CHAPTER ELEVEN

Multiple Functional Targets of the Immunoregulatory Activity of Galectin-1: Control of Immune Cell Trafficking, Dendritic Cell Physiology, and T-Cell Fate

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Contents

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Abstract

In the postgenomic era, the study of the glycome—the whole repertoire of saccharides in cells and tissues—has enabled the association of unique glycan structures with specific physiological and pathological processes. The responsibility for deciphering this biological information belongs to endogenous glycan-binding proteins or lectins. Galectin-1, a prototypic member of a family of structurally related proteins, has demonstrated selective antiinflammatory and immunoregulatory effects either by controlling immune cell trafficking, "fine-tuning" dendritic cell physiology and regulating T-cell fate. These regulatory functions mediated by an endogenous glycan-binding protein may contribute to fulfill the needs for immune cell homeostasis, including preservation of feto-maternal tolerance and prevention of collateral damage as a result of microbial invasion or autoimmune pathology. We will discuss here the conceptual framework which led to the study of galectin–glycan lattices as a novel paradigm of immune cell communication in physiological and pathological processes and will highlight selected methods and experimental strategies which have contributed to the study of the immunoregulatory activities of this multifaceted glycan-binding protein both in *in vitro* and *in vivo* biological settings.

1. General Introduction

The immune system has evolved as a highly effective and dynamic cellular network which signals the presence of invading pathogens and growing tumors and initiates a protective response that is specific for danger signals; yet maintaining tolerance to self. Active investigation performed over the last decade has disclosed multiple regulatory pathways composed of several checkpoints and fail–safe processes preventing self-reactivity and limiting aberrant or unfaithful immune responses. Important developments include the identification of a number of gene products responsible for central and peripheral T-cell deletion, T-cell anergy, and cytokine deviation, as well as the dissection of the molecular mechanisms underlying the differentiation and function of T regulatory (T_{reg}) cells and tolerogenic dendritic cells (DCs; Bluestone *et al.*, 2007; Tarner and Fathman, 2006). These regulatory processes involve a number of receptors, cytokines, and inhibitory signaling pathways, which may act in concert during the lifespan of immune cells to achieve homeostasis (Fife and Bluestone, 2008; Maynard and Weaver, 2008; Peggs *et al.*, 2008).

In spite of the well-established mechanisms governing cognate ligand–receptor interactions during cytokine signaling, chemotaxis, and cell adhesion paradigms, the immunological relevance of supramolecular lattices established by lectin–saccharide interactions and their role in immune cell tolerance and homeostasis is just emerging (Toscano *et al.*, 2007a; van Kooyk and Rabinovich, 2008). Galectins, a family of soluble lectins widely distributed in the animal kingdom, have emerged as pleiotropic regulators of innate and adaptive immune responses (Rabinovich *et al.*, 2007a,b; Yang *et al.*, 2008). To date, 15 galectins have been identified in mammals, most of them with wide tissue distribution; yet expression of some galectins is confined to a restricted set of tissues. Galectins share a common structural fold and contain a conserved carbohydrate-recognition domain (CRD) of about 130 amino acids that mediates carbohydrate binding. A traditional classification based on structural similarities includes: (a) "proto-type" galectins (galectin-1, -2, -5, -7, -10, -11, -13, -14, and -15) which have one CRD and may exist as monomers or dimers; (b) "tandem-repeat type" galectins (galectin-4, -6, -8, -9, and -12) which contain two different CRDs separated by a linker of up to 70 amino acids, and (c) the unique "chimera-type" galectin-3 which contains a CRD connected to a nonlectin N-terminal region. Most galectins are either bivalent or multivalent with regard to their carbohydrate-binding activities, which enable recognition of multiple binding partners and activation of distinct signaling pathways; one-CRD galectins can dimerize, two-CRD galectins are at least bivalent; and galectin-3 can form oligomers upon binding to multivalent glycoproteins (Cummings and Liu, 2008; Rabinovich *et al.*, 2007a,b). Although galectins do not contain a classical signal sequence, they are frequently found in the extracellular compartment and are released through an unusual route which requires intact β-galactoside-specific activity of the secreted protein (Cummings and Liu, 2008). Once outside the cell, galectins can bind multiple glycosylated-binding partners, form distinct types of multivalent lectin–glycoprotein lattices and convey glycan-containing information into immune cell activation, differentiation, trafficking, and signaling programs (Brewer *et al.*, 2002; Rabinovich *et al.*, 2007b). In this way, galectins are capable of eliciting autocrine or paracrine regulatory effects in acute and chronic inflammatory microenvironments.

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 typical hemagglutinating activity (Levi and Teichberg, 1981). Since then, several studies have identified and characterized galectin-1 in many different tissues of several species, demonstrating its widespread distribution in multiple cells and tissues. 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 (Rabinovich *et al.*, 2002a). Within the immune system, galectin-1 is synthesized and secreted by activated but not resting T and B cells (Blaser *et al.*, 1998; Fuertes *et al.*, 2004; Rabinovich *et al.*, 2002b; Zuñiga *et al.*, 2001a) and it is significantly upregulated in activated endothelial cells, activated

macrophages, $CD4^+CD25^+$ T_{reg} cells and decidual natural killer (NK) cells (Garín *et al.*, 2007; Kopcow *et al.*, 2008; Rabinovich *et al.*, 1996, 1998; Sugimoto *et al.*, 2006). This regulated secretion and preferential localization suggested a potential function for galectin-1 in negative regulation of leukocyte trafficking and inhibition of effector T-cell responses. Relevantly to the latter, expression of galectin-1 is abundant in immune privileged sites such as placenta (Blois *et al.*, 2007; Iglesias *et al.*, 1998; Kopcow *et al.*, 2008), testis (Dettin *et al.*, 2003; Wollina *et al.*, 1999), and retina (Ishida *et al.*, 2003; Romero *et al.*, 2006) and is significantly altered (up- or downregulated) during several pathological conditions, including cancer, infections, and autoimmunity (Harjacek *et al.*, 2001; Rabinovich, 2005; Rabinovich and Gruppi, 2005; Zuñiga *et al.*, 2001b).

Research over the past years using experimental models of autoimmunity, acute and chronic inflammation, fetomaternal tolerance and cancer, has provided proof-of-concept of the pivotal role of galectin-1 and its specific saccharide ligands in immune tolerance and homeostasis, highlighting multiple functional targets of its immunoregulatory activity. While galectin-1-based gene and protein therapy strategies suppress chronic inflammation and autoimmunity in experimental models of arthritis, diabetes, inflammatory bowel disease, multiple sclerosis, and uveitis (reviewed in Rabinovich and Toscano, 2009), targeted disruption of galectin-1 gene expression results in heightened T-cell-mediated tumor rejection in B16 melanoma (Rubinstein *et al.*, 2004) and classical Hodgkin lymphoma (Juszczynski *et al.*, 2007), highlighting novel therapeutic opportunities for immune-intervention based on the selective manipulation of galectin-1-glycan interactions. Here, we will discuss the most important methods and experimental strategies used to investigate the role of galectin-1 in acute and chronic inflammatory microenvironments. Particularly, we will focus on three well-established functions exerted by this glycan-binding protein, namely modulation of immune cell trafficking and recruitment, control of DC physiology and selective regulation of T-cell fate, which have led to the notion of galectin-1 as a selective and "nonredundant" regulator of immune cell homeostasis.

2. Regulation of Immune Cell Trafficking, Recruitment, and Chemotaxis

2.1. Conceptual framework

The trafficking and selective recruitment of leukocytes throughout the vasculature toward inflamed tissues is a life-saving process. Three decades of intense research have allowed the development of a model whereby different molecules, spanning from proteins, lipids, glycans, and autacoids, may act in concert to orchestrate the process of leukocyte migration and extravasation.

Concomitantly, a wealth of knowledge on the structural features and functional properties of adhesion molecules, chemoattractants, and extracellular matrix proteins has been acquired. The spatiotemporal regulation of this response is accomplished through the coordinated action of pro- and antiinflammatory mediators that appear in the inflammation scene in a tightly controlled sequential fashion. In this way, the complex series of events "switched-on" by proinflammatory signals are slowly "switched-off" by the action of the latter until tissue homeostasis is reestablished. Interestingly, galectin-1 has been shown to downmodulate innate immune responses by inhibiting leukocyte trafficking and extravasation. Illustrating this concept, in a model of noninfective and resolving acute inflammation, high levels of galectin-1 were detected in peritoneal macrophages as well as in inflammatory exudates only at later stages, when the inflammatory reaction is being extinguished, but not at earlier time periods (Gil *et al.*, 2006).

Initially, studies on the biological properties of galectin-1 on T cells have revealed important modulatory functions of this endogenous lectin in the context of adaptive immunity (see Sections 3 and 4 for more details); in contrast, the study of galectin-1's effects on specific innate immune components has lagged behind. In a model of rat paw oedema, administration of recombinant galectin-1 resulted in a robust antiinflammatory effect, evidenced both in terms of decreased paw swelling and diminished cellular infiltration (Rabinovich *et al.*, 2000). Histological analysis of galectin-1-treated paws showed significantly reduced numbers of infiltrated polymorphonuclear neutrophils (PMN) and degranulated mast cells when compared to controls. Furthermore, galectin-1 was also found to inhibit arachidonic acid release and prostaglandin E_2 production by lipopolisaccharide (LPS)-stimulated macrophages (Rabinovich *et al.*, 2000), suggesting a potential mechanism for the beneficial effects observed in this model. The effects of galectin-1 in the process of leukocyte migration and extravasation have been further studied in detail in a mouse model (La *et al.*, 2003). Here, administration of low doses of recombinant galectin-1 ($\sim$0.3–1 μg per mouse equivalent to 20–66 pmol) induced potent inhibition of PMN migration elicited by interleukin (IL)-1β (see below for technical details on the peritonitis protocol). Studies *in vivo* were complemented by dose-response analyses using an established *in vitro* system to study leukocyte chemotaxis. These assays demonstrated that addition of low concentrations of galectin-1 (ranging from 0.04 to 4 μg/ml) induced downregulation of IL-8-induced human PMN locomotion (La *et al.*, 2003). These inhibitory properties were also evident when human PMN transmigration across endothelial cell monolayers was assessed under static conditions. In these studies, addition of low concentrations of galectin-1 (0.04 μg/ml corresponding to $\sim$3 pmol/ml) induced a sustained inhibitory effect. Thus, galectin-1-induced inhibition of PMN chemotaxis and

transmigration followed a bell shape response, being pronounced at very low and high concentrations (La *et al.*, 2003). The same was observed when cell–cell interactions were studied under flow (Cooper *et al.*, 2008).

Cummings and colleagues obtained provocative results when studying the effects of galectin-1 on human PMN physiology. In contrast to the proapoptotic effects observed on T cells (Perillo *et al.*, 1997), exposure of activated human PMN resulted in sustained phosphatidylserine (PS) exposure, an effect which might reflect changes in scramblase and/or flipase activation in the absence of overt apoptosis (Dias-Baruffi *et al.*, 2003). Although PMN remained viable over time, binding to annexin V was significantly enhanced in a time-dependent fashion. Moreover, induction of PS exposure was found to be reversible upon removal of galectin-1 suggesting that this lectin does not initiate irreversible cell death in human PMN, but rather prepares PMN for phagocytosis (Dias-Baruffi *et al.*, 2003; Stowell *et al.*, 2009). Remarkably, binding of galectin-1 to PMN was markedly increased when PMN were activated, suggesting exposure of lectin-binding sites during the activation process, an effect which might reflect changes in the cell surface "glycome" of these cells (Dahlgren *et al.*, 2000; Dias-Baruffi *et al.*, 2003; La *et al.*, 2003; Sengeløv *et al.*, 1995). Collectively, these data identify PMN as unequivocal targets of galectin-1 activity, but do not shed light as to its modes of action. In order to address this question, protocols of intravital microscopy (IVM), which allow direct visualization of the inflamed microcirculation, were performed. Following administration of an antiinflammatory dose of galectin-1, inhibition of all three processes typical of an inflamed microcirculation, namely cell rolling, adhesion, and migration was observed (La *et al.*, 2003). These results were confirmed for human PMN by monitoring interactions of these cells with monolayers of human umbilical vein endothelial cells (HUVECs) under flow (using the flow chamber system; protocol detailed below). This study showed that preincubation of PMN with recombinant galectin-1 significantly decreased the extent of capture, rolling, and adhesion of these cells on activated endothelial cell monolayers (Cooper *et al.*, 2008). Of note, these assays were done with increasing concentrations of galectin-1 (0.04, 0.4, and 4 μg/ml), showing that the lowest concentrations affected both PMN rolling and adhesion, while higher concentrations only abolished rolling. The fact that distinct cellular processes are affected by galectin-1 in a concentration-dependent fashion suggests that unique receptors could mediate different biological processes during neutrophil activation. Following this reasoning, PMN rolling on endothelial cell monolayers might induce cell surface exposure of a second and distinct glyco-receptor which might be subsequently activated by a different concentration of this lectin, leading to inhibition of PMN adhesion and transmigration. Additional work is needed in order to define and characterize specific receptors involved in these effects and their selective glycosylation pattern during the lifespan of PMN. In addition, galectin-1-binding sites have also been described on platelets

which are involved in the aggregation and activation of these cells (Pacienza *et al.*, 2008). Future studies are warranted to determine whether galectin-1 affects platelet adhesion to the endothelium and to define whether galectin-1-mediated platelet/leukocyte interactions are critical to delineate the fate of systemic inflammatory responses.

