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Sean R. Stowell Date

Modulation of Innate and Adaptive Immunity by Galectin Family Members

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Immunology and Molecular Pathogenesis

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Modulation of Innate and Adaptive Immunity by Galectin Family Members

By

Sean Robinson Stowell
B.S., Brigham Young University, 2001

Advisor: Richard D. Cummings, Ph.D.

An abstract of
a dissertation submitted to the Faculty of the Graduate
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ABSTRACT

Modulation of Innate and Adaptive Immunity by Galectin Family Members

Sean Robinson Stowell

Regulation of leukocyte viability is a key mechanism responsible for inflammatory resolution. Many factors, including members of the TNF and galectin families, appear to regulate fundamental activities of leukocytes involved in both innate and adaptive immunity. However, the mechanisms responsible for this regulation remain enigmatic. Therefore, in this study we sought to clarify the mechanisms underlying the immunomodulatory activities of galectins. Our studies demonstrate varied effects of galectin on different leukocyte populations as well as innate antimicrobial activity. Several galectins, including Galectin-1 (Gal-1), galectin-2 (Gal-2), galectin-3 (Gal-3), galectin-4 (Gal-4) and galectin-8 (Gal-8) induce phosphatidylserine (PS) exposure, a common feature of apoptotic cell death, in activated neutrophils independently of cell death. Induction of PS exposure sensitizes cells to phagocytic removal, yet fails to induce alterations in cell viability. This process, preaparesis, prepares live cells for removal, in contrast with pathways induced by other immunomodulatory factors such as TNF family members that induce apoptotic cell removal. In addition to effects in neutrophils, galectins also regulate T cells. Gal-3 signals T cell apoptosis while other galectin family members signal secretion of the cytokine IL-10. Unlike TNF family members, which regulate leukocyte turnover through protein-protein interactions, galectins uniquely recognize cell surface carbohydrates. Selective modification of these carbohydrate ligands significantly and differentially alters cellular sensitivity to galectin signaling of leukocytes. In addition to regulating leukocyte function, several galectins, including galectin-8, appear to also possess critical innate immune capacity, directly killing blood group B positive pathogens independent of complement. Taken together, these studies demonstrate that galectins possess critical activities in both innate and adaptive immunity.

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Chapter 1

INTRODUCTION

In addition to regulatory mechanisms governing cellular trafficking, cytokine secretion and cellular differentiation, cell turnover, and removal during homeostasis and inflammation provides an important regulatory point in controlling immune function. Leukocyte removal often requires the induction of apoptosis by effecter molecules, such as members of the TNF family, including Fas, TRAIL and tumor necrosis factor-alpha (TNFα) [1-3]. Recent studies also implicate the galectin family of β-galactoside binding proteins in the regulation of leukocyte turnover [4].

Galectins represent an evolutionary conserved protein family found in all metazoans [4]. Currently, galectin family members reside in three groups based on similarities in quaternary structure: prototypical, chimera, and tandem repeat [5]. The prototypical galectins, Gal-1, Gal-2, Gal-7, Gal-10, Gal-13 and Gal-14, exist primarily as homodimers with each carbohydrate recognition domain (CRD) arranged in opposing directions. Gal-3, the only chimeric galectin, possesses one CRD and a large non-lectin amino terminal collagen-like domain, which mediates homo-oligomerization following ligand binding. Tandem repeat galectins, which include Gal-4, Gal-8, Gal-9 and Gal-12, consist of one polypeptide that folds into two distinct CRDs connected by a short linker peptide (Fig. 1-1A). Each galectin family member shares conserved residues within the CRD that allow overlapping recognition of the simple dissacharide Galβ1-4GlcNAc. However, adjacent residues in and around each CRD differ between family members, suggesting potential differences may exist in glycan binding following Galβ1-4GlcNAc modification (Fig. 1-1).

Many biological functions have been attributed to various galectin family members, which range from muscle development to RNA splicing [6, 7]. The vast majority of previous studies on galectin function, however, have examined their possible roles in the regulation of adaptive immunity [4, 8-10]. Gal-1, Gal-2, Gal-3, and Gal-9 are thought to induce apoptosis in activated T cells through discrete receptor recognition and distinct pathways [11-14]. In addition, galectins may also moderate thymocyte selection during T cell development; Gal-1, Gal-3 and Gal-9 induce apoptosis in both CD4/CD8 double negative and double positive thymocytes [15-17]. Galectin-induced apoptosis of effector T cells *in vivo* appears to provide therapeutic potential; administration of Gal-1 ameliorates most clinical sequelae associated with a wide range of autoimmune disease in animal models [18-28].

Cells undergoing apoptosis expose phosphatidylserine (PS), a phospholipid normally confined to the inner leaflet of the plasma membrane [29, 30]. At the cellular level, signals initiated during apoptosis activate phospholipid scramblases, which allows PS to mobilize to the outer leaflet of the plasma membrane [31, 32]. Concomitant inactivation of aminophospholipid translocase, which normally translocates PS and phosphatidylethanolamine (PE) inside the cell, allows net movement of PS to the outer membrane of the cell [31]. Once exposed, PS serves as a ligand for receptor mediated phagocytosis [29, 32]. In contrast to necrosis, engagement of apoptotic cells by macrophages reduces their activation and induces the secretion of anti-inflammatory cytokines [33]. As a result, apoptotic cell death not only reduces the number of activated T cells or other leukocytes following an inflammatory episode, but also signals paracrine events which facilitate inflammatory resolution [33].

In contrast to T cells, several studies demonstrate that neutrophils may possess distinct pathways of removal. For example, mice genetically deficient in either Fas or Fas L exhibit lymphocytosis while maintaining normal neutrophil numbers [34]. Similarly, patients with autoimmune lymphoproliferative disease, who also possess mutations in either Fas or FasL, likewise exhibit defects in lymphocyte removal while maintaining normal neutrophil numbers [35, 36]. Neutrophils transgenetically engineered to express bcl-2 fail to undergo apoptosis, yet appear to remain equally sensitive to phagocytic removal [37]. In addition, Kupffer cells phagocytose non-apoptotic neutrophils *in vivo* [38]. Thus, while apoptotic cellular removal of T cells appears to be a requirement for appropriate turnover, neutrophils likely possess apoptosis-independent pathways for removal.

Until recently, potential factors responsible for regulating non-apoptotic turnover of neutrophils remained unknown [29, 39]. We recently demonstrated that Gal-1 induces PS exposure in the absence of apoptosis in activated, but not resting, neutrophils [40]. Although Gal-1 induces PS exposure in the absence of cell death, it sensitizes cells for phagocytic removal [40, 41], providing the first description of a single factor capable of inducing cellular phagocytosis of living cells. Gal-1 induces PS exposure through a transient intracellular Ca^{2+} mobilization and Src kinase activation [42] (Fig. 1-2,1-3). **However, whether other features of apoptosis, in addition to PS exposure, occur following Gal-1 treatment is unknown. In addition, whether other galectin family members also possess the ability to induce PS exposure independently of apoptosis in neutrophils or other leukocyte populations remains untested.**

Although the putative immunoregulatory roles of Gal-1 require extracellular engagement of leukocyte ligands, Gal-1 displays a unique sensitivity to oxidative inactivation once

outside the cell [43-46], suggesting a potential redox regulatory mechanism governing galectin function. Gal-1 has only free, unpaired Cys residues, but upon oxidation, Gal-1 forms three specific disulfide bonds that result in significant conformational changes that preclude further glycan binding [47-49]. Several studies suggest that oxidized Gal-1 may possess unique functions independent of carbohydrate recognition [50], suggesting that oxidation may regulate distinct Gal-1 effector functions. Engagement of carbohydrate ligand protects Gal-1 from oxidative inactivation [45, 46, 51], allowing Gal-1 to retain activity once bound to leukocyte or ECM ligands. **However, the mechanism whereby ligand protects Gal-1 remains unclear.**

In addition to their putative roles in the regulation of leukocyte viability, several galectin family members display important and often disparate immunoregulatory activities independently of inducing cell death or phagocytic removal. For example, Gal-1 inhibits mast cell degranulation [25], but other studies demonstrate that Gal-3 induces degranulation in mast cells independently of IgE-mediated antigen stimulation [52]. Furthermore, while Gal-1 blocks leukocyte chemotaxis [53], Gal-3 has the opposite effect, inducing leukocyte chemotaxis [54] and the release of pre-formed IL-8 from neutrophils [55], which further augments chemotaxis of leukocytes [56]. In addition, while Gal-1 inhibits acute inflammatory responses through various mechanisms, including suppression of PLA-2-induced edema [57] and inhibition of neutrophil extravasation [53], Gal-3 enhances the extravasation of neutrophils, and Gal-3-null mice also exhibit attenuated leukocyte infiltration following challenge [58]. Interestingly, patients with reduced Gal-2 expression were found to have reduced risk for myocardial infarction, suggesting that Gal-2 may also have pro-inflammatory roles [59] (Fig. 1-4). Furthermore, recent studies demonstrate that Gal-3, but not Gal-1, induces cell death in *Candida albicans [60]*. Gal-3 also appears to recognize specific antigens on *Leishmania major*

and *Schistosoma mansoni* [61, 62]. **However, the potential involvement of other galectins in innate immunity, either as pattern recognition receptors or as factors that directly eliminate pathogens, remains to be determined.**

The differential activity of different galectin family members toward distinct leukocyte populations suggests that each member likely possess unique carbohydrate recognition properties. However, previous studies on galectin-glycan interactions fail to demonstrate significant differences in glycan recognition [63, 64]. Limitations in the availability of glycan libraries made it difficult to assess subtle, yet important differences in glycan binding. Common methods used to assess galectin-ligand interactions may also preclude accurate reflection of glycan presentation *in vivo*. For example, Gal-1 recognizes N-acetyllactosamine (LacNAc) and poly-N-acetyllactoamsine (polyLacNAc) with similar affinity in solution [65]. However, when glycans are surface immobilized, in a fashion that may more closely mimic the natural membrane presentation, Gal-1 displays a 10-fold increase in affinity for polyLacNAc glycans over LacNAc alone [65]. This preference also holds true for cell surface polyLacNAc glycans and likely reflects the need for favorable terminal LacNAc presentation [65]. **However, whether other galectin family members display a unique preference for glycans following immobilization remains unknown.**

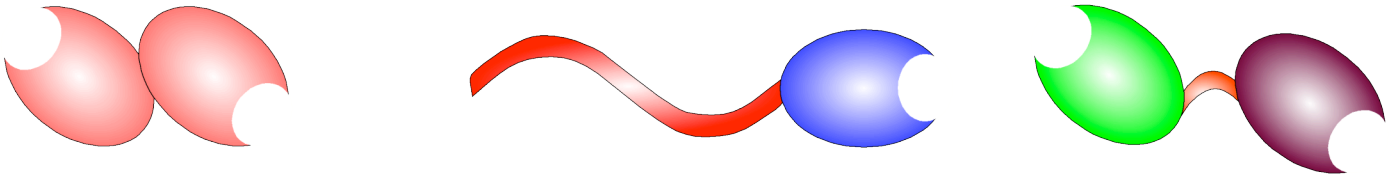
In this work, we sought to answer several questions concerning galectin function in an effort to understand at a mechanistic level, the potential overlapping and distinct activities of different galectin family members *in vivo*. Our studies provide several key findings concerning galectin function: 1) PS exposure induced by Gal-1 fundamentally differs from PS exposure accompanying apoptosis, both spatially and temporally. 2) Ligand regulates Gal-1 sensitivity to oxidative inactivation by modulating monomer-dimer

equilibrium. 3) Gal-1 displays broad expression in adult tissue with unique localization in striated tissue. 4) Gal-1, Gal-2, and Gal-4 induce PS exposure in neutrophils independently of apoptosis, yet fail to alter T cell viability. 5) Gal-3 induces PS exposure independently of apoptosis in neutrophils, while it induces apoptosis in T cells. 6) Gal-1, Gal-2, and Gal-3 display distinct binding preferences for cell surface polyLacNAc glycans, which results in differential recognition of sialylated glycans. 7) Gal-8 specifically kills blood group B positive *E. coli* strain O86.

It is our expectation that these collective results will clarify and extend previous studies in the field and provide valuable insight into the potential mechanisms whereby galectin family members modulate immunity *in vivo*.

Figure 1-1

Galectins in Humans



Prototypical	Chimeric	Tandem Repeat
Galectin-1	Galectin-3	Galectin-4
Galectin-2		Galectin-8
Galectin-7		Galectin-9
Galectin-10		Galectin-12
Galectin-13		
Galectin-14		

Sequence Alignments of Some Human Galectins

```
                41   *  *  *                 56      *     66   *   71*  *
Galectin-1   -NLCLHFNPRFNAHGD------ANTIVCNSKD-GGAWG—TEQRE-
Galectin-2   -KLNLHFNPRFS----------ESTIVCNSLD-GSNWG-QEQRE-
Galectin-3   -DVAFHFNPRFNENN-------RRVIVCNTKL-DNNWG-REERQ-
Galectin-4   -DVAFHFNPRFDG---------WDKVVFNTLQ-GGKWG-SEERK-  (N-term)
             -DIALHINPRMG----------NGTVVRNSLL-NGSWG-SEEKK-  (C-term)
Galectin-7   -DAALHFNPRLD----------TSEVVFNSKE-QGSWG-REERG
```

red = conserved hydrophilic residues;
blue = conserved hydrophobic residues
* = residues directly involved in binding sugars

Figure 1-2

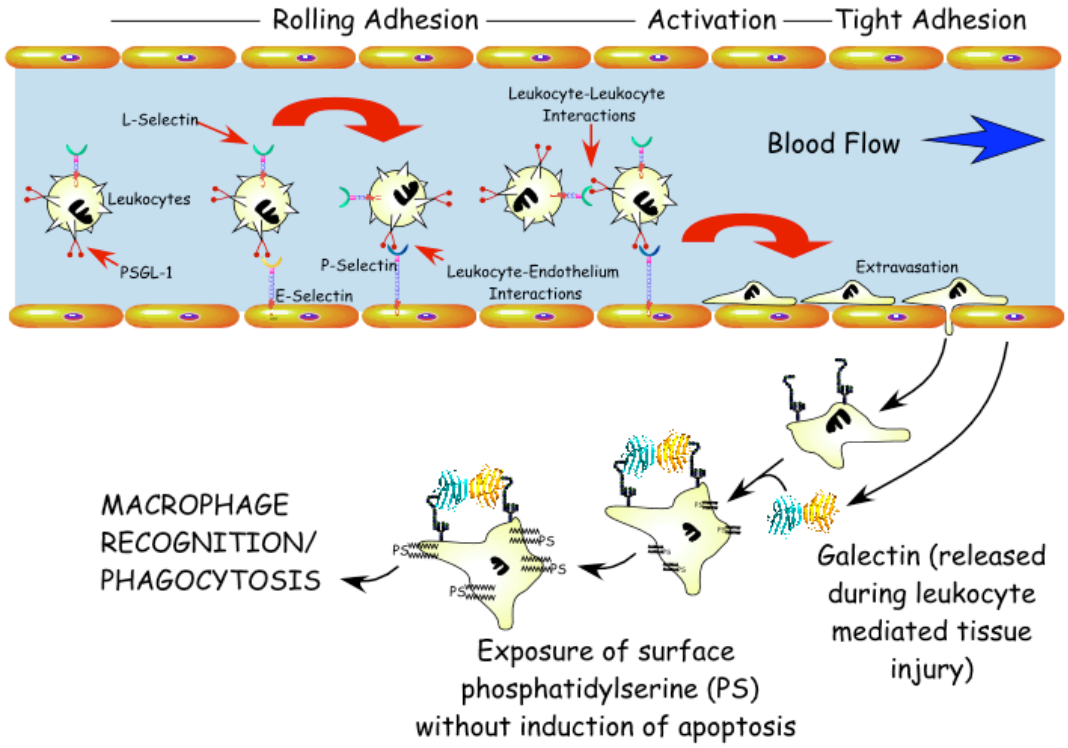
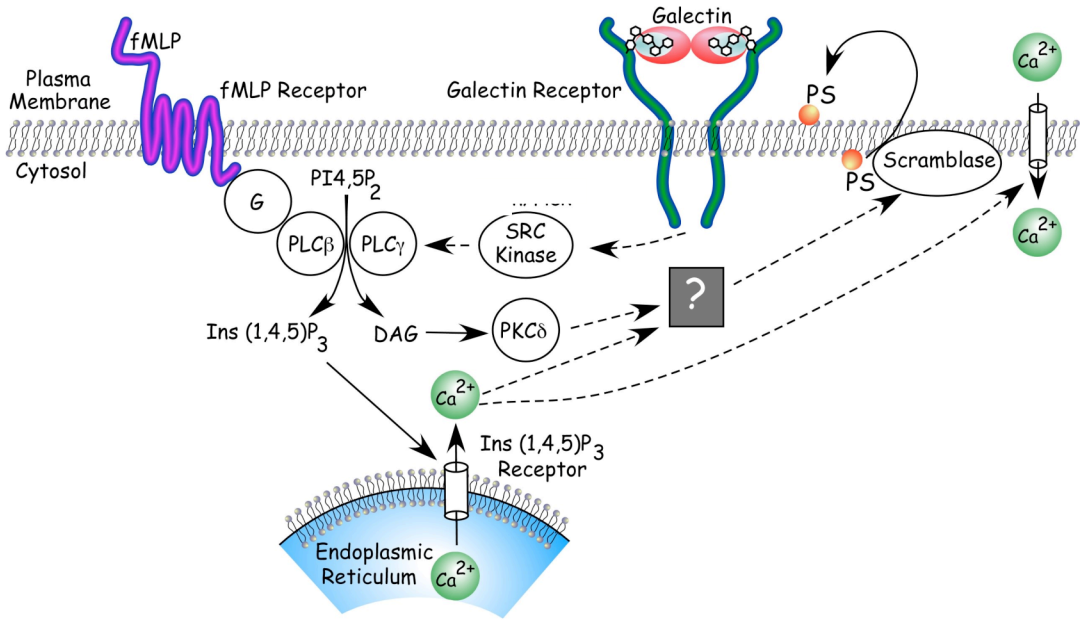

Rolling Adhesion
Activation
Tight Adhesion
L-Selectin
Leukocytes
PSGL-1
E-Selectin
P-Selectin
Leukocyte-Leukocyte Interactions
Leukocyte-Endothelium Interactions
Blood Flow
Extravasation
MACROPHAGE RECOGNITION/ PHAGOCYTOSIS
PS
Exposure of surface phosphatidylserine (PS) without induction of apoptosis
Galectin (released during leukocyte mediated tissue injury)

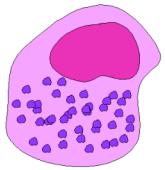
Figure 1-3


Galectin
fMLP
Plasma Membrane
fMLP Receptor
Galectin Receptor
PS
Ca2+
Cytosol
PS
Scramblase
G
PI4,5P2
PLCβ
PLCγ
SRC Kinase
Ca2+
?
Ins (1,4,5)P3
DAG
PKCδ
Ca2+
Ins (1,4,5)P3 Receptor
Endoplasmic Reticulum
Ca2+

Galectin Activities Toward Leukocytes

General Immune Functions

Gal-1 reduces pathology associated GVHD, ConA-induced hepatitis, EAE, Myasthenia Gravies and RA.
Gal-1 reduces acute inflammatory responses
Gal-2 is involved in the pathogenesis of artheroma formation
Gal-3 exacerbates Th2 immune responses (Asthma)

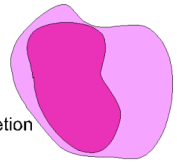


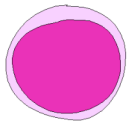
Mast Cells

Gal-1 inhibits degranulation
Gal-3 induces degranulation independent of antigen-mediated IgE stimulation.

