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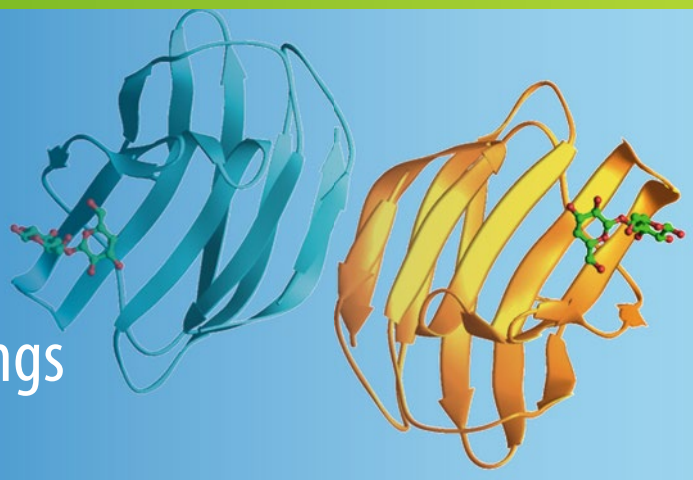

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Richard D. Cummings
Editors

Galectins

Methods and Protocols

Humana Press

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Galectins

Methods and Protocols

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Preface

Although the discovery of galectins over 30 years ago stemmed from interest in understanding the roles of carbohydrates in fundamental biological processes, this finding ultimately uncovered an entire family of potent regulatory proteins. Given their nearly ubiquitous expression and ability to bind highly modifiable carbohydrate ligands, in addition to a variety of other regulatory proteins, these glycan-binding proteins (GBPs) possess the capacity to regulate a wide variety of biological processes. As a result, galectins may not only be some of the most ancient GBPs known but, given their history throughout evolution, also appear to have been a unique evolutionary substrate in many different biological processes. Consistent with this, the galectin family likely represents one of the most pleiotropic families described, with individual members having been implicated in various aspects of nearly every biological process, from RNA splicing to complex regulatory circuits that orchestrate adaptive immunity. Given the diverse roles of galectins in a variety of biological systems, studying these GBPs often requires the assimilation of diverse technical skills to fully appreciate their biological function. Furthermore, as perhaps the most unique and defining feature of galectins lies in their ability to regulate cell behavior through the recognition of carbohydrates, examination of galectin behavior often requires the utilization of carbohydrate biochemistry techniques that may not be familiar to individuals just entering the field. In this volume, individual chapters are dedicated to examining salient features of galectin functions. Each chapter has been written by the world's experts in the field and features clear protocols with notes that provide important considerations that will help you avoid common pitfalls when examining galectin biology. As the first volume solely dedicated to methodological approaches designed to study galectin function, we hope that this work will provide a useful framework when examining galectin function for many years to come.

Atlanta, GA

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Chapter 1

Evolving Mechanistic Insights into Galectin Functions

Connie M. Arthur, Marcelo Dias Baruffi, Richard D. Cummings, and Sean R. Stowell

Abstract

Galectins are an evolutionarily ancient family of glycan-binding proteins (GBPs) and are found in all animals. Although they were discovered over 30 years ago, ideas about their biological functions continue to evolve. Current evidence indicates that galectins, which are the only known GBPs that occur free in the cytoplasm and extracellularly, are involved in a variety of intracellular and extracellular pathways contributing to homeostasis, cellular turnover, cell adhesion, and immunity. Here we review evolving insights into galectin biology from a historical perspective and explore current evidence regarding biological roles of galectins.

Key words Galectin, Glycan binging protein (GBP), Homeostasis, Cellular turnover, Cell adhesion, Immunity, Neoplasia, Carbohydrates

Abbreviations

APC	Antigen presenting cell
DC	Dendritic cell
DTT	Dithiothreitol
εBP	Epsilon binding protein
ER	Endoplasmic reticulum
FITC	Fluorescein isothiocyanate
Gal-1	Galectin-1
Gal-2	Galectin-2
Gal-3	Galectin-3
Gal-4	Galectin-4
Gal-7	Galectin-7
Gal-8	Galectin-8
Gal-9	Galectin-9
Gal-10	Galectin-10
Gal-12	Galectin-12
GBP	Glycan binding protein
IFN-γ	Interferon gamma
IgE	Immunoglobulin E

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IL-10	Interleukin-10
IL-5	Interleukin-5
IL-6	Interleukin 6
KO	Knockout
LacNAc	*N*-acetyllactosamine
LPS	Lipopolysaccharide
MOG	Myelin oligodendrocyte protein
NK cell	Natural Killer Cell
NKT cell	Natural Killer T cell
PKC	Protein kinase C
PLC-γ	Phospholipase C-gamma
polyLacNAc	Poly-*N*-acetyllactosamine
PS	Phosphatidylserine
TCR	T cell receptor
TGF-β	Transforming growth factor beta
TH1	T helper cells type 1
TH2	T helper cells type 2
TNF	Tumor necrosis actor
Tregs	Regulatory T cells
vWF	von Willebrand Factor

1 Introduction

Although the importance of cell surface carbohydrates in normal cellular physiology remained elusive for many years, recent studies demonstrate that these highly complex macromolecules possess diverse roles in many cellular processes [1]. In addition to directly impacting glycoprotein function, complex carbohydrate structures serve as ligands for glycan binding proteins (GBPs), which enable cell surface glycan-dependent signaling [2–4]. Recent studies demonstrate that GBP–cell surface carbohydrate interactions play key roles in a variety of processes ranging from cellular turnover to innate immunity. Given the plasticity of immune cells, which not only enables differentiation of cellular function but also results in distinct alterations in cell surface glycosylation [5], GBP–glycan interactions appear to be especially important in the regulation of immunity.

Among GBPs with immunoregulatory activities, galectin family members appear to regulate a wide variety of immunological processes in addition to serving as factors directly involved in microbial killing [6–9]. In this review, we attempt to link original descriptions concerning the immunological activities of galectins to new mechanistic insights into their regulatory activities within immunity and beyond. In doing so, we do not seek to provide an exhaustive review regarding the biological activities of the entire galectin family, especially given the rapidly expanding nature of this field. However, we seek to highlight salient features of galectins that illustrate the broad regulatory capacity of likely one of the most pleiotropic protein families described.

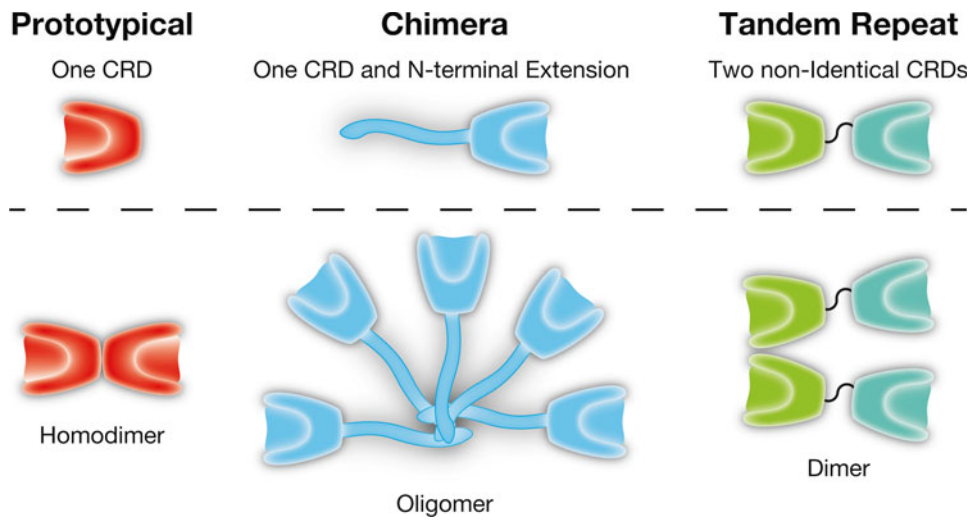

Fig. 1 The galectin family of β-galactoside binding proteins. Galectins are classified into three distinct groups based on their quaternary structure: prototypical, chimeric, and tandem repeat. Prototypical: Gal-1, Gal-2, Gal-7, Gal-10, Gal-13, and Gal-14. Chimeric: Gal-3. Tandem repeat: Gal-4, Gal-8, Gal-9, and Gal-12

2 Discovery of Galectins

Galectins were identified within a few years after the pioneering work in the 1960s of Ashwell and Morrell who discovered the asialoglycoprotein receptor, the first GBP described in vertebrates [10, 11]. Although some of the physiological functions of that receptor have only recently been elucidated [2], these early studies suggested the potential existence of other mammalian GBPs. In 1975, the first vertebrate galectin (an ortholog of mammalian galectin-1) was discovered in the electric organ of the electric eel *Electrophorus electricus* by Teichberg and colleagues [12]. This was followed shortly thereafter by isolation of galectins in the laboratories of Barondes and Kornfeld, who reported similar proteins in avian and mammalian sources, respectively [12–14]. There are now 15 described members of the galectin family (Gal-1 through -15) encoded in mammals (11 are known in humans), and all range in subunit size from 14 to 39 kDa [15] (Fig. 1). They are found in all metazoans and are the only known GBPs in animals that occur both in the cytoplasm, where they are synthesized on free polyribosomes, and on the plasma membrane and extracellular matrix [15].

3 Galectin-1: Regulator of Adaptive Immunity

Although the discovery of this ancient galectin family of GBPs was important in confirming the existence of multiple mammalian GBPs, the physiological functions of galectins were more difficult to define. Early studies suggested that Gal-1 might regulate the

development of muscle, the first mammalian organ from which the protein was isolated [14], including maintenance of the neuromuscular junction [16–18]. Teichberg and colleagues examined whether administration of Gal-1 might affect the pathological sequelae associated with neuromuscular junction pathology. They employed an animal model of myasthenia gravis induced by autoantibody formation against the acetylcholine receptor. Consistent with the potential involvement of Gal-1 in the neuromuscular junction, exogenously added Gal-1 appeared to cause a significant enhancement of muscle function. However, the apparent amelioration of disease actually resulted from the ability of Gal-1 to suppress the autoimmunity needed to generate a myasthenia gravis model [16–18]. Thus, an indirect result of these experiments was the first evidence for what continues to be one of the more intriguing properties of Gal-1, namely, its ability to significantly suppress immune function [7, 12].

4 Gal-1 Regulation of T Cells

While several studies suggested that Gal-1 might regulate adaptive immunity [6, 7, 19–25], it was not until nearly a decade later that studies began to provide a mechanistic insight into Gal-1-mediated immunosuppression. Early studies suggested that Gal-1 may actually induce lymphocyte proliferation, which suggested to the authors that Gal-1 may enhance the development of suppressor T cells [7]. Gal-1 also appeared to mediate adhesion of thymocytes to epithelial cells, which supported a possible role for Gal-1 in the regulation of T cell development [26, 27]. However, it was a seminal paper by Baum and colleagues that suggested that Gal-1 might directly impact T cell viability by inducing apoptosis, that provided the most substantial mechanistic insight into the immunomodulatory activities of Gal-1 [28]. That study first reported that Gal-1 could induce apoptosis of primary activated T cells and several T cell lines, including MOLT-4 and ARR cells. Thus, this study suggested that Gal-1 might regulate adaptive immunity through directly inducing apoptotic death of effector T cells [28].

In addition to directly inducing cell death in activated T cells, subsequent studies suggested that Gal-1 might also serve as a key regulator of a variety of other T cell functions (Fig. 2). For example, Gal-1 induces robust IL-10 production in both CD4+ and CD8+ T cells while inhibiting IFN-γ formation, which suggests that Gal-1 may reduce adaptive immune responses by altering T cell cytokine production [29, 30]. Consistent with this, adoptive transfer of CD4+ T cells from Gal-1 treated mice, which displayed similar cytokine profiles observed following in vitro incubation with Gal-1, protected mice from uveitis with the same efficiency as injection of Gal-1 alone [23]. In addition, injection of Gal-1 into IL-10 null mice fails to convey the immunoprotective properties of

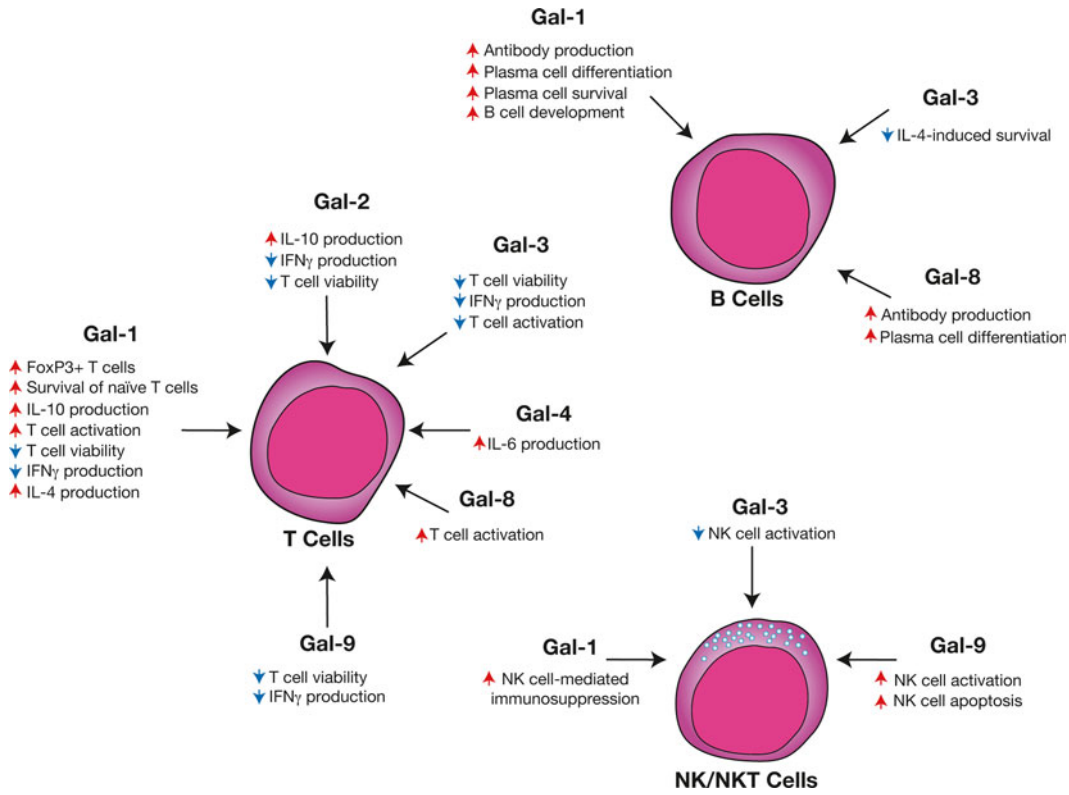

Fig. 2 Distinct galectin family members possess the capacity to differentially regulate lymphocytes. Some of the earliest studies suggested that galectins appear to possess immunomodulatory activity by directly impacting T cell viability. Subsequent studies suggested that galectin might not only regulate T cell viability but also regulate cytokine secretion and activation. Several galectins might also play a variety of roles in the development, activation and differentiation of B cells. Similar galectin-mediated regulation may play a role in NK and NKT cell activation and viability. Representative galectin-regulated activities are shown. *Red arrows* indicate an activity that the respective galectin increases, while *blue arrows* signify galectin-induced decreases in the accompanying activity

Gal-1, strongly suggesting a role for IL-10 and possibly other cytokines, in mediating the immunosuppressive activities of this protein [25]. Similarly, Gal-1-Ig chimera constructs can induce significant IL-10 by T cells, providing a useful therapeutic approach to enhancing Gal-1-mediated immunosuppression [31, 32]. Regulatory T cells (Tregs) may also utilize Gal-1 to induce tolerance, as Tregs from Gal-1 null mice exhibit an impaired capacity to suppress T cell activation [33]. Furthermore, Gal-1 itself appears to facilitate the formation of induced Tregs (iTregs) by upregulating FOXP3 expression in peripheral activated T cells, strongly suggesting a key role for Gal-1 in Treg effector function and development [34]. Perturbation of the cytokine milieu by Gal-1 may indirectly impact T cell viability, as distinct T cell populations often rely on specific cytokines to maintain viability in vivo [35, 36].

In addition to directly altering T cell cytokine production, several studies suggest that Gal-1 might also inhibit T cell activation,

both in vitro and following injection during T cell activation in vivo, providing an additional mechanism whereby Gal-1 might inhibit adaptive immunity [6, 19–24, 37]. Recent studies also demonstrate an in vivo role for Gal-1 in T cell development. TCR transgenic Gal-1 null mice appear to possess altered central selection, which favors the generation of CD8αα T cells, among other alterations in T cell behavior, providing further evidence that Gal-1 affects multiple aspects of T cell biology [38]. In contrast to the ability of Gal-1 to inhibit activation and induce apoptosis of activated T cells, Gal-1 might actually sustain naive T cell survival in peripheral tissue [39]. In addition, recent studies suggest that under certain circumstances Gal-1 may actually facilitate T cell activation in the absence or presence of cognate ligand on antigen presenting cells [40] (Fig. 2).

5 T Cell Regulation: A General Theme of the Galectin Family

In the wake of early studies demonstrating that Gal-1 possessed potent immunoregulatory activities, many studies began to examine the potential role of other galectin family members in the regulation of T cell viability and function (Fig. 2). For example, subsequent studies demonstrated that Gal-3, Gal-8, and Gal-9 regulate thymocyte and T cell viability [41–45]. While different galectin family members appear to signal apoptosis in T cells, individual galectin-mediated regulation of T cell viability likely occurs through engagement of distinct counter receptors. For example, Gal-1 fails to inhibit Gal-3 induced Ca^{2+} flux or apoptosis in primary activated T cells, although both Gal-1 and Gal-3 recognize their respective receptors with similar affinity [30], strongly suggesting engagement of distinct ligands [46]. Similarly, recent work demonstrated that Gal-9, but not Gal-1 or Gal-3, specifically induces apoptosis in CD4+ TH1 cells through engagement of Tim-3 [42], although Tim-3 fails to relay Gal-9 signaling in several T leukemic cell lines [47].

Similar to Gal-1, other galectin family members appear to regulate other aspects of T cell biology in addition to T cell viability. For example, Gal-3 appears to regulate T cell activation and the induction of T cell anergy by restricting lateral mobilization of the TCR into the immunological synapse or by dissociating CD8 from the TCR, respectively [48, 49]. Similar to Gal-1, Gal-2 induces IL-10 production, along with IL-5 and TGF-β, while also decreasing IFN-γ and IL-2, which can significantly impact T cell activation and differentiation [50]. Gal-10, originally known as Charcot–Leyden protein, appears to also be a key player in proper Treg function [51]. In contrast, several studies suggest that Gal-4 may actually exhibit pro-inflammatory activity, inducing IL-6 production and expansion of CD4+ T cells [52], likely following specific colitis-associated changes in CD4+ T cell glycosylation [53]. Gal-3

may also regulate CD4+ T cell differentiation by reducing IL-5 secretion [54]. Interestingly, in contrast to Gal-8-induced apoptosis in activated T cells [55], similar to Gal-1, recent studies suggest that Gal-8 may likewise enhance T cell proliferation [40] (Fig. 2).

6 Galectin Regulation of B Cells

Galectins also appear to serve as key regulators of B cells (Fig. 2). Similar to the ability of Gal-1-mediated thymocyte engagement of thymic epithelial cells, work by Schiff and colleagues demonstrated that Gal-1 can likewise facilitate developing B cell interactions with key stromal constituents within the bone marrow [56]. Subsequent studies demonstrated that Gal-1 mediates B cell stromal contacts by facilitating interactions between the surrogate light chain, α5β1, α4β1, and α4β7 integrins, and ADAM15/fibronectin [57, 58]. These interactions ultimately result in relocalization of the B cell receptor to the B cell-stromal interface to form an immunological synapse that induces critical signals necessary for B cell development [57, 59]. Stromal cells expressing Gal-1 appear to reflect a subset distinct from those expressing IL-7 [60], suggesting a unique role for Gal-1 in B cell maturation. Consistent with this, Gal-1 KO mice experience impaired pre-BII-cell development following marrow challenge [59].

In addition to facilitating B cell development, galectins may also play an important role in B cell activation, antibody secretion and differentiation into plasma cells. Gal-1 null mice generate reduced antibody levels following immunization with T cell dependent antigens [61]. Although this could in part reflect Gal-1-mediated regulation of T cell activation and differentiation, Gal-1 KO mice also display reduced antibody formation following challenge with T cell-independent antigens [61], strongly suggesting a direct role for Gal-1 in B cell activation and antibody secretion. Consistent with this, ectopic expression of Gal-1 within B cells facilitates B cell activation and antibody secretion in vitro, likely through engagement of cell surface glycans [62]. Endogenous Gal-1 also appears to be significantly upregulated during B cell differentiation into plasma cells, likely through a BLIMP1-dependent mechanism [61, 62], strongly suggesting a role for B cell-derived Gal-1 in plasma cell biology. Indeed, Gal-1 KO plasma cells display enhanced sensitivity to apoptosis [61], suggesting that Gal-1 may in part maintain antibody secretion by sustaining plasma cell survival. In addition to Gal-1, recent studies suggest that Gal-8 may also play a key role in B cell differentiation [63]. Knockdown of Gal-8 in Gal-1 KO B cells results in further inhibition of plasma cell differentiation and antibody production [63]. Additional studies suggest that Gal-3 may also impact B cell differentiation and survival [64].

7 Galectins and Natural Killer (NK)/Natural Killer T (NKT) Cells

Following embryo implantation, unique NK cell subsets within the decidua may utilize Gal-1 as an effector molecule in the maintenance of fetal–maternal tolerance [65, 66]. NK cells in this setting secrete Gal-1 that, in turn, appears to engage infiltrating T cells, inducing their apoptosis [67]. In contrast, Gal-3 appears to serve as a neoplastic-derived inhibitor of NK function by inhibiting NKG2D-mediated NK cell activation [68, 69], providing a potential mechanism of inhibiting antitumor immunity. In contrast to the inhibitory activity of Gal-3 on NK cells, Gal-9 induces NK cell activation through ligation of Tim3, possibly enhancing antiviral immunity in the setting of HIV infection [70]. However, Gal-9 appears to exert the opposite effect on NKT cells [71], by directly inducing apoptotic cell death [72]. Gal-9 may also facilitate NKT cell activation indirectly by inducing IL-15-dependent NKT cell activation by neighboring macrophages [72]. Similar to Gal-1, alterations in Gal-9-mediated NK and NKT cell function may contribute to the pathogenesis of preeclampsia [73] (Fig. 2).

8 Galectins and DCs/Macrophages

Given the regulatory network responsible for lymphocyte activation and the intimate connection between innate immunity and the development of an adaptive immune response [74], it is not surprising that galectin family members also appear to regulate a variety of antigen presenting cells (APCs) (Fig. 3). Early studies suggested that individual galectin family members might induce macrophage chemotaxis, macrophage activation, and monocyte to macrophage differentiation [75–78]. In addition, galectins appear to directly enhance macrophage phagocytosis [79, 80]. While all of these features would be predicted to enhance overall antigen uptake and presentation, more recent studies suggest that several galectin family members may also directly impact the ability of APCs to activate antigen specific T cells. For example, Gal-3 appears to regulate immunological synapse formation by engaging beta1,6 *N*-acetylglucosaminyltransferase V (Mgat5)-dependent *N* glycans on various glycoproteins within the immunological synapse [48]. Gal-3-mediated interactions appear to reduce synapse formation and therefore inhibit T cell activation [48]. Consistent with this, Mgat5 KO recipients display heightened T cell activation and an increased propensity to develop autoimmunity [48]. In contrast, Gal-9 appears to enhance T cell activation in the setting of autoimmunity [81]. Similar results suggest that Gal-1 and Gal-8 may enhance T cell activation by facilitating T cell interactions with APCs in the presence of cognate antigen [40].

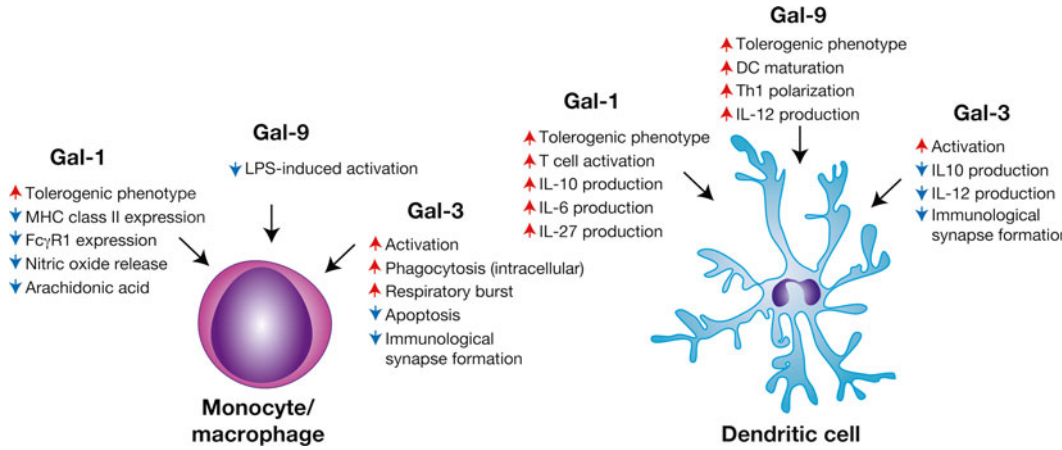

Fig. 3 Galectins differentially regulate monocytes/macrophages and dendritic cells. Different galectin family members appear to possess the ability to directly impact the activity of monocytes/macrophages and dendritic cells with significant implications not only on generalized inflammation but also on the ability of these cells to engage cells involved in adaptive immunity. Several galectins also appear to directly regulate T cell-antigen presenting cell interactions with implications on subsequent T cell activation and differentiation. Representative galectin-regulated activities are shown. *Red arrows* indicate an activity that the respective galectin increases, while *blue arrows* signify galectin-induced decreases in the accompanying activity

In addition to potentially regulating the formation and stability of the immunological synapse, individual galectins may also differentially impact the immunological outcome of APC-T cell interactions by altering APC cytokine profiles following activation. Incubation of DCs in the presence of Gal-1 results in the formation of tolerogenic DCs that subsequently induce IL-10-expressing T cells following TCR ligation [82]. Transfer of Gal-1-treated DCs similarly results in tolerance induction in a variety of settings. For example, transfer of Gal-1-treated DCs reduces IFNγ and increases IL-10, ultimately resulting in enhanced maintenance of fetal-maternal tolerance following maternal stress [82]. Similarly, Gal-1 reduces cell surface MHC class II levels following IFNγ treatment, yet differentially regulates FcγR1 expression depending on the presence or absence of other cytokines [83]. Gal-9 appears to also possess the capacity to induce tolerogenic APC function. Gal-9 suppresses LPS-induced TNF secretion by macrophages and transfer of DCs from Gal-9-treated mice attenuates acute lung injury [84]. These findings do not appear to be limited to murine models as Gal-1 inhibits DC production of IFNγ in patients with psoriasis [85] (Fig. 3).

In contrast to the ability of Gal-1 and Gal-9 to induce tolerogenic activity in APCs, Gal-3 appears to engage pro-inflammatory programs in various APC populations (Fig. 3). For example, in vitro studies demonstrate that Gal-3 induces macrophage activation and significant TNFα secretion [86]. Consistent with

this, Gal-3 KO mice display reduced macrophage activation and inflammatory sequelae associated with liver injury [87]. Similarly, DCs isolated from Gal-3 KO mice immunized with MOG in the setting of experimental autoimmune encephalomyelitis likewise exhibit increased IL-10 production and decreased IFNγ [88]. Importantly, similar changes in DC activation and cytokine secretion can be observed in Gal-3 KO mice in the setting of reperfusion injury, stroke and Con-A-induced hepatitis [20, 89, 90], strongly suggesting that Gal-3 enhances APC activation and pro-inflammatory cytokine secretion in a variety of settings [91]. Although incubation of recombinant Gal-3 can regulate macrophage function, transfer of macrophages isolated from Gal-3 KO mice into WT recipients results in a similar outcome [91], strongly suggesting that Gal-3-mediated regulation can be a cell intrinsic phenomenon. Similar to Gal-3, recent results suggest that in certain settings, Gal-9 may also promote DC maturation with subsequent enhancement of NK cell activation [92].

9 Galectin Regulation of Granulocytes/Mast Cells

The apparent ability of Gal-1 to induce apoptotic cell death in T cells, coupled with growing evidence that Gal-1 may be involved in many aspects of immune regulation, strongly suggested that Gal-1 might be a general regulator of leukocyte activation, trafficking, and viability (Fig. 4). Consistent with this, early studies demonstrated

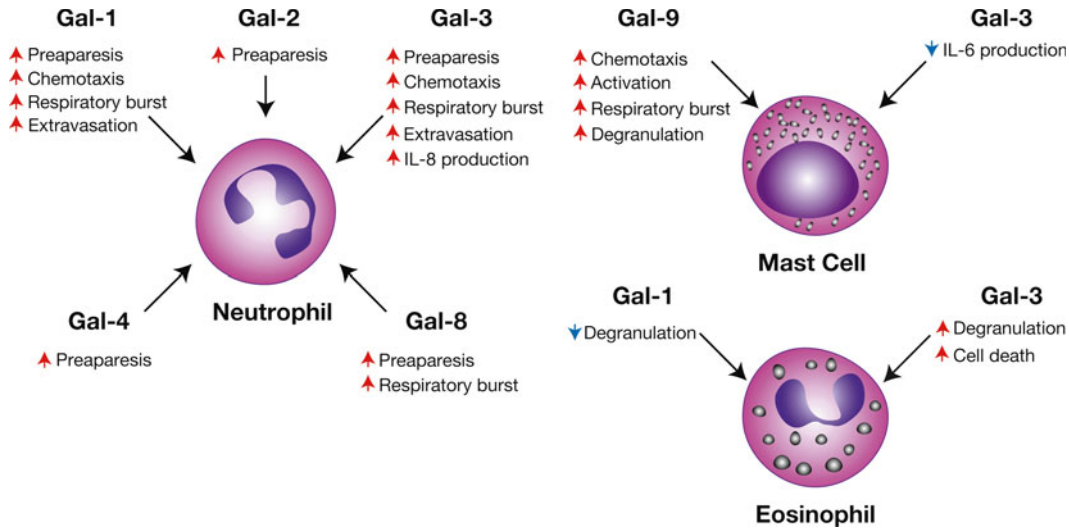

Fig. 4 Galectins regulate granulocyte activation and turnover. Galectin family members appear to possess the ability to differentially activate various granulocytes, including neutrophils, eosinophils, and mast cells. In addition, galectins appear to regulate the turnover and chemotaxis of various granulocyte populations. Representative galectin-regulated activities are shown. *Red arrows* indicate an activity that the respective galectin increases, while *blue arrows* signify galectin-induced decreases in the accompanying activity

that Gal-1 can induce neutrophil NADPH activation and enhance superoxide production, possibly through interactions with CD66a and CD66b [93, 94]. In addition to facilitating neutrophil activation, different galectin family members also appear to regulate neutrophil extravasation and induce differential effects on chemotaxis, depending on the activation state of the cells [131–134]. Similar to what had been observed in activated T cells [28], several studies suggest that galectins may also aid in neutrophil turnover. For example, recent results suggested that Gal-1 induces exposure of PS, a common ligand responsible for triggering cellular removal in cells undergoing apoptosis, in activated neutrophils and the promyelocytic cell line, HL60 [30, 95, 96]. However, in contrast to the effects of Gal-1 on T cells, induction of PS exposure in activated neutrophils by Gal-1 appeared to occur in the conspicuous absence of cell death [30, 95–97]. Despite the inability of Gal-1 to induce apoptosis in activated neutrophils, Gal-1-induced PS exposure sensitized cells to phagocytic removal [95, 97]. This form of removal may be unique to neutrophils [98] as it prepares cells for removal without inducing cell death, a process recently termed "preaparesis" [30]. As late apoptosis is often accompanied by loss of membrane integrity, this form of cellular turnover may facilitate the maintenance of membrane integrity in the setting of acute inflammation until appropriate phagocytic removal occurs. The ability to induce PS exposure in the absence of apoptosis in neutrophils does not appear to be limited to Gal-1, as subsequent studies demonstrated that Gal-2, Gal-3, Gal-4, and Gal-8 also possess a similar ability to induce apoptosis-independent expression of PS [96, 99, 100], although distinct signaling pathways appear to be engaged [96].

In addition to potentially regulating neutrophils, early studies suggested that galectins may also regulate other granulocyte populations. Galectin-10, the first galectin actually described, but originally known as Charcot-Leyden protein [101, 102], accumulates as crystals in vivo under allergic or parasitic conditions associated with hypereosinophilia [102]. Although the exact stimulus and purpose of copious Gal-10 accumulation in this setting remains to be elucidated, these results suggest that Gal-10 may serve a regulatory or effector role in eosinophil function. Originally described as one of several T cell-derived factors capable of regulating eosinophils [103], Gal-9 secreted by activated T cells appears to drive eosinophil chemotaxis [104]. Subsequent studies suggested that Gal-9 may not only facilitate eosinophil migration, but may also regulate extravasation and viability [105–107].

In addition to regulating neutrophils and eosinophils, some of the earliest work examining the potential immunomodulatory activity of galectins involved mast cell biology. Pioneering work by Fu-Tong Liu's group demonstrated that a unique protein, the epsilon binding protein, recognized IgE. Subsequent studies identified

epsilon binding protein as Gal-3 [108, 109]. Gal-3 not only appeared by bind IgE [110] but also recognized the Fcε receptor (FcεR) on mast cells [111], suggesting a general regulatory role for Gal-3 in mast cell function. Indeed, Gal-3 appears to directly engage the FcεR and induce mast cell degranulation [111]. Consistent with this, Gal-3 KO mast cells display significantly impaired degranulation [112]. In addition, recent studies suggest that Gal-3 may actually regulate mast cell viability [113]. In contrast to Gal-3, Gal-1 may induce the opposite effect on mast cells, as injection of Gal-1 appears to inhibit mast cell degranulation following inflammatory challenge in vivo [114] (Fig. 4).

10 Galectins Regulate Hemostasis, Tissue Repair, and Angiogenesis

Given the breadth of activities attributed to individual galectin family members in the regulation of immunity and the intimate association of immunity with coagulation [115], it appears that galectins also evolved important roles in hemostasis (Fig. 5).

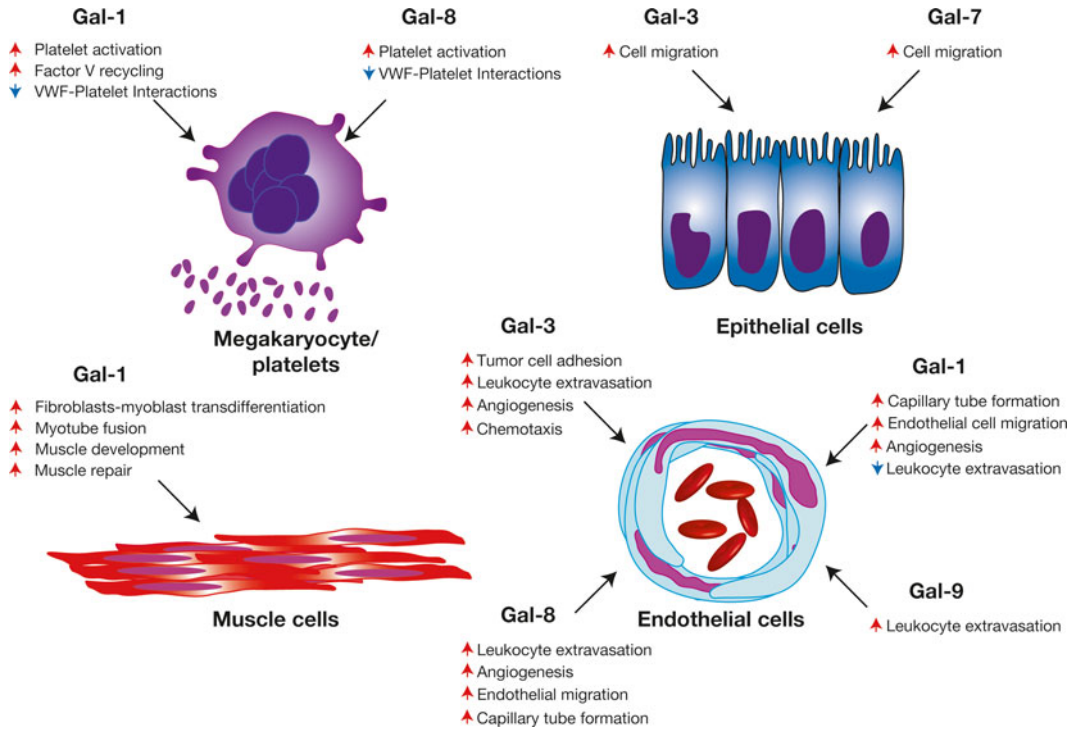

Fig. 5 Galectins regulate hemostasis, angiogenesis and tissue repair. Various members of the galectin family regulate megakaryocyte activity, hemostasis, angiogenesis, epithelial migration, and general tissue repair following injury. Representative galectin-regulated activities are shown. *Red arrows* indicate an activity that the respective galectin increases, while *blue arrows* signify galectin-induced decreases in the accompanying activity. Plt = platelet, vWF = von Willebrand Factor

Several studies demonstrated that Gal-1 directly triggers platelet activation and aggregation in vitro, likely through interactions with αIIβ3 integrins [116]. In addition, Gal-1 appears to enhance platelet sensitivity to activation by other agonists [117]. Although the addition of exogenous Gal-1 can induce platelet activation, co-incubation of platelets with a Gal-1 blocking antibody significantly inhibits ADP-induced platelet aggregation [117], strongly suggesting a role for platelet-derived Gal-1 in platelet activation. Consistent with these in vitro results, Gal-1 KO mice experience increased bleeding time [116], suggesting that Gal-1 plays an important role in hemostasis in vivo. Galectin-mediated platelet activation does not appear to be limited to Gal-1, as Gal-8 similarly activates platelets [118]. However, in contrast to Gal-1, Gal-8 signals through a GP1β-dependent pathway, although a similar downstream Erk pathway becomes activated following engagement by either galectin [116, 118]. In addition to regulating platelet function, Gal-8 appears to regulate Factor V levels by enhancing its endocytic recycling in megakaryocytes [119]. In contrast, Gal-1 and Gal-3 appear to negatively regulate von Willebrand factor (vWF) interactions with platelets through direct interactions with vWF *N* glycans [120].

In addition to regulating hemostasis, different galectin family members appear to play a critical role in tissue repair. Work by Panjwani and colleagues demonstrated that Gal-7 in particular plays a critical role in epithelial repair, as Gal-7 induces the cell migration and proliferation necessary for wound closure in an in vitro wound healing model [121, 122] (Fig. 5). Consistent with this, Gal-7 KO mice displayed reduced epithelial repair following wounding in vivo [123]. Gal-3 likewise appears to play a critical role in this process, while Gal-1 failed to exhibit similar activity [121]. In addition to epithelial repair, early studies suggested that Gal-1 might facilitate muscle development and repair by inducing myotube fusion and fibroblast to myocyte transdifferentiation [124, 125]. Gal-1 displays a unique striated localization pattern within muscle and muscle injury results in a significant increase in Gal-1 expression [126, 127]. Gal-1 KO mice exhibit impaired muscle regeneration following $BaCl_2$-induced muscle injury [124]. Furthermore, knockdown of Gal-1 in zebrafish resulted in impaired muscle formation, suggesting a role in embryonic muscle development [128].

An important component of injury repair is the development of blood vessels, in which endothelial cells play a critical role [129]. Endothelial cells express several galectin family members, including Gal-1, Gal-3, and Gal-9 and activation increases galectin expression [130]. While enhanced galectin expression may regulate endothelial interactions with leukocytes and subsequent leukocyte extravasation [131–134], several galectins appear to directly impact endothelial cell-mediated regulation of angiogenesis [135, 136].

Incubation of endothelial cells with Gal-3 results in microtube development [137], possibly through NG2, a driver of angiogenesis [138], by enhancing NG2 interactions with α3β1 integrins [138]. Several studies suggest that Gal-1 may also facilitate angiogenesis, as knockdown of Gal-1 reduces endothelial migration and expansion. Unlike Gal-3, Gal-1-mediated regulation of angiogenesis appears to occur through interactions with VEGFR1 and VEGFR2 [139]. Disruption of this interaction and subsequent loss of VEGFR2 signaling likely contributes to the pathogenesis of preeclampsia, as Gal-1 KO mice exhibit lower levels of desidual vascularization and an increased frequency of preeclampsia [140]. Consistent with this, individuals with preeclampsia display reduced levels of Gal-1 expression [140]. Similar studies suggested that Gal-8 might induce endothelial cell migration and angiogenesis through interactions with CD166 [141]. In contrast, a recent study suggested that Gal-1 might engage CD146 to induce endothelial cell apoptosis [142].

11 Intracellular Roles of Galectins

Although the extracellular carbohydrate binding activities of galectins became their defining feature [143], galectin synthesis occurs within the cytosol, where individual galectin family members often accumulate prior to export through a noncanonical Golgi-independent pathway [144–146]. Given their location within the cytosol, several studies have implicated various galectin family members as key players in the regulation of a variety of intracellular processes [147] (Fig. 6). For example, Gal-3 binds GSK-β and β-catenin and can facilitate GSK-β-mediated signaling and transcriptional upregulation of downstream targets, such as fascin-1 [148, 149]. Phosphorylation of Gal-3 appears to modulate Gal-3-mediated regulation of cellular signaling and survival, possibly through altering potential interactions with K-Ras-GTP [150, 151]. Additional studies suggest that similar intracellular phosphorylation events may also regulate Gal-3-mediated extracellular activities by impacting the ability of Gal-3 to recognize carbohydrate ligands [152]. Intracellular Gal-3 can also regulate signaling pathways involved in TRAIL, Fas, and TNF-induced apoptosis [153–155]. As Gal-3 contains the bcl-2 NWGR motif, several studies suggest that Gal-3-mediated regulation of cell survival may occur through a bcl-2 pathway [155, 156]. In contrast, intracellular Gal-7 appears to induce keratinocyte apoptosis following UVB-induced damage [157]. Gal-1 also appears to directly regulate cellular signaling by modulating Ras localization and signaling [158]. Gal-12 plays a critical role in adipocyte signaling [159], which likely contributes to the increased lipolysis and reduced adiposity displayed by Gal-12 KO mice [160]. While studies suggest

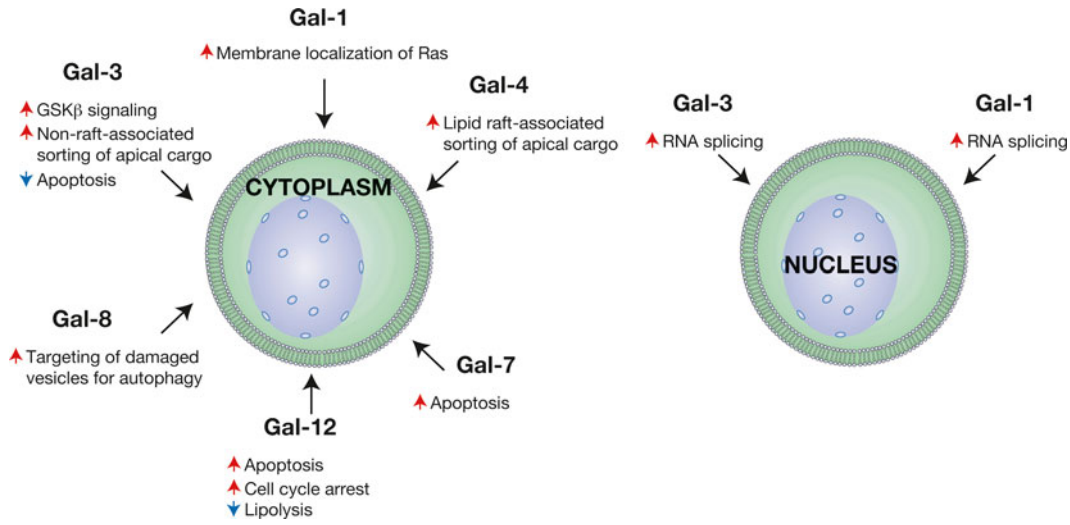

Fig. 6 Galectin family members regulate various intracellular processes. While the earliest studies on galectins demonstrated that these proteins recognize extracellular carbohydrate ligands, subsequent studies have demonstrated that galectins possess significant roles in the regulation of a variety of fundamental intracellular activities, both in the cytoplasm and the nucleus. Representative galectin-regulated activities are shown. *Red arrows* indicate an activity that the respective galectin increases, while *blue arrows* signify galectin-induced decreases in the accompanying activity

that several members of the galectin family, such as Gal-3 and Gal-4, likely regulate membrane trafficking through carbohydrate recognition in distinct membrane microdomains outside the cell [161, 162], recent studies suggest that Gal-8 may recognize extracellular carbohydrate ligands exposed during vesicular damage following bacterial infection intracellularly [163]. Gal-8-mediated recognition of damaged vesicles appears to target these contents for autophagy, providing a unique form of intracellular antimicrobial defense [163].

In addition to regulating signaling pathways in the cytosol, galectins appear to modulate nuclear activities within the cell (Fig. 6). For example, some of the earliest work suggesting an intracellular role for galectins demonstrated that Gal-1 and Gal-3 might regulate a process as fundamental as RNA splicing [164]. Using nuclear extracts isolated from HeLa cells to examine RNA processing, depletion of Gal-1 or Gal-3 significantly inhibited RNA splicing, while reconstitution of only one of these galectins would largely restore this activity, strongly suggesting potential compensatory activity [164]. Subsequent studies demonstrated that Gal-1 and Gal-3 localize not only in the nucleus but also co-localize and directly interact with known factors involved in RNA splicing [165–167]. Consistent with their potential role in nuclear activities, including transcriptional

regulation [168], nuclear import of galectin family members appears to be a highly regulated process [169, 170]. Inhibition of Gal-3 nuclear trafficking, by preventing serine phosphorylation via site directed mutagenesis, appears to significantly impact its ability to regulate cell survival [171].

12 Galectins and Neoplastic Disease

As galectins appear to play key regulatory roles in general immunity, hemostasis, vascular biology, cell signaling and viability, it is not surprising that altered galectin expression may impact neoplastic transformation and progression. Given the fundamental contribution of cell growth dysregulation in neoplastic transformation, it is fitting that some of the earliest studies suggesting that galectins may facilitate neoplastic transformation examined the potential impact of intracellular galectins on neoplastic cell survival. For example, seminal studies by Liu, Raz and others demonstrated that upregulation of Gal-3 can significantly reduce cellular sensitivity to apoptotic stimuli [150, 151, 153–155]. In contrast, neoplastic transformation of keratinized epithelial cells appears to result in downregulation of Gal-7 [157], consistent with its ability to induce apoptosis following cellular injury [172]. Furthermore, dysregulation of other galectin pathways also appears to likewise impact cellular signaling, with a significant effect on cell growth and neoplastic progression [173]. In addition to directly regulating neoplastic transformation at the cellular level, galectin family members may also facilitate overall tumor growth and dissemination by enhancing tumor angiogenesis [174, 175]. Several galectins, in particular Gal-3, appear to engage circulating neoplastic cells and enhance their extravasation, directly facilitating metastasis [176].

Given the role of immunosurveillance in the inhibition of neoplastic disease [177], transformed cells may also utilize galectins to inhibit immunological rejection of neoplastic lesions. Early studies demonstrated that overexpression of Gal-1 by neoplastic cells likely enhances neoplastic cell survival by directly inhibiting antitumor immunity. For example, overexpression of Gal-1 in a variety of settings, including neuroblastoma and breast cancer, appears to impair CD4+ T cells, CD8+ T cells and DC function, strongly suggesting a role for Gal-1 in the suppression of antitumor immunity [178, 179]. Consistent with this, knockdown of Gal-1 in neoplastic cells prior to transfer in vivo results in considerable expansion and enhanced function of CD4+ and CD8+ T cells, decreased levels of Tregs and enhanced DC function [178, 179]. Gal-1-mediated regulation of antitumor immunity may similarly impact neoplastic hematological disease. Increased expression of Gal-1 in patients with Hodgkin's lymphoma correlates with a poorer prognosis and

may likewise reflect significant modulation of antitumor immunity [34, 180]. In contrast, other galectins may also actually facilitate antitumor immunity. For example, Gal-9-mediated alterations in macrophage activation appear to result in significant NK cell activation, thereby enhancing NK cell-mediated antitumor immunity [92].

13 The Biochemistry of Galectins

Sensitivity to oxidative inactivation—In addition to the ability of galectins to recognize carbohydrates, the unique sensitivity of several galectin family members to oxidative inactivation represents one of their earliest recognized features. Indeed, the recognition of galectin sensitivity to redox environments provided the first name of this class of GBPs: the S-type, or thiol-dependent, lectins [181–185]. Some of the first studies on Gal-1 demonstrated that in the absence of reducing conditions Gal-1 gradually oxidizes [182], which results in profound conformational changes that preclude dimerization and recognition of carbohydrate ligands [186–188] (Fig. 7). However, redox regulation is not limited to Gal-1, as several other galectins, including Gal-2 and Gal-7, appeared to also be sensitive to oxidative inactivation.

While Gal-1 oxidation does occur following removal of reducing agents, several factors appear to regulate this process. Gal-1 engagement of ligand stabilizes dimer formation and reduces Gal-1 sensitivity to oxidative inactivation [12, 182, 188] (Fig. 7). As a result, the unique sensitivity of Gal-1 to oxidative inactivation may be critical in regulating the spatial and temporal activity of this and related family members. Rapid oxidation of Gal-1 following tissue damage likely reduces the ability of Gal-1 and other galectins to inhibit neutrophils and other leukocytes immediately following injury, allowing these cells to neutralize potential pathogens and remove necrotic debris. However, as neutrophils and other leukocytes encroach on viable tissue surrounding an area of inflammation, leukocyte mediated-damage may release reduced, and therefore active, Gal-1, allowing engagement of leukocyte ligands and signaling of leukocyte turnover [127, 188]. Furthermore, while the redox environment can directly impact Gal-1 function, several studies suggest that the redox status of the cells themselves may also alter cellular sensitivity to Gal-1 signaling. For example, while Gal-1 typically induces apoptosis in activated T cells in the presence of reducing conditions [28], similar alterations in viability may be less apparent in the absence of reducing agents [30, 96, 97]. As a result, the outcome of Gal-1 engagement of cellular ligands appears to reflect the integration of a variety of signals that determine whether cellular death occurs. It remains possible that reducing conditions in vitro may also serve as a surrogate for other

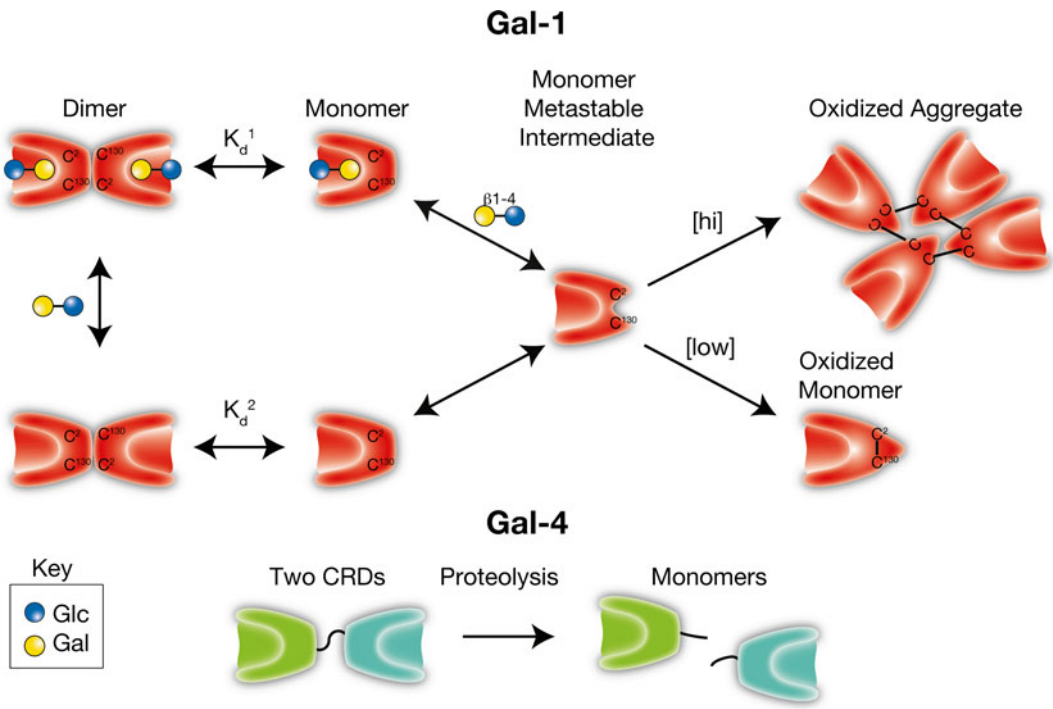

Fig. 7 Galectin activity is regulated by oxidation and proteolysis. Galectins, first called S-type lectins secondary to the requirement of several galectins for reduced thiols to maintain carbohydrate recognition activity, can form intramolecular and intermolecular disulfide bridges that often result in significant conformational changes that preclude carbohydrate recognition. As monomers appear to be a key intermediate in oxidative inactivation and carbohydrates can drive dimerization, ligand appears to reduce oxidative inactivation by facilitating dimer formation ($K_d^1 < K_d^2$). [hi] = higher concentrations of Gal-1. [low] = lower concentrations of Gal-1. In contrast, several galectins, especially tandem repeat and chimeric galectins, rely on linker peptide bound carbohydrate recognition domains or N-terminal collagen-like domains to facilitate dimerization/oligomerization. Cleavage of intervening peptides that connect oligomerization domains to functional carbohydrate recognition domains can render carbohydrate recognition domains monomeric and therefore incapable of generating molecular lattices typically thought to be required for optimal galectin-mediated signaling

unknown factors in vivo that similarly regulate cellular fate following Gal-1 engagement. While Gal-1 oxidation results in loss of carbohydrate binding activity, oxidized Gal-1 itself appears to possess distinct biological activity. Several studies demonstrate that oxidized Gal-1 can facilitate neuronal regeneration [189, 190], suggesting that oxidation may not only limit Gal-1 carbohydrate binding activity, but also result in acquisition of alternative biological properties. In this way, Gal-1, and perhaps other galectins behave as morpheeins, proteins that possess the unique ability to regulate diverse biological processes depending on the conformational state of the protein [191].

Although oxidative inactivation likely plays a critical role in the regulation of many prototypical galectins, additional regulatory mechanisms likely govern the activity of other galectin family members.

For example, chimeric Gal-3 and tandem repeat galectins, unlike prototypical galectins, possess unique domains or linker peptides that facilitate oligomerization or functional bivalency. Sequences between the collagen-like "oligomerization" domain and the carbohydrate recognition domain of Gal-3, can be cleaved by several proteases, including matrix metalloproteinases 2 and 9 and collagenase 3 [192, 193]. As Gal-3-mediated signaling and galectin signaling in general often requires clustering and lattice formation of counter receptors, collagen domain removal-induced loss of Gal-3 oligomerization likely contributes to the inability of the CRD of Gal-3 alone to signal cells [194]. While recent studies suggest that the individual domains of tandem repeat galectins may retain unique biological activity, many of the putative functions of tandem repeat galectins also require intact full length proteins for optimal signaling [100, 195], suggesting that proteolytic cleavage of the linker peptide responsible for tethering carbohydrate recognition domains would likewise inhibit this subgroup's function [196] (Fig. 7).

Carbohydrate recognition—Perhaps the most unique and defining feature of galectins lies in their ability to regulate cell behavior through the recognition of highly modifiable carbohydrate structures [5]. Although it should be noted that subtle, but fundamental differences in carbohydrate binding preferences were noted in elegant studies utilizing only a few defined carbohydrates [197, 198], these early studies were limited by the number of available test glycans [197, 198]. Using frontal affinity chromatography coupled with a large library of target compounds, Hirabayashi and colleagues demonstrated that individual galectin family members possess unique specificity for many different carbohydrates [199]. Additional studies, using a wide variety of different modalities in addition to frontal affinity chromatography, including isothermal calorimetry, fluorescence polarization, and more recently, glycan microarrays [99, 199–201], strongly suggest that individual galectin family members exhibit unique and overlapping carbohydrate binding specificity that in addition to differences in quaternary structure [99, 100, 202], likely contributes to the distinct and overlapping biological activities displayed by this protein family.

Although galectins recognize the common disaccharide motif Galβ1-4GlcNAc, numerous studies suggest that many galectin family members prefer polymers of this structure in the form of poly-*N*-acetyllactosamine (polyLacNAc), when expressed on the cell surface. However, the mode and mechanisms of polyLacNAc interactions appear to vary among different galectin family members, resulting in a differential impact of polyLacNAc modification on galectin recognition [100, 203]. For example, while Gal-1 and Gal-2 recognize the terminal *N*-acetyllactosamine (LacNAc) determinant [204], these prototypical galectins prefer the motif expressed on extended glycans, either as *N*-glycans or polyLacNAc

glycans bearing this terminal LacNAc structure [203–205]. As Gal-1 and Gal-2 exists as homodimers, yet only recognize the terminal LacNAc motif, this preference likely only occurs following glycan immobilization, where extended glycans may make cross-linking interactions more favorable. Consistent with this, in solution-based assays, Gal-1 and Gal-2 do not display the same preference for extended ligands [203, 205]. Not only may homodimerization influence carbohydrate binding specificity, but binding of ligand itself can actually enhance Gal-1 dimerization [188]. Similar results demonstrate that ligand may also influence the quaternary state of other galectins, such as Gal-3 [202]. In contrast to Gal-1 and Gal-2, Gal-3 and Gal-8, which do not exist as rigid homodimers, recognize internal LacNAc motifs within extended polyLacNAc [100, 203]. As these two galectin family members possess oligomeric carbohydrate recognition domains organized through flexible linker peptides, conformational constraints that provide the specificity of Gal-1 and Gal-2 toward extended glycans terminating in LacNAc following immobilization may not be relevant. Consistent with this, Gal-3 displays a preference for polyLacNAc repeats in solid phase and solution-based assays [203].

Distinct recognition of polyLacNAc results in a differential impact of polyLacNAc sialylation, a highly regulatable glycan modification, on the binding and signaling of these galectin family members [22]. Sialylation of the terminal galactose of LacNAc commonly occurs in two distinct linkages. The α2-3 sialylation linkage reflects addition of the sialic acid to the 3-OH of galactose, while α2-6 sialylation refers to the attachment of sialic acid to the 6-OH of galactose. As Gal-1 and Gal-2 recognize the terminal LacNAc, sialylation can significantly and differentially impact glycan recognition [206]. For example, although Gal-1 recognizes α2-3 sialylated glycans, it fails to recognize glycans following α2-6 sialylation [22, 203]. In contrast, Gal-2 fails to recognize glycans following sialylation with either linkage [203]. Gal-3 binds internal LacNAc within polyLacNAc, and sialylation of polyLacNAc with either linkage fails to significantly alter Gal-3 binding and signaling [22, 203], although in different cellular contexts sialylation may also impact Gal-3 binding and signaling [206]. Unlike Gal-1, Gal-2, and Gal-3, Gal-4, Gal-8, and Gal-9 possesses two unique carbohydrate recognition domains. Intriguingly, the N-terminal domain of Gal-8 recognizes sialic acid, while the C-terminal domain prefers non-sialylated glycans [207]. In contrast, the individual domains of Gal-4 and Gal-9 do not appear to possess drastic differences in glycan binding. Recent studies suggest that Gal-8 may form dimers and signal leukocytes entirely through the C-terminal domain [100], suggesting that tandem repeat galectins may in general form higher order quaternary structures that may be important in signaling. Additional studies suggest that Gal-9 can also associate with Gal-3 and Gal-8, suggesting not only that galectins may form higher order homotypic oligomers, but that complex

hetero-oligomers may also occur [208]. Given the ability of galectins to form highly ordered lattices on cell surfaces and the potential impact of galectin-induced lattice formation in galectin signaling [194], these results suggest that in addition to distinct carbohydrate recognition preferences, differences in quaternary structure also likely impact the potential outcome of galectin engagement of a cell. Alterations in galectin binding to glycoconjugate ligands may not only regulate cellular signaling, but could also significantly impact galectin-mediated regulation of the sorting of cell surface glycoproteins [209–211]. In addition to common glycoprotein modifications, galectins appear to also possess affinity for glycolipids, including GM1 [212]. Given the proclivity of GM1 for unique lipid microdomains [213], galectin–glycolipid interactions may directly impact cellular signaling [214].

14 Galectins in Innate Immunity

As galectin family members recognize a wide variety of highly modifiable carbohydrate structures, it is not surprising that individual galectin family members appear to be implicated in nearly every biological process described. However, recent studies provide insight into what may be one of the first biological roles of galectin family members—their ability to recognize pathogens [215]. Similar to the ability of mannose-binding protein, complement and other soluble immune factors [216, 217], Gal-3 recognizes a variety of microbes, including *Neisseria gonorrhoeae*, *Candida Albicans*, *Toxoplasma gondii*, *Leishmania major*, *Schistosoma mansoni*, and *Trypanosoma cruzi* [8, 215, 218–222], while Gal-9 also appears to recognize *Leishmania major* [223, 224]. Similarly, several studies suggest that Gal-1 binds Nipah, Influenza, and HIV viruses [225, 223] [226, 227] and that other galectins may specifically recognize unique microbial species [9].

Galectin-mediated interactions with pathogens appear to result in a wide variety of consequences, from enhanced phagocytosis to direct killing (Fig. 8). For example, Gal-3 recognition of *Candida albicans* induces direct microbial killing [8]. In contrast, Gal-3 binding of *Toxoplasma gondii* glycosylphosphatidylinositols facilitates parasite recognition and TNF production by macrophages [218]. In addition to recognizing glycans unique to pathogens, recent results suggest that several galectins may be able to protect individuals from microbes that express self-like antigens. For example, Gal-4 and Gal-8 recognize and kill microbes expressing the blood group B antigen, providing a potential mechanism of protection against blood group B expressing pathogens in blood group B positive individuals [9]. Recent studies utilizing microbial glycan microarrays suggest that Gal-4 and Gal-8 recognition of self-like microbial determinants may not be limited to blood group antigens, but that galectins may recognize a variety of microbes,

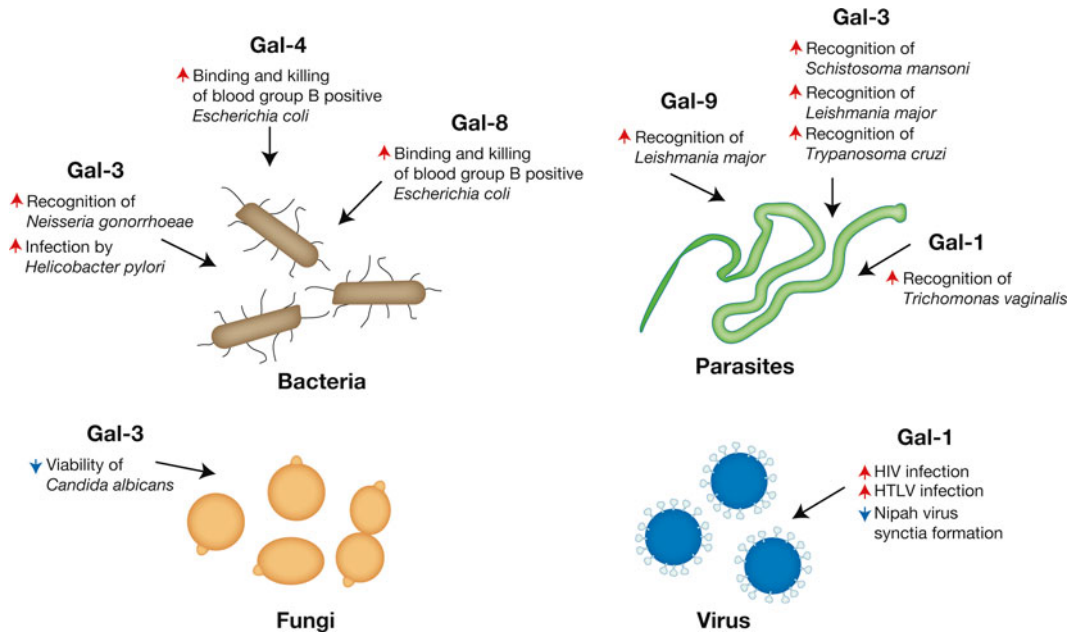

Fig. 8 Galectins recognize a diverse range of pathogens. While the immunoregulatory roles of galectins likely represent some of their most well known functions, the ability of galectins to recognize a diverse range of pathogens may reflect some of their earliest evolutionary activities. Galectin recognition of pathogens can result in opsonization or direct microbial killing. In contrast, pathogens may utilize galectins to facilitate attachment and invasion. Representative galectin-regulated activities are shown. *Red arrows* indicate an activity that the respective galectin increases, while *blue arrows* signify galectin-induced decreases in the accompanying activity

both gram negative and gram positive, that share the unique feature of generating mammalian-like carbohydrate determinants [228, 229]. These results suggest that galectins may provide a broad form of innate immunity against molecular mimicry. Several studies also suggest that Gal-1 binds and inhibits entry of Nipah and Influenza viruses [225, 223]. Thus, galectin recognition of pathogens appears to provide innate immunity through a variety of mechanisms.

In contrast to innate immune activity of galectins, several pathogens appear to have subverted galectin function and use these proteins as a mechanism of host attachment and invasion (Fig. 8). For example, key work by Sato and colleagues demonstrated that Gal-1 may actually facilitate HIV entry during infection [226, 227], likely through direct glycan interactions between gp120 and CD4 [230]. Gal-1 also appears to facilitate *Trichomonas vaginalis* infection by enhancing attachment to the vaginal epithelium [231], while Gal-3 appears to similarly aid in the colonization of *Neisseria meningitidis* [222]. Galectin facilitation of pathogen survival does not appear to be limited to mammals, as a Gal-9 orthologue, PpGalec, found in the midgut of the sandfly *Phlebotomus papatasi*, mediates attachment and facilitates survival of *Leishmania major* in this key vector [232].

15 Summary

Although the discovery of galectins over 30 years ago stemmed from interest in understanding the roles of carbohydrates in fundamental biological processes, this finding ultimately uncovered an entire family of potent regulatory proteins [12–14]. Given their nearly ubiquitous expression and ability to bind highly modifiable carbohydrate ligands expressed on nearly every cell, in addition to a variety of other intracellular and extracellular partners, these proteins appear to be uniquely poised to regulate a wide variety of biological activities. Consistent with this, individual members of the galectin family have been implicated in various aspects of nearly every biological process described. As a result, these proteins may not only be some of the most ancient lectins known, but given their history throughout evolution, also appear to have been a unique evolutionary substrate in many different biological systems. Future studies will undoubtedly continue to unravel additional biological functions of this complex and intriguing protein family.

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Chapter 2

Cloning, Expression, and Purification of Galectins for In Vitro Studies

Paul A. Poland, Carol L. Kinlough, and Rebecca P. Hughey

Abstract

Galectins are best known for their ability to bind glycoconjugates containing β-galactose, but classification of these small proteins within the galectin family is also defined by amino acid homology within structural domains and exon/intron junctions within genes. As galectins are expressed by organisms as diverse as some fungi, *C. elegans*, fish, birds, and mammals, and biological activities attributed to galectins are equally diverse, it becomes essential to identify, clone, and characterize galectins from many sources. Glutathione S-transferase (GST) fused to the amino-terminus of galectin cDNAs has proven to be especially useful for preparation of recombinant galectins in bacteria for use on glycan arrays, in experiments with cultured or isolated cells, and in pull-down assays with immunopurified glycoproteins. Many galectins are stabilized by reducing reagents, such that binding and elution of GST-galectins from glutathione-conjugated Sepharose with excess glutathione is both efficient and innocuous. The ability to bind and elute GST-galectins from lactose-conjugated Sepharose with excess lactose provides a relatively easy means to insure that galectins are competent for glycoconjugate binding prior to experimentation. This chapter focuses primarily on the varied approaches to use GST-galectin binding to glutathione- and lactose-conjugated Sepharose to purify recombinant galectins and then develop effective experimental protocols to characterize the specificity, interactions, and function of galectins cloned from any source. We provide one example where a pull-down assay with all the GST-tagged canine galectins reveals that the C-terminal carbohydrate recognition domain of galectin-9 (Gal-9C) specifically recognizes the glycan-dependent apical targeting signal from the glycoprotein MUC1.

Key words Galectin, GST-galectin, Recombinant galectin, Pull-down assay

1 Introduction

Our interest in the role of galectins (Gal) in glycan-dependent apical targeting of transmembrane MUC1, in the well-characterized model system of polarized Madin Darby canine kidney (MDCK) epithelial cells, led us down the unforeseen path of cloning and characterizing all of the canine galectins [1, 2].

At the start of our odyssey, Gal-3 and Gal-4 were implicated in glycan-dependent and lipid raft-dependent sorting of apical proteins, respectively, in polarized epithelial cells [3–7]. Gal-3 was

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purported to cross-link glycoproteins while Gal-4 was thought to cross-link glycolipids, thereby producing sorting platforms for clustering of proteins. As our preliminary data were most consistent with glycan-dependent targeting of MUC1 [2], we focused on knockdown of Gal-3 with siRNAs in MDCK cells but found no effect on MUC1 apical targeting [8]. In order to consider a role for other galectins in apical targeting in MDCK cells, we had to both determine which galectins were actually expressed by dogs and which were present in MDCK cells [9].

Using Nucleotide BLAST and TBLASTIN programs with both the dog genome and the NCBI Nucleotide Database, we identified sequences which aligned with nucleotide sequences and exon profiles, or amino acid sequences, for mammalian Gal-1, -2, -3, -4, -7, -8, -9, and -12, as well as the galectin-related HSPC159. HSPC159 lacks essential residues in the carbohydrate recognition domains for sugar binding. We found no canine genes homologous to rat Gal-5, mouse Gal-6, human Gal-10, human Gal-13, ovine Gal-14, ovine Gal-15, human PP13, or human PPL13.

We estimated the levels of transcripts for the canine galectins in MDCK cells using RT-PCR and primers designed to yield ~200 bp sequences. RNA from canine jejunum was used as a positive control. Amplification of RNA for Gal-1, -3, -8, and -9 was readily achieved by this approach while amplification of RNA for Gal-4 and -7 required nested primers to produce a band on an agarose gel with ethidium bromide staining. Based on these data we concluded that transcript levels varied (Gal-3 > Gal-9 > Gal-8 > Gal-1 >>> Gal-4 > Gal-7). While we did not obtain evidence for Gal-2 or Gal-12 transcripts in MDCK cells, a subsequent study by Friedrichs et al. [10] using real-time PCR reported that transcripts for Gal-3 were 100 times more abundant than transcripts for Gal-1, -8, and -9, and 1,000 times more abundant than for Gal-12, essentially consistent with our findings [9].

We first attempted to clone the canine galectins by RT-PCR from RNA isolated from MDCK cells using primers designed to match nucleotide sequences overlapping the predicted translational start and stop codons. Full-length cDNAs for canine Gal-1, -3, and -8 were obtained by this approach, while cloning of Gal-4 and -7 required amplification with nested primers where external primers matched nucleotide sequences within the 3′ or 5′ UTR, and internal primers overlapped the start and stop codons. The cDNAs for Gal-2 and -9 required amplification from a commercial source of canine jejunum RNA, and Gal-12 cDNA was eventually obtained by amplification from canine heart RNA. The cDNAs were cloned and sequenced for alignment with the canine genome and determination of the exon/intron structure of the genes for comparison to other mammalian galectin genes. We found 73–86 % identity

between canine and human amino acid sequences of the galectin with missing or extra exons representing previously described short (Gal-8 and Gal-12) and long (Gal-9) isoforms, respectively. We found five residues within exon 1 of Gal-7 as described for cow, but unique from that of mouse (two) and human (six), and six 9-mer amino acid repeats in Gal-3 as compared to cow (four), mouse (three), pig (three), human (two), rat (two), and rabbit (one). Although these differences are small, they could affect galectin biological activities and we did proceed to isolate the GST-tagged recombinant canine galectins for characterization on glycan arrays of Core H of the NIH Consortium for Functional Glycomics. This involved expression of GST-galectins in bacteria and the realization that GST-Gal-9 and -12 formed aggregates in bacteria and were not useful constructs. We subsequently subcloned the two different carbohydrate recognition domains from Gal-9 as it was prevalent in MDCK, while Gal-12 was not, producing GST-Gal-9N and GST-Gal-9C. GST-Gal-1, -3, -4, -7, -8, -9N, and -9C were purified from bacterial extracts by affinity binding to glutathione-conjugated and then lactose-conjugated Sepharose. Binding of GST-galectins on glycan arrays was then followed with an Alexa 488-conjugated anti-GST antibody [9].

Finally, to assess which endogenous galectins are likely interacting with MUC1 expressed in polarized MDCK cells, we carried out preliminary experiments where human MUC1 immunopurified from stably transfected MDCK cell extracts was incubated with recombinant GST-tagged Gal-1, -3, -4, -7, -8, -9N, and -9C in pull-down assays. Interestingly, we found that MUC1, whether metabolically labeled with [^{35}S]Met/Cys or followed by immunoblotting, interacted best with Gal-3 and Gal-9, the two most abundant galectins expressed in MDCK cells [9]. In subsequent experiments we found that core-glycosylated mucin-like tandem repeats (0TR) from MUC1 are an apical targeting signal in polarized MDCK cells when appended to a non-polarized protein (Tac) [8]. We now present data from galectin pull-down assays comparing interactions with Tac and 0TR-Tac that clearly shows that Gal-9C specifically binds to the apical targeting motif of MUC1.

2 Materials

2.1 Cloning of Galectins

1. RNA from specified organisms and/or tissues (Zyagen) (*see* **Note 1**).
2. RNA Superscript II reverse transcriptase and Taq DNA polymerase (Invitrogen).
3. PCR primers (Integrated DNA Technologies).

4. pCR2.1 TOPO vector for DNA cloning (Invitrogen).
5. pGEX-6P-1 for production of glutathione S-transferase (GST) fusion galectins (GE Health Care Life Sciences) (*see* **Note 2**).
6. Bacteria XL-1 Blue (Agilent Technologies).

2.2 Expression and Purification of GST-Galectins

1. *E. coli* strain BL21 (EMD Millipore).
2. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma).
3. Luria Bertani (LB) broth.
4. Ampicillin (Sigma).
5. Lysis-Sonication buffer: 300 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 % Triton X-100 and 50 mM Tris-HCl, 0.1 mM phenylmethanesulfonylfluoride (PMSF), pH 8.0.
6. Glutathione conjugated to Sepharose (GE Healthcare).
7. PD-10 gel filtration columns (GE Healthcare).
8. Sepharose CL-6B (Sigma) (*see* **Note 3**).
9. Lactose-conjugated Sepharose (Sigma).
10. Elution buffer (with glutathione): 100 mM Tris–HCl, pH 8.0, 15 mM glutathione, 1 mM dithiothreitol (*see* **Note 4**).
11. Tris buffered saline: 0.1 M Tris–HCl, pH 8, 150 mM NaCl, 14 mM beta-mercaptoethanol (βME).
12. Elution buffer (with lactose or sucrose): 0.1 M Tris–HCl, pH 8, 150 mM NaCl, 14 mM beta-mercaptoethanol (βME), 0.1 mM α-lactose (Sigma) (*see* **Note 4**).
13. 1-cm diameter column (8 cm tall) containing a sintered glass filter.
14. 15-ml conical plastic tubes.

2.3 Preparation of GST-Galectins for Pull-Down Assays

1. Lactose-conjugated Sepharose (Sigma).
2. Glutathione conjugated to Sepharose (GE Healthcare).
3. Sepharose CL-6B (Sigma).
4. Hamilton syringe, 100 μl size with a 22S-gauge needle (Gastight).
5. PreScission protease (GE Healthcare).
6. 15-ml conical tubes.

2.4 Preparation of Immunopurified Proteins

1. Glutathione conjugated to Sepharose (GE Healthcare).
2. Protein G or Protein A conjugated to Sepharose (Invitrogen).
3. [^{35}S]Met/Cys (Perkin Elmer).
4. Hepes-buffered saline" (10 mM Hepes, pH 7.4, 150 mM NaCl) with 1 % Triton X-100 or 0.1 % SDS.

5. Hamilton syringe, 100 μl size with a 22S-gauge needle (Gastight).
6. Criterion Precast Gels (4–15 % Tris–HCl, 1 mm).
7. BioRad Criterion Gel system.
8. SDS-PAGE sample buffer (BioRad).
9. Nitrocellulose (Millipore, 0.45 μm).
10. Bio-safe Coomassie (BioRad).
11. 1.5-ml tubes with snap cap.

2.5 Special Equipment

1. Thermocycler.
2. BioRad Phosphorimager.
3. Quantity One Software.

3 Methods

3.1 Cloning

1. First strand cDNA is synthesized from 1 μg RNA using RNA Superscript II reverse transcriptase and amplified using Taq DNA polymerase as described by the manufacturer.
2. Primers for PCR to amplify the full-length transcripts are designed to overlap the predicted start and stop codons using nucleotide sequence information from database queries (*see* **Note 5**).
3. Amplify DNA using standard PCR technique.
4. Amplified DNA is cloned into pCR2.1 TOPO vector as described by the supplier.
5. Transform bacteria XL-1 Blue by the heat shock protocol.
6. Culture bacteria and isolate colonies.
7. Plasmid preps from multiple colonies should be generated.
8. Sequence isolated cDNAs from individual colonies in order to verify the predicted amino acid sequence of new galectins.

3.2 Expression and Purification of GST-Galectins

1. Galectin cDNAs are subcloned into pGEX-6P-1 for expression in bacteria as N-terminal GST-fusion proteins based on a previous protocol [11]. Details are found below.
2. LB medium (20 ml) containing ampicillin is inoculated with 200 μl of glycerol stock of *E. coli* strain BL21 transformed with pGEX-6P-1 encoding GST-galectin and grown overnight with shaking at 37 °C.
3. This overnight 20-ml starter culture is used to inoculate 2 l of culture medium with ampicillin (divided between two 2 l flasks) and grown with shaking for 2–3 h at 37 °C until it reaches an OD_{600} of 0.8.

4. Isopropyl β-D-1-thiogalactopyranoside is then added to 0.1 mM to induce expression of the fusion protein, and bacteria are grown with shaking overnight at room temperature.
5. Bacteria are collected by centrifugation at 25,000×*g* for 12 min at 4 °C in 250-ml centrifuge bottles. Pellets of bacteria can be frozen at –20 °C at this step for later use.
6. Pellets of bacteria are subsequently resuspended in 10 ml of Lysis-Sonication buffer (with PMSF) using a glass rod and transfer pipettes.
7. Bacteria are further lysed by sonication on ice with a Fisher Sonic Dismembrator Model 100 (five times, at setting 5 (8–12 W) for 30 s with 30 s intervals) (see **Note 12**).
8. The lysate is centrifuged for 30 min at 10,000×*g* at 4 °C to pellet cell debris.
9. The supernatant from the lysed bacteria is incubated at 4 °C overnight with 0.75 ml of a 50 % slurry of glutathione-conjugated Sepharose in a 15-ml conical tube with end-over-end mixing.
10. Beads are subsequently pelleted after centrifugation for 2 min at 500×*g* (Eppendorf model 5702 swinging bucket centrifuge) and the supernatant removed with a transfer pipette.
11. Beads are washed three times by addition of 5 ml of Lysis-Sonication buffer, mixing and centrifugation, as already described.
12. GST-galectins are released from the washed glutathione-conjugated Sepharose beads by adding 5 ml of Elution buffer (with glutathione), mixing, and incubation at 4 °C for 15 min.
13. After centrifugation for 2 min at 500×*g*, supernatant (eluate) is removed with a transfer pipette and saved.

Alternative protocol for purifying galectin from glutathione eluate (*see* **Notes 6** and **7**).

14. The entire eluate from the glutathione-conjugated Sepharose beads is incubated with 1 ml of a 50 % slurry of lactose-conjugated Sepharose in a 15-ml conical tube at 4 °C for 30 min with end-over-end mixing.
15. The slurry is then poured into a 1-cm diameter column containing a sintered glass filter and the packed column bed is overlayed with 1 ml of a 50 % slurry of Sepharose 6B. The column bed is washed with 5 ml Tris buffered saline.
16. To elute the GST-galectins from the column, 2 ml of elution buffer (with lactose).
17. 0.5 ml is collected from the bottom, and the column closed and capped for 30 min at 4 °C.

18. Fractions of 0.25 ml are subsequently collected after adding buffer to the top of the column.
19. The absorbance at 280 nm is determined with a spectrophotometer for each fraction.
20. The peak fractions are pooled and lactose is removed on a PD-10 gel filtration column as described by the manufacturer (*see* Fig. 1 for representative elution profiles) (*see* **Note 8**).

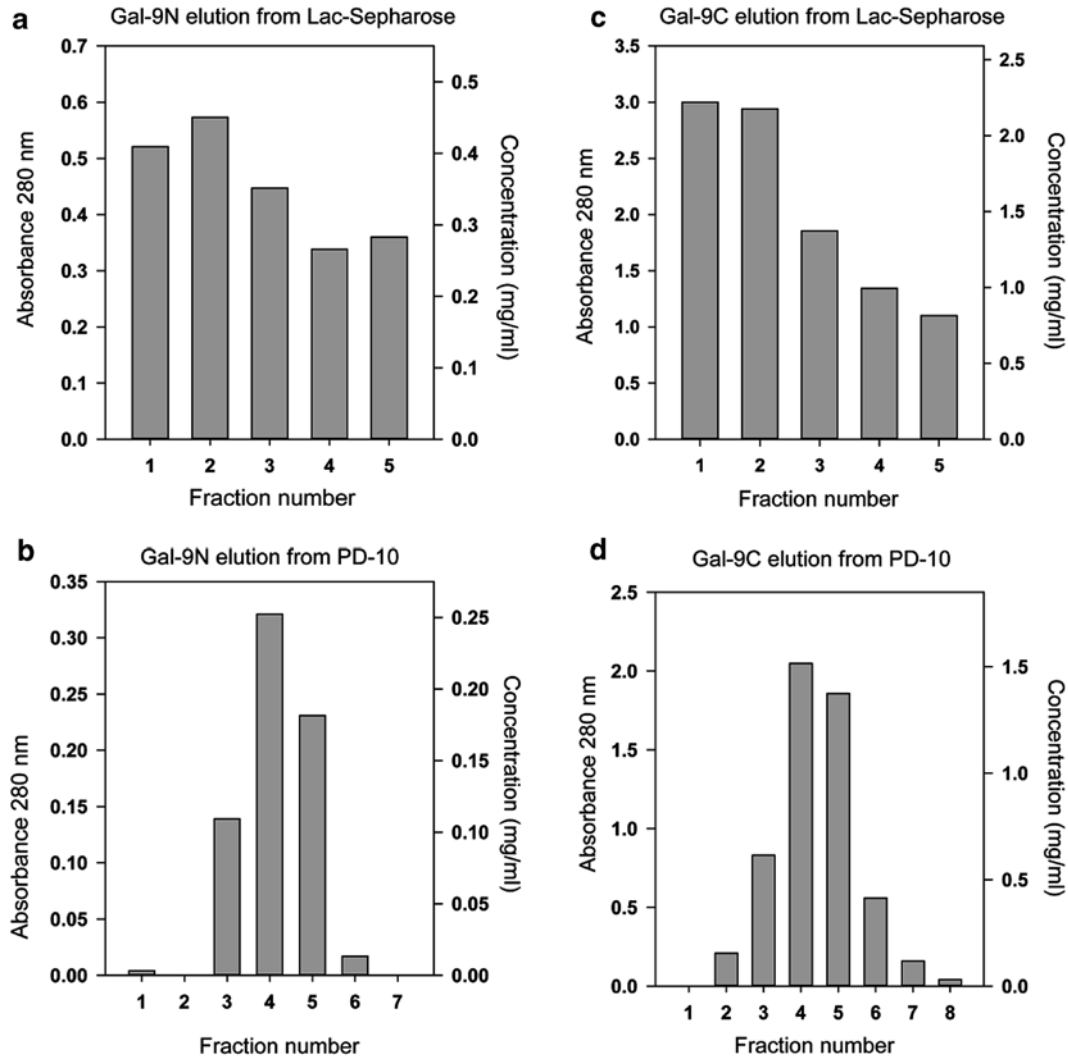

Fig. 1 Affinity purification of GST-galectins on Lac-Sepharose. After batch elution of recombinant GST-galectins from glutathione conjugated to Sepharose, the GST-galectins can be stored at −80 °C. Before experimental use, GST-galectins are affinity purified by incubation with lactose conjugated to Sepharose (Lac-Sepharose) and eluted after incubation in a column equilibrated with α-lactose (**a** and **c**). Lactose is removed from the GST-galectins using a PD-10 gel filtration column (**b** and **d**). See text for protocol details. Example profiles for GST-Gal-9N (**a** and **b**) and GST-Gal-9C (**c** and **d**) are shown here using calculated extinction coefficients of 55010M-1 and 56620M-1, respectively (A_{280} on *left* axis and concentration on *right* axis).

3.3 Preparation of GST-Galectins for Pull-Down Assays

1. Aliquots of thawed GST-galectins (*see* **Note 7**) for pull-down assays are first affinity purified on lactose-conjugated Sepharose before binding to glutathione beads and incubation with immunopurified proteins (*see* Subheading 3.4). Tris buffered saline containing βME is used throughout the protocol (*see* **Note 4**).
2. An aliquot of GST-galectin (1 μg) is added to 30 μl of a mixed slurry of lactose-conjugated Sepharose and carrier Sepharose CL-6B in a 1.5-ml tube with snap cap and incubated for 30 min at 4 °C with end-over-end mixing (*see* **Note 9**).
3. After centrifugation at 500 × *g* in a table-top microcentrifuge for 2 min, the supernatant is removed and discarded and the pellet is washed two times with 150 μl Tris buffered saline to remove any unbound GST-galectin that is potentially "inactive." Liquid is always removed from the beads with a Hamilton syringe (100 μl size with a 22S-gauge needle) (*see* **Note 10**).
4. GST-galectin is eluted from the lactose-conjugated Sepharose by incubation with 30 μl of Elution buffer (with lactose) for 15 min at 4 °C with end-over-end mixing.
5. After centrifugation at 500 × *g* in a table-top microcentrifuge for 2 min, the supernatant containing the "active" GST-galectin is transferred to a mixed slurry of glutathione-conjugated Sepharose and carrier Sepharose CL-6B in a 1.5-ml tube with snap cap, and incubated for 15 min at 4 °C with end-over-end mixing (*see* **Note 11**).
6. After centrifugation at 500 × *g* in a table-top microcentrifuge for 2 min, the beads are washed twice with 200 μl of Tris buffered saline and all liquid removed from the pellet with a Hamilton syringe.
7. The final pellet of GST-galectin bound to glutathione-conjugated beads is subsequently mixed with immunopurified protein already prepared from cell extracts (*see* Subheading 3.4). Alternatively, the beads can be resuspended into 100 μl of Tris buffered saline and 30–45 μl can be transferred to different tubes of immunopurified proteins to produce duplicate or triplicate samples.
8. The GST tag on GST-galectin prepared in the pGEX-6P-1 vector can also be removed by treatment of GST-galectin prebound to glutathione-conjugated beads using PreScission protease to cut at the single engineered consensus sequence LEVLFQ/GP between the GST and galectin as directed by the manufacturer (where / is the cleavage site and letters are abbreviations for amino acids).

3.4 Pull-Down Assays with GST-Galectins

1. Pull-down assays can be carried out either by following protein binding by immunoblotting or by following binding of [^{35}S] proteins recovered by metabolic labeling of cell cultures

(described here). The exact protocol for preparation of each immunopurified protein should be optimized by the researcher.

2. Multiple wells (or permeable supports) of cultured cells expressing the protein of interest are metabolically labeled with [^{35}S]Met/Cys for 15–60 min and chased in medium containing Met and Cys for 60–120 min (*see* **Note 12**). Cells are extracted from each well in Hepes buffered saline with a mild detergent such as octyl-glucoside or Triton X-100, centrifuged to remove cell debris and nuclei, and the supernatants combined.
3. [^{35}S]Proteins are recovered by immunoprecipitation from the combined supernatants in 1.5-ml tube with snap cap using specific antibodies and either Protein G or Protein A conjugated to Sepharose beads. Proteins are eluted from the beads by heating at 90 °C for 2 min in 200 μl Hepes buffered saline containing 0.1 % SDS (heating is optional). The samples are centrifuged at 9,300 × *g* in a table-top microcentrifuge twice for 30 s to pellet the beads.
4. Equal aliquots of eluant (20 μl) are transferred to clean snap cap tubes. One of the aliquots is retained as the *input* sample and mixed with 10 μl SDS gel sample buffer. The remainder of the aliquots is diluted tenfold with Hepes buffered saline containing βME and 1 % Triton X-100 to neutralize the 0.1 % SDS prior to addition of freshly prepared GST-galectins prebound to glutathione-conjugated Sepharose beads. One tube receives Sepharose CL-6B beads as a negative control (i.e., there should be no binding to the beads alone). *See* Fig. 2 for a representative experiment with eight different GST-galectins, beads alone and *input* sample (total).
5. [^{35}S]Proteins are incubated with the GST-galectins prebound to glutathione beads overnight at 4 °C with end-over-end mixing. After centrifugation at 500 × *g* in a table-top microcentrifuge for 2 min, the supernatant is removed and the pellet washed two times with 200 μl Tris buffered saline containing βME to remove any unbound [^{35}S]proteins.
6. Beads are incubated for 30 min at 4 °C with 10 μl Elution buffer (with sucrose) βME and 100 mM *sucrose* to elute proteins bound nonspecifically. After centrifugation at 500 × *g* in a table-top microcentrifuge for 2 min, the eluant is removed with a Hamilton syringe and saved.
7. Beads are incubated for 30 min at 4 °C with 10 μl Elution buffer (with lactose) containing βME and 100 mM *lactose* to elute bound proteins. After centrifugation at 500 × *g* in a table-top microcentrifuge for 2 min, the eluant is removed with a Hamilton syringe and saved.
8. Beads are incubated for 30 min at 4 °C with 10 μl Elution buffer (with glutathione) containing βME and 15 mM *glutathione*

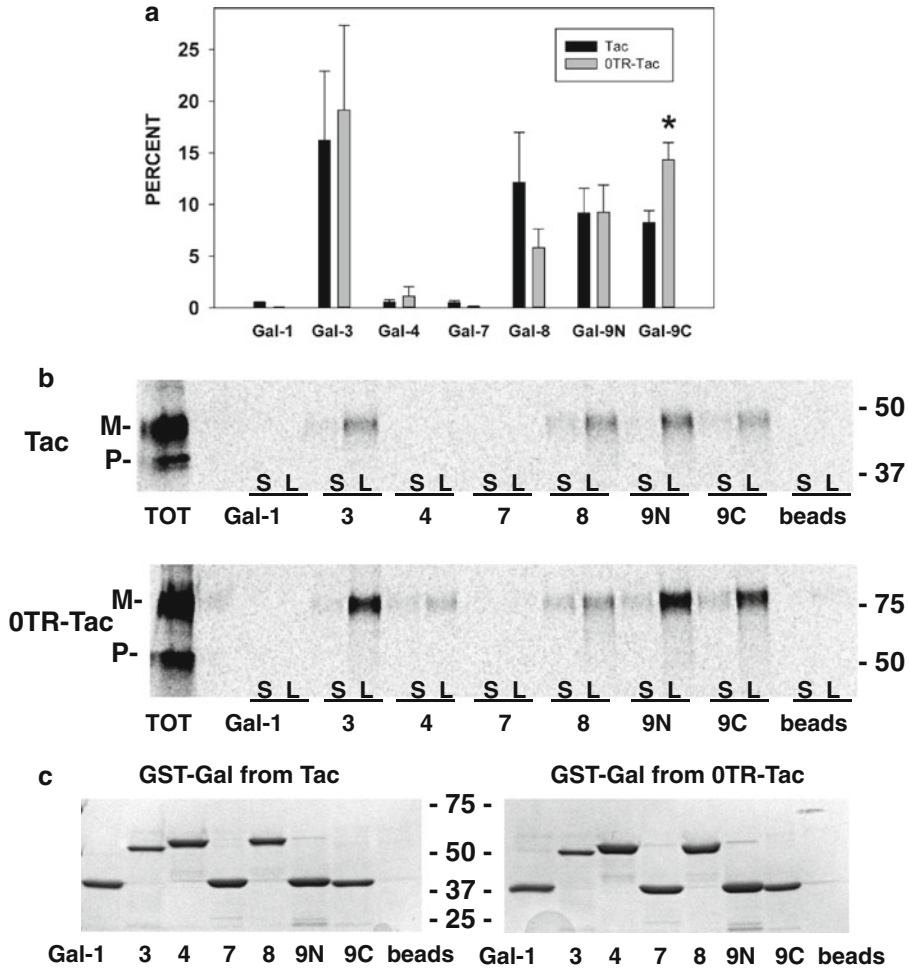

Fig. 2 Pull-down experiments with GST-galectins reveal specific binding of GST-Gal-9C to the apical targeting signal from MUC1. Core-glycosylated mucin-like tandem repeats (0TR) from MUC1 are an apical targeting signal in polarized MDCK cells when appended to a non-polarized protein (Tac) [8]. To identify specific galectins that bind to 0TR, we compared Tac and 0TR-Tac binding to GST-galectins in pull-down assays. (**a**) Equal aliquots of immunopurified [^{35}S]Tac or [^{35}S]0TR-Tac were incubated overnight with fresh GST-galectins bound to GSH-beads (or beads alone) and eluted with sucrose (S), then lactose (L), and analyzed after SDS-PAGE with a BioRad imager. The percent [^{35}S]Tac or [^{35}S]0TR-Tac eluted with lactose was calculated from the input total (TOT) aliquot and is presented as the mean and SEM for three experiments. [^{35}S]0TR-Tac binding to only GST-Gal-9C was consistently greater than [^{35}S]Tac binding ($p < 0.05$). Note that mature (M), but not precursor (P), forms were bound. One representative experiment is presented in (**b**) showing a side-by-side comparison of [^{35}S]Tac or [^{35}S]0TR-Tac binding. The GST-galectins from each sample were eluted with glutathione for SDS-PAGE, and analyzed by scanning the Coomassie-stained SDS-gel. Mobility of MW markers in kDa is noted between the two gels (**c**). See text for protocol details

to elute GST-galectins. After centrifugation at 500×*g* in a table-top microcentrifuge for 2 min, the eluant is removed with a Hamilton syringe and saved.

9. Eluants from *sucrose*, *lactose*, and *glutathione* incubations are subjected to SDS-PAGE by mixing 10 µl of sample with 10 µl SDS sample buffer and heating at 90 °C for 2 min.
10. After electrophoresis, the proteins eluted by *sucrose* and *lactose* can be electrophoretically transferred to nitrocellulose. Nitrocellulose is dried for 1 h at room temperature, stored 0.5–3 days with a Kodak TR screen and bands analyzed with a BioRad Phosphorimager and Quantity One Software. The SDS-gel containing proteins eluted with *glutathione* (GST-galectins) is stained with Bio-safe Coomassie. The stained gel is scanned and bands analyzed with Quantity One Software. *See* Fig. 2 for a representative experiment with immunopurified [^{35}S]proteins and pull-down with eight different GST-galectins.
11. The dry nitrocellulose can be subsequently hydrated and used for immunoblotting after blocking using standard protocols. Bands are visualized with a BioRad Versadoc or by scanning bands on exposed film, and quantified using Quantity One Software.
12. The fraction of immunopurified protein bound and specifically eluted from each GST-galectin is calculated using the *input* aliquot as 100 %. These values are normalized with the amount of each GST-galectin bound to the glutathione-conjugated beads (based on the Coomassie stained gel and divided by the individual formula weight of the GST-galectin).

4 Notes

1. Alternatively, RNA can be isolated from tissues or cell lines using commercial kits (Ambion RNAqueous 4PCR Kit) following the directions of the manufacturer.
2. It may be necessary to express carbohydrate binding domains from tandem repeat galectins separately to assess binding specificities individually. On the other hand, we found that canine GST-Gal-9 repeatedly aggregated in the bacteria, so we subcloned the N-terminal and C-terminal CRDs separately as GST-Gal-9N (residues 1–148) and GST-Gal-9C (residues 225–355), respectively, for further study. In more recent studies it appears that GST on the C-terminus rather than the N-terminus of Gal-9 permits expression of the full-lenght galectin.
3. Sepharose CL-6B is used because it is cross-linked and will not be altered by heating in 1 % SDS.

4. Beta-mercaptoethanol or dithiothreitol is included in buffers to stabilize the galectins as many have reactive Cys residues.
5. When either the start or stop codon cannot be located in the database, the full-length cDNA should be obtained using standard approaches such as RACE-PCR [12]. Amplification of rare transcripts may also require the use of nested primers whereby an aliquot (1 %) of the amplified DNA obtained by PCR with external primers after 28 cycles is amplified a second time for 28 cycles with internal primers.
6. At this stage, the GST-galectin can be divided into aliquots and frozen at −80 °C after addition of βME to 14 mM. Before any further use, GST-galectins should be affinity purified on lactose-conjugated Sepharose to be sure that the galectin is active (i.e., competent for binding glycans). Protein concentration of GST-galectin is estimated from Coomassie staining after SDS-PAGE as glutathione interferes with spectrophotometer readings at A_{280}.
7. GST-galectins can be batch eluted from the lactose-conjugated Sepharose. Elution from the column allows retrieval of the most concentrated fractions as needed for protocols such as screening glycan arrays. Sufficient GST-galectin for pull-down assays can be obtained from a 50 ml culture of bacteria (~5 μg is eluted from the glutathione-conjugated Sepharose).
8. Protein concentrations of GST-galectins are calculated from A_{280} using individual extinction coefficients determined from the amino acid content of each GST-Gal using the Peptide Property Calculator found online (http://www.basic.northwestern.edu/biotools/proteincalc.html). Be sure to include the amino acid content of GST in the calculation. Pooled peak samples (1–1.5 ml) routinely contain ~0.25–1 mg GST-galectin. This active concentrated GST-galectin is optimal for use on glycan arrays.
9. A 50 % slurry of lactose-conjugated Sepharose (25 μl) is mixed with a 50 % slurry of carrier Sepharose CL-6B (225 μl), and 30 μl of the mixed slurry is transferred to a 1.5-ml conical tube with snap cap for subsequent addition of 1 μg of GST-galectin.
10. This size of Hamilton syringe allows removal of all the liquid while excluding the Sepharose beads when the tip is placed at the bottom of the tube in the middle of the pellet.
11. A 50 % slurry of glutathione-conjugated Sepharose (90 μl) is mixed with a 50 % slurry of Sepharose CL-6B (270 μl). A 40 μl aliquot of the mixed slurry is used for each aliquot of GST-galectin eluted from the mixed slurry of lactose-conjugated Sepharose.
12. Our investigation of MUC1 binding to canine GST-galectins was carried out by metabolic labeling of polarized cultures of

MDCK epithelial cells with [^{35}S]Met/Cys for 30 min and a chase in normal culture medium for 90 min, a time profile previously determined for efficient surface expression of MUC1. [^{35}S]MUC1 eluted from GST-galectins (or the input control) was subjected to SDS-PAGE and transferred to nitrocellulose such that we were able to analyze both newly synthesized radiolabeled MUC1 and then steady-state MUC1 by immunoblotting. Data can also be obtained from analysis of a dried SDS-gel.

Acknowledgments

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Chapter 3

Alkylation of Galectin-1 with Iodoacetamide and Mass Spectrometric Mapping of the Sites of Incorporation

Sean R. Stowell, Connie M. Arthur, Richard D. Cummings, and Christa L. Feasley

Abstract

Galectins can display unique sensitivity to oxidative changes that result in significant conformational alterations that prevent carbohydrate recognition. While a variety of approaches can be utilized to prevent galectin oxidation, several of these require inclusion of reducing agents that not only prevent galectins from undergoing oxidative inactivation, but can also interfere with normal redox potentials required for fundamental cellular processes. To overcome limitations associated with placing cells in an artificial reducing environment, cysteine residues on galectins can be directly alkylated with iodoacetamide to form a stable thioether adduct that is resistant to further modification. Iodoacetamide alkylated galectin remains stable over prolonged periods of time and retains the carbohydrate binding and biological activities of the native protein. As a result, this approach allows examination of the biological roles of a stabilized form of galectin-1 without introducing the confounding variables that can occur when typical soluble reducing agents are employed.

Key words Alkylation, Galectin, Mass spectrometry, Oxidation, Reducing agents

1 Introduction

Unlike most proteins secreted from cells, galectins primarily reside within the cytosol where they can be secreted into the extracellular space through an endoplasmic reticulum and Golgi apparatus-independent pathway [1, 2]. Synthesized on free ribosomes in the cytosol of cells, galectins are maintained in a reduced state prior to secretion from the cell secondary to the reducing environment within the cell [3]. Following secretion, galectins can engage carbohydrate ligands outside the cells [4], which appears to reduce galectin sensitivity to oxidative inactivation in the extracellular environment and allows galectins to participate in a wide range of biological activities, from regulating a variety of cellular activities to directly providing innate immunity [5–10]. However, in the absence of carbohydrate ligand, some members of the galectin

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family, in particular galectin-1, can undergo intramolecular disulfide oxidation, which results in significant alteration in the protein's conformation that precludes dimerization and carbohydrate recognition [5, 11–14]. However, in the presence of ligand, galectin-1 experiences enhanced dimerization, which limits formation of monomers that appear to be an intermediate for effective galectin-1 oxidation [5].

While galectin-1 oxidation likely reflects a normal pathway that regulates protein function [15, 16], oxidation can complicate the assessment of galectin-1 activity in the context of other biological systems. As a result, many studies include reducing agents in treatment buffers to reduce the impact of oxidation when assessing the carbohydrate recognition-dependent activity of galectin-1 [17, 18]. While this approach facilitates biochemical assessment of galectin-1 activity, such as carbohydrate binding specificity, when added to biological systems, reducing agents can unfortunately complicate the interpretation of results. For example, while inclusion of reducing agents such as dithiothreitol (DTT) and betamercaptoethanol (βME) can prevent galectin oxidation, these reagents also possess the ability to cross cell membranes where they can induce the unfolded protein response within the endoplasmic reticulum [19–21], likely secondary to preventing appropriate oxidation during protein folding [20]. While utilization of membrane impermeable reducing agents, such as reduced glutathione, can reduce the deleterious intracellular consequences associated with DTT or βME inclusion, these reagents can also reduce proteins normally oxidized on the cell surface, which can likewise alter their biological activity. As a result, a method to prevent galectin oxidation in the absence of residual soluble reducing agents is needed to maintain galectin-1 activity while avoiding the confounding influence of reducing agent inclusion.

Cysteine oxidation often reflects disulfide bond formation, as occurs during galectin-1 oxidation, which can be readily reversed upon inclusion of free thiol reducing agents, such as βME, DTT, or reduced glutathione [22]. As a result, these reducing agents form distinct molecular adducts that do not typically reflect the type of stable interactions commonly observed among other covalent linkages in biological systems [22] (Fig. 1). Thus, reduction of galectin-1 with βME, DTT, or reduced glutathione, followed by elimination of excess reducing agent, results in spontaneous adduct loss as reciprocal intramolecular disulfides form. In contrast, cysteine modification driven by reaction with iodoacetamide or maleimide results in the formation of a thioether, which represents a relatively irreversible adduct that

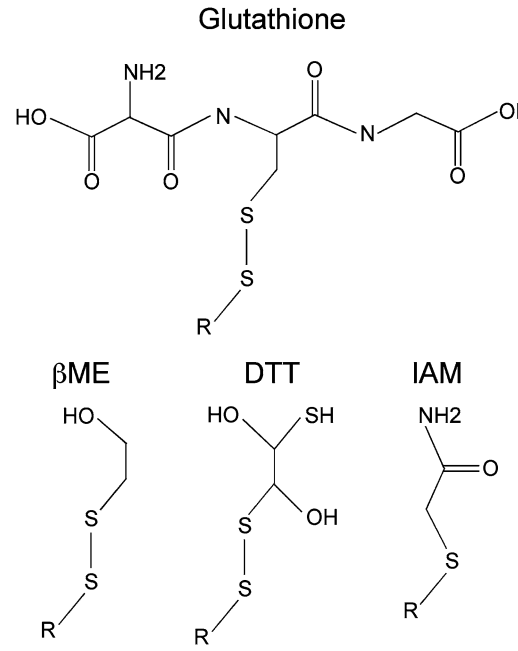

Fig. 1 Cysteine residues can be modified with various adducts to reduce intramolecular disulfide bond formation. Reduced glutathione, betamercaptoethanol (βME), and dithiothreitol (DTT) behave in a similar way, forming reversible disulfides with free thiols that can reduce intramolecular disulfide bond formation. In contrast, iodoacetamide (IAM) forms a stable thioether with cysteine that does not readily reverse upon removal of excess iodoacetamide

can prevent disulfide bond formation [23] (Fig. 1). Thus, following iodoacetamide alkylation, excess iodoacetamide can be removed from the alkylated protein, allowing galectin-1 to retain a stable adduct that prevents intramolecular disulfide bond formation and inactivation of the protein [23].

In this chapter, we describe the process of galectin-1 alkylation with iodoacetamide, including a description of methods required for mapping iodoacetamide incorporation (Fig. 2). In addition, we will discuss approaches for examining the effect of iodoacetamide alkylation on carbohydrate binding activity in addition to methods used to assess the protein's sensitivity to oxidation (Fig. 3). These methods should be of significant value when seeking to understand the potential role of galectin-1 in a variety of contexts while controlling for protein oxidation.

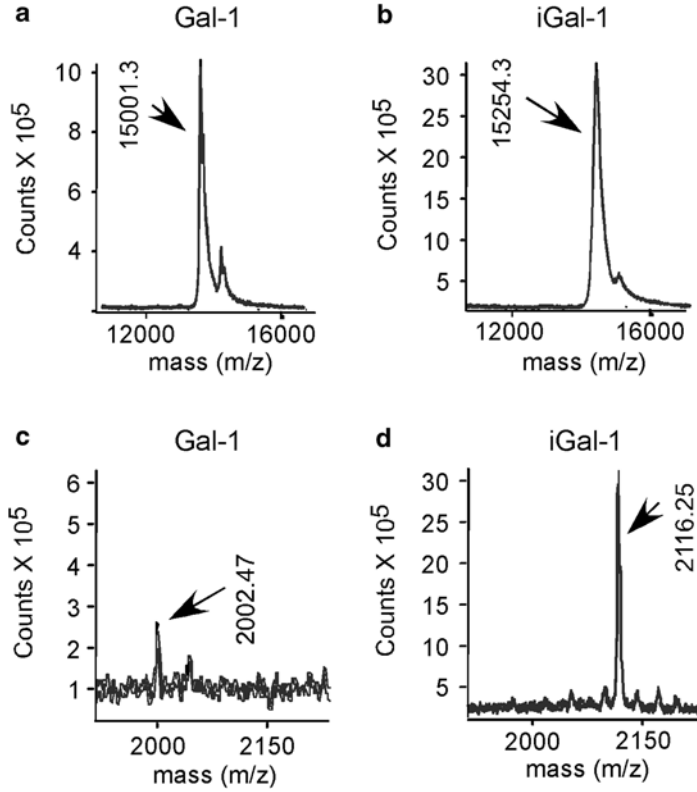

Fig. 2 Incubation of Gal-1 with iodoacetamide results in acetamide incorporation. (**a**, **b**) Mass spectrometry of galectin-1 (Gal-1) (**a**) or iodoacetamide-treated galectin-1 (iGal-1) (**b**). (**c**, **d**) Mass spectrometry of tryptic fragments of Gal-1 or iGal-1. Peak 2002.47 corresponds to the tryptic peptide fragment from Gal-1, while peak 2116.25 corresponds to same peptide after reaction with iodoacetamide. This research was originally published in the Journal of Biological Chemistry. Stowell SR, Cho M, Feasley CL, Arthur CM, Song X, Colucci JK, Karmakar S, Mehta P, Dias-Baruffi M, McEver RP, Cummings RD. Ligand reduces galectin-1 sensitivity to oxidative inactivation by enhancing dimer formation. 2009 Feb 20;284(8):4989-99 © the American Society for Biochemistry and Molecular Biology

2 Materials

2.1 Iodoacetamide Alkylation of Free Sulfhydryls on Galectin-1

1. Recombinant galectin (*see* **Note 1**).
2. PD10 gel filtration column (GE Healthcare) (*see* **Note 2**).
3. Phosphate Buffered Saline (PBS), standard pH 7.4 (Hyclone).
4. α-D-(+) Lactose, ACS grade (Fisher).
5. Iodoacetamide (IAM): 1 M stock freshly made in water (Sigma-Aldrich).
6. Purified water (dH_2O): The use of Barnstead/Millipore water (deionized, UV, and carbon filtered H_2O) for all buffer preparations is recommended.

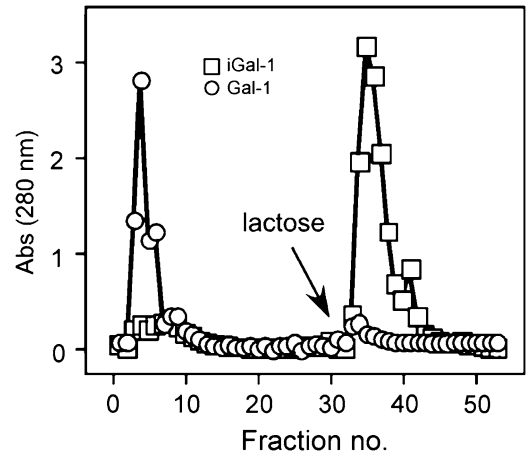

Fig. 3 Alkylation protects Gal-1 from oxidative inactivation. Galectin-1 (Gal-1) or iodoacetamide-treated Gal-1 (iGal-1) was incubated for 24 h in PBS at 37 °C followed by subjection to affinity chromatography over lactosyl-Sepharose. This research was originally published in the Journal of Biological Chemistry. Stowell SR, Cho M, Feasley CL, Arthur CM, Song X, Colucci JK, Karmakar S, Mehta P, Dias-Baruffi M, McEver RP, Cummings RD. Ligand reduces galectin-1 sensitivity to oxidative inactivation by enhancing dimer formation. 2009 Feb 20;284(8):4989-99 © the American Society for Biochemistry and Molecular Biology

2.2 Galectin Activity Assay

1. Lactosyl-Agarose column (Sigma-Aldrich) (*see* **Note 3**).
2. 2-Mercaptoethanol (2-ME) (Fisher) (*see* **Note 4**).
3. Affinity column loading buffer: Phosphate buffered saline with 2-mercaptoethanol (MEPBS)—0.01 M Na_2HPO_4, 0.85 % NaCl, pH 7.4 with 14 mM 2-ME.
4. Affinity column elution buffer: MEPBS with 100 mM lactose.
5. PD-10 gel filtration column (GE Healthcare) (*see* **Note 2**).

2.3 Proteolytic Digestion of Alkylated Galectin-1

1. Sequencing grade modified trypsin, 1 mg/mL in 50 mM acetic acid, store at −80 °C (Promega).
2. C_{18} Sep-Pak: 100 mg cartridge (Waters).
3. C_{18} Sep-Pak pre-equilibration solution: HPLC grade methanol, water, 50 % ACN, 0.1 % TFA in water.

2.4 HPLC Separation and Fraction Collection

1. Vydac analytical C_{18} column.
2. HPLC and MS solvents: Purified, deionized H_2O, HPLC grade or better is recommended (*see* **Note 5**).
3. Acetonitrile (ACN), LC-MS grade.
4. Trifluoroacetic acid (TFA), Sequencing grade 99.5 % (Thermo Scientific).
5. Formic acid, LC-MS grade, 99 % (Thermo Scientific).

HPLC buffers (see **Note 5***)*

6. HPLC buffer A: 94.9 % dH_2O, 5 % ACN, 0.1 % TFA. Add 50 mL ACN to 1 mL ampoule TFA and dilute to 1 L with dH_2O.
7. HPLC buffer B: 94.9 % ACN, 5 % dH_2O, 0.1 % TFA.

2.5 Mass Spectrometry Reagents and Software

1. Alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix solution.
2. MALDI target plate (Bruker).
3. Methanol: MS grade (Fisher, Optima grade or better).
4. Acetic acid (Fisher).
5. Flow splitter (Upchurch).
6. Peptide standard mixture for external mass calibration: bradykinin, angiotensin I, angiotensin II, ACTH clip 18-39 (Anaspec; Fremont, CA) at 1 nmol/μL in 50 % ACN, 0.1 % formic acid in dH_2O.
7. α-Cyano-4-hydroxycinnamic acid (CHCA) matrix solution, recrystallized (*see* **Note 6**): Make fresh daily, 10 mg/mL in 50 % ACN, 0.1 % TFA in dH_2O (Sigma-Aldrich).
8. C18 column (Vydac).
9. LC-MS buffers: (*see* **Note 5**).
   (a) LC-MS buffer A: 94.9 % dH_2O, 5 % ACN, 0.1 % formic acid.
   (b) LC-MS buffer B: 94.9 % ACN, 5 % dH_2O, 0.1 % formic acid.

Mass spectrometry analysis software

10. GRAMS: Perseptive (*see* **Note 7**).
11. flexAnalysis 2.4, BioTools 3.0: Bruker Daltonics.
12. ChemStation (Agilent Technology).
13. BioTools 3.0.

2.6 Special Equipment

1. Centrifugal vacuum concentrator, centrivap (Labconco) (*see* **Note 8**).
2. Fast Protein Liquid Chromatography (FPLC) (*see* **Note 9**).
3. High Performance Liquid Chromatography (HPLC) (*see* **Note 10**).
4. MALDI-TOF(/TOF)-MS: Voyager DE-STR (Perseptive Biosystems) or Ultraflex II (Bruker Daltonics).
5. ESI-MS: Agilent 1100 MSD ion trap with quaternary pump, diode array detector, and flow splitter into the ion trap (*see* **Note 11**).
6. Syringe pump (Harvard Apparatus).
7. Fraction collector, such as BioRad 2110.

3 Methods

3.1 Iodoacetamide Alkylation of Free Sulfhydryls on Galectin-1

1. Frozen galectin stocks should be stored in PBS with 100 mM lactose and 14 mM 2-ME (or other appropriate reducing agent) (*see* **Note 4**). Remove stock from storage at –80 °C and thaw on ice.
2. Equilibrate a PD-10 column with PBS and buffer exchange galectin over equilibrated column into PBS buffer.
3. Collect 0.5 mL fractions from PD-10 column and determine protein concentration. Pool fractions with protein concentrations greater than 1 mg/mL and add PBS such that the final galectin-1 (Gal-1) concentration is ~2–5 mg/mL in PBS.
4. To the purified galectin (2–5 mg/mL) sample, add 10 % v/v of 1 M IAM stock and incubate at 4 °C overnight (*see* **Note 12**).
5. Remove excess iodoacetamide by applying the protein solution to a PD-10 column pre-equilibrated with PBS. Elute Gal-1 from PD-10 column with PBS.
6. Verify Gal-1 activity after iodoacetamide treatment.

3.2 Galectin Activity Assay

1. Dilute recombinant galectin, both alkylated and non-alkylated, in PBS to the desired concentration (typically 1–20 μM) (*see* **Note 13**).
2. Add 250 μL of galectin PBS solution to a 48 well sterile tissue culture plate.
3. Incubate this plate at 37 °C in a humidified chamber for preestablished time intervals (*see* **Note 14**).
4. To determine residual activity, remove recombinant galectin solution and apply galectin to 1 mL packed column of lactosyl-agarose that has been equilibrated with PBS (*see* **Note 15**).
5. Once the galectin solution has penetrated the column, add five column volumes of PBS to the column and collect 0.5 mL fractions.
6. Add 100 mM Lactose in PBS as an elution buffer and continue to collect 0.5 mL fractions.
7. Determine the approximate protein concentration of each fraction by measuring the OD280 nm. Evaluate the percent of galectin in total lactose eluted fractions as a percent of the total starting material (*see* **Note 16**).

3.3 Proteolytic Digestion of Alkylated Galectin-1

1. To iodoacetamide-treated Galectin-1 samples add sequencing grade trypsin (1 mg/mL stock) at a 1:50 enzyme:substrate (w/w) ratio and incubate digest at 37 °C for 14 h.
2. To desalt peptides by C_{18} Sep-Pak, apply digested Galectin-1 peptide sample to a Sep-Pak cartridge pre-equilibrated with 3 mL sequential washes of C_{18} Sep-Pak pre-equilibration solution.
3. Then elute unbound species from the Sep-Pak by washing with 3×1 mL of C_{18} Sep-Pak pre-equilibration solution.
4. Finally, elute peptides with 3×1 mL of 50 % ACN, 0.1 % TFA in water.
5. Dry samples by rotary evaporation to 10–20 μL. Tryptic peptides from this step can be analyzed directly (go to Subheading 3.6 below) or after further purification (continue to Subheading 3.4) by LC-MS(/MS).

3.4 HPLC Separation and Fraction Collection

1. Add an equal volume (10–20 μL) of HPLC buffer A to Galectin-1 tryptic peptides.
2. Inject tryptic peptides onto the Vydac analytical C_{18} column pre-equilibrated with HPLC buffer A.
3. Elute buffer salts and unbound peptides with a two column volume of 98 % buffer A/2 % buffer B wash at 1 mL/min flow rate.
4. Separate peptides using a 1 mL/min linear gradient from 2 % buffer B to 100 % HPLC buffer B over 80 min, monitoring UV absorption at 215 nm. Collect 1 min fractions.
5. Concentrate fractions by vacuum centrifugation to a uniform volume of 100 μL.

3.5 MALDI-TOF and TOF/TOF Analysis

1. To analyze HPLC separated peptide fractions in positive or negative ion mode, spot 1 μL of each peptide fraction on a ground steel MALDI target plate (Bruker), add 1 μL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution, and dry under vacuum.
2. In parallel, prepare a peptide standard spot with 1 μL of the peptide calibration mixture and 1 μL of CHCA matrix solution.
3. Analyze galectin peptides with a Ultraflex II MALDI-TOF-TOF MS in reflectron positive ion mode with an accelerating voltage of 23 kV. Acquire spectra with 66 Hz laser frequency and sum 1,000–2,000 individual spectra for each sample.
4. Externally calibrate the TOF with the peptide calibration mixture using flexControl 2.4 software.
5. Galectin-1 peptides observed in the MS are accelerated by 8.5 kV and selected by a timed ion gate for MS/MS fragmentation.
6. TOF/TOF fragmentation is achieved by accelerating the fragment ions to 19 kV using the LIFT apparatus.

3.6 ESI-MS, Direct Infusion

1. Combine peptide fractions with an equal volume of methanol with 1 % acetic acid (100 μL) and directly infuse (5 μL/min) into the Agilent 1100 LC/MSD instrument equipped with an ion trap mass analyzer by a syringe pump.
2. Trap parameters are set as follows: Dry nitrogen is introduced into the capillary region at a flow rate of 5 L/min and heated to 220 °C. Compound stability is set to 35 % and trap drive level parameters are 70 %. Nebulizer gas pressure is set to 15 psi and the spray voltage is adjusted to 3 kV.
3. Use helium for collision-induced dissociation.
4. MS and tandem MS (MS/MS) data were acquired and processed using Bruker Trap software 4.1 in our lab.

3.7 LC-MS Analysis (see Note 17)

1. Inject galectin-1 tryptic peptides (10–20 μL) on an Agilent 1100 HPLC-MSD-Trap with a 4.6×250 mm C18 column pre-equilibrated with LC-MS buffer A.
2. Use a flow rate of 0.5 ml/min and a linear gradient 2 % LC-MS buffer B to 95 % LC-MS buffer B over 95 min and split the flow 1:10 using a flow splitter and direct 1/10 total column elution into the MSD trap.
3. Trap parameters should be set the same as in Subheading 3.6 with the following exceptions: Dry nitrogen is introduced into the capillary region at a flow rate of 8 L/min and heated to 300 °C. Compound stability is set to 30 %. Nebulizer gas pressure is set to 25 psi.
4. MS/MS fragmentation should be data driven to select and fragment the most abundant three ions.

3.8 Data Processing and Analysis

1. Use flexAnalysis 2.1 software for MALDI-MS spectra processing and peak picking.
2. Use ChemStation software to process ESI-MSD trap data.
3. Use BioTools 3.0 software for peptide MS/MS database searching and de novo sequencing.

4 Notes

1. Purification of recombinant galectin is reported elsewhere [5].
2. Alternative gel filtration or desalting columns can be utilized (spin columns, etc.) but this desalting step should be rapid and remove excess lactose used to affinity purify galectin.
3. Buffer exchange protein into buffers containing no lactose before application of cellular extracts or purified proteins.
4. 2-Mercaptoethanol or DTT can be utilized as a reducing agent; however, both should be removed from galectin before use in biological assays as both of these thiols also possess the

ability to cross cell membranes where they can induce the unfolded protein response within the endoplasmic reticulum [19–21] likely secondary to preventing appropriate oxidation of proteins during protein folding [20]. Thus, each may cause cell damage if not removed prior to biological assays.

5. All FPLC and HPLC buffers should be vacuum filtered with a nylon filter (0.22 μm, 47 mm) and degassed with a helium sparge or vacuum degassed with sonication.
6. Recrystallize CHCA matrix as follows: Mass 5 g CHCA into a clean, dry beaker. Add 10 mL hot methanol and heat the matrix/methanol solution. Add enough methanol (the minimum amount required) to dissolve all of the CHCA. Add 100 mg of activated charcoal and filter the CHCA/methanol solution. Cool the filtrate to room temperature, then cool to 4 °C on ice. Vacuum filter CHCA crystals and wash with ice-cold water. Store at −20 °C in a desiccated container.
7. Data processing and handling on mass spectrometers is instrument and vendor dependent. A variety of software packages are available and may be utilized. Software tools that de novo sequence peptides from MS/MS fragmentation data can also be used for determining sites of iodoacetamide incorporation.
8. We used Labconco centrivap containing a glass lined cold trap chilled to −50 °C with an Edwards vacuum pump capable of reaching 50 mTorr or equivalent.
9. Our lab used AKTA FPLC for protein purification and gel filtration.
10. Use a HPLC system capable of high pressure pumping, flow rates of 0.1–10 ml/min and a detector capable of monitoring at 215 nm or other UV-Vis wavelengths. We used a Beckman System Gold with a UV-Vis detector system equipped with an analytical C_{18} column (Vydac 4.6×250 mm) for protein separations.
11. There are many additional systems available including nanoLC-MS versions capable of mass spectrometric peptide mapping. MS^n studies can be performed (if needed) by direct infusion of peptide fractions collected from the HPLC.
12. Alkylation of free sulfhydryls can be achieved in shorter incubation times at elevated temperatures. This may be determined empirically.
13. This is commonly done by examining galectin at a similar concentration as employed in biological assays, which can range from 1 to 20 μM. As a control, wells containing 14 mM βME or 3 mM DTT should also be included in separate plates.
14. This can range from examining galectin hours to days following initiation of the incubation period. There are many

methodological approaches to inducing protein oxidation. We prefer to measure the potential impact of protein oxidation following protein modification by incubating galectins in a similar environment to which they will be exposed during a typical incubation with cells as this will be the commonly employed experimental situation where galectin oxidation may significantly impact the biological outcome of galectin–ligand interactions, but where inclusion of reducing agents can obfuscate results.

15. It is possible, depending on the protein concentration employed, that oxidative inactivation will result in protein aggregate formation. If this occurs, the final concentration of soluble galectin should be noted and may be used to calculate the overall loss of protein activity secondary to galectin oxidation.
16. If determining galectin protein concentration by measurement of OD 280 then be sure to determine the concentration using the extinction coefficient. Ensure that the column is not saturated such that active galectin is found in the flow through simply because the column limit has been reached. This can be achieved by examining for potential flow through in the reducing agent included control and/or by examining the saturation limit prior to experimental setup.
17. Peptides analyzed by this method are described in an off-line LC-MS manner. There are numerous other high-resolution LC-MS systems that can be substituted for the ones described here.

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Chapter 4

Evaluation of Galectin Binding by Frontal Affinity Chromatography (FAC)

Jun Iwaki and Jun Hirabayashi

Abstract

Frontal affinity chromatography (FAC) is a simple and versatile procedure enabling quantitative determination of diverse biological interactions in terms of dissociation constants (K_d), even though these interactions are relatively weak. The method is best applied to glycans and their binding proteins, with the analytical system operating on the basis of highly reproducible isocratic elution by liquid chromatography. Its application to galectins has been successfully developed to characterize their binding specificities in detail. As a result, their minimal requirements for recognition of disaccharides, i.e., β-galactosides, as well as characteristic features of individual galectins, have been elucidated. In this chapter, we describe standard procedures to determine the K_d's for interactions between a series of standard glycans and various galectins.

Key words Oligosaccharide specificity, Carbohydrate-recognition domain, Comparative glycomics, Dissociation constant, Frontal affinity chromatography, Pyridylamination

1 Introduction

Frontal affinity chromatography (FAC) is a chromatographic method used to determine biomolecular interactions between analyte (A) and immobilized ligand (B) in terms of a dissociation constant (K_d) or association constant (K_a), where $K_d = 1/K_a$. Historically, the essence of quantitative affinity chromatography was discovered by two independent groups at almost the same time; i.e., by Dunn and Chaiken [1] and Kasai and Ishii [2]. The procedures described differ in their characteristics, and are generally referred to as "zonal" [3] and "frontal" analyses, respectively [4]. However, both are based on the classic principle of Langmuir's adsorption isotherm, and are quantitative methods for the precise analysis of biomolecular interactions using a conventional liquid chromatography system. It is generally difficult to determine accurate K_d values for weak interactions (>10^{-6} M), because high concentrations (e.g., >1 mM) of saccharides are required.

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In contrast, FAC can utilize extremely low concentrations of saccharide (e.g., $<10^{-8}$ M), usually in the fluorescently labeled form, for each analysis. Moreover, at such low concentrations, FAC measurements are not compromised by experimental errors in concentration (described below). As a fluorescence-labeling method for glycans, Hase et al. exploited pyridylamination, in which 2-aminopyridine is used for coupling to reducing termini of glycans and the derived Schiff base is reduced [5]. The resultant pyridylaminated (PA) glycans are sufficiently stable and sensitive for separation and analysis in high-performance liquid chromatography (HPLC). Such PA-glycans are perfect for high-sensitivity analysis by FAC [6].

