22, 23). By triggering multivalent interactions with cell surface glycoconjugates, galectins can regulate immune cell trafficking, activation, cytokine secretion, and apoptosis. However, galectins can also regulate intracellular processes including pre-messenger RNA (mRNA) splicing, cell-cycle progression, and survival through still poorly understood mechanisms involving either protein–protein or protein–saccharide interactions (4). The capacity of galectins to modulate such a broad range of biological processes is supported by their considerable plasticity within intracellular and extracellular microenvironments, as well as their capacity to establish multivalent interactions with a repertoire of glycan structures displayed on an assortment of cell surface glycoconjugates (7, 24). In this regard, the galectin family appears to be much more conservative than other families of glycan-binding proteins (e.g. C-type lectins), as they exhibit a more restricted glycan specificity, with multiple tasks handled by a limited number of family members (16).

Galectin-1 as a new player on the scene of immunoregulation: from biochemistry to physiology and back again

Galectin-1, a prototypical member of the galectin family, was discovered more than 20 years ago as a β-galactoside-binding lectin of 14.5 kDa with hemagglutinating activity (25). Since then, several studies have identified and characterized galectin-1 in many different tissues of several species, demonstrat-

ing its widespread distribution in the animal kingdom. However, it was only in the last decade that this endogenous lectin appeared in the center of the scene as a fine-tuner of innate and adaptive immune responses (24).

Within the immune system, galectin-1 is synthesized and secreted by activated but not resting T and B cells (26–29), and it is significantly upregulated in activated macrophages, $CD4^+CD25^+$ regulatory T cells (Tregs) and decidual natural killer (NK) cells (30–34). This regulated secretion and preferential localization suggests a candidate function for galectin-1 in negative regulation of effector T-cell responses. Remarkably, expression of galectin-1 is abundant in immune privileged sites such as placenta (34–37), testis (38, 39), and retina (40, 41) and is significantly altered (up or downregulated) during several pathological conditions including cancer, infections, and autoimmunity (42–47).

Understanding the biochemical and biophysical features of galectin-1 is of critical importance for the further dissection of its immunoregulatory functions. Galectin-1 can be found as a monomer as well as a non-covalent homodimer composed of subunits of 14.5 kDa, each containing an identical CRD which is characterized by its specificity for poly-LacNAc structures displayed on both N- and O-glycans (48, 49). The presence of more than one CRD in a galectin-1 homodimer makes it well-suited for mediating cell–cell and cell–matrix interactions, triggering intracellular signaling, and forming lattices (7, 8). Yet, galectin-1 exhibits unique biochemical properties which make its functional analysis even more complex. This protein contains unpaired cysteine residues in the CRD that, in the absence of carbohydrate binding activity, can form intramolecular disulfide bonds and thereby diminish its known biological functions (48). This is probably one of the most challenging obstacles to overcome before considering galectin-1 as a potential therapeutic target. In this regard, a recent study has provided a connection linking these biochemical features, demonstrating that glycan recognition partially protects galectin-1 from oxidative inactivation and enhances galectin-1 dimerization (10). However, if optimal galectin-1 activities are imposed by a reducing microenvironment, a still unanswered question is why galectin-1 is preferentially localized to the extracellular milieu where the risk of oxidative inactivation is extremely high. In addition, another particular feature of galectin-1, which also characterizes other members of the galectin family, relates to the divergent functions of this protein, as galectin-1 may act either as a pro-survival signal or pro-apoptotic factor in different cell types and likewise may display either pro- or anti-inflammatory effects in different microenvironments. Hence, challenges for the future will include a thorough analysis of the interplay between biochemical and biophysical features of galectin-1 and its immunological effects in different tissues, as well as the relevance of different physicochemical parameters in regulating galectin-1 functions in physiological and pathological settings. These include but are not limited to (i) the influence of oxidative versus reducing microenvironments in regulating galectin-1 activity (10), (ii) the contextual regulation of galectin-1–glycan interactions (e.g. prevalence of cytokines, temperature, pH at sites of inflammation, etc) (50–53), (iii) a careful analysis of the specificity of galectin-1 toward complex glycans at physiological concentrations and re-examination of its avidity for common glycoconjugates (9, 54, 55), (iv) the levels of galectin-1 attained *in vivo* in physiological and pathological settings (56), and (v) the independent and often contrasting functions exerted by monomeric or dimeric forms of this protein (57–59). In addition, it is of critical importance to discriminate between the prevailing extracellular or intracellular activities of galectin-1 *in vivo*. Thus, a multidisciplinary approach connecting the expertise of biochemists, glycobiologists, and immunologists is essential to establish a definitive role of endogenous galectin-1 during the development and resolution of immune responses.

In the present review we dissect the functional significance of galectin-1–glycan interactions in key regulatory processes involved in T-cell physiology, including T-cell survival, signaling, cytokine secretion, and migration. In addition, we underscore the emerging role of galectin-1 as a critical mediator of the immunosuppressive activity of Tregs (*Fig.* 1). Lastly, we highlight the landmarks which set the basis for postulating galectin-1 as a novel therapeutic target, particularly the role of this glycan-binding protein in delineating an immunosuppressive microenvironment at the maternal-fetal interface, its ability to restore T-cell tolerance in inflammatory and autoimmune disorders, and the pitfalls of its role in hampering T-cell-dependent immunity in neoplastic settings.