Monocytes/Macrophages

Gal-3
- -enhances phagocytosis
- -enhances respiratory burst
- -enhances LPS-induced IL-1β secretion
- -inhibits apoptosis (intracellularly)





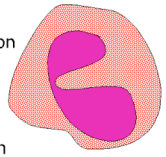
B cells

Gal-3 blocks IL-4-induced survival of activated B cells
- -favors plasma cell differentiation
- -exhibits an anti-apoptotic role in B-cell lymphomas

Eosinophils

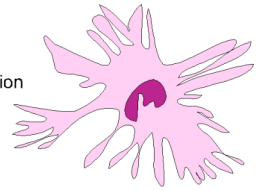
Gal-3 inhibits IL-5 production
Gal-9 induces
- -chemotaxis
- -activation
- -superoxide generation
- -moderate degranulation

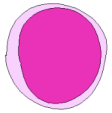
Gal-10 (Charcot-Leyden crystal protein) is highly expressed; function unknown
Gal-14 expressed but function unknown



Dendritic Cells

Gal-1and Gal-9 induce maturation





Thymocytes/T cells

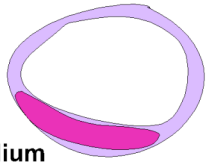
Gal-1 involved in Treg effector function.
- -conflicting results on effects on viability
- -mediates adhesion of thymocytes to thymic epithelium
- -induces apoptosis in $CD4^+CD8^+$ double positive thymocytes (DTT present)
- -induces shift in Th1 response to Th2 (decreases IFNγ; increases IL-5)
- -reduces IL-2, TNFα, IL-1β, IL-12, and IFNγ
- -enhances Treg formation
- -increases IL-10 production in both niave and activated T cells

Gal-2-induces T cell apoptosis (DTT present)
- - decreases IFNγ and TNFα while increasing IL-10 and IL-5

Gal-3 blocks apoptosis when overexpressed intracellularly
- -conflicting results on the effects of endogenous intracellular involvement in viability
- -conflicting reports on the effects of extracellular effects on viability
- -promotes adhesion of thymocytes to thymic epithelium
- -enhances Th2 immune responses
- -enhances adhesion of niave T cells to DCs
- -binds TCR, reducing TCR-mediated T cell activation

Gal-4 induces IL-6 production
Gal-8 activates Rac-1
Gal-9 induces apoptosis in thymocytes and T cells (DTT present) .
- -induces selective loss of $CD4^+$ Th1 cells
- -induces selective loss of $CD8^+$ T cells

Gal-10 involved in Treg effector function.



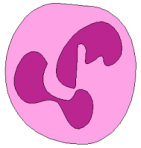
Endothelium

Gal-3 enhances pro-inflammatory cytokine release

Neutrophils

Gal-1 induces apoptosis-independent PS exposure (Ca^{2+} dependent)
- -inhibits chemotaxis
- -inhibits extravasation
- -activates NADPH dependent respiratory burst.

Gal-3 induces chemotaxis
- -enhances extravasation
- -activates NADPH dependent respiratory burst.
- -induces neutrophil activation
- -induces release of IL-8
- -mediates interaction with lamnin and fibronectin (both directly and indirectly)
- -enhances leukocyte adhesion to endothelium



Gal-2 induces apoptosis-independent PS exposure (Ca^{2+} dependent)
Gal-4 induces apoptosis-independent PS exposure (Ca^{2+} independent)
Gal-8-activates NADPH dependent respiratory burst.
- - modulates integrin-mediated neutrophil adhesion

Figure 1-4

FIGURE LEGENDS

Figure 1-1. The galectin family of β-galactoside binding proteins. Galectins reside in three distinct groups based on their quaternary structure: prototypical, chimera, and tandem repeat. Sequence alignment for Gal-1, Gal-2, Gal-3, Gal-4 and Gal-7 CRD is shown. Critical residues involved in recognition of the core Galβ1-4GlcNAc are highlighted.

Figure 1-2. Model depicting potential involvement of galectin in neutrophil turnover. Following extravasation of neutrophils to an area of injury, neutrophil mediated injury of viable tissue likely releases active Gal-1. Gal-1 engages leukocyte receptors and signals PS independent of apoptosis that prepares neutrophils for phagocytic removal

Figure 1-3. Gal-1 signals PS exposure in activated neutrophils. Gal-1 induces the exposure of PS in activated neutrophils through proximal signaling pathways that involve transient Ca^{2+} mobilization and Src kinase activation.

Figure 1-4. Known immunoregulatory functions of galectins. Previous published findings regarding galectin modulation of the immune system including findings published in this work. Adapted from Interactions of Leukocytes with Galectins [66].

Chapter 2

Galectin-1 induces reversible phosphatidylserine exposure at the plasma membrane.

Cells normally undergo physiologic turnover through the induction of apoptosis and phagocytic removal, partly through exposure of cell surface phosphatidylserine (PS). In contrast, neutrophils appear to possess apoptosis-independent mechanisms of removal. Here we show that Galectin-1 (Gal-1) induces PS exposure independent of alterations in mitochondrial potential, caspase activation, or cell death. Furthermore, Gal-1-induced PS exposure reverts following Gal-1 removal without altering cell viability. Gal-1-induced PS exposure is uniquely microdomain restricted, yet cells exposing PS do not display evident alterations in membrane morphology nor do they exhibit bleb formation, typically seen in apoptotic cells. Long-term exposure to Gal-1 prolongs PS exposure with no alteration in cell cycle progression or cell growth. These results demonstrate that Gal-1-induced PS exposure and subsequent phagocytic removal of living cells represents a new paradigm in cellular turnover.

INTRODUCTION

Cellular turnover represents one of the most fundamental homeostatic processes of multi-cellular organisms. Although many tissues experience cellular division and removal, cells of the immune system possess a unique capacity to rapidly proliferate in response to pathogenic challenge [67]. Significant expansion of leukocytes involved in both innate and adaptive immunity ultimately results in neutralization and removal of invading pathogens [68]. However, for effective immunological homeostasis to be maintained, efficient contraction of activated leukocyte populations must occur [69]. Failure to appropriately eliminate activated leukocytes not only enhances the probability of cellular transformation, but also results in leukocyte-mediated damage of viable tissue and can eventually result in autoimmunity [70, 71].

Many factors regulate leukocyte turnover, including members of the tumor necrosis factor (TNF) and galectin families [10, 22]. TNF family members, including Fas, TRAIL, and TNFα effect leukocyte contraction through the induction of apoptotic cell death. Similarly, several galectin family members, including galectin-3 and galectin-9 [12, 26] (Gal-3 and Gal-9), also induce leukocyte removal through apoptosis [1, 26, 72]. Cells undergoing apoptotic cell death typically express phosphatidylserine (PS), a phospholipid normally confined to the inner leaflet of the plasma membrane, which serves as a ligand for receptor mediated phagocytosis [29]. Apoptotic cell death also results in DNA degradation and eventual cellular fragmentation [73, 74]. Importantly, apoptosis occurs in a coordinated fashion, ultimately resulting in homeostatic cellular removal without inciting the deleterious consequences of an inflammatory response [73].

In contrast to Gal-3, Gal-9, and members of the TNF family, several studies suggest that galectin-1 (Gal-1) may induce PS exposure in leukocytes independent of apoptosis. For example, Gal-1 induces PS exposure in leukocytes in the absence of detectable DNA fragmentation [40]. Externalization of PS exposure in viable cells may not be limited to Gal-1, as activation of some leukocytes, in particular T cells and mast cells, can also result in apoptosis-independent PS exposure [75-79]. Activation-induced PS exposure in viable cells appears to regulate diverse cellular processes ranging from cellular trafficking to proper formation of the immunological synapse [78, 79]. Taken together, these previous studies strongly suggest that cellular pathways exist whereby PS exposure may be induced in viable cells with diverse consequences.

Although Gal-1 induced PS exposure may occur in viable cells and therefore alter cellular processes as described previously [75-79], Gal-1-induced PS exposure sensitizes cells to phagocytic removal [40], in a similar fashion to cells undergoing apoptotic cell death [30]. Such results suggests that Gal-1 induced PS exposure may accompany apoptotic cell death, consistent with several other studies suggesting that Gal-1 actually induces apoptotic cell death in leukocytes [14, 80]. However, Gal-1 is uniquely sensitive to oxidative inactivation. As a result, studies showing Gal-1-induced apoptosis included dithiothreitol (DTT) in cell treatments [17, 22, 80]. Indeed, utilization of DTT appears to render cells susceptible to Gal-1-induced cell death [72, 81, 82]. However, the treatment of living cells with DTT can induce the unfolded protein response and can directly induce apoptotic cell death [83-85], making interpretations about the effects of Gal-1 on leukocyte viability in the presence of DTT difficult.

Although the inclusion of DTT may complicate assays on cellular viability, the ability of Gal-1 to induce PS externalization and prime cells for phagocytic removal, both of which

represent characteristic features of cells undergoing apoptotic cell death [29, 40], suggests that Gal-1 could induce apoptotic cell death, even in the absence of DTT. To help resolve these conflicting interpretations, we have more thoroughly examined whether Gal-1-induced PS exposure occurs in the presence or absence of cell death. In this study we demonstrate that Gal-1-induced PS exposure occurs in the absence of cell death, which suggest a new paradigm in cellular removal.

RESULTS

Gal-1 induces reversible PS exposure

To determine whether Gal-1 induces PS exposure in the presence or absence of cell death, we first sought to examine whether Gal-1-induced PS exposure exhibits reversibility following Gal-1 removal, as PS exposure represents a terminal process in cells undergoing apoptosis[29, 39]. Consistent with previous results, Gal-1 induced robust PS exposure in leukocytes (Fig. 2-1A,B) as measured by Annexin V (An-V) staining following prolonged incubation, similar to cells undergoing apoptotic cell death. However, Gal-1 treated cells displayed complete *reversion* of PS exposure following Gal-1 removal (Fig. 2-1A,B), with a cell surface half-life of approximately 10 h (Fig. 2-1D-F). By contrast, cells induced to undergo apoptosis by camptothecin (Camp) treatment displayed near complete *conversion* from a PS positive/PI negative state to a PS positive/PI positive state over the same period of time (Fig. 2-1A,C,F).

PS reversion does not represent PS-positive cellular removal

These results strongly suggest that Gal-1-induced PS exposure may be reversible following Gal-1 removal. PS externalization sensitizes neutrophils for phagocytic removal by surrounding macrophages[40]. Although neutrophils do not normally phagocytose other neutrophils undergoing apoptotic cell death[68], we considered the possibility that Gal-1 induced PS exposure may potentially sensitize cells for phagocytic removal by PS-negative cells, similar to recent reports showing that transformed epithelial cells can cannibalize neighboring cells[86]. In addition, because previous studies suggested that Gal-1 may be mitogenic in some cell populations[87, 88], we determined whether PS reversion might reflect outgrowth of PS-negative cells. To differentiate between these possibilities, we labeled cells with the intracellular dye carboxyfluorescein succinimidyl

ester (CFSE) to enable tracking of two distinct cell populations within a mixture. To ensure that CFSE did not alter cellular sensitivity to Gal-1-induced PS exposure, cells were labeled with or without CFSE followed by incubation with Gal-1. Importantly, CFSE did not alter cellular sensitivity to Gal-1 (Fig. 2-2A,B) and CFSE-labeled cells could be easily distinguished following Gal-1 treatment within a mixture of cells (Fig. 2-2C). Gal-1 treated PS-positive cells experienced full reversion with no alteration in the percent of cells within each population (Fig. 2-2D-F). This demonstrates that there was no removal or loss of cells[89]. In addition, the Gal-1 treated cell population, compared to non-treated cells, displayed nearly identical CFSE dilution (Fig. 2-3A-C) which is a mark of cell proliferation[67]. These results demonstrate that PS reversion does not represent overgrowth or engulfment by a subpopulation of PS-negative cells and that cells undergoing reversion of PS experience normal cell growth kinetics. Furthermore, physically sorted PS-positive cells following initial Gal-1 treatment reverted PS upon removal of Gal-1, similar to cells mixed with untreated cells (Fig. 2-3A-E). This reversion occurred without alterations in DNA degradation or cell growth (Fig. 2-3D,F,G), which further demonstrated that Gal-1-induced PS exposure autonomously reverts following Gal-1 removal.

Cells previously positive for PS remain sensitive to restimulation by Gal-1

We next determined whether cells previously positive for PS remain sensitive to Gal-1 signaling following PS reversion. Cells retreated with Gal-1 displayed virtually identical sensitivity to Gal-1 in terms of re-expression of PS (Fig. 2-4A), thus demonstrating that cells remain sensitive to Gal-1 following PS reversion. However, PS re-expression may represent PS exposure in cells previously PS-negative following primary incubation with Gal-1. To address this possibility, we physically sorted cells following initial Gal-1 treatment into PS-positive and PS-negative populations (Fig. 2-3D, Gal-1 Day 0).

Following reversion of PS exposure in PS-positive cells (Fig. 2-4B), we incubated previously PS-positive or PS-negative cells with Gal-1. PS-positive cells not only remained sensitive to Gal-1 re-treatment after PS reversion, but displayed enhanced sensitivity to Gal-1 when compared to PS-negative cells (Fig. 2-4B,C), with no detectable DNA fragmentation following re-incubation with Gal-1 (Fig. 2-4B,D).

Gal-1 induces PS exposure through a caspase-independent process

The reversible nature of Gal-1-induced PS exposure suggests that Gal-1 does not engage irreversible cellular pathways that normally accompany apoptotic cell death, such as mitochondrial potential depolarization and caspase activation[90]. Consistent with this, Gal-1 failed to alter mitochondrial potential, although cells undergoing apoptotic cell death demonstrated significant depolarization (Fig. 5A,C). Furthermore, treatment of cells with the pan-caspase inhibitor, zVAD-fmk, did not inhibit Gal-1 induced PS exposure (Fig. 2-5B,D), although it inhibited PS exposure in cells undergoing apoptotic cell death (data not shown). In addition, cells treated with Gal-1 failed to display cleavage of the common caspase substrate PARP[91]; by contrast, cells undergoing apoptotic cell death displayed significant PARP cleavage (Fig. 2-5E). These results show that Gal-1 induces PS exposure independent of irreversible processes, such as mitochondrial potential loss or caspase activation, consistent with the ability of PS to revert in its surface exposure following Gal-1 treatment and withdrawal and strongly suggesting that Gal-1-induced PS exposure occurs independent of cell death.

Gal-1-induced PS exposure resides in punctate microdomains

In contrast to Gal-1-induced PS exposure, apoptotic cells generated by camptothecin treatment not only converted to a double PS/PI positive state over time, which indicated conversion to late apoptosis, but also displayed a fraction of cells which displayed a

greater magnitude of PS positivity during the single PS positive state (Fig. 2-1A). By contrast, significant numbers of apoptotic cells also exhibited similar PS positivity to Gal-1 treated cells during early apoptosis (Fig. 2-1A). These differences may reflect the amount and distribution of PS on the cell surface, as previous studies demonstrated that cells undergoing apoptosis often display punctate PS exposure during early phases of apoptosis which progresses to encompass the entire cell surface over time[92]. Previous results suggested that Gal-1 induced PS exposure may exhibit some asymmetric distribution on the plasma membrane, similar to early phases of apoptotic cell death[92], suggesting that cells incubated with Gal-1 may fail to transition to later phases of apoptosis where greater PS positivity occurs. To examine this in more detail, we analyzed cells by confocal analysis for localization of PS expression following incubation with Gal-1 at later time points. In contrast to cells undergoing apoptotic cell death, Gal-1 treated cells retained punctate PS exposure, while cells undergoing apoptosis progressed to nearly uniform PS exposure (Fig. 2-6A).

In addition to alterations in phospholipid asymmetry, cells undergoing apoptosis experience gross morphological change in membrane architecture, including surface flattening of microvilli and the formation of apoptotic blebs[93]. We examined whether cell surface alterations that normally accompany apoptosis might also occur during Gal-1-induced PS exposure. Consistent with previous results[93], SEM analysis of apoptotic cells showed significant retraction of microvilli and cell surface flattening with prominent formation of apoptotic blebs (Fig. 2-6D,G,H). By contrast, cells exposed to Gal-1 displayed morphological features indistinguishable from non-apoptotic controls (Fig. 2-6B,C,E,F). These results support the conclusion that Gal-1 induces PS exposure independent of cell death and without changes in cellular morphology.

iGal-1 induces sustained PS exposure without altering cell division

Although PS exposure induced by Gal-1 clearly reverts following Gal-1 removal, we sought to define whether continuous Gal-1 incubation could induce sustained PS exposure over prolonged incubation periods. However, Gal-1 loses significant activity following prolonged incubation periods (days) [46, 51]. Previous studies utilized DTT in cell treatments to prevent Gal-1 oxidation[14, 22, 80]. However, DTT alone can induce significant alterations in cellular viability and artificially sensitize cells to Gal-1-induced apoptosis (Fig. 2-7A,B)[72, 82, 85]. As a result, we stabilized Gal-1 by derivatization with iodoacetamide (iGal-1), as outlined previously[72, 94]. Similar to the unmodified protein, iGal-1 induced significant PS exposure in cells (Fig. 2-7C). iGal-1-induced PS exposure required binding to cell surface carbohydrates, since lactose, a general inhibitor of galectin-ligand interactions[95], completely blocked iGal-1-induced PS exposure (Fig. 2-7C). Furthermore, iGal-1-induced PS exposure displayed equivalent kinetics, occurred over a similar dose response, and also sensitized cells to phagocytosis at comparable levels to cells undergoing apoptotic cell death as observed previously (Fig. 2-7D-F)[40].

Since iGal-1 had similar activity to Gal-1 in short term treatments, we examined the effects of iGal-1 on cell viability and PS exposure over prolonged time in the absence of DTT [72, 82, 85]. Cells incubated with iGal-1 in the absence of DTT maintained continual PS exposure over 3 days (Fig. 2-8A,D). Importantly, although iGal-1 induced sustained PS exposure, no detectable changes in PI staining, cellular fragmentation, or DNA degradation occurred (Fig. 2-8A-F). By contrast, cells undergoing apoptosis displayed significant increases in PI staining, cellular fragmentation, and DNA degradation over the same time period (Fig. 2-8A-F).

The ability of iGal-1 to induce unaltered PS exposure over a prolonged period may represent iGal-1-induced cell cycle arrest, despite normal cell division following Gal-1 removal during PS reversion (Fig. 2-3A). To determine whether cells incubated with iGal-1 continued to divide or whether iGal-1 induced cell cycle arrest in the presence of sustained PS exposure, we labeled cells with CFSE[67]. Cells treated with iGal-1 continued to experience CFSE dilution while maintaining uniform PS exposure over time (Fig. 2-9A,C). PS exposure also remained punctate over 2 days of continuous iGal-1 incubation (Fig. 2-9B) with no alterations in cell cycle progression or viable cell number (Fig. 2-9D,E). Taken together, these results demonstrate that iGal-1 induces sustained PS exposure without altering cell growth or viability.

DISCUSSION

The ability of Gal-1 to induce sustained PS exposure with no detectable alterations in DNA degradation, cell size, membrane integrity, or cell division demonstrates that Gal-1-induced PS exposure occurs in the absence of cell death. The reversion of PS exposure following Gal-1 removal demonstrates that unlike apoptotic PS exposure, Gal-1-induced PS exposure does not represent a terminal event and further demonstrates that Gal-1 induces PS exposure in viable cells. Given the ability of Gal-1 to sensitize cells to phagocytic removal[40] (and Figure 7D-F), these results demonstrate that Gal-1 induces phagocytic removal of living cells, as opposed to apoptotic cells, suggesting a new paradigm in cellular turnover.

Although many studies suggested that Gal-1 affects leukocyte turnover and function[96], whether Gal-1 directly alters leukocyte viability remained enigmatic. Many previous studies utilized the reducing agent DTT when evaluating the effect of Gal-1 on leukocyte viability[14, 17, 22, 80]. However, DTT induces the unfolded protein response, directly induces apoptosis, and sensitizes cells to Gal-1-induced cell death[72, 82-85]. Because the extracellular environment is largely oxidative and DTT artificially penetrates cell membranes[97], the physiological relevance of DTT inclusion remains unclear. Other studies demonstrate that Gal-1 induces PS exposure and sensitizes cells to phagocytic removal in the absence of DTT[40], suggesting that Gal-1 may alter cell viability regardless of DTT inclusion. However, these studies failed to detect DNA fragmentation or alterations in membrane integrity following Gal-1 incubation[40, 72, 82], suggesting that either definitive signs of Gal-1-induced cell death required prolonged Gal-1 incubation or that Gal-1 induces phagocytic removal of living cells. To clarify the effect of Gal-1 on cell viability we utilized iGal-1, which retains previously defined activities of

non-derivatized Gal-1, yet remains resistant to oxidation in the absence of DTT[40, 72, 94, 98]. Cells treated with iGal-1 maintained PS exposure for 3 continuous days without inducing changes in membrane integrity, DNA degradation, or cell size. Furthermore, cells continued to growth normally as indicated by absolute viable cell numbers, CFSE dilution, and cell cycle analysis. These results not only demonstrate that Gal-1 fails to alter cell viability while maintaining prolonged PS externalization, but also suggest that PS asymmetry is not a strict requirement for cell division.