As an advanced system for FAC, Hirabayashi et al. combined a conventional HPLC system with a fluorescence detector, utilizing a relatively large sample loop (2 ml capacity) and a small column (4 mm × 10 mm, volume 0.126 ml) [6]. The system is user-friendly in terms of both simple operation (isocratic elution) and clear theory (described later), and further it has many practical merits. Another example of the successful application of FAC is its connection to a mass spectrometer (FAC-MS) for detection of multiple analytes [7–9]. The FAC-MS method is useful for high-throughput screening of the library derived by combinatorial chemistry, as it allows simultaneous analyses of compounds having different m/z values.

Over the past decade, the former system using fluorescence detection has been dramatically improved in terms of precision, sensitivity, and throughput on the basis of system automation (*see* Fig. 1). Consequently, it has become a highly reproducible and accurate means for comprehensive quantitative interaction analysis targeting lectins and fluorescently labeled glycans. Therefore, FAC

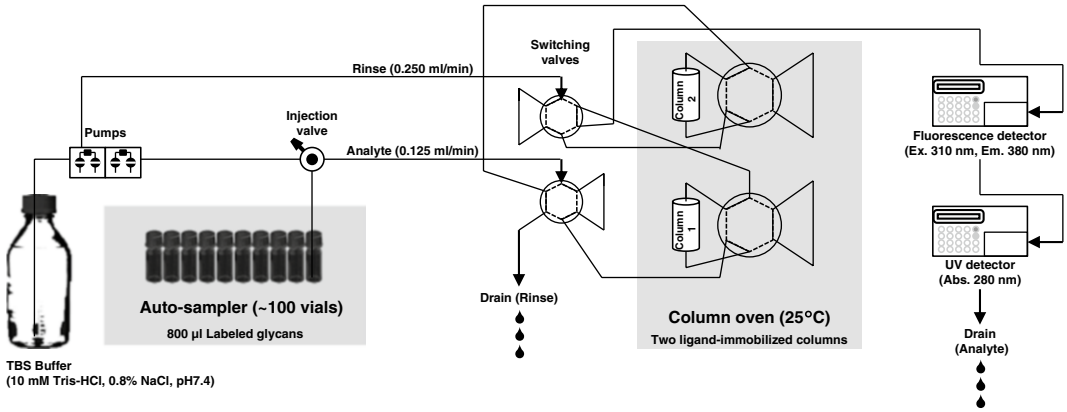


Fig. 1 A representative system for automated FAC. The analytical system comprises a pumping system for the mobile phase, an analyte injection port connecting an auto-sampler, one or two ligand columns clamped in a thermo-regulated oven, fluorescence and UV detectors, and an administration computer for control of the system and to record elution profiles. A wash and equilibration of column 2 is carried out while an analysis is being performed by column 1

provides us with a most reliable data set to elucidate complicated features of binding specificities of galectins [10]. In fact, galectins derived from diverse organisms including humans and nematodes have been analyzed and the K_d values against 41 PA-glycans have been determined [11, 12]. More recently, FAC was successfully used to differentiate binding features of galectins from those of other galactose-binding lectins [13]. These basic insights, together with likely future results, will provide a platform for the understanding of more complex mechanisms, including biological regulation by galectins under physiological conditions.

2 Materials

2.1 Equipment

1. Frontal Affinity Chromatography (FAC) system: An automated FAC system (Shimadzu) (Fig. 1) (*see* **Notes 1** and **2**).

2.2 Fluorescence- and UV-Labeled Glycans

1. The reducing terminal of a glycan can be labeled with any appropriate group such as PA, *p*NP (*p*-nitrophenyl), or *p*MP (*p*-methoxyphenyl) (Fig. 2). The latter two are often used for concentration-dependence analysis. PA can be substituted with other fluorescence groups if they show no apparent nonspecific binding to the system materials, including the column and line tubes (*see* **Note 3**).
2. Commercially available PA-glycans (Masuda Chemical Industries, Seikagaku Kogyo Co. and Takara Bio Inc).
3. Nonlabeled glycans (Funakoshi Co., Ltd, Dextra Laboratories Ltd. and Calbiochem) can also be used after pyridylamination either manually or automatically, e.g., with GlycoTag (Takara Bio Inc.) [14].
4. *p*NP-labeled glycans (Sigma, Calbiochem, Funakoshi Co., Ltd. or Toronto Research Chemicals, Inc.).
5. *p*MP-LDN (Tokyo Chemical Industry Co. Ltd.).
6. For the concentration-dependence analysis, ligand solutions of various concentrations (e.g., 5–300 μM) of an appropriate *p*NP-glycan are used.

2.3 Preparation of a Galectin-Immobilized Miniature Column

1. Either natural or recombinant galectin (100–1,000 μg).
2. Coupling buffer (1 ml): 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3.
3. *N*-hydroxysuccinimide (NHS)-activated Sepharose 4FF (100–200 μl; GE).
4. Ice-cold 1 mM HCl (1 ml).
5. Stop solution (1 ml): 0.5 M monoethanolamine, 0.5 M NaCl, pH 8.3.
6. TBS (1 l): 10 mM Tris–HCl, 0.8 % NaCl, pH 7.4.

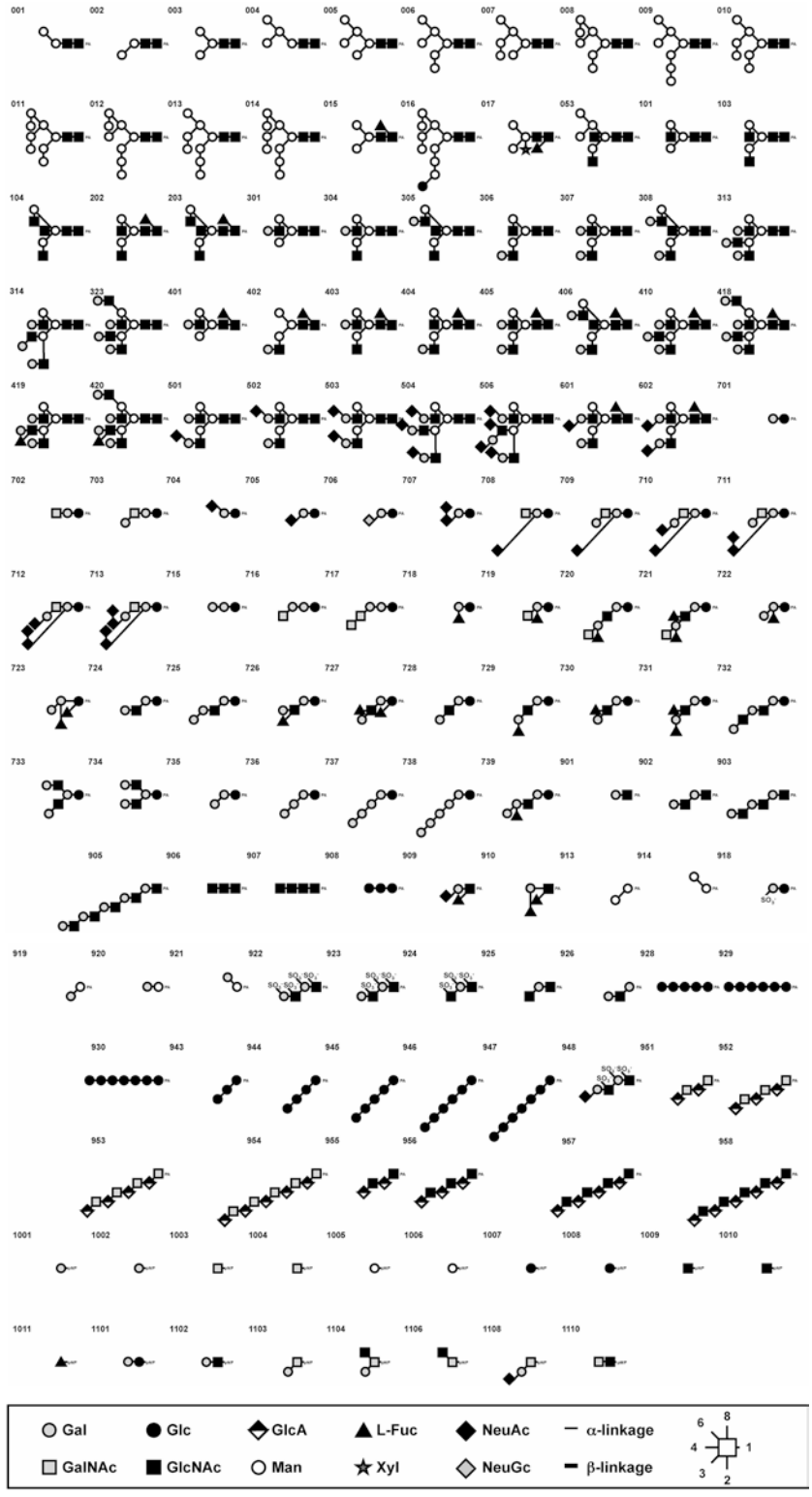


Fig. 2 Schematic representation of fluorescence- and UV-labeled glycan structures**.** *Symbols* are shown in the *box* at the *bottom*. The reducing terminal is labeled with either PA, *p*NP, or *p*MP. The glycan library is mainly composed of *N*-glycans, *O*-glycans, glycolipids, milk oligosaccharides, glycosaminoglycans (GAGs), and monosaccharides

7. Miniature column (2 mm × 10 mm, 31.4 μl) with a silicon filter cap.
8. 1.5 ml tubes.
9. 1 cm silicon tube.
10. 1 ml syringe.

2.4 Determination of B_t Value of a Galectin-Immobilized Column

1. 1.5 ml glass vials.
2. TBS (1 l): 10 mM Tris–HCl, 0.8 % NaCl, pH 7.4.
3. *p*NP-Lac or *p*NP-LacNAc
4. Galectin columns (prepared in Subheading 3.3).

2.5 Comprehensive Analysis with a Panel of Labeled Glycans

1. 1.5 ml glass vials.
2. TBS (1 l): 10 mM Tris–HCl, 0.8 % NaCl, pH 7.4.
3. Test pyridylaminated (PA)-glycans.
4. Galectin columns (prepared in Subheading 3.3).

3 Methods

3.1 Principle of FAC and a Procedure to Determine Dissociation Constant (K_d)

An advanced procedure for FAC is simply operated on the basis of isocratic elution HPLC. Elution of a series of fluorescently labeled glycans is monitored by a fluorescence detector. When a relatively large volume of diluted glycan A solution is continuously applied to a miniature column packed with galectin B covalently immobilized on Sepharose, an incompatible glycan will pass through the column without interaction, and the elution front should appear at V_0 ml (Fig. 3). On the other hand, a glycan with significant affinity should exhibit significant delay of the elution front caused by repeated interaction between the glycan and the immobilized galectin at V ml. Thus, the observed retardation of elution is $V - V_0$. If an initial concentration of the diluted glycan A is $[A]_0$ M, the amount of glycan A retained in the galectin column under dynamic equilibrium can be shown as $[A]_0 (V - V_0)$. When the effective ligand content is expressed as B_t, the basic equation of FAC (Eq. 1) resembles the well-known Michaelis–Menten equation.

$$[A]_0 (V - V_0) = \frac{B_t [A]_0}{[A]_0 + K_d} \ldots \text{Cf. Michaelis–Menten equation: } \left(v = \frac{V_{max}[S]}{[S] + K_m} \right) \quad (1)$$

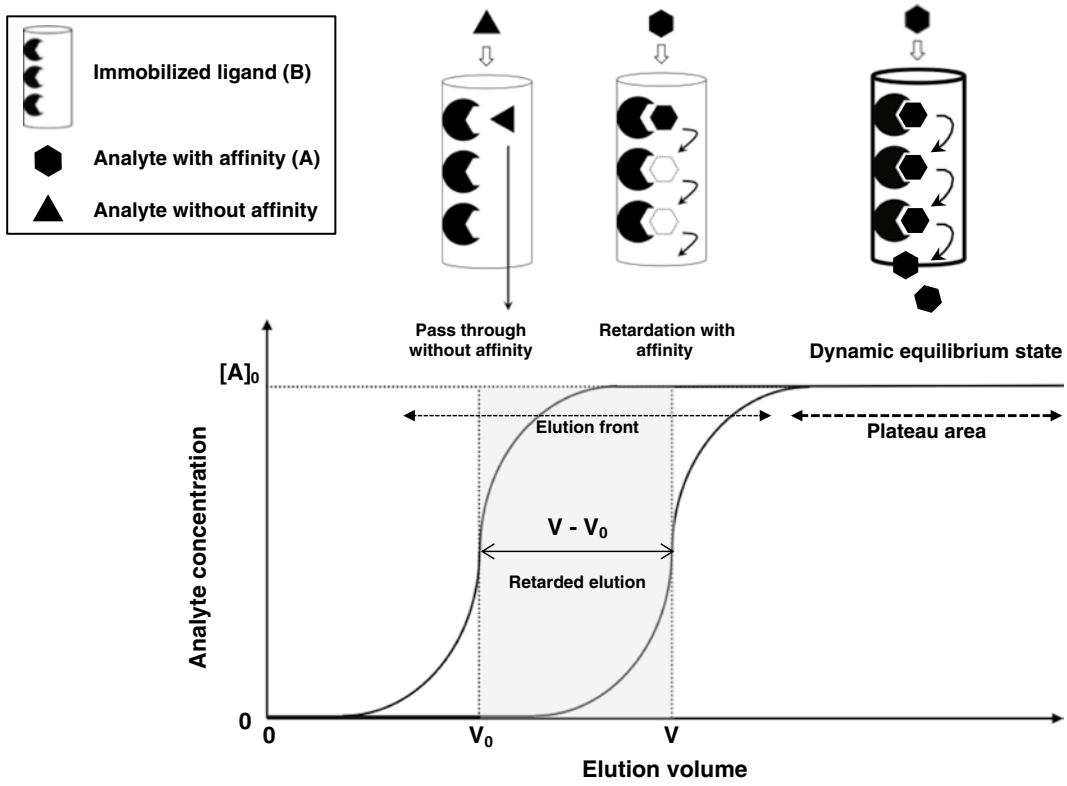


Fig. 3 The principle of FAC**.** An analyte with affinity (A) is continuously applied to column with immobilized ligand (B) assuming that the initial concentration of the analyte and the total binding of immobilized ligand are $[A]_0$ and B_t, respectively. When analyte (A) significantly interacts with ligand (B), the retarded volume ($V - V_0$) is represented by the delta of the elution fronts between an analyte with affinity for the ligand and an analyte with no affinity. If $K_d \gg [A]_0$, then $V - V_0$ is inversely related to K_d because B_t is constant depending on each immobilized ligand (Eq. 2)

Transformation of the formula results in Eq. 2.

$$K_d = \frac{B_t}{V - V_0} - [A]_0 \tag{2}$$

If an initial concentration of the diluted glycan A solution $[A]_0$ is negligibly low, Eq. 2 is simplified to Eq. 3

$$K_d = \frac{B_t}{V - V_0} \qquad \left(K_d >> [A]_0\right) \tag{3}$$

Here, Eq. 3 is no longer dependent on the initial concentration of glycan A solution $[A]_0$, which means K_d determination is free from experimental errors in $[A]_0$. In many cases, this condition is fulfilled, considering that lectin–glycan interactions are generally weak (i.e., $K_d \gg 10^{-8}$ M) and $[A]_0$ can be extremely low (~10^{-9} M) for fluorescence detection. The B_t value of each lectin column is

obtained by concentration-dependence analysis using an appropriate ligand (in case of galectins, generally *p*NP-Lac or *p*NP-LacNAc) as described below. Once a B_t value is determined, K_d values toward a panel of glycans are readily calculated according to Eq. 2. An established procedure for FAC is also described by Tateno et al. [15].

3.2 Preparation of Diluted PA-Glycans

1. Dilute the stock solutions of PA-glycans (10 pmol/μl) into TBS to a final volume of 2.0 ml in a 4 ml glass vial (*see* **Note 4**).

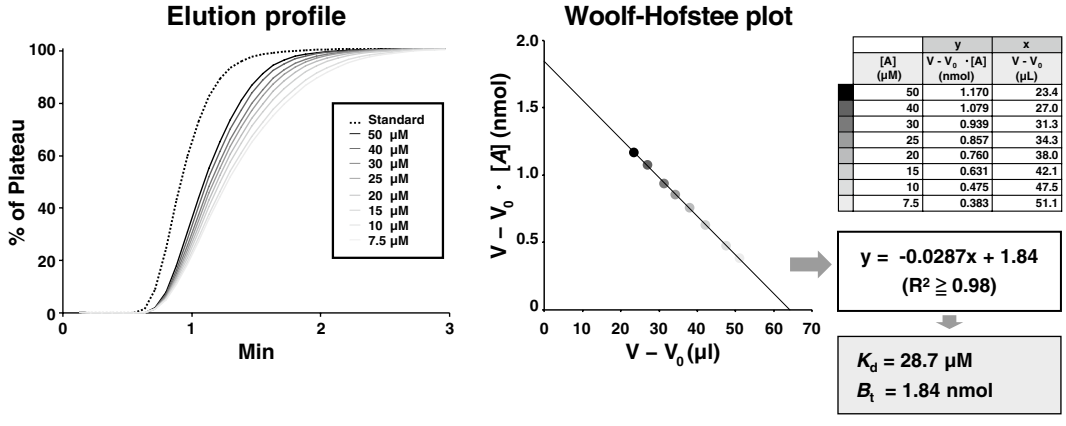
3.3 Preparation of a Galectin-Immobilized Miniature Column

1. Dissolve 100 μg of galectin in 350 μl of coupling buffer in a 1.5 ml tube (e.g., target immobilization concentration, 1.0 mg/ml). Check the concentration by an appropriate protein assay (e.g., Bradford protein assay).
2. Take 200 μl of the 50 % (v/v) suspension of NHS-Sepharose in a new 1.5 ml tube. Discard the supernatant after centrifugation of the tube at 820 × *g* for 3 min. To activate the resin, add 1 ml of ice-cold 1 mM HCl into the tube, quickly suspend, and discard the supernatant immediately after centrifugation as above.
3. Add 350 μl of the galectin solution into the 1.5 ml tube containing the above activated NHS-Sepharose and rotate the tube at room temperature for 3 h. After the coupling reaction, centrifuge the tube at 820 × *g* for 3 min and remove the supernatant into a new tube. Check the amount of unbound galectin by protein assay to calculate the amount of immobilized ligand.
4. If a sufficient amount of protein is immobilized, add 1 ml of the stop solution provided by the manufacturer, and rotate the tube at room temperature for 30 min. Centrifuge the tube at 820 × *g* for 3 min and discard the supernatant.
5. Wash the resin three times with 1 ml of TBS.
6. Suspend the resin in an equal volume (100 μl) of TBS.
7. For packing of the resin, set a miniature column on a silicon tube (about 1 cm) connected to a 1 ml syringe as described by Nakamura-Tsuruta et al. [16]. Add TBS to the column, and remove bubbles in the bottom (if any) by carefully aspirating the column with the syringe.
8. Add a 50 % suspension of the galectin-immobilized resin to the top of the column. Fill the column with resin by continuously applying the suspension while aspirating the buffer from the bottom. After completion of the resin packing, cap the top of the miniature column with a silicon filter (when not in immediate use, store the packed column immersed in TBS in a closed tube at 4 °C).
9. Set the miniature column in a column holder before use.

10. To ensure acquisition of reliable V-V_0 values with a stable "plateau," several galectin columns with different immobilization concentrations should be used. In this case, data obtained from different columns can be combined under the assumption that K_d values for a certain glycan are the same despite the different immobilization concentrations (*see* **Note 5**).

3.4 Determination of B_t Value of a Galectin-Immobilized Column

1. Prepare 1.0 ml each of 5–300 μM *p*NP-Lac or *p*NP-LacNAc (in general, 8–10 concentrations are necessary for the concentration-dependence analysis) in 1.5 ml glass vials. It is important to note that when $[A]_0$ is K_d the elution curve should show just a half extent of the maximal retardation from Eq. 2.
2. Turn on the UV detector and equilibrate the flow lines with TBS.
3. After equilibration with TBS for 15 min at 0.250 ml/min, set an appropriate galectin column in a column oven at 25 °C, and equilibrate the column for 30 min at a reduced flow rate of 0.125 ml/min.
4. Set a program for 800 μl injection of a series of *p*NP-glycan solutions.
5. Set samples on the auto-sampler and run the program.
6. After the run, check the data collection.
7. Using the obtained data (Fig. 4, left panel), perform Woolf-Hofstee-type plots; i.e., "$V - V_0 \cdot [A]_0$" and "$V - V_0$" as *x*- and *y*-axes, respectively (Fig. 4, right panel). The *y*-intercept of the derived line represents an effective ligand content (B_t; nmol), while the slope gives the negative of the dissociation constant ($-K_d$; mM).



	y	x
[A] (μM)	V - V₀ ·[A] (nmol)	V - V₀ (μL)
50	1.170	23.4
40	1.079	27.0
30	0.939	31.3
25	0.857	34.3
20	0.760	38.0
15	0.631	42.1
10	0.475	47.5
7.5	0.383	51.1

Fig. 4 Determination of an effective ligand content (B_t). The B_t and K_d values of a galectin-3C column (2.9 mg/ml) are determined by the concentration-dependence analysis using *p*NP-LacNAc. Elution profiles from the galectin-3C column with eight concentrations of *p*NP-LacNAc are shown (*solid lines*; from the *left* to the *right*, 50, 40, 30, 25, 20, 15, 10, and 7.5 μM). *p*NP-α-glucose was used as a negative control (*dotted line*). The *y*-intercept of the derived line represents B_t, while the slope shows $-K_d$. The *R*-squared value of not lower than 0.98 is considered to support the conclusion that the determined B_t and K_d values are reliable

3.5 Comprehensive Analysis with a Panel of Labeled Glycans

1. Prepare a panel of diluted fluorescently labeled (e.g., PA-) glycans, and set on the auto-sampler.
2. Turn on the fluorescence detector and equilibrate the flow path of the FAC system with TBS.
3. After equilibration of the FAC system for 15 min at a flow rate of 0.250 ml/min, install a galectin column (when appropriate, two) in the column oven set at 25 °C, and equilibrate the column for 30 min at a reduced flow rate of 0.125 ml/min.
4. Set a program for 800 μl injection of a series of diluted PA-glycans.
5. Run the program. The analysis is carried out at a constant flow rate of 0.125 ml/min at 25 °C for 8 min. After the run, check whether each plateau area is sufficient for analysis or not. If too strong binding is observed and the plateau is insufficient, injection of a larger volume (e.g., 1 ml) of the same solution should be attempted. In this case, monitoring time should be extended appropriately (e.g., for 10 min).
6. After the run for the initial set of ~100 samples, run an additional set if necessary.
7. Calculate the $V - V_0$ values using the FAC analyzer followed by processing the obtained $V - V_0$ values into K_a values ($K_a = 1/K_d$) using Eq. 3 and the B_t values determined above (Fig. 5) (*see* **Notes 6** and **7**).

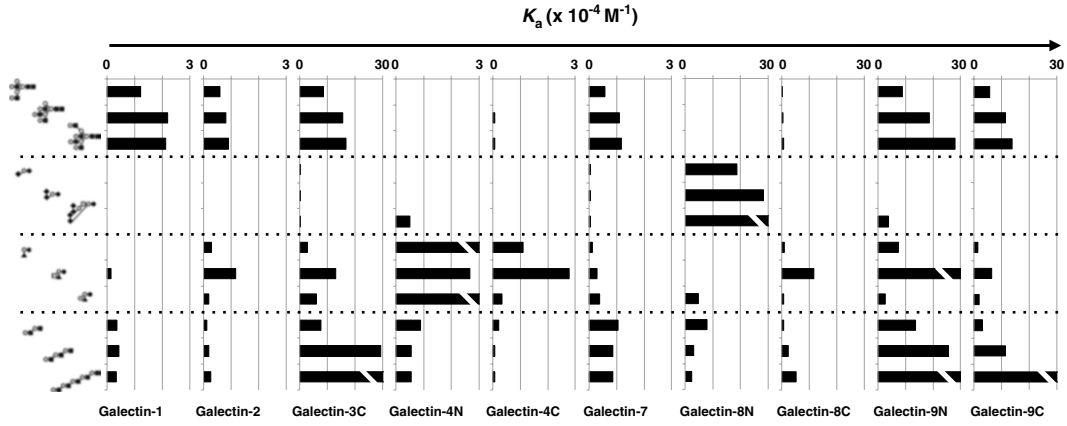


Fig. 5 Comparison of K_a values of human galectin CRDs. Only representative glycans, i.e., *N*-glycans, ganglioside-type glycans, blood-type antigens, and repetitive lactosamine units (poly-*N*-acetyllactosamine), showing significant affinity to some galectins are shown. Note that binding affinities and specificity preferences are quite different between the galectin members as well as between individual CRDs, whereas the recognition consensus has been preserved. Galectins-1, -2, -3C, -7, -9N, and -9C show substantial binding to *N*-glycans in a branch-dependent manner. By contrast, galectins-4N, -4C, -8N, and -8C seem to be strongly associated with glycolipid-type glycans. The binding affinities of galectins-3C, -9N, and -9C toward poly-*N*-acetyllactosamine are greatly enhanced in proportion to repetitive lactosamine units, whereas those of galectins-1 and -7 are not

4 Notes

1. The typical system comprises a controller (computer), two parallel pumps for isocratic elution, each connecting to a miniature column packed with galectin-immobilized resin clamped in a column oven (25 °C), an auto-sampler for setting a series (~100 vials) of labeled glycans, and either fluorescence (excitation: 310 nm, emission: 380 nm) or UV (absorbance: 280 nm) detectors (Fig. 1). Flow rates of TBS buffer for sample injection and rinse are employed at 0.125 ml/min and 0.250 ml/min, respectively. To ensure a dynamic equilibrium state in the miniature column, the linear flow rate (defined as flow rate per sectional area) is set at a minimum value (e.g., 0.66 mm/s). An ordinary computer is used to process the elution profile data (signal intensity vs. elution time). Even when individual glycans are manually injected, the results obtained show no basic difference in data reliability, as various researchers have demonstrated [11, 17–19].
2. All possible impurities should be removed before analysis. Otherwise, fluorescence-active ingredients will interfere with elution curves and sometimes considerably disturb the precision of the analysis. Any detergent on glass bottles and plastic tubes used in the system should also be removed by extensive washing with pure water.
3. As fluorescent groups, either of 2-aminobenzoic acid and 2-aminobenzamide can be used. The use of 2-aminoacridone and pyrene hydrazide is not recommended as they show substantial nonspecific interaction with the agarose resin on which ligands are immobilized, and even with PEEK tubes used for the line.
4. To obtain a steady-state "plateau," e.g., for 8 min, at least 800 μl is necessary for a single injection.
5. In some cases, a so-called "cluster effect" may appear, where lectins immobilized at a higher concentration show much stronger retardation than expected from the data obtained at a lower concentration. In such a case, determination of a consistent K_d value is difficult, and is more correctly a "conditional" one under the given concentration.
6. Data obtained by FAC analysis do not necessarily coincide with those derived by other methods (e.g., glycan array) because of significant differences in experimental conditions: for instance, situations are quite different between cases where lectins are immobilized and those where glycans are immobilized. FAC is performed using a series of diluted glycans to fill the condition, $K_d \gg [A]_0$, where the basic equation of FAC is preferably simplified to Eq. 3. On the other hand, glycan array generally

exhibits a multivalent feature mimicking to some degree of cell surface structures, where a cluster effect will more easily be observed. In this respect, FAC is considered to provide a fundamental data set of lectin–glycan interactions with no probable higher-ordered recognitions. Nevertheless, both data should have much in common regarding basic saccharide specificity, e.g., requirement of 4-OH of β-galactosides and preference for 3*O*-sialylation, etc. As a practical merit of FAC, extended analysis, when additional glycans are available, is quite possible in contrast to glycan array analysis, where a set of glycans is fixed.

7. The Lectin *Frontier* DataBase (L*f*DB, http://jcggdb.jp/rcmg/glycodb/LectinSearch) provides K_a (K_d) value-based data sets of galectins as well as their CRDs in the bar graph format, where the function "One parameter difference" will be helpful for users to understand which parameter (e.g., difference in anomeric configuration, linkage, substitution, monosaccharide type) is critical for each galectin (CRD) binding.

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Chapter 5

Probing Lectin–Mucin Interactions by Isothermal Titration Microcalorimetry

Tarun K. Dam and C. Fred Brewer

Abstract

Isothermal titration microcalorimetry (ITC) can directly determine the thermodynamic binding parameters of biological molecules including affinity constant, binding stoichiometry, and heat of binding (enthalpy) and indirectly the entropy and free energy of binding. ITC has been extensively used to study the binding of lectins to mono- and oligosaccharides, but limited applications to lectin–glycoprotein interactions. Inherent experimental challenges to ITC include sample precipitation during the experiment and relative high amount of sample required, but careful design of experiments can minimize these problems and allow valuable information to be obtained. For example, the thermodynamics of binding of lectins to multivalent globular and linear glycoproteins (mucins) have been described. The results are consistent with a dynamic binding mechanism in which lectins bind and jump from carbohydrate to carbohydrate epitope in these molecules leading to increased affinity. Importantly, the mechanism of binding of lectins to mucins appears similar to that for a variety of protein ligands binding to DNA. Recent results also show that high affinity lectin–mucin cross-linking interactions are driven by favorable entropy of binding that is associated with the bind and jump mechanism. The results suggest that the binding of ligands to biopolymers, in general, may involve a common mechanism that involves enhanced entropic effects that facilitate binding interactions.

Key words Lectins, Multivalent carbohydrates, Glycoproteins, Mucins, Thermodynamics

1 Introduction

1.1 General Overview

Lectins are ubiquitous proteins that recognize specific carbohydrate residues of glycoproteins and glycolipids. Lectin–glycoprotein and lectin–glycolipid interactions are central to a variety of biological activities including receptor-mediated endocytosis, cellular recognition and adhesion [1], inflammation [2], cell growth, and metastasis [3, 4]. Glycoproteins are broadly classified as N-linked and O-linked glycoproteins, including heavily O-linked glycoproteins known as mucins, and multidomain glycoproteins with mucin domains (mucin-type glycoproteins).

The glycoprotein receptors for a number of lectins including selectins, siglecs, and galectins are often mucin domains with large numbers of glycan epitopes [5]. Mucins are secreted by higher

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organisms to protect and lubricate epithelia cell surfaces from biological, chemical, and mechanical insults. Mucin and mucin-type molecules exist as soluble and transmembrane molecules that are involved in modulating immune response, inflammation, adhesion, and tumorigenesis [6–8]. The O-glycosylated domains of mucins and mucin-type glycoproteins contain 50–80 % carbohydrate and possess expanded linear conformations. Polypeptide tandem repeats are found in mucins that contain clusters of Ser and Thr residues in high content. The O-linked carbohydrate chains in these domains are attached via α-GalNAc residues to Ser and Thr residues, which have been shown to induce a threefold expansion of the polypeptide chain of these molecules [9]. The oligosaccharide chains on mucins have been shown to be important for a variety of their biological properties including their interactions with animal lectins such as the selectins and galectins, as well as their physical properties including their extended linear structures [10].

There are at least 17 mucin gene products (MUC1-MUC17) reported in the literature [7]. These gene products represent two structurally and functionally distinct classes of mucins: secreted gel-forming mucins and transmembrane mucins, although there are a few mucin gene products that do not appear to fit into either category. The transmembrane mucins include MUC1 whose structure includes a cytoplasmic domain in addition to the extracellular O-glycosylated polypeptide tandem repeat domains [11]. Evidence indicates that the C-terminal cytoplasmic domain of MUC1 is involved in signal transduction mechanisms including T-cell activation and inhibition, and adhesion signaling responses [12, 13].

Mucins are also useful in the diagnosis of a variety of diseases [10]. In particular, the level of expression of mucin peptide antigens and type of carbohydrate chains of mucins has proven to be useful diagnostic markers for a variety of cancers [7, 14]. For example, MUC1 expression as detected immunologically is increased in colon cancers and is associated with a poor diagnosis [7]. Colon cancer-associated mucins also have differences associated with their core carbohydrate structures, often presenting shorter chain versions of normal mucins. Colon cancer mucins often have increased expression of the GalNAcαThr/Ser (Tn-antigen), Galβ3GalNAc (T or TF antigen), and NeuAcα6GalNAc (sialyl Tn-antigen) [7]. Importantly, recent studies have shown that binding of galectin-3, an endogenous Gal-specific animal lectin, to cancer-associated MUC1 causes increased endothelial cancer cell adhesion [15]. Thus, the molecular recognition properties of cancer-related mucins including their truncated carbohydrates are important in terms of gaining insight into their structure–activity properties.

The binding and cross-linking of cell surface mucins and mucin-like glycoproteins by lectins is known to lead to signal transduction effects including cell growth and cell death [16, 17]. For example, galectin-1 cross-linking of CD43, a transmembrane mucin-type glycoprotein receptor that possesses approximately 80

O-linked chains with terminal LacNAc epitopes [18], along with CD45 induces apoptosis in susceptible T cells [19]. However, details of the energetics and mechanisms of lectin binding and cross-linking of mucins and mucin-type receptors have been lacking.

Quantitative binding studies such as ITC often require relatively high amount of samples. Obtaining recombinant human mucins sufficient for thermodynamic studies is difficult. Therefore, other mammalian mucins that show some structural similarities with human mucins can be used in such studies. One such mucin is the porcine submaxillary mucin (PSM), which is a physically well-characterized mucin, and the subject of studies of the regulation of O-glycosylation with glycosyl transferases [20]. The cDNA sequence of PSM has been determined [21], and the 81 amino acid tandem repeat domain that is present in 100 copies (Fig. 1a). The structures of the carbohydrate chains were determined by chemical [22] and NMR techniques [23]. Gerken and coworkers

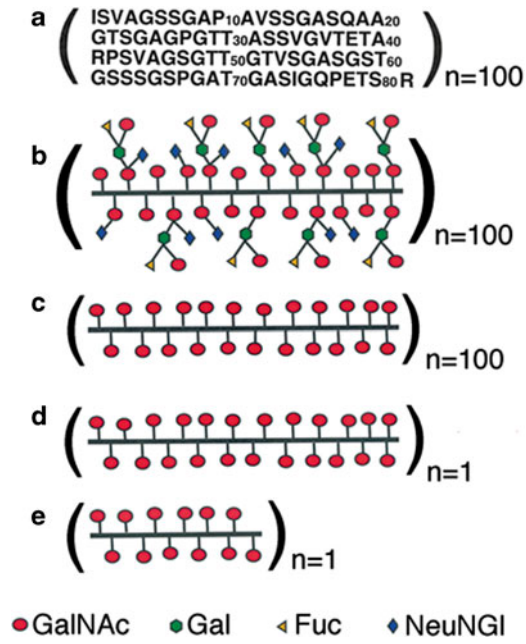

Fig. 1 Structural representations of (**a**) the amino acid sequence of the 100-repeat 81-residue polypeptide O-glycosylation domain of intact PSM; (**b**) the fully carbohydrate decorated form (described in the text) of the 100-repeat 81-residue polypeptide O-glycosylation domain of PSM (Fd-PSM); (**c**) the 100-repeat 81-residue polypeptide O-glycosylation domain of PSM containing only peptide linked α-GalNAc residues (Tn-PSM); (**d**) the single 81-residue polypeptide O-glycosylation domain of PSM containing peptide linked α-GalNAc residues (81-mer Tn-PSM); (**e**) the 38/40-residue polypeptide(s) derived from the 81-residue polypeptide O-glycosylation domain of PSM containing peptide linked α-GalNAc residues (38/40-mer Tn-PSM). The number of glycan chains in Fd-PSM and Tn-PSM is ~2,300. The number of α-GalNAc residues in 81-mer Tn-PSM is ~23, while the number of α-GalNAc residues in 38/40-mer Tn-PSM is ~11 to 12

[23] isolated the O-glycosylated domain of PSM that possesses a molecular mass of ~10^6 Da and is fully decorated with naturally occurring carbohydrates (Fd-PSM) (Fig. 1b). The O-glycosylated domain of PSM possessing only α-GalNAc residues (Tn-PSM) was also obtained using chemical and enzymatic treatments [24]. The GalNAcα1-*O*-Ser/Thr residue(s) in Tn-PSM (Fig. 1c) is the pan-carcinoma carbohydrate antigen Tn that is aberrantly expressed in mucins such as MUC1 in adenocarcinomas [25]. The 81-mer tandem repeat domain of Tn-PSM (81-mer Tn-PSM) (Fig. 1d) and the 38/40-mer digest of this domain (38/40-mer Tn-PSM) (Fig. 1e) also have been obtained using enzymatic digests [24].

Historically, the relative affinities and specificities of lectins for carbohydrates were first addressed using hemagglutination inhibition, equilibrium dialysis, and quantitative precipitation inhibition techniques [26]. While these methods are still employed, additional techniques are often used including gradient affinity chromatography, nuclear magnetic resonance, and surface plasmon resonance. However, all of the above methods suffer from either being indirect measurements of binding constants or have special conditions required for the measurements. For example, hemagglutination inhibition and quantitative precipitation inhibition techniques are indirect binding methods, while equilibrium dialysis typically requires radioactivity or a chromophore in the molecules. Affinity chromatography suffers from potential matrix interactions with ligands in solution, and covalent attachment of a receptor or ligand to the matrix. Nuclear magnetic resonance measurements require the binding between two molecules to be in certain kinetic conditions. Surface plasmon resonance mandates covalent attachment of one of the binding molecules on a solid-state chip. Equilibrium constants are derived from kinetic on-off rate measurements of a ligand in a flowing system. Thus, all of the techniques have limitations in determining quantitative binding constants for carbohydrate–lectin interactions in solution and none of these methods can provide comprehensive thermodynamic and hence mechanistic information on lectin–glycoprotein interactions. ITC has been employed extensively to study the binding interactions of lectins with glycans, and, more recently, lectins with glycoproteins.

Using ITC measurements, the affinities of a series of galectins for asialofetuin (ASF), a nonavalent globular glycoprotein with terminal LacNAc residues, were observed to be ~50- to 80-fold greater than LacNAc [27]. However, the affinity of the GalNAc-specific soybean agglutinin (SBA) for a mucin that possesses ~2,300 GalNAc residues was reported to be ~10^6-fold greater than the corresponding monovalent carbohydrate [28]. Based on the thermodynamic data, a model called the "bind and jump mechanism" has been proposed to provide a mechanistic basis for the higher affinities of the lectins for these glycoproteins [28].

Table 1
Thermodynamic binding parameters for SBA and VML at pH 7.2, 27 °C

Ligand	K_d[a] (μM)	K(rel)[b]	$-\Delta G$[c] (kcal/mol)	$-\Delta H$[d] (kcal/mol)	$-T\Delta S$[e] (kcal/mol)	n[f]
SBA						
GalNAcα1-*O*-Ser	170	1	5.2	7.9	2.7	1.0
38/40-mer Tn-PSM	1.4	120	8.0	32.2	24.2	0.2
81-mer Tn-PSM	0.06	2,800	9.8	56.1	46.3	0.12
Tn-PSM	0.0002	850,000	13.1	4,310	4,297	0.0018
Fd-PSM	0.024	7,100	10.4	703	693	0.008
VML						
GalNAcα1-*O*-Ser	130	1	5.3	6.4	1.1	1.0
38/40-mer Tn-PSM	0.20	650	9.1	36.8	27.7	0.2
81-mer Tn-PSM	0.012	11,000	10.8	52.7	41.9	0.1
Tn-PSM	0.0001	1,300,000	13.6	5,274	5,260	0.0012
Fd-PSM	0.0014	93,000	12.1	1,251	1,240	0.005

[a] Errors in K_d range from 1 to 7 %
[b] Relative to GalNAcα1-*O*-Ser
[c] Errors in ΔG are less than 2 %
[d] Errors in ΔH are 1–4 %
[e] Errors in $T\Delta S$ are 1–7 %
[f] Errors in n are less than 4 %

Previous reviews of multivalent interactions in biological systems including protein–carbohydrate interactions have been reported [29–31]. This review will focus primarily on studies of the thermodynamics of binding of lectins to mucins since the results have provided new insights into the energetics and mechanisms of binding of lectins to multi- and polyvalent glycoproteins. Furthermore, the results have suggested common energetics and mechanisms of binding of ligands to biopolymers in general.

1.2 Affinities of SBA and VML for Mucins

ITC measurements demonstrate that SBA binds to Tn-PSM possessing ~2,300 α-GalNAc residues and a molecular mass of ~10^6 Da (Fig. 1c) with a K_d of 0.2 nM (Table 1), which is ~10^6-fold enhanced affinity relative to monovalent GalNAcα1-*O*-Ser. The 81 amino acid tandem repeat domain of Tn-PSM containing ~23 GalNAc residues (81-mer Tn-PSM) (Fig. 1d) binds with ~10^3-fold enhanced affinity, while the 38/40-mer fragment of Tn-PSM containing ~11 to 12 GalNAc residues (38/40-mer Tn-PSM) (Fig. 1e) shows ~10^2-fold enhanced affinity (Table 1). Fd-PSM, the fully decorated form of PSM containing 40 % of the core 1 blood group type A tetrasaccharide, and 58 % peptide linked GalNAcα1-*O*-Ser/Thr residues, with 45 % of the peptide linked GalNAc residues linked α [2, 6] to *N*-glycolylneuraminic acid (Fig. 1b),

shows ~10^4 enhanced affinity for SBA (Table 1). VML displays a similar pattern of affinities for the PSM analogs although there are differences in the absolute affinities (Table 1) [28]. The higher affinities of SBA and VML for Tn-PSM relative to Fd-PSM indicate the importance of carbohydrate composition and epitope density of the mucins on their affinities for the lectins. The higher affinities of both lectins for Tn-PSM relative to its two shorter chain analogs demonstrate that the length of a mucin polypeptide and hence total carbohydrate valence determines the affinities of the lectins for the three Tn-PSM analogs.

Kiessling and coworkers [32] reported a similar increase in the inhibitory activity of synthetic polymers of increasing lengths that possess Man residues in a hemagglutination assay with ConA. The authors concluded that the enhanced inhibitory activities of the longer chain polymers were "largely due to a combination of statistical and chelation effects" and therefore lower dissociation rates.

1.3 Thermodynamics of SBA Binding Tn-PSM

The ITC data also include the thermodynamics of binding of SBA and VML to the PSM analogs, including their stoichiometries of binding (Table 1) [28]. The n value for SBA binding to Tn-PSM, which is the binding stoichiometry expressed as number of binding sites per subunit of lectin, is 0.0018 (Table 1). The value of $1/n$ has been shown to provide the functional valence of multivalent carbohydrates and glycoproteins binding to lectins [27, 33]. The $1/n$ for Tn-PSM binding SBA is 540, which indicates that 540 α-GalNAc residues of the ~2,300 α-GalNAc residues of Tn-PSM bind 540 monomers of SBA since each monomer binds one GalNAcα1-*O*-Ser. The $1/n$ value indicates that the functional valence of Tn-PSM for SBA is less than the structural valence of Tn-PSM. The reason for the fractional binding of SBA to the carbohydrate epitopes in Tn-PSM appears to be the size of the SBA tetramer, which possesses a molecular mass of 120 kDa and one N-linked Man-9 oligomannose chain per monomer [34, 35]. Kiessling and coworkers have reported similar fractional occupancies for the binding of ConA, a tetrameric Man-specific lectin similar in size to SBA, to synthetic polymers containing α-mannose residues [36]. Fractional occupancy has also been reported for SBA binding to the nine LacNAc epitopes of ASF [37].

SBA binding to Tn-PSM yields a ΔH of –4,310 kcal/mol as compared to –7.9 kcal/mol for GalNAcα1-*O*-Ser (Table 1). If the ΔH for SBA binding to Tn-PSM is divided by the $1/n$ value of 540 α-GalNAc residues on Tn-PSM that bind to the lectin, the resulting ΔH per α-GalNAc residue of Tn-PSM is –7.98 kcal/mol, which is very similar to the ΔH of –7.9 kcal/mol for GalNAcα1-O-Ser. This indicates that each α-GalNAc residue of Tn-PSM that binds to SBA possesses the same enthalpy of binding as that of GalNAcα1-*O*-Ser. The observed ΔH for SBA binding to Tn-PSM is thus the sum of the individual ΔH values of the α-GalNAc binding

residues of the mucin. Similar observations have been made for the binding of ConA and DGL to synthetic bi-, tri-, and tetraantennary glycosides [38].

The calculated $T\Delta S$ for SBA binding to Tn-PSM is –4,297 kcal/mol (Table 1). If $T\Delta S$ is divided by the $1/n$ value of 540, the resulting $T\Delta S$ value per α-GalNAc residue of Tn-PSM is –7.96 kcal/mol, which is more unfavorable than the –2.7 kcal/mol for GalNAcα1-*O*-Ser (Table 1). If $T\Delta S$ were proportional to the number of binding epitopes in Tn-PSM, the observed $T\Delta S$ value would be –1,458 kcal/mol, and ΔG would therefore be equal to $\Delta H - T\Delta S$ or –2,852 kcal/mol, an impossibly large value. The observation that, unlike ΔH, $T\Delta S$ does not scale in proportion to the number of binding epitopes in multivalent carbohydrates binding to lectins but instead is much more negative has been previously observed in the binding of ConA and DGL to bi-, tri-, and tetraantennary carbohydrates [38]. The non-proportionality of $T\Delta S$ is characteristic of different lectin molecules binding to separate epitopes of a multivalent carbohydrate instead of a single lectin binding to multiple epitopes of a multivalent carbohydrate [39]. In the latter case, both ΔH and $T\Delta S$ increase in proportion to the number of binding epitopes in the multivalent ligand, with concomitantly larger increases in affinity [39]. Nevertheless, the observed $T\Delta S$ value of –4,297 kcal/mol when subtracted from the observed ΔH value of –4,310 kcal/mol for SBA binding to Tn-PSM gives a ΔG value of –13.1 kcal/mol, which is –7.9 kcal/mol greater than the ΔG of –5.2 kcal/mol for GalNAcα1-*O*-Ser (Table 1).

1.4 Thermodynamics of SBA Binding the 81-mer Tn-PSM

The ITC-derived K_d value for SBA binding to 81-mer Tn-PSM is 0.06 μM. This can be compared with the value of 0.15 μM obtained by hemagglutination inhibition [28]. The K(rel) for 81-mer Tn-PSM is 2,800 as compared to GalNAcα1-*O*-Ser. The affinity of SBA for 81-mer Tn-PSM is ~300-fold weaker than that of Tn-PSM.

The n value for SBA binding to 81-mer Tn-PSM is 0.12, and $1/n = 8$, which suggests that ~8 α-GalNAc residues of 81-mer Tn-PSM bind to ~8 monomers of SBA. Hence, the functional valence of 81-mer PSM is less than its structural valence for SBA binding, as observed for Tn-PSM.

The ΔH for SBA binding to 81-mer Tn-PSM is –56.1 kcal/mol. If ΔH is divided by the $1/n$ value of 8, the number of bound SBA monomers per ~23 α-GalNAc residues, the resulting value is –7.0 kcal/mol per α-GalNAc binding residue, which is slightly less than the value of –7.9 kcal/mol for GalNAcα1-*O*-Ser. This indicates that each α-GalNAc binding residue of 81-mer Tn-PSM binds with nearly the same ΔH as that of GalNAcα1-*O*-Ser. This finding is similar to that observed for SBA binding to Tn-PSM.

SBA binding to 81-mer Tn-PSM gives a $T\Delta S$ of –46.3 kcal/mol which is greater than the calculated value of –21.6 kcal/mol if $T\Delta S$ were proportional to the number of α-GalNAc residues

involved in binding to SBA. Thus, $T\Delta S$ for SBA binding to 81-mer Tn-PSM does not increase in proportion to the number of α-GalNAc residues that bind, but rather has a larger negative value. Similar results are observed for SBA binding to Tn-PSM.

1.5 Thermodynamics of SBA Binding 38/40-mer Tn-PSM

ITC data for the binding of SBA to 38/40-mer Tn-PSM shows a K_d of 1.4 μM (Table 1), which can be compared with ~6 μM obtained by hemagglutination inhibition [28]. The K_d for 38/40-mer Tn-PSM is reduced nearly 20-fold relative to that for 81-mer Tn-PSM. Thus, SBA shows lowest affinity for the shortest fragment of Tn-PSM.

The n value for SBA binding to 38/40-mer Tn-PSM is 0.2, and $1/n = 5$. This indicates that ~5 α-GalNAc residues of 38/40-mer Tn-PSM bind to five SBA monomers.

The ΔH and $T\Delta S$ values of –32.2 kcal/mol and –24.2 kcal/mol, respectively, are consistent with the lower affinity of SBA for 38/40-mer Tn-PSM as compared to the longer polypeptide chain analogs. If ΔH is divided by the $1/n$ value of 5, then ΔH per α-GalNAc residue of 38/40-mer is –6.44 kcal/mol, which is somewhat lower than the –7.9 kcal/mol for GalNAcα1-*O*-Ser. The lower ΔH per α-GalNAc residue may be due to the lower affinity of SBA for 38/40-mer Tn-PSM as compared to Tn-PSM and 81-mer Tn-PSM (discussed further below). If the observed $T\Delta S$ is divided by $1/n$ value of 5, the $T\Delta S$ per α-GalNAc residue of 38/40-mer is –4.84 kcal/mol, which is larger than –2.7 kcal/mol for GalNAcα1-*O*-Ser. Similar results are observed for SBA binding to Tn-PSM and 81-mer Tn-PSM, suggesting similar binding mechanisms of SBA with all three PSM analogs.

1.6 Thermodynamics of SBA Binding Fd-PSM

The ITC-derived K_d for SBA binding to Fd-PSM is 0.024 μM (Table 1), which can be compared to the inhibition constant of 0.08 μM obtained by hemagglutination inhibition [28]. The loss in affinity of SBA for Fd-PSM relative to Tn-PSM may be due to the lower density of free α-GalNAc residues in Fd-PSM, due to NeuNGl present on ~45 % of the total α-GalNAc residues.

The n value for SBA binding to Fd-PSM is 0.008 (Table 1), and $1/n$ is 125, which is the number of α-GalNAc residues of Fd-PSM bound to SBA monomers. This number of α-GalNAc residues is consistent with a reduced number of binding sites for SBA on Fd-PSM relative to the 540 α-GalNAc residues on Tn-PSM. The factor of ~4 reduction in the number of binding sites on Fd-PSM suggests that in addition to 45 % of the total α-GalNAc residues capped with NeuNGl, binding of SBA to the nonreducing α-GalNAc residue of the tetrasaccharide chains of PSM may alter the accessibility of SBA binding to the single peptide linked α-GalNAc residues by a further factor of 2. Similar effects are observed for VML binding to Tn-PSM and Fd-PSM (below).

The ΔH and $T\Delta S$ values for Fd-PSM binding to SBA are −703 kcal/mol and −693 kcal/mol, respectively, and are lower than the corresponding values for Tn-PSM (Table 1). If ΔH is divided by $1/n = 125$, the resulting ΔH per binding α-GalNAc residue is −5.6 kcal/mol which is somewhat lower than that for GalNAcα1-*O*-Ser (Table 1). If $T\Delta S$ is divided by $1/n = 125$, the resulting $T\Delta S$ per binding α-GalNAc residue is −5.54 kcal/mol which is greater than that for GalNAcα1-*O*-Ser. Similar results are observed for SBA binding to Tn-PSM, 81-mer Tn-PSM, and 38/40-mer PSM.

1.7 Thermodynamics of VML Binding Tn-PSM

The ITC data for VML binding to Tn-PSM is similar to that for SBA [28]. The main difference is the stoichiometry of binding of VML, which shows that $1/n = 833$ α-GalNAc residues binding to 833 monomers of VML. This can be compared with 540 α-GalNAc residues of Tn-PSM binding to 540 monomers of SBA. Importantly, the size of the VML tetramer is similar to that of SBA [40], and VML also possesses covalently bound carbohydrate like SBA [40].

1.8 Thermodynamics of VML Binding 81-mer Tn-PSM and 38/40-mer Tn-PSM

The ITC data for VML binding to 81-mer Tn-PSM and 38/40-mer Tn-PSM in Table 1 are similar to that observed for SBA [28]. Differences between VML and SBA binding to the two fragments of Tn-PSM are principally in their K(rel) values which are somewhat greater for VML. The binding stoichiometries of VML to the two fragments are also similar to those observed for SBA. The results support the conclusion that the affinity of VML, like SBA, decreases with shorter fragments of Tn-PSM.

1.9 Thermodynamics of VML Binding Fd-PSM

The ITC data for VML binding to Fd-PSM is also similar to that for SBA. Differences between the two lectins are in their K(rel) values which are greater for VML. Like SBA, VML binds with greater affinity to Fd-PSM than to 81-mer Tn-PSM and 38/40-mer PSM, but with less affinity to Fd-PSM than to Tn-PSM. The binding stoichiometry of VML to Fd-PSM indicates that there are 200 α-GalNAc residues of Fd-PSM that bind to VML monomers. This can be compared to the 125 α-GalNAc residues of Fd-PSM that bind to SBA.

1.10 Analysis of the Stoichiometry of Binding of SBA to the Mucins

Using the $1/n$ value for Tn-PSM indicates that ~540 GalNAc residues out of the total of ~2,300 GalNAc residues of the mucin bind to SBA under saturation conditions. SBA is a tetramer with four binding sites per molecule [35]. Thus, all four sites of SBA are occupied at the end of the ITC experiment, and hence SBA is completely cross-linked with Tn-PSM. This is also true for the ITC experiments for SBA binding to the shorter mucin fragments.

1.11 Mechanisms of Binding of SBA and VML to PSM: The "Bind and Jump Model"

The increasing affinities of SBA and VML for 38/40-mer Tn-PSM, 81-mer Tn-PSM, and Tn-PSM, respectively, indicate that the lengths of the polypeptide chains and hence total carbohydrate valences of these mucin analogs regulate their affinities for the lectins. The higher affinities of SBA and VML for Tn-PSM relative to Fd-PSM also indicate the importance of carbohydrate composition and epitope density of the mucins on their affinities for lectins. The similarities in the ΔH per α-GalNAc binding residue of Tn-PSM and Fd-PSM with that of GalNAcα1-*O*-Ser for the two lectins, respectively, also suggest a common mechanism of binding. Similar correlations with 81-mer Tn-PSM and 38/40-mer Tn-PSM suggest that the binding mechanisms of these analogs are common.

A model to explain these results is similar to that proposed for the binding of lectins to multivalent carbohydrates [38] and globular glycoproteins [27]. Namely, binding of a lectin to a multivalent carbohydrate or glycoprotein involves internal diffusion "jumps" of the lectin from carbohydrate epitope to epitope before complete dissociation. Kinetically, this has the effect of reducing the macroscopic off-rate of the lectin and hence increasing its affinity, since the affinity constant is the ratio of the forward and reverse rate constants. The forward rate constant for lectin binding may also be enhanced as a result of the larger number of binding epitopes on the glycoprotein.

The diffusion jump model for SBA and VML binding to Tn-PSM and the other PSM analogs can be envisioned as occurring with one subunit of SBA or VML bound to one α-GalNAc residue of Tn-PSM at a time (Fig. 2a). (Two subunits of individual SBA or VML molecules simultaneously binding to a single Tn-PSM chain are not supported by the enhanced affinities of both lectins to 38/40-mer Tn-PSM relative to GalNAcα1-*O*-Ser (Table 1). If two subunits of an SBA tetramer bound to 38/40-mer Tn-PSM, the affinity enhancement would be approximately the product of the individual GalNAcα1-*O*-Ser dissociation constant which would be ~10^{-8} M instead of the observed 10^{-6} M.) This model provides a molecular mechanism to account for the dependence of the affinities of the two lectins on the total carbohydrate valences of Tn-PSM, 81-mer Tn-PSM, and 38/40-mer Tn-PSM. This model further suggests that as more lectin molecules bind to a mucin chain, the affinity of the lectin will decrease because of steric crowding and shorter diffusion distances on the polypeptide chain of the mucin (Fig. 2b). Indeed, Hill plots for SBA binding to Tn-PSM show evidence for negative binding cooperativity (data below). Similar Hill plots have been interpreted as evidence for increasing negative cooperativity in the binding of galectins to ASF [27] and ConA and DGL to synthetic di-, tri-, and tetravalent carbohydrates [38]. In these cases, binding of the lectins to the multivalent carbohydrates and glycoprotein was associated with gradients of

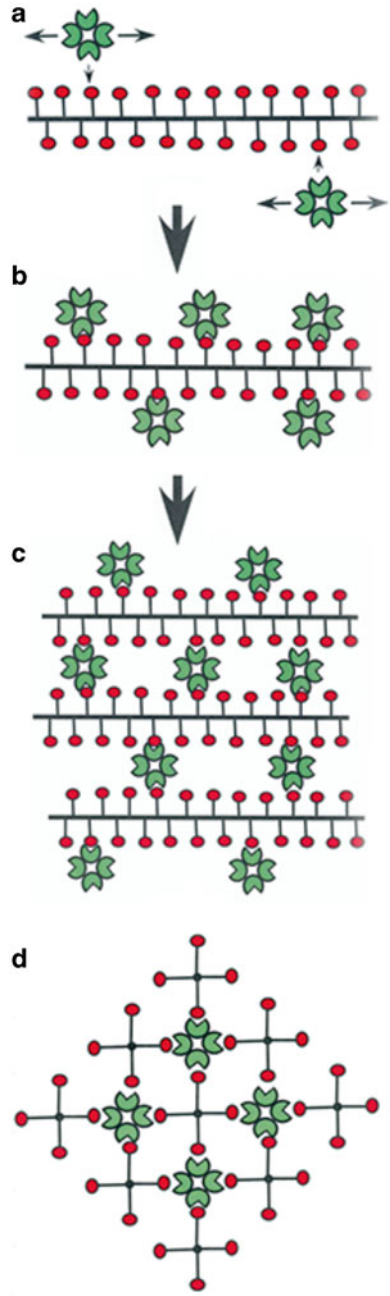

Fig. 2 Schematic representations of (**a**) SBA or VML binding at low density to Tn-PSM; (**b**) SBA or VML binding at higher density to Tn-PSM; (**c**) SBA and VML binding at higher density to Tn-PSM and initiating cross-linking of the complexes; (**d**) SBA cross-linked complexes with Tn-PSM under saturation binding conditions. The view is on the end of the polypeptide chains of Tn-PSM in Fig. 1c. α-GalNAc residues extend out from the polypeptide chains of Tn-PSM in three dimensions. Lectin tetramers are bound to four separate Tn-PSM chains, with staggered binding down the length of the mucin chains

decreasing microaffinity constants of the lectins for the multiple epitopes of the ligands. This suggests that the observed dissociation constants for SBA and VML binding to the four PSM analogs in Table 1 represent a composite of the gradient of binding constants present in each case. Such gradients have been estimated to be as large as 3,000- to 6,000-fold for galectins binding to ASF [27]. These gradient effects may explain the lower average ΔH values per α-GalNAc residue for 81-mer Tn-PSM and 38/40-mer PSM binding to SBA and VML.

The model for SBA and VML binding must also agree with the final saturation density of SBA and VML molecules bound to single PSM analogs as reflected in ITC n values. For example, the $1/n$ value for SBA binding to Tn-PSM indicates 540 α-GalNAc residues bound to 540 SBA monomers. The $1/n$ value for VML binding to Tn-PSM indicates 833 α-GalNAc residues bound to 833 VML monomers. Another requirement of the binding model is the observation that at the end of ITC experiments, solutions of SBA and VML with all four PSM analogs begin to precipitate out of solution (data not shown). This indicates that lectin-mediated cross-linking of the mucins occurs following saturation binding.

Both of these observations are accounted for in the schematic representation in Fig. 2c, d of SBA cross-linked with Tn-PSM under saturation conditions. The schematic shows individual SBA molecules cross-linked to four different Tn-PSM molecules. Importantly, in order to form the cross-linked complex shown in Fig. 2d, lectin molecules bind to α-GalNAc residues on all four sides of a Tn-PSM polypeptide chain. This allows staggering of individual SBA molecules along the Tn-PSM polypeptide chain, with concomitant reduction in steric interactions between lectin molecules. This is important since calculations of the density of SBA molecules bound to Tn-PSM (knowing the diameter of SBA from x-ray crystal studies [35] and the length of the Tn-PSM polypeptide chain) suggest that only ~300 SBA tetramers can bind to the same side of a Tn-PSM polypeptide chain, which is less than the 540 bound monomers of SBA and 833 bound monomers of VML derived from ITC n values. The apparent steric crowding is overcome by having lectin molecules bound to all four sides of Tn-PSM.

In summary, the binding models first show a fraction of SBA or VML molecules that bind to Tn-PSM and "jump" between different α-GalNAc residues of the mucin. As the number of bound lectin molecules increases, the affinity of the lectin decreases because of shorter diffusion distances on the mucin chain due to steric crowding and cross-linking by multiple bound lectin molecules. Finally, upon saturation binding, full lectin-mediated cross-linking of the complexes takes place.

2 Materials

1. Soybean agglutinin (SBA) (EY Biochemicals, Inc.) (*see* **Note 1**).
2. *Vatairea macrocarpa* lectin (VML) (*see* **Note 2**).
3. Guargum column.
4. Preparations of Fd-PSM, Tn-PSM, 81-mer Tn-PSM, and 38/40-mer Tn-PSM (prepared as previously described [24]) (*see* **Note 3**).
5. GalNAcα1-*O*-Ser (Sigma).
6. Rabbit erythrocytes.
7. 96 well round bottom microtiter plate.

Buffers

8. Hepes buffer: 0.1 M Hepes, 0.15 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$, pH 7.2.
9. Phosphate Buffered Saline (PBS) (Hyclone).

Special Equipment

10. VP-ITC instrument from Microcal, Inc. (Northampton, MA).
11. Computer-controlled microsyringe.

3 Methods

3.1 Hemagglutination Inhibition Assay to Determine the Binding Affinity of Mucins

1. Add 0.3 mL of packed rabbit red blood cells to 9.7 mL of Hepes buffer (0.1 M Hepes, 0.15 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$, pH 7.2) for a final concentration of 3 %.
2. At room temperature, prepare a twofold serial dilution of lectin sample as well as a twofold serial dilution of saccharide sample with PBS (*see* **Note 4**).
3. Mix 25 μL of diluted lectin samples with 25 μL of each dilution of saccharide sample in wells of a 96 well round bottom microtiter plate. Final volume in each well should be 0.05 mL.
4. Add 0.05 mL of 3 % erythrocyte suspension to each well and allow erythrocytes to settle for 20–30 min.
5. Note agglutination. A positive pattern, indicating agglutination, is a uniform covering of the bottom of the round bottom well by erythrocytes. A negative pattern, indicating no agglutination, is a circular clump of erythrocytes surrounded by a clear zone.
6. The minimum concentration of saccharide required for complete inhibition of four hemagglutination doses is determined.

3.2 ITC Studies of Lectin–Mucin Interactions

The thermodynamic binding properties of modified forms of porcine submaxillary mucin (PSM) were investigated with SBA and VML [28]. ITC experiments were performed using a VP-ITC instrument from Microcal, Inc. (Northampton, MA) (*see* **Note 5**).

1. At room temperature, prepare a twofold serial dilution of lectin sample as well as a twofold serial dilution of saccharide sample with PBS (*see* **Note 6**).
2. Inject 4 μl of carbohydrate solution from a computer-controlled microsyringe at an interval of 4 min into the sample solution of lectin (cell volume = 1.43 ml) with stirring at 350 rpm. Control experiments should be performed by injection of the mucin into a cell containing only buffer.
3. Fit the experimental data to a theoretical titration curve using software supplied by Microcal, with ΔH (binding enthalpy in kcal/mol) (lectin monomer units), K_a (association constant), and n (number of binding sites per monomer), as adjustable parameters.
4. Calculate the K_d (dissociation constant) from $1/K_a$. The quantity $c = K_a$ Mt (0), where Mt (0) is the initial macromolecule concentration, is of importance in titration microcalorimetry. All experiments were performed with c values $1 < c < 500$ (*see* **Note 7**).

4 Notes

1. The concentration of SBA was determined spectrophotometrically using $A_{1\%,\,1\text{ cm}} = 12.8$ and expressed in terms of monomer.
2. Purified by affinity chromatography on a guargum column. The concentration of VML was determined spectrophotometrically using $A_{1\,\%,\,1\text{ cm}} = 13.0$ and expressed in terms of monomer.
3. The 81-mer Tn-PSM was obtained by trypsin digestion of Tn-PSM, and 38/40-mer Tn-PSM obtained by GluC digestion of 81-mer PSM.
4. Appropriate starting concentration can be determined empirically.
5. The instrument was calibrated using the calibration kit containing ribonuclease A (RNase A) and cytidine 2′-monophosphate (2′-CMP) supplied by the manufacturer. Thermodynamic parameters were calculated from the Gibbs Free Energy equation, $\Delta G = \Delta H - T\Delta S = -RT \ln(K_a)$, where ΔG, ΔH, and ΔS are the changes in free energy, enthalpy, and entropy of binding, respectively. T is the absolute temperature and $R = 1.98$ cal/mol/K.
6. Titrations were done at pH 7.2 using 20 mM PBS buffer. The concentration range of the lectins should be 5–100 μM and for

the mucins should be 0.6 μM to 0.50 mM based on the dry weight of the latter molecules. Concentration of the mucins was based on their molecular weights.

7. Multivalent lectin–mucin interactions often generate insoluble precipitates under proper stoichiometric condition. Such precipitation during ITC experiments affects the quality of the binding isotherm. This problem can be minimized by choosing proper experimental conditions, including buffers and low concentration of reactants (lectins and mucins in this case). ITC can be done at low sample concentration if the affinity of interaction is high. Newer ITC instruments also possess smaller sample volumes, thereby reducing the amount of sample required.

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Chapter 6

Examination of Whole Cell Galectin Binding by Solid Phase and Flow Cytometric Analysis

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Abstract

We have utilized simple flow cytometric and fluorescence-based solid phase assays to study the interaction of glycan-binding proteins (GBP) to cell surface glycoconjugates. These methods utilize commonly employed flow cytometry techniques and commercially available streptavidin-coated microplates to immobilize various biotinylated ligands, such as glycopeptides, oligosaccharides, and whole cells. Using this approach, fluorescently labeled GBPs, in particular, members of the galectin family, can be interrogated for potential interactions with cell surface carbohydrates, including elucidation of the potential impact of alterations in glycosylation on carbohydrate recognition. Using these approaches, we present examples of flow cytometric and fluorescence-based solid phase assays to study galectin–carbohydrate interactions.

Key words Galectin-1, Solid phase assay, Biotinylation, Fluorescence labeling, Immobilization, Binding affinity

1 Introduction

Glycan-binding proteins (GBPs) often regulate a wide variety of biological processes through the recognition of highly modifiable terminal residue recognition. However, in addition to the potential impact of terminal modifications, the core backbone of many glycan structures can influence terminal glycan binding [1–3]. As a result, binding data obtained following analysis on glycan microarrays that primarily contain relatively simple terminal glycan motifs may not fully illustrate the impact of core glycan structure on overall binding interactions [4, 5]. To overcome these limitations, various approaches have employed natural carbohydrates harvested from glycoproteins or glycolipids [4, 6–11]. However, in addition to the glycan backbone, cell surface presentation of glycans can also significantly impact galectin–carbohydrate interactions [12]. As a result, in addition to examining galectin–carbohydrate specificity using defined arrays populated with synthetic or naturally

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occurring glycans, interrogating galectin-binding specificity toward intact cells can provide a complementary tool to investigate the potential impact of cell surface glycan alterations on the binding interactions of various galectin family members.

Although previous studies have employed a variety of formats to examine galectin binding toward cell surface carbohydrates, flow cytometric analysis of galectin interactions with mammalian cells or microbes likely reflects the most commonly employed method [13, 14]. This approach enables a relatively high-throughput method of evaluating significant changes in galectin engagement following genetic or enzymatic alterations in cell surface glycans (Fig. 1) [12, 15–20]. As different cell populations may express distinct galectin ligands, this approach also allows examination of potential galectin interactions with distinct populations of cells within a complex mixture, providing a useful tool to not only examine the potential impact of changes in glycosylation on galectin binding and function but also to characterize previously unrecognized cellular subsets that may express distinct carbohydrate ligand for various members of the galectin family.

While flow cytometric approaches provide a useful tool to examine general interactions between galectins and cell surface carbohydrates, the concentrations of galectins utilized in this format rarely saturate cell surface counter receptors prior to inducing significant agglutination. Galectin-induced agglutination can induce artifacts during flow cytometric analysis secondary to cell fragmentation, as previously shown for antibodies capable of agglutinating cells [21], which can significantly alter the cellular profile and apparent density of ligands. Although sub-agglutinating concentrations of galectins are often employed to avoid this limitation, these sub-saturating concentrations also limit complete analysis of binding as saturation can rarely be achieved in order to fully appreciate the impact of ligand density and other potential changes on binding affinity. In addition, the examination of galectin binding in the linear range of ligand engagement can result in significant inter-assay variability as a result of subtle differences in the concentration of galectins employed in different assay conditions. By contrast, most flow cytometric analyses purposely employ monoclonal antibodies at saturating levels to ensure complete engagement of target ligands in order to adequately identify potential alteration in ligand density between cellular subsets and following potential ligand modifications [22–24]. In addition, flow cytometric analysis does not necessarily provide a measurement of affinity to determine whether, in addition to alterations in ligand density, the actual affinity of galectins toward carbohydrate ligands may differ between unique cell populations decorated with different glycan ligands.

To overcome inherent limitations associated with flow cytometric analysis, we have also utilized a simple fluorescence-based solid phase assay on a microplate format to study interactions between galectins and cell surface glycoconjugates. This approach

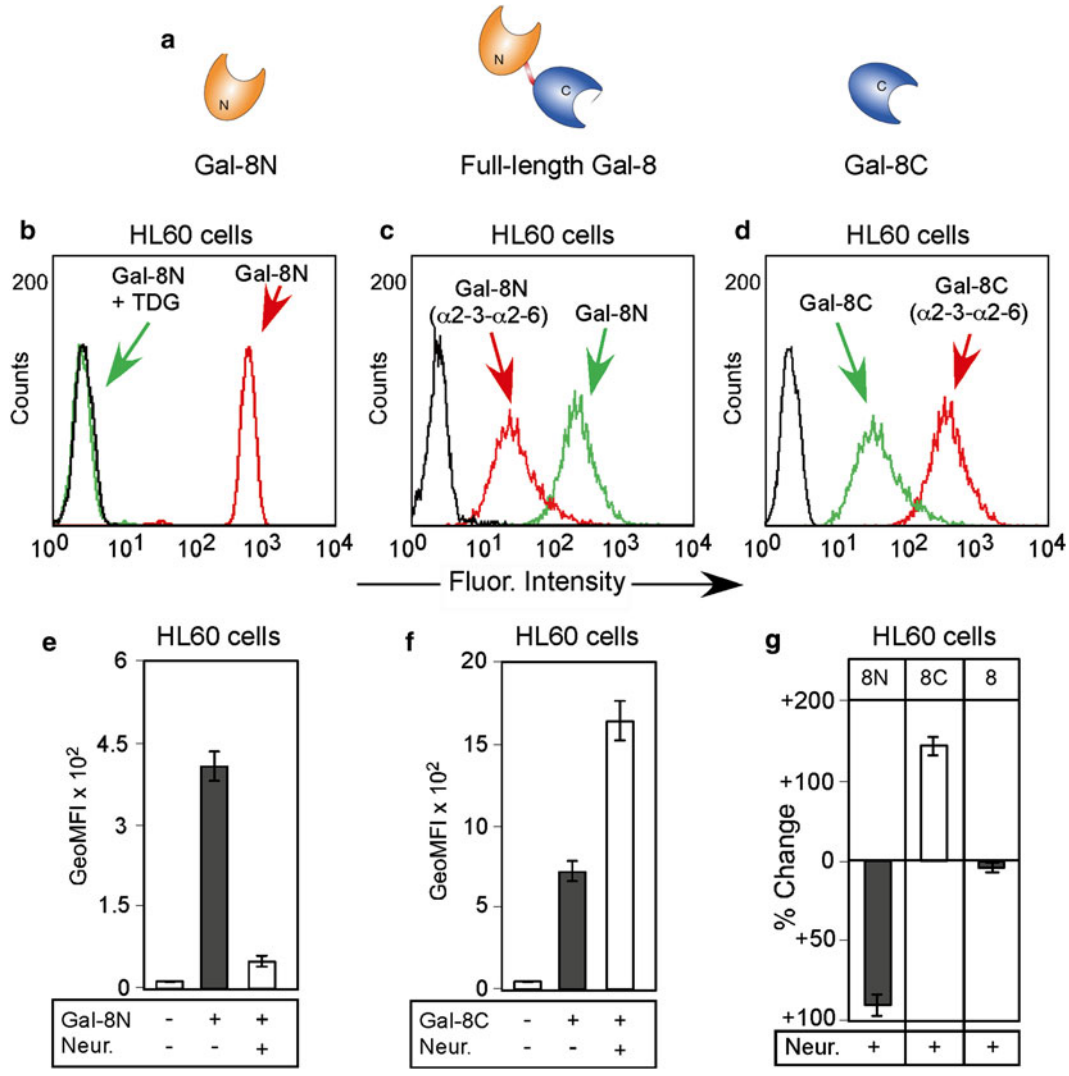

Fig. 1 Gal-8N and Gal-8C display differential recognition of cell surface glycans. (**a**) Schematic representation of full-length Gal-8 and individual domains. (**b**) Binding of Gal-8N toward HL-60 cells with or without incubation 20 mM TDG or 20 mM sucrose. (**c**) Binding of Gal-8N toward HL-60 cells treated with *A. ureafaciens* neuraminidase. (**d**) Binding of Gal-8C toward HL-60 cells treated with *A. ureafaciens* neuraminidase. (**e**) Geometric mean fluorescent intensities (GeoMFI), a measure of mean fluorescent intensity on logarithmic scales, of Gal-8N binding before and after treatment of cells with *A. ureafaciens* neuraminidase. (**f**) GeoMFI of Gal-8C binding before and after treatment of cells with *A. ureafaciens* neuraminidase. (**g**) Comparison of Gal-8, Gal-8N, or Gal-8C binding toward HL-60 cells following treatment with *A. ureafaciens* neuraminidase. *Bars* represent the percent change in cell surface binding when compared with the mean fluorescent intensity of nontreated cells ± S.D. This research was originally published in the Journal of Biological Chemistry [16] with permission from the American Society for Biochemistry and Molecular Biology

appears to avoid potential artifacts that may be introduced when using saturating concentrations of galectins [25–27]. To accomplish this, biotinylated human HL-60 cells or T cells were immobilized onto the streptavidin-coated microplates and probed with

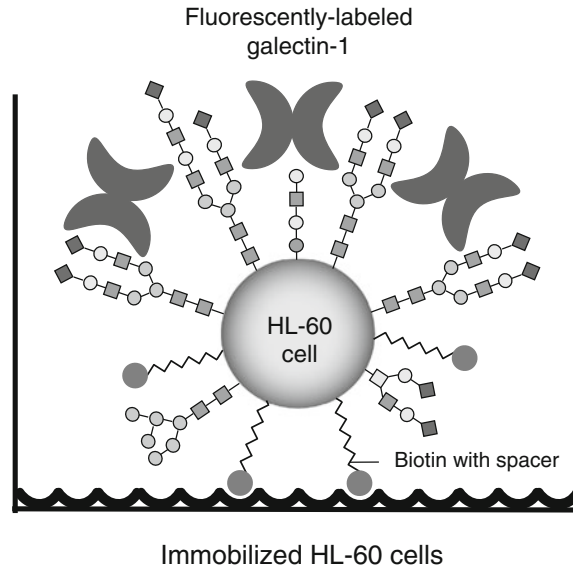

Fig. 2 Galectin-1 binding to immobilized HL-60 cells as an example of fluorescence-based solid phase assay to study GBP–glycoconjugate interactions on streptavidin-coated microplates

fluorescently labeled galectins (Fig. 2) [26, 15]. A portion of cells were also treated sequentially with different glycosidases before immobilization into the microtiter plates to gather structural information regarding the impact of carbohydrate modification on cell surface binding [26, 15]. Parallel binding experiments were performed with fluorescently labeled plant lectins with known binding specificity to control for the effect of glycosidases on cells [26, 15, 28, 29]. By using serially diluted galectins in solid phase assay with immobilized cells, apparent binding affinity of galectin to cell surface glycoconjugates was obtained [26, 27]. Thus, this approach not only facilitates examination of the impact of alterations in glycosylation on galectin binding but also provides a useful approach to determine the relative affinity of galectins toward various cell surface-associated glycans.

Regardless of the approach used, galectin typically requires labeling using a variety of fluorescent probes that react with different functional groups of a protein to facilitate flow cytometric or fluorimeter detection following potential binding reactions. Fluorescent probes can be covalently attached in a variety of ways, including via primary amines, reduced cysteine residues, or oxidized carbohydrate residues. The fluorescent probe should be selected carefully, because covalent derivatization of amino acid residues involved in ligand binding may result in galectin inactivation. Therefore, it is important to test the activity of the labeled galectins before using it in an assay. If a monoclonal antibody is

available to a galectin under study, an antibody can be fluorescently labeled and used to detect the bound galectin in the assay. Alternatively, if a recombinant galectin has been produced as an IgG fusion protein [30], commercial fluorescently labeled anti-IgG monoclonal antibody can be used for detection. Taken together, these methods provide a variety of approaches to elucidate galectin interactions with highly modifiable cell surface glycans.

2 Materials

2.1 Biotinylation and Fixation of HL-60 Cells

1. HL-60 cells (other types of cells can also be used).
2. Phosphate buffered saline (PBS) and PBS, pH 8.0
3. 8 % paraformaldehyde in PBS, pH 7.2 (prepare fresh or store at –20 °C).
4. EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce).
5. Burker chamber (or suitable equipment) for counting cells.

2.2 Glycosidase Treatments of Biotinylated and Fixed HL-60 Cells

1. *Arthrobacter ureafaciens* neuraminidase (e.g., Roche Diagnostics, Mannheim, Germany).
2. *Escherichia freundii* endo-β-galactosidase (e.g., V-Labs, Inc., Covington, LA).
3. Jack bean β-galactosidase (e.g., Glyko).
4. PBS (standard) pH 7.4.
5. PBS, pH 5.0 containing 1 mg/ml BSA.

2.3 Fluorescence Labeling of Gal-1 Through Primary Amines

1. Recombinant human dimeric Galectin-1.
2. Alexa Fluor 488 carboxylic acid succinimidyl ester (Invitrogen).
3. 0.1 M lactose in PBS.
4. 14 mM β-mercaptoethanol in PBS.
5. PD-10 (or equivalent) small gel filtration column (GE Healthcare).
6. α-Lactosyl-agarose (Sigma) (1–2 ml volume).

2.4 Fluorescence Labeling of Gal-1 Through Cysteines

1. Recombinant human dimeric galectin-1.
2. Alexa Fluor 488 C_5-maleimide (Invitrogen).
3. 0.1 M lactose in PBS.
4. 14 mM β-mercaptoethanol in PBS.
5. PD-10 (or equivalent) small gel filtration column (GE Healthcare).
6. α-Lactosyl-agarose (Sigma) (1–2 ml volume).

2.5 Fluorescence Labeling of Tomato Lectin (LEA) Through Carbohydrates

1. *Lycopersicon esculentum* (tomato) agglutinin (LEA) (Vector Laboratories).
2. Sodium *m*-periodate.
3. Alexa Fluor 488 hydrazide (Invitrogen), prepare fresh solution 2 mg/ml in PBS.
4. PBS (standard) pH 7.4.
5. PD-10 (or equivalent) small gel filtration column (GE Healthcare).

2.6 Gal-1, LEA, and GS-II Binding to Immobilized HL-60 Cells

1. Streptavidin-coated high-binding-capacity 96-well microtiter plates (Pierce).
2. PBS (standard) pH 7.4.
3. 1 % BSA in PBS.
4. Fluorescently labeled lectins: Alexa Fluor 488-labeled Gal-1, Alexa Fluor 488-labeled LEA, and fluorescein-labeled *Griffonia simplicifolia* lectin II (GS-II) (EY Laboratories Inc., San Mateo, CA).
5. Fluorescence microtiter plate reader with suitable filters (for Alexa Fluor 488 and fluorescein excitation at 485 nm and emission at 535 nm).
6. 8-channel (or 12-channel) manual pipet (250–300 μl).

2.7 Determination of Gal-1 Binding Using Flow Cytometric Analysis

1. PBS.
2. 1 % BSA in PBS.
3. Fluorescently labeled lectins: Alexa Fluor 488-labeled Gal-1, Alexa Fluor 488-labeled LEA, and fluorescein-labeled *Griffonia simplicifolia* lectin II (GS-II) (EY Laboratories Inc., San Mateo, CA).
4. BD FACSCalibur.

2.8 Determination of Apparent Binding Affinity of Gal-1 for Immobilized HL-60 Cells

1. Streptavidin-coated high-binding-capacity 96-well microtiter plates (Pierce).
2. PBS (standard) pH 7.4.
3. 1 % BSA in PBS.
4. 1 % BSA and 20 mM lactose in PBS.
5. Alexa Fluor 488-labeled Gal-1.
6. Fluorescence microtiter plate reader with suitable filters (for Alexa Fluor 488 and fluorescein excitation at 485 nm and emission at 535 nm).
7. 8-channel (or 12-channel) manual pipet (250–300 μl).
8. Computer software capable to calculate nonlinear curve fitting (e.g., SigmaPlot).

3 Methods

3.1 Biotinylation and Fixation of HL-60 Cells

1. Wash HL-60 cells three times with PBS (*see* **Note 1**).
2. Suspend cells at a concentration of 20×10^6 cells/ml in PBS, pH 8.0.
3. Add 1.3 mg EZ-Link Sulfo-NHS-LC-Biotin per ml of cells and incubate at RT for 30 min.
4. Wash cells three times with ice cold PBS.
5. Add 8 % paraformaldehyde in PBS to final concentration of 2 % and incubate at RT for 30 min.
6. Wash cells three times with ice cold PBS.
7. After final wash suspend cells to 1 ml of PBS and count cells.

3.2 Fixation Alone of HL-60 Cells (for Enzymatic Treatment Followed by Flow Cytometric Analysis)

1. Wash cells three times with ice cold PBS.
2. Add 8 % paraformaldehyde in PBS to final concentration of 2 % and incubate at RT for 30 min.
3. Wash cells three times with ice cold PBS.
4. After final wash suspend cells to 1 ml of PBS and count cells.

3.3 Glycosidase Treatments of Biotinylated and Fixed (or Fixed Alone) HL-60 Cells

1. Suspend biotinylated and fixed HL-60 cells at the concentration of 5×10^6 cells/ml in PBS. Take a portion of cells for neuraminidase treatment (proceed to **step 2**), and leave the rest of cells untreated (proceed directly to **step 4**).
2. Add *Arthrobacter ureafaciens* neuraminidase to a final concentration of 100 milliunits/ml of cells in PBS and incubate at 37 ° C for 3 h.
3. Wash neuraminidase-treated cells three times with PBS and count cells.
4. Suspend neuraminidase-treated and neuraminidase-untreated cells (from **step 1**) into PBS, pH 5.0 containing 1 mg/ml BSA at the concentration of $2–5 \times 10^6$ cells/ml, and divide each into three equal aliquots.
5. Add *Escherichia freundii* endo-β-galactosidase (250 milliunits/ml, final concentration) to the first sample, Jack bean β-galactosidase (100 milliunits/ml, final concentration) to the second sample, and buffer alone (PBS, pH 5.0 containing 1 mg/ml BSA) to the third sample.
6. Incubate overnight at 37 ° C.
7. Glycosidase-treated cells and control cells can be immobilized directly into streptavidin-coated microtiter plates (Subheading 3.7) (*see* **Note 2**).

3.4 Fluorescence Labeling of Gal-1 Through Primary Amines

1. Human galectin-1 (Gal-1) (1–2 mg) can be labeled through primary amines using Alexa Fluor 488 carboxylic acid succinimidyl ester according to manufacturer's instructions with minor modifications as described in the following steps.
2. Incubate Gal-1 with reactive dye in PBS containing 0.1 M lactose for 1 h at room temperature or by continuing incubation overnight at 4 °C with stirring.
3. Remove free dye and lactose from the labeled Gal-1 using a PD-10 column equilibrated in PBS containing 14 mM β-mercaptoethanol.
4. To separate functionally active Gal-1 from inactive protein, pass labeled Gal-1 over a small lactosyl-agarose column (1–2 ml volume) in PBS. Wash unbound (inactive) Gal-1 from the column with 3–5 column volumes of PBS. Bound (active) Gal-1 is eluted with 0.1 M lactose in PBS. Before each experiment, lactose must be removed using a PD-10 column equilibrated in PBS containing 14 mM β-mercaptoethanol.
5. Gal-1 samples labeled with Alexa Fluor 488 carboxylic acid succinimidyl ester can be stored in 0.1 M lactose in PBS at +4 °C for few months. Lactose must be removed using a PD-10 column in PBS containing 14 mM β-mercaptoethanol before each experiment (*see* **Note 3**).

3.5 Fluorescence Labeling of Gal-1 Through Cysteines

1. Accessible cysteine residues on human galectin-1 (Gal-1) (1–1.5 mg) can be labeled using thiol reactive Alexa Fluor 488 C_5-maleimide according to manufacturer's instructions with minor modifications as described in the following steps.
2. Incubate Gal-1 with tenfold molar excess of thiol reactive Alexa Fluor 488 C_5-maleimide in PBS containing 0.1 M lactose overnight at 4 °C under stirring.
3. Remove free dye and lactose from the labeled Gal-1 using a PD-10 column in PBS containing 14 mM β-mercaptoethanol.
4. To separate functionally active Gal-1 from inactive protein, pass labeled Gal-1 over a small lactosyl-agarose column (1–2 ml volume) in PBS. Wash unbound (inactive) Gal-1 from the column with 3–5 column volumes of PBS. Bound (active) Gal-1 is eluted with 0.1 M lactose in PBS. Before each experiment, lactose must be removed using a PD-10 column equilibrated in PBS containing 14 mM β-mercaptoethanol.
5. Gal-1 samples labeled with Alexa Fluor 488 C_5-maleimide can be stored in PBS containing 14 mM β-mercaptoethanol at 4 °C for at least 3 months without losing activity (*see* **Note 4**).

3.6 Fluorescence Labeling of Tomato Lectin (LEA) Through Carbohydrates

1. Dissolve *Lycopersicon esculentum* (tomato) agglutinin (LEA) in PBS to the final concentration of 4 mg/ml.
2. Weight out solid sodium *m*-periodate (to obtain final concentration of 100 mM in the sample).
3. Add LEA in PBS to the tube containing solid sodium *m-periodate*, and incubate for 30 min at room temperature in the dark to oxidize *cis*-diols of carbohydrates to aldehydes.
4. Remove sodium *m*-periodate using a PD-10 gel filtration column in PBS. Collect 0.5 ml fractions and measure absorbance at 280 nm for each fraction. Pool fractions containing protein (=oxidized LEA).
5. Add Alexa Fluor 488 hydrazide solution (2 mg/ml in PBS) to oxidized LEA sample to the final concentration of 100 μg/mg lectin.
6. Incubate for 1.5–2 h at room temperature under stirring.
7. Remove free dye using a PD-10 column in PBS. Collect 0.5 ml fractions and measure absorbance at 280 nm and 494 nm for each fraction. Pool fractions containing protein (*see* **Note 5**).

3.7 Gal-1, LEA, and GS-II Binding to Immobilized HL-60 Cells

1. To immobilize equal amount of biotinylated HL-60 cells in microtiter well, adjust cell density in each sample to 2×10^6 cells/ml using PBS (*see* **Note 6**).
2. Wash streptavidin-coated microtiter plates three times with 200 μl of PBS.
3. Immobilize glycosidase digested and nondigested HL-60 cells to streptavidin-coated microtiter wells at equivalent densities (100,000 cells/well) in 50 μl of PBS for 1.5 h at room temperature.
4. Wash the wells three times with 200 μl of PBS containing 1 % BSA. After last wash remove buffer carefully by tapping the plate upside down gently against paper towel. Do not let the plate dry (*see* **Note 7**).
5. Add 50 μl of fluorescently labeled Gal-1 (40 μg/ml), LEA (100 μg/ml), or GS-II (100 μg/ml) in PBS containing 1 % BSA, and incubate for 1 h at room temperature.
6. Wash the wells four times with 250–300 μl of PBS containing 1 % BSA (*see* **Note 7**).
7. Add 100 μl of PBS to each well and read the fluorescence by fluorescence microplate reader. Figure 3 shows results on the binding of Gal-1, LEA, and GS-II to immobilized HL-60 cells.

3.8 Determination of Gal-1 Binding to HL-60 Cell Using Flow Cytometric Analysis

1. Prepare dilutions 0.04, 0.08, 0.15, 0.31, 0.75, 1.25, 2.5, and 5.0 μM of fluorescently labeled Gal-1 in PBS with 1 % BSA and in PBS with 1 % BSA and 20 mM lactose (*see* **Note 8**).
2. Add 50 μl of fluorescently labeled Gal-1 dilutions in PBS with1 % BSA or PBS with1 % BSA and 20 mM lactose to each sample containing 1×10^6 cells/ml (final concentration), and incubate for 1 h at room temperature. When analyzing non-fixed live cells, incubate the indicated concentrations of fluorescently labeled Gal-1 with 1×10^6 cells/ml for 30 min. at 4 ° C to avoid Gal-1 internalization or other alterations that may occur at room or higher temperatures during the binding assay.
3. Wash the cells three times with 250–300 μl of PBS containing 1 % BSA.
4. Following the wash steps, evaluate cells using a microscope to ensure that the cells are not agglutinated.

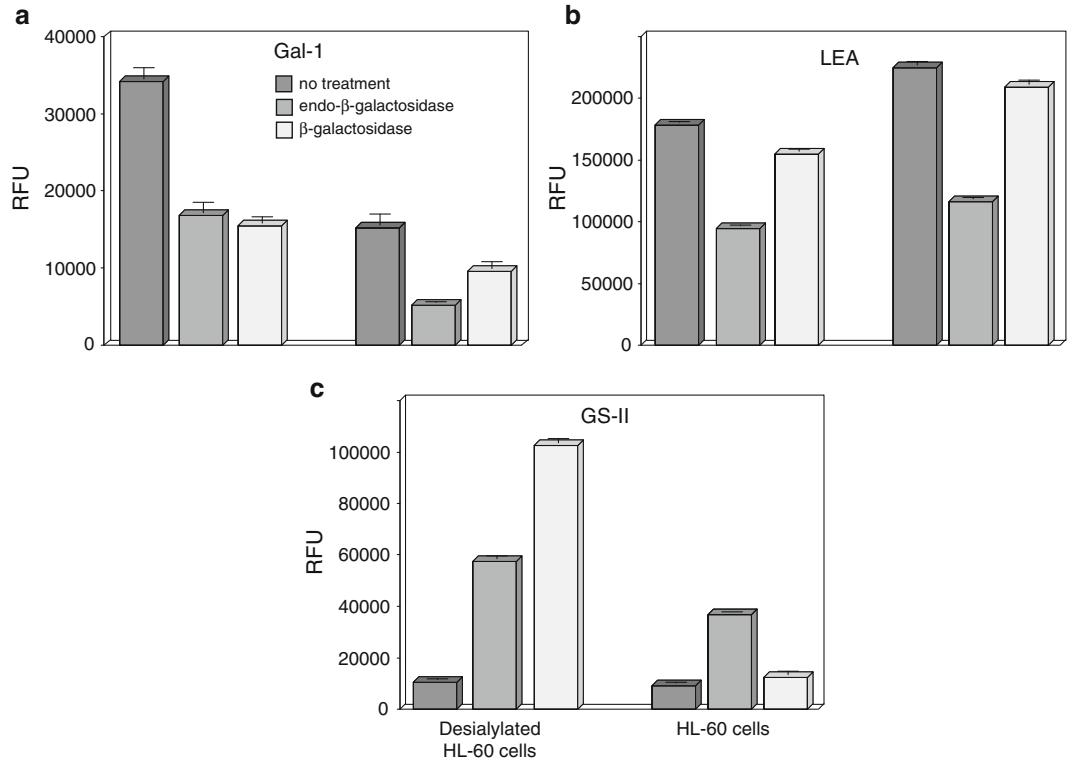

Fig. 3 Binding of Gal-1, LEA, and GS-II to immobilized desialylated and nontreated HL-60 cells. A portion of biotinylated and fixed HL-60 cells first were desialylated. Nontreated and desialylated HL-60 cells were treated with endo-β-galactosidase or β-galactosidase and immobilized on streptavidin-coated microtiter wells (100,000 cells/well). Fluorescently labeled A, Gal-1 (40 μg/ml); B, LEA (100 μg/ml); and C, GS-II (100 μg/ml) were incubated with the immobilized cells. All assays were performed in triplicate, and the results are the means ± S.D. of three determinations. This research was originally published in the Journal of Biological Chemistry [26] with permission from the American Society for Biochemistry and Molecular Biology

5. Add 300 μl of PBS to each well and examine bound galectin by evaluating the mean fluorescence intensity of appropriately gated cells by using commonly employed flow cytometric analysis techniques. Figure 1 shows results on the determination of the binding of Gal-1 to HL-60 cells by flow cytometry.

3.9 Determination of Apparent Binding Affinity of Gal-1 for Immobilized HL-60 Cells

1. Prepare dilutions 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, and 20.0 μM of fluorescently labeled Gal-1 in PBS with 1 % BSA and in PBS with 1 % BSA and 20 mM lactose (*see* **Note 9**).
2. Wash streptavidin-coated microtiter plates three times with 200–300 μl of PBS.
3. Immobilize neuraminidase digested and nondigested HL-60 cells to streptavidin-coated microtiter wells at equivalent density (100,000 cells/well) in 50 μl of PBS for 1.5 h at room temperature.
4. Wash the wells three times with 200 μl of PBS containing 1 % BSA. After last wash remove buffer carefully by tapping the plate upside down gently against paper towel. Do not let the plate dry (*see* **Note 7**).
5. Add 50 μl of fluorescently labeled Gal-1 dilutions in PBS with1 % BSA or PBS with1 % BSA and 20 mM lactose to each well, and incubate for 1 h at room temperature.
6. Wash the wells four times with 250–300 μl of PBS containing 1 % BSA. After last wash remove buffer carefully by tapping the plate upside down gently against paper towel (*see* **Note 7**).
7. Add 100 μl of PBS to each well and read the fluorescence by fluorescence microplate reader.
8. Calculate the apparent dissociation constants (K_d) using nonlinear curve fitting program. Figure 4 shows results on the determination of the binding affinity of Gal-1 for immobilized HL-60 cells.

4 Notes

1. When planning the experiment, it should be considered that relatively a large number of cells are required for solid phase assay. Here we use 100,000 cells/well on 96-well plate. Initial amount of harvested cells should be considerably larger because a number of centrifugations during the course of the experiment result in loss of cells.
2. In the present experiments, we treated biotinylated and fixed cells with glycosidases before capturing cells on streptavidin plates. We did not observe significant differences in the binding assays, if glycosidase treatments were performed on the plate after capturing biotinylated and fixed cells.
3. Alternatively, labeled Gal-1 can be stored without lactose in PBS containing 14 mM β-mercaptoethanol at 4 °C for a few days.

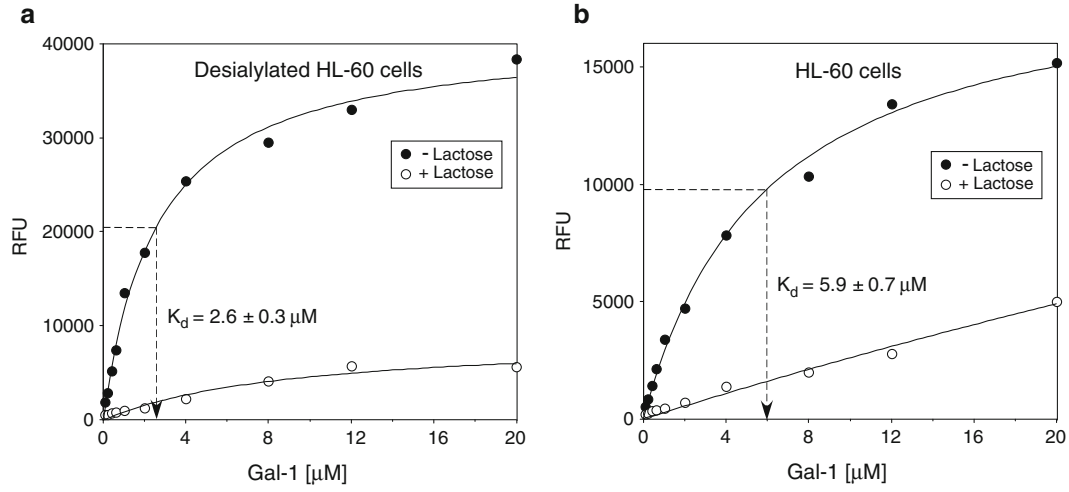

Fig. 4 Binding affinity of Gal-1 for immobilized desialylated and nontreated HL-60 cells. Biotinylated, fixed, desialylated HL-60 cells (**a**) and biotinylated, fixed, nontreated HL-60 cells (**b**) were immobilized on streptavidin-coated microtiter wells (100,000 cells/well). Various concentrations of Gal-1 were incubated with the immobilized cells in buffer with or without 20 mM lactose. All assays were performed in duplicate, and the results are the average of two determinations. This research was originally published in the Journal of Biological Chemistry [26] with permission from the American Society for Biochemistry and Molecular Biology

4. Gal-1 labeled with Alexa Fluor 488 C_5-maleimide is more stable during long-term storage than Gal-1 labeled with Alexa Fluor 488 carboxylic acid succinimidyl ester.
5. The protein concentration and degree of labeling can be calculated using absorbance readings at 280 and 494 nm according to Alexa Fluor 488 hydrazide manufacturer's instructions. The degree of labeling of LEA was significantly higher by labeling through carbohydrates than in commercial fluorescently labeled LEA.
6. Samples should be prepared at least in triplicate, and the results should be calculated as a mean ± S.D.
7. Microtiter plate washing machines should be avoided, and microtiter wells should be washed manually using 8-channel (or 12-channel) pipet. Fill wells gently with wash buffer using pipet, and empty wells into the waste by inverting the plate. After the last wash, remove buffer carefully by gently tapping the plate against paper towel. Do not let the wells to dry at any point during the experiment.
8. Examination of potential Gal-1 interactions over a range of concentrations will allow a linear and sub-agglutinating range of concentrations to be established. Comparison between different potential cellular subsets, enzymatic treatments, or genetic changes in glycosylation should be done by examining

potential differences in binding at the same concentration of galectin. As subtle changes in concentration of galectin may result in some degree of inter-assay variability, differences in binding between cell subsets within an assay may be best enumerated by examining percent changes in comparison to control cells.

9. Samples should be prepared at least in triplicate, and the results should be calculated as an average of three determinations.

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Chapter 7

Evaluation of Galectin Binding by Surface Plasmon Resonance

Padmaja Mehta-D'souza

Abstract

Surface Plasmon Resonance (SPR) instruments, like the BIAcore 3000, are useful for studying the binding between macromolecules in real time. The high sensitivity and low sample consumption in the BIAcore enables the measurement of rapid kinetics and low affinities characteristic of many biological interactions. This chapter describes the affinity measurement of Galectins-1, -2, and -3 and their glycoside ligands using this approach.

Key words Surface plasmon resonance (SPR), Streptavidin (SA) sensor chip, Affinity, *Ligand*, *Analyte*, *Flow cell* (fc), Galectin, Glycosides

1 Introduction

The BIAcore 3000 measures the interaction between two macromolecules using the principle of Surface Plasmon Resonance (SPR). One molecule is coupled to a sensor surface, and is referred to as the *ligand*, the other is in free flow and is referred to as the *analyte* [1–3]. Each sensor chip creates four reaction chambers, referred to as *flow cells* (fc). The integrated microfluidic cartridge (IFC) is designed such that samples can be run over each individual flow cell, over selected pairs of flow cells, or serially over all four flow cells. It is always recommended that one flow cell be used as a control surface to minimize nonspecific binding to the carboxymethylated dextran surface of the sensor chip. An ideal control surface is created by immobilizing a nonbinding ligand control and should always be on fc1 so that it is upstream of the test ligands.

Ligands can be covalently coupled to the sensor surface or captured with an affinity tag such that all immobilized molecules are oriented the same way. However, if it is possible to label a protein with biotin or synthesize a ligand with a biotin tag, that should be the method of choice, as the ligand can then be conveniently captured on a sensor chip pre-coupled with streptavidin (SA).

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Interactions on the BIAcore require small amounts of each macromolecule; the analyte molecule does not need to be labeled, and data is obtained in a relatively short time. Choice of *ligand* and *analyte* depends on several factors, but especially on relative molecular size, availability, and relative purity.

Given the potential roles of individual galectin family members in a wide variety of biological processes, many studies have sought to identify the carbohydrate ligands through which galectins mediate their regulatory activities [4, 5]. Many studies have evaluated galectin–ligand interactions using a variety of approaches, including frontal affinity chromatography, isothermal calorimetry, fluorescence polarization, and more recently glycan microarrays [6–11]. While each of these approaches can enable unique insight into the specificity and biological activities of galectin family members [10–20], SPR also provides a distinct approach with unique advantages when evaluating galectin–glycan interactions [2, 9].

When using BIAcore to evaluate galectin–glycan interactions, samples can be injected onto the sensor chip surface using several different flow rates (from 1 to 100 μl/min). During analyte injection, a constant concentration of analyte is delivered to the ligand surface. The flow rate selected and the volume of the sample injected determines the contact time of the analyte with the ligand. Binding is measured in arbitrary Resonance Units (RU) and recorded in real time in a *sensorgram*, which is the raw data output from a BIAcore experiment. A sensorgram always provides information about the association and dissociation phases of an interaction, and often contains information about steady state binding as well (depending on the affinity of the interaction, the analyte concentration used, and the length of the injection). Antigen–antibody interactions are usually depicted by a typical profile shown in panel "a" of Fig. 1. For some low-affinity interactions with rapid kinetics, a representative sensorgram often looks like that depicted in panel "b" of Fig. 1. In binding studies for these interactions, a control surface is very essential so that the nonspecific binding response arising from changes in the bulk refractive index can be subtracted [21].

One of the advantages of using the BIAcore to look at binding of macromolecules is that the same surface can be used repeatedly. After each analyte injection, the bound analyte is removed with a regeneration solution that disrupts the ligand–analyte interaction but does not affect the functionality of the ligand. When the interaction between analyte and ligand is fairly weak, as in most lectin–carbohydrate interactions, the bound analyte is often washed away with continuous buffer flow alone without the need for regeneration. In this study [9], the BIAcore 3000 was used to compare the binding affinities of Galectin-1, -2, and -3 for lactose, $(LacNAc)_2$ and $(LacNAc)_3$.

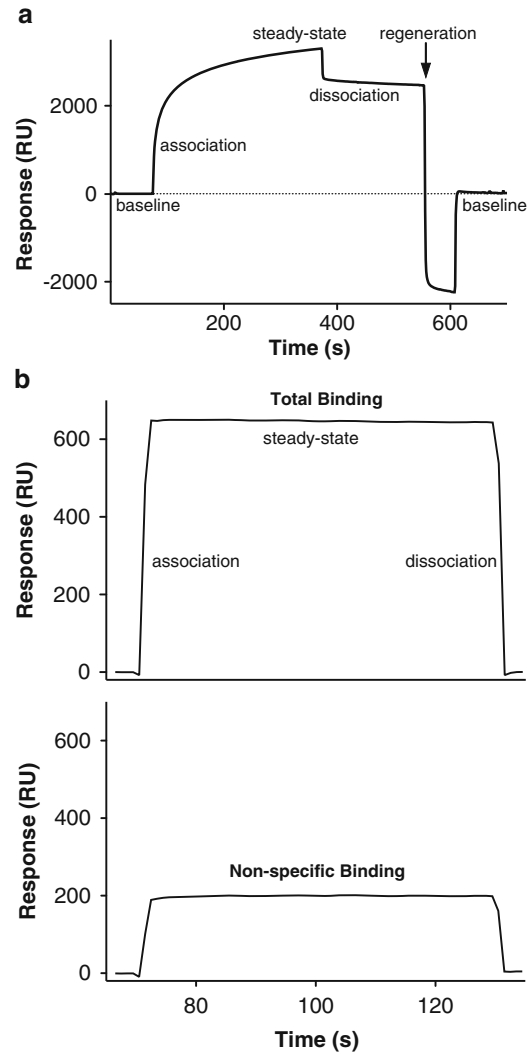

Fig. 1 Sensorgrams showing different phases of the interaction. (**a**) Typical profile with measurable association and dissociation kinetics. (**b**) Rapid association and dissociation kinetics showing total binding (*top*) on ligand surface and nonspecific binding (*bottom*) on control surface

2 Materials (*See* Notes 1 and 2)

2.1 Starting the BIAcore 3000

1. BIAcore 3000 instrument (GE Healthcare Life Sciences): a high-performance research system that enables label-free measurements of biomolecular interactions.

2.2 Preparing the Sensor Surface

1. Sensor Chip SA: sensor chip that is pre-coated with streptavidin.
2. Biotinylated proteins, glycosides, or peptides (National Institutes for Health/NIGMS-funded Consortium for Functional Glycomics) (*see* **Note 3**).

3. Plastic sample vials: 7 mm plastic vials with caps (*see* **Note 4**).
4. Preconditioning solution: 1 M NaCl in 50 mM NaOH.
5. 40 % Glycerol for the *Normalize* routine.

2.3 Galectin Binding

1. Recombinant human Galectin-1, Galectin-2, and Galectin-3 were purified by affinity chromatography on lactosyl-Sepharosey [22].
2. BIAcore Running Buffer: Phosphate buffered saline (*see* **Notes 5** and **6**).

2.4 Data Analysis

1. BIAevaluation software.

3 Methods

3.1 Starting Up BIAcore 3000 (See Notes 7 and 8)

1. Stop the *Standby* routine.
2. Undock, then remove the maintenance chip and replace it with a fresh, unused research grade SA chip (*see* **Note 9**).
3. Remove the D/W bottle used for *Standby* and replace it with room temperature filtered and degassed running buffer (*see* **Note 10**).
4. Empty the waste bottle.
5. *Prime* the system twice using running buffer (*see* **Note 11**).
6. *Normalize* the signal in all flow cells according to the manufacturer's instructions.
7. *Prime* the system once more with running buffer.
8. The instrument and the docked sensor chip are now ready to use.

3.2 Preparing the Sensor Surface

1. Start a new sensorgram at a flow rate of 5 µl/min.
2. Set flow path to fc 1, 2, 3, 4, over all four flow cells (*see* **Note 12**).
3. Inject 5 µl of preconditioning solution, 1 M NaCl in 50 mM NaOH, three times (*see* **Note 13**) (Fig. 2).
4. Dilute each biotinylated glycoside to a concentration of 10 fmol/ml in running buffer.
5. Change the flow rate to 2 µl/min.
6. Change the flow path to fc1.
7. Inject 12 µl of the control glycoside arabinose on fc1.
8. Change the flow path first to fc2, then to fc3, and then to fc4, injecting lactose, $(LacNAC)_2$ and $(LacNAc)_3$, respectively (*see* **Note 14**).
9. Stop the sensorgram and run *Standby* for 3–4 h or overnight to stabilize the baseline (*see* **Note 15**).

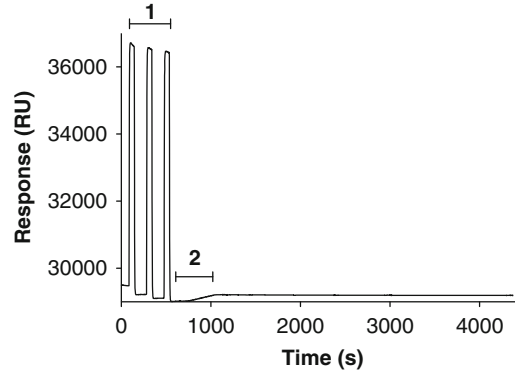

Fig. 2 Ligand capture on an SA chip. Sensorgram shows a typical profile for capturing ligand on an SA chip. The first three injections (1) represented by a large increase in response are for the 1 M NaCl/50 mM NaOH pre-conditioning solution. Subsequent injection of the glycoside ligand (2) produces a very small response

3.3 Galectin Binding

1. To test the ligand surface, run *Prime* before injecting the analyte.
2. First run a few pilot experiments in manual mode to test the prepared surfaces.
3. Start a new sensorgram at a flow rate of 30 μl/min.
4. Set the flow path to fc 1, 2, 3, 4, with inline reference subtraction of 2-1, 3-1, 4-1 (*see* **Note 16**).
5. Make three serial dilutions of Galectin-1 (or one of the analytes being tested; a wild-type protein if testing binding mutants).
6. Inject 30 μl of the lowest concentration over the sensor chip (*see* **Notes 17** and **18**).
7. Inject the remaining concentrations in turn, waiting for the protein from each prior injection to be completely washed away before the next injection.
8. Examine the subtracted sensorgrams showing the 2-1, 3-1, and 4-1 profiles. Dose-dependent specific binding on each surface indicates that the biotinylated glycosides are functional and the surfaces are ready for the kinetic experiments.
9. For the affinity measurements, make a set of serial dilutions from 100 to 0.1 μM; for Galectin-1, Galectin-2, and Galectin-3.
10. Write out a customized wizard setting the flow rate to 60 μl/min, a flow path of fc1, 2, 3, 4 and a detection readout for fc 2-1, 3-1, 4-1 (*see* **Note 19**).

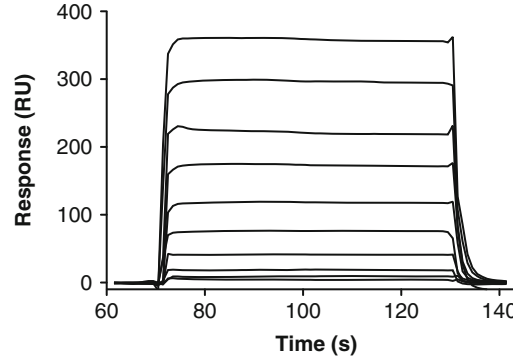

Fig. 3 Specific Binding of Galectin-1 to $(LacNAc)_2$. An overlay of binding sensorgrams. For each concentration of Galectin-1 obtained after subtracting nonspecific binding on the control glycoside

11. Specify sample injections for 60 µl using the *Kinject* command, allowing adequate dissociation time for the response to come back to baseline after each injection.
12. Check the boxes to *Prime* the instrument before starting the wizard and to run the *Standby* routine once all the injections have been completed.
13. Start the wizard and enter a file name for saving the results.
14. Each Galectin concentration will be recorded as a new cycle with a new sensorgram.

3.4 Data Analysis

1. Using the BIAevaluation software, open the results file from the kinetic experiment.
2. Import the 2-1, 3-1, and 4-1 subtracted sensorgrams for all three Galectins.
3. Overlay all sensorgrams for Galectin-1 (fc 2-1) and determine the apparent K_d as described below (Fig. 3).
4. Select a 30 s section of the curves just before the end of the injection, representing the steady-state binding.
5. Get the average value for each concentration of Galectin-1, using Fit: *Average*.
6. Using this average response for each concentration, in the menu bar, select *Plot Parameters* and generate a plot of *Response Units* vs. *Concentration*.
7. Select all the points on the plot. In the menu bar select Fit: *Steady State Analysis* (*see* **Note 20**) (Fig. 4).
8. The table below the curve fitting gives the apparent K_d value and other statistics associated with the curve fitting.
9. Repeat analysis for Galectins-2 and -3.

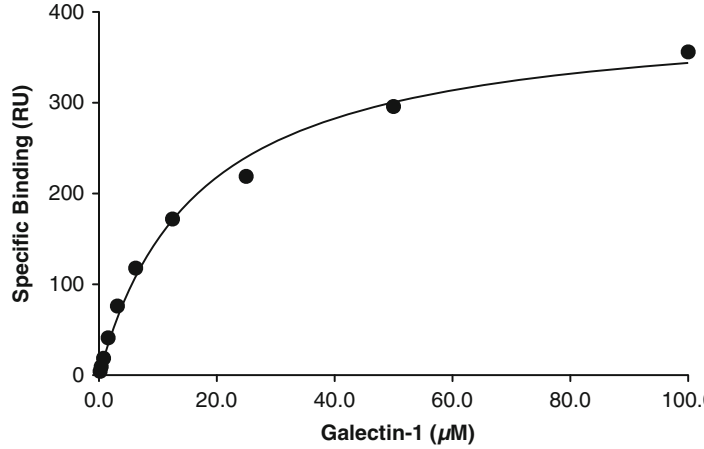

Fig. 4 Affinity of binding of Galectin-1 to $(LacNAc)_2$. Average specific binding responses were plotted against each Galectin-1 concentration. The equilibrium affinity of binding was determined from nonlinear curve fitting of this data

4 Notes

1. All aqueous reagents were made with MilliQ water. Common reagents were all of analytical grade. All buffers and other solutions meant for the BIAcore were filtered using 0.45 μm filter units. After filtration was complete, the buffers were subjected to vacuum for an additional 20 min to effectively degas them.
2. Buffers should be thoroughly degassed prior to using them on the BIAcore. Even though air bubbles are not likely to cause damage due to pressure changes, the small diameter of the channels in the IFC can be easily blocked by a large air bubble, causing a spike in the sensorgram, thereby interfering with the signal.
3. It is advantageous to couple or capture proteins on the sensor chip such that all molecules are uniformly oriented and hence present a homogenous ligand surface. Attaching a biotin label to one end of a glycoside helps to orient all molecules in the same uniform presentation. A pre-coated SA chip can be purchased or a streptavidin chip can be made by covalently coupling streptavidin to a CM5 sensor chip.
4. All 7 mm plastic vials are supplied with caps and should be capped to prevent evaporation of sample. Sample evaporation changes the concentration and hence the refractive index of the sample solution thereby altering the magnitude of the signal obtained.
5. Although several pre-made buffers are available from GE for using as running buffers on the BIAcore, we preferred to use Phosphate-buffered saline (PBS) in which the galectins were

known to be stable. The PBS was filtered using a 0.45 μm filter unit and P20 (GE Healthcare), which is a 10 % filtered solution of Tween 20) was added to a final concentration of 0.05 % to generate the running buffer (*see* **Note 4**).

6. The addition of a small amount of Tween20 is highly recommended to prevent nonspecific adsorption to the IFC that delivers sample to the sensor surface.
7. The B3000 instrument should be routinely maintained according to the manufacturer's instructions so that no additional cleaning is required prior to the start of the experiment.
8. *Desorb* and *Sanitize* routines should be performed on a weekly basis to ensure that there is no build-up in the IFC. For multiuser instruments, a more rigorous maintenance schedule may be required depending on frequency of usage and the nature of samples used.
9. Prior to docking a brand new sensor chip, always ensure that the chip is pushed all the way into the cassette assembly, as even a slight protrusion of the chip can interfere with the docking process.
10. Reserve 10 ml of running buffer in a separate tube before inserting the tubing into the container. This tube of buffer will be useful for diluting analyte samples.
11. The *Prime* routine should be used whenever the signal quality is erratic or seen to deteriorate suddenly. *Prime* should always be used each morning and whenever the buffer is changed. It is always recommended to *Prime* the system twice before starting up a new experiment aimed at collecting data for kinetic analysis.
12. There are several different injection options available for use on the BIAcore 3000. Typically, injections that reduce dispersion from the buffer, require a larger volume of sample. For pilot tests or for samples that are in limiting amounts, use the Quickinject command. For kinetic experiments and when the dissociation phase is to be monitored, use the Kinject command.
13. The SA chip is convenient because it comes pre-coupled with streptavidin. However, before a biotinylated ligand is injected, it is necessary to precondition the sensor surface in order to remove any loosely bound streptavidin. This ensures that subsequent injection of the biotinylated ligand will couple it only to the immobilized streptavidin.
14. Each time, inject a different glycoside on the respective flow cell. Inject 12 μl first and then if the ligand density is lower than the control flow cell, inject some more ligand to get approximately the same molar amount as the control glycoside. The *manual inject* command may be used to control ligand density.
15. Especially when a surface is prepared for running kinetic experiments, or when the magnitude of the signal obtained is likely to be low, it is advisable to stabilize the baseline before injecting analyte.

16. When immobilizing several ligands, it is always recommended that the control ligand be immobilized on fc1, upstream of the remaining ligands to be tested. If ligands are to be tested in pairs, the control ligand can be immobilized on fc1 and fc3; in which case, when collecting data, detection should be set to fc2-1 and fc4-3.
17. Galectin-1 dissociates rapidly from the sensor chip, hence regenerating the surface is not necessary for this analyte.
18. In this instance, when evaluating galectin binding to glycoside ligands, the bound galectin dissociated fairly quickly and it was not necessary to regenerate the surface. However, when regeneration is required, it should be carried out with the mildest solution and for the shortest time necessary. This will ensure that the bound analyte is completely dissociated and the ligand functionality has been minimally affected.
19. Analyte injections can be performed at flow rates ranging from 1 to 100 µl/min. For experiments where the data is to be analyzed for determining kinetic parameters, however, the highest flow-rate possible should be selected. The flow rate should be at least 30 µl/min and will depend on the amount of analyte that is available; higher flow rates require more analyte.
20. Kinetic analysis for a 1:1 binding model using the BIAevaluation software uses the Levenberg-Marquardt algorithm for nonlinear curve fitting.

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Chapter 8

Examining Galectin Binding Specificity Using Glycan Microarrays

Connie M. Arthur, Lílian Cataldi Rodrigues, Marcelo Dias Baruffi, Harold C. Sullivan, Jamie Heimburg-Molinaro, Dave F. Smith, Richard D. Cummings, and Sean R. Stowell

Abstract

Glycan binding proteins (GBPs) possess the unique ability to regulate a wide variety of biological processes through interactions with highly modifiable cell surface glycans. While many studies demonstrate the impact of glycan modification on GBP recognition and activity, the relative contribution of subtle changes in glycan structure on GBP binding can be difficult to define. To overcome limitations in the analysis of GBP-glycan interactions, recent studies utilized glycan microarray platforms containing hundreds of structurally defined glycans. These studies not only provided important information regarding GBP–glycan interactions, but have also resulted in significant insight into the binding specificity and biological activity of the galectin family. We will describe the methods used when employing glycan microarray platforms to examine galectin–glycan binding specificity and function.

Key words Glycan binding protein (GBP), Galectin, Glycan microarray, GBP–glycan interactions

1 Introduction

Members of the galectin family of carbohydrate binding proteins have been implicated in a wide variety of biological processes including roles in cell signaling, development, and immunity [1–3]. While galectins initially reside within the cytosol where they can regulate various intracellular processes [4–6], most documented activities of galectin family members reflect modulation of cellular behavior following release from cells, where they recognize and crosslink highly modifiable cell surface carbohydrates [3, 7, 8]. Although all galectins share a common affinity for β-galactose terminating carbohydrate structures, variations within the carbohydrate-binding domains of individual galectin family members can lead to differential binding following β-galactose modification [9–11]. Thus, knowledge of the specific binding preferences

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of individual galectins can provide significant insight into the function and regulatory activity of this protein family.

Various methods have been employed to determine the binding preferences of galectins. Early studies utilized simple monosaccharides, typically in the setting of hemagglutination inhibition assays, to understand general binding preferences of glycan binding proteins (GBPs), including individual members of the galectin family [12–14]. These early studies suggested that galectins typically recognize terminal β-galactoside-containing glycans, although the fine specificity for these ligands and the potential impact of β-galactose modification appeared to differ between individual family members [14, 15]. Subsequent studies utilized a variety of biochemical assays, including isothermal calorimetry, fluorescence polarization, surface plasmon resonance, and frontal affinity chromatography [10, 11, 16–18]. These approaches often used expanded libraries of glycans, which not only contributed to a greater understanding of galectin-carbohydate binding properties, but also provided significant insight into the thermodynamics of galectin–glycan interactions [17, 18].

In an effort to expand on previous findings [12–14, 19], several groups began to generate larger libraries of test glycans [20–22]. These libraries typically contained combinations of naturally occurring and uniquely modified glycans designed to specifically elucidate the binding specificity of GBPs [20, 21, 23–25]. Although these libraries can be utilized in a variety of formats, glycan microarrays allow interrogation of hundreds of structurally distinct glycans while employing small amounts of target glycan and test GBPs [10, 18, 26, 27]. As synthetic limitations may reduce the overall breadth of a particular glycan library, recent strategies also generated glycan microarrays from glycans directly harvested from natural sources [24, 25, 28–34]. Using a combined approach of different glycan microarray strategies, significant insight into galectin–glycan binding specificity and overall biological function can be obtained [11, 16, 35–39].

In this work we will discuss the methods used for examining galectin-glycan binding specificity using a glycan microarray format. The potential limitations and impact of galectin concentration on glycan microarray results and analysis will also be explored. Given the utility of this tool, not only in elucidating the binding specificity, but also in facilitating the discovery of previously unrecognized biological activities of individual members of the galectin family [11, 37, 39], additional studies using various glycan microarray approaches will likely continue to provide significant insight into the biochemical and biological roles of galectins and other GBPs.

2 Materials

2.1 Galectin Preparation

1. Lactosyl-sepharose (Sigma-Aldrich).
2. Isopropyl β- D -1-thiogalactopyranoside (IPTG) (Thermo Scientific).
3. Phosphate Buffered Saline (PBS) (Hyclone).
4. Lactose (Fisher).
5. β-mercaptoethanol (βME) (Fisher).
6. CelLytic B-II Bacterial Cell Lysis/Extraction reagent (Sigma).
7. Complete Mini EDTA-free Protease Inhibitor cocktail tablets (Roche).
8. Lysozyme (Sigma Aldrich).
9. RNase (Thermo Scientific).
10. DNase (Thermo Scientific).
11. Sodium Azide (Sigma Aldrich).
12. Tris Base.
13. Glycine.
14. Sodium Dodecyl Sulfate.
15. Loading buffer (Thermo Scientific).
16. Broad Range STD Molecular Weight Markers (Santa Cruz).
17. 4–20 % or 16 % Tris-Glycine Gel (10 Well) (Life Technologies).
18. Coomassie staining solution (Life Technologies).
19. Methanol.
20. Glacial Acetic Acid.
21. 1.7 mL snap cap microcentrifuge tube (Sigma-Aldrich).
22. 2 L Erlenmeyer flask.
23. 50 mL Erlenmeyer flask.
24. 1 L centrifuge bottles.

Special Equipment

25. Centrifuge.
26. Sonicator.
27. Fraction collector (BioRad).
28. Gel apparatus including voltage source.

Buffers

29. Lysis Buffer = (for 1 L of culture) Use 10 mL of CelLytic B-II Bacterial Cell Lysis/Extraction reagent, 14 mM β-Mercaptoethanol (βME), one Complete Mini EDTA-free Protease Inhibitor cocktail tablet, Lysozyme (1 mg), RNase with a stock of 10 mg/mL (10 μL) and DNase with a stock of 10 mg/mL (10 μL).

30. Wash Buffer = (1 L) 1× PBS with 14 mM βME.
31. Elution Buffer = (1 L) 1× PBS, 14 mM βME and 100 mM Lactose (36 g).
32. Column Storage Buffer = (total volume 500 mL) 1× PBS w/0.02 % Azide, 14 mM βME.
33. 1× Electrophoresis Buffer = 3 g Tris Base, 14.4 g Glycine and 1 g Sodium Dodecyl Sulfate in total volume of 1 L dH_2O.
34. Destain Solution = 40 % Methanol and 10 % Glacial Acetic Acid in dH_2O.

2.2 Galectin Derivatiztion

1. PD10 column (GE Healthcare).
2. PBS (Hyclone).
3. NHS-LC-Biotin (Thermo-Scientific).
4. Lactose (Fisher).
5. β-mercaptoethanol (Fisher).
6. Lactosyl-sepharose (Fisher).

2.3 Glycan Array Screening

1. Glycan printed slides (NIH Consortium for Functional Glycomics Core D), printed on the side of the slide with the white etched bar code and black marks—*DO NOT TOUCH THIS AREA*.
2. Cover slips, 24 × 50 (Fisher scientific).
3. Humidified Slide processing chambers (Fisher), or homemade system made with Petri Dish, with wet paper towels in the bottom of the chamber.
4. 100 mL Coplin jars for slide washing.
5. Tris–HCl.
6. NaCl.
7. $CaCl_2$.
8. $MgCl_2$.
9. Potassium Phosphate Monobasic.
10. dH_2O.
11. BSA (Fisher scientific).
12. Alexa Fluor-488-Streptavidin (Invitrogen).
13. Tween-20 (EMD Biosciences).

Special Equipment

14. ProScanArray Scanner (Perkin Elmer).

Buffers

15. TSM = 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$.

16. TSM Wash Buffer (TSMW) = TSM Buffer + 0.05 % Tween-20.
17. TSM Binding Buffer (TSMBB) = TSM buffer + 0.05 % Tween 20 + 1 % BSA.

3 Methods

3.1 Galectin Preparation

1. In a laminar flow hood pour 10 mL of autoclaved LB Media into a sterile 50 mL Erlenmeyer flask or other appropriate container. Add 10 μL of Ampicillin (stock 50 μg/mL) to media (*see* **Note 1**).
2. Remove the previously prepared and validated glycerol stock of bacteria transformed with the appropriate galectin expression vector from the −80 °C freezer and place on ice. Place a loopful of bacteria into the LB/Amp media to inoculate starter culture.
3. Place cover on flask loosely and incubate overnight at 37 °C, shaking at 250 revolutions per minute (rpm).
4. Prepare a 2 L Erlenmeyer flask containing 1 L of LB media by adding 20 g of LB Broth powder in 1 L of dH_2O in the flask. Mix well and then autoclave on a 30 min liquid cycle to sterilize. Following autoclave cycle, remove flask and allow it to cool to room temperature.
5. Place the 2 L Erlenmeyer flask containing 1 L LB media into the incubator/shaker and allow it to warm to 37 °C before proceeding.
6. Once media is warmed, move media to a culture hood and add 1 mL of Ampicillin (stock concentration 50 μg/mL) to the 1 L of LB media in each flask (*see* **Note 1**).
7. Add 10 mL of starter culture to the flask containing 1 L of sterile LB media and antibiotic to inoculate cultures.
8. Place cap on flask loosely and incubate shaking for 2–2.5 h at 37 °C and 250 rpm.
9. Check the cultures OD at 600 nm (OD600) using visible light approximately every 30 min. Blank with autoclaved sterile LB Media. When OD600 of each culture reaches between 0.45 and 0.50, induce the bacteria in each culture to express the recombinant galectin by addition of 0.36 g of IPTG to each flask (*see* **Note 2**).
10. Continue IPTG induced growth for 4–5 h at 37 °C and 250 rpm (*see* **Note 3**).
11. Collect bacteria by pouring 1 L of culture into 2 (500 mL in each) clean 1 L plastic centrifuge bottles. Spin down @ 4,200 × *g* for 30 min at 4 °C.
12. Pour off the supernatant and place pelleted bacteria at −80 °C. (Pellet can be stored at −80 °C for up to 2 weeks).