Regulatory checkpoints targeted by galectin-1 during the lifespan of T cells

Regulatory checkpoint 1: control of T-cell survival

Apoptotic mechanisms are critical to regulate the development and shaping of the T-cell repertoire in the thymus (60). Moreover, they are at the heart of peripheral tolerance, serving to control self-reactive T cells, restore T-cell number following execution of effector functions, and prevent immune-mediated pathology. Several regulatory pathways may act in concert to regulate T-cell death programs including endogenous

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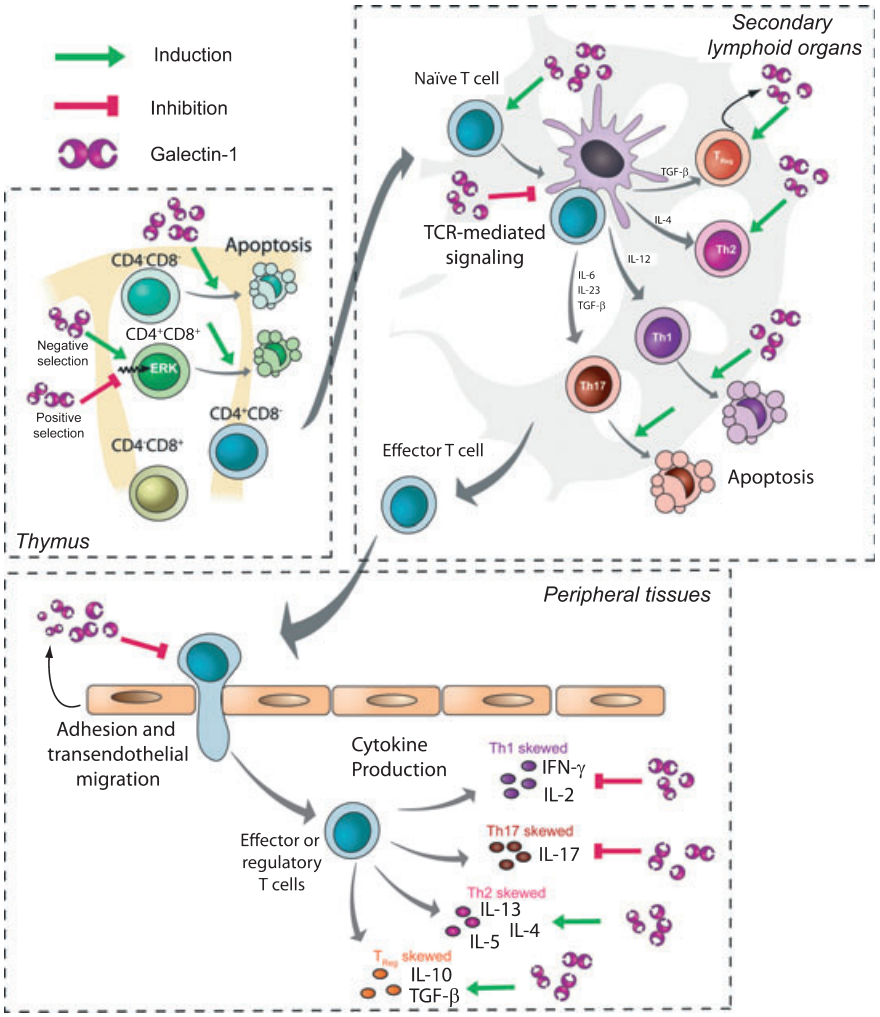

***Fig. 1.* Regulatory effects of galectin-1 at different checkpoints during the lifespan of T cells.** Galectin-1–glycan interactions may influence T-cell physiology through modulation of a variety of regulatory checkpoints including T-cell survival, TCR-mediated signaling, T-cell trafficking, and cytokine secretion. These cellular processes take place along the entire lifespan of T cells including T-cell development in the thymic compartment, activation and differentiation into effector or regulatory T cell subsets in secondary lymphoid organs, and execution or regulation of effector T-cell functions in peripheral tissues. ERK, extracellular signal regulated kinase; TCR, T-cell receptor; Th, T-helper; Treg, regulatory T cells.

glucocorticoids and T-cell receptor (TCR) signals within the thymic microenvironment, as well as members of the tumor necrosis factor receptor (TNFR) family within the peripheral compartment (60). Genetic disruption of these pathways leading to the occurrence of autoimmune pathology has provided critical insights into the role of apoptosis in peripheral T-cell homeostasis (61).

Considerable evidence has been accumulating regarding the role of galectin-1 in the control of T-cell viability along the whole lifespan of T cells from developing thymocytes to activated and fully-differentiated effector T cells (28, 31, 34, 62–79) (*Fig. 1*). T-cell susceptibility to galectin-1-induced cell death may be regulated at least at three distinct levels. First, galectin-1 sensitivity may be influenced by the presence of specific glycoprotein receptors. Interestingly, while many cell surface glycoproteins contain substantial amounts of LacNAc glycans, galectin-1 binds to a restricted set of T-cell surface glycoproteins (i.e. CD45, CD43, CD2, CD3 and CD7) (64, 73, 80). Of these, CD7 appears to be essential for galectin-1-induced cell death (81) and Sezary cells, the malignant T cells in mycosis fungoides that lack CD7 expression are resistant to the pro-apoptotic effects of this protein (82, 83). Of note, galectin-1 binding to T cells induces redistribution of specific glycoproteins into membrane microdomains, thus allowing signaling and activation of specific downstream effectors molecules (64). On the other hand, galectin-1 binding is limited to those cells that are able to generate specific saccharide ligands by expressing a set of particular glycosyltransferases responsible of creating or modifying cell surface glycoconjugates. In this regard, cell death triggered by galectin-1–glycan

interactions involves the expression and activity of the core 2 β-1,6 N-acetylglucosaminyltransferase (GCNT1) (66), an enzyme responsible of creating the core 2 branch on O-glycans, thus allowing the exposure of poly-N-acetyl-lactosamine sequences, which are the preferred saccharide ligands of galectin-1. In this regard, lymphoma T cells lacking core-2-O-glycans are resistant to galectin-1-induced cell death (84). Moreover, T-cell susceptibility to galectin-1-induced death may be also determined by the expression of the α2,6-sialyltransferase (ST6Gal1), which is responsible for the addition of sialic acid in α2,6 position of terminal galactose. Increased ST6Gal1 activity results in masked galactose residues on T-cell surface glycoproteins which are no longer able to bind galectin-1, thus rendering T cells resistant to cell death (85). However, a given glycosylation profile is not always permissive or restrictive for galectin-1 as $CD45^+$ T cells lacking GCNT1, which are not able to generate core 2-O-glycans, are resistant to galectin-1-induced cell death (66), while galectin-1 binds to CD43 modified with either unbranched core 1 or branched core 2 O-glycans (86). In this regard, previous work claimed that GCNT1 activity may not account for galectin-1-mediated contraction of the CD8 T-cell compartment, as neither galectin-1 binding nor cell death was altered in $CD8^+$ T cells lacking GCNT1 (87). Thus, a given glycosylation profile may impact differently on galectin-1-mediated effects depending on the cell type and the target glycoprotein implicated. These apparent discrepancies and experimental differences remain to be reconciled in future work.