The ability of Gal-1 to induce PS exposure despite failing to alter cellular viability presented the unusual possibility that cells incubated with Gal-1 may reverse PS exposure following Gal-1 removal. This was important to definitively evaluate, as PS externalization is commonly thought to be a terminal event of, and directly correlated with, cells undergoing apoptotic cell death[29, 39]. Indeed, removal of Gal-1 resulted in PS reversion without detectable alterations in cell viability, which further demonstrated the non-apoptotic nature of Gal-1-induced PS exposure. Importantly, although several studies suggest that Gal-1 may be mitogenic at low concentrations[87, 88], reversion did not represent mitogenic outgrowth of a PS negative population, as differentially labeled cells and physically sorted cells autonomously reverted PS. The ability of PS positive cells to autonomously revert PS also ruled out the possibility that PS reversion reflected cannibalism by bystander cells, as recently reported to occur in transformed epithelial cells[86]. The ability of Gal-1 to induce PS exposure despite failing to engage irreversible pathways commonly associated with apoptotic cell death, such as caspase activation and loss of mitochondrial potential[99], also corroborates the reversible nature of Gal-1 induced PS exposure. Equally important, while some studies demonstrate that caspases may directly induce PS exposure[100, 101], caspase activation is not a prerequisite or necessary for PS exposure to occur.

In addition to inducing PS exposure in the absence of apoptosis, Gal-1-induced PS exposure morphologically differed from PS exposure accompanying cell death. Gal-1 induced PS exposure remained punctate regardless of the duration of Gal-1 incubation, while cells undergoing apoptotic cell death displayed uniform PS exposure prior to transition to late apoptosis and loss of membrane integrity. Previous results demonstrate that apoptotic cells also exhibit punctate PS exposure during early phases of apoptosis with gradual progression over the entire cell surface with time[92]. Although the exact players responsible for PS mobilization remain uncertain [102], PS asymmetry does require an ATP driven aminophospholipid translocase (APT) activity that translocates any external PS back to the inner leaflet of the plasma membrane[103]. By contrast, putative phospholipid scramblases, which are thought to facilitate PS externalization, often occur in membrane microdomains[104]. Failure of cells incubated with Gal-1 to undergo cell death may prevent full inactivation of ATP-driven APT[105, 106], suggesting that Gal-1 may activate PS exposure in areas near lipid rafts followed by re-internalization by incompletely inactivated APT, resulting in the appearance of punctate PS. Although Gal-1-induced PS exposure occurs independent of caspase activation, cells undergoing apoptosis activate putative scramblases through caspase-mediated activation of PKC that results in scramblase phosphorylation and activation[107]. The ability of Gal-1 to activate PKC[42], suggests that Gal-1 may be able to induce PS exposure independent of caspase activation by directly activating scramblase.

Gal-1 displays unique sensitivity to oxidative inactivation. Thus, increases in environmental oxidation associated with failure to remove pathogens may trigger reversal of PS exposure in leukocytes originally targeted for removal to allow re-

engagement in host defense[45, 51, 68, 97]. Such normally oxidative environments may be especially important for granulocytes, which do not possess antigen specific immunity and therefore have limited capacity to directly detect pathogen levels[68]. Failure to remove pathogens results in tissue damage and injury, which causes additional leukocyte recruitment and enhanced oxidation of the extracellular environment. Enhanced oxidation leads to Gal-1 inactivation[44], allowing cells originally targeted for removal to revert PS and become re-engaged in host defense. Consistent with this, continual incubation of cells with non-stabilized Gal-1 results in spontaneous Gal-1 oxidation and PS reversion (Stowell and Cummings, unpublished observations). Thus, by such mechanisms Gal-1 may be uniquely suited to sense oxidative environments and alter leukocyte turnover and accumulation.

Although apoptosis generally prevents inflammation during cellular turnover, exuberant apoptosis, as can occur during inflammation, can actually be pro-inflammatory[108]. Consistent with this, several studies suggest that granulocytes have a unique apoptosis-independent pathway(s) of removal. Granulocytes transgenically overexpressing bcl-2 fail to undergo apoptotic cell death, yet display normal sensitivity to phagocytic removal *in vivo* and *in vitro*[37]. Furthermore, Kupffer cells phagocytose non-apoptotic neutrophils *in vivo*[38]. In addition, Fas and FasL null mice, which display significant defects in lymphocyte turnover, display no alterations in homeostatic granulocyte turnover, both in resting and inflammatory conditions[109]. In contrast to the reversible nature of Gal-1-induced PS exposure, the ability of Gal-1 to sustain PS exposure may reflect successful pathogen removal, inflammation resolution, and therefore reduced Gal-1 oxidation. In this way, Gal-1-induced turnover of viable cells allows granulocytes to actively maintain membrane integrity until successfully phagocytosed.

In addition to regulating leukocyte turnover, recent studies suggest that Gal-1-induced PS exposure in viable cells may also regulate other fundamental cellular processes. For example, PS exposure during T cell activation appears to alter ion transport, cellular trafficking and immunological synapse formation [78, 79], although the extent to which PS directly modulates these processes remains uncertain[110]. In addition to T cells, several studies suggest that B cells may also externalize PS independent of apoptosis[111-114]. Brief PS externalization may also facilitate the release of preformed inflammatory mediators, as macrophages and mast cells display transient PS exposure following activation [115, 116]. By contrast, recent studies demonstrated that ligation of cell surface GPI anchored proteins on mast cells can induce sustained PS exposure independent of cell death[75, 76]. Similar to Gal-1-induced PS exposure, sustained PS exposure in mast cells may contribute to the removal of viable cells harboring pathogens during infection[75].

Before the discovery of alterations in PS asymmetry in cells undergoing apoptosis[117], many studies documented a key role for PS asymmetry on activated platelets in thrombosis[118]. Similar to platelets, PS exposure on cells can also induce thrombosis[119, 120], which suggests that in addition to regulating neutrophil turnover, Gal-1-induced PS exposure may also contribute to fibrin deposition. Consistent with this, improper resolution of inflammatory responses often results in significant peripheral fibrin deposition[121], suggesting a role for PS positive neutrophils awaiting removal in this process.

Figure 2-1

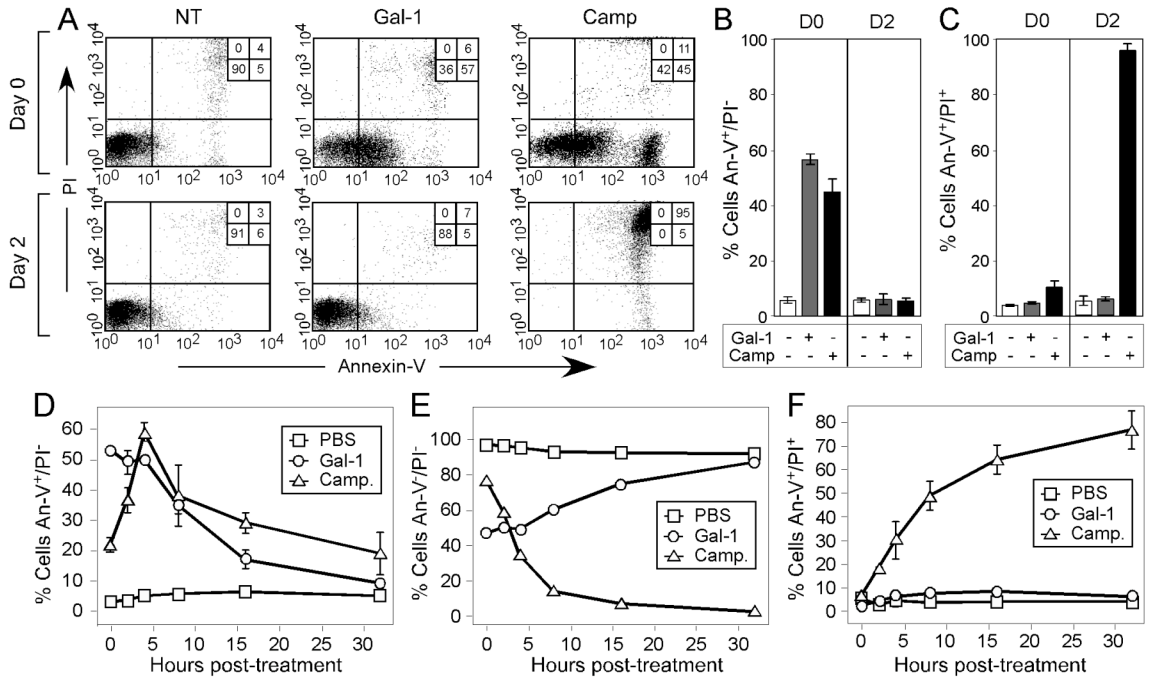

A
NT
Gal-1
Camp
Day 0
Day 2
PI
Annexin-V
B
D0
D2
% Cells An-V+/PI-
Gal-1
Camp
C
% Cells An-V+/PI+
D
% Cells An-V+/PI-
PBS
Gal-1
Camp.
Hours post-treatment
E
% Cells An-V-/PI-
F
% Cells An-V+/PI+

Figure 2-2

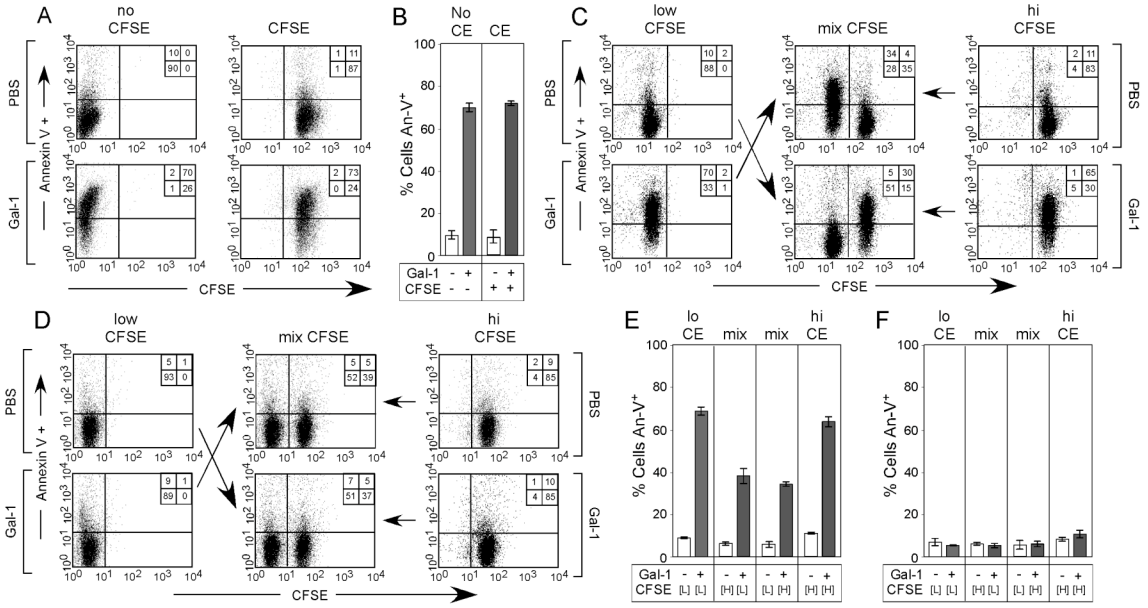

A
no CFSE
CFSE
PBS
Gal-1
Annexin V +
CFSE
B
No CE
CE
% Cells An-V+
Gal-1
CFSE
C
low CFSE
mix CFSE
hi CFSE
PBS
Gal-1
Annexin V +
CFSE
D
low CFSE
mix CFSE
hi CFSE
PBS
Gal-1
Annexin V +
CFSE
E
lo CE
mix
mix
hi CE
% Cells An-V+
Gal-1
CFSE
F
lo CE
mix
mix
hi CE
% Cells An-V+
Gal-1
CFSE

Figure 2-3

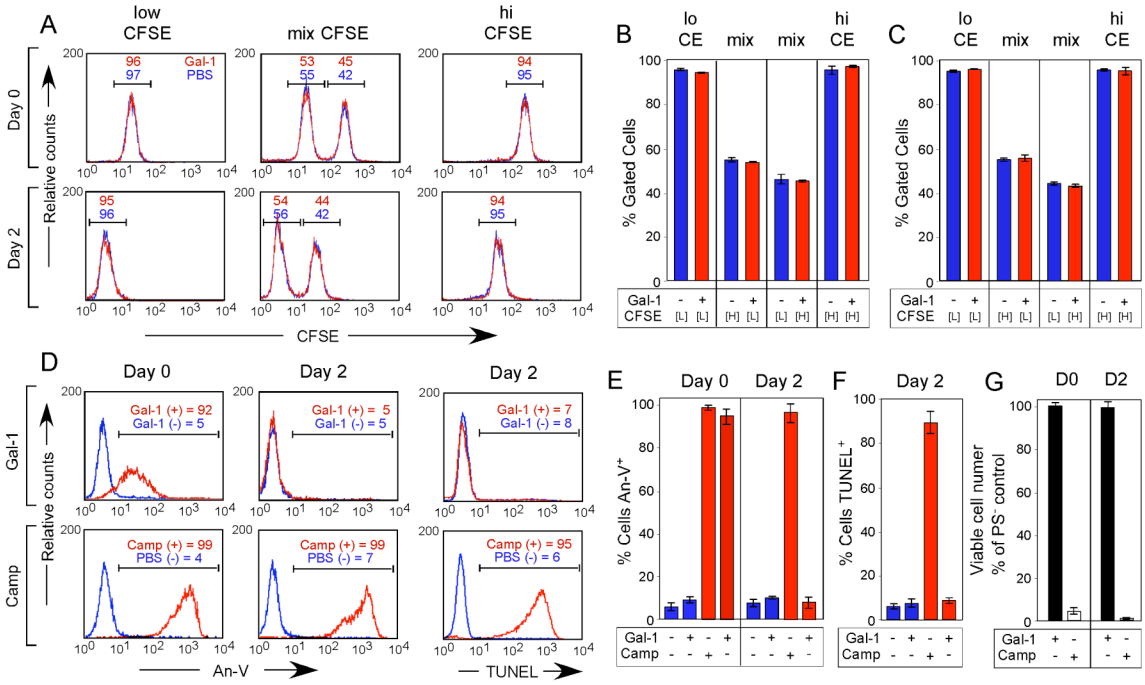

A
low CFSE
mix CFSE
hi CFSE
Day 0
Day 2
Relative counts
Gal-1
PBS
96
97
53
55
45
42
94
95
54
56
44
42
CFSE
B
lo CE
mix
mix
hi CE
% Gated Cells
Gal-1
CFSE
C
D
Day 0
Day 2
Day 2
Gal-1
Camp
Gal-1 (+) = 92
Gal-1 (-) = 5
Gal-1 (+) = 5
Gal-1 (-) = 5
Gal-1 (+) = 7
Gal-1 (-) = 8
Camp (+) = 99
PBS (-) = 4
Camp (+) = 99
PBS (-) = 7
Camp (+) = 95
PBS (-) = 6
An-V
TUNEL
E
Day 0
Day 2
% Cells An-V+
F
Day 2
% Cells TUNEL+
G
D0
D2
Viable cell numer
% of PS- control

Figure 2-4

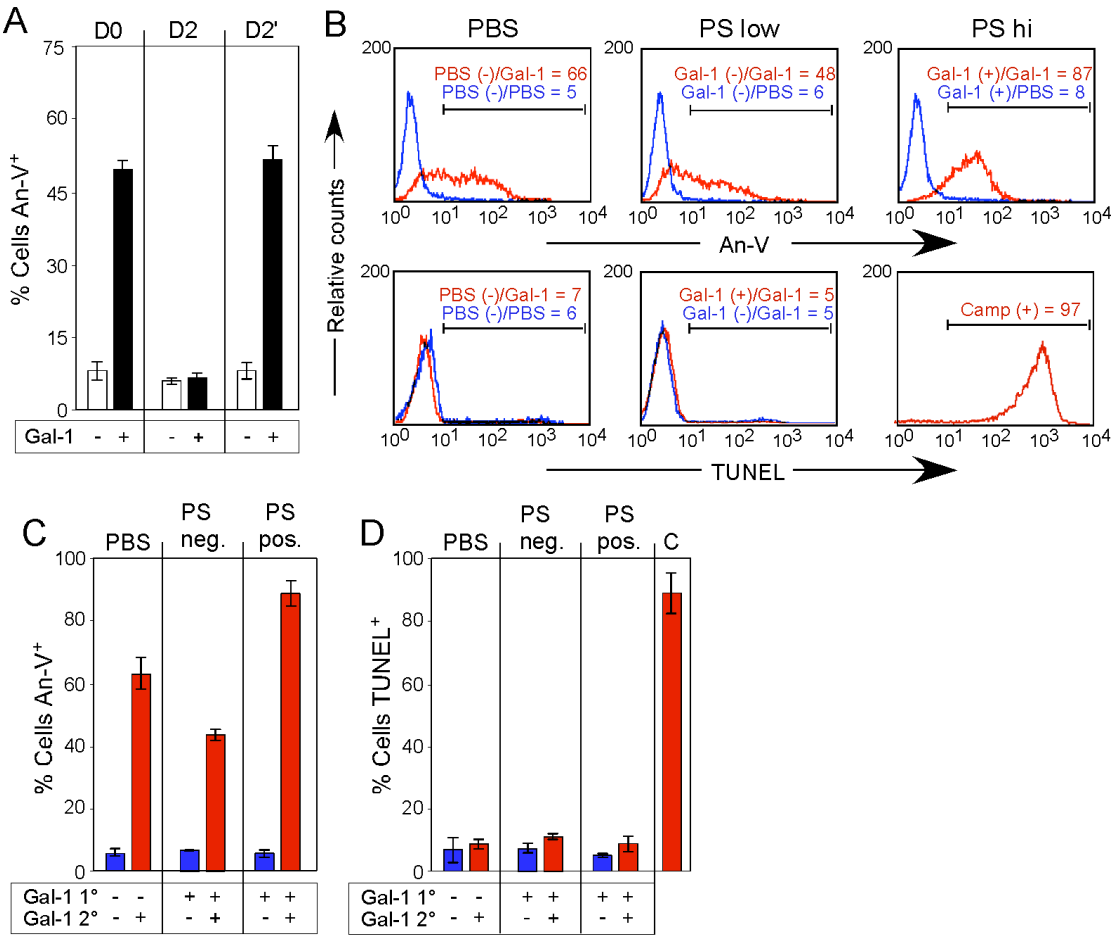

A
D0
D2
D2'
% Cells An-V+
Gal-1
B
PBS
PS low
PS hi
PBS (-)/Gal-1 = 66
PBS (-)/PBS = 5
Gal-1 (-)/Gal-1 = 48
Gal-1 (-)/PBS = 6
Gal-1 (+)/Gal-1 = 87
Gal-1 (+)/PBS = 8
Relative counts
An-V
PBS (-)/Gal-1 = 7
PBS (-)/PBS = 6
Gal-1 (+)/Gal-1 = 5
Gal-1 (-)/Gal-1 = 5
Camp (+) = 97
TUNEL
C
PBS
PS neg.
PS pos.
% Cells An-V+
Gal-1 1°
Gal-1 2°
D
PBS
PS neg.
PS pos.
C
% Cells TUNEL+
Gal-1 1°
Gal-1 2°