13. Thaw pellets on ice. Add 5 mL of Lysis Buffer to each bottle and resuspend pellets (homogenization). Let resuspension sit at RT for 30 min or at 37 °C for 15 min (*see* **Note 4**).
14. Pool the resuspended pellets in a 250 mL plastic centrifuge bottle on ice and sonicate the pellets two to three times for 20 s per cycle (*see* **Note 5**).
15. Spin the lysate @ 13,000 × *g*/4 °C for 30 min.
16. Collect the supernatant without disrupting the pelleted cell debris and place on ice.

Purification

17. Prepare the Lactosyl Sepharose column by washing with 10 column volumes of wash buffer (*see* **Note 6**).
18. Apply the supernatant to the column (*see* **Note 7**).
19. Collect the supernatant flow through (Sample Flow Through) (*see* **Note 8**).
20. Wash the column with 10 more column volumes of Wash Buffer (*see* **Note 9**).
21. Elute the recombinant protein by preparing 3 column volumes of Elution Buffer and adding it to the column.
22. Begin to collect 1–2 mL fractions immediately following addition of Elution Buffer.
23. Take 10 μL from each fraction and dilute tenfold (i.e. 10 μL of each fraction in 90 μL Elution Buffer). Read the OD of each dilution at OD at 280 nm (OD280) using a spectrophotometer. Blank with Elution Buffer.
24. Calculate protein concentration using the following equation: OD280 × 10 × extinction coefficient ratio (*see* **Note 10**).
25. Identify the peak fractions (the three to five fractions with the highest concentration of protein as determined by OD280).
26. Prepare samples for an SDS PAGE using 10 μL of the "Start Material" and "Sample Flow Through" and the volume that equals 2 μg of total protein for each of the "Peak Fractions". Use 7.5 μL of 4× Loading Buffer. Bring up the total load volume to 30 μL with dH_2O. Load 30 μL into each well.
27. Use Broad Range STD Molecular Weight Markers (Total load volume should be 10 μL).
28. Over a Bunsen burner, allow a pot of dH_2O to come to a boil. Place samples in the water and boil for 10 min.
29. Set up gel apparatus and fill with Electrophoresis Buffer.
30. Spin down samples in a table top centrifuge for 5 s before loading on Gel.
31. Load samples into a 4–20 % or 16 % Tris-Glycine Gel (10 Well).

32. Perform SDS-PAGE at 125 V for about 1.5 h.
33. Remove the gel from gel apparatus and stain the gel with Coomassie Stain for 30 min then de-stain for 2 h with de-stain solution. Verify the "Peak Fractions" are the appropriate molecular weight. Take a picture of the gel and dry.
34. Once the protein identity is verified by SDS PAGE, pool all of the fractions that contain galectin protein. Examine protein content by measuring the OD280 of each fraction. This is typically done by diluting each fraction tenfold (i.e. 10 μL of each fraction in 90 μL PBS) followed by examination of the OD280. Blank with Elution Buffer.
35. Calculate protein concentration using following equation: OD280 × 10 × extinction coefficient ratio (*see* **Note 10**).
36. Make 500 μL aliquots of the purified galectin protein in 1.7 mL microcentrifuge tubes. Store aliquots at −80 °C.

3.2 Galectin Biotinylation

1. To remove βME, prepare a PD10 column for gel filtration by equilibration with 5 column volumes of cold PBS (pH 7.4) (*see* **Note 11**).
2. Thaw frozen stock aliquots of galectins at 4 °C.
3. Add 1 mL of recombinant galectin solution (containing Elution Buffer) to the PBS re-equilibrated PD10 column and collect 0.5 mL fractions.
4. Following complete penetration of the galectin solution into the PD10 column, add 2 mL cold PBS.
5. Continue to collect 0.5 mL fractions while adding additional PBS as needed to elute all protein and prevent the column from drying.
6. To determine which fractions may have galectin protein, examine protein content by measuring the OD280 of each fraction. This is typically done by diluting each fraction tenfold (i.e. 10 μL of each fraction in 90 μL PBS) followed by examination of the OD280 (*see* **Note 10**).
7. Once positive fractions are identified, pool galectin containing fractions and reevaluate the OD280 to determine the final concentration of pooled galectin (*see* **Note 12**).
8. Add lactose in PBS at a final concentration of 100 mM to facilitate the maintenance of galectin activity during the labeling procedure (*see* **Note 13**).
9. Add NHS biotin at the concentration recommended by the manufacturer followed by incubation at RT for 1 h or at 4 °C for 2 h (*see* **Note 14**).
10. Remove Lactose and nonreacted NHS biotin by passing the reaction mixture over a new PD10 column, re-equilibrated

with cold PBS, using the same approach as outlined in **steps 1–7** above.

11. Once pooled biotinylated galectin fractions are collected and evaluated for concentration, add βME at a final concentration of 14 mM to sustain galectin activity for the duration of the additional purification steps and actual experiment (*see* **Note 15**).
12. To separate potentially inactive galectin from active galectin, pass pooled biotinylated galectin over a lactosyl-sepharose column re-equilibrated in PBS containing 14 mM βME.
13. Wash the column with 10 column volumes of PBS containing 14 mM βME.
14. Elute bound protein with PBS containing 14 mM βME and 100 mM lactose. Collect 0.5 mL fractions as soon as the elution buffer is added to the column.
15. Evaluate galectin concentration within each collected fraction by diluting each fraction tenfold in PBS containing βME and 100 mM lactose (i.e. 10 μL of each fraction in 90 μL PBS). Be sure to use PBS with βME and 100 mM lactose as a baseline measure of OD280.
16. Once protein-positive fractions are identified, pool galectin containing fractions and reevaluate the OD280 to determine the final galectin concentration.
17. The degree of biotin incorporation can be evaluated by commonly employed mass spectrometric analysis, western blot analysis using HRP-streptavidin or by examining whether engagement of cell surface ligands by biotinylated galectin can be detected using FITC-streptavidin using standard flow cytometric analysis (*see* **Note 16**).
18. Once biotinylation is documented on active recombinant galectin, these proteins can then be analyzed on the glycan microarray.

3.3 Microarray Probing with Biotinylated Galectin

1. Make TSM, TSMW, and TSMBB or bring them to room temperature if they have made previously and stored at 4 °C.
2. Prepare 100 μL of sample by diluting biotin-labeled galectin protein in TSMBB with 14 mM βME added if needed to maintain galectin activity (*see* **Note 17**).
3. Remove slide(s) from desiccator and hydrate by placing in a glass Coplin staining jar containing 100 mL of TSMW for 5 min.
4. Remove excess liquid from slide by setting the slide upright on a paper towel to drain the liquid off.
5. Carefully apply 70 μL of sample made in **step 2** above close to the left edge of the slide in between the black marks found on the slide surface.

6. Slowly lower the cover slip onto the slide and gently remove any bubbles trapped between the slide and cover slip by soft tapping with a pipette tip. Be sure the cover slip stays between the black marks on the slide since this is the portion of the slide where glycans are printed.
7. Incubate slide in a humidified tray in the dark for 1 h at RT.
8. After 1 h incubation, remove cover slip by turning the slide onto its side and allowing the cover slip to slip off into the glass trash/biohazard trash.
9. To wash, dip the slide four times into 100 mL of first TSMW and then TSM in Coplin Jars.
10. Set the slide upright on a paper towel to remove excess TSM buffer.
11. Add 70 μL of Streptavidin-AlexaFluor-488 and apply cover slip as outlined above in **step 6** and incubate in a humidified tray in the dark for 1 h at RT.
12. After 1 h incubation, remove cover slip by turning the slide onto its side and allowing the cover slip to slip off into the glass trash/biohazard trash.
13. To wash, dip the slide four times into 100 mL of first TSMW followed by TSM and then dH_2O in Coplin Jars.
14. Spin slide in slide centrifuge for ~15 s to remove excess water.
15. Scan in a fluorescent Scanner and obtain mean fluorescent intensities for each glycan binding event on the array (*see* **Note 18**).

4 Notes

1. Use the appropriate antibiotic selection protocol based on the antibiotic resistance gene encoded in the expression vector for each recombinant galectin.
2. IPTG can be dissolved directly into 10 mL of sterile autoclaved media for each 1 L culture. Use the appropriate inducing agent and concentration as outlined by the manufacture for the expression vector used to generate recombinant galectin.
3. While induction at 0.4–0.5 OD600 followed by 4–5 h of continued incubation yields optimal galectin production for most galectins, variations may occur depending on expression vector, galectin stability, and other expression parameters. In these cases, optimal conditions should be empirically determined.
4. Use of bacterial lytic reagent when seeking to isolate galectin-2 will results in loss of galectin-2 activity. Instead use PBS when isolating galectin-2. It should also be noted that several methods of bacterial lysis have been published and employed by our lab. Each appears to yield relatively similar overall protein amounts.

5. Although this may help bacterial lysis and appears to increase the ultimate protein yield, this step is not absolutely required when using the Bacterial lytic reagent.
6. Assign separate lactosyl sepharose columns for the purification of individual galectin family members and/or mutants to avoid potential contamination between galectins when isolating each protein.
7. Save some of the supernatant to be analyzed by SDS-PAGE as the "Start Material".
8. Some of the "Sample Flow Through" will also be analyzed by SDS-PAGE.
9. Collect the "Wash Flow Through" in case the column capacity is exceeded. Keep the "Wash Flow Through" separate from the "Sample Flow Through".
10. To extrapolate the protein concentration from the OD 280 nm values, use the extinction coefficient for the particular galectin being examined to calculate actual concentration in mg/mL. The following websites http://www.basic.northwestern.edu/biotools/proteincalc.html and http://web.expasy.org/protparam/protparam-doc.html, offer explanation and assistance in calculating the extinction coefficient and using this calculation to determine the actual concentration of a given protein in mg/mL, including caveats as to how these numbers may differ from the actual number. As methods of calculating the extinction coefficient only provide estimates, alternative approaches can be used to empirically determine these values. These include using a Bradford assay to calculate protein concentration or simply re-equilibrating the recombinant protein directly into water, lyophilizing, weighing, and then resuspending in the buffer of choice followed by empirically determining the extinction coefficients for a particular galectin family member.
11. βME can readily inactivate NHS biotin and therefore significantly reduce the efficacy of galectin biotin incorporation.
12. Reaction efficiency is typically a function of the concentration of the biotin labeling reagent and the protein. We typically label galectins at a concentration of >1 mg/mL to optimize labeling efficiency. If the final concentration is not sufficient, galectins can be readily concentrated using centricon concentrating devices (Millipore) according to the manufacturer's protocol.
13. The biotin and galectin reaction mixture will pass over a PD10 column at least once more after the labeling reaction is completed. Since some galectin can be lost with each pass over a column, it is important to start with sufficient galectin to achieve the required amount of end product needed to conduct the experiment.
14. Many different labeling strategies can be employed when seeking to provide a method of detecting recombinant galectin

binding. However, we have found that biotinylation is the least likely to induce inactivation of the galectins following derivatization. In contrast, several galectins readily loose carbohydrate binding activity following direct labeling with fluorescent adducts. As a result, care should be taken to determine the potential impact of galectin derivatization on carbohydrate binding activity. The impact of the reactive label and galectin concentrations and the duration of the labeling reaction, as well as the type of label should be empirically evaluated to assess the potential impact of the label on galectin activity. Partially denatured, yet labeled, galectin can result in nonspecific binding on array platforms and therefore result in false positive hits that may significantly impact the interpretation of the apparent binding preferences for an individual galectin.

15. Not all galectins are equally sensitive to oxidative inactivation. For example, Gal-3 does not appear to display significant sensitivity to oxidative inactivation in the absence of reducing agents. In contrast, Gal-1, Gal-2, and Gal-7 can display significant loss of activity over time. Other galectins can also lose activity in the absence of reducing conditions, albeit at a reduce rate compared to Gal-1. As a result, the requirement of βME to maintain galectin activity should be empirically determined for each galectin following the labeling procedure.
16. While mass spectrometric analysis and western blot approaches may provide a general sense of the degree of protein biotinylation and the passage of the protein over a column would in theory allow isolation of active protein, examination of cell surface binding by flow cytometry simultaneously tests whether the galectin is biotinylated and retains gross carbohydrate binding properties.
17. Cy5-labeled Streptavidin for aligning subarray grids for analysis can be added as a separate step on the slide before adding galectin.
18. Although glycan microarrays allow rapid examination of GBP–glycan interactions on a single platform, several considerations should be taken when using this approach to study GBP binding specificity. For example, while microarray presentation of glycans fixed on a solid support may be analogous to glycans similarly fixed on a cell surface, they still reflect artificial presentation of potential ligands. As a result, potential alterations in printing density, presentation, coupling formats, and methods of GBP binding detection can impact the overall binding specificity of a particular GBP. Furthermore, some GBPs, such as selectins, exhibit complex interactions with glycan ligands that also include the peptide backbone and other posttranslational modifications [31, 40]. In these situations, more complex libraries of glycopeptides can be used to evaluate the potential influence of non-glycan moieties on GBP–glycan interactions [31, 40].

In addition to potential differences in glycan presentation between individual glycan microarrays and cell surface glycans, very few arrays possess well-defined concentrations of the glycans actually coupled to the array. As a result, differences in GBP binding to unique glycans can also be significantly influenced by variation in glycan coupling efficiency. To reduce the impact of this limitation, analysis of GBP binding over a variety of concentrations can reduce the impact of alterations in printing by providing apparent Kd values toward individual glycans [16, 37]. Although GBPs may be in equilibrium with many different glycans on a microarray and therefore limit the overall accuracy of this approach, evaluating the binding of GBPs at different concentrations still appears to reduce bias based on alterations in printing efficiency. This is especially important when B_{max} values between individual glycans differ significantly [16, 37] (Figs. 1, 2, and 3). Regardless of the

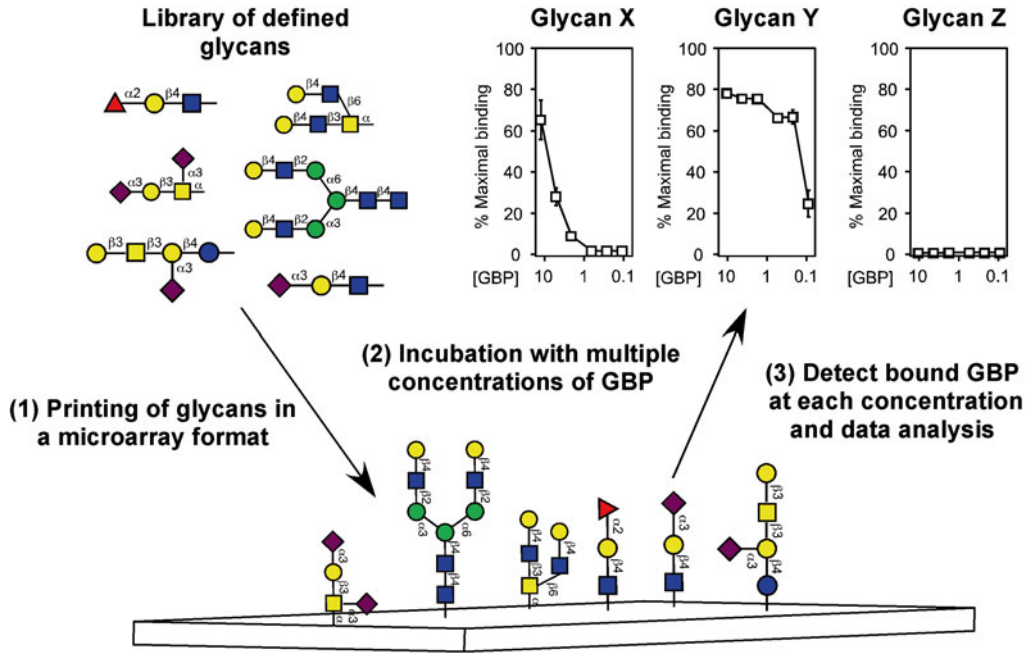

Fig. 1 Utilization of defined glycan microarrays to elucidate GBP specificity. Libraries of well-characterized glycans generated by release of defined glycans from glycoproteins or other natural sources or by chemical or chemoenzymatic synthesis are used to populate well-defined glycan microarrays. Structures reflect naturally occurring glycans and modifications of glycans not typically found in nature. Glycan libraries undergo derivatization with a functional coupling moiety, followed by printing in a microarray format to generate the glycan microarray. GBPs are incubated with the glycan microarrays over different concentrations and detected by fluorescence emission if directly labeled or by a similarly labeled suitable secondary detecting agent. While many approaches can be taken to analyze glycan array data, examination of GBP binding over a variety of concentrations for individual glycans is shown. This research was originally published in the Journal of Biological Chemistry [37] with permission from the American Society for Biochemistry and Molecular Biology

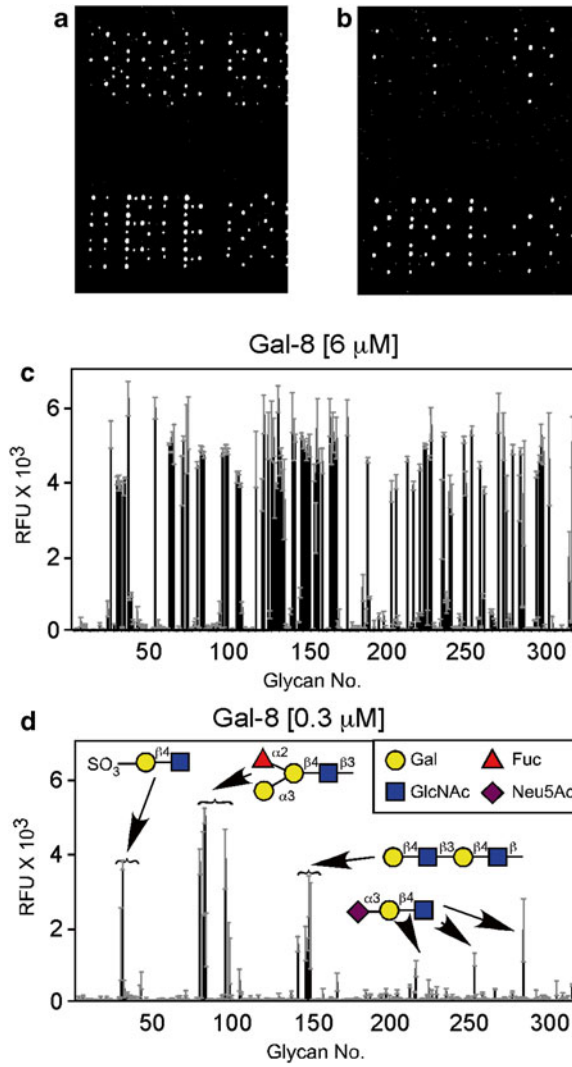

Fig. 2 Galectins interact with glycan microarray in a concentration-dependent manner. Gal-8 recognizes distinct classes of glycans. (**a** and **b**) Examination of the glycan microarray followed incubation of the glycan microarray with 6 μM Gal-8 (**a**) or 0.3 μM Gal-8 (**b**). (**c** and **d**) Glycan microarray data obtained following incubation with 6 μM Gal-8 (**c**) or 0.3 μM Gal-8 (**d**). **d** *inset*: Legend of symbols for monosaccharides. This research was originally published in the Journal of Biological Chemistry [37] with permission from the American Society for Biochemistry and Molecular Biology

microarray approach used, validation of findings using alternative methods, including evaluation of cell surface glycans, provides a useful strategy to insure that array findings reflect real interactions with native glycan ligands [11, 16, 19, 37, 39].

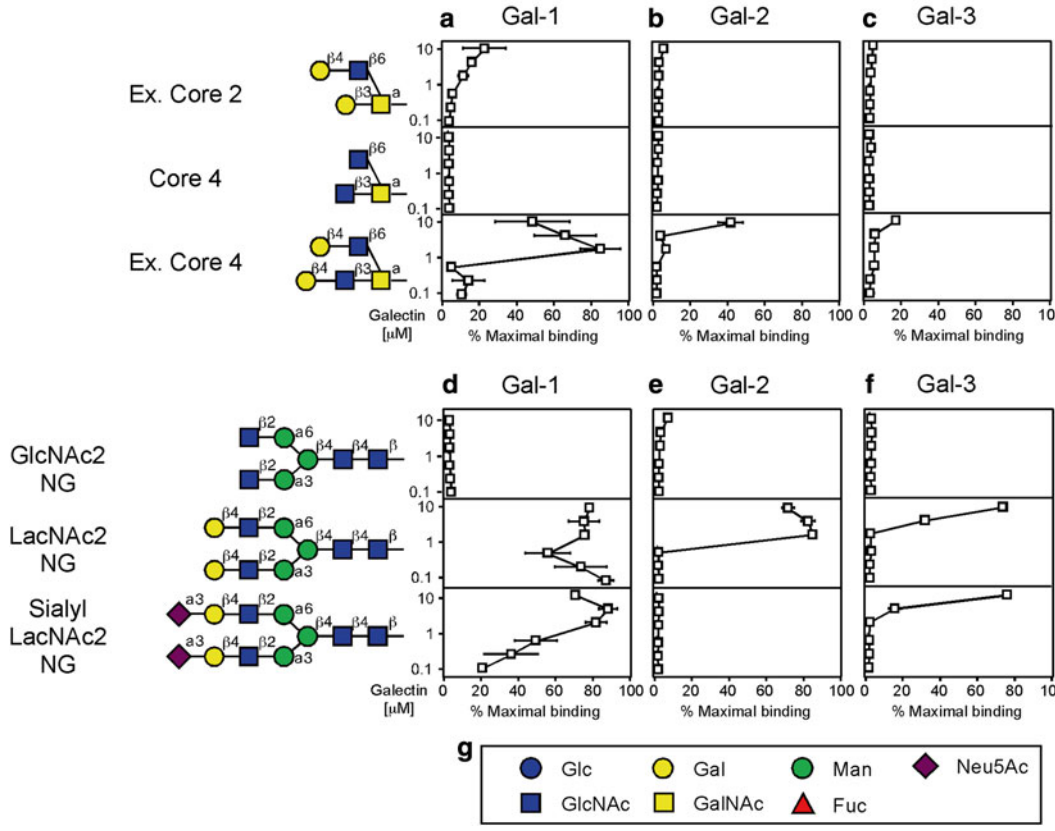

Fig. 3 Examination of galectins over a range of concentrations can provide a relative affinity for individual ligands on the glycan microarray. Trivial names followed by the structures of each glycan tested are shown. Recognition of each representative glycan is displayed as the percent bound when compared with the highest bound ligand for each respective galectin tested in this study. Glycan recognition of O-glycans is shown for Gal-1 (**a**), Gal-2 (**b**), and Gal-3 (**c**). Glycan recognition of N-glycans is shown for Gal-1 (**d**), Gal-2 (**e**), and Gal-3 (**f**). (**g**) Legend of symbols for monosaccharides. This research was originally published in the Journal of Biological Chemistry [16] with permission from the American Society for Biochemistry and Molecular Biology

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Chapter 9

Methods for Assessing the Effects of Galectins on Leukocyte Trafficking

Beatrice R. Gittens, Rachael D. Wright, and Dianne Cooper

Abstract

Numerous protocols exist for investigating leukocyte recruitment both in vitro and in vivo. Here we describe three of these methods; an in vitro flow chamber assay, intravital microscopy, and zymosan-induced peritonitis, and give details as to how they can be used to study the actions of galectins on this crucial process.

Key words Leukocyte, Neutrophil, Endothelium, Galectin, Transmigration

1 Introduction

Inflammation has evolved to maintain tissue homeostasis in response to injury or infection. It is a complex and coordinated response mediated primarily by leukocytes, which arrive at the site of injury in stages and carry out subtype-specific roles. Regardless of their specific function, an important hallmark of all leukocytes is their ability to migrate quickly to the area of inflammation. Consequently, the mechanisms underlying leukocyte recruitment have been extensively studied both in vivo and in vitro; from this a model of cell movement across the endothelial barrier has been formed to include initial capture and rolling, activation, firm adhesion, crawling, and finally transmigration [1, 2].

Several methods exist for investigating leukocyte recruitment and here we describe three assays commonly used in our laboratory: the in vitro flow chamber assay, intravital microscopy, and zymosan-induced peritonitis. In vitro flow chamber assays allow analysis of the leukocyte recruitment cascade under flow and several systems have been designed for this purpose. The method detailed below describes how to use Ibidi® chamber slides. This relies on culturing endothelial cells in the chamber slide and flowing freshly isolated peripheral blood neutrophils over the endothelial monolayer.

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The next level of complexity is to study leukocyte–endothelial interactions in real time in vivo, for this we use the technique of intra-vital microscopy which involves exposure of a microvascular bed in a living animal, usually a mouse [3]. Finally we describe the method of zymosan-induced peritonitis, which differs from the previous two models in that it does not allow visualization of the leukocyte recruitment cascade; however, it does allow leukocytes that have migrated into the peritoneal cavity to be collected and analyzed [4]. We have utilized these methodologies for investigating the role of galectins on leukocyte recruitment both in vitro and in vivo [5, 6]. This chapter will therefore provide information of the reagants and technical know-how required to perform these techniques and will indicate how they can be adapted to study the effect of proteins such as galectins on the leukocyte recruitment cascade.

2 Materials

2.1 In Vitro Flow Chamber Assay

2.1.1 Isolation and Culture of Human Umbilical Vein Endothelial Cells

1. 23 g butterfly needle.
2. 30 ml syringe with luer lock.
3. Hartman hemostat forceps, straight 10 cm (Fine Science Tools, Inc.)
4. Medium M199 (Sigma Aldrich).
5. Type II collagenase from *Clostridium histolyticum* (Lorne laboratories) (*see* **Note 1**).
6. Human serum (Sigma Aldrich) (*see* **Note 2**).
7. 0.5 % bovine gelatin (Sigma Aldrich).
8. T75 tissue culture flasks (*see* **Note 3**).

Buffers

9. Complete medium: M199 containing 20 % human serum, penicillin (100 U), streptomycin (100 mg/ml), and fungizone (2.5 μg/ml).
10. Hank's balanced salt solution (HBSS) containing penicillin (100 U), streptomycin (100 mg/ml), and fungizone (2.5 μg/ml).
11. Phosphate buffered saline (PBS) containing penicillin (100 U), streptomycin (100 mg/ml) and Fungizone (2.5 μg/ml).

2.1.2 Subculturing of HUVEC

1. Medium M199 (Sigma Aldrich).
2. Phosphate buffered saline (PBS) sterile.
3. Trypsin/EDTA.

Buffers

4. Complete medium: M199 containing 20 % human serum, penicillin (100 U), streptomycin (100 mg/ml) and fungizone (2.5 μg/ml).

2.1.3 Seeding Cells into Ibidi Slides

1. Ibidi μ-Slides VI $^{0.4}$ (*see* **Note 4**).
2. 0.5 % bovine gelatin (Sigma Aldrich).
3. Trypsin.
4. Subcultured HUVEC (*see* Subheading 3.1.1).
5. hrTNFα.

Buffer

6. Complete medium: M199 containing 20 % human serum, penicillin (100 U), streptomycin (100 mg/ml) and fungizone (2.5 μg/ml).

Special Equipment

7. Humidified incubator with 5 % CO_2 at 37 °C.

2.1.4 Isolation of Neutrophils from Human Blood

1. 15 ml Falcon tubes.
2. 50 ml Falcon tubes.
3. Histopaque 1119 (Sigma Aldrich).
4. Histopaque 1077 (Sigma Aldrich).
5. 21 g butterfly needle.
6. RPMI 1640 medium.
7. Sterile pasteur pipettes.
8. Double distilled H_2O.
9. Dulbecco's phosphate buffered saline (DPBS) (Life Technologies).

Buffers

10. Sodium Chloride (NaCl) (3.6 % in dH_2O).
11. Sodium citrate (3.2 % in dH_2O).
12. Turk's solution: 0.01 % crystal violet in 3 % glacial acetic acid.

Special Equipment

13. Haemocytometer.
14. Inverted Microscope.

2.1.5 In Vitro Flow Chamber Assay

1. Human PMN (*see* Subheading 3.1.4).
2. 50 ml Falcon tubes.
3. Dulbecco's phosphate buffered saline (DPBS) (with calcium and magnesium) (Life Technologies) (*see* **Note 5**).
4. Dulbecco's phosphate buffered saline (DPBS) (without calcium and magnesium).
5. Ibidi μ-Slides VI $^{0.4}$ (*see* **Note 4**).
6. Tygon tubing (Saint Gobain Performance Plastics, T5002-13) with an internal diameter of 1.6 mm and an external diameter of 3.2 mm.

7. Luer lock syringes.

Special Equipment

8. Automated syringe pump (e.g. PHD 2000 programmable pump, Harvard Apparatus).
9. A heated and humidified chamber with an active gas mixer to ensure that the temperature and carbon dioxide levels stay constant at 37 °C with 5 % CO_2 throughout the experiment.

2.1.6 In Vitro Flow Chamber Assay

1. Inverted phase contrast microscope fitted with ×10 and ×20 phase contrast objectives.
2. A video camera (e.g. Q-Imaging Retiga EXi Digital).
3. Software for analysis (*see* **Note 6**).

2.2 Intravital Microscopy

2.2.1 Sample Preparation and Image Capture

1. Bicarbonate buffered saline (BBS): 132 nM sodium chloride, 5 mM Potassium Chloride, 2 mM Calcium Chloride, 1 mM Magnesium Sulfate and 20 mM Sodium Hydrogen Carbonate in distilled water (*see* **Note 7**).
2. Distilled H_2O.
3. Xylazine Hydrochloride (23.32 mg/ml, Rompun).
4. Ketamine Hydrochloride (100 mg/ml, Vetoquinol).
5. Inflammogen (*see* **Notes 8** and **9**).
6. Isoflourane for inhalation anesthesia if performing intra-scrotal injections.

Special Equipment

7. Dissecting microscope and heat pad for initial tissue preparation.
8. Surgical tools including vannas scissors, Iris curved serated forceps, dissecting scissors, and Dumont #5 fine forceps (Mouse dissecting Kit, World Precision Instruments).
9. Round bodied, 9.3 mm sterile suture (BV-1, Ethicon).
10. Cauterizer.
11. A heated water bath connected to a heated coil for heating BBS.
12. Peristaltic pump for superfusion of BBS and inflammogens.

2.2.2 Data Analysis

1. An upright microscope with water immersion objectives (fluorescence capabilities are required if individual leukocyte subtypes are to be analyzed). We use an Olympus BX61 WI microscope with ×20 and ×40 objectives.
2. A Perspex viewing board.
3. A video camera (e.g. Hamamatsu C9300-201 CCD camera).
4. Computer and software for analysis and image acquisition (e.g. Slidebook; Intelligent Imaging Innovations) (*see* **Note 10**).

2.3 Zymosan Peritonitis

1. Dulbecco's phosphate buffered saline (DPBS) (Life Technologies).
2. Zymosan A from *Saccharomyces cerevisiae* (Sigma).
3. 29 g needles.
4. 23 g needles.
5. Carbon Dioxide (CO_2).
6. 5 ml syringes.
7. 15 ml Falcon tubes.
8. 96-Well round bottom plate.
9. 4 % Paraformaldehyde.
10. Bovine serum albumin (10 % in DPBS) (*see* **Note 11**).

Antibodies

11. Anti-Ly6g (clone 1A8) and respective isotype control.
12. Anti-F4/80 (clone BM8) and respective isotype control.
13. Anti-GR-1 (clone RB6-8C5) and respective isotype control.
14. Anti-mouse FcγRII/III.

Buffers

15. Lavage fluid: sterile PBS containing 3 mM EDTA.
16. PBS + 0.1 % bovine serum albumin.
17. Turk's solution: 0.01 % crystal violet in 3 % glacial acetic acid

Special Equipment

18. Haemocytometer.
19. Inverted Microscope.
20. Surgical tools including vannas scissors, Iris curved serated forceps, dissecting scissors, and Dumont #5 fine forceps (Mouse dissecting Kit, World Precision Instruments).

3 Methods

3.1 In Vitro Flow Chamber

3.1.1 Isolation of Human Umbilical Vein Endothelial Cells

1. Collect cords in HBSS containing antibiotics and store at 4 °C until processing.
2. Insert a butterfly needle (sheath on) into one end of the umbilical vein (*see* **Note 12**) and clamp around needle to hold in place.
3. Attach a 30 ml syringe containing PBS to the needle and flush cord with approximately 30 ml PBS to wash away residual blood and identify any perforations in the cord.
4. Clamp the bottom of the cord and infuse vein with approximately 20–25 ml collagenase (*see* Fig. 1).

Fig. 1 Isolation of human umbilical vein endothelial cells from umbilical cords. (**a**) Cross section of the umbilical cord showing the two umbilical arteries and the umbilical vein. (**b**) The umbilical vein is filled with collagenase and clamped at either end prior to incubation at 37 °C for 15 min

5. Incubate the clamped cord for 15 min in a humidified chamber in 5 % carbon dioxide at 37 °C.
6. Transfer the collagenase solution to a 50 ml falcon tube and flush the cord with 30 ml PBS, also adding this to the tube.
7. Push air through the cord to collect any remaining PBS.
8. Centrifuge the cells at 300 × *g* for 10 min, remove the supernatant, and resuspend the cell pellet in 15 ml complete medium.
9. Transfer the cells to a gelatin-coated (*see* **Note 13**) T75 flask (75 cm^2) and place in a humidified incubator in 5 % carbon dioxide at 37 °C.
10. Change the culture medium 24 h later and then every other day until approximately 95 % confluent.
11. Once at 95 % confluence the cells can be sub-cultured or used for experimentation (*see* **Note 14**).

3.1.2 Subculturing of HUVEC

1. Remove culture medium and rinse cells in 10 ml sterile PBS.
2. Add 2 ml Trypsin/EDTA to the cells.
3. Monitor cells microscopically for signs of "rounding up".

4. Remove 1.5 ml Trypsin/EDTA solution from flask and discard. Leave cells a further 2 min at room temperature.
5. Observe cells microscopically to assess detachment. Tap side of flask gently if required (*see* **Note 15**).
6. Add required amount of complete culture medium and resuspend cells.
7. Transfer required volume of cells to new culture vessel.

3.1.3 Seeding Cells into Ibidi Slides

1. Coat Ibidi µ-Slides VI$^{0.4}$ flow chamber slides with 100 µl 0.5 % gelatin for at least 30 min at room temperature.
2. Trypsinise one confluent T75 flask of HUVEC and resuspend in 2 ml complete medium.
3. Add 150 µl cell suspension to each channel, replace the lid, and culture the cells overnight in a humidified incubator with 5 % CO_2 at 37 °C.
4. Stimulate the HUVEC (*see* **Note 16**) 4 h prior to carrying out the flow assay with 10 ng/ml hrTNFα in complete media.

3.1.4 PMN Isolation

1. Prepare 15 ml Falcon tubes containing 3 ml histopaque 1119 overlaid with 3 ml histopaque 1077 (*see* **Note 17**).
2. Collect blood from healthy volunteers using a 21-gauge butterfly needle and transfer to a 50 ml falcon tube containing a 1/10 volume of 3.2 % sodium citrate to prevent clotting (*see* **Note 18**).
3. Dilute blood 1:2 with warm RPMI.
4. Layer 6 ml blood on top of double histopaque gradient.
5. Centrifuge blood at 400 × *g* for 30 min (*see* **Note 19**)
6. Remove plasma and PBMC layer and discard (*see* **Note 20** and Fig. 2).

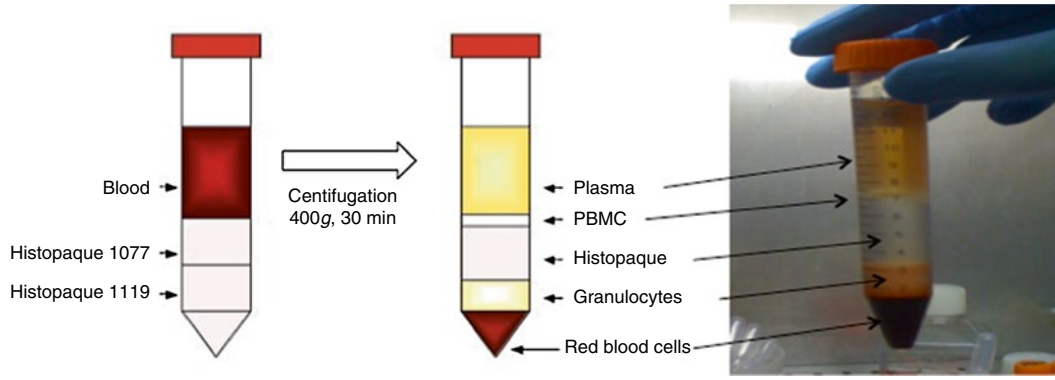

Fig. 2 Blood separation using a double density gradient of histopaque. Blood is layered on top of the histopaque and centrifuged at 400 × *g* for 30 min (no brake) to separate it into its constituent parts

7. Remove granulocyte layer using a sterile pasteur pipette and transfer to new 15 ml Falcon tubes (maximum volume 6 ml/tube). Top tubes up to 12 ml with RPMI.
8. Centrifuge at 300 × *g* for 15 min.
9. During this centrifugation place 50 ml double distilled water at −80 °C.
10. Discard supernatant and flick tubes to resuspend neutrophil pellet.
11. Add 7.5 ml ice-cold water per tube and ensure cell pellet is fully resuspended. Invert tube several times for a period of 10 s (*see* **Note 21**).
12. Add 2.5 ml 3.6 % NaCl per tube and invert tube twice to mix.
13. Centrifuge at 300 × *g* for 10 min.
14. Discard supernatant and resuspend cell pellet in residual fluid. Combine all tubes into one.
15. Take 10 µl cell suspension and add to 990 µl Turk's solution.
16. Count number of neutrophils based on nuclear morphology using a haemocytometer.
17. For flow chamber assays dilute PMN to a concentration of 1×10^7/ml in DPBS without calcium and magnesium and place cells on ice until use.

3.1.5 Flow Chamber Assay

1. Dilute isolated human PMN in a 50 ml falcon tube to a concentration of 1×10^6 cells/ml in DPBS (with calcium and magnesium) and incubate for 10 min at 37 °C (*see* **Note 22**).
2. Place ibidi slide in heated chamber and attach tygon tubing to outlet reservoir.
3. Preload 30 ml leur lock syringes with 10 ml 37 °C DPBS (with calcium and magnesium; *see* **Note 5**) and place in syringe pump.
4. Fill the inlet reservoir of the ibidi slide with DPBS and then attach the tubing from the syringes to the ibidi slide (*see* Fig. 3) and pump through DPBS at 1 dyn/cm^2, taking extreme care not to introduce any air bubbles into the system.
5. Once the DPBS has reached the end of the outlet tubing, pause the syringe pump and transfer the tubing to the 50 ml falcon containing the isolated leukocyte suspension.
6. Start the syringe-pump, this time withdrawing the cells over the HUVEC at 1 dyn/cm^2 for 8 min (*see* **Notes 23** and **24**).
7. Record random 6 frames per channel along the centreline, each lasting 10 s. Frames should be recorded starting at the end of the slide nearest to the syringe pump to avoid recording the same leukocyte population twice.

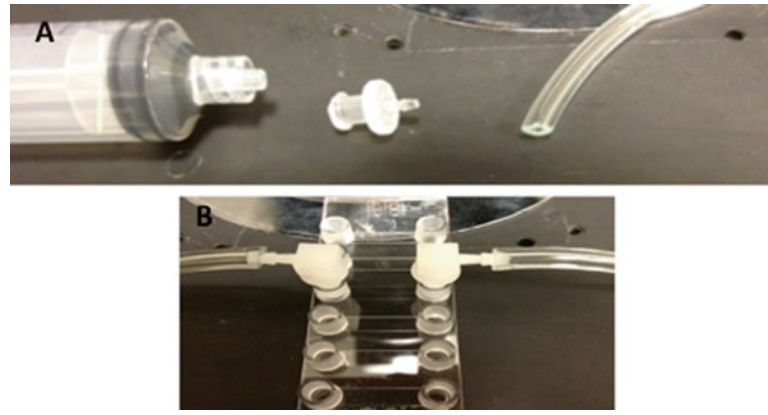

Fig. 3 Assembly of Ibidi® μ slides. (**a**) A leur lock syringe is connected to a female leur connector which in turn is connected to tygon tubing. (**b**) Male leur connectors attach the tubing to the inlet and outlet ports of the ibidi slide

3.1.6 Data Analysis

Most commonly, the analysis parameters include the number of cells initially captured, which is then divided into two groups—those cells that are rolling or those that are firmly adherent and remain stationary for the duration of the recording (10 s) as well as the number of transmigrated cells (*see* **Note 25**).

1. To analyze the frames in Image Pro Plus, use the Measure > Manual Tag option to count the phase light cells seen in the first still of the frame, which are those that are captured on the surface of the endothelia.
2. Move to the last frame and count how many of the cells have moved throughout the recording—these are the rolling cells.
3. The total captured cells minus the rolling cells equal the adherent cells.
4. Finally, use the manual tag to count the transmigrated cells, which will appear as flattened and phase dark cells underneath the monolayer (*see* Fig. 4).

3.2 Intravital Microscopy

3.2.1 Sample Preparation and Image Capture

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

Different protocols can be followed to inflame the tissue of study. Commonly we inject an inflammogen or compound of interest intra-scrotally a number of hours prior to observation of the tissue under isoflourane inhalation anesthesia. To investigate the role of galectins in vivo, intravital microscopy is performed in galectin null mice [5]. Alternatively, recombinant galectins may be administered either intra-scrotally or intravenously to assess the

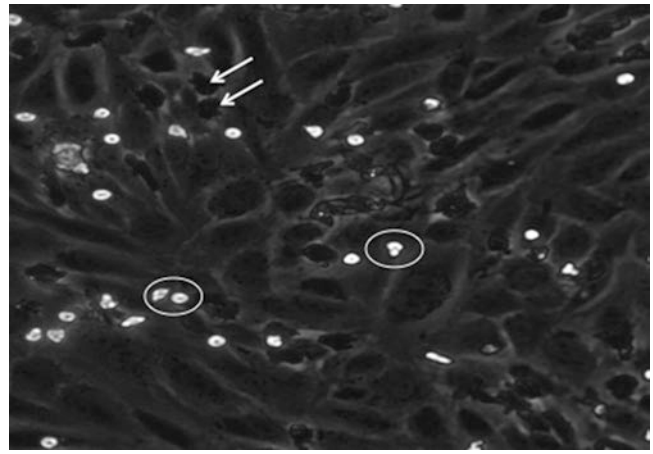

Fig. 4 Image from a flow chamber experiment. Phase bright neutrophils can be observed on the surface of the endothelial monolayer (*circled*). Neutrophils that have transmigrated underneath the endothelium appear phase dark (*arrow*)

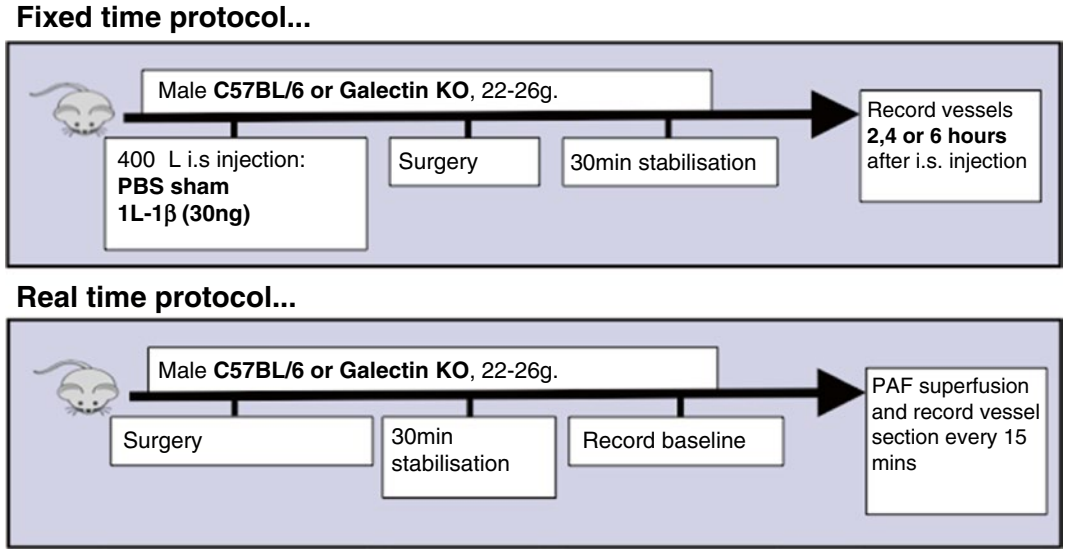

Fig. 5 Two different protocols for observing the inflammatory response in the cremaster. The fixed time protocol involves an intra-scrotal injection of an inflammogen such as TNF-α a number of hours prior to visulisation of the microcirculation by intra-vital microscopy. The real time protocol involves superfusing an inflammogen, such as platelet activating factor onto the tissue. This protocol has the advantage of allowing a baseline reading to be taken and then the development of the inflammatory response can be monitored over time

effects of the exogenous protein. A real-time protocol can alternatively be followed and an inflammogen can be superfused in BBS over the exposed tissue. Figure 5 outlines these protocols. To visualize a specific leukocyte subtype fluorescently conjugated antibodies can be administered i.v. prior to observation. We typically use a

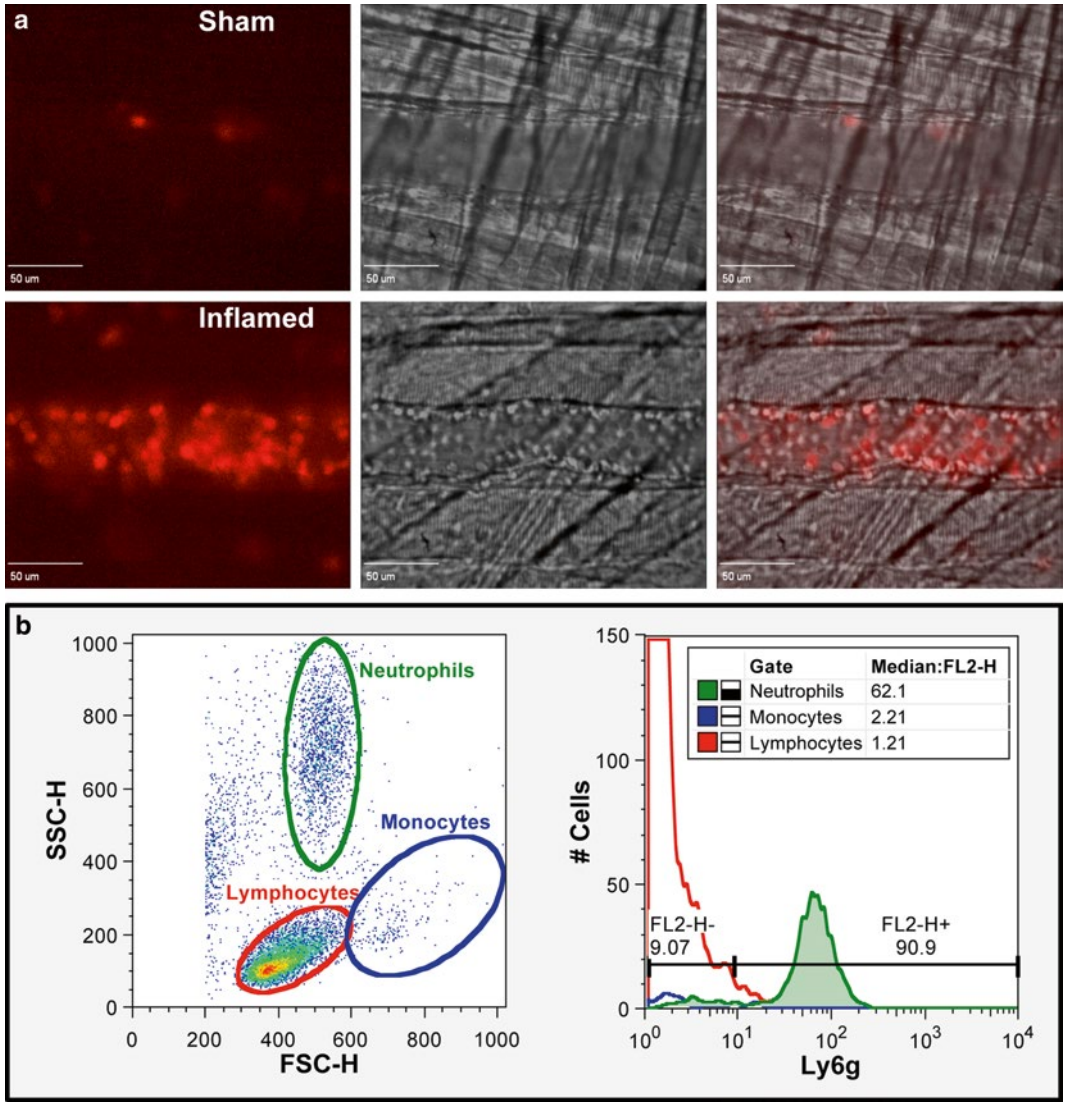

Fig. 6 The use of fluorescent antibodies to label leukocyte subsets for analysis by intravital microscopy. The cremasteric microcirculation in C57/BL6 mice was assessed 4 h after intrascrotal injection of murine recombinant TNFα (300 ng in 400 μl PBS) and i.v. (tail vein) administration of anti-mouse Ly6G (2 μg in 200 μl saline) to label murine neutrophils. (**a**) Stills are representative images showing Ly6G only in the *left panel*, brightfield in the center and merged Ly6G and brightfield channels on the right. (**b**) Following image capture, whole blood was collected from the animal via cardiac puncture and the red cells lysed before flow cytometric analysis. The neutrophils, monocytes, and lymphocytes were gated based on their characteristic forward and side scatter (*left panel*) and each cell population viewed using the FL-2 Ly6G channel. Approximately 90.9 % of the neutrophils were labeled with Ly6G and there was no staining of monocytes and lymphocytes (*right panel*)

phycoerythrin-conjugated anti-Ly6g antibody (clone 1A8) to identify neutrophils (*see* Fig. 6) administered via the tail vein.

1. Dilute 10× stock of BBS to 1× with distilled water and adjust pH to 7.4. The BBS is heated to 37 °C and used to wet the tissue throughout surgery.

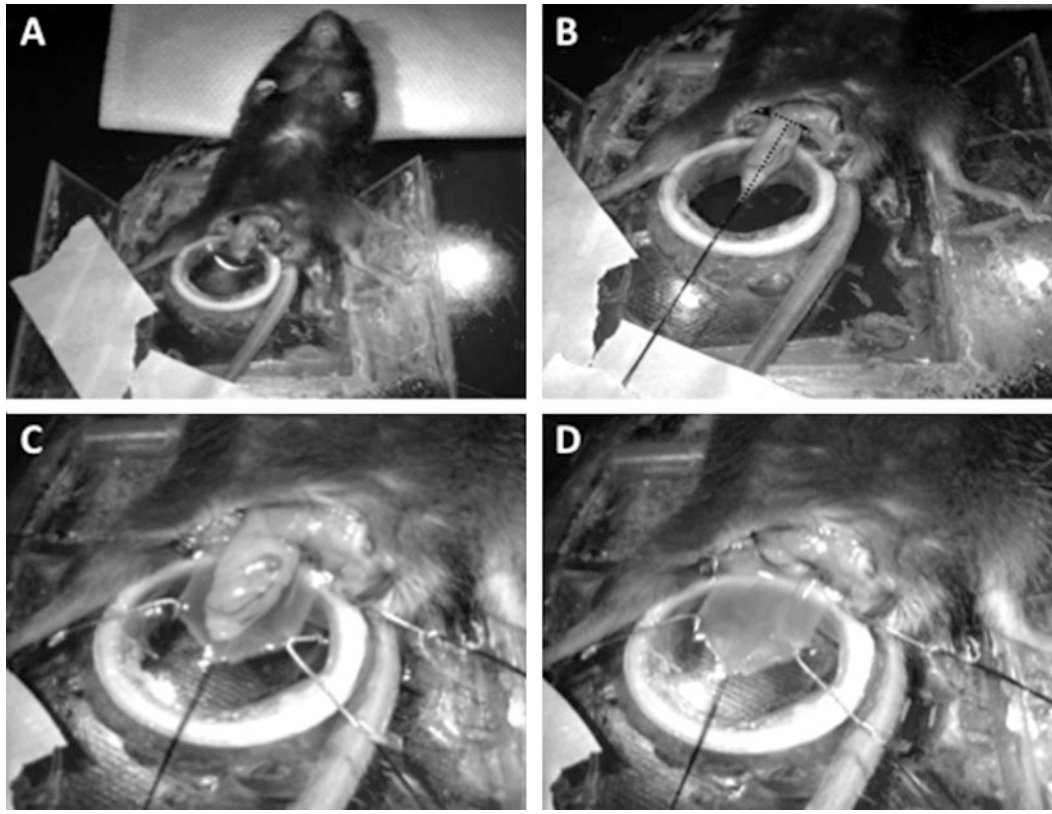

Fig. 7 Preparation of the cremaster muscle for intravital microscopy. (**a**) The skin and fascia covering the ventral aspect of the right scrotum is removed and the cremaster cleared of fatty deposits before the mouse is moved into position with the cremaster resting on the glass coverslip of the viewing stage and supported by a heat pack. (**b**) A suture is threaded through the distal point of the cremaster sack and secured to slightly extend the tissue. The cauteriser is used to score a T-section down the center of the cremaster (*dotted line*). (**c**) The T-section is cut and the cremaster spread flat over the glass coverslip, four hooks are used to secure the cremaster in place. (**d**) The testicle is then separated from the cremaster and secured out of the way of the viewing area

2. Prepare anesthetic by mixing stock solutions of xylazine and ketamine in a 1:1 ratio prior to injection.
3. Anasthetise mouse by intraperitoneal injection of xylazine/ketamine stock solutions diluted to 2.5 mg/ml (xylazine) and 50 mg/ml (ketamine) in sterile dH_2O and administered to give a final dose of 7.5 mg/kg xylazine and 150 mg/kg ketamine.
4. Cremaster preparation (*see* Fig. 7).
   (a) Remove skin and fascia covering the ventral aspect of the right scrotum, exposing an area extending from the inguinal fold and to the distal end of the scrotum (*see* **Note 26**).
   (b) Any remaining fascia should be separated from the cremaster muscle using Dumont forceps and Vannas scissors.

Once the cremaster is cleared of fatty deposits the mouse is moved into position with the cremaster resting on the glass coverslip of the viewing stage and supported by a heat pack (*see* **Note 27**).

(c) Thread suture (BV1; Ethicon) through the distal point of the cremaster sack and use to secure and slightly extend the tissue.

(d) Score a line down the center of the cremaster with a cauterizer and also two lengths at the top edge of the muscle, to form a T.

(e) Use Vannas scissors to cut over the T-section resulting in the cremaster opening such that it can be spread flat over the glass coverslip.

(f) Use four hooks to secure the cremaster in place (*see* **Note 28**).

(g) Use the cauteriser to cut through the vessel connecting the cremaster and the testicle, which is then separated from the cremaster using Vannas scissors and secured out of the way of the viewing area.

(h) Move the viewing stage to the microscope and begin the 30-min stabilization period (*see* **Note 29**).

5. Mesentery preparation.

(a) Perform a midline laparotomy and exteriorise the small bowel using moist cotton buds (*see* **Note 30**).

(b) Place a loop of bowel over the viewing platform and move the viewing stage to the microscope.

6. Post-capillary venules with a diameter of 20–40 μm, an adequate centerline velocity (≥500S^{-1}; *see* **Note 31**) and no branches within 100 μm either side of the segment to be analyzed are chosen (*see* **Note 32**).

7. Vessel segments of 100 μm in 3–5 vessels per mouse and of 3–5 mice per group are recorded for offline analysis.

3.2.2 Data Analysis

Leukocyte flux, adhesion, rolling velocity, and transmigration are quantified off-line (*see* Fig. 8).

1. The video files can be analyzed using the Slidebook software or alternatively exported by going to View > Export (TIFF) or View > Create series movie… (Apple Quicktime or Microsoft AVI).
2. If using multichannel recordings go to Image > Blend background to merge frames.
3. Use the rectangle tool to draw a box measuring 50× 100 μm.
4. Go to Annotations > Timestamp.

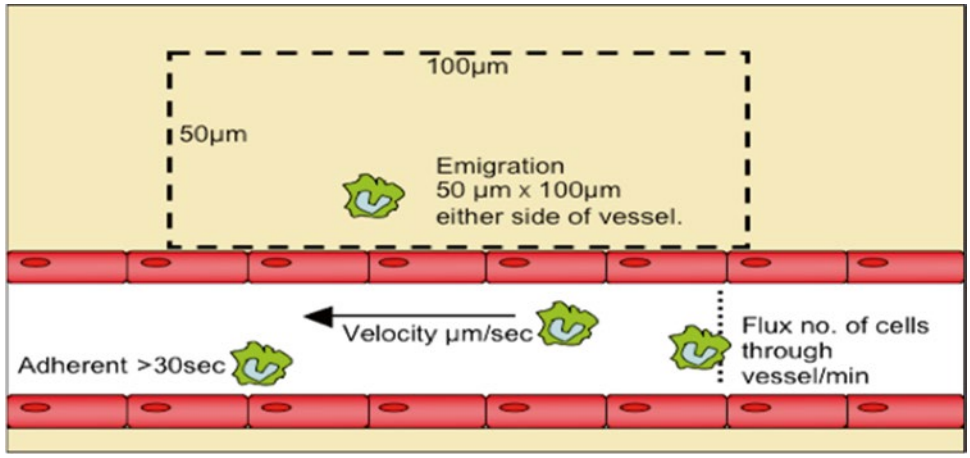

Fig. 8 Parameters measured by intravital microscopy. Rolling flux is quantified as the number of cells passing through a fixed point in the vessel per minute, rolling velocity is determined from the time taken for a cell to roll 100 µm. Adherent cells are those which remain stationary for ≥30 s in a 100 µm vessel section and emigrated cells are quantified as those within an area 100 µm by 50 µm either side of the vessel

5. In Slidebook four analysis parameters can be examined.
   (a) Position the box on either side of the vessel to count transmigrated cells.
   (b) Position the box over the vessel to measure adherent cells, which are those that remain stationary for ≥30 s.
   (c) Keeping the box in the same position on the frame, rolling flux is recorded as the number of leukocytes rolling through the vessel per minute.
   (d) Rolling velocity is determined from the time taken for the leukocytes to travel 100 µm, to do this freeze the video when a leukocyte enters the box and make a note of the timestamp. Follow the leukocyte through the vessel until it reaches the end of the box, freeze the video, and subtract the time on entering the box from the time leaving. The rolling velocity (µm/s) is calculated by dividing 100 µm by the time taken (sec).

3.3 Zymosan Peritonitis

To investigate the role of galectins in this model, mice are either treated with an intraperitoneal injection of recombinant galectin in 0.5 ml PBS, 15 min to 1 h prior to zymosan administration [8] or the experiment is performed in galectin null mice.

1. Inject mice i.p. with 1 mg Zymosan in 0.5 ml sterile PBS using a 29 g needle (*see* **Note 33**).
2. At required time-point sacrifice mice by CO_2 inhalation (*see* **Note 34**).
3. Perform a midline laparotomy to expose the abdominal muscles, inject 4 ml lavage fluid into the peritoneal cavity using a 23 g needle and gently massage abdomen.

4. Collect lavage fluid and store in a 15 ml falcon tube on ice (*see* **Note 35**).
5. Ensure cells are fully resuspended and then remove 100 μl of fluid and add to 100 μl Turks. Count cells using a light microscope to obtain a total cell count in a Neubauer chamber.
6. Remove 1,600 μl of fluid for analysis of cell types recruited by flow cytometry.
7. Add 200 μl fluid per well on a 96-well round bottomed plate for 8 wells.
8. Wash the cells by centrifuging at 850 × *g* and resuspending the pellet in 200 μl PBS (+0.1 % BSA) and repeating.
9. Resuspend pellet in 30 μl PBS (+0.1 % BSA) containing 1/100 anti-mouse FcγRII/III to block against nonspecific binding and incubate on ice for 10 min.
10. Add 30 μl PBS (+0.1 % BSA) containing the relevant antibody for the wells as outlined below (*see* **Note 36**):
    (a) Unlabelled well—no antibody.
    (b) Isotype control for Ly6G (Rat IgG2a FITC).
    (c) Isotype control for F4/80 (Rat IgG2a PE).
    (d) Isotype control for Gr-1 (Rat IgG2b APC).
    (e) Single Stain for Ly6G.
    (f) Single Stain for F4/80.
    (g) Single Stain for Gr-1.
    (h) Double Stain F4/80 and Gr-1.
11. Incubate with the antibodies for 30 min on ice in the dark.
12. Wash ×2 as above.
13. Resuspend pellet in 150 μl PBS (+0.1 % BSA) and 50 μl 4 % paraformaldehyde and transfer to tubes for analysis by flow cytometry.
14. Centrifuge remaining lavage fluid at 850 × *g* and transfer to eppendorfs for storage at ≤−20 °C. This can be analyzed subsequently for soluble mediators by ELISA.
15. Store cell pellets at ≤−20 °C for further analysis by real-time RT-PCR.

4 Notes

1. A stock solution of 1 mg/ml collagenase is prepared in medium M199 and aliquots are stored at −20 °C until required. These aliquots are further diluted to 0.1 mg/ml in M199 for use. Usually around 30 ml is required per cord.

2. HUVEC can also be cultured using fetal calf serum, though cell growth and surface protein expression levels should be assessed in-house.
3. Flasks are coated in a solution of 0.5 % bovine gelatin for 30 min at room temperature prior to use.
4. Comprehensive information about the Ibidi flow chamber systems including the different chamber slides available can be found on their website at www.ibidi.de. Other flow systems widely used are the Glycotech circular and rectangular gasket sets, though these require higher numbers of HUVEC per one chamber (www.glycotech.com/apparatus/parallel.htm).
5. Use of calcium/magnesium free DPBS will result in detachment of endothelial cells.
6. Image Pro Plus 7.0 (media Cybernetics) enables frame capture and subsequent analysis using the manual tagging and object tracking tools; alternatively, ImageJ (NIH) can be used.
7. A 10 × stock of BBS is stable at 4 °C and can be prepared by adding 154.2 g NaCl, 7.0 g KCl and 2.8 g $MgSO_4$ to 12 l dH_2O.
8. Commonly used inflammogens for the cremaster preparation include: recombinant mouse TNF-α (300 ng/400 μl PBS), IL-1β (30 ng/400 μl PBS), injected intrascrotally 2–6 h pre-recording or alternatively platelet activating factor (C16 form: $C_{26}H_{54}NO_7P$; Sigma) can be superfused over the preparation throughout the recording (100 nM in BBS over 2 h).
9. Commonly used inflammogens for the mesentery preparation include: intraperitoneal injection of IL-1β (5 ng), TNFα (500 ng) or proinflammatory agents such as zymosan (1 mg/500 μl sterile saline) or LPS (0.5 mg/kg/500 μl sterile PBS; derived from *Escherichia Coli* serotype 0111:B4), 2–4 h prior to vessel recording.
10. A computer and software are not required for basic intra-vital microscopy; a recordable hard drive or DVD player and a monitor is sufficient.
11. BSA is most stable when stored as frozen aliquots at −20 °C as it may form aggregates at higher temperatures; it is best to avoid refreezing the aliquots once thawed.
12. The umbilical cord contains three blood vessels, one large vein and two smaller arteries surrounded by a proteoglycan-rich matrix known as Wharton's jelly.
13. Flasks are coated in a solution of 0.5 % bovine gelatin for 30 min at room temperature prior to use.
14. Typically the yield from this procedure is $0.5–1.5 \times 10^6$ cells per cord. The cells are used in experimental procedures up to

passage 3 when they are discarded due to changes in their cell surface protein expression profile.

15. If the cells start to detach too quickly, an equal volume of trypsin deactivating media (complete media) can be added, the cells collected and centrifuged to remove trypsin-containing media before resuspension in the required volume of complete culture medium.
16. Check confluency of the endothelial monolayer prior to stimulation. Monolayers must be 100 % confluent for flow chamber assays. If not fully confluent the cells can be incubated in the ibidi slides for a further 24 h, however the culture media should be renewed every 24 h due to the small volume per channel. During this incubation stage, endothelial cells can also be treated with recombinant galectins.
17. Histopaque should be allowed to warm to room temperature prior to use and tubes should be prepared immediately prior to blood withdrawal. The second layer of histopaque (1077) should be layered on top of the bottom layer carefully to avoid mixing. This is best achieved by using either a sterile pasteur pipette or a pipette-boy set to low and gravity.
18. The yield of neutrophils isolated varies between donors but a typical yield is 1×10^6 neutrophils/ml of blood taken. Typically for a flow chamber assay 50 ml of blood is sufficient.
19. The brake on the centrifuge should be switched off for this spin.
20. At this point the PBMC layer can be retained if required for analysis of monocyte and or lymphocyte recruitment.
21. Timing is critical when lysing red cells to avoid activation of neutrophils.
22. At this point leukocytes can be incubated with recombinant galectins prior to flow.
23. When ready to flow leukocytes, it is important that the syringe-pump acts to pull as opposed to push the cell suspension through the flow chamber, as the latter will result in uneven pulsate flow.
24. We routinely perform our flow chamber experiments at 1 dyn/cm^2 as this is roughly equivalent to the shear rates found in inflamed post-capillary venules. The mean flow velocity and thus the shear stress within the chamber can be altered by the syringe pump and is calculated according to an adaptation of Poiseulle's law, that states:

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

25. Treatment of leukocytes with galectins may cause aggregation of cells. Such an effect will skew data acquired in the flow chamber assay. Lower concentrations of the galectin should be used or cells should be treated with an inhibitor such as lactose to disaggregate leukocytes prior to flow.
26. Throughout the surgery, extreme care should be taken to avoid agitating the cremaster muscle by touching the underlying tissue with any of the instruments used.
27. It is possible to use palm-sized reusable hot gel hand warmers which are widely available.
28. Hooks can be made by removing approximately 1.5 cm from the end of a 26 g needle, bending the pointed end to form an L-shape and then bending the blunt end back on itself to attach surgical thread.
29. The surgery itself results in low-level activation of the tissue causing an increase in leukocyte rolling via partial degranulation of perivascular mast cells and rapid upregulation of endothelial P-Selectin levels; thus for the cremaster preparation, a stabilization period of approximately 30 min is required.
30. Care should be taken to avoid pulling on the bowel as this may activate the tissue and reduce blood flow to the area.
31. Shear rate should ideally be monitored in vessels chosen for analysis. Low shear rates may skew results through shear-dependent recruitment of vascular cells [7].
32. The mesentery preparation has a maximum time on the stage of approximately 30 min due to mast cell activation in the tissue. If using fluorescent antibodies or flourochromes, once on the stage it is important not to expose the preparation to excessive fluorescent excitation as the fluorophores, particularly those labeling transmigrated cells, can fade quickly and may result in activation of the leukocytes.
33. Mix zymosan immediately prior to injection to ensure it is fully suspended in the PBS. The dose is injected into the bottom right hand side of the abdomen of the mouse to avoid damaging the liver.
34. Typically peritonitis experiments are performed for up to 96 h with the leukocyte subtype recruited varying over time. At time zero F4/80 high resident macrophages are the dominant cell type. From around 4–12 h Ly6g positive neutrophils become dominant and the macrophage population diminishes. At later time-points, the number of neutrophils decline and monocytes become the dominant cell type. Over time these monocytes differentiate into macrophages.
35. Lavage fluid that is bloody should be excluded from the analysis as leukocyte counts may be skewed due to contamination with peripheral leukocytes.

36. Antibodies are prepared as 2× concentrations as they are added to the 30 µl already in the well. Antibody titrations should be performed in-house to determine optimal antibody dilutions.

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Chapter 10

Examination of the Role of Galectins in Plasma Cell Differentiation

Chih-Ming Tsai and Kuo-I Lin

Abstract

Plasma cells are terminally differentiated B cells that develop via the stimulation of mature B cells with various agents such as antigens and mitogens. Recently, we found that plasma cell differentiation can be modulated by galectin-1 and galectin-8; these galectins appear to play additive and redundant roles in promoting the production of antibody. Here, we describe the protocols for how to investigate the roles of galectins in plasma cell differentiation. These methods include the preparation of recombinant galectins from *Escherichia coli* for exogenously treating primary B cells, generation of galectin_Fc^{m} fusion proteins for determining their binding to B cells, introduction of ectopic galectins in primary B cells using retroviral vectors, and inhibition of the binding of galectins to B cells by synthetic disaccharides.

Key words Mouse splenic B cells, Plasma cell, Recombinant galectin, Retroviral vector, Flow cytometry

1 Introduction

Galectins are a family of evolutionarily conserved animal lectins that recognize β-galactoside [1, 2]. As a prototypical galectin, galectin-1 has affinity for *N*-acetyllactosamine (Gal-GlcNAc, LacNAc) and is expressed as a noncovalently linked homodimer in a variety of cell types [3]. The role of galectin-1 in B cell development has been investigated; galectin-1 is expressed by bone marrow stromal cells and promotes the formation of developmental synapses in the interphase between pre-B cells and bone marrow stromal cells by mediating the interaction between pre-B cell integrins and pre-B cell receptors (BCRs), which leads to the initiation of pre-BCR signaling and the proliferation of pre-B cells [4, 5]. Congruently, B cell development is impaired at the stage of pre-BII-cells in mice deficient for *Lgals1*, the gene encoding galectin-1 [6]. Galectin-1 is secreted by activated mature B cells from mice infected with *Trypanosoma cruzi* [7]. The secreted galectin-1 induces apoptosis of T cells, but not of B cells, which explains the

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effect of B cells on the regulation of T cell homeostasis and function in *Trypanosoma cruzi* infection [7]. Among the other galectins, galectin-3 expression is lower in germinal center B cells and plasma cells than in naïve B cells [8]. Galectin-3 negatively regulates B1 and B2 B cell differentiation into plasma cells [9, 10].

We previously demonstrated that, among galectin family members, galectin-1, galectin-3, and galectin-8 are prevalently expressed in mature B cells [11, 12]. Following stimulus-mediated plasma cell differentiation, the expression of galectin-1 and galectin-8 is increased, whereas the level of galectin-3 is reduced. The induction of galectin-1 depends on B lymphocyte-induced maturation protein-1 (Blimp-1), a master regulator of plasma cell differentiation. Ectopic expression of galectin-1 or galectin-8, but not galectin-3, by retroviral vectors in lipopolysaccharide (LPS)-stimulated mouse primary splenic B cells causes increased numbers of $B220^{lo}CD138^{+}$ antibody-secreting plasma cells, indicating that galectin-1 or galectin-8, but not galectin-3, can promote stimulus-mediated plasma cell differentiation. Ectopic expression of a mutant form of galectin-1 that cannot be exported was compared with exogenously added recombinant galectin-1 to demonstrate that plasma cell differentiation is primarily promoted by extracellular galectin-1. In fact, during differentiation, extracellular galectin-1 and galectin-8 bind mature B cells preferentially over plasma cells. Addition of LacNAc blocks the binding of galectin-1 to mouse splenic B cells and inhibits the generation of plasma cells. Moreover, depletion of galectin-8 by short hairpin RNA expressed by a lentiviral vector, or inhibition of galectin-8 function by a LacNAc analog impairs the formation of plasma cells derived from B cells lacking galectin-1, indicating redundancy in the effects of galectin-1 and galectin-8 on plasma cell formation. Herein, we use galectin-1 as an example to describe methods for studying the role of galectins in plasma cell differentiation. We describe how to prepare recombinant galectin, examine the binding of galectin to B cells, and assess the biological effect of galectin on plasma cell formation.

2 Materials

2.1 Reagents for Preparation of Recombinant Galectin-1

2.1.1 Transformation of Bacteria and Expression of Recombinant Galectin-1

1. *Escherichia coli* BL21(DE3) competent cells (Sigma).
2. pET15b expression vector (Invitrogen) encoding galectin-1 cDNA.
3. Luria-Bertani medium supplemented with 100 μg/ml ampicillin (LB-Amp).
4. 1 M Isopropyl β-D-thiogalactoside (IPTG).
5. Bacterial protein extraction reagent (B-PER) lysis buffer: B-PER (Pierce), 8 mM Dithiothreitol (DTT) and 1 mM Phenylmethylsulfonyl fluoride (PMSF).

2.1.2 Purification of Recombinant Galectin-1

1. 10-ml β-Lactosyl-Sepharose 6B packed in a column (1.5 × 12 cm) [13].
2. Blocking buffer: 3 % BSA in PBS; sterilize through 0.22-μm filter and store at 4 °C.
3. Phosphate-buffered saline (PBS).
4. Wash buffer: 8 mM DTT in sterile PBS.
5. Lactose elution buffer: 0.1 M β-lactose, 8 mM DTT in PBS; sterilize through 0.22-μm filter and store at 4 °C (*see* **Note 1**).
6. Sodium Dodecyl Sulfate.
7. Loading buffer (Thermo Scientific).
8. Broad Range STD Molecular Weight Markers (Santa Cruz).
9. 15 % polyacrylamide gel.
10. Coomassie staining solution (Life Technologies).

2.1.3 Gel Filtration of Galectin-1

1. Centrifugal concentrator: Vivaspin Turbo 15, 5 kDa MWCO (Sartorius).
2. Gel filtration column: Column XK 16/100 (18-8776-01; GE) packed with 200 ml Superdex™ 75 (Amersham-Pharmacia) equipped with a flow adapter (*see* **Note 2**).

2.1.4 Removal of Endotoxin in Recombinant Galectin-1 Solution

1. EndoTrap HD column: 1-ml EndoTrap HD (Hyglos) packed in a column (0.5 × 15 cm, BioRad).
2. Endpoint chromogenic Limulus amebocyte lysate assays (QCL-1000, Lonza).

2.1.5 Quality Test of Recombinant Galectin-1 by T Cell Death Assay

1. MOLT-4 cells cultured in RPMI 1640 medium containing 10 % fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin.
2. Recombinant galectin-1 in PBS containing 8 mM DTT.
3. 0.5 M β-lactose in PBS.
4. R-Phycoerythrin-conjugated annexin V (BD Pharmingen).
5. 7-Amino-actinomycin D (7-AAD) (BD Pharmingen).
6. Annexin V binding buffer, 10× concentrate (BD Pharmingen).
7. Phosphate buffered saline.

2.2 Preparation of Galectin-1_Fc^m Expressed by Mammalian Cells

1. FreeStyle™ 293-F cells (Invitrogen).
2. FreeStyle™ 293 expression medium (Invitrogen).
3. pSecTag2Fc^m expression vector encoding human galectin-1 cDNA [12].
4. Opti-MEM (Invitrogen).
5. 25-kD linear polyethylenimine (PEI) (1 mg/ml, Polysciences, Warrington, PA), prepared in water and adjusted to pH < 2.0

to dissolve PEI and then neutralized to pH 7.0. Sterilize through 0.22-μm filter and store at −80 °C.

6. Protein A Sepharose CL-4B (GE Healthcare).
7. 1 M Tris–HCl, pH 8.0.
8. 0.1 M glycine, pH 3.0.
9. Centrifugal concentrator: Vivaspin Turbo 15, 5 kDa MWCO (Sartorius).

2.3 Preparation of Retroviral Vector Expressing Galectin-1

1. pGC-YFP (yellow fluorescent protein) expression vector encoding human galectin-1 cDNA [14, 12].
2. Packaging DNA from pSV-ψ$^-$-E-MLV [15].
3. Vesicular stomatitis virus G glycoprotein-encoding plasmid, pMD.G [16, 17].
4. 293 T cells cultured in DMEM containing 10 % FBS, 50 U/ml penicillin and 50 μg/ml streptomycin.
5. 2 × HBS: 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, and 50 mM HEPES, pH 7.0.
6. 2 M $CaCl_2$.
7. 25 mM Chloroquine.
8. 3 T3 cells cultured in DMEM containing 10 % FBS, 50 U/ml penicillin and 50 μg/ml streptomycin.

2.4 Examination of the Binding of Galectin-1 to B Cells

1. B cell medium: RPMI medium containing 10 % charcoal-dextran (CD)-treated FBS, 50 μM 2-ME, 50 U/ml penicillin and 50 μg/ml streptomycin.
2. Red blood cell lysis buffer (Sigma).
3. B cell isolation kit (Miltenyi Biotec).
4. Recombinant galectin-1.
5. Lightning-Link Fluorescein kit (Innova Biosciences).
6. Purified galectin-1_Fc^m.
7. Goat anti-human IgG-fluorescein isothiocyanate (FITC) (Sigma).

2.5 Assessment of the Effects of Galectin-1 on Plasma Cell Differentiation

1. R-phycoerythrin-conjugated rat anti-mouse CD138 (BD Pharmingen).
2. Allophycocyanin-conjugated rat anti-mouse CD45R/B220 (BD Pharmingen).
3. Carboxyfluorescein succinimidyl ester (CFSE) (5 mM, eBioscience).

2.6 Special Equipment

1. Ultracentrifuge (Optima L90K; Beckman Coulter).
2. SW28 rotor.

3 Methods

3.1 Preparation of Recombinant Galectin-1

3.1.1 Transformation of Bacteria and Expression of Recombinant Galectin-1

1. Transform pET15b-Gal-1 vector into BL21(DE3) competent cells using standard transformation procedures and then plate the transformed bacteria on LB-Amp plates overnight (*see* **Note 3**).
2. Pick one colony and inoculate in a flask containing 250 ml LB-Amp broth and shake the culture at 250 rpm at 37 °C overnight.
3. Dilute the overnight culture of *E. coli* with 500 ml LB-Amp (a 1:3 ratio), and shake the culture at 250 rpm at 37 °C for ~1 h until OD_{600} = ~0.6.
4. Induce galectin-1 expression by adding IPTG to a final concentration of 1 mM and then culture the bacteria in a 37 °C shaker at 250 rpm for an additional 4 h.
5. Harvest bacteria by centrifugation at 3,800 × *g* for 10 min at 4 °C.
6. Decant the supernatant, and resuspend the bacterial cell pellet by vortexing.
7. Lyse the bacteria with 10 ml B-PER lysis buffer with constant agitation for 8–10 min.
8. Separate soluble proteins from insoluble proteins and other cell debris by centrifugation at 15,000 × *g* for 15 min at 4 °C. Save the supernatant for further purification.

3.1.2 Purification of Recombinant Galectin-1

1. In a cold room/cabinet at 4 °C, wash the β-Lactosyl-Sepharose 6B column with 3 column volumes of blocking buffer followed by 5 column volumes of wash buffer to prevent potential nonspecific binding.
2. Load the soluble bacterial lysate onto the column, and let the lysate remain on the column overnight at 4 °C.
3. Wash the column thoroughly with 5 column volumes of wash buffer to remove the unbound proteins.
4. Elute bound galectin-1 from the column with 3 column volumes of lactose elution buffer.
5. Collect the eluent fractions in 1-ml aliquots, and check for the presence of galectin-1 by SDS-PAGE (15 % polyacrylamide) with subsequent Coomassie blue staining.
6. Wash the column with 5 column volumes of wash buffer and then with 3 column volumes of PBS.
7. Keep the column in PBS containing 0.02 % sodium azide at 4 °C for future use.

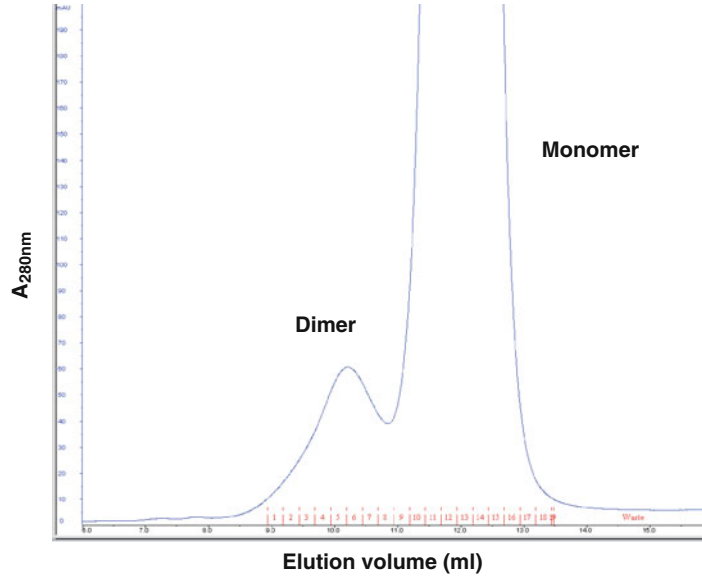

Fig. 1 Purification of the dimer form of galectin-1 by gel filtration chromatography. Gel filtration chromatography shows the separation of dimer and monomer forms of galectin-1. Dimer form of galectin-1 is present in fractions 1 to 8

3.1.3 Gel Filtration Chromatography of Recombinant Galectin-1

1. Pool the β-Lactosyl-Sepharose 6B column fractions containing galectin-1, and then concentrate the combined fractions to a final volume of 1 ml using Vivaspin Turbo 5 kDa MWCO spin columns by centrifugation at 2,000 × *g* at 4 °C for 30 min.
2. In a cold room/cabinet at 4 °C, wash a gel filtration column with 1.5 column volumes of lactose elution buffer, set the pump speed to 1 ml/min, inject the samples, and elute galectin-1. Representative chromatographic results are shown in Fig. 1.
3. Concentrate fractions containing the dimer form of galectin-1 to 1 ml using spin column, and then add 1 column volume of 8 mM DTT in PBS to the spin column.
4. Spin the column at 2,000 × *g* at 4 °C for 30 min to bring the volume of the dimer form of galectin-1 to 1 ml in 8 mM DTT/PBS.

3.1.4 Removal of Endotoxin in Recombinant Galectin-1 Solution

1. Load 1 ml of the dimer form of recombinant galectin-1 from Subheading 3.1.3 into an EndoTrap HD column to remove endotoxin.
2. Apply 3 ml 8 mM DTT/PBS into EndoTrap HD column to elute galectin-1.
3. Concentrate the eluted dimer form of galectin-1 as described in Subheading 3.1.3.

4. Perform a chromogenic Limulus amebocyte lysate assay to ensure that there is no residual endotoxin left in the galectin-1 dimer eluants (*see* **Note 4**).
5. Aliquot the purified, endotoxin-free dimer-form of galectin-1 into 0.5 ml aliquots and store at −80 °C (*see* **Note 5**).

3.1.5 Quality Test of Recombinant Galectin-1 by T Cell Treatment

1. To assess the activity of galectin-1, add various doses of galectin-1 from Subheading 3.1.4 to MOLT-4 cells, incubate for 4 h at 37 °C, and stain with annexin V and 7-AAD. The latter is used to stain for necrotic cells (Fig. 2). The extent of phospha-

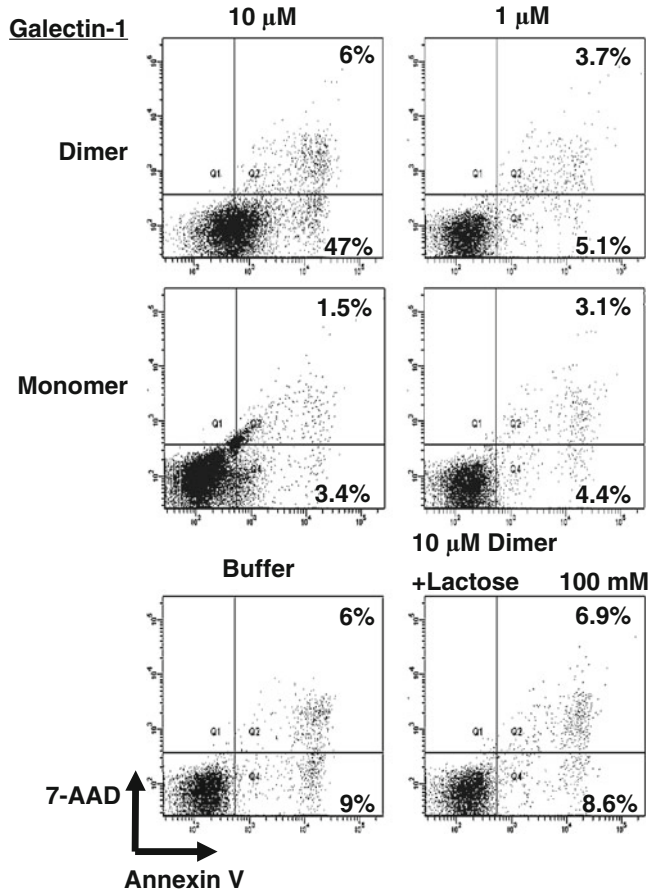

Fig. 2 Test of the activity of galectin-1 on inducing apoptosis of T cells. Dot plots of FACS analysis of annexin V and 7-AAD staining are shown. The lower-left quadrant shows that viable cells are negative for both annexin V and 7-AAD. The lower-right quadrant contains early apoptotic cells that bind annexin V but exclude 7-AAD. The upper-right quadrant contains late apoptotic cells that bind annexin V and are permeable to 7-AAD. Results shown here demonstrate that galectin-1 dimer at 10 μM or 1 μM, but not monomer, induce apoptosis of MOLT-4 cells. Buffer (8 mM DTT in PBS) was used as a control. Addition of 100 mM lactose prevents the apoptosis of T cells caused by the treatment with 10 μM galectin-1 dimer (*bottom-right panel*)

tidylserine exposure can be measured by fluorescence-activated cell sorting (FACS) (*see* **Notes 6** and **7**) The effect of galectin-1 on T cell apoptosis can be blocked by adding 100 mM lactose to the incubation with galectin-1.

3.2 Preparation of Galectin-1_Fc^m Expressed by Mammalian Cells

3.2.1 Transfection and Expression of Galectin-1_Fc^m in 293 T Cells

1. Passage FreeStyle™ 293 F cells at 6×10^5 cells/ml for 24 h before transfection. Rotate the flasks on an orbital shaker platform at 125 rpm at 37 °C in an incubator with an atmosphere of 8 % CO_2 in air.
2. Dilute the cells to 1×10^7/ml in FreeStyle™ 293 expression medium on the day of transfection. Pipet 2.4 ml (2.4×10^7 cells) into a 15-ml centrifuge tube for one transfection.
3. Dilute 18 μg pSecTagGalectin-1Fc^m DNA into Opti-MEM to a total volume of 300 μl, and mix by pipetting up and down.
4. In a separate tube, dilute 54 μl of 1 mg/ml PEI in Opti-MEM to a total volume of 300 μl, and mix gently by inverting the tube (do not vortex).
5. Immediately add diluted PEI to the diluted DNA solution to obtain a total volume of 600 μl, and mix gently.
6. Incubate the DNA/PEI mixture for 10 min at room temperature to allow the formation of complexes.
7. Add 600 μl of the DNA/PEI mixture to 2.4 ml of 293 F cells, and swirl the tube.
8. Transfer this 3 ml of transfected 293 F cells to a 125-ml tissue culture flask and rotate the flask on an orbital shaker platform at 125 rpm at 37 °C in an incubator (8 % CO_2) for 4 h.
9. Add another 30 ml of FreeStyle™ 293 expression medium into the flask.
10. Harvest the culture supernatants daily on days 2, 3, and 4 for further purification of galectin-1_Fc^m.

3.2.2 Purification of Galectin-1_Fc^m

1. Add 1.5 ml of 1.0 M Tris–HCl, pH 8.0, to the supernatants collected in Subheading 3.2.1 that are then subjected to centrifugation at $15{,}000\times g$ to remove debris.
2. Wash protein A Sepharose columns with 5 column volumes of 20 mM Tris–HCl, pH 8.0.
3. Load supernatants onto prewashed columns.
4. Elute bound galectin-1_Fc^m with 3 column volumes of 0.1 M glycine, pH 3.0.
5. Concentrate galectin-1_Fc^m using Vivaspin Turbo spin columns, and store the concentrated galectin-1_Fc^m in PBS in 50-μl aliquots at –20 °C.

3.3 Preparation of Retroviral Vector Expressing Galectin-1

1. Seed 5×10^6 293 T cells in 10 ml in a 10-cm tissue culture plate before transfection, and incubate for 16–18 h at 37 °C in an incubator (5 % CO_2) (*see* **Note 8**).
2. Add 10 μg each of pGC-Gal-1-YFP, packaging DNA, and pMD.G and 186 μl of 2 M $CaCl_2$ stock for each transfection in 1.5 ml H_2O, then add in a dropwise manner into 1.5 ml of 2× HBS followed by incubation at room temperature for 30 min.
3. Replace cell culture medium of 293 T cells with fresh complete medium, and add chloroquine to a final concentration of 25 μM.
4. Add the transfection mixtures dropwise to 293 T cells, and incubate for 6–8 h at 37 °C in an incubator (5 % CO_2).
5. Save the culture media 48 and 72 h posttransfection for further concentration of retrovirus by ultracentrifugation (Optima L90K; Beckman Coulter) at 113,000 × *g* at 4 °C for 1.5 h with a SW28 rotor.
6. Measure the retroviral titers in 3T3 cells. In general, retroviral vectors with titers ranging from 2×10^7 cfu/ml to 9×10^7 cfu/ml can be generated using this protocol.

3.4 Examining the Binding of Galectin-1 to B Cells

3.4.1 Purification of Primary Murine Splenic B Cells

1. Sacrifice C57BL/6 mice at 6–8 weeks of age, and remove spleen.
2. Cut spleen tissue into 5-mm pieces and immerse in 10 ml RPMI medium, and then press the tissue gently between two glass microscope slides to obtain a splenocyte suspension.
3. Add 5 ml of 1× red blood cell lysis buffer for 3 min at room temperature to remove red blood cells, followed by a centrifugation step at 300 × *g*.
4. Wash remaining cells once with 5 ml RPMI medium, pellet cells, and resuspend cells in 10 ml B cell medium, plate in a 10-cm tissue culture dish, and further incubate at 37 °C for 2 h in an incubator (5 % CO_2) to remove the adherent macrophages.
5. Isolate B cells by positive selection with B220 microbeads (Miltenyi Biotec).
6. Resuspend purified B cells with B cell medium at 2×10^6 cells/ml (*see* **Note 9**).

3.4.2 Testing the Binding of Galectin-1 to B Cells Using Galectin-1_Fc^m

1. Wash B cells, described in Subheading 3.4.1, with 0.1 M β-lactose in PBS, and resuspend 2×10^5 B cells in 100 μl PBS.
2. Add 10 μg of galectin-1_Fc^m to B cells and incubate at 4 °C for 30 min. To block galectin-1 binding, use 0.1 M β-lactose; 0.1 M sucrose can be used as a sugar control that does not affect the binding of galectin-1.

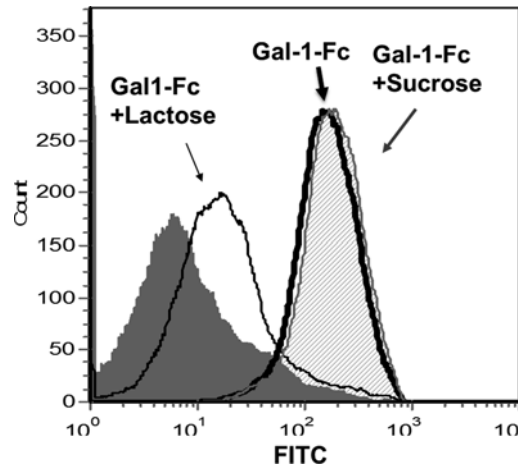

Fig. 3 Galectin-1_Fc^m binding to B cells. Primary murine B cells were incubated with galectin-1_Fc^m (Gal1-Fc) in the presence or absence of 100 mM lactose or sucrose. Histogram shows the FITC fluorescence intensity resulting from galectin-1_Fc^m binding to B cells. The *dark shaded* area represents B cells in the absence of galectin-1_Fc^m and anti-human IgGFc-FITC

3. Wash cells with 500 μl PBS. Spin down the cells, and further incubate with anti-human IgGFc-FITC (2 μg/ml) in 100 μl PBS at 4 °C for 30 min to detect the bound galectin-1_Fc^m.
4. Wash cells three times with 500 μl PBS, and then analyze the binding of galectin-1_Fc^m by FACS (Fig. 3).

3.4.3 Testing the Binding of Recombinant Galectin-1 to B Cells

1. Label 200 μg recombinant galectin-1, as purified in Subheading 3.1.3, using the Lightning-Link Fluorescein kit.
2. Wash B cells (2×10^6 cells/ml) with 0.1 M β-lactose in PBS, and resuspend 2×10^5 B cells in 100 μl PBS.
3. Incubate 0.5 μg FITC-labeled galectin-1 with 2×10^5 B cells in 100 μl PBS at 4 °C for 30 min.
4. Wash B cells three times with 500 μl PBS, and analyze the binding of FITC-labeled galectin-1 to B cells by FACS.

3.5 Assessing the Effects of Galectin-1 on Plasma Cell Differentiation

3.5.1 Examine the Effect of Recombinant Galectin-1

1. Incubate various amounts (2–5 μM) of recombinant galectin-1 with purified murine B cells (described in Subheading 3.4.1) at a density of 2×10^6 cells/ml. As recombinant galectin-1 is dissolved in 8 mM DTT/PBS from Subheading 3.1.4, a solvent control containing 8 mM DTT/PBS is suggested.
2. Harvest cells 72 h later. Cell supernatants can be saved for enzyme-linked immunosorbent assay (ELISA) to determine the amount of Ig, according to the previously described protocol [18]. An aliquot of the cells can also be used for enzyme-linked immunospot assay (ELISPOT) [19].

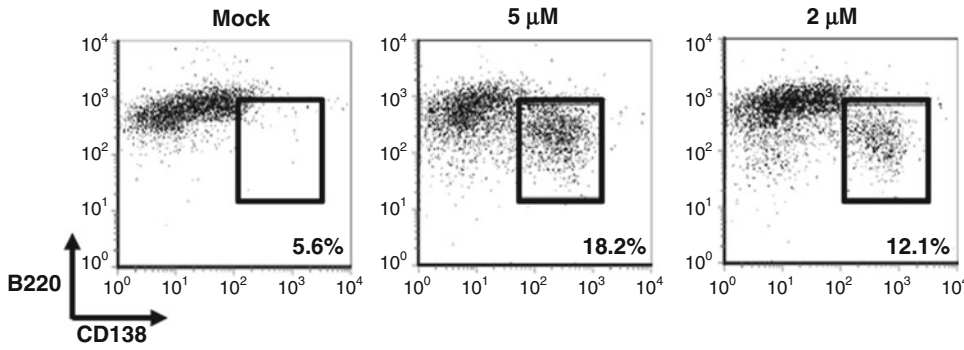

Fig. 4 Galectin-1 induces plasma cell differentiation. Dot plots of FACS analysis show the percentage of the B220^{lo} and CD138^{+} plasma cell population (boxed regions) derived from purified murine B cells incubated with the indicated doses of recombinant galectin-1 or buffer only (mock)

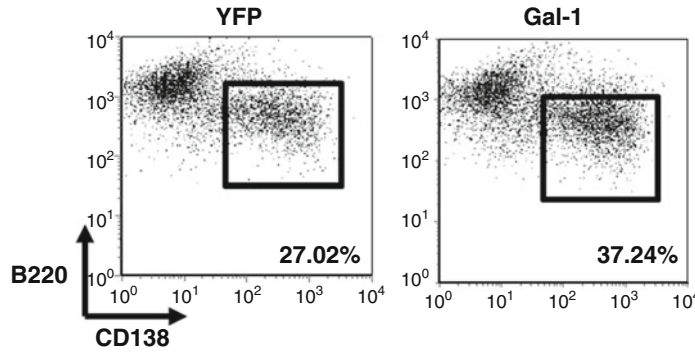

Fig. 5 Ectopic expression of galectin-1 by retroviral vectors promotes plasma cell differentiation. Dot plots of FACS analysis show the percentage of B220^{lo} and CD138^{+} plasma cells (boxed regions) in the YFP^{+} gate. Stimulated splenic B cells were transduced with retroviral vector encoding galectin-1 and YFP (Gal-1) or YFP alone. FACS was conducted 48 h after transduction

Meanwhile, cells can be subjected to FACS analysis to examine the plasma cell population characterized by B220^{lo} and CD138^{+} (Fig. 4) (*see* **Note 10**).

3.5.2 Examining the Effect of Galectin-1 by Retroviral Transduction of Galectin-1

1. Stimulate 2×10^6 cells/ml B cells (described in Subheading 3.4.1) with LPS (0.5 μg/ml) to improve the cycling status of cells, which facilitates the transduction of retrovirus.
2. After 12–16 h, add retroviral vectors described in Subheading 3.3 at the multiplicity of infection of 5 in the presence of 5 μg/ml polybrene.
3. Harvest the cells 48 h after retroviral transduction. Retrovirally transduced cells can be monitored by FACS according to the YFP expression. Subsequently, the expression of B220 and CD138 on the cells of the YFP^{+} gate can be determined (Fig. 5).

4. Sort the retrovirally transduced cells by cell sorter based on YFP expression. The sorted cells can be further subjected to ELISPOT or ELISA to determine the level of Ig.

3.5.3 Examining the Effect of Sugar Compounds on Blocking Galectin-1-Mediated Plasma Cell Differentiation

1. Label B cells (described in Subheading 3.4.1) with 2 μM CFSE at 25 °C for 8 min (*see* **Note 11**).
2. Add sugar compounds into CFSE-labeled B cell culture (2×10^6 cells/ml) in the presence of LPS (2 μg/ml). The effect of the sugar compound on cell proliferation and differentiation can be determined 3 days later by FACS (Fig. 6). To examine the redundant effect of various galectins in this context, sugar compounds preferentially recognized by a particular type of galectin can be used in genetically modified galectin knockout animals. For example, 6SI, a compound that has a monosulfate modification at the C6 of GlcNAc of type 1 LacNAc, selectively blocks galectin-8 binding to B cells [11], and thus it can be used to treat LPS-stimulated B cells from galectin-1 knockout mice to examine the redundant role of galectin-1 and galectin-8 in plasma cell differentiation (Fig. 7).

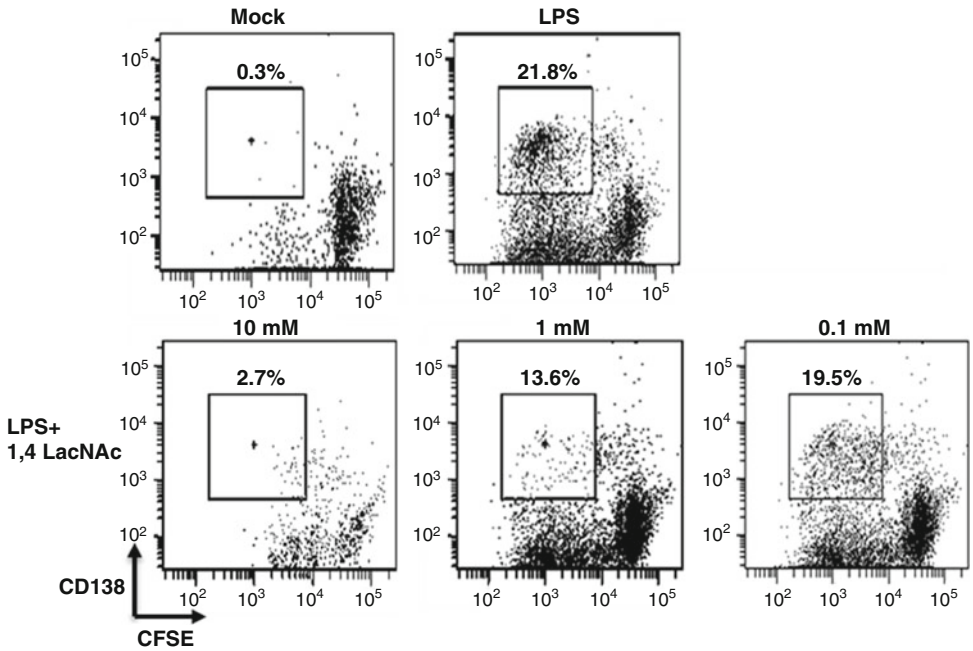

Fig. 6 Type 2 LacNAc blocks plasma cell differentiation. Dot plots of FACS analysis show the percentage of $CD138^+$ cells that have divided (boxed regions). CFSE-labeled splenic B cells were stimulated with LPS (2 μg/ml) in the presence of various amounts (0.1 – 10 mM) of 1,4 LacNAc (type 2 LacNAc). Three days later, cells were subjected to FACS analysis. Mock refers to the unstimulated and CFSE-labeled cells

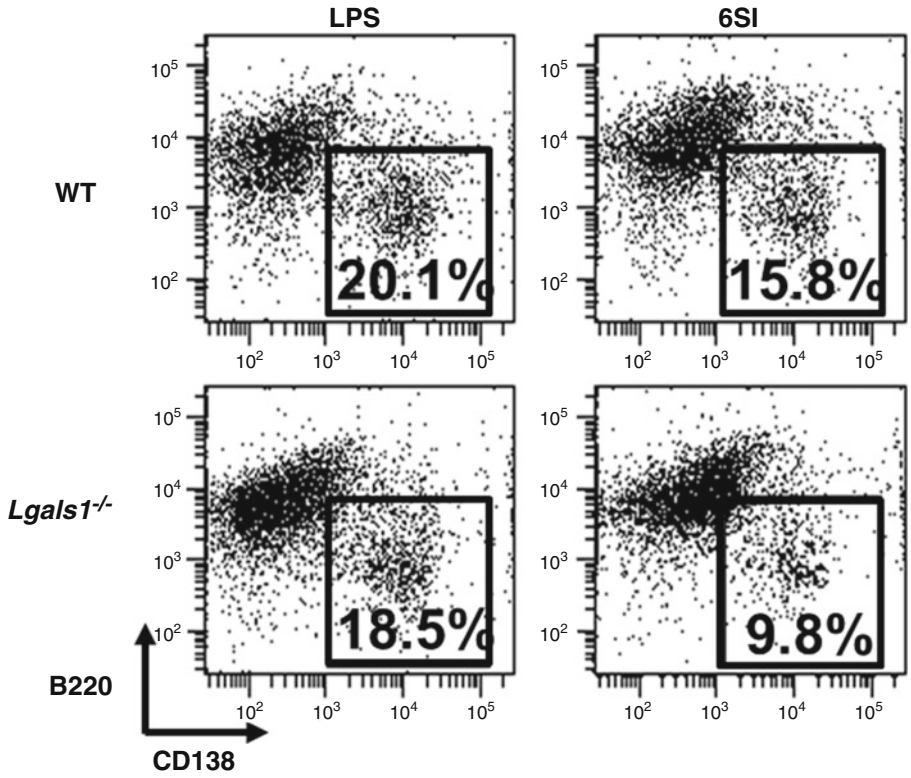

Fig. 7 In the absence of the galectin-1, 6SI blocks plasma cell differentiation. Dot plots of FACS analysis show the percentage of B220^{lo} and CD138^{+} cells (boxed regions). Purified splenic B cells from galectin-1 knockout (*Lgals1*$^{-/-}$) or littermate wild type (WT) control mice were stimulated with LPS (2 μg/ml) in the presence of 6SI (0.1 mM). Three days later, cells were subjected to ACS analysis

4 Notes

1. Buffers containing DTT should be freshly prepared.
2. The column should be equilibrated with elution buffer and kept at 4 °C.
3. We recommend the use of freshly transformed colonies.
4. The steps of removal endotoxin should be repeated until endotoxin is removed entirely.
5. Do not freeze and thaw the proteins. Repeated freeze/thaws will result in significant loss of protein activity.
6. Galectin-1 can induce phosphatidylserine exposure in MOLT-4 T cells [20]. To ensure the biological activity of recombinant galectin-1, perform annexin V staining, which can quickly detect the exposure of phosphatidylserine following treatment with purified active recombinant galectin-1.

7. Although DTT has been used by many laboratories to maintain galectin-1 activity in a variety of assay conditions [20, 21], several studies suggest that DTT may alter cellular responses to galectin-1 [22, 23]. Care should be taken with interpreting results in the presence DTT not only for potential changes in cellular response, but also for the possible influence of loss of Gal-1 activity when DTT is not included.
8. To produce a retroviral vector expressing galectin-1, retroviral vector pGC-Gal-1-YFP [12], packaging DNA, and pMD.G are used [17, 16]. Protocols for preparation of retroviral vectors and retroviral transduction in murine primary B cells have been described [24]. The protocol is described briefly below. Further details regarding the preparation of retroviral vectors can be found at the website maintained by Dr. Garry Nolan's laboratory from Stanford University: http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html.).
9. The purity of B cells should be evaluated by FACS.
10. To study whether multiple extracellular galectin-family proteins are involved, a combination of various types of recombinant galectins can be added to the B cell culture. For instance, we have shown that simultaneous treatment of murine splenic B cells with galectin-1 and galectin-8 led to a more significant effect of each galectin on promoting plasma cell formation than treatment with each galectin alone [11].
11. To examine the effect of extracellular galectin-1 on plasma cell differentiation, sugar compounds that contain LacNAc for galectin-1 recognition can be used as competitors to block galectin-1 binding to B cells. Various galectin family proteins have different carbohydrate ligand specificities [11], although some overlap in binding can exist and should be considered when interpreting results [25]. Thus, various sugar compounds can be applied to test the potential impact of individual galectins on plasma cell differentiation. To avoid the potential problem of cytotoxicity caused by treatment with sugar compounds, monitor the status of B cell division by CFSE staining.

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Chapter 11

Examination of Galectin-Induced Lattice Formation on Early B-Cell Development

Stéphane J.C. Mancini, Latifa Elantak, Annie Boned, Marion Espéli, Françoise Guerlesquin, and Claudine Schiff

Abstract

Galectin-1 (GAL1) is a pre-B cell receptor (pre-BCR) ligand that induces pre-BCR clustering and leads to efficient pre-B cell proliferation and differentiation in the bone marrow. To study pre-BCR–GAL1 interactions and its functional consequence on the early steps of the B cell development, we combine structural nuclear magnetic resonance (NMR) approaches and B cell biology techniques. NMR is applied to identify the residues involved in pre-BCR–GAL1 interactions by monitoring chemical shift perturbations when the complex is formed. This structural information is then used at the cellular level to target specifically the complex formation during GAL1-induced pre-BCR clustering and lattice formation, using immunofluorescence techniques. Moreover, an in vivo assay was set up to study the consequence of synapse formation on the early steps of B cell development.

Key words Galectin-1, pre-B cell receptor, Lattice formation, Protein interactions, B cell development, Nuclear magnetic resonance, Immunofluorescence, Flow cytometry

1 Introduction

In many biological processes, ligand-mediated clustering of protein receptors is required for optimal transmission of signals into a cell. This is the case for the galectin-1 (GAL1) dependent pre-B cell receptor (pre-BCR) activation. The pre-BCR is expressed by pre-B lymphocytes during B cell differentiation in the bone marrow (BM) and its GAL1 ligand is produced by BM stromal cells [1, 2]. We have reported on the formation of an immune developmental synapse between pre-B and stromal cells where pre-BCRs relocalize at the cellular contact zone, leading to pre-BCR activation and to the optimal development of normal pre-B cells [3]. The formation of the synapse takes place through a network of interactions involving GAL1, which binds to the pre-BCR through a protein–protein

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interaction and to glycosylated integrins on stromal and pre-B cells via protein–carbohydrate interactions [2]. In general, protein–protein interactions involving GAL1 mainly occur in the intracellular compartments whereas its carbohydrate binding activity is mostly extracellular. So far, the pre-BCR is a unique example of a nonglycosylated protein partner of GAL1 described in the extracellular matrix. The pre-BCR consists of two immunoglobulin heavy chains (Igμ), two surrogate light chains (SLC), composed of the invariant λ5 and VpreB proteins, and the CD79a/b signaling molecules. The SLC exhibits a structure similar to IgL chains and the λ5 and VpreB proteins contain unique regions (UR) at their N and C terminus, respectively. GAL1 interacts with the pre-BCR via the unique region of λ5 (λ5-UR) [1, 4].

In order to study the pre-BCR/GAL1 lattice and its functional consequence on the early steps of the B cell development, we combine structural nuclear magnetic resonance (NMR) approaches and B cell biology techniques. NMR is a technique of choice for the study of protein–ligand interactions since the binding interface can be efficiently identified by monitoring chemical shift perturbations, when a complex is formed. Atomic level information on the pre-BCR–GAL1 interaction can then be used to target specifically this interaction at the cellular level for evaluating its role in lattice formation by immunofluorescence and to study the consequence of synapse formation on the early steps of B cell development in the BM using an in vivo assay.

2 Materials

2.1 Protein Expression and Purification

1. BL21 (DE3) *E. coli* competent cells.
2. Protein expression plasmids for human GAL1 (pQE60 plasmid which produces a protein without any tag) and λ5-UR (modified pET28a plasmid in order to produce λ5-UR fused to a His6-tagged lipoyl domain which is cleavable by the TEV protease).
3. pGEX2T-λ5-UR.
4. pGEX2T.
5. Kanamycin (50 mg/ml), ampicillin (100 mg/ml) filter-sterile stocks.
6. LB medium: Dissolve 5 g yeast extract, 10 g trypton, and 10 g NaCl in 1 L H_2O. Autoclave. Add 1 ml of the appropriate antibiotic stock solution.
7. M9 medium: Dissolve 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl in 1 L H_2O. Autoclave. Add 1 ml of sterile 0.1 M $CaCl_2$, 1 ml of 1 M $MgCl_2$, 10 ml of 20 % glucose, 5 ml of 0.2 g/ml $^{15}NH_4Cl$ (purchased from Cambridge Isotope Laboratories), 1 ml of antibiotic stock solution.
8. IPTG 1 M stock solution in H_2O.

9. BugBuster protein extraction reagent (Novagen).
10. 100 ml β-lactosyl-Sepharose 6B (Sigma) column prepared as described in the detailed protocol published previously in Methods in Enzymology [5].
11. Glutathione Sepharose 4B (GE Healthcare).
12. Washing buffer for β-lactosyl-Sepharose column: 8 mM DTT, 0.02 % sodium azide, in sterile PBS.
13. Elution buffer for β-lactosyl-Sepharose column: 0.1 M β-lactose, 8 mM DTT, 0.02 % sodium azide in PBS. Filter on 0.22 μm to sterilize and store at 4 °C.
14. Elution buffer for glutathione column: 10 mM reduced glutathione, 50 mM Tris–HCl pH 8.
15. Resuspension buffer (for λ5-UR fused to GST and of the GST purification): Phosphate buffered saline, 0.36 M NaCl, 1/1,000 protease inhibitors (stock 1 mg/ml of pepstatin A, leupeptin, aprotinin and antipain), and 5 % glycerol.
16. PBS, 0.36 M NaCl.
17. Syringe with 22G needle.
18. HiTrap 5 ml prepacked column (GE Healthcare).
19. Washing buffer for HiTrap column: 20 mM Hepes pH 7.5, 10 % glycerol, 500 mM NaCl, 1 tablet of protease inhibitors cocktail (Roche).
20. Elution buffer for HiTrap column: 20 mM Hepes pH 7.5, 10 % glycerol, 500 mM NaCl, 300 mM Imidazole.
21. AKTAPrime purification system.
22. Amicon Ultra-15 concentrator (Millipore).
23. Superdex 75 10/300 GL prepacked gel filtration column (GE Healthcare).

2.2 NMR Experiments (Identification of GAL1 Interacting Residues)

1. Bruker Avance III 600 MHz spectrometer equipped with a TCI cryoprobe.
2. NMR tubes.
3. NMR buffer: 20 mM KPO_4 pH 5.2 (*see* **Note 1**), 100 mM NaCl.
4. Deuterium oxide (D_2O) solution.

2.3 Immunofluorescence Assay of the pre-BCR–GAL1 Interaction and Lattice Formation

1. Supplemented MEMα medium.
2. 0.05 % Trypsin-EDTA (Life Technologies).

2.3.1 Preparation of the OP9 BM Stromal Cell Line (RIKEN; [6])

2.3.2 Preparation of Large pre-B Cells

1. 6-Week-old C57Bl/6 mice.
2. CD45R MicroBeads (Miltenyi).
3. AutoMacs system (Miltenyi).
4. Antibody Mix1.
5. FACS buffer.
6. Sytox Blue.

2.3.3 Immunfluorescence Assay

1. Supplemented MEMα medium: MEMα (Life Technologies), 20 % decomplemented fetal calf serum (FCS), 100 U/ml Penicillin and 100 µg/ml Streptomycin.
2. Supplemented DMEM medium: DMEM (Life Technologies), 10 % decomplemented FCS, 100 U/ml Penicillin and 100 µg/ml Streptomycin.
3. FACS buffer: PBS, 1 % FCS, 2 mM EDTA.
4. Antibody Mix1: FITC rat anti-mouse Igκ, FITC rat anti-mouse Igλ1, λ2, λ3 (BD biosciences), PE rat anti-mouse CD117, PE-Cy5 rat anti-mouse CD19 (eBioscience) in FACS buffer (*see* **Note 2**).
5. Sytox Blue 5 mM (Life Technologies).
6. 4 % Paraformaldehyde (PFA) in PBS, prepared from 16 % PFA aqueous solution (Electron Microscopy Sciences).
7. Saturation buffer: PBS, 0.5 % Bovine Serum Albumin (BSA).
8. Goat anti-mouse IgM antibody, 1 mg/ml (Southern Biotech).
9. Rabbit anti-mouse/human GAL1 antiserum [1].
10. Alexa Fluor® 555 donkey anti-goat IgG (H + L) antibody, 2 mg/ml (Life Technologies).
11. Cy5 donkey anti-rabbit IgG (H + L) antibody (Jackson Immunoresearch Laboratories).
12. Mowiol 4-88 with DABCO: Put in a beaker 6 g of glycerol with 2.4 g of Mowiol 4-88 mounting medium (Calbiochem) and leave 2 h at room temperature. Add 6 ml of MilliQ water and incubate overnight at room temperature under gentle shaking. Add 12 ml Tris–HCl 0.2 M pH 8.5 and homogenize. Heat at 55 °C up to complete dissolution. Cool down the solution and add 2.5 % w/v DABCO (Sigma Aldrich). Centrifuge at 5,000 × *g* during 15 min at room temperature. Take the supernatant (some crystals of Mowiol can remain) and prepare 0.5 or 1 ml aliquots. Store at −20 °C.
13. 12 mm circular glass coverslips (Marienfeld Laboratory) (*see* **Note 3**).
14. SuperFrost Plus slides (Thermo Scientific).

2.4 In Vivo Assay to Analyze the Physiological Role of the pre-BCR–GAL1 Interaction

1. Hydroxyurea (Calbiochem) 100 mg/ml in PBS.
2. FACS buffer.
3. Antibody Mix2: PE rat anti-mouse CD25 (BD Biosciences); PE-Cy7 rat anti-mouse CD23; APC rat anti-mouse IgM (eBioscience) (*see* **Note 2**).
4. Antibody Mix3: FITC rat anti-mouse CD2; PE-Cy5 rat anti-mouse CD19 (eBioscience); APC-Cy7 rat anti-mouse CD45R (BD Biosciences) (*see* **Note 2**).
5. Sytox Blue 5 mM.
6. 10 ml syringe.
7. 25G needle.

2.5 Special Equipment

1. French press.
2. Confocal microscope. Use any confocal microscope with the appropriate lasers and detectors. We used a LSM510 (Carl Zeiss) with lasers at 543 nm and at 633 nm.
3. FACS apparatus:
   (a) FACS Sorter: We used a BD FACSAriaIII. The staining proposed implies the use of lasers at 405 nm, 488 nm and eventually 561 nm (PE and PE tandems can be excited at 488 nm or 561 nm) with adapted detectors.
   (b) FACS Analyzer: We used a BD FACSCantoII. The staining proposed implies the use of lasers at 405 nm, 488 nm and 640 nm with adapted detectors.

3 Methods

3.1 Preparation of Proteins

3.1.1 Human GAL1

1. Transform the expression construct into BL21 (DE3) *E. coli* cells.
2. Streak cells onto an LB plate containing appropriate antibiotic, incubate overnight at 37 ° C.
3. Select a single colony. Inoculate 1 ml of LB medium with the colony, and grow 4 h at 37 ° C in a shaker. Inoculate 100 ml of M9 medium (*see* **Note 4**) with the 1 ml starter culture and let it grow overnight at 37 ° C.
4. Inoculate 900 ml of M9 medium with the 100 ml overnight starter culture.
5. Shake in a 37 ° C incubator at 220 rpm, 2–3 h until $OD_{600} = 0.6–0.8$.
6. Induce protein expression by adding 1 mM IPTG (1 ml of sterile 1 M stock solution). Incubate 3 h at 37 °C.
7. Harvest by centrifuging cells (3,000 × *g*, 15 min) and remove supernatant.

8. Resuspend the cell pellet with Bugbuster Protein extraction reagent (~50 ml for 1 L culture). Incubate the cell suspension on a shaking platform or rotating mixer at a slow setting for 10–20 min at room temperature as recommended by the manufacturer.
9. Centrifuge the suspension (125,000 × *g*, 30 min) and retain supernatant.
10. Equilibrate the β-lactosyl sepharose column with washing buffer.
11. Apply supernatant to the column at a flow-rate of 0.5 ml/min and let the lysate on the column overnight.
12. Wash the column with washing buffer (~200 ml).
13. Elute bound protein with elution buffer. Collect 1 ml per fraction.
14. Check protein purity by Coomassie blue staining of a SDS-PAGE gel.
15. After elution of the GAL1 protein from the β-lactosyl sepharose column, the protein can be aliquoted and stored at –80 °C (*see* **Note 5**).
16. Right before use for the NMR experiments, load the aliquot onto a gel filtration column (Superdex S75 10/300GL) equilibrated with the NMR buffer.
17. Concentrate the ^{15}N-labeled GAL1 containing fractions to a final concentration of 200 μM using an Amicon Ultra-15 concentrator (cutoff 10 kDa).

3.1.2 Human λ5-UR

1. Transformed BL21 (DE3) *E. coli* cells with expression plasmid containing the human gene sequence of λ5-UR are treated as detailed above for GAL-1 production, except that LB medium is used instead of M9 medium. When OD at 600 nm of the culture reaches 0.7, add 1 ml of 1 M IPTG to induce protein expression. After 3 h incubation at 37 °C, harvest the cells by centrifugation (3,000 × *g*, 15 min).
2. Resuspend cells in a solution of 25 ml washing buffer. Cell lysis is achieved with a French press.
3. Centrifuge the suspension (125,000 × *g*, 30 min) and retain supernatant.
4. Purify the His_6-tagged fusion proteins from the supernatant by affinity chromatography using HiTrap 5 ml prepacked column plugged to an AKTAPrime purifying system (please refer to the instructions by the manufacturer).
5. Check protein purity by Coomassie blue staining of a SDS-PAGE gel.

6. Pool fractions containing the λ5-UR fused to the His_6-tagged lipoyl domain.
7. Take OD at 280 nm of the pooled fractions and add 3 mg of His_6-tagged-TEV protease for 10 OD of proteins. The protease cleaves the fusion protein into two proteins: the His_6-tagged lipoyl domain and the λ5-UR.
8. Place the mixture into dialysis tubing and dialyze in 2 L of washing buffer for 3 h at room temperature.
9. Load the dialyzed proteins onto a HiTrap column. The λ5-UR does not bind to the column and is found in the flow through whereas the tagged lipoyl binds to the column and is eluted with the imidazole gradient.
10. Dialyze the flow through against NMR buffer and concentrate using Amicon Ultra-15 concentrator (cutoff 3 kDa).

3.1.3 Preparation of Recombinant λ5-UR Fused to GST and of the GST Control Protein (the λ5-UR was Cloned into pGEX2T, a Protein Expression Plasmid Allowing the Fusion to GST)

1. Grow *E.coli* BL21 bacteria transformed with pGEX2T-λ5-UR (for the production of recombinant λ5-UR-GST) or with pGEX2T (for the production of the GST control protein) in LB medium containing 100 μg/ml ampicillin overnight at 37 °C.
2. Dilute to 1/10 in 1 L of LB containing 100 μg/ml ampicillin and grow at 37 °C until the absorbance at 600 nm reaches between 0.6 and 1.
3. Induce protein expression with 1 mM IPTG during 4 h at 37 °C.
4. Centrifuge the bacteria at 6,000 × *g* during 15 min at 4 °C.
5. Resuspend the bacteria in 16 ml of resuspension buffer.
6. Bacteria lysis is performed with two passages in a French Press.
7. The lysate is then passed six times through a syringe with a 23G needle.
8. Centrifuge at 12,000 × *g* for 20 min at 4 °C. Retain the supernatant and add 2 ml of Glutathione Sepharose 4B at 50 %
9. Wash twice with cold PBS, 0.36 M NaCl, centrifuge at 500 × *g* for 5 min, remove the supernatant, and add 1 ml of PBS.
10. Incubate 30 min on a wheel at room temperature and centrifuge at 500 × *g* during 5 min.
11. Wash three times with 10 ml PBS, 0.36 M NaCl.
12. Remove the supernatant, add 2 ml of elution buffer.
13. Incubate on a wheel during 10 min at room temperature.
14. Centrifuge at 500 × *g* during 5 min and get the supernatant.
15. Repeat the elution twice and determine the protein concentration for each sample.
16. Check the purity of the protein by SDS-PAGE.

3.2 Identification of GAL1 Interacting Residues (See Note 6)

1. Prepare 500 µl of ^{15}N-labeled GAL1 in NMR buffer and place it in a NMR tube. Add 50 µl of D_2O.
2. Take an initial ^{1}H-^{15}N HSQC spectrum of this GAL1 sample. This will serve as your unbound control.
3. Add unlabeled λ5-UR to the sample to specific molar equivalences (e.g. 1:0.5, 1:0.75, 1:1, 1:1.5, 1:2), mix well, and take a new ^{1}H-^{15}N HSQC spectrum after each addition.
4. Process the spectra using programs such as Topspin (Bruker).
5. Calculate the chemical shift perturbations for each resonance using the equation: $\Delta\delta obs = [(\Delta\delta H_N^2 + \Delta\delta N^2/25)]^{1/2}$ where $\Delta\delta H_N$ and $\Delta\delta N$ are the proton and nitrogen chemical shifts variation of each residue.
6. Map the affected residues onto the 3D structure of the human GAL1. This requires resonance assignment of the protein, which has been published previously [7] and is available in the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/) under the accession number 15800.
7. This mapping may aid in confirming the interacting site on GAL1 and differentiating between local and long-range structural changes. The residues highlighted as key interacting residues can then be used as restraints for GAL1/λ5-UR docking calculations using Haddock software in order to get a structural model of the GAL1/λ5-UR complex. These structural information are crucial for the design of GAL1 and λ5-UR mutants that will be used in cellular assays to assess specifically the role of this complex formation during B cell development.

3.3 Immunofluorescence Assay of the pre-BCR–GAL1 Interaction and Lattice Formation

3.3.1 Preparation of OP9 Cells

1. Culture OP9 cells in supplemented MEMα medium at 37 °C with 5 % CO_2 in a humid atmosphere (*see* **Note 7**).
2. The day before the pre-B/OP9 cell coculture, put circular glass coverslips in 24-well plates and add 2×10^4 OP9 cells in 1 ml of supplemented MEMα in the number of wells required.

3.3.2 Preparation of Large pre-B Cells

1. The large pre-B cells are sorted from the BM of 6-week-old C57Bl/6 mice. (For the preparation of the BM cells, see the **steps 3–9** of the Subheading 3.5).
2. B cell pre-enrichment is performed using magnetic beads coupled to an anti-CD45R antibody (*see* **Notes 8** and **9**).
3. Stain the cells with the Antibody Mix1 at a concentration of 3×10^6 cells/100 µl, at 4°c in the dark during 20 min.
4. Wash with 10 volumes of FACS buffer, centrifuge at $450 \times g$ for 5 min at 4 °C and resuspend the cell pellet in FACS buffer

containing 1/20,000 Sytox Blue (the cellular concentration depends on the FACS sorter used).

5. Before starting the sorting, procedures common to the different cytometers have to be followed (*see* **Note 10**).
6. The large pre-B cells are sorted negatively by using antibodies specific for the different B cell subpopulations except for the pre-B cells (*see* Fig. 1a). The procedure to gate out cell doublets and dead cells on the cytometer is detailed in the Subheading 3.6, **steps 2–4**.