Interestingly, N- and O-glycosylation can dramatically change all the way through the lifespan of T cells (18, 19), thus allowing or restricting binding of galectin-1. This effect is clearly evident during T-cell development (63), activation (88) and T-helper cell differentiation (76). In addition, galectin-1-induced death may be controlled by upstream or downstream intracellular events, which may amplify or prevent the apoptotic signal triggered by this protein. In this regard, a functional cross-talk has been reported between different members of the galectin family, as expression of intracellular galectin-3 rendered T cells resistant to galectin-1-induced cell death (69, 89). Hence, the susceptibility to galectin-1 is tightly controlled by the selective expression of a preferred set of glycoreceptors, the spatiotemporal expression, and activity of different glycosyltransferases creating or masking specific galectin-1 ligands, and the activation or silencing of intracellular pathways.

The signal transduction events that lead to apoptosis induced by galectin-1 involve several intracellular mediators of apoptosis, including in some cases induction of specific transcription factors [i.e. activator protein 1 (AP-1)] and modulation of B-cell leukemia/lymphoma 2 (Bcl2) protein expression (28, 65, 72, 90), sphingomyelinase-mediated release of ceramide (70, 72), and the involvement of proximal signals such as lymphocyte protein tyrosine kinase (p56lck) and ζ-chain-associated protein kinase of 70 kDa (ZAP70) (91). In addition, galectin-1 triggers a cell death program which involves mitochondrial morphogenetic changes including mitochondrial coalescence, budding, and fission (70). Yet, a still unresolved issue is whether galectin-1-induced death involves activation of a caspase-dependent or independent pathways. While some studies found induction of apoptosis through Fas (TNFR superfamily member 6)-, cytochrome *c*-, and caspase-independent mechanisms (69), others demonstrated the ability of galectin-1 to trigger activation of caspases-9 and -3 (72) and sensitize T cells to a Fas/caspase-8-mediated apoptotic pathway (28, 70, 78).

In spite of considerable efforts toward elucidating the intricate mechanisms involved in galectin-1-mediated death, the physiological relevance of this biological effect is less understood. While galectin-3 selectively kills $CD4^-CD8^-$ thymocytes, galectin-1 deletes double negative and double positive thymocytes with equal efficiency (73), suggesting the potential involvement of this protein as a pro-apoptotic signal in thymocytes who fail to survive positive selection and those undergoing negative selection. In addition, the preferential expression of galectin-1 in activated but not resting T cells (26, 27), suggests a potential autocrine inhibitory mechanism by which galectin-1 may blunt T-cell responses after the completion of an immune response. These interesting functions of galectin-1 during T-cell development and activation still remain to be fully elucidated in *in vivo* settings.

In this regard, we have provided a proof-of-concept of the critical role of endogenous galectin-1 in the control of T-helper cells in antigen-specific and inflammatory settings. Using *in vitro* and *in vivo* experiments, we found a link between differential glycosylation of T-helper cells, susceptibility to galectin-1-induced cell death, and termination of the inflammatory response (76). While T-helper type 1 (Th1)- and Th17-differentiated cells expressed the repertoire of cell surface glycans that are critical for galectin-1 binding and cell death, Th2 cells were protected from galectin-1 through differential α2,6 sialylation of cell surface glycoproteins (76). Remarkably, *in vivo*-differentiated antigen-specific T-helper cells (i.e. Th1 cells generated *in vivo* by dendritic cells pulsed by the bacteria *Propionibacterium acnes* and Th2 cells driven by dendritic cells pulsed with *Schistosoma mansoni* egg antigen) displayed comparable glycophenotypes and susceptibility to

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galectin-1 as *in vitro* human polarized T-helper cells (76). Accordingly, galectin-1-deficient mice showed greater Th1 and Th-17 responses and enhanced susceptibility to autoimmune brain inflammation than their wildtype counterpart (76), demonstrating the critical role of endogenous galectin-1 in controlling T-cell homeostasis. Collectively, these data indicate that differential glycosylation of cell surface glycoproteins can selectively control the survival of T-helper cells by modulating their susceptibility to galectin-1. In line with this evidence, Motran *et al.* (89) showed that Th2 cells can promote Th1 cell apoptosis through secretion of galectin-1, suggesting a lectin-dependent mechanism of cross-regulation between distinct T-helper subsets. Furthermore, recent studies, using *in vivo* injections of recombinant galectin-1 in NOD diabetic mice, confirmed the ability of galectin-1 to eliminate selectively Th1 and Th17 effector cells, while sparing Th2 cells (92). Likewise, $CD8^+$ T cells also appeared to be sensitive to galectin-1-induced immunoregulation as shown in autoimmune and cancer settings (92, 93).