Figure 2-5

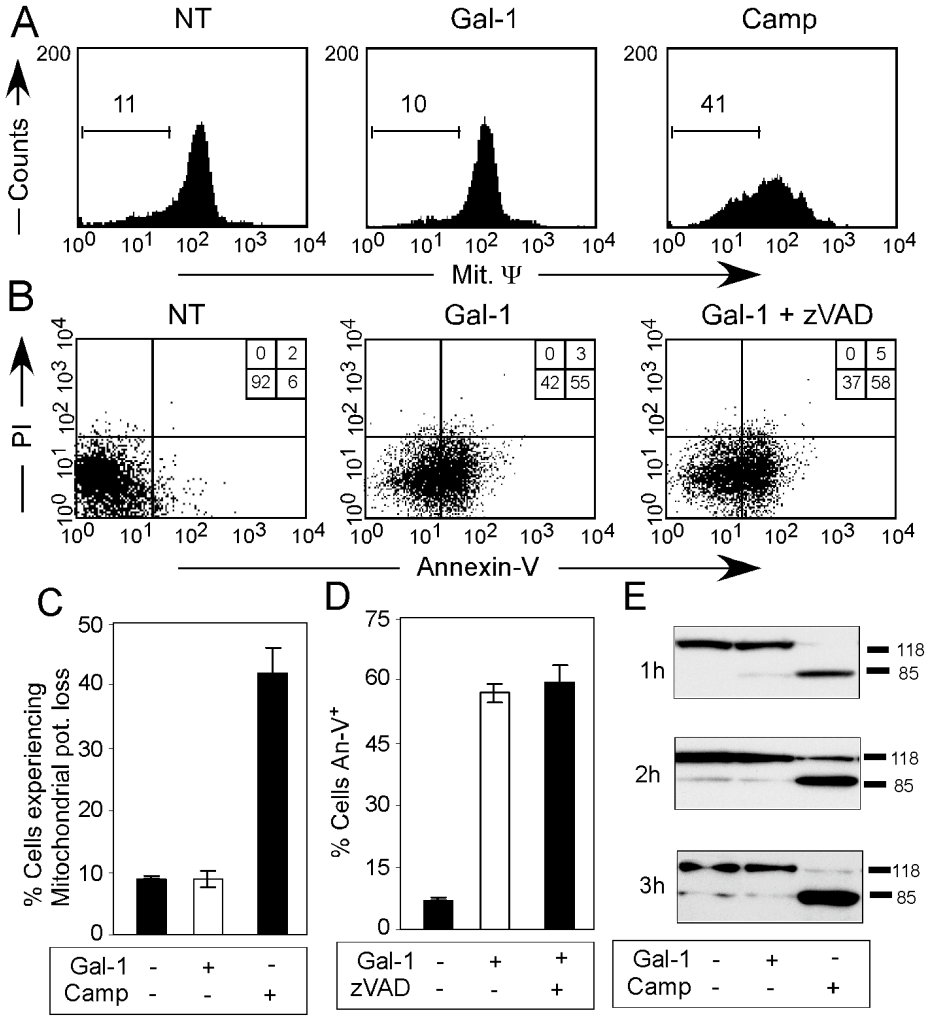

A
NT
Gal-1
Camp
200
11
10
41
Counts
Mit. Ψ
B
NT
Gal-1
Gal-1 + zVAD
0 2
92 6
0 3
42 55
0 5
37 58
PI
Annexin-V
C
% Cells experiencing Mitochondrial pot. loss
Gal-1 - + -
Camp - - +
D
% Cells An-V+
Gal-1 - + +
zVAD - - +
E
1h
2h
3h
118
85
Gal-1 - + -
Camp - - +

Figure 2-6

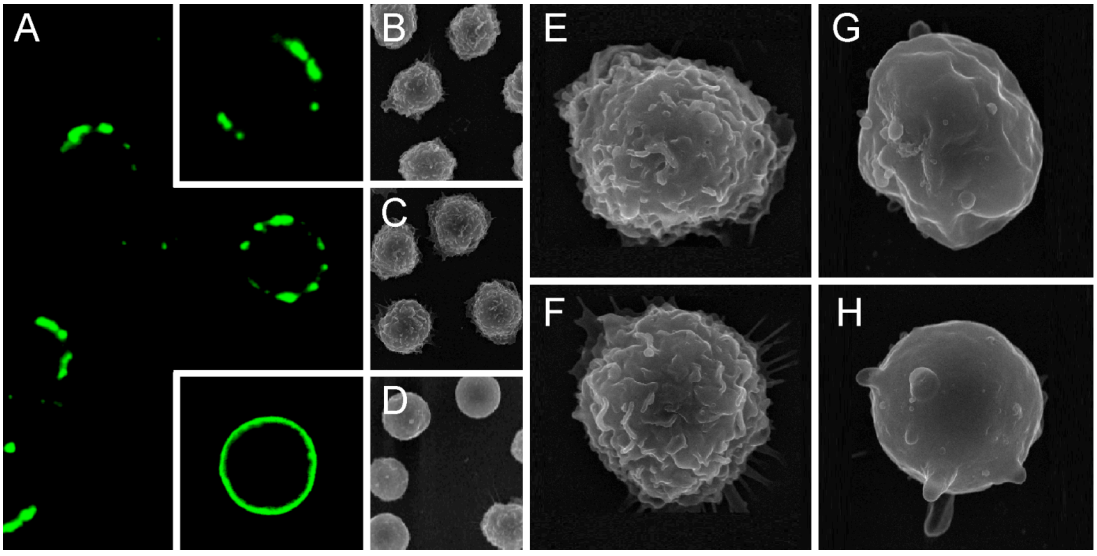


Figure 2-7

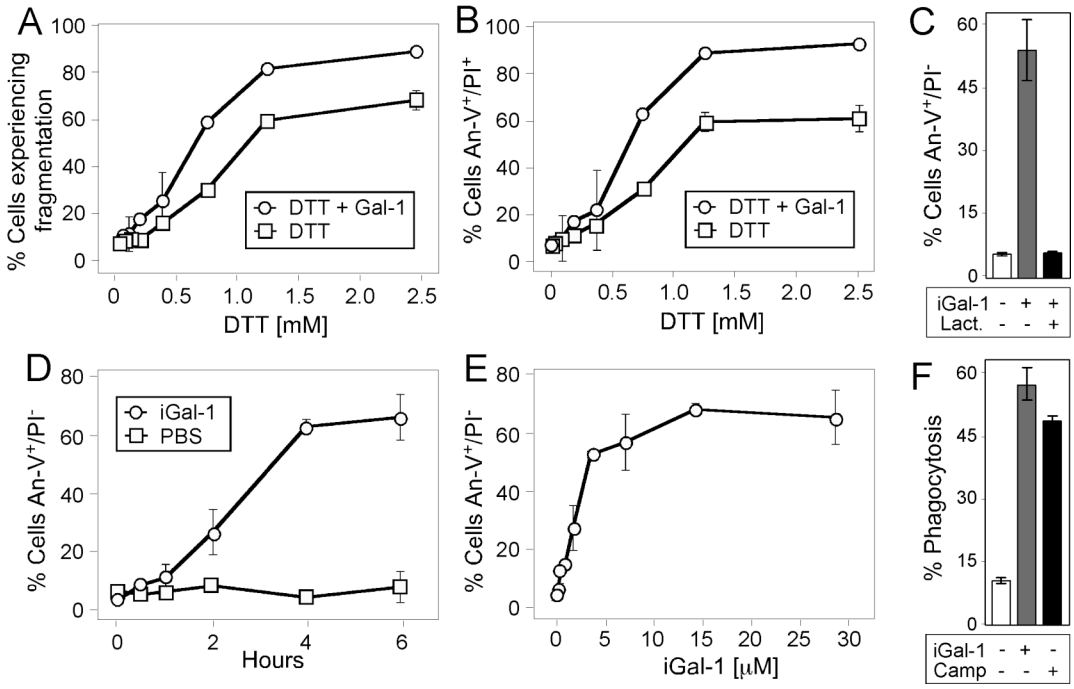

A
% Cells experiencing fragmentation
DTT [mM]
DTT + Gal-1
DTT
B
% Cells An-V+/PI+
DTT [mM]
DTT + Gal-1
DTT
C
% Cells An-V+/PI-
iGal-1 - + +
Lact. - - +
D
% Cells An-V+/PI-
Hours
iGal-1
PBS
E
% Cells An-V+/PI-
iGal-1 [μM]
F
% Phagocytosis
iGal-1 - + -
Camp - - +

Figure 2-8

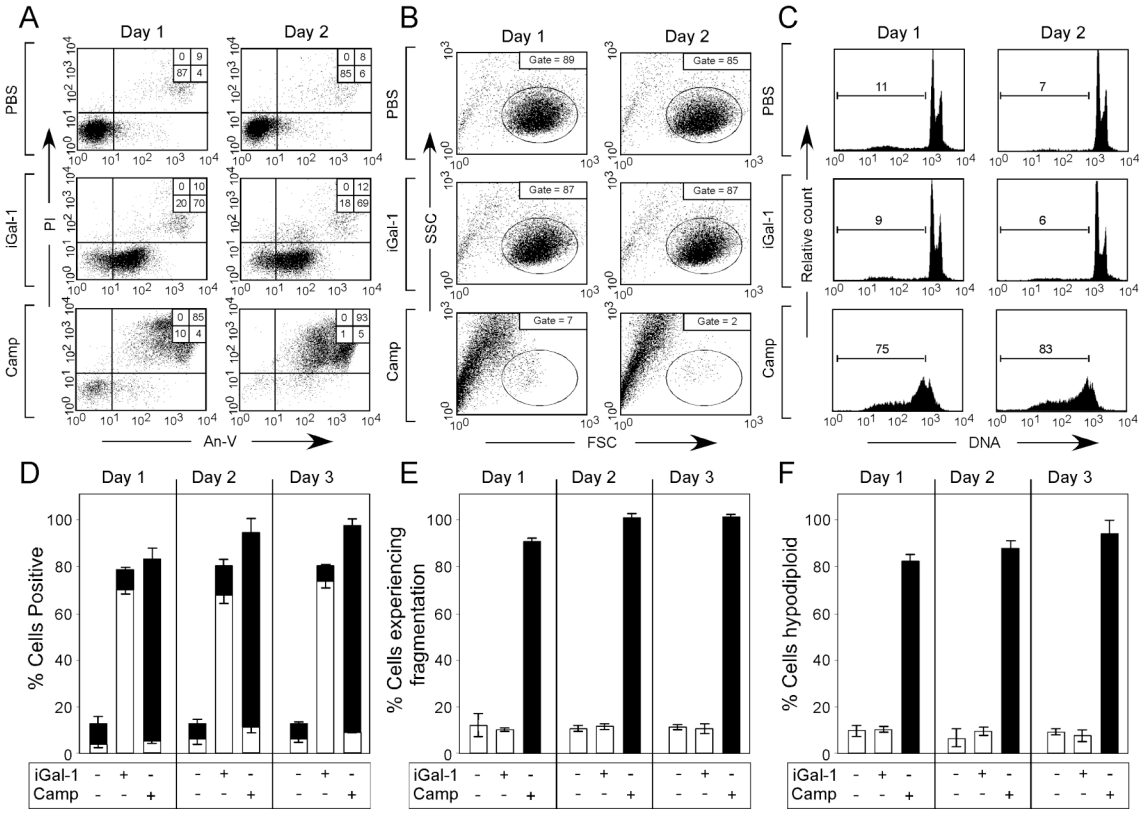

A
Day 1
Day 2
PBS
iGal-1
Camp
PI
An-V
0 9
87 4
0 8
85 6
0 10
20 70
0 12
18 69
0 85
10 4
0 93
1 5
B
Day 1
Day 2
Gate = 89
Gate = 85
Gate = 87
Gate = 87
Gate = 7
Gate = 2
SSC
FSC
C
Day 1
Day 2
11
7
9
6
75
83
Relative count
DNA
D
Day 1
Day 2
Day 3
% Cells Positive
E
% Cells experiencing fragmentation
F
% Cells hypodiploid
iGal-1 - + - - + - - + -
Camp - - + - - + - - +

Figure 2-9

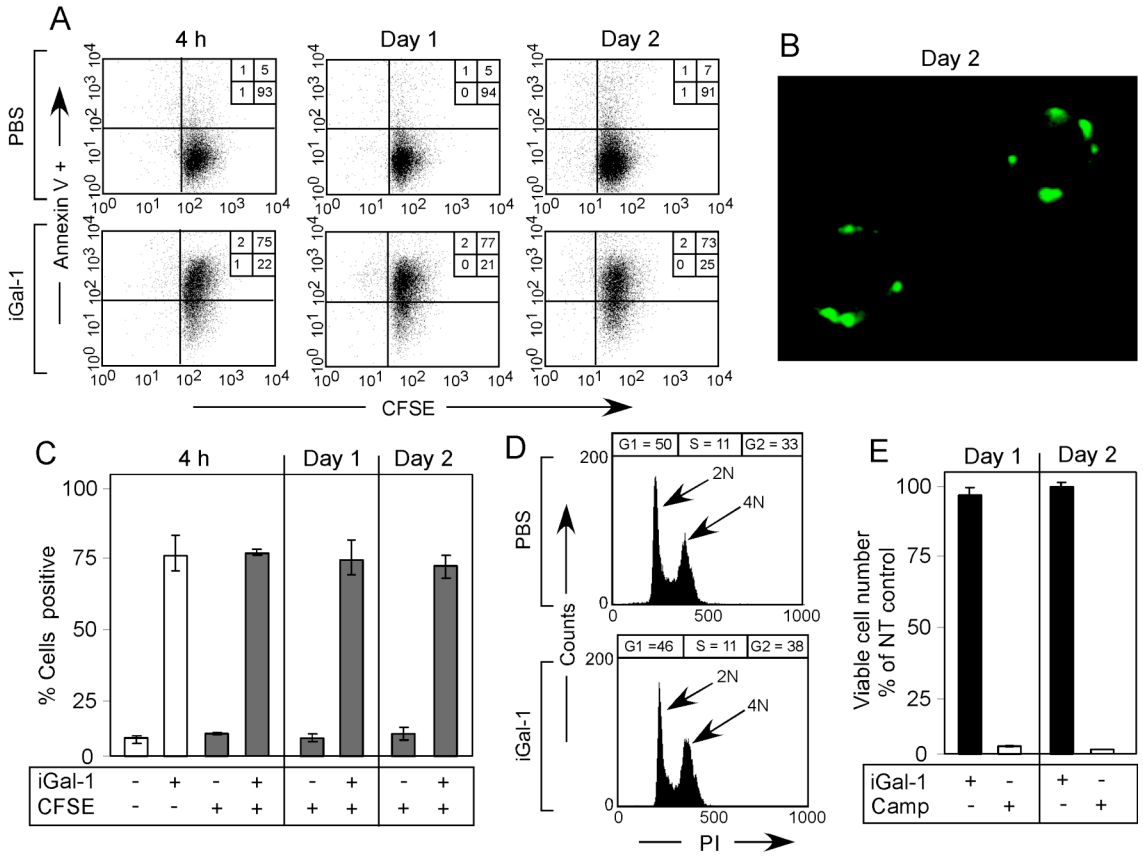

A
4 h
Day 1
Day 2
PBS
iGal-1
Annexin V +
CFSE
B
Day 2
C
4 h
Day 1
Day 2
% Cells positive
iGal-1
CFSE
D
PBS
iGal-1
Counts
G1 = 50
S = 11
G2 = 33
G1 =46
S = 11
G2 = 38
2N
4N
PI
E
Day 1
Day 2
Viable cell number
% of NT control
iGal-1
Camp

FIGURE LEGENDS

Figure 2-1. Gal-1 induces reversible PS exposure. (A) HL60 cells were treated with PBS (NT), 10 μM Gal-1, or 10 μM camptothecin (Camp) for 12 h followed by immediate detection for PS exposure using Annexin-V (An-V)-FITC, propidium iodide (PI), and subsequent flow cytometric analysis, or washed in lactose and re-incubated in complete RPMI for 2 days followed by detection for PS exposure using An-V-FITC, PI, and subsequent flow cytometric analysis. (B-C) As outlined in A, quantification of PS exposure (B) by An-V staining or cell death (C) indicated by the PI positivity of cells evaluated immediately following treatment for 12 hours (D0) or following removal of Gal-1 and incubation for 2 days (D2). (D-F) HL60 cells were treated with PBS (NT), 10 μM Gal-1, or 10 μM camptothecin (Camp) for 12 h followed by Gal-1 removal and detection for PS exposure using An-V-FITC, PI, and subsequent flow cytometric analysis at the indicated times following Gal-1 removal, with (D) representing those cells which only stain with An-V-FITC, (E) representing those cells staining with neither An-V-FITC or PI, and (F) representing those cells staining with both An-V-FITC and PI.

Figure 2-2. PS reversion does not represent PS positive cellular removal. (A) Cells were incubated with or without CFSE followed by incubation with Gal-1 and detection for PS exposure using An-V-FITC. (B) Quantification of PS exposure of cells treated with or without CFSE (CE). (C) Cells were differentially stained with CFSE followed by incubation with PBS or 10 μM Gal-1 for 4 h. Following the 4 h incubation, cells were incubated with 50 mM lactose to remove Gal-1. Cells were then either incubated alone or mixed followed by immediate examination of PS exposure using An-V-FITC (C) or re-suspended in complete RPMI and allowed to incubate alone or mixed as outlined for 2

days followed by detection for PS using An-V-FITC (D). (E) Quantification of PS exposure of cells treated in (C). (F) Quantification of PS exposure of cells treated in (D).

Figure 2-3. PS-positive cells display unaltered cell division during PS reversion. (A) Cells were differentially stained with CFSE followed by incubation with PBS or 10 μM Gal-1 for 4 h. Following the 4 h incubation, cells were incubated with 50 mM lactose to remove Gal-1. Cells were then either not mixed or mixed followed either by immediate enumeration of cell percentages in each population (Day 0), or re-suspended in complete RPMI and allowed to incubate alone or mixed as outlined for 2 days followed by enumeration of cell percentages in each population (Day 2) with red representing those cells treated with Gal-1 and blue representing those cells treated with PBS. (B-C) Quantification of the percent of cells in each population at Day 0 (B) and Day 2 (C) with red representing those cells treated with Gal-1 and blue representing those cells treated with PBS. (D) Upper panels: Cells were sorted into PS positive (red = Gal-1 treated PS positive) or PS negative (blue = Gal-1 treated PS negative) fractions followed by either re-staining for PS using An-V-FITC or re-incubating in complete RPMI for 2 days to allow PS reversion. Following the two-day incubation, cells were stained for PS using An-V-FITC or analyzed for DNA degradation using TUNEL. Lower panels: Cells were treated with PBS or 10 μM Camp followed by sorting into PS positive (red = Camp treated PS positive) or PS negative (blue = PBS treated PS negative) fractions, followed by either re-staining for PS using An-V-FITC or re-incubating in complete RPMI for 2 days to allow PS reversion. Following the two- day incubation, cells were stained for PS using An-V-FITC or analyzed for DNA degradation using TUNEL. (E-F) Quantification of cells treated as outlined in (D) for either PS exposure (E) or DNA fragmentation using TUNEL (F). (G) PS positive cells sorted following treatment with Gal-1 or Camp as outlined in (D) were resuspended in RPMI for 2 days followed by enumeration of viable cell number

using a hemocytometer and trypan blue exclusion. Cell numbers are reported as the percent of the PBS treated PS negative control.

Figure 2-4. Cells previously positive for PS remain sensitive to restimulation by Gal-1. (A) Cells were incubated with 10 μM Gal-1 for 4 h followed by removal of Gal-1 with 50 mM lactose and either detection of PS exposure by An-V-FITC and PI staining or re-suspension in complete RPMI. Resuspended cells were allowed to incubate for 2 days (Day 2) to revert PS followed by incubation with 10 μM Gal-1 for 4 h and detection for PS (Day 2'). (B) Upper panels: PS positive or negative cells previously incubated with Gal-1 or PS negative cells treated with PBS were re-treated with 10 μM Gal-1 or PBS for 4 h (Primary treatment/Secondary treatment) followed by detection of PS with An-V-FITC. Gate values are shown. Lower panels: PS positive or negative cells previously incubated with Gal-1 or PS negative cells treated with PBS were re-treated with 10 μM Gal-1 or PBS for 10 h (Primary treatment/Secondary treatment) followed by analysis for DNA fragmentation. Gate values are shown. (C-D) Quantification of cells treated as outlined in (B) for either PS exposure (C) or DNA fragmentation using TUNEL (D).

Figure 2-5. Gal-1 induces PS exposure through a caspase-independent process. (A) Cells were incubated with PBS (NT), 10 μM Gal-1, or 10 μM Camp for 8 h as indicated followed by examination for alterations in mitochondrial potential change. (B) Cells were incubated with or without zVAD-fmk followed by incubation with 10 μM Gal-1 for 4 h and analyzed for PS exposure by staining with An-V-FITC. (C) Quantification of cells treated as outlined in (A). (D) Quantification of cells treated as outlined in (B). (E) Cells were incubated with 10 μM Gal-1 or 10 μM Camp for the indicated times followed

by cell lysis and examination of PARP cleavage by SDS-PAGE and western blot analysis.