The role of endogenous galectin-1 in immune cell trafficking may be assessed experimentally using two different approaches: (a) siRNA-mediated silencing of galectin-1 *in vitro* and *in vivo*; (b) gene deleted mice lacking the galectin-1 gene (*Lgals1*$^{-/-}$). As determined by flow chamber assays, downregulation of galectin-1 in endothelial cells using siRNA strategies favored their interaction with leukocytes resulting in increased number of captured cells (Cooper *et al.*, 2008). Collectively, these findings indicate that galectin-1 functions to limit PMN recruitment onto a tumor necrosis factor (TNF)-treated endothelium, a property that may underline its inhibitory effects during acute inflammation. To verify the effects of galectin-1 in an *in vivo* system, IVM of *Lgals1*$^{-/-}$ mice was performed in comparison with their wild-type counterpart (Poirier and Robertson, 1993). Consistent with an inhibitory function of galectin-1 in acute inflammation, leukocyte trafficking, adhesion, and migration were significantly increased in the cremasteric circulation of galectin-1 null mice (Cooper *et al.*, 2008). Of interest, these inhibitory properties of endothelial galectin-1 were not restricted to the PMN compartment, but were also evident on flowing lymphocytes (Norling *et al.*, 2008). In addition to regulating cell trafficking, endothelial cell-derived galectin-1 has been shown to play an important role in angiogenesis (Thijssen *et al.*, 2006). Hence, targeting endothelial galectin-1 may represent a novel strategy with potential anti-cancer applications (Thijssen *et al.*, 2007). Furthermore, studies performed in galectin-1-deficient mice showed that endothelial-derived galectin-1 activates inhibitory signals to reduce lymphocyte trafficking during the early phases of an inflammatory response (Norling *et al.*, 2008). The same "buffering" effect was seen, as mentioned above, when the cremaster microcirculation was exposed to IL-1β (Cooper *et al.*, 2008), indicating again the potent inhibitory effects of galectin-1 on cell migration and endothelial–leukocyte interactions. Thus, mimicking endothelial galectin-1 during the development of inflammatory responses, would represent a novel strategy to control aberrant cell trafficking. Figure 11.1 illustrates the working model that prompted us to investigate whether exogenous and endogenous galectin-1 acts as a tonic inhibitory mediator of leukocyte recruitment. This effect could also underlie the antiinflammatory effects of this glycan-binding protein or its genetic delivery in experimental models of autoimmune disease, including collagen-induced arthritis, diabetes, uveitis, concanavalin A-induced hepatitis, hapten-induced colitis, and experimental autoimmune encephalomyelitis (EAE; reviewed in Rabinovich and Ilarregui, 2009; Rabinovich and Toscano, 2009).

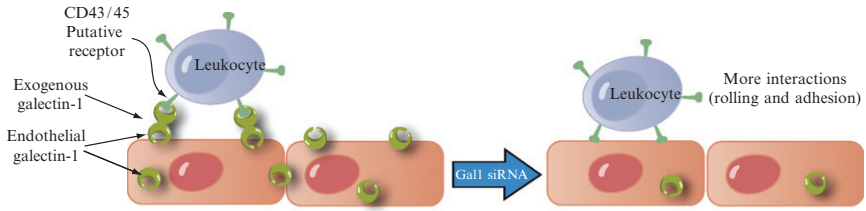

Figure 11.1 Galectin-1 acts as a tonic inhibitor of leukocyte interaction with endothelial cells. Endothelial cells express high amounts of galectin-1, a good proportion of which is exposed on the luminal face. Exposure to exogenous galectin-1 inhibits leukocyte rolling and adhesion. In contrast, reducing galectin-1 expression by using silencing RNA approaches (or genetically modified animals, or cells thereof) would augment the extent of white blood cell interaction with the endothelial monolayer.

2.2. *In vitro* approaches to study galectins in leukocyte chemotaxis, trafficking, and recruitment

A number of experimental approaches *in vitro* have allowed the investigation of the mechanisms underlying leukocyte chemoattraction and migration toward inflamed tissues. Among them, the chemotaxis assay can assess the migration of a single population of leukocytes through a filter in response to a chemotactic stimulus whereas the flow chamber assay allows examination of the interactions between endothelial cells and leukocytes under flow. Details of these assays are described below.

2.2.1. Human blood leukocyte isolation

The isolation of pure leukocyte populations is required for chemotaxis, as well as for flow chamber assays. We typically collect blood from healthy volunteers with a 21-gauge needle and transfer to a 50-ml centrifuge tube containing 1/10 volume of 3.2% (w/v) sodium citrate. This is further diluted 1:1 with warm RPMI 1640 medium. A double density gradient is formed by layering an equal volume (3 ml) of Histopaque 1077 over Histopaque 1119. This must be prepared immediately before use to avoid any diffusion occurring between the two layers. Using a sterile Pasteur pipette prediluted blood should be layered onto the Histopaque and centrifuged at room temperature for 30 min at 400×*g*. Two distinct layers of leukocytes can be seen after centrifugation, with red blood cells (RBCs) pelleted to the bottom of the centrifuge tube. PMN are found above the RBCs and peripheral blood mononuclear cells (PBMCs) are found at the plasma/Histopaque interface.

2.2.2. Lymphocyte isolation

For lymphocyte isolation, the PBMC layer should be removed first with a Pasteur pipette to avoid crosscontamination with PMN. Aliquots of 25 ml should be placed in 50 ml centrifuge tubes and an equal volume of RPMI 1640

should be added. Following centrifugation at 300×*g* for 15 min the supernatant should be discarded. PBMCs are resuspended in 50 ml RPMI 1640 and centrifuged at 300×*g* for 10 min. This step is repeated until the supernatant is clear so that contaminating platelets are removed. The cell pellet is then resuspended in 2 ml RPMI medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 m*M* L-glutamine, 100 U penicillin and 100 mg/ml streptomycin (complete media) to count (see below). To separate monocytes and lymphocytes, PBMC are plated into 6-well culture plates (4×10^6 cells/well) and placed at 37 °C for 1 h to allow monocytes to adhere. Lymphocytes can then be removed for use in chemotaxis or flow assays.

2.2.3. PMN isolation

Following collection of the PMN layer with a Pasteur pipette (6 ml PMN/15 ml falcon tube) an equal volume (6 ml) of RPMI 1640 is added. Cells are then centrifuged at 300×*g* for 15 min, the supernatants are discarded and the pellet is resuspended by flicking tubes gently. If the pellet is not completely resuspended red cell lysis is not achieved. Next, 7.5 ml of ice-cold water is added, the tube is inverted three times and 2.5 ml of 3.6% (w/v) sodium chloride solution is subsequently added. The ice-cold water is employed to induce lysis of contaminating erythrocytes, whilst sodium chloride is added to preserve PMN. Cells are finally centrifuged at 300×*g* for 10 min and resuspended in 2 ml RPMI medium supplemented with 0.1% (w/v) BSA to count.

2.2.4. Cell counting

For cell counting, we remove a 10-μl aliquot of cells and add to 990 μl Turk's [0.01% (w/v) crystal violet in 3% (v/v) acetic acid]. This allows a differential cell count (distinguishing between PMN, monocytes, and lymphocytes based on nuclear morphology) to be performed.

2.2.5. Chemotaxis Assay

Chemotaxis assays can be performed using any purified leukocyte subset and chemoattractant of choice. IL-8 (30 ng/ml) is commonly used for testing potential inhibitors of PMN chemotaxis, whilst SDF-1α (10 ng/ml) or MIP-1α (10 ng/ml) is typically used for T cells or monocytes respectively. Chemotaxis plates are commercially available (e.g., the Neuroprobe ChemoTxplate™). The plate is based on a 96-well microplate format with an upper filter and lid. The filters are available with different pore sizes that can be used for studying different cell populations (3 μm for PMN, 5 μm for T lymphocytes and 8 μm for monocytes and macrophages). PMN are isolated as described above and resuspended in RPMI containing 0.1% (w/v) BSA at a concentration of 4×10^6 ml^{-1}. Twenty-seven microliters of the chemoattractant or medium alone (negative control) is pipetted into the bottom well of the chamber. Care must be taken to avoid development of any air bubbles. The upper filter is then carefully placed on top of the plate

and fixed in place at each corner. The filter should be checked visibly to ensure that the membrane is in contact with the fluid in the bottom compartment of every well and that there are no air bubbles between the filter and the media in the bottom well as this will cause false negative results. Twenty-five microliters of cell suspension is then carefully pipetted onto the top wells and the lid put on the plate. The plate is then placed in a humidified chamber in 5% CO_2 at 37 °C for the desired time, which in the case of neutrophil transmigration is 1.5 h. At the end of this experimental period, the remaining cells/media are removed from the top of the membrane using a cotton bud. Each well is then washed with 25 μl RPMI to remove any remaining cells. The plate and membrane is centrifuged for 1 min at 312×*g* for 5 min to pellet cells in the bottom well, the filter is then removed and the cell pellet resuspended. Leukocytes that have migrated to the bottom chamber are quantified by diluting in Turk's dye and cells are counted as described above.

To test the effect of galectin-1 in this model, leukocytes are preincubated with recombinant galectin-1 for 10 min at 37 °C prior to addition to the filter. We have found that PMN chemotaxis is inhibited when PMN are preincubated with 0.04–4 μg/ml galectin-1 (La *et al.*, 2003). To determine whether galectin-1 itself has chemotactic potential, a range of galectin-1 concentrations may be placed in the lower well and chemotaxis may be compared to that induced by a known chemoattractant such as IL-8 for PMN. As galectins are known to induce agglutination of some cell types this should be assessed prior to performing chemotaxis assays as false negative results could be obtained if leukocytes agglutinate in the top well of the chamber.

2.2.6. Flow chamber assay: Studying the effects of galectin-1 on human PMN and lymphocyte interactions with human endothelial cells under flow

The major advantage of the *in vitro* flow chamber over static assays such as the chemotaxis assay described above is that it is performed under flow. This enables a more physiologically relevant examination of the effects of single agents and compounds on the various steps of the leukocyte recruitment cascade. Flow assays are required to investigate mechanisms by which adhesion molecules such as selectins capture leukocytes so that they initiate rolling across the endothelium. If the correct stimulations are applied, all aspects of leukocyte recruitment, such as capture, rolling, adhesion, and transmigration, can be monitored. A detailed protocol for carrying out these assays is detailed below.

2.2.7. Isolation and culture of primary HUVECs

Primary HUVECs are isolated from umbilical cords. Cords are collected in cord buffer [PBS containing penicillin (100 U), streptomycin (100 mg/ml), and fungizone (2.5 μg/ml)] and stored at 4 °C until endothelium isolation.

Endothelial cells are purified from umbilical cords by collagenase digestion of the interior umbilical vein. The veins are perfused with approximately 50 ml of cord buffer using a sterile syringe to wash out the residual blood. The other end of the cord is then clamped and approximately 20 ml of 0.1% (w/v) collagenase type II in serum-free medium 199 containing penicillin (100 U), streptomycin (100 mg/ml), fungizone (2.5 μg/ml), and L-glutamine (2 m*M*) is added. Another clamp is then placed at the top end of the cord and the vein is incubated in a humidified chamber in 5% CO_2 at 37 °C for 15 min. Following incubation, the collagenase solution is collected into a 50-ml centrifuge tube and the vein flushed with 30 ml of cord buffer to remove endothelial cells. Cells are then centrifuged at 560×*g* for 5 min, supernatants are removed and the pellet is resuspended in 15 ml of complete medium (M199 containing 20% human serum, 100 U penicillin, 100 mg/ml streptomycin, 2.5 μg/ml fungizone, and 2 m*M* L-glutamine) and transferred to a T75 flask. Typically the yield of this procedure is in the range of $0.5–1.5 \times 10^6$ cells per cord.

Cells are seeded into T75 flasks or 35 mm plates (for flow assays) precoated in 0.5% (w/v) bovine gelatin prior to use. Briefly, 2% (w/v) gelatin is diluted 1:4 with PBS and tissue culture plastics are coated with the 0.5% solution for 20 min at room temperature. Following this coating procedure, gelatin is aspirated before seeding. Then, cells are incubated in a humidified chamber in 5% CO_2 at 37 °C, medium is replaced after 24 h to remove residual erythrocytes and changed every 48 h thereafter. When cells cultured in the flask reach approximately 80% confluency they are rinsed once with PBS and subcultured using 0.025% (w/v) trypsin/0.01% (v/v) EDTA solution (2 ml for a 75-cm^2 flask). When 90% of the cells have rounded and started to detach, the flask is then tapped firmly on the side to release the cells and an appropriate volume of complete media is added. For flow chamber assays, HUVEC should ideally be used at passage 1–2 and not beyond passage 3; as high passage HUVECs begin to lose their responsiveness and expression levels of adhesion molecules.