In contrast to the pro-apoptotic effects of galectin-1 on activated T cells, Endharti *et al.* (71) demonstrated that secretion of this protein by lymph-node stromal cells supports the survival of naive T cells without promoting their proliferation. Hence, in addition to the unique biochemical features of galectin-1 and its regulated expression, the immunoregulatory effects of galectin-1 may be also controlled by extrinsic factors including the nature of target T cells (i.e. whether they are naive, activated, or effector T cells). In addition, Stowell *et al.* (94, 95) claimed that in the absence of a reducing agent such as dithiotreitol (DTT), galectin-1 does not alter the viability of T cells. To eliminate the potential confounding effects of DTT on cell viability, the authors stabilized the protein using the alkylating agent iodoacetamide, and verified that, under these conditions, galectin-1 did not affect T-cell apoptosis, but still retained biological activity toward T cells to favor the synthesis of interleukin-10 (IL-10) (95). These results suggested caution in the assignment of intrinsic pro-apoptotic activities to galectin-1 independent of extrinsic factors, including the surrounding microenvironment. In this regard, the demonstration of galectin-1 effects *in vivo* at sites of inflammation, where the risk of oxidative inactivation is high, is of critical importance to understand the mechanisms used by galectin-1 to overcome oxidative inactivation, while keeping its biological activities. Specifically, it is worth mentioning that the thiol-redox state of lymphoid organs or peripheral tissues is dramatically altered during ongoing T-cell responses (96) suggesting that endogenous galectin-1 might trigger T-cell death depending on the fluctuations of the redox state in inflammatory or tolerogenic microenvironments. Thus, while putting forward galectin-1 as a potential candidate for therapeutic manipulation (see below), efforts are being made to overcome these drawbacks, including the design of leucine-zipper based or covalently linked stable galectin-1 homodimers (67, 97) as well as the generation of cysteine-free mutants of galectin-1, obtained by substituting all cysteines with serine residues (98). Of interest these variants can signal T cells, suppress T-cell proliferation, induce T-cell apoptosis, or modulate cytokine production with equal or even more potency than wildtype recombinant galectin-1. However, despite considerable advances, more work is still necessary to address the role of endogenous galectin-1 in the regulation of apoptosis *in vivo* and to determine the effects of different microenvironments in controlling the immunoregulatory activities of this glycan-binding protein.

Regulatory checkpoint 2: control of TCR-mediated signaling and activation

Negative regulation of T-cell signaling delivered by the antigen receptors and co-receptors plays an important role in T-cell development and activation. Cell surface inhibitory receptors including cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1), and other molecules associated to immunoreceptor tyrosine-based inhibition motifs (ITIMs) play a crucial role in delivering negative signals that regulate the balance between T-cell activation, tolerance, and immunopathology (2). Although limited information is available on the role of galectin-1 in TCR-mediated T-cell activation, this protein has been reported to modulate T-cell signaling at sites of immunological synapse (Fig. 1). Liu *et al.* (99) found that galectin-1 favors TCR-mediated negative selection in the thymus by promoting a rapid and transient extracellular signal-regulated kinase (ERK) activation, while antagonizing ERK activity in thymocytes undergoing positive selection. In this way, galectin-1, which is synthesized by cortical and medullary thymic epithelial cells (100, 101), can differentially regulate the fate of TCR signaling during negative or positive selection and contribute to shape the nature of the selected T-cell repertoire. Furthermore, galectin-1 can antagonize TCR signals that require costimulation such as proliferation and IL-2 production, while allowing TCR responses that only require partial TCR signals such as apoptosis (102), thereby providing an alternative explanation for the pro-apoptotic effects of this glycan-binding protein. Thus, galectin-1 may regulate T-cell fate at sites of the immunological synapse by modulating TCR/costimulator-dependent clustering and signaling. Similar to galectin-3-*N*-glycan lattices which have

been shown to restrict spontaneous TCR clustering and down-modulate TCR responses (103, 104), galectin-1 might also contribute to this phenoptype by interacting with N-glycans modified by the enzyme N-acetylglucosaminyltransferase 5 (Mgat5). In this regard, galectin-3 N-glycan lattices suppress Lck activity and TCR signaling (105). These effects might have potential implications in the regulation of T-cell physiology at the cross roads of T-cell activation, tolerance, and immunopathology.

Regulatory checkpoint 3: fine-tuning T-cell adhesion and trafficking

T-cell homeostasis largely depends on a selective combinatorial process involving sequential engagement of specific receptors which can positively or negatively regulate T-cell adhesion and trafficking. This multistep process is responsible for targeting effector and regulatory T cells to sites of inflammation, microbial invasion, or tumor growth (106). Cell surface carbohydrates and their specific glycan-binding proteins (e.g. selectins) play a determinant role in the control of lymphocyte homing and the recruitment of leukocytes to secondary lymphoid organs and inflamed tissues (107). In keeping with its anti-inflammatory activities, a critical inhibitory role has been described for galectin-1 in T-cell adhesion and transendothelial migration (108–110) (*Fig. 1*). Exposure of T cells to galectin-1 blocked adhesion of activated T cells to extracellular matrix glycoproteins such as fibronectin and laminin (108). In addition, endothelial cell expression of galectin-1 inhibited T-cell transendothelial migration through mechanisms involving clustering of CD43 (109). Furthermore, using small interfering RNA (siRNA)-mediated silencing strategies, Norling *et al.* (110) found that galectin-1 limits T-cell capture, rolling, and adhesion to activated endothelial cells under flow. These anti-inflammatory effects were confirmed *in vivo* in galectin-1-deficient mice, where trafficking to mesenteric lymphoid organs and inflamed tissues was significantly augmented compared with their wildtype counterpart. These results entail a different regulatory checkpoint by which galectin-1 may contribute to T-cell homeostasis during inflammatory reactions.

Regulatory checkpoint 4: modulation of the cytokine balance

An imbalance of pro-inflammatory and anti-inflammatory cytokines results in the loss of immune tolerance and the subsequent appearance of inflammatory autoimmune conditions (111). Although the underlying mechanisms are still poorly understood, different members of the galectin family can regulate the balance of pro- or anti-inflammatory cytokines. In this regard, one of the most consistent findings in the literature is the ability of galectin-1 to skew the balance from a Th1- and Th17- toward a Th2-polarized immune profile in several experimental models of chronic inflammation, autoimmunity, and cancer (see next section). Although this effect may be clearly explained by our findings on the selective pro-apoptotic effect of galectin-1 on Th1 and Th17 effector cells and the sialylation-dependent resistance of Th2 cell subsets (76), galectin-1 may also suppress Th1-type cytokines and promote the synthesis of Th2-derived cytokines through non-apoptotic mechanisms (28, 89, 95, 97, 108) (*Fig. 1*). Early studies from our group demonstrated that treatment of T cells with low concentrations (approximately 0.01–0.10 μM) of recombinant galectin-1 blunts pro-inflammatory and Th1 cytokine production without affecting T-cell viability (28, 108). More recently, several reports demonstrated a consistent induction of IL-10 in non-activated and activated $CD4^+$ and $CD8^+$ T cells upon exposure to recombinant galectin-1 *in vitro* (95, 97) and following its administration *in vivo* (37, 92, 112). As mentioned above, galectin-1 secreted by Th1 cells also contributed to immunoregulation by sustaining TCR-induced Th2 cytokine production (89). These results argue for several immunoregulatory mechanisms, including T-helper cytokine regulation, accounting for the broad anti-inflammatory effects of this glycan-binding protein *in vivo* (see below).