Figure 2-6. Gal-1-induced PS exposure resides in punctate microdomains. (A) Cells were incubated with 10 μM Gal-1 for 12h followed by staining with An-V. Upper inset: Representative cell treated with 10 μM Gal-1. Lower inset: Representative cell treated with 10 μM Camp. (B-H) SEM analysis of cells treated with PBS (B,E), 10 mM Gal-1 (C,F), or anti-Fas (D,G,H).

Figure 2-7. DTT sensitizes cells to Gal-1 induced apoptosis. (A) Cells were incubated with PBS, 10 μM Gal-1, or the indicated concentration of DTT for 9 hours 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 the indicated concentration of DTT for 9 hours 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 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 the indicated concentration of iGal-1 for 8 hours followed by detection for PS exposure by An-V-FITC staining and PI exclusion. (E) 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. (F) Cells were incubated with PBS, 10 μM iGal-1, or 10 μM Camp for 8 hours followed by incubation of peritoneal macrophages for 1 hour and microscopic examination of phagocytosis.

Figure 2-8. iGal-1 induces continuous PS exposure. (A) Cells were incubated with PBS, 10 μM iGal-1, or 10 μM Camp for 1 or 2 days 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 days 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) Cells were incubated with PBS, 10 μM iGal-1, or 10 μM Camp for 1 or 2 days as indicated followed by measuring DNA fragmentation by hypodiploid analysis. Gate = % of cells experiencing DNA fragmentation. (D) Quantification of cells treated in (A). White = % An-V+, Black = % PI+. (E) Quantification of cells treated in (B). (F) Quantification of cells treated in (C).

Figure 2-9. iGal-1 induces sustained PS exposure without altering cell division. (A) Cells were labeled with CFSE followed by incubation with 10 μM iGal-1 for 4 h, 1 day, or 2 days as indicated followed by detection for PS exposure by An-V-FITC. (B) Cells were incubated with 10 μM iGal-1 for 2 days followed by confocal analysis for PS exposure by An-V. (C) Quantification of data in (A). (D) Cells were incubated with 10 μM iGal-1 for 24 hours followed by cell cycle analysis. (E) Cells were incubated with PBS, 10 μM iGal-1 or 10 μM Camp for 1 or 2 days following by enumerating viable cell number using trypan blue exclusion.

Chapter 3

Ligand Reduces Galectin-1 Sensitivity to Oxidative Inactivation by Enhancing Dimer Formation.

Galectin-1 (Gal-1) regulates leukocyte turnover by inducing the cell surface exposure of phosphatidylserine (PS), a ligand that targets cells for phagocytic removal, in the absence of apoptosis. Gal-1 monomer-dimer equilibrium appears to modulate Gal-1-induced PS exposure, although the mechanism underlying this regulation remains unclear. Here we show that monomer-dimer equilibrium regulates Gal-1 sensitivity to oxidation. A mutant form of Gal-1 containing C2S and V5D mutations (mGal-1), exhibits impaired dimerization and fails to induce cell surface PS exposure, while retaining the ability to recognize carbohydrates and signal Ca^{2+} flux in leukocytes. mGal-1 also displayed enhanced sensitivity to oxidation, while ligand, which partially protected Gal-1 from oxidation, enhanced Gal-1 dimerization. Continual incubation of leukocytes with Gal-1 resulted in gradual oxidative inactivation with concomitant loss of cell surface PS, while rapid oxidation prevented mGal-1 from inducing PS exposure. Stabilization of Gal-1 or mGal-1 with iodoacetamide fully protected Gal-1 and mGal-1 from oxidation. Alkylation-induced stabilization allowed Gal-1 to signal sustained PS exposure in leukocytes and mGal-1 to signal both Ca^{2+} flux and PS exposure. Taken together, these results demonstrate that monomer-dimer equilibrium regulates Gal-1 sensitivity to oxidative inactivation and provides a mechanism whereby ligand partially protects Gal-1 from oxidation.

INTRODUCTION

Immunological homeostasis relies on efficient contraction of activated leukocytes following an inflammatory episode. Several factors, including members of the galectin and TNF families [2, 22], regulate leukocyte turnover by inducing apoptotic cell death. In contrast, several galectin family members, in particular galectin-1 (Gal-1), uniquely regulate neutrophil turnover by inducing phosphatidylserine (PS) exposure, which normally sensitizes apoptotic cells to phagocytic removal [82, 122], independent of apoptosis, a process recently termed preaparesis [72].

Previous studies suggested that dimerization may be required for Gal-1-induced PS exposure, as a mutant form of Gal-1 (mGal-1) containing two point mutations within the dimer interface, C2S and V5D (C2SV5D), displays impaired Gal-1 dimerization and fails to induce PS exposure [40]. However, the manner in which monomer-dimer equilibrium regulates Gal-1 signaling remains unclear. Previous studies suggest that dimerization may be required for efficient crosslinking of functional receptors or the formation of signaling lattices [123-125]. Consistent with this, monomeric mutants of several other galectins fail to induce PS exposure or signal leukocytes [122, 124]. Gal-1 signaling of PS exposure requires initial signaling events, such as mobilization of intracellular Ca^{2+} followed by sustained receptor engagement [42]. Although mGal-1 fails to induce PS exposure [40], whether mGal-1 can induce these initial signaling events remains unknown [42].

In addition to directly regulating signaling, monomer-dimer equilibrium may also regulate other aspects of Gal-1 function. Unlike many other proteins involved in the regulation of immunity, Gal-1 displays unique sensitivity to oxidative inactivation [44, 46, 49, 51, 126].

Although engagement of ligand partially protects Gal-1 from oxidation [46], the impact of Gal-1 oxidation on signaling remains enigmatic. During oxidation, Gal-1 forms three distinct intramolecular disulfide bridges which facilitate profound conformational changes that preclude ligand binding and Gal-1 dimerization [44, 49, 126], suggesting that monomer-dimer equilibrium may also regulate Gal-1 sensitivity to oxidative inactivation.

Previous studies utilized dithiothreitol (DTT) in treatment conditions to protect Gal-1 from oxidative inactivation [14, 80]. Indeed, failure to include DTT precluded Gal-1-induced death in T cells [81, 82], suggesting that Gal-1 undergoes rapid oxidation *in vivo* in the absence of reducing conditions. However, DTT itself can induce apoptosis in leukocytes [85], leaving questions regarding the impact of Gal-1 oxidation on these signaling events. In contrast, recent studies utilizing iodoacetamide alkylated Gal-1 (iGal-1), previously shown to protect Gal-1 from oxidative inactivation[48, 94, 98, 127-133], demonstrated that DTT actually primes cells to become sensitive to Gal-1-induced apoptosis regardless of Gal-1 sensitivity to oxidation [72].

As the engagement of leukocyte ligands requires glycan recognition and oxidation precludes this binding [46, 51], understanding the impact of oxidation on Gal-1 signals will facilitate a greater appreciation of the factors which govern Gal-1 oxidation and therefore function. Our results demonstrate that Gal-1 monomer-dimer equilibrium provides a key regulatory point controlling both Gal-1 sensitivity to oxidation and its ability to signal PS exposure in leukocytes. These results provide novel insights into Gal-1 function and explain at a biochemical level the mechanisms regulating Gal-1 oxidative inactivation and signaling.

RESULTS

mGal-1 induces Ca^{2+} flux yet fails to induce PS exposure in HL60 cells

Although Gal-1-induced PS exposure requires the induction of proximal signaling events, such as intracellular Ca^{2+} flux [40, 42], previous studies also demonstrated that Gal-1-induced PS exposure requires continuous engagement of functional cell surface receptors for PS to be realized [42]. In contrast to Gal-1, a mutant form of Gal-1 (mGal-1) which contains two point mutations in the dimer interface that impair Gal-1 dimerization, Cys-2-Ser and Val-5-Asp (C2SV5D), fails to induce PS exposure regardless of the length of treatment [40]. However, whether mGal-1 can engage early signaling events remains unknown [40]. Thus, we determined whether mGal-1 induces Ca^{2+} mobilization in HL60 cells. While mGal-1 failed to induce significant Ca^{2+} mobilization at 10mM (Fig. 3-1D), mGal-1 produced robust Ca^{2+} mobilization at 20mM (Fig. 3-1F), although at a reduced magnitude when compared to Ca^{2+} flux induced by Gal-1. (Fig. 3-1C,E). However, consistent with previous results, mGal-1 failed to induce PS exposure over a wide range of concentrations (Fig. 3-1H,J,K) in contrast to Gal-1, which induces PS exposure at both 10mM and 20mM (Fig. 3-1G,I). Taken together, these results demonstrate that although mGal-1 fails to induce PS exposure, it can induce significant Ca^{2+} flux in HL60 cells.

Gal-1 and mGal-1 display similar glycan recognition properties

In an effort to elucidate the underlying mechanism responsible for the discordance between the ability of mGal-1 to induce Ca^{2+} flux while failing to induce PS externalization, we first examined the possibility that mutations in the dimer interface might significantly alter the carbohydrate binding specificity of Gal-1. To test this, we first examined Gal-1 and mGal-1 binding to cells following enzymatic removal of cell surface

glycans as done previously to elucidate the binding specificity of different galectin family members [122, 134]. Gal-1 and mGal-1 both bound leukocytes and inclusion of lactose inhibited binding, which demonstrated that both proteins required glycan recognition for cell surface binding (data not shown). Treatment of cells with neuraminidase, which enhances Gal-1 binding and cellular sensitivity to Gal-1-induced PS exposure [40, 134], resulted in a comparable increase in both Gal-1 and mGal-1 cell surface glycan recognition (Fig. 3-2A). Furthermore, treatment of cells with endo-b-galactosidase, which cleaves cell surface linear poly-N-acetyllactosamine sequences, a common galectin ligand [122, 134], resulted in comparable reduction in both Gal-1 and mGal-1 binding (Fig. 3-2B), suggesting that both proteins display a similar general preference for cell surface glycans. Furthermore, Gal-1 and mGal-1 displayed similar binding to carbohydrates on a chemically defined glycan microarray (Fig. 3-2C,D). These results show that Gal-1 and mGal-1 possess similar glycan recognition, and thus the observed differences in signaling between Gal-1 and mGal-1 do not likely reflect significant alterations in carbohydrate binding specificity of Gal-1 following introduction of the C2SV5D mutations.

mGal-1 displays enhanced sensitivity to oxidative inactivation

Although previous studies suggested that mGal-1 fails to dimerize [40, 46], we noticed that during incubation with mGal-1, leukocytes appeared to display varying levels of cellular agglutination, a process which requires functional bivalency of Gal-1. These results suggested that the ability of mGal-1 to signal Ca^{2+} flux might reflect residual dimerization not fully eliminated by the C2SV5D mutation. To examine this in more detail, we incubated leukocytes with different concentrations of Gal-1 or mGal-1 followed by determination of agglutination. mGal-1 induced significant agglutination of cells at concentrations similar to those at which mGal-1 induced Ca^{2+} flux, although mGal-1

displayed significantly impaired agglutination when compared to Gal-1 (Fig. 3-3A inset). Indeed, 10 mM mGal-1 produced very little agglutination (Fig. 3-3A inset) and also failed to induce significant Ca^{2+} flux (Fig. 3-1D), which strongly suggested that proximal signaling requires Gal-1 dimerization.

Although mGal-1 treated cells induced significant initial agglutination of cells, after 4h, the time point at which evaluation for cell surface PS occurs, the cells incubated with mGal-1 were no longer agglutinated. Consistent with this, examination of mGal-1 treated cells over time demonstrated that although cells displayed significant agglutination when examined at earlier time points, cells displayed a gradual and spontaneous disengagement over time, with no detectable agglutination at 4h (Fig 3-3A,B), suggesting that mGal-1 may display an enhanced sensitivity to oxidative inactivation, a process which precludes Gal-1 recognition of ligand [45, 51]. Consistent with this, we also observed significant protein precipitation following prolonged incubation of cells with mGal-1, a hallmark of Gal-1 oxidation at higher concentrations. To examine this in more detail, we directly examined binding of Gal-1 or mGal-1 to lactosyl-Sepharose. Incubation of mGal-1 for 3h at 37°C degrees resulted in a 50% reduction in activity, while Gal-1 lost less than 15% activity over the same time period (Fig. 3-3C,D). Importantly, inclusion of 2̃ME protected both proteins from activity loss (data not shown), which demonstrated that mGal-1 activity loss reflected oxidative inactivation.

Prolonged incubation of HL60 cells with Gal-1 results in PS reversion

The enhanced sensitivity of mGal-1 to oxidative inactivation suggested that the inability of mGal-1 to signal PS exposure may reflect rapid oxidation and therefore inability to engage functional leukocyte receptors for the prolonged periods of time needed for full

realization of PS exposure to occur [42](Fig. 3-3B). However, previous studies also demonstrated that Gal-1 displays significant sensitivity to oxidation [45, 51]. As a result, we next sought to examine the potential impact of oxidation on Gal-1-induced PS exposure. As expected, incubation of cells with Gal-1 for 4h resulted in significant PS exposure (Fig. 3-4A,B,E). However, cells incubated in parallel for 24h with Gal-1 displayed a significant reduction in cell surface PS (Fig. 3-4C,D,E), without loss in cell viability (Fig. 3-4F). Similar to cells incubated with mGal-1 evaluated at 4h, cells incubated with Gal-1 displayed a reversion of cellular agglutination at 24h (data not shown), suggesting that reversion in Gal-1 signaling may also reflect Gal-1 oxidation. Taken together, these results demonstrate that while both Gal-1 and mGal-1 display sensitivity to oxidative inactivation, mGal-1 exhibits a significantly enhanced sensitivity to oxidation, although oxidative inactivation may impact signaling induced by both proteins.

Ligand shifts Gal-1 monomer-dimer equilibrium in favor of dimerization

As mGal-1 displays an enhanced sensitivity to oxidative inactivation and ligand partially protects Gal-1 from oxidation [46], dimerization itself may be a mechanism through which ligand inhibits Gal-1 oxidation. Indeed, Gal-1 was found to exist in a reversible monomer-dimer equilibrium (Fig. 3-5A-C,E,F), while ligand shifted this equilibrium in favor of dimer formation (Fig. 3-5D). By contrast, mGal-1 behaved exclusively as a monomer in this assay and ligand failed to enhance dimerization (Fig. 3-5G,H), suggesting that protection of Gal-1 from oxidation occurs through ligand-induced dimerization. Consistent with this, ligand not only failed to enhance mGal-1 dimerization, but also displayed a reduced capacity to protect mGal-1 from oxidative inactivation (Fig. 3-6A,B), although mGal-1 and Gal-1 displayed similar binding to ligand (Fig. 3-6C,D).

The inability of mGal-1 to appear as a dimer following HPLC gel filtration analyses likely reflects weak dimerization and rapid dissociation. In contrast, the ability of mGal-1 to agglutinate cells at higher concentrations suggested that mGal-1 might dimerize. To test this, we examined Gal-1 and mGal-1 following chemical crosslinking of the dimer using water-soluble BS^3, a homobifunctional, water-soluble, non-cleavable crosslinker with a diameter of 11.4 Å, which allows trapping of weakly associated molecules. Crosslinking can alter protein detection when utilizing common protein staining procedures. To overcome this, we labeled Gal-1 and mGal-1 with Alexa-488 maleimide to insure that dimer and monomer fractions retained detection sensitivity irrespective of crosslinking. Although reduced when compared to Gal-1, significant mGal-1 dimers could be trapped at higher concentrations (Fig. 3-6E), which indicated that although mGal-1 displays reduced dimer formation, mGal-1 can dimerize.

Carboxymethylation protects Gal-1 from oxidative inactivation

These results suggest that the inability of mGal-1 to induce PS exposure and Gal-1 to sustain PS exposure likely reflects spontaneous oxidative inactivation during continual leukocyte incubation. Many previous studies utilized DTT in treatment conditions to prevent Gal-1 oxidative inactivation [14, 80, 81]. Indeed, inclusion of 1 mM DTT prevented spontaneous disengagement of cells incubated with either Gal-1 or mGal-1 (data not shown), strongly suggesting that loss of agglutination reflected Gal-1 oxidation. However, DTT can induce significant alterations in cellular responses to Gal-1, making it difficult to separate the impact of DTT on cellular function from its ability to prevent Gal-1 oxidation [82, 85, 135]. In contrast, previous studies alkylated Gal-1 with iodoacetamide followed by removal of free iodoacetamide, which protects Gal-1 from oxidation without introducing cells to a reducing environment [48, 94, 98, 127-133]. Importantly, these studies demonstrated that alkylation not only protects Gal-1 from oxidation, but also fails

to alter biological activity or quaternary structure [48, 94, 98, 127-133]. Similar to previous results, alkylation with iodoacetamide failed to quantitatively alkylate all Cys residue, since we observed approximately 5.4 moles of incorporation per subunit (Fig. 3-7A,B), rather than 6.0 as expected [48]. Examination of tryptic fragments of alkylated Gal-1 demonstrated a preference of iodoacetamide for Cys 2, 16 and 130 (data not shown), similar to previous findings [48]. Importantly, alkylated Gal-1 (iGal-1) retained > 90% activity and induced robust PS exposure in leukocytes (Fig. 3-7C,D), consistent with previous findings [48, 94, 98, 127-133]. Taken together, these results demonstrate that alkylation can significantly protect Gal-1 from oxidative inactivation.

Carboxymethylation enhances Gal-1 and mGal-1 signaling of HL60 cells

As alkylation protected Gal-1 from oxidative inactivation, we also alkylated mGal-1 (imGal-1) to directly examine whether alterations in cellular signaling reflected differential sensitivity of these proteins to oxidative inactivation. Similar to Gal-1, alkylation of mGal-1 with iodoacetamide resulted in incomplete Cys modification, with one mole less iodoacetamide incorporation than Gal-1, likely due to the C2S mutation in mGal-1 (data now shown). Similar to iGal-1, imGal-1 prevented oxidation-induced precipitation and when examined over lactosyl-Sepharose retained >90% activity following a 24h incubation in the absence of 2-ME (Fig 3-8A,B). Furthermore, alkylation failed to alter mGal-1 dimerization as assessed following HPLC analysis (Fig. 3-8C,D). To compare mGal-1 and imGal-1 directly, we examined PS exposure as done previously [22, 40, 75, 76, 136]. Although Gal-1 and iGal-1 displayed similar signaling capacity following a 4 h incubation (Fig. 3-8G,H), 20 and 40 mM imGal-1 now signaled significant PS exposure (Fig. 3-8F), although 20 mM signaled PS externalization at a reduced magnitude when compared to 20 mM Gal-1 (Fig. 3-8F), suggesting a preference for Gal-1 dimerization for full realization of PS exposure. In contrast, neither concentration of mGal-1 induced PS

exposure (Fig. 3-8E). Furthermore, although cells incubated with mGal-1 spontaneously disengaged over time, imGal-1 sustained agglutination over the duration of the incubation (data not shown), further suggesting that mGal-1 fails to induce PS exposure due to rapid oxidation.

In contrast to mGal-1, Gal-1 induces PS exposure following a 4 hour incubation, although this PS externalization gradually reverted, a process that paralleled gradual and spontaneous disengagement of cells (Fig 3-4). To examine whether the inability of Gal-1 to sustain PS exposure over a prolonged incubation period also reflected oxidation, we evaluated cells after 48h of treatment with Gal-1 or iGal-1. Although Gal-1 treated cells completely reverted PS exposure (Fig. 3-8I), iGal-1 treated cells displayed significant PS positivity (Fig. 3-8J). Indeed, iGal-1 treated cells displayed continuous PS exposure for 72h of treatment, while Gal-1 treated cells displayed a significant loss of PS following 24h accompanied by spontaneous disengagement (Fig. 3-8K,L).

Gal-1 signals PS exposure independent of cellular agglutination

Although alkylation allowed mGal-1 to signal PS, imGal-1-induced PS exposure occurred at a reduced magnitude when compared to cells incubated with Gal-1, which suggested that dimerization not only protects Gal-1 from oxidation but also facilitates Gal-1 signaling as suggested previously [40]. Although the requirement for Gal-1 dimerization strongly suggests crosslinking of functional cell surface receptors, we observed that Gal-1-induced agglutination paralleled Gal-1-induced PS exposure. Agglutination may mediate the association of other receptors not directly bound by Gal-1, allowing Gal-1 to indirectly signal PS exposure through receptor approximation instead of directly inducing signaling. To test this, we serially diluted cells in the presence of uniform Gal-1 that resulted in reduced agglutination as a function of reduced

cell number. If Gal-1-induced PS exposure required agglutination, a significant reduction in agglutination would be expected to reduce Gal-1-induced PS externalization. However, Gal-1 induced equivalent PS exposure regardless of the agglutination state of the cells (Fig. 3-9A-I). Similarly, cytospin-induced adhesion of leukocytes followed by Gal-1 incubation resulted in PS positivity of single cells following Gal-1 treatment (Fig. 3-9J). These results demonstrate that Gal-1-induced PS exposure likely reflects a need for crosslinking of functional receptors and can occur independently of cell-cell agglutination.