2.2.8. *In vitro* flow chamber assay

HUVECs are seeded in 35 mm^2 gelatin-coated dishes at a density of 3×10^5 cells in complete medium and used 24 h after plating at confluence. Confluent monolayers can be stimulated with TNF (10 ng/ml) for 4 h to upregulate adhesion molecules such as E-Selectin, ICAM-1, and VCAM-1 (Cooper *et al.*, 2008). Human PMN, isolated as outlined above, are resuspended to 1×10^7 cells/ml in Dulbecco phosphate buffered saline (DPBS; without calcium and magnesium) containing 0.1% (w/v) BSA and kept on ice before use. Immediately prior to flow, 5×10^6 (0.5 ml) PMN are diluted to 5 ml in DPBS supplemented with Ca^{2+} and Mg^{2+}, yielding a cell suspension of 1×10^6 cells/ml. PMN are then incubated for 10 min

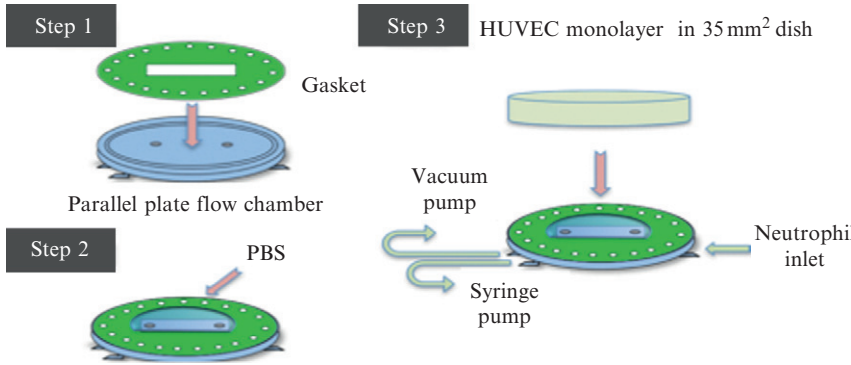

Figure 11.2 Assembly of the parallel plate flow chamber. The chamber is first covered in vacuum grease to hold the gasket in place, and residual grease is removed using an alcohol wipe. PBS is then added to the window in the gasket to cover both the inlet and outlet, ensuring no air bubbles are present. Finally, the HUVEC monolayer is added to the vacuum-sealed chamber, inverted, and placed under an inverted microscope.

at 37 °C following which the endothelium is rinsed with PBS prior to attachment to the parallel plate laminar flow chamber (e.g., the one from GlycoTech), as shown in Figure 11.2, and the cells are flowed for 8 min prior to recording. A shear stress of 1 dyne/cm^2 is generated using an automated syringe pump (Harvard Apparatus, South Natick, MA). This is calculated according to an adaptation of Poiseuille's law that states:

$$\text{Wall shear stress(dyne/cm}^2) = \text{Mean flow velocity(mm/s)} \times [8/\text{tube diameter(mm)}] \times \text{viscosity(Poise)}$$

This approximation takes into account the flow rate through a cylindrical vessel; the equation relating wall shear stress to volumetric flow rate through the chamber is given by:

$$\text{Tw} = 6\mu Q/a2b$$

where τw, wall shear stress (dynes/cm^2); μ, coefficient of viscosity (P); Q, volumetric flow rate (ml/s); *a*, channel height (i.e., gasket thickness, cm); b, channel width (i.e., gasket width, cm).

Experiments are usually performed at a constant volumetric flow rate of 0.00707 ml/s. The coefficient of viscosity is determined by the fluid in which the PMN are resuspended and the temperature of the solution. For PBS at a constant temperature of 37 °C, the viscosity is 0.0076 Poise.

- HUVEC stimulated with 10 ng/ml of hr-TNF for 4h
- 5×10^6 PMN/ml flowed over a HUVEC mono-layer 8-min
- Six 10s clips recorded for offline analysis

Figure 11.3 Equipment required for the flow chamber assay. An example of a full set up used to run the flow chamber assay with human PMN and HUVEC and used, for instance, to determine the effect of exogenously applied or endogenous (endothelial) galectin-1.

The flow channel has a set size determined by the dimensions of the gasket; the channel height relates to the thickness of the gasket, which is 0.0254 cm with a channel width of 0.5 cm. These conditions create a wall shear stress of precisely 0.9994172 dyne/cm^2.

The entire flow chamber is placed under an inverted microscope fitted with 10× and 20× phase contrast objectives (Nikon, Melville, NY). Figure 11.3 shows a complete flow chamber setup. PMN are perfused over HUVEC monolayers for a period of 8 min, and six, 10-s frames are chosen from random fields of view using a Q-imaging Retiga EXi digital video camera (Q-imaging) and recorded in Streampix capture software (Norpix) ready for off-line analysis. Sequences are loaded into ImagePro-Plus software (Media Cybernetics, Wokingham), PMN can be manually tagged and their migration monitored. Three measurements are usually made in the analysis: the total number of interacting cells are quantified as number of cells initially captured during the 10-s frame, which is further

classified as either rolling or firmly adherent (those which remain stationary for the 10-s observation period); the total number of interacting cells for each category (capture, rolling, and adhesion) is expressed as cells per field, counting six fields per treatment.

To determine the effect of galectin-1 on leukocyte recruitment either the leukocyte population to be studied or the endothelial cells can be preincubated with the recombinant lectin. We have found that preincubation of PMN with low concentrations of galectin-1 (0.04 μg/ml) significantly reduced the number of captured and rolling PMN (Cooper *et al.*, 2008), whereas lymphocyte interactions are inhibited by preincubating either lymphocytes or endothelial cells with recombinant galectin-1 (Norling *et al.*, 2008).

2.3. *In vivo* methods to study the role of galectin-1 in leukocyte trafficking and recruitment

All animal studies are conducted following local Ethics Committee's approval and in accordance with national regulations (in the United Kingdom, following the Home Office Guidance on the Operation of Animals Scientific Procedures Act, 1986).

2.3.1. Experimental model of mouse peritonitis: Studying the effect of galectin-1 on PMN trafficking

Peritonitis is a clinical feature observed in patients undergoing long-term peritoneal dialysis or in complications following peritoneal surgery. Experimental peritonitis can help to understand the mechanisms of inflammation in that particular cavity; in some cases, the local inflammatory reaction in the peritoneum can rapidly disseminate and lead to a systemic syndrome associated with high morbidity. Experimental peritonitis is widely used as an inflammatory model for drug screening and testing. Depending on the triggering agent used, specific inflammatory mediators, enzymes, or receptors can contribute to the leukocyte recruitment process, facilitating assessment of the effects of compounds or drugs of potential interest. More recently, this self-resolving model has been used to study the mechanisms and molecules that contribute to the resolution of inflammation.

Rodents are usually the species of choice for peritonitis induction, although the use of larger animals has also been reported. The peritoneal cavity is a suitable model for injection of different biological or chemical agents capable of mediating the recruitment of specific leukocyte subsets. For example, nonspecific inflamogens such as zymosan or carrageenan (Ajuebor *et al.*, 1998a) attract multiple cell types, whereas more specific inflammatory mediators, such as IL-1β or chemokines (CCL2, CXCL1, and so forth; Ajuebor *et al.*, 1998a,b; La *et al.*, 2003) induce selective recruitment of specific subsets. IL-1β-induced PMN recruitment represents an *in vivo* model where chemotaxis occurs in the absence of many features that characterize acute

inflammation, for example, exudation. In fact, PMN recruitment observed in response to application of IL-1β is not strictly direct; that is, it does not result from a direct activation of the circulating PMN, at variance from TNF (Young *et al.*, 2002) and it may require activation of intermediate cells, including endothelial cells and mesothelial cells.

2.3.2. Induction of peritonits

IL-1β injection induces peritonitis in both mice and rats; commonly doses of 0.5–50 ng per mouse cavity and 10–100 ng per rat cavity are given. On the day of the experiment we remove an aliquot of IL-1β from the freezer and resuspend to a concentration suitable for injecting a volume of 0.5–1 ml per mouse cavity or 1–5 ml per rat cavity. We inject the lower part of the abdominal cavity, being careful not to damage the liver in order to minimize a potential hemorrhage that will interfere with the evaluation of leukocyte recruitment.

2.3.3. Harvesting of recruited cells

After a given time period, animals are sacrificed and the skin is separated from the abdomen wall with help of forceps via a careful incision; ensure the abdominal wall is not perforated. Next, the abdomen is exposed and up to 2–3 or 6–10 ml of ice-cold PBS per mouse or rat respectively is injected using a syringe and 21-gauge needle. The abdomen is gently massaged to enable complete washing of the cavity and recovery of recruited leukocytes. The use of heparin, EDTA (or both) in the wash buffer minimizes leukocyte aggregation (25 U/ml heparin, 2 m*M* EDTA in sterile PBS). The recovery of the wash fluid can be performed via an incision and by the use of a plastic Pasteur pipette. If sterile conditions are required, lavage fluid can be recovered by using a syringe without opening the cavity. For best practice, access to a cell culture hood will ensure the most likely sterile conditions. Migrated cells can be determined by light microscopy after staining in Turk's dye, which allows a differential cell count (distinguishing PMN from mononuclear cells based on nuclear morphology) to be performed. For more detailed identification of migrating cell populations, peritoneal cells harvested from the cavities may be stained with specific monoclonal antibodies (mAb) and analyzed by flow cytometry. For instance Gr-1^{+} cells can be determined with a Ly6C/Ly6G mAb (Clone RB6-8C5; BD Pharmingen). However, since this mAb can also react with a monocyte population with intermediate level of Gr-1 expression, it is advisable to quantify Gr-1^{bright} cells as PMN. Alternatively, the Ly6G mAb (Clone 1A8; BD Pharmingen), which only stains mouse PMN may be used.

2.3.4. Summary of the peritonitis model in the mouse

1. Inject IL-1β (5–10 ng/cavity) in a final volume of 500 μl pyrogen-free PBS, with control animals receiving an identical volume of PBS.

2. At the selected timepoint, use a humane procedure to sacrifice the animals according to the most rigorous ethical guidelines.
3. Expose the abdominal cavity via an incision in the skin, without opening the cavity.
4. Detach the skin from the abdominal wall with the help of forceps.
5. Inject 3 ml of ice-cold wash buffer with a syringe equipped with a 21-gauge needle.
6. Perform a gentle massage of the abdomen (for 10–20 s).
7. Make a small incision and carefully insert a Pasteur pipette for lavage fluid collection.
8. Transfer the collected fluid to a 15-ml tube on ice.
9. Repeat the collection step until the fluid has been completely harvested. Note that usually the volume recovered is $\leq$2.5 ml.
10. Take a 100-μl aliquot and dilute in 900 μl of Turk's solution for total leukocyte count in a Neubauer chamber.
11. The remaining wash fluid can be centrifuged for collection of the cell-free lavage fluid and subsequent determination of soluble mediators. The remaining cells can be pelleted and used for cellular analysis by flow cytometry, Western blot and real time RT-PCR.

2.3.5. IVM: Studying leukocyte trafficking *in vivo*

IVM is used to directly visualize the microcirculation of animals *in vivo*, thus allowing assessment of leukocyte–endothelial cell interactions. Since many immunological disease mechanisms are reflected by primary interactions at the microcirculation level, IVM can be used to study the underlying mechanisms, their contributions to leukocyte trafficking and implications for tissue damage and immunopathology.

2.3.6. Microvascular beds

Early IVM studies are typically carried out in an array of tissue beds and animals with the bat wing, hamster cheek, rabbit ear, and cat mesentery all being used. The advent of genetically modified animals has led to rodents now being the preferred choice with the cremaster (the muscle surrounding the testicle) and mesentery being the most studied tissues due to their thin and transparent nature. However, the surgical preparation required to analyze these microvascular beds may result in activation of the tissue causing rapid and pronounced upregulation of rolling through partial degranulation of perivascular mast cells and endothelial expression of P-selectin; yet this effect has been exploited for studies investigating selectin-dependent recruitment. A further limitation of IVM has been the inability to differentiate granulocyte responses from those of mononuclear cells. However, this problem has been overcome recently with the use of leukocyte subtype-specific antibodies.

2.3.7. Intravital setup

The basic setup for IVM requires a microscope suitable for epi- or transillumination, a camera, a video, or DVD recorder for recording images to be analyzed off-line and a Doppler velocimeter for measuring blood flow within vessels.

2.3.8. Mesentery preparation

Mice are anesthetized using xylazine (7.5 mg/kg) and ketamine (150 mg/kg) i.p. Once anesthetized the mouse is placed on a specialized plexiglas board and the right jugular vein is cannulated for administration of saline, pharmacological agents or antibodies. A midline laparotomy is then performed and the small bowel is gently exteriorized using moist cotton buds. Care should be taken to avoid pulling on the bowel as this may activate the tissue and reduce blood flow within the microcirculation. A loop of bowel is placed over the viewing platform and the mouse is placed on the microscope. The exposed tissue is constantly perfused with 37 °C bicarbonate buffered saline (BBS: 132 n*M* NaCl, 5 m*M* KCl, 2 m*M* $CaCl_2$, 1 m*M* $MgSO_4$, 20 m*M* $NaHCO_3$) and the microcirculation is observed.

Common methods for inducing inflammation in the murine mesentery include i.p. injection of cytokines such as IL-1β (5 ng) or TNF (500 ng) or proinflammatory agents such as zymosan (1 mg in 0.5 ml sterile saline) or LPS (0.5 mg/kg derived from *Escherichia Coli* serotype 0111:B4 in a volume of 0.5 ml sterile PBS) for 2–4 h prior to observation. The mesentery is also a good option for studying ischemia reperfusion injury as ischemia can be easily established through clamping the superior mesenteric artery (45 min ischemia is typical with reperfusion times ranging from 30 min to 4 h). The major limitations of the mesentery are that young mice weighing no more than 12 g should be used, as beyond this age the postcapillary venules become obscured by fat limiting visibility of transmigrated cells. Movement of the bowel due to peristalsis can also prove challenging although this can be limited to some degree by overnight fasting of mice prior to IVM.

2.3.9. Cremaster preparation

Mice are anesthetized as described above and the skin over the ventral aspect of the right scrotum is removed. The fascia surrounding the cremasteric sack is removed with care being taken to minimize manipulation of the cremaster itself. At this point the animal is positioned using a gel heating pack in a supine position with the cremaster sack resting on the central glass region of the stage. Throughout the isolation procedure the preparation is kept moist with 37 °C BBS. A suture is placed through the distal end of the cremaster and secured to the Plexiglas board with tape. A longitudinal line down the center and a line from left to right along the top edge of the sack is then scored in the cremaster using a cauterizer so that it can be opened flat against the viewing pedestal. The muscle is held in place using four hooks made from 6-gauge needles.