Regulatory checkpoint 5: control of Treg cell function

The investigation of suppressor or Tregs has witnessed a renaissance in the past few years (113). Endowed with the ability to suppress T-cell responses, Tregs including naturally-occurring and inducible CD^+CD25^+forkhead box p3 $(Foxp3)^+$ Tregs, as well as IL-10-producing Tr1 cells, hold the promise of preventing allograft rejection, restoring tolerance at the maternal-fetal interface and limiting pathogenic inflammatory responses (113). In addition, they may represent a significant hurdle for successful tumor immunity and resolution of microbial infections (114). The dramatic immunosuppressive effects of galectin-1 *in vivo* (5) prompted us to investigate the ability of this glycan-binding protein to modulate the Treg compartment. Remarkably, administration of galectin-1 in experimental models of autoimmune ocular inflammation (112) and stress-induced pregnancy failure (37) restored T-cell tolerance and resulted in considerable expansion of IL-10-producing $CD4^+CD25^{high}$ Tregs (*Fig. 1*). Interestingly,

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these cells showed no significant variations in the levels of Foxp3 expression but had considerable immunosuppressive activity *in vivo*. However, using *in vitro* differentiation systems, exposure of T cells to galectin-1 resulted in significant expansion of a population of $CD4^+CD25^{high}$ Tregs with high Foxp3 expression (77). Whether galectin-1 stimulates the differentiation and/or expansion of both $Foxp3^+$ and $Foxp3^-$ Tregs still remain to be ascertained.

Interestingly, analysis of gene expression profiles of regulatory versus effector T cells revealed a substantial increase in *Lgals1* mRNA (the transcript encoding galectin-1 protein) in naturally occurring Treg cells (32, 33). Notably, *Lgals1* overexpression was found to be Foxp3-independent similar to other upregulated genes such as *granzyme B* and *Helios* (32). Remarkably, antibody-mediated blockade of galectin-1 significantly reduced the suppressive effects of human and mouse $CD4^+CD25^+$ Tregs, indicating that endogenous galectin-1 was required for maximal Treg function (33). Thus, galectin-1 may influence the Treg compartment by modulating the expansion and/or survival of these cells or by contributing to their immunosuppressive activity. Further studies are warranted that will dissect the molecular pathways leading to galectin-1 overexpression in Tregs, examining the molecular mechanisms leading to galectin-1-induced Treg expansion and evaluating the contribution of this endogenous lectin to Treg-induced immunosuppression in settings of pathophysiologic relevance. How galectin-1 decodes glycan-containing information to selectively amplify Th2-and Treg-cell homeostatic programs still remains to be explored.

Galectin-1 *in vivo*: lessons from experimental models at the cross roads of health and disease

The cross-talk among different cellular processes and checkpoints modulated by galectin-1, including T-cell survival, signaling, trafficking, cytokine production, and Treg function, may lead to critical although unexpected immunoregulatory phenotypes when examined in the context of physiological and pathological scenarios. In addition, galectin-1 may act in concert with other regulatory pathways (i.e. PD-1/PD-L1, CTLA-4, indoleamine 2,3-dioxygenase, Fas ligand) to achieve T-cell homeostasis. In this section we summarize our knowledge on the tolerogenic effects of galectin-1 *in vivo* in different pathophysiologic settings, including maternal-fetal tolerance, autoimmunity, and cancer. In particular, we highlight emerging evidence on the functional significance of galectin-1 or N-glycans deficiencies *in vivo*, which clearly illustrate the relevance of galectin-1–glycan interactions in immune cell homeostasis and their contribution to immunopathology (Fig. 2).

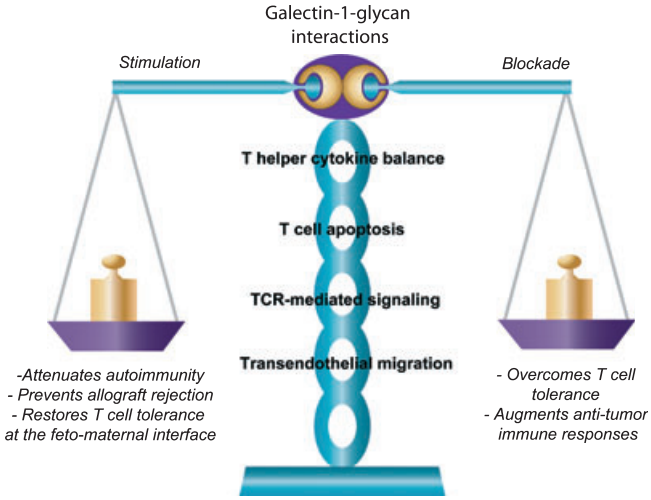

Fig. 2. The dual effects of galectin-1–glycan lattices in the control of immunopathology: two sides of the same coin? Given thes broad spectrum of T-cell inhibitory activities of galectin-1, this glycan-binding protein as well as its specific saccharide ligands have been postulated as candidate therapeutic targets for attenuating immune responses in autoimmune diseases, preventing graft rejection and restoring T-cell tolerance at the fetomaternal interface, thus preventing recurrent fetal loss. On the other hand, blockade of galectin-1–glycan interactions may contribute to overcome immune tolerance in tumor microenvironments, thus stimulating the development of tumor-specific immune responses.