DISCUSSION

These results demonstrate that Gal-1 undergoes oxidation when co-incubated with leukocytes and that this oxidation significantly impacts Gal-1 signaling. Furthermore, our results demonstrate that glycan ligand regulates Gal-1 sensitivity to oxidation by shifting the monomer-dimer equilibrium in favor of dimerization, providing an explanation whereby ligand may protect Gal-1 from oxidative inactivation.

Although the unique sensitivity of Gal-1 to oxidative inactivation has been known for many years [46, 51, 137-139], the underlying mechanism responsible for this sensitivity remained enigmatic. Previous studies demonstrated that following oxidation, each subunit of Gal-1 forms three discrete intramolecular disulfide bridges [44, 49]. Disulfide bridge formation results in a significant conformational change [43, 47, 48], which prohibits ligand recognition and prevents dimerization [44]. Crystallographic studies strongly suggest impaired conformational rotation of Cys residues during dimerization [140-142]. As a result, dimerization likely limits the conformational freedom needed to successfully form intramolecular disulfide bonds. Taken together, these results suggest that the dimer interface itself likely protects Gal-1 from oxidation by locking Cys residues in positions that make intramolecular disulfide bond formation unfavorable.

The ability of ligand to enhance dimerization provides a mechanism whereby ligand protects Gal-1 from oxidative inactivation, an observation made shortly after the initial discovery of Gal-1. Although crystallographic and solution based CD experiments failed to detect significant changes in Gal-1 conformation following ligand binding [140-143], previous studies utilizing chaotropic denaturation demonstrated that ligand can significantly impact the nature and pathway of folding intermediates [144], providing

some insight into Gal-1 oxidation. Introduction of ligand enhances Gal-1 stability and results in denaturation of dimeric Gal-1 directly into unfolded monomers [144]. By contrast, in the absence of ligand, Gal-1 undergoes an unfolding reaction that involves the formation of monomeric intermediates prior to full denaturation and required lower concentration of chaotropic denaturant [144], suggesting that monomer formation and alterations in monomer conformation are favored in the absence of ligand. Given the present results, Gal-1 oxidation likely proceeds through a similar pathway. Ligand enhanced dimerization likely precludes conformational changes needed to form critical intramolecular disulfide bridges. However, absence of ligand allows increased formation of monomeric Gal-1 and therefore monomeric metastable intermediates. These monomeric intermediates likely possess greater conformational freedom, increasing the likelihood of intramolecular disulfide bond formation. The monomeric nature of oxidized Gal-1 corroborates this finding and strongly suggests a monomeric intermediate in this pathway [44]. Furthermore, the enhanced sensitivity of mGal-1 to oxidation strongly suggests that, just as ligand enhances dimerization and thereby reduces sensitivity to oxidative inactivation, mutations that impair dimerization and therefore increase monomer formation favor oxidation.

The ability of ligand to enhance dimerization not only suggests a pathway for protecting Gal-1 oxidation, but also demonstrates that Gal-1 exists in a monomer-dimer equilibrium, in contrast to previous studies which suggested that Gal-1 exists as an irreversible dimer [145]. Gal-1 dilution resulted in monomer formation, while concentrating Gal-1 allowed monomeric Gal-1 to reform dimers. As the present results suggest that monomer-dimer equilibrium likely regulates both Gal-1 activity and the ability to signal, regulation of monomer-dimer equilibrium likely provides a key regulatory point governing Gal-1 function.

Although alkylation protected mGal-1 from oxidative inactivation, imGal-1 still exhibited impaired signaling compared to iGal-1, which suggests a requirement for dimerization to effect full signaling. Furthermore, the inability of mGal-1 to induce PS externalization corroborates previous results demonstrating a requirement for continual engagement of leukocyte ligands for PS to be realized [42]. Once PS exposure occurs, continual ligand binding must also occur for sustained PS exposure, as cells treated with Gal-1 failed to maintain PS following Gal-1 oxidation. Reversion of PS during Gal-1 incubation does not likely reflect removal of cells following potential Gal-1-induced death, as cells undergoing apoptosis were readily detected in this assay and iGal-1 induced sustained PS exposure over prolonged periods. These results also demonstrate that loss of PS following prolonged Gal-1 incubation does not likely reflect cellular insensitivity to Gal-1 over time. Given the oxidative nature of the extracellular environment, oxidation of Gal-1 may be an irreversible event. As a result, cellular movement into different redox environments may actually facilitate Gal-1 oxidation and therefore allow cells initially targeted for phagocytic removal to become reengaged in host defense following PS reversion. In contrast, leukocyte-mediated damage of viable tissue may facilitate the release of reduced Gal-1 from intracellular stores that then engage leukocytes and induce their turnover [40, 146].

In addition to regulating Gal-1 signaling, monomer-dimer equilibrium may also regulate Gal-1 secretion. Gal-1 exists in a monomer-dimer equilibrium both inside and outside the cell [46], although secreted Gal-1, which exits through an incompletely defined ER-independent pathway or pathways, occurs primarily as a monomer. [6, 45, 46, 147]. In the absence of extracellular ligand, Gal-1 readily undergoes inactivation into oxidized monomers following secretion [44, 45], suggesting that Gal-1 may be secreted as an

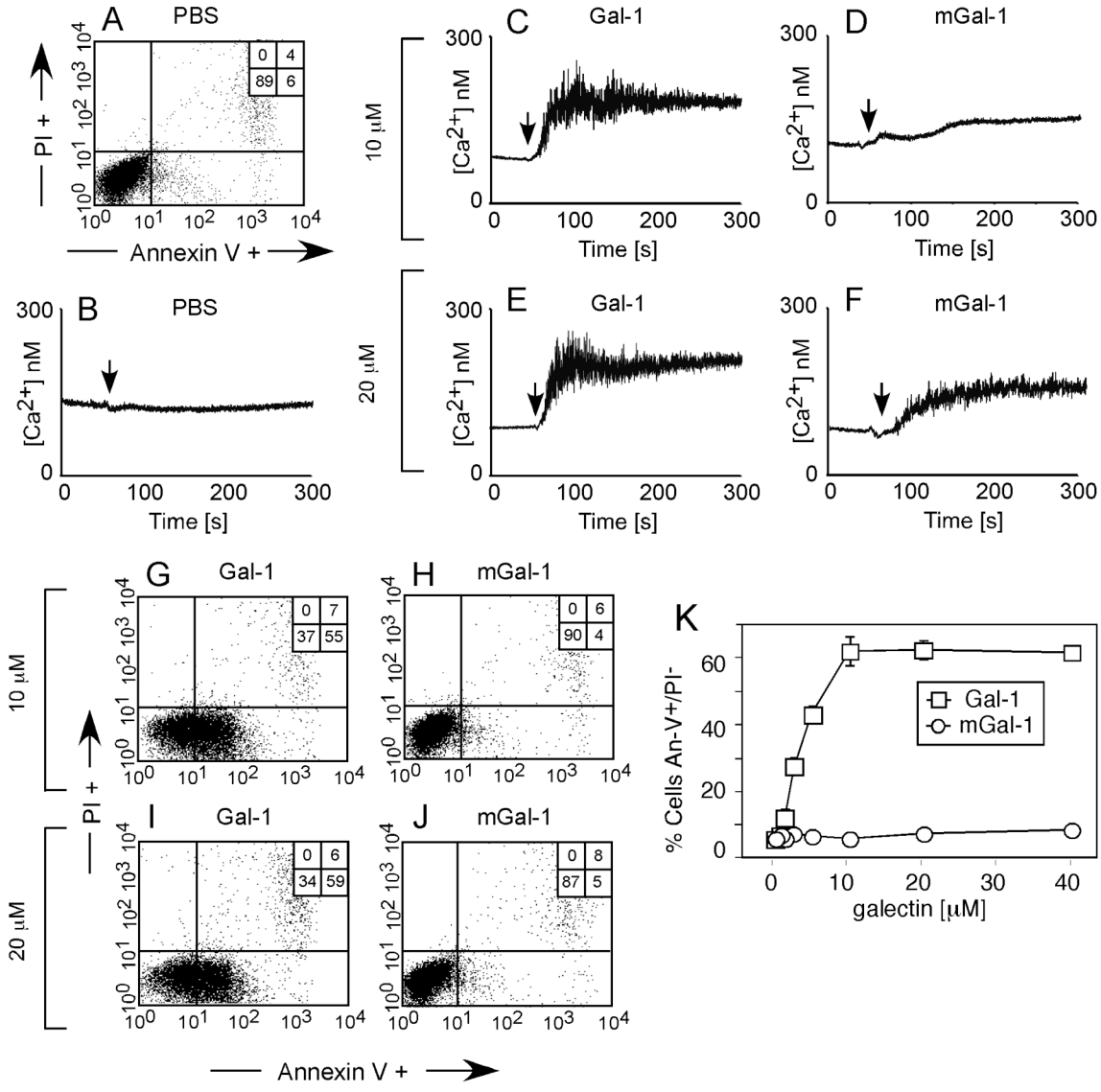
inactive or partially folded monomer. Engagement of ligand, either on the secreting cell or target cell, likely facilitates dimerization, thereby protecting Gal-1 from oxidative inactivation while also facilitating signaling events. In this way, ligand may not only stabilize Gal-1, but also enhance Gal-1-induced signaling.

Although the present study focused on Gal-1 oxidation as an inactivating process, oxidized Gal-1 appears to have significant biological activity independent of glycan ligand recognition. Oxidized Gal-1 enhances peripheral nerve regeneration both *in vitro* and *in vivo* [50]. As a result, oxidation not only regulates the immunomodulary lectin-dependent activities of Gal-1, but also determines when the bioactivities of oxidized Gal-1 become apparent. In this way, Gal-1 provides another example of a morpheein, a protein capable of adopting different conformations capable of regulating distinct biological processes [148].

Given the significant number of studies suggesting a role for Gal-1 in the regulation of immunity, the sensitivity of Gal-1 to oxidative inactivation likely evolved as an intrinsic regulatory mechanism responsible for governing Gal-1 activity once secreted from the cell [149]. Such a sensor may be important in dictating the distribution and longevity of Gal-1 signaling. For example, while Gal-1 induces turnover of neutrophils [40, 82, 135], inhibits leukocyte chemotaxis and induces immunosuppressive cytokine secretion in both naive and activated T cells [53, 135], premature engagement of infiltrating leukocyte by Gal-1 could ameliorate an otherwise productive and necessary inflammatory response. The highly oxidative environment surrounding inflammation likely facilitates the oxidation of Gal-1 released during primary tissue injury prior to significant leukocyte recruitment [2], allowing leukocytes to successful neutralize pathogen or remove necrotic tissue without being impeded by the immunosuppressive

effects of Gal-1. However, as leukocytes encroach on viable tissue surrounding an area of tissue injury, leukocyte-mediated damage may release reduced and therefore active Gal-1, allowing Gal-1 to inhibit leukocyte chemotaxis and induce their turnover [150, 151]. Future studies will explore these intriguing possibilities.

Figure 3-1


A
PBS
0
4
89
6
PI +
Annexin V +
B
PBS
C
Gal-1
D
mGal-1
10 μM
20 μM
[Ca2+] nM
Time [s]
E
Gal-1
F
mGal-1
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Gal-1
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7
37
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H
mGal-1
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90
4
I
Gal-1
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59
J
mGal-1
0
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K
% Cells An-V+/PI-
galectin [μM]
Gal-1
mGal-1

Figure 3-2

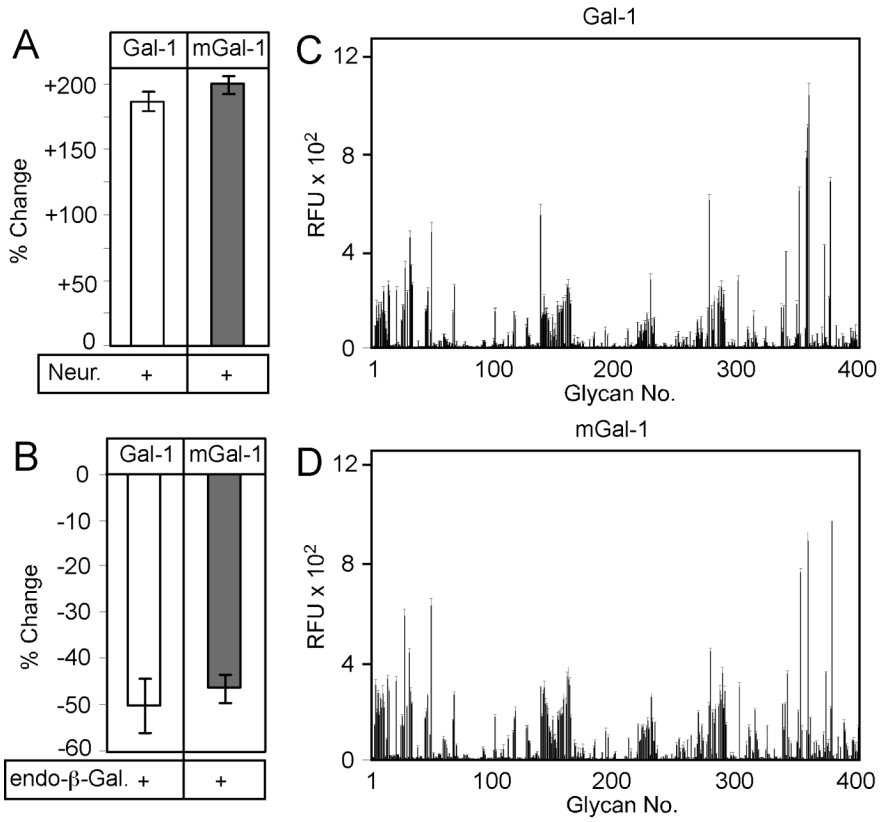

A
Gal-1
mGal-1
+200
+150
+100
+50
0
% Change
Neur. +
+
B
Gal-1
mGal-1
0
-10
-20
-30
-40
-50
-60
% Change
endo-β-Gal. +
+
C
Gal-1
12
8
4
0
RFU x 10²
1
100
200
300
400
Glycan No.
D
mGal-1
12
8
4
0
RFU x 10²
1
100
200
300
400
Glycan No.

Figure 3-3

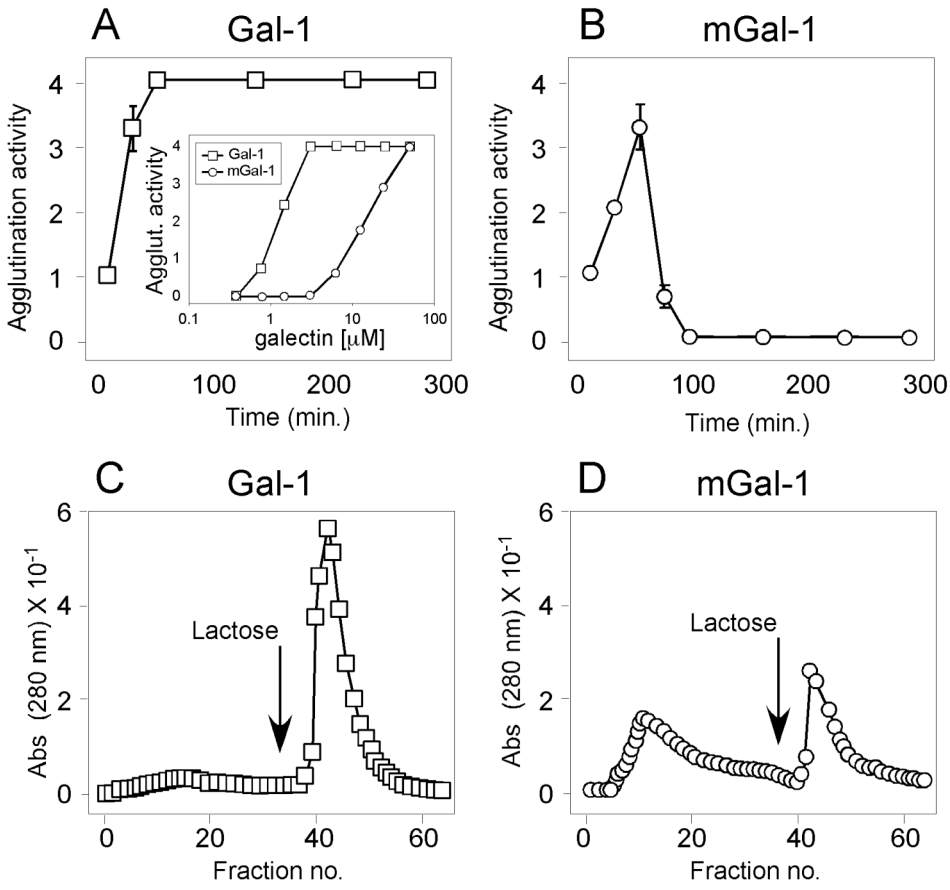

A
Gal-1
Agglutination activity
Time (min.)
Agglut. activity
Gal-1
mGal-1
galectin [μM]
B
mGal-1
Agglutination activity
Time (min.)
C
Gal-1
Abs (280 nm) X 10-1
Lactose
Fraction no.
D
mGal-1
Abs (280 nm) X 10-1
Lactose
Fraction no.