The vessel connecting the testicle to the cremaster is cauterized and the remaining connective tissue is cut so that the testicle can be moved aside leaving a clear view of the cremasteric microcirculation.

Larger mice can be used for studies in the cremaster as fat deposits are not an issue in this preparation. It is good practice to let the cremaster stabilize for 30 min once surgery is finished. In comparison to the mesentery the cremaster preparation is stable and can be observed for a number of hours. This is advantageous for studies involving superfusion of inflamogens such as PAF (100 n*M* superfused in BBS for up to 2 h). Superfusion protocols allow recordings to be made prior to induction of inflammation and the inflammatory response is then monitored over time usually in one vessel section. End-point experiments are performed by inducing inflammation through injection of proinflammatory agents, such as TNF (300 ng in 400 μl sterile saline) or IL-1β (30 ng in 400 μl sterile saline) intrascrotally 2–6 h prior to observation. In this case numerous vessels and vessel sections can be analyzed and compared to mice injected with saline alone.

2.3.10. Parameters analyzed by IVM

Various parameters can be analyzed by IVM. These include leukocyte rolling, velocity, adhesion, and transmigration (vascular permeability can also be quantified although fluorescence is required to measure this parameter). Figure 11.4 illustrates how these parameters are quantified. The shear rate within postcapillary venules can also be calculated using a Doppler velocimeter to measure mean RBC velocity (shear-dependent leukocyte recruitment has been shown to occur at rates lower than 500 s^{-1} (Bienvenu and Granger, 1993). Mean RBC velocity is calculated from the formula V_{mean} = centerline velocity/1.6. Wall shear rate (WSR) is then derived from the Newtonian formula: WSR = 8000(V_{mean}/diameter).

With the availability of galectin-1-deficient mice, these experiments allow both the endogenous and exogenous proteins to be studied. Figure 11.5 shows data from IVM experiments performed in galectin-1-deficient mice with significantly increased leukocyte transmigration apparent in the absence of this endogenous lectin.

3. Galectin–Glycan Lattices in the Control of DC Physiology

3.1. Conceptual framework

During the past years great progress has been made in our understanding of the cellular and molecular pathways that regulate immune cell tolerance and homeostasis (Rabinovich *et al.*, 2007c). Active immunosuppression can be achieved through the secretion of antiinflammatory cytokines or through

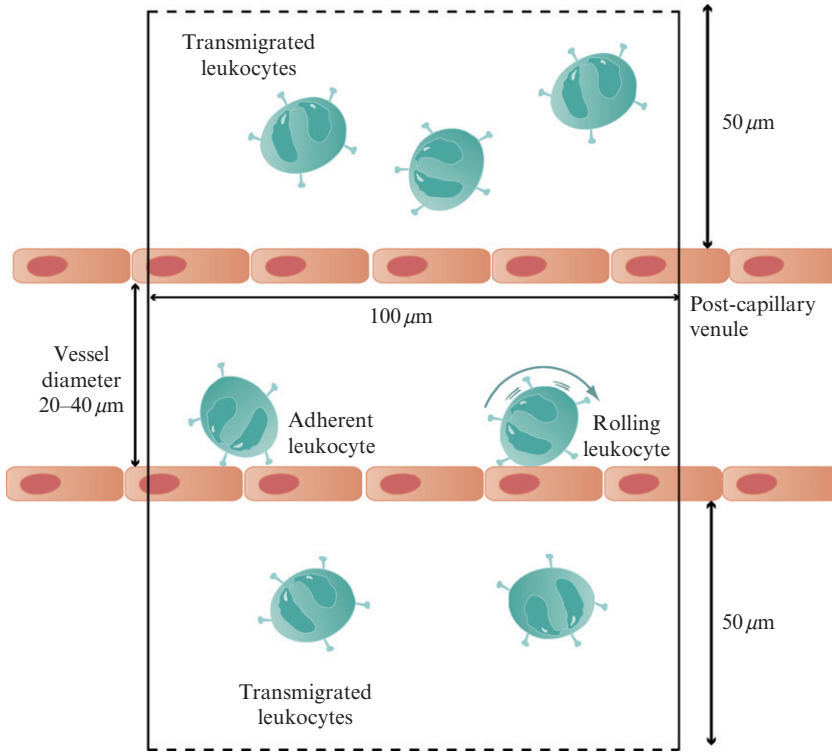

Figure 11.4 Parameters analyzed by IVM. Postcapillary venules with diameters between 20 and 40 μm are selected for analysis by IVM. Leukocyte flux is quantified by counting the number of cells that role past a fixed point over 1 min and is expressed as number of cells/min. Rolling velocity is calculated from the time taken for a given leukocyte to travel a fixed distance and is expressed as μm/s. Adherent cells are classified as those being stationary for a 30-s period. The number of adherent cells within a 100-μm vessel section are quantified over a period of 1 min. Transmigrated cells are quantified at both sides of the selected 100 μm vessel section in an area of $100 \times 50\ \mu m^2$.

specialized suppressor cells, including $CD4^+CD25^+FoxP3^+$ T_{reg} cells, IL-10-producing type-1 $FoxP3^-$ T_{reg} cells, myeloid-derived suppressor cells, "alternatively activated" macrophages, and tolerogenic DCs (Rabinovich *et al.*, 2007c). DCs are the central players in all immune responses, both innate and adaptive (Steinman *et al.*, 2003). Conventional DC subsets described in humans include myeloid DCs and plasmacytoid DCs. Through antigen recognition, processing, and presentation, DCs can orchestrate adaptive immune responses; yet these cells can also attenuate inflammatory reactions irrespective of their maturation status by promoting T-cell anergy or by favoring the expansion and/or differentiation of T_{reg} cells (Steinman

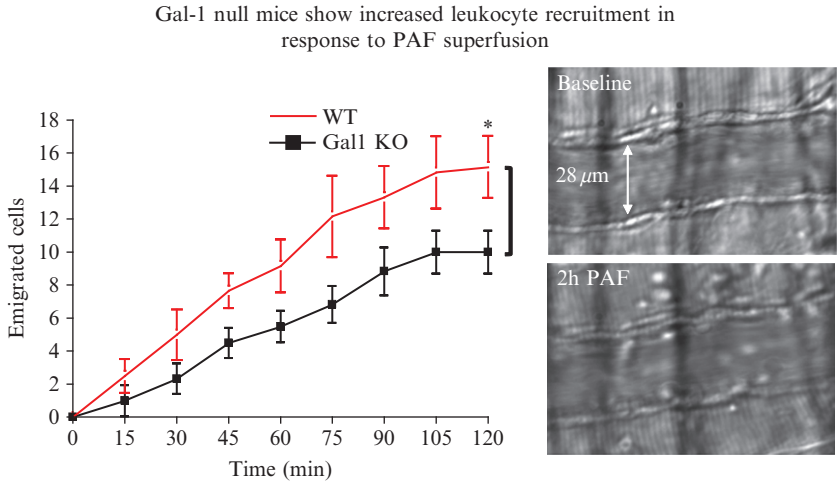

Figure 11.5 Data obtained by IVM of the cremasteric microcirculation. Cremasters were exteriorized and baseline parameters in one vessel section were quantified. PAF (100 n*M*) was then superfused onto the tissue for 2 h and the vessel was recorded and analyzed every 15 min. Significantly increased numbers of leukocytes were observed to transmigrate in galectin-1 knockout mice compared to their wild-type counterparts. Images of vessels in galectin-1 knockout mice at the 0 and 2 h timepoint post-PAF are also shown.

et al., 2003). Research over the past decade has identified a number of upstream and downstream signaling events on DCs which can be manipulated in a selective manner to amplify either an immunogenic or a tolerogenic response (Rabinovich *et al.*, 2007c). Several stimuli may influence the decision of DCs to become tolerogenic, including transforming growth factor-β (TGF-β), IL-10, vasoactive intestinal peptide (VIP), and 1,25-dihydroxyvitamin D3 (Steinman *et al.*, 2003). In spite of early observations assigning a predominant immunogenic function to mature DCs (which express high levels of major histocompatibility complex (MHC) II and costimulatory molecules CD80/CD86), recent evidence challenged this paradigm showing that DC maturation itself is neither an immunogenic nor a tolerogenic hallmark of DCs (Reis e Sousa, 2006). Supporting this concept, fully mature DCs are abundant throughout the peak and resolution phase of autoimmune inflammation and are capable of promoting the expansion of T_{reg} cells instead of inciting a primary or memory T-cell response (Reis e Sousa, 2006). Hence, it is the flexibility of specialized DCs to respond to selective environmental signals, which may determine the amplification or silencing of adaptive immunity, which may in turn shape the course of chronic inflammation. The mechanisms and pathways underlying these regulatory processes are the subject of intensive research with still more questions than answers. While DCs producing high amounts

of IL-12 favor the differentiation of Th1 effector cells, IL-23-secreting DCs favor a Th17 pathogenic phenotype, and those producing high amounts of IL-27 determine the differentiation of IL-10-producing FoxP3$^-$ T_{reg} (Tr1) cells (Ilarregui and Rabinovich, 2010; Steinman *et al.*, 2003). Hence, promotion of T_{reg} cell expansion and induction of tolerogenic DCs have emerged as rational therapeutic strategies aimed at tempering autoimmune inflammation and protecting from immune-mediated pathology.

To understand the cellular and molecular mechanisms underlying the broad immunosuppressive activity of galectin-1 in autoimmunity and tumor settings, we investigated the impact of this glycan-binding protein on human and mouse DC physiology using a series of *in vivo* models (Ilarregui *et al.*, 2009). Notably, DCs differentiated or matured in a galectin-1-enriched microenvironment acquired a distinctive "regulatory signature" characterized by high expression of the cell surface marker CD45RB, phosphorylation of the transcription factor STAT3 and abundant secretion of IL-27 and IL-10. More importantly, when transferred *in vivo*, these DCs promoted T-cell tolerance in antigen-specific and neoplastic settings, blunted Th1 and Th17 responses and halted autoimmune neuroinflammation through mechanisms involving DC-derived IL-27 and T-cell-derived IL-10. Thus, using IL-27 receptor-deficient (*Il27ra*$^{-/-}$) and IL-10-deficient (*Il10*$^{-/-}$) mice, we have identified an immunoregulatory circuit linking galectin-1 signaling, IL-27-producing tolerogenic DCs and IL-10 secreting Tr1 cells (Fig. 11.6; Ilarregui

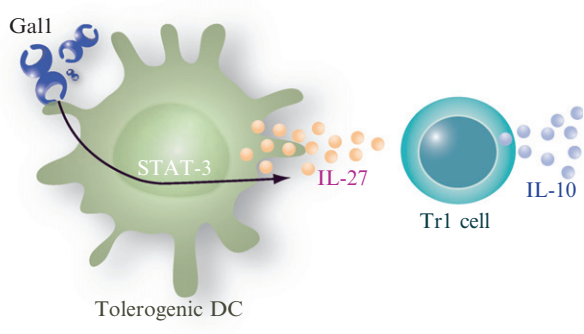

Figure 11.6 Identification of an immunoregulatory circuit linking galectin-1 signaling, IL-27-producing DCs and IL-10-secreting Tr1 cells. During the peak and resolution stages of autoimmune inflammation, galectin-1 expression augments and promotes the differentiation of CD11c^{lo}, CD45RB$^+$ tolerogenic DCs which express high levels of phosphorylated STAT3 (pSTAT3) and IL-10. These DCs also secrete high amounts of IL-27, a heterodimeric cytokine composed of the p28 and the EBI3 subunits, which interacts with its specific receptor (gp130/WSX1) and promotes the expansion of IL-10-producing FoxP3$^-$ regulatory type 1 T cells (Tr1 cells). Delivery of these tolerogenic signals from DCs to T cells blunt Th1 and Th17 responses and promotes the resolution of autoimmune inflammation.

et al., 2009). Other studies have found that DCs engineered to overexpress galectin-1 can induce contrasting effects on naïve and stimulated T cells similar to direct exposure of T cells to soluble recombinant galectin-1 (Perone *et al.*, 2006), suggesting that these cells could also be used as vehicles of immunomodulatory target genes. Moreover, exposure to galectin-1 also promoted the migration of DCs through mechanisms involving Syk and PKC signaling (Fulcher *et al.*, 2009), suggesting that DCs exposed to galectin-1 may acquire a distinctive immunomodulatory program characterized by a "mature" or "semimature" cell surface phenotype, increased migration profile, and enhanced tolerogenic potential.

Given its tolerogenic effects, we also investigated the relevance of endogenous galectin-1 during the evolution of central nervous system (CNS) autoimmune inflammation. Galectin-1 expression augmented during the peak and recovery phases of EAE (see below) and was dramatically upregulated by tolerogenic stimuli including VIP, vitamin D3, and IL-10, but significantly downmodulated by proinflammatory agents such as TNF, IFN-γ, and most Toll-like receptor (TLR) agonists (Ilarregui *et al.*, 2009). Moreover, DCs lacking *Lgals1* gene had lower expression of IL-27, higher production of IL-23 and reduced STAT3 phosphorylation and were not capable of promoting T-cell tolerance during ongoing EAE. In contrast, galectin-1-expressing DCs restored T-cell tolerance and contributed to the resolution of autoimmune neuroinflammation, suggesting a crucial role of endogenous galectin-1 in "fine-tuning" the immunogenicity of DCs. Thus, galectin-1-differentiated DCs producing IL-27 can be harnessed to silence Th1- and Th17-mediated responses and promote the differentiation of IL-10-producing T_{reg} type 1 (Tr1) cells, suggesting a hierarchy of tolerogenic signals which may represent potential targets in T-cell-mediated immunopathology.