Preserving homeostasis at the maternal-fetal interface

An essential feature of successful mammalian reproduction is maternal tolerance to the presence of semiallogeneic fetus (115). Multiple tolerance mechanisms operate at the fetomaternal interface to induce immune tolerance, including modulation of the Th1–Th2 cytokine balance, specific recruitment of $CD4^+CD25^+$ Tregs, expansion of decidual NK cells, induction of decidual T-cell apoptosis, and activation of negative regulatory pathways such as those triggered by PD-1/PD-L1 (115, 116). Given the ability of galectin-1 to control multiple regulatory checkpoints, we examined the role of this glycan-binding protein in immune cell tolerance at the fetomaternal interface. Galectin-1 is abundant in the human and mouse female reproductive tracts (117, 118) and is differentially expressed in normal and pathological placenta (119) and uterine decidual NK cells (34). Using an established mouse model of stress-induced pregnancy failure, we found that recombinant galectin-1 prevents fetal loss and restores tolerance *in vivo* (37). Consistent with those findings, galectin-1-deficient female mice showed higher rates of fetal loss compared with their wildtype counterpart in allogeneic but not syngeneic matings; yet, no changes were observed in

placentation or decidualization processes (37). Investigation of the mechanisms involved in these regulatory effects revealed the ability of galectin-1 to restore the Th1/Th2 cytokine balance, promote expansion of IL-10-producing Tregs and favor the recruitment of uterine dendritic cells with a regulatory phenotype (37). The differential expansion of dendritic cells in regional lymph nodes and uterine tissue suggested the ability of galectin-1 to selectively regulate the trafficking of these antigen-presenting cells, in line with previous observations indicating a role for this glycan-binding protein in promoting dendritic cell migration (120). Remarkably, a functional cross-regulation between progesterone and galectin-1 has been reported at the fetomaternal interface (37), indicating a novel immune-endocrine mechanism to regulate fetal tolerance during pregnancy. In line with these findings, Kopcow *et al.* (34) found that human decidual NK cells secrete considerable amounts of galectin-1 which induce apoptosis of decidual but not peripheral T cells. Similar to Th1 and Th17 subsets (76), decidual T cells expressed the repertoire of cell surface glycans compatible with high sensitivity to galectin-1 (34). Strikingly, the role of galectin-1 in pregnancy preservation has been further supported by phylogenetic analysis showing the acquisition of steroid responsive elements in the *Lgals1* promoter as well as selective gain of cysteine residues involved in redox regulation early during the emergence of placental mammals (121). Strikingly, the most intense selection process in *Lgals1* gene was found on residues localized within the CRD and the dimerization interface (121), suggesting adaptation of these biochemical features to immune regulatory effects. Collectively, these data underscore an evolutionarily-conserved function of progesterone-regulated galectin-1 in establishing maternal-fetal immune tolerance through modulation of a hierarchy of regulatory pathways. In addition, these results emphasize a potential approach for therapeutic intervention aimed at re-establishing immune cell homeostasis in failing pregnancies (*Fig.* 2).

Restoring tolerance in chronic inflammation and autoimmunity

Altered activity of key regulatory checkpoints, including galectin-1–glycan interactions, may contribute to the pathophysiology of autoimmune diseases by tipping the balance toward inflammation (*Fig.* 2). With the increased availability of null mutant mice and the emerging strategies for studying the cell surface 'glycome', the investigation of glycan-binding proteins and glycan structures in tolerance induction and autoimmunity is becoming more and more exciting (122). In this context, the immunoregulatory activities of galectin-1 and its specific glycan receptors have been widely acknowledged and extensively studied in several models of chronic inflammation and autoimmunity (5, 7).

Early in the 1980s, Levi *et al.* (123) described the protective effects of electrolectin, a galectin-1 homologue purified from electric eel, in experimental autoimmune myasthenia gravis in rabbits. In 1990, Offner *et al.* (124) showed that galectin-1 can protect Lewis rats from experimental autoimmune encephalomyelitis (EAE) induction. Although these studies set the basis for the study of galectin-1 in immunoregulation, the precise mechanisms of action of this protein were then uncertain.

In 1999, we demonstrated that a single cell injection of syngeneic fibroblasts engineered to secrete galectin-1 on the day of the disease onset abrogated clinical manifestations of collagen-induced arthritis (CIA), an experimental model of rheumatoid arthritis in DBA/1 mice (125). A comparable suppressive effect in CIA was also observed in response to daily intraperitoneal injections of recombinant galectin-1 (125). Investigation of the mechanisms involved in this therapeutic effect revealed a dramatic role of galectin-1 in skewing the cytokine balance toward Th2-polarized immune responses. In addition, lymph node cells from mice engaged in the galectin-1 gene therapy protocol showed enhanced susceptibility to TCR-induced apoptosis (125). Similarly, Santucci *et al.* found inhibition of tissue injury and attenuation of Th1-mediated inflammation in animal models of concanavalin A-induced hepatitis (126) and trinitrobenzenesulfonic acid (TNBS)-induced colitis (127) following intravenous administration of galectin-1. In both models, the authors found consistent reduction in the frequency of antigen-activated T cells and decreased production of pro-inflammatory and Th1-type cytokines [TNF-α, interferon-γ (IFN-γ)] following injection of galectin-1 (126, 127). Similar reduction of Th1-type responses was found in a mouse model of graft versus host disease (GVHD), suggesting the potential use of galectin-1 as an immunosuppressive agent in allogeneic bone marrow transplantation (128). Interestingly, in this model, galectin-1 suppressed GVHD without compromising engraftment or immune reconstitution following allogeneic hematopoietic stem cell graft (128).