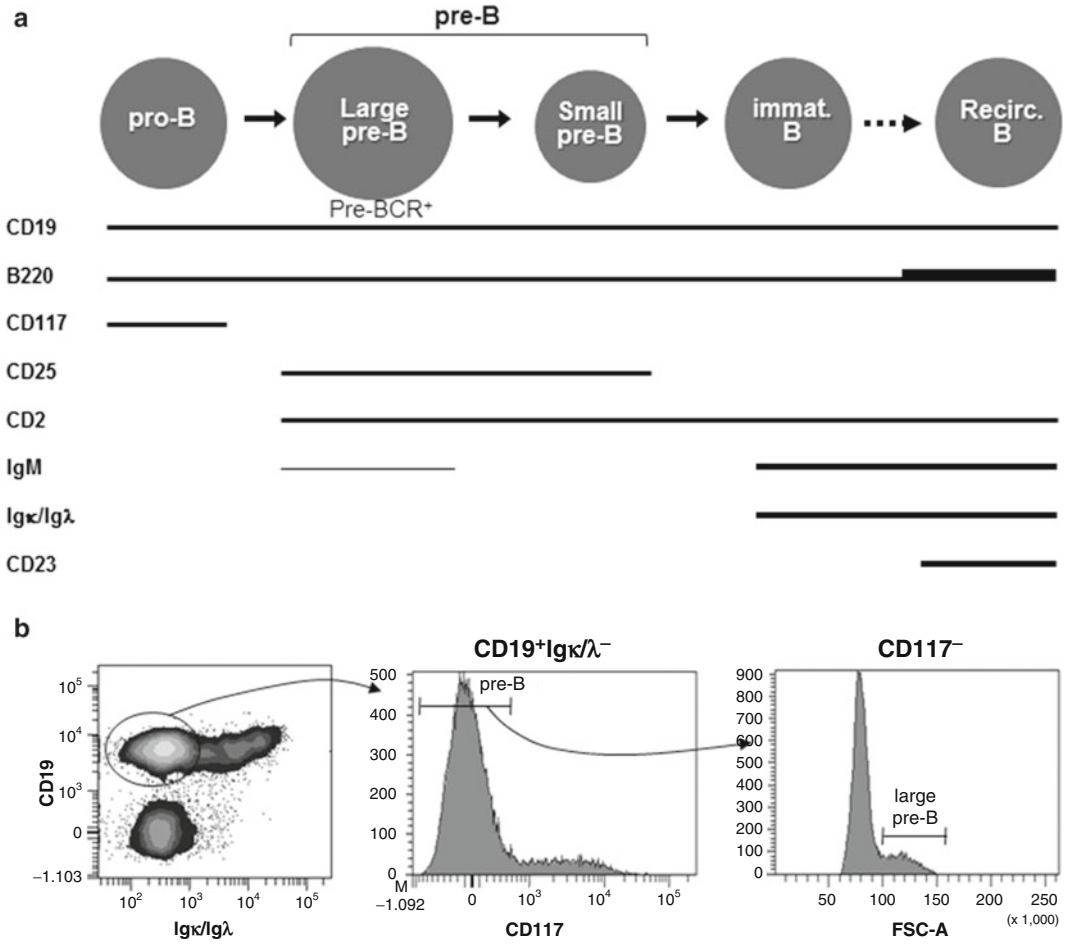

Fig. 1 (**a**) The different B cell subsets observed during B cell differentiation in the BM are represented. Pre-B cells are composed of large proliferating cells expressing the pre-BCR and of small nonproliferating cells, which have down-regulated the pre-BCR. The *discontinuous arrow* between the immature and the recirculating B cells indicates that the immature B cells differentiate into naive B cells in the periphery, called recirculating B cells when they home back to the BM. The expression pattern of the different surface markers used in Figs. 1b and 3 is shown below the B cell subsets. The thickness of the lines is representative of the level of expression. The immunoglobulin heavy chain IgM can be expressed as part as the pre-BCR (associated to the SLC) or as part of the BCR (associated to Igκ or Igλ). Imm. B = Immature B cells; Recirc. B = recirculating B cells. (**b**) Gating strategy used for the sorting of the large pre-B cells

7. For the sorting, a gate is performed on the $CD19^{+}Ig\kappa/\lambda^{-}$ B cells to eliminate $Ig\kappa^{+}$ or $Ig\lambda^{+}$ immature and recirculating B cells (Fig. 1b).
8. A gate is then performed on the $CD117^{-}$ pre-B cells (in opposition to the $CD117^{+}$ pro-B cells).
9. Finally, the large pre-B cells, which express the pre-BCR are selected on the size criterion using the Forward Scatter (FSC) parameter.
10. After sorting, the large pre-B cells are resuspended in supplemented DMEM at a concentration of 4×10^5 cells/ml.

3.3.3 Immunfluorescence Assay

1. Remove the MEMα medium from the OP9 cells cultivated on circular glass coverslips in the 24-well plates and replace by 500 µl of supplemented DMEM.
2. Prepare 2×10^5 large pre-B cells in 500 µl DMEM in presence of 100 µg/ml of λ5UR-GST or 100 µg/ml of the GST control protein (*see* **Note 11**).
3. Add 500 µl to the wells to have a final concentration of 2×10^5 pre-B cells/ml and 50 µg/ml of λ5UR-GST or of GST control protein.
4. Incubate the cells for 2 h at 37 °C with 5 % CO_2.
5. Wash the cells with 500 µl of PBS and then fix by adding 250 µl of 4 % PFA during 10 min at room temperature (*see* **Note 12**).
6. Gently wash twice with 1 ml of PBS. The fixed cells can be kept at least 7 days in PBS at 4 °C.
7. Perform a saturation step by transferring the glass coverslips with the cells face down onto a drop of 60 µl PBS/0.5 % BSA into a humid black box (*see* **Note 13**). Incubate 30 min at room temperature.
8. Transfer the glass coverslips onto drops of 50 µl of saturation buffer containing 5 µg/ml of goat anti-mouse IgM and 1/100 of rabbit anti-mouse GAL1 antiserum. Incubate 1 h at room temperature.
9. Wash by soaking the glass coverslips in 4 successive beakers containing PBS and repeat once (*see* **Note 14**).
10. Transfer the glass coverslips onto drops of 50 µl of saturation buffer containing 1/1,000 of Alexa Fluor-555 donkey anti-goat IgG (H + L) antibody and 1/1,000 Cy5 donkey anti-rabbit IgG (H + L) antibody. Incubate 30 min at room temperature.
11. Wash twice in PBS as in **step 9**.
12. Put a drop of 10 µl of Mowiol 4-88 with DABCO (*see* **Note 15**) on glass slides washed with 70 % ethanol. Put the coverslips with the cells face down on the mounting medium.

Keep the slides in the dark up to the following day to allow the polymerization of the mounting medium. Slides can be kept at least 2 weeks in the fridge and in the dark.

3.4 Analysis of the Immunofluorescence Assay

1. The morphology of the cells is observed by differential interference contrast microscopy (DIC) with a 63×/1.4 NA oil objective.
2. The fluorescence analysis is performed with a 40×/1.3 NA oil objective or a 63×/1.4 NA oil objective. Alexa Fluor 555 is excited with a 543 nm laser and Cy5 with a 633 nm laser.
3. The interaction between the pre-BCR and GAL1 induces the relocalization of both proteins at the contact zone between the pre-B cell and the OP9 stromal cell, forming a crescent at the surface of the pre-B cells in opposition to a continuous ring when the pre-BCR is not relocalized (Fig. 2). For each experiment, we count between 120 and 500 cells for statistical significance.

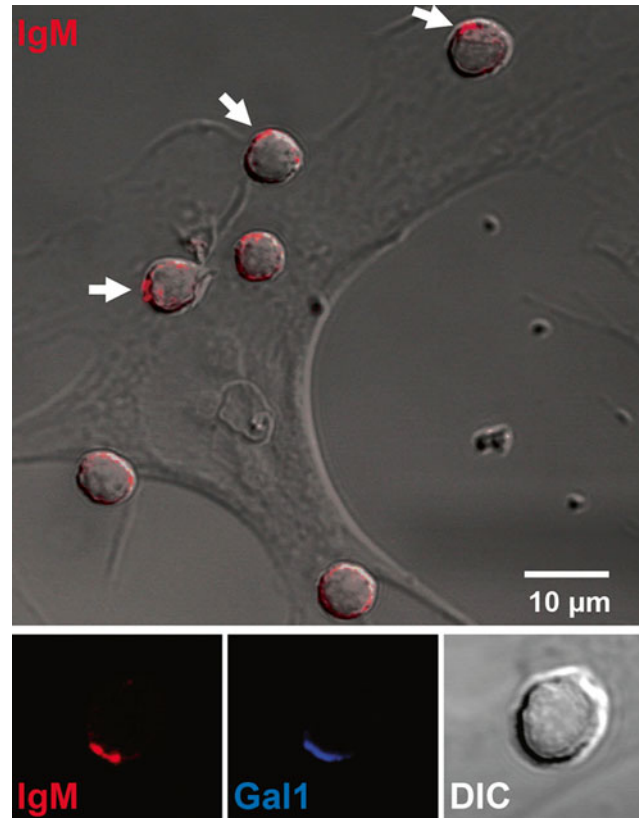

Fig. 2 Image of large pre-B cells in contact with an OP9 stromal cell. The *white arrows* show the pre-B cells for which the pre-BCR is relocalized. A magnification of a pre-B cell for which the pre-BCR and GAL1 are colocalized is shown in the *lower panel*

In a typical experiment, about 55 % of pre-B cells present relocalized pre-BCRs [3]. The relocalization is decreased by about 25 % when the pre-BCR–GAL1 interaction is antagonized with the soluble λ5UR-GST.

3.5 In Vivo Assay to Analyze the Physiological Role of the Pre-BCR–GAL1 Interaction

1. Perform 4 intraperitoneal injections of hydroxyurea (1 mg/g of body weight) to WT and GAL1 deficient mice at the rate of 2 injections per day (leave 7 h between the injections) for 2 days (*see* **Note 16**).
2. Kill the mice 7 days after hydroxyurea injections and cut out the rear legs (tibias and femurs). Keep the legs on ice in FACS buffer.
3. Catch the bones with towel paper and tear the muscles up to detach them completely from the bones.
4. Prepare a 10 ml syringe filled with FACS buffer and then attach a 25G needle. Cut out the extremities of the bones, insert the needle in the bone cavity and flush out the BM. The cells must be kept on ice during all the experiment.
5. Centrifuge the cells at 450×*g* for 5 min at 4 °C.
6. Resuspend the cells in 1 ml of red blood cell lysis buffer (eBioscience) and incubate 5 min at room temperature.
7. Add 9 ml of FACS buffer and filter the cells on 70 μm nylon cell strainers (BD Falcon). Centrifuge the cells as in **step 6**.
8. Resuspend the cells in 3 ml of FACS buffer and count the cells (*see* **Note 17**).
9. The staining is performed in 96-well plates with V bottom. Put 10^6 BM cells from each mouse into wells (to avoid cross contamination between samples, always leave an empty well between each of them).
10. Centrifuge at 450×*g* for 3 min at 4 °C. Remove the supernatant by flicking firmly the 96-well plate. Dry the border of the plate by taping it gently on a towel paper.
11. Resuspend each of the samples with 50 μl of the Antibody Mix2 and incubate 20 min on ice and in the dark.
12. Fill completely the wells with FACS buffer to wash, centrifuge, and remove the supernatant as in **step 11**.
13. Repeat the **step 12** (with 50 μl of Antibody Mix3 per sample) and the **step 13**.
14. Resuspend the samples in 300 μl of FACS buffer containing 1/20,000 of Sytox Blue (*see* **Note 18**).
15. Acquire the samples on the FACS analyzer as recommended by the manufacturer (*see* **Note 10**).

3.6 Data Analysis of the In Vivo Assay

1. Use any software dedicated to the analysis of flow cytometry data. The results presented here were analyzed using the BD FACSDiva 6 software (Fig. 3). We strongly recommend the use of the logical display (biexponential representation), which allows the visualization of the cells with minimal fluorescence "piled up" on the axis with the traditional logarithmic display [8]. With the procedure proposed below, the different B cell subpopulations from the BM can be visualized. The expression pattern of the different markers used is shown in Fig. 1a.
2. In a FSC-A/SSC-A dot plot, gate on the BM cells.
3. From this "BM cells" gate, perform a gate in a SSC-W/SSC-H dot plot to remove the doublets on the granulosity parameter ("singlets1" gate) and then a FSC-W/FSC-H dot plot to remove the doublets on the size parameter ("singlets2" gate). This step is facultative.
4. In a Sytox Blue/FSC-A dot plot, do a "live cells" gate by excluding the Sytox Blue positive dead cells.
5. In a B220/CD19 dot plot, gate on the $CD19^{+}B220^{+}$ population which corresponds to the B cells from the pro-B cell to

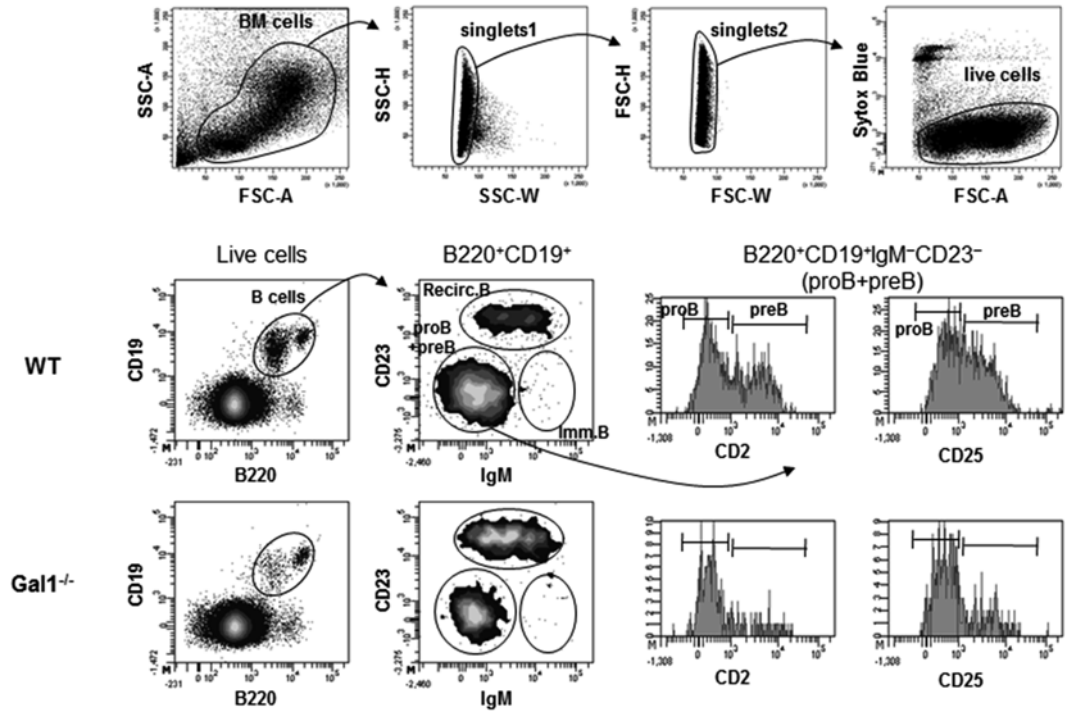

Fig. 3 Gating strategy used for the analysis of the different BM B cell subsets in the case of WT and GAL1$^{-/-}$ mice, 7 days after hydroxyurea injections

the immature B cell stages and also includes the recirculating B cells, homing back from the periphery to the BM.

6. From an IgM/CD23 dot plot, the immature B cells (IgM^+CD23^-) and the recirculating B cells (IgM^+CD23^+) are separated from the pro-B and the pre-B cells (IgM^-CD23^-).
7. From the IgM^-CD23^- subpopulation, draw histograms showing the CD2 and CD25 markers. In opposition to the pro-B cells, the pre-B cells express both CD2 and CD25.

4 Notes

1. Acidic pH is used to slow the exchange rate of backbone amide protons with the solvent.
2. In order to get an optimal staining, the antibodies must be carefully titrated. Depending on the antibody, the fluorochrome, and the batch, the dilution can vary from 1/50 to 1/5,000. The titration is therefore a crucial step.
3. Before use, soak the circular glass coverslips in acetone, dry them, and then soak them in 100 % ethanol. Let them dry under the hood in a sterile dish onto a blotting paper.
4. The M9 medium prepared here contains as sole nitrogen source $^{15}NH_4Cl$ in order to produce ^{15}N-labeled proteins only.
5. Once purified, the GAL1 protein must be stored at –80 °C since the protein tends to precipitate. It must not be freezed and thawed more than once.
6. Using ^{15}N-labeled protein, ^{1}H-^{15}N HSQC experiments can be recorded. In these experiments, a 2D spectrum is produced where peaks represent correlations between ^{15}N and ^{1}H covalently bound. Position of each peak on the spectrum, the chemical shifts, is highly sensitivity to the electronic environment of a nucleus. One can monitor perturbations in the chemical shift caused by a change through noncovalent interactions with binding partners and thus identify interaction surfaces in protein complexes. Usually a series of ^{1}H-^{15}N HSQC experiments are recorded in which increasing amounts of a binding partner are added to the ^{15}N-labeled protein. Upon addition of the binding partner, some peak chemical shifts will be perturbed. Usually, these belong to amino acids close to the interaction surface.
7. The OP9 cells are adherent and are dissociated using a solution of 0.05 % Trypsin-EDTA.
8. The B cells represent between 20 and 25 % of the BM, and the large pre-B cell subset only 5 % of the B cells. In order to increase the efficiency of the sorting, a pre-enrichment step is

performed on the B cells. Although not essential, this step is highly recommended.

9. Several systems for magnetic separation are commercially available. We used the CD45R MicroBeads and the AutoMacs system (Miltenyi) and strictly followed the recommendations of the manufacturer. From 300×10^6 BM cells, we generally recover 50×10^6 cells from which more than 90 % are $CD45R^+$.
10. When using multiple fluorochromes, overlaps between the emission spectrum of consecutive fluorochromes are observed. This phenomenon called "spillover" can be corrected by applying compensations, which will remove the signal from a given fluorochrome from the neighboring channels. In that purpose, each fluorochrome used has first to be acquired individually. For the acquisition of the single-stained controls, the calculation of the compensations and then the acquisition of the samples on the cytometer, refer to the manufacturer's instructions. For the emission signal of the different fluorochromes, display them with a log scale. For the size (Forward Scatter, FSC) and the granulosity (Side Scatter, SSC) of the cells, use a linear scale. Save the FSC-A (Area), FSC-H (Height), FSC-W (Width), and the SSC-A, SSC-H, SSC-W parameters. The acquisition and the analysis can be performed by using only The FSC-A and SSC-A (or FSC-H and SSC-H) but the use of all the parameters allows removing cellular doublets and therefore improves the quality of the data.
11. In the experiment proposed, the λ5-UR is added in the medium to compete out the interaction between the pre-BCR and GAL1 and thus demonstrate the importance of this interaction for the pre-BCR relocalization. The amino acids of λ5-UR identified by NMR as being involved in the interaction with GAL1 can be targeted to produce a mutant protein. The use of mutants in the cocultures would allow determining which of the interacting residues are crucial in the process of relocalization.
12. In order to avoid disruption of the interactions set-up between stromal cells and pre-B cells, the medium and solutions have to be added and removed very gently.
13. In order to lift easily the glass coverslips from the bottom of the wells of the 24-well plates, use a 25G needle from which you bend the tip to form a hook. Lift the coverslip with the hook and catch it with pliers. To perform the immunofluorescence staining, use a black box in which you put wet towel paper on the bottom to be in a humid atmosphere. Saturation and staining are performed by putting the drops on parafilm into the black box.
14. Remove the excess PBS remaining on the coverslips after the washes by taping them vertically on a towel paper.

15. The Mowiol 4-88 is an anti-fade mounting medium, which decreases the photobleaching of the fluorophores. DABCO, another anti-fade agent, further improves the anti-fade effect.
16. No alteration in BM B-cell subpopulations can be observed in adult GAL1$^{-/-}$ compared to WT mice [3]. As redundancies exist between galectin family members for the interaction with the pre-BCR, we have made the assumption that the influence of GAL1 on pre-B cell differentiation could be visualized in the case of de novo differentiation. In that purpose, the cytostatic agent hydroxyurea was injected to mice to kill BM differentiating B cells which are in a high cycling state. Two days after the treatment, the only B cell subsets observed are the earliest pre pro-B and pro-B cells [3].
17. For 6- to 12-week old C57Bl/6 mice, we typically get 60×10^6 cells from the rear legs. Seven days after hydroxyurea injection, we recover about 25×10^6 cells.
18. If the samples are not processed immediately with the cytometer, resuspend them in 150 μl of FACS buffer, and at the last moment, add 150 μl of FACS buffer containing 1/10,000 of Sytox Blue.

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Chapter 12

Detection of Phosphatidylserine Exposure on Leukocytes Following Treatment with Human Galectins

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Abstract

Cellular turnover represents a fundamental aspect of immunological homeostasis. While many factors appear to regulate leukocyte removal during inflammatory resolution, recent studies suggest that members of the galectin family play a unique role in orchestrating this process. Unlike cellular removal through apoptotic cell death, several members of the galectin family induce surface expression of phosphatidylserine (PS), a phagocytic marker on cells undergoing apoptosis, in the absence of cell death. However, similar to PS on cells undergoing apoptosis, galectin-induced PS exposure sensitizes cells to phagocytic removal. As galectins appear to prepare cells for phagocytic removal without actually inducing apoptotic cell death, this process has recently been coined preaparesis. Given the unique characteristics of galectin-induced PS exposure in the context of preaparesis, we will examine important considerations when evaluating the potential impact of different galectin family members on PS exposure and cell viability.

Key words Galectin, Phosphatidylserine (PS), Inflammatory resolution, Leukocyte turnover, Preaparesis

1 Introduction

Appropriate removal of leukocytes during inflammatory resolution represents a fundamental process in immunological homeostasis [1, 2]. Although many factors, including members of the TNF family [3], appear to regulate this process, recent studies suggest that several members of the galectin family also regulate leukocyte turnover [4]. While most immunoregulatory factors induce leukocyte removal by initiating apoptotic cell death, galectins possess a unique capacity to trigger phagocytic removal in the absence of apoptosis [5–7]. Galectin engagement of neutrophil ligands induces surface expression of phosphatidylserine (PS), similar to cells undergoing apoptotic cell death [8]. However, unlike apoptosis, galectin-induced PS exposure occurs in the conspicuous absence of cell death [5–7, 9, 10]. While galectins fail to induce

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apoptotic cell death in neutrophils, galectin-induced PS exposure maintains the ability to sensitize cells to phagocytic removal [7]. Thus, galectins possess a unique ability to prepare cells for phagocytic removal without inducing cell death, a process recently termed preaparesis [6].

During cellular turnover, apoptotic cell death often provides a distinct pathway for enabling cell removal without inducing significant inflammation [1, 2]. This largely reflects the ability of cells undergoing apoptosis to maintain membrane integrity prior to successful phagocytic removal. However, once an apoptotic program is engaged, cells only maintain membrane integrity for a limited time before membrane integrity becomes compromised and late apoptosis/necrosis occurs [1, 2]. In the setting of acute inflammation, not only are inflammatory signals already present, but the number of neutrophils often far exceeds the number of phagocytic cells needed for rapid removal during inflammatory resolution [11]. As a result, induction of apoptosis in neutrophils would be predicted to result in a significant number of late apoptotic events prior to efficient phagocytic removal, which may actually increase inflammatory stimuli [11, 12]. Furthermore, given the inflammatory environment in which neutrophils often reside, the likelihood of maintaining membrane integrity in this setting is also reduced [12, 13]. Consistent with this, previous studies suggested that neutrophils evolved a unique mechanism of turnover that occurs independent of apoptosis [11, 14, 15]. The ability of galectins to induce PS exposure in the absence of cell death, while retaining the ability to sensitize these cells for phagocytic removal, likely enables neutrophils to maintain membrane integrity until successfully phagocytosed.

As neutrophils do not typically utilize the same level of directed immunological activity of T cells and other cells involved in adaptive immunity, the ability of galectins to induce preaparesis in neutrophils, while typically inducing apoptosis in T cells [6, 16], may in part reflect engagement of unique pathways not only important in turnover but also important in the spatial and temporal regulation of neutrophil function. Galectins possess a unique sensitivity to oxidative or proteolytic inactivation once outside the cell [7, 9, 17–19]. As a result, galectin released from damaged tissue at the time of an initial inflammatory insult likely undergoes inactivation prior to significant neutrophil accumulation. However, as neutrophils begin to encroach on viable tissue following the removal of necrotic debris, release of reduced, and therefore active, galectin likely results in galectin engagement and induction of preaparesis in neutrophils, thereby facilitating macrophage-mediated removal (Fig. 1) [9, 20, 21]. In contrast, as T cells often do not accumulate at the same rate or magnitude as neutrophils and also display a targeted approach to their activity [22, 23], apoptotic cell death is likely sufficient to induce cellular removal without causing deleterious consequences in the setting of inflammation.

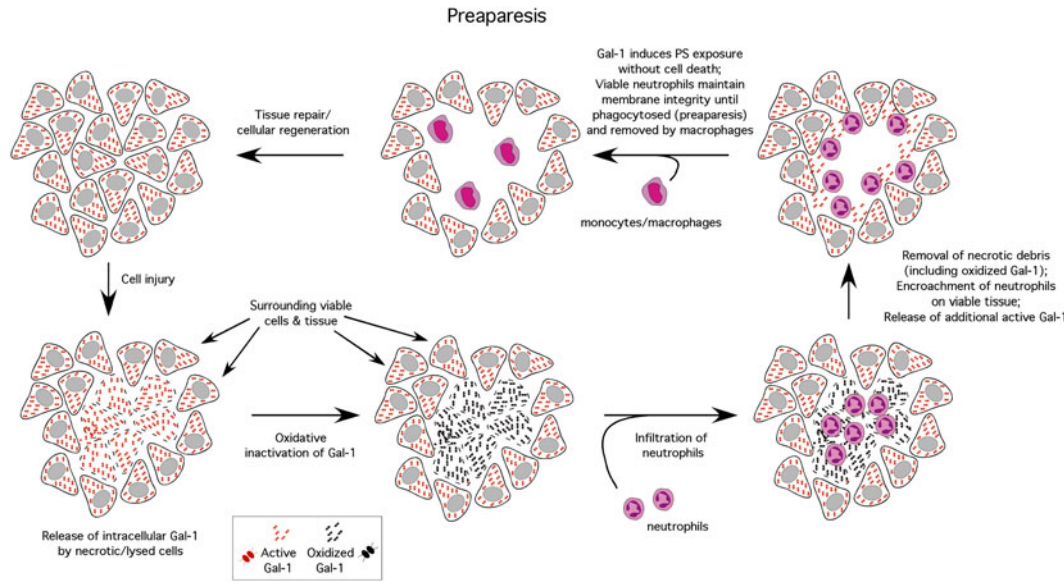

Fig. 1 Galectins may contribute to the spatial and temporal regulation of neutrophil turnover. Intracellular galectin remains reduced and active. However, following cellular injury, intracellular galectin becomes exposed to the extracellular oxidizing environment, oxidizes and becomes inactive. Following galectin inactivation, neutrophils infiltrate, which allows for neutralization of potential pathogens and removal of necrotic tissue. During this inflammatory episode, most of the galectin released during the primary injury likely becomes oxidized, preventing galectins from inhibiting a productive inflammatory response. Following removal of necrotic tissue and removal of pathogens, encroachment of neutrophils on surrounding viable tissue results in cellular damage and release of reduced and therefore active galectin. Galectin engages neutrophil receptors and induces PS exposure without altering cellular viability, a process termed preaparesis, which allows neutrophils to maintain membrane integrity until successfully phagocytosed

Given the unique sensitivity of galectins to oxidative inactivation coupled with their unprecedented ability to induce leukocyte turnover independent of cell death [7, 9, 17], examination of the potential impact of galectins on cellular viability requires careful analysis of galectin activity and cellular function. In this chapter, we will describe methods used to examine the potential impact of galectins on cellular viability and PS exposure, including potential pitfalls commonly associated with these approaches.

2 Materials

2.1 Neutrophil Isolation

1. Dextran (6 % dextran 70 in 0.9 % Sodium Chloride injection USP—Braun Medical Inc.).
2. 0.2 % NaCl.
3. 1.6 % NaCl.
4. Hanks' Balanced Salts Solution (HBSS) (standard with Mg and Ca) (HyClone).

5. human serum albumin (HSA) (Fisher scientific).
6. Ficoll (GE Healthcare).
7. Butterfly needle (BD).
8. 60 mL syringe (Cole-Parmer).
9. 15 mL Falcon tube (BD).
10. 50 mL Falcon tube (BD).
11. Wright-Giemsa stain (Sigma-Aldrich).
12. fMLP (Sigma-Aldrich).

2.2 Cell Culture

1. HL60 cells (ATCC CCL240).
2. RPMI (Gibco).
3. FBS (Gibco, Life Technologies).
4. Glutamine (Life Technologies).
5. Penicillin/streptomycin (Sigma-Aldrich).
6. Supplemented RPMI media: RPMI with 10 % FBS, 1 % glutamine, 1 % penicillin/streptomycin.

2.3 Galectin Preparation and Incubation with Cells

1. Recombinant galectin.
2. PBS standard pH 7.4 (HyClone).
3. β-Mercaptoethanol (βME) (Fisher).
4. PD-10 (or equivalent) small gel filtration column (GE Healthcare).
5. RPMI media (Gibco).
6. Lactose (Fisher).
7. Thiodigalactoside (TDG) (Carbosynth).
8. 0.1 M lactose in HBSS.
9. Sterile 48-well tissue culture plate.

2.4 Annexin V Staining of Galectin-Treated Cells

1. HEPES/NaOH.
2. NaCl.
3. $CaCl_2$.
4. Hanks' Balanced Salts Solution (HBSS) (standard with Mg and Ca) (HyClone).
5. Annexin V, Alexa Fluor 488 (Life Technologies).
6. Propidium iodide (PI) (Life Technologies).

Prepared Buffers

7. Annexin V staining buffer: 10 mM HEPES/NaOH, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4.
8. Annexin V and propidium iodide (PI) staining solution: Add 2 μL of Annexin V-FITC conjugate and PI (final concentration of 1 μg/mL) for every 100 μL of Annexin V staining buffer (*see* **Notes 1** and **2**).

3 Methods

3.1 Isolation of Human Neutrophils from Whole Blood

1. Using a 60 mL syringe, draw 300 μL of heparin (1,000 U/mL) (*see* **Note 3**).
2. Draw 30 mLs of whole blood into this 60 ml syringe using a butterfly needle by employing standard phlebotomy procedures (*see* **Note 4**).
3. Carefully rotate capped syringe in order to adequately mix heparin with blood to prevent clotting.
4. Draw 15 mLs of dextran into the whole blood-heparin mixture (*see* **Note 5**).
5. Carefully mix the dextran solution with the whole blood solution.
6. Affix the syringe with the opening facing upright against a wall or other support in a sterile hood. The dextran/whole blood solution will begin to separate. The top layer, which appears to become devoid of red blood cells (RBCs), is the layer that contains neutrophils.
7. At 30 min, 45 min, and 1 h after affixing the syringe in the upright position, remove this top layer (*see* **Note 6**).
8. Once the top layer of solution is completely removed, place this solution in a 50 mL sterile tube and centrifuge for 7 min at 600 × *g* at RT (*see* **Note 7)**.
9. After centrifugation, discard the supernatant (*see* **Note 8**).
10. Resuspend the pellet in 25 mL of a 0.2 % NaCl solution, and incubate at RT for 20 s (*see* **Note 9**).
11. Immediately add 25 mL 1.6 % NaCl and mix well.
12. Place cells in a centrifuge for 7 min at 600 × *g* at RT.
13. Discard supernatant and resuspend pellet in 50 mL of HBSS with 0.5 % human serum albumin.
14. Place cells in a centrifuge for 7 min at 600 × *g* at RT.
15. Add 6 mL Ficoll to a sterile 15 mL Falcon tube.
16. Resuspend pellet isolated in **step 14** in 6 mL of HBSS with 0.5 % human serum albumin.
17. Place the leukocyte solution on top of the Ficoll by tilting the tube at a 45° angle and carefully layer the leukocyte solution on top of the Ficoll (*see* **Note 10**).
18. Centrifuge the layered leukocyte/Ficoll solution for 30 min at 600 × *g* at RT (*see* **Note 11**).
19. Remove the middle band (this layer primarily consists of lymphocytes).
20. Remove the pellet (this primarily consists of neutrophils) and resuspend in 12 mL HBSS with 0.5 % human serum albumin.

21. Centrifuge cells for 7 min at $600 \times g$ at RT.
22. Wash two more times in 12 mL HBSS with 0.5 % human serum albumin by centrifugation for 7 min at $600 \times g$ at RT.
23. Examine the cells for neutrophil content by staining with Wright-Giemsa stain per the manufacturer's protocol, followed by morphological analysis of neutrophils (*see* **Note 12**).
24. Following the final wash, cells can be resuspended in supplemented RPMI.
25. Count the number of cells per mL using a standard hemocytometer.
26. For activation, resuspend the cells in RPMI at 1×10^6/mL, and add 1 μg of fMLP for every mL. Incubate at 37 °C for 15 min. Following incubation, wash cells in supplemented RPMI by centrifuging for 7 min at $600 \times g$ at RT twice.

3.2 Maintaining Cell Culture of Non-adherent HL60 Cells

1. HL60 cells should be purchased from ATCC.
2. Frozen aliquots of cells should be prepared and cultured according the recommended ATCC protocol (*see* **Note 13**).
3. Culture cells in supplemented RPMI at 37 °C in 5 % CO_2.
4. Typically cells are maintained at a concentration of 2×10^5 cells/mL (*see* **Note 14**).

3.3 Galectin Preparation and Incubation with Cells

1. Thaw frozen vial of recombinant galectin by placing on ice (4 °C) (*see* **Note 15**).
2. Re-equilibrate a PD-10 column with 5 column volumes of cold PBS.
3. To remove lactose and βME from galectin, add 1 mL of recombinant galectin solution to the re-equilibrated PD-10 column (*see* **Note 16**).
4. Collect 0.5 mL fractions from the PD-10 column following the addition of recombinant galectin.
5. Once the recombinant galectin solution has completely penetrated the column, add 2 mL of cold PBS.
6. Continue collecting 0.5 mL fractions from the PD-10 column following the addition of cold PBS, adding additional PBS as needed to elute all galectin from the column and to prevent the column from drying.
7. Examine protein content of each fraction by measuring the OD 280 nm. This is typically done by diluting each fraction tenfold (i.e., 10 μL of each fraction in 90 μL PBS) followed by examination of the OD 280 nm on a standard spectrophotometer (*see* **Note 17**).
8. Place pooled recombinant galectin on ice until ready to use.

9. Prepare galectins in PBS at five times the desired final concentration.
10. Pipet 200 μL of cells in supplemented RPMI media at a concentration of 1×10^6 cells/mL into each well of a 48-well plate (*see* **Note 18**).
11. Pre-warm galectin to 37 °C and add 50 μL of recombinant galectin solution or PBS control to each well (*see* **Note 19**).
12. Incubate at 37 °C for the indicated time (*see* **Note 20**).

3.4 Staining Cells with Annexin V

1. At the end of the incubation time point, add 50 μL of 120 mM lactose in RPMI to remove bound galectin and disengage cells (*see* **Note 21**).
2. Incubate for 5 min at 37 °C.
3. Examine cells for agglutination following incubation (*see* **Note 22**).
4. Once cells appear to be sufficiently disengaged, wash cells three times in HBSS by centrifuging cells at $600 \times g$ for 7 min.
5. Place cells on ice.
6. Resuspend cells in 100 μL Annexin V and PI staining solution precooled to 4 °C.
7. Incubate at 4 °C for 15 min in the dark.
8. Add 400 μL of Annexin V staining buffer precooled at 4 °C to each sample.
9. Analyze cells by flow cytometry (*see* **Notes 23** and **24**).

4 Notes

1. Following buffer preparation, cool buffers to 4 °C prior to staining the cells.
2. The amount of Annexin V used may vary based on the manufacturer of the product.
3. Typically 100 μL of heparin stock solution (1,000 U/mL) is used for every 10 mL of whole blood drawn.
4. Institutional review board (IRB) or equivalent approval must be obtained prior to drawing blood from healthy human volunteers.
5. The total amount of dextran added typically equals half the total starting volume of whole blood. This is often done by drawing the dextran solution into a separate container followed by injecting the dextran solution into the whole blood-heparin mixture to prevent contamination of the dextran stock.

6. The 30 and 45 min interval removal of the top layer appears to facilitate additional separation. However, these steps are not absolutely necessary.
7. Although protocols can differ, we found that placing neutrophils on ice, followed by rewarming, can result in significant neutrophil activation. As a result, a concerted effort is made to avoid cold temperatures during neutrophil isolation. All procedures should be done under a sterile laminar flow hood.
8. The pellet will look red due to significant red blood cell contamination.
9. This should lyse the red blood cells. As this is a hypotonic RBC lysis method, care should be taken to not over-incubate the cells.
10. Care should be taken to not allow the leukocyte HBSS solution to penetrate the Ficoll solution.
11. Do not use the brakes on the centrifuge as this can disrupt the desired interfaces generated during centrifugation.
12. Using this protocol typically >90 % of the isolated cells are neutrophils.
13. As HL60 cells will differentiate in the presence of dimethyl sulfoxide (DMSO) and DMSO is a commonly employed cryopreservant, care should be taken to remove DMSO as soon as possible following initiation of the HL60 culture.
14. Care should be taken to not allow cells to exceed 1×10^6 cells/mL. Cells can be counted using a standard hemocytometer. Equally important, the media should be regularly changed to allow cells to remain in a physiologic pH. Using this approach, typically >90 % of the cells remain viable at baseline. When cells are allowed to become overcrowded and/or the media is changed infrequently, cellular viability suffers and the overall outcome of galectin-HL60 cell interactions can be altered.
15. All galectin preparation should be done under a sterile laminar flow hood. Potential lipopolysaccharide (LPS) contamination should be assessed prior to preparing galectin for incubation with cells. This can be done using the commercially available limulus amebocyte lysate (LAL) assay (Pierce). If significant LPS contamination is noted, LPS removal can be achieved by passing the endotoxin-contaminated recombinant galectin sample over a polymyxin B column (Sigma-Aldrich) according to the manufacturer's protocol. Repeat removal should be employed until LPS is undetectable.
16. Several galectins display unique sensitivity to oxidative inactivation [5, 9]. This should be considered when assessing the potential ability of an individual galectin to induce PS exposure

when incubated with a given cell. Methods to alkylate galectin-1 and protect it from oxidative inactivation have been developed [9]. However, it should be noted that for most galectins, oxidative inactivation is a gradual process and that biological activity of these proteins can be assessed in the absence of reducing conditions or other chemical modifications if care is taken to use them immediately following removal of βME (or other reducing agent) and ligand (*see* Fig. 2 for impact of DTT on cellular viability and sensitivity to Gal-1-induced PS exposure).

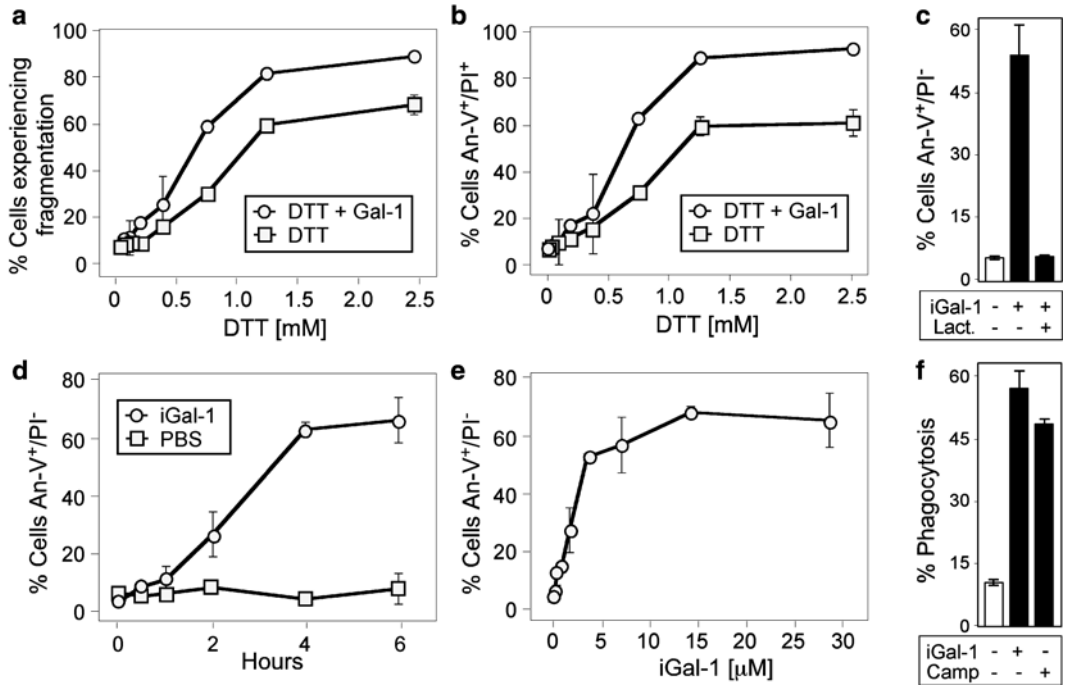

Fig. 2 DTT sensitizes cells to Gal-1-induced apoptosis. (**a**) Promyelocytic HL60 cells (cells) were incubated with PBS, 10 μM Gal-1, or various concentrations of DTT with or without 10 μ M Gal-1 as indicated for 9 h followed by detection for cellular fragmentation as indicated by changes in forward (FSC) and side scatter (SSC) profiles of cells. (**b**) Cells were incubated with PBS, 10 μM Gal-1, or various concentrations of DTT with or without 10 μ M Gal-1 as indicated for 9 h followed by detection for cell death by PS exposure and membrane integrity loss by An-V-FITC and PI staining. (**c**) Cells were incubated with PBS, 10 μM iodoacetamide-treated Gal-1 (iGal-1), or 10 μM iGal-1 with 20 mM lactose followed by detection for PS exposure by An-V-FITC staining and PI exclusion. (**d**) Cells were incubated with PBS or 10 μM iGal-1 for the indicated time followed by detection for PS exposure by An-V-FITC staining and PI exclusion. (**e**) Cells were incubated with PBS or the indicated concentration of iGal-1 for 8 h followed by detection for PS exposure by An-V-FITC staining and PI exclusion. (**f**) Cells were incubated with PBS, 10 μM iGal-1, or 10 μM Camp for 8 h followed by incubation of peritoneal macrophages for 1 h and microscopic examination of phagocytosis. This research was originally published in Molecular Biology of the Cell. Stowell SR, Karmakar S, Arthur CM, Ju T, Rodrigues LC, Riul TB, Dias-Baruffi M, Miner J, McEver RP, Cummings RD. Galectin-1 induces reversible phosphatidylserine exposure at the plasma membrane. 2009 Mar;20(5):1408–18

17. To extrapolate the protein concentration from the OD 280 nm values, we use the extinction coefficient for the particular galectin being examined to calculate actual concentration in mg/mL. The following Web sites http://www.basic.northwestern.edu/biotools/proteincalc.html and http://web.expasy.org/protparam/protparam-doc.html offer explanation and assistance in calculating the extinction coefficient and using this calculation to determine the actual concentration of a given protein in mg/mL, including caveats as to how these numbers may differ from the actual number. As methods of calculating the extinction coefficient only provide estimates, alternative approaches can be used to empirically determine these values. These include using a Bradford assay to calculate protein concentration or simply re-equilibrating the recombinant protein directly into water, lyophilizing, weighing, and then resuspending in the buffer of choice followed by empirically determining the extinction coefficients for a particular galectin family member.
18. When counting cells, use trypan blue (Sigma-Aldrich) to determine the percent of viable and nonviable cells. If the nonviable cell count is higher than 10 %, these cells are not sufficiently healthy to use in this assay.
19. Control wells containing galectin along with a final concentration of 20 mM lactose or 20 mM TDG (both hapten inhibitors of galectin-carbohydrate binding) should be included as controls to determine whether positive signaling events are likely due to galectin-carbohydrate interactions. Additionally, it is critical to incorporate an apoptosis-positive control. Typically Fas for neutrophils and camptothecin for HL60 cells can be used as an apoptosis-positive control, although a variety of proapoptotic agents can be employed.
20. PS exposure typically peaks between 2 and 4 h. A range of galectin concentrations and times should be employed to determine the optimal time for PS exposure to be realized. To determine whether PS exposure occurs in the absence of cell death, later time points, including 8, 12, and even 24 h, are recommended to determine whether PS exposure continues to occur in the absence of apoptosis using additional methods in parallel that are capable of detecting more definitive markers of apoptotic cell death (examination of DNA fragmentation, changes in mitochondrial potential, cellular fragmentation, etc.).
21. Unlike the use of antibodies for staining cell surface CD markers, galectins typically agglutinate cells in suspension. Agglutinated cells not only possess the ability to interfere with and potentially occlude appropriate fluid flow during flow cytometric analysis, but cellular fragmentation of agglutinated cells during flow cytometric analysis can result in false-positive results

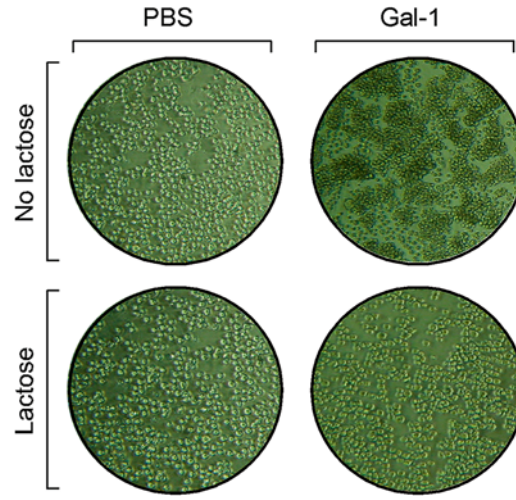

Fig. 3 Incubation of leukocytes with galectins induces agglutination. Promyelocytic HL60 cells were incubated with PBS or 10 μM galectin-1 (Gal-1) for 4 h followed by microscopic evaluation for agglutination (no lactose). Following incubation with PBS or Gal-1, lactose was added at a final concentration of 20 mM lactose for 15 min at 37 °C followed by microscopic evaluation as indicated

(*see* Figs. 3, 4, and 5). Although most antibodies used for flow cytometric examination of antigen reactivity at saturating concentrations do not induce agglutination [24–26], antibodies capable of inducing agglutination can induce similar fragmentation and PS positivity [27].

22. Each galectin family member has different affinity for lactose and leukocyte ligands. As a result, the concentration of lactose and the duration of incubation needs to be empirically determined for each galectin and cell population being analyzed. Thiodigalactoside (TDG) can be a more efficient inhibitor of galectins and therefore can occasionally be used when lactose appears to be insufficient to induce galectin release and disengagement of galectin-induced agglutinated aggregates.
23. When analyzing cells undergoing apoptosis, it is important to consider potential changes in the forward and side scatter profiles that may occur. The changes can be diagnostic of cells undergoing apoptosis, but may be inadvertently gated out during data acquisition and analysis. As a result, care should be taken when analyzing cells by flow cytometry to ensure that apoptosis-/late apoptosis-positive events are not gated out during data acquisition or analysis (Fig. 6).
24. Keep cells on ice and analyze as soon as possible (typically <1 h following completion of staining to avoid potential changes in viability associated simply with prolonged incubation in Annexin V staining buffer.) Fixation of cells can result in artificial exposure of PS and should be avoided.

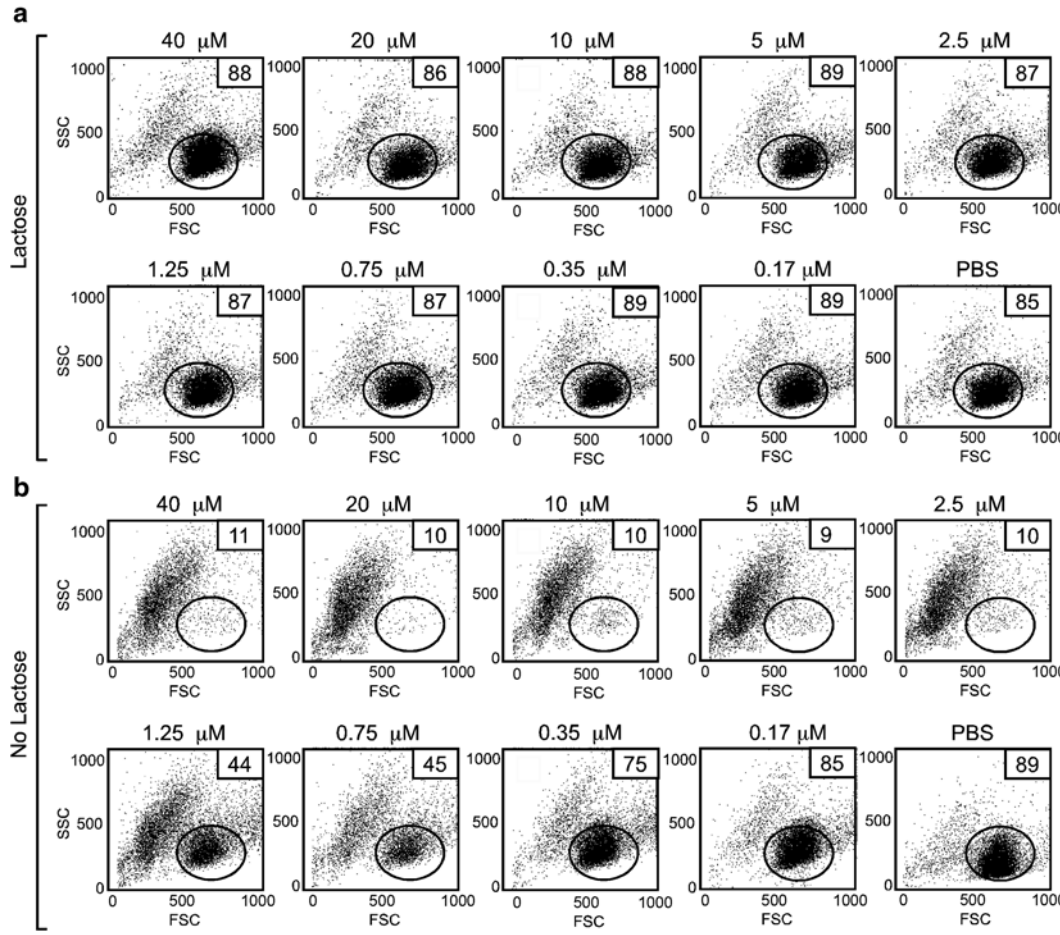

Fig. 4 Failure to disengage cells can result in significant changes in the forward and side scatter profiles following flow cytometric analysis. Promyelocytic HL60 cells were incubated at the indicated concentrations of galectin-1 (Gal-1) for 4 h followed by addition of PBS (no lactose) or lactose at a final concentration of 20 mM (lactose) and incubated for an additional 15 min. Following incubation, cells were washed and analyzed for potential alterations in the forward and side scatter profiles as indicated. Gate = % of cells experiencing no fragmentation. The percent of cells in the gate for each condition is shown

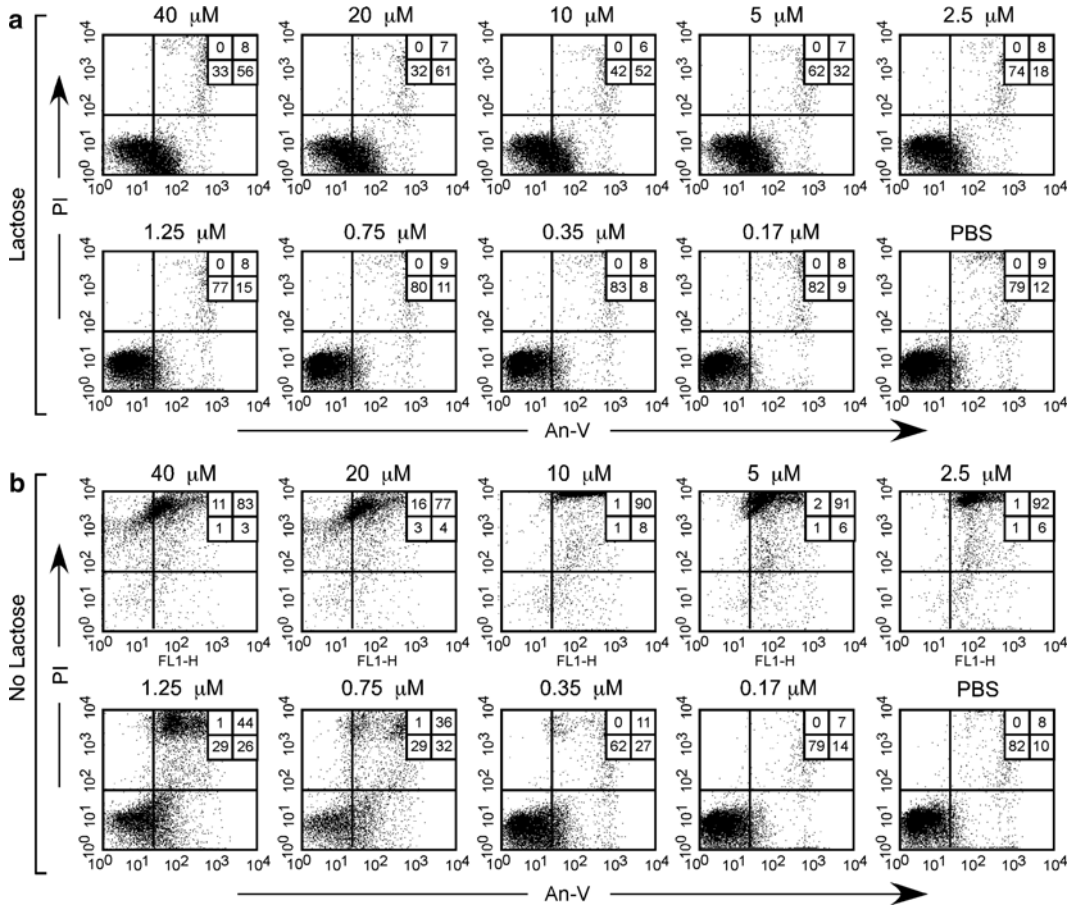

Fig. 5 Failure to disengage cells can result in significant Annexin V and propidium iodide double-positive staining. Promyelocytic HL60 cells were incubated at the indicated concentrations of galectin-1 (Gal-1) for 4 h followed by addition of PBS (no lactose) or lactose at a final concentration of 20 mM (lactose) and incubated for an additional 15 min. Following incubation, cells were washed and stained with Annexin V (An-V) and propidium iodide (PI) as outlined. The percent of cells in each quadrant is shown

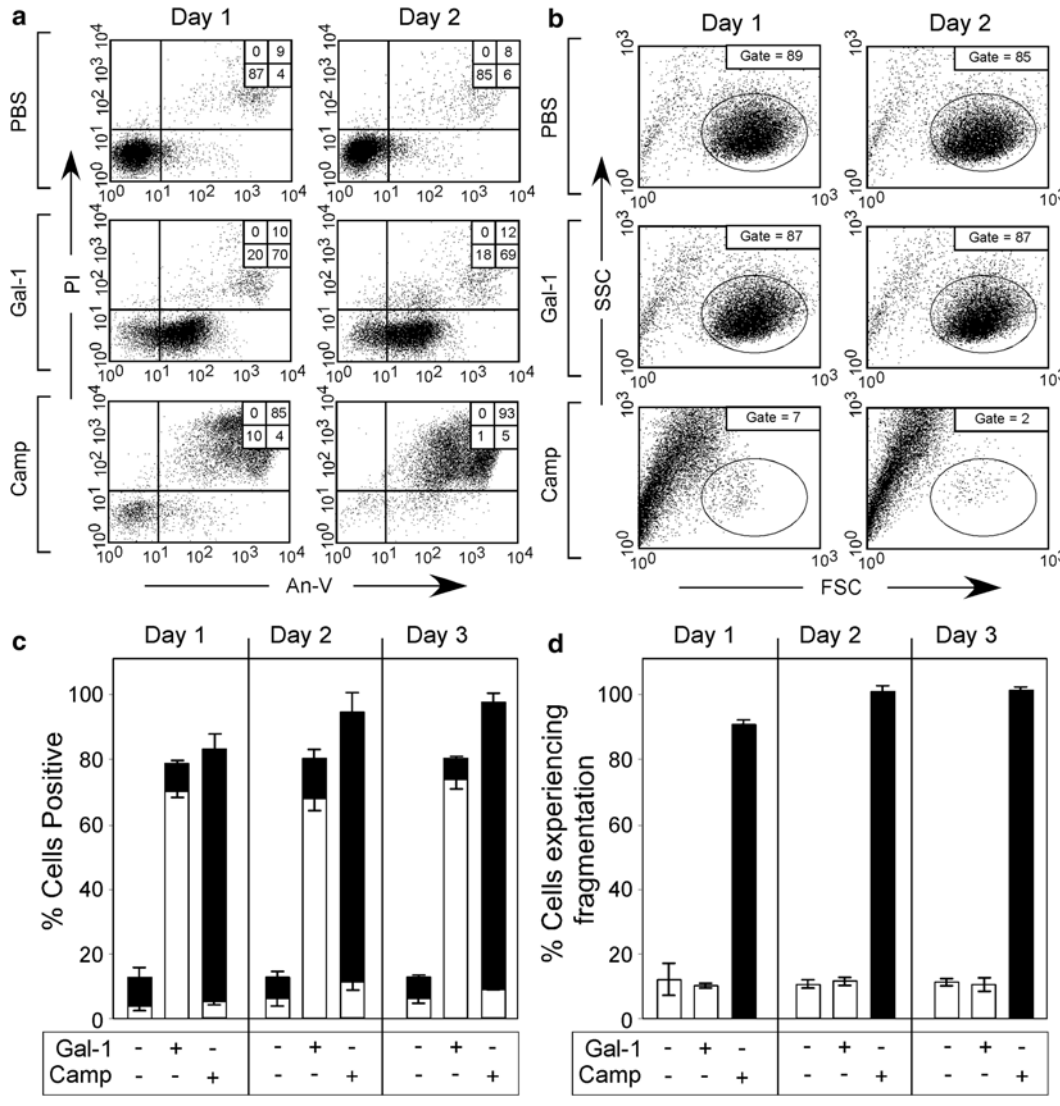

Fig. 6 Gal-1 induces continuous PS exposure in the absence of cellular fragmentation. (**a**) Cells were incubated with PBS, 10 μM iodoacetamide-alkylated Gal-1 (iGal-1), or 10 μM Camp for 1 or 2 d as indicated, followed by detection for PS exposure by An-V-FITC staining and PI exclusion. (**b**) Cells were incubated with PBS, 10 μM iGal-1, or 10 μM Camp for 1 or 2 d as indicated, followed by examination for cellular fragmentation as indicated by changes in forward (FSC) and side scatter (SSC) profiles of cells. Gate = % of cells experiencing no fragmentation. (**c**) Quantification of cells treated in (**a**). *White bars* = % An-V+, PI–; *black bars* = % An-V+, PI+. (**d**) Quantification of cells treated in (**b**). This research was originally published in Molecular Biology of the Cell. Stowell SR, Karmakar S, Arthur CM, Ju T, Rodrigues LC, Riul TB, Dias-Baruffi M, Miner J, McEver RP, Cummings RD. Galectin-1 induces reversible phosphatidylserine exposure at the plasma membrane. 2009 Mar;20(5):1408–18

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Chapter 13

Examination of Galectins in Phagocytosis

Huan-Yuan Chen, I-Chun Weng, Chi-Shan Li, Lei Wan, and Fu-Tong Liu

Abstract

Galectins, a family of β-galactoside-binding proteins, are expressed in many different phagocytic leukocytes (granulocytes, monocytes, and macrophages). A number of family members have been shown to play an important role in ingestion of particles (phagocytosis), thus contributing to clearance of damaged cells and host defense against pathogens. Here we describe procedures for analysis of the roles of galectins in phagocytosis by using galectin-3 as an example. We emphasize the function of endogenous galectin-3 as determined by comparison of phagocytosis by macrophages from galectin-3 knockout mice and wild-type mice. We focus on the role of galectin-3 in phagocytosis of pathogens and Fcγ receptor-mediated phagocytosis of opsonized cells and particles.

Key words Galectin, Phagocytosis, Opsonization, Macrophage

1 Introduction

Phagocytosis is defined by ingestion of particles (usually larger than 0.5 μm in diameter) by cells that results in invagination of the plasma membrane, followed by formation of phagosomes. Examples of particles ingested by phagocytes include dead cells (apoptotic bodies), pathogens, and inert beads (reviewed in [1]). Ingestion of dead cells results in digestion and clearance of self and unwanted cells/tissues which is critical in maintenance of tissue homeostasis. On the other hand, uptake of foreign pathogens and subsequent destruction/digestion of these microorganisms represents the protective role of phagocytosis. Phagocytosis assays can be used to identify the receptors involved in the recognition and ingestion of particles as well as elucidate the cellular mechanisms. The ingestion of particles by phagocytes can be through a nonspecific manner or via specific receptors. For example, Fcγ receptors on phagocytes can bind to IgG-coated particles and mediate the ingestion (opsonization).

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Galectins are a β-galactoside-binding lectin family; some of the members are highly expressed by phagocytic leukocytes, such as macrophages and granulocytes. Galectin-3 was also reported to augment neutrophil phagocytosis of bacteria [2] and fungi such as *Candida albicans* [3]. Similarly, recombinant galectin-1 can enhance FcγRI expression on human monocytes and FcγRI-dependent phagocytosis [4]. In addition, galectin-1 can induce cell surface exposure of phosphatidylserine (PS) in neutrophils, thus facilitating phagocytosis of neutrophils by macrophages [5]. Of particular note is that the above-mentioned functions of galectins are mainly investigated with exogenously added galectins, which may not reveal the functions of endogenous galectins. Our work focuses on the roles of endogenous galectins in phagocytosis. We found that galectin-3 plays an important role in phagocytosis of opsonized red blood cells (RBC) by macrophages and it translocates to the cytosolic site of the phagosomes [6]. Galectin-9 was also found in the phagosomes as revealed by proteomic analysis [7]. Our preliminary data showed that galectin-9 is involved in phagocytosis by human monocytes (unpublished data).

This chapter describes assays to study the functions of galectin-3 in phagocytosis. Detailed procedures are provided for the Fcγ receptor-mediated phagocytosis of opsonized sheep red blood cells (SRBC) and inert latex beads (Subheading 3.1). The roles of galectin-3 in phagocytosis of *Listeria monocytogenes* by macrophages are also described (Subheading 3.2).

2 Materials

2.1 Fcγ Receptor-Mediated Phagocytosis

2.1.1 Phagocytosis of IgG-Opsonized Sheep Red Blood Cells (SRBC)

1. Wild-type bone marrow-derived macrophages (WT BMM) and galectin-3 knockout bone marrow-derived macrophages (Gal3KO BMM) (*see* ref. 6).
2. Culture medium: RPMI1640 medium with 10 % fetal bovine serum (FBS), 100 U penicillin, and 100 μg/mL streptomycin.
3. Sheep red blood cells.
4. Rabbit polyclonal IgG anti-SRBC antibody.
5. ACK lysing buffer: 0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2 EDTA, pH 7.2.
6. Phosphate-buffered saline (PBS): 140 mM NaCl, 5 mM KCl, 8 mM NaH_2PO_4, and 2 mM KH_2PO_4, adjusted to pH 7.4.
7. 2.5 % (v/v) glutaraldehyde in PBS.
8. 1 % eosin solution.
9. 96-well flat-bottom tissue culture plates.
10. 15 mL conical plastic centrifuge tubes.
11. 8-channel pipettor and disposable reservoirs.

12. Hemocytometer.
13. Mini-rotator.
14. Refrigerated centrifuge.
15. Inverted microscope.
16. Digital camera.
17. Incubator at 37 °C and 5 % CO_2.

2.1.2 Phagocytosis of Inert Latex Beads

1. Latex beads (*see* **Note 1**).
2. Phosphate-buffered saline (PBS): 140 mM NaCl, 5 mM KCl, 8 mM NaH_2PO_4, and 2 mM KH_2PO_4, adjusted to pH 7.4.

Opsonization and Fluorescence Labeling

3. Total IgG.
4. Secondary antibody conjugated with fluorochrome.
5. DAPI or Hoechst 33342.
6. 25G needle.
7. Syringe.

Phagocytosis

1. Primary neutrophils, macrophages, or macrophage cell lines, such as RAW264.7 mouse macrophage cell line and THP-1 human monocytic leukemia cell line (*see* **Note 2**).
2. Latex beads prepared in Subheading "Opsonization and Fluorescence Labeling."
3. 24-well plate.
4. Incubator at 37 °C and 5 % CO_2.
5. Media: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum and 4.5 g/L of glucose, L-glutamine, and antibiotics.

Internal/External Particles Discrimination

1. Media: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum and 4.5 g/L of glucose, L-glutamine, and antibiotics.
2. Rhodamine labeled secondary antibody.
3. Phosphate-buffered saline (PBS): 140 mM NaCl, 5 mM KCl, 8 mM NaH_2PO_4, and 2 mM KH_2PO_4, adjusted to pH 7.4.

Cell Identification

1. 4 % paraformaldehyde.
2. Hoechst 33342 or DAPI.
3. Phosphate-buffered saline (PBS): 140 mM NaCl, 5 mM KCl, 8 mM NaH_2PO_4, and 2 mM KH_2PO_4, adjusted to pH 7.4.

Determination of the Phagocytic Index

1. Imaging processing software.
2. Fluorescence microscope.

2.2 Phagocytosis of Bacteria

2.2.1 Preparation and Phagocytes and Phagocytosis Assay

1. Monocytes/macrophages: Primary monocytes/macrophages (e.g., bone marrow-derived macrophages or peritoneal macrophages) and monocytic cell lines (e.g., RAW264.7 or J774A.1 cells).
2. Mid-log phase (OD600 = 0.4–0.7) bacterial culture (e.g., *Listeria monocytogenes* 10403S).
3. Complete RPMI medium: RPMI1640 supplemented with 20 mM HEPES, 1× nonessential amino acids (NEAA), and 10 % FBS.
4. Phosphate-buffered saline (PBS): 140 mM NaCl, 5 mM KCl, 8 mM NaH_2PO_4, and 2 mM KH_2PO_4, adjusted to pH 7.4.
5. 24-well tissue culture plates containing sterilized glass coverslips (12 mm diameter).

2.2.2 Double-Cycle Immunofluorescence Staining

1. 5 % casein blocking reagent (refer to Current Protocols in Immunology 18.13.23).
2. Antibody or antisera against the targeted bacteria (e.g., *Listeria* antisera, Denka Seiken).
3. Fluorochrome-conjugated secondary antibodies (e.g., Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 647-conjugated goat anti-rabbit IgG).
4. Washing buffer: PBS containing 1 % BSA.
5. Blocking/staining buffer: PBS containing 1 % BSA and 2.5 % casein.
6. Fixation buffer: 4 % paraformaldehyde in PBS.
7. Phosphate-buffered saline (PBS): 140 mM NaCl, 5 mM KCl, 8 mM NaH_2PO_4, and 2 mM KH_2PO_4, adjusted to pH 7.4.
8. Permeabilization buffer: PBS containing 1 % BSA, 2.5 % casein, and 0.05 % saponin.
9. Glass microscope slides.
10. Anti-fade mounting medium with DAPI (ProLong® Gold Antifade Reagent with DAPI, Molecular Probe).
11. Rhodamine-conjugated phalloidin.
12. Fluorescence microscope equipped with lasers and filter sets suitable for collecting fluorescence signals of 488, 555, and 647 nm.

2.3 Flow Cytometric Phagocytosis Assay

2.3.1 Label Bacteria with FITC

1. 1 mg/mL FITC (fluorescein isothiocyanate) isomer 1 (Sigma) in PBS, pH 8.0.
2. 1 % (v/v) glutaraldehyde in PBS.
3. 1 M glycine in PBS.

2.3.2 Initiation of Phagocytosis and Analysis with Flow Cytometry

1. FITC-labeled bacteria prepared in Subheading 3.2.2, **step 1**.
2. Adherent phagocytes in 24-well culture plates prepared in Subheading "Opsonization and Fluorescence Labeling."
3. Complete RPMI medium: RPMI1640 supplemented with 20 mM HEPES, 1× nonessential amino acids (NEAA), and 10 % FBS.
4. Phosphate-buffered saline (PBS): 140 mM NaCl, 5 mM KCl, 8 mM NaH_2PO_4, and 2 mM KH_2PO_4, adjusted to pH 7.4.
5. 0.02 % EDTA in PBS.
6. 1.25 mg/mL trypan blue in PBS.
7. 0.5 % trypsin/EDTA or nonenzymatic cell dissociation solution such as Cell Stripper (Cellgro).
8. 1 % FBS in PBS.
9. Polypropylene tube suitable for flow cytometry.
10. Flow cytometry.

3 Methods

3.1 Fcγ Receptor-Mediated Phagocytosis

3.1.1 Phagocytosis of IgG-Opsonized Sheep Red Blood Cells (SRBC)

1. Place wild-type bone marrow-derived macrophages (WT BMM) and galectin-3 knockout bone marrow-derived macrophages (Gal3KO BMM) in wells of 96-well plates (10^5 cells per well) and cultured at 37 °C in a CO_2 incubator overnight.
2. Dilute 5 mL of sheep whole blood with 10 mL of PBS and centrifuge the cells at 800×*g* for 10 min at room temperature.
3. Remove the supernatant and buffy coat layer, and wash SRBC with 15 mL of PBS twice (*see* **Notes 3** and **4**).
4. Count SRBC in a hemocytometer and adjust the cell number to 10^8/mL in PBS.
5. Mix 10^8/mL SRBC with rabbit polyclonal anti-SRBC antibody (subagglutination concentration, *see* **Note 5**) and incubate at room temperature for 30 min with gentle rotation on a mini-rotator.
6. Wash the opsonized SRBC twice with 10 mL of PBS and resuspend the opsonized SRBC into 10^8/mL in culture medium.
7. Place the opsonized SRBC on ice for later usage (*see* **Note 6**).
8. Take out the 96-well plates containing WT BMM and Gal3KO BMM from the incubator and place the 96-well plates on ice for 30 min.
9. Remove the medium from the plate and add 100 μL of ice-cold opsonized SRBC (10^8/mL).

10. Centrifuge the plates at 300 × *g* for 5 min at 4 °C to increase the contact between SRBC and macrophages.
11. Place the plates back into the 37 °C incubator and incubate the plates for different time periods (e.g., 0, 5, 10, 20, 40, and 60 min).
12. Remove the plates from the incubator and place on ice, after the indicated time periods.
13. Remove the cell medium and wash the cell monolayers with 200 μL of cold PBS followed by adding 100 μL of ACK lysis buffer and placing the plates on ice for 1 min to remove the SRBC not phagocytosed (*see* **Note 7**).
14. Remove the ACK lysis buffer and wash the cells with cold PBS again and fix cells by adding 100 μL of fresh cold 2.5 % glutaraldehyde for 15 min on ice.
15. After fixation, the fixed cells are washed twice with PBS and stain the cells with eosin for 5 min at room temperature.
16. Next, wash the cells with PBS three times and leave PBS in the wells.
17. Capture digital images under an inverted microscope.
18. Calculate the phagocytic index according to the following formula: Phagocytic index = (total number of engulfed cells/number of macrophages containing engulfed cells) × (number of macrophages containing engulfed cells/total number of counted macrophages) × 100.

3.1.2 Phagocytosis of Inert Latex Beads

Opsonization and Fluorescence Labeling (*See* **Note 8**)

1. Resuspend 40 μL of 10 % suspension of latex beads in 500 μL of PBS and pellet the beads by centrifugation for 30 s at 5,000 × *g* in a microcentrifuge, discard the supernatant, and resuspend the beads in 500 μL of fresh PBS.
2. Repeat **step 1** twice to wash the beads.
3. At the third wash, resuspend the beads in 190 μL of PBS and add 10 μL of IgG (stock concentration: 20 mg/mL) (*see* **Note 9**).
4. Mix gently and incubate the beads at room temperature for 60 min.
5. Wash the mixtures three times with PBS to remove unbounded antibody and resuspend the beads in 500 μL PBS.
6. Label the beads with fluorochrome for detection by fluorescence microscopy or flow cytometry by adding 5 μL of fluorochrome-conjugated (e.g., FITC) secondary antibody to 500 μL opsonized beads.
7. Mix gently and incubate at room temperature for 60 min.
8. Wash the mixtures four times with PBS to remove unbounded antibody and resuspend in 500 μL PBS (*see* **Note 10**).

Phagocytosis

1. Place cells in the wells of 24-well plates at 1×10^5 cells/well and incubate at 37 °C, 5 % CO_2 overnight.
2. Add 15 μL of the labeled opsonized bead suspension to each well.
3. Shake the plate to ensure even distribution of the beads.
4. Centrifuge the plates at $300 \times g$ for 1 min to sink the beads onto the cells. Alternatively, place the plate on ice for 10 min to allow the beads to slowly settle on the cells without being internalized. Wash the cells once with media prewarmed to 37 °C and proceed to the following step.
5. Incubate the plates at 37 °C for 15 min to allow proper phagocytosis process. Different incubation times should be optimized for different experiments.

Internal/External Particles Discrimination (*See* Notes 11 and 12)

1. Place the plates on ice to stop phagocytosis and wash the cells with ice-cold DMEM three times to remove unbound beads.
2. Stain the external beads with a secondary antibody labeled with a fluorochrome different from the one used in Subheading "Opsonization and Fluorescence Labeling" (e.g., Rhodamine labeled). Dilute the secondary antibody 100-fold in cold PBS and add 200 μL of diluted antibody to each well and incubate the cells on ice for 10 min.
3. Wash the cells five times with ice-cold PBS.

Cell Identification (*See* Note 13)

1. Remove supernatant from cultured cells and then add 500 μL of 4 % paraformaldehyde in PBS into each well and incubate at room temperature for 20 min to fix the cell.
2. Stain the cell nuclei with Hoechst 33342 (8 μg/mL) in PBS for 10 min (*see* **Note 8**) (DAPI can also be used to stain nuclei).
3. Remove the excess dye by washing two times with PBS.
4. Maintain the cells in PBS until analysis by fluorescence microscopy.

Determination of Phagocytic Index

1. After phagocytosis, external particle discrimination, and cell identification, the images of macrophages are acquired by fluorescence microscopy.
2. Quantitation can be performed by using image processing software or simply counting cells one by one.
3. The efficiency can be expressed as the phagocytic index, which is defined as the number of latex beads ingested by 100 macrophages:

$$\text{Phagocytic index} = \left(\text{total number of latex beads} - \text{external beads}\right) / 100 \text{ macrophages.}$$

3.2 Phagocytosis of Bacteria

Microscopic quantification of phagocytosis of bacteria by double-cycle immunofluorescence staining (*see* **Note 14**).

3.2.1 Preparation of Phagocytes and Phagocytosis Assay

1. The day before phagocytosis assay, seed macrophages at a density of $1.8–2 \times 10^5$ cells/mL in complete RPMI medium on sterile glass coverslips contained in 24-well cultures plates. It is best for the cells to reach ~75 % confluency at the time of experiment.
2. Measure optical density at 600 nm (OD600) of a mid-log phase bacteria culture with a spectrophotometer and estimate the bacteria number.
3. Transfer 10^9 bacteria to a new microcentrifuge tube. Sediment bacteria by centrifuging at maximum speed (~20,000 × *g*) for 1 min with a table-top centrifuge.
4. Discard supernatant and resuspend with 1 mL sterile PBS.
5. Repeat the wash by sedimentation and resuspension in sterile PBS.
6. Dilute bacteria with prechilled complete RPMI medium to the desired density so that the multiplicity of infection (MOI) will be 5 or 10 when added to the cells.
7. Place the 24-well culture plates with macrophages on ice.
8. Aspirate culture medium and add the prepared bacteria suspension to the cells (MOI = 5 or 10 in 1 mL/well).
9. Centrifuge the culture plate at 600 × *g*, 4 °C for 5 min to facilitate bacteria adherence to cells.
10. Wash the cells gently with ice-cold medium to remove unbound bacteria.
11. Remove the culture plate from ice. Initiate phagocytosis by replacing cold medium with 1 mL prewarmed (37 °C) medium per well. Place the culture plate in 37 °C incubator.
12. At 5, 10, 20, 30, 40, 60, 90, and 120 min postinfection (or any other desired time points), stop phagocytosis by placing the culture plate back on ice.
13. Wash the cells twice with ice-cold PBS and proceed to immunofluorescence staining.

3.2.2 Double-Cycle Immunofluorescence Staining

1. To discriminate between extracellular bacteria that adhere to the cell surface and intracellular bacteria that have been phagocytosed, two cycles of staining will be performed on the infected cells.
2. Keep cells on ice. Add blocking buffer to the cells and incubate for 10 min.
3. Remove blocking buffer and replace with primary antibody (e.g., rabbit-anti-Listeria) diluted in blocking buffer.

Incubate for 45 min on ice, and then wash for three times with washing buffer.

4. Add appropriate fluorochrome-conjugated secondary antibody (e.g., Alexa Fluor 488-conjugated goat anti-rabbit IgG) diluted in blocking buffer. Incubate for 30 min on ice followed by three washes with washing buffer.
5. Fix cells with 4 % paraformaldehyde for 10 min on ice. Wash cells three times with washing buffer.
6. Incubate cells with permeabilization buffer for 10 min at room temperature (RT).
7. Remove blocking buffer and incubate cells with primary antibody (e.g., rabbit-anti-Listeria) diluted in blocking buffer for 45 min at RT. Wash three times with washing buffer.
8. Add secondary antibody conjugated with a fluorochrome different from that used in the first cycle of staining (e.g., Alexa Fluor 647-conjugated goat anti-rabbit IgG) diluted in blocking buffer. Incubate for 30 min at RT.
9. (Optional) Add rhodamine-conjugated phalloidin simultaneously with secondary antibody to assist identification of cell boundaries (*see* **Note 15**).
10. Wash cells for three times with washing buffer.
11. Mount samples on glass coverslides with antifade mounting medium.
12. View samples with a fluorescence microscope. Examine cells by using a 40× or greater oil-immersion objective. Count the number of single-labeled bacteria in at least 200 cells. Single-labeled bacteria represent intracellular bacteria, while double-labeled bacteria are extracellular bacteria.
13. Calculate phagocytic index according to this formula: (total number of engulfed bacteria/total number of counted macrophages) × (number of macrophages containing at least one bacterium/total number of counted macrophages) × 100.

3.3 Flow Cytometric Phagocytosis Assay (See Note 16)

3.3.1 Label Bacteria with FITC

1. Transfer 5×10^8 bacteria to a new microcentrifuge tube. Sediment bacteria by centrifuging at ~20,000 × *g* for 1 min with a table-top centrifuge. Discard supernatant and resuspend with 1 mL sterile PBS.
2. Centrifuge again, discard supernatant, and resuspend in 1 mL of 1 mg/mL FITC in PBS, pH 8.0. Incubate at RT on an end-over-end rotator for 40 min.
3. Wash labeled-bacteria for three times with PBS as in **step 1**. After last wash, fix bacteria in 1 mL of 1 % glutaraldehyde. Incubate for 20 min at room temperature.

4. Quench free glutaraldehyde by adding 100 μL of 1 M glycine. Incubate for 10 min at RT.
5. Wash with PBS as in **step 1** and resuspend in 500 μL sterile PBS. Keep on ice.

*3.3.2 Initiation of Phagocytosis and Analysis with Flow Cytometry (See **Note 17**)*

1. Dilute FITC-labeled bacteria with ice-cold complete medium and add to adherent phagocytes grown in wells of a 24-well culture plate with a MOI of 10.
2. Centrifuge the culture plate at 600 × *g*, 4 °C for 5 min to accelerate bacteria deposition and adherence to cells.
3. Wash cells gently with ice-cold medium to remove unbound bacteria.
4. Remove the culture plate from ice. Initiate phagocytosis by replacing cold medium with 1 mL prewarmed (37 °C) medium per well. Place the culture plate in 37 °C incubator. As a negative control, keep one sample at 4 °C the whole time so phagocytosis is not initiated.
5. At desired time points (e.g., 30 and 60 min postinfection), stop phagocytosis by adding 200 μL per well of ice-cold 0.02 % EDTA/PBS and move the culture plate back on ice. Wash the cells twice with ice-cold PBS.
6. Detach cells from the culture plate with 0.5 % trypsin/EDTA or nonenzymatic cell dissociation buffer and collect cells in a new microcentrifuge tube. Spin down cells and resuspend in 240 μL of PBS containing 1 % FBS. Pipet gently repeatedly to ensure cells are dispersed into a single-cell suspension.
7. Keep cells on ice and add 60 μL (1/5 total volume) of 1.25 mg/mL trypan blue to quench extracellular FITC.
8. Transfer cells to a polypropylene tube suitable for use with flow cytometry and analyze immediately.

4 Notes

1. The particle size used for phagocytosis ranges from 0.5 to 3 μm, which needs to be optimized for different experiments.
2. The assays described in the section of phagocytosis of inert beads are performed with any of these phagocytic cells.
3. After centrifugation, SRBC will only form loose pellets. Be careful when removing the supernatant.
4. It is better to use SRBC less than 1 week old. Aged SRBC are sticky. It is better to include a set of non-opsonized SRBC as a negative control (Fig. 1). In addition to nuclear staining, cell identification can also be accomplished by using cytosol/cytoplasm labeling. There are several different methods to

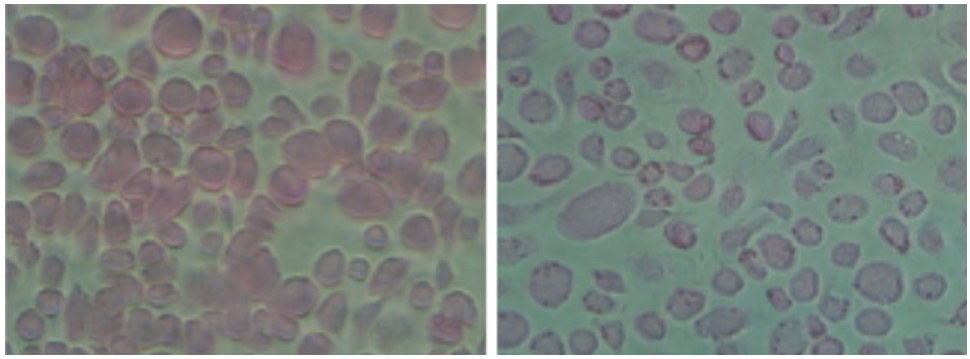

Fig. 1 Non-opsonized SRBC (*left* panel) and opsonized SRBC (*right* panel) incubated with macrophages for 10 min at 37 °C

label the cytosol, for example, the Qtracker kits from Life Technologies can be used to label the cell and the labeling is stable through several generations. The cells can be labeled before or after phagocytosis experiments.

5. Usually the agglutination titer of the anti-SRBC antibody is provided from the commercial source. Use the concentration just below the agglutination titer.
6. Opsonized SRBC can be stored at 4 °C and should be used within several hours.
7. Macrophages are also destroyed by ACK lysis buffer, if the cells are exposed to the buffer for too long.
8. The opsonization of inert latex beads is important for the phagocytes to recognize the inert beads as foreign particles. By the use of fluorochrome-conjugated antibodies that recognize the antibodies coated on the surface of the beads, we can determine the phagocytic index.
9. In this protocol, we mainly focus on using IgG to coat the latex beads for FcγR-mediated phagocytosis. One can also use complement component C3bi to opsonize the beads to study complement-mediated phagocytosis.
10. The opsonized beads can be stored at 4 °C for 5 days. Before performing phagocytosis, the particles should be vortexed vigorously for 5 s and pass through a 25-gauge needle to disrupt any text aggregates.
11. The fluorescence can also be quenched by using trypan blue at a concentration of 250 μg/mL in PBS. 0.5 mL of trypan blue is added to each well and the mixtures are kept for 2 min at room temperature.
12. When phagocytosis is complete, it is important to determine whether those particles are internalized or just adhere to cell

membrane. This can be achieved by using differential labeling the external beads with different fluorochrome-labeled secondary antibody. Since the internal beads cannot be detected by the secondary antibody, the internal beads will show only the color of the first fluorochrome, while the external beads will show two different fluorescence signals.

13. To quantitate phagocytosis, it is important to determine the exact number of phagocytes as well as the internal and external particles. There are several different ways to identify the cells. Here in this protocol, we use nuclear staining to identify individual cells.
14. This protocol describes a method to measure the ability of adhered phagocytes to ingest bacteria. Bacterial suspensions are added to cells grown on glass coverslips and centrifuged to enhance contact between the bacteria and cells. Extracellular bacteria are removed by washing (these steps are performed at 4 °C). Phagocytosis is initiated by adding a fresh medium prewarmed at 37 °C and continued at 37 °C, and it is terminated at different time points by placing the cells back on ice. Bacteria are then immunostained and visualized by using a fluorescence microscope. Despite the washing step, some extracellular bacteria may remain adhered to the cell surface, and it may be difficult for the experimenter to distinguish these extracellular bacteria from those truly internalized. A double-cycle immunofluorescence staining method solves this problem by staining extracellular bacteria first, followed by cell permeabilization and a second round of staining of bacteria. Using secondary antibodies conjugated with different fluorochromes that emit different colors, one can easily discriminate intracellular single-stained bacteria from extracellular double-stained bacteria.
15. Phalloidin selectively binds to F-actin with high affinity. Combining phalloidin staining with bacteria staining allows visualization of the cell boundaries and quantitation of the number of engulfed bacteria per cell. Some intracellular bacteria (e.g., *Listeria monocytogenes* and *Shigella flexneri*) are capable of escaping from the vacuole and polymerizing host actin to facilitate their movement inside the cells. Phalloidin also stains the actin tail of these bacteria.
16. An alternative approach to analyzing bacterial phagocytosis is to allow the cells to phagocytose fluorescence-labeled bacteria and measure the fluorescence intensity of the cells by flow cytometry. Most gram-positive bacteria can be easily stained by fluorescein isothiocyanate (FITC). Following internalization of FITC-labeled bacteria by cells, extracellular FITC on the bacteria bound to the cell surface but not internalized can be quenched by trypan blue. Therefore, the fluorescence detected is from the phagocytosed FITC-labeled bacteria only.

17. Compared to the control samples kept at 4 °C throughout the experiment, cells that have phagocytosed FITC-labeled bacteria show a shift in fluorescence intensity. Although this method does not reveal the absolute number of bacteria phagocytosed by each cell, the relative phagocytosis ability can be compared according to the fluorescence intensity shift.

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Chapter 14

Assessing the Roles of Galectins in Regulating Dendritic Cell Migration Through Extracellular Matrix and Across Lymphatic Endothelial Cells

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Abstract

Leukocyte migration from the bloodstream into tissues, and from tissues to lymph nodes, depends on expression of specific adhesion and signaling molecules by vascular endothelial cells and lymphatic endothelial cells. Tissue damage and microbial infection induce vascular endothelial cells to up-regulate expression of adhesion molecules to facilitate entry of several leukocyte populations from blood into tissues. Many of these cells then leave inflamed tissue and migrate to regional lymph nodes. A critical population that emigrates from inflamed tissue is dendritic cells. Dendritic cells in tissue have to migrate through extracellular matrix and across a layer of lymphatic endothelial cells to enter the lymphatic vasculature. Little is known about the adhesion molecules expressed by lymphatic endothelial cells or the processes required for the critical step of dendritic cell exit from tissues, specifically migration through the extracellular matrix and basal-to-apical migration across the lymphatic endothelial cell layer into lymphatic vasculature.

Members of the galectin family of carbohydrate binding proteins are expressed in both vascular and lymphatic endothelial cells. Dynamic changes in galectin expression during inflammation are known to regulate leukocyte tissue entry during inflammation. However, the roles of galectin family members expressed by lymphatic endothelial cells in leukocyte tissue exit remain to be explored.

Here, we describe an in vitro transmigration assay that mimics dendritic cell tissue exit in the presence and absence of galectin protein. Fluorescently labeled human dendritic cell migration through extracellular matrix and across human lymphatic endothelial cells is examined in the presence and absence of recombinant human galectin protein.

Key words Leukocyte migration, Human dendritic cell, Human lymphatic endothelial cell, Tissue exit, Extracellular matrix, Galectin, Transmigration insert

1 Introduction

1.1 Leukocyte Migration During Inflammation

Tissue damage and microbial infection induce migration of leukocytes from the blood stream to sites of inflammation (Fig. 1). To enter damaged tissue, leukocytes exit blood vessels by migrating across vascular endothelial cells (VECs) and through the subjacent extracellular matrix (ECM). The process of leukocyte migration across VECs into tissues is fairly well described; production of

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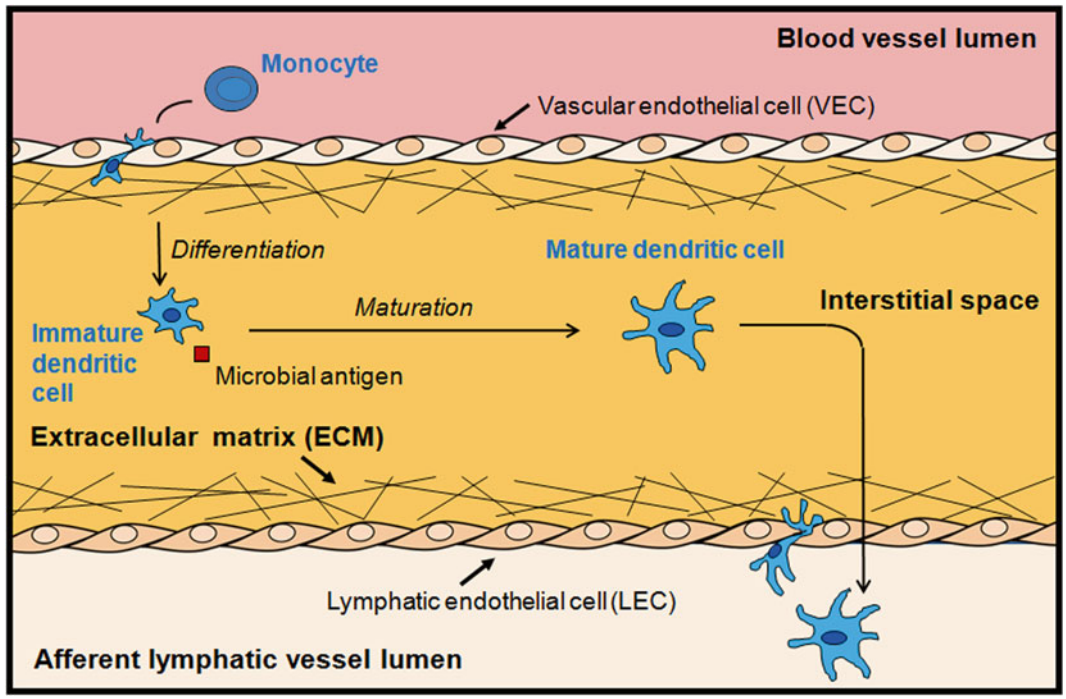

Fig. 1 Leukocyte entry into and exit from tissue. Schematic representation of leukocyte entry from the blood vasculature into inflamed tissue and exit into the lymphatic vasculature

pro-inflammatory cytokines at sites of inflammation induces VECs to increase expression of adhesion molecules, e.g., selectins and integrins, which facilitate tissue entry of lymphocytes [1, 2].

Once leukocytes have crossed the VEC layer, the cells migrate through the subjacent ECM, a dense network of glycoproteins [3]. Leukocyte migration through ECM during inflammation is regulated by binding of ECM glycoproteins to carbohydrate binding proteins, as well as modulation of the ECM composition by inflammatory cytokines and chemokines [3, 4].

Various types of immune cells, including neutrophils, monocytes, and lymphocytes, enter sites of inflammation in response to migration stimuli expressed by VECs. In contrast, one of the primary populations that leaves tissue is antigen-experienced mature dendritic cells (DCs) (Fig. 1) [5, 6]. However, little is known about the mechanism that DCs use to exit inflamed tissues or how the cells travel to lymph nodes [7]. During tissue exit, DCs have to migrate through ECM towards the lymphatic endothelium and in a basal-to-apical direction across a layer of lymphatic endothelial cells (LECs) that form the lymphatic vessel wall. DCs that exit tissue and enter the lymphatic vasculature travel to tissue-draining lymph nodes where the cells initiate adaptive immune responses [6].

DC migration across LECs and to the lymph node can be analyzed by in vivo and in vitro methods. The in vivo system typically analyzes DC mobilization and migration from the skin; a fluorescent dye, e.g., fluorescein-isothiocyanate (FITC), solubilized in an agent causing skin inflammation, is applied to the skin and phagocytosed by DCs. As the labeled DCs leave tissue, FITC-positive DCs in the draining lymph node are counted and/or phenotyped [8, 9]. Although this type of analysis can be used to accurately examine which DC populations travel to skin draining lymph nodes in the presence and absence of skin inflammation, it is not optimal for dissecting individual molecular mechanisms that regulate migration of DCs into the lymphatic vasculature and then to the lymph nodes.

In contrast, in vitro assays, although reductionist, are best suited for interrogating the roles of individual molecular interactions that control tissue exit of specific DC populations. For example, in vitro studies revealed that human LECs, similar to VECs, up-regulate expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), and secrete the chemokine CCL21 under inflammatory conditions. Interestingly, all three stimuli promoted human inflammatory DC migration across human LECs in vitro, a result that was confirmed for VCAM-1 and ICAM-1 using the murine in vivo system described above [10, 11].

Although these reports have started to reveal some of the molecular interactions that regulate inflammatory DC tissue exit, there are likely many other molecular interactions involved in this process. Thus, the in vitro migration assay provides a simple, yet powerful, method to study the effect of individual molecules that may play a role in emigration of specific DC populations [12] across ECM and LECs.

1.2 In Vitro Migration Assays to Study Leukocyte Tissue Entry and Exit

In vitro migration assays can be used to study leukocyte tissue entry or leukocyte tissue exit, the difference being the cell types used and the direction of migration (Fig. 2). To mimic tissue entry, VECs are seeded on top of the ECM-compound Matrigel™ or other ECM components, such as collagen, and leukocytes, e.g., monocytes, migrate from the apical to the basal side of VECs before migrating through the ECM [13, 14]. To mimic tissue exit, LECs are seeded on a filter underneath the ECM-compound Matrigel™ and leukocytes known to exit tissue, such as antigen-experienced DCs, migrate through the ECM before migrating across LECs from the basal to the apical side [10, 15].

Not only are in vitro models generally more cost- and time-efficient than in vivo models, there are also several technical advantages in using in vitro models to study DC tissue exit, specifically

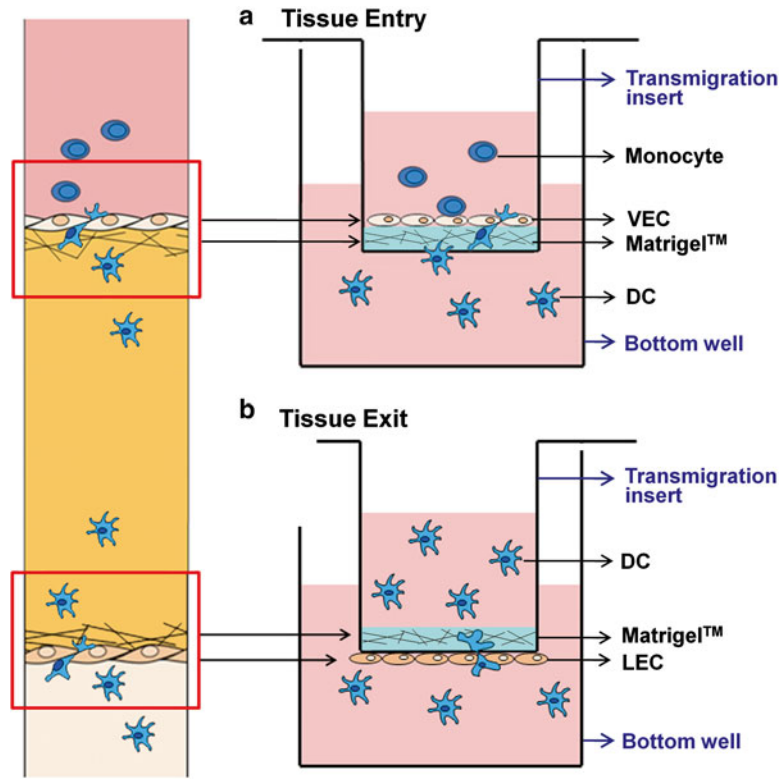

Fig. 2 In vitro transmigration assays that mimic leukocyte tissue entry and exit. Schematic representation of leukocyte tissue entry and exit in vivo (*left*) and corresponding in vitro transmigration assays (*right*). To mimic tissue entry, VECs are seeded on top of the ECM-compound Matrigel™ or other ECM components, such as collagen, and leukocytes, e.g., monocytes, migrate from the apical to the basal side of VECs before migrating through the ECM (*top right*, Panel **a**). To mimic tissue exit, LECs are seeded on a filter underneath the ECM-compound Matrigel™ and leukocytes known to exit tissue, such as antigen-experienced DCs, migrate through the ECM before migrating across LECs from the basal to the apical side (*bottom right*, Panel **b**)

for analyzing migration of specific human DC populations across ECM plus human LECs:

1. Use of primary human LECs prepared from dermal tissue [16, 17].
2. Use of different human DC populations matured in vitro [12, 18] or purified from blood [19].
3. Separate and optimal exposure of each cell type to specific inflammatory stimuli; e.g., human DC maturation in the presence of TNF-α or lipopolysaccharide (LPS) [20]; LEC treatment with TNF-α or LPS [21, 22].

4. Dissection of individual roles of ECM, LECs, and leukocytes; e.g., DC migration through ECM alone or across ECM plus LEC layer.
5. Manipulation of protein expression in different cell types; e.g., protein over-expression or "knock-down" in DCs, LECs, or both.
6. Manipulation of protein concentration in ECM; e.g., supplementation of Matrigel™ with different concentrations of recombinant protein [23–25].
7. Exclusion of bystander cell effects; e.g., exclusion of interactions of one DC population with another at endothelial cell barrier.

1.3 The Role of Galectins in Leukocyte Migration and Tissue Inflammation

Members of the galectin family of carbohydrate binding proteins are expressed in VECs and galectin expression changes dynamically during inflammation [26, 27]. Using various in vivo systems, it has been established that changes in galectin expression in VECs regulate leukocyte tissue entry during inflammation [28].

In contrast, little is known about galectin expression in LECs and whether galectin expression in LECs may regulate leukocyte tissue exit [28, 29]. Given the complex regulation of galectin expression in VECs, it is plausible that LECs express several galectin family members and that galectin expression in LECs also changes dynamically during inflammation. Recent work from our laboratory and others has provided in vivo evidence suggesting a role for galectins in regulating inflammatory DC migration to the lymph node [9, 30]. However, these in vivo models do not address the molecular mechanism(s) that the carbohydrate binding galectins utilize to regulate DC tissue exit and subsequent migration to the lymph node.

To determine the molecular mechanism(s) by which galectin family members may regulate DC exit, it is important to understand that all galectin family members contain conserved carbohydrate-recognition domains (CRDs) which bind discrete but overlapping ligands displayed on glycoproteins [31–35]. Once secreted, all galectins can functionally oligomerize, thus potentially binding several glycoproteins simultaneously [36, 37]. Since galectin family members are secreted into the extracellular space by many different cell types [38–40], these multivalent lectins may regulate DC tissue exit by binding to glycoproteins expressed on the surface of DCs and/or LECs as well as binding to glycoproteins in the ECM.

To further analyze which galectin family members regulate tissue exit of specific human DC populations and which molecular mechanism(s) play a role in this process, we developed an in vitro transmigration assay. Fluorescently labeled human DC

migration through ECM and across human LECs is examined in the presence and absence of recombinant human galectin protein added to the ECM.

2 Materials

2.1 Cell Culture

1. Lymphatic endothelial cells (LECs): Human dermal microvascular lymphatic endothelial cells (HMVEC-dLyAd-Der Lym Endo cells) (Lonza, Walkersville, MD).
2. Primary human DCs matured from peripheral blood monocytes isolated using standard protocols [41].
3. EGM™-2MV medium (Lonza, Walkersville, MD), warmed to 37 °C.
4. 1× PBS: 1× phosphate-buffered saline (use standard formulation), warmed to 37 °C.
5. Trypsin-EDTA: GIBCO® 0.25 % Trypsin-EDTA (1×) (Invitrogen, Grand Island, NY).
6. Recovery™-Cell Culture Freezing medium (GIBCO®; Invitrogen, Grand Island, NY).
7. T75 flask: 75 cm^2 cell culture flask, 0.2 μm vent cap, tissue-culture treated (Corning, Corning, NY).

2.2 Matrigel™ Migration Assays

1. Transmigration inserts: BD Biocoat™ BD Matrigel™ invasion chambers in 24-well plates, 8 μm pore size (BD Biosciences, San Jose, CA).
2. BD Falcon™ 24-well companion plates (BD Biosciences, San Jose, CA).
3. Hydration medium: GIBCO® DMEM medium, serum-free, high glucose (Invitrogen, Grand Island, NY), warmed to 37 °C.
4. DC medium: GIBCO® RPMI medium (Invitrogen, Grand Island, NY), 10 % fetal bovine serum (FBS), 25 mM HEPES, 2 mM GIBCO® GlutaMAX™, warmed to 37 °C.
5. Serum-free DC medium: GIBCO® RPMI medium (Invitrogen, Grand Island, NY), 25 mM HEPES, 2 mM GIBCO® GlutaMAX™, warmed to 37 °C.
6. 150 mm × 25 mm round suspension culture dish (Corning, Corning, NY).
7. Diff-Quick solutions (Dade Behring, Switzerland).
8. Recombinant human MIP-3β/CCL19 (Peprotech, Rocky Hill, NJ): stock concentration 20 μg/ml.
9. CellTrace™ CFSE Proliferation Kit (Invitrogen, Grand Island, NY).

3 Methods

Please refer to Fig. 3 for a time line of transmigration assay preparation and analysis.

3.1 Culturing Human Lymphatic Endothelial Cells

1. Thaw primary human LECs (HMVEC-dLyAd-Der Lym Endo cells; Lonza) according to the manufacturer's protocol into 10 ml EGM-2MV medium in a T75 flask.
2. Incubate at 37 °C, 5 % CO_2.
3. Change EGM-2MV medium (10 ml) carefully every 48 h until cells grow to an 80 % confluent layer (*see* **Note 1**).
4. Dissociate LEC monolayer from the bottom of the flask: Remove EGM-2MV medium and wash cells by adding 10 ml warm 1× PBS into the flask, carefully swirling the liquid around before removing it. Add 8 ml of Trypsin-EDTA (1×) to the flask and incubate for 45–60 s at 37 °C (*see* **Note 2**). Then, check whether cells are dissociated under light microscope. Add 12 ml EGM-2MV medium to dissociated cells and collect cells into a 50 ml plastic tube. Pellet cells by centrifugation (350 × *g*, 5 min, room temperature (RT)) and resuspend pellet in 10 ml EGM-2MV medium.
5. Seed LECs for expansion: Add 9 ml EGM-2MV medium to each of ten T75 tissue culture flasks. Then, add 1 ml LECs in EGM-2MV medium to each flask (it is not necessary to count

Day 0	Day 1	Day 2
Method	**Method**	**Method**
(Section 3.3) Preparing Matrigel™ transmigration inserts for LEC seeding	(Section 3.5) Testing confluency of LECs grown on the bottom of Matrigel™ transmigration inserts	(Section 3.10) Migration assay analysis
(Section 3.4) Preparing LECs for seeding on Matrigel™ transmigration inserts	(Section 3.6) Preparing Matrigel™/LEC transmigration inserts for use in migration assay	
	(Section 3.7) Saturating Matrigel™ with recombinant galectin	
	(Section 3.8) CFSE-labeling of DCs	
	(Section 3.9) Assembly of transmigration assay plate	

Fig. 3 Time line for transmigration assay preparation and analysis

LECs prior to seeding). Care for the cells as described in **step 3** until cells grow to an 80 % confluent layer. Dissociate LEC monolayer as described in **step 4** and freeze 1×10^6 cells in 1 ml Recovery™-Cell Culture Freezing medium in liquid nitrogen (using a standard protocol).

6. Thaw 1×10^6 LECs in 10 ml EGM-2MV medium in a 10 cm-diameter, round tissue culture plate. Care for the cells as described in **steps 3** and **4** (and adjust liquid volumes to the smaller plate size).

3.2 Isolation and Maturation of Human Dendritic Cells

1. Primary human DCs are matured from peripheral blood monocytes isolated using standard protocols [41] (*see* **Notes 3–5**).

3.3 Preparing Matrigel™ Transmigration Inserts for LEC Seeding

1. Prepare the desired number of Matrigel™-coated transmigration inserts by thawing Matrigel™-coated inserts inside the companion plate for 20 min at room temperature (*see* **Notes 6** and **7**).
2. To hydrate Matrigel™ before use, add 700 µl hydration medium to the bottom well of a companion plate. Place transmigration insert into the well and add 400 µl hydration medium to each insert before placing the plate in a 37 °C tissue culture incubator (5 % CO_2) for 2 h.
3. Upon hydration, lift transmigration inserts out of the companion plate using forceps and carefully remove all hydration medium from inside the inserts with a P1000 pipet.
4. Using a second pair of forceps, turn inserts upside down and place into a large (150 mm × 25 mm), round suspension culture dish. If there is residual hydration medium on the bottom of a transmigration insert (now the side facing up), remove it carefully with a small pipet tip.

3.4 Preparing LECs for Seeding on Matrigel™ Transmigration Inserts

1. Dissociate 80 % confluent LEC monolayer from plates as described above (Subheading 3.1, **step 4**) and prepare 2×10^5 LECs in 100 µl EGM-2MV medium for each transmigration insert you wish to use (*see* **Note 8**).
2. Very slowly, using a circular motion and a P200 pipet, layer LECs in 100 µl medium on top of each inverted transmigration insert (Subheading 3.3, **step 4**) inside the large suspension culture dish. Place the lid on the dish, carefully move the dish into a 37 °C incubator (5 % CO_2), and incubate for 2 h (*see* **Notes 9** and **10**).
3. Check cells every 15–20 min to make sure that the medium bubble is still on top of the insert; otherwise the cells may dry out.
4. After incubation, prepare a companion plate by placing 700 µl EGM-2MV medium in one well for each insert you are using.

Then, turn inserts around with forceps and place them into the companion plate. Add 400 µl EGM-2MV medium to each insert and incubate the cells overnight in a 37 °C incubator (5 % CO_2).

5. By the next day (16–24 h later), LECs on the bottom of the inserts will have grown into a confluent layer.

3.5 Testing Confluency of LECs Grown on the Bottom Membrane of Matrigel™ Transmigration Inserts

Stain LECs grown on the bottom membrane of a transmigration insert (Subheading 3.4, **step 5**) with Diff-Quick solutions in a companion plate (*see* **Note 11**).

1. Add 700 µl of each solution into a well of a companion plate: two wells of 1× PBS, one well of Diff-Quick Fix, one well of Diff-Quick Stain I, one well of Diff-Quick Stain II, three wells of H_2O.
2. In this order, hang transmigration inserts into each solution for about 30 s before moving to the next well.
3. Dry transmigration inserts upside down in a large, round suspension culture dish without a lid in the chemical hood for 30–45 min. (Diff-Quick solutions are toxic.)
4. Cut membrane out of each insert using a scalpel and add membrane with cells facing down on a microscope slide where some immersion oil has been placed.
5. Add cover slip. Keep slides at 4 °C and analyze LEC confluency under a light microscope.

3.6 Preparing Matrigel™/LEC Transmigration Inserts for Use in Migration Assay

1. Remove EGM-2MV medium from the Matrigel™/LEC transmigration inserts (Subheading 3.4, **step 5**) with a P1000 pipet (the recovered volume should be around 400 µl).
2. Wash inserts twice with 400 µl warm 1× PBS by carefully adding and removing the solution from inserts.

The washed inserts can either be used directly in a migration assay or Matrigel™ can be saturated with recombinant galectin before using inserts in a migration assay.

3.7 Saturating Matrigel™ with Recombinant Galectin

1. Dilute recombinant galectin to the concentration of your choice in 100 µl warm 1× PBS and place inside the washed transmigration inserts so that the galectin is on top of the Matrigel™ (Subheading 3.6, **step 2**) (*see* **Note 12**).
2. Place inserts in a companion plate with 700 µl EGM-2MV medium in the bottom well so that LECs on the bottom of the inserts do not dry out.
3. Incubate plate at 37 °C for 30 min.
4. Carefully remove residual solution containing galectin (should be around 100 µl) and wash inserts in warm 1× PBS as described above (Subheading 3.6).

The washed inserts can now be used in a migration assay.

3.8 CFSE-Labeling of DCs

The number of DCs migrating across ECM and LECs is analyzed by counting the number of DCs that have migrated from the transmigration insert into the bottom well of the companion plate. However, we noticed that, occasionally, LECs detach from transmigration insert membranes and fall into the bottom well during the duration of this migration assay. To avoid LEC contamination of the migrating DC population, we reasoned that fluorescent labeling of DCs prior to migration assay assembly would distinguish the two cell populations. Thus, DCs are fluorescently labeled with the component carboxyfluorescein succinimidyl ester (CFSE) to distinguish DCs from LEC contamination in the bottom well. For DC labeling, we use the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, Grand Island, NY) (*see* **Note 13**).

1. Solubilize one vial of CellTrace™ CFSE in 18 µl dimethyl sulfoxide (DMSO) to make a 5 mM stock solution (according to manufacturer's instructions).
2. Dilute 5 mM stock 1:500 into warm 1× PBS to make a 10 µM working solution.
3. Resuspend 1×10^6 DCs in 500 µl CFSE working solution in a 1.5 ml microcentrifuge tube (*see* **Note 14**).
4. Incubate cells in CFSE solution for 15 min at 37 °C (5 % CO_2).
5. Pellet cells by centrifugation (350 × *g*, 5 min, RT).
6. Resuspend the pellet in 500 µl serum-free DC medium and incubate for 30 min at 37 °C (5 % CO_2).
7. Pellet cells by centrifugation (350 × *g*, 5 min, RT).
8. Wash DCs twice in 10 ml of serum-free DC medium and adjust concentration to 5×10^4 DC per 400 µl in serum-free DC medium (*see* **Notes 15** and **16**).

3.9 Assembly of Transmigration Assay Plate

Each transmigration insert will be filled with 5×10^4 CFSE-labeled DCs in 400 µl serum-free DC medium (Subheading 3.8, **step 8**).

1. Prepare a fresh companion plate by adding 700 µl DC medium plus 200 ng/ml of the recombinant human chemokine MIP-3β/CCL19 [42] into a well for each transmigration insert (*see* **Note 17**).
2. Place washed transmigration inserts (with or without galectin in Matrigel™ (Subheadings 3.6 and 3.7)) into wells of the companion plate using forceps.
3. To each transmigration insert, carefully add 5×10^4 CFSE-labeled DCs in 400 µl serum-free DC medium (Subheading 3.8, **step 8**).
4. Incubate migration assays for up to 24 h at 37 °C before analyzing the number of cells in transmigration inserts and in bottom wells (*see* **Note 18**).

3.10 Migration Assay Analysis

1. Prepare one labeled 1.5 ml microcentrifuge tube for each transmigration insert and each bottom well.
2. Dismantle migration assay setup by removing medium from transmigration insert (pipet up and down several times to ensure that all cells are taken up; the volume should be around 400 μl) and placing it in the corresponding tube. Add 250 μl warm 1× PBS into each insert and repeat.
3. Lift inserts from plate and discard.
4. Remove medium from bottom wells (pipet up and down several times to ensure that all cells are taken up; the volume should be around 700 μl) and place into the corresponding tube. Add 400 μl warm 1× PBS into each insert and repeat.
5. Spin tubes at 350 × *g* for 5 min at RT and carefully remove the supernatant from each tube without disturbing the cell pellet.
6. Add 100 μl 1× PBS to each tube.
7. Count cells in a hemocytometer and record the numbers.
8. Some LECs will fall into the bottom well. To determine the number of migrated DCs, combine triplicate samples for each insert or each bottom well and analyze the percentage of CFSE-positive DCs in each sample by flow cytometry (using a standard flow cytometry protocol).
9. Calculate the number of migrated DCs by multiplying the total hemocytometer cell count by the percentage of CFSE-positive DCs.
10. Display the result as "percent migration" by calculating the number of DCs in the bottom well as a percentage of the total DC number in the transmigration insert plus the bottom well (*see* **Notes 19** and **20**).

4 Notes

1. Each frozen vial of LECs can be thawed in 10 ml EGM-2MV medium, plated into a 10 cm-diameter, round tissue culture plate, and should grow to 80 % confluence in 24–72 h, depending on the donor. One 10 cm-diameter, round plate yields between 1 and 2.5×10^6 LECs. Commercially available LECs are purified from human donors and thus can vary in their growth behavior. We suggest testing how fast each lot of cells grows and how many cells can be harvested from a 10 cm-diameter, round tissue culture plate before using LECs in a migration assay. In addition, we recommend not using LECs that have been passaged more than eight times. It is also imperative to test the cells occasionally for the expression of LEC markers, such as podoplanin or LYVE-1 [43].

2. LEC incubation in Trypsin-EDTA (1×) for less than 1 min is preferred.
3. We recommend analyzing human mature DCs for complete maturation before preparing the cells for migration assays. We use a standard panel of fluorochrome-labeled antibodies against surface molecules differentially expressed upon DC maturation (e.g., CD14, CD86, CD83, and MHC class II) [44, 21] and analyze the cells by flow cytometry.
4. Different leukocyte populations, directly purified from human blood or generated in vitro, can be used if desired.
5. If manipulation of DC cell surface glycosylation is desired prior to using DCs in a migration assay, standard small interfering RNA (siRNA) technology can be used [45, 46].
6. Although we recommend using pre-coated Matrigel™ transmigration inserts for this particular assay, our laboratory has previously published a protocol on how to isolate ECM secreted from cells and how to use this ECM substrate to coat transmigration inserts [23].
7. It is imperative that all work should be carried out under sterile conditions and that each sample should be analyzed in triplicate to ensure reproducibility.
8. We found that LECs are best harvested and prepared for seeding during the last 45 min of Matrigel™ hydration.
9. It is important to use large, round suspension culture dishes (150 mm × 25 mm) for seeding LECs on inverted Matrigel™ transmigration inserts since the height of smaller plates is too low to close the lid and keep inserts sterile during incubation.
10. During the 2 h incubation in which LECs adhere to the bottom membrane of the transmigration insert, some of the medium (used to disperse and seed the cells in) will drip down on the side of Matrigel™ transmigration inserts. This is not a concern as long as there is some medium left on the membrane. LECs are seeded in excess and cell loss during this process is minimal. However, when inserts are turned around and placed into the companion plate, it is important to ensure that there is no medium left on top of the insert facing the companion plate lid.
11. To test whether LECs grew into a confluent layer, prepare three extra transmigration inserts with LECs for this purpose. Diff-Quick stain is a modified Romanowski stain that labels the cytoplasm and nucleus and thus visualizes the LEC monolayer on the bottom of the transmigration insert membrane.
12. Recombinant galectins are commercially available or galectin protein can be produced in bacteria and purified [47].

13. We suggest starting CFSE treatment of DCs while Matrigel™ transmigration inserts are being hydrated (2 h at 37 °C).
14. We recommend starting the CFSE-labeling with an excess number of DCs since there will be cell loss during the labeling process.
15. The CFSE-labeled cell pellet should appear green in room light. If there is a question about labeling efficiency, we recommend analyzing a CFSE-labeled DC sample by flow cytometry.
16. We recommend harvesting and counting CFSE-treated DCs while (or before) Matrigel™ transmigration inserts are incubating with recombinant galectin solution.
17. We find it helpful to label the lid of the companion plate with an identifier for each sample before assembling the migration assay.
18. We chose the 24 h time point for this analysis, since the maximal number of DCs will have migrated to the bottom well at this point. The number of migrated DCs can be analyzed at earlier time points if desired.
19. If a fluorescent plate reader is available, transmigration of fluorescently labeled DCs across ECM plus LECs can be determined automatically [10].
20. To analyze the number of migratory events that reflect DC tissue exit, we suggest counting the number of cells in medium in the bottom well. This is different from some published analyses of cell migration, where the filter is removed from the transmigration insert and the number of cells adherent to the underside of the filter is counted [13, 23, 25]. We have found that the majority of DCs detach and fall to the bottom of the well and thus this counting method yields the best reproducibility.

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Chapter 15

Examination of the Role of Galectins in Intestinal Inflammation

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Abstract

The intestine, which provides the first line of defense against over trillion of enteric microorganisms, suffers from broad range of inflammatory conditions caused by infectious, autoimmune, allergic, neurological, and ischemic mechanisms. Recent data have suggested dual roles (protective versus deleterious) for galectins in the pathogenesis of some intestinal inflammations, highlighting the importance of this area of research. A potential problem with the research of intestinal inflammation may be the requirement of some unique techniques. Therefore, we herein describe how to induce intestinal inflammation and how to isolate lymphocyte, myeloid cell, follicular cell, and epithelial cell populations separately from the intestine for the study of intestinal inflammations.

Key words Colitis, Epithelial cell isolation, Galectins, Inflammatory Bowel Disease, Lamina propria cell isolation, Mouse models

1 Introduction

The intestine provides the first line of defense against more than a trillion enteric microorganisms. However, when the microbial interaction is dysregulated, the intestine can suffer from a broad range of inflammatory conditions such as infectious colitis/ileitis, food allergy, celiac disease, ischemic enteritis, and inflammatory bowel disease (IBD) that is classified into two major forms, Crohn's disease (CD) and ulcerative colitis (UC) [1–5]. The enormous exposure to enteric microorganisms makes mucosal immune system distinct from its systemic counterpart [6]. An endogenous lectin family, galectins, is composed of 15 members that have the ability to recognize multiple carbohydrate epitopes and play several different roles (e.g., anti- versus pro-inflammatory) in adaptive and innate immune responses [7–9]. Although little information is currently available, some data suggest dual roles of galectins in the pathogenesis of some intestinal inflammations. For example, acute colitis, which can be induced in mice by rectal administration of

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2,4,6-trinitrobenzene sulfonic acid (TNBS), is attenuated by administration of human recombinant galectin-1 (prototype) through induction of apoptosis in effector T cells producing IFN-γ [10]. Oral administration of dextran sulfate sodium (DSS) through drinking water induces acute intestinal injury, and the inflammation spontaneously recovers after termination of DSS treatment. Innate immune response is primarily responsible for the acute (induction) phase of inflammation [11], while adaptive and innate responses both participate in the wound healing process [12]. The acute phase of DSS colitis has previously been shown to be suppressed by administration of recombinant galectin-2, galectin-4, or galectin-9 [13–15]. Consistent with this, galectin-4 has an ability to suppress the innate immune response by killing blood group antigen-expressing bacteria [16] and by activating epithelial cell function through induction of super raft formation [17]. In contrast, the healing process from DSS-induced acute colitis is suppressed by galectin-4 [18]. In addition, galectin-4 serves as an exacerbation factor in T cell-mediated chronic colitis by stimulating the proliferation of memory effector $CD4^+$ T cells through stabilization of immunological synapse signaling [19]. These data highlight the contrasting roles of galectins in colitis depending upon several factors such as involvement of innate versus adaptive immunity. Therefore, further extensive studies are necessary to fully understand the role of galectins in intestinal inflammation and to determine whether galectins can be targeted for the treatment of certain intestinal inflammatory conditions. However, the requirement of unique methods for isolation of intestinal cells tends to hamper investigators who are unfamiliar with intestinal biology in their ability to closely study the role of galectins in intestinal inflammation at cellular level. Therefore, we herein describe key protocols that may be indispensable to study the role of galectins in the intestinal inflammation.

2 Materials

2.1 CD45RB Colitis Model

1. Dulbecco's phosphate-buffer saline (DPBS).
2. Cell isolation medium: RPMI1640 medium, 0.3 mg/ml L-glutamine, 4 % Fetal Bovine Serum (FBS, Hybridoma grade, Sigma), 25 mM HEPES buffer, and antibiotics (120 IU/ml Penicillin, 120 μg/ml Streptomycin, and 0.3 μg/ml Amphotericin B). Sterilize through 0.22 μm filter.
3. Sorting buffer: 500 ml cold (4 °C) DPBS, 1 g bovine serum albumin (Sigma), de-gas on ice for 10 min and sterilize through 0.22 μm filter.
4. AKC Lysis Buffer: 0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA in distilled water, pH 7.4.

5. Mouse T cell Enrichment Column (R&D systems).
6. T cell enrich column buffer: 1 volume 10× column buffer (supplied with column), 9 volumes distilled water.
7. Scissors.
8. Curved and Straight Jewelers style forceps.
9. 100 × 5 mm petri dish.
10. 60 μm pore size nylon mesh.
11. 15 ml polystyrene conical tube (BD Falcon).
12. FITC-CD4 (RM4.5) (eBioscience).
13. PE-CD45RB (C363.16A) (eBioscience).
14. C57BL/6 mice.
15. Recombinant activating gene (RAG) 1 knockout mice (C57BL/6 background, Jackson Laboratory).

2.2 Self-Limiting Colitis Model

1. Dextran sulfate sodium salt (DSS) (MP Biomedicals).
2. C57BL/6 mice.
3. 10 % buffered formalin.

2.3 Isolation of Colonic Lamina Propria Lymphocytes (T Cells and B Cells)

1. Cell isolation medium: RPMI 1640, 0.3 mg/ml L-glutamine, 4 % FBS, 25 mM HEPES buffer, and antibiotics (120 IU/ml Penicillin, 120 μg/ml Streptomycin, and 0.3 μg/ml Amphotericin B).
2. Cell isolation solution #1: cell isolation medium, Dispase (1.5 mg/ml, Life Technology). *Freshly prepare 20 ml for each sample. Keep at room temperature for at least an hour prior to use and sterile before use.*
3. Cell isolation solution #2: cell isolation medium, Dispase (1.0 mg/ml) and Collagenase type II (1.5 mg/ml, Life Technology). *Freshly prepare 25 ml for each sample. Keep at room temperature and sterile before use.*
4. 90 % Percoll solution: 1 volume of 10 × RPMI 1640 (Life Technology), 9 volumes Percoll to make 90 % Percoll solution.
5. 45 % Percoll solution: 1 volume of 90 % Percoll solution (GE Healthcare), 1 volume of sterile RPMI 1640 medium without FBS or additive. *4 ml of 45 % Percoll solution is needed for each sample.*
6. 72 % Percoll solution: 4 volumes of 90 % Percoll solution to 1 volume of sterile RPMI 1640 medium without FBS or additive. *3 ml of 72 % Percoll solution is needed for each sample.*
7. Anatomical scissors.
8. Curved and Straight Jewelers style forceps.

9. Glass wool column: five layers of glass wool (PYREX 9989 fiber glass) in 10 ml syringe (*see* **Note 1**).
10. 19G and 27G needles.
11. Stop cock.
12. Tendersorb (Kendall).
13. Surgical blades #21 (HMD Healthcare Ltd.).
14. Bacterial (rotary) shaker.

2.4 Isolation of Colonic Epithelial Crypts

1. Pre-warmed (37 °C) 30 mM EDTA in Hank's balanced salt solution (HBSS).
2. Cold 2 mM EDTA in HBSS.
3. Isoflurane.
4. 22G catheter.
5. Mini-Beadbeater (Biospec Products).
6. 1.8 ml microtube (Biospec Products).
7. Syringe pump (Harvard Apparatus).

2.5 Isolation of Large Cells (e.g., Macrophages and Fibroblasts) from the Colon

1. Cell isolation medium: RPMI 1640, 0.3 mg/ml L-glutamine, 4 % FBS, 25 mM HEPES buffer, antibiotics (120 IU/ml Penicillin, 120 μg/ml Streptomycin, 0.3 μg/ml of Amphotericin B).
2. 10 % medium: RPMI 1640, 0.3 mg/ml L-glutamine, 10 % FBS, 25 mM HEPES buffer, antibiotics (120 IU/ml Penicillin, 120 μg/ml Streptomycin, and 0.3 μg/ml Amphotericin B).
3. Cell isolation solution #3: cell isolation medium, 2 mg/ml collagenase type II, 1 mg/ml dispase, 15 μg/ml DNase. *Freshly prepare 20 ml for each sample. Keep at room temperature for at least an hour prior to use and sterilize before use.*
4. Pre-warmed (37 °C) 30 mM EDTA in Hank's balanced salt solution (HBSS).
5. Pre-warmed 5 mM EDTA in HBSS.
6. Isoflurane.
7. 22G catheter.
8. Mini-Beadbeater (Biospec Products).
9. 1.8 ml microtube for beadbeater (Biospec Products).
10. Syringe pump (Harvard Apparatus).
11. Bacterial (rotary) shaker.
12. 60 μm pore size nylon mesh.

2.6 Isolation of Appendix Lymphoid Cells

1. Cell isolation medium: RPMI 1640 supplemented with 0.3 mg/ml L-glutamine, 4 % FBS, 25 mM HEPES buffer, and antibiotics (120 IU/ml of Penicillin, 120 μg/ml of Streptomycin, and 0.3 μg/ml of Amphotericin B).

2. Curved Jewelers style forceps.
3. Surgical blade #21.
4. 27G needles.
5. One layer glass wool column: one layer glass wool (PYREX 9989 fiber glass) in 3 ml syringe.

3 Methods

3.1 CD45RB Colitis Model (See Note 2)

1. Euthanize C57BL/6 mouse and carefully take out spleen using aseptic technique. Subsequent steps should be performed under sterile condition.
2. Place the spleen in a petri dish with cold DPBS, make several cuts on the capsule of spleen, and loosen the spleen using Curved Jewelers style forceps to release splenic cells into DPBS.
3. Collect cells into 15 ml conical tube and vortex vigorously for 15–30 s.
4. After centrifugation at 1,500 rpm for 5 min at 4 °C, discard supernatant and resuspend cells with 7 ml ACK lysis buffer.
5. Incubate for 5 min at room temperature with occasional mixing and then add 7 ml of cell isolation medium.
6. After centrifugation at 1,150 rpm for 5 min at 4 °C, discard supernatant and resuspend cells with 10 ml cell isolation medium.
7. Pass cells through 60 μm pore size nylon mesh to remove cell aggregations and splenic capsule.
8. After counting cell numbers, enrich T cells using T cell enrichment column according to the manufacturer's instruction.
9. Prepare a cocktail mix of antibodies by adding FITC-CD4 antibody at 12 μl/1×10^7 cells and PE-CD45RB antibody at 5 μl/1×10^7 cells into cold sorting buffer (100 μl/1×10^7 cells). Sterilize with a 0.22 μm pore size syringe filter unit.
10. After washing cells from T cell enrichment step with cold sorting buffer, discard supernatant and resuspend cells with the antibody mix prepared.
11. Incubate cells on ice in the dark for 20 min.
12. Wash cells with sorting buffer twice and resuspend cells with appropriate volume of sorting buffer (the concentration of cells will depend on the sorting machine used).
13. Sort cells into $CD4^+$ $CD45RB^{hi}$ under FACS sorter.
14. After washing cells, resuspend the sorted cells with cold DPBS at 5×10^5 cells/200–300 μl/recipient mouse.
15. Measure body weight of recipient RAG1 knockout mouse.
16. Inject 5×10^5 cells/200–300 μl intravenously into RAG1 knockout mouse through tail vein (*see* **Note 3**).

17. Monitor the body weight change twice a week. Body weight loss has been widely used to monitor the development of colitis in this model [20]. Almost 100 % of recipient mice will develop colitis within 8 weeks after cell transfer if viable, pure $CD4^+$ $CD45RB^{hi}$ T cells are successfully transferred into recipients.

3.2 Self-Limiting Colitis Model (See Note 2)

1. Resolve the desired concentration (v/v) of DSS in distilled water and sterile using 0.22 μm filter system (*see* **Note 4**).
2. Measure body weight of C57BL/6 mouse (*see* **Note 5**).
3. Replace normal drinking water with DSS water (200 ml for 4–5 mice). DSS concentration and treatment period should be predetermined based on the purpose of study (*see* **Note 6**).
4. Monitor body weight every day.
5. After end of DSS treatment, change DSS water to normal drinking water. The body weight will still decrease within few days after termination of DSS treatment, and it will gradually gain afterward (this is a sign of wound healing).
6. Euthanize the mouse by CO_2 inhalation.
7. Cut off colon from ileocecal junction and anus regions.
8. Measure the length of colon. Severity of DSS colitis is well correlated with the shortening of colon (Fig. 1a).