3.2. *In vitro* strategies to study the role of galectins in DC physiology

3.2.1. Preparation of recombinant galectin-1

For all the studies described in this chapter, including those detailed above, recombinant galectin-1 is purified as described (Barrionuevo *et al.*, 2007; Pace *et al.*, 2003). Briefly, soluble fractions are obtained for subsequent purification of the recombinant protein by affinity chromatography on a lactosyl-Sepharose column (Sigma). LPS should be carefully removed using Detoxi-Gel^{TM} (Pierce) and LPS content should be tested using Gel Clot Limulus Test (Cape Code) until levels are lower than 0.5 IU/mg. Removal of LPS is particularly important in the case of DCs as low traces of endotoxin contamination may promote DC maturation and mask galectin-1 inhibitory effects. Endotoxin-free recombinant galectin-1 is lyophilized for long-term storage, and when needed reconstituted at 1 mg/ml either in PBS alone or in PBS containing 1 m*M* 2-mercaptoethanol to avoid intramolecular

disulfide bonding between unpaired cysteines present in the carbohydrate-binding site and consequent reduction of biological activity. Remarkably, in our assays both preparations showed a comparable capacity to modulate DC physiology.

3.2.2. Differentiation and maturation of human DCs

PBMCs are isolated from healthy volunteers by standard density gradient centrifugation on Ficoll-Paque^{TM} Plus (GE Healthcare). Monocytes are purified by centrifugation on a discontinuous Percoll^{TM} Plus (GE Healthcare) gradient. In brief, PBMCs are suspended in Ca_2^+/Mg^{2+}-free tyrode solution supplemented with 0.2% (w/v) EDTA and incubated for 30 min at 37 °C. Three different Percoll^{TM} fractions are layered in polypropylene tubes: 50% at the bottom, followed by 46% and 40%. PBMCs (5×10^6 ml^{-1}) are layered at the top and centrifuged at 400×*g* for 20 min at 4 °C. Monocytes are recovered at the 50/46% interface. The purity can be checked by flow cytometry using an anti-CD14 mAb (>85% is appropriate). To differentiate immature DCs, monocytes are plated onto 6-well culture plates or Petri dishes at 1×10^6 cells/ml in complete medium [RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 40 μg/ml gentamicin, 50 μ*M* 2-mercaptoethanol and 2 m*M* L-glutamine (all from Gibco)] containing 5 ng/ml IL-4 (Sigma) and 35 ng/ml recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF; Sigma) in the absence or presence of galectin-1 at concentrations ranging from 0.3 to 3 μ*M*. Cells are fed on day 3 by adding 50% fresh medium, restoring the same concentration of IL-4, GM-CSF, and galectin-1.

To obtain human mature DCs, immature DCs differentiated in the absence of galectin-1 are harvested at day 6, washed with PBS, resuspended in complete medium and counted. Cells are then plated at a concentration of 1×10^6 cells/ml and exposed to 1 μg/ml LPS (0111:B4 *E. coli* strain; Sigma) in the absence (DC) or presence (DC_{Gal1}) of galectin-1 at concentrations ranging from 0.3 to 3 μ*M*. To analyze the role of the JAK2-STAT3 signaling pathway in the immunoregulatory activity of galectin-1, immature DCs are cocultured with LPS, recombinant galectin-1, and different concentrations (0.25, 2.5, and 25 μ*M*) of the pharmacological inhibitor AG490 (Calbiochem).

3.2.3. Differentiation and maturation of mouse DCs

Six- to eight-week-old female or male C57BL/6 or BALB/c mice are used for these assays. The protocol is performed as described by Inaba *et al.* (1992) with minor modifications. After removing all muscle tissues with gauze from the femurs and tibias, the bones are transferred into a fresh dish with Dulbecco's modified Eagle's medium (DMEM). Both ends of the bones are cut with scissors and then the marrow is flushed out using 1 ml syringe and 25-gauge needle filled with DMEM. Bone marrow cells are then passed

through a 21-gauge needle to obtain a single cell suspension. After washing, RBCs are lysed in 5 ml of ACK buffer (NH_4Cl 0.15 *M*; $KHCO_3$ 10 m*M*; Na_2EDTA 0.1 m*M*) for 5 min, washed with PBS and resuspended in DMEM complete medium [DMEM supplemented with 10% FCS, 40 μg/ml gentamicin, 50 μM 2-mercaptoethanol, and 2 m*M* L-glutamine (all from Gibco)] containing 20 ng/ml recombinant mouse GM-CSF (BD Biosciences) or 10% conditioned medium from the GM-CSF-producing J588L cell line. Cells are counted and plated at 2×10^6 cells/ml into 6-well culture plates or Petri dishes in the absence or presence of recombinant galectin-1 (0.3–3 μM). On day 2 and 5, floating cells are gently removed and resuspended in fresh medium restoring the same GM-CSF and galectin-1 concentrations. On day 7 or 8 of cell culture, nonadherent cells and loosely adherent DC aggregates are harvested for analysis or stimulation (approximately 80% of nonadherent cells should express CD11c). In certain experiments, to obtain highly purified populations for subsequent analysis, DCs are isolated using bead-conjugated anti-CD11c mAb (Miltenyi Biotec) followed by positive selection through paramagnetic columns (LS columns; Miltenyi Biotec). The purity of the selected cell fraction is typically $>95\%$. For maturation, immature DCs (1×10^6 cells/ml) are exposed for 48 h to 1 μg/ml LPS in DMEM complete medium.

To study the regulated expression of endogenous galectin-1, we selected a panel of tolerogenic stimuli such as VIP (10^{-8} *M*; Calbiochem), 1,25-dihydroxyvitamin D3 (10^{-8} *M*; Sigma), IL-10 (50 ng/ml; R&D), or proinflammatory signals such as IFN-γ (50 ng/ml; R&D), TNF (20 ng/ml; Sigma), CD40-specific agonistic antibody (10 μg/ml HM40-3;BD Biosciences), and TLR agonists including synthetic bacterial lipoproteins Pam2CSK4 (100 ng/ml; Invivogen) and Pam3CSK4 (1 μg/ml; Invivogen), *Bacillus subtilis* PGN (10 μg/ml; Invivogen), poly(I:C) (10 μg/ml; Invivogen), zymosan (10 μg/ml; Invivogen), heat-killed *Proprionibacterium acnes* (20 μg/ml; van Kampen Group), *B. subtilis* flagellin (200 ng/ml; Invivogen), endotoxin-free Schistosome egg antigen (SEA; 50 μg/ml), or apoptotic splenocytes (irradiated with 12,000 rad) in a 5:1 ratio. To study the intracellular mechanisms underlying these processes, immature DCs are exposed to pharmacological inhibitors of JAK2-STAT3 (2.5 μM AG490; Calbiochem), JNK-SAP (20 μM SP600125; Calbiochem), ERK1/2 (5 μM U0126; Sigma), NF-κB (1 μM BAY11-7082; Sigma), PI3K-AKT (2 μM Ly294002; Sigma), and p38 (10 μM SB202190; Calbiochem) signaling pathways.

For phenotypic and functional analyses of *in vivo*-expanded DCs, spleens are typically placed in 60 mm Petri dishes and injected with 1 mg/ml collagenase IV (Invitrogen) in DMEM (500 μl per spleen), minced into small fragments, transferred into 15 ml polypropylene tubes and incubated for 30 min at 37 °C with 5 ml DMEM plus 1 mg/ml collagenase IV. The reaction is stopped with 500 μl FCS and 50 U/ml DNase I (Roche). Splenic DCs are purified with magnetic beads (MACS) as described above,

resuspended in DMEM complete medium and cell viability is assessed using 0.2% (w/v) Trypan blue dye exclusion.

3.2.4. Galectin-1-binding assays

To use in binding assays, galectin-1 is biotinylated with the EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce; cat #21435) according to manufacturer's instructions. DCs (5×10^5) are washed with PBS in 1.5 ml tubes and resuspended in 50 μl PBS plus 50 μM 2-mercaptoethanol. Biotinylated galectin-1 is added to cells at concentrations ranging from 0.3 to 3 μM and incubated for 1 h at 4 °C in the absence or presence of 30 mM lactose or 30 mM sucrose as specific or nonspecific disaccharides respectively. Cells are then washed with 1 ml PBS, incubated for 30 min at 4 °C with fluorescently labeled streptavidin, washed, and analyzed in a FACSAria^{TM} cytometer (BD Biosciences). Nonspecific binding is assessed with conjugated streptavidin alone.

3.2.5. Cell surface phenotypic analysis

To analyze cell surface markers, DCs (1×10^5) are normally washed with PBS and resuspended in 20 μl PBS plus 1% (v/v) FCS and 0.05% (w/v) sodium azide. Then, cells are incubated for 30 min at 4 °C with fluorochrome-labeled mAb (all from BD Biosciences) at 10 μg/ml. Human cells are typically stained with fluorochrome (phycoerythrin, PE, or fluorescein isothiocyanate, FICT)-conjugated anti-CD1a (HI149), anti-CD14 (M5E2), anti-CD86 (2331-FUN-1), anti-HLA-DR (G46-6), and anti-CD83 (HB15e) mAb. Mouse cells are typically stained with fluorochrome (PE or FITC)-labeled anti-CD11c (HL3), anti-CD40 (HM40-3), anti-I-A^b (AF6-120.1), anti-H-$2K^b$ (AF6-88.5), anti-CD80 (16-10A1), anti-CD86 (GL1), and anti-CD45RB (16A) mAb. Nonspecific binding is determined using appropriate fluorochrome-conjugated, isotype-matched irrelevant mAb. Cells are acquired on a FACSAria^{TM} cytometer (BD Biosciences) and analyzed by FACSDiva software (BD Biosciences).

3.2.6. Endocytosis assays

Human immature DCs (1×10^6) are incubated in RPMI 1640 in the presence or absence of 300 μg/ml FITC-conjugated ovalbumin (OVA) for different time periods either at 37 or 4 °C as a negative control. After incubation, cells are washed twice with cold PBS and resuspended in 200 μl of PBS. Endocytosis is analyzed by flow cytometry.

3.2.7. Receptor segregation

To analyze receptor segregation, DCs (2×10^6) are washed with PBS in 1.5 ml tubes, resuspended in 50 μl PBS containing 50 μM 2-mercaptoethanol, treated with optimal doses of recombinant galectin-1 or buffer control for 1 h and fixed for 30 min at 4 °C with 2% (w/v) paraformaldehyde. Then, cells are washed with 1 ml PBS, resuspended in 50 μl PBS containing 1% (v/v) FCS and

incubated for 1 h with anti-CD43 (8.4 μg/ml; DF-T1, Dako) or anti-CD45 (14.5 mg/ml; 2B11, Dako) human mAb at 4 °C. Next, cells are washed with 1 ml PBS containing 1% (v/v) FCS and incubated with FITC-conjugated antimouse IgG (F0479; Dako) for 30 min at 4 °C. Cells are then washed and resuspended in 20 μl ProLong Gold antifade Reagent (Invitrogen, Cat. No. P36930) with 1 μg/ml propidium iodide and 5 μl of these preparations are mounted on each slide. Receptor segregation is analyzed on a Nikon laser confocal microscope (Eclipse E800).

3.2.8. Real-time quantitative RT-PCR

Total RNA is prepared using Trizol (Invitrogen) following manufacturer's instructions. Real-time quantitative PCR is performed using the SYBR Green PCR Master Mix (Applied Biosystem) in an ABI PRISM 7500 Sequence Detection Software (Applied Biosystem) by means of the absolute quantification protocol. Primers and conditions used are as follows: mouse galectin-1 forward: 5′-TGAACCTGGGAAAAGACAGC-3′; mouse galectin-1 reverse: 5′-TCAGCCTGGTCAAAGGTGAT-3′, Tm = 62 °C. Mouse GAPDH forward: 5′-CCAGAACATCATCCCTGCAT-3′; mouse GAPDH reverse: 5′-GTTCAGCTCTGGGATGACCTT-3′, Tm = 62 °C.

3.2.9. Western blot analysis

Cells (1×10^6) are extensively washed and lysed in 30 μl lysis buffer (5 m*M* EDTA, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.15 *M* NaCl, 50 m*M* Tris–HCl) in the presence of a mixture of protease inhibitors (Sigma). For detection of protein phosphorylation, 1 m*M* Na_3VO_4, 50 m*M* NaF and 10 m*M* β-glycerophosphate is added to the lysis buffer. Protein concentration is measured using the MicroBCA™ Protein Assay Reagent Kit (Pierce) as described by the manufacturer. Equal amounts of protein are resolved by SDS-PAGE, blotted onto nitrocellulose membranes (GE Healthcare) and probed with a series of commercially available antibodies or a rabbit anti-galectin-1 IgG (1.5 μg/ml) generated and used as described (Juszczynski *et al.*, 2007; Rubinstein *et al.*, 2004; Toscano *et al.*, 2007a) in PBS containing 1% BSA (w/v). Bound antibodies are detected using peroxidase-labeled anti-IgG (BioRad) and immunoreactivity is developed using ECL Plus Western blotting detection system (GE Healthcare) following the manufacturer's guidelines. Films are analyzed using Scion Image Analysis software (Scion Corp.). The intensity of each band is recorded and expressed as relative expression (RE) to the expression of actin or the unphosphorylated forms of signaling molecules when appropriate.