Extending the anti-inflammatory effects of galectin-1 to immune privileged sites, the immunosuppressive activity of galectin-1 was further explored in experimental autoimmune uveitis (EAU), a T-cell mediated model of ocular inflammation induced by interphotoreceptor retinoid-binding protein (IRBP). Treatment with recombinant galectin-1 either early or

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late during the course of EAU was sufficient to limit ocular inflammation and counteract pathogenic Th1 responses (112). This glycan-binding protein ameliorated retinal inflammation by skewing the uveitogenic response toward a Th2- or Treg-mediated anti-inflammatory response. Remarkably, adoptive transfer of IL-10-producing $CD4^+$ T cells obtained from galectin-1-treated mice prevented the development of active EAU in syngeneic recipients (112). Of note, we observed apoptosis in this model only in those mice receiving galectin-1 during efferent phase of the disease (112), suggesting the activation of distinct but potentially overlapping mechanisms operating during galectin-1-induced immunosuppression. Moreover, in an adoptive transfer model of autoimmune diabetes, Perone *et al.* (129) found that transgenic dendritic cells engineered to overexpress galectin-1 delayed the onset of diabetes and insulitis in non-obese diabetic (NOD)-recombination activating gene 1 $(Rag1)^{-/-}$ mice (129). This effect was accompanied by increased percentage of apoptotic T cells and blunted Th1 responses in pancreatic lymph nodes (129). More recently, the authors found that soluble galectin-1 can prevent the onset of hyperglycemia and revert β-cell-specific autoimmunity in NOD mice (92), indicating the ability of this protein to suppress autoimmune inflammation not only in experimentally-induced, but also in spontaneous models of autoimmune pathology. Although there is still limited clinical data in human tissues, decreased expression of galectin-1 was found in patients with juvenile rheumatoid arthritis (47) and increased occurrence of anti-galectin-1 autoantibodies was found in sera from patients with distinct inflammatory disorders which correlated with poor clinical outcome (41, 130). The biological relevance of these autoantibodies – whether they have blocking or pathogenic activity – still remains to be explored.

While the aforementioned models broadly support the concept of anti-inflammatory effects of galectin-1 when delivered to sites of inflammation, the function of endogenous galectin-1 in the evolution of autoimmune inflammation was lacking until recently, when novel findings emerged with the careful analysis of null mutant mice (76). Remarkably, galectin-1-deficient mice showed greater antigen-specific Th1 and Th-17 responses and exhibited more severe autoimmune inflammation and demyelination than their wildtype counterpart. Hence, together with increased fetal loss in female pregnant mice lacking galectin-1 (37), our findings indicate an essential role of endogenous galectin-1 in limiting pathogenic T-cell responses. Whether these biological effects are associated with the intracellular or extracellular functions of the endogenous lectin still remains to be established. Strikingly, clinical features observed in $Lgals1^{-/-}$ mice, including T-cell hyperreactivity and accelerated demyelination, phenocopied those found spontaneously in mice lacking GlcNAc-branched N-glycan structures responsible of forming lattices with multivalent galectins (104). These observations further emphasize the critical role of galectin-1–glycan interactions in regulating T-cell homeostasis.

Thwarting T-cell responses in cancer

Similar to immune privileged tissues, cancer cells display multiple immunosuppressive mechanisms to elude T-cell responses, either to avoid immune recognition or to disable effector T cells (114, 131). These include alterations of components of the antigen processing machinery, defects in proximal TCR signals, activation of negative regulatory pathways (e.g. CTLA-4, PD-1/PD-L1, IDO), specific recruitment of regulatory cell populations and secretion of immunosuppressive factors, such as transforming growth factor-β (TGF-β) and IL-10 (114, 131). These mechanisms cooperate in advanced stages of cancer to limit the immune system's ability to restrain the tumor and the effectiveness of immunotherapy strategies to successfully eradicate malignant cells (114).

Recent efforts toward deciphering the 'poor prognosis' signature of different tumor types had recurrently led in microarray and proteomic analyses to the identification of galectin-1 as a 'typical' protein, whose expression is altered in several tumors and metastatic lessions (42, 43). However, in sharp contrast with the beneficial effects of galectin-1 in autoimmune pathology, this endogenous lectin contributes to tumor malignancy by modulating different steps of tumor progression including homotypic cell aggregation, tumor cell migration, and angiogenesis (43, 132). We found that human and mouse melanoma cells secrete high amounts of galectin-1, which substantially contributes to the immunosuppressive activity of these cells (93). Remarkably, silencing of galectin-1 expression within the tumor microenvironment rendered mice resistant to tumor challenge and stimulated the generation of a melanoma-specific Th1-type response in tumor-draining lymph nodes (93). Accordingly, Reed Sternberg cells, the pathognomic cells in classical Hogdkin lymphoma, selectively overexpressed galectin-1 which favored the secretion of Th2-type cytokines, induced Treg expansion and suppressed Epstein–Barr virus specific T-cell immunity (77, 133). Interestingly, a detailed molecular dissection of *Lgals1* gene revealed a critical role for a cell-type specific AP-1-dependent enhancer in driving the selective expression of galectin-1 in classical Hodgkin lymphoma and anaplastic large

cell lymphoma compared with other types of lymphoma cells (77, 134). In addition, prostate cancer cells which have low expression of core-2-O-glycans were resistant to galectin-1 but were capable of killing T cells in co-culture experiments through expression of this glycan-binding protein (135). Interestingly, the T-cell inhibitory activities of galectin-1 were confirmed in human tumor tissue, as Le *et al.* (136) found a strong inverse correlation between galectin-1 expression and the presence of viable T cells in tumor sections from head and neck squamous carcinoma patients. Remarkably, several stimuli including a hypoxic microenvironment (136), the immunosuppressive cytokine TGF-β1 (137), the immunomodulatory drug cyclophosphamide (138), and the differentiating agent retinoic acid (139) were capable of upregulating galectin-1 expression in different tumor types. Thus, galectin-1–glycan interactions may contribute to create an immunosuppressive microenvironment at sites of tumor growth by tilting the balance of T-helper responses, antagonizing T-cell signaling, promoting Treg expansion and negatively regulating T-cell viability (*Fig.* 2). An increased understanding of the role of galectin-1 in tumor biology will provide new insights into how the regulation of galectin-1 expression might be exploited for therapeutic purposes.