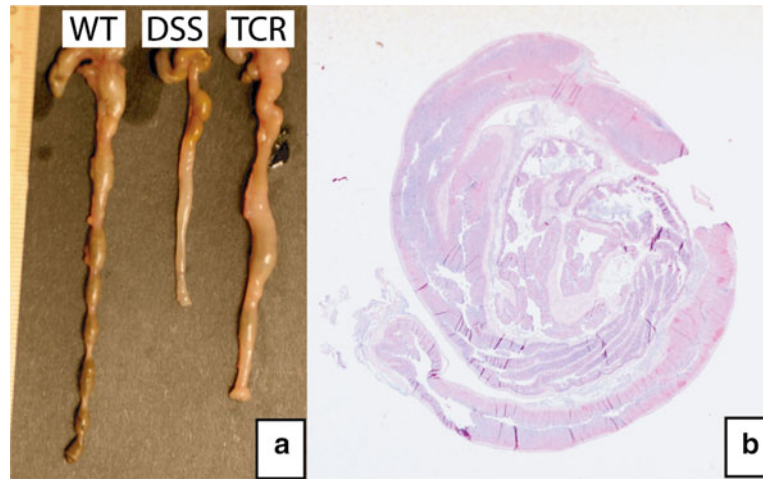

Fig. 1 (**a**) Macroscopic findings of normal colon from WT mice (WT), acutely inflamed colon from DSS model (DSS), and chronically inflamed colon from a chronic colitis model of T cell receptor α knockout mice (TCR) are shown. (**b**) Histology of "Swiss Roll" colon made from DSS model is shown. Normal colon is characterized by the appearance of beaded formation (presence of normal stool pellets). Inflamed colon of DSS model is characterized by shortened colonic length. Inflamed colon in chronic colitis models is generally characterized by the enlargement of colonic diameter due to increased thickness of colonic wall

9. Flush PBS into colonic lumen to remove luminal contents and then flush 300 μl of 10 % formalin.
10. Immediately make a colon "Swiss roll" on empty 10 cm petri dish (*see* **Note 7**). Spray 10 % formalin around the colon and put 2.5 cm petri dish as a weight on the colon (this needs to preserve the Swiss roll formation) for 1 h.
11. Remove 2.5 cm petri dish and add 10 % formalin into 10 cm petri dish until entire colon is soaked. After 2 days of fixation under this condition, the Swiss rolled colon can be subjected to H&E staining (Fig. 1b).

3.3 Isolation of Colonic Lamina Propria Lymphocytes (T Cells and B Cells)

1. Euthanize mouse by CO_2 inhalation.
2. Open the chest and draw blood by cutting heart to minimize the contamination of circulating lymphocytes and red blood cells to colonic lamina propria lymphocytes.
3. Fill 10 ml syringe/27G needle with DPBS. Insert 27G needle directly into colonic lumen from the colonic wall around ileocecal area, and hold the colonic wall and needle altogether with forceps to prevent the backward flow of DPBS into small intestine. To remove luminal contents, flush DPBS into colonic lumen until full excretion of stool can be observed.
4. Take out colon from abdominal cavity.
5. Invert colon (inside out) by using curved and straight Jewelers style forceps. To do so, insert a curved forceps carefully into lumen from one edge of colon (edge #1) until the tip of forceps reaches another edge (edge #2) (Fig. 2a). Open the inserted curved forceps slightly and force the colonic edge #2 into the open area of curved forceps by using the tip of straight forceps (Fig. 2b, allow). Close curved forceps to allow it to grasp the colonic edge #2 tightly. Push out colon from colonic edge #1 to edge #2 using straight forceps to turn the colon inside out (Fig. 2c).
6. Wash the inverted colon carefully in DPBS.
7. Transfer the colon to a 50 ml tube containing 20 ml cell isolation solution #1.
8. Incubate under constant rotary shaking at 100 rpm for 30 min at 37 °C. Vortex briefly every 10 min. For the shaking, we have used a bacterial shaker.
9. Take colon from the tube, place it on a Tendersorb, and roll it on the Tendersorb with a surgical knife to remove epithelial layer (Fig. 2d).
10. Transfer the colon onto a petri dish and cut into tiny pieces using two surgical knives (Fig. 2e). Do not use any buffers in this step to fully break the tissue into pieces because floating tissues on buffer will be difficult to cut. To prevent the drying

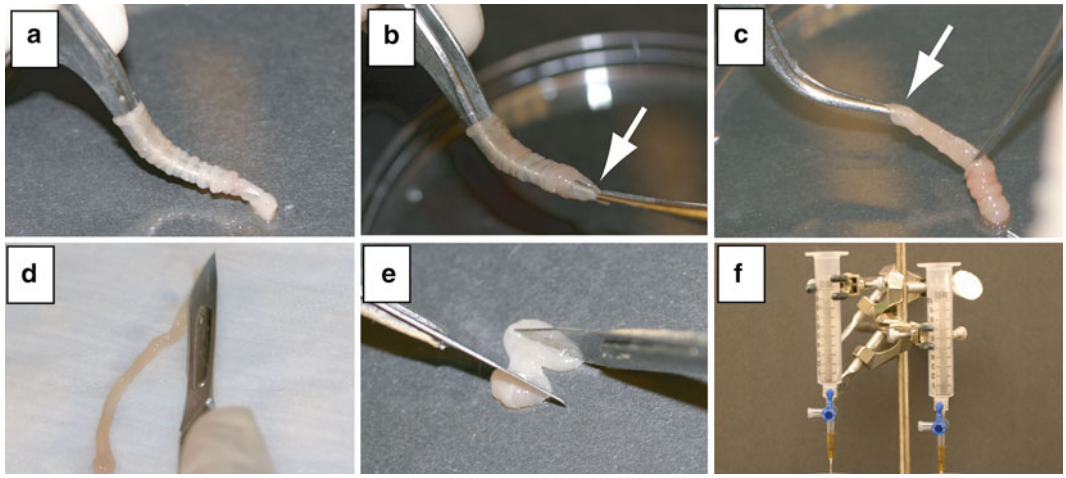

Fig. 2 Procedure to handle colon. (**a**) Curved Jewelers style forceps was inserted into colonic lumen from one open edge until the tip of forceps reached another open edge. (**b**) The tissue at open edge was forced into the open area of curved Jewelers style forceps (*arrow*) by using the tip of straight Jewelers style forceps. (**c**) After grasping the colonic edge by Curved Jewelers style forceps (*arrow*), the colon was inverted to inside out. (**d**) Epithelial layer was removed by rolling the inside-out colon on Tendersorb using a surgical blade. (**e**) Colonic tissue was cut into tiny pieces by using two surgical blades. (**f**) Glass wool column was set on a stand

of tissue, this step should be done very quickly (approximately within 1 min).

11. Collect the colonic pieces using 25 ml of cell isolation solution #2, and transfer into 50 ml tube.
12. Digest the colon under constant rotary shaking at 210 rpm for 30 min at 37 °C. Vortex briefly every 10 min.
13. At the end of incubation, vortex vigorously for 30 s and centrifuge at 1,500 rpm for 5 min at room temperature.
14. Discard the supernatant, resuspend cells with 10 ml room temperature isolation medium, and transfer into 15 ml tube.
15. Centrifuge at 1,500 rpm for 5 min at room temperature. Repeat the wash one more time. Since exposure of cells to cold temperature significantly reduces the efficacy of next step to remove potentially adherent cells, this step should be done at room temperature.
16. During the wash step above, prepare glass wool column. Attach a stopcock to a glass wool column and then connect 19G needle to the stopcock. Set the glass wool column to the stand and wash the column with RT-DPBS (Fig. 2f).
17. Add 10 ml RT isolation medium, and close stopcock to soak the glass column with the isolation medium.
18. Discard the supernatant from **step 14**, resuspend cells with 5 ml of RT cell isolation medium, and vortex vigorously for several seconds to make single cell suspension.

19. Add cells onto the glass wool column and slightly open stopcock to allow slow drain. Collect the drain in 50 ml tube.
20. Add cell isolation medium continuously onto the column until 25 ml is collected. After collecting, place the tube on ice for 30 min (*see* **Note 8**).
21. Centrifuge at 1,500 rpm for 5 min at 4 °C. Afterwards, discard the supernatant, resuspend cells with 10 ml of cold isolation medium, and centrifuge again.
22. Place 3 ml of 72 % Percoll solution into 15 ml conical tube.
23. Discard supernatant from **step 17** and resuspend cells well with 4 ml of 45 % Percoll solution.
24. Overlay the cell suspension on the top of 72 % Percoll solution carefully.
25. Centrifuge at 2,100 rpm for 15 min at 4 °C with no brake.
26. Discard approximately 1 ml of top layer (this top layer contains damaged/aggregated epithelial cells) and collect the interfacial white layer between 45 and 72 % Percoll layer carefully using 1 ml serological pipet.
27. Transfer the cells to 15 ml tube containing 10 ml cold cell isolation medium.
28. Wash cells twice with cold cell isolation medium.
29. After final wash, the cells, which contain T cells and B cells mainly [19, 21], can be used for any experiments that have been widely used in non-intestinal cells (*see* **Notes 9** and **10**). Since expansion and homing of lymphocytes are significantly enhanced in the context of inflammation, the yield of isolated cells will depend on the severity of colitis (*see* **Notes 11** and **12**).

3.4 Isolation of Colonic Epithelial Crypts

1. Anesthetize mice by inhalation of isoflurane. To do so, put surgical sponge into 14 ml round-bottom tube (BD Falcon #352059) and absorb 0.5 ml of isoflurane into the sponge. Hold mouse by a single hand and mask the nose with the isoflurane-containing tube. Confirm there is no response of mouse by tail pinch and other stimuli. This procedure should be done in a safety cabinet to prevent the exposure of operator to isoflurane.
2. Open abdomen carefully without damaging recognizable blood vessels. Major bleeding would significantly reduce the efficiency of following systemic perfusion.
3. As shown in Subheading 3.3, **step 3**, flush colonic lumen with pre-warmed DPBS to remove luminal contents.
4. Open thoracic cavity by making left parasagittal thoracic incision.
5. Cut the tip of catheter at a 45° angle. The 45° angled catheter tip, like needle, can be directly inserted into the heart.

6. Fill 50 ml syringe with pre-warmed 30 mM EDTA/HBSS and connect the catheter to the syringe.
7. Remove air from catheter and syringe and place syringe on Syringe pump and set the speed at 10 ml/min.
8. Hold heart gently with forceps and insert 45° angled catheter tip directly into the left ventricle. The left versus right ventricles can be distinguished by the difference in color darkness.
9. Start perfusion with 15 ml of 30 mM EDTA/HBSS at 10 ml/min.
10. Cut portal vein a few seconds after initiation of perfusion to allow the drain of excess body fluids. The success of this method fully depends on this step (*see* **Note 13**).
11. Fill a 10 cm petri dish with cold 2 mM EDTA/HBSS and place it on ice.
12. Take colon out from abdominal cavity and wash gently in the petri dish.
13. As described in Subheading 3.3, **step 5**, invert colon (inside out) by using curved and straight Jewelers style forceps.
14. Cut the colon into approximately 1 cm length pieces.
15. Prepare 1.8 ml microtubes filled with cold 2 mM EDTA/HBSS and keep them on ice.
16. Put colon pieces into the tube and place it on Mini-Beadbeater.
17. Shake the tube at 2,500 rpm for 30 s.
18. Take colonic tissues out from the tube, and place the tube on ice to allow epithelial crypts to settle to the bottom.
19. Aspirate the supernatant carefully, and add new 2 mM EDTA/HBSS. Repeat this step twice for washing purpose.

Through this method, epithelial cells can be isolated as crypt units (Fig. 3, left panel). The crypt units can be used for a short-term in vitro stimulation and other assays that are required for the study of galectins [22, 23] (*see* **Note 14**).

3.5 Isolation of Large Cells (e.g., Macrophages and Fibroblasts) from the Colon

The method described in Subheading 3.3 is useful to enrich lymphocyte population including T and B cells. In contrast, the steps necessary for depletion of epithelial cells also remove some large cells with adherent capability such as macrophages, dendritic cells, and fibroblasts. Therefore, we have used the method described in this section when such large cells are required for our study [24].

1. Anesthetize mouse by inhalation of isoflurane.
2. Open abdomen carefully without damaging recognizable blood vessels.

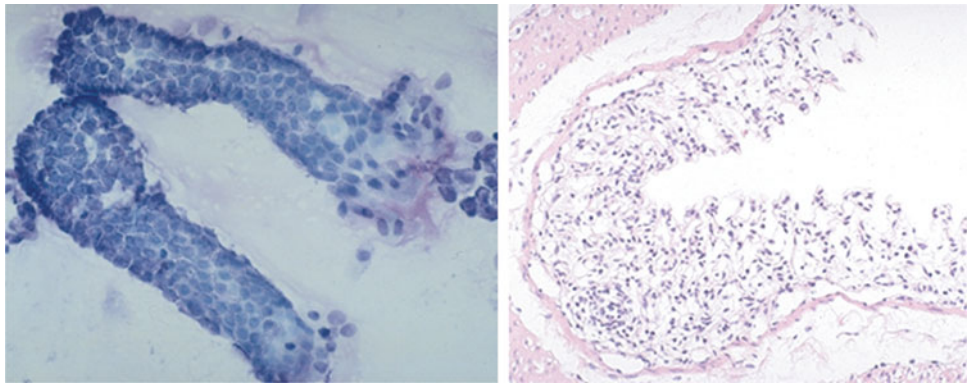

Fig. 3 Epithelial crypt units (*left panel*) and colonic tissues after extraction of epithelial crypts (*right panel*). After 30 mM EDTA perfusion, epithelial cells can be extracted as crypt units (*left panel*) and lamina propria and muscular layers remain in the colonic tissue (*right panel*)

3. As shown in Subheading 3.3, **step 3**, flush colonic lumen with pre-warmed DPBS to remove luminal contents.
4. Open thoracic cavity by making left parasagittal thoracic incision.
5. Cut the tip of catheter at a 45° angle. Fill 50 ml syringe with pre-warmed 30 mM EDTA/HBSS and connect the catheter to the syringe.
6. After removal of air from catheter and syringe, place syringe on Syringe pump.
7. Hold heart gently by a forceps and insert 45° angled catheter tip into the left ventricle.
8. Start perfusion with 15 ml of 30 mM EDTA/HBSS at 10 ml/min.
9. Cut portal vein at few seconds after initiation of perfusion. Please *see* Subheading 3.4, **step 5**, for more detail.
10. Take colon out from abdominal cavity. As described in Subheading 3.3, **step 5**, invert colon (inside out) by using curved and straight Jewelers style forceps.
11. Cut the colon into approximately 1 cm length pieces.
12. Put the colon pieces into a 1.8 ml microtube filled with cold 2 mM EDTA/HBSS.
13. Place tube on Mini-Beadbeater and shake the tube at 5,000 rpm for 1 min.
14. Take colonic tissues from the tube and roll the colon on the surgical sponge to completely remove epithelial cells from the tissue. After this step, the majority of epithelial cells should be removed from the tissues (Fig. 3, right panel).

15. Transfer the tissue into 50 ml tube containing 25 ml of 10 % medium and incubate at room temperature for 30 min (*see* **Note 15**).
16. Take colonic tissues out from the tube and cut into tiny pieces with two surgical knives as shown in Subheading 3.3, **step 10**.
17. Transfer the pieces into 50 ml tube containing 20 ml of pre-warmed cell isolation solution #3.
18. Digest the colonic tissues under constant rotary shaking at 210 rpm at 37 °C for 30 min. The incubation time depends on the thickness of colon. Occasional vortex is needed during the incubation.
19. Centrifuge at 1,500 rpm for 5 min at RT.
20. Discard the supernatant and resuspend the digested tissues with 15 ml of pre-warmed 5 mM EDTA/HBSS.
21. Incubate with constant rotation (210 rpm) at 37 °C for 20 min to make single cell suspension.
22. Pour all contents from the tube into 60 μm pore size nylon mesh to remove undigested tissues.
23. Collect the drain into 15 ml tube and centrifuge at 1,500 rpm for 5 min at 4 °C.
24. Discard the supernatant and resuspend the cells with 15 ml of cold cell isolation medium. Centrifuge at 1,500 rpm for 5 min at 4 °C. Repeat this step twice. Final cell pellet contains enriched large cells as shown in Fig. 4 (*see* **Note 16**).

3.6 Isolation of Appendix Lymphoid Cells

1. Euthanize mouse by CO_2 inhalation.
2. Open the chest and draw blood by cutting heart to minimize the contamination of circulating lymphocytes and red blood cells to appendix lymphoid follicle.
3. Open abdomen and find the cecum. Unlike humans, in which cecum is located in bottom left of abdomen, cecum in mouse may be anywhere in the abdominal cavity.
4. Find the appendix lymphoid follicle that can be recognized as a white ellipse structure around the tip (blunt end) of cecum.
5. Hold appendix follicle along the bottom edge by using Curved Jewelers style forceps.
6. Make incision carefully along the Curved Jewelers style forceps using the tip of a surgical blade. After making the incision, the appendix follicle can be extracted from the cecum tissue.
7. Put two drops of cold cell isolation medium onto 10 cm petri dish that is placed on ice.
8. Place extracted follicle into the medium.
9. Secure the follicle using a 27G needle to prevent the movement. Loosen the follicle by using another 27G needle. The

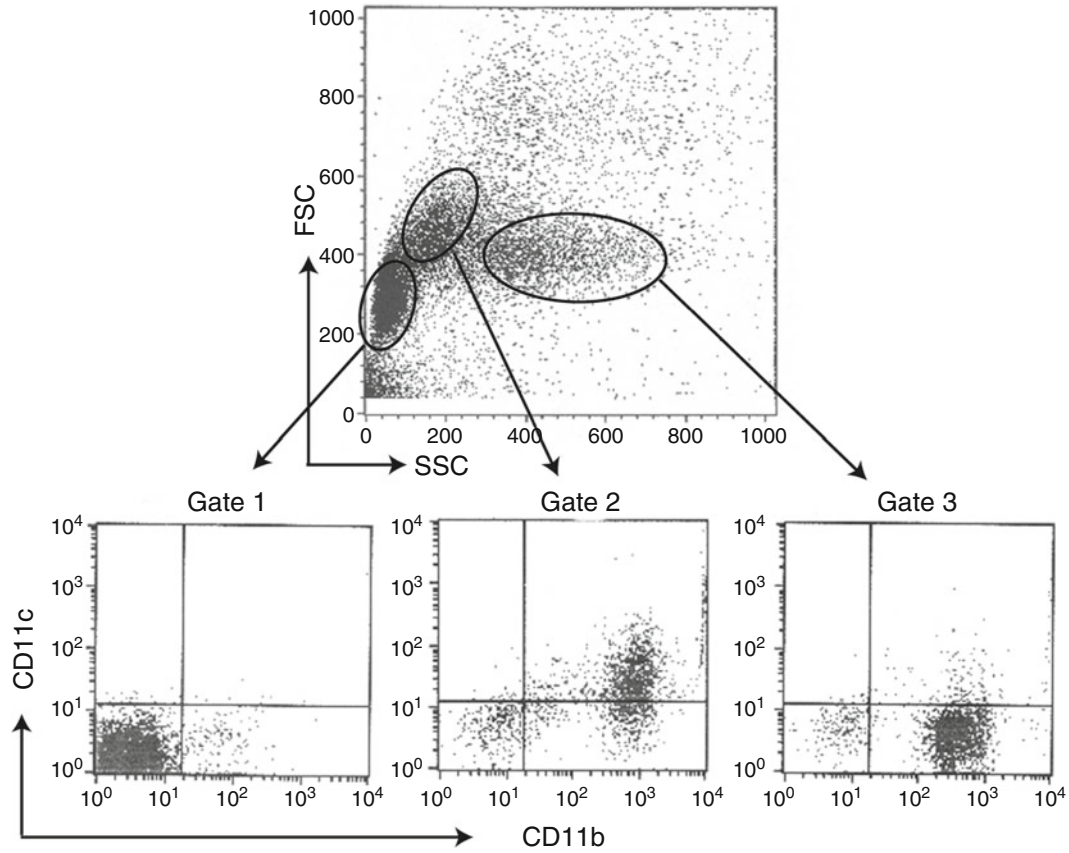

Fig. 4 Cytogram of colonic cells isolated using large cell isolation protocol is shown in *upper panel*. Expression patterns of CD11c versus CD11b are shown using different gates in the cytogram

medium color will be changed from pink transparent to opaque white due to release of cells from the follicle into the medium.

10. Collect cells and transfer to 3 ml FACS tube. Vortex well.
11. Wash one layer glass wool column with 3 ml of cold cell isolation medium.
12. Pour the cells from **step 10** into the glass wool column and collect the drain into 3 ml FACS tube.
13. Centrifuge at 1,500 rpm for 5 min at 4 °C. Use the pellet as appendix lymphoid cells [25] (*see* **Note 17**).

4 Notes

1. Do not tightly pack glass wool. Autoclave and keep at room temperature. Per your request, we will be able to provide the sample glass columns.

2. There are currently more than 66 different mouse models available to use for studying IBD [12]. Each model has its specific advantage(s) and disadvantage(s) over other models [12]. Of note, none of the models can fully reflect human IBD. Therefore, it is important for the investigator to select appropriate colitis model(s) for his/her intended purpose of research and also confirm the outcome using other colitis model(s). Among the models described in this chapter, CD45RB model has been widely used to study adaptive immune mechanism underlying Crohn's disease [2, 20]. Alternatively, DSS model has allowed investigators to closely examine the wound healing process from intestinal damage due to the nature of self-limiting inflammation [12].
3. For CD45RB model, intravenous, rather than intraperitoneal, injection of cells tends to generate more reproducible results. Purified $CD4^+$ $CD45RB^{high}$ T cells should be brought on ice to mouse's procedure room to preserve the viability of cells. Immediately before injection, the cells should be suspended well and transferred into tuberculin (1 ml) syringe to prevent the aggregation of cells—cell aggregation may cause the death of mouse during the intravenous injection. To transfer of cells from tube to syringe, cells should be aspirated directly into syringe "without" needle. The aspiration of cells through 27G needle has a risk to damage cells. To minimize such risk, use 27G needle only for injection of cells.
4. There is still a controversial issue as to whether DSS can be resolved in distilled water or drinking (tap) water. Since biological activity of DSS is impaired by a long-term exposure to acidic pH [26], we have used pH 7 distilled water for resolving DSS. Since drinking water in many animal facilities has been acidified to prevent the contamination of bacteria, each investigator needs to check the drinking water that is used for resolving DSS.
5. Susceptibility to DSS-induced colitis fully depends on the mouse strain. For example, C3H/HeJ mice are highly susceptible to this colitis model as compared to other strains of mice such as C57BL/6 [27]. In addition, males tend to be more susceptible than females [27]. In our experience, variations in the data (e.g., body weight change) tend to minimize when females C57BL/6 are used. In addition, to further minimize the variations of data, we have selected mice that exhibited similar degree of body weight loss at the end of DSS treatment and then used only the selected mice for the evaluation of their wound healing [18].
6. For DSS colitis model, some groups have used a short-term treatment (4 days) with high concentration (3.5–4 %) of DSS, and others have used a long-term treatment (7–8 days) with low

concentration (1.5–2 %). For the long-term treatment, DSS water needs to be replaced with a new one every 3 days [28].

7. Since severity of DSS colitis considerably varies from area to area within a same individual colon [12], histological evaluation of entire colon using the Swiss roll system is highly recommended [22, 29]. In contrast, colonic wall of chronic colitis models generally becomes much thicker (Fig. 1a), hampering to make such Swiss roll. Therefore, both macroscopic (for evaluation of disease expansion within an individual colon) and microscopic examinations would be necessary for chronic colitis models.
8. This cooling step is necessary to stabilize the cell density, allowing the successful separation of Low (epithelial cells), intermediate (lymphocytes), versus high (apoptotic cells) density cell populations in next step.
9. The enriched intestinal lymphocytes can be applied to any types of galectin studies such as galectin binding assay, lectin blot, immunoprecipitation with galectin, and in vitro stimulation with galectins [18, 19]. In addition, majority of methods described in this "Protocols in Galectin Biology" will be applicable to the intestinal cells isolated.
10. Collagenase digestion may have a potential risk to reduce the expression level of some surface molecules. Although we have confirmed that majority of colonic T and B cell-associated classical surface molecules, except CD23, are unaffected by collagenase digestion, it would be strongly recommended that each investigator confirms his/her data from flow cytometric analysis by an in situ assay such as immunofluorescent or immunohistochemical analysis.
11. The yield of isolated colonic cells fully depends on the type and severity of colitis. In general, $1–3 \times 10^7$ cells will be obtained from an inflamed colon of Th1/Th17-mediated chronic colitis models such as CD45RB model and IL-10 knockout mouse. The yield is reduced to $0.5–1.5 \times 10^7$ cells when Th2-mediated chronic colitis model such as T cell receptor α knockout mouse is used. In contrast, only 0.1×10^7 cells can be obtained from a normal colon of WT mouse. Therefore, it will be necessary to pool approximately 10–15 WT mice for a sample particularly when cell sorting is planned for subsequent experiment. After obtaining a great deal of skill with this protocol, the absolute number of isolated cells will be closely correlated with the severity of colitis that is assessed by histological examination [30].
12. As shown in the FACS panels (Fig. 5), both percoll and glass wool steps are necessary to fully enrich the lymphocyte population by minimizing the contamination of myeloid cells and removing epithelial cells and dead cells, both of which often

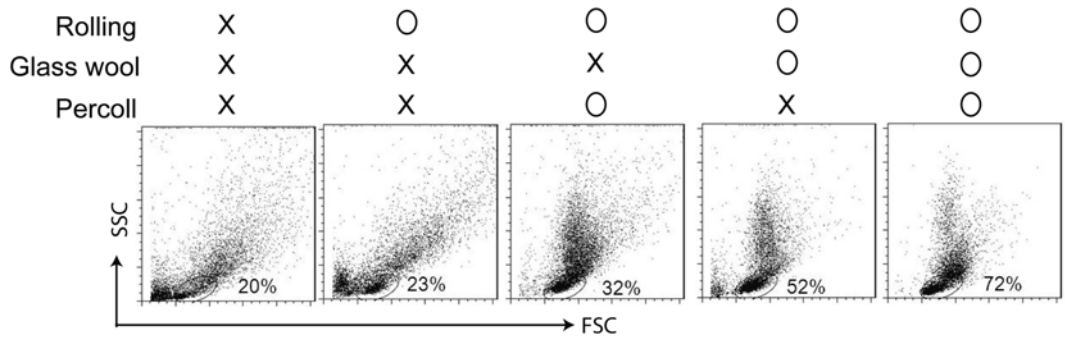

Fig. 5 Cytogram of isolated colonic lamina propria cells. The cytograms are shown using the lymphocyte isolation protocol with omission (X) of colon-rolling, glass wool, and/or percoll step. The *number* indicates the percentage of lymphocytes in total cells isolated

cause nonspecific signals in surface phenotype analysis (this would also reduce the purity of target cells when the isolated cells are subjected to cell sorting). Glass wool step is required to fully avoid the contamination of epithelial cells, and percoll step is necessary particularly to remove dead cells. Therefore, it is possible to omit one or more steps depending upon the objective of experiment, but we highly recommend all steps to be included for the study focusing on T and/or B cells.

13. If tip of 45° angled catheter is correctly placed in left ventricle, EDTA will be circulated through systemic circulation. In this case, muscle cramps will be observed throughout entire body. This is a sign of success of perfusion. If tip of 45° angled catheter is wrongly placed in right ventricle, it will induce pulmonary circulation of EDTA, resulting in the failure of epithelial cell isolation. In this case, swelling and color change (from pink to white) of lungs will be seen shortly after initiation of perfusion.
14. The epithelial cells, which are isolated as crypt units, can be used for in vitro functional assays [22]. A concern with this method is a minor contamination of interepithelial lymphocytes (IELs) in the isolated crypt. We have confirmed that CD3 signal was detectable in the crypt when sensitive methods such as PCR amplification were used. In contrast, CD3 signal was undetectable when nonsensitive methods such as RNA protection assay were used. Therefore, additional negative selection using anti-CD3 mAb to remove IELs [24] may be necessary depending on the purpose of experiment. To do so, single cell suspension should be made from the epithelial crypt units by an enzymatic treatment such as collagenase [23]. However, the further purified epithelial cells cannot be used for any functional assays because apoptosis is induced immediately after single cell suspension is made from the crypt unit.

To overcome the problem, utilization of colonic epithelial stem cells may be helpful [31].

15. This step is necessary to inactivate EDTA and supply Ca^{2+} that needs for the enzymatic activity in the next step.
16. As shown in gate 1 of Fig. 4, lymphocytes can be obtained from inflamed (thickened) colon using the large cell isolation method. However, it would be important to mention that this method does not work well for lymphocyte isolation when normal (thin) colon is used presumably due to the damage of lymphocytes (T and B cells) by a combination of 30 mM EDTA exposure and collagenase digestion.
17. The isolation method described in Subheading 3.6 can be applied to isolate "lymphoid follicular" cells from macroscopically recognizable patches such as appendix lymphoid follicles and small intestinal Peyer's patches [26]. If cells from "T cell zone" are needed, an additional short-term incubation with 0.5 mg/ml collagenase will be required. The yield of isolated cells ranges from 0.5×10^6 to 8×10^6 cells/a follicle depending on the size of patch. There are other organized lymphoid structures in the colon such as colonic patches, isolated lymphoid follicles, and crypt patches [32, 33]. Since our method is applicable only to macroscopically recognizable follicles, isolation of cells from crypt patches and isolated lymphoid follicles should be done using other methods [32, 33]. Interestingly, some colonic patches may be macroscopically recognizable in BALB/c, but not C57BL/6, mouse strain.

Acknowledgements

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Chapter 16

Study of Galectins in Tumor Immunity: Strategies and Methods

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Abstract

During the past decade, a better understanding of the cellular and molecular mechanisms underlying tumor immunity has provided the appropriate framework for the development of therapeutic strategies for cancer immunotherapy. Under this complex scenario, galectins have emerged as promising molecular targets for cancer therapy responsible of creating immunosuppressive microenvironments at sites of tumor growth and metastasis. Galectins, expressed in tumor, stromal, and endothelial cells, contribute to thwart the development of immune responses by favoring the expansion of T regulatory cells and contributing to their immunosuppressive activity, driving the differentiation of tolerogenic dendritic cells, limiting T cell viability, and maintaining T cell anergy. The emerging data promise a future scenario in which the selective blockade of individual members of the galectin family, either alone or in combination with other therapeutic regimens, will contribute to halt tumor progression by counteracting tumor-immune escape. Here we describe a selection of methods used to investigate the role of galectin-1 in tumor-immune escape.

Key words Galectin, Tumor immunity, Immunosuppressive, Tumor growth, Metastasis, T regulatory cells, Tolerogenic dendritic cells

1 Introduction

Despite major advances in understanding the mechanisms leading to tumor immunity, a number of obstacles hinder the successful translation of mechanistic insights into effective cancer immunotherapy [1]. Such obstacles include the ability of tumors to display multiple immunosuppressive mechanisms to avoid immune recognition or to disarm effector T cell function [2]. These mechanisms involve alterations of the antigen presentation machinery; secretion of immunosuppressive cytokines such as transforming growth factor-β (TGF-β) and interleukin-10 (IL-10);

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expression of inhibitory molecules such as programmed death ligand-1 (PD-L1), cytotoxic T-lymphocyte antigen-4 (CTLA-4), and indoleamine 2,3 dioxygenase (IDO); and specific recruitment of regulatory cell populations including FoxP3$^+$ T regulatory (Treg) cells, IL-10-producing type-1 T regulatory (Tr1) cells, tolerogenic dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs), and M2-type macrophages [2].

The prominent immunological phenotypes observed upon disruption of genes encoding components of the glycosylation machinery, including glycosyltransferases, glycosidases, and lectins, reflect the central role played by glycosylation in the control of immune tolerance and inflammation. In fact, glycosylation regulates a variety of immune cell processes including immune cell activation, differentiation, homing, and survival [3, 4]. In addition, aberrant expression of glycans during the transition from normal to inflamed or neoplastic tissue provides a vast potential for information display [5]. Although these alterations have been mostly documented in cancer cells during tumor progression, cell surface glycosylation is also dramatically altered in the tumor microenvironment, particularly in stromal and immune cell compartments. Endogenous glycan-binding proteins, including C-type lectins, siglecs, and galectins, can selectively recognize neo-glycoepitopes and convey this structural information into functional cellular responses, including the modulation of immunological and vascular signaling programs [6].

Galectins show prominent expression in inflammatory and tumor microenvironments [7]. Through regulation of cellular signaling programs, galectin–glycan interactions provide "on-and-off" signals that control the decisions between immune cell responsiveness and tolerance. Particularly, galectin-1 suppresses chronic inflammation, blunts Th1 and Th17 responses, and skews the immune response toward a Th2 profile [8]. In addition, this lectin instructs DCs to become tolerogenic [9], induces alternatively activated "M2-type" macrophages and microglia [10, 11], inhibits T cell trafficking [12, 13], and favors the expansion of FoxP3$^+$ Treg and FoxP3(−) Tr1 cells [14, 15] further limiting the magnitude of an effective immune response.

Galectin-1 expression correlates with tumor burden and adverse clinical features in several tumor types including laryngeal squamous cell carcinoma [16], prostate adenocarcinoma [17], colon adenocarcinoma [18, 19], ovarian carcinoma [20, 21], breast carcinoma [14, 22], melanoma [23], Hodgkin lymphoma [24, 25], cervical cancer [26], T cell lymphoma [27], pancreatic ductal adenocarcinoma [28], neuroblastoma [29], hepatocellular carcinoma [30, 31], chronic lymphocytic leukemia [32], glioblastomas [33, 34], MLL-rearranged B lymphoblastic leukemias [35], and thyroid carcinoma [36].

Through galectin-1-driven inhibitory mechanisms, cancer cells can evade and thwart immune attack [37]. In several tumors

galectin-1 selectively blunts tumor-specific T cell responses through modulation of T cell apoptosis and skewing of the cytokine balance toward a Th2 profile [23, 28, 29, 38, 39]. Furthermore, in breast adenocarcinoma, this lectin favors the differentiation and recruitment of FoxP3$^+$ Treg cells [14] or modulates the survival of effector T cells [38]. Moreover, galectin-1 promotes the differentiation of tolerogenic DCs in settings of melanoma, lung adenocarcinoma, and neuroblastoma [9, 29, 40]. Although we will focus here on galectin-1, it should be mentioned that other galectins, including galectin-3 and galectin-9, also influence tumor-immune escape mechanisms including T cell apoptosis, T cell anergy, NK cell activation, and expansion of myeloid-derived suppressor cells [41–44]. Thus, the spatiotemporal regulation of different galectins in conjunction with other immune escape mechanisms will dictate the decisions between immune cell responsiveness and tolerance in tumor microenvironments. Here we describe a selection of methods used to study the role of galectins, particularly galectin-1, in tumor immunity.

2 Materials

2.1 Methods to Study the Regulatory T Cell Compartment in the Tumor Microenvironment

2.1.1 Collection of Tumor Tissue, Draining Lymph Nodes, and Spleen from Tumor-Bearing Mice

1. 8- to 12-week-old Balb/c and C57Bl/6 tumor-bearing mice.
2. RPMI 1640 (GIBCO).
3. 1 ml syringe (Neojet).
4. Sterile scissors.
5. P60 Petri dishes (GBO).
6. Sterile 70 μm filter (BD Pharmingen).
7. Phosphate buffered saline (PBS): 136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
8. Sterile red blood lysis buffer (ACK buffer): 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in distilled H_2O.
9. Frosted microscope slides (BioTraza).
10. 15 ml conical tubes (BD Pharmingen)
11. FACS buffer I (PBS with 0.1 % BSA and 2 mM EDTA).

2.1.2 Staining and Purification of CD4$^+$ Treg Cells, Naïve T Cells, and Responder T Cells

1. Allophycocyanin (APC)-conjugated CD4 antibody (clone GK1.5), Alexa Fluor 488-conjugated CD25 antibody (clone PC61.5), Phycoerythrin (PE)-conjugated CD62L antibody (clone MEL-14), PE-conjugated Foxp3 antibody (clone FJK-16s) (all from eBiosciences).
2. Fix/Perm buffer (eBiosciences).
3. Permeabilization Buffer 10× (eBiosciences).
4. Dynal® Mouse CD4 Cell Negative Isolation Kit (Invitrogen).

5. Heat-inactivated fetal bovine serum (Gibco, FBS).
6. FACS buffer I: PBS with 0.1 % BSA and 2 mM EDTA.
7. Sorted cells collection medium: RPMI 1640 supplemented with 20 % FBS.
8. 15 ml conical tubes.
9. PBS (136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4).
10. Mouse spleen.
11. FACSaria sorter

2.1.3 Coating of 24- and 96-Well Plates with Anti-CD3 Antibodies

1. 96- and 24-well round bottom plates (GBO).
2. Purified NA/LE hamster anti-mouse CD3ε monoclonal antibody (clone 145-2C11, BD Pharmingen).
3. Phosphate buffered saline (PBS): 136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
4. Humidified incubator at 37 °C.

2.1.4 Differentiation of Treg Cells In Vitro in the Presence of Conditioned Media

1. Recombinant TGF-β (R&D Systems) diluted in phosphate buffered saline (PBS), pH 7.4 (30 μg/ml).
2. Incubator at 5 % CO_2 and 37 °C.
3. Recombinant IL-2 (R&D Systems) in PBS (10 μg/ml).
4. RPMI 1640 supplemented with 50 μM β-mercaptoethanol and antibiotic-antimycotic (Invitrogen).
5. Gal-1 wild type (WT) or Gal-1 knockdown (shRNA-Gal-1) tumor cells.
6. P60 dishes (GBO).
7. Twenty-four well plates (GBO).
8. 0.22 μm syringe filter (Millipore).
9. Naïve T cells.
10. Purified NA/LE Hamster anti-mouse CD28 monoclonal antibody (clone 37.51, BD Pharmingen).
11. Twenty-four well plates coated with anti-mouse CD3 monoclonal antibody (*see* **Note 1**).

2.1.5 Purification of Tumor-Associated Treg Cells and $CD3^+$ Responder T Cells (Tresp)

1. 8- to 12-week-old Balb/c and C57Bl/6 tumor-bearing and tumor-free mice.
2. RPMI 1640 (GIBCO) supplemented with 20 % FBS.
3. 5 ml polystyrene tubes.
4. 15 ml conical tubes (BD Pharmingen).
5. Allophycocyanin (APC)-conjugated CD4 antibody (clone GK1.5), Alexa Fluor 488-conjugated CD25 antibody (clone PC61.5), Phycoerythrin Cyanine-7 (PECy7)-conjugated FR4

antibody (clone eBio12A5), Fluorescein Isothiocyanate (FITC)-conjugated CD3 antibody (clone 145-2C11).

6. Phosphate buffered saline (PBS): 136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
7. FACS buffer I: PBS with 0.1 % BSA and 2 mM EDTA.
8. FACSAria flow cytometer.

2.1.6 Assessment of the Suppressive Activity of Treg Cells

1. Tregs and Tresp purified as described in Subheading 3.1.5.
2. Ninety-six plates coated with anti-CD3 monoclonal antibody (as described in Subheading 2.1.3).
3. Recombinant mIL-2 (R&D Systems) in PBS (10 μg/ml).
4. Purified NA/LE Hamster anti-mouse CD28 monoclonal antibody (clone 37.51, BD Pharmingen).
5. RPMI supplemented with 5 % FBS, 50 μM β-mercaptoethanol, and 1 μg/ml CD28 mAb.
6. Incubator at 5 % CO_2 and 37 °C.
7. [^{3}H]-thymidine solution (PerkinElmer).
8. Direct β-counter.
9. 1 ml syringe (Neojet).
10. Cell harvester.
11. Scintillation liquid (Perkin Elmer).
12. Scintillation vials.
13. Absorbent glass filter paper (3 M).

2.1.7 Adoptive Transfer of Treg Cells

1. Treg cells from the desired source.
2. Phosphate buffered saline (PBS): 136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
3. Mice.
4. Anesthetic.
5. Warm water.
6. Sterile 27G needles.
7. 1 ml syringe (Neojet).

2.1.8 Tumor Antigen-Specific T Cell Proliferation

1. Tumor cell lines (B16, 4T1) as an antigenic source.
2. Liquid nitrogen.
3. 37 °C water bath.
4. Phosphate buffered saline (PBS): 136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
5. RPMI 1640, 10 % FBS supplemented with 50 μM β-mercaptoethanol and an antibiotics-antimycotics.
6. Single cell suspension of mouse spleen and draining lymph nodes.

7. 96-well round bottom plates.
8. [^{3}H]-thymidine solution (PerkinElmer).
9. Direct β-counter.
10. Scintillation liquid (Perkin Elmer).
11. Scintillation vials.
12. Absorbent glass filter paper (3 M).

2.1.9 Cytokine Determination by ELISA

1. DuoSet ELISA Mouse IFN-γ (R&D).
2. BD OptEIA™ Mouse IL-10 ELISA Set (BD Biosciences).
3. BD OptEIA™ Mouse IL-5 ELISA Set (BD Biosciences).

2.2 Study of Galectins in DC Compartment

2.2.1 Differentiation of Bone Marrow-Derived Tolerogenic DCs

1. Complete RPMI (cRPMI): RPMI 1640 (Invitrogen) medium with 10 % heat inactivated fetal bovine serum (FBS) (GIBCO), 40 μg/ml of gentamicin, 50 μM β-mercaptoethanol, 2 mM L-glutamine, and 10 mM HEPES.
2. Recombinant mouse GM-CSF (rGM-CSF) (R&D System).
3. Recombinant human Gal-1 (rGal-1).
4. 8- to 12-week-old C57BL/6 mice.
5. P60 and P100 non-adherent Petri dishes (Greiner-GBO).
6. Sterile red blood lysis buffer (ACK buffer): 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in distilled H_2O.
7. rGal-1 (in-house production) as described [10].
8. Phosphate buffered saline (PBS): 136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
9. Sterile red blood lysis buffer (ACK buffer): 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in distilled H_2O.
10. rGM-CSF (R&D System).
11. 21- or 25-gauge needles (BD PrecisionGlide).
12. Scissors and scalpel.
13. 1 ml syringe (Neojet).

2.2.2 Determination of DC Markers by Flow Cytometry

1. FACS buffer II: PBS (136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4) with 0.1 % FBS (Gibco).
2. PE-conjugated anti-CD11c antibody (clone HL3), PE-conjugated anti-MHC II (I A^b) antibody (clone AF6-120.1), FITC-conjugated anti-CD86 antibody (clone GL1), FITC-conjugated anti-CD45RB antibody (clone 16A).
3. 1.5 ml tubes.

2.2.3 Determination of IL-27 by ELISA

1. ELISA for mouse IL-27 p28 (R&D).

2.2.4 Evaluation of STAT-3 Phosphorylation by Western Blot

1. Dendritic cells.
2. Protein Extraction Buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 10 mM EDTA; 1 % v/v NP-40) with protease and phosphatase inhibitors (Pierce).
3. Bradford reagent.
4. 2× Laemmli sample buffer (BioRad).
5. Amersham Hybond-ECL (GE Healthcare).
6. Tris-buffered saline (TBS): 150 mM NaCl, 50 mM Tris–HCl, pH 7.4.
7. tTBS (TBS with 0.05 % Tween 20).
8. Blocking buffer: tTBS with 5 % nonfat milk or BSA (Sigma).
9. Anti-phospho-STAT3 antibody (Santa Cruz Biotechnology, sc-8059).
10. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Vector Labs).
11. Immobilon chemiluminescent HRP substrate (WBKLS01-00, Millipore).
12. G-Box.

2.3 Profiling Galectin Expression in the Tumor Microenvironment

2.3.1 Galectins Immunostaining in Paraffin Embedded Tissues

1. Rabbit anti-galectin-1 (H-45), anti-galectin-8 (H-80), anti-galectin-3 (H-160), anti-galectin-12 (H-166), and goat anti-galectin-9 (C-20) (Santa Cruz Ref).
2. Saponin (Sigma).
3. PBS (136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4).
4. PBS-0.05 % w/v saponin.
5. Ethanol (Cicarelli).
6. Xylene (Cicarelli).
7. Vectastain Universal Elite ABC Kit (Vector).
8. Normal horse serum.
9. Dako DAB + substrate system (Dako).
10. Giemsa (Sigma).
11. Dako Ultramount Aqueous Permanent Mounting medium (Dako).
12. Cover Glass 24 × 40 mm.
13. H_2O_2 30 % solution (Cicarelli) (stored at 4 °C and protected from light).
14. Wet chamber.
15. ImmEdge Pen (Vector).
16. HRP-Label anti-goat (Sigma).
17. Humidified chamber.

2.4 Lentiviral-Mediated Silencing of Galectin Expression

2.4.1 Silencing Galectin Expression. Lentiviral Production (See **Note 2**)

1. HEK 293 T cell line (ATCC).
2. Vector—(pLVTHM-shRNA).
3. Packaging plasmid—pMD2.G (Addgene).
4. Envelope plasmid—pCMVR8.74 (Addgene).
5. P100 Petri dishes (Greiner, GBO).
6. PBS (136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4).
7. DMEM (Gibco) supplemented with 10 % heat-inactivated FBS (Gibco).
8. 2× HBSS solution (for 500 ml final solution include 8 g NaCl; 0.38 g KCl; 0.1 g Na_2HPO_4; 5 g HEPES; 1 g glucose in 400 ml of bi-distilled water. pH: 7.05–7.12, complete to 500 ml bi-distilled water) (*see* **Note 3**).
9. 2.5 M $CaCl_2$ (Sigma) (in bi-distilled water).
10. Incubator at 5 % CO_2 and 37 °C.
11. Sterile RNAse-free DNAse (Invitrogen) in bi-distilled water.
12. 15 ml conical tubes (BD Falcon).
13. Syringe filters 0.45 μm (MilliPore).

2.4.2 Titration of Lentiviral Vectors and Transduction of Target Cells

1. HEK 293 T cells.
2. 24-well plates.
3. DMEM (Gibco) supplemented with 10 % heat-inactivated FBS (Gibco).
4. Polybrene (4 % solution).
5. Thawed virus solution collected (Subheading 3.4.1).
6. FACSAria flow cytometer.
7. Snap lock 1.5 ml tubes (Axygen).

3 Methods

3.1 Methods to Study the Regulatory T Cell Compartment in the Tumor Microenvironment

3.1.1 Collection of Tumor Tissue, Draining Lymph Nodes, and Spleen from Tumor-Bearing Mice

1. Euthanize tumor-bearing mice and harvest spleen, draining lymph nodes, peripheral lymph nodes, and irrigated tumor tissue.
2. Disrupt the spleen and draining lymph nodes (both axilar and inguinal) with the plunger of a 1 ml syringe against a 70 μm filter in a P60 Petri dish filled with 2 ml RPMI.
3. Cut the tumor tissue with sterile scissors and grind it using the frosted sides of two microscope slides in a P60 Petri dish with 2 ml of RPMI. Filter the suspension with a 70 μm filter. Centrifuge single cell suspensions in 15 ml conical tubes for 8 min at no more than 300 × *g*.

4. Resuspend splenocytes with 5 ml of ACK buffer and incubate for 5 min at RT. Dilute it with PBS and centrifuge for 8 min at no more than $300 \times g$.
5. Resuspend the cell pellets in FACS buffer I or RPMI.

3.1.2 Staining and Purification of CD4+ Treg Cells, Naïve T Cells, and Responder T Cells

1. Staining of CD4 and CD25 molecules is performed for 30 min in the dark at 4 °C. For 2×10^6 cells, use 0.03 μg of APC-conjugated anti-CD4 antibody and 0.075 μg of Alexa Fluor 488-conjugated anti-CD25 antibody in 100 μl of FACS buffer I.
2. Wash cells with PBS and centrifuge for 8 min at no more than $300 \times g$. Fix and permeabilize cells using Fix & Perm buffer in 100 μl for 30 min to 18 h in the dark at 4 °C.
3. Wash cells with Permeabilization Buffer 1×. Foxp3 staining is performed in 100 μl Permeabilization Buffer 1× using 0.225 μg PE-conjugated anti-Foxp3 antibody for 1 h at 4 °C in the dark.
4. Wash cells with Permeabilization buffer 1×, centrifuge for 10 min at $300 \times g$, and resuspend in FACS buffer I.
5. For flow cytometry analysis a two-laser cytometer must be used and five additional tubes containing the appropriate compensation samples should be considered (*see* **Note 4**).
6. For isolation of $CD4^+CD62L^+$ naïve T cells prepare a single cell suspension from mouse spleens.
7. Purification of $CD4^+$ T cells is performed by negative selection using Dynal® Mouse CD4 Cell Negative Isolation Kit (Invitrogen). This procedure is thoroughly detailed in the protocol provided by manufacturer (*see* **Note 5**). Protocol yield is usually 20–25 % of spleen cells.
8. After purification of $CD4^+$ T cells adjust the cell concentration to 4×10^7/ml in FACS buffer I and proceed to CD4 and CD62L surface immunostaining.
9. Use 0.2 μg APC-conjugated anti-CD4 antibody and 0.3 μg PE-conjugated anti-CD62L antibody per 200 μl of lymphocyte suspension. Incubate for 30 min at 4 °C in the dark.
10. Wash cells with FACS buffer I, centrifuge for 8 min at no more than $300 \times g$, and resuspend cell pellet at a concentration of 3×10^7/ml.
11. Using a FACSAria cell sorter proceed with the selection and sorting of the $CD4^+CD62L^{high}$ population (*see* **Note 6**).
12. Use 15 ml conical tubes to collect sorted population with 2.5 ml of collection medium. Prior to use, vortex the tubes (*see* **Note 7**).
13. Keep the sorted population on ice.

3.1.3 Coating of 24- and 96-Well Plates with Anti-CD3 Antibody

1. Prepare a 5 μg/ml solution from the stock of CD3ε antibody (1 mg/ml) in sterile PBS and vortex. For 24-well and 96-well round bottom plates use 150 μl and 40 μl, respectively.
2. Incubate at 37 °C in a humidified atmosphere for at least 2 h.
3. Before use rinse wells with PBS and aspirate twice.

3.1.4 Differentiation of Treg Cells In Vitro in the Presence of Conditioned Media

1. For preparing conditioned media (CM) from wild-type or Gal-1 knockdown tumor cells, plate wild-type (WT) or Gal-1 knockdown tumor cells in P60 dishes at 50 % confluence with 2 ml of serum-free RPMI. Incubate for 18 h at 37 °C with 5 % CO_2 and then collect CM. Filter with 0.22 μm syringe filter, aliquot, and store at −70 °C.
2. The stimuli required for Treg cell differentiation in vitro are TGF-β and IL-2. To assess the role of Gal-1 in Treg cell differentiation, it is important to use a limiting concentration of TGF-β. Adjust the number of naïve T cells to 1×10^6/ml in serum-free RPMI supplemented with 1–2 ng/ml hTGFβ, 100 U/ml mIL-2, 1 μg/ml CD28 mAb, and a combination of antibiotic-antimycotic.
3. Plate 1 ml of a suspension of naïve T cells per well in 24-well plates coated with anti-CD3 monoclonal antibody (obtained in Subheading 3.1.2).
4. Add CM from WT or Gal-1 knockdown tumor cells (*see* **Note 8**).
5. Incubate at 37 °C with 5 % CO_2 for 4 days (*see* **Note 9**).
6. Asses Treg cell frequency by flow cytometry after staining of CD4, CD25, and FoxP3.

3.1.5 Purification of Tumor-Associated Treg Cells and $CD3^+$ Responder T Cells

1. For Treg cell purification, prepare a lymphocyte suspension from the tumor, draining lymph nodes, or spleen collected from Balb/c or C57BL/6 tumor-bearing mice. Adjust lymphocyte number to 4×10^7/ml cells in FACS buffer I.
2. Regulatory T cells are characterized by surface expression of CD4, CD25, and $FR4^{high}$ [45]. Use 0.2 μg of APC-conjugated anti-CD4 antibody, 0.5 μg of Alexa Fluor 488-conjugated anti-CD25 antibody and 0.4 μg of PECy7-conjugated anti-FR4 antibody per 0.2 ml cells. Incubate for 30 min at 4 °C in the dark.
3. Wash cells with FACS buffer I, centrifuge for 8 min at no more than $300 \times g$, and resuspend cell pellet at a concentration of 3×10^7 cells/ml.
4. Using a FACSAria cell sorter, select within the lymphocyte gate the $CD4^+CD25^+FR4^{high}$ population (*see* **Note 10**).
5. Collect in 5 ml polystyrene round bottom tube containing 2 ml collection medium. Vortex tube prior to use (*see* **Note 7**).
6. Keep the sorted population on ice.

7. For T responder cells, prepare a single cell suspension of mouse spleen from tumor-free mice as described (Subheading 3.1.1).
8. Adjust splenocyte concentration to 4×10^7/ml with FACS buffer I and stain for the CD3 surface marker using 0.2 μg of FITC-conjugated anti-CD3 antibody per 200 μl. Incubate for 30 min at 4 °C in the dark.
9. Wash cells and resuspend the cell pellet in FACS buffer I at a concentration of 3×10^7/ml.
10. Using a FACSAria cell sorter select within the lymphocyte gate the $CD3^+$ population (*see* **Note 10**).
11. Collect cells in 15 ml conical tubes containing 2.5 ml of collection buffer. Vortex tubes prior to use (*see* **Note 7**).
12. Keep the sorted population on ice.

3.1.6 Assessment of the Suppressive Activity of Treg Cells

1. Purify Treg cells and T responder cells from the desired source.
2. Count Treg and T responder cells and adjust to 5×10^5 cells/ml in RPMI 5 % FBS supplemented with 50 μM β-mercaptoethanol and 1 μg/ml anti-CD28 monoclonal antibody. Authors have reported that sorted Treg cells remain partially anergic after purification [46]. It is therefore recommended to supplement culture medium with 20 U/ml IL-2.
3. In 96-well round bottom plates coated with anti-CD3 monoclonal antibody, add Treg cells and prepare twofold serial dilutions of these cells. It is recommended that at least three serial dilutions are performed. Treg cell proliferation should be also evaluated.
4. Add 50 μl of T responder cells to all the required wells. Make sure to evaluate proliferation of T responder cells in the absence of Treg cells. The Tresp:Treg ratio should be 1:1, 1:0.5, 1:0.25, 1:0.125, etc.
5. Incubate plates at 37 °C, 5 % CO_2 for 4 days.
6. Pulse plates with 1 μCi [^{3}H]-thymidine per well 18 h prior to completion of the experiment. Since proliferation by [^{3}H]-thymidine incorporation is often variable, wells must be processed in triplicate.
7. Harvest cultures with a commercial cell harvester and determine counts per minute (cpm) with a direct β-counter.
8. Data are reported as cpm or percent of suppression considering T responder cells alone as 100 % of proliferation.

3.1.7 Adoptive Transfer of Treg Cells

1. Purify Treg cells from the desired source and resuspend cells in sterile PBS at a concentration of 6×10^6/ml.
2. Anesthetize mice and gently warm the tail vein that is located laterally.
3. Inoculate 50 μl of Treg cells intravenously with 27G needles using a 1 ml syringe.

3.1.8 Tumor Antigen-Specific Proliferation

1. Prepare cell lysates from cultured tumor cell lines (B16, 4T1) as an antigenic source by four freeze-thaw cycles (liquid nitrogen and 37 °C water bath) at a concentration of 2×10^7 cells/ml in PBS.
2. For ex vivo antigen stimulation prepare a working solution by diluting the tumor lysates 1:400 in RPMI 1640, 10 % FBS supplemented with 50 μM β-mercaptoethanol and a mixture of antibiotics-antimycotics.
3. Prepare a single cell suspension of mouse spleen and draining lymph nodes as described in Subheading 3.1.5.
4. Adjust cells concentration to 2×10^6/ml in RPMI 10 % FBS supplemented with 50 μM β-mercaptoethanol and antibiotic-antimycotic.
5. In 96-well round bottom plates add 50 μl of cells and 50 μl of the working dilution of tumor antigen or 50 μl of RPMI as a control. Calculate three wells for proliferation and two wells for determination of each cytokine by ELISA.
6. For proliferation assay, incubate plates at 37 °C, 5 % CO_2 for 4 days and pulse plates with 1 μCi [^{3}H]-thymidine per well for 18 h.
7. For cytokine determination, incubate plates at 37 °C, 5 % CO_2 for 24–48 h, harvest culture supernatants in 100 μl aliquots, and keep at −70 °C until use.

3.1.9 Cytokine Determination by ELISA

1. ELISAs for mouse IFN-γ, IL-10, IL-5 were performed according to the manufacturer's instructions.

3.2 Study of the Role of Galectins in the DC Compartment

3.2.1 Differentiation of Bone Marrow-Derived Tolerogenic DCs

1. Remove both femurs and tibias from C57Bl/6 mice and place them in a P60 Petri dish with cRPMI.
2. Remove excess muscle with forceps and scalpel. Cut bone's epiphysis.
3. Load 1 ml syringe with cRPMI.
4. Insert 21- or 25G needle into the bone marrow cavity. Flush the bone cavity with 2 ml cRPMI until the cavity is emptied.
5. Homogenize marrow suspension vigorously to disaggregate clusters that may be present in the suspension with a 21G needle.
6. Centrifuge cells for 10 min at 200 × *g*.
7. Discard supernatant. Resuspend cells with 5 ml ACK lysis buffer and incubate for 10 min. Dilute with 20 ml PBS.
8. Centrifuge cells for 10 min at 200 × *g*.
9. Discard supernatant and resuspend cells (10^6 cells/ml) in 10 ml cRPMI medium supplemented with 20 ng/ml rGM-CSF and 3 μM rGal-1 in P100 petri dish.

10. Feed cultures on days 2, 5, and 7 without discarding any cells: swirl plates and aspirate 75 % of the medium. Add fresh medium containing rGM-CSF and rGal-1.
11. After 8–9 days purify the non-adherent bone marrow-derived cells obtained in the supernatant.

*3.2.2 Determination of DC Markers by Flow Cytometry (See **Note 11**)*

1. Add cell suspension (5×10^5 cells) to a 1.5 ml tube. An isotype control antibody for each marker should be included.
2. Centrifuge cells at $200 \times g$ for 10 min at 4 °C and discard the supernatant.
3. Wash cells with 1 ml FACS buffer II.
4. Centrifuge cells at $200 \times g$ for 10 min at 4 °C and discard the supernatant.
5. Resuspend cells in 100 μl FACS buffer II and add 10 μl of a cocktail of antibodies (MHC II, CD11c, CD86, CD45RB). All the antibodies must be diluted with FACS buffer II (0.2 μg/tube).
6. Incubate for 30 min at 4 °C.
7. Wash cells with 1 ml FACS buffer II.
8. Centrifuge at $200 \times g$ for 10 min at 4 °C; discard the supernatant and resuspend the stained cells pellet in 500 μl PBS.
9. Analyze the sample(s) using a flow cytometer.

3.2.3 Determination of IL-27 by ELISA

1. IL-27 p28 ELISA protocol is thoroughly detailed in the data sheet provided by the manufacturer (*see* **Note 12**).

3.2.4 Evaluation of STAT-3 Phosphorylation by Western Blot

1. To prepare cell lysates, centrifuge DCs (1×10^7) at $200 \times g$ for 10 min at 4 °C. Discard the supernatant.
2. Resuspend cells in 200 μl ice-cold protein extraction buffer (200 μl per 10^7 cells).
3. Keep stirring for 30 min at 4 °C.
4. Centrifuge at $16{,}000 \times g$ for 20 min in a 4 °C pre-cooled centrifuge.
5. Transfer the supernatant to a fresh tube on ice and discard the pellet.
6. Remove a small volume (10 μl) of cell lysate to perform Bradford assay.
7. Determine the protein concentration for each cell lysate.
8. Prepare 30 μg of total protein from cell lysate with 2× Laemmli Sample Buffer.
9. Incubate each cell lysate at 100 °C for 5 min.
10. Load samples on an SDS-PAGE gel.
11. Run the gel for 1–2 h at 100 V.

12. Transfer proteins from the gel to a nitrocellulose or PVDF membrane.
13. Block the membrane for 1 h at room temperature using blocking buffer.
14. Incubate the membrane with the anti-phospho-STAT3 primary antibody (0.2 μg/ml) in blocking solution overnight at 4 °C.
15. Wash the membrane with tTBS for 5 min three times.
16. Incubate the membrane with the HRP-conjugated anti-rabbit IgG diluted 1/3,000 in tTBS at RT for 1 h.
17. Wash the membrane with tTBS for 5 min three times.
18. Incubate with Immobilon chemiluminescent HRP substrate and capture the luminescent image in a GBOX incubator.

3.3 Profiling Galectin Expression in Tumor Microenvironments

3.3.1 Galectins Immunostaining in Paraffin-Embedded Tissues

Deparaffinization of tissue sections

1. 30 min in xylene at RT.
2. 10 min in 100 % ethanol at RT.
3. 10 min in 95 % ethanol at RT.
4. 10 min in 75 % ethanol at RT.
5. 5 min in distilled H_2O three times.

Quenching of endogenous peroxidase activity

6. 10 min in 1 % H_2O_2 in H_2O.

Blocking and antigen retrieval

7. Incubate tissue samples with normal horse serum (2 drops in 40 ml PBS 0.05 % saponin) overnight at 4 °C.
8. Circumscribe the tissue section with the ImmEdge Pen.
9. Incubate with antibody dilutions for 1 h at RT in a humidified atmosphere. Antibodies are diluted 1:100 in PBS-saponin (in the case of the anti-galectin-9 antibody, dilution should be 1:50). Volume = 100 μl/condition. Antibodies should be centrifuged for 2 min at 9,600 × *g* before use.
10. Wash twice in PBS-0.05 % saponin (5 min each).
11. Incubate with biotinylated antibodies (1 drop/1 ml in PBS-saponin) for 1 h at RT in a humidified chamber. Volume = 100 μl/condition. Biotinylated antibodies (anti-rabbit or anti-mouse are used). In case of galectin-9 staining, use HRP-labeled anti-goat dilution 1/100. Incubate for 1 h at RT in a humidified atmosphere.
12. Wash twice in PBS-0.05 % saponin for 5 min.

Amplification reaction: Avidin-Peroxidase-Biotyn system

13. Incubate with ABC reagent for 1 h at RT in a humidified atmosphere (1 drop of reagent A in 1 ml PBS-0.05 % saponin, incubate for 5 min. Add 1 drop of reagent B, vortex, and incubate for 5 min). Volume = 100 µl/condition.
14. Wash twice in PBS-0.05 % saponin for 5 min.
15. Add 100 µl/condition of DAKO substrate system. Prepare substrate adding 2 drops of chromogen in 2 ml buffer. Incubate for 5 min at RT (*see* **Note 13**).
16. Stop reaction by rinsing with distilled H_2O.
17. Incubate with Giemsa for 30 min at RT (30 drops in 10 ml of distilled H_2O).
18. Mount by using Dako Ultramount aqueous mounting medium (*see* **Note 13**).

3.4 Lentiviral-Mediated Silencing of Galectin Expression

3.4.1 Silencing Galectin Expression. Lentiviral Production (See **Note 2**)

Day 1: Plate 2–2.5 × 10^6 of HEK 293T cells (low passage) per P100 Petri dish in 10 ml of DMEM medium supplemented with 10 % FBS.

Day 2: Transfection

1. Change culture medium at least 2 h before transfection.
2. Prepare calcium-phosphate precipitate (1 ml/P100 Petri dish):
   (a) Transfer vector—(pLVTHM-shRNA): 20 µg [47].
   (b) Packaging plasmid pMD2.G (Addgene #12259) (plasmid encoding capsid and polymerase genes) 15 µg.
   (c) Envelope plasmid—pCMVR8.74 (Addgene #22036) (plasmid encoding amphotropic envelop VSVG) 6 µg.
   (d) Complete to 500 µl with bi-distilled water, and then add 50 µl of 2.5 M $CaCl_2$ (prepared in bi-distilled water). Add dropwise 500 µl of 2× HBSS while gently vortexing. Incubate at RT for 15–25 min.
   (e) Add dropwise on a plate and mix gently with culture medium.
3. After 6–8 h of culture in CO_2-controlled incubator at 37 °C, change medium; wash cells two times with pre-warmed PBS and add 6 ml/plate of fresh complete medium (*see* **Note 14**).
4. At day 4 collect medium containing virus particles in 15 ml conical tubes.
5. Spin at 200 × *g* for 5 min at RT to remove all cells and contamination.
6. Filter supernatant with a 0.45 µm syringe filter. Virus can then be used for transduction or stored at −70 °C until use.

*3.4.2 Titration of Lentiviral Vectors and Transduction of Target Cells (See **Note 15**)*

1. Day 1: Plate 30,000 HEK 293T cells in 24-well plates in 1 ml of complete DMEM.
2. Day 2:
   (a) Count cells: To count cells, evaluate the cell number in one well to evaluate the number of cells at the day of infection. In typical culture conditions, this number should be around 60,000–80,000 cells.
   (b) Infection cells: The infection of cells should be performed in DMEM complete medium—4 % of polybrene solution (250 μl final volume) with six serial dilutions of virus solution; use for example 10–200 μl of thawed virus solution and complete to 250 μl of DMEM complete medium in 1.5 snap lock tubes.
3. Day 3: Add 1 ml of complete DMEM.
4. Day 4: Split cells and assess transduction efficiency as the percentage of green fluorescent protein $(GFP)^+$ cells (transfer vector contains a GFP-coding sequence as a marker of viral integration) and analyze fluorescence by FACS. Read the percentage from linear values (usually 5–10 % to no more than 20 % of GFP^+ cells is considered as linear values) (*see* **Note 16**).
5. To infect your target cells with a ratio cells/virus between 1 and 10, follow the steps in **step 2**, and then wait two passages of transduced cells before analyzing the transduction efficiency (*see* **Note 17**).
6. Transduced cells should be amplified to allow purification of GFP^+ cells by FACS (*see* **Note 18**).

4 Notes

1. Store antibodies in aliquots at −70 °C. Avoid repeated freeze-thaw cycles as it may lead to loss of activity.
2. *WARNING*! Production of lentivirus is not a simple and 100 % safe procedure. You should always keep in mind that the production process allows you to produce high titers of mammal's unreplicative but infectious virus solutions. Thus, viral production should be done following safety instructions, in authorized locations. It is essential to follow safety and security guidelines of your institution.
3. As transfection efficiency depends on the cell type, solutions at different pH should be tested to optimize transfection efficiency).
4. For three-color flow cytometry including APC, FITC, and PE staining, three individual additional tubes (each with a different fluorochrome-conjugated antibody) are needed in order to properly compensate the experiment. This is because FITC

usually bleeds considerably into PE channel. For FoxP3 staining as well as for detection of intracellular cytokines it is highly recommended to add a FM1 tube. Briefly cells are only stained with surface antibodies. After fixation and permeabilization add PE-conjugated isotype antibody (corresponding to PE-conjugated Foxp3 antibody) in permeabilization buffer 1×. This allows the correct definition of $Foxp3^-$ populations. It is highly recommended to exclude cell doublets using FSC-H vs. FSC-W and SSC-H vs. SSC-W dot plots.

5. Invitrogen's protocol is detailed for 1×10^7 leukocytes in 100 µl. Both antibody mix and Dynabeads yield up to three times the values that are specified. It is therefore recommended to use one-third of the reagent's volume corresponding to 1×10^7 leukocytes. Then, for a mouse spleen (usually around 10×10^7 leukocytes) one should use 70 µl antibody mix and 700 µl Dynabeads.
6. Exclude cell doublets using FSC-H vs. FSC-W and SSC-H vs. SSC-W dot plots. Percent of total cells should range between 60 and 70 % for Balb/c mice and 50–60 % for C57Bl/6 mice. Flow rate is recommended to be adjusted to around 1–3. Sort precision could be set to "yield."
7. Vortexing tubes will ensure that the tube will be covered by a thin layer of fluid to avoid cell death when cells are deflected to the tube.
8. It is advised to perform a dose-dependent curve using CM at different dilutions. Dilutions ranging from 1:10 to 1:100 are recommended.
9. Incubation beyond 4 days will only result in increased cell death.
10. Percentage of total cells depends on the tumor model and the time of tumor burden. Sort precision should be set to "purity" and flow rate around 1–3.
11. Surface markers of tolerogenic DCs are evaluated by flow cytometry. The typical markers are CD11c, MHC II ($I\text{-}A^b$), CD86, and CD45RB.
12. Use this approach to evaluate the secretion of IL-27 by tolerogenic vs. immunogenic DCs.
13. Avoid using buffers and solutions with sodium azide since this compound inhibits peroxidase activity.
14. All material used after transfection of HEK 293T cells should be washed twice in 2 % bleach solution to avoid viral contamination and prevent subsequent health risk for the personnel and the environment.
15. As transduction of cells lines with lentivirus is a rather efficient technique and allows integration of high number of copies of viral genome, target cells should be infected with low number of virus particles to ensure no more than two or three copies of

integrated shRNA cassette. There are important reasons for this. First, less of the target cell's genome will be modified, fewer side effects will be caused by virus integration, and fewer shRNA molecules will be produced to ensure no saturation of the miRNA natural program.

16. Titer corresponds to the percentage of cells (GFP^+ cells) transduced by a given virus suspension volume used on day 2, e.g., 50 μl of a dilution 5 leads to 10 % of positive cells, and the number of cells on day 2 is 70,000, then the titer of the viral solution is 5,000 TU/μl > 1.4×10^5 TU/ml.
17. Avoiding more than 10 % of infection allows one to keep the line heterogeneity, and no more than 15 % ensures minimum high copy integration number and thus genome integrity.
18. In this chapter we detail some of the strategies used to study the role of galectins, particularly galectin-1 in tumor immunity, including the study of the differentiation and frequency of Treg cells and tolerogenic DCs, the profile of galectin expression in the tumor microenvironment, and the production of lentiviral vectors to manipulate galectin expression selectively in different cell types (tumor, stromal, and immune compartments). Other methods including promotion of T cell apoptosis, cytokine detection, and T cell trafficking are described in detail in recent papers and excellent review articles [8–14, 17]. During the past decade, a better understanding of the cellular and molecular mechanisms underlying tumor immunity has provided the appropriate framework for the development of novel therapeutic strategies in cancer. Under this complex scenario, galectins and their glycosylated ligands have emerged as promising molecular targets and galectin antagonists have the potential to be used as anti-tumor and anti-metastatic agents in those cases in which galectins are up-regulated in tumor microenvironments. The emerging data promise a future scenario in which the selective blockade of galectin-1, either alone or in combination with other therapeutic regimens, will contribute to halt tumor progression by counteracting cancer immunosuppression [48, 49]. Blockade of galectin-1–glycan interactions may also influence the efficacy of tumor vaccines, and other immunotherapeutic approaches. We hope that the strategies and methods described here will facilitate and encourage scientists to further evaluate the role of galectins in tumor immunity.

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Chapter 17

Galectins in the Regulation of Platelet Biology

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Abstract

Platelets are anucleated blood cells derived from megakaryocytes, and although they are essential for proper hemostasis, their function extends to physiologic processes such as tissue repair, wound remodeling, and antimicrobial host defense, or pathologic conditions such as thrombosis, atherosclerosis, chronic inflammatory diseases, and cancer. Recently, we demonstrated that two structurally divergent members of the galectin family, galectin-1 and galectin-8, are potent platelet agonists. The emergence of galectins as soluble mediators capable of triggering platelet activation opens a new field of research that will provide further insights into the mechanisms linking inflammatory responses to thrombus formation and could expand our view of the role of platelets much beyond hemostasis to their pathophysiologic role during inflammation and cancer. The present article details the various protocols and reagents currently used in our laboratory to study the role of galectins in human platelet function.

Key words Platelets, Galectins, Glycobiology, P-selectin, Thromboxane A_2, Inflammation, Platelet aggregation, Adhesion, Hemostasis, Thrombosis

1 Introduction

Platelet activation and subsequent accumulation at sites of vascular injury are the first steps in hemostasis. Excessive platelet activation after atherosclerotic plaque rupture or endothelial cell erosion may also lead to the formation of occlusive thrombi, which are responsible for acute ischemic events. Platelets play important roles in several physiopathological processes beyond hemostasis and thrombosis, including promotion of inflammatory responses and the maintenance of vascular integrity, wound healing. Furthermore, platelets are important players in the development of atherosclerosis, sepsis, hepatitis, vascular restenosis, acute lung injury, and transplant rejection [1–4]. When platelets perceive activating signals through their cell surface receptors, they undergo dramatic structural and chemical changes, involving a complex interplay of cell adhesion and signaling molecules. Platelet activation can be

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triggered by a broad spectrum of vascular adhesive proteins such as von Willebrand factor (WF), collagen, fibronectin, and soluble agonists including adenosine diphosphate (ADP), thromboxane A_2 (TXA_2), thrombin, and serotonin [5]. We have recently described that two structurally divergent galectins (galectin-1 and galectin-8), either in a soluble or immobilized form, are capable of triggering a broad range of platelet responses including adhesion and spreading, aggregation, release of granule content, and P-selectin expression through the interaction with the carbohydrate backbone of the major platelet receptors involved in hemostasis: e.g., GPIbVIX complex and integrin $\alpha_{IIb}\beta_3$, [6–8]. Moreover, the relevance of galectin-1 in hemostasis has been studied in galectin-1 null mutant mice, and it was shown that animals deficient in galectin-1 had prolonged bleeding time, an effect which was not associated with a decrease in platelet number but rather to a platelet dysfunction. In fact, galectin-1-deficient platelets showed a restricted adhesion to immobilized fibrinogen and a delayed clot retraction, two platelet activation responses dependent on outside–in signaling of platelet integrin $\alpha_{IIb}\beta_3$ [6]. Thus, an impairment of this activation pathway appears to be a major cause of the altered bleeding time in mice lacking galectin-1.

Platelets are readily available, easily separated from other blood cells, and contain a signaling apparatus common to other cells. Here we detail the optimized methods to study the role of galectins in platelet signaling and activation.

2 Materials

2.1 Preparation of Platelet and Polymorphonuclear Leukocytes (PMN) Suspensions

2.1.1 Platelet-Rich Plasma, Platelet-Poor Plasma, and Washed Platelets

1. Syringe with 19-G needle.
2. 15 ml tubes.
3. Plastic transfer pipette.
4. Sodium citrate (3.8 % w/v) or ACD (65 mM trisodium citrate, 70 mM citric acid, 100 mM dextrose, pH 4.4) dissolved in sterile water.
5. Prostacyclin (PGI_2, Cayman Chemical) dissolved at 1 mg/ml in 50 mM Tris buffer, pH 9.1 (*see* **Note 1**).
6. Washing buffer: 90 mM NaCl, 5 mM KCl, 36 mM Na_3 citrate, 5 mM glucose, pH 6.5.
7. Tyrode's buffer: 134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, 0.3 %, bovine serum albumin (BSA), pH 7.4.

2.1.2 PMN Isolation

1. Syringe with 19-G needle.
2. Sodium citrate 3.8 % (w/v) dissolved in sterile water.
3. 15 ml tubes.
4. Plastic transfer pipette.

5. Hypaque–Ficoll solution.
6. 6 % dextran.
7. Phosphate-buffered saline.
8. Sterile H_2O.
9. 10× PBS.
10. RPMI (Life Technologies).
11. Hemocytometer.

2.2 Platelet Count

1. EDTA or ammonium oxalate for platelet-rich plasma (PRP) or whole blood respectively.
2. Hemocytometer.

2.3 Binding of Galectins to Platelets

1. PBS: 4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH 7.4.
2. EZ-Link Micro NHS-PEO4-Biotinylation Kit (Pierce).
3. Purified recombinant galectin.
4. Tyrode's buffer: 134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, 0.3 %, bovine serum albumin (BSA), pH 7.4.
5. PBS supplemented with 2 mM EDTA.
6. FITC-conjugated streptavidin (BD Biosciences).
7. 4 % paraformaldehyde (PFA) solution prepared in phosphate-buffered saline.
8. 5 ml polystyrene round-bottom tubes.
9. Cytometer sheath fluid (BD Biosciences).

2.4 Adhesion and Spreading of Platelets on Immobilized Galectins

1. 8-well multitest glass slides.
2. PBS pH 7.4.
3. 1 mM $CaCl_2$ solution.
4. Prepare a heat-inactivated 5 mg/ml BSA solution in PBS by incubating 3 h at 58 °C. Filter through a 0.45 μm filter and store on ice until use.
5. Purified recombinant galectins.
6. Fibrinogen from human plasma (Sigma-Aldrich).
7. 4 % paraformaldehyde (PFA) solution prepared in PBS.
8. Triton X100 0.3 % in PBS.
9. TRITC-conjugated phalloidin (Sigma-Aldrich).
10. Mounting media: Aqua-Poly/Mount (Polysciences Inc.).
11. Glass coverslips (60 × 24 mm).

2.5 Integrin $\alpha_{IIb}\beta_3$ Activation

1. Alexa 488-conjugated fibrinogen.
2. 5 ml polystyrene round-bottom tubes.

3. FITC-PAC-1 and FITC-IgM isotype antibodies.
4. 10 mM $CaCl_2$ solution.
5. 4 % PFA solution prepared in phosphate-buffered saline.
6. Purified recombinant galectins.
7. Human alpha thrombin.
8. Cytometer sheath fluid (BD Biosciences).

2.6 Platelet Aggregation

1. Glass cuvettes for aggregometer.
2. Platelet poor plasma.
3. Tyrode's buffer: 134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, 0.3 %, BSA, pH 7.4.
4. Clean stir bar.
5. $CaCl_2$ solution.
6. Purified recombinant galectins.
7. Human alpha thrombin.

2.7 ATP Release from Dense Granule

1. Glass cuvettes for aggregometer.
2. $CaCl_2$ solution.
3. Firefly Lantern Extract (luciferin–luciferase, Sigma-Aldrich) (20 mg/ml) resuspended in PBS.
4. Purified recombinant galectins.
5. Human alpha thrombin.
6. ATP standard solution (Sigma-Aldrich).

2.8 P-Selectin Expression

1. 10 mM $CaCl_2$ solution.
2. Microtubes.
3. PBS with 1 % fetal bovine serum (FBS).
4. Purified recombinant galectins.
5. Human alpha thrombin.
6. 4 % PFA solution prepared in phosphate-buffered saline.
7. Fetal bovine serum (FBS).
8. FITC-conjugated mouse antihuman CD62P.
9. FITC-conjugated mouse IgG isotype control.
10. Cytometer sheath fluid (BD Biosciences).

2.9 Thromboxane A_2 Release

1. Glass cuvettes for aggregometer.
2. 10 mM $CaCl_2$ solution.
3. Purified recombinant galectins.

4. Human alpha thrombin.
5. Ice-cold solution of PBS/2 mM EDTA/0 μM acetyl salicylic acid (ASA).
6. A plate reader capable of measuring absorbance between 405 and 420 nm.
7. Thromboxane B_2 EIA Kit (Cayman Chemicals).

2.10 Mixed Aggregates Platelets and Neutrophils

1. PBS 1×.
2. PBS 10×.
3. 10 nM $CaCl_2$ solution.
4. Ficoll–Hypaque solution.
5. 2 % PFA solution prepared in phosphate-buffered saline.
6. 6 % (w/v) dextran solution: dissolve 60 g/L of Dextran-500 (average molecular weight 200,000–500,000) in endotoxin-free, sterile 0.9 % NaCl.
7. RPMI culture medium.
8. Purified recombinant galectins.
9. Human alpha thrombin.
10. FBS.
11. FITC-conjugated mouse antihuman CD45.
12. PE-conjugated mouse antihuman CD61.
13. FITC and PE-conjugated mouse IgG isotype controls.
14. 5 ml polystyrene round-bottom tubes.

2.11 Special Equipment

1. Flow cytometry (*see* **Note 2**).
2. Aggregometer (*see* **Note 3**).
3. Hematological Analyzer (*see* **Note 4**).
4. Fluorescence microscope (*see* **Note 5**).

3 Methods

3.1 Preparation of Platelet and Polymorphonuclear Leukocytes (PMN) Suspensions

3.1.1 Preparation of Platelet-Rich Plasma, Platelet-Poor Plasma, and Washed Platelets

For Platelet-Rich Plasma (PRP)

1. Blood is collected from the forearm vein using a 19-G needle from healthy donors who have not taken any medication for at least 10 days. Draw blood directly into polypropylene tubes or into syringes containing anticoagulant (sodium citrate 3.8 %, 9:1 v/v) (*see* **Notes 6** and **7**).
2. Transfer anticoagulated whole blood into 15 ml tubes to a volume of 10 ml in each tube.
3. Centrifuge the blood sample at 200 × *g* for 15 min with minimum brake at room temperature (RT).

4. Aspirate PRP using a plastic pipette and transfer to a fresh plastic tube. Care should be taken not to disturb either the buffy coat or red cells to prevent contamination of the platelet preparation.

For Platelet-Poor Plasma (PPP)

5. After aspirating PRP, centrifuge the remnant blood sample at high speed (3,000 × *g* for 10 min.).

For Washed Platelets

6. Prepare PRP but from blood anticoagulated with ACD, 6:1, v/v.
7. Add prostacyclin (75 nM) to the PRP to avoid platelet activation during the centrifugation. Mix thoroughly (*see* **Note 1**).
8. Centrifuge the PRP at 900 × *g* for 10 min with minimum brake at RT.
9. Aspirate and discard the supernatant and gently resuspend the platelet pellet in washing buffer at a volume equivalent to that of the PRP.
10. Centrifuge at 900 × *g* for 10 min with minimum brake at RT.
11. Aspirate and discard the supernatant and gently resuspend the platelets in Tyrode's buffer.
12. Count platelets using a hemocytometer or an automated hematologic analyzer and adjust to the desired number.
13. Purified platelets should be kept at RT and used within 4 h of isolation (*see* **Note 8**).

3.1.2 PMN Isolation

1. After PRP separation, add 2.5 volume of PBS to the remaining blood.
2. Gently transfer 35 ml of the diluted blood to a 50 ml tube containing 15 ml of Hypaque–Ficoll solution.
3. Centrifuge at 480 × *g* for 25 min without brake at 4 °C.
4. Aspirate and discard the remaining gradient above the PMN–erythrocyte pellet.
5. Add to the pellet one volume of 6 % dextran and two volumes of PBS. Mix by repeated inversion and set tubes upright for 18–20 min at RT.
6. Aspirate the straw-colored, leukocyte-rich upper layer with a sterile plastic pipette and transfer the aspirate to a sterile 50 ml tube.
7. Add PBS to a volume of 50 ml and centrifuge at 480 × *g* for 10 min at 4 °C.
8. Resuspend the pellet in 9 ml of cold sterile H_2O and mix well for 40 s to lyse red cells.

9. Add 1 ml of cold 10× PBS to restore tonicity.
10. Add 40 ml of PBS and centrifuge at 480 × *g* for 10 min at 4 °C.
11. Resuspend cells in RPMI.
12. Determine the cell concentration by counting using a hemocytometer and adjust to 5×10^6 PMN/ml.

3.2 Platelet Count in Whole Blood or PRP

1. Dilute 1/100 whole blood with 1 % ammonium oxalate or the PRP in EDTA 2 %.
2. Mix well the dilution and incubate to allow lysis of the erythrocytes. Following the incubation period, the dilution is mounted on a hemocytometer. The cells are allowed to settle and then are counted in the entire center large square (1 mm^2, 25 small squares) of the hemocytometer chamber under the microscope (×400 magnification).
3. The total number of cells is determined by using the following formula: total number of cells counted × dilution factor × 10.

3.3 Binding of Galectin to Platelets

1. Add 50 μl of washed platelets (5×10^7/ml) to a microtube.
2. Incubate with biotinylated galectin (4 μM Gal-1 or 0.4 μM for Gal-8) or vehicle for 15 min (*see* **Note 9**).
3. Add 1 ml of PBS/2 mM EDTA and centrifuge at 350 × *g* for 10 min at RT.
4. Resuspend the pellet in 50 μl of Tyrode's buffer.
5. Incubate with FITC streptavidin (1 μM) for 30 min.
6. Add 1 ml of PBS/2 mM EDTA and centrifuge at 350 × *g* for 10 min at RT.
7. Resuspend in 50 μl of Tyrode's buffer.
8. Fix with 12.5 μl of 4 % PFA for 15 min.
9. Add the fixed platelets to a 5 ml polystyrene round-bottom tube containing 500 μl of flow cytometer sheath fluid.
10. Analyze by flow cytometry.

3.4 Adhesion and Spreading of Platelets on Immobilized Galectins (See Note 10)

1. Prepare a solution of galectin (1 and 0.25 μM for Gal-1 and Gal-8, respectively). A solution of 5 mg/ml of heat-inactivated BSA must be used as a negative control, and a solution of 100 μg/ml of fibrinogen can be used as a positive control.
2. Pipette 40 μl of the protein solution onto the center region of the glass slide well, and incubate for 2 h at 4 °C.
3. After washing slides three times with PBS, pipette 40 μl of BSA blocking buffer (5 mg/ml of heat-inactivated BSA). Incubate for 1 h at RT and then wash three times in PBS.
4. Pipette 50 μl of washed platelets (3×10^7/ml with 1 mM $CaCl_2$) into each well, and incubate for 40 min at 37 °C.

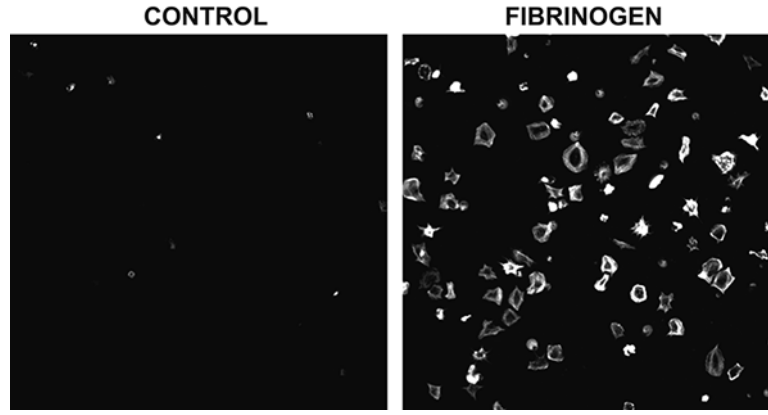

Fig. 1 Platelet adhesion and spreading. Platelets (5×10^7/ml) were plated on BSA 2 % (negative control) or 100 μg/ml fibrinogen-coated slides for 20 min and then fixed and stained with TRITC-phalloidin. Platelet spreading was visualized by confocal microscopy (Olympus FV1000)

5. Wash gently twice with PBS to remove unbound platelets.
6. Pipette 12.5 μl of 4 % PFA into each well, and incubate 20 min at RT to fix adherent platelets.
7. Wash three times with PBS and permeabilize cells by adding 40 μl of 0.3 % Triton-X100 for 15 min at RT. Afterwards wash three times with PBS.
8. Pipette 40 μl of TRITC-conjugated phalloidin (50 μM in PBS) on each well, and incubate for 1 h at RT in the dark. Wash three times with PBS.
9. Remove PBS and add a drop of mounting media on each well and place the coverslip. Let dry for 24 h.
10. The slides can be viewed by fluorescence microscopy (Fig. 1). Software can be used to quantify the degree of adhesion and the surface area of adherent platelets.

3.5 Integrin $\alpha_{IIb}\beta_3$ Activation (See Note 11)

1. Add 32 μl of washed platelets (3×10^7/ml) to a 5 ml polystyrene round-bottom tube.
2. Add 2 μl of a 200 μM solution of Alexa 488-labeled fibrinogen or 2 μl of a saturating concentration of FITC-PAC-1 or FITC-IgM isotype control in PBS containing 1 % FBS.
3. Add 4 μl of 10 mM $CaCl_2$.
4. Add 2 μl of agonist (4 μM galectin-1 or 0.5 μM galectin-8) or vehicle control, and incubate for 10 min at RT. Thrombin (0.1 U/ml) can be used as a positive control.
5. Fix with 15 μl of 4 % PFA for 20 min.
6. Add 400 μl of flow cytometer sheath fluid.
7. Analyze by flow cytometry.

3.6 Platelet Aggregation (See Note 12)

1. Allow the aggregometer cuvette chamber to reach 37 °C. Set the stirrer to 1,000 rpm.
2. Set the chart recorder at 1 cm/min.
3. Add 400 μl of the appropriate blank (PPP or Tyrode's buffer when measuring aggregation in PRP or washed platelets, respectively) into a siliconized glass cuvette, and place it in the designated chamber of the aggregometer.
4. Add 400 μl of the platelet suspension into an aggregometer cuvette, and place it into the designated chamber of the aggregometer. Platelet density can range from 2 to 4×10^8/ml. If PRP is being studied, this dilution should be done with the donors' own PPP.
5. Set the aggregation baseline.
6. Add a clean stir bar to the platelet suspension.
7. Add 4 μl of 10 mM $CaCl_2$ (only if washed platelets are being used).

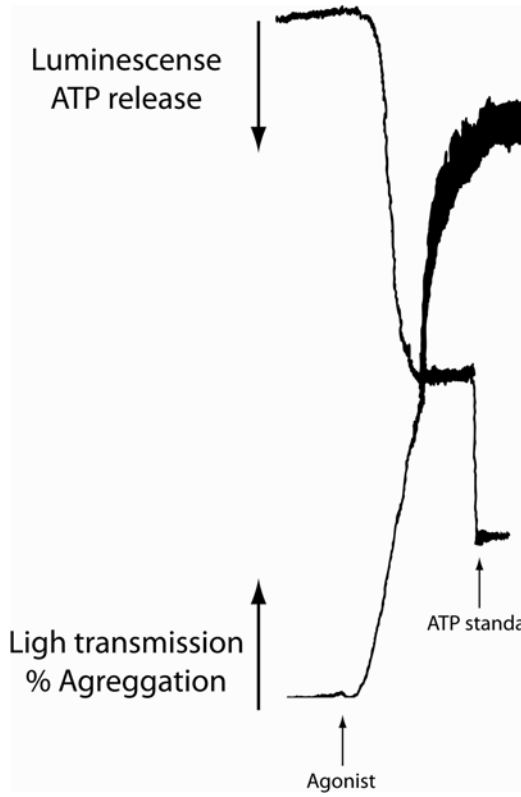

Fig. 2 Simultaneous measurement of platelet aggregation and ATP release using a lumi-aggregometer. Following addition of an agonist, the light transmission transiently decreases consistent with platelet shape change. The subsequent increase in light transmission reflects platelet aggregation. ATP levels are calculated at the end of the assay by adding a known amount of ATP

8. Turn on the chart recorder.
9. Monitor for 1 min to ensure baseline is stable.
10. Add 4 µl of galectin (1–4 µM for galectin-1 and 0.1–0.5 µM for galectin-8 final concentration).
11. Monitor aggregation for 5–10 min (Fig. 2).

3.7 ATP Release from Dense Granule (See Note 13)

1. Begin with Subheading 3.6, **steps 1–7**.
2. Add 25 µl of the luciferin–luciferase solution (20 mg/ml), and close the lid to ensure light-free environment.
3. Turn on the chart recorder.
4. Set the luminescence baseline.
5. Monitor for 1 min to ensure baseline is stable.
6. Add 4 µl of galectin (1–4 µM for galectin-1 and 0.1–0.5 µM for galectin-8 final concentration).
7. When the maximal deflection (i.e., ATP release) has been reached, add 4 µl of 200 µM ATP to give a final concentration of 2 µM ATP. Close the lid.
8. Measure the distance from the baseline to the first plateau. This is the agonist-induced ATP release.
9. Measure the distance induced by the standard ATP.
10. The amount of ATP released by the agonist is calculated by

$$\frac{\text{Agonist} - \text{induced} \times \text{ATP standard amount added}\,(2\ \mu\text{M})}{\text{ATP standard distance}} = \text{ATP released}\,(\mu\text{M}).$$

3.8 P-Selectin Externalization from Alpha Granules (See Note 14)

1. Add 32 µl of washed platelets (3×10^7/ml) to a microtube.
2. Add 2 µl of a saturating concentration of FITC-labeled antihuman CD62P or FITC-labeled IgG isotype in PBS containing 1 % FBS.
3. Add 4 µl of 10 mM $CaCl_2$.
4. Add 2 µl of agonist (4 µM galectin-1 or 0.5 µM galectin-8) or vehicle and incubate for 10 min at RT. Thrombin (0.1 U/ml) can be used as a positive control.
5. Fix with 4 µl of 4 % PFA for 20 min.
6. Add 400 µl of flow cytometer sheath fluid.
7. Analyze by flow cytometry.

3.9 Thromboxane A_2 Release (See Note 15)

1. Begin with Subheading 3.6, **steps 1–11**.
2. Monitor aggregation for 5 min.
3. Stop the reaction by adding 400 µl of ice-cold PBS/2 mM EDTA/1 mM ASA.

4. Transfer the cell suspension to a microtube and centrifuge at $350 \times g$ for 10 min at 4 °C.
5. Keep the supernatant and centrifuge again at $1{,}000 \times g$ for 10 min at 4 °C.
6. Remove the supernatant and store at –80 °C until used.
7. TXB2 determination is performed with a TXB2 EIA Kit (Cayman Chemicals) following the recommendations of the manufacturer (*see* **Note 16**).

3.10 Platelet–PMN Aggregates Formation (See Note 17)

1. Add to a microtube 50 µl of platelets (2×10^8/ml) and 50 µl of PMN (5×10^6 PMN/ml) to obtain a PMN–platelet relation 1:40.
2. Add 10 µl of a 10 nM $CaCl_2$ solution.
3. Add galectin (4 µM galectin1 or 0.5 µM galectin-8) or vehicle and incubate for 10 min at RT. Thrombin (0.1 U/ml) can be used as a positive control.
4. Fix with 100 µl of 2 % PFA.
5. Transfer 50 µl of cells to another tube, and add 2.5 µl of FBS, 2 µl of FITC-labeled antihuman CD45 (PMN antigen), and 2 µl of PE-labeled antihuman CD61 (platelet antigen), and incubate 20 min at RT. For isotype controls, add to non-stimulated cells equivalent protein concentrations of FITC-labeled IgG and PE-labeled IgG.
6. Transfer the samples to a 5 ml polystyrene round-bottom tube containing 400 µl of flow cytometer sheath fluid.
7. Select a PMN region according to their specific light scatter profiles and the levels of CD45 expression.
8. PMN–platelet aggregates are detected as the presence of platelet CD61-positive fluorescence in 10,000 CD45-positive PMN.

4 Notes

1. PGI_2 is a very unstable molecule that gets rapidly degraded at RT. It is recommended to keep aliquots at –80 °C, and once defrosted, keep on ice and use it immediately. PGE_1 can also be used instead of PGI_2, but the former is dissolved in methanol, so this alcohol should be used as a vehicle.
2. Labeling of platelets with antibodies directed against surface membrane glycoproteins and then analyzing the binding by flow cytometry is a rapid and sensitive technique for measuring several platelet activation responses including among others P-selectin externalization, phosphatidylserine exposure, conformational changes of $\alpha_{IIb}\beta_3$integrin, and formation of aggregates between platelets and leukocytes. Assessment of these parameters

can be done in whole blood, platelet-rich plasma (PRP), or washed platelets. A basic two-color flow cytometer is suitable for most platelet function assays. Platelet acquisition by flow cytometry requires a particular set up, including: (1) Set the forward and side scatters in log scale (to show the classical distribution of resting platelet population in the center of the FSC vs SSC dot plot). (2)The rate of sample acquisition must be adjusted as low as possible (to diminish the electronic noise).

3. This equipment has the capability to compare light transmission through a stirred platelet suspension and display the results either on a chart recorder or computer. The aggregometer is calibrated so that the light transmission is 10 % through the unstimulated platelet suspension and 90 % through a control blank. Certain aggregometers can concomitantly measure luminescence and therefore. ATP release could be detected simultaneously with platelet aggregation.
4. This is a rapid method for counting platelets. If it is not available, platelet counts could be performed by optical microscopy using hemocytometers.
5. This equipment is necessary for the observation of platelet morphology during the platelet spreading assay, as adherent cells can be stained with phalloidin conjugated with TRITC fluorochrome. A confocal microscope gives better information and resolution of the images, although an epifluorescence microscope can be used as well.
6. Blood should be drawn in an even manner and continuously mixed with the anticoagulant. Low temperatures can activate platelets. Therefore, do not expose whole blood or platelet suspensions to temperatures below 25 °C during incubations or centrifugation.
7. There are several anticoagulants used for measuring platelet functions including ACD, sodium citrate, or EDTA. The difference between them is their potency as calcium-chelating agents (EDTA>ACD>sodium citrate). Thus, the choice of each one depends on the platelet activation response to be measured. For example, platelet aggregation in PRP or washed platelets must be performed using blood samples anticoagulated with sodium citrate or ACD, respectively.
8. Prior to any experimental procedure, platelets are typically left at RT for 30 min; after 30 min, most of the PGI_2 has become inactive. The platelet suspension is supplemented with 1 mM $CaCl_2$ before assays are performed.
9. Chemical biotinylation of galectin can be performed with EZ-Link Micro NHSPEO4-Biotinylation Kit (Pierce) following the recommendation of the manufacturer.
10. The adhesion of platelets to the subendothelial matrix is the initial step in primary hemostasis. Platelets interact with extracellular

matrix proteins via specific adhesive glycoproteins (GP) which leads to receptor cross-linking, inducing a complex cascade of signals transmitted from the membrane into the cytoplasm. This results in platelet adhesion activation (outside–in signaling). Activated platelets show a change in the assembly of cytoskeleton proteins resulting in a shape change with extensive formation of pseudopodia originating from the plasma membrane [9]. This assay in which resting platelets are added to an immobilized substrate allows studying platelet adhesion and activation by staining platelet actin with phalloidin.

11. Integrin $\alpha_{IIb}\beta_3$ is the major integral plasma membrane protein on platelets. On resting platelets this integrin is unable to bind its ligand. When a platelet agonist binds its specific receptor, it triggers a signaling pathway (inside-out signaling) that promotes a conformational change of the integrin from a low-affinity state (resting state) to a high-affinity state (active state) for its extracellular ligands. This transformation of $\alpha_{IIb}\beta_3$ allows it to bind divalent fibrinogen or multivalent vWF, which can act as bridging molecules between platelets to form aggregates. Therefore, it is possible to monitor the binding of fluorophore-labeled soluble fibrinogen as a function of integrin activation on the cell surface. Because fibrinogen is a polyvalent ligand, more discriminating tools have also been developed in order to monitor the changes in integrin affinity at a single molecule level. One such tool is PAC-1-FITC antibody.
12. The aggregation of platelets is characterized by the accumulation of platelets into a hemostatic plug. Aggregation can be monitored in vitro by measuring light transmission through a stirred platelet suspension using an aggregometer. In this assay, a suspension of resting platelets is placed in a tube into the aggregometer. Following exposure to an agonist, platelets are activated and form small clumps leading to an increase in light transmission which is registered by the aggregometer [10].
13. Platelets contain several types of secretory organelles being dense granules, alpha granules, and lysosomes the most important. Dense granules (or dense bodies) contain a variety of hemostatically active substances that are released upon platelet activation, including serotonin, catecholamines, ADP, adenosine 5′-triphosphate (ATP), and calcium [11]. The most commonly used method to study dense granule release is determination of ATP release by luciferin–luciferase using a lumi-aggregometer. The basis of this reaction is the emission of light following the interaction of the firefly extract substrate luciferin with the enzyme luciferase in the presence of ATP. The emitted light can be detected and quantified. (Fig. 3).
14. The alpha granules are the major storage organelles for secreted proteins. Proteomic analysis of the platelet secretome suggests that more than 300 proteins are released, including adhesive

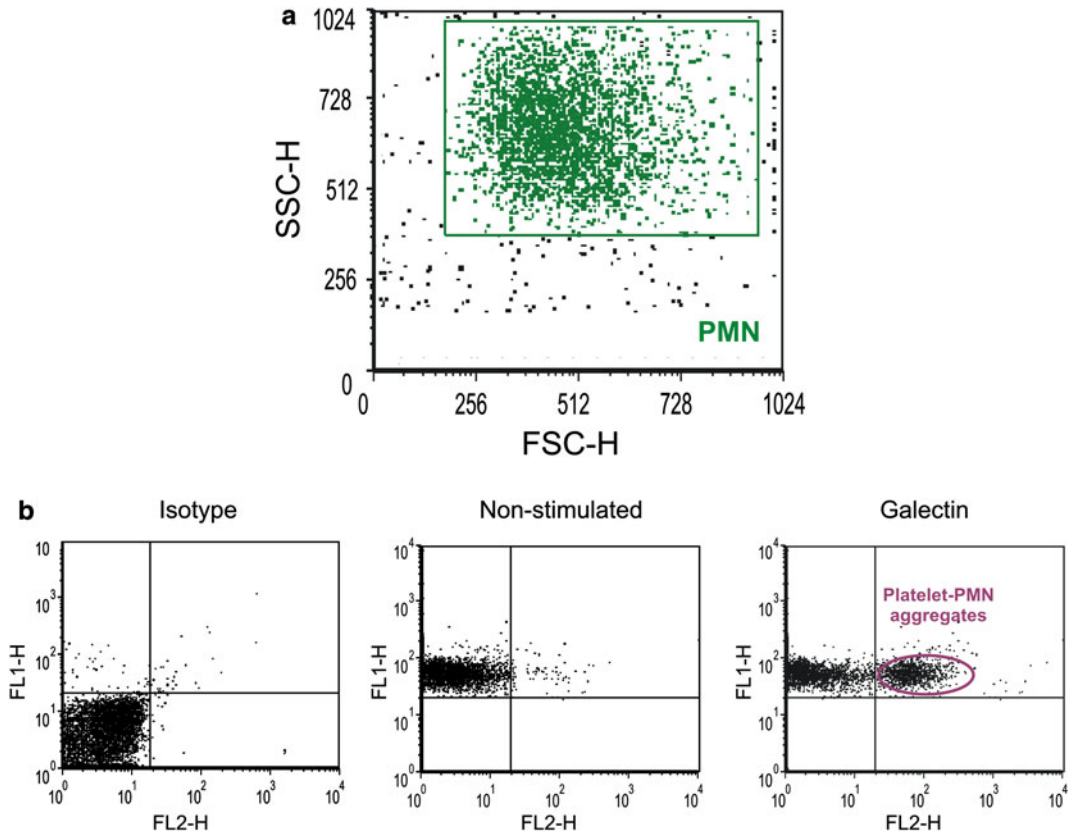

Fig. 3 Detection of platelet–PMN aggregates by flow cytometry. (**a**) The PMN population was identified by their characteristic size and granularity and a region gate was established. (**b**) *Dot plot* analysis for FL1 (CD45, leukocyte antigen) vs. FL2 (CD61, platelet antigen) was used to quantify the platelet–PMN mixed aggregates

proteins, clotting factors, fibrinolytic factors, cytokines, antimicrobial proteins, and membrane glycoproteins [12]. P-selectin (CD62P) is a membrane protein and upon platelet activation is released from granules and exposed in the platelet membrane. Due to its abundance in alpha granules, P-selectin constitutes a suitable marker to assess platelet degranulation/activation.

15. Platelets can respond to stimulation by secreting newly synthesized soluble metabolites. Thromboxane A_2 (TXA_2) is produced from arachidonic acid and is a strong amplifier of platelet activation and a powerful vasoconstrictor. TXA_2 is a very unstable molecule and is rapidly hydrolyzed to form TXB_2 [13]. Therefore, measurement of TXB_2 gives accurate estimates of TXA_2 production.
16. For TXB_2 determination, supernatants of activated platelets in the absence of stirring can also be used, although thromboxane generation is more efficient in stirring conditions. A similar technique compared to that described for TXB_2 can be used to measure several proteins released from alpha granules such as

vascular endothelial growth factor, vWF, platelet factor 4, and endostatin, among others using commercial ELISA Kits.

17. Activated platelets may adhere to leukocytes and form mixed aggregates. The molecular mechanisms responsible of this cellular interaction include a central role of platelet P-selectin and of P-selectin glycoprotein ligand-1 (PSGL-1) its counter receptor on leukocytes. The interaction of PSGL-1 with P-selectin activates a signaling cascade, resulting in the activation of the beta-2 integrin Mac-1 and in the firm adhesion between the two cell types. Platelet–leukocyte aggregates provide a novel link between inflammation and thrombosis; two central processes in atherogenesis and circulating mixed aggregates are considered a reliable marker of a prothrombotic state and are associated with several cardiovascular conditions [14].

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Chapter 18

Examination of the Role of Galectins and Galectin Inhibitors in Endothelial Cell Biology

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Abstract

The growth of new blood vessels is a key event in many (patho)physiological processes, including embryogenesis, wound healing, inflammatory diseases, and cancer. Neovascularization requires different, well-coordinated actions of endothelial cells, i.e., the cells lining the luminal side of all blood vessels. Galectins are involved in several of these activities. In this chapter we describe methods to study galectins and galectin inhibition in three key functions of endothelial cells during angiogenesis, i.e., endothelial cell migration, endothelial cell sprouting, and endothelial cell network formation.

Key words Angiogenesis, Endothelial cell, Migration, Network formation, Sprouting, Galectin

1 Introduction

Blood vessels are the infrastructure of an organism, as they facilitate the transport of nutrients and oxygen throughout the body. The vascular infrastructure is formed during embryogenesis by two main processes, vasculogenesis and angiogenesis. Vasculogenesis refers to the early de novo formation of a primitive vasculature by angioblasts. During further development, new vessels are continuously sprouting from existing capillaries in this vasculature by a process called angiogenesis [3]. Angiogenesis is a complex process that involves different well-coordinated and stepwise activities of endothelial cells (EC), e.g., the cells that cover the luminal side of blood vessels. The angiogenesis cascade is initiated by activation of EC by pro-angiogenic growth factors like vascular endothelial growth factor (VEGF). Once activated, supportive cells (pericytes) detach from the capillary and supportive structures (basal membrane and the underlying extracellular matrix) are degraded. Next, the endothelial cells sense the direction of the angiogenic stimulus and start to proliferate and migrate in that direction.

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The growing EC sprouts form primitive tubular structures that are eventually stabilized by deposition of a new basal membrane and attraction of pericytes. This allows the EC in these newly formed capillaries to return to a quiescent state [2, 4].

As evident from their pleiotropic functions in cell biology, several galectins are involved in different steps of the angiogenesis cascade. This is not restricted to physiological angiogenesis during, for example, wound healing and embryogenesis, but extends to different pathologies that are characterized by dysregulated angiogenesis, including cancer [8]. Galectins have been shown to activate EC and to promote angiogenic signaling in EC [5, 7]. In addition, galectins have been associated with EC adhesion, migration, and proliferation during angiogenesis [1, 7, 8]. Thus, galectins are now recognized as regulators of angiogenesis and even as targets for angiostatic therapy [6, 8, 9]. In this chapter, we provide methods to assess the effect of galectins on endothelial cell migration, network formation, and sprouting in vitro. Apart from studying the direct effects of galectins, these methods can also be used to determine the effects of galectin-blocking compounds on endothelial cell function.

2 Materials

2.1 Migration

1. Endothelial cells (*see* **Note 1**).
2. Flat-bottom 96-well plates (Costar) (*see* **Note 2**).
3. Pipetman with 1.0–10 mL sterile pipettes.
4. 10–1,000 μL pipettes with sterile tips.
5. Phosphate-buffered saline (PBS).
6. 0.2 % gelatin in PBS.
7. Recombinant galectins and/or galectin inhibitors.
8. Humidified 5 % CO_2 incubator at 37 °C/99.5 °F.
9. A 96-well pin tool scratcher (*see* **Note 3**).
10. UniversalGrab 6.2 software (DCIlabs) for image acquirement.
11. Image analysis software, e.g., ScratchAssay 6.2 (DCIlabs), ImageJ, or Photoshop.

2.2 Network Formation

1. Flat-bottom 96-well plates.
2. BD Matrigel basement membrane matrix (*see* **Note 4**).
3. Endothelial cells (*see* **Note 1**).
4. Recombinant galectins and/or galectin inhibitors.
5. Humidified 5 % CO_2 incubator at 37 °C/99.5 °F.
6. UniversalGrab 6.2 software (DCIlabs) for image acquirement.

2.3 Sprouting

1. Endothelial cells (*see* **Note 1**).
2. Flat-bottom 24-well plates.
3. Non-adhesive square Petri dishes (10 × 10 cm).
4. Methocel medium: RPMI with 20 % methocel and 10 % heat-inactivated human serum.
5. PBS with 10 % heat-inactivated human serum.
6. BD Matrigel basement membrane matrix (*see* **Note 4**).
7. Humidified 5 % CO_2 incubator at 37 °C/99.5 °F.
8. UniversalGrab 6.2 software (DCIlabs) for image acquirement.
9. Image analysis software, ImageJ.

2.4 Special Equipment

1. Inverted microscope equipped with a camera: We use a Leica DMI3000B microscope equipped with an automated *xyz* stage and a Hitachi 1.4 Mb GiGE color camera.

3 Methods

3.1 Migration

1. Coat a flat-bottom 96-well plate with 0.2 % gelatin/PBS for at least 30 min at 37 °C/99.5 °F or 2 h at room temperature. Aspirate gelatin before seeding the cells. Alternatively, the wells can be coated with the recombinant galectin of interest in PBS (optimal concentration should be determined empirically).
2. Seed cells and grow to confluence in 2–3 days (*see* **Note 5**).
3. Scratch the confluent monolayer with a 96-well pin tool (*see* **Note 3**).
4. Aspirate/drain the culture medium.
5. Carefully wash cells with 100 μL PBS.
6. Apply the appropriate medium containing recombinant galectin or galectin inhibitors (*see* **Notes 6** and **7**).
7. Take images of the scratch at $t = 0$, $t = 2$, $t = 4$, $t = 6$, and $t = 8$ h (*see* **Note 8** and Fig. 1a).
8. Measure wound width or scratch area with automated ScratchAnalysis software. Alternatively use ImageJ (use straight line selection tool to measure wound width or free hand selection tool for measuring scratch area) or Photoshop (use ruler for wound width measurement or wand tool for scratch area). Calculate percentage of wound closure or remaining wound width/area (% of $t = 0$).

3.2 Network Formation

1. Coat each well of a flat-bottom 96-well plate with 40 μL Matrigel (*see* **Note 4**) and incubate at 37 °C/99.5 °F for 30 min.

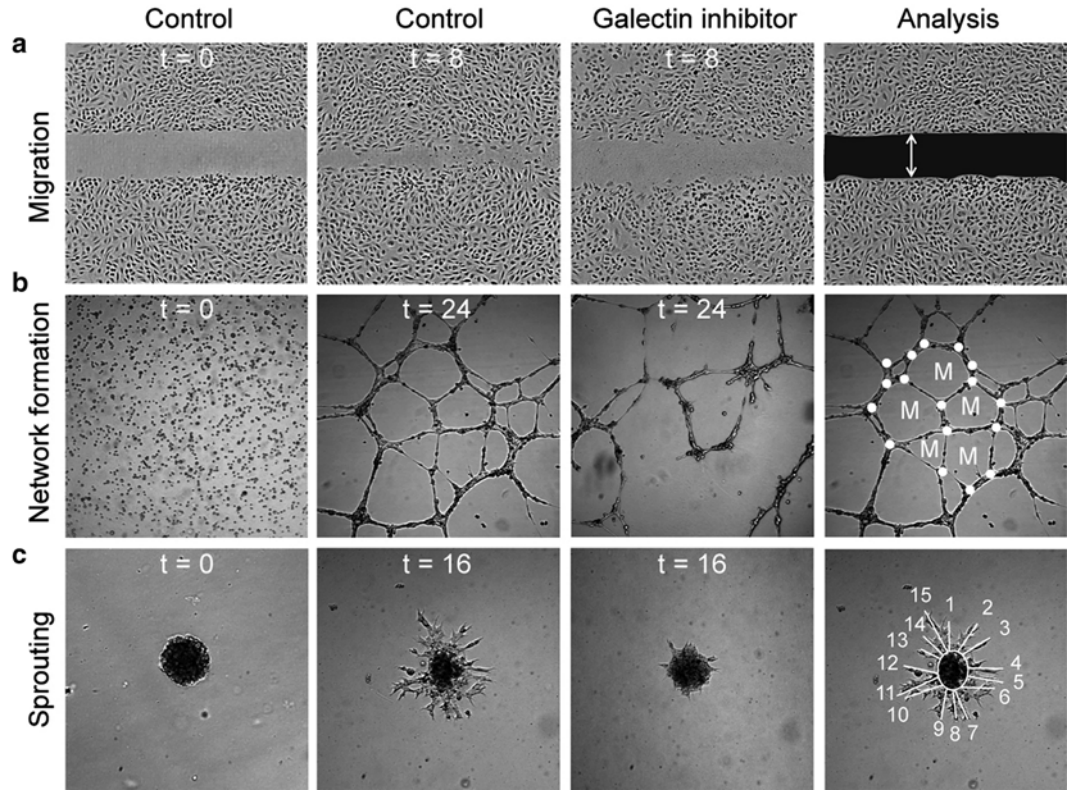

Fig. 1 In vitro angiogenesis assays. Representative pictures of migration (**a**), network formation (**b**), and sprouting (**c**) of non-treated (control) and galectin inhibitor-treated HUVEC. The first panels represent the starting conditions at $t=0$. The second and third panels indicate typical results at the end point of the experiment of non-treated and galectin inhibitor-treated cells, respectively. The last panels illustrate different ways of analyzing the respective results: (**a**) For analysis of migration, wound width (*white arrow*) or wound area (in *black*) can be measured at different time points and compared with $t=0$. (**b**) Tube formation can be quantified by counting the number of meshes (*M*) and branch points (*white dots*). For clarity only part of the image is scored. (**c**) Sprouting is analyzed by measuring the sprout length (*white lines*) and counting the number of sprouts

2. Apply the required number of cells in 50 μL medium per well (*see* **Note 9**). Add recombinant galectin or galectin inhibitors to the medium.
3. Incubate overnight in a humidified incubator at 37 °C/99.5 °F, 5 % CO_2.
4. Acquire images.
5. Endothelial cell network formation can be quantified by counting the number of branch points and number of meshes (*see* Fig. 1b).

3.3 Sprouting

1. Harvest cells and resuspend to a final concentration of 40,000 cells/mL in methocel medium (*see* **Note 10**).
2. Distribute 25 μL drops with a multichannel pipet on the lid of a non-adhesive square petri dish (*see* **Note 11**).

3. Add 10 mL PBS to the bottom of the petri dish to prevent evaporation of the drops.
4. Turn the lid with drops carefully upside down and place on the bottom dish containing PBS.
5. Incubate overnight in a humidified incubator at 37 °C/99.5 °F, 5 % CO_2.
6. Harvest the spheroids by rinsing the plate twice with 6 mL PBS containing 10 % heat-inactivated human serum.
7. Collect the spheroids in a 15 mL tube and centrifuge for 1 min at 250 rcf.
8. Aspirate the PBS and carefully flick the tube to prevent the spheroids from sticking together.
9. Resuspend 60 spheroids per condition in 200 µL Matrigel (*see* **Note 4**) and transfer the suspension to a 24-well plate (*see* **Note 12**).
10. Incubate the plate for 15 min at 37 °C/99.5 °F until the Matrigel is solidified.
11. Apply 500 µL medium, containing recombinant galectin proteins and/or galectin inhibitors on top of the Matrigel (*see* **Note 13**).
12. Incubate in a humidified incubator at 37 °C/99.5 °F, 5 % CO_2, for 16–24 h and take pictures of the spheroids (*see* **Note 14** and Fig. 1c).
13. Analyze sprout length and sprout number per spheroid using ImageJ.

4 Notes

1. Different sources of endothelial cells are available [10]. Human umbilical vein endothelial cells (HUVEC) are frequently used and these cells can be isolated in-house or purchased from a commercial source. Apart from HUVEC, we also use the EC lines EC-RF24 (HUVEC origin) and HMEC (human microvascular endothelial cells). For network formation and sprouting assays we generally use HUVEC while migration assays can be performed with any source of EC. If required, cells can be transfected with galectin-targeting siRNA or galectin expression constructs according to standard protocols. All EC are cultured in RPMI1640 (Lonza) supplemented with 10 % fetal bovine serum (FBS), 10 % human serum, 1 % L-glutamine, and 1 % penicillin/streptomycin. At confluency, passage the cells 1:3.
2. We prefer the use of Costar flat-bottom plates since plates from other suppliers sometimes contain markings on their back due to the production process. These markings can interfere with the scratch analysis.

3. A pin tool scratcher is preferred since this gives low variability and high reproducibility in wound width. We use the Peira HTSScratcher. However, it is also possible to apply a scratch manually using a small pipetting tip, e.g., 10 μL tips. Take a fresh tip for each well to avoid increasing scratch width due to cells that stick to the tip.
4. Matrigel is derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma and contains several extracellular matrix proteins that facilitate endothelial cell adhesion and migration. However, standard Matrigel also contains several growth factors that stimulate the angiogenic activity of endothelial cells. Therefore, depending on the research question, e.g., test effect of inhibitors on galectin-induced network formation or sprouting, it is better to use growth factor-reduced Matrigel. Note that Matrigel stock solutions should be kept at temperatures below 4 °C/39.2 °F to prevent solidification. Thawing should therefore be performed on ice in a fridge (this takes approximately 1 h).
5. It is important to start with a confluent monolayer of cells in order to induce unidirectional migration. For RF24 cells seed 15,000 cells/well, and for HUVEC/HMEC use 5,000 cells/well.
6. Evaluation of inhibitory activity can be performed in normal EC culture medium. To evaluate stimulatory activity, low serum (0.5 % FBS and/or 0.5 % human serum) can be used. Furthermore, we have observed a biphasic activity of galectin-1 depending on the concentration [7]. Therefore, we suggest using a broad concentration range to optimize your assay.
7. Always include proper controls, e.g., Sutent (Sunitinib malate) (Sigma-Aldrich) for inhibition or Fibroblast Growth Factor-Basic (bFGF) (Sigma-Aldrich) for stimulation of migration. Use as directed by the manufacturer.
8. For analysis it is important that the images of the scratch within the time series are taken at the exact same position. If no appropriate software and automated *xyz* table are available to automate this, make sure to mark the culture plate in such a way that the area in the well where the images are taken can be easily found. For example, prior to plating the cells, use a scalpel to make a scratch on the backside of each well in the plate. Make sure that the scratch is perpendicular to the direction of the "wound." The perpendicular scratch can then be used to identify the region where the wound width is measured.
9. The optimal number of cells used in this assay should be individually determined for each cell line. Too high cell numbers will result in robust branches or confluent areas at the center of the well. Too few cells will yield incomplete network structures.

10. Dilute to 40,000 cells/mL to obtain 1,000 cells/25 μL drop. Prepare 60 drops (60,000 cells in 1,500 μL methocel medium) per test condition since not all drops will successfully form a spheroid.
11. Use reverse pipetting technique to avoid air bubbles disturbing the spheroid formation.
12. When transferring the spheroid suspension to a 24-well plate, make sure that the whole well is covered by pipetting the suspension "swirl-wise," starting in the middle of the well. Avoid air bubbles.
13. As a positive control for induction of sprouting, add 100 nM of the γ-secretase inhibitor dibenzazepine (DBZ) to the medium. As a positive control for inhibition of sprouting 10 μM sutent can be added to the medium.
14. For reliable analysis of sprouting at least ten spheroids per treatment condition should be photographed and analyzed.

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Chapter 19

Regulation of Galectins by Hypoxia and Their Relevance in Angiogenesis: Strategies and Methods

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Abstract

Formation of an aberrant and heterogeneous vascular network is a key pathological event in the multistep process of tumor growth and metastasis. Pro-angiogenic factors are synthesized and released from tumor, stromal, endothelial, and myeloid cells in response to hypoxic and immunosuppressive microenvironments which are commonly found during cancer progression. Emerging data indicate key roles for galectins, particularly galectin-1, -3, -8, and -9 in the regulation of angiogenesis in different pathophysiologic settings. Each galectin interacts with a preferred set of glycosylated receptors, triggers different signaling pathway, and promotes sprouting angiogenesis through different mechanisms. Understanding the role of galectins in tumor neovascularization will contribute to the design of novel anti-angiogenic therapies aimed at complementing current clinical approaches. Here we describe selected strategies and methods used to study the galectin-1 regulation by hypoxia and its role in blood vessel formation.

Key words Galectin, Angiogenesis, Tumor neovascularization, Hypoxia

1 Introduction

Angiogenesis is the physiologic mechanism that leads to formation of new blood vessels from preexisting ones and involves the coordinated action of different soluble factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-1 and -2, angiopoietins, and cell adhesion molecules such as integrins [1]. This process can be examined in vitro by studying three critical steps: endothelial cell proliferation, migration, and tube formation in response to different extracellular or intracellular stimuli [1]. Angiogenesis is a hallmark of cancer and various ischemic diseases like retinopathies [2]. The identification of new players of angiogenic

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programs and the elucidation of the precise molecular pathways linking tumor hypoxia to angiogenesis are essential for the design of rational anti-angiogenic therapies.

In addition to modulation of tumor immunity (reviewed by Salatino et al. in this issue), emerging evidence indicates a key role for galectin-1 in the modulation of vascular signaling programs. This glycan-binding protein is up-regulated in hypoxic microenvironments [3, 4] through hypoxia-inducible factor (HIF)-dependent [5] or HIF-independent pathways involving activation of nuclear factor (NF)-κB and production of reactive oxygen species (ROS) [3]. Thijssen and colleagues demonstrated that galectin-1 is expressed in tumor-associated endothelial cells, an effect which is associated with the promotion of an angiogenic phenotype [6]. In addition, endothelial cells can also take up galectin-1 which activates H-Ras signaling and Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase (Erk) kinase (Mek)/Erk cascade, thus stimulating endothelial cell proliferation and migration [7]. This pathway has been proposed to be activated through binding to neuropilin-1 on the surface of endothelial cells [8]. Interestingly, galectin-1 promotes tumor angiogenesis in different tumor models including Kaposi's sarcoma, melanoma, and prostate cancer [3, 6, 7, 9, 10]. Disruption of galectin-1-*N*-glycan interactions, using a galectin-1-specific monoclonal antibody or through inhibition of complex *N*-glycan branching, abrogates hypoxia-driven angiogenesis and tumorigenesis in a model of Kaposi's sarcoma [3], suggesting that blockade of galectin-1 may contribute not only to potentiate tumor immunity, but also to ameliorate hypoxia and block neovascularization in different tumor types. Furthermore, other galectins including galectin-3 and galectin-8 also contribute to tumor angiogenesis [11–13]. The $\alpha_v\beta_3$ integrin has been proposed to be a major galectin-3-binding protein [11] and CD166 (activated leukocyte cell adhesion molecule; ALCAM) has been identified as a candidate receptor for galectin-8 in normal vascular ECs [14]. Here we describe a selection of methods used to study the role of galectins, particularly galectin-1, in the modulation of tumor angiogenesis and their regulated expression by hypoxic microenvironments [3, 15].

2 Materials (*See* Note 1)

2.1 Hypoxia Induction in Modular Incubation Chamber

1. Modular Incubator Chamber (MIC-10, Billups-Rothenberg).
2. Petri dishes.
3. Cells to be evaluated.
4. Appropriate cell culture medium (follow guidelines for individual cell culture).
5. O_2 gas cylinder.

6. N_2 gas cylinder.
7. CO_2 gas cylinder.
8. Closing clamps.
9. Oxygen sensor.
10. Conventional incubator.

2.2 Evaluation of Hypoxia. Hif-1α Detection by Western Blot

1. Cells to be evaluated (e.g., tumor cells; endothelial cells).
2. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4.
3. Protein Extraction Buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 10 mM EDTA; 1 % v/v NP-40) with protease and phosphatase inhibitors cocktails (Sigma).
4. 18 mm cell scraper (Corning).
5. 2× Laemmli sample buffer (BioRad).
6. Amersham Hybond-ECL (GE Healthcare).
7. 1.6 ml tubes (Axygen).
8. Tris-buffered saline (TBS): 150 mM NaCl, 50 mM Tris, pH: 7.4.
9. tTBS (TBS with 0.1 % Tween 20).
10. Blocking buffer: tTBS with 5 % nonfat milk or bovine serum albumin (BSA).
11. HIF-1α primary antibody (MA1-516, Pierce).
12. HRP-conjugated secondary antibody (Vector Labs).
13. Immobilon chemiluminescent HRP substrate (WBKLS01-00, Millipore).
14. PVDF membrane (Millipore).
15. 7.5 % SDS-polyacrylamide electrophoresis gel.
16. GBOX incubator (Syngene).
17. Bradford assay kit (Pierce).

2.3 Detection of Soluble VEGF

1. Cells to be evaluated.
2. Cell culture medium.
3. 15 ml tubes (BD).
4. P60 petri dish (GBO).
5. Human VEGF DuoSet ELISA Kit (R&D System).

2.4 Assessment of Angiogenesis In Vitro

2.4.1 Endothelial Cell Tubulogenesis

1. Conditioned media (*see* **Note 2**).
2. Primary Human Umbilical Vein Endothelial Cells (HUVEC) or Bovine Aortic Endothelial Cells (BAEC) (*see* **Note 3**).
3. Matrigel Reduced Growth Factor Basement Membrane Matrix (BD Biosciences).

4. DMEM medium (D1) supplemented with 1 % heat-inactivated FBS (PAA, the Cell Culture Company), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (Life Technologies).
5. 24-well plates (GBO).
6. Crystal Violet aqueous solution 0.1 % (Sigma-Aldrich).
7. Recombinant human Gal-1 (rGal-1).
8. Anti-Gal-1 monoclonal antibody (Clone F8.G7).
9. Lactose (Sigma).
10. Incubator set at 37 °C, 5 % CO_2.
11. Inverted phase microscope.
12. Digital camera (Nikon).

2.4.2 Endothelial Cell Migration

1. Conditioned media (*see* **Note 2**).
2. Primary Human Umbilical Vein Endothelial Cells (HUVEC) or Bovine Aortic Endothelial Cells (BAEC) (*see* **Note 3**).
3. DMEM medium (D1) supplemented with 1 % heat-inactivated FBS (PAA, the Cell Culture Company), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (Life Technologies).
4. Endothelial cell migration 24-multiwell transwells, 8 μm (BD Biosciences).
5. 24-well plates (GBO).
6. rGal-1.
7. Lactose (Sigma).
8. Incubator set at 37 °C, 5 % CO_2.
9. 0.1 % crystal violet solution (Sigma-Aldrich).
10. Distilled water.
11. Q-tips.
12. Inverted microscope.
13. Chemoattractant (*see* **Note 4**).
14. rVEGF (R&D).