3.2.10. Human allogeneic mixed leukocyte reaction

Human CD4 T cells are purified from PBMCs of healthy donors by negative selection using the CD4 T Cell Isolation kit (RosetteSep™; StemCell Technologies) as specified by the manufacturer. Human control DC (DC)

or DC differentiated in the presence of galectin-1 (DC_{Gal1}) are extensively washed with PBS and resuspended in RPMI complete medium, irradiated (3000 rad, ^{137}Cs source) and cocultured with allogeneic CD4 T cells (1×10^5) in RPMI complete medium at various DC:T ratios (1:1, 1:5, 1:10, 1:50) in U-bottom 96-well culture plates in duplicates. At day 3, supernatants are harvested and used for cytokine assessment. After 4 days of culture, 1 μCi/well [^{3}H]-thymidine (specific activity 5 Ci/m*M*; PerkinElmer NEN®) is added during the last 18 h of culture, cells are then harvested and thymidine incorporation is monitored in a liquid scintillation counter.

To determine whether DC_{Gal1} have regulatory function, human allogeneic CD4 T cells (1×10^5) isolated as above are cocultured in RPMI complete medium for 5 days with LPS-matured DCs (1×10^4) in the absence or presence of variable numbers of DC_{Gal1} (1×10^3, 1×10^4, and 3×10^4). Cells are then plated in U-bottom 96-well culture plates in a final volume of 200 μl/well in duplicates. At day 3, supernatants are harvested for cytokine determination. After 4 days of culture, 1 μCi/well [^{3}H]-thymidine (specific activity 5 Ci/m*M*) is added during the last 18 h of culture and thymidine incorporation is monitored in a liquid scintillation counter.

To examine whether allogeneic T cells are rendered regulatory following exposure to DC_{Gal1}, CD4 T cells are purified from mixed leukocyte reaction (MLR) cultures and their suppressive capacity is analyzed. Human CD4 T cells primed with allogeneic DC or DC_{Gal1} for 5 days (as stated above) are further purified by cell sorting. For this, 10^7 cells from MLR are washed with PBS and resuspended in 100 μl of PBS plus 1% (v/v) FCS. Then, cells are incubated for 30 min at 4 °C with fluorochrome-conjugated anti-human CD4 mAb at a concentration of 10 μg/ml. Next, cells are washed with 2 ml PBS plus 1% (v/v) FCS, resuspended in 1 ml of RPMI complete medium, sorted in a FACSAria™ (BD Biosciences) and collected in 1 ml FCS. CD4 T cells are finally resuspended in RPMI complete medium and their viability and recovery is assessed by 0.2% (w/v) Trypan blue staining. To evaluate the regulatory capacity of allogeneic T cells on subsequent MLR cultures, CD4 T cells primed with DC or DC_{Gal1} (10^4 or 10^5) are cocultured in a secondary MLR involving 10^5 fresh CD4 T cells (isolated as stated above) and 2×10^4 DCs. In some experiments, anti-human cytokine neutralizing Abs can be added to the MLR in order to evaluate the requirements of endogenous cytokines for the observed inhibitory effects.

3.2.11. Mouse allogeneic MLR

Mouse naïve CD4 T cells ($CD62L^+CD44^{lo}$) are isolated from spleens of 6–8-week-old BALB/c or C57BL/6 wild-type mice using the MagCellect Isolation kit (R&D) as described by the manufacturer and resuspended in DMEM complete medium. For this, spleens are removed, transferred to a well of a 6-well culture plate with 1 ml DMEM and cut in small pieces. Then, tissue is placed on a mesh and gently pressed; the obtained suspension

(splenocytes) is washed with DMEM (200 × *g*, 10 min at room temperature) and treated with the MagCellect Isolation kit.

DCs differentiated in the absence or presence of galectin-1 or obtained from *Lgals1*$^{-/-}$ mice are extensively washed with PBS and resuspended in DMEM complete medium, irradiated (3000 rad, ^{137}Cs source) and cocultured for 5 days with naïve CD4 splenocytes (2×10^5) in DMEM complete medium at various DC:T cell ratios (1:5, 1:10, 1:20, 1:50), in U-bottom 96-well culture plates in duplicates. At day 3, supernatants are collected for cytokine determination. After 4 days of culture, 1 μCi/well [^{3}H]-thymidine (specific activity 5 Ci/m*M*) is added during the last 18 h of culture, cells are harvested and thymidine incorporation is analyzed in a liquid scintillation counter.

In selected experiments, anti-IL-27p28 (AF1834, R&D), anti-TGF-β_1 (1D11, R&D), or anti-IL-10 receptor (CD210; 1B1.3a, BD Biosciences) neutralizing mAbs (10 μg/ml) are incorporated at the beginning of MLR to determine potential cytokines and mediators responsible for the tolerogenic activity of galectin-1-differentiated DCs.

3.3. *In vivo* strategies to study the role of galectins in DC physiology

All animal work should be approved by the Institutional Ethics Committees in agreement with Institutional Animal House and NIH guidelines.

3.3.1. Adoptive transfer experiments

For adoptive transfer, DCs are washed with PBS, resuspended in DMEM complete medium at 1×10^6 cells/ml and pulsed with OVA (200 μg/ml; Sigma) overnight. Thereafter, cells are extensively washed, resuspended in PBS and injected (3×10^5 in 200 μl per mouse) i.p. with a 23-gauge needle into recipient mice. After 7 days, mice are challenged subcutaneously with OVA (100 μg) in CFA (IFA plus 2 mg/ml of *Mycobacterium tuberculosis*, H37Ra; Difco). Typically 7 days later, splenocytes are isolated as stated above and 1×10^5 cells are plated in U-bottom 96-well culture plates in DMEM complete medium in the absence or presence of 75 μg/ml OVA (final volume 200 μl/well). After 2 days of culture, 1 μCi/well [^{3}H]-thymidine (specific activity 5 Ci/m*M*) is added during the last 18 h of culture, cells are harvested and thymidine incorporation is analyzed with a liquid scintillation counter. Additionally, at day 3 supernatants are harvested and either used immediately to measure cytokine production or frozen at −80 °C until further use.

3.3.2. Tumor protection assays

The mouse B16/F0 or F1 melanoma cell line (C57BL/6 background) obtained from ATCC is used for these experiments. Cells are typically cultured in DMEM complete medium and subcultured every 2 days after

treatment with TrypLE^{TM} Express solution (Invitrogen). For induction of B16 lysates (i.e., necrotic cells), cells are collected using TrypLE^{TM} Express solution, washed twice in PBS and resuspended in PBS at 10^7 cells/ml in 2 ml cryotubes. Then, B16 melanoma cells are subjected to four cycles of rapid freeze (liquid nitrogen) and thaw (42 °C water bath), spun down at 200 × *g* at 4 °C for 10 min to remove cellular debris and resuspended at 1×10^7 cells/ml. Tumor cells are then examined for the degree of necrosis using FITC-annexin-V and propidium iodide (typically necrotic cells are annexin-V$^+$, propidium iodide$^+$).

For immunization C57BL/6 control DC or DC_{Gal1} are washed with PBS, resuspended in DMEM complete medium and cocultured in the presence or absence of necrotic cells at a 1:3 DC:B16 cell ratio in round-bottom 12 ml polypropylene tubes overnight. Then, DCs are extensively washed, resuspended with PBS at 2.5×10^6 cells/ml and injected (200 μl, 5×10^5 DCs) in C57BL/6 mice twice subcutaneously at 7-days intervals. After 14 days of the last immunization, mice are challenged subcutaneously with 2×10^5 viable B16 cells in 200 μl with a 25-gauge needle. B16 melanoma cells are collected using TrypLE^{TM} Express solution, washed twice in PBS and resuspended in PBS at 10^6 cells/ml prior to injection.

Tumor development is monitored every second day by measuring tumor perpendicular diameters with a digital caliper. Tumor volume is estimated as ($d^2 \times D \times 0.5$), where *d* and *D* are the minor and major diameters, respectively. For ethical reasons, animals should be sacrificed when tumors reach a volume greater than 2 cm^3. Mice with tumor volumes of less than 0.5 cm^3 are normally considered as tumor free. Some animals from each group are sacrificed at day 12 following challenge to analyze immunological parameters. Briefly, tumor-draining lymph nodes are isolated, transferred to a 24-well culture plate in 200 μl DMEM and cut in small pieces. Then, tissue is placed into a mesh and gently pressed, the suspension obtained (lymph node cells) is washed with DMEM (200 × *g*, 10 min). Lymph node cells (2×10^5) are cocultured with 1×10^4 irradiated (5000 rad, ^{137}Cs source) B16 melanoma cells for 72 h in F-bottom 96-well culture plates in DMEM complete medium. After 2 days of culture, 1 μCi/well [^{3}H]-thymidine (specific activity 5 Ci/m*M*) is added during the last 18 h of culture, cells are then harvested and thymidine incorporation is monitored in a liquid scintillation counter. At day 3, supernatants are harvested and either used immediately or frozen at −80 °C until further use.

3.3.3. Induction and assessment of EAE

Multiple sclerosis is a major inflammatory and demyelinating disease of the CNS characterized by a relapsing-remitting stage followed by a secondary progressive phase (Ilarregui and Rabinovich, 2010; Sospedra and Martin, 2005). Animal models of experimental EAE recapitulate the clinical and immunological features of the disease and have been of crucial importance

for the validation of many therapeutic targets (Sospedra and Martin, 2005). From an immunological standpoint, the activity of the disease is controlled through a delicate balance of Th1, Th2, Th17, and T_{reg} cells (Lopez-Diego and Weiner, 2008). There are several models of EAE which may reflect the relapsing-remitting, primary progressive or secondary progressive disease and may be induced in Lewis rats, SJL mice, 129/Sv mice, C57BL/6 mice, or guinea pigs. In addition, for EAE induction a number of different immunogens may be used including a peptide (35–55) of myelin oligodendrocyte glycoprotein (MOG), proteolypid peptide (PLP), and myelin basic protein (MBP; Sospedra and Martin, 2005).

MOG-induced EAE is typically induced in 6–8-week-old female mice. While C57BL/6 mice are highly susceptible to disease development, 129/Sv are quite resistance to disease induction. Therefore different strains should be used based on the requirements of each particular experiment. For example, to evaluate whether endogenous galectin-1 is critical for limiting autoimmune brain inflammation, we used 129/Sv *Lgals1*$^{-/-}$ and wild-type mice as this mouse strain provides a window of opportunity to visualize an aggravation of the disease. In contrast, we used mice of the C57BL/6 background to assess the ameliorating and therapeutic activity of recombinant galectin-1 (i.p. injections from days 3–9) or DC_{Gal1} (a single injection at the day of disease onset) as it allows clear visualization of an antiinflammatory effect (Ilarregui *et al.*, 2009; Toscano *et al.*, 2007a). MOG-induced EAE is induced by s.c. immunization with 200 μg $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) in CFA 4 mg/ml of *M. tuberculosis* (H37Ra; Difco) using a 23-gauge needle. On days 0 and 2, mice receive 200 ng of pertussis toxin (List Biological Labs) in 100 μl PBS i.p. using a 27-gauge needle. Mice are examined daily for signs of EAE and assigned scores as follows: 1, limp tail; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; and 5, moribund. Mice with established EAE (clinical score 1) are injected with syngeneic $MOG_{35–55}$-pulsed or unpulsed DC or DC_{Gal1} (Ilarregui *et al.*, 2009). For this, DC or DC_{Gal1} are washed with PBS, resuspended in DMEM complete medium and cocultured with 75 μg/ml $MOG_{35–55}$ in round bottom 12 ml polypropylene tubes overnight. Then, DCs are extensively washed, resuspended with PBS at 1×10^6 cells/ml and injected (200 μl, 2×10^5 DCs) i.p. with a 25-gauge needle.

At day 25 after immunization, lymph nodes draining sites of immunization are isolated (as stated above) and 1×10^5 cells are plated in U-bottom 96-well culture plates in DMEM complete medium in the absence or presence of 50 μg/ml $MOG_{35–55}$ (final volume 200 μl/well). After 2 days of culture, 1 μCi/well [^{3}H]-thymidine (specific activity 5 Ci/m*M*) is added for the last 18 h of culture, cells are harvested and thymidine incorporation is monitored in a liquid scintillation counter. Following 3 days of *ex vivo* restimulation, supernatants from lymph node cells are harvested and either used immediately or frozen at -80 °C until further use for cytokine

determination. For histopathologic analysis, spinal cords are removed on days 25–30 after the first immunization and immediately fixed with neutral 10% (v/v) formalin. Six micrometer paraffin-embedded sections are examined histopathologically following staining with hematoxilin & eosin (H&E) to evaluate inflammatory infiltrates and Luxol Fast blue to assess demyelination.

4. Galectin–Glycan Lattices in the Control of T Helper Cell Fate

4.1. Conceptual framework

In addition to their tolerogenic effects on DC biology, galectin–glycan interactions can also modulate the T-cell compartment. In fact, a number of regulatory checkpoints may be targeted by galectin-1 during the lifespan of T cells including T-cell maturation, activation, cytokine secretion, and survival (Rabinovich and Toscano, 2009).