Resolving acute inflammation: an earlier homeostatic checkpoint

Resolution of acute inflammation involves the activation of endogenous biochemical programs and key regulatory components that enable inflamed tissues to return to homeostasis (140). In spite of its essential role in regulating T-cell homeostasis, galectin-1 may also contribute to terminate acute inflammation as a 'gatekeeper' at the cross-roads of innate and adaptive immune responses. We found, using a model of paw oedema in rats that injection of recombinant galectin-1 suppressed bee venom phospholipase A_2-induced inflammation in a manner independent of its carbohydrate-binding activity (141). In this model, galectin-1 treatment resulted in significant reduction of the number of infiltrated polymorphonuclear neutrophils (PMN) and reduced mast cell degranulation, suggesting a role for this protein in limiting acute inflammation (141). The mechanisms underlying these anti-inflammatory effects have been further examined. In fact, La *et al.* (142) found that PMN exposed to human recombinant galectin-1 experienced impaired chemotaxis and reduced transendothelial migration. Accordingly, galectin-1 administration resulted in reduced IL-1β-induced PMN recruitment into the mouse peritoneal cavity (142), suggesting an essential role of galectin-1 in controlling PMN trafficking similar to the effects observed in T cells. On the other hand, Stowell *et al.* (94) found that galectin-1 treatment induces exposure of cell surface phosphatidylserine in PMN, thus preparing these cells for phagocytic removal. Remarkably, this effect did not involve typical alterations of apoptosis such as DNA fragmentation, changes in mitochondrial membrane potential or caspase activation (94, 143). However, induction of phosphatidylserine exposure was cell-type specific, required calcium (Ca^{2+}) mobilization, involved galectin-1 interaction with complex-type N-glycans (144) and was overturned following galectin-1 removal (143). Thus, galectin-1 may also contribute to the resolution of acute inflammation by preparing PMN for phagocytic removal, thus favoring their physiologic turnover independently of apoptosis.

In addition, galectin-1 may also target the function of other innate immune cells including monocytes and macrophages. Galectin-1 treatment caused reduction in prostaglandin E2 secretion and nitric oxide production, but tilted the balance to favor activation of the L-arginase pathway in rat peritoneal macrophages (141, 145). In keeping with these findings, galectin-1 significantly inhibited IFN-γ-induced major histocompatibility complex-II (MHC-II) expression in human monocytes *in vitro* and in mouse macrophages recruited *in vivo* in response to inflammatory stimuli (59). Furthermore, we have recently identified a previously unappreciated role for galectin-1 in the control of platelet physiology (146). Galectin-1 synergized with adenosine diphosphate (ADP) or thrombin to induce platelet aggregation and adenosine triphosphate (ATP) release, promoted shedding of platelet microvesicles and favored the generation of leukocyte-platelet aggregates (146), events which have potential implications in the modulation of thrombosis, inflammation and metastasis. Thus, galectin-1 may also control immune cell homeostasis through regulation of early events during the inflammatory response.

Concluding remarks and future directions

In the present review we described the consequences of galectin-1 signaling as it relates to T-cell survival, TCR-mediated clustering and activation, T-cell mobility, cytokine production, and Treg function. Similar to what has been observed for many cytokines and growth factors, it is not surprising that galectin-1 exhibits a 'double-edged sword' effect with opposing biological outcomes depending on different intrinsic factors such as the physicochemical properties of the protein (monomer/dimer equilibrium), stability of the protein in oxidative versus reducing microenvironments, as well as extrinsic factors such as the target cell type and its activation and/or differentiation status.

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In light of the broad spectrum of immunoregulatory effects, challenges for the future will embrace a rational manipulation of galectin-1–glycan interactions towards attenuating immune responses in autoimmune diseases, graft rejection, and recurrent fetal loss (*Fig.* 2). As illustrated in the present review, *in vivo* studies, including those using galectin-1-deficient mice, have begun to provide relevant information on the selective function of this endogenous lectin in negative regulation of the inflammatory response. Moreover, with the diverse range of glycosyltransferase knock out mice that are available it will now be feasible to determine the impact of glycosylation in galectin-1-mediated effects. However, before galectin-1-based therapeutic agents can be extrapolated to clinical settings, a more thorough understanding of the mechanisms involved in galectin-1 functions is essential. In this regard, it will be critical to evaluate the results of side-by-side studies of the anti-inflammatory activities of different members of the galectin family, dissect the biological activity of different galectin-1 variants, evaluate the influence of pro-inflammatory and tolerogenic microenvironments, and establish the most adequate routes of administration as well as the underlying toxicity of this glycan-binding protein *in vivo*. Also, it would be of particular interest to examine the cross-talk between galectin-1 and other established inhibitory pathways including CTLA-4, PD-1/PD-L1, and IDO.

As a reverse side of the same coin, interrupting galectin-1–glycan interactions may contribute to overcome T-cell tolerance (*Fig.* 2). Hence, galectin-1 inhibitors may serve as adjuvants in preventive or therapeutic vaccines against chronic infection and cancer. In order to validate this concept, the design of specific galectin-1 antagonists as well as a comparative study of already established inhibitors (e.g. natural polysaccharides, synthetic glycoamines, glycodendrimers or blocking antibodies) is essential (147–154).

One general concern of the use of immune modulators is that some approaches may cause generalized immunosuppression and may leave patients immunocompromised. For instance, antibody-mediated $CD4^+$ T-cell depletion targets more efficiently circulating naive T cells than tissue-infiltrating pathogenic T cells and causes protracted lymphopenia in treated patients (155). Moreover, TNF-α blockade as a treatment for organ-specific autoimmune disorders can leave individuals prone to a variety of opportunistic infections (156). As galectin-1 selectively targets Th1 and Th17 cells but spares Th2 or naive T cells (76), it is less likely that it would compromise protective immunity when compared with other more generalized immunosuppressive therapies. Finally, the definitive proof-of-concept of the significance of galectin-1–glycan interactions in immunopathology will arise from the identification of individuals with relevant primary genetic defects or polymorphisms associated with inflammatory or neoplastic settings similarly to those found for galectin-2 in myocardial infarction (157) and for galectin-3 in breast cancer (158). Given the complexity of galectin-1–glycan interactions and the multiple parameters influencing these molecular contacts, further work is required, involving multidisciplinary approaches, to achieve a global comprehensive view of the role of endogenous galectin-1 and its specific carbohydrate ligands in immunoregulation.

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