2.5 Assessment of Angiogenesis In Vivo

2.5.1 Matrigel Plug Assay

1. Matrigel Reduced Growth Factor Basement Membrane Matrix (BD Biosciences).
2. 1 ml syringe (Neojet).
3. 23 G needle (BD).
4. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4.
5. rVEGF-A (R&D system).
6. FGF-2 (R&D system).

7. TNF-α (R&D system).
8. Heparin (Fluka, Sigma).
9. Serum-Free Conditioned Media from Kaposi's sarcoma.
10. Gal-1 shRNA.
11. rGal-1.
12. Anti-Gal-1 monoclonal Ab (Clone F8.G7).
13. 1.6 ml tubes (Axygen).

2.5.2 Inoculation of Matrigel Plugs to Evaluate Angiogenesis In Vivo

1. 23 G needle.
2. Matrigel mix (*see* Subheading 3.5.1).
3. Athymic nude mice.
4. C57BL/6 *Lgals1*$^{-/-}$ (Gal-1 KO).
5. C57BL/6 WT (Jackson).

2.5.3 Determination of Angiogenesis In Vivo in Matrigel Plugs

1. Scale.
2. H_2O.
3. P60 Petri dish (GBO).
4. Drapkin's reagent (Sigma).
5. Spectrophotometer.
6. Mouse hemoglobin (Sigma).
7. RPMI (Gibco).
8. 50 ml conical tubes (BD/Falcon).
9. Collagenase II solution (0.03 % in PBS/Sigma).
10. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4.
11. 100 μm cell strainer (BD bioscience).
12. 1 % fetal bovine serum (FBS) (Gibco).
13. Paraformaldehyde (1 % and 4 % w/v buffer).
14. PE-conjugated anti-CD34 antibody (clone RAM34 BD biosciences).
15. Phosphate buffered saline with 1 % FBS and 0.05 % NaN_3.
16. Ketamine (Holliday scott).
17. Xylazine (Richmond).
18. Optimum cutting temperature (OCT) medium (Biopack).
19. Cryostat.
20. PBS 10 % normal rat serum (Sigma).
21. Acetone (Cicarelli).
22. Anti-PECAM-1/CD31 antibody (clone MEC13.3 Novus Biologicals).

23. PBS 1 % BSA.
24. Slides.
25. AlexaFluor 596-conjugated goat anti-rat antibody (Cell Signaling).
26. Fluoromount slide mounting media (Southern Biotech).
27. DAPI (Life technologies).

2.6 Special Equipment

1. Flow cytometer.
2. Fluorescence microscope.

3 Methods

3.1 Induction of Hypoxia in Modular Incubator Chamber

1. Prepare cell cultures at 60–70 % of confluence.
2. Open the chamber incubator, place a Petri dish containing water and place the cells inside the incubator. Close the incubator ensuring that it is hermetically closed.
3. Maintain a separate cell culture in normoxia as control.
4. To generate an hypoxic atmosphere, flush a mixture of 1 % O_2, 5 % CO_2, and 94 % N_2 at 2 psi during 10 min. Turn off the gas flow and isolate the chamber by closing clamps (*see* **Notes 5** and **6**).
5. Place the chamber in a conventional incubator for 18–24 h (*see* **Notes 5** and **6**).

3.2 Evaluation of Hypoxia. HIF-1α Detection by Western Blot

1. Open the chamber and immediately place cell cultures on ice.
2. Remove culture medium, and wash with PBS twice.
3. Add protein extraction buffer (30 μl for 60 mm dishes) and use a scraper on hypoxia-treated and control cells.
4. Collect the total volume and centrifuge at 16,000 × *g* for 20 min in a 4 °C precooled centrifuge.
5. Transfer the supernatant to a fresh 1.6 ml tube on ice and discard the pellet.
6. Remove a small volume (5 μl) of lysate to perform Bradford assay according the manufacturer's recommended protocol.
7. Determine the protein concentration for each cell lysate. Prepare 20–40 μg of total cell lysate in 2× Laemmli sample buffer and boil it for 3 min.
8. Run samples on a 7.5 % SDS-PAGE gel and transfer to a PDVF membrane.
9. Block the membrane with TBS 0.1 % Tween-20 (tTBS) with 5 % nonfat milk at room temperature for 1 h on constant stirring.

10. Incubate for 18 h with HIF-1α primary antibody diluted 1:500 in tTBS 1 % nonfat milk at 4 °C on constant stirring.
11. Wash three times with tTBS at room temperature for 10 min and incubate with HRP-conjugated secondary anti-mouse antibody diluted 1:3,000 in tTBS for 1 h at room temperature.
12. Wash three times, incubate with Immobilon chemiluminescent HRP substrate, and capture the luminescent image in a GBOX incubator.

3.3 Detection of Soluble VEGF by ELISA

1. Collect culture medium supernatants from 24 h cell cultures in P60 Petri dishes. Subject samples to quick centrifugation (spin) in 15 ml tubes to eliminate cellular debris. Store supernatants at −80 °C until use.
2. Determine VEGF concentration in the supernatants with a VEGF ELISA kit following the manufacturer's instructions.

3.4 Assessment of Angiogenesis In Vitro

3.4.1 Endothelial Cell Tubulogenesis (See **Note 7**)

1. Seed 150 μl of Matrigel per well in prechilled 24 well plate. Incubate 2 h at 37 °C.
2. Add the conditioned media (CM) to be tested. Avoid freeze/thaw cycles of the CM. Different dilutions of the CM should be assayed.
3. Adjust to a final volume of 400 μl.
4. Add 25,000 endothelial cells in 100 μl D1 per well.
5. Add 1 μM recombinant galectin-1 (rGal-1) or other relevant galectin to evaluate tubulogenesis. Use 30 mM lactose or anti-Gal-1 monoclonal antibody to selectively block Gal-1 function (*see* **Note 8**).
6. Incubate at 37 °C and 5 % CO_2. Visualize slides at phase contrast microscope every hour for 24 h.
7. When tubular structures are apparent, take photomicrographs of several fields.
8. Quantify tubular structures (*see* **Note 8**).

3.4.2 Endothelial Cell Migration

1. Seed 40,000 endothelial cells per well in 250 μl D1 in the upper chamber of the endothelial cell migration 24-multiwell transwells (*see* **Note 9**).
2. The bottom well is filled with 750 μl CM containing the chemotactic factor to be tested, or other modulators of endothelial cell migration.
3. Add 1 μM rGal-1 or 1 μM rGal-1 plus 30 mM lactose to evaluate the effects of Gal-1 on endothelial cell migration.
4. Incubate for 18–24 h at 37 °C, 5 %CO_2.

5. Stain transwells with 0.1 % crystal violet solution for 10 min.
6. Wash transwells with distilled water.
7. Remove excess Matrigel with a Q-tip.
8. Examine at inverted microscope and count the number of cells. Data are expressed as cells per cm^2. A "fold-migration" value may be calculated as the number of cells migrating in response to rGal1 or VEGF (positive control), relative to the number of cells in the absence of mediator.

3.5 Assessment of Angiogenesis In Vivo

3.5.1 Matrigel Plug Assay

Matrigel preparation:

1. It is important to keep Matrigel HC as cold as possible (lower than 10 °C). It is recommended to maintain all materials and reagent (syringes, needles, solutions, pipettes, etc.) on ice prior to use.
2. Mix 300 μl of Matrigel with 200 μl of experimental solution in 1.6 ml tubes.
   (a) Experimental solutions:
   - Positive control: PBS + VEGF (10 ng/ml) + FGF-2 (20 ng/ml) + TNF-α (5 ng/ml), + heparin 10,000 IU (positive control mix) [16].
   - Serum-Free Conditioned Media (SFCM) from Kaposi's sarcoma (KS) cells exposed or not to a hypoxic atmosphere (1 % O_2, 5 % CO_2, and 94 % N_2 during 18 h) and transduced or not with Gal-1 shRNA encoded retrovirus [3].
   - PBS + rGal-1 (1.5 μM).
   - PBS + rGal-1 (1.5 μM) + anti-Gal-1 monoclonal antibody Clone F8.G7 (1 μM).
3. Vortex tubes.
4. Inject the solution subcutaneously using a 23 G precooled needle. Injections should be done quickly to prevent the gel from solidifying.

3.5.2 Inoculation of Matrigel Plugs to Evaluate Angiogenesis In Vivo

1. Using 23G precooled needle inject 0.5 ml of Matrigel mix subcutaneously into anesthetized female athymic nude mice (for SFCM studies) or female C57BL/6 *Lgals1*$^{-/-}$ (KO) or WT (for rGal-1 studies).
2. After 7 days, euthanize mice and remove the Matrigel plugs.
3. Angiogenesis can be evaluated by studying three independent parameters (*see* Subheading 3.5.3):
   (a) Hemoglobin content in pellets.
   (b) Number of endothelial cells.
   (c) Microvascular density.

3.5.3 Determination of Angiogenesis In Vivo in Matrigel Plugs

Hemoglobin content

1. Remove Matrigel plugs, weight and mechanically disaggregate them in 1.5 ml of H_2O in a Petri dish.
2. Incubate for 20 min at RT.
3. Centrifuge for 5 min at 10,000 × *g*.
4. Discard cell pellet.
5. Incubate supernatant with Drabkin's reagent according to the manufacturer's instructions.
6. Read absorbance of tubes at 540 nm. A standard curve of mouse hemoglobin should be simultaneously performed. Plot absorbance vs. cyanmethemoglobin concentration (mg/ml) and interpolate. The final concentration of hemoglobin in Matrigel pellets is calculated as mg/ml per 100 mg of pellets.

Number of endothelial cells

1. Remove Matrigel pellets, and mechanically disaggregate them in 5 ml of RPMI medium in a Petri dish.
2. Transfer to a 50 ml conical tube, add 5 ml of collagenase II solution, and incubate for 10 min at 37 °C water bath. After incubation, add 25 ml of PBS and filter the suspension through a 100 μm cell strainer.
3. Wash the filtered solution by adding 5 ml PBS and centrifuge for 5 min at 800 × *g*.
4. Remove the supernatant and wash the pellet with PBS 1 % FBS.
5. Stain cells with 1 μg of PE-conjugated anti-CD34 antibody in 100 μl of PBS 1 % FBS 0.05 % NaN_3 for 45 min in ice.
6. Wash cells with PBS and centrifuge for 5 min at 800 × *g*.
7. Fix cells with 1 % paraformaldehyde.
8. Analyze the percentage of PE^+ $CD34^+$ cells by flow cytometry.

Analysis of microvascular density (MVD)

1. Anesthetize animals (ketamine/xylazine, 140/14 mg/kg) and perfuse with PBS and 4 % paraformaldehyde.
2. Remove Matrigel pellets and embed them in frozen Optimum Cutting Temperature (OCT) medium and freeze at –70 °C.
3. Cut frozen Matrigel into 40–100 μm sections with a cryostat.
4. Air-dry sections at RT and fix in acetone for 10 min at –20 °C.
5. Air-dry for 5 min. Wash three times with PBS.
6. Block nonspecific binding through incubation for 1 h at RT with PBS 10 % normal rat serum.
7. Incubate sections with 1.5 μg of anti-PECAM-1/CD31 antibody in 200 μl of PBS 1 % BSA ON at 4 °C.
8. Wash with PBS three times.

9. Incubate for 1 h at RT with 0.2 μg of AlexaFluor 596-conjugated goat anti-rat antibody in 100 μl PBS.
10. Wash slides and mount in Fluoromount slide mounting medium containing DAPI as counterstaining fluorophore.
11. Determine microvessel density (MVD) by counting the number of microvessels per mm^2 in ten randomly selected fields (200×).

4 Notes

1. Galectins, particularly galectin-1, -3, and -8, have recently emerged as novel pro-angiogenic molecules responsible of the generation of tumor vascular networks [3, 6–15]. In this chapter we enumerate and discuss some of the strategies used to study the regulation of galectin-1 by hypoxic microenvironments and the stimulatory function of galectins in angiogenesis in vitro and in vivo. These lectins may act as "cytokine-like molecules" and contribute to angioproliferative and immunosuppressive nature of different pathologic conditions [3, 15, 17]. Understanding the molecular and cellular mechanisms underlying the pro-angiogenic function of galectins will contribute to delineate novel therapeutic strategies. We hope that the strategies and methods described here will facilitate and encourage scientists to further evaluate the role of galectins in neovascularization processes in different pathophysiologic settings.
2. To obtain serum-free conditioned media (CM), cells are cultured in normal culture media (RPMI, 10 % FCS) until reaching ~80 % confluency. Then, medium is discarded, cells are washed three times with sterile PBS, and serum-free (SF) RPMI media is added. After ~24 h, CM is collected, filtered with 0.22 μm syringe, filter and distributed in 1 ml aliquots.
3. For Primary Human Umbilical Vein Endothelial Cells (HUVEC) or Bovine Aortic Endothelial Cells, passages 8 or lower are recommended.
4. We recommend the use of oxygen sensors for more precise measurement of the intra-chamber O_2 levels during the experiment.
5. In order to eliminate the O_2 diluted in the medium it is recommended to re-gas the chamber once after 2 h or bubbling the gas into the cell culture medium before starting the experiment.
6. Hypoxia regulates a large number of genes through the binding of HIF-1α to Hypoxia Response Element (HRE) sequences. Many genes are under the regulation of HRE such as VEGF-A and erythropoietin. Therefore, measurement of up-regulation of pro-angiogenic mediators is an effective and reliable method to evaluate induction of HIF-dependent hypoxia.

7. Suggested Experimental Controls: Negative control: CM is replaced by D1, Positive control: 10 ng/ml rVEGF diluted in D1
8. Quantification can be done through multiple ways. One is based on counting the number of tubules/cm^2. On the other hand, morphometric analysis can be performed and tubules length can be measured using the ImageJ software.
9. The filter pores are small enough ~8 μm to allow passage of actively migrating cells; otherwise they rest upon the filter.

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Chapter 20

Examination of the Role of Galectins During In Vivo Angiogenesis Using the Chick Chorioallantoic Membrane Assay

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Abstract

Angiogenesis is a complex multi-process involving various activities of endothelial cells. These activities are influenced in vivo by environmental conditions like interactions with other cell types and the microenvironment. Galectins play a role in several of these interactions and are therefore required for proper execution of in vivo angiogenesis. In this chapter we describe a method to study galectins and galectin inhibitors during physiologic and pathophysiologic angiogenesis in vivo using the chicken chorioallantoic membrane (CAM) assay.

Key words Chorioallantoic membrane (CAM) assay, Chicken, Angiogenesis, Galectin, Tumor graft, Blood vessel, Vasculature

1 Introduction

Angiogenesis is a complex multi-process involving different activities of endothelial cells. The function of endothelial cells can be influenced by environmental conditions like changing flow dynamics, interactions with other cell types, and interactions with specific extracellular matrix components [1, 2]. Thus, while in vitro assays can provide insights in the effects of molecules like galectins and/or galectin inhibitors on endothelial cell function, further assessment of their role in angiogenesis in vivo is important. A commonly used assay to study angiogenesis in vivo is the chick chorioallantoic membrane (CAM) assay. The CAM is a highly vascularized extraembryonic membrane that mediates exchange of gas and nutrients during embryonic chick development. It is formed between embryonic day of development (EDD) 3–10 of

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the 21-day gestation period by fusion of the allantois mesodermal layer—extending out of the embryo—with the mesodermal layer of the chorion. Within the resultant double layer a dense vascular network develops up to EDD11 after which endothelial cell proliferation drops rapidly allowing further maturation of the vascular bed [7, 9, 14].

Apart from the rapid vascular development there are numerous advantages that warrant the use of the CAM assay for in vivo angiogenesis studies. First, the assay is low in cost, reproducible, reliable, and fairly simple to perform [9]. Furthermore, there are a variety of methods for the application of testing compounds using CAM and several methods are available to monitor the subsequent response in the vasculature. For example, we have used the CAM assay to study the effects of galectin-1 and galectin-1 inhibition on angiogenesis [12, 13]. The CAM assay can also be used to test the effects of other treatment modalities like radiotherapy or photodynamic therapy [5, 6]. Finally, the model does not require a sterile work environment and since the immune system of the chicken embryo is not fully developed until ± EDD18 the CAM assay can also be used for grafting xenograft cells and tissues. On the other hand, nonspecific reactions can occur due to contamination with egg shell itself or due to the use of reactive carrier vehicles [14]. In addition, CAM development is sensitive to alterations in environmental conditions like temperature, oxygen tension, and osmolarity. This indicates that experiments using the CAM assay should be carefully executed. In this chapter we will describe a method for the topical application of soluble compounds (galectins and/or galectin inhibitors) on the CAM in order to study their effects on angiogenesis in vivo. In addition, we provide a method to graft tumor cells onto the CAM which can provide information on the role of galectins in tumor growth and tumor angiogenesis.

2 Materials

We use fertilized eggs of white leghorn chicken that are purchased from a local commercial supplier. The eggs can be stored for several days at 4 °C/39.2 °F but after more than 1 week the quality and viability of the eggs decreases affecting the quality of the data. Please be aware that depending on the legislation of your country regarding animal use in research experiments, a license might be needed to perform the described experiments.

2.1 Incubation of the Chicken Eggs

1. 70 % Ethanol.
2. Sterilized fine tweezers.
3. Scotch "Magic" adhesive tape (*see* **Note 1**).

2.2 Application of Galectins/Galectin Inhibitors onto the CAM

1. Non-latex elastic rings (*see* **Note 2**).
2. Saline.
3. Test compounds, i.e., galectins and/or galectin inhibitors.
4. 10–100 μL pipette with sterile filter tips.

2.3 Data Acquisition and Analysis

1. Fridge or cold room.
2. 20 mL syringe.
3. 33 G injection needle.
4. Contrast solution (4 g of zinc oxide in 50 mL pure vegetable oil).

2.4 Data Acquisition and Analysis

1. Image analysis software (HetCAM, DCIlabs) or Adobe Photoshop/MS Office.

2.5 Grafting Tumor Cells onto the CAM

1. Approximately 5×10^6 cells (*see* **Note 3**).
2. Matrigel (*see* **Note 4**).
3. Soft paper tissues.
4. Ice.
5. 100 μL pipette with sterile filter tips.
6. Ruler with mm scaling.
7. Small surgical scissors.
8. Balance.
9. Phosphate buffered saline (PBS).
10. Fixative, e.g., 4 % paraformaldehyde in PBS or zinc fixative.

2.6 Special Equipment

1. Fan-assisted humidified (egg) incubator, 37.5 °C/99.5 °F (*see* **Note 5**). We use a FIEM MG140/200 Rural which allows us to switch between tilting racks and non-tilting racks (*see* **Note 6**). The incubator should be well humidified throughout the whole experiment to prevent dehydration of the CAM. We achieve this by putting water basins on the floor of the incubator (*see* **Note 7**).
2. Fiber optic illuminator. We use a Schott KL 1500 Electronic Light Source (*see* **Note 8**).
3. Stereo microscope equipped with a camera (e.g., Leica M125 stereomicroscope with 12.5:1 zoom which is equipped with a Leica KL1500 LED ring illumination system and a Leica 5 Megapixel DFC425 CCD camera).

3 Methods

A schematic drawing of the chicken gestation period is shown in Fig. 1a. A typical CAM assay takes approximately 10 days and a CAM tumor graft experiment takes 17 days. Thus, depending on

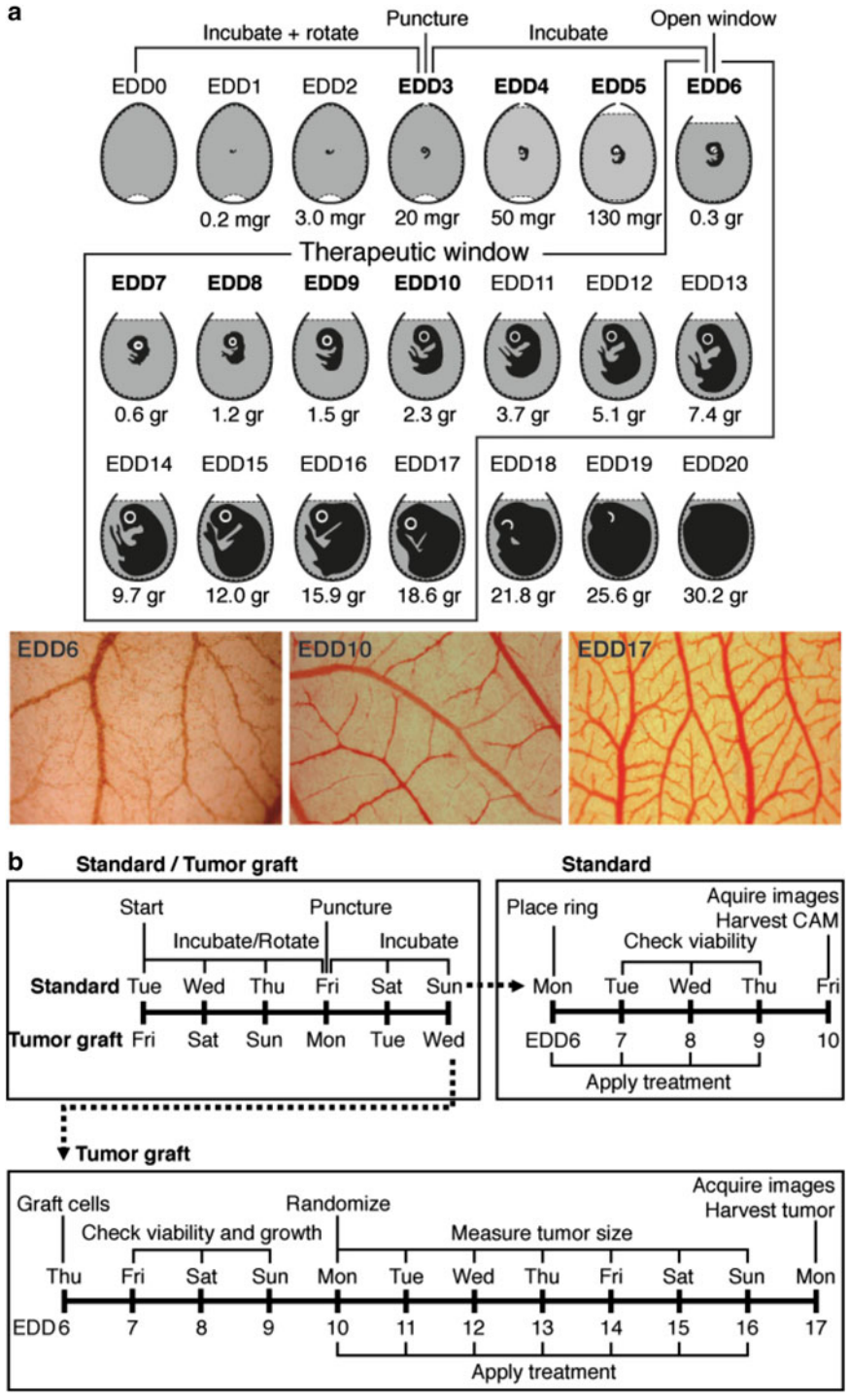

Fig. 1 Time schedule for the CAM assay. **(a)** Schematic representation of the CAM assay schedule during embryonic chicken development from embryonic day of development (EDD) 0 until EDD20, i.e., the day before hatching. The EDD during which extensive angiogenesis takes place in the CAM is shown in **bold**. The images show the CAM vasculature at different EDD. (**b**) Scheme of a standard CAM assay (*upper panels*) and of the tumor graft assay on the CAM (*lower panel*)

the type and frequency of treatment, careful planning of the experiments is important. The time schedules that are used in our lab for a normal CAM experiment as well as for a tumor graft CAM experiment are shown in Fig. 1b.

3.1 Incubation of the Chicken Eggs

1. Transfer the eggs from the cold storage to room temperature for at least 12 h prior to the incubation (*see* **Note 9**).
2. Clean the shell of each egg with a tissue soaked in 70 % ethanol.
3. Place the eggs horizontally on a 90° tilting rack, which rotates minimally six times per 24 h. Place the rack in a pre-warmed and humidified fan-assisted egg incubator at 37.5 °C/99.5 °F (*see* **Notes 5–7**). The starting day of the incubation is regarded as EDD0.
4. On EDD3, put the eggs in an upright position and make a small hole in the narrow end of the shell with fine tweezers. This will translocate the air compartment in the egg to the top of the egg. Seal the hole with adhesive tape using as little tape as possible (*see* **Note 10**). Stop the rotation of the racks and place the eggs back in the incubator, with the sealed hole at the top.
5. On EDD6, check the eggs for fertilization. Point the fiber optic light source towards one side of the egg. Vasculature should become visible at the opposite side of the egg. If not, the egg is not fertilized and can be discarded.
6. Create a window of ±1 cm^3 in the top of the shell with fine tweezers (*see* **Note 11**). The CAM vasculature can now be observed through the window.
7. Proceed with direct application of galectins/galectin inhibitors onto the CAM (Subheading 3.2) or with grafting of tumor cells onto the CAM (Subheading 3.5).

3.2 Application of Galectins/Galectin Inhibitors onto the CAM

1. On EDD6, carefully place a sterilized non-latex plastic dental ring through the window on top and in the center of the CAM. Seal the window with adhesive tape (*see* **Note 10**) and place the egg back in the incubator for at least 2 h. This allows the ring to settle down on the CAM (*see* **Note 12**).
2. Prepare the treatment solution by diluting the appropriate galectin with or without the specific inhibitor in saline. The total amount of solution depends on the number of eggs, the diameter of the ring, and the duration/frequency of the treatment (*see* **Note 13**).
3. Take an egg out of the incubator. Open the sealed window and check if the embryo is still alive. Apply 50–80 μL of galectin/galectin inhibitors within the ring without touching the CAM itself. Reseal the window and place the eggs back in the incubator (*see* **Note 10**).

4. Repeat the addition of galectin/galectin inhibitors depending on your required treatment schedule. Usually, treatment is performed on a daily basis until EDD9.

3.3 Data Acquisition

1. On EDD10, place the eggs at 4 °C/39.2 °F for 30 min to induce hypothermia (*see* **Note 14**).
2. Prepare contrast solution by mixing 4 g of zinc oxide with 50 mL of pure vegetable oil in a 50 mL tube. Shake vigorously and leave it on a roller platform for 20 min.
3. Fill a 20 mL syringe with the zinc oxide/oil mixture. Make sure to remove any air bubbles.
4. Open the shell of a hypothermic egg as far as possible without disrupting the CAM.
5. Carefully inject ± 1 mL of the contrast solution directly under the CAM where the ring is located.
6. Use the microscope with camera to acquire images of CAM vasculature within the ring area (*see* **Note 15**).
7. If necessary, the treated CAM area can be collected for further analysis, e.g., gene expression, immunohistochemistry. Following acquisition of images, isolate the CAM area under the ring using fine tweezers and small surgical scissors. Wash the freshly isolated CAM in PBS and transfer it to the desired fixation buffer or liquid nitrogen. Further processing of the tissue is not described in this chapter.
8. Finally, euthanize the chicken embryo by transferring the egg to −20 °C/−4°°F for 24 h.

3.4 Data Analysis

Several methods have been published to analyze the CAM images [3, 4, 11, 14]. Nowadays, software-based image analysis is often used for rapid, objective, and extensive image analysis. The software uses specific algorithms to recognize and skeletonize the vascular bed from which different vascular parameters can be extracted like vessel length, vessel branchpoints, vessel endpoints, and total vessel area (Fig. 2a). We use HetCAM software (DCIlabs, Belgium) but other software packages might be used as well. However, we are aware that such software is expensive and not always available. Therefore, we here describe a widely accepted morphometric method to analyze images of the CAM, using software that is available in most research labs (Fig. 2b) (*see* **Note 16**).

1. Open the desired CAM image in a graphics editing program like Adobe Photoshop (or any comparable software package).
2. Set the image to grayscale to enhance the contrast between the vessels and the background. If necessary, use the image autocontrast function to improve contrast. Note that this should be performed for all images within a single experiment and that this should not be used to obscure or remove any unwanted data.

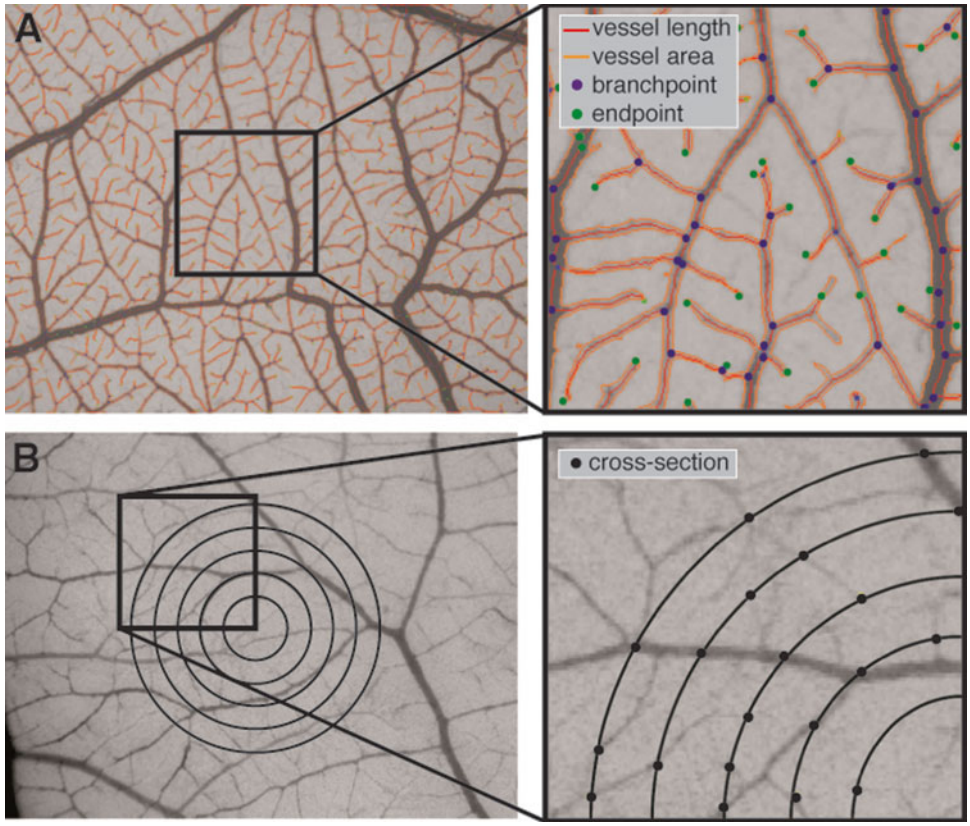

Fig. 2 Analysis of the CAM vasculature. (**a**) CAM analysis by skeletonization-based method. The HetCAM software (DCI labs) automatically analyses the skeleton length, the vessel area, the number of endpoints, and the number of branchpoints in each CAM picture. This method provides a highly objective and precise analysis of the vascular bed, which is quick and allows for high-throughput analysis. (**b**) Morphometric CAM analysis. In this method, five concentric rings are projected over the CAM image and the cross sections of the vessels with the rings are counted. This will give insight into the vessel density of the CAM

3. Place the CAM image in a graphic design program like Adobe Illustrator or a presentation program like Powerpoint.
4. Project five concentric rings over the CAM image and count the cross sections between the vessels and the rings. The sum of these counts is a morphometric measurement for vessel density in the CAM.

3.5 Grafting Tumor Cells onto the CAM

While the method described above provides information on the direct effects of galectin/galectin inhibitors on angiogenesis, much galectin research is performed in the context of tumor biology. Consequently, it is important to determine how galectin expression in tumor cells or treatment with galectin-targeting compounds affects tumor growth and tumor angiogenesis. This can be readily studied using the CAM assay since it is possible to graft (human) tumor cells onto the CAM, most of which will rapidly grow into well-vascularized tumors.

1. On EDD6, harvest the tumor cells. Count the cells and aliquot them in separate tubes, each tube containing 5×10^6 cells and spin them down. Discard the medium.
2. On ice, mix 5×10^6 cells with 50 µL Matrigel (*see* **Note 3**).
3. Carefully "damage" a small area of the CAM with a soft tissue.
4. Transfer the Matrigel/cell mix onto the damaged area.
5. Close window and place egg back into incubator.
6. Check growth of tumor daily and measure size and width using a ruler (*see* **Note 17**).
7. If necessary, start treatment on EDD10 by applying the appropriate galectin inhibitor topically onto the tumor or by direct injection into the tumor tissue.
8. Measure size on a daily basis until EDD14 or maximally until EDD17 (*see* **Note 17**).
9. At the end of the experiment, harvest, photograph, and weigh the tumor and subsequently place it in the appropriate fixative for further processing (Fig. 3).
10. Discard the eggs as described in Subheading 3.3.

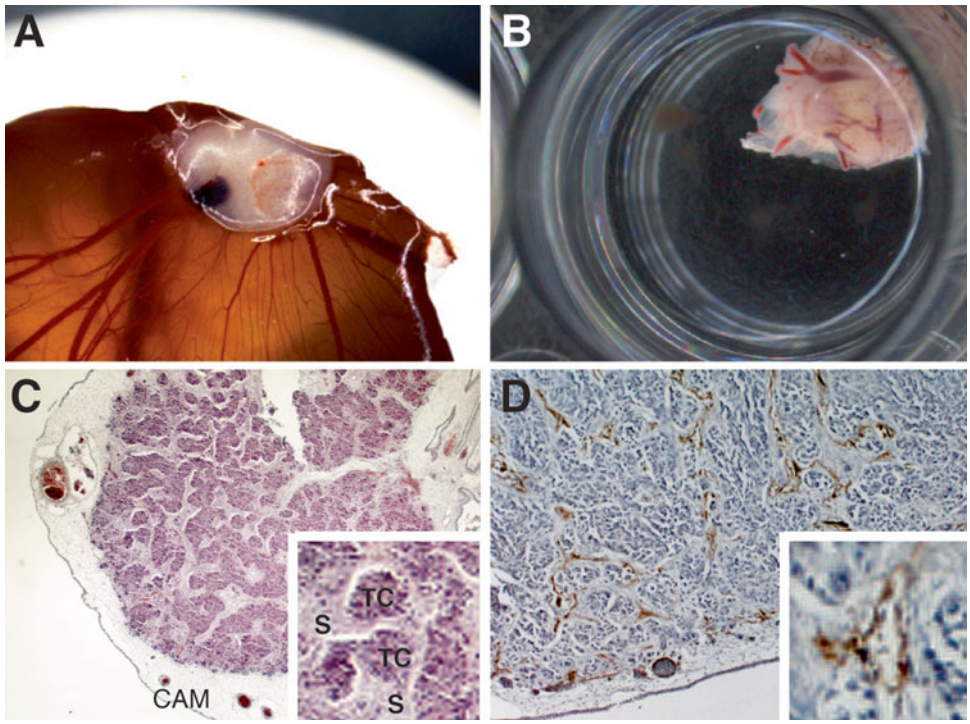

Fig. 3 Tumor grafts on the CAM. (**a**) Image of a HT29 tumor on the CAM at EDD14. The tumor cells were grafted on EDD6. (**b**) HT29 tumor after resection. The tumor is well vascularized indicating adequate tumor angiogenesis. (**c**) Image of hematoxylin/eosin staining on a paraformaldehyde-fixed and paraffin-embedded HT29 tumor graft. Both the CAM and nest of tumor cells (TC) surrounded by tumor stroma (S) are clearly visible. (**d**) Image of vessel staining (CD31, *brown*) on a paraformaldehyde-fixed and paraffin-embedded HT29 tumor graft

4 Notes

1. Other brands of tape can be used but we have good experience with the Scotch Magic tape because it is not too sticky which makes it easy to repeatedly open and close the window in the egg shell.
2. Non-latex rings are commercially available or they can be custom-made. It is important that the weight of the ring is as low as possible to avoid aspecific responses in the vasculature due to occlusion of vessels by the ring. We have found that orthodontic dental elastic bands (non-latex, Ø 9.5 mm) are a good and cheap alternative. Varying the diameter of the rings allows larger areas to be treated but also requires more compound. In addition, with increasing diameter the weight of the ring also increases. A diameter of± Ø 9.5 mm typically allows application of 50–80 μL solution.
3. The number of cells required for adequate grafting depends on the specific cell line and should be tested. However, we have found that increasing the cell number increases the success of grafting and most cell lines tested in our lab show successful grafting when five million cells are used.
4. We have successfully used both normal and growth factor reduced Matrigel. The latter is preferred if the angiostimulatory effect of cells is tested since normal Matrigel already contains more stimulatory factors by itself.
5. Temperature setting depends on the type of incubator. For a still-air incubator (no fan): 38.5 °C (101.3 °F) measured at the top of the eggs. For a fan-assisted incubator: 37.5 °C (99.5 °F) measured anywhere in the incubator.
6. If the incubator has no tilting racks, manual rotation of the eggs is also possible. Turn the eggs through 180° by hand at least twice a day.
7. Humidity should be maintained at ±50 % during incubation. Excessive humidity could result in an increased rate of infections in the eggs.
8. The fiber optic illuminator is used to check successful fertilization on EDD3. However, if no such device is available successful fertilization can also be readily checked following opening of the egg shell.
9. We use anywhere between 8 and 10 eggs per treatment condition. For experienced users a total number of about 80 eggs per experiment are manageable which thus allows for 8–10 different groups per experiment.
10. The adhesive tape prevents the CAM from dehydration. However, the tape also prevents gas exchange through the egg shell and should therefore be kept to a minimum.

11. The egg shell should be removed carefully since debris of the shell can induce a response in the CAM.
12. Instead of a ring to define the therapeutic area, some people prefer to use for example gelatin or methylcellulose discs [8, 10]. Also other absorbent materials might be used as long as these do not induce a response in the CAM.
13. In general, 50–80 μL of compound is applied daily from EDD6 until EDD9. The solution can be prepared fresh daily or stored at 4 °C/39.2 °F. Note that long-term storage at 4 °C/39.2 °F of galectins in solution can affect protein stability and activity. Furthermore, solutions stored at 4 °C/39.2 °F should be allowed to return to room temperature before applying them to the CAM.
14. Movements of the embryo are very likely to disturb you while taking pictures of the CAM. The induced hypothermia will result in less movements, by slowing down the metabolism of the embryo. However, if the aim is to measure blood flow, hypothermia should not be applied.
15. The magnification will determine the level of detail that can be analyzed. At 25-fold magnification (2.5×10) mainly the larger, mature vessels will be visible while at 100-fold magnification (10×10) detailed images of the capillary bed can be obtained. We usually acquire images at both magnifications in order to distinguish between both vessel types.
16. This quantification method is laborious, less accurate, and more sensitive to subjective errors. In addition, it will only provide information about the vascular density. Nevertheless, it is a cheap method and available to everyone.
17. Be aware that not all tumors will grow on top of the CAM. We have observed that some tumors will grow just underneath the CAM.

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Chapter 21

Examination of the Role of Galectins in Cell Migration and Re-epithelialization of Wounds

Zhiyi Cao, Chandrassegar Saravanan, Wei-Sheng Chen, and Noorjahan Panjwani

Abstract

Re-epithelialization is a crucial step for wound healing. As galectins play important roles in re-epithelialization, we describe here protocols for in vivo, ex vivo and in vitro examination of the role of galectins in cell migration and in re-epithelialization of wounds. For in vivo models, mouse corneas are wounded by a variety of techniques and the rate of re-epithelialization is quantified. For ex vivo organ culture models, mouse corneas are wounded in situ, the eyes are enucleated, the eyeballs are cultured in the presence or absence of galectins and the rate of re-epithelialization is quantified. For cell cultured-based in vitro assays, we examine formation of lamellipodia and activation of focal adhesion kinase in various epithelial cells.

Key words Galectins, Cell migration, Re-epithelialization, Wound healing, Cornea, Epithelial cells, Lamellipodia, Focal adhesion kinase

1 Introduction

Impaired or delayed re-epithelialization underlies serious disorders of wound healing that are painful, difficult to treat, and affect a variety of human tissues [7, 9, 10, 23, 26, 30]. In the cornea, epithelial defects may persist and threaten the integrity of the anterior stroma, causing ulceration and in the worst cases perforation of the stromal tissue resulting in significant visual loss. Chronic wounds in the elderly, decubitus ulcer, and venous stasis ulcer of the skin also are attributable to delayed re-epithelialization and resultant persistent epithelial defects. Damage and impairment of the intestinal surface barrier are commonly observed in a variety of GI diseases including inflammatory bowel diseases (IBDs). The treatment goal is prompt re-epithelialization of the wound, essential for rapid resealing of the epithelial surface barrier to control inflammation

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and to restore intestinal homeostasis. Failure to re-epithelialize is generally caused not by inadequate cell proliferation but due to a reduced potential of the epithelium to migrate across the wound bed [6, 14, 29]. Cell migration involves sequential adhesion to and release from the substrate, a complex process of cell-matrix interactions [1, 13, 17, 20]. Results of recent studies suggest that members of the galectin class of β-galactoside-binding proteins have a critical role in modulating cell–matrix interactions and re-epithelialization of wounds through novel carbohydrate-based recognition systems [3, 4, 11, 18, 24, 25, 27, 28]. Our group reported in 2002 that the rate of re-epithelialization of corneal wounds is significantly slower in galectin-3-deficient mice compared to the wild type mice and that exogenous galectin-3 enhances the re-epithelialization of wounds in mouse corneas [3]. Subsequent studies aimed at characterization of the role of galectins in wound healing have shown that galectins-2 and -4 promote re-epithelialization of intestinal wounds [24], galectin-3 promotes re-epithelialization of corneal and skin wounds [3, 21], and galectin-7 promotes re-epithelialization of corneal, skin and kidney wounds [3, 4, 11, 27].

Molecular mechanisms by which galectins promote re-epithelialization are still under investigation. Galectin-3 promotes re-epithelialization by (1) interacting with *N*-glycans of laminin-332 [18], (2) modulating cell surface EGFR (epidermal growth factor receptor) expression by interacting with Alix [21], and (3) activating α3β1-integrin/focal adhesion kinase (FAK)/Rac1 signaling axis [28]. FAK is a crucial intracellular signaling molecule that integrates signals transduced by a variety of growth factors and integrins to promote cell migration [22]. Therefore, the demonstration of activation (i.e., phosphorylation of Y397) of FAK is an important biochemical corroboration of galectin-induced signal transduction events involved in cell migration. To study the molecular mechanisms of galectin-mediated cellular functions, we describe protocols for examination of lamellipodia formation and FAK activation using various epithelial cell lines. Knocking down endogenous galectins and glycosyltransferases is another approach to study galectin-mediated cellular functions. For example, knocking down *MGAT5*, which synthesizes β-1,6-*N*-acetyl-D-glucosamine-branched complex *N*-glycans that are preferentially recognized by galectin-3, attenuates galectin-3-induced lamellipodia formation in epithelial cells [28].

Findings that galectins stimulate the re-epithelialization of corneal, dermal, intestinal, and kidney wounds have broad implications for developing novel therapeutic strategies for the treatment of non-healing wounds. The methods that we describe in this chapter are useful to study other emerging roles of galectins in cell migration and in re-epithelialization of wounds.

2 Materials

2.1 In Vivo Corneal Re-epithelialization Assay: Scrape Wounds by Algerbrush

1. Phosphate Buffered Saline (PBS).
2. Proparacaine eye drops (Bausch + Lomb, Tampa, FL).
3. Ketamine (Fort Dodge Animal Health, Fort dodge, IA).
4. Xylazine (LLOYD, Shenandoah, Iowa).
5. Buprenorphine (Reckitt Benckiser, Richmond, VA).
6. Trephines (2 mm) (Bausch + Lomb, Tampa, FL).
7. Fluorescein (Ayerst Laboratories Inc, Philadelphia, PA). Dissolve fluorescein in PBS (1 mg/mL).
8. Ophthalmic sponges (Beaver-Visitec International, Inc. Waltham, MA).
9. Algerbrush (Ambler surgical, Exton, PA).

2.2 In Vivo Corneal Re-epithelialization Assay: Alkali Burn Wound

1. Filter disks (*see* **Note 1**).
2. Sodium hydroxide (NaOH): 0.5 N NaOH in autoclaved distilled water.

2.3 Eye Organ Culture

1. Paraplast wax (Monoject, St. Louis, MO) (*see* **Note 2**).
2. 24-well plate.
3. Minimum Essential Medium (MEM).
4. Recombinant galectins (prepared as previously described) [5, 8, 15, 16, 19] (*see* **Note 3**).
5. Lactose and sucrose (0.1 M) dissolved in MEM (*see* **Note 4**).
6. Needles, 20-G (*see* **Note 5**).

2.4 Examination of Lamellipodia Formation in Epithelial Cells

1. Telomerase reverse transcriptase-immortalized human corneal epithelial (HCLE) cells [12] (*see* **Note 6**).
2. HaCaT cells (spontaneously immortalized, nontransformed epithelial cells derived from adult human skin [2]) (*see* **Note 7**).
3. Fibronectin-collagen (FNC) Coating Mix (AthenaES, Baltimore, MD).
4. SFB media: keratinocyte serum free medium (K-SFM) supplemented with 0.4 % bovine serum albumin (BSA).
5. Paraformaldehyde (4 % in PBS) (EMS, Hatfield, PA).
6. Triton X-100 (0.5 % in PBS).
7. Tetramethylrhodamine (TRITC)-conjugated phalloidin (Sigma, St Louis, MO) (prepared as 0.1 μg/mL in PBS).
8. VECTASHIELD mounting medium (Vector Labs, Burlingame, CA).

9. α3 integrin function blocking monoclonal antibody (mAb) (P1B5) (Chemicon, Temecula, CA).
10. Mouse IgG (Chemicon, Temecula, CA).

2.5 Activation of Focal Adhesion Kinase in Epithelial Cells

1. PBST: PBS containing 0.1 % Tween-20.
2. Skim milk (5 % in PBST) (Bio-Rad, Hercules, CA).
3. Rabbit anti-FAK pY397 monoclonal antibody (Invitrogen, Carlsbad, CA).
4. HRP-conjugated anti-rabbit IgG secondary antibody (Invitrogen, Carlsbad, CA).
5. Mouse anti-FAK monoclonal antibody (clone 77) (BD, San Jose, CA).
6. HRP-conjugated anti-mouse IgG secondary antibody (BD, San Jose, CA).
7. Chemiluminescence detection system (PerkinElmer, Waltham, MA).
8. Western blot stripping buffer (Thermo Scientific, Rockford, IL).
9. Kodak X-Omat AR5 (Kodak, Rochester, NY).

3 Methods

3.1 In Vivo Corneal Re-epithelialization Assay: Scrape Wounds by Algerbrush

1. Anesthetize mice by intra-peritoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg), and topically anesthetize the cornea by application of proparacaine eye drops. In addition, provide analgesia by a subcutaneous injection of buprenorphine (0.05 mg/kg) before the surgery.
2. Use a 2-mm trephine to demarcate the area for de-epithelialization (*see* **Note 8**).
3. Apply 5 μL of fluorescein (1 mg/mL) and stain for 10 s. Remove excessive liquid with ophthalmic sponges.
4. Remove epithelium with in the demarcated area using an Algerbrush with a 0.5 mm burr (corneal rust ring remover) under a stereomicroscope.
5. Repeat **step 3** to visualize wound areas.
6. Take pictures within one minute (Fig. 1a).
7. Apply antibiotic ointment on cornea to prevent infection.
8. After 16 h, anesthetize mice as described in **step 1** and stain cornea as described in **step 3**. Take pictures within 1 min. (Fig. 1b).
9. Use ImageJ or other software to quantify wound areas and calculate the wound healing rate using the following formula:

$$\text{Rate of re-ephithilialization of wounds}\left(\text{mm}^2/\text{h}\right) = \left(\text{initial wound area} - \text{wound area at 16 h}\right) \div \text{time in hours.}$$

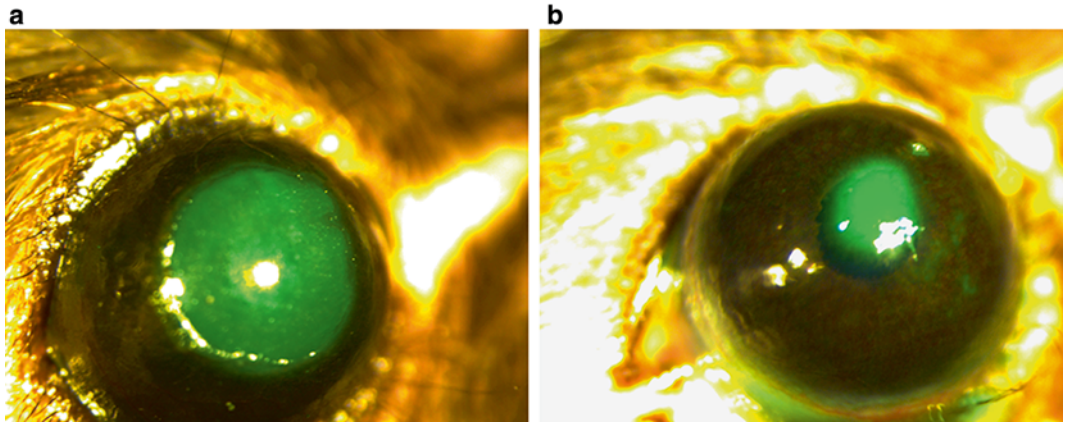

Fig. 1 An in vivo mouse model of re-epithelialization. (**a**) A mouse cornea is stained with fluorescein immediately after ablation by Algerbrush. (**b**) At 16 h after ablation, the same mouse cornea is stained with fluorescein. *Green* areas indicate wounded areas of corneal epithelium

3.2 In Vivo Corneal Re-epithelialization Assay: Alkali Burn Wound

1. Anesthetize mice as described in Subheading 3.1.
2. Soak 2-mm filter disks in 0.5 N NaOH and place the filter disk on the surface of the cornea for 2 min.
3. Immediately wash the cornea extensively with saline solution.
4. Apply antibiotic ointment on cornea and allow the wounds to partially heal for 16–18 h. Then stain the corneas with fluorescein, take pictures, and quantify wound healing rate as described in Subheading 3.1 (*see* **Note 9**).

3.3 Eye Organ Culture

1. Add 0.5 mL of melted Paraplast wax into each well of a 24-well plate.
2. Produce 2-mm corneal wounds on both eyes of each mouse as described in Subheadings 3.1 and 3.2.
3. Excise and pin down the eye balls on Paraplast wax in individual wells of a 24-well plate using 0.5–1.0 cm long tips of 20-G needles (one eye per well) (Fig. 2) (*see* **Note 10**).
4. Use left eyes as controls. Incubate control eyes in 0.5 mL/well of serum-free MEM. Incubate rights eyes in 0.5 mL/well of serum-free MEM containing different test reagents, including (1) galectin alone (0.5–25 μg/mL), (2) galectin plus 0.1 M β-lactose, and (3) galectin plus 0.1 M sucrose. Each group should have a minimum of six eyes.
5. After 24 h in a humidified incubator with 5 % CO_2 at 37 °C, visualize wound areas by staining with fluorescein (1 mg/mL).
6. Take pictures and quantify wound-healing rate as described in Subheading 3.1.

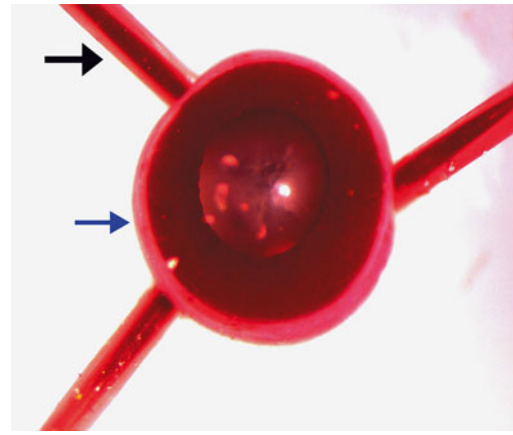

Fig. 2 An enucleated eye is pinned down on Paraplast wax with three sterilized 1-cm long tips of 20-G needles and incubated in 0.5 mL of culture medium. *Black arrow* indicates the pins; *blue arrow* indicates the eye ball

3.4 Examination of the Role of Galectins in Lamellipodia Formation

1. Coat the sterile 8-well culture slides with FNC Coating Mix (0.25 mL per chamber) for 30 min at room temperature in the tissue culture hood.
2. Remove FNC Coating Mix and air-dry the plates for 5 min.
3. Plate the epithelial cells (e.g., HCLE, HaCaT; 2×10^4 cells per chamber) on FNC coated eight-well culture slides.
4. Incubate overnight and then wash the cells twice with SFB media. Incubate the cells in SFB (growth factor starvation) for 2 h.
5. Incubate the growth factor starved cells in SFB in the absence or presence of recombinant human galectins (5–25 μg/mL) for 30 min.
6. In some experiments, to characterize the role of the carbohydrate recognition domain, expose the cells to galectin-3 in the presence of a competing disaccharide, β-lactose (0.1 M) or a non-competing disaccharide, sucrose (0.1 M). Prepare the disaccharides in SFB (*see* **Note 11**).
7. At the end of incubation period, fix the cells with 4 % paraformaldehyde for 20 min at room temperature.
8. Wash three times with PBS.
9. Permeabilize the fixed cells with 0.5 % Triton X-100 for 10 min.
10. Wash the cells three times with PBS and stain with TRITC-conjugated phalloidin for 1 h at room temperature.
11. Mount the chamber slides using VECTASHIELD mounting medium. The slides can be stored in dark at 4 °C.

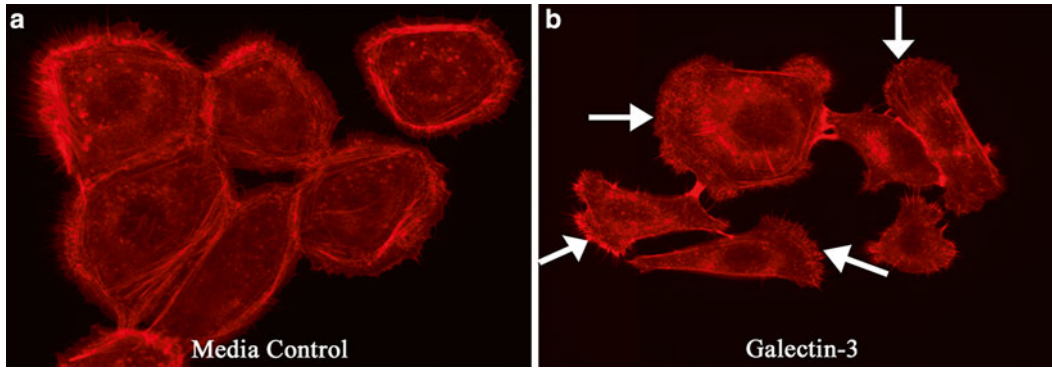

Fig. 3 HCLE cells were treated with or without galectin-3 and stained with TRITC-conjugated phalloidin. *White arrows* indicate the lamellipodia formation

12. View the slides with a 40× Zeiss objective lens under a Leica Optigrid confocal microscope using Rhodamine (502 nm) filter and capture images.
13. Count at least 250 cells from several nonoverlapping microscopic fields and estimate the percentage of cells with lamellipodia (actin-rich, fan-shaped, membrane protrusions at the leading edge) (Fig. 3).

3.5 Activation of Focal Adhesion Kinase in Epithelial Cells

1. Prepare cell lysates using RIPA buffer from HCLE cells exposed to 10–25 μg/mL of galectin for various time periods (0, 2, 5, and 10 min).
2. Electrophorese aliquots of cell lysates (containing 35 μg of total protein) in 4–12 % SDS-polyacrylamide gels.
3. Transfer the proteins onto a nitrocellulose membrane and check for successful transfer of proteins using Ponceau S stain. Blots can be stored overnight in PBS at 4 °C.
4. Block the membrane with 5 % skim milk in PBST for 1 h at room temperature with gentle shaking on a rocker.
5. Probe the protein blots with rabbit anti-FAK pY397 (1:1,000 in 5 % skim milk) for 1 h at room temperature.
6. Wash three times with PBST.
7. Probe the protein blots with HRP-conjugated anti-rabbit IgG secondary antibody (1:20,000 in 5 % skim milk) for 1 h at room temperature.
8. Wash three times with PBST.
9. Develop the blot using a chemiluminescence detection system as per the manufacturer's recommendations.
10. Use stripping buffer to strip the protein blots according to the manufacturer's recommendations.

11. Probe the same protein blot with mouse anti-FAK mAb (1:1,000 in 5 % skim milk) for 1 h at room temperature.
12. Wash three times with PBST.
13. Probe the protein blots with HRP-conjugated anti-mouse IgG secondary antibody (1:50,000 5 % in skim milk) for 1 h at room temperature.
14. Wash three times with PBST.
15. Develop the blot as described in **step 7** above.
16. Quantify the intensity of each band using the ImageJ program.
17. Calculate the ratios of p-FAK to total FAK and normalize to the values obtained from the cells not exposed to galectin-3.

4 Notes

1. Use a 2-mm trephine to punch a Whatman filter paper then autoclave the filter disks.
2. Add Paraplast wax chips in a 50-mL tube and secure the cap. Allow the wax to melt by pacing the tube in a water bath at 60 °C. Add 0.5 mL of wax to each well of a 24-well plate.
3. Also *see* Chapter 2 of this volume on "Cloning, expression, and purification of galectins for in vitro studies" for details on methods to prepare recombinant galectins. Galectins can be purchased from a number of companies (e.g., Abcam, Cambridge, MA). All galectins commercially purchased must be tested for: (1) carbohydrate binding activity and (2) endotoxin levels using published procedures [5].
4. To prepare solutions, dissolve 342.3 mg of lactose or sucrose in 10 mL MEM, and sterilize the medium by filtration through 0.22 μm disk filters.
5. Using a wire cutter cut off 0.5–1.0 cm tips of 20-G needles. Autoclave the tips before use.
6. Maintain HCLE cells in keratinocyte serum-free medium supplemented with 0.2 ng/mL epidermal growth factor (EGF), 25 μg/mL bovine pituitary extract (K-SFM), and 0.4 mM $CaCl_2$ at 37 °C in a 5 % CO_2 incubator.
7. Maintain HaCaT cells in Dulbecco's modified Eagle's medium (DMEM) containing 5 % fetal calf serum and 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer at 37 °C in a 5 % CO_2 incubator.
8. Touch trephine to the corneal surface gently. Excessive pressure may result in excision or perforation of the cornea.
9. The initial wound area will be 2-mm, as this is the size of the filter disks used to create the wound.

10. Rinse the enucleated eyes in PBS to remove blood. The rinsed eyes are placed on top of Paraplast wax without any medium before pinning. Avoid damaging corneas while pinning. After pinning the eyes on the wax, add media slowly to cover the entire eye ball.
11. To assess the role of integrins in galectin-3-induced migratory phenotype, incubate the growth factor starved cells with a selected function-blocking anti-integrin mAb (e.g., 10 μg/mL of α3 integrin mAb (P1B5)) or control IgG, prepared in SFB, for 30 min at 37 °C. Cells are then washed with PBS and treated with galectin-3 in SFB for 30 min at 37 °C. Cells are fixed with 4 % paraformaldehyde and stained with TRITC-phalloidin as described.

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Chapter 22

Manipulating Galectin Expression in Zebrafish (*Danio rerio*)

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Abstract

Techniques for disrupting gene expression are invaluable tools for the analysis of the biological role(s) of a gene product. Because of its genetic tractability and multiple advantages over conventional mammalian models, the zebrafish (*Danio rerio*) is recognized as a powerful system for gaining new insight into diverse aspects of human health and disease. Among the multiple mammalian gene families for which the zebrafish has shown promise as an invaluable model for functional studies, the galectins have attracted great interest due to their participation in early development, regulation of immune homeostasis, and recognition of microbial pathogens. Galectins are β-galactosyl-binding lectins with a characteristic sequence motif in their carbohydrate recognition domains (CRDs), which comprise an evolutionary conserved family ubiquitous in eukaryotic taxa. Galectins are emerging as key players in the modulation of many important pathological processes, which include acute and chronic inflammatory diseases, autoimmunity and cancer, thus making them potential molecular targets for innovative drug discovery. Here, we provide a review of the current methods available for the manipulation of gene expression in the zebrafish, with a focus on gene knockdown [morpholino (MO)-derived antisense oligonucleotides] and knockout (CRISPR-Cas) technologies.

Key words Galectins, Zebrafish, Morpholino, CRISPR-Cas, Gene expression, Microinjection

1 Introduction

The zebrafish (*Danio rerio*) is a widely used model organism to study a broad spectrum of human normal and pathological processes, including development, autoimmune, neoplastic, and infectious diseases [1–3]. The growing interest in using zebrafish as a genetically tractable model system is due to its multiple advantages relative to the mammalian models, which include high fecundity, external fertilization, rapid development, transparent embryo, low maintenance cost, easy genetic manipulation, and an extensive collection of mutants currently available [4–6]. Zebrafish and human genomes have been shown to share highly conserved structural

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and functional features [7]. Thousands of zebrafish genes have been identified and its full genome sequence is now available online (http://www.ncbi.nlm.nih.gov/genome/guide/zebrafish/). Moreover, *D. rerio* exhibits some higher-level behaviors previously observed only in mammals, such as memory, conditioned responses, and schooling [8, 9]. In recent years, the zebrafish model has proven useful to gain new insight into the functional aspects of protein–carbohydrate interactions, such as those mediated by galectins [10–12]. Galectins are β-galactosyl-binding lectins, which share primary structural homology in their carbohydrate-recognition domains (CRDs) [13]. They are classified into three major structural types: (1) proto-type galectins, which contain one CRD and form homodimers; (2) chimera-type galectins, which have a single CRD and can oligomerize forming trimers and pentamers; (3) tandem-repeat-type galectins, which are composed of two CRDs joined by a linker peptide [14]. Galectins participate in a multitude of biological processes, such as development, apoptosis, tumor metastasis, and regulation of immune responses [15–19]. All three structural types of galectins have been identified and characterized in various tissues, plasma, and mucus of teleost fish, such as zebrafish [12, 20–23]. Among the various methodologies for elucidating the biological role(s) of a particular protein of interest, disruption of gene expression represents a useful approach. Multiple strategies have been developed to modulate gene expression at a genetic or epigenetic level (Table 1). Several of these methodologies have been recently applied to functional studies of galectins [10, 24, 25].

Morpholino (MO) oligos are antisense oligonucleotides derivatized with morpholine rings to increase stability, and designed to anneal proximal to the start codon of the selected gene and disrupt its translation, or to the splice acceptor sequence to induce incorrectly spliced mRNA [26]. The MO oligos do not degrade their mRNA targets, but (1) block mRNA translation by targeting the 5′-UTR through the first 25 bases of coding sequence [10], or (2) alter the translation modifying pre-mRNA processing by targeting splice junctions or regulatory sites (Fig. 1) [27, 28]. Thus, the "morphant" phenotype results from disrupted protein expression levels. Although their effects are only transient, MO oligos are relatively long-lived inside the cell upon delivery, with the effect on pre-mRNA splicing or translation lasting for up to 5 days following microinjection. This technique allows for the rapid manipulation and interrogation of complex processes such as embryonic development, organ formation, innate immunity, and host–pathogen interactions [3, 10, 28]. However, since the effects on mRNA processing and translation by MO oligos are temporary and not inheritable, and there is no suitable way to deliver them systemically in adult fish, this system is not suitable for more integrated approaches. For inheritable genetic modulation, a set of other techniques has been introduced in the last 15 years (Table 1).

Table 1
Reverse genetic tools and transgenic methodologies for disruption of gene expression

Procedure	Mechanism	References
MO (Morpholino) oligos	Custom-designed synthetic oligonucleotides block translation or pre-mRNA processing of the targeted gene	Summerton 2009 [33] Nasevicius et al., 2000 [26] Tallafuss et al., 2012 [25]
TILLING (*T*argeting *I*nduced *L*ocal *L*esions in *G*enomes)	Formation of DNA hetero-duplexes between multiple alleles amplified by PCR and cleaved by single stranded nucleases. Mutagenized products are screened by size	McCallum et al., 2000 [34] Wienholds et al., 2003 [35]
Heat shock promoters	Targeted gene is engineered to be under inducible control of a heat shock promoter. Transgene expression induced by heat shock	Halloran et al., 2000 [24] Hardy et al., 2007 [36]
Targeted cell ablation	Expression of bacterial nitro reductase (NTR) under a promoter that controls the proliferation and survival of a particular cell type	Curado et al., 2007 [37]
ZFN (*Z*inc-*F*inger *N*uclease)	DNA-binding endonucleases engineered to target double-stranded DNA for specific site mutagenesis	Ekker 2008 [38]
Gal4/UAS	Targeted gene is engineered to be under inducible control of UAS promoter. Transgene expression induced by tissue-specific Gal4/UAS system	Scheer et al., 1999 [39] Scott 2009 [40]
TALEN (*T*ranscription *A*ctivator-*L*ike *E*ffector *N*uclease)	Engineered TAL effector DNA domain fused to a DNA restriction enzyme binds and cleaves targeted gene, introducing a custom-designed mutagenesis site	Clark et al., 2011 [41]
CRISPR-Cas (*C*lustered *R*egularly *I*nterspaced *S*hort *P*alindromic *R*epeats)	Engineered CRISPR system with Cas9 endo nuclease directed by custom-designed guide RNA to introduce double-strand break in the targeted DNA sequence, prompting error-prone DNA repair	Jinek et al., 2012 [29] Hwang et al., 2013 [32]

CRISPR-Cas is a genome editing approach based on the prokaryotic immune system. By using a segment of virus-derived DNA from its CRISPR array and processing to crRNA targeting the viral genome, the system leads to the inactivation or degradation of targeted DNA by the CAS-crRNA complex [29]. Cas9 nuclease transgenically expressed in vertebrates is active and able to cleave target DNA when directed by a single guide RNA (sgRNA), which at its 5′-end contains 20 base pairs of complementary target DNA [30–32].

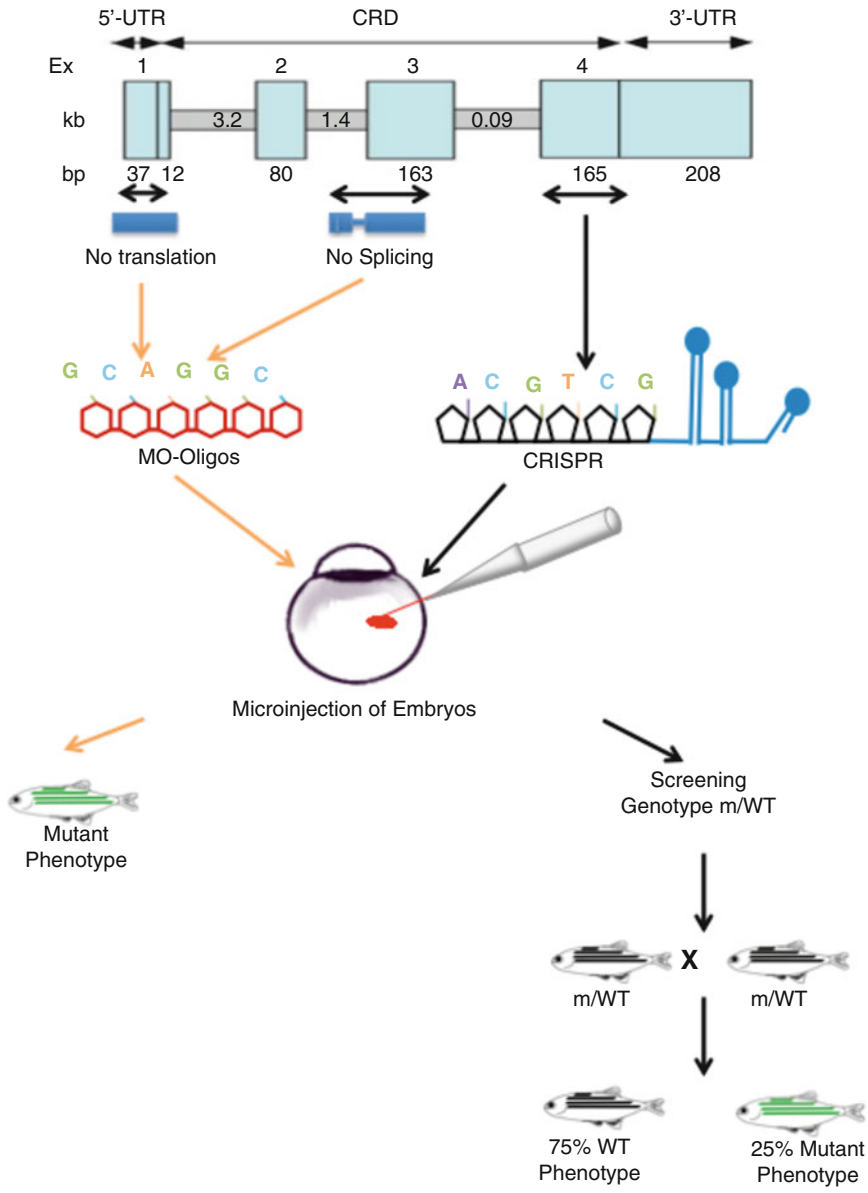

Fig. 1 Genetic modulation of galectins by morpholinos and CRISPR-Cas microinjection. MO oligos may be designed to target an exonic sequence, thus blocking mRNA translation. Alternatively, MO oligos may be designed to target an exon–intron junction, preventing the maturation of the targeted pre-mRNAs. MO oligos and CRISPR transcripts are microinjected into the embryo yolk. In the case of morpholinos (*orange arrows*), the microinjection will result in a mutant phenotype either/both in embryo and/or adult. When injecting CRISPR transcripts, the screening of the microinjected embryos will result in the selection of the mutant genotypes (usually heterozygous). After crossing individuals with mutant genotypes, fish with mutant phenotypes (homozygous) will be selected from the resultant progeny (Color figure online)

Here we describe in detail the use of MO oligos and CRISPR-Cas to modulate galectin expression in the zebrafish model to elucidate their functions in development and immunity. The MO oligos Drgal1-L1-MO and Drgal1-L2-MO were designed to block

Table 2
Verified morpholino-modified antisense oligonucleotides

Designation	Sequence	Reference
DrGal1-L1-MO	5′- TCTATAAGCACAGTCTCATGCA -3′	Ahmed et al. (2009) [10]
DrGal1-L2-MO	5′- ATAAGCACACCGGCCATTTTGACGT -3′	

translation initiation based on 5′-UTR sequences of Drgal1-L1 and Drgal1-L2, respectively (Table 2). MO oligos are usually validated by in vitro blockade of the corresponding protein expression using the TNT SP6 Coupled Rabbit Reticulocyte Lysate System. Following MO oligo injection of zebrafish embryos, expression of the corresponding protein is inspected by whole mount antibody staining. Potential phenotypes are inspected under microscope and by whole mount antibody staining of a specific marker. Zebrafish embryos injected with validated Drgal1-L2-MO (Fig. 2a) show dramatically reduced Drgal1-L2 expression as observed by whole mount antibody staining (Fig. 2c). Drgal1-L2 is strongly expressed in the notochord during early embryogenesis (Fig. 2b) and Drgal1-L2 knockdown results in a characteristic phenotype with a short and bent tail (Fig. 2c). Microscopically, the phenotype exhibits disrupted muscle fiber organization as observed by whole mount immunostaining with the F59 antibody (monoclonal anti-myosin antibody), a marker for slow muscle (Fig. 2e).

2 Materials

2.1 Preparation and Validation of Morpholino (MO) Oligos

1. MO oligos (Gene tools) (*see* **Note 1**).
2. pCS2^{+} vector (Addgene).
3. Drgal 1-L2 cDNA.
4. TNT SP6 Coupled Rabbit Reticulocyte Lysate System (Promega).
5. [^{35}S]methionine.
6. 15 % polyacrylamide gel.

2.2 Preparation of sgRNA

1. Single guide RNA (sgRNA) expression vector pDR274 (Addgene) (*see* **Note 2**).
2. Cas9 nuclease expression plasmid pMLM3613 (Addgene).
3. Gene specific oligos (Sigma) (*see* **Notes 3** and **4**).
4. Agarose gel.
5. LB agar.
6. 42 °C water bath.
7. Kanamycin.

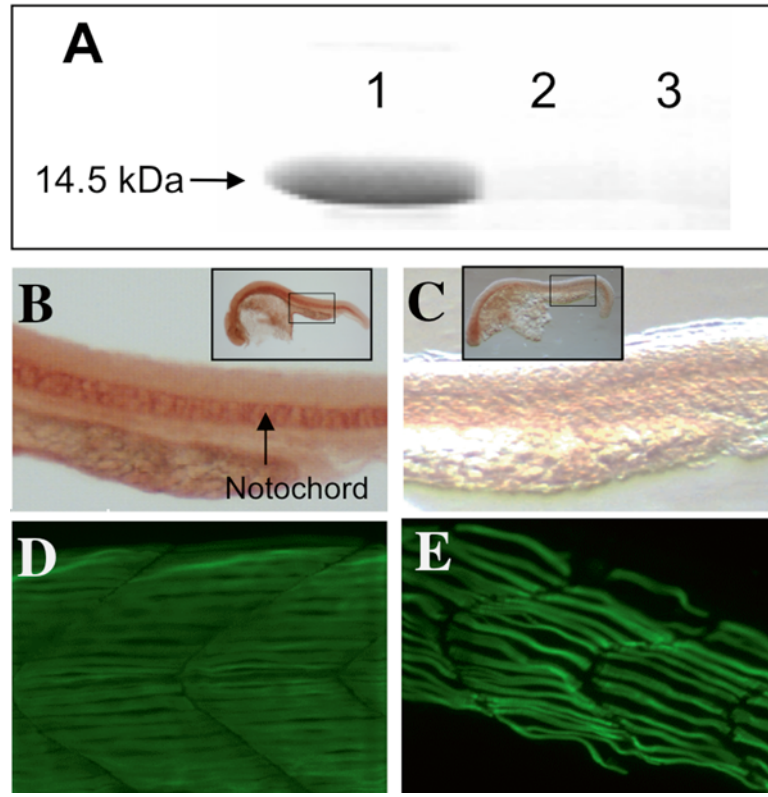

Fig. 2 Validation of Drgal1-L2-MO. (**a**) In vitro blockade of Drgal1-L2 protein in a rabbit reticulocyte system. SDS-PAGE/autoradiography analysis of the in vitro labeled Drgal1-L2 translation products. *Lane 1*: translation reaction in the absence of Drgal1-L2-MO (positive control); *lanes 2* and *3*: reactions in the presence of 170 and 340 ng of Drgal1-L2-MO, respectively. (**b**) Whole mount immunostaining of an uninjected embryo probed with anti-Drgal1-L2 antibodies (positive control). Lateral and dorsal view of a 27 h post fertilization (hpf) embryo. Drgal1-L2 was seen expressed in the notochord. (**c**) In vivo blocking of Drgal1-L2 protein as shown by whole mount immunostaining of an embryo injected with Drgal1-L2-MO, and probed with anti-Drgal1-L2 antibodies. Lateral and dorsal view of a 27 hpf embryo. Whole mount immunostaining of an uninjected embryo (**d**) and an embryo injected with Drgal1-L2-MO (**e**) probed with F59 antibody. Lateral and dorsal view of 48 hpf embryos. In (**e**), muscle fibers were found disorganized

8. 30 % glycerol.
9. QIAprep Spin Miniprep Kit/QIAfilter Plasmid Midi Kit (Qiagen).
10. MinElute Gel Extraction Kit (Qiagen).
11. Rapid DNA Ligation kit (Roche).
12. DH5α Competent cells (Invitrogen).

13. SOC medium (Sigma).
14. Restriction enzymes: BsaI, PmeI, DraI (New England Biolabs).
15. MAXIscript T7 kit (Invitrogen).

2.3 Embryo Preparation

1. Morpholino oligo (Drgal 1-L2-MO, etc.) (*see* Subheading 3.1).
2. RNAse-free water.
3. Danieau's solution: 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES, pH 7.6.
4. 1 % phenol red dye (Sigma).
5. Glass microinjection needles (Tritech Research) (*see* **Notes 5** and **6**).
6. Borosilicate glass capillary tubes (Narishige).
7. Micropipette puller (Sutter Instrument Co., Model P-97).
8. Agarose (Sigma).
9. 90 mm Petri dishes.
10. Adult male and female zebrafish.
11. Crossing Tank Kit (Thoren Aquatics Housing Systems).
12. Tank divider.

2.4 Embryo Microinjections

1. Dissecting forceps.
2. Micropipettes and disposable tips.
3. PBS (Bio-Rad).
4. Incubator at 28 °C.
5. 15 and 50 ml Conical tube (BD Biosciences).
6. Eppendorf tube (Thermo Scientific).
7. ECL Plus detection kit (Amersham Biosciences).
8. Polyclonal antibodies to Drgal1, 3 and 9 were custom-produced (Thermo Scientific).

2.5 Whole Mount Antibody Staining

1. 4 % paraformaldehyde.
2. PBS-Tween: Phosphate buffered saline with 0.1 % Tween 20.
3. 0.1 % BSA/1 % dimethyl sulfoxide/phosphate buffered saline (BDP).
4. Avidin (Vector Laboratories).
5. 10 % goat serum in BDP.
6. Anti-Drgal1-L2 antibody.
7. Biotin-labeled goat anti-rabbit IgG (Vector).
8. 1:1 diluted avidin-biotin complex solution (Vector).
9. DAB substrate (Vector).

2.6 Rescuing the Phenotypes by Co-injection of Embryos with Drgal1-L2 cRNA and Drgal1-L2-MO (Ectopic Expression of Native Drgal1-L2 on Drgal1-L2-MO Injected Embryos)

1. 4 % paraformaldehyde.
2. In vitro transcription kit (mMESSAGE mMACHINE SP6).
3. Formaldehyde 1.5 % agarose gels.
4. Distilled water.
5. Micropipettes and disposable tips.

2.7 Special Instruments

1. Microinjection system: Pico-injector PLI-100 (Harvard Medical Systems Research Products). It is connected to compress nitrogen gas and to adjust the air pressure for reagent delivery. Modulator M152 (Narishige).

2.8 General Instruments

1. Microscope Stemi 2000 (Zeiss).
2. Microcentrifuge (Beckman Coulter).
3. Incubator (Fisher Scientific).

3 Methods

3.1 Preparation and Validation of Morpholino (MO) Oligos

1. Synthesis of MO oligos: (*see* **Notes 1, 7,** and **8**).
2. Prepare an expression construct for a particular gene for in vitro protein expression (*see* **Note 9**).
3. Perform in vitro direct translation of Drgal1-L2 from pCS-Drgal1-L2 plasmid DNA (0.5 μg) in the presence or absence of the Drgal1-L2-MO using TNT SP6 Coupled Rabbit Reticulocyte Lysate System according to manufacturer's instructions.
4. To detect the translated product, use [^{35}S]methionine with the methionine free amino acid mixture.
5. After completion of the reaction, analyze the translated product on 15 % SDS-PAGE followed by autoradiography. As shown in Fig. 2a, Drgal1-L2-MO blocked translation of Drgal1-L2 protein.

3.2 Preparation of sgRNA

1. Digest the pDR274 with BsaI for 1 h at 37 °C. The linearized fragment is separated in an agarose gel and recovered using Gel Extraction kit.
2. Mix the 50 ng of linearized vector with annealed targeted sequences (1:3 M ratio) in 20 μl of ligation solution from Quick Ligation kit and incubate for 5–15 min at room temperature. The ligates can be used immediately or stored at −20 °C for future use.

3. Add 2 μl of the above ligate in 50 μl of DH5α competent cells and incubate on ice for 20 min.
4. Heat-shock the mixture for 45 s in 42 °C water bath, and incubate on ice for at least 2 min.
5. Afterward, add 500 μl of SOC medium and incubate the bacteria for 1 h at 37 °C.
6. Spread the bacteria onto LB agar with 50 μg/ml of kanamycin for overnight incubation at 37 °C.
7. Inoculate each colony into one 15 ml tube with 3 ml of LB broth supplemented with 50 μg/ml of kanamycin for overnight incubation at 37 °C.
8. Extract plasmid using Mini prep kit for sequence analysis.
9. Stock colonies in 30 % glycerol for future use.
10. Select the colony with expected sequence for growth and purification using Midi kit.
11. Prepare sgRNA using DraI-digested sgRNA expression vector and the MAXIscript T7 kit as manufacturer's recommendation; prepare Cas9 mRNA using PmeI-digested Cas9 expression vector and mMESSAGE mMACHINE T7 ULTRA kit.
12. Purify RNA by lithium chloride (LiCl) precipitation and use for microinjection.

3.3 Embryo Preparation

1. Prepare MO oligo stocks: Aliquots of 5 mM MO oligo stock solution are prepared by adding RNAse-free water (or Danieau's solution) and stored at −20 °C. Before use, mixed with Danieau's solution and 1 % phenol red dye (final 0.1 %) to make 0.5–2 mM working solution (*see* **Note 10**). Keep working solution at room temperature.
2. Prepare agarose injection bed: In a 90 mm Petri dish, lay carefully several glass micropipettes in parallel onto a molten 1 % agarose surface to make the troughs. After cooling down, remove and discard micropipettes and the injection bed is ready for use or for storage at 4 °C.
3. The day before spawning, separate adult male and female zebrafish into spawning tanks with divider. Set up 2–4 pairs of animals for each experiment.
4. In the morning of the day for injection, remove the divider to allow mating. Usually the females will begin to lay eggs within 15–45 min.
5. Within 30 min of spawning, collect embryos into a fish tray, wash thoroughly several times with fresh water.
6. Place embryos onto the injection bed. Carefully arrange the eggs on the troughs of injection bed.
7. Centrifuge working solution (e.g., morpholino solution for 30 s) at room temperature and use the supernatant for microinjection.

3.4 Embryo Microinjections

1. Set up the microinjector: Attach the microinjection needles to the needle holder of the microinjector. (*see* **Notes 5** and **6**).
2. Place the injection bed on the stage, and adjust the modulator to appropriate position for injection.
3. Load the microinjection needle with 2 μl of working solution on it. (Optional: Test injection in a few embryos. Adjust pressure values if needed) (*see* **Note 11**).
4. Penetrate the needle to the yolk and deliver 1 nl of working solution. The flow is visible due to the phenol red added to the working solution (*see* **Note 12**).
5. Retract the pipette and repeat on remaining embryos.
6. After microinjection, wash the embryos into a petri dish with fresh water and place them in incubator at 28 °C.
7. Evaluate the injected embryos under the microscope a few hours after injections, and remove the damaged embryos. If the water in the dish is cloudy, transfer the embryos to a new dish with fresh water.
8. After 24 h, dechorionate the embryos under microscope using forceps.
9. Visually inspect embryos for any potential phenotypes. Further investigation to verify the effect of MO oligo injection on protein expression is done by Western blot and whole mount antibody staining (*see* below).
10. Following Drgal1-L2-MO injection, we performed whole mount immunostaining with Drgal1-L2 antibody (to validate the Drgal1-L2-MO by in vivo blocking of Drgal1-L2 protein expression) and F59 antibody (to observe potential defects in muscle fiber organization).
11. Once we confirmed the phenotype, we then rescued the defect by injection of embryos with Drgal1-L2 cRNA (for ectopic expression of Drgal1-L2) along with Drgal1-L2-MO.

3.5 Whole Mount Antibody Staining

1. Fix embryos with 4 % paraformaldehyde for 1 h at room temperature, wash twice with PBS-Tween for 5 min each, and soak in cold acetone for 10 min at –20 °C.
2. Wash embryos twice with PBS-Tween for 5 min. each followed by washing once with 0.1 % BSA/1 % dimethyl sulfoxide/PBS (BDP) for 5 min.
3. Incubate with avidin (four drops/ml) in blocking buffer (10 % of goat serum in BDP) for 30 min at room temperature.
4. Wash the embryos twice with BDP for 5 min each and incubate with 1:10,000 dilution of anti-Drgal1-L2 antibody in blocking buffer containing biotin (Vector) (four drops/ml) overnight at 4 °C.

5. Wash the embryos three times for 30 min each with BDP, followed by incubation with diluted (1:1,000) biotin-labeled secondary antibody (goat anti-rabbit IgG) (Vector) in BDP for 1 h at room temperature.
6. Wash the embryos three times for 30 min each with BDP and incubate with 1:1 diluted avidin-biotin complex solution (Vector) for 30 min at room temperature.
7. Wash the embryos three times for 30 min each in BDP and develop color with DAB substrate according to the manufacturer's protocol.

3.6 Rescuing the Phenotypes by Co-injection of Embryos with Drgal1-L2 cRNA and Drgal1-L2-MO (Ectopic Expression of Native Drgal1-L2 on Drgal1-L2-MO Injected Embryos)

1. To determine if the ectopic expression of native Drgal1-L2 rescues the phenotypes observed, Drgal1-L2 mRNAs were synthesized from a pCS-Drgal1-L2 construct using an in vitro transcription kit (*see* **Note 13**).
2. The integrity of the transcribed RNA was examined on formaldehyde 1.5 % agarose gels.
3. For microinjection, mRNA was dissolved in distilled water to a final concentration of 100 μg/ml. The transcribed RNA solution (approximately 2 nl) was microinjected into the cytoplasm of zebrafish embryos at the one- or two-cell stage, and subsequently, the Drgal1-L2-MO was microinjected into the yolk sac.
4. After 24 h, we visually inspected embryos for any phenotypes and performed whole mount immunostaining with Drgal1-L2 and F59 antibodies as described before.

4 Notes

1. GeneTools, LLC (Philomath, OR; https://oligodesign.genetools.com/request/) provides a free design service that does not require prior knowledge of the start codon of the mRNA target. Despite being very user-friendly and not being held to a mandatory order online from the same site, this software provides very limited sequence design and analysis option to the user. In that regard, Vector NTI® software (Life Technologies: Grand Island, NY) offers an excellent alternative to MO oligo design provided the user has previous knowledge of the target sequence. Additionally, MOrpholino DataBase (http://www.morpholinodatabase.org/) is a public Web-based database with more than 700 morpholinos to date against zebrafish genomic sequences.
2. Selected 20 variable nucleotides to base pair with a target genomic DNA sequence will be inserted at its 5′ end of pDR274 to produce a short 102 nucleotides guide RNA (*see* Subheading 3.2).

3. One pair of oligos for each target gene is designed using ZiFiT Targeter. The ZiFiT Targeter Web site (http://zifit.partners.org/) offers an online option to identify potential target sites for the described CRISPR-Cas system. By default, the sequences that meet the following criteria: 5′-GG- (N)18-NGG-3′ will be identified. ZiFiT Targeter will analyze the user-input sequences and returns a list of recommended target sites and sequences of oligonucleotides that need to be synthesized for cloning into the pDR274 vector.
4. The single strand oligos are synthesized, mixed at 95 °C for 10 min, then cooled down gradually at room temperature to allow annealing. The annealed oligos contain unique overhangs on each site for directional cloning into BsaI-digested pDR274 (*see* Subheading 3.2).
5. Break off the tip of the microinjection needle with your forceps under the microscope, so that they have an open tip.
6. Prepare the microinjection needles by heating and pulling borosilicate glass capillary tubes in a micropipette puller. Store the needles in a petri dish on top of small amount of clay or adhesive tape.
7. MO oligos for each gene are designed based on the gene sequence to obtain *translational blocker* (designed to bind close to start codon disrupting the translation) or *splice blocker* (designed to bind to splice acceptor sequence to induce incorrectly spliced mRNA). For example, Drgal1-L1-MO and Drgal1-L2-MO shown in Table 2 are translational blockers of the Drgal1-L1 and Drgal1-L2, respectively. MO oligos were custom synthesized by Gene Tools (www.gene-tools.com).
8. The ZiFiT Targeter Web site (http://zifit.partners.org/) offers an online option to identify potential target sites for the described CRISPR-Cas system. By default, the sequences that meet the following criteria: 5′-GG- (N)18-NGG-3′ will be identified. ZiFiT Targeter will analyze the user-input sequences and returns a list of recommended target sites and sequences of oligonucleotides that need to be synthesized for cloning into the pDR274 vector.
9. For example, we cloned Drgal1-L2 with 5′-UTR sequences into a pCS2^{+} vector (a gift from D. Turner, R. Rupp, J. Lee, and H. Weintraub, Fred Hutchinson Cancer Research Center, Seattle, WA) to obtain the pCS-Drgal1-L2 construct. In this construct, a 27-nucleotide untranslated 5′ leader (derived from the *Xenopus* β-globin mRNA 5′-end) is introduced between the SP6 promoter and the Drgal1-L2 insert. The pCS-Drgal1-L2 construct is expected to generate protein when added to a cell-free protein synthesis system that is initiated by SP6 RNA polymerase.

10. The first step in using a new MO oligo is to determine the optimum delivery dose. Thus, MO oligos can be initially injected at different doses and the dosages are increased or decreased to optimize the phenotype to toxicity ratio. Working stocks of MO oligos were prepared to use as near-isotonic solutions for the zebrafish (i.e., Danieau solution).
11. Alternatively, "freehand" injection can be practiced without a micromanipulator in a normal petri dish. It is more robust but time-consuming as proper embryo orientation and microinjection technique is required.
12. The phenol red color from the injected sample will not change if sample delivered into the embryonic cells. A shift to pink in phenol red color will indicate the microinjection was delivered outside the embryo.
13. In this construct, 27 nucleotides derived from the *Xenopus* β-globin 5′-UTR were used to replace the Drgal1-L2 5′-UTR as the Drgal1-L2-MO was specifically targeted to the Drgal1-L2 5′-UTR. Thus, the Drgal1-L2-MO would only inhibit expression of the endogenous Drgal1-L2, but not of the injected Drgal1-L2 mRNA.

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Chapter 23

Examination of Galectin Localization Using Confocal Microscopy

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Abstract

Confocal microscopy provides a unique modality to examine the expression and localization of biomolecules in a variety of settings. Using this technique, an image is acquired from the focal plane of the objective using focused laser light, making it possible to work within the resolution limit of the optical system. In addition, by acquiring multiple images from a variety of focal planes, stacked series of images can provide clear spatial localization of a probed structure or protein. We describe herein the immunofluorescence methods for galectin staining in frozen sections of tissue for galectin localization using confocal microscopy.

Key words Confocal microscopy, Galectin, Tissue, Antibody, Immunofluorescence

1 Introduction

Galectins are ancient lectins that bind to specific carbohydrate chains containing β-galactosides through their carbohydrate recognition domains (CRD) [1, 2]. These proteins are found in both the intracellular (cytoplasm and nucleus) as well as the extracellular (cell surface and serum) compartments [3]. Depending on expression, secretion, and local concentration, these lectins can mediate cell–cell, cell–matrix, or cell–pathogen interactions, displaying various functions in physiological and pathological processes [4–8]. Although 15 members of the galectin family have been described [1], galectin-1 appears to be the most ubiquitously expressed member of the family. Mammalian galectin-1 is found in skeletal and striated muscle, liver, lung, brain, kidney, spleen, and intestine; in arterial walls, and in cytosol and nucleus of cultured human endothelial cells [9, 10]. Additionally, galectin-1, along with galectin-3 is expressed in cells of myeloid and lymphoid lineage and appears to be involved in regulation of immune cell development

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and inflammatory processes [11]. Alternatively, some other galectins show more selective expression patterns. For example, galectin-7 and -12 are localized to epithelial and adipose tissue, respectively [12–14], while galectin-4 resides primarily within tissue of the alimentary tract [15]. Thus, some galectins can be preferentially localized to selective tissues while others are more widely expressed.

Fluorescence microscopy offers a sensitive technique for observation of biomolecules in cells and tissues. This basic approach involves targeting the desired molecule with a probe that can either be fluorescently labeled itself or recognized by a secondary fluorescent probe. However, when using traditional fluorescence microscopy, out-of-focus light remains in the light path blurring the image of the sample and causing loss of resolution [16]. Laser Scanning Confocal Microscopy (LSCM) overcomes such technical limitations, providing the ability to collect light solely from the objective's focal plane. This eliminates blurring due to out-of-focus light to reveal finer detail than conventional systems could provide [16]. Furthermore, as the emitted light comes from a spatially determined location from the sample, it is possible to determine the discrete position of structures and protein expression within a cell or tissue. If the sample is moved through the focal plane a stack of images, referred to as a *Z*-stack, a three-dimensional localization and reconstruction of structures can be obtained [16]. The use of this technique to spatially locate and analyze the expression of galectin family members can contribute greatly to knowledge of relationships between galectins and their ligands as well as normal expression patterns within an organism.

As confocal microscopy offers an exceptionally specific, high resolution method for protein localization, several studies have recently utilized this method for the localization and functional study of galectin family members [17–19]. LCSM can provide unprecedented levels of detail regarding the location and function of galectin family members (*see* Figs. 1 and 2), but careful technique is important to achieve accurate information. Therefore, this chapter outlines methods for preparing and imaging tissue sections and cellular samples to analyze galectin localization and function by confocal microscopy.

2 Materials

2.1 Preparation of Glass Slides

1. Gelatin from bovine source, Bloom 225 (Sigma, St Louis, MO).
2. ddH_2O.
3. Chromium (III) potassium sulfate [chrome alum (Sigma, St Louis, MO)].
4. General-purpose filter paper (Sigma, St Louis, MO).
5. Glass slides (Knitel, Braunschweig, Germany).
6. Poly-L-lysine 0.1 % (w/v) in water (Sigma, St Louis, MO).

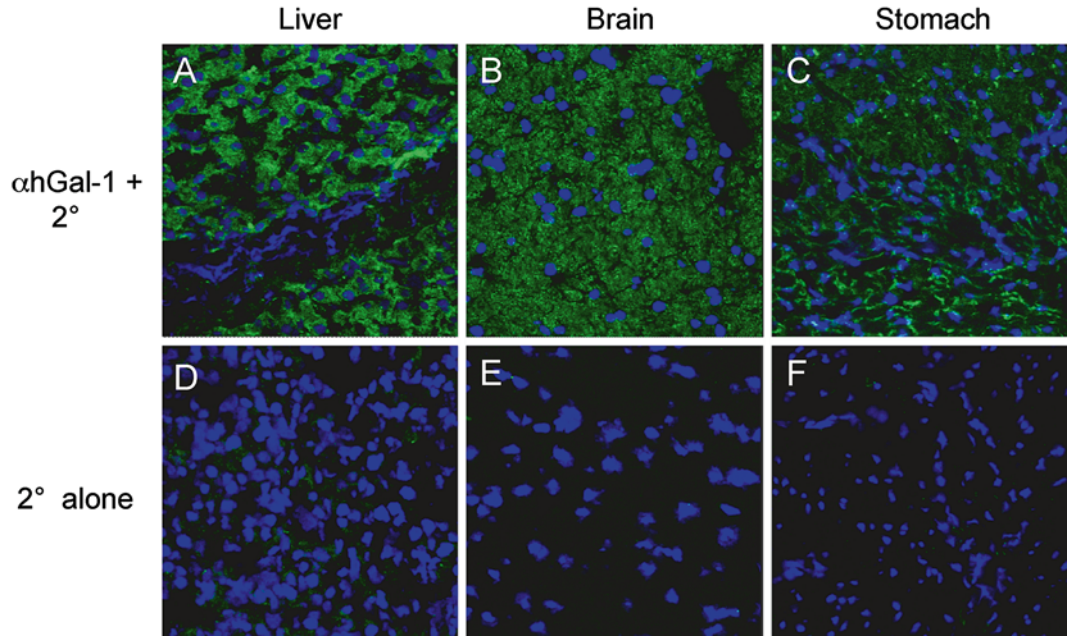

Fig. 1 Gal-1 exhibits diffuse cytosolic localization in various adult tissues. Frozen sections of porcine tissue were subjected to confocal analysis using anti-Gal-1 (αhGal-1) and secondary antibody (αhGal-1 + 2°) or secondary alone (2° alone) as indicated. (**a–c**) αhGal-1 followed by secondary stain of liver (**a**), brain (**b**), or stomach (**c**). (**d–f**) Staining with the secondary antibody alone for liver (**d**), brain (**e**), or stomach (**f**). This research was originally published in Glycobiology. Dias-Baruffi M, Stowell SR, Song SC, Arthur CM, Cho M, Rodrigues LC, Montes MA, Rossi MA, James JA, McEver RP, Cummings RD. Differential expression of immunomodulatory galectin-1 in peripheral leukocytes and adult tissues and its cytosolic organization in striated muscle. Glycobiology. 2010 May;20 (5):507–20. Oxford University Press [10]

2.2 Processing of Tissue

1. Surgical material for tissue sampling (tweezers, scissors, etc.).
2. PBS 1× (137 mM NaCl, 2.7 mM KCl, 4.4 mM Na_2HPO_4, and 1.4 mM KH_2PO_4 pH = 7.3) and PBS 10×.
3. Paraformaldehyde 4 % in PBS (Sigma, St Louis, MO).
4. Sucrose 30–50 % in PBS (w/v) (Fisher, Hampton, NH).

2.3 Cryogenic Technique

1. OCT Compound—Tissue-Tek (Sakura Finetek, Netherlands).
2. [Isopentane (Fisher, Hampton, NH) and liquid nitrogen] or [acetone (Fisher, Hampton, NH) and dry ice].
3. Polyethylene embedding cassettes (Fisher, Hampton, NH) or cryogenic vials (Corning Incorporated Life Sciences, Tewksbury, MA).

2.4 Preparation of Sample Sectioning and Mounting

1. OCT Compound—Tissue-Tek (Sakura Finetek, Netherlands).
2. Pencil.
3. Cryostat (Leica CM 1850, Wetzlar, Germany).
4. Gelatin coated glass slides (Subheading 2.1).

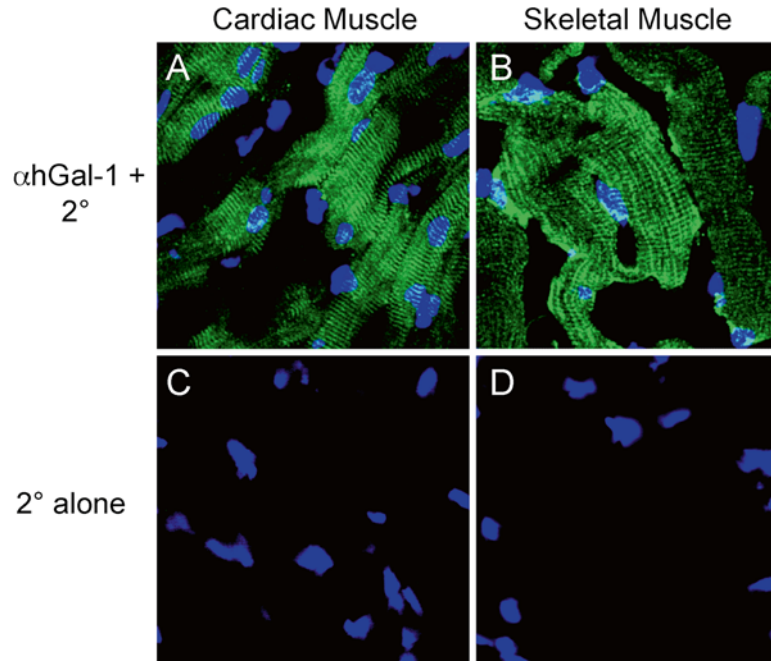

Fig. 2 Gal-1 displays organized cytosolic localization in striated tissues. Frozen sections of cardiac or skeletal porcine muscle tissue were subjected to confocal analysis using anti-Gal-1 (αhGal-1) and secondary antibody (αhGal-1 + 2°) or secondary alone (2° alone) as indicated. (**a**, **b**) αhGal-1 followed by secondary stain of (**a**) cardiac muscle and (**b**) skeletal muscle. (**c**, **d**) Staining with the secondary antibody alone for (**c**) cardiac muscle and (**d**) skeletal muscle. This research was originally published in Glycobiology. Dias-Baruffi M, Stowell SR, Song SC, Arthur CM, Cho M, Rodrigues LC, Montes MA, Rossi MA, James JA, McEver RP, Cummings RD. Differential expression of immunomodulatory galectin-1 in peripheral leukocytes and adult tissues and its cytosolic organization in striated muscle. Glycobiology. 2010 May;20 (5):507–20. Oxford University Press [10]

2.5 Tissue Immunofluorescence

1. 0.25 M glycine in PBS 1×.
2. Hydrophobic barrier pen (Vector Laboratories, Burlingame, CA).
3. Bovine Serum Albumin—Fraction V (BSA) (Sigma, St Louis, MO).
4. DAPI- 4,6-diamidino-2-phenylindole, dihydrochloride (Molecular Probes/Life Technologies, Grand Island, NY).
5. Triton X-100 (Fisher, Hampton, NH).
6. Tween™ 20 (Fisher, Hampton, NH).
7. Fc receptor Blocker—ChromPure whole molecule non-immune IgG from the same animal source as your secondary antibody (Jackson ImmunoResearch, West Groove, PA).
8. Fluoromount G (Electron Microscopy Sciences, Hatfield, PA).

9. Primary antibody.
10. Secondary antibody.
11. PBS 1× (*see* **Note 1**).
12. Refrigerated microcentrifuge.

3 Methods

3.1 Preparation of Glass Slides

1. Clean glass slides thoroughly with ddH_2O and identify individual slides using a pencil (*see* **Note 2**).
2. Prepare the gelatin solution by dissolving 2.5 g of gelatin into 500 mL of ddH_2O heated to 60 °C to make a 0.5 % solution (Do not exceed 60 °C).
3. After gelatin is dissolved, add 0.25 g of Chromium (III) potassium sulfate (chrome alum) to make 0.05 % chrome alum solution (*see* **Note 3**).
4. Filter the solution using general-purpose filter paper.
5. Dip the slide once into the gelatin-coating solution for few seconds.
6. Place gelatinized glass slides at approximately an 80° angle to drain the excess solution onto a paper towel. Cover the slides with aluminum foil to keep off dust.
7. Allow the slides to dry at room temperature for 2 days or overnight at 37 °C. After drying, the slides can be stored in the refrigerator (4–8 °C).
8. Discard gelatin solution after use.
9. Alternatively, the glass slides maybe prepared by coating with 0.1 % Poly-L-lysine (*see* **Note 4**).

3.2 Processing of Tissue

1. *Tissue sampling*: Excise the desired tissue and wash the biopsy briefly in PBS. If freezing tissue on the same day as tissue excision then proceed directly to Subheading 3.3 Cryogenic technique. Otherwise proceed to **step 2** (*see* **Note 5**).
2. *Tissue fixation*: Place excised tissue into a tissue cassette and fix in fresh 4 % paraformaldehyde in PBS for 4 h at room temperature (*see* **Notes 6–8**).
3. *Post-fixation treatment:* To improve the cryopreservation of paraformaldehyde-fixed tissue, these materials should be incubated in sucrose solution (5–30%) and optimal cutting temperature (OCT) compound Tissue-Tek (*see* **Note 9**).

3.3 Cryogenic Technique

1. *Cryogenic technique*—Samples should be placed into a polyethylene embedding cassette (alternatively, cryogenic tubes can be used) filled with OCT. Be sure that the tissue biopsy is

in the correct orientation for future sectioning (transversal, longitudinal, or oblique) (*see* **Notes 10** and **11**).

2. Freeze the embedded sample using one of the two freezing techniques described below: (*see* **Note 12**).
   (a) *Acetone plus dry ice:* Place dry ice into a single 250–500 mL Becker and very slowly add acetone to cover the ice. Wait for 5 min for acetone to chill and then, with the aid of tweezers, dip the cassette (or cryogenic tube) and wait for at least 2 min to ensure the sample has frozen completely (*see* **Note 13**).
   (b) *Liquid nitrogen plus isopentane*: Fill a polystyrene container (Nalgene, Rochester, NY) with liquid nitrogen to approximately ¾ of its total volume. Use tongs to lower a stainless steel or polypropylene container of isopentane into the liquid nitrogen. Begin chilling isopentane, at least 30 min before freezing samples. As isopentane nears freezing it will become opaque. With the aid of tweezers, dip the cassette (or cryogenic tube) into the chilled isopentane and wait for at least 2 min to ensure the sample has frozen completely.

3.4 Preparation of Sample Sectioning and Mounting

1. Carefully detach the frozen sample block from the cassette and identify it with a small piece of paper or other appropriate marking.
2. Handle the cryostat according to the manufacturer guidelines.
3. Fasten the block into the block holder of the cryostat and adjust the tissue thickness for 5–10 μm.
4. Slice 5–10 μm repeatedly until a ribbon includes tissue forms (*see* **Note 14**).
5. Place a previously gelatin-coated slide into position so that the ribbon containing the desired tissue section settles onto the slide as it is sliced.
6. If your primary antibody is sensitive to paraformaldehyde fixation antigen retrieval may be required. Otherwise proceed directly to Subheading 3.5 (*see* **Note 15**).

3.5 Tissue Immunofluorescence

1. If tissue was fixed in formaldehyde/paraformaldehyde (**step 2** of Subheading 3.2), then block free formaldehyde/paraformaldehyde residues by placing tissue in 0.25 M glycine in PBS for 5 min at room temperature. Otherwise, proceed as follows:
2. Determine appropriate antibody conditions (*see* **Notes 16–19**).
3. With a hydrophobic barrier pen, manually circumvent the tissue to restrain the antibody solution around the tissue during the incubation period.

All subsequent steps should be performed in a moisture chamber at room temperature.

4. Block tissue sections in 500 μL PBS containing 2 % BSA and 0.5 % (v/v) Triton X-100 plus Fc blocker (we used 50 μg/mL donkey IgG) for 45 min.
5. Primary antibody should be prepared in PBS with 2 % BSA.
6. Remove blocking buffer and add primary antibody at appropriate concentration to slide. Incubate for 1 h.
7. Wash slides two times for 15 min in PBS plus 0.1 % (v/v) Triton X-100 and then two times in PBS plus 0.05 % (v/v) Tween 20.
8. Secondary antibody should be prepared in PBS with 2 % BSA and 50 ng/mL DAPI. The optimal concentration of secondary antibody should be provided by the manufacturer.
9. Incubate slide with secondary antibody for 1 h.
10. Wash slides four times for 15 min each in PBS plus 0.05 % (v/v) Tween 20.
11. *Mounting slides:* Dip the slide briefly in Milli-Q water. With the aid of a soft paper towel, dry around the sample very carefully.
12. To mount the coverslip over the tissue section, gently add 20–30 μL of Fluoromount, directly to the slide and carefully lay the coverslip over the tissue section taking care to avoid bubbles.
13. Dry gently around the coverslip and seal with colorless nail polish (with no formaldehyde in its formulation) to prevent drying and displacement of biological specimen.
14. Observe the prepared tissue slide using a conventional fluorescence microscope to determine whether fluorescence is detectable in the sample.
15. In the case of positive results, place them in a LCSM and proceed as indicated by manufacturer (*see* **Note 20**).

4 Notes

1. Alternatively, you may use TBS (10 mM Tris and 100 mM NaCl pH = 8.0).
2. When marking glass slides pencil should always be used. Markings made using pens are frequently erased during immunofluorescence techniques.
3. Chrome Alum will positively charge the slides allowing them to attract negatively charged tissue sections.

4. For coating glass slides with Poly-L-lysine, drop 10 μL of solution in the middle of a glass slide and distribute it evenly over the surface by the aid of another moving glass slide. Air-dry for 5 min at room temperature.
5. As this chapter focuses on confocal analysis of galectin localization within tissue, unique considerations should be employed when examining galectin localization in cells in suspension. Cells in suspension can be spread on a glass slide using routine blood smear techniques or overlaid on a poly-L-lysine coated coverslip [20, 21]. When using this approach, evaluation of antibody engagement over a range of concentrations using flow cytometric examination may be beneficial to insure adequate saturation of the target antigen when seeking to ascertain potential changes under different conditions [22, 23]. If fixation is required, this process typically follows adhesion of cells to the slide or coverslip followed by employing similar considerations as are utilized when staining a tissue section [24]. However, as cells may not adhere to the slide or coverslip as well as a tissue section, great care should be taken when incubating and washing the slide to not remove the cells.
6. Several galectins, including galectin-1, galectin-2, and galectin-3 can undergo rapid oxidation following removal from a reducing environment [21, 25, 26]. Galectin oxidation results in significant conformational changes that may preclude antibody recognition depending on the antibody employed and the galectin being examined [21, 27]. As a result, significant care should be taken to rapidly process tissue immediately after harvesting to reduce the potential impact of galectin oxidation on detection and localization of these proteins.
7. To prepare 100 mL of 8 % paraformaldehyde (2×) solution, add 100 μL 1.0 M NaOH into a glass beaker containing 80 mL of water. Add 8.0 g of paraformaldehyde with agitation and heating to 60 °C (be sure to keep a cap on or heat under a fume hood). Wait until complete solubilization (clear solution). Cool solution in ice until it reaches room temperature. Add 10 mL of PBS 10× and complete to 100 mL with water. Mix and filter the resulting solution with a 0.2 μm pore size filter. Prepare a fresh 4 % paraformaldehyde solution by diluting 8 % paraformaldehyde in PBS (1×). The time of incubation may vary according to the kind and size of tissue (over 4 h to several days). Search literature to determine the ideal time for tissue fixation.
8. The desired concentration and content of paraformaldehyde solution may vary from depending on the chosen protein or structure that is being observed. (e.g., for microtubules visualization 4 % paraformaldehyde plus 50 mM EGTA and 50 μM Taxol preserves cytoskeleton structure). Always consult literature for appropriate conditions.

9. The post-fixation step in sucrose solution should not be performed if your tissue was not fixed in formaldehyde/paraformaldehyde, to avoid shrinking of non-fixed tissue. Following chemical fixation, tissue samples are sequentially immersed in solutions of sucrose (5, 10, 20, and 30 %) in PBS, pH 7.2. The transition from a concentration of sucrose solution to another should occur after chemical tissue fixation. Then transfer the tissue sample into a cassette (or cryogenic tube) filled with OCT. This procedure is indicated for tissues such as brain and skeletal muscle.
10. Make sure to mark the facilities identification and correct orientation of particular tissue pieces or surfaces during embedding and subsequent microscopical examination. Cassettes should be marked before embedding to establish orientation. Tissue blocks are simply marked after freezing by cutting a notch on the reverse side of the block face to be sectioned, or by trimming the block to a particular shape.
11. For some special tissues such as bones, wait for at least 1 h at 4 °C in OCT for best infiltration, before freezing.
12. It is important to freeze biological sample until it has been completely frozen. Freezing will begin on the outside and move inward. The process will be complete when tissue plus OCT becomes milky-white in appearance.
13. Be careful when adding acetone directly to dry ice. It will "boil" fast, splashing acetone in all directions. Do not permit the acetone to contact your sample directly.
14. Be sure to obtain additional sections for hematoxylin and eosin staining using commonly employed approaches to provide a histological reference for each slide [28].
15. Fixation should immobilize antigens while retaining cellular and subcellular structure and permeabilize samples to allow for access of antibodies to all cells and subcellular compartments. Cross-linking reagents such as paraformaldehyde serve this purpose, but may reduce the antigenicity of some cell components as the cross-linking can obstruct antibody binding. As a result, antigen retrieval techniques may be required. We use an additional acetone fixation if antigen retrieval is needed. For acetone fixation, dip the slide into −20 °C acetone for 10 min. Before proceeding with immunofluorescence, rehydrate sections with PBS (1×). Remember that acetone fixation will promote cell permeabilization.
16. To confirm antibody specificity, perform immunoblotting with its specific purified protein and/or tissue/cell extract to verify if the protein recognized is the expected size. In addition, antibody specificity may be demonstrated by performing the immunofluorescence stain with primary antibody that has been previously neutralized with its specific purified protein.

17. Centrifuging the diluted solutions of antibodies at maximum speed (~20,000 × *g*) for 30 min at 4 °C before using them can help to avoid undesired background during the immunofluorescence procedure.
18. It is important to use the minimal concentration of primary antibody to achieve a good staining. As a result, it is recommended to perform some test staining with multiple concentrations. If the antibody is a purchased antibody, begin with the minimum concentration indicated on the product datasheet. Usually, we see good staining by using 10 μg/mL of purified antibody.
19. Negative controls such as absence of primary antibody or use of an isotype control should be included in the staining protocol in order to achieve interpretable results.
20. When using the confocal microscope, select the best parameters for your samples, such as a low laser power and a quick scan, to avoid photobleaching. The pinhole should be set to the optimal size for each objective. Care should be taken to avoid overlapping signals between fluorochromes by setting each channel excitation power and emission bandwidth correctly. The PMT (Photo Multiplier Tube) gain should be high enough to have a bright image without saturated pixels. If the signal is weak, increasing laser power at maximum may be useless, because you probably will create a false positive image.

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Chapter 24

Examination of the Regulation of Galectin-3 Expression in Cancer

Hafiz Ahmed and Gargi Bandyopadhyaya

Abstract

Galectin-3, a member of a β-galactoside-binding protein family, is involved in normal growth development as well as cancer progression and metastasis, but the detailed mechanisms of its functions or its transcriptional regulations are not well understood. Besides, several regulatory elements such as GC box, CRE motif, AP-1 site, and NF-κB sites, the promoter of galectin-3 gene (*LGALS3*) contains several CpG islands that can be methylated during tumorigenesis of prostate leading to the gene silencing. Here we describe protocols for identification of galectin-3 DNA methylation, suppression of DNA methyltransferases to reactivate galectin-3 expression, and development of methylation-specific polymerase chain reaction (MS-PCR) to assess galectin-3 expression in various biological specimens such as tissue, serum, and urine samples.

Key words Galectin-3, Gene regulation, Gene silencing, DNA methylation, Prostate cancer

1 Introduction

Interactions between cells or between cell and the extracellular matrix (ECM) are pivotal for proper cellular function. In recent years, protein–carbohydrate interactions have been considered as very important for modulation cell–cell and cell–ECM interactions, which, in turn, mediate various biological processes such as cell activation, growth regulation, cancer metastasis, and apoptosis. Thus, the identification of carbohydrate-binding proteins (lectins) and their partners (carbohydrate ligands), and the detailed understanding of the molecular mechanisms and downstream effects of these protein-carbohydrate interactions are subjects of current intense research. Galectin-3, a member of at least 15 β-galactoside-binding proteins, is involved in cancer progression and metastasis [1–5]. The intensity of the galectin-3 expression in tumors depends on the type of tumor, its invasiveness and metastatic potential [6, 7]. For example, increased expression of galectin-3 is observed in colon, head and neck, gastric, endometrial, thyroid, liver, bladder cancers and breast carcinomas [8–13]. In contrast, decreased

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expression of galectin-3 was observed in prostate [14–16], kidney [17], and pituitary cancers [18]. Moreover, change in cellular localization of galectin-3 is observed during progression of various cancers [6, 7, 19]. For example, in tongue cancer nuclear galectin-3 is decreased, but cytoplasmic galectin-3 is increased during progression from normal to cancer [6, 7]. In prostate cancer although overall galectin-3 expression is decreased, the gradual change in nuclear to cytoplasmic expression of galectin-3 is evident during stage evolution [16].

Although a large body of data about galectin-3 expression is available in the literature, the mechanisms of regulation of galectin-3 expression are not well understood. However, the expression of galectin-3 depends on cell type, external stimuli and environmental conditions and involves numerous transcription factors and signaling pathways [20]. In the promoter region of the galectin-3 gene, several regulatory elements such as five putative Sp1 binding sites (GC boxes), five cAMP-dependent response element (CRE) motifs, four AP-1- and one AP-4-like sites, two NF-κB-like sites, one sis-inducible element (SIE), and a consensus basic helix–loop–helix (bHLH) core sequence are found [21]. The presence of multiple GC box motifs for binding ubiquitous expressed Sp1 transcription factor is a characteristic of constitutively expressed "housekeeping" genes. The activation of the Sp1 binding transcription factor is responsible for galectin-3 induction by Tat protein of HIV [22]. The SIE that binds sis inducible factors was suggested to be a possible candidate for the growth-induced activation of galectin-3 gene expression, caused by the addition of serum. The presence of CRE and NF-κB-like site in the galectin-3 promoter suggests that the activation of galectin-3 expression could be regulated through the signaling pathways involving the cAMP-response element-binding protein (CREB) or the NF-κB transcription factor. The CREB/ATF and the NF-κB/Rel transcription factors pathways may be involved in the regulation of galectin-3 expression by the Tax protein during HTLV-I infection of T cells [23]. The involvement of the NF-κB transcription factor in regulation of galectin-3 expression, as well as the Jun protein, a component of AP-1 transcription factor has recently been confirmed [24]. The regulation of galectin-3 expression through the NF-κB transcription factor was shown to be mediated by nucling, a novel apoptosis-associated protein that interferes with NF-κB via the nuclear translocation process of NF-κB/p65, thus inhibiting galectin-3 expression on both protein and mRNA level [24]. In skeletal tissues, the regulation of galectin-3 expression is mediated by the transcription factor Runx2 [25–27]. Very recently, galectin-3 expression is found to be regulated in pituitary and prostate tumors by methylation of CpG islands in the promoter region [16, 18, 28, 29].

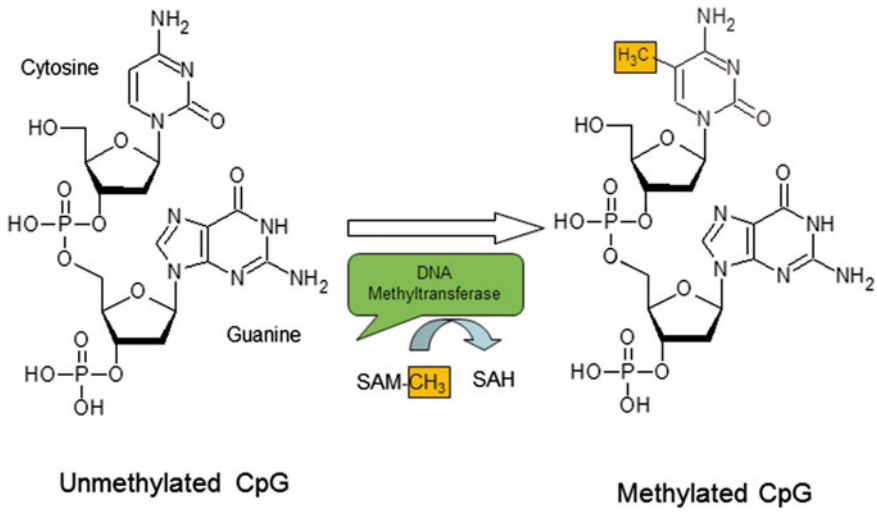

Fig. 1 DNA methylation catalyzed by DNA methyltransferase. DNA methyltransferase transfers methyl group from S-adenosyl methionine (SAM-CH_3) to cytosine yielding S-adenosyl homocysteine (SAH) and 5-methylcytosine

DNA methylation refers to the covalent binding of a methyl group specifically to the carbon-5 position of cytosine residues of the dinucleotide CpG (Fig. 1), and DNA methylation plays essential roles in many molecular and cellular alterations associated with the development and progression of cancer [30]. DNA methylation is catalyzed by a family of enzymes, the DNA methyltransferases (DNMTs). Although the importance of CpG island methylation has been demonstrated in cancer, the mechanisms that lead to these changes in cancer are not yet understood. Of three members (DNMT1, DNMT3a, and DNMT3b) of the DNA methyltransferase family, DNMT1 is believed to be primarily involved in the maintenance of CpG methylation [31, 32]. However, other studies suggest that DNMT3b, independently or in cooperation with DNMT1, also contributes to hypermethylation [33–35]. The suppression of transcription by DNA methylation may occur by either direct inhibition [36] or indirect inhibition [37] of transcription factor binding (Fig. 2). For the latter, a family of proteins known as methyl binding domain (MBD) proteins is believed to specifically bind DNA containing methylated CpG sites [38, 39]. At least three of the five known members of this family (MeCP2, MBD2, and MBD3) have been shown to be associated with large protein complexes containing histone deacetylase (HDAC1 and HDAC2) and chromatin-remodeling (Sin3a and mi-2) activities [40, 41]. Histone deacetylase (HDAC1 and 2) and chromatin remodeling activities (Mi-2 and Sin3a) produce alterations in chromatin structure that make it

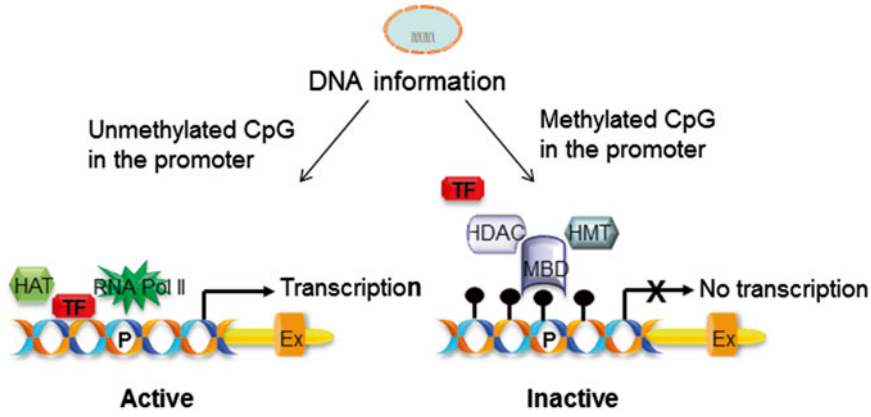

Fig. 2 Simplified cartoon showing gene transcription by unmethylated promoter and gene silencing by the methylated promoter. In normal cells, promoter of some genes such as tumor suppressor protein, DNA repair proteins is unmethylated and accessible to binding to the transcription factors (TF) allowing transcription. But, in many cancers these genes are methylated by DNA methyltransferase 1 and therefore bound by the methyl-CpG binding proteins (MBD) and histone deacetylase (HDAC). Thus the methylated promoter is not accessible to binding to the transcription factors and inactive. In tumor tissues and biological fluids such as serum and urine, the methylated DNA is measured by various methods for the development of diagnostic and prognostic tools for the cancer. *HAT* indicates histone acetyltransferase, *RNA pol II* RNA polymerase II, and *HMT* histone methyltransferase

refractory to transcriptional activation. In addition to the large protein complexes, the MBD proteins may associate with several other complexes involved in transcriptional repression.

Here we describe protocols for identification of galectin-3 DNA methylation, suppression of DNA methyltransferases to reactivate galectin-3 expression, and development of methylation-specific polymerase chain reaction (MS-PCR) to assess galectin-3 expression in various biological specimens such as tissue, serum, and urine samples of prostate cancer patients. First we investigate expression of galectin-3 in prostate cell lines (normal and malignant). We treat galectin-3 non-expressing cells, if any, with 5-aza-2′-deoxycytidine (inhibitor of DNA methyltransferase) to investigate if the silencing is due to the promoter methylation. Since azacytidine blocks cytidine methyl transferase activity, treatment of cells with azacytidine should reactivate the genes. We then investigate DNA methylation of the galectin-3 promoter in the galectin-3 non-expressing cells (before and after azacytidine treatment) by cloning and sequencing. Finally we investigate DNA methylation of the galectin-3 promoter in the tissues and other biological specimens such as serum and urine from normal and prostate cancer patients by directly cloning/sequencing and/or by MS-PCR.

2 Materials

2.1 Cell Culture

1. PrEC cells (Clonetics/Cambrex).
2. Supplemented keratinocyte-serum free medium: Keratinocyte-serum free medium with 2 mM glutamine, 5 ng/ml epidermal growth factor, 50 μg/ml bovine pituitary extract, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (Gibco/Invitrogen Corp. and Sigma).
3. Incubator at 5 % CO_2 at 37 °C.
4. BPH-1 (benign prostatic hyperplasia) cell line (gift from Dr. Simon Hayward).
5. IMEM media (Biofluids).
6. RPMI media.
7. DMEM media.
8. 10 % fetal bovine serum (Quality Biologicals).
9. LNCaP cells (American Type Culture Collection).
10. PC-3 cells (American Type Culture Collection).
11. DU-145 cells (American Type Culture Collection).

2.2 Treatment of Cells with Azacytidine

1. 5-aza-2′-deoxycytidine, hydroquinone, and sodium bisulfite (Sigma-Aldrich).

2.3 Preparation of RNA and RT-PCR

1. All-in-One Purification kit (Norgen Biotek Corporation).
2. NanoDrop 2000 spectrophotometer (Thermo Scientific).
3. RT^2 First Strand Kit (SA Biosciences).
4. Galectin-3 specific primers.
5. Actin or glyceraldehyde 3-phosphate dehydrogenase control primers.
6. MJ Thermal Cycler.
7. ABI Fast 7500.

2.4 Validation of Protein Expression by Western Blot

1. Mammalian Cell Lysis kit (Sigma).
2. Bradford dye assay kit.
3. Bovine serum albumin.
4. 10 % polyacrylamide gel.
5. Nitrocellulose.
6. Protein A.
7. Rabbit anti-Galectin-3 antibody.
8. ECL Western Blotting Substrate (Thermo Scientific).

2.5 Bisulfite Modification of Genomic DNA Followed by PCR, Cloning, and Sequencing

1. NucleoSpin Genomic DNA Extraction Kit (Macherey-Nagel).
2. DNA Easy kit (Qiagen).
3. NanoDrop 2000 spectrophotometer (Thermo Scientific).
4. 0.3 M NaOH.
5. 10 mM hydroquinone.
6. 3.6 M sodium bisulfite (pH 5).
7. Desalting column (Magic DNA Clean-Up System; Promega).
8. 3 M ammonium acetate (pH 7).
9. EZ Gold Methylation Kit (Zymo Research).
10. Multiplex PCR Kit (Qiagen, Inc.).
11. DNA Extraction kit (Qiagen).
12. pGEM-T vector (Promega).
13. JM109 competent cells.
14. T7 or sp6 primers.

2.6 Investigation of Galectin-3 Promoter Methylation in Prostate Tissues (Normal and Malignant)

1. Fresh frozen tissues (Cooperative Human Tissue Network (CHTN) of National Cancer Institute).
2. Post mortem normal prostate tissues (National Disease Research Interchange—NDRI).
3. Affinity purified anti-galectin-3.

2.7 Direct Assessment of Galectin-3 Promoter Methylation in Biological Specimens Using Methylation Specific PCR (MS-PCR)

1. Methylated primers.
2. Unmethyated primers.
3. 5 % Top Vision LE GQ agarose (Fermentas).

2.8 Special Equipment

1. Thermal cycler (MJ, Thermo Scientific).
2. Real time PCR System (Applied Biosystems 7500 Fast).
3. NanoDrop 2000 spectrophotometer (Thermo Scientific).

3 Methods

3.1 Cell Culture

1. Culture PrEC cells in supplemented keratinocyte-serum free medium with 2 mM glutamine, 5 ng/ml epidermal growth factor and 50 μg/ml bovine pituitary extract and 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate (Sigma, St. Louis, MO) in the presence of 5 % CO_2 at 37 °C.