Apoptotic mechanisms are critical to regulate the development and shaping of the T-cell repertoire in the thymus (Strasser and Bouillet, 2003). Moreover, they are also crucial to peripheral tolerance by dampening self-reactive T cells, restoring T-cell number following execution of effector functions and/or preventing immune-mediated pathology (Bidère *et al.*, 2006). Compelling evidence has been accumulated regarding the role of galectin-1 in the control of T-cell viability from developing thymocytes to activated and fully differentiated effector T cells (Kopcow *et al.*, 2008; Perillo *et al.*, 1997; Rabinovich and Ilarregui, 2009; Rabinovich *et al.*, 1997, 1998, 2002b; Toscano *et al.*, 2007a,b). T-cell susceptibility to galectin-1 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 (e.g., CD45, CD43, CD2, CD3, CD7; Pace *et al.*, 1999; Stillman *et al.*, 2006; Walzel *et al.*, 2000) and glycolipids such as GM1 (Wang *et al.*, 2009). Second, galectin-1 binding is limited to those cells that are able to generate specific saccharide ligands by expressing a particular repertoire of glycosyltransferases responsible for creating or modifying cell surface glycoconjugates (Fig. 11.7). In this regard, cell death triggered by galectin-1 involves the expression and activity of the core 2 β-1,6-*N*-acetylglucosaminyltransferase (GCNT1), an enzyme responsible for 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 (Cabrera *et al.*, 2006). Moreover,

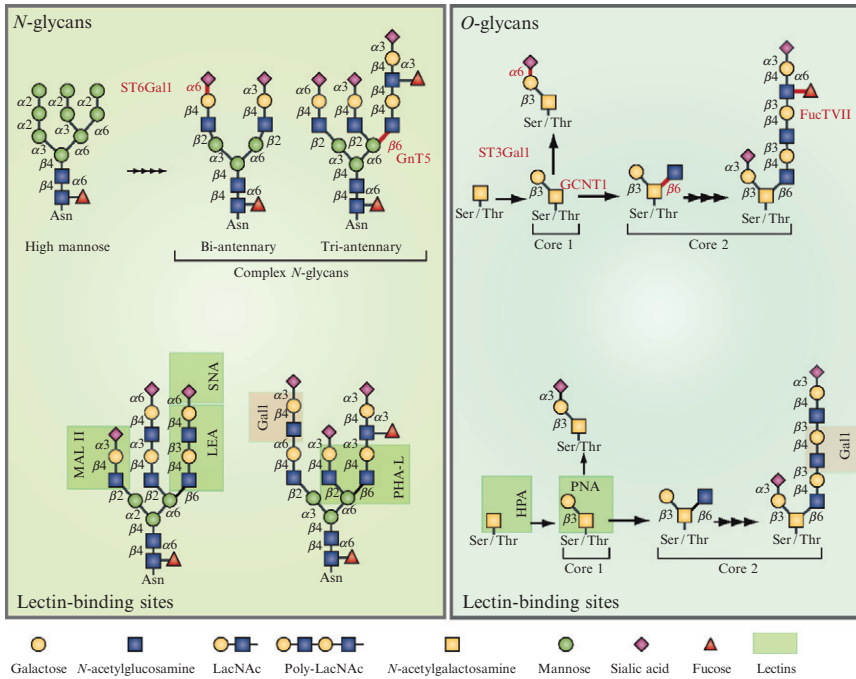

Figure 11.7 Schematic representation of *N*- and *O*-glycan biosynthesis. This scheme includes relevant glycosyltransferases, such as GCNT1, GnT5, ST3Gal1, and ST6Gal1, the coordinated actions of which lead to the generation or masking of common glycosylated ligands for galectin-1 (LacNAc). Galectin-1 binding to specific glycoepitopes is indicated in orange and lectins used as tools for glycophenotyping are depicted in green.

T-cell susceptibility to galectin-1-induced death may also be regulated by the controlled expression of the α2,6-sialyltransferase 1 (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 (Amano *et al.*, 2003; Earl *et al.*, 2010). 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, while galectin-1 binds to CD43 modified with either unbranched core 1 or branched core 2-*O*-glycans (Hernandez *et al.*, 2006).

We have provided 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 (Toscano *et al.*, 2007a). While Th1- and Th17-differentiated cells express the repertoire of cell surface glycans that are critical for galectin-1 binding and cell death, Th2 cells are protected from galectin-1 through differential α2,6-sialylation of cell surface glycoproteins (Toscano *et al.*, 2007a), demonstrating the critical role of endogenous galectin-1 in controlling T-cell homeostasis. Remarkably, *in vivo*-differentiated antigen-specific T helper cells (i.e., Th1 cells generated *in vivo* by adoptive transfer of DCs pulsed with the bacteria *P. acnes* and Th2 cells driven by DCs pulsed with SEA) exhibited comparable glycophenotypes and susceptibility to galectin-1 as *in vitro* human polarized T helper cells. Accordingly, in the EAE model, galectin-1-deficient mice showed greater Th1 and Th17 responses and enhanced susceptibility to autoimmune brain inflammation than their wild-type counterpart (Toscano *et al.*, 2007a). 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 (Fig. 11.8). In line with this evidence, Motran *et al.* (2008) 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 cell subsets. Consistent with the ability of galectin-1 to dampen Th1 responses, silencing of *Lgals1* gene expression resulted in increased IFN-γ and IL-2 production at sites of tumor growth, an effect which was associated with heightened tumor rejection (Juszczynski *et al.*, 2007; Rubinstein *et al.*, 2004).

Finally, the dramatic immunosuppressive effects of galectin-1 *in vivo* in experimental models of autoimmunity and cancer (Rabinovich *et al.*, 2007b), prompted us to investigate the ability of this glycan-binding protein to modulate the T_{reg} cell compartment. Remarkably, administration of galectin-1 in experimental models of autoimmune ocular inflammation (Toscano *et al.*, 2006) and stress-induced pregnancy failure (Blois *et al.*, 2007) restored T-cell tolerance and resulted in considerable expansion of

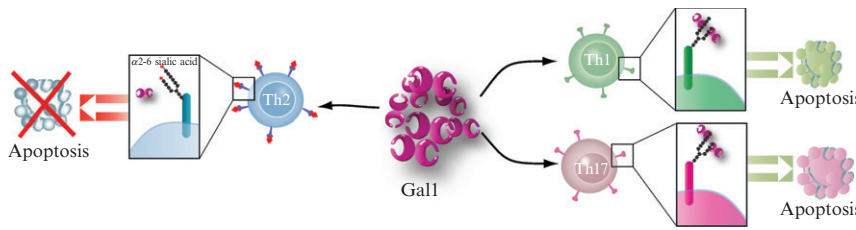

Figure 11.8 Differential glycosylation of T helper cell subsets and susceptibility to cell death. While Th1 and Th17 cells share the repertoire of cell surface glycans that are critical for galectin-1 binding and cell death, Th2 cells are protected from galectin-1 through differential α2–6 sialylation of cell surface glycoproteins.

IL-10-producing $CD4^+CD25^{high}$ T_{reg} cells. Interestingly, these cells showed no significant variations in the levels of Foxp3 expression; yet they displayed 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 $CD4^+CD25^{high}$ T_{reg} cells with high expression of FoxP3 (Juszczynski *et al.*, 2007). Whether galectin-1 stimulates the differentiation and/or expansion of both $Foxp3^+$ and $Foxp3^-$ T_{reg} cells still remains unclear. Interestingly, analysis of gene expression profiles of regulatory versus effector T cells revealed a substantial increase in *Lgals1* mRNA in naturally occurring T_{reg} cells (Garín *et al.*, 2007; Sugimoto *et al.*, 2006). Notably, *Lgals1* overexpression was found to be Foxp3-independent similar to other upregulated genes such as granzyme B and Helios (Sugimoto *et al.*, 2006). Remarkably, Ab-mediated blockade of galectin-1 significantly reduced the suppressive effects of human and mouse T_{reg} cells indicating that endogenous galectin-1 is required for maximal T_{reg} cell function (Garín *et al.*, 2007). This effect appeared to be mediated by galectin-1 cross-linking of the GM1 ganglioside and activation of the TRPC5 ion channel on effector T cells (Wang *et al.*, 2009). These results suggest that protein–glycan systems can also operate within the T_{reg} cell compartment to modulate their expansion and immunosuppressive activity. As discussed here, like many cytokines and growth factors, galectins and their specific glycan partners are critical regulators of innate and adaptive immune cells (particularly DCs and T $CD4^+$ cell subsets; Toscano *et al.*, 2006), which makes them attractive therapeutic targets for limiting autoimmune inflammation, preventing allograft rejection, and potentiating antitumor responses.

4.2. *In vitro* studies to study the role of galectins in Thelper cell survival

4.2.1. Polarization of Th1, Th2, and Th17 cells

When naïve CD4 T cells are primed with appropriate antigens, they undergo a process of activation, proliferation (clonal expansion) and differentiation. In response to IL-12 and the adequate activation stimuli (anti-CD3 mAb, anti-CD28 mAb, and IL-2), naïve T cells differentiate into a Th1-type phenotype which typically produce IFN-γ, IL-2, and TNF and express high levels of the T-bet and STAT1 transcription factors, while in the presence of IL-4, T cells differentiate to a Th2 subset which produces considerable amounts of IL-4, IL-13, and IL-5 and expresses high levels of GATA-3 and c-Maf. While Th1 cells are involved in the defense against intracellular pathogens and tumors and play a pathogenic role in the development of autoimmune reactions, Th2 responses are critical in fighting helminthic infections and play a pathogenic role in allergic disorders (Rabinovich and Toscano, 2009). In addition, other T helper subsets have been incorporated in recent years to the portfolio of T helper cells that regulate adaptive immunity; these include Th17 cells that are

generated in the presence of IL-6 and TGF-β_1, produce IL-17 and IL-6, express considerable amounts of the RORγt and STAT3 transcription factors and are involved in autoimmune reactions and defense against certain types of bacteria and fungi. More recently other cell subsets have been identified including Th9 and Th22 cells, the pathophysiologic relevance of which still remains to be unveiled. Although a variety of factors such as antigen dose, costimulatory molecules and genetic polymorphism may play a role in dictating differentiation of T helper cells, the cytokine microenvironment encountered by the naïve CD4 T cells during activation, plays a dominant role in the subsequent T helper profile. In this section we will focus on current methods used to polarize human and mouse Th1, Th2, and Th17 cells *in vitro*.

4.2.2. Polarization of human T helper cells

Human CD4 T cells are purified from PBMCs of healthy donors by negative selection with magnetic beads (Dynabeads CD4 T Cell Isolation kit Invitrogen) according to the manufacturer's protocol. After purification, CD4 T cells are cultured at a concentration of 1×10^6 cells/ml for 5 days with phytohemagglutinin (PHA; 1 μg/ml) and IL-2 (8 ng/ml; BD Biosciences) in neutral or polarizing conditions essentially as described (Hannier *et al.*, 2002; Fitch *et al.*, 2008).

In order to polarize cells toward a Th1 cytokine profile, RPMI 1640 complete medium supplemented with PHA (2 μg/ml), IL-2 (16 ng/ml; R&D), IL-12 (4 ng/ml; BD Biosciences), plus IL-4-specific neutralizing mAb (200 ng/ml; clone MP4-25D2; BD Biosciences) must be used. Five hundred microliters of this Th1 supplemented medium is added to the purified CD4 T-cell suspension cultured in 24-well plates. To obtain Th2-polarized cells, RPMI 1640 complete medium supplemented with PHA (2 μg/ml), IL-2 (16 ng/ml; R&D), IL-4 (10 ng/ml; Sigma) plus anti-IL-12 mAb (4 μg/ml; clone C8.6; BD Biosciences) is prepared. As above, 500 μl of the Th2 supplemented medium is added to the same volume of a purified $CD4^+$ T-cell suspension in a 24-well plate. In either case, cells are cultured at 37 °C for 4–5 days. CD4 T cells will develop blast morphology after 1–2 days. In cases where cells have overgrown and consume medium, additional fresh supplemented medium without PHA should be added. Of note, the presence of IL-4 in the Th2 culture medium for the first 72 h is critical to sustain IL-4 production by Th2 cells upon subsequent reactivation. After 4 or 5 days of culture, cells are harvested in 15–50 ml tubes in PBS and centrifuged. The cell pellet is resuspended, washed, and counted and the viability is assessed using the Trypan blue dye exclusion method. Polarization toward a Th1 cytokine profile is checked by the expression levels of IFN-γ (ELISA) or the T-bet transcription factor (Western blot), while Th2 differentiation is characterized by the expression of IL-5 and GATA-3 (Toscano *et al.*, 2007a).

For evaluation of the function of sialylation, cells were pretreated for 1 h at 37 °C with *Clostridium perfringens* α2–6/α2–3 neuraminidase (500 mU/ml),

Salmonella typhimurium α2–3 neuraminidase (500 mU/ml; New England Biolab), or buffer control (50 m*M* sodium citrate, pH 6.0) before lectin-binding and cell death assays.

4.2.3. Polarization of mouse T helper cells

Naïve $CD4^+$ T cells ($CD62L^+CD44^{lo}$) are isolated from mouse spleens using the MagCellect Isolation kit (R&D Systems) following the manufacturer's recommended protocol. Purified cells are resuspended at a concentration of 2×10^6 cells/ml and stimulated with plate-bound anti-CD3 mAb (5 μg/ml; clone 145-2C11; BD Biosciences) and soluble anti-CD28 mAb (1 μg/ml; clone 37.51; BD Biosciences) in neutral or polarizing conditions as described (Morgan *et al.*, 2004). Briefly, 150 μl (5 μg/ml in PBS) of anti-CD3 mAb is added to each well of 24-well plates and incubated for 2 h at 37 °C. Subsequently, plates are washed three times with PBS. Care should be taken to avoid plates getting dry and touching the bottom of the wells during the washing step. Then, 500 μl of cell suspensions and 500 μl of Th1, Th2, or Th17 polarizing media (see below) are added to each well and cultures are placed in the incubator for 5 days. Polarized cells are then harvested and their number and viability are assessed as described above.