Figure 3-4

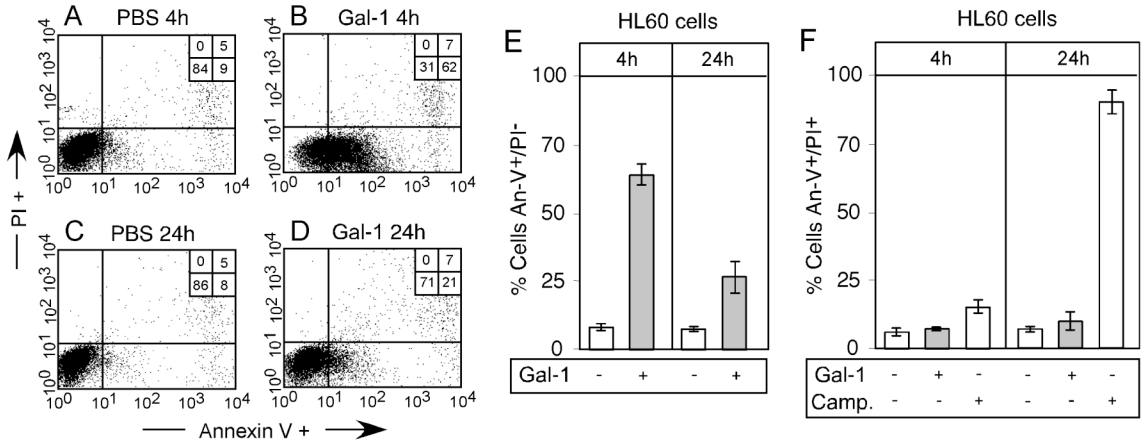

A PBS 4h
B Gal-1 4h
C PBS 24h
D Gal-1 24h
0 5
84 9
0 7
31 62
0 5
86 8
0 7
71 21
PI +
Annexin V +
E HL60 cells
4h
24h
% Cells An-V+/PI-
Gal-1 - + - +
F HL60 cells
4h
24h
% Cells An-V+/PI+
Gal-1 - + - - + -
Camp. - - + - - +

Figure 3-5

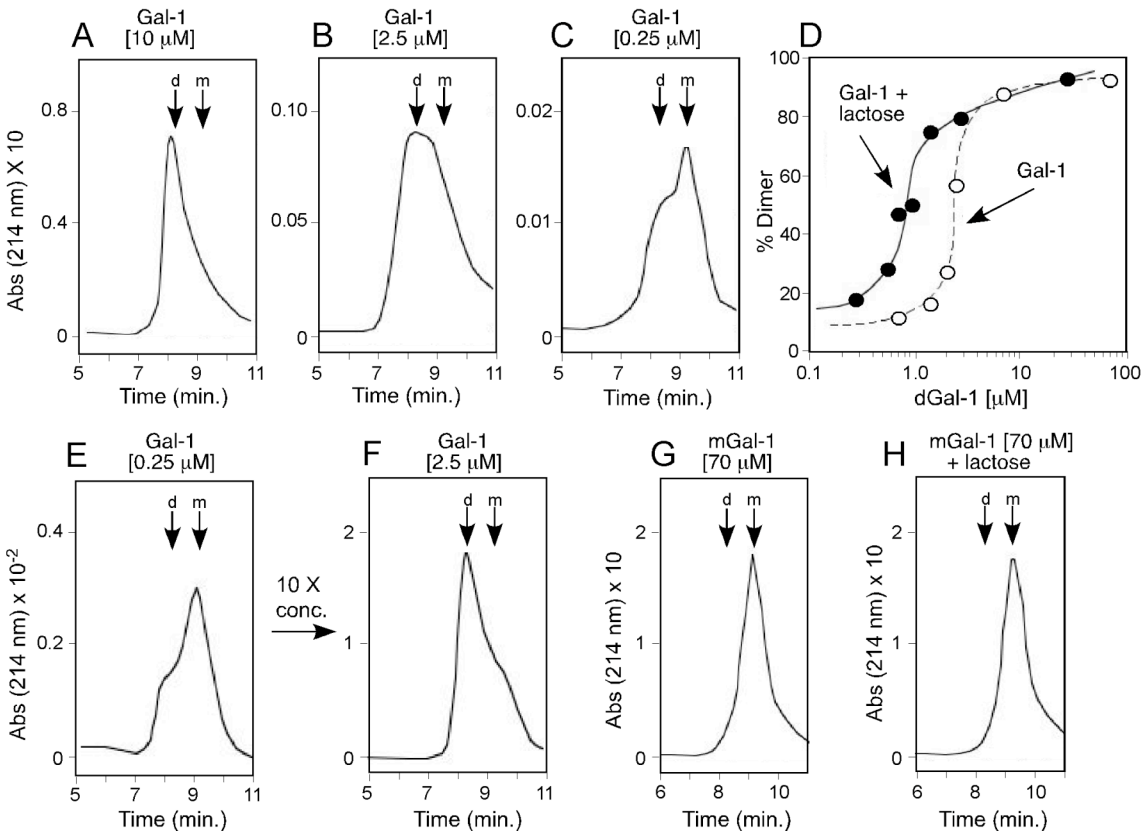

A
Gal-1
[10 μM]
B
Gal-1
[2.5 μM]
C
Gal-1
[0.25 μM]
D
Gal-1 +
lactose
Gal-1
% Dimer
dGal-1 [μM]
Abs (214 nm) X 10
Time (min.)
E
Gal-1
[0.25 μM]
F
Gal-1
[2.5 μM]
10 X
conc.
G
mGal-1
[70 μM]
H
mGal-1 [70 μM]
+ lactose
Abs (214 nm) x 10-2
Abs (214 nm) x 10
d
m

Figure 3-6

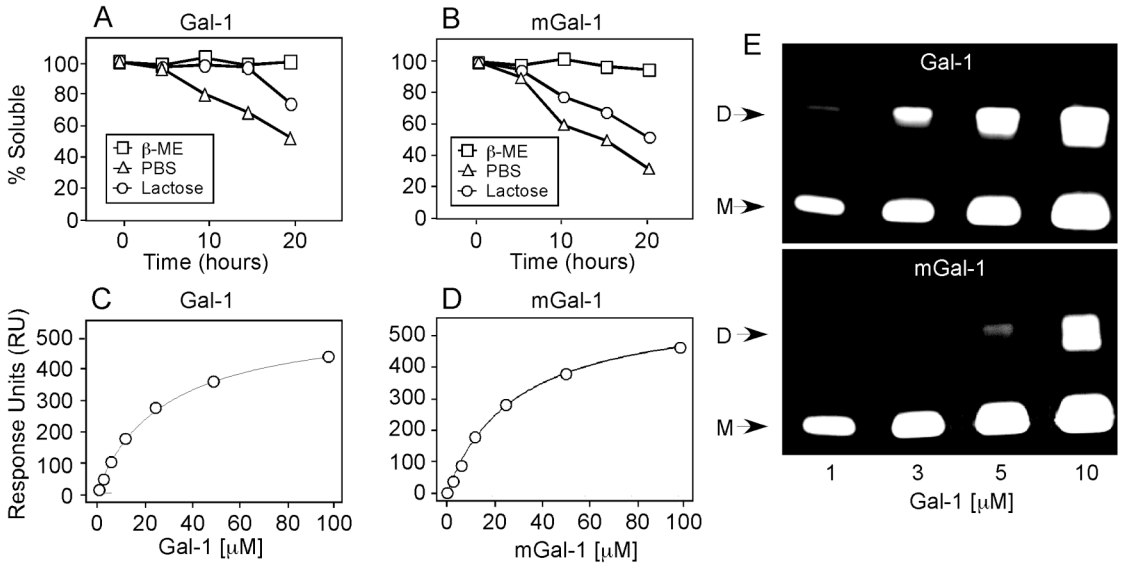

A
Gal-1
% Soluble
β-ME
PBS
Lactose
Time (hours)
B
mGal-1
β-ME
PBS
Lactose
Time (hours)
E
Gal-1
D
M
mGal-1
D
M
1
3
5
10
Gal-1 [μM]
C
Gal-1
Response Units (RU)
Gal-1 [μM]
D
mGal-1
mGal-1 [μM]

Figure 3-7

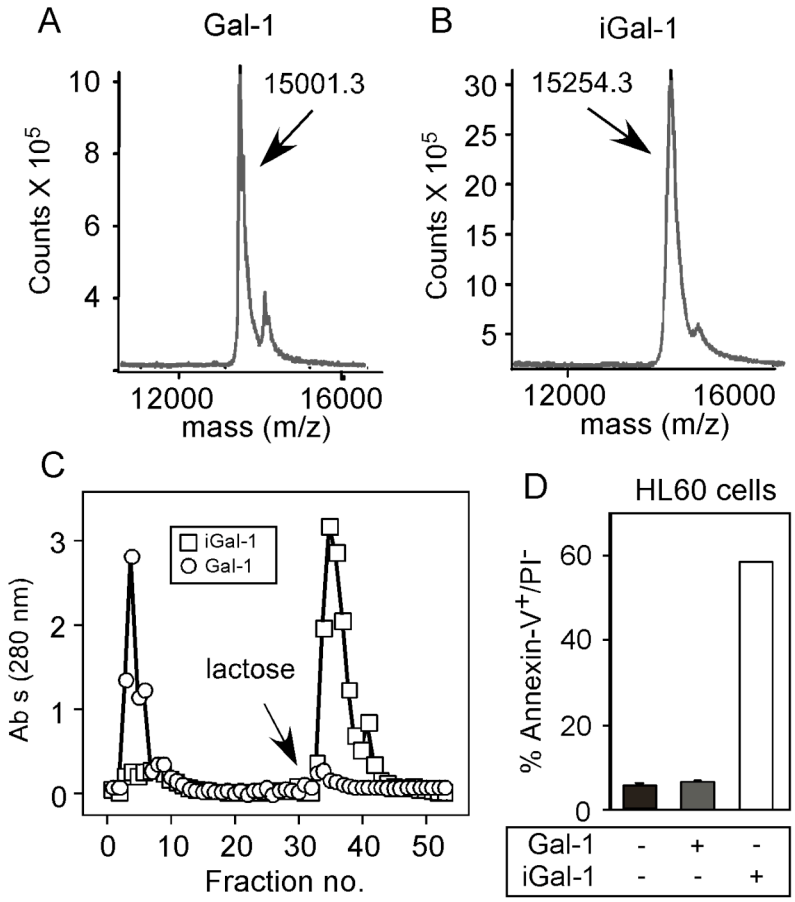

A
Gal-1
15001.3
Counts X 10^5
mass (m/z)
B
iGal-1
15254.3
Counts X 10^5
mass (m/z)
C
iGal-1
Gal-1
lactose
Ab s (280 nm)
Fraction no.
D
HL60 cells
% Annexin-V+/PI-
Gal-1 - + -
iGal-1 - - +

Figure 3-8

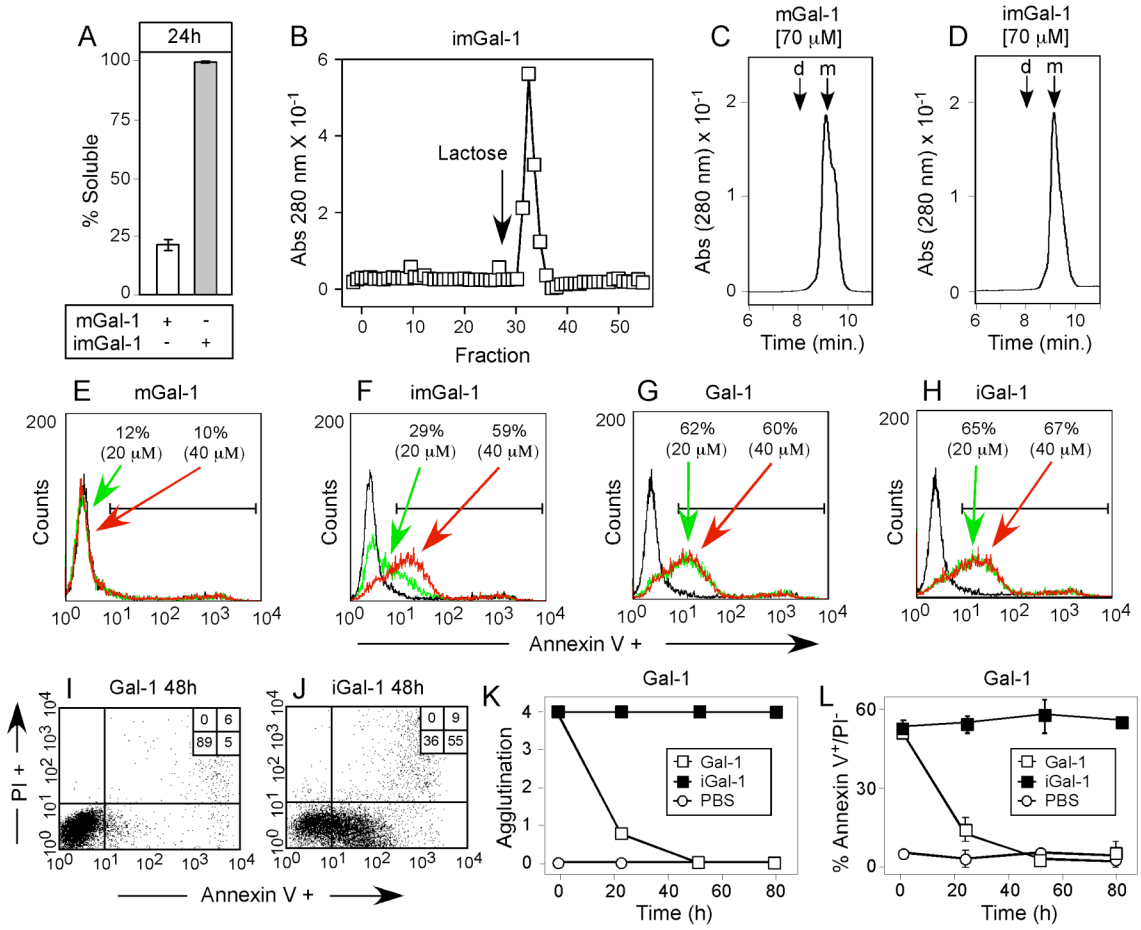

A
24h
% Soluble
mGal-1 + -
imGal-1 - +
B
imGal-1
Lactose
Abs 280 nm X 10-1
Fraction
C
mGal-1
[70 μM]
d m
Abs (280 nm) x 10-1
Time (min.)
D
imGal-1
[70 μM]
d m
Abs (280 nm) x 10-1
Time (min.)
E mGal-1
F imGal-1
G Gal-1
H iGal-1
12% (20 μM) 10% (40 μM)
29% (20 μM) 59% (40 μM)
62% (20 μM) 60% (40 μM)
65% (20 μM) 67% (40 μM)
Counts
Annexin V +
I Gal-1 48h
J iGal-1 48h
PI +
Annexin V +
K Gal-1
Agglutination
Gal-1
iGal-1
PBS
Time (h)
L Gal-1
% Annexin V+/PI-
Gal-1
iGal-1
PBS
Time (h)

Figure 3-9

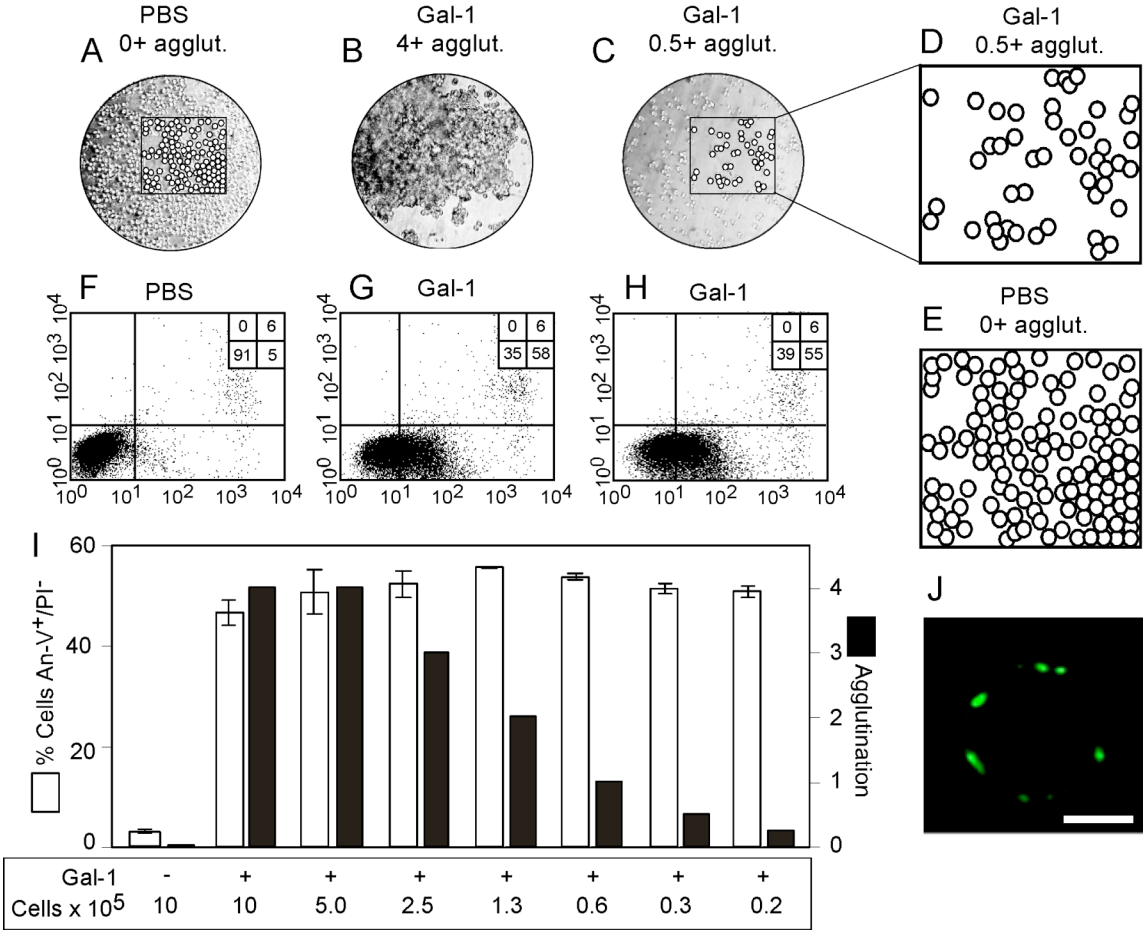

A
PBS
0+ agglut.
B
Gal-1
4+ agglut.
C
Gal-1
0.5+ agglut.
D
Gal-1
0.5+ agglut.
E
PBS
0+ agglut.
F
PBS
G
Gal-1
H
Gal-1
0 6
91 5
0 6
35 58
0 6
39 55
I
% Cells An-V+/PI-
Agglutination
Gal-1 - + + + + + + +
Cells x 10^5 10 10 5.0 2.5 1.3 0.6 0.3 0.2
J

FIGURE LEGENDS

Figure 3-1. mGal-1 induces proximal signaling events in leukocytes but fails to induce PS exposure. (A) HL60 cells treated with PBS for 4h were stained with Annexin-V (An-V) FITC and propidium iodide (PI) to detect PS exposure followed by flow cytometric analysis. (B) Cells were loaded with Fluo-4 and analyzed for changes in intracellular Ca^{2+} using a fluorometer following addition of PBS at the indicated time (vertical arrow). (C-F) HL60 cells were loaded with Fluo-4 as in B, followed by addition of (C) 10mM Gal-1, (D), 10mM mGal-1, (E), 20mM Gal-1 or (F) 20mM mGal-1 as indicated by the arrows. (G-J) HL60 cells were treated with (G) 10mM Gal-1, (H) 10mM mGal-1, (I) 20mM Gal-1 or (J) 20mM mGal-1 for 4h were labeling cells with An-V FITC and PI to detect PS exposure followed by flow cytometric analysis. (K) HL60 cells were treated with the indicated concentrations of Gal-1 or mGal-1 for 4h followed by detection for PS exposure.

Figure 3-2. Gal-1 and mGal-1 display similar glycan binding properties. (A) Quantification of mGal-1 and Gal-1 binding before and after treatment of cells with neuraminidase. (B) Quantification of mGal-1 and Gal-1 binding before and after treatment of cells with endo-b-galactosidase. (C) Gal-1 binding toward distinct classes of glycan ligands on the glycan microarray. (D) mGal-1 binding toward distinct classes of glycan ligands on the glycan microarray.

Figure 3-3. mGal-1 displays enhanced sensitivity to oxidative inactivation. (A) HL60 cells were incubated with 20mM Gal-1 at 37°C for the times indicated followed by assessment for the degree of agglutination. Inset: HL60 cells were incubated for 30 min.

at 37°C with the concentrations of Gal-1 or mGal-1 indicated followed by assessment for agglutination. (B) HL60 cells were incubated with 20mM mGal-1 at 37°C for the times indicated followed by assessment for the degree of agglutination. (C) Gal-1 was incubated in PBS at 37°C for 3h followed by subjection to affinity chromatography over lactosyl-Sepharose. (D) mGal-1 was incubated in PBS at 37°C for 3h followed by subjection to affinity chromatography over lactosyl-Sepharose.

Figure 3-4. Oxidation of Gal-1 results in reversion of PS exposure. (A-D) HL60 cells were treated with 20mM Gal-1 for 4h or 24h as indicated, followed by detection of PS exposure by flow cytometric analysis. (E) Quantification of the number of An-V^+/PI^- cells following Gal-1 treatment for the length of time indicated. (F) Quantification of the number of An-V^+/PI^+ cells following treatment of cells with either 20mM Gal-1 or 20mM Camptothecin (Camp.) treatment for the length of time indicated.

Figure 3-5. Ligand modulates monomer-dimer equilibrium in favor of dimerization. (A-C) Gel filtration of Gal-1 at (A) 10mM, (B) 2.5mM and (C) 0.25mM. (D) Quantification of the percent dimer formation following gel filtration for each indicated concentration in the presence or absence of ligand. (E) Gel filtration analysis of 0.25mM Gal-1. Following analysis, monomeric fractions were concentrated to 2.5mM Gal-1 and reanalyzed in (F). (G-H) mGal-1 was subjected to gel filtration analysis in either the absence (G) or presence (H) of ligand. D=Dimer, M=Monomer.

Figure 3-6. mGal-1 displays impaired capacity to be stabilized by ligand. (A) Incubation of 40 mM Gal-1 in PBS, with or without $\tilde{2}$ME or lactose as indicated followed by determination of the soluble fraction. (B) Incubation of 40 mM mGal-1 in PBS, with or without $\tilde{2}$ME or lactose as indicated followed by determination of the soluble fraction.

(C-D) SPR analysis of the binding of (C) Gal-1 or (D) mGal-1 to lactose. (E) Fluorescent image acquired following SDS-PAGE of BS^3 chemically crosslinked Alexa Fluor 488-labeled Gal-1 or mGal-1. D=dimer, M=monomer.