2. Culture BPH-1 (benign prostatic hyperplasia) cell line in IMEM supplemented with 10 % fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate in the presence of 5 % CO_2 at 37 °C.
3. Culture LNCaP cells in phenol red free RPMI 1640 medium supplemented with 10 % FBS, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate in the presence of 5 % CO_2 at 37 °C.
4. Culture PC-3 and DU-145 cells in a mixture of DMEM (Sigma) and F-12 (Sigma) (1:1) supplemented with 10 % FBS, penicillin and streptomycin in the presence of 5 % CO_2 at 37 °C.

3.2 Treatment of Cells with Azacytidine

1. Treat the cells with 10 μM 5-aza-2′-deoxycytidine (Sigma) for 7 days replacing the medium every day [28]. Control wells receive only medium with vehicle.

3.3 Preparation of RNA and RT-PCR

1. Harvest cells (untreated or treated) for extraction of total RNA using all-in-One Purification kit.
2. Quantify RNA spectrophotometrically using NanoDrop 2000 spectrophotometer.
3. Take 4 μg of total RNA and synthesize first strand cDNA using RT^2 First Strand.
4. Perform standard (such as on MJ Thermal Cycler) or quantitative (such as on ABI Fast 7500) PCR for galectin-3 expression using gene specific primers (galectin-3: forward 5′-CACGGT GAAGCCCAATGCA-3′ and reverse 5′ GTGAGCATCATTC ACTGCAACC-3′).
5. Use actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for control reaction. Expected results of galectin-3 expression in the untreated and azacytidine treated prostate cell lines are shown in Fig. 3.

3.4 Validation of Protein Expression by Western Blot (See Note 1)

1. Proteins are extracted from the cells using Mammalian Cell Lysis kit using manufacturer's instructions.
2. Concentration of protein is measured by Bradford dye assay using BSA as a protein standard.
3. Protein samples are denatured and subjected to SDS-PAGE (10 %) gel electrophoresis and transfer to nitrocellulose membrane [16].
4. Proteins on the membrane are visualized by probing with affinity purified (over Protein A-Sepharose) rabbit anti-galectin-3 antibody followed by addition of goat anti-rabbit IgG-horse radish peroxidase conjugate and development with ECL Western Blotting Substrate.

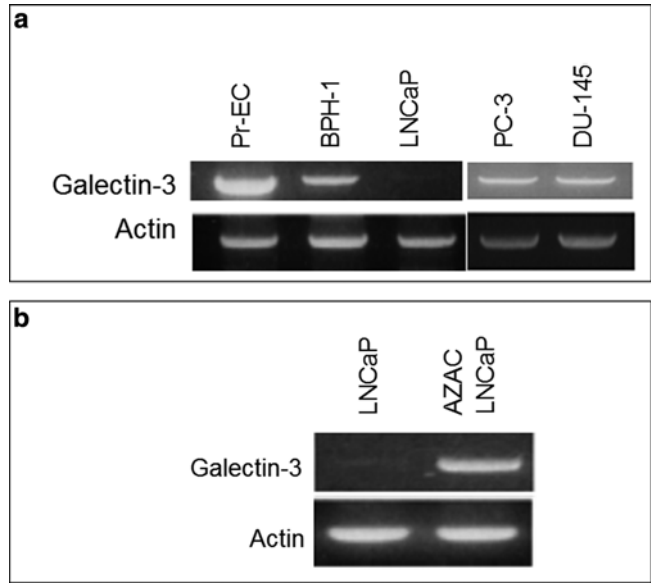

Fig. 3 Expression of galectin-3 determined by reverse transcriptase polymerase chain reaction (RT-PCR). (**a**) Expression of galectin-3 in normal prostate cells (Pr-EC), benign prostatic hyperplasia cells (BPH-1), and androgen-dependent (LNCaP) and androgen-independent (PC-3, DU-145) prostate cancer cells. (**b**) Expression of galectin-3 in azacytidine (AZAC)-treated LNCaP

3.5 Bisulfite Modification of Genomic DNA Followed by PCR, Cloning and Sequencing

1. To prepare genomic DNA from untreated and treated cells, use the NucleoSpin Genomic DNA Extraction Kit following the manufacturer's instructions (*see* **Note 2**).
2. Quantify DNA spectrophotometrically using NanoDrop 2000 spectrophotometer.
3. For bisulfite treatment, genomic DNA (8 μg) is denatured in 0.3 M NaOH for 15 min at 37 °C in a volume of 100 μl, and after adding 60 μl of 10 mM hydroquinone and 1.04 ml of 3.6 M sodium bisulfite (pH 5), the reaction mixture is incubated at 50 °C for 16 h in the dark (*see* **Note 3**).
4. The DNA is then purified on a desalting column (Magic DNA Clean-Up System; Promega).
5. DNA is then denatured with 0.3 M NaOH for 15 min at 37 °C, neutralized with 3 M ammonium acetate (pH 7), and ethanol-precipitated. Occasionally, bisulfite modification of genomic DNA is performed by EZ Gold Methylation Kit according to the manufacturer's instructions.
6. The sequence of interest in the bisulfite-treated DNA is amplified by PCR using primer pairs located outside the CpG sites (*see* **Note 4**).

7. The amplification reaction is normally carried out by using Multiplex PCR Kit (Qiagen).
8. The PCR is set for 25–30 cycles using standard conditions with denaturation at 94 °C, annealing at 50–55 °C, and extension at 72 °C at thermocycler.
9. The amplified fragment is then subjected to electrophoreses on a 2 % agarose gel and the separated product was cut out from the gel by razor blade.
10. Extract the DNA from the gel using DNA Extraction kit following the manufacturer's instructions.
11. The purified PCR product is then cloned into pGEM-T vector following ligation and transformation of JM109 competent cells according to the manufacturer's instructions.
12. To sequence the cloned insert, white colonies are selected and subjected to plasmid DNA preparation.
13. Plasmids DNAs are sequenced using vector primers T7 or sp6 (*see* **Note 5**).

3.6 Investigation of Galectin-3 Promoter Methylation in Prostate Tissues (Normal and Malignant)

1. Fresh frozen tissues are obtained from the Cooperative Human Tissue Network (CHTN) of National Cancer Institute (*see* **Note 6**).
2. Post mortem normal prostate tissues are obtained through National Disease Research Interchange (NDRI) (*see* **Note 7**).
3. To prepare genomic DNA from normal, benign, and malignant prostate tissues are prepared and bisulfite treated as described in Subheading 3.5.
4. Bisulfite-treated DNA from each specimen is subjected to PCR amplification, cloning, and sequencing as described in Subheading 3.5 (*see* **Note 8**).
5. To investigate a correlation between galectin-3 promoter methylation in tissues and its tissue expression, we usually perform immunohistochemical staining. For this purpose, we use affinity purified (over Protein A-Sepharose column) anti-galectin-3 [16].
6. FFPE (Formaldehyde fixed paraffin embedded) tissues are deparaffinized followed by antigen retrieval and immunostaining following standard procedures.
7. Staining is visualized with the diaminobenzidine (DAB) chromogen and counterstained with Mayer's hematoxylin.
8. For statistical analyses, at least eight slides for each stage of tumor are evaluated by two independent observers to semi-quantify the percentage of galectin-3 immunopositivity [16] (*see* **Note 9**).

3.7 Direct Assessment of Galectin-3 Promoter Methylation in Biological Specimens Using Methylation Specific PCR (MS-PCR)

1. To perform MS-PCR, primers are designed on the CpG sites to enable discrimination between methylated and unmethylated alleles after bisulfite treatment, and thus, the methylation status of the CpG island is assessed directly from the presence or absence of the PCR product (Fig. 6).
2. To identify any unmethylated DNA allele, PCR is also performed using "unmethylated" primers (*see* **Note 10**).
3. For the bisulfite conversion reaction, the "methylated" primers are designed as follows: forward primer HuG3BPMF1, 5′-CGTTTCGTCGGCGTTCG-3′ (ranging from –9 to +8 of the promoter sequence) and reverse primer HuG3BPMR1, 5′-CACGCAACTCACCGCTCG-3′ (ranging from +47 to +64 of the promoter sequence).
4. The unmethylated primers, designed for bisulfite conversion reaction as follows: forward primer HuG3BPUF1, 5′-GA GGTTTGGAGTTATTGTTTTGTTGGTG-3′ (ranging from –24 to +4 of the promoter sequence) and reverse primer HuG3BPUR1, 5′-CCCCACACAACTCACCACTCA-3′ (ranging from +47 to +67 of the promoter sequence) (*see* **Note 11**).
5. An equal amount of bisulfite treated DNA (75 ng) is used for each PCR condition.
6. For separation, the PCR product is subjected to agarose gel electrophoresis on a 5 % Top Vision LE GQ agarose (*see* **Note 12**).

4 Notes

1. To validate RT-PCR results, we perform Western blot.
2. Occasionally, the genomic DNAs are extracted using DNA Easy kit following the manufacturer's protocol.
3. To identify cytosine methylation by analyzing bisulfite modification in the galectin-3 promoter, genomic DNAs are treated with sodium bisulfite. Sodium bisulfite converts unmethylated cytosine to "uracil" (reads as "thymine" in DNA sequencing) as shown in Fig. 4, but methylated cytosine remains unchanged. Thus, sodium bisulfite can distinguish methylated cytosine from unmethylated cytosine.
4. This method allows precise analysis of methylation in a selected region by converting all non-methylated cytosine (C) into uracil (U) while methylated cytosine remains unchanged. The primers for galectin-3 taken for this bisulfite conversion reaction were as follows: (a) forward primer (HuG3BPF3), 5′-GGAGA-GGGTGGGGGATAG-3′ derived from the wild-type sequence 5′-GGAGAGGGCGGGGGACAG-3′ (ranging from –277 to –260 nt of the promoter sequence) and (b) reverse primer (HuG3BPR3), 5′-ACACCTCTCCCCTACCC-3′ derived from

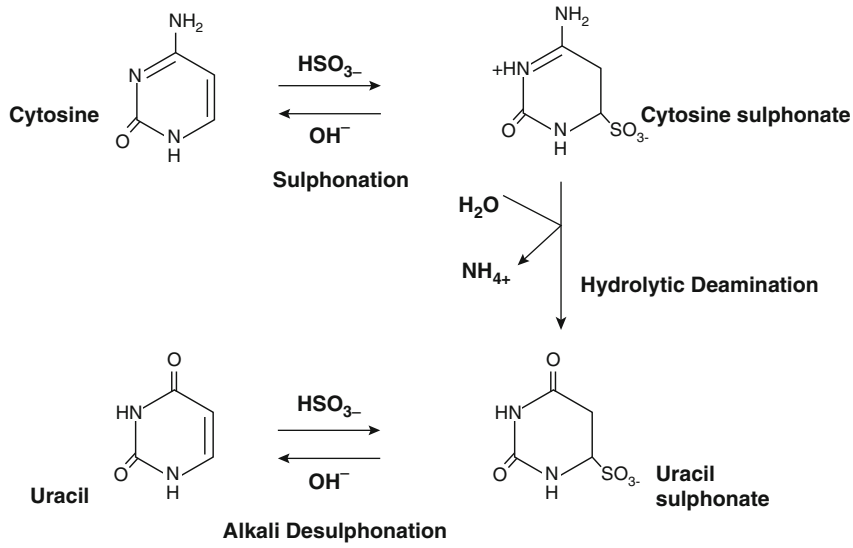

Fig. 4 Cartoon showing conversion of unmethylated cytosine to uracil in the presence of sodium bisulfite

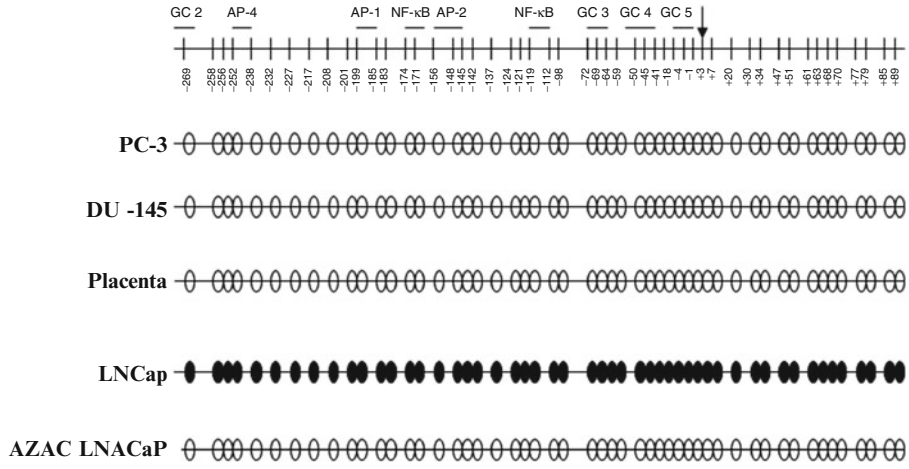

Fig. 5 Methylation profile of galectin-3 promoter region (384 bp) from prostate cancer cell lines LNCaP (untreated and AZAC-treated), PC-3, and DU-145. Each *row* represents a single cloned allele, and each *oval* represents a single CpG site (*open oval*, unmethylated; *closed oval*, methylated). The *numbering* in the schematic diagram at the top represents the position relative to the published transcription site (+1, indicated by the *arrow*)

the wild-type sequence 5′-GCGCCCTCTCCCTGCCC-3′ (ranging from +90 to +107 nt of the promoter sequence).

5. As shown in Fig. 3a, galectin-3 is not expressed in LNCaP, but its expression is observed after azacytidine treatment suggesting silencing of galectin-3 in LNCaP is due to promoter DNA methylation. So, cytosine methylation of the galectin-3 promoter in LNCaP cells is expected upon sequencing (Fig. 5).
6. All CHTN tissues are obtained through radical prostatectomy and associated with synoptic report of detailed pathological

staging of the tumor as follows: histologic type, histologic grade (both primary and secondary patterns), and tumor quantification, any indication of extraprostatic extension, seminal vesicle invasion, margins involvement, and information about the primary tumor, regional lymph nodes, and distant metastases. Moreover, donor's age, race, and serum PSA value are recorded prior to the radical prostatectomy and added to the synoptic report. For our study, we use only pathologically defined radical prostatectomy tissues and their matching serum and urine specimens whenever applicable.

7. All specimens are procured under approved IRB protocol.
8. As tumor is heterogeneous, we usually sequence at least 20 clones from each stage of prostate cancer to obtain statistically significant data. Our investigation of galectin-3 promoter in various stages of prostate cancer resulted differential methylation. Particularly, galectin-3 promoter in organ confined prostate cancer is found heavily methylated (throughout the entire 384 bp promoter) [16]. In locally advanced prostate cancer, galectin-3 promoter shows a few methylation sites, mostly between −199 and −252 nt; whereas the galectin-3 promoter from advanced prostate cancer is methylated between −112 and −227 nt [16]. Galectin-3 promoter from either normal prostate or BPH is not methylated [16].
9. In our investigations, galectin-3 is found strongly expressed both in nucleus and cytoplasm in normal, BPH (benign prostatic hyperplasia), and HGPIN (high-grade prostatic intraepithelial neoplasia, a precursor lesion to development of invasive prostatic adenocarcinoma) tissues. Galectin-3 shows decreasing immunopositivity during stage evolution [16]. However, localization of galectin-3 is interesting during stage evolution. In particular, stage I tumors showed a strong immunopositivity both in nucleus and cytoplasm, while in more advanced stages immunostaining was less intense and localized mainly in cytoplasm, with rare, occasional nucleus positivity [16]. However, two stage I specimens (out of 10) showed little or no gal3 immunopositivity. Overall, galectin-3 immunostaining results of malignant prostate tissues do not seem to correlate with the promoter methylation status of the galectin-3. Probable reasons for this anomaly are that we use macroscopic tumor tissue for the promoter methylation analysis and also prostate adenocarcinoma by nature is heterogeneous. For a better correlation, investigation of galectin-3 promoter methylation from laser-capture microdissected tissues should be performed.
10. The PCR product obtained with the unmethylated primers serve as also as a positive control for the presence of DNA in the PCR reaction, which is particularly relevant when MS-PCR based sensitive assay with the methylated primers yield a negative result.

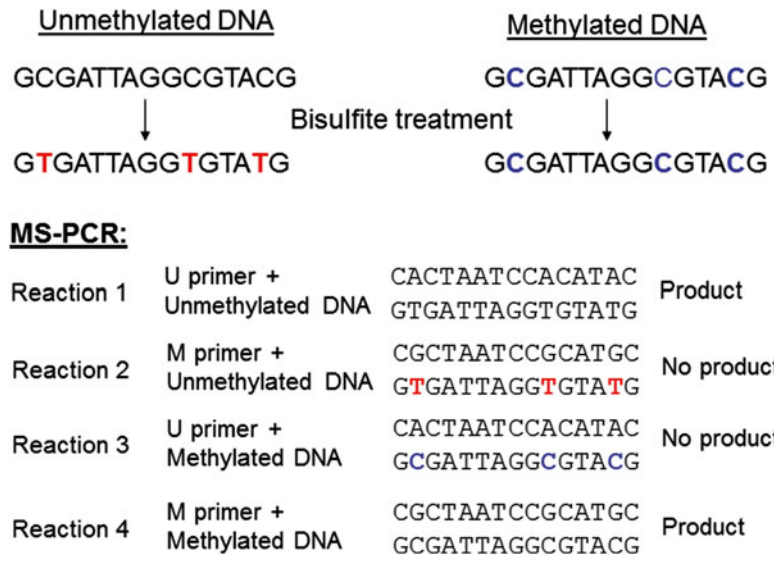

Fig. 6 Schematic representation of MS-PCR. In MS-PCR, primers are designed on the CpG sites to discriminate between methylated and unmethylated alleles following bisulfite treatment (unmethylated "C" changes to "U," but methylated "C" remains unchanged) and thus, the methylation status of the CpG islands is assessed directly from the presence of a PCR product. PCR product is expected only in those samples where methylated DNA is mixed with the methylated primer and unmethylated DNA is mixed with the unmethylated primer

11. The PCR product is formed where methylated primers interact with the methylated DNA and where unmethylated primers interact with the unmethylated DNA (*see* Fig. 6). Methylated primers can also be designed using MethPrimer program [42]. The specificities of the methylated and unmethylated primers are tested using known methylated and unmethylated DNA under the same PCR conditions.
12. Our MS-PCR is expected to detect methylation of the galectin-3 promoter in various biological specimens (*see* Fig. 7 for an example of MS-PCR results). As our MS-PCR assay involves the galectin-3 promoter ranging from −9 to +64 nt, it identifies only organ confined prostate cancer (*see* Fig. 7) and thus our MS-PCR serves as a tool for early detection of prostate cancer [29, 43].

Acknowledgments

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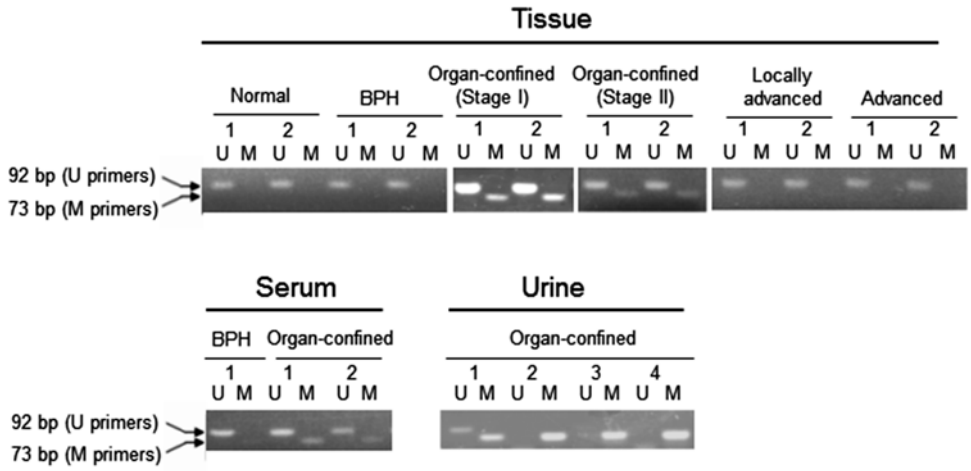

Fig. 7 PCR of bisulfite-treated DNA from tissue, serum, and urine specimens from normal, BPH, and tumor prostate tissues with galectin-3 unmethylated (U) and methylated (M) primer pairs. The products 92 and 73 bp obtained from U and M primers, respectively, are shown by *arrows*. An equal amount of bisulfite-treated DNA (75 ng) was used for each PCR reaction and the product was subjected to electrophoresis on a 5 % TopVision LE GQ agarose (Fermentas, Glen Burnie, MD)

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Chapter 25

Evaluation of the Role of Galectins in Parasite Immunity

Sarah Preston, Jillian Dunphy, Travis Beddoe, Els Meeusen, and Anna Young

Abstract

Galectin-11 and galectin-14 are ruminant galectins involved in parasitic infections. Although their roles in parasite immunity are still being elucidated, its appears that their functions are parasite specific. In gastrointestinal infections with the nematode *Haemonchus contortus*, both galectin-11 and galectin-14 appear to be protective. However, in a chronic infection of liver fluke, *Fasciola hepatica*, these galectins may aid parasite survival. This chapter discusses the methods designed to study parasitic infections in sheep, which have provided us with insight into the functions of galectin-11 and galectin-14 during host–parasite interactions. These methods include parasite cultivation and infection, galectin staining of host and parasite tissue, surface staining of parasites with recombinant galectins and in vitro assays to monitor the effect of galectins on larval development.

Key words Galectin-11, Galectin-14, *Haemonchus contortus*, *Fasciola hepatica*, Larval culture, Immunohistochemistry, Larval molting assay, Larval growth assay, Larval feeding assay

1 Introduction

Galectins are produced by parasites and by the host in response to parasite infections, suggesting that they play an important role in host–parasite interactions [1]. Of the 15 mammalian galectins described to date, at least five are upregulated during parasitic infection (galectin-1, -3, -9, -11, and -14). Galectin-3 has been implicated in a range of parasitic infections, including malaria [2]; Leishmania [3]; *Trypanosmoa cruzi*; *Toxoplasma gondii* [4]; and *Schistosoma mansoni* [5]. Galectin-1 is involved in infections with the sexually transmitted protozoan parasite *Trichomonas vaginalis*, and galectin-9 reduced eosinophilia in a mouse model of *Ascaris suum* infection [6, 7]. Since the initial discovery of galectin-11 and -14 in parasite-infected sheep by our laboratory, we have focused on elucidating their functional role in parasitic infections.

Parasites that infect ruminants are a primary research focus of our group due to their significant worldwide economic impact and

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implications for food security. In particular, we have focused on the gastrointestinal nematode parasite *Haemonchus contortus* (*H. contortus*) and the trematode parasite, the liver fluke *Fasciola hepatica* (*F. hepatica*). Our laboratory explores the immune response to these parasites to aid vaccine design and disease diagnostics. As it is believed that the larval stages of parasitic helminths are the most vulnerable to host immune attack, parasitic larvae are the main focus of our studies.

Sheep are naturally infected while grazing on pasture by the third-stage *H. contortus* larvae (L3) attached to grass blades. This is replicated experimentally by oral infection with parasitic larvae using a gelatin capsule delivered by a dosing gun. The L3 for infection can be initially obtained from veterinary departments or companies, but once an infection is established the cultivated L3 can be used to reinfect, creating a continuous infection cycle. Once ingested, the L3 will migrate through the rumen (first stomach of ruminants) to the abomasum (fourth or true stomach) where they develop through one more larval stage, the fourth larval stage (L4), prior to developing into an immature adult stage that gradually grows and becomes sexually mature. Under optimal conditions, 28 days is the time required for larvae to develop into sexually mature adults and start producing eggs. When the infection has reached this stage, feces are collected to cultivate the eggs which in nature would be excreted to contaminate pasture and cause reinfection.

H. contortus eggs are hatched by replicating optimal conditions, i.e., a warm and humid environment. The first and second-stage larvae develop by eating bacteria from the feces. When the larvae develop into the L3 stage, they can be collected and stored for either reinfection or use in in vitro assays. The L3 collected from the feces still retain their second larval stage (L2) cuticle as it provides protection from the environment until a suitable host is found. Under natural conditions, L3 will lose their L2 cuticle (exsheath) when passing through the rumen on their migration to the abomasum. Exsheathment can be induced artificially by two methods, either by treatment with carbon dioxide (CO_2) or incubation with sodium hypochlorite. Treatment with CO_2 replicates the environment L3 would be exposed to while passing through the sheep's rumen. If the L3 tegument is the focus of the study, for example binding studies with galectins, then CO_2 exsheathment is used as this is a more gentle process and leaves the lipids, proteins, and carbohydrates covering the cuticle (epicuticle) unmodified.

For assays involving the L4 stage, the exsheathment of L3 is performed using sodium hypochlorite as this treatment kills bacteria and fungi that would cause contaminations. As the L4 stage is where a mouth develops and *H. contortus* starts feeding from the host, assays measuring growth and feeding can be set up to explore factors of the host immune response that may interfere

with this process. It was using this system that the role of galectin-11 in inhibiting growth and feeding was noted (Preston et al., in preparation).

To study the immune response to *H. contortus* infections in vivo, naïve sheep (young animals with no or little prior exposure to parasites) are usually primed to the infection. Priming involves a series of infections which aims to mimic natural kinetics where animals would undergo constant reinfection from pasture grazing. Following repeated priming and a rest period, a challenge infection is administered, which in resistant animals will provoke a strong adaptive immune response resulting in rejection of the parasites. Immediate challenge without a rest period may result in a rapid rejection of incoming larvae without recalling an adaptive immune response [10]. The size of the animals is advantageous as it allows for collection of large tissue samples and in vivo cannulation studies during the development of parasite rejection [8, 9]. Using this experimental model, the immune response can be strategically investigated to study either the larval or adult parasitic stage, as differential immune responses are known to exist [10].

Sheep are infected with liver fluke when they ingest encysted larvae (metacercariae) attached to vegetation. The metacercariae excyst in the small intestines and the resulting newly excysted juvenile flukes migrate through the intestinal wall to the liver. In the liver they feed for several weeks prior to migration to the biliary ducts where they develop into egg-producing adults resulting in chronic infection [11]. Eggs produced in the biliary ducts are then excreted in the feces. Unlike the gastrointestinal nematodes, liver flukes require an intermediate host (snails) to complete their life cycle and produce the infective metacercariae. For our laboratory, metacercariae collected from the snails are usually purchased and are orally delivered into animals to study the immune response to acute and chronic infection.

One immune cell which targets the larval stages of *H. contortus* is the eosinophil [12] and it was this cell that was found to secrete galectin-14 [13, 14]. Galectin-14 is thought to be the ortholog of human galectin-10, which is also secreted by eosinophils [15, 16]. Analysis of *H. contortus* infected sheep revealed release of galectin-14 into the gastrointestinal mucus, the interface of host and parasite interaction [14]. In addition, kinetic studies of galectin-14 showed that release into the mucus occurred soon after challenge infection, and correlated with a reduction in parasitic burden [8].

The above work suggests that galectin-14 plays a role in resistance to *H. contortus*, but its role in infection in *F. hepatica* may tell a different story. Eosinophilia is also a hallmark of *F. hepatica* infections, and recently the secretion of galectin-14 from eosinophils has been found to occur in the bile duct during chronic liver fluke infection [11]. Although endogenous galectin-14 was not found attached to the surface of *F. hepatica*, recombinant galectin-14 was

shown to bind to the fluke surface in frozen tissue sections. This is most likely due to an evasion strategy of helminths which involves shedding of adhered host antibodies [17–19]. This process can be a hindrance when trying to stain live parasites but can be counteracted by snap-freezing the parasites prior to antibody incubations. As resistance to chronic *F. hepatica* infections in sheep is not believed to exist [20] it is likely that these parasites have "high-jacked" eosinophilia and galectin-14 secretion for their own benefit, unlike the probable protective role of eosinophils in *H. contortus* infections.

Galectin-11 was discovered to be an inducible galectin, specifically expressed and secreted during *H. contortus* infections in sheep [21]. Immunohistochemistry revealed that galectin-11 was secreted by epithelial cells lining the gastrointestinal tract, where it was localized to the nucleus and cytoplasm of cells (Fig. 1). Analysis of the mucosal contents lining the gastrointestinal tract also revealed secretion of galectin-11 into the mucus. An observation of increased mucus adhesiveness, corresponding with the production of galectin-11, suggested this may be a mechanism to

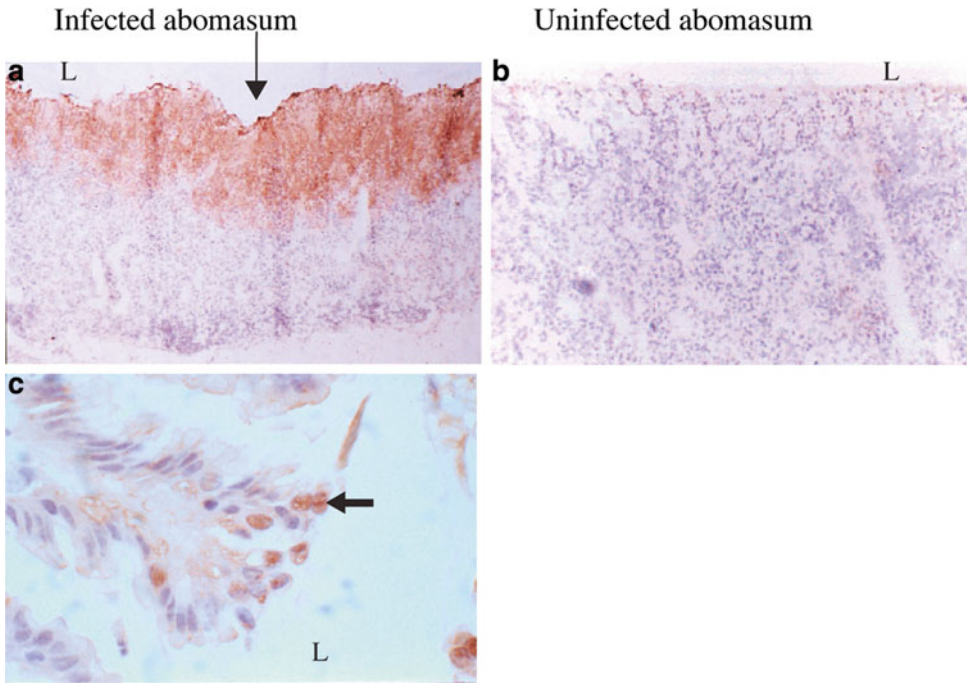

Fig. 1 Immunohistochemistry of OCT embedded tissue using rabbit polyclonal anti-galectin-11 antibody and indirect immunoperoxidase staining. *H. contortus* infected abomasum collected 5 days post-challenge (**a**, **c**) shows strong galectin-11 staining in the upper epithelial layer. In (**a**) the *thin arrow* points to the position of an L3 larva. In (**c**) a *thick arrow* points to nuclear staining. In contrast, parasite-free (primed but not challenged) abomasum lacks galectin-11 staining (**b**). "L" indicates the abomasal lumen. Magnification: (**a**) and (**b**) 100×; (**c**) 200×

impede *H. contortus* motility [8]. Recent immunofluorescent staining techniques using a recombinant form of galectin-11 have revealed binding to the fourth larval stage and adult *H. contortus* (Preston et al., in preparation). These studies suggest a more direct or additional role for galectin-11 during *H. contortus* infections. Since its discovery, galectin-11 has been found to be upregulated in response to other gastrointestinal parasitic infections [22–24]. Interestingly, using the same antibodies produced in our laboratory, Thomas Spencer's laboratory showed that galectin-11 was also expressed in ovine endometrial uterine tissue during pregnancy [25]. In this study the protein was renamed galectin-15, and subsequent characterization studies have shown the importance of galectin-11/15 in ovine reproduction [26].

In our own work, galectin-14 and galectin-11 were initially produced as recombinant GST-fusion proteins (rGST–galectin-14 and -11). Using a red blood cell agglutination assay, rGST–galectin-14 was shown to be active but rGST–galectin-11 was not. Cleaving the GST from rGST–galectin-14 resulted in protein precipitation and the full fusion protein was therefore used in all functional studies. However, to produce monoclonal and polyclonal antibodies the GST tags were removed from both these recombinant galectins. Prolonged freezing of rGST–galectin-14 also results in the formation of large aggregates, similar to its human galectin-10 orthologue. Recently, galectin-11 has been produced as a soluble active recombinant containing an N-terminal His-tag (rgalectin-11). Activity of rgalectin-11 was confirmed by binding to an immobilized galactose column and subsequently eluted with galactose. This rgalectin-11 was used in the *H. contortus* binding studies and larval in vitro assays.

The following chapter describes in detail the methods that our laboratory has developed and performed to understand the role of galectin-11 and galectin-14 in *H. contortus* and *F. hepatica* infections.

2 Materials

2.1 Cultivation of the Larval Stages of H. contortus

2.1.1 Hatching H. contortus Eggs from Infected Sheep to the Third Larval Stage (L3)

1. 3–4 Merino wethers aged between 3 months and 1 year old (*see* **Note 1**).
2. 20,000 *H. contortus* L3 larvae per sheep for inoculation.
3. Gelatin capsules.
4. Plastic dosing gun (Torpac, USA).
5. 0.9 % (w/v) sterile sodium chloride (saline).
6. Fecal collection bags (*see* **Note 2**).
7. Plastic trays.
8. Clear plastic sheets (long enough to cover plastic trays).

9. A room with morning sunlight.
10. Spray bottle containing tap water.
11. Large glass jar.
12. 75 cm^2 tissue culture flask.
13. Light microscope.
14. Centrifuge.

2.1.2 Fecal Egg Count for Determining H. contortus Infection

1. 5 g of feces from each sheep to be tested (collected fresh from rectum).
2. Electronic balance.
3. Saturated salt solution (0.32 kg/l).
4. Whitlock McMaster counting chamber.
5. Plastic pipettes.
6. Glass jar.
7. Handheld blender.
8. 2 mm sieve.
9. Metal teaspoon.
10. Light microscope.

2.1.3 Storage and Monitoring of H. contortus L3 Larvae

1. Cool room or fridge at 10 °C.
2. Centrifuge.
3. Tap water.
4. 75 cm^2 tissue culture flasks.

2.1.4 Exsheathment of H. contortus Third Larval Stage (L3) Using Sodium Hypochlorite

1. *H. contortus* L3 larvae.
2. Pyrogen free saline (PFS).
3. 0.15 % (v/v) sodium hypochlorite in PFS.
4. Centrifuge.
5. 50 ml tubes.
6. Water bath set at 37 °C.
7. 0.9 % (w/v) sterile sodium chloride (saline).
8. Penicillin–streptomycin solution (Invitrogen; 5,000 units penicillin and 5,000 μg streptomycin/ml).

2.1.5 Exsheathment of H. contortus L3 Using CO_2

1. *H. contortus* L3 larvae (1,000/ml).
2. Phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
3. 100 ml 0.09 % (w/v) glucose.
4. 250 ml glass bottle which can be sealed tightly with a lid.
5. Gas cylinder of 100 % CO_2.
6. Orbital incubator set to 40 °C.

7. Centrifuge.
8. Light microscope.

2.1.6 Culturing of H. contortus L3 to the Fourth Larval Stage (L4)

1. L3 *H. contortus* exsheathed by sodium hypochlorite (*see* Subheading 3.1.4).
2. Incubator set at 40 °C, 10 % CO_2.
3. Centrifuge.
4. 0.9 % (w/v) sterile sodium chloride (saline).
5. Tissue culture medium {Dulbecco's modified Eagle Medium + GlutaMAX (DMEM) supplemented with 1 % penicillin–streptomycin and 1 % Fungisome}.
6. 75 cm^2 tissue culture flask.
7. Light microscope.

2.1.7 Collection of Adult H. contortus

1. *H. contortus* infected sheep (at stage where parasitic eggs are being detected in the feces) (*see* Subheading 3.2 for infection protocols).
2. Sodium pentobarbital.
3. Dissection equipment (including blunt nose tweezers).
4. Phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) warmed to 40 °C.
5. Plastic tray.
6. 1 L Beaker.
7. Incubator set to 40 °C.

2.1.8 Infection of Sheep with Liver Fluke (F. hepatica)

1. *F. hepatica* metacercariae (*see* **Note 3**).
2. Filter paper.
3. Gelatin capsules.
4. Parasite free adult sheep.
5. Plastic dosing gun (Torpac, USA).
6. Sodium pentobarbital.
7. Dissection equipment and tweezers.
8. Tissue homogenizer.
9. Phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
10. 2 × 2 mm sieves.
11. 250 μm sieve.
12. Tweezers.
13. Optimal cutting temperature (OCT) compound.
14. Liquid nitrogen and aluminum foil boats.

2.2 Preparation of Parasite Infected Gastrointestinal Tissues (H. contortus Infected Abomasum) from Primary and Primed Infections

1. Parasite-free sheep.
2. *Haemonchus contortus* L3 larvae gelatine capsules (prepared in Subheading 3.1.1, **steps 1–3**).
3. Plastic dosing gun (Torpac, USA).
4. Antihelminthic, levamisole.
5. Commercial feed pellets (such as lucerne pellets).
6. Sodium pentobarbital.
7. Dissection equipment.

2.3 Detection of Endogenous Galectins in Tissues (Immuno-histochemistry in OCT Embedded Frozen Tissues)

1. Infected and control tissues (prepared in Subheading 3.2).
2. Optimal cutting temperature (OCT) compound.
3. Containers to hold OCT embedded tissue (we use small plastic weigh boats).
4. Liquid nitrogen.
5. Aluminum foil.
6. Cryostat.
7. Microscope slides.
8. 95 % (v/v) ethanol.
9. Peroxidase blocking reagent (DAKO).
10. Phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
11. Equipment to create a humidity chamber (water-saturated material such as wet tissues in the bottom of an enclosed container helps to prevent sections from drying out).
12. Primary anti-galectin antibody (we have produced our own rabbit polyclonal anti-galectin-11 and mouse monoclonal anti-galectin-14 antibodies).
13. Horseradish peroxidase-conjugated secondary antibody (e.g., horseradish peroxidase-conjugated anti-rabbit IgG).
14. 1.5 mM 3,3′-diaminobenzidine tetrahydrochloride (DAB).
15. Hematoxylin or Hematoxylin and Eosin Y (H and E).
16. Light microscope.

2.4 Recombinant Galectin Binding to Frozen Tissue Sections

2.4.1 Fluorescent Labelling of Galectin Probes, Assessment of Activity by Hemagglutination Assay

1. 5-iodoacetamidofluorescein (Molecular Probes, Invitrogen).
2. Recombinant GST–galectin-14 (rGST–galectin-14) and recombinant GST (rGST).
3. Rabbit erythrocytes–rabbit blood collected 1:1 with Alsevers.
4. 50 ml sterile tubes.
5. Trypsin.
6. Phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).

7. 0.15 M sodium chloride (NaCl) in phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
8. 1 % glutaraldehyde in phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
9. 0.1 M glycine in phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
10. V-well microtiter plates.
11. DES (0.15 M NaCl, 2 mM EDTA, 2 mM DTT made fresh daily).
12. 0.5 % (w/v) bovine serum albumin (BSA) in 0.15 M NaCl.

2.4.2 Binding of Fluorescent Galectin to Frozen Tissue Sections

1. Blocking solution: 3 % BSA (w/v) in phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
2. Fluorescence microscope.
3. 100 mM lactose.
4. Fluorescein-labelled recombinant GST–galectin-14 (rGST–galectin-14) and recombinant GST (rGST) (*see* Subheading 3.4.1).
5. Phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).

2.4.3 Binding of Nonfluorescent Galectin to Frozen Tissue Sections

1. rGST–galectin-14, inactive rGST–galectin-11, and rGST.
2. Anti-GST horseradish peroxidase (HRP)-conjugated secondary antibody.
3. 1.5 mM 3,3′-diaminobenzidine tetrahydrochloride (DAB).
4. Hematoxylin or Hematoxylin and Eosin Y (H and E).
5. Humidity chamber (*see* Subheading 2.3, **item 11**).
6. Phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
7. Light microscope.

2.5 Galectin Binding to Parasites (*H. contortus* and *F. hepatica*)

2.5.1 Fluorescent Staining of *H. contortus* L3, L4, and Adult Stage Parasites

1. L3 (CO_2 exsheathed), L4, and adult stage *H. contortus* parasites.
2. Liquid nitrogen.
3. Phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
4. Eppendorf tubes (1.5 ml).
5. Eppendorf centrifuge.
6. Water bath set at 37 °C.
7. 100 mM D-galactose.
8. Recombinant galectin-11 (5 μg/ml-100 μg/ml).

9. Rabbit anti-ovine galectin-11 polyclonal antibody (1:1,000).
10. Goat anti-rabbit conjugated to Alexa Fluor 647 (1:1,000).
11. Aluminum foil.
12. Microscopic glass slide and coverslips.
13. Fluorescent microscope.

2.5.2 Binding of Nonfluorescent Galectin Probes to F. hepatica

1. Liver fluke *F. hepatica* collected from infected sheep livers and bile ducts (from the local abattoir, or collected from deliberately infected sheep, as described in Subheading 3.1.8).
2. Optimal cutting temperature compound (OCT).
3. Cryostat.
4. 95 % (v/v) ethanol.
5. 1.5 % (v/v) hydrogen peroxide diluted in phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
6. 3 % (w/v) Bovine serum albumin (BSA) in phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
7. Galectin probe (rGST–galectin-14) and control probes (inactive rGST–galectin-11, and rGST).
8. Anti-GST HRP-conjugated antibody.
9. DAB (3,3′-diaminobenzidine tetrahydrochloride).
10. Hematoxylin.

2.6 Larvae Molting Assay Using H. contortus L3

1. Tissue culture media {Dulbecco's modified Eagle Medium + GlutaMAX (DMEM) supplemented with 1 % penicillin–streptomycin and 1 % Fungisome}.
2. Sodium hypochlorite exsheathed L3 *H. contortus* parasites (*see* Subheading 3.1.4, **steps 1–5**).
3. 24-well plates.
4. Recombinant galectin-11 (5–100 μ/ml).
5. Tissue culture incubator set to 40 °C and 10 % CO_2 flow.
6. 1.5 ml eppendorf tubes.
7. Iodine.
8. Eppendorf centrifuge.
9. Microscopic glass slide and coverslips.
10. Light microscope with camera.

2.7 Larvae Growth Assay Using H. contortus L4

1. Tissue culture media {Dulbecco's modified Eagle Medium + GlutaMAX (DMEM) supplemented with 1 % penicillin–streptomycin and 1 % Fungisome}.
2. L4 *H. contortus* parasites (*see* Subheading 3.1.6).

3. 24-well plates.
4. Tissue culture incubator set to 40 °C and 10 % CO_2 flow.
5. 1.5 ml eppendorf tubes.
6. Eppendorf centrifuge.
7. Iodine.
8. Recombinant galectin-11 (5–100 μg/ml).
9. Microscopic glass slide and coverslips.
10. Light microscope with camera.
11. Imaging software such as Image J.

2.8 Larvae Feeding Assay Using H. contortus L4

1. Tissue culture media {Dulbecco's modified Eagle Medium + GlutaMAX (DMEM) supplemented with 1 % penicillin–streptomycin and 1 % Fungisome}.
2. L4 *H. contortus* parasites (*see* Subheading 3.1.6).
3. 24-well plates.
4. Recombinant galectin-11 (5–100 μg/ml).
5. 1.5 ml eppendorf tubes.
6. Eppendorf centrifuge.
7. Tissue culture incubator set to 40 °C and 10 % CO_2 flow.
8. Protein labelled to fluorescence [we used chicken ovalbumin–fluorescein isothiocyanate (OVA-FITC) concentrated at 2 mg/ml].
9. Phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
10. Microscopic glass slide and coverslips.
11. Fluorescent microscope.

3 Methods

3.1 Cultivation of the Larval Stages of H. contortus

3.1.1 Hatching H. contortus Eggs from Infected Sheep to the Third Larval Stage (L3)

1. Wash *H. contortus* L3 larvae three times in sterile saline by spinning at 350 × *g*.
2. Resuspend L3 in sterile saline at concentration of 10,000/ml (*see* **Note 4**).
3. Transfer 1 ml into each gelatin capsule (You will need to prepare two capsules per sheep—*see* **Note 5**).
4. Infect sheep with two capsules each, using a dosing gun (*see* **Note 6**).
5. After 4 weeks check if *H. contortus* infection has established by performing fecal egg counts (FEC) (*see* Subheading 3.1.2).
6. Perform FEC weekly until infection level is approximately 1,000 eggs per gram. Once infection level is 1,000 eggs per gram, collect feces.

7. To collect feces, attach fecal collection bags to the animals. Feces should be collected twice daily.
8. Place collected feces into plastic trays, moisten with tap water using a spray bottle and cover with clear plastic sheets. Place in a position where morning sunlight will be received.
9. After 10 days, check for L3 (*see* **Note 7**). To collect L3, rinse plastic sheet with tap water into large glass jar. Transfer contents into a 75 cm^2 tissue culture flasks (100 ml/flask) and store at 10 °C. L3 can be visualized under a light microscope. Replace system with fresh feces when L3 collection begins to decline or feces become overgrown with fungi.

3.1.2 Fecal egg Count for Determining H. contortus Infection

1. Collect approximately 5 g of fresh feces from rectum of the animal.
2. Weigh out 5 g of feces.
3. Place 5 g of feces in large glass jar with 50 ml of saturated salt solution and create a homogenous mixture using handheld blender.
4. Strain mixture through sieve to remove particulates. Push down on particulates in sieve with teaspoon to collect remaining liquid.
5. Using a plastic pipette transfer 5 ml of the solution into a Whitlock McMaster counting chamber and count the number of eggs in each chamber under a light microscope at 20× magnification.
6. To calculate the number of eggs per gram (EPG) use the following formula:

$$Eggs\ per\ gram\ (EPG) = (egg\ number \times solution\ volume\ (ml))/(feces\ (g) \times volume\ in\ counting\ chamber) = (egg\ \# \times 50\ ml)/(5\ g \times 0.5\ ml).$$

3.1.3 Storage and Monitoring of H. contortus L3

1. Stored L3 will need to be washed every month to prevent fungal/bacteria buildup and to remove dead parasite larvae.
2. Wash L3 by centrifuging at 350 × *g* (with no break) and resuspend in tap water with total volume of approximately 100 ml/flask.

3.1.4 Exsheathment of H. contortus Third Larval Stage (L3) Using Sodium Hypochlorite

1. Transfer ~10,000 *H. contortus* L3 larvae to a 50 ml tube containing 10 ml of 0.15 % sodium hypochlorite in PFS.
2. Incubate at 37 °C for 15 min with occasional agitation.
3. Add more PFS up to a total volume of 50 ml.
4. Centrifuge at 500 × *g* for 5 min (with no brake).
5. Wash larvae three times in PFS.

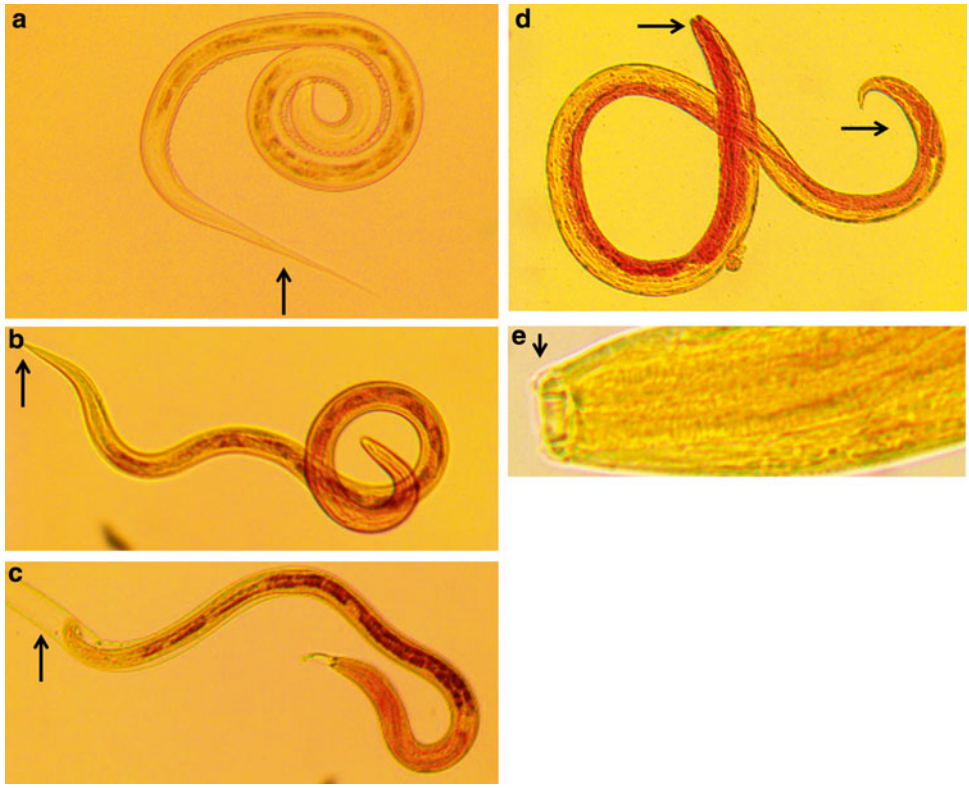

Fig. 2 *Haemonchus contortus* larvae illustrating the morphological features distinguishing third stage larvae (L3), exsheathed L3 (xL3), and fourth stage larvae (L4). (**a**) Sheathed L3 collected from fecal culture. *Arrow* indicates sharp tail used to distinguish between L3 and xL3. (**b**) xL3, *arrow* indicates a rounder tail compared to L3 which is a morphological feature used to estimate percentage of exsheathed larvae post sodium hypochlorite or carbon dioxide treatment. (**c**) xL3 undergoing exsheathment to L4, *arrow* indicates molting sheath. (**d**) L4 *H. contortus*, *arrows* indicate characteristic mouth and tail morphological development used to distinguish between xL3 and L4. (**e**) *Arrow* indicates L4 mouth piece. Magnification: (**a**)–(**d**) 20×; (**e**) 100×

6. Count the percentage of exsheathed larvae (*see* **Note 8** and Fig. 2).
7. Resuspend ~1,000 larvae/ml in saline containing 0.5 % penicillin–streptomycin solution.

3.1.5 Exsheathment of H. contortus L3 Using CO_2

1. Wash L3 *H. contortus* three times in PBS by centrifuging at 350 × *g* (with no break) for 5 min.
2. Resuspend in 50 ml PBS.
3. Transfer into 250 ml glass bottle containing 100 ml of 0.09 % glucose warmed to 40 °C.
4. Bubble solution with CO_2 gas for 20 s, immediately seal with lid.
5. Place in orbital incubator heated to 40 °C at a speed of 100 rpm for 15 min.

6. Re-bubble L3 solution with CO_2 for 2 min and place back into incubator for 90 min or leave overnight.
7. Examine exsheathment under a light microscope (*see* **Note 8** and Fig. 2). A substantial exsheathment rate is 75 %.

3.1.6 Culturing H. contortus L3 to the Fourth Larval Stage (L4)

1. Exsheath L3 with sodium hypochlorite (*see* Subheading 3.1.4).
2. At **step 5**, wash exsheathed L3 (xL3) five times with saline by spinning at 350×*g* (with no break) and resuspend at 1,000 xL3/ml into tissue culture medium.
3. Transfer medium containing xL3 into a 75 cm^2 tissue culture flask and place in tissue culture incubator set to 40 °C, 10 % CO_2 flow.
4. Incubate for 5 days or until molting to L4 greater than 80 %.
5. L4 can be distinguished under a light microscope by the development of mouth parts and distinct markings on the tail (*see* Fig. 2).

3.1.7 Collection of Adult H. contortus

1. Sacrifice infected sheep with an overdose of sodium pentobarbital.
2. Open up stomach and remove abomasum.
3. Open abomasum by cutting along the greater curvature.
4. Place abomasum in plastic tray filled with warm PBS and rinse.
5. Use tweezers to collect dislodged adult worms from solution and transfer into a beaker containing a warm PBS solution.
6. Use tweezers to remove adult worms still lodged inside the tissue, check under abomasal fold.
7. Keep at 40 °C if performing staining straight away or preserve for later use by snap freezing (*see* **Note 9**).

3.1.8 Infection of Sheep with Liver Fluke (F. hepatica)

1. Obtain *F. hepatica* metacercariae (*see* **Note 3**).
2. Using a pipette, load the metacercariae onto filter paper.
3. Insert loaded filter paper into gelatine capsules.
4. Infect sheep with 250 metacercariae of *F. hepatica* by orally administering the metacercariae gelatin capsules with a dosing gun.
5. 3 months post-infection with *F. hepatica* metacercariae, sacrifice sheep by lethal overdose with sodium pentobarbital to obtain infected liver and bile duct (*see* **Note 10**).
6. Collect juvenile *F. hepatica* parasites from the infected liver by slicing tissue, homogenizing the tissue slices in 4 °C PBS, and then washing this homogenized tissue consecutively through two sieves of 2 mm and 250 μm mesh. Liver flukes (between 2 and 5 mm long) will be caught by the 250 μm screen.

Adult liver flukes in the bile duct can be visualized by eye and removed with tweezers. Isolated liver fluke and infected tissue samples can be embedded in OCT, frozen on the surface of liquid nitrogen, and stored frozen for subsequent immunohistology or galectin binding assays (Subheadings 3.3 and 3.4) (*see* **Note 11**).

3.2 Preparation of Parasite Infected Gastrointestinal Tissues (H. contortus Infected Abomasum)

3.2.1 Preparation of Parasite Infected Abomasal Tissue in Primed Sheep

1. Prime nematode-free adult merino sheep by oral infection with 5,000 *H. contortus* L3 larvae once a week for approximately 12 weeks (*see* **Notes 12** and **13**).
2. Following the manufacturer's instructions, drench the sheep with levamisole (10 ml/sheep).
3. Maintain the sheep nematode free for approximately 12 weeks (by keeping in pens and feeding with commercial pellets).
4. Challenge by oral administration of 5×10^4 *H. contortus* L3 larvae (*see* **Note 13**).
5. At different times after challenge (depending on stage of infection to be examined) sacrifice the sheep via an overdose of sodium pentobarbital and collect tissue samples. Open the abomasum along the greater curvature and gently wash out the stomach contents with slow-running tap water, before collecting tissue from the fundic folds (*see* **Notes 14** and **15**).

3.2.2 Preparation of Abomasal Tissue from Sheep with a Primary Infection

1. Orally infect 3-month old worm-free sheep once with $1–5 \times 10^4$ *H. contortus* L3 larvae (Using young sheep, particularly those raised in an animal house, makes previous exposure to the parasite unlikely. Control sheep are uninfected).
2. Sacrifice the sheep at different time post-infection and collect tissue samples as in Subheading 3.2.1, **step 5**.

3.3 Detection of Endogenous Galectins in Tissues (Immunohistochemistry in OCT Embedded Frozen Tissues)

1. Cut tissue into small pieces and embed in OCT (Tissue pieces can vary in size from approximately 7 mm^3–1 cm^3 as long as they are fully covered by the OCT medium).
2. Freeze embedded tissues by floating samples on the surface of liquid nitrogen in foil boats.
3. Store frozen tissue blocks at –80 °C.
4. Cut 5–10-μm sections of the OCT-embedded frozen tissue blocks using a cryostat and transfer sections onto microscope slides.
5. Allow sections to dry at room temperature.
6. Fix sections in 95 % ethanol for 10 min at 4 °C.
7. Allow fixed sections to dry, wrap slides in tissue and foil, and store at –80 °C.
8. Prior to use, bring slides to room temperature.
9. Quench endogenous peroxidase by submerging slides in peroxidase blocking reagent for 10 min.

10. Wash slides by immersing in PBS.
11. Incubate slides with primary anti-galectin antibody in PBS for 1–2 h at room temperature in a humidified chamber (It is important from this stage onwards to not allow the slides to dry).
12. Wash slides in PBS.
13. Incubate slides with horseradish peroxidase-conjugated secondary antibody in PBS for 1 h at room temperature in a humidified chamber (*see* **Note 16**).
14. Wash slides in PBS.
15. Incubate slides in 1.5 mM DAB for 10 min at room temperature.
16. Counterstain slides with Hematoxylin or Hematoxylin and Eosin Y (H and E) as directed by the manufacturer and view under a light microscope (*see* Fig. 1).

3.4 Recombinant Galectin Binding to Frozen Tissue Sections

3.4.1 Fluorescent Labelling of Galectin Probes and Assessment of Activity by Hemagglutination Assay

1. Following the manufacturer's instructions, label the galectin and control probes with the thiol reactive probe 5-iodoacetamidofluorescein. (We used rGST–galectin-14, and rGST alone as the control probe.)
2. Check that labelling has not interfered with the activity of the recombinant galectin by performing a hemagglutination assay post-labelling. This assay requires trypsin-treated, glutaraldehyde-fixed rabbit erythrocytes that can be prepared as follows in **steps 3–8**.
3. Wash erythrocytes four times with 5 vol (*see* **Note 17**) of 0.15 M NaCl and resuspend as a 4 % suspension (v/v) in PBS (usually produces 2–2.5 ml of erythrocytes).
4. Add 1 mg/ml trypsin and incubate at 37 °C with very mild shaking for 1 h.
5. Wash four times with 5 vol of 0.15 M NaCl.
6. Fix in 5 vol of 1 % glutaraldehyde in PBS for 1 h at room temperature.
7. Terminate fixation with 5 vol of 0.1 M glycine in PBS at 4 °C.
8. Wash the erythrocytes twice with 0.1 M glycine in PBS and twice in PBS. The fixed erythrocytes can be stored as a 10 % suspension in PBS for ≤1 month.
9. For the hemagglutination assay use V-well microtiter plates with serial twofold dilutions of samples in DES. To each well, in order, add

   25 μl of sample diluted in DES.

   50 μl of 0.5 % BSA in 0.15 M NaCl.

   25 μl of 4 % erythrocytes in PBS.

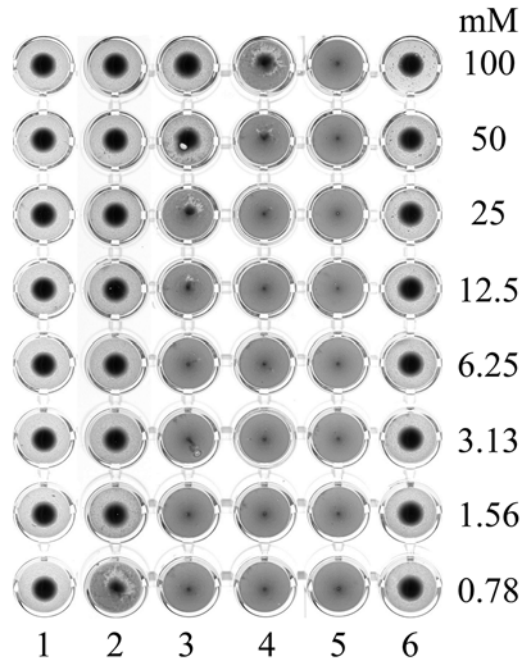

Fig. 3 Hemagglutination activity using rGST–galectin-14 fusion protein. Agglutination of rabbit erythrocytes was assayed in a 96-well microtiter plate. Assays were conducted in the presence (*2*, *3*, *4*, and *5*) or absence (*1* and *6*) of 0.28 μM rGST–galectin-14, the minimum concentration to induce agglutination. Increasing concentrations (0.78–100 mM) of lactose (*2*), galactose (*3*), or *N*-acetyl-glucosamine (*4*) were added to inhibit agglutination. In the presence of rGST–galectin-14 without sugar (*5*) the rabbit erythrocytes form a "mat" at the bottom of the wells. The assay buffer DES alone (*1*) and 3 μM rGST (*6*) were included as negative controls

Then shake the plate vigorously for 30 s. Read agglutination after 90 min.

(a) Cells as a dot = no agglutination.

(b) Cells as a mat = agglutination (*see* Fig. 3).

3.4.2 Binding of Fluorescent Galectin to Frozen Tissues Sections

1. Prepare OCT embedded tissue sections as in Subheading 3.3 (**steps 1–8**).
2. Block frozen tissue sections in 3 % BSA PBS for 30 min.
3. Incubate sections with fluorescein-labelled recombinant galectin (e.g., rGST–galectin-14) or control (e.g., rGST) diluted to 10 μg/ml in blocking solution for 30 min at room temperature.

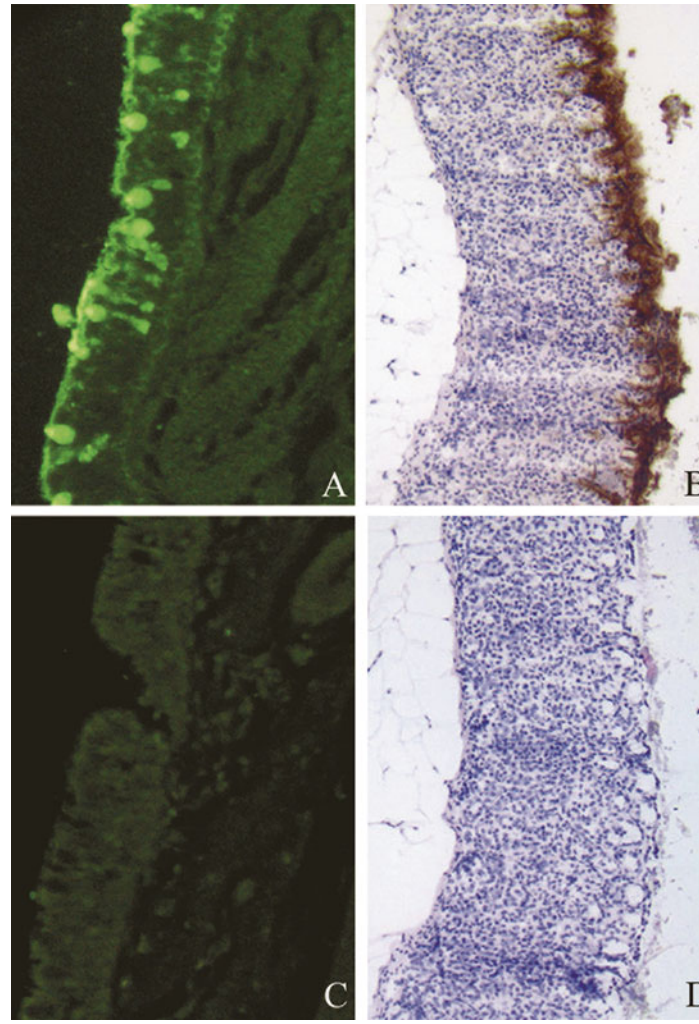

Fig. 4 Binding of galectin-14 on lung and intestinal tissue sections. Frozen tissue sections from healthy sheep were incubated with recombinant proteins; lung tissue with rGST–galectin-14–fluorescein (**a**) or rGST–fluorescein (**c**) and intestinal tract tissue with rGST–galectin-14 (**b**) or inactive rGST–galectin-11 (**d**). Protein binding was monitored directly by fluorescence microscopy (**a**, **c**) or with an anti-GST HRP-conjugated antibody (**b**, **d**). The tissues (**b**, **d**) were counterstained with hematoxylin. Magnification: (**a**) and (**c**), 200×; (**b**) and (**d**), 100×. Originally published in the Glycoconjugate Journal, volume 26, 2009, page 430, Functional characterization of an eosinophil-specific galectin, ovine galectin-14, Anna R. Young, Garry J. Barcham, Joanna M. Kemp, Jillian L. Dunphy, Andrew Nash, Els N. Meeusen, figure 5, original copyright notice 2008. Reprinted here with kind permission from Springer Science and Business Media

4. Wash in PBS three times for 5 min, and examine under an epifluorescence microscope (*see* Fig. 4).
5. To check that the galectin is binding to parasite glycans, include 100 mM lactose in the blocking solution in **step 3**; if glycan-specific this should inhibit galectin binding.

3.4.3 Binding of Nonfluorescent Galectins to Frozen Tissues Sections

1. Prepare tissue section slides as for immunohistochemistry (Subheading 3.3, **steps 1–10**).
2. Incubate the slides with recombinant GST–galectin (e.g., rGST–galectin-14) or control probes (e.g., inactive recombinant GST–galectin-11 or rGST alone) at 10 μg/ml in a humidity chamber for 1–2 h at room temperature.
3. Wash the slides in PBS.
4. Incubate the slides with anti-GST HRP-conjugated antibody in PBS for 1 h at room temperature in a humidified chamber.
5. Wash the slides in PBS.
6. Incubate the slides in 1.5 mM DAB for 10 min at room temperature.
7. Counterstain the slides with hematoxylin or hematoxylin and Eosin Y (H and E) as directed by the manufacturer and view under a light microscope (*see* Fig. 4).

3.5 Galectin Binding to Parasites (H. contortus and F. hepatica)

3.5.1 Fluorescent Staining of H. contortus L3, L4, and Adult Stage Parasites

1. Aliquot 1 ml of parasites at concentration of 1,000/ml in PBS into 1.5 ml eppendorf tubes. For adult *H. contortus*, 3–4 worms/ml.
2. Wash the parasites three times in PBS by spinning at 500 × *g* for 30 s. /The parasites will pellet at the bottom. Replace supernatant with 1 ml PBS.
3. To prevent antibody shedding, snap-freeze the parasites by placing in liquid nitrogen for 1 min.
4. Thaw in water bath at 37 °C and wash once in PBS by spinning at 500 × *g* for 30 s.
5. Incubate the parasites with 200 μl of recombinant galectin-11 for 30 min–1 h at room temperature (concentration of recombinant galectin-11 used ranges from 5 to 100 μg/ml). For a negative control, pre-incubate recombinant galectin-11 with 100 mM of D-galactose.
6. Wash the parasites to remove any unbound protein as outlined in **step 2**.
7. Add 200 μl of secondary antibody, rabbit anti-ovine galectin-11 at concentration of 1:1,000. Incubate for 30 min–1 h at room temperature.
8. Wash (as outlined in **step 2**) and incubate with detection antibody, goat anti-rabbit Alexa Fluor 647 (1:1,000) for 30 min–1 h at room temperature in the dark (wrap in foil).
9. Wash (outlined in **step 2**) and resuspend in 100 μl of PBS. Place 50 μl onto glass slide and coverslip.
10. Examine fluorescent binding under microscope using a water lens to enhance focus.

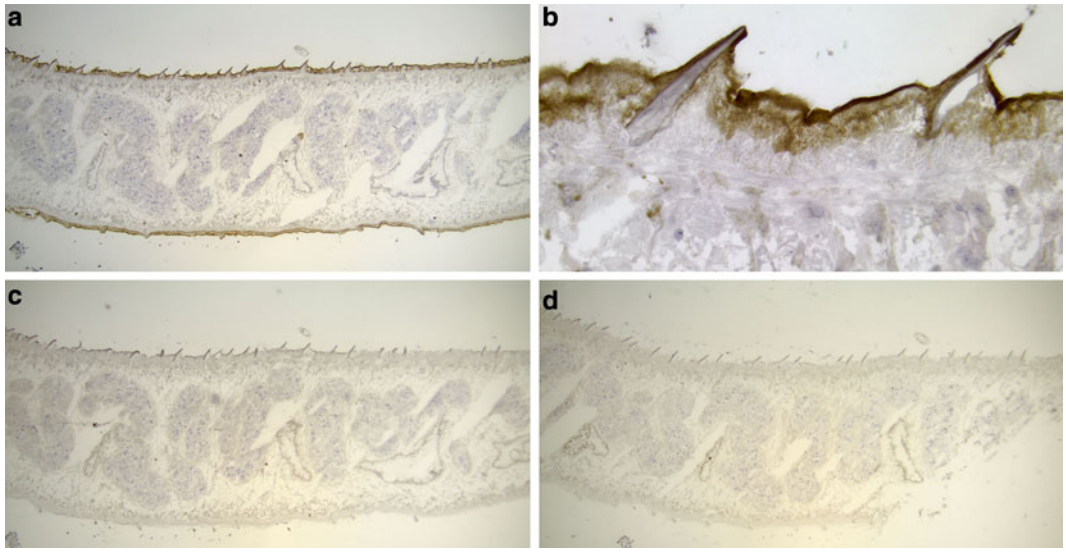

Fig. 5 Galectin-binding to the parasite surface. Longitudinal sections of adult *F. hepatica* were incubated with rGST–Galectin-14 (**a**, **b**), rGST–Galectin-14 + 100 mM lactose (**c**) or inactive rGST–Galectin-11 (**d**) and protein binding detected with an anti-GST HRP-conjugated antibody. The flukes were counterstained with hematoxylin. Magnification: (**a**), (**c**) and (**d**) 40×; (**b**) 400×. Reprinted from Veterinary Immunology and Immunopathology, Vol 145, Anna R. Young, Garry J. Barcham, Hamish E. McWilliam, David M. Piedrafita, Els N. Meeusen, Galectin secretion and binding to adult *Fasciola hepatica* during chronic liver fluke infection of sheep, Pages No. 362–367, Copyright (2011), with permission from Elsevier

3.5.2 Binding of Nonfluorescent Galectin Probes to F. hepatica

1. Embed freshly collected liver flukes in OCT and store frozen until use (Subheading 3.1.8, **step 6**).
2. Cut 5–10 μm sections, dry and fix in 95 % ethanol for 10 min at room temperature.
3. Reduce endogenous peroxidase by submerging slides in 1.5 % hydrogen peroxide/PBS for 10 min.
4. Block sections with 3 % BSA PBS for 30 min.
5. Incubate for 1 h with 10 μg/ml rGST–Galectin-14, inactive rGST–Galectin-11, or rGST.
6. Detect recombinant protein binding by incubation with anti-GST HRP-conjugated antibody and visualize with DAB as described in Subheading 3.4.3 **steps 3**–**7** (*see* Fig. 5).

3.6 Larvae Molting Assay Using H. contortus L3

1. Exsheath *H. contortus* L3 with sodium hypochlorite (Subheading 3.1.4, **steps 1**–**5**).
2. Resuspend xL3 at concentration of 1,000/ml in tissue culture media and transfer 1 ml into a 24-well plate.
3. Add 1 ml of rgalectin-11 at concentrations between 5 and 100 μg/ml and 1 ml of appropriate controls.
4. Incubate at 40 °C and 10 % CO_2 for 5 days.

5. Monitor molting rate to L4 parasites by imaging samples on day 3 and 5. Remove 500 μl of media containing the parasites into 1.5 ml eppendorfs and spin down at 1,000 × *g* for 1 min to resuspend parasites into 50 μl of Iodine.
6. Aliquot 10 μl onto microscopic slide and cover with coverslip.
7. Image parasites at 20× magnification, 20–30 parasites per treatment.
8. Molting into L4 can be distinctly identified by the morphological development of mouth parts (*see* Fig. 2).

3.7 Larvae Growth Assay Using *H. contortus* L4

1. Culture *H. contortus* L3 to L4 parasites by following culture protocol (Subheading 3.1.6). Once L4 is greater than 80 % begin growth assay.
2. In 24-well plate, add 1 ml of L4 parasites at concentration of 1,000/ml in tissue culture medium.
3. Add 1 ml of rgalectin-11 at concentrations between 5 and 100 μg/ml or appropriate controls (e.g., rGST).
4. Incubate parasites at 40 °C, 10 % CO_2 for 5 days.
5. On day 3 and 5, remove 500 μl of media containing the parasites into 1.5 ml eppendorfs and spin down at 1,000 × *g* for 1 min to resuspend parasites into 50 μl of Iodine.
6. Aliquot 10 μl onto microscopic glass slide and cover with coverslip.
7. Image parasites at 20× magnification, 20–30 parasites per treatment.
8. Use software such as Image J to measure the area of the worms as an indicator of growth.

3.8 Larvae Feeding Assay Using *H. contortus* L4

1. Culture *H. contortus* L3 to L4 parasites by following culture protocol (Subheading 3.1.6). Once L4 is greater than 80 % begin feeding assay.
2. In 24-well plate, add 1 ml of *H. contortus* L4 larvae at concentration of 1,000/ml in tissue culture medium.
3. Add 1 ml rgalectin-11 at concentrations between 5 and 100 μg/ml, or appropriate controls (e.g., rGST).
4. Incubate parasites at 40 °C, CO_2 10 % for 24 h.
5. Add 50 μl of fluorescent protein such as OVA-FITC (2 mg/ml) for 2 h. (This will be ingested if parasites are feeding.)
6. Transfer parasites into eppendorf tubes and wash three times with PBS by spinning suspension down at 100 × *g* for 1 min. Resuspend in 200 μl of PBS.
7. Aliquot 10 μl onto microscopic slide and cover with coverslip.
8. Image on fluorescent microscope and count number of worms with fluorescent esophagus/intestines (*see* Fig. 6).

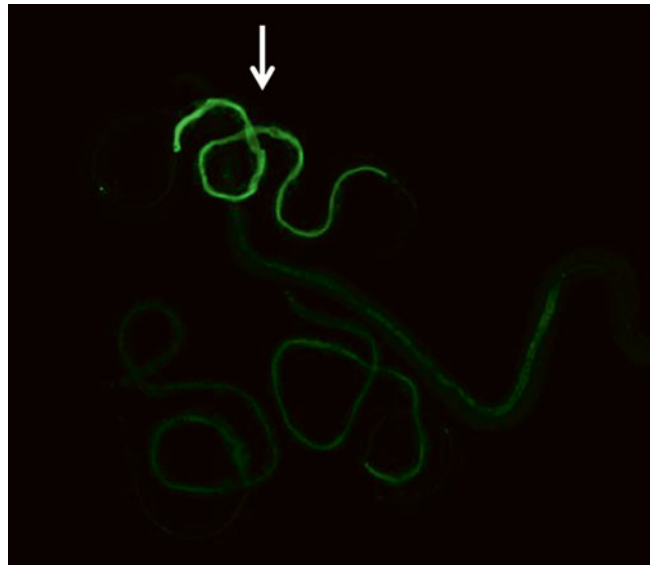

Fig. 6 L4 *H. contortus* with fluorescently labelled protein located to intestines. *H. contortus* L4 was incubated with 50 μl of 2 mg/ml of chicken ovalbumin–fluorescein isothiocyanate (OVA-FITC) for 2 h and visualized under fluorescent microscope. *White arrow* indicates bright fluorescence localized to intestine of a single L4 indicating ingestion of fluorescent protein. Faint fluorescence of other L4 represents autofluorescence and in the feeding assay is recorded as L4 which are not feeding on fluorescently labelled protein. Magnification: 20×

4 Notes

1. Ewes can be used but egg hatching is less successful due to urine contamination. Young (between 3 months and 2 years) animals are preferred as they are more susceptible to infection and less likely to be pasture primed.
2. These bags were handmade using shade cloth material and elastic banding to fit around the sheep.
3. Metacercariae can be purchased or collected from *F. hepatica* infected snails (obtained from laboratory snail cultures). We used *F. hepatica* infected *Lymnaea tomentosa* snails from the Elizabeth Macarthur Agricultural Institute (NSW, Australia). Others have purchased *F. hepatica* metacercariae from Compton Paddock Laboratories (UK; VIAS strain) and Baldwin Aquatics (USA; Oregon strain).
4. L3 are counted by taking 100 μl of solution and pipetting the volume as microliter droplets onto a clear plastic tray. L3 can be visualized at 10× magnification and calculated per milliliter.
5. Transfer L3 preparation into gelatin capsules just prior to administration as capsule will quickly start to dissolve.

6. Dosing gun needs to be thoroughly pushed down the animals' throat before capsules are released. This is done by allowing the animal to swallow part of the dosing gun.
7. When many larvae have hatched and developed into L3, a peanut butter-colored solution will form on the clear plastic sheets.
8. Exsheathed L3 can be distinguished from unexsheathed L3 by a rounder tail (*see* Fig. 2).
9. Adult worms can be incubated to collect eggs but this has not been done in our laboratory.
10. *F. hepatica* infected livers and bile ducts can also be sourced from local abattoirs for the isolation of juvenile and adult liver flukes as described in Subheading 3.1.8.
11. In addition to collecting and freezing parasites for immunohistology or exogenous galectin binding, live parasites can be collected for in vitro culture.
12. Primed but not challenged sheep can provide control abomasum samples for comparison.
13. For oral dosing technique refer to Subheading 3.1.1, **steps 1–4**.
14. We have also collected gastrointestinal mucus from control and parasite-infected sheep for analysis as follows. Gently scrape mucus off the surface of the abomasum, dilute it 1/3 (w/v) with 10 mM citric acid pH 5, centrifuge ~4,000 × *g* for 30 min, collect the supernatant, and store at −20 °C.
15. Abomasal tissue samples can be processed for subsequent RNA preparations, protein extractions, and/or immunohistology to study galectin expression during parasite infections, or can be used to test exogenous galectin binding. Preparation of immunohistology sections is described in Subheading 3.3 and exogenous galectin binding in Subheading 3.4.
16. We have used horseradish peroxidase-conjugated anti-rabbit IgG to detect primary rabbit polyclonal anti-galectin antibodies, and horseradish peroxidase-conjugated anti-mouse secondary antibodies to detect primary murine monoclonal anti-galectin antibodies.
17. Collect 5 ml of rabbit blood to 5 ml Alsever's solution (to prevent coagulation). Then 1 vol is 10 ml and you can use a 50 ml tube for all the 5 vol washes.

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Chapter 26

Effect of Galectins on Viral Transmission

Michel Ouellet, Christian St-Pierre, Michel J. Tremblay, and Sachiko Sato

Abstract

Recent reports suggest that some galectins bind to enveloped viruses. They include influenza virus, human immunodeficiency virus-1 (HIV-1), human T-cell leukemia virus-1 (HTLV-1), and Nipah virus. It is also suggested that the interaction between viruses and galectins influences viral attachment to their susceptible cells, affecting the viral infectivity. Our work suggests that galectin-1 increases the infectivity of HIV-1 and HTVL-1. Indeed, galectin-1 promotes the initial adsorption of HIV-1 to $CD4^+$ cells through its binding to viral envelope gp120 and facilitates HIV-1 infection in a manner that is dependent on its recognition of β-galactoside residues. Thus, as galectin-1 can be considered as a pattern recognition receptor, HIV-1 exploits this host factor to promote its transmission or replication. In this chapter, we describe methods used to investigate this potential role of galectins in HIV-1 infection as a case in point for future studies on galectin–virus interactions.

Key words Virus, HIV-1, Galectins

1 Introduction

Galectin-1 is reported to directly bind to several enveloped viruses [1–9]. For Nipah and Hendra viruses (paramyxoviruses), their recognition by galectin-1 leads to inhibition of virus-induced cell fusion [1, 2]. Galectin-1 binds to different subtypes of influenza A and reduces their infectivity [3]. In sharp contrast, interaction of galectin-1 with HIV-1 and HTLV-1 facilitates infection of target cells [4–10]. In the case of HTLV-1, galectin-3 was recently reported to be incorporated to the biofilm-like extracellular viral assemblies that stabilize cell–cell interactions and promote HTLV-1 transmission [11]. Interestingly, galectin-3 neither recognizes HIV-1 virus particles nor interferes with the galectin-1-promoting effect observed on viral infectivity, suggesting a highly specific recognition of HIV-1 by galectin-1. Since this chapter describes methods related to HIV-1 as a case in point for future studies on

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the lectin–virus interaction, a brief biochemical background of the role of galectin-1 in HIV-1 is first explained.

Initial binding of HIV-1 to a susceptible CD4$^+$ cells is one of the limiting steps for the establishment of infection. The number of viral envelope spikes on the surface of HIV-1 is limited compared to highly infectious viruses such as influenza A [12] so that the avidity of viral envelope gp120 for CD4 is relatively low. Recent studies suggest that a very small proportion of infectious HIV-1 particles reach the lamina propria of the genital mucosa where a small number of "resting yet previously activated" effector memory CD4$^+$ T lymphocytes reside. These highly susceptible cells, once infected, represent a founder HIV-1$^+$ cell population [13–15] able to amplify the initial inoculum. Within 4–10 days, viral production leads to the destruction of a significant number of CD4$^+$ memory T lymphocytes found in the gut-associated lymphoid tissue. By 3 weeks post-infection, close to 80 % of CD4$^+$ T lymphocytes are depleted and the virus has already spread to the whole organism while entering the chronic stage of the infection [16, 13, 17, 18]. Since viral spread and memory CD4$^+$ T lymphocytes depletion arise from infection of such a limited number of susceptible cells initially, this phase represents a major vulnerability for HIV-1. Thus, information related to the very early stage of HIV-1 infection could provide other avenues of prevention at this bottleneck of viral transmission.

While HIV-1 gp120 is known to carry many oligomannose-type glycans (56–79 % of the *N*-glycans), intact virus-associated or recombinant gp120 also contains steady levels of complex-type glycans (21–44 % of the *N*-glycans) [8, 19]. Galectin-1 binds to those complex-type glycans and cross-links gp120 with host membrane proteins such as CD4, the receptor for gp120. Our recent data suggest that the peculiar spatial arrangement of glycans on gp120 plays a critical role to allow binding of galectin-1 while limiting binding of galectin-3. Therefore, the study on the interaction of galectins with viral glycoproteins/virus particles requires special attention on the tertiary protein structure of virus particles since clustering of glycans may be crucial for the binding specificity of galectins.

2 Materials (*See* Notes 1 and 2)

2.1 Preparation of Primary Cells

2.1.1 Primary Cells for HIV-1 Study (Peripheral Blood Mononuclear Cells, PBMCs)

1. 50 ml sterile conical tube with anticoagulant (heparin or citrate).
2. Ficoll-Hypaque (GE Healthcare).
3. Hank's Balanced Salt Solution (HBSS).
4. RPMI 1640 with 10 % FBS.

2.1.2 Primary Cells for HIV-1 Study (CD4+ T Lymphocytes)

1. EasySep CD4+ T cell enrichment kit (STEMCELL Technologies Inc.).
2. EasySep Magnet (STEMCELL Technologies Inc.).
3. 5 ml polystyrene tube.
4. PBS supplemented with 2 % FBS.

2.1.3 Primary Cells for HIV-1 Study (Monocyte-Derived-Macrophages, MDMs)

1. T-75 flask.
2. Incubator at 37 °C.
3. RPMI 1640 with 5 % FBS.
4. Macrophage-colony stimulating factor (M-CSF, GenScript Corporation).
5. Accutase® (Sigma-Aldrich).
6. Cell scrapers.
7. 48-well plates.

2.1.4 Activation of PBMCs or CD4 T Cells

1. PHA-L.
2. Recombinant human IL-2.
3. OKT3 (1 μg/ml, ATCC).
4. Anti-CD28 antibody (clone 9.3 at 1 μg/ml) provided by J. A. Ledbetter (Bristol-Myers Squibb Pharmaceutical Research Institute).
5. Goat anti-mouse IgG.

2.2 HIV Production

2.2.1 HIV-1 Production in Human Embryonic Kidney (HEK) 293T Cells

1. HEK cells.
2. T-75 flasks.
3. Incubator set at 37 °C and 5 % CO_2.
4. HIV-1 molecular clones (pNL4-3 (X4 tropic) or pNL4-3 Bal env (R5 tropic)).
5. 0.25 M calcium chloride in sterile distilled water.
6. 2× HEPES-buffered saline: 280 mM NaCl supplemented with 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, 50 mM HEPES (pH 7.05–7.12).
7. DMEM supplemented with 10 % FBS.
8. Phosphate buffered saline.
9. 0.22 μm sterile syringe filter.

2.2.2 HIV-1 Production in CD4+ T Lymphocytes

1. CD4+ T lymphocytes cells.
2. Phytohemagglutinin-L (PHA-L, Sigma-Aldrich).
3. Recombinant human IL-2 (NIH AIDS Research and Reference Reagent Program).
4. RPMI 1640 supplemented with 10 % FBS.

5. HIV-1 (NL4-3, X4 tropic virus).
6. OptiPrep™ gradient.

2.2.3 HIV-1 Production in Monocyte-Derived Macrophages (MDMs)

1. Monocyte-derived macrophages (MDMs).
2. T-75 flasks.
3. HIV-1 (NL-4-3 Bal env).
4. Phosphate buffered saline.
5. RPMI 1640 supplemented with 5 % FBS.

2.3 HIV Detection, Concentration and Purification

2.3.1 p24 ELISA

1. 96-well plate (high binding, Immuno Plate MaxiSorp, NUNC).
2. Anti-p24 antibody (183-H12-5C, 2.5 μg/ml, 100 μl/well, NIH AIDS Research and Reference Reagent Program).
3. PBST: PBS with 0.05 % Tween-20.
4. 1 % bovine serum albumin (BSA) in PBST.
5. p25gag/SF2 (31.25–2,000 pg/ml, supplied by Chiron Corporation).
6. Disruption buffer: 2.5 % Triton X-100, 0.05 % Tween-20, Thimerosal 0.02 % in PBS.
7. Biotinylated anti-p24 antibody (clone 31-90-25, ATCC #HB-9725).
8. Streptavidin-conjugated horseradish peroxidase (100 μl/well, 66 ng/ml, HRP40-streptavidin, Fitzgerald Industries International).
9. Peroxidase substrate, TMB-S (100 μl/well, Fitzgerald industries international).
10. 1 M H_3PO_4.
11. Microplate reader.

2.3.2 Concentration of HIV-1 Preparation by an Ultracentrifugation Step

1. Ultracentrifuge with Beckman 70 Ti rotor.
2. Phosphate buffered saline.

2.3.3 Purification of HIV-1 with OptiPrep™

1. OptiPrep™ (Axis-shield).
2. Laminar flow hood.
3. Phosphate buffered saline.
4. OptiSeal™ tube (Beckman).
5. Serological 1 ml pipette.
6. NVT65 rotor and caps.
7. Dynamometer.
8. Ultracentrifuge.

2.3.4 AT-2 Inactivation of HIV-1

1. 2-Aldrithiol (2,2′-dithiodipyridine or AT-2, Sigma-Aldrich).
2. Methanol.
3. 0.22 μm syringe filter.
4. Ultracentrifuge with Beckman rotor type 70 Ti.
5. Phosphate buffered saline.
6. LuSIV cell line (NIH AIDS Research and Reference Reagent Program) (*see* **Note 3**).
7. TZM-bl cell line (NIH AIDS Research and Reference Reagent Program) (*see* **Note 4**).
8. Hygromycin B.
9. DMEM supplemented with 10 % FBS.
10. 0.25 % trypsin solution.

2.4 Galectins

2.4.1 Quality Control of Galectins Purity

1. 12 or 15 % polyacrylamide gel.
2. Gel electrophoresis system.
3. Limulus Amebocyte Lysate (LAL) assay kit (Associates of Cape Cod Incorporated).
4. ActiClean Etox endotoxin removing gel (Sterogene).

2.4.2 Quality Control of Galectins Activity per Weight of Protein

1. Heparin treated human peripheral blood.
2. Phosphate buffered saline.
3. Glutaraldehyde (3 % final concentration).
4. 0.025 % NaN_3 in PBS.
5. U-shape 96-well plate.
6. Incubator at 37 °C.
7. Recombinant galectin.

2.4.3 S-Carboxyamido-methylation of Galectin-1

1. Galectin-1 (2–5 mg/ml).
2. 100 mM lactose–phosphate buffered saline (final concentration).
3. Iodoacetamide.
4. Dialysis membrane tubing with 3,500 molecular weight cutoff (Spectra/Por 3 from Spectrum Labs).

2.5 Virus Attachment Assays

2.5.1 Indirect Evaluation of HIV-1 Binding by Luciferase Activity

1. Recombinant galectin.
2. Lactose–phosphate buffered saline.
3. HIV-1 (virus suspension containing 10 ng of p24).
4. Enfuvirtide (T-20) (NIH AIDS Research and Reference Reagent Program, 100 ng/ml).
5. Phosphate buffered saline.
6. RPMI 1640-10 % FBS.

7. Incubator at 37 °C.
8. LuSIV cell line (NIH AIDS Research and Reference Reagent Program).
9. Luciferase lysis buffer: Tris–HCl, pH 7.8 supplemented with 10 mM DTT, 5 % Triton X-100 and 50 % glycerol.
10. 96-well luminometer plate.
11. Dynex MLX microplate luminometer.
12. Luciferase assay buffer: 20 mM Tricine supplemented with 1.07 mM $(MgCO_3)_4{\cdot}Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 220 μM Coenzyme A, 4.7 μM D-Luciferin potassium salt, 530 μM ATP, and 33.3 mM DTT.
13. Hygromycin B.

2.5.2 Direct Evaluation of HIV-1 Binding by Measuring p24

1. Recombinant galectin.
2. Lactose–phosphate buffered saline.
3. HIV-1 (R5-tropic virus at 10 ng of p24).
4. Enfuvirtide (T-20) (NIH AIDS Research and Reference Reagent Program, 100 ng/ml).
5. Phosphate buffered saline.
6. RPMI 1640-10 % FBS.
7. Incubator at 37 °C.
8. LuSIV cell line (NIH AIDS Research and Reference Reagent Program).
9. Disruption buffer: 2.5 % Triton X-100, 0.05 % Tween-20, Thimerosal 0.02 % in PBS.
10. 48-well plate.
11. Hygromycin B.

2.6 HIV-1 Infection Assay

2.6.1 One-Cycle Infection of LuSIV Reporter Cells

1. Recombinant galectin.
2. Lactose–phosphate buffered saline.
3. HIV-1 (virus suspension containing 10 ng of p24).
4. Enfuvirtide (T-20) (NIH AIDS Research and Reference Reagent Program, 100 ng/ml).
5. Phosphate buffered saline.
6. RPMI 1640-10 % FBS.
7. Incubator at 37 °C.
8. LuSIV cell line (NIH AIDS Research and Reference Reagent Program).
9. Luciferase lysis buffer: Tris–HCl, pH 7.8 supplemented with 10 mM DTT, 5 % Triton X-100, and 50 % glycerol.
10. 96-well luminometer plate.

11. Dynex MLX microplate luminometer.
12. Luciferase assay buffer: 20 mM Tricine supplemented with 1.07 mM $(MgCO_3)_4 \cdot Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 220 μM Coenzyme A, 4.7 μM D-Luciferin potassium salt, 530 μM ATP, and 33.3 mM DTT.
13. Hygromycin B.

2.6.2 Multiple Cycle Infection of Primary Cells

1. Peripheral blood mononuclear cells (PBMCs) or CD4 T cells.
2. Recombinant galectin.
3. Lactose–phosphate buffered saline.
4. HIV-1 (virus suspension containing 10 ng of p24).
5. Phosphate buffered saline.
6. RPMI 1640-10 % FBS.
7. Incubator at 37 °C.

2.7 Microscale Affinity Chromatography to Detect Interactions of Virus or Viral Components with Galectins

1. Recombinant galectin.
2. Phosphate buffered saline (pH 7.5).
3. Lactose–phosphate buffered saline (pH 7.5).
4. AminoLink Plus (1 ml) (Pierce).
5. 5 M cyanoborohydride solution.
6. End-over-end rotator.
7. Fume hood.
8. 1 M Tris–HCl pH 7.5.
9. PBS–0.02 % NaN_3.
10. PBS with 0.1–0.5 % Triton X-100.
11. AT-2 treated HIV-1, virus lysates, or gp120.
12. Polypropylene disposable column.
13. 100 mM mannose–PBS.
14. 100 mM lactose–PBS.

3 Methods

3.1 Preparation of Primary Cells

*3.1.1 Primary Cells for HIV-1 Study (Peripheral Blood Mononuclear Cells, PBMCs) (See **Note 5**)*

1. Collect fresh blood to the container in the presence of anticoagulants, heparin or citrate.
2. Apply carefully 25 ml of fresh blood over 20 ml of Ficoll-Hypaque in a 50 ml conical tube.
3. Centrifuge at 400 × *g* for 30 min at room temperature (RT) without brake.
4. Remove the upper layer, containing diluted autologous plasma, by aspiration to leave the interphase layer, rich in mononuclear cells, undisturbed.

5. Transfer carefully the interphase to a new conical tube, which is filled with Hank's Balanced Salt Solution (HBSS), and centrifuge at $300 \times g$ for 10 min at RT.
6. Remove the supernatant.
7. Resuspend the cells with 50 ml of HBSS and centrifuge at $200 \times g$ for 10 min at RT. Repeat this washing step twice.
8. Resuspend the cells at 1×10^6 cells/ml in RPMI 1640 with 10 % FBS.
9. Use PBMCs immediately or activate them (*see* Subheading 3.1.4).

3.1.2 Primary Cells for HIV-1 Study (CD4⁺ T Lymphocytes)

CD4⁺ T lymphocytes are purified from PBMCs using the human CD4⁺ T lymphocytes enrichment kit (STEMCELL Technologies Inc., EasySep CD4⁺ T cell enrichment kit Cat#19052) and EasySep Magnet (Cat# 18000) by following the manufacturer's protocol.

1. Resuspend PBMCs at a concentration of 5×10^7 cells/ml in PBS supplemented with 2 % FBS and place in a 5 ml polystyrene tube.
2. Add the enrichment cocktail (50 µl/ml), mix and incubate at RT for 10 min.
3. Vortex EasySep D magnetic Particles at high setting for at least 30 s to prevent aggregation.
4. Add the particles (100 µl/ml) to resuspended PBMCs, mix and incubate at RT for 5 min.
5. Add PBS supplemented with 2 % FBS to the cell suspension to obtain a total volume of 2.5 ml.
6. Mix gently the entire suspension and place in the magnet, without cap, for 5 min.
7. Pour the cell suspension into a new tube by inversion of the magnet in one continuous motion. Do not shake the last drop loose.
8. Use the enriched CD4⁺ T cells immediately or activate them (*see* Subheading 3.1.4).

3.1.3 Primary Cells for HIV-1 Study (Monocyte-Derived Macrophages, MDMs)

1. Plate PBMCs at 1.25×10^8 cells in a ventilated 75 cm^2 cell culture flask (T-75 flask).
2. Incubate at 37 °C for 2 h.
3. Remove non-adherent cells and wash adherent monocytes twice with RPMI 1640-5 % FBS.
4. Culture the cells for a week in medium supplemented with 100 ng/ml macrophage-colony stimulating factor for differentiation into MDMs.

5. Remove MDMs from the flask by briefly incubating with Accutase® followed by gentle scraping with a cell scraper.
6. Replate the cells in a 48-well plate at 5×10^4 cells/well for infection studies [6].

*3.1.4 Activation of PBMCs or CD4 T Cells (See **Note 6**)*

1. There are two primary activation strategies employed for these assays:
   (a) Activate $CD4^+$ T lymphocytes with 1 μg/ml of PHA-L and 50 U/ml of recombinant human IL-2 [20].
   (b) Activate PBMCs or $CD4^+$ T lymphocytes through the T cell receptor with the antibody OKT3 (1 μg/ml) and a co-stimulation signal with an anti-CD28 antibody (clone 9.3 at 1 μg/ml), followed by cross-linking with goat anti-mouse IgG 5 μg/ml [21].

3.2 HIV Production

*3.2.1 HIV-1 Production in Human Embryonic Kidney (HEK) 293T Cells (See **Note 7**)*

1. Seed HEK cells at 2×10^6 cells in a T-75 flask for 16 h.
2. Allow all solution for the calcium phosphate transfection to reach room temperature.
3. Use infectious HIV-1 molecular clones, such as pNL4-3 (X4 tropic) or pNL4-3 Bal env (R5 tropic) to prepare HIV-1 virus particles [22, 23].
4. Suspend the plasmid DNA (30 μg) in 500 μl of 0.25 M calcium chloride in sterile distilled water.
5. Slowly add DNA solution drop-wise into 500 μl of 2× HEPES-buffered saline under continuous mild agitation.
6. Incubate the mixed solution for 20 min at room temperature. Once these reagents are mixed, milky cloudiness should appear during the incubation.
7. Replace the culture medium of HEK cells in the flask with 9 ml of fresh DMEM supplemented with 10 % FBS.
8. Add slowly the premixed transfection reagent to cells.
9. Move gently the flask back and forth (but not to use rotation movements) to distribute the transfection solution evenly.
10. Remove the medium after 4–16 h at 37 °C.
11. Wash cells gently with PBS and then add 10 ml of fresh DMEM-10 % FBS.
12. Collect virus-containing cell-free medium 2 days post-transfection.
13. Filter the viral preparation through a 0.22 μm sterile syringe filter.
14. Make aliquots to prepare frozen stocks at −80 °C.
15. If necessary, this viral preparation can be further concentrated and/or purified (*see* Subheading 3.3).
16. Measure both p24 capsid protein and infectivity as quality control of prepared virus (*see* Subheading 3.3.1).

*3.2.2 HIV-1 Production in CD4⁺ T Lymphocytes (See **Note 8**)*

1. Activate $CD4^+$ T lymphocytes cells ($1–2 \times 10^6$ cells/ml) with 1 μg/ml of Phytohemagglutinin-L and 50 U/ml of recombinant human IL-2 (NIH AIDS Research and Reference Reagent Program) for 3 days in RPMI 1640 supplemented with 10 % FBS.
2. Infect cells with HIV-1 (NL4-3, X4 tropic virus) at a concentration of 10 ng of p24 equivalent per 1×10^5 cells for 4–10 days.
3. Collect the virus-containing cell-free medium at 4, 7, and 10 days post-infection, and replace by fresh culture medium to continue viral harvest.
4. Concentrate supernatants by ultracentrifugation and further purified by OptiPrep™ gradient (*see* Subheading 3.3.3).

3.2.3 HIV-1 Production in Monocyte-Derived Macrophages (MDMs)

1. Plate MDMs cells at a density of $5–10 \times 10^6$ cells per T-75 flask.
2. Infect with HIV-1 (NL-4-3 Bal env) at a dose of 20 ng of p24 per 1×10^5 cells for 2–4 h at 37 °C.
3. Wash MDMs twice with PBS, followed by a brief wash with RPMI 1640 supplemented with 5 % FBS.
4. Incubate MDM with RPMI 1640 supplemented with 5 % FBS for the production of virus.
5. Collect half of the cell-free medium at day 7, 14, 21, and 28 post-infection and replace the collected medium with fresh medium. Peak of virus production (based on p24 levels in the medium) is between 10 and 21 days post-infection.
6. If necessary, purify or concentrate virus from this cell-free supernatant.

3.3 HIV Detection, Concentration and Purification

*3.3.1 p24 ELISA (See **Note 9**)*

1. Coat wells of an ELISA 96-well plate with a monoclonal anti-p24 antibody either overnight at 4 °C or for 1 h at 37 °C.
2. Wash the wells three times with 300 μl PBST (PBS with 0.05 % Tween-20).
3. Add 200 μl of 1 % bovine serum albumin (BSA) in PBST to the well at RT for 30 min to block nonspecific binding sites.
4. Wash the well three times.
5. Add viral preparations to the wells (100 μl/well) at various dilutions in 1 % BSA–PBST.
6. Use recombinant purified p25gag/SF2 (31.25–2,000 pg/ml as a standard).
7. Add 25 μl/well of disruption buffer to lyse viral particles.
8. Incubate the well for 1 h at RT followed by three washes.
9. Add 100 μl/well of 0.5 μg/ml biotinylated anti-p24 antibody and incubate for 1 h at RT.
10. Wash three times to remove unbound antibody.

11. Add streptavidin-conjugated horseradish peroxidase (100 µl/well, 66 ng/ml) and incubate at RT for 20 min.
12. Wash three times and incubate for 10 min with a peroxidase substrate, TMB-S (100 µl/well).
13. Terminate the reaction by adding 50 µl of 1 M H_3PO_4.
14. Measure the absorbance using a microplate reader at 450 nm with a reference at 630 nm.
15. Estimate p24 values based on regression analysis of p24 standards over a linear range [24].

3.3.2 Concentration of HIV-1 Preparation by an Ultracentrifugation Step

1. Mark the expected pellet position on the centrifuge tube since the virus pellet is not visible at the end of the run.
2. Centrifuge the cell-free supernatant containing HIV-1 by ultracentrifugation at 58,000 × *g* (28,000 rpm with Beckman 70 Ti rotor) for 45 min at 4 °C without brake to obtain higher titer viral preparations.
3. Discard supernatant by pouring it off in one continuous motion, being careful not to shake or blot off any drop that may remain hanging.
4. Resuspend thoroughly the virus pellet with 1 ml of PBS (the virus pellet can be located both at the bottom and on the side of the tube).
5. Reevaluate the concentration of virus with a p24 ELISA.

*3.3.3 Purification of HIV-1 with OptiPrep™ (See **Note 10**)*

1. Prepare the stock solutions 6.0, 7.2, 8.4, 9.6, 10.8, 12.0, 13.2, 14.4, 15.6, 16.8, 18.0 % of iodixanol under a laminar flow hood. The solutions can be kept at 4 °C.
2. Place 0.9 ml of 18 % iodixanol–PBS is placed at the bottom of an OptiSeal™ tube by using a serological 1 ml pipette.
3. Mark the top position of the 18 % iodixanol on the tube to facilitate the collection of a desired fraction after centrifugation.
4. Tilt the tube at 45 °C and add slowly 0.9 ml of the 16.8 % solution but continuously deposited onto the 18 % iodixanol layer. Maintain the end of the pipette in a position during the step to keep it at the position where the pipette barely touches the 18 % layer. After the addition of a new layer, the interface of the layers becomes visible for a short period of time.
5. Place nine more layers in a similar manner to obtain a 6–18 % gradient of iodixanol.
6. Apply slowly 400 µl of virus suspension, which was concentrated by ultracentrifugation to the top of the iodixanol gradient (6 %).
7. Insert aluminum caps and close firmly on OptiSeal™ tubes to seal them in the NVT65 rotor and caps are then screwed over

the filled positions of the rotor by applying pressure between 80 and 100 psi using the provided tool (Allen key with a dynamometer).

8. Centrifuge the gradient by using ultracentrifugation for 1 h 15 min at 219,000 × *g* (52,000 rpm) at 4 °C with slow acceleration and deceleration.
9. Remove gently each gradient fraction with a 1 ml pipette. HIV-1 is normally found between fractions 14.4 and 16.8 %.
10. Pool the fractions of interest (~3 ml) and dilute ~10 fold with PBS followed by ultracentrifugation at 58,000 × *g* as outlined in Subheading 3.3.2 to obtain purified HIV.
11. Carry out quality control of prepared virus following each purification using both the p24 ELISA described in Subheading 3.3.1 and infectivity assays with reporter cell lines described in Subheading 3.6.

*3.3.4 AT-2 Inactivation of HIV-1 (See **Note 11**)*

1. Prepare the stock solution of AT-2 (100 mM in methanol) under chemical hood. This solution can be stored at −20 °C for 1 month. (AT-2 powder should be kept at 4 °C and protected from moisture.)
2. Add AT-2 stock solution (10 μl per 1 ml of virus solution, final concentration 1 mM) to the virus containing solution that is filtered through a 0.22 μm syringe filter.
3. Incubat the solution overnight at 4 °C (protect it from light).
4. Purify virus particles by ultracentrifugation at 58,000 × *g* (Beckman rotor type 70 Ti at 28,000 rpm) at 4 °C for 45 min without brake.
5. Remove carefully the supernatant. It is important to remove the AT-2 containing supernatant as much as possible since concentrations of more than 8 μM have been observed to induce apoptosis of PBMCs after 24 h of treatment.
6. Suspend the virus pellet in PBS.
7. Estimate the virus concentration by monitoring the p24 content.
8. Confirm the lack of infectivity of each viral preparation treated with AT-2 if the experiments with AT-2-treated HIV-1 are to be performed outside a BSL3 facility by using a sensitive luciferase-based HIV-1 infection assay involving reporter cell lines, such as LuSIV or TZMBL cells. Typically, concentrations of HIV-1 between 1 and 100 ng of p24 per 1×10^5 cells are used to evaluate inactivation. Luciferase activity is checked after 24–48 h to be sure that viral particles do not carry any residual infectivity. It is only after this verification that the viral preparations can be used outside a BSL3 facility.

3.4 Galectins (See Notes 12 and 13)

3.4.1 Quality Control of Galectins Purity

1. Several procedures are used to ensure the quality of purified galectins:
   (a) *SDS-PAGE*: Purified galectins are subjected to a 12 or 15 % SDS-PAGE to confirm that galectin-1 and -3 migrate at their respective position of 14 and 30 kDa, without any additional protein band.
   (b) *Endotoxin levels*: The endotoxin level is measured using Limulus Amebocyte Lysate (LAL) assay kit (Associates of Cape Cod Incorporated). Protein preparations that exceed 10 EU/mg of protein are rejected or passed through the ActiClean Etox endotoxin-removing gel.

3.4.2 Quality Control of Galectins Activity per Weight of Protein (See **Note 14**)

1. Centrifuge heparin-treated human peripheral blood (10 ml) at 2,000 × *g* for 5 min.
2. Remove buffy coat as much as possible.
3. Wash red blood cells (RBCs) three times with 50 ml PBS.
4. Dilute RBCs with PBS to obtain 100 ml of an 8 % cell suspension.
5. Treat RBC suspension with glutaraldehyde (at a final concentration of 3 %) under rotation for 1 h at RT.
6. Wash RBC with 0.025 % NaN_3 in PBS.
7. Resuspend the fixed RBCs at 3–4 % RBCs in PBS–NaN_3 (*see* **Note 15**).

   Calibration of RBCs to obtain the appropriate concentration for lectin-mediated hemagglutination

8. Make series of RBCs dilutions (2–20 fold).
9. Distribute them into a U-shape 96-well plate (100 µl/well).
10. Incubate at 37 °C for 30 min. RBCs form very tight button-like precipitation at the bottom of the well when there is no aggregation. We then use the minimum concentration of RBCs that results in this very tight button-like precipitation.
11. Prepare serial dilutions of galectins (from 10 to 0 µM) and mix with the appropriate quantity of RBCs in the wells of a U-shape 96-well plate.
12. Incubat at 37 °C for 30 min. When a lectin induces aggregation of RBCs (hemagglutination), they are spread out like a sheet covering the entire surface of the well. Galectin-1 and galectin-3 induce hemagglutination at concentrations of around 15 µg/ml and 10 µg/ml, respectively [25, 26].

3.4.3 S-Carboxyamidomethylation of Galectin-1 (See **Note 16**)

1. Prepare galectin-1 (2–5 mg/ml) in 100 mM lactose–PBS solution to protect the carbohydrate recognition domain of the protein.
2. Incubate galectin-1 overnight at 4 °C with iodoacetamide at a final concentration of 100 mM.

3. Remove extensively free iodoacetamide and lactose by series of dialysis against PBS, followed by sterilization.
4. Control for the quality of galectin-1 by hemagglutination assay.
5. The treated protein remains stable over prolonged period at 4 °C or can be kept frozen [27, 28].

3.5 Virus Attachment Assays (See Note 17)

3.5.1 Indirect Evaluation of HIV-1 Binding by Luciferase Activity

1. Pretreat cells with various concentrations of galectins (0–4 μM) for 15 min at 4 °C. In some cases, a galectin antagonist, lactose (50 mM), is included to verify specificity (*see* **Note 13**).
2. Add HIV-1 (virus suspension containing 10 ng of p24) to the well with 1×10^5 target cells.
3. Incubate cells for various times (0–2 h) at 4 °C (*see* **Note 18**).
4. Wash cells three times with PBS to remove both unbound lectin and virus.
5. Resuspend cells in RPMI 1640-10 % FBS (100 μl/well) and culture at 37 °C for 24 h (*see* **Note 19**).
6. Add 25 μl of 5× luciferase lysis buffer to cells.
7. Incubate for 30 min under agitation.
8. The cell lysate can be frozen at −20 °C for a few weeks or at −80 °C for a few months.
9. Transfer an aliquot of the cell lysate (20 μl per well) to a 96-well luminometer plate.
10. Place the plate in a Dynex MLX microplate luminometer. The luminometer injects 100 μl per well of luciferase assay buffer. After a 2 s delay, the luciferase activity is monitored and integrated for 20 s per well [29].

3.5.2 Direct Evaluation of HIV-1 Binding by Measuring p24 (See **Note 20**)

1. Carry out a similar type of viral binding at 4 °C in Subheading 3.5.1.
2. Wash the cells extensively to remove unbound virus particles.
3. Lyse cells with p24 ELISA disruption buffer to obtain the sample for p24 ELISA. For example, MDMs at 5×10^4 cells/well are plated in a 48-well plate and incubated with R5-tropic virus (the viral input is usually 10 ng of p24) with or without galectin-1 for 1 h.
4. After being washed, cells are lysed with 25 μl/well of disruption buffer and p24 levels are measured (*see* Subheading 3.3.1) [4].

3.6 HIV-1 Infection Assay (See Note 21)

3.6.1 One-Cycle Infection of LuSIV Reporter Cells (**See** Note 22)

1. Add various concentrations of galectins (0–4 μM) to LuSIV cells (1×10^5 cells per well) in RPMI 1640-10 % FBS (100 μl per well). As a negative control, add a galectin antagonist, such as lactose (50 mM) to verify whether the effects of galectins on HIV-1 infection are dependent on the galectin's glycan binding activity.

2. Immediately add HIV-1 (virus suspension containing 10 ng of p24).
3. Incubate cells at 37 °C for 24 h to initiate a single round of viral replication. Washing step is not necessary in this infectivity assay.
4. Lyse the cells by adding 20 μl of 5× luciferase lysis buffer, and measure luciferase activity as mentioned in Subheading 3.5.1 [29].

*3.6.2 Multiple Cycle Infection of Primary Cells (See **Note 23**)*

1. Infect 1×10^5 cells (such as PBMC or $CD4^+$ T lymphocytes) per well with HIV-1 (10 ng of p24 per 1×10^5 cells) with or without galectin as described in Subheading 3.5.1.
2. Collect the medium at day 3, 6, and 9 post-treatment with HIV-1.
3. Measure p24 in the medium by using p24 ELISA as described in Subheading 3.3.1 (p24 ELISA) (*see* **Note 24**).

3.7 Microscale Affinity Chromatography to Detect Interactions of Virus or Viral Components with Galectins (See Note 25)

1. To prepare a galectin-agarose column make sure that all solutions do not contain any primary amine, including Tris base. Prepare galectin in PBS (make sure that pH is around 7.5).
2. Add lactose to purified galectin to protect the carbohydrate binding domain from modification.
3. Wash AminoLink Plus (1 ml) with 5 ml of PBS (pH 7.5).
4. Add lactose–galectin (1–2 mg/ml) and 20 μl of 5 M cyanoborohydride solution (final concentration of the cyanoborohydride is 50 mM) in a fume hood.
5. Incubate the mixture for 4 h at room temperature using end-over-end rotator.
6. Separate the slurry from the solution and remove the solution in a fume hood. Keep the solution to check the coupling efficiency.
7. Wash the slurry with 2 ml of quenching buffer (0.1 M Tris–HCl pH 7.5).
8. Add 1 ml of 1 M Tris–HCl pH 7.5 and cyanoborohydride solution to the slurry to block the active sites of AminoLink. Incubate for 30 min.
9. Wash galectin–AminoLink gel extensively (at least 20 ml) with PBS. For preservation, keep the column in PBS–0.02 % NaN_3.
10. Use the galectin–AminoLink column within a week.
11. For affinity chromatography, AT-2 treated HIV-1, virus lysates, or gp120 can be applied to the galectin–AminoLink column to evaluate their potential interaction with the lectin. When virus lysates or gp120 is applied to the column, 0.1–0.5 % Triton X-100 can be added to PBS to avoid any nonspecific hydrophobic

interaction. However, for AT-2 treated HIV-1, to maintain the intact virus structure, no detergent should be added to the column.

12. Add 0.5 ml of galectin–AminoLink to a small polypropylene disposable column and wash well with PBS.
13. Apply AT-2 treated virus particles (1–1.5 μg of p24), viral lysates prepared from HIV-1 (p24, 500 ng), or gp120 (2 μg) to the column. Collect the eluate (the void fraction).
14. Apply 0. 5 ml of PBS and collect the fraction #1. Continue to apply 2 × 0.5 ml of PBS and collect the fraction #2 and 3.
15. Apply 0.3 ml of 100 mM mannose–PBS three times and collect the fraction #4–6. This step is to verify if the interaction between the galectin and bound materials is dependent on the recognition of β-galactoside.
16. Elute the bound material by applying 0.3 ml of 100 mM lactose–PBS three times to collect the fraction #7–9.
17. Estimate the levels of HIV-1, or gp120 in the fractions by using p24 ELISA, or western blotting with anti-gp120 antibody (provided by NIH AIDS Research and Reference Reagent Program and polyclonal antibody is purified from sheep antiserum).

4 Notes

1. We use Milli-Q water for all aqueous buffer and medium preparation. Virus and aliquots of purified galectins are stored at −80 °C. Since repeated freeze–thaw cycles significantly compromise the quality of virus and galectins, it should be avoided. When galectin is thawed, it can be kept at 4 °C for several days. However, it is strongly recommended to control the quality of the lectin activity of galectins just before their usage. Galectin-1 is highly sensitive to oxidation, and some recommend that it be kept in the presence of reducing agents, such as 2-mercaptoethanol or DTT. However, a tiny amount of those reducing agents may compromise the tertiary structure of viral and cellular proteins and may modify cell biology. Thus, it is highly recommended to omit those reagents in the latter steps of the purification of galectins or to remove completely the reducing agents from purified galectins. Some companies provide recombinant galectins with a reducing agent; thus, special attention should be paid when galectins are purchased from a third party. Unless using a cysteine-modified version of galectin-1, as described in Subheading 2.4.3, galectin-1 should be used within 48 h of its purification.

2. Special attention regarding biosafety level Selection of an appropriate biosafety level for work with a particular pathogen depends on a number of factors, such as its virulence, pathogenicity, route of spread [30], manipulations involving the agent and the availability of effective therapeutic measures. In the case of HIV-1, routine diagnostic work with clinical specimens to detect the presence of antibodies against HIV-1 can be performed in a biosafety level 2 (BSL2) facility using practices and procedures relevant to this biosafety level [31]. However, the majority of experimental procedures involved in fundamental research of HIV-1, including infection of susceptible cells, replication and harvest of the virus, or the use of concentrated viral preparations must be carried out using BSL3 practices and procedures. Further, medium-to-large-scale viral production and concentration of viral preparation by ultracentrifugation also require a BSL3 facility and use of BSL3 practices and procedures [32]. It is strongly recommended to strictly follow the laboratory safety and biosecurity measures recommended by both your country and institution. Some information can be found on Web sites such as http://www.cdc.gov and http://www.phac-aspc.gc.ca.
3. The LuSIV cell line (NIH AIDS Research and Reference Reagent Program) is derived from the CEMx174 cell line, which stably expresses a firefly luciferase reporter gene driven by the SIVmac 239 long terminal repeat (LTR) region. This cell line is highly sensitive to infection by primary and laboratory T-cell-tropic strains of HIV and SIV, resulting in Tat-mediated expression of luciferase correlating with viral infectivity. The LuSIV system is a powerful tool to analyze HIV/SIV infectivity, providing a unique assay system that can detect virus replication as soon as 24 h, due to the initial presence and early expression of Tat in infected cells [33]. Since primate lentiviruses, such as HIV-1 and SIV, require an average of 24 h to complete one cycle of replication, LuSIV enables measurements after a single round of infection [34]. Cells are cultured in RPMI 1640 supplemented with 10 % FBS, 300 μg/ml of hygromycin B at a cellular concentration between 2.5×10^5 and 2×10^6 cells/ml. Hygromycin B is used to maintain cells containing the episomal reporter plasmid [35].
4. The TZM-bl cell line, also called JC53BL-13, is a CXCR4 positive HeLa cell clone that is engineered to express both CD4 and CCR5 [36]. These cells have been further modified to contain integrated reporter genes for firefly luciferase and β-galactosidase under the control of the HIV LTR sequence [37]. TZM-bl cells are permissive to infection by a wide variety of HIV, SIV and SHIV strains, including primary HIV isolates and molecularly cloned Env-pseudotyped viruses.

These adherent cells are grown in DMEM supplemented with 10 % FBS. For the passage of this cell line, a 0.25 % trypsin solution is used [38].

5. PBMCs are purified from blood of healthy donors by Ficoll-Hypaque centrifugation.
6. It is known that resting $CD4^+$ T lymphocytes are only weakly permissive to productive HIV-1 infection due to host factors affecting different steps of the virus life cycle [39]. Thus, to maximize infection, activated cells must be used.
7. HEK cells are normally used to obtain highly concentrated and infectious HIV-1 preparations, as this cell line consistently produces higher virus yields compared to methods using primary $CD4^+$ cells.
8. Once a lectin has been observed to influence HIV-1 infection or alter HIV-1 binding to susceptible cells, it is highly recommended to verify whether these effects can be observed with HIV-1 produced by human primary target cells, as different glycosylation patterns of the viral membrane which contain both viral and host proteins may have an influence on the role of the lectin. In the case of HIV-1, however, it is important to mention that the expected yield in primary $CD4^+$ cells such as $CD4^+$ T lymphocytes and macrophages is often several orders of magnitude less than with HEK cells.
9. Our previous observations indicate that the majority of the p24 capsid protein (>90 %) is associated with HIV-1 particles [40]. Thus, virus stocks are normalized using p24 levels as estimated by an in-house p24 ELISA.
10. For highly purified HIV-1, an OptiPrep™ gradient is used to separate virus particles from cell-released exosomes. OptiPrep™ is a 60 % (w/v) iodixanol solution in water (Axis-shield Cat# 1030061) with a density of 1.32 g/ml. The gradient is composed of 11 fractions of iodixanol in PBS with concentrations starting from 6 to 18 % with 1.2 % increments, i.e., 6.0, 7.2, 8.4, 9.6, 10.8, 12.0, 13.2, 14.4, 15.6, 16.8, 18.0 %.
11. For some studies that do not require infectious HIV-1 virus particles, such as virion structure studies or virus–lectin interaction studies, a treatment with 2-Aldrithiol (AT-2) can be used to inactivate HIV-1, making it possible to perform experiments in a BSL2 facility using BSL2 practices and procedures. AT-2 covalently modifies the essential zinc fingers in the viral nucleocapsid proteins, reverse transcriptase, and integrase, thereby inactivating HIV-1 while preserving the integrity of viral envelope proteins [41, 42].
12. To get consistent data obtained from experiments with galectins, it is essential to be fully aware that galectins are not necessarily

stable [43, 44] (unpublished observations in the laboratory of SS). Thus, constant monitoring of the quality of purified galectins is extremely important. Refer to the purification methods of galectins presented in Chapter 2 and these refs. 7, 6, 4, 45–48. It is important to completely remove EDTA and reducing agents which are used during the initial stage of purification at the step of lactose-Sepharose affinity chromatography since any trace of reducing agents such as DTT (as low as 1 μM) inhibits HIV-1 infection assays (unpublished data, CSP, SS) [49]. In addition, lactose or other sugar ligands, which are used for the elution of galectin from the column, should be removed completely as well. In some cases, it is important to remove endotoxin from the purified galectins; thus, purified galectins are passed through ActiClean Etox endotoxin removing gels following manufacturer recommendations (Sterogene, Carlsbad, California) or any other type of column as long as the flow rate is well-controlled to enable maximal interaction of samples with ActiClean. Endotoxin binding capacity of this matrix is 20,000 EU/ml.

13. Sugar antagonists to inhibit galectin's lectin activity: Being a β-galactoside binding protein, the lectin activity of galectins can be readily inhibited by relatively small β-galactoside-containing sugars, such as lactose (Galβ1-4glucose) or other appropriate β-galactoside-containing oligosaccharides. While lactose is one of the most used inhibitors for galectins, affinity of galectins for lactose is often low and thereby high concentrations of lactose are necessary to inhibit the lectin's activity. In our laboratory, 50–150 mM lactose is routinely used to ensure inhibition. The isotonicity of the medium has to be taken into account when such high concentrations of saccharide are used for cell assays as cells exposed to hypertonic solutions would become fragile to any successive treatment. To overcome this problem, the concentration of sodium chloride in PBS is reduced for the accommodation of high doses of lactose so that the lactose-containing PBS has the same osmolarity as saline (i.e., 317 mosml/l).
14. Specific lectin activity per protein concentration is evaluated using a hemagglutination assay.
15. RBC suspension can be kept at 4 °C for more than 3 months, although a routine check of the quality of RBCs is necessary.
16. Among members of the galectin family, galectin-1 is a unique lectin that is sensitive to oxidation due to a cysteine residue proximal to its dimerization site [50]. Oxidation leads to its inactivation as a lectin and therefore loss of its hemagglutinin activity. In order to prevent its oxidative inactivation and to maintain carbohydrate-binding activity upon purification and storage, relatively high concentrations of thiol-reducing agent

such as DTT and 2-mercaptoethanol is added in some commercially available galectins. However, treatment of live cells with DTT could seriously hamper cell viability and make interpretations about the effects of galectin-1 difficult as previously pointed out by the group of Cummings [51]. Indeed, as little as 5 μM of DTT was sufficient to halt virtually all natural migration and movement of T lymphocytes (time-lapse live cell imaging, unpublished observations by CST and SS). Moreover, the presence of 1 μM of DTT inhibited HIV-1 infection (unpublished observations by CST and SS). Thus, it is highly recommended to avoid any reducing agents in assays related to galectins. Other commercial sources provide preservative-free lyophilized form of galectin-1, although the state of oxidation of these reagents remains unknown. As recent works suggest, the oxidized form of galectin-1 also possesses some biological functions but they are independent of its lectin binding activity [52, 53]. Thus, caution must be applied for the analysis of data obtained with commercially available galectin-1. Recently, Stowell and colleagues found that S-carboxyamidomethylation of galectin-1 by mild treatment with iodoacetamide makes galectin-1 resistant to atmospheric oxygen in absence of disulfide-reducing reagents and yet preserves some of its biological functions [54]. We also confirm that S-carboxyamidomethylated galectin-1 facilitates HIV-1 infection to similar levels than freshly purified galectin-1 and its glycan binding specificity towards HIV-1 is similar with unmodified freshly purified galectin-1. Thus, in some cases, we have begun to use this method to stabilize galectin-1, although it is highly recommended that each laboratory verifies whether this treatment has an impact on their own assays.

17. To study whether a lectin could increase HIV-1 binding to target cells or not, HIV-1 permissive cells, such as LuSIV cells, primary PBMC or primary $CD4^+$ cells are incubated with HIV-1 virions in the presence or absence of a lectin. Several approaches can be used to estimate the levels of HIV-1 attached to the target cells; for LuSIV, luciferase activity is evaluated while direct measurement of cell-associated p24 can be performed when primary cells are used.
18. In some cases, higher temperatures such as RT or 37 °C can be used, especially when the dynamics or the tertiary structures of membrane proteins on the viral surfaces are a concern, although those temperatures also induce both viral–host membrane fusion and endocytosis, which may make the analysis of the impact of a lectin on binding difficult. Fusion inhibitors, such as Enfuvirtide (T-20) can be included in such assays.
19. The initial binding of HIV-1 to LuSIV followed by incubation at 37 °C leads to fusion, reverse transcription, integration, and production of the viral Tat protein. This viral transactivator

binds to the TAR region of the integrated LTR located upstream of the luciferase gene, thereby increasing by 1,000-fold the biosynthesis of the luciferase protein within 24 h. Since LuSIV cells are highly permissive to HIV-1 infection, the level of HIV-1 binding is directly correlated with the level of infection and therefore to the level of luciferase activity.

20. When natural cellular reservoirs, PBMCs, $CD4^+$ T lymphocytes or MDMs as well as LuSIV cells, are used, HIV-1 binding to target cells are estimated by measuring cell-associated p24 with a p24 ELISA.
21. For the function of a lectin in HIV-1 infection, it is recommended to initiate the study with a sensitive method like the LuSIV reporter cell system. This assay allows the quantitative evaluation of single-cycle infection events through activation of integrated LTR sequences driving the luciferase reporter gene following the production of the viral protein Tat by de novo viral infection. Once the lectin has been observed to influence HIV-1 infection in this reporter cell system, it is recommended to verify whether these effects can be observed in other HIV-1 susceptible cells, as different glycosylation patterns expressed by different cells may have an influence on the role of the lectin. For galectin-1, we have observed that galectin-1, but not galectin-3, facilitates HIV-1 infection in LuSIV cells, PBMC, both activated and naïve $CD4^+$ T lymphocytes and macrophages [6, 4, 8]. We have also verified that this effect could be reproduced using HIV-1 produced in various cellular settings such as HEK cells, PBMCs or macrophages [9, 8]. Thus, at least for galectin-1 activity in the context of HIV-1 infection, the involved glycan ligands appear to be similarly glycosylated, regardless of the cell type used for virus production and/or infectivity assays. This further suggests that, in the case of the galectin-1 ligands involved in HIV infection, the dominant glycosylation pattern of surface proteins and the presentation of glycan on the surface of viral gp120 could be mainly determined by their structure, rather than by the type of cells that produced the proteins [9, 8].
22. Infectivity assays are performed in a similar manner to the HIV-1 binding assay described in Subheadings 3.5.1 and 3.5.2 with some modifications.
23. For infection in primary cells, PBMCs, $CD4^+$ T lymphocytes, and MDMs, the amount of HIV-1 p24capsid protein in the culture medium is used to estimate viral replication as papers previously published by others and us have shown that the amount of p24 in the culture medium is closely correlated with the amount of HIV-1 produced by infected cells.

24. This multi-round of infection assay allows us to study the role of a lectin in a more physiological context, since it provides an opportunity to address not only the role in a single viral replication (LuSIV cell system) but also to examine whether a lectin is involved in later steps of viral replication or in further rounds of infection.
25. Direct binding of galectin to HIV-1 virus particles or viral envelop protein gp120 can be studied through various approaches. We describe a method that employs microscale galectin–agarose column chromatography as an example.

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Chapter 27

Evaluation of the Bactericidal Activity of Galectins

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Abstract

Over a century ago, Karl Landsteiner discovered that blood group antigens could predict the immunological outcome of red blood cell transfusion. While the discovery of ABO(H) blood group antigens revolutionized transfusion medicine, many questions remain regarding the development and regulation of naturally occurring anti-blood group antibody formation. Early studies suggested that blood group antibodies develop following stimulation by bacteria that express blood group antigens. While this may explain the development of anti-blood group antibodies in blood group negative individuals, how blood group positive individuals, who cannot generate anti-blood group antibodies, protect themselves against blood group positive microbes remained unknown. Recent studies suggest that several members of the galectin family specifically target blood group positive microbes, thereby providing innate immune protection against blood group antigen positive microbes regardless of the blood group status of an individual. Importantly, subsequent studies suggest that this unique form of immunity may not be limited to blood group expressing microbes, but may reflect a more generalized form of innate immunity against molecular mimicry. As this form of antimicrobial activity represents a unique and unprecedented form of immunity, we will examine important considerations and methodological approaches that can be used when seeking to ascertain the potential antimicrobial activity of various members of the galectin family.