- Culture medium for Th1 cell polarization contains RPMI 1640, IL-12 (10 ng/ml; BD Biosciences), anti-IL-4 Ab (2 μg/ml; clone 11B11; BD Biosciences), and IL-2 (100 U/ml; R&D).
- Culture medium for Th2 cell polarization contains RPMI 1640, IL-4 (20 ng/ml; BD Biosciences), anti-IL-12 Ab (2 μg/ml; clone C 17.8; BD Biosciences), anti-IFN-γ Ab (20 μg/ml; clone XMG1.2; BD Biosciences), and IL-2 (100 U/ml; R&D).
- Culture medium for Th17 cell polarization contains RPMI 1640, TGF-β_1 (6 ng/ml; R&D), IL-6 (40 ng/ml; R&D), IL-23 (40 ng/ml; R&D), anti-IL-4 mAb (20 μg/ml; clone 11B11), and anti-IFN-γ neutralizing mAb (20 μg/ml; clone XMG1.2). Cultures were supplemented with IL-2 (50 U/ml) on days 2 and 4 of the polarization protocol as described (Bettelli *et al.*, 2006; Mangan *et al.*, 2006).

4.2.4. Glycophenotype analysis

The binding activity of endogenous lectins is limited to those cells that are able to generate specific saccharide ligands by expressing a set of particular glycosyltransferases (Fig. 11.7). The cell surface glycophenotype can be assessed by flow cytometry using a panel of plant lectins (conjugated with either biotin or different fluorescent probes) with specificity for particular saccharide structures (Fig. 11.7). Also, some mAb which recognize specific carbohydrate structures decorating particular glycoproteins may be used for glycophenotyping; this is the case of the 1D4 mAb which recognizes the core 2-*O*-glycan epitope and the 1B11 mAb which recognizes the same saccharide

decorating CD43 on mouse T cells (Toscano *et al.*, 2007a,b). Although these approaches are highly reliable and offer a broad overview of the cell surface glycans expressed in a given cell population, the systematic study of glycans in cells and tissues (glycomic analysis) relies on effective analytical techniques for correlation of glycan structure with function (Hsu and Mahal, 2009). Hence, chromatographic analysis, mass spectrometry, and nuclear magnetic resonance have been traditionally used for glycan profiling; yet lectin-based flow cytometry and lectin microarrays have emerged as simple, rapid, and reliable methods for deciphering the complex nature of glycan-mediated recognition. While lectin cytometry detects a restricted number of cell surface glycans in an individual fashion and provides a broad idea of the ''glycosylation signature'' of different cell types, it is recommended to combine this technique with other high-throughput profiling methods of glycan detection including lectin-based microarrays, glyco-gene arrays and MALDI-MS analysis (Tateno *et al.*, 2007).

For lectin cytometry, polarized Th1, Th2, and Th17 cells (5×10^5) are incubated with a panel of biotinylated or fluorescent-conjugated plant lectins and processed for flow cytometry as described (Toscano *et al.*, 2007a). The specificity of these lectins is illustrated in Fig. 11.7. In brief, 5×10^5 polarized CD4 T cells are suspended in 50 μl of PBS containing 1% (w/v) BSA and biotinylated lectins (20 μg/ml) including *Sambucus nigra* agglutinin (SNA; E-Y Labs), Peanut agglutinin (PNA; Sigma), *Maackia amurensis* agglutinin (MAL II; Vector), *Helix pomatia* agglutinin (HPA; Vector), *Lycopersicon esculentum* agglutinin (LEA; Vector), and L-phytohemagglutinin (L-PHA; Sigma) for 1 h at room temperature. After incubation, cells are washed with PBS containing 1% (w/v) BSA and further incubated with FITC-conjugated streptavidin (10 μg/ml in PBS-1% (w/v) BSA) for 30 min in the dark. After washing twice, cells are analyzed in a FACScalibur flow cytometer (BD Bioscience). Nonspecific binding is determined with conjugated streptavidin alone. Galectin-1 binding to T cells is evaluated essentially as described in Section 3 for DCs.

4.2.5. Cell death assays

Polarized CD4 T cells (1×10^6 cells/ml) are incubated for various time periods with galectin-1 (at concentrations of ranging from 5 to 10 m*M*). These assays are traditionally performed in 1 m*M* dithiothreitol in RPMI medium in order to keep galectin-1's CRD in reducing conditions. However, it is our experience that, when cells are polarized toward Th1 or Th17 profiles, such concentrations of a reducing agent may not be required to induce cell death. Apoptotic cells can be identified by double staining using annexin V and propidium iodide (BD Biosciences) or by using the TUNEL assay (ApopTag kit; Chemicon) according to manufacturers' recommended protocols. At least three types of assays including morphological, cytofluorometric, and biochemical analyses are recommended for the study of

a given cell death pathway as recently indicated (Galluzzi *et al.*, 2009). Using the annexin V-propidium iodide kit for analysis of early and late apoptotic cells, polarized T cells (1×10^5) are exposed to recombinant galectin-1, washed twice with cold PBS and resuspended in 100 μl of 1× binding buffer containing with 2.5 μl FITC-Annexin V according to the manufacturer's recommended protocol (BD Biosciences). Cell death is determined as the percent annexin V-positive polarized cells with stimulus minus the percent annexin V-positive polarized cells without stimulus. These assays are often complemented by assessment of cell viability using the Trypan blue dye exclusion test described above.

4.3. *In vivo* assays to study the role of galectin-1 in T helper cell fate

In order to validate the *in vitro* methods of T-helper cell polarization, different approaches can be used to differentiate T helper cells *in vivo* and further evaluate their glycophenotype and susceptibility to cell death. These approaches include experimental models of autoimmunity such as EAE (see Section 3.3) and adoptive transfer experiments of antigen-pulsed DCs. Although experimental models of autoimmunity offer the possibility of analyzing T helper cell differentiation in a complete organism in a setting of pathophysiologic relevance, adoptive transfer experiments, although more artificial, can raise cleaner and unequivocal results based on the administration of DCs pulsed with Th1 or Th2-polarizing antigens.

For these experiments, we take advantage of the ability of DCs to tailor T helper cell responses. Hence, bone marrow-derived DCs pulsed with the bacteria *P. acnes* (Pa) selectively promote Th1 responses when adoptively transferred into naïve mice, whereas DCs pulsed with *Schistosoma mansoni* egg antigen (SEA) selectively induce Th2-polarized responses (Cervi *et al.*, 2004). The experimental strategy is illustrated in Fig. 11.9. Briefly, bone marrow-derived DCs (C57BL/6 mice) are differentiated in the presence of GM-CSF as described in Section 3.2. On day 8, when 80% or more of nonadherent cells express CD11c, immature DCs are exposed to 20 μg/ml heat-killed *P. acnes* (Van Kampen Group) or 50 μg/ml of endotoxin-free SEA (supplied by Dr. M. Doenhoff or Dr. E. Pearce for our experiments) for 18 h. As control, DCs are exposed to medium alone. The activated phenotype of DCs is controlled by expression of CD11c, MHC II, and costimulatory molecules using flow cytometry as described (see Section 3.2).

To generate *in vivo* Th1 and Th2 responses, syngeneic naïve mice are injected i.p. with 5×10^5 DCs that had been pulsed with Pa, SEA, or neither antigen as described above. Seven days later, mice are sacrificed and spleens are removed. Splenocytes are then restimulated with Pa (25 μg/ml) or SEA (50 μg/ml) for 48 h and polarization toward Th1 and Th2 profiles are verified by measuring in culture supernatants the levels of IFN-γ and IL-5

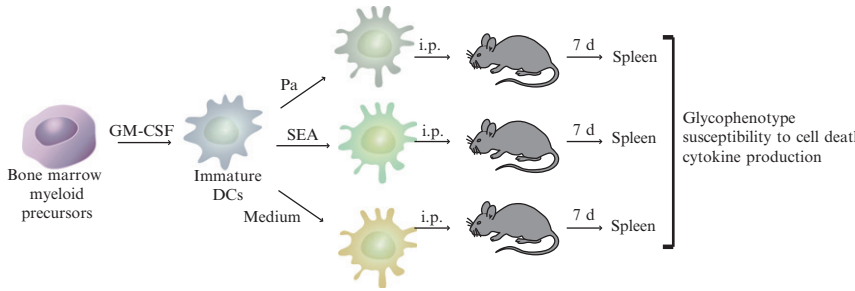

Figure 11.9 Schematic representation of adoptive transfer experiments used to analyze the glycophenotype and function of T helper cells *in vivo*. Bone marrow-derived DCs pulsed with *P. acnes* (Pa) promote selective Th1 responses when adoptively transferred into naïve mice, whereas DCs pulsed with *Schistosoma mansoni* egg antigen (SEA) induce selective Th2 responses. Mice transferred with DCs pulsed with medium alone are used as controls. After 7 days, CD4 T cells are purified and analyzed for glycophenotype, susceptibility to cell death and antigen-specific cytokine production to check the *in vivo* generated Th1 and Th2 responses.

by capture ELISA. CD4 T cells are further purified and processed for glycophenotypic analysis and cell death assays as described in Section 4.2.

5. Final Remarks and Future Directions

In the present review, we summarize emerging evidence on the immunoregulatory activity of galectin-1, its most important cellular targets within the innate and adaptive immune cell compartments and a number of experimental strategies used to determine its biological activities both in *in vitro* and *in vivo* settings. Galectin-1 signaling has been consistently associated with T helper cell death, generation of tolerogenic DCs, leukocyte mobility, cytokine production, and T_{reg} cell function. Similar to many cytokines, autacoids and growth factors, it is not surprising that galectin-1 exhibits a ''double-edge 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. Thus, an integrated study of galectin-1 functions should comprise: (a) generation of endotoxin-free recombinant protein (and evaluation of its biochemical and biophysical properties); (b) *in vitro* assays including reliable readouts such as PMN chemotaxis and transmigration, DC differentiation, T helper cell apoptosis, cytokine production (particularly IL-10 which is a very consistent readout), and endothelial cell morphogenesis (angiogenesis);

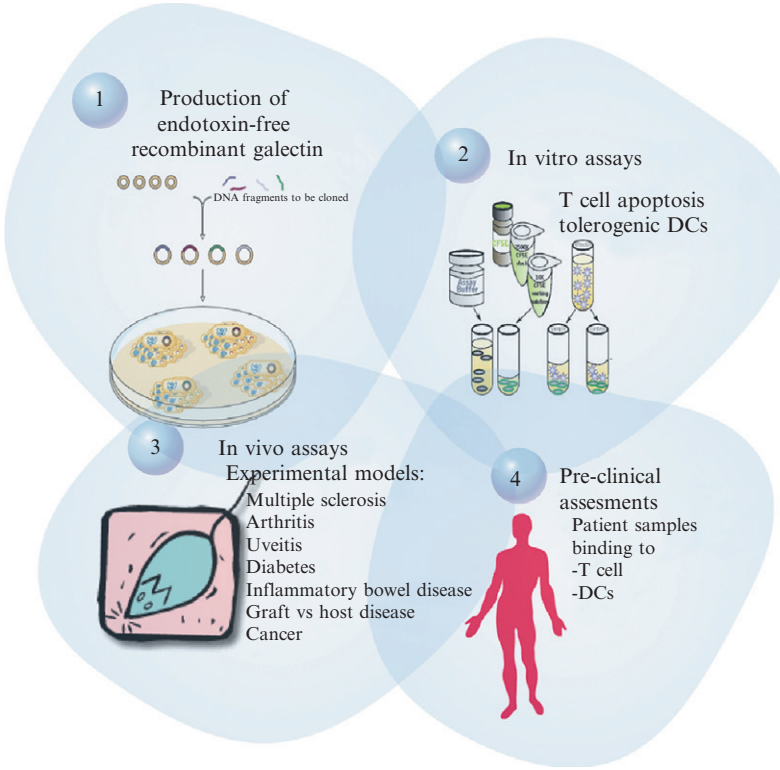

Figure 11.10 **Multidisciplinary approach to study the immunoregulatory activity of galectin-1. In general, the extracellular functions of this lectin are studied using endotoxin-free recombinant galectin-1 while the biological effects of the endogenous protein are typically studied following antisense- or siRNA-mediated silencing of cells expressing high levels of galectin-1 or overexpression of the *Lgals1* gene in cells which do not express or express low levels of this protein. A variety of *in vitro* readouts and *in vivo* experimental models are illustrated and mentioned in detail in the text.**

(c) *in vivo* assays in different experimental mouse models; and (d) correlation of the glycophenotype of immune cells (DCs and T cells) of patients with different pathological conditions with galectin-1-binding and signaling capacity (Fig. 11.10).

In light of the broad spectrum of immunoregulatory effects, challenges for the future will embrace a rational manipulation of galectin-1-glycan interactions toward attenuating immune responses in autoimmune diseases, graft rejection and recurrent fetal loss. Important proof-of-concept data must be generated using galectin-1-deficient mice and the application of predictive disease models, as emerged from initial analyses of the inflammatory response. Moreover, with the diverse range of glycosyltransferase knockout 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 antiinflammatory activities of different members of the galectin family, dissect the biological activity of different galectin-1 variants, evaluate the influence of proinflammatory and tolerogenic microenvironments, and establish the most adequate routes of administration as well as the underlying toxicity of this glycan-binding protein *in vivo*. As a reverse side of the same coin, interrupting galectin-1-glycan interactions may contribute to overcome T-cell tolerance. Hence, galectin-1 inhibitors/antagonists may serve as adjuvants in preventive or therapeutic vaccines against chronic infections and cancer (Liu and Rabinovich, 2005). In order to validate this concept, the design of specific antagonists as well as a comparative study of already established inhibitors is essential. Given the complexity of galectin-1-glycan interactions and the multiple parameters influencing these molecular contacts, further work is required, involving multidisciplinary efforts from different laboratories, to achieve a global comprehensive view of the role of endogenous galectin-1 and its specific carbohydrate ligands in immunoregulation. It is our hope that this conceptual and methodological review will contribute to this goal.

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