Key words Galectin, Blood group positive microbes, Innate immune lectin, Molecular mimicry, Antimicrobial

1 Introduction

While the discovery of ABO(H) blood groups by Karl Landsteiner over a century ago provided an unprecedented ability to predict the immunological outcome of red blood cell transfusion, many questions remain regarding the development and regulation of naturally occurring anti-blood group antibodies. Several early studies suggested that naturally occurring antibody formation results from the colonization of microbes that express blood group antigens within the first few months of life [1–3]. While the ability of blood group positive microbes to stimulate naturally occurring antibodies provides a mechanism for naturally occurring antibody formation, the factors that regulate immunity to blood group positive microbes in

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blood group positive individuals remain undefined [4]. As tolerance prevents adaptive immunity against blood group antigens in blood group positive individuals, the factors that are responsible for providing protection against blood group positive microbes in blood group positive people likely reside within the realm of innate immunity.

Because ABO(H) blood group antigens reflect carbohydrate posttranslational modifications [5], factors responsible for providing immunity toward blood group positive microbes likely possess carbohydrate-binding activity. As a result, over 100 different carbohydrate-binding proteins with putative immunological activity were examined on a glycan microarray in an effort to determine whether innate immune factors exist that possess significant blood group-binding activity [6–9].While many carbohydrate binding proteins displayed reactivity toward non-blood group antigens [10–12], several members of the galectin family, in particular galectin-4 (Gal-4) and galectin-8 (Gal-8), exhibited significant preference for blood group antigens [9, 13, 14]. Gal-4 and Gal-8 not only recognized blood group antigens on the glycan microarray, but these innate immune proteins also exhibited similar specificity toward blood group positive microbes [14]. These results strongly suggested that galectins might be uniquely poised to provide innate immunity against blood group positive microbes.

Although galectins have been implicated in a wide variety of biological processes [15], including antimicrobial immunity against mycotic organisms [16], whether galectins could directly impact bacterial viability remained untested. Gal-4 and Gal-8 not only recognized blood group positive microbes, but this recognition also resulted in direct killing [14]. These results suggest that Gal-4 and Gal-8 provide innate immunity against blood group positive microbes and thus fill an important gap in adaptive immunity against blood group positive microbes in blood group positive individuals. In addition to recognizing blood group positive microbes, recent studies using microbial glycan microarrays suggest that Gal-4 and Gal-8 display very specific interactions with a variety of microbes that share the common feature of expressing mammalian-like carbohydrate antigens, while failing to recognize microbes with unrelated antigens [17, 18]. Similar to the impact of Gal-4 and Gal-8 on blood group positive microbes, galectin engagement of additional gram-negative and gram-positive microbes that express other mammalian-like antigens likewise results in impaired microbial viability [17, 18]. These results strongly suggest that galectins may provide a unique and broad form of innate immunity against molecular mimicry. In contrast to their ability to recognize and kill microbes expressing self-like antigens, Gal-4 and Gal-8 recognize the same antigenic determinants on mammalian cells, yet fail to induce detectable changes in membrane integrity or cellular viability [9, 14, 18]. While complex

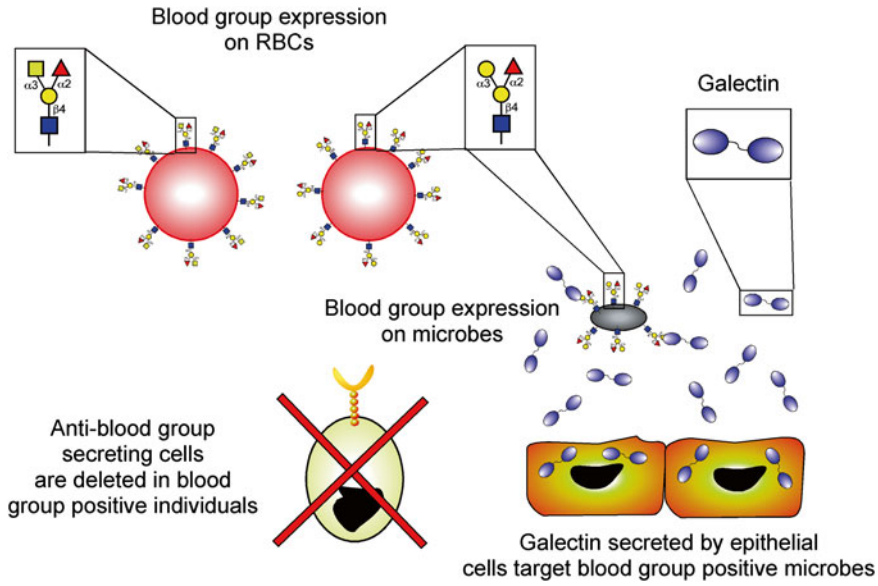

Fig. 1 Galectins provide innate immunity against blood group positive microbes. While elimination of self-reactive immune cells reduces the probability of autoimmunity, a fitness cost would be anticipated due to the reduced ability of an individual to respond to self-like antigens on pathogens. However, compensatory mechanisms at the level of innate immunity may exist whereby individuals protect themselves against self-like antigens. Gal-4 and Gal-8 uniquely recognize pathogens baring self-antigens, providing a possible mechanism whereby individuals protect themselves against self-antigen positive pathogens despite reduced adaptive immunity toward these microbes

pathways, such as those that regulate complement, typically protect mammalian cells from injury [19], Gal-4 and Gal-8 appear to possess the intrinsic ability to recognize the exact same antigenic determinant on a microbe and mammalian cell, while only killing the microbe. As a result, galectin antimicrobial activity represents a unique form of immunity with significant implications in innate immune protection against molecular mimicry (Fig. 1).

As Gal-4- and Gal-8-mediated immunity requires recognition of self-like antigens on microbes, this form of immunity fundamentally differs from defensins and other cationic antimicrobial peptides that engage features unique to an intact microbial membrane [20, 21]. The requirement of Gal-4 and Gal-8 for distinct antigen recognition not only suggests their potential role in immunological protection against molecular mimicry, but also requires unique considerations when assessing microbial recognition and killing. In this work, we describe the methods employed to examine the antimicrobial activity of Gal-4 and Gal-8 toward microbes, using blood group positive microbes as a model system.

2 Materials

2.1 Bacteria Preparation

1. Luria Bertani (LB) media (Difco).
2. *E. coli* O86 (ATCC 12701).
3. Luria Bertani agar (Difco).
4. 100 × 15 mm sterile disposable petri dishes (Fisher).
5. 17 × 100 mm polystyrene tubes, dual-position cap, sterile (USA Scientific).
6. 100 mL Erlenmeyer flask.
7. 200 mL Erlenmeyer flask.
5. Bacteria cell spreader (sterile).
9. Spectrophotometer.
10. 37 °C degree incubator/shaker.
11. 37 °C incubator (stationary).
12. Autoclave.

2.2 Galectin Preparation

1. Recombinant galectin.
2. PD10 column (GE Healthcare).
3. 0.2 μm filter (Fisher).
4. Phosphate-buffered saline standard pH 7.4 (Hyclone).
5. Spectrophotometer.

2.3 Assessing Antimicrobial Activity

1. Luria Bertani media (Difco).
2. Luria Bertani agar (Difco).
3. Phosphate-buffered saline standard pH 7.4 (Hyclone).
4. Bacteria cell spreader (sterile).
6. Sterile 96-well, round-bottom plate.
7. 12 × 75 mm polystyrene tubes with dual-position polyethylene caps (USA Scientific).
8. 1.7 mL snap cap microcentrifuge tube (Sigma-Aldrich).
9. 37 °C degree incubator/shaker.
10. 37 °C incubator (stationary).

3 Methods

3.1 Bacteria Preparation

1. Bacterial glycerol stocks should be stored at −80 °C.
2. Using a sterilized loop under a sterile hood, transfer a loop of glycerol stock to 1 mL of sterile LB media in a sterile 15 mL culture tube (17 × 100 mm polystyrene tube).

3. Incubate culture at 37 °C overnight on an orbital shaker at 250 revolutions per minute (rpm).
4. Following the overnight incubation, dilute culture 1:100 into LB media by placing 0.05 mL of the overnight culture in 5 mL of sterile LB media.
5. Allow this culture to incubate at 37 °C on an orbital shaker for 2.5–3 h.
6. Remove 0.5 mL of culture from the sample and determine the OD at 600 nm (OD600). If the OD600 is between 0.3 and 0.5 then proceed to **step 7**. If the value is below 0.3, continue to check the culture every 30 min until an OD600 of between 0.3 and 0.5 is achieved. If the culture is over 0.5 OD600 then repeat **step 4** with examination of the OD600 at 30-min intervals until an OD600 of 0.3 is achieved.
7. Dilute the culture 1:100 by placing 0.5 mL of the culture into 50 mL of sterile LB media in an autoclaved 200 mL Erlenmeyer flask.
8. Every 30 min, remove 0.6 mL of this culture. Place 100 μL of this sample into a sterile Eppendorf tube and use the rest to record the OD600 of the culture.
9. To determine the corresponding CFU value of the culture to the OD600 reading, perform 6 tenfold serial dilutions in sterile LB media.
10. Plate 50 μL of each dilution onto three separate LB agar plates and spread the suspension over the entire face of the agar plate using a sterile bacterial cell spreader.
11. Allow plate to dry for 2–3 min under tissue culture hood.
12. Incubate plates face down at 37 °C for 12 h or overnight and then remove plates from incubator.
13. Repeat **steps 7–10** every 30 min to obtain an OD600 value with a corresponding set of dilutions plated on LB agar for CFU enumeration.
14. Following **step 12**, count the number of colony-forming units on the corresponding plate (*see* **Note 1**).
15. Use the dilution calculations to extrapolate the number of CFUs in the starting material of each sample.
16. Once the relationship between OD600 and CFU has been established, use the OD600 readings to provide an estimate of the number of bacteria typically present at a particular OD600 value. Additionally, use these data to determine the normal growth kinetics of the bacterial culture.

3.1.1 Preparing E. coli 086 for Bacteria Killing Assay

17. Inoculate a fresh overnight culture of *E. coli* O86 and grow overnight shaking at 37 °C as outlined in **steps 1–3** of Subheading 3.1.

18. Use 100 μL of this overnight culture to re-inoculate 2 mL of sterile culture media.
19. Grow bacteria to midlog phase (approximately OD600 0.3–0.5, or as determined empirically in **steps 1–16**).
20. Re-inoculate fresh sterile culture media for killing assay at a 1:100 dilution (*see* **Note 2**).
21. Grow fresh culture to midlog phase.
22. Dilute culture in sterile LB mediate to 10^8 cells/mL (as determined empirically in **steps 1–16**) (*see* **Note 3**).

3.2 Galectin Preparation

1. Thaw frozen galectin stocks on ice at 4 °C.
2. To remove βME and lactose, prepare a PD10 column for gel filtration by equilibrating the column with 5 column volumes of cold PBS (pH 7.4) (*see* **Note 4**).
3. Add 1 mL of the recombinant galectin solution to the PBS-re-equilibrated PD10 column and collect 0.5 mL fractions.
4. Following complete penetration of the galectin solution into the PD10 column, add 2 mL of cold PBS.
5. Continue to collect 0.5 mL fractions while adding additional PBS as needed to prevent the column from drying.
6. To determine which fractions may have galectin protein, examine protein content by simply measuring the OD at 280 nm (OD280) of each fraction. This is typically done by diluting each fraction tenfold (i.e., 10 μL of each fraction in 90 μL PBS) followed by examination of the OD280 (*see* **Note 5**).
7. Once positive fractions are identified, pool galectin-containing fractions and reevaluate the OD280 to determine the final concentration of pooled galectin.
8. Filter-sterilize galectin using 0.2 μm filter. Maintain galectin sterility after this point (*see* **Note 6**).
9. Once the galectin has been filtered, reevaluate the OD280 to determine the final galectin concentration.
10. Dilute galectin to the appropriate concentration with sterile PBS using aseptic technique under a sterile hood (*see* **Note 7**).
11. In addition, hapten inclusion control solutions, which consist of LB solution containing 100 mM TDG (galectin inhibitor) or 100 mM sucrose (non-inhibitor control), may be added to control samples. Control solutions are diluted fivefold to achieve a final concentration of 20 mM in control samples (*see* **Note 8**).

3.3 Assessing Antimicrobial Activity

1. Once galectin is diluted to the appropriate concentration and bacterial cultures are grown to midlog phase, a final bacteria concentration of 10^8 bacteria/mL should be obtained.

2. Mix galectin and bacteria and place in optimal growth conditions for the microbe being examined.
3. Following 2-h incubation, bacteria should be immediately removed (*see* **Note 9**).
4. To determine the corresponding CFU value of each condition, perform 6 tenfold serial dilutions in sterile LB media.
5. Plate 50 μL of each dilution onto three separate LB agar plates and spread the suspension over the entire face of the agar plate using a sterile bacteria cell spreader (*see* **Note 1**).
6. Allow plate to dry for 2–3 min under tissue culture hood.
7. Incubate plates face down at 37 °C for 12 h or overnight and then remove plates from incubator.
8. Count the number of colony-forming units for each dilution.
9. Use the dilution calculations to extrapolate the number of CFUs in the starting material of each sample (*see* **Notes 10** and **11**).

4 Notes

1. Meticulous care should be employed when enumerating CFUs using these methods to ensure that the potential antimicrobial activity of galectins is not over- or underestimated. In addition, the drop plate method can be used in place of the spread method for CFU enumeration [22]. Each of these methods possesses intrinsic advantages and limitations [22].
2. The number of samples needed to do a particular experiment will dictate the amount of media used for this step. When examining alternative microbes, follow recommended guidelines for ideal growth conditions and empirically determine the optimal conditions for examining potential galectin antimicrobial activity.
3. Bacteria used in killing assays should be in midlog phase. This is the reason for repeat inoculations. Inoculation with overnight culture will contain mostly dead or stationary post-log-phase bacteria. However, following a second inoculation, bacteria that are mostly in the log phase of growth will be present. This may be important during the killing assay, as many bacteria are susceptible to particular killing mechanisms during active growth. In addition, examination of microbes harvested from post-log growth conditions often results in cultures with significant lipopolysaccharide (LPS) contamination. As the blood group antigen on blood group positive microbes resides on LPS, significant increases in LPS concentrations secondary to overgrowth conditions may artificially inhibit galectin-mediated antimicrobial activity.

4. βME may interfere with the biological activity of the microbe. In addition, lactose can readily inhibit galectin-carbohydrate interactions. Inclusion of lactose can be used as a control but must be removed to assess the potential biological activity of a particular galectin in the absence of hapten inhibitors. When assessing the potential dependency of galectin microbial interactions or antimicrobial activity, thiodigalactoside (TDG) may be employed as an inhibitor of galectin-carbohydrate interaction instead of lactose, as it is relatively inert to metabolism.
5. To extrapolate the protein concentration from the OD280 values, use the extinction coefficient for the particular galectin being examined to calculate actual concentration in mg/ml. The following websites, http://www.basic.northwestern.edu/biotools/proteincalc.html and http://web.expasy.org/protparam/protparam-doc.html, offer explanation and assistance in calculating the extinction coefficient and using this calculation to determine the actual concentration of a given protein in mg/ml, including caveats as to how these numbers may differ from the actual values. As methods of calculating the extinction coefficient only provide estimates, alternative approaches can be used to empirically determine these values. These include using a Bradford assay to calculate protein concentration or simply re-equilibrating the recombinant protein directly into water, lyophilizing, weighing, and then resuspending in the buffer of choice followed by empirically determining the extinction coefficients for a particular galectin family member.
6. This should be done before a final concentration is determined and final dilutions are made since some galectin could be lost during the sterile filtration step.
7. Typically a target concentration of five times the desired final concentration is employed to allow for a 1:5 dilution of galectin with the target bacteria.
8. Both lactose and TDG are effective inhibitors of galectin carbohydrate binding. Lactose is typically easier to obtain, but also can be metabolized with a variety of potential alterations in the test system. Incubation of hapten solutions with recombinant galectins prior to introducing microbes should facilitate hapten engagement of galectin prior to galectin incubation with microbes.
9. A 2-h incubation is not necessary to observe antimicrobial activity. Shorter intervals may be used and can be emperically determined. Longer intervals allow easy visual determination of the potential antimicrobial activity of a particular galectin for a given microbe before plating.
10. Plating accuracy is the most challenging step of this assay. As a result, significant care should be taken when plating samples

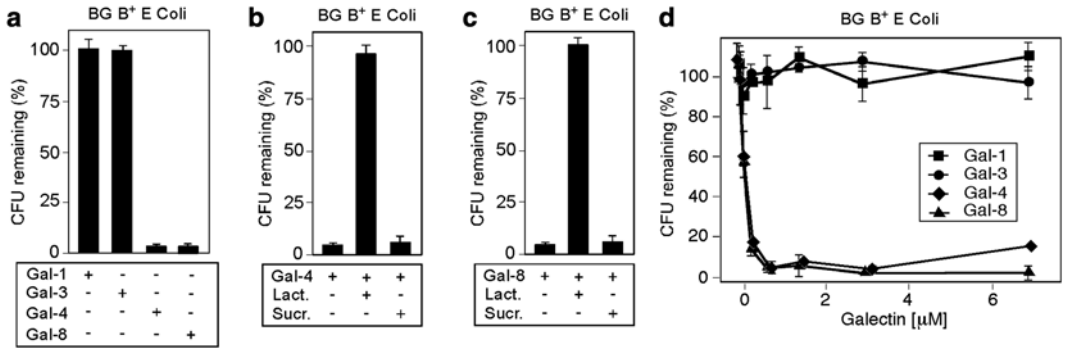

Fig. 2 Galectin-4 and galectin-8 killing of blood group positive microbes requires carbohydrate recognition. Quantified viable bacteria after BGB$^+$ *E. coli* were mixed with 5 μM Gal-1, Gal-3, Gal-4, or Gal-8 (**a**), 5 μM Gal-4 with or without 20 mM lactose (Lact.) or 20 mM sucrose (Sucr.) (**b**), 5 μM Gal-8 with or without 20 mM lactose (Lact.) or 20 mM sucrose (Sucr.) (**c**), or the indicated concentrations of Gal-1, Gal-3, Gal-4, and Gal-8 (**d**). In each experiment, bacteria were quantified by dilution plating. Error bars represent means ± s.d. This research was originally published in Nature Medicine. Stowell SR, Arthur CM, Dias-Baruffi M, Rodrigues LC, Gourdine JP, Heimburg-Molinaro J, Ju T, Molinaro RJ, Rivera-Marrero C, Xia B, Smith DF, Cummings RD. Innate immune lectins kill bacteria expressing blood group antigen. 2010 Mar;16(3):295-301

on LB agar to ensure even distribution of the sample across the entire agar plate.

11. CFU counts in PBS-treated control samples can be used to represent 100 % of normal growth for a given bacteria. Galectin antimicrobial activity may be shown as an extrapolation of the difference in CFU between PBS-treated and galectin-treated bacteria, typically shown as "CFU remaining (%)" (*see* Fig. 2).

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Chapter 28

Examination of the Role of Galectins in Pre-mRNA Splicing

Ronald J. Patterson, Kevin C. Haudek, Patricia G. Voss, and John L. Wang

Abstract

Several lines of evidence have been accumulated to indicate that galectin-1 and galectin-3 are two of the many proteins involved in nuclear splicing of pre-mRNA. First, nuclear extracts, capable of carrying out splicing of pre-mRNA in a cell-free assay, contain both of the galectins. Second, depletion of the galectins from nuclear extracts, using either lactose affinity chromatography or immunoadsorption with antibodies, results in concomitant loss of splicing activity. Third, addition of either galectin-1 or galectin-3 to the galectin-depleted extract reconstitutes the splicing activity. Fourth, the addition of saccharides that bind to galectin-1 and galectin-3 with high affinity (e.g., lactose or thiodigalactoside) to nuclear extract results in inhibition of splicing whereas parallel addition of saccharides that do not bind to the galectins (e.g., cellobiose) fail to yield the same effect. Finally, when a splicing reaction is subjected to immunoprecipitation by antibodies directed against galectin-1, radiolabeled RNA species corresponding to the starting pre-mRNA substrate, the mature mRNA product, and intermediates of the splicing reaction are coprecipitated with the galectin. Similar results were also obtained with antibodies against galectin-3. This chapter describes two key assays used in our studies: one reports on the splicing activity by looking at product formation on a denaturing gel; the other reports on the intermediates of spliceosome assembly using non-denaturing or native gels.

Key words RNA processing, Pre-mRNA splicing, Spliceosome, Cell-free splicing assay, Galectin

1 Introduction

Early immunofluorescence studies noted that, in addition to a diffuse distribution in the nucleoplasm, galectin-3 (Gal3) appears to be localized with a number of discrete punctate structures designated as speckles [1]. Gal3 is colocalized in certain of these speckles with two other proteins identified as components of the splicing machinery: the Sm core polypeptides of small nuclear ribonucleoprotein complexes (snRNPs) and the serine- and arginine-rich (SR) family of splicing factors [2, 3]. At the ultrastructural level, these speckled structures have been found to correspond to interchromatin granule clusters and perichromatin fibrils [4]. Indeed, immunogold labeling at the ultrastructural level has localized Gal3

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in perichromatin fibrils [5], which represent nascent transcripts at the site of mRNA synthesis and early events of pre-mRNA processing. Moreover, when nucleoplasm was subjected to sedimentation on a cesium sulfate gradient (1.25–1.75 g/ml), immunoblotting yielded Gal3 in fractions with densities of 1.3–1.35 g/ml [4], matching the reported densities of hnRNP (heterogeneous nuclear RNP) and snRNP [6]. Inasmuch as both hnRNP and snRNP have been documented to play a role in nuclear pre-mRNA splicing [7, 8], we tested for a similar role for Gal3.

Our knowledge on the splicing of pre-mRNAs has advanced rapidly due to the availability of nuclear extracts (NEs) that can execute the cleavage and ligation reactions of intron removal and exon joining. The NEs we used are derived from HeLa cells [9]. In the course of our studies, we found that galectin-1 (Gal1) was also in these NEs (along with Gal3) and therefore, our studies have included Gal1 as well. The pre-mRNA substrate used in our experiments is a synthetic gene construct, containing two exons separated by an intervening sequence [10]. In the course of the splicing reaction, the various components of the splicing machinery assemble into a macromolecular complex, designated as the spliceosome. Various intermediates in this assembly process can be distinguished, on the basis of their protein and RNA content. Initially, the pre-mRNA is complexed with hnRNP to form the so-called H-complex. Addition of U1 snRNP in the absence of ATP results in an E-complex, committed to the splicing pathway. In the presence of ATP, various other uracil-rich snRNPs are added to form the A-complex, B-complex, and the active spliceosome in the C-complex [11].

This chapter describes two key assays used in our studies: one reports on the splicing activity by looking at product formation on a denaturing gel (Fig. 1, left); the other reports on the intermediates of spliceosome assembly on non-denaturing or native gels (Fig. 1, right). The nuclear extract and the ^{32}P-labeled pre-mRNA substrate are mixed at 30 °C. After a given time period for the splicing reaction, the RNA components of the reaction mixture are extracted and resolved on a 13 % acrylamide-8.3 M urea denaturing gel system. The starting pre-mRNA substrate, the mature RNA product, and the intermediates of the splicing reaction can all be discriminated by this gel system. As shown on the extreme left-hand side of Fig. 1, this splicing reaction is strictly dependent on ATP as, in its absence, the pre-mRNA is recovered. The second assay, which reports on the intermediates of spliceosome assembly, corresponds to a gel mobility shift assay. In our gel system, naked ^{32}P-labeled pre-mRNA migrates off the bottom of the gel. When complexed with proteins and RNP, the H- and E-complexes are observed to retard the mobility of the pre-mRNA and the radioactive species is detected at the bottom of the gel. Our gel system cannot distinguish H-complex and E-complex, so they are lumped together at

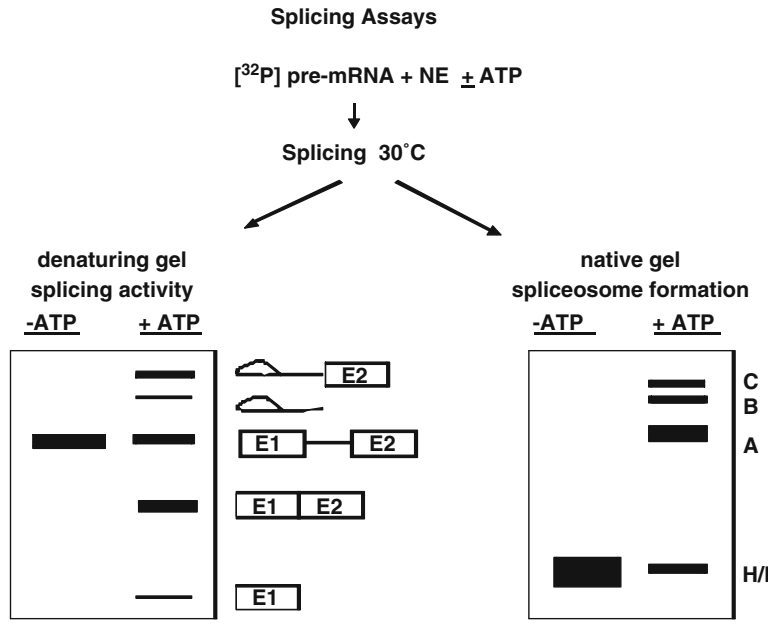

Fig. 1 Schematic diagram illustrating the two key assays used in documenting a role for Gal1 and Gal3 in pre-mRNA splicing. Nuclear extract (NE) derived from HeLa cells are incubated with ^{32}P-labeled pre-mRNA substrate in a splicing reaction. When the RNA components of the splicing reaction are analyzed on a denaturing gel (*left*), radioactive bands corresponding to the starting pre-mRNA substrate, the mature mRNA product, as well as intermediates of the splicing reaction can be resolved. For the starting pre-mRNA substrate, the two rectangles labeled E1 and E2 represent exons and the *solid line* connecting the rectangles represents the intron. There are two products of the splicing reaction: (a) the mature mRNA product is represented by two contiguous rectangles (E1 spliced onto E2); (b) the excised intron is shown as a lariat. Two intermediates of the splicing reaction are also shown: (a) the lariat intron linked to E2; (b) isolated E1. When the splicing reaction is analyzed on a native (non-denaturing) gel (*right*), intermediates in the assembly of the spliceosome are revealed by the positions of migration of the radioactive pre-mRNA. The letters on the right designate distinct complexes: H, E, A, B, and C

the bottom of the gel. The H- and E-complexes are well resolved, however, from the higher order A-, B-, and C-complexes. The formation of these higher order complexes is strictly ATP dependent, as is the splicing reaction itself (Fig. 1, right).

2 Materials

2.1 Nuclear Extract (NE) Preparation

1. Human HeLa S3 cells (National Cell Culture Center, Minneapolis, MN).
2. Dounce homogenizer (tight-fitting pestle).
3. Light microscope.

4. Nalgene Oak Ridge high-speed centrifuge tube, 10 ml.
5. Sorvall SS34 rotor.
6. Dialysis tubing (6–8 K molecular weight cutoff membrane).
7. Bradford assay kit.

Buffers (*see* **Notes 1–3**)

8. RSB: 10 mM Tris, pH 7.2, 10 mM NaCl, 1 mM $MgCl_2$.
9. Buffer A: 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT).
10. Buffer C: 20 mM HEPES, pH 7.9, 25 % (vol/vol) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethyl-sulfonyl fluoride (PMSF), 0.5 mM DTT.
11. Buffer D: 10 mM HEPES, pH 7.9, 20 % (vol/vol) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT.

2.2 Splicing Substrate

1. BamHI restriction endonuclease.
2. MINX plasmid DNA [10].
3. SP6 RNA polymerase (20,000 U/ml) (New England Biolabs).
4. SP6 buffer, provided with SP6 RNA polymerase (40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine) (New England Biolabs).
5. Ribonucleoside triphosphate solution (6.25 mM CTP; 6.25 mM ATP; 6.25 mM UTP; 1.25 mM GTP).
6. 7-Methylguanosine cap analog (10 mM) (New England Biolabs).
7. RNasin (40 U/μl) (Promega).
8. Dithiothreitol (DTT) (10 mM).
9. α-^{32}P[GTP] (3,000 Ci/mmol; 50 μCi) (PerkinElmer).
10. DEPC-treated H_2O (*see* **Note 3**).
11. Sodium acetate (3 M).
12. Ethanol (200 proof).
13. Phenol-chloroform (50:50 v/v).
14. Chloroform-isoamyl alcohol (25:1 v/v).
15. 5 % Trichloroacetic acid.

Buffer (*see* **Notes 1–3**)

16. TE: 1.25 mM Tris, pH 8, 1 mM EDTA.

Special equipment

17. Scintillation counter.

2.3 Assembly of Splicing Reaction Mixture and Analysis of Products

2.3.1 Splicing Reaction

1. Nuclear extract (NE) (Subheading 3.1).
2. DEPC H_2O (*see* **Note 3**).
3. ^{32}P-MINX splicing substrate (approximately 10,000 cpm in final reaction volume) (Subheading 3.2).
4. $MgCl_2$.
5. ATP.
6. Creatine phosphate (20 mM).
7. Dithiothreitol (DTT).
8. RNasin (20 U) (Promega).
9. Proteinase K (20 mg/ml)-SDS (0.5 %) in DEPC H2O.

Buffers (*see* **Notes 1–3**)

10. Buffer D: 10 mM HEPES, pH 7.9, 20 % glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT.

2.3.2 RNA Extraction

1. Sodium acetate (3 M).
2. Phenol-chloroform (50:50 v/v).
3. Chloroform-isoamyl alcohol (25:1 v/v).
4. Ethanol (200 proof).

Buffers (*see* **Notes 1–3**)

5. TE: 1.25 mM Tris, pH 8, 1 mM EDTA.

Special equipment

6. Speed Vac Concentrator (Savant).

2.3.3 Denaturing Gel Electrophoresis

1. 13 % Polyacrylamide (bisacrylamide:acrylamide, 1.9:50 [wt/wt]) with 8.3 M urea in TBE.
2. 3M Whatman filter paper.

Buffers

3. RNA loading buffer: 90 % formamide, 20 mM EDTA, pH 8.0, 0.05 % bromophenol blue (w/v).
4. TBE buffer for RNA gels: 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA.

Special equipment

5. Vacuum gel dryer system.

2.3.4 Native Gel Electrophoresis for Analyzing Complex Formation

1. Heparin.
2. Glycerol (containing 0.2 % each bromophenol blue, xylene cyanol, and phenol red).
3. 4 % Polyacrylamide gel (bisacrylamide-acrylamide, 1:80 [wt/wt]).

Buffers

4. RNA loading buffer: 90 % formamide, 20 mM EDTA, pH 8.0, 0.05 % bromophenol blue (w/v).
5. TBE buffer for RNA gels: 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA.

Special equipment

6. Vacuum gel dryer system.

2.4 Perturbation of the Splicing Reaction by Reagents Related to Gal3

2.4.1 Testing the Effect of Addition of the Amino-Terminal Domain (ND), Carboxyl-Terminal Domain (CD), or the Full-Length Polypeptide (rGal3)

1. Nuclear extract (NE) (Subheading 3.1).
2. Sodium chloride (NaCl) (5 M).
3. rGal3 amino-terminal domain (ND).
4. rGal3 carboxyl-terminal domain (CD).
5. rGal3 full-length polypeptide.
6. ^{32}P-MINX splicing substrate (approximately 10,000 cpm in final reaction volume) (Subheading 3.2).
7. $MgCl_2$.
8. ATP.
9. Creatine phosphate (20 mM).
10. Dithiothreitol (DTT).
11. RNasin (40 U/μl) (Promega).
12. Proteinase K (20 mg/ml) – SDS (0.5 %) in DEPC H_2O.

Buffers

13. 60 %D: 60 % Buffer D and 40 % H_2O.
14. RNA loading buffer: 90 % formamide, 20 mM EDTA, pH 8.0, 0.05 % bromophenol blue (w/v).
15. TBE buffer for RNA gels: 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA.

Special equipment

16. 12-Well microdialyzer with a 6–8 K molecular weight cutoff dialysis membrane.

2.4.2 Testing the Effect of Addition of Anti-Gal3 Antibodies (Same as Subheading 2.4.1)

All materials listed in Subheading 2.4.1 are needed in addition to the materials listed below.

1. Rat monoclonal anti-Mac-2.
2. Mouse monoclonal NCL-GAL3.

2.4.3 Testing the Effect of Addition of Synthetic Peptides Containing Gal3 Sequences

All materials listed in Subheading 2.4.1 are needed in addition to the materials listed below

1. DEPC H_2O (*see* **Note 3**).
2. Synthetic peptides: (a) 9-mer, (b) 18-mer, and (c) 27-mer synthetic peptides (*see* **Note 7**).

3. Sodium acetate (3 M).
4. Chloroform-isoamyl alcohol.
5. Ethanol (200 proof).
6. ^{32}P-MINX splicing substrate (approximately 10,000 cpm in final reaction volume).
7. $MgCl_2$.
8. ATP.
9. Creatine phosphate (20 mM).
10. Dithiothreitol (DTT).
11. RNasin (40 U/μl) (Promega).
12. Proteinase K (20 mg/ml)-SDS (0.5 %).
13. 13 % polyacrylamide (bisacrylamide:acrylamide, 1.9:50 [wt/wt]) with 8.3 M urea in TBE.
14. 3M Whatman filter paper.

Buffers (*see* **Notes 1–3**)

15. Buffer D.
16. TE: 1.25 mM Tris, pH 8, 1 mM EDTA.
17. Phenol-chloroform (50:50 v/v).
18. RNA loading buffer: 90 % formamide, 20 mM EDTA, pH 8.0, 0.05 % bromophenol blue (w/v).
19. TBE buffer for RNA gels: 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA.

Special equipment

20. Speed vacuum.
21. Vacuum gel dryer system.

2.4.4 Testing the Effect of Addition of Saccharides

All materials listed in Subheading 2.4.1 are needed in addition to the materials listed below

1. DEPC H_2O (*see* **Note 3**).
2. Lactose.
3. Cellobiose.
4. Thiodigalactoside.
5. Sodium acetate (3 M).
6. Chloroform-isoamyl alcohol.
7. Ethanol (200 proof).
8. ^{32}P-MINX splicing substrate (approximately 10,000 cpm in final reaction volume).
9. $MgCl_2$.
10. ATP.

11. Creatine phosphate (20 mM).
12. Dithiothreitol (DTT).
13. RNasin (40 U/μl) (Promega).
14. Proteinase K (20 mg/ml)-SDS (0.5 %).
15. 13 % polyacrylamide (bisacrylamide:acrylamide, 1.9:50 [wt/wt]) with 8.3 M urea in TBE.
16. 3M Whatman filter paper.

Buffers (*see* **Notes 1–3**)

17. Buffer D.
18. TE: 1.25 mM Tris, pH 8, 1 mM EDTA.
19. Phenol-chloroform (50:50 v/v).
20. RNA loading buffer: 90 % formamide, 20 mM EDTA, pH 8.0, 0.05 % bromophenol blue (w/v).
21. TBE buffer for RNA gels: 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA.

Special equipment

22. Speed vacuum.
23. Vacuum gel dryer system.

2.5 Gal1 and Gal3: Depletion and Reconstitution of Splicing Activity in NE

2.5.1 Preparation of Beads for Immunoadsorption

1. Protein G-Sepharose beads.
2. Deionized H_2O.
3. Antiserum (*see* **Note 8**).
4. Dimethylpimelimidate (Sigma-Aldrich).
5. Sodium chloride (NaCl) (6.5 M).

Buffers (*see* **Notes 1–3**)

6. Wash buffer: 20 mM HEPES, pH 7.9, 0.5 M NaCl.
7. HEPES buffer, pH 7.9.
8. 0.2 M sodium borate (pH 9).
9. 0.2 M ethanolamine (pH 8).

Special equipment

10. Microdialyzer, with a dialysis membrane with 10 K cutoff.

2.5.2 Depletion of Gal1 and Gal3 from NE

1. Nuclear extract (NE) (Subheading 3.1).
2. α-Lactose (Lac) immobilized to 6 % beaded agarose (Lac-A).
3. Cellobiose immobilized to agarose (Cello-A).
4. Wash buffer: 20 mM HEPES, pH 7.9, 0.5 M NaCl.
5. Hamilton syringe.
6. 60 %D containing 2.5 mM $MgCl_2$, 1 mM ATP, and 5 mM creatine phosphate.

7. Sodium chloride (NaCl).
8. 13 % Polyacrylamide (bisacrylamide:acrylamide, 1.9:50 [wt/wt]) with 8.3 M urea in TBE.

Buffers

9. RNA loading buffer: 90 % formamide, 20 mM EDTA, pH 8.0, 0.05 % bromophenol blue (w/v).
10. TBE buffer for RNA gels: 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA.

2.5.3 Reconstitution of Splicing Activity by Addition of Recombinant Gal1 and Gal3

1. Recombinant proteins tested for reconstitution of splicing activity (*see* **Note 11**).
2. Depleted and non-depleted NE.
3. ^{32}P-MINX splicing substrate (approximately 10,000 cpm in final reaction volume) (Subheading 3.2).
4. $MgCl_2$.
5. ATP.
6. Creatine phosphate (20 mM).
7. Dithiothreitol (DTT).
8. RNasin (40 U/μl) (Promega).
9. Proteinase K (20 mg/ml)-SDS (0.5 %) in DEPC H_2O.
10. 13 % polyacrylamide (bisacrylamide:acrylamide, 1.9:50 [wt/wt]) with 8.3 M urea in TBE.
11. 3M Whatman filter paper.

Buffers (*see* **Notes 1–3**)

12. TE: 1.25 mM Tris, pH 8, 1 mM EDTA.
13. Phenol-chloroform (50:50 v/v).
14. RNA loading buffer: 90 % formamide, 20 mM EDTA, pH 8.0, 0.05 % bromophenol blue (w/v).
15. TBE buffer for RNA gels: 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA.

Special equipment

16. Speed vacuum.
17. Vacuum gel dryer system.

3 Methods

3.1 Nuclear Extract (NE) Preparation

Human HeLa S3 cells were grown in suspension culture by the National Cell Culture Center and were shipped as a cell pellet (generally derived from 10 to 20 L cultures) on wet ice. The following procedure is described for 20 ml of packed cells. NE was

prepared essentially as described by Dignam et al. [9]. All steps described below for the preparation of nuclear extract should be carried out at 4 °C.

1. Resuspend the HeLa S3 cell pellet as received from NCCC in 100 ml of RSB and re-pellet by centrifugation at 1,000 × *g* for 5 min.
2. Remove supernatant, resuspend the cell pellet in 40 ml of Buffer A, and allow cells to swell on ice for 10 min.
3. Lyse cells by Dounce homogenization (tight-fitting pestle) 10–15 times and check for lysis under a light microscope using a 40× objective (should have >95 % cell lysis).
4. Pellet nuclei from homogenized cells by centrifugation at 2,500 × *g* for 10 min.
5. Remove supernatant and resuspend the nuclear pellet in 5 ml of Buffer A. Transfer the resuspended pellet to a 10 ml Nalgene Oak Ridge high-speed centrifuge tube.
6. Pellet nuclei by centrifuging using in a Sorvall SS34 rotor at 15,000 rpm for 10 min.
7. Discard the supernatant and resuspend the nuclear pellet in 10 ml of Buffer C.
8. Disperse the nuclear pellet by Dounce homogenization (1–2 times) and stir on ice for 30 min (to extract nuclear components).
9. Pellet the nuclei by centrifugation in a Sorvall SS34 rotor at 15,000 rpm for 15 min.
10. Dialyze the supernatant (which contains the NE) against 1 L of Buffer D for 4–5 h, aliquot into 100–200 μl samples, and snap-freeze in a dry ice–liquid N_2 bath before storing at −80 °C.
11. Determine protein concentrations by the Bradford assay [12]. The protein concentration of NE is generally 4–6 mg/ml.

3.2 Splicing Substrate

Pre-mRNA substrates are transcribed from plasmids containing the splicing substrate DNA sequence under the control of T3, T7, or SP6 RNA polymerase promoters. The MINX substrate contains two exonic sequences and one intronic sequence from adenovirus; the plasmid was obtained from Dr. Susan Berget (Baylor College of Medicine, Houston) [10]. A β-globin splicing substrate, obtained from Dr. Jeff Patton (Medical College of South Carolina, Columbia), and a control splicing substrate (SP65 RNA) with no consensus splice site sequences have been previously described [13, 14]. We have carried out most of our experiments with the MINX substrate and the procedures described below pertain to purified MINX plasmid DNA linearized with BamHI restriction endonuclease.

1. Mix the following components for a transcription reaction: (a) 2 μl containing 3 μg of linearized MINX DNA; (b) 2 μl of SP6 RNA polymerase (20,000 U/ml); (c) 2.5 μl of manufacturer's SP6 buffer (40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine); (d) 1 μl of a stock ribonucleoside triphosphate solution (6.25 mM CTP; 6.25 mM ATP; 6.25 mM UTP; 1.25 mM GTP); (e) 5 μl of 7-methylguanosine cap analog (10 mM); (f) 1 μl of RNasin (40 U/μl); (g) 2.5 μl DTT (10 mM); (h) 5 μl containing α-^{32}P[GTP] (3,000 Ci/mmol; 50 μCi); and (i) 5 μl DEPC-treated H_2O. The transcription reaction is incubated for 70 min at 37 °C.
2. Treat the transcription mixture with 2 μl of RNase-free DNaseI (200 mg/ml) for 15 min at 37 °C to digest the DNA template.
3. After the digestion, add 65 μl TE and 10 μl of 3 M sodium acetate.
4. The transcribed RNA is then isolated by extraction with 200 μl of phenol-chloroform (50:50 v/v) followed by extraction 180 μl of chloroform-isoamyl alcohol (25:1 v/v).
5. After removal of the organic phase, approximately 100 μl of aqueous phase should remain. Spot 1 μl of this aqueous phase on each of the two Whatman GF/C filters.
6. Allow one filter to dry. Subject the second filter to precipitation by immersion in a 50 ml solution of 5 % trichloroacetic acid for 10 min.
7. After air-drying, subject the radioactivity on each filter to scintillation counting. The radioactivity of the precipitated filter should be at least 40 % of the radioactivity on the filter without precipitation.
8. After the 1 μl samples have been aliquoted for the determination of radioactivity, immediately precipitate the RNA with 300 μl of 200 proof cold ethanol and store at –20 °C. Radiolabeled splicing substrate can be used for 4–6 weeks post-transcription.

3.3 Assembly of Splicing Reaction Mixture and Analysis of Products

The splicing reaction is generally carried out in a total volume of 10 μl.

3.3.1 Splicing Reaction

1. Mix 4 μl of NE, 2 μl of Buffer D, 3 μl of DEPC H_2O, and 1 μl of a mix containing ^{32}P-MINX splicing substrate (approximately 10,000 cpm in final reaction volume), 2.5 mM $MgCl_2$, 1.5 mM ATP, 20 mM creatine phosphate, 0.5 mM DTT, and 20 U RNasin (Promega).
2. Incubate splicing mixture at 30 °C for 45 min.
3. Stop the splicing reaction by the addition of 2.5 μl of proteinase K (20 mg/ml)-SDS (0.5 %) and incubation at 37 °C for 20 min.

3.3.2 RNA Extraction

1. Dilute each sample with 75 µl of TE (1.25 mM Tris, pH 8, 1 mM EDTA) and 10 µl of sodium acetate (3 M).
2. Extract the RNAs in each sample by adding 200 µl of phenol-chloroform (50:50 v/v). The aqueous upper phase is subjected to a second extraction with 180 µl of chloroform-isoamyl alcohol (25:1 v/v).
3. Precipitate RNAs by adding 300 µl of 200 proof cold ethanol, invert to mix, and store overnight at –20 °C.
4. Collect the ethanol-precipitated RNA by centrifugation at 12,000 × *g* for 10 min at 4 °C.
5. Wash pellets with 150 µl of cold 200 proof ethanol and centrifuge at 4 °C for 15 min.
6. Carefully remove the supernatant by pipette and dry the pellets in a speed vac for 10–15 min.

3.3.3 Denaturing Gel Electrophoresis

The starting pre-mRNA substrate, the splicing intermediates, and the products of the splicing reaction can be resolved by denaturing gel electrophoresis (Fig. 1, left).

1. Resuspend the dried RNA pellet in 10 µl RNA loading buffer, gently vortex, heat to 75–85 °C for 1.5 min, and incubate on ice for 2 min.
2. Prepare a 20 ml solution containing 13 % polyacrylamide (bisacrylamide:acrylamide, 1.9:50 [wt/wt])—8.3 M urea in TBE and use this to cast gels 15 cm in length.
3. Using TBE as the running buffer, pre-run the gel at 400 V for 20 min.
4. Wash the wells with TBE running buffer, load the RNA samples, and electrophorese at constant 400 V for 3.5 to 4 h.
5. After electrophoresis, immerse the gel in distilled water and rotate for 10 min to remove the urea.
6. Vacuum dry the gel on 3 M Whatman filter paper and subject it to film for autoradiography. A typical result documenting the splicing activity of NE on the MINX substrate is illustrated in Fig. 2, lane 1.

3.3.4 Native Gel Electrophoresis for Analyzing Complex Formation

The formation of splicing complexes may be analyzed by native gel electrophoresis (Fig. 1, right).

1. Assemble splicing reaction mixtures as in Subheading 3.3.1 but incubate at 30 °C for 15–20 min rather than 45 min.
2. Add heparin (final concentration 0.6 mg/ml) and incubate the mixture at 30 °C for 15 min.
3. Before electrophoresis, add 1 µl glycerol (containing 0.2 % each bromophenol blue, xylene cyanol, and phenol red) to the sample.

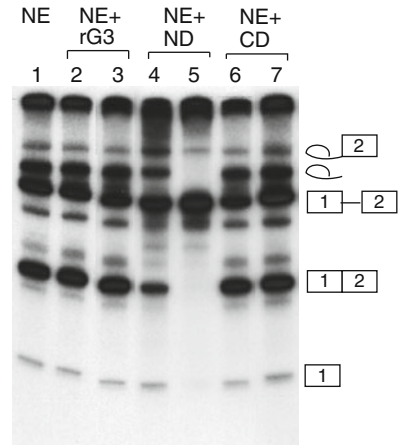

Fig. 2 Representative results of splicing reactions analyzed on denaturing gels, showing the effect of addition of Gal3 or its domains on product formation. NE: nuclear extract; rG3: recombinant Gal3; ND: the amino-terminal domain of Gal3; CD: the carboxyl-terminal domain of Gal3. Lane 1: NE only; lane 2: NE + 3 μM rG3; lane 3: NE + 12 μM rG3; lane 4: NE + 1.3 μM ND; lane 5: NE + 10 μM ND; lane 6: NE + 3.5 μM CD; lane 7: NE + 14 μM CD. The positions of migration of the pre-mRNA substrate, splicing intermediates, and RNA products are highlighted on the *right*

4. Load samples onto a pre-run (*see* **step 3** of Subheading 3.3.3) 4 % polyacrylamide gel (bisacrylamide-acrylamide, 1:80 [wt/wt]) and electrophorese in 0.5 M Tris and 0.5 M glycine, pH 8.8 at 4 °C and 25 V/cm for about 105 min.
5. Determine the migration of the splicing complexes by autoradiography following drying as in **steps 5** and **6** of Subheading 3.3.3.

3.4 Perturbation of the Splicing Reaction by Reagents Related to Gal3

3.4.1 Testing the Effect of Addition of the Amino-Terminal Domain (ND), Carboxyl-Terminal Domain (CD), or the Full-Length Polypeptide (rGal3)

1. Thaw NE, frozen and stored in Buffer D, on ice for 10–15 min and then centrifuge for 5 min at 12,000 × *g*.
2. Transfer the supernatant to a fresh 1.2 ml microfuge tube.
3. Add NaCl from a stock 5 M solution to a final concentration of 0.5 M and incubate on ice for 40 min.
4. Add 7 μl of the NE (in Buffer D containing 0.5 M NaCl) to each of several microfuge tubes containing (a) 5 μl of 60 %D; (b) 5 μl of rGal3ND ranging in final concentrations of 1.25–10 μM; (c) 5 μl of rGal3CD ranging in final concentrations of 3.5–57 μM; and (d) 5 μl of rGal3 ranging in final concentrations of 3–18 μM. Each sample contained a total volume of 12 μl (*see* **Note 4**).
5. Load each sample into a 12-well microdialyzer with a 6–8 K molecular weight cutoff dialysis membrane and dialyze against 60 %D for 75 min at 4 °C. Cover the top with parafilm to prevent evaporation.

6. Tilt the microdialyzer, remove 9 μl from each well, and transfer to a fresh microfuge tube.
7. Assemble splicing reactions in each of the microfuge tubes containing dialyzed NE from **step 6** above by adding 1 μl of a mix containing ^{32}P-MINX pre-mRNA (10,000 cpm), 2.5 mM $MgCl_2$, 1.5 mM ATP, 20 mM creatine phosphate, 0.5 mM DTT, and 20 U of RNasin.
8. Incubate at 30 °C for 45 min.
9. Stop the splicing reaction by the addition of 2.5 μl of proteinase K (20 mg/ml)-SDS (0.5 %) and incubate at 37 °C for 20 min.
10. After splicing reaction is stopped, analyze by following the steps outlined in Subheadings 3.3.2 and 3.3.3 (*see* **Note 5**).

3.4.2 Testing the Effect of Addition of Anti-Gal3 Antibodies

1. Thaw NE, frozen and stored in Buffer D, on ice for 10–15 min and then centrifuge for 5 min at 12,000 × *g*.
2. Transfer the supernatant to a fresh 1.2 ml microfuge tube.
3. Add NaCl from a stock 5 M solution to a final concentration of 0.5 M and incubate on ice for 40 min.
4. Add 7 μl of the NE (in Buffer D containing 0.5 M NaCl) to each of several microfuge tubes containing (a) 5 μl of 60 %D; (b) 5 μl of rat monoclonal anti-Mac-2 ranging in final concentrations of 30–150 nM; and (c) 5 μl of mouse monoclonal NCL-GAL3 ranging in final concentrations of 30–150 nM. Each reaction should contain a total volume of 12 μl (*see* **Note 6**).
5. Load each sample into a 12-well microdialyzer with a 6–8 K molecular weight cutoff dialysis membrane and dialyze against 60 %D for 75 min at 4 °C. Cover the top with parafilm to prevent evaporation.
6. Tilt the microdialyzer, remove 9 μl from each well, and transfer to a fresh microfuge tube.
7. Assemble splicing reactions in each of the microfuge tubes containing dialyzed NE from **step 6** above by adding 1 μl of a mix containing ^{32}P-MINX pre-mRNA (10,000 cpm), 2.5 mM $MgCl_2$, 1.5 mM ATP, 20 mM creatine phosphate, 0.5 mM DTT, and 20 U of RNasin. Incubate the splicing reactions at 30 °C for 45 min.
8. Stop the splicing reaction by the addition of 2.5 μl of proteinase K (20 mg/ml)-SDS (0.5 %) and incubate at 37 °C for 20 min.
9. After splicing reaction is stopped, analyze by following the steps outlined in Subheadings 3.3.2 and 3.3.3.

3.4.3 Testing the Effect of Addition of Synthetic Peptides Containing Gal3 Sequences

The ND contains multiple repeats of a 9-residue motif, PGAYPGXXX. Since the ND showed a dominant negative effect on the splicing activity of NE, synthetic peptides containing one, two, or three iterations of the PGAYPGQAP repeat may be tested for effects on the splicing reaction (*see* **Note 7**).

1. Add 4.6 μl of NE in Buffer D and 1.4 μl of Buffer D to each of several microfuge tubes containing (a) 9-mer, (b) 18-mer, and (c) 27-mer synthetic peptides in DEPC H_2O ranging in final concentration of 100–1,000 μM. The total volume in each tube should be 10 μl.
2. Incubate the tubes at 30 °C for 20 min.
3. Assemble splicing reactions in each of the microfuge tubes containing NE preincubated with synthetic peptides in **step 1** above by adding 1 μl of a mix containing ^{32}P-MINX pre-mRNA (10,000 cpm), 2.5 mM $MgCl_2$, 1.5 mM ATP, 20 mM creatine phosphate, 0.5 mM DTT, and 20 U of RNasin.
4. Incubate the splicing reactions at 30 °C for 45 min.
5. Stop the splicing reaction by the addition of 2.5 μl of proteinase K (20 mg/ml)-SDS (0.5 %) and incubate at 37 °C for 20 min.
6. After splicing reaction is stopped, analyze by following the steps outlined in Subheadings 3.3.2 and 3.3.3.

3.4.4 Testing the Effect of Addition of Saccharides

1. Add 3 μl aliquot of NE in Buffer D and 3 μl of Buffer D to each of several microfuge tubes containing (a) lactose, (b) cellobiose, and (c) thiodigalactoside in DEPC H_2O ranging in final concentration of 25–200 mM. The total volume in each tube should be 10 μl.
2. Incubate the tubes at 4 °C for 30 min.
3. Assemble splicing reactions in each of the microfuge tubes containing NE preincubated with saccharides in **step 1** above by adding 1 μl of a mix containing ^{32}P-MINX pre-mRNA (10,000 cpm), 2.5 mM $MgCl_2$, 1.5 mM ATP, 20 mM creatine phosphate, 0.5 mM DTT, and 20 U of RNasin.
4. Incubate the splicing reactions at 30 °C for 45 min.
5. Stop the splicing reaction by the addition of 2.5 μl of proteinase K (20 mg/ml)-SDS (0.5 %) and incubate at 37 °C for 20 min.
6. After splicing reaction is stopped, analyze by following the steps outlined in Subheadings 3.3.2 and 3.3.3.

3.5 Gal1 and Gal3: Depletion and Reconstitution of Splicing Activity in NE

3.5.1 Preparation of Beads for Immunoadsorption

1. Pre-swell Protein G-Sepharose beads in H_2O and then wash in wash buffer.
2. Mix 150 μl of washed beads with 150–300 μl of antiserum (*see* **Note 8**).
3. Based on the total volume, adjust the mixture to 20 mM HEPES, pH 7.9, and 0.5 M NaCl and incubate for 60 min with continuous rocking at room temperature.
4. Wash the antibody-bound beads with 1 ml of 0.2 M sodium borate (pH 9) and resuspend in the same buffer.
5. Add dimethylpimelimidate to a final concentration of 20 mM to covalently cross-link the antibodies to the Protein G-Sepharose.

6. After 1-h incubation at room temperature, wash the beads with 0.2 M sodium borate (pH 9).
7. To block the unreacted cross-linker, add 1 ml of 0.2 M ethanolamine (pH 8) and incubate for 1 h at room temperature.
8. Wash the coupled antibody-Sepharose mixture five times (1 ml each) with wash buffer and store refrigerated in this buffer.

3.5.2 Depletion of Gal1 and Gal3 from NE

NEs can be depleted of Gal1 and Gal3 by adsorption on either a saccharide affinity column or an antibody column. The procedure is described below for saccharide affinity adsorption using α-lactose (Lac) immobilized to 6 % beaded agarose (Lac-A) to deplete Gal1 and Gal3 from NE. Agarose derivatized with cellobiose (Cello-A) was used as a control. *See* **Note 9** *for immunoadsorption using the antibody-coupled beads described in* Subheading 3.5.1.

1. Wash 200–250 μl of Lac-A or Cello-A beads with 20 volumes of wash buffer and remove all fluid from the beads using a Hamilton syringe.
2. To disassemble any spliceosomes in the NE, preincubate NE (30 μl) in 60 %D containing 2.5 mM $MgCl_2$, 1 mM ATP, and 5 mM creatine phosphate in a total volume of 50 μl for 30 min at 30 °C. Then add NaCl to a final concentration of 0.5 M.
4. Add the preincubated NE (50 μl) to the washed saccharide-agarose (150 μl) and incubate for 30 min at 4 °C with rotation.
5. Pellet the agarose beads by gentle centrifugation (1,000 × *g* in a swinging bucket rotor at 4 °C) and collect the unbound material using a Hamilton syringe.
6. Wash the packed agarose with 50 μl of wash buffer and remove this wash also by Hamilton syringe. Add this to the unbound fraction. *This represents NE depleted of Gal1 and Gal3 (from the Lac-A column) or control-depleted NE (from Cello-A column).*
7. Dialyze aliquots (20 μl) of nondepleted NE and the unbound fractions of the saccharide adsorption procedure in a microdialyzer, with a dialysis membrane with 10 K molecular weight cutoff, for 40 min against 60 %D.
8. Use NEs for splicing assays with and without reconstitution, immediately after dialysis (*see* **Note 10**).

3.5.3 Reconstitution of Splicing Activity by Addition of Recombinant Gal1 and Gal3

1. To reconstitute splicing activity in a galectin-depleted NE, add the recombinant protein to be tested (*see* **Notes 11** and **12**) to the depleted or non-depleted NE prior to dialysis (**step 7** of Subheading 3.5.2).
2. Dialyze as outlined in **step 7** of Subheading 3.5.2.
3. After dialysis, incubate ~8 μl of the dialyzed NE with 2 μl of a mixture of the following components to bring the final

concentration of $MgCl_2$ to 2.5 mM, ATP to 1.5 mM, creatine phosphate to 20 mM, DTT to 0.5 mM, and RNasin to 20 units.

4. Finally, add 1 μl of ^{32}P-labeled splicing substrate (~10,000 cpm) and initiate splicing by incubation at 30 °C for 45–90 min.
5. Stop the splicing reaction by the addition of 2.5 μl of proteinase K (20 mg/ml)-SDS (0.5 %) and incubate at 37 °C for 20 min.
6. After splicing reaction is stopped, analyze by following the steps outlined in Subheadings 3.3.2 and 3.3.3.

4 Notes

1. All chemicals (buffer components, enzymes, etc.) are to be kept free of ribonuclease (RNase). The commercially purchased reagent bottles are sequestered from general lab use. Use RNase-free spatulas (*see* **Note 2** below) to aliquot chemicals from these reagent bottles. Wear gloves for all procedures.
2. All glassware (beakers, flasks, bottles, pipettes, etc.) are baked for a minimum of 4 h at 350 °C. Other utensils (spatulas, stir bars, etc.) are wrapped in aluminum foil before baking under the same conditions.
3. DEPC (diethylpyrocarbonate) was added to double-distilled water (ddH_2O) to final concentration of 0.1 % vol/vol. The solution was stirred overnight using a magnetic stir bar at room temperature and then autoclaved. All solutions containing Tris are prepared using DEPC-treated H_2O and then filter sterilized. Other solutions (without Tris) can be made with regular ddH_2O, then subsequently treated with DEPC, and then autoclaved.
4. The preparation and characterization of recombinant full-length Gal3 (rGal3), ND, and CD have been reported in reference 15. In some instances, rGal3ND and rGal3CD were lyophilized and rehydrated in 60 %D to achieve appropriate concentrations.
5. A typical result documenting that ND exerts a dominant negative effect when added to a splicing competent NE is illustrated in Fig. 2, lanes 4 and 5. Parallel addition of full-length rGal3 or the CD failed to yield the same effect (Fig. 2, lanes 2 and 3; lanes 6 and 7).
6. The derivation and epitope mapping of the monoclonal antibodies anti-Mac-2 and NCL-GAL3 have been reported in reference 16.
7. The synthesis and characterization of peptides corresponding to the 9-mer (PGAYPGQAP), 18-mer, and 27-mer have been reported in reference 16.
8. The derivation and characterization of rabbit polyclonal antiserum directed against Gal1 and Gal3 have been reported in

references 17 and 15, respectively. Rat monoclonal anti-Gal3 (anti-Mac-2) can be substituted for rabbit polyclonal anti-Gal3; in this case, 100 μg of the purified monoclonal is used in place of 100 μl of antiserum.

9. For immunoadsorption, 30 μl of NE was incubated with either 60 μl of anti-Gal3-Protein G-Sepharose beads or 90 μl of anti-Gal1-Protein G-Sepharose beads for single-antibody depletions and with a mixture of both for double-antibody depletions.
10. The nondepleted NE, the depleted NE (unbound fraction of the saccharide-agarose adsorption), and the material bound to the beads (removed from the beads by SDS-PAGE sample buffer) can be compared in terms of protein concentration (Bradford assay [12]) and in terms of Gal1 and Gal3 (immunoblotting).
11. We have tested the reconstitution of splicing activity in galectin-depleted NE with recombinant Gal1, recombinant Gal3, the ND of Gal3, the CD of Gal3, and various fusion proteins consisting of glutathione S-transferase (GST) and Gal1 and Gal3 derivatives, as reported in references 2, 17 and 18. As controls, we also tested the ability to reconstitute splicing activity in a galectin-depleted NE using plant lectins exhibiting similar carbohydrate-binding specificity as the galectins (soy bean agglutinin) or different specificity from the galectins (wheat germ agglutinin).
12. The reconstituting protein was tested in amounts ranging from 0.5 to 8 μg per splicing reaction.

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Chapter 29

Analysis of the Intracellular Role of Galectins in Cell Growth and Apoptosis

Daniel K. Hsu, Ri-Yao Yang, Jun Saegusa, and Fu-Tong Liu

Abstract

Galectins are a family of animal lectins with conserved carbohydrate-recognition domains that recognize β-galactosides. Despite structural similarities, these proteins have diverse functions in a variety of cellular processes. While a large number of extracellular functions have been demonstrated for galectins, the existence of intracellular functions has been clearly shown for a number of galectins, including regulation of cell growth and apoptosis; these latter functions may not involve glycan binding. There is considerable interest in intracellular regulation by galectins of cell growth and apoptosis, as these are fundamental cellular processes in normal homeostasis. Their dysregulation can cause pathologies such as autoimmune disorders, cancer, and neural degenerative diseases. Here we describe methods that we routinely perform in the laboratory to investigate the role of galectins in cell growth and apoptosis. These include methods for cell isolation, cell maintenance, and genetic manipulations to perturb galectin gene expression, as well as assays for cell growth and apoptosis.

Key words Galectin, Intracellular regulation, Cell growth, Apoptosis

1 Introduction

The galectin family has presented an intriguing set of proteins located inside and outside the cell compartment. Early studies of galectin-1 and galectin-3 biology revealed predominant nuclear and cytoplasmic localization [9, 10], but externalization of galectins was also observed and was recognized to occur through unconventional secretion [9, 10]. Analyses of glycan recognition by galectin-1, -2, and -3 revealed ligand specificity that suggested differential extracellular functions could be attributed to each galectin [2, 3]. Almost a decade later, subsequent studies in a seminal report that described how recombinant galectin-1 could regulate T cell function through its ability to induce apoptosis in a carbohydrate-dependent manner by recognition of cell surface CD45 on activated T cells [4]. Along with its effect in causing cell

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death, galectin-1 was also observed to mediate adhesion to ECM components [5] and regulate cell growth [6, 7].

In the meantime, examination of the function of galectin-3 in T cells revealed that it possessed an intracellular function. When expressed, this lectin conferred resistance to apoptotic stimuli and beneficial influence on cell growth under suboptimal conditions [8]. Furthermore, galectin-3 was observed to interact directly with Bcl-2 in a manner that was sensitive to inhibition by lactose. As Bcl-2 is not known to be glycosylated these observations suggested that galectin-3 could interact with other proteins in a lectin-independent fashion. Although galectin-3 possesses a non-lectin N-terminal domain, this lectin appears to have the ability to function through peptide-peptide interaction with its lectin domain, and this mode of action may be a common property of galectins. Galectins are likely to perform intracellular functions because they are present in substantial quantities in intracellular compartments, including the nuclei and the subcellular localization (i.e., cytosol vs. nucleus), and corresponds to the proliferation status of the cell [9, 10].

Further investigations by others have since revealed other intracellular signaling partners of galectin-3, exemplified by gemin-4, K-Ras, β-catenin, and Alix [11–15]. Additionally, galectin-3 participates in cross-signaling with p53-induced apoptosis whereby the levels of galectin-3, as an anti-apoptotic protein, are reduced in order to coordinate p53 function [16]. The anti-apoptotic function of galectin-3 has since been observed in many independent studies and a variety of cell types (reviewed in [17, 18]). Interestingly, in contrast to anti-apoptotic galectin-3, galectin-7 is highly induced by p53 [19], and was described to possess an intracellular pro-apoptotic function that operates by activation of JNK and cytochrome *c* release [20]. In this regard, a prominent theme to intracellular functions of galectins may be their participation in the regulation of cell growth and survival. On the other hand, a large number of functions unrelated to cell growth and apoptosis have been described for galectins (reviewed in [21–28]).

Like galectin-1, galectin-3 has been observed to induce apoptosis in T cells, through cell surface glycan binding [29, 30]. Thus, galectin-3 inhibits apoptosis through an intracellular mechanism independent of glycan binding, while it promotes apoptosis through an extracellular glycan-binding mechanism. It is to be noted that other galectins, including galectin-2, -4, -8, and -9, also kill T cells, as well as other cell types [31–34], each likely acting by binding to distinct cell surface glycoproteins in accordance to their respective glycan specificities. Whether they also regulate apoptosis through intracellular mechanisms is unknown.

The protocols below describe the analyses of anti-apoptotic properties of galectin-3 in different cell types. Unactivated T cells express insignificant levels of galectin-3, and this is represented by

the Jurkat E6-1 cell line. In this cell line, the function of galectin-3 was analyzed after transfection with a galectin-3 construct to induce ectopic expression. Studies in two other cell types are described—macrophages and keratinocytes. These cells are derived from galectin-3-deficient (KO) mice, which are available from the authors' laboratory.

2 Materials

2.1 Analysis of the Intracellular Role of Galectins in Cell Growth and Apoptosis by Altering Gene Expression Levels in Cell Lines

2.1.1 Examination of the Role of Galectin-3 Expression in Jurkat E6-1 Cells

1. Jurkat E6.1 cells (ATCC).
2. Human galectin-3 cDNA cloned into an eukaryotic vector conferring G418 resistance.
3. 0.25 M NaCl.
4. Sterile TE: 10 mM Tris-HCl, 1 mM EDTA, pH 7.5.
5. 100 % ethanol.
6. 5 M NaCl stock.
7. 1 M Tris–HCl, 100 mM EDTA, pH 7.5 (100×).
8. Complete culture media: RPMI 1640 medium, 10 % FBS.
9. Incubator at 37 °C and 5 % CO_2.
10. Clinical centrifuge.
11. BioRad Gene Pulser electroporator.
12. G418 100 mg/ml in PBS.
13. Triton X-100.
14. Lysis buffer: 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 140 mM NaCl, 1 % Triton X-100, 0.2 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM 2-mercaptoethanol (commercial cocktail for eukaryotic cells, *see* **Note 1**).
15. Protease inhibitor stocks (20 mM PMSF/ethanol, 100 μg/ml leupeptin, 100 μg/ml pepstatin/DMSO).
16. Mercaptoethanol.
17. BCA assay (Pierce/Thermo Fisher).
18. Refrigerated microcentrifuge.
19. PVDF membrane (Immobilon P, Millipore).
20. PAGE apparatus and 12 % polyacrylamide gels.

2.1.2 Galectin-3 in HeLa Cells (ATCC)

1. siRNA (Stealth RNAi, Invitrogen) (*see* **Notes 2** and **3**).
2. RNase-free water.
3. 1 M Tris–HCl, 100 mM EDTA, 2 M NaCl, pH 8.0 (100×).
4. HeLa cells (ATCC).
5. PolyFect or Effectene transfection reagent (Qiagen).

6. 24-Well culture plates.
7. Media: DMEM with 10 % FBS.

2.1.3 Assays of Cell Growth and Apoptosis in Cell Lines

1. MTS[(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] or CellTiter 96 Aqueous Cell Proliferation Assay kit (Promega).
2. Anti-human Fas (eBioscience/Affymetrix).
3. TNF-α (eBioscience/Affymetrix).
4. Staurosporine (Sigma).
5. Actinomycin D (Sigma).
6. Etoposide (Sigma).
7. Camptothecin (Sigma).
8. Cycloheximide (Sigma).
9. Fluorescein-labeled Annexin V (eBioscience/Affymetrix).
10. Propidium Iodide (Life Technologies).
11. Flow cytometer.

2.2 Analysis of the Intracellular Role of Galectins in Apoptosis Using Primary Cells from Galectin-Deficient Mice

1. Galectin-3 KO mice (*see* **Note 4**).
2. C57BL/6 wt mice.
3. Brewer's thioglycollate broth (Difco Laboratories).
4. 10 ml syringe.
5. 25 gauge needles.
6. 18 gauge needle.
7. PBS, 1 mM EDTA.
8. Clinical centrifuge.
9. Media: DMEM supplemented with 10 % FBS.

2.2.1 Isolation and Culture of Primary Macrophages from Galectin-3 Knockout Mice

2.2.2 Induction and Measurement of Apoptosis in Activated Macrophages

1. 5 ml Teflon beaker (Thermo Fisher).
2. IFNγ (Roche Biochemicals).
3. DMEM supplemented with 10 % FBS.
4. *E. coli* lipopolysaccharide (LIST Biologicals).
5. Fluorescein-labeled Annexin V (eBioscience/Affymetrix).
6. Propidium Iodide (Life Technologies).

2.2.3 Isolation and Culture of Primary Keratinocytes from Galectin-3 Knockout Mice

1. Neonatal wild-type and galectin-3 KO C57BL/6 mice (12–48 h after birth).
2. 70 % ethanol.
3. 100 mm Petri culture dishes.
4. Small scissors.

5. Curved scissors.
6. Blunt forceps.
7. Curved forceps.
8. Surgical scalpel handle.
9. Sterile surgical blades.
10. Dispase II (Roche Biochemicals).
11. Epilife medium (Invitrogen).
12. Supplemented Epilife Medium: Epilife Medium supplemented with 0.06 mM $CaCl_2$, 10 ng/ml mouse epidermal growth factor, 10^{-10} M cholera toxin, and human keratinocyte growth supplement-V2.
13. Phosphate-buffered saline.

2.2.4 Induction and Measurement of UV-Induced Apoptosis in Keratinocytes

1. Phosphate-buffered saline.
2. 10 cm dishes.
3. UV crosslinker (Stratagene/Agilent) fitted with BLE-8T312 UVB lamps (Spectronics Corp.).
4. UVB-Meter model UVB-500C (National Biological Corporation).
5. CO_2 incubator.

2.2.5 Treatment with Hydrogen Peroxide and Etoposide to Induce Apoptosis

1. Hydrogen peroxide.
2. Etoposide.
3. CO_2 incubator.

2.2.6 Detection of Apoptosis by Flow Cytometry

1. 100 mm Tissue culture plates.
2. Trypsin 0.25 %.
3. EDTA 0.1 %.
4. Trypsin and trypsin inhibitor (Cascade Biologics/Invitrogen).
5. Fluorescein-labeled Annexin V (eBioscience/Affymetrix).
6. Propidium Iodide (Life Technologies).
7. F FACS Calibur (Becton Dickinson) or comparable flow cytometer.

2.2.7 Detection of Apoptosis by Nuclear Staining with Hoechst 33342

1. Lab Tek Chamber Slide (Thermo Scientific Nunc).
2. Phosphate-buffered Saline.
3. 4 % paraformaldehyde.
4. Hoechst 33342.
5. Fluoromount-G (Southern Biotech).
6. Cover slips.
7. Fluorescence microscope.

2.2.8 Detection of Apoptosis by Quantitative Estimation of Histone-Associated DNA Fragmentation by ELISA

1. Flat-bottomed six-well plates (Falcon, Becton Dickinson).
2. ELISA Kit (Cell Detection ELISAPLUS, Roche Diagnostics Co.).
3. Cell Detection ELISA^{PLUS} Kit (Roche Biochemicals).
4. ELISA Microplate Reader.

3 Methods

3.1 Analysis of the Intracellular Role of Galectins in Cell Growth and Apoptosis by Altering Gene Expression Levels in Cell Lines

*3.1.1 Examination of the Role of Galectin-3 Expression in Jurkat E6-1 Cells (See **Note 5**)*

1. Precipitate linearized plasmid DNA with ethanol in the presence of 0.25 M NaCl.
2. Add 0.5 ml 70 % ethanol and rinse the internal surface of the tube.
3. Aspirate ethanol under sterile conditions in a laminar flow hood and briefly air-dry for 5 min.
4. Resuspend DNA in sterile TE at 2 mg/ml (*see* **Note 6**).
5. Spin down 1–2 × 10^6 actively proliferating cells at 1,100 rpm (280 × *g*) for 5 min at 4 °C, and decant supernatant (*see* **Note 7**).
6. Resuspend cells in 0.4 ml complete culture medium. Transfer cells to a 0.4 cm cuvette and add 15 µl of DNA (2 mg/ml).
7. Electroporate with BioRad Gene Pulser with Capacitance Extender, no pulse extender, at 260 V, 1,000 µF.
8. Transfer cells to 5 ml medium and return to the incubator.
9. Initiate drug selection 1 or 2 days after transfection with 1 mg/ml G418 (*see* **Note 8**).
10. Replace selection medium on alternating days in order to establish growth of transfected cells.
11. Continue cultivation with selection pressure for 2 weeks, expanding the culture as necessary, and freeze cell aliquots periodically after the 2-week period post-electroporation.
12. To determine galectin-3 expression from successful cell transfectants by immunoblotting, wash cells in PBS and add lysis buffer at 10^7 cells/ml on ice.
13. Vortex well, then return to ice, and repeat two times.
14. Microcentrifuge at >13,000 × *g*, 15 min, 4 °C, collect supernatant, and determine protein concentration by the BCA assay.
15. Run 20 µg extract on 12 % PAGE, transfer to a PVDF membrane (e.g., Immobilon P, Millipore), and process for detection with galectin-3 antibody.

*3.1.2 Role of Galectin-3 by RNAi Suppression in HeLa Cells (See **Note 9**)*

1. Resuspend Stealth RNAi siRNA duplexes in RNase-free water to make a 20 µM solution in 10 mM Tris–HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA.
2. Transfect 2 pmol of siRNA into HeLa cells in a 24-well plate with Effectene reagent.

3.1.3 Assays of Cell Growth and Apoptosis in Cell Lines

1. Cells are assessed for cell survival under suboptimal conditions in low-serum medium (10^5 cells/ml in RPMI 1640/1 % FBS for Jurkat E6-1, or 10^5 cells in 3 ml DMEM/1 % FBS in a 6-well plate for HeLa cells).
2. Induction of apoptosis in Jurkat cells can be performed by the following methods:
   (a) Incubation with 50 ng/ml anti-human Fas (eBioscience/Affymetrix) for 12–24 h.
   (b) Overnight culture with 0.5 μM of staurosporine (Sigma).
3. Induction of apoptosis in HeLa cells can be performed by culturing 10^5 cells/well in 12-well dishes followed by overnight treatment with any of the following: actinomycin D (1 μM), etoposide (50 μM), camptothecin (1 μM), or a combination of TNF-α (10 ng/ml) and cycloheximide (10 μg/ml).
4. Surviving cells are determined by the MTS assay as previously described [35] (*see* **Note 10**).
5. Alternatively, early and late apoptosis can be differentiated by flow cytometry by detection of externalized phosphatidylserine and compromised membrane integrity by incubation with fluorescein-labeled Annexin V (10 μl/10^5 cells) and propidium iodide (PI, 1 μg/ml), and analyzed by flow cytometry in FL1 and FL3 channels, respectively [36].

3.2 Analysis of the Intracellular Role of Galectins in Apoptosis Using Primary Cells from Galectin-Deficient Mice

3.2.1 Isolation and Culture of Primary Macrophages from Galectin-3 Knockout Mice

1. Inject 3 ml thioglycollate broth using a 25 gauge needle into the peritoneum of each mouse, penetrating at the left lower quadrant [1] (*see* **Note 11**).
2. Three days later, wipe the abdomen well with alcohol, and then perform peritoneal lavage by injecting 10 ml ice-cold PBS/1 mM EDTA using a 10 ml syringe fitted with an 18 gauge needle into the peritoneum through the lower left quadrant with bevel side down (*see* **Note 12**).
3. Slowly begin to aspirate by withdrawing the syringe plunger while tenting the abdominal skin with the needle to avoid obstructing the needle opening.
4. Centrifuge the aspirate at $200 \times g$ for 10 min at 4 °C.
5. Wash once in cold PBS.
6. Enumerate cells to determine leukocyte concentration.
7. Centrifuge required number of cells needed for the assay and resuspend cells at 2×10^6 cells/ml DMEM/10 % FBS.

3.2.2 Induction and Measurement of Apoptosis in Activated Macrophages

1. Culture 2×10^6 macrophages in a 5 ml Teflon beaker.
2. Add an equal volume of IFNγ from 0 to 50 U/ml and culture in CO_2 incubator for 6 h.
3. Recover cells by gently pipetting around the beaker to dislodge cells.

4. Centrifuge cells at 200 × *g* for 10 min at 4 °C.
5. Wash once in DMEM supplemented with 10 % FBS.
6. Reculture cells in Teflon beakers at 10^6 cells/ml in DMEM supplemented with 10 % FBS and 1 μg/ml *E. coli* lipopolysaccharide for 3 days.
7. Harvest by gentle pipetting to dislodge macrophages (lightly adhered), and remove one-fourth culture daily for detection of apoptosis.
8. Cool cells and centrifuge at 4 °C.
9. Resuspend in annexin V staining buffer, add 10 μl FITC-annexin V per 10^6 cells, and incubate for 15 min.
10. Prior to analysis, add propidium iodide to 1 μg/ml and analyze for FITC and PI staining as described above.

3.2.3 Isolation and Culture of Primary Keratinocytes from Galectin-3 Knockout Mice

1. Euthanize neonatal wild-type and galectin-3 KO C57BL/6 mice (12–48 h after birth) by CO_2 inhalation from a tank of compressed gas.
2. Rinse them in 70 % ethanol.
3. Move the pups onto the cover of one 100 mm sterile plate.
4. Amputate limbs and tails with small sharp scissors.
5. Slit along vertical (mid) section from nose to tail.
6. Gently peel off the entire skin; avoid damaging the skin.
7. Flatten skins with the dermis side down on 10 mg/ml of Dispase II in Epilife medium at 4 °C overnight.
8. Separate epidermis from the dermis.
9. Mince the epidermis with sterile curved scissors.
10. Shake 50 times in medium and then discard large pieces.
11. Centrifuge at 200 × *g* for 5 min, and resuspend the cells in supplemented Epilife medium.
12. Plate the cells at a density of 5×10^4 cells/cm^2.
13. Incubate the cells at 37 °C, 5 % CO_2, for 48 h undisturbed, and then gently add additional supplemented Epilife medium to the plates.
14. Mouse epidermal keratinocyte foci can be seen after 3 days.
15. Change medium every 2–3 days.

3.2.4 Induction and Measurement of UV-Induced Apoptosis in Keratinocytes

1. Seed wild-type and galectin-3 KO mouse keratinocytes at a semi-confluent density, 24 h before irradiation.
2. Incubate the cells at 37 °C, 5 % CO_2, for 24 h.
3. Wash cells with PBS and add 5 ml PBS to cover cells (for 10 cm dish).
4. Irradiate cells with a dosage of 100–200 J/m^2.

5. Reconstitute the cells into fresh culture medium. Incubate the cells at 37 °C, 5 % CO_2.
6. Assay for cell death 8–24 h later.

3.2.5 Treatment with Hydrogen Peroxide and Etoposide to Induce Apoptosis

1. Seed wild-type and galectin-3 KO mouse keratinocytes at a semi-confluent density, 24 h before treatment.
2. Incubate the cells at 37 °C, 5 % CO_2.
3. Reconstitute with fresh culture medium.
4. Culture the cells with hydrogen peroxide (25 μM) or etoposide (25 μM) at 37 °C, 5 % CO_2, for 24 h.

3.2.6 Detection of Apoptosis by Flow Cytometry

1. Culture wild-type and galectin-3 KO mouse keratinocytes in 100 mm tissue culture plates.
2. Induce apoptosis with UVB irradiation, or hydrogen peroxide or etoposide treatment.
3. Twenty-four hours after induction of apoptosis, harvest both detached and attached cells.
4. Use mild trypsinization to collect the attached cells.
5. Neutralize with defined trypsin inhibitor.
6. Cool on ice, and then centrifuge the cells at 200 × *g* for 5 min.
7. Wash once with cold PBS.
8. Stain the cells with FITC-conjugated annexin V and propidium iodide (1 μg/ml).
9. Examine cells by two-color analysis using FACS Calibur in FL1 and FL3 channels, respectively.

3.2.7 Detection of Apoptosis by Nuclear Staining with Hoechst 33342

1. Plate wild-type and galectin-3 KO mouse keratinocytes on Lab Tek Chamber Slides.
2. Induce apoptosis with UVB irradiation, hydrogen peroxide, or etoposide treatment.
3. Twenty-four hours after induction of apoptosis, wash cells with PBS.
4. Fix the cells with 4 % paraformaldehyde.
5. Incubate for 10 min with Hoechst 33342 (5 μg/ml) in the dark at room temperature.
6. Wash with PBS.
7. Mount cover slips with Fluoromount-G.
8. Identify the morphological characteristics of apoptotic cells with the aid of a fluorescence microscope using the excitation wavelength of 340 nm.
9. Cells with fragmented and/or condensed nuclei are considered as apoptotic cells.

3.2.8 Detection of Apoptosis by Quantitative Estimation of Histone-Associated DNA Fragmentation by ELISA

1. Plate wild-type and galectin-3 KO mouse keratinocytes in flat-bottomed six-well plates.
2. Induce apoptosis with UVB irradiation, hydrogen peroxide, or etoposide treatment.
3. Twenty-four hours later, centrifuge the plates at 200 × *g* for 10 min.
4. Lyse the cell pellets in buffer provided with the ELISA Kit (Cell Detection ELISAPLUS, Roche Diagnostics Co.).
5. Measure soluble histone–DNA complex in the lysates as a result of DNA fragmentation by ELISA. Absorbance at 405 nm (reference at 492 nm) is measured in each well.

4 Notes

1. Stock concentrations of inhibitors are 0.2 mM PMSF in absolute ethanol (due to limited stability, add to lysis buffer just before use and vortex thoroughly), 1 mg/ml pepstatin in dimethyl sulfoxide, and 1 mg/ml leupeptin in H_2O. The concentration of undiluted mercaptoethanol is 14 M. All reagents are available from Sigma.
2. Order three pairs of complementary Stealth RNAi siRNA oligonucleotides from Invitrogen for control, and galectin-3. The three pairs of galectin-3 siRNA strands are as follows: CCAUGAUGCGUUAUCUGGGUCUGGA, UCCAGACCC AGAUAACGCAUCAUGG; UGCUGGGCCACUGAUUGU GCCUUAU, AUAAGGCACAAUCAGUGGCCCAGCA; UG GUGCCUCGCAUGCUGAUAACAAU, and AUUGUUAU CAGCAUGCGAGGCACCA. Dissolve in RNase-free water.
3. The use of Stealth siRNA minimizes off-target effects. These siRNAs provide higher specificity and increased stability compared to standard siRNA.
4. Galectin-3 KO mice on a C57BL/6 or BALB/c background are distributed worldwide and available from the author's laboratory on request.
5. As the human T-cell line Jurkat clone E6-1 does not express endogenous galectin-3 this cell line can be used as recipient for ectopic galectin-3 expression. The cells can be transfected by electroporation with a protocol generally applicable to a wide range of cell lines without pre-optimization [37].
6. It is critical that the DNA for transfection is of high quality and purity. DNA is traditionally prepared by CsCl (cesium chloride) centrifugation. A number of reagent kits are commercially available for this purpose, such as Qiagen Plasmid Kits for various scales of plasmid DNA purification. We routinely use a

cost-efficient method involving alkaline lysis, phenol extraction, and polyethylene glycol precipitation with satisfactory results [38, 39].

7. The proliferative state of the cells greatly affects the transfection efficiency. We usually dilute near-saturated culture 1:3 with fresh growth medium the day before transfection to make sure that they are actively proliferating on the day of transfection.
8. We use 1 mg/ml G418 for selection of Jurkat cells transfected with constructs carrying a neo-resistance cassette. Conditions need to be empirically determined for other selection markers and other cell lines.
9. The human cervical cancer cell line HeLa expresses galectin-3 and can be used as a model to test the intracellular role of galectin-3 by suppressing endogenous galectin-3 using RNAi.
10. The MTS method is similar to the MTT assay but avoids the need for solubilization with solvent and detergent.
11. Estimate the numbers of mice required by calculating approximate yields of 8×10^6 macrophages/mouse.
12. While the needle is inserted into the peritoneal cavity, gently roll (vigorous rolling may cause leakage of fluid from the highly distended abdomen) the mouse side to side with the needle in order to gently disturb the fluid within the peritoneum while maintaining a good needle seal against the mouse skin so as to prevent fluid leakage. This can increase cell yields. Avoid downward orientation of the tip of the needle in order to prevent rupturing the intestines or causing bleeding.

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Chapter 30

Nucleocytoplasmic Shuttling of Galectin-3

Eric J. Arnoys, Cheri M. Ackerman, and John L. Wang

Abstract

A large number of observations on the nuclear versus cytoplasmic distribution of galectin-3 have been reported, correlating the presence or absence of the protein in a particular compartment of the cell to various parameters such as source of the cells under study, specific cell type, culture conditions, proliferation status of the cell/culture, or neoplastic transformation. In fact, galectin-3 exhibits the phenomenon of nucleocytoplasmic shuttling, defined as the repeated bidirectional movement of a protein across the nuclear pore complex. Nevertheless, the finding that galectin-3 can show a predominantly nuclear localization under one set of conditions and a prominent cytoplasmic localization under other conditions suggests specific and regulated mechanisms of balance between cytoplasmic anchorage, nuclear import, nuclear retention, and nuclear export. One key consideration in the understanding of these processes is the definition of the signals and receptors that mediate the transport. In this chapter, we describe the experimental procedures that have allowed us to document the phenomenon of nucleocytoplasmic shuttling and the identification of the nuclear localization signal as well as the nuclear export signal.

Key words Nuclear import, Nuclear export, Nuclear localization signal, Nuclear export signal

1 Introduction

Of the 15 members of the galectin family, 11 have been reported to be in the nucleus and cytoplasm of cells [1]. Several distinct activities have been reported for galectins in the cytoplasm, including membrane anchorage of the H-Ras oncogene product by galectin-1 (Gal1) [2], a similar direct interaction between galectin-3 (Gal3) and K-Ras [3], the centrosome organizing activity of Gal3 [4], and either pro- or anti-apoptotic activities of Gal3, galectin-7, and galectin-12 [5–7]. On the other hand, Gal1 and Gal3 have been localized in nuclear speckles under immunofluorescence microscopy [8, 9] and to corresponding structures by electron microscopy [10] and, using a cell-free assay, depletion and reconstitution experiments showed that these two galectins were factors involved in the splicing of pre-mRNA [8, 11]. The nuclear versus cytoplasmic distribution reported for each galectin most likely reflects the specific cell type and experimental conditions (source of

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cells under study, culture conditions, proliferation status of the cell/culture, or neoplastic transformation). For example, Gal3 has been reported to be exclusively cytoplasmic, predominantly nuclear, or distributed between the two subcellular compartments.

In fact, Gal3 shuttles between the cytoplasm and the nucleus [12] and an importin-α/β-mediated nuclear localization signal (NLS) [13, 14] as well as an exportin-1-mediated nuclear export signal (NES) [15] have been identified on the polypeptide. This chapter describes the procedures used in these studies of Gal3 in terms of: (a) analysis of nucleocytoplasmic shuttling using a heterokaryon assay; (b) identification of a NLS using fusion constructs containing Green Fluorescent Protein (GFP); and (c) documentation and identification of an NES sensitive to inhibition by leptomycin B (LMB).

2 Materials

2.1 Analysis of Nucleocytoplasmic Shuttling

2.1.1 Bead Tagging and Polyethylene Glycol-Mediated Cell Fusion

1. Human diploid fibroblast LG-1 (*see* **Note 1** for culture conditions).
2. NIH mouse 3T3 fibroblasts (American Type Culture Collection) (*see* **Note 2** for culture conditions).
3. MEM-ASP: minimum essential Eagle's medium.
4. Fetal calf serum (FCS).
5. L-aspartic acid.
6. L-serine.
7. Sodium pyruvate.
8. Penicillin.
9. Streptomycin.
10. Calf serum.
11. Mouse monoclonal antibody NCL-GAL3 (Vector Laboratories; hybridoma clone 9C4) (*see* **Note 3**).
12. 100 × 20 mm tissue culture plates.
13. Polysciences styrene microspheres; ~2 μm average diameter.
14. Polysciences Fluoresbrite NYO carboxylate microspheres; ~1.75 μm average diameter.
15. Trypsin (Invitrogen) (*see* **Note 4**).
16. 15-ml centrifuge tubes.
17. 22-mm^2 glass coverslips.
18. Ethanol.
19. Bunsen burner.
20. 35-mm culture dishes.
21. Polyethylene glycol-1000 (Fluka Chemical).

22. MEM-ASP: minimum essential Eagle's medium.
23. 0.22-μm Millex syringe driven filter unit.

Buffers

24. PBS: 140 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4.
25. Phosphate-buffered saline (PBS)-Versene solution: 140 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.68 mM ethylenediamine tetracetic acid, 0.15 % phenol red, pH 7.2.
26. Culture medium: minimum essential Eagle's medium (MEM-ASP) + 10 % fetal calf serum (FCS).
27. PEG fusion solution: 45 % polyethylene glycol-1000 in serum-free MEM-ASP.

2.1.2 Immunostaining and Fluorescence Microscopy

1. Fused cells (Subheading 3.1.1).
2. 4 % paraformaldehyde in PBS (*see* **Note 5**).
3. 0.2 % Triton X-100 in PBS.
4. 0.2 % gelatin in PBS.
5. Mouse monoclonal antibody NCL-GAL3 (Vector Laboratories; hybridoma clone 9C4) (*see* **Note 3**).
6. FITC (fluorescein isothiocyanate)-conjugated goat anti-mouse immunoglobulin.
7. Propidium iodide (PI).
8. Microscope slides.
9. Perma-Fluor (Thermo Scientific).

Buffers

10. PBS: 140 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4.
11. T-TBS: 10 mM Tris, pH 7.5, 0.5 M NaCl, 0.05 % Tween 20.

Special Equipment

12. Insight Plus laser scanning confocal microscope (Meridian Instruments).

2.2 Identification of NLS on the Gal3 Polypeptide

2.2.1 Preparation and Characterization of the Expression Constructs

1. Plasmid pEGFP-c_1 (Clontech) (*see* **Note 6**).
2. Plasmid pWJ31 (containing the cDNA of murine Gal3) [18].
3. pMAL-c_2x (New England Biolabs).
4. Polymerase pfu (Stratagene).
5. PCR Purification Kit (Qiagen).
6. QIAquick Gel Extraction Kit (Qiagen) (*see* **Note 7**).
7. Appropriately designed PCR primers (as indicated).

8. Quick-Change Site-Directed Mutagenesis kit (Stratagene).
9. T4 DNA ligase (*see* **Note 8**).
10. Alkaline phosphatase.
11. DH5α cells.

Restriction Enzymes (*see* **Note 8**)

12. *Kpn*I restriction enzyme.
13. *Bam*HI restriction enzyme.
14. *Eco*RI restriction enzyme.
15. *Sac*I restriction enzyme.
16. *Pst*I restriction enzyme.

Special Equipment

17. Thermocycler.

2.2.2 Transfection of Cells with the DNA Constructs

1. NIH mouse 3T3 fibroblasts (American Type Culture Collection) (*see* **Note 2** for culture conditions and materials).
2. 22-mm^2 glass coverslips.
3. 35-mm tissue culture dishes.
4. 500 μl Eppendorf tubes.
5. Serum-free Dulbecco modified Eagle's Medium (DMEM).
6. Lipofectamine (Invitrogen).

Special Equipment

7. Inverted microscope.

2.2.3 Examination of Transfected Cells for GFP Fluorescence and a Scoring System for Designating Subcellular Localization

1. 4 % paraformaldehyde in PBS (*see* **Note 5**).
2. Microscope slides.
3. Perma-Fluor (Thermo Scientific).

Buffers

4. PBS: 140 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4.

Special Equipment

5. Insight Plus laser scanning confocal microscope (Meridian Instruments).

2.3 Analysis of a Functional NES

2.3.1 Construction of Rev(1.4)-GFP Vectors

1. Appropriately designed PCR primers (as indicated).
2. NEB Buffer 1 (New England Biolabs).
3. 100× BSA (New England Biolabs).
4. Sterile H_2O.

5. dNTPs (New England Biolabs).
6. Klenow DNA polymerase (New England Biolabs).
7. T4 DNA ligase (New England Biolabs).
8. pRev(1.4)-GFP (Nuclear localization control vector) (*see* **Note 9**).
9. Polymerase pfu (Stratagene).
10. QIAquick Gel Extraction Kit (Qiagen) (*see* **Note 7**).
11. Quick-Change Site-Directed Mutagenesis Kit (Stratagene).
12. DH5α cells.

Restriction Enzymes (*see* **Note 8**)

12. *Age*I restriction enzyme.
13. *Bam*HI restriction enzyme.

Special Equipment

14. Thermocycler.

2.3.2 Transfection, Observation, and Analysis

Materials needed include all required materials listed in Subheadings 2.2.2 *and* 2.2.3.

3 Methods

3.1 Analysis of Gal3 Nucleocytoplasmic Shuttling

3.1.1 Bead Tagging and Polyethylene Glycol-Mediated Cell Fusion

Figure 1 schematically illustrates the experimental strategy used to test for shuttling of Gal3. Heterokaryons are generated through fusion of human LG-1 fibroblasts (H in Fig. 1) and mouse 3T3 fibroblasts (M in Fig. 1). The presence of human Gal3 in both nuclei of a heterodikaryon (path I) could arise due to nuclear import from three sources: (a) human Gal3 in the cytoplasm; (b) newly synthesized human Gal3; and (c) human Gal3 from the human nucleus. The last of these three possibilities requires export of human Gal3 from the human nucleus into the cytoplasm of the heterkaryon and subsequent import into the mouse nucleus. Therefore, if shuttling makes a significant contribution to the percentage of heterodikaryons exhibiting human Gal3 staining in both nuclei, it should be sensitive to inhibition of nuclear export. On this basis, we expect the percentage of heterodikaryons showing human Gal3 in both nuclei (path I) to be decreased and, correspondingly, the percentage of heterodikaryons showing human Gal3 in only one nucleus (path II) to be increased by inhibitors of nuclear export. This experimental scheme has two key requirements. First, we needed to be able to distinguish heterodikaryons (human-mouse cell hybrids) from homodikaryons (human-human and mouse-mouse cell hybrids). This was accomplished by tagging the two cell types with distinguishable microsphere beads prior to

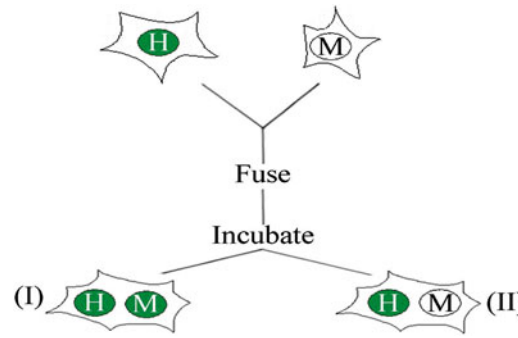


Fig. 1 Schematic illustrating the use of heterokaryons to study shuttling. Human LG-1 cells (H) were first tagged with nonfluorescent (*black*) beads and mouse 3T3 fibroblasts (M) were tagged with fluorescent beads. Heterodikaryons were generated by fusing human and mouse cells and were recognized by the presence of both fluorescent and nonfluorescent beads. The presence of human Gal3 in both nuclei of a heterokaryon (path I yielding *green color* in both nuclei) and the presence of human Gal3 in only one nuclei of a heterokaryon (path II) can be determined using the NCL-GAL3 monoclonal antibody which specifically recognizes human Gal3. One mechanism leading to path I requires that human Gal3 first gets exported from the human nucleus into the cytoplasm of the heterodikaryon and subsequently gets imported into the mouse nucleus (Color figure online)

fusion. Second, we needed to be able to immunostain for human Gal3 with an antibody that fails to recognize mouse Gal3.

1. Seed human LG-1 cells in 100 × 20 mm tissue culture plates at 5×10^3 cells/cm^2 and allow the cells to attach for 6 h. Add nonfluorescent beads (Polysciences styrene microspheres; ~2 μm average diameter) to the culture medium at a concentration of 250 beads/cell and incubate overnight.
2. Concurrently, seed mouse 3T3 cells in 100 × 20 mm tissue cultures plates at 5×10^3 cells/cm^2 and allow the cells to attach for 6 h. Add fluorescent beads (Polysciences Fluoresbrite NYO carboxylate microspheres; ~1.75 μm average diameter) to the culture medium at a concentration of 250 beads/cell and incubate overnight.
3. Rinse each plate with phosphate-buffered saline (PBS)-Versene solution and dislodge the cells from the tissue culture dish by incubation in 2 ml of 0.25 % trypsin (*see* **Note 4**) PBS-Versene solution at 37 °C for 5 min.
4. Dilute the trypsinized cell suspension into a 15-ml centrifuge tube containing 10 ml of culture medium (MEM-ASP + 10 % serum).
5. Pellet the cells by centrifugation (*see* **Note 10**) and discard supernatant.
6. Resuspend in 5 ml of culture medium and determine the cell concentration.

7. Sterilize 22-mm^2 glass coverslips by dipping in ethanol and quickly passing over a Bunsen burner flame and place one coverslip into a 35-mm culture dish.
8. Mix suspensions containing the two cell types, each at 3.7×10^4 cells/ml. Place 4 ml of the mixed suspension into one dish containing a coverslip (the seeding density of each cell type ~3.9×10^3 cells/cm^2) and coculture the cells overnight.
9. Warm ~50 ml of serum-free MEM-ASP in the incubator, then melt polyethylene glycol-1000, dilute to 45 % with warmed MEM-ASP, and filter through a 0.22-μm Millex syringe driven filter unit. *The sterilized solution is designated as PEG fusion solution.*
10. Rinse the cocultured LG-1 and 3T3 cells in warmed PBS.
11. Add 2 ml of PEG fusion solution to each well of cluster dish. After 55 s, promptly remove the PEG fusion solution and rinse the cells three times in warmed PBS.
12. Add 2 ml of MEM-ASP containing 10 % fetal calf serum to each well and return the coculture to the incubator until immunostaining (*see* **Note 11**).

3.1.2 Immunostaining and Fluorescence Microscopy

1. Cells are processed for immunostaining 5–10 h post fusion. Wash the cells twice with PBS and then fix for 20 min at 4 °C with 4 % paraformaldehyde in PBS (*see* **Note 5**).
2. Wash cells twice with PBS and then permeabilize with 0.2 % Triton X-100 in PBS for 5 min at 4 °C.
3. After permeabilization, wash the cells twice with PBS and incubate with 0.2 % gelatin in PBS for 1 h on an orbital shaker at room temperature.
4. Stain the coverslips with NCL-GAL3 (2.1 μg/ml in PBS containing 0.2 % gelatin) for 1 h at room temperature (*see* **Notes 3** and **12**).
5. Wash the cells with T-TBS three times (15 min each) on an orbital shaker at room temperature.

All subsequent steps should be carried out in the dimmest light possible.

6. Stain the coverslips with FITC (fluorescein isothiocyanate)-conjugated goat anti-mouse immunoglobulin (in PBS containing 0.2 % gelatin) for 30 min at room temperature (*see* **Note 12**).
7. Wash the cells with T-TBS three times, 15 min each, on an orbital shaker at room temperature.
8. Stain the coverslips with propidium iodide (32 μg/ml in PBS containing 0.2 % gelatin) and wash again in T-TBS (three times, 15 min each).

9. Invert the coverslips onto clean microscope slides with a drop (~150 μl) of Perma-Fluor and allow to dry in the dark overnight at room temperature.
10. Fluorescent cells may be viewed using an Insight Plus laser scanning confocal microscope (*see* **Notes 13** and **14**).

3.2 Identification of NLS on the Gal3 Polypeptide

To define the residues on the Gal3 polypeptide critical for nuclear import, we developed a reporter construct expressing a fusion protein containing Gal3 (~33 kDa) and green fluorescent protein (GFP) (~27 kDa). This fusion protein also contained bacterial maltose-binding protein (MBP) (~40 kDa) to serve as a "spacer" that increases the molecular weight of the reporter polypeptide. This was done to ensure that the size of the reporter would exceed the exclusion limit of nuclear pores (40–60 kDa), even when the Gal3 polypeptide sequences were decreased significantly though deletion mutagenesis. Mouse 3T3 fibroblasts were transfected with this expression construct, as well as specific deletion mutants, and the nuclear versus cytoplasmic distribution of the protein products were compared on the basis of GFP fluorescence.

3.2.1 Preparation and Characterization of the Expression Constructs

The plasmid pEGFP-c_1 is used to generate GFP fusion proteins and as a control for localization of proteins without localization signals (*see* **Notes 6** and **15**).

1. *Construction of GFP-Gal3(1–263)*:
   (a) Using standard PCR procedures, amplify murine Gal3 from the pWJ31 plasmid [18], a construct from a λgt11 library which does not contain the first amino acid in Gal3. Use primers designed to add a *Kpn*I restriction site in frame with that in pEGFP-c_1 at the 5′ end and a *Bam*HI site at the 3′ end.
   (b) Clean up the PCR product with a PCR Purification Kit (Qiagen), and digest both it and pEGFP-c_1 with *Kpn*I and *Bam*HI according to supplier's guidelines.
   (c) Gel-purify both fragments with the QIAquick Gel Extraction Kit (Qiagen) per manufacturer's protocol and ligate Gal3 into the digested pEGFP-c_1 with T4 DNA ligase as described by supplier.
   (d) Transform DH5α cells with the ligated DNA via heat shock, plate, and miniprep the resulting colonies using standard miniprep procedures.
2. *Construction of the MBP-Gal3 plasmid*.
   (a) Digest both the expression vector (pMAL-c_2x) and the vector containing murine Gal3 (pWJ31) [18] with *Eco*RI.
   (b) Gel-purify the bands of the appropriate size using the QIAquick Gel Extraction Kit per manufacturer's protocol,

a 5'-CGA GCT CAA GCT TC**C** AAT TCT GCA G-3'

b 5'-CC CTC ACC AGC **TAG** AAC CAC GCC ATG ATC-3'

Fig. 2 Examples of oligonucleotides to remove a restriction site within the coding sequence or to add a stop codon. Site-directed mutagenesis requires a forward primer to remove the *Eco*RI restriction site between GFP and MBP in GFP-MBP-Gal3(1–263) (**a**). The sequence is shown with the codons in the reading frame of the expressed protein and the mutated nucleotide shown as **C**. For deletion mutants at the carboxyl end of a fusion construct, insertion of a stop codon via site-directed mutagenesis is straightforward (**b**). It is possible to mutate multiple bases at once, as demonstrated by the example shown here, in which the GCT codon for A258 was replaced with the TAG stop codon (*see* **Notes 16** and **17**)

treat the expression vector with alkaline phosphatase, and then ligate the vector and insert with T4 DNA ligase as described by supplier.

(c) Transform DH5α cells with the ligated DNA via heat shock, plate, and then miniprep the resulting colonies.

(d) To verify the directionality of the insert, perform a double digest with *Sac*I and the internal *Pst*I site in Gal3.

3. *Construction of the GFP-MBP-Gal3 plasmid.*

(a) Use MBP-Gal3 (above) as a template for PCR, using primers to insert a *Kpn*I restriction site in frame with that in pEGFP-c_1 at the 5′ end of MBP and keeping the *Bam*HI site at the 3′ end of Gal3 in MBP-Gal3.

(b) Clean up the PCR product with a PCR Purification Kit (Qiagen) and cut both the product and pEGFP-c_1 with *Kpn*I and *Bam*HI as described by the supplier.

(c) Gel-purify the bands of the appropriate size using the QIAquick Gel Extraction Kit per manufacturer's protocol, treat the expression vector with alkaline phosphatase, and then ligate the vector and insert with T4 DNA ligase as described by supplier.

(d) Transform DH5α cells with the ligated DNA via heat shock, plate, and then miniprep the resulting colonies.

4. *Construction of N-terminal deletion mutants.*

(a) The parent GFP-MBP-Gal3 vector (above) contains three *Eco*RI restriction sites, including one overlapping with the start codon of Gal3. To delete coding sequence from the 5′ end of the gene, first remove the other two *Eco*RI sites via site-directed mutagenesis using a standard QuickChange Site-Directed Mutagenesis Kit (*see* Fig. 2 for an example).

(b) Once the two *Eco*RI sites are removed, replace the two codons immediately upstream of the new first Gal3 residue

with an *Eco*RI site via mutagenesis, also using a standard Quick-Change Site-Directed Mutagenesis kit as described above.

(c) Cut the resulting vector with *Eco*RI.

(d) Gel-purify the bands of the appropriate size using the QIAquick Gel Extraction Kit per manufacturer's protocol. Religate the vector with T4 DNA ligase as described by supplier to produce mutants truncated at the amino terminus.

(e) Transform DH5α cells with the ligated DNA via heat shock, plate, and then miniprep the resulting colonies.

5. *Construction of C-terminal deletion mutants.*

(a) Use site-directed mutagenesis (as described above) to insert stop codons in mutants truncated at the carboxyl terminus. An example of oligo design is given in Fig. 2B (*see* Fig. 2 and **Note 16** and **17**).

6. *Generation of point mutants.*

(a) To evaluate the role of individual residues in nuclear import, change each amino acid in and surrounding a putative NLS to alanine via site-directed mutagenesis as described above using the Quick-Change Site-Directed Mutagenesis Kit per manufacturer's protocol.

(b) For Gal3 in GFP-MBP-Gal3(1–263), generate additional alanine mutants throughout the carbohydrate binding domain to serve as negative controls.

3.2.2 Transfection of Cells with the DNA Constructs

1. On the day before transfection, seed NIH 3T3 fibroblasts to be transfected with DNA constructs onto 22-mm^2 glass coverslips in a 35-mm culture dish at 1×10^4 cells/cm^2 and culture cells as described in Subheading 3.1.1 (*see* **Note 18**).
2. Using an inverted microscope, check to make sure the cells are attached after overnight culture.
3. Into a 500 μl Eppendorf tube, pipet 100 μl of serum-free Dulbecco modified MEM (DMEM) and 3 μl of Lipofectamine (Invitrogen). Allow the solution to equilibrate on the bench top for 5 min.
4. Into a separate 500 μl Eppendorf tube, pipet 100 μl of serum-free DMEM and 1 μl of the DNA construct (*see* Subheading 3.2.1).
5. Once the Lipofectamine has equilibrated, pipet the DMEM-DNA solution into the DMEM-Lipofectamine solution, and pipet up and down three to five times to fully mix the two solutions.

6. Allow this mixture to equilibrate for 20 min on the bench top so that DNA-Lipofectamine complexes can form.
7. When there are 5 min of equilibration time left, aspirate the medium off of the cells and wash the cells once with serum-free DMEM, being sure to apply the wash along the wall of the dish to avoid disturbing the cells.
8. Add 0.8 ml of serum-free DMEM to the cells and add the 200 μl of equilibrated DNA-Lipofectamine mixture to the cells dropwise over the top of the medium while shaking the dish gently. Do not add this along the side of the dish, but rather drip it directly into the medium, spreading it evenly over the entire plate in order to ensure equal DNA uptake in all the cells.
9. Incubate the cells in a CO_2 incubator at 37 °C for 3 h.
10. After 3 h of incubation, add 1 ml of DMEM containing 20 % calf serum, bringing the total serum concentration to 10 %.
11. Incubate the cells in the CO_2 incubator for another 6 h, then aspirate the medium off and add 2 ml of fresh DMEM containing 10 % calf serum.
12. Incubate the cells for an additional 5 h, and proceed to fluorescence analysis.

3.2.3 Examination of Transfected Cells for GFP Fluorescence and a Scoring System for Designating Subcellular Localization

1. To examine the transfected cells by fluorescence microscopy, wash the coverslips (from Subheading 3.2.2) three times with ice-cold PBS and incubate in 2 ml of 4 % paraformaldehyde (*see* **Note 5**) in PBS at room temperature for 20 min.
2. Wash twice for 10 min in 3 ml PBS at room temperature. Then invert coverslips onto clean microscope slides with a drop (~150 μl) of Perma-Fluor and allow to dry in the dark overnight at room temperature.
3. View fluorescence with a laser-scanning confocal microscope according to manufacturer guidelines, with as small a pinhole setting as possible while still maintaining the ability to view fluorescence.
4. For each observed cell, classify the subcellular localization into one of the categories outlined in Fig. 3, panels a–e (*see* **Note 19**).
5. Plot the mean number of cells scored to each localization as a histogram including standard error of the mean. An example of the histogram obtained for GFP-MBP-Gal3(1–263) is given in Fig. 3, panel f (*see* **Note 20**).

3.3 Documentation of an Exportin-Mediated NES on Gal3

The addition of LMB to cultured 3T3 cells resulted in accumulation of Gal3 in the nucleus [12, 13]. LMB inhibits exportin-mediated nuclear export of proteins bearing leucine-rich NESs [19, 20], and Gal3 contains a conserved sequence consistent with

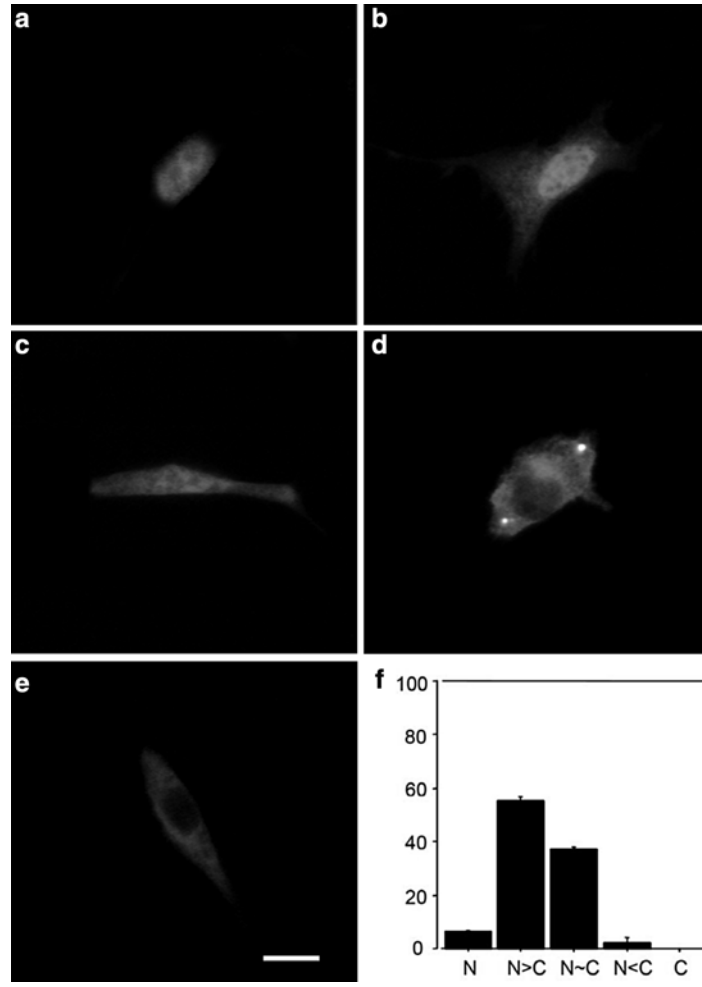
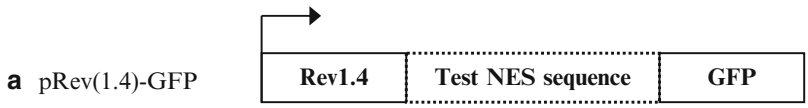


Fig. 3 Comparison of the subcellular localization in individual cells. Each observed cell was classified into one of the following categories according to the subcellular localization of fusion proteins. Representative micrographs show (**a**) N: exclusively nuclear; (**b**) N > C: intensely nuclear over a cytoplasmic background; (**c**) N ~ C: equal distribution between the nucleus and cytoplasm; (**d**) N < C: less nuclear labeling than in the cytoplasm; (**e**) C: exclusively cytoplasmic. A representative histogram of GFP-MBP-Gal3(1–263) is shown in **f**

such an NES. Therefore, we employed the pRev(1.4)-GFP vector [21] to determine whether Gal3 contains a functional NES. The pRev(1.4)-GFP system contains the following (Fig. 4): (1) NLS from the Rev protein of HIV-1, whose import activity can be decreased by addition of actinomycin D (ActD), thus providing a means to assay even a weak NES; (2) a multiple cloning site for the insertion of either test or control NESs; (3) the coding sequence for GFP, allowing easy visualization of the intracellular localization of expressed protein. We transfected mouse 3T3 fibroblasts with



b

Construct	Test NES sequence
GFP	none (and no NLS)
Rev1.4	none
PKI NES	33-N S N E **L** A L K **L** A G **L** D **I** N K T E
IκB-α NES	261-P S T R **I** Q Q Q **L** G Q **L** E **L** E N L Q
Gal3 NES	240-N **L** R E **I** S Q **L** G **I** S G D I T L

Fig. 4 Overview of the pRev(1.4)-GFP system to evaluate suspected NESs. (**a**) The pRev(1.4)-GFP vector includes the coding sequence for a modified Rev protein from HIV-1 containing: (1) a NLS whose activity can be reduced by addition of ActD and lacking a functional NES; (2) a site bordered by *Bam*HI and *Age*I in which to clone test NESs; and (3) an in-frame GFP for detection of localization within the cell of fusion protein. (**b**) Example test NES vectors that we have used in our experiments include negative controls (GFP and empty Rev1.4), positive controls (the PKI NES and IκB-α NES), and our Gal3 test sequence. The hydrophobic leucine-rich amino acids are highlighted in **bold**

constructs containing the putative Gal3 NES, two mutant Gal3 NESs (I244A; L247A and L247A; I249A), or the sequence from two previously characterized NESs, cAMP-dependent protein kinase (PKI) or IκB-α, and then we compared the intracellular localization of the fusion proteins.

3.3.1 Construction of Rev(1.4)-GFP Vectors for Analysis of a Functional NES

1. *Generation of Rev(1.4)-GFP vectors containing known NESs from (a) cAMP-dependent protein kinase (PKI) and (b) IκBα* (Fig. 5).
   (a) The first primer consists of a long single stranded oligonucleotide corresponding to the reverse complement of the NES flanked by the common sequences containing *Age*I and *Bam*HI restriction sites, as shown in Fig. 5B. The second primer corresponds to the sequence of BH977 [21] (Fig. 5C).
   (b) Mix 1 μg of the long primer with 1 μg of the common primer BH977 in 10 μl of NEB Buffer 1 with 1 μl of 100× BSA in a sterile tube.
   (c) Add sterile water so that final reaction volume will be 100 μl (*see* **Note 21** and the addition of dNTPs and Klenow DNA polymerase below).
   (d) Place the tube in boiling water for a few seconds and let it cool on the bench top to room temperature to anneal the primers.

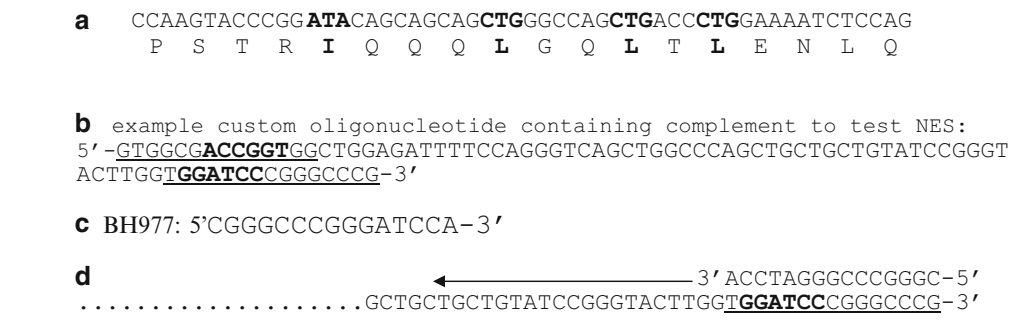


Fig. 5 Subcloning NES test sequences into the pRev(1.4)-GFP system. (**a**) The sequence of the DNA coding for the NES of IκB-α for insertion into the pRev(1.4)-GFP vector is given, along with its one-letter amino acid translation *underneath*. The hydrophobic amino acids critical for exportin-1 transport, as well as their codons, are highlighted in **bold**. (**b**) To subclone a test NES into the pRev(1.4)-GFP vector, the reverse complement to the test sequence is inserted between the two *underlined* flanking regions containing an *Age*I restriction site on the 5′ end (in **bold**) and a *Bam*HI restriction site on the 3′ end (in **bold**); shown here is that of IκB-α. This oligonucleotide can be ordered from a third-party supplier. (**c**) A second oligonucleotide, identical to the BH977 [21] common primer, must also be purchased. (**d**) When BH977 (on *top*, reversed) and the custom oligonucleotide containing the reverse complement to the test NES are annealed, a double-stranded insert can be synthesized with the Klenow DNA polymerase fragment, which can then be cut with *Bam*HI and *Age*I and inserted into the pRev(1.4)-GFP vector. Only a portion of the custom oligonucleotide is shown

(e) Add dNTPs to 33 μM of each and mix in 2 U of Klenow DNA polymerase (NEB). Incubate 1 h at 37 °C to extend.

(f) Heat-inactivate the enzyme by incubating for 20 min at 75 °C.

(g) Add 1 μl each of *Bam*HI and *Age*I (NEB) to the reaction mixture, mix well, and incubate for at least 2 h at 37 °C.

(h) Ligate with T4 ligase (NEB) into the pRev(1.4)-GFP vector that has also been cut with *Bam*HI and *Age*I and purify with the QIAquick Gel Extraction Kit according to the manufacurer's protocol.

(i) Transform DH5α cells with the isolated DNA via heat shock, plate, and then miniprep the resulting colonies.

2. *Generation of vectors containing the putative NES of Gal3.*

   (a) Starting with the pRev(1.4)-GFP vector containing the NES of PKI (from **step 1** of Subheading 3.3.1 above), carry out sequential site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit to convert the test NES sequence to that of Gal3 (*see* **Note 22**). NES test sequences are given in Fig. 4.

3. *Generation of vectors containing point mutants of the Gal3 NES to evaluate the importance of specific hydrophobic residues*—Starting with pRev(1.4)-GFP vector containing the wild-type Gal3 NES, use the following oligonucleotides to generate

point mutations of the NES using the QuickChange Site-Directed Mutagenesis Kit.

(a) For the mutant Gal3 NES (I244A, L247A), we used the forward and reverse primers 5′-CCA AAC CTT CGA GAG GCA TCT CAG GCA GGT ATC AGT GGG-3′ and 5′-CCC ACT GAT ACC TGC CTG AGA TGC CTC TCG AAG GTT TGG-3′

(b) For Gal3 NES (L247A, I249A), we used the forward and reverse primers 5′-CGA GAG ATA TCT CAG GCA GGT GCC AGT GGG GAC ATC ACA C-3′ and 5′-G TGT GAT GTC CCC ACT GGC ACC TGC CTG AGA TAT CTC TCG-3′.

(c) Transform DH5α cells with the isolated DNA via heat shock, plate, and then miniprep the resulting colonies.

4. Verify all constructs by DNA sequencing.

3.3.2 Transfection, Observation, and Analysis

1. Transfect cells with Rev(1.4)-NES-GFP constructs as described in Subheading 3.2.2.
2. To assess the potency of each NES in the Rev(1.4) reporter system, evaluate the localization of the expressed fusion protein relative to that in the presence of inhibitors of either nuclear import (*see* **Note 23**) or nuclear export (*see* **Note 24**). Add inhibitors during the step described in **step 11** of Subheading 3.2.2.
3. Continue with the **step 12** of Subheading 3.2.2 through **step 5** of Subheading 3.2.3.

4 Notes

1. The human diploid fibroblast strain, designated LG-1 [16], was obtained at passage 8, with a calculated cumulative population doubling of 16. Through passage 20 (corresponding to a cumulative population doubling of 40), the cells were seeded at 3.5×10^3 cells/cm^2 and serially passaged at a split ratio of 1:4. The cells were cultured in MEM-ASP supplemented with 10 % fetal calf serum at 37 °C and 5 % CO_2 and were used up to passage 20.
2. NIH mouse 3T3 fibroblasts were obtained from the American Type Culture Collection and were cultured in MEM-ASP plus 10 % calf serum at 37 °C and 5 % CO_2. MEM-ASP: minimum essential Eagle's medium, 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 0.1 μg/ml streptomycin.
3. The epitope of the mouse monoclonal antibody NCL-GAL3 (Vector Laboratories; hybridoma clone 9C4) has been mapped

to the amino-terminal 14 amino acids of the human Gal3 polypeptide [17]. NCL-GAL3 (21 ng/ml) immunoblots a band corresponding to Gal3 in lysates (50 μg total protein) derived from human cells (e.g. LG-1 fibroblasts) but fails to yield the Gal3 band in lysates (50 μg total protein) derived from mouse cells (e.g. 3T3 fibroblasts). Under immunofluorescence microscopy, NCL-GAL3 (2.1 μg/ml) stains the nucleus and cytoplasm of human cells (e.g. HeLa) but fails to yield detectable staining of mouse cells (e.g. 3T3). On this basis, it was concluded that NCL-GAL3 preferentially recognizes human Gal3 over the murine homolog.

4. It is important to use trypsin for cell culture (Gibco Life Technologies; www.invitrogen.com) rather than purified trypsin. The purified enzyme with high specific activity will damage the cells.
5. Preparation of 4 % paraformaldehyde solution in PBS: (a) heat 200 ml of H_2O (in an Erlenmeyer flask with stirrer) to 60 °C in a fume hood; (b) add 16 g paraformaldehyde and cover the flask; (c) stir at 60 °C for 10 min; (d) add, with a Pasteur pipette, one to three drops of 2 N NaOH and the solution should clear in ~2 min (although some fine particles may remain); (e) remove from heat and add 80 ml 5× PBS (pH 7); (f) adjust pH to 7.2 and adjust volume to 400 ml; (g) filter with a 0.45 μm filter (ZapCap); store at 4 °C.
6. The polypeptide expressed from the Clontech plasmid pEGFP-c1 was used to determine the intracellular localization of GFP without any targeting signal.
7. You will need all appropriate materials for DNA gels according to standard procedures.
8. The restriction enzymes and T4 DNA ligase were all from New England Biolabs.
9. The pRev(1.4)-GFP vector from Henderson and Elftheriou [21] expresses a labeled fusion protein containing GFP and a mutant HIV-1 Rev that carries a functional NLS but lacks an NES.
10. Table-top centrifuge with a swinging bucket rotor; 2,700 rpm corresponding to ~570 × *g*; 5 min.
11. Two variations of the general procedure in Subheading 3.1.1 can be carried out in parallel: (a) To inhibit de novo protein synthesis, add cycloheximide (CHX) to each cell type (10 μg/ml in MEM-ASP containing serum) 1 h prior to fusion, remove during fusion, and return after fusion. (b) To inhibit nuclear export, add leptomycin B (LMB) to each cell type (2 ng/ml or 3.8 nM in MEM-ASP containing serum) 10 h prior to fusion, remove during fusion, and returned after fusion.
12. The following procedure is used to minimize the amount of antibody solution required for staining. A 150-μl droplet of

antibody solution is deposited on a piece of parafilm. The coverslip is removed from the culture dish, blotted edgewise on a Kimwipe, and inverted over the droplet of antibody solution. After the staining period, the coverslip is returned to the culture dish for the washing steps.

13. The maximum and minimum fluorescence intensities of each nucleus were determined using the image analysis subroutine. By viewing over 100 images, including human and mouse monokaryons as well as homodikaryons and heterodikaryons, it was determined that a nucleus devoid of human Gal3 (not stained by NCL-GAL3) yielded a maximum fluorescence of 250 (arbitrary units). In contrast, a nucleus containing human Gal3 always yielded a fluorescence intensity of 300 or greater. On this basis, a nucleus whose maximum fluorescence intensity was 250 or lower is scored negative for the presence of human Gal3 and a nucleus whose minimum fluorescence intensity was 300 or higher was scored positive for the presence of human Gal3.
14. The inclusion of CHX decreased the percentage of heterodikaryons exhibiting human Gal3 in both nuclei from 72 to 50 %. Thus, newly synthesized human Gal3 was not the only source for the mouse nucleus in the heterodikaryon. The percentage of heterodikaryons exhibiting human Gal3 in both nuclei was further decreased in the presence of CHX + LMB to 19 %. Thus, at least some of the human Gal3 appearing in the mouse nucleus of a heterodikaryon had to first exit the human nucleus into the common cytoplasm followed by entry into the mouse nucleus, fulfilling the definition of shuttling.
15. *Characterization of fusion constructs.* Verify all constructs with DNA sequencing, SDS PAGE and Western blotting of cell extracts for both GFP and Gal3, and binding of fusion proteins to α-lactose-agarose.
16. Mutation of a single base is adequate; in the example in Fig. 2A, the first G of the GAATTC *Eco*RI site will be mutated to a C (shown as **C**). For removing or adding restriction sites within coding frames, silent mutations are preferred, with conservative mutations the next best option. When designing oligonucleotides, include a minimum of nine nucleotides identical to the sequence of the template on either side of the mutated residue, ending with a G or C from the template sequence. If more than one nucleotide is to be replaced, increase the length of the oligo by a few nucleotides on either side of the mutated sequence. Ideal oligos will possess 40–70 % GC content. We highly recommend using an oligo analysis tool to check for secondary structure and the likelihood of homodimer formation, as we have had little trouble with oligos with a ΔG that does not exceed −15 kcal/mol for any single interaction.

17. Evaluation of the success of the mutagenesis reaction for this particular mutation was made easier by loss of an *Eco*R47III restriction site (AGCGCT).
18. In some experiments, Lab-Tek 2-well chamber slides (Nalge Nunc International) were used instead of coverslips in 35-mm culture dishes.
19. Collect data from three independent experiments with at least 100 fluorescent cells each.
20. We used a program such as StatView, version 5.01 (SAS Institute) to perform chi-square analysis to determine whether any two histograms were significantly different. The analyses were performed using the "Contingency Table" function, selecting "Coded summary data" and deselecting "Fisher's Exact Test."
21. In Subheading 3.3.1, the final reaction volume should be 100 µl (volume primer 1 + volume primer 2 + volume of NEB Buffer 1 + volume of 100× BSA + volume of dNTPs + volume of Klenow DNA polymerase).
22. Test NESs could also be inserted following the method described in Subheading 3.3.1.
23. In step **11** of Subheading 3.2.2, the 2 ml of fresh DMEM containing 10 % calf serum should include 5 µg/ml ActD (to limit the nuclear import activity of the NLS of HIV-1 Rev) and 10 µg/ml CHX (to block new protein synthesis).
24. To assess whether nuclear export is exportin-1 mediated, requiring a leucine-rich NES, include in the medium change (step **11** of Subheading 3.2.2) 10 nM LMB to inhibit nuclear export and 10 µg/ml CHX to block synthesis of new fusion protein.

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