Figure 3-7. Alkylation protects Gal-1 from oxidative inactivation. (A-B) Mass spectrometry analysis using MALDI-TOF of Gal-1 (A) without treatment or (B) following treatment with iodoacetamide. (C) Gal-1 or iodoacetamide treated Gal-1 (iGal-1) were incubated for 24h in PBS at 37°C followed by subjection to affinity chromatography over lactosyl-Sepharose. (D) Gal-1 or iodoacetamide treated Gal-1 (iGal-1) were incubated for 24h in PBS at 37°C followed by addition of 20μM of each galectin to HL60 cells for 4h. Cells were then stained with An-V FITC and PI and analyzed for PS positivity by flow cytometric analysis.

Figure 3-8. Alkylation rescues Gal-1 and mGal-1 from oxidation. (A) Incubation of 40 mM mGal-1 or imGal-1 in PBS for 24h followed by determination of the soluble fraction. (B) imGal-1 was incubated in PBS at 37°C for 24h followed by subjection to affinity chromatography over lactosyl-Sepharose. (C-D) Gel filtration of (C) mGal-1 or (D) imGal-1 at 70 mM. D=Dimer, M=Monomer. (E-H) HL60 cells were treated with either the indicated concentrations of (E) mGal-1, (F) imGal-1, (G) Gal-1 or (H) iGal-1 or PBS (black histogram in each data set) for 4h followed by detection of PS exposure by flow cytometric analysis. (I-J) HL60 cells were treated with (I) PBS or (J) 20mM iGal-1 for 48h followed by detection for PS exposure by flow cytometric analysis. (K-L) HL60 cells were incubated with 20mM iGal-1 or 20mM Gal-1 for the indicated time at 37°C followed by examination for (K) the agglutination state of cells and (L) detection for PS exposure by flow cytometric analysis.

Figure 3-9. Gal-1-induced PS exposure occurs independent of Gal-1-induced agglutination. (A-C) Pictures of cells treated with (A) PBS (10^6 cells), (B) 20mM Gal-1 (10^6 cells), or (C) 20mM Gal-1 (0.3×10^5 cells) at 10x magnification. (D) Demarcation of cells in C. (E) Demarcation of cells in A. (F-H) HL60 cells treated with (F) PBS (10^6 cells), (G) 20mM Gal-1 (10^6 cells) or (H) 20mM Gal-1 (0.3×10^5 cells) were incubated for 4h followed by detection for PS exposure by flow cytometric analysis. (I) The indicated number of HL60 cells was treated with 20mM Gal-1 followed by detection for agglutination (black bars). Following determination of cellular agglutination, cells were analyzed for PS exposure by flow cytometric analysis (white bars). (J) HL60 cells were fixed to slides using cytospin followed by incubation with 20mM Gal-1 for 4h and confocal analysis using An-V-Alexa Fluor-488. White bar = 5mm.

Chapter 4

Immunoregulatory Galectin-1 Exhibits Diffuse Expression in Adult Tissue with Cytosolic Organization in Striated Muscle Tissues

Many studies demonstrate key roles for Galectin-1 (Gal-1) in immune function and muscle development and regeneration. However, the expression and localization of Gal-1 in adult tissue remains unknown. To examine the expression and localization of Gal-1, we generated an epitope-defined monoclonal antibody against Gal-1, aGal-1. Examination of Gal-1 expression utilizing aGal-1 demonstrated detectable Gal-1 expression in a wide range of tissues, including striated muscle, liver, lung, brain and intestine. Immunolocalization of Gal-1 using aGal-1 demonstrated diffuse cytosolic staining of Gal-1 in the cerebrum, gastric mucosa, and kidney. In contrast, Gal-1 exhibited organized cytosolic staining pattern within striated muscle tissue pf cardiac and skeletal muscle. aGal-1 also detected Gal-1 expression in in large vessels, with prominent cytosolic staining in cultured endothelial cells, although several different leukocyte populations failed to express detectable Gal-1. These results provide the first description of Gal-1 expression and localization in adult tissue and provide important insight intro previously defined roles for the protein.

INTRODUCTION

Proper regulation of host immunity occurs through the coordinated effects of many factors that modulate leukocyte trafficking, cytokine secretion, and cellular differentiation. Effective removal of leukocytes following inflammatory episodes also enables important control of immune function, especially during the later stages of an immunological response. Phosphatidylserine (PS), a phospholipid normally confined to the inner leaflet of the plasma membrane, becomes exposed in the outer membrane leaflet in cells undergoing apoptotic cell death, as a signal for phagocytic clearance [29, 39]. Apoptotic cell removal occurs by the engagement of several effector molecules such as Fas and tumor necrosis factor-alpha (TNFα) [1-3]. However, several studies demonstrate that cells may undergo removal through apoptosis-independent mechanisms. For example, neutrophil populations, which harbor significant destructive potential [152-154], can undergo non-apoptotic turnover *in vivo*, [37, 38] possibly reducing unregulated release of deleterious contents during late apoptotic cell death prior to phagocytic removal [108, 155, 156].

Several recent studies suggest that members of the galectin family of β-galactoside binding proteins may be involved in regulating leukocyte function and turnover [19-21, 25, 53, 157-160]. We recently demonstrated that galectin-1 (Gal-1) induces PS exposure in the absence of apoptosis, yet sensitizes cells for phagocytic removal [40, 41], providing one of the first potential pathways whereby neutrophils may be induced to undergo removal by apoptosis-independent pathways. We also found that Gal-1 induces PS exposure through recognition of polylactosamine glycans, signaling elevated cytosolic Ca^{2+}, and activation of Src kinases and phospholipase Cg [42]. In contrast to neutrophils, Gal-1 fails to alter PS redistribution or viability in T cells [82], yet signals

robust alterations in T cell cytokine production which favor TH2 responses [161], suggesting a unique pathway for neutrophil removal.

In addition to the effects of Gal-1 on leukocyte function [4], many studies suggest a role for Gal-1 in non-immunological processes [162]. For example, Gal-1 induces myoblast differentiation *in vitro* and Gal-1 null mice experience reduced myofiber formation *in vivo* [163, 164]. Gal-1 also regulates olfactory axon targeting during development and may be involved in peripheral nerve regeneration[162]. Intracellularly, Gal-1 modulates Ras membrane localization and signaling [165]. The unique ability of Gal-1 to recognize a diverse array of glycan structures that likely decorate many different functional counter receptors likely underlies the plieotrophic activity of this protein[64].

Although many studies have evaluated potential roles of Gal-1 in various processes, including putative ligands responsible for mediating these processes, surprisingly little is known about the localization and expression of Gal-1 in adult tissue. In this study we examined the expression and localization of Gal-1 using an epitope defined monoclonal antibody. Gal-1 expression occurs in every tissue examined and localizes primarily to the cytosol. In contrast to non-striated tissue, which exhibited diffuse cytosolic Gal-1 localization, Gal-1 displayed a unique organization within skeletal muscle and cardiac tissue, suggesting an unprecedented intracellular function for this protein. These results provide the first description of Gal-1 expression and localization in adult tissue and provide important insight intro previously defined roles for the protein.

RESULTS

Monoclonal antibody, αGal-1, displays specificity for hGal-1 in multiple formats

In order to accurately define the location of Gal-1 in tissue, we developed a highly specific monoclonal antibody. To accomplish this, we immunized mice with recombinant human Gal-1, followed by isolation of splenocytes and fusion with SP2/O mouse myeloma cells to form monoclonal antibody producing hybridomas. Of the many hybridomas generated from immunized mice, media isolated from one clone showed positive reactivity toward Gal-1, while failing to react with other human galectins, Gal-2, Gal-3, Gal-4, and Gal-7, during primary screening (data not shown). We next tested the purified monoclonal antibody from this clone, αGal-1, and determined its specificity toward Gal-1 over defined concentrations. Importantly, αGal-1 exhibited a dose dependent response to Gal-1 in a solid phase assay format, while failing to react with Gal-2, Gal-3, Gal-4, and Gal-7 under the same conditions (Fig. 4-1A), demonstrating that αGal-1 specifically recognized natively folded Gal-1. As αGal-1 clearly recognizes folded Gal-1, we next sought to determine whether αGal-1 required a conformational epitope for Gal-1 recognition, or if αGal-1 binds Gal-1 following denaturation. To accomplish this, we determined whether αGal-1 recognizes Gal-1 using Western blot analysis. αGal-1 detected recombinant Gal-1 utilizing Western blot, while it failed to display similar reactivity toward Gal-2, Gal-3, Gal-4, and Gal-7 (Fig. 4-1B), which demonstrated that αGal-1 specifically recognizes Gal-1 in this format. Taken together, these results demonstrate that αGal-1 specifically recognizes Gal-1 and that this recognition can occur toward the denatured protein or under conditions which allow Gal-1 to retain its native conformation.

The ability of αGal-1 to react with Gal-1 under a denaturing condition suggested recognition of a surface exposed linear epitope within the Gal-1 sequence. To determine which sequence supported αGal-1 recognition, we utilized a PIN map system of overlapping octapeptide sequences covering the entire sequence of Gal-1. αGal-1 specifically reacted with the amino acid sequence SKDGGAWG (Fig 4-1C). Importantly, SKDGGAWG lies on the surface of Gal-1, which corroborated the ability of aGal-1 to recognize denatured and native Gal-1. Sequence comparison analysis between Gal-1 and other galectin family members demonstrated that Gal-2 displays the highest similarity over this epitope region (Table 4-1). However, αGal-1 failed to react with Gal-2, both in a solid phase assay system and Western blot analysis (Fig. 4-1A,B), which demonstrated the specificity of αGal-1 toward Gal-1.

αGal-1 recognizes Gal-1 bound to ligand

The αGal-1 epitope, SKDGGAWG, lies in close proximity to the carbohydrate recognition domain of the protein (Fig. 4-2A), which raised the possibility that αGal-1 may preclude Gal-1 ligand recognition and therefore serve as a functional blocking antibody. To test this, we pre-incubated biotinylated Gal-1 with αGal-1, followed by incubation of this complex with laminin, a previously described ligand for Gal-1 [166], to determine whether αGal-1 blocks the ability of Gal-1 to bind ligand. Pre-incubation of aGal-1 with Gal-1 failed to reduce Gal-1 recognition of laminin (Fig. 4-2B). Importantly, Gal-1 binding to laminin required glycan recognition as lactose, but not maltose, inhibited binding (Fig. 4-2B). Similar results occurred over a wide range of aGal-1 concentrations (Fig. 4-2C). The inability of αGal-1 to prohibit Gal-1-ligand interactions suggested that αGal-1 likely recognizes Gal-1 following ligand binding. To test this, we examined the ability of aGal-1 to directly detect Gal-1 once bound to laminin. αGal-1 detected Gal-1

following pre-incubation of Gal-1 alone with laminin (Fig. 4-2D). Inclusion of lactose precluded detection (Fig. 4-2D), which demonstrated that aGal-1 specificity recognized Gal-1 in this assay. Taken together, these results demonstrate that aGal-1 fails to inhibit Gal-1 ligand interactions and that αGal-1 recognizes Gal-1 bound to ligand.

Gal-1 displays broad expression in adult tissue

As our purpose in developing a highly specific monoclonal antibody against Gal-1 was to determine the expression and localization of Gal-1 in adult tissue, we next examined the distribution of Gal-1 expression among different tissues. To accomplish this, we examined porcine tissue due to the ease of obtaining sufficient quantities of tissue needed for analysis and the conservation of aGal-1 epitope between porcine and human Gal-1. Western blot analysis of tissue extracts revealed a specific immunoreactive 14.5 Kd band, which displayed identical electrophoretic mobility as recombinant Gal-1 (data not shown). Importantly, aGal-1 specifically detected Gal-1, as incubation of extracts with the secondary antibody alone failed to produce similar immunoreactivity (Fig. 4-3A,B). Comparison of equal wet weight of total tissue demonstrated Gal-1 expression in all tissues examined, with varying levels of expression between different tissues (Fig. 4-3A), although similar amounts of protein extracted from each tissue were compared (Fig. 4-3C). Gal-1 displayed higher expression in tissues lining the alimentary canal and striated muscle tissue, with less, but detectable, expression in tissue isolated from the kidney and cerebrum (Fig. 4-3A). These results demonstrate that aGal-1 specifically recognized Gal-1 and that Gal-1 expression occurred in all tissues examined.

Galectin-1 exhibits diffuse cytosolic localization liver, brain and stomach but organized localization in striated tissue

As western blot analysis clearly demonstrated Gal-1 expression in multiple tissues, we next sought to determine the localization of Gal-1. Gal-1 displayed a diffuse localization, with significant cytosolic staining in most tissues examined, as shown for liver, brain, and stomach (Fig. 4-4A-C). Importantly, αGal-1 specifically detected Gal-1, as tissue sections stained with the secondary antibody alone (Fig. 4-4D-F) or with an isotype control followed by secondary failed to produce similar reactivity (data not shown). In contrast to these tissues, Gal-1 displayed an unprecedented staining pattern in striated tissue. In cardiac and skeletal muscle, Gal-1 also localized primarily to the cytosol (Fig. 4-5A,B). However, in contrast to liver, brain, and kidney tissues, Gal-1 displayed an organized, striated staining pattern within the cytosol (Fig. 4-5A,B). To determine whether this was specific to striated tissue, we examined Gal-1 localization in smooth muscle. Smooth muscle within the tunica media of large vessels stained strongly with Gal-1 (Fig. 4-6A). However, unlike Gal-1 localization in striated muscle, Gal-1 displayed a diffuse staining pattern in smooth muscle (Fig. 4-6A), similar to that observed in liver, kidney, and brain (Fig. 4-4A-C). Importantly, Gal-1 also appeared to display significant staining of endothelial cells lining the vessel lumen. To examine Gal-1 staining in the endothelium in more detail, we examined Gal-1 expression in human umbilical vein endothelial cells (HUVECs). Similar to previous results [167], Gal-1 exhibited significant expression within HUVECs (Fig. 4-6B). As Gal-1 exhibits significant effects on leukocytes, we next examined Gal-1 expression in several leukocyte populations. Interestingly, αGal-1 failed to detect Gal-1 expression in neutrophils, HL60 cells, or MOLT-4 cells, although αGal-1 detected Gal-1 in HUVECs (Fig. 4-6C). Taken together, these results demonstrate that Gal-1 exhibits high expression in multiple tissues, which remains primarily cytosolic, yet exhibits an unprecedented organization within striated muscle cells.

To ensure that the unique intracellular localization of Gal-1 in striated tissue did not reflect cross reactivity between αGal-1 and an unknown muscle specific antigen, we next examined localization of Gal-1 using an alternative affinity purified polyclonal antibody preparation against Gal-1 (PαGal-1) and compared this to the staining observed following detection with αGal-1. To accomplish this, we first sought to determine whether PαaGal-1 might react with epitopes on Gal-1 distinct from that recognized by αGal-1. The αGal-1 epitope, SKDGGAWG, contains a critical lysine residue, which suggested that lysine modification might preclude Gal-1 recognition by αGal-1. To test this, we fixed Gal-1 in a solid phase assay. Fixation of Gal-1 significantly reduced αGal-1 recognition of Gal-1, while failing to exhibit a similar affect on PαGal-1 detection (Fig. 4-7A), demonstrating that PaGal-1 recognizes epitopes not affected by fixation and therefore unique from SKDGGAWG. To further test whether αGal-1 cross-reacted with a muscle specific antigen within porcine tissue, we examined Gal-1 localization in fixed skeletal muscle. Similar to fixation of recombinant Gal-1, αGal-1 failed to detect protein extracted from fixed skeletal muscle using Western blot analysis (Fig. 4-7B). Importantly, PαGal-1 specifically recognized a 14.5 kD band, which demonstrated that PαGal-1 recognizes Gal-1 isolated from fixed tissue and that this preparation appears to be specific to Gal-1 (Fig. 4-7B). As PαGal-1 specifically recognizes Gal-1, yet reacted with a distinct epitope from αGal-1, we next determined the localization of Gal-1 in skeletal muscle using PαGal-1. Staining of fixed tissue with aGal-1 failed to demonstrate Gal-1 localization (Fig. 4-7G), similar to the inability of αGal-1 to recognize fixed Gal-1 utilizing Western blot analysis. However, staining of skeletal muscle with PαGal-1 produced the same staining pattern observed following staining of frozen sections with αGal-1 (Fig. 4-7H). To determine whether PαGal-1 specifically recognized Gal-1 within muscle tissue, we pre-incubated PαGal-1 with recombinant Gal-1, or Gal-4,

as a control. Importantly, pre-incubation of PαGal-1 with Gal-1 blocked staining, while pre-incubation with Gal-4 had no effect (Fig. 4-7I,J). Taken together, these results demonstrate Gal-1 displays a unique organized staining pattern within striated tissue.

DISSCUSION

Previous studies demonstrate that Gal-1 experiences differential expression during embryogenesis, suggesting a critical role for Gal-1 in development [168]. However, early studies failed to demonstrate significant developmental defects in Gal-1 null mice [169]. In contrast, recent results demonstrate significant roles for Gal-1 *in vivo* in both immunological regulation and muscle development and regeneration [161, 163]. However, few studies examined the expression and localization of Gal-1 in adult tissue. The present results provide one of the first examinations of Gal-1 expression and localization in adult tissue and provide important insight into previously documented biological activities of Gal-1.

The expression and localization of Gal-1 suggests unique roles for Gal-1 in the regulation of immunity. Most factors involved in immune regulation, such as TNFa and FasL, experience relatively restricted expression, most often expressed on leukocyte subpopulations [170]. In contrast, the present results demonstrate constitutive expression of Gal-1 in every tissue examined with predominate localization to the cytosol of parenchymal cells in each respective organ, consistent with previous results in CHO cells [171]. Furthermore, in contrast to TNFa and FasL which are commonly expressed on leukocytes [170], aGal-1 failed to detect Gal-1 expression in leukocytes, consistent with recent finding demonstrating that activated cytotoxic T cells fail to express significant Gal-1 [159], although some leukocyte subpopulations may express Gal-1 [159]. In contrast, human endothelial cells, which regulate critical processes in leukocyte activity and trafficking [172], express significant Gal-1, corroborating previous results [167] and suggest potential roles for Gal-1 in regulating leukocyte trafficking, as previously reported [53].

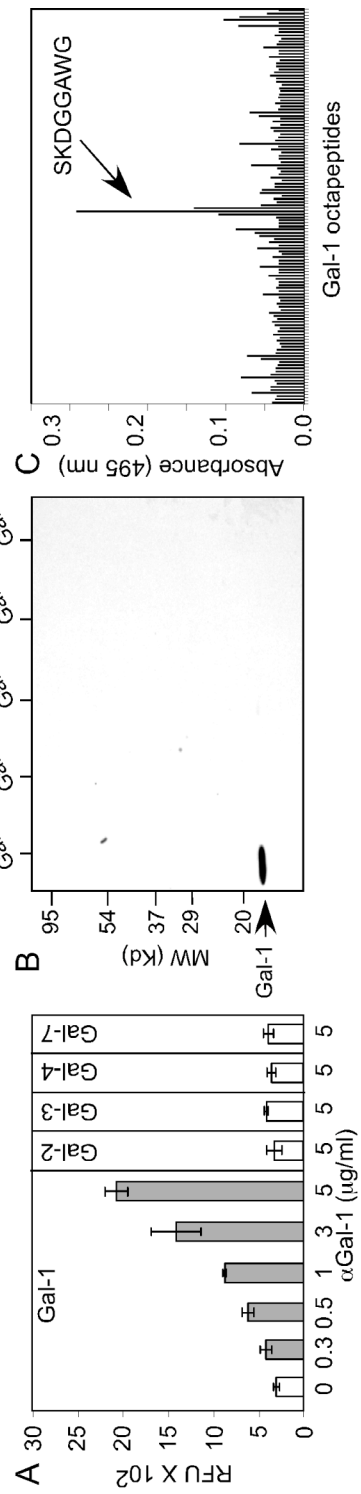
Leukocytes harbor a significant threat to the viable tissue surrounding areas of active inflammation [173]. Proper containment of activated leukocytes to areas of tissue infection and injury allows proper demarcation between necrotic and viable tissue [173], allowing proper protection of surrounding tissue. As Gal-1 induces leukocyte turnover and inhibits chemotaxis [53, 82], the expression of Gal-1 in parenchymal tissue suggests a potential role in regulating leukocyte function and turnover at the boundary between active inflammation and surrounding viable tissue. Release of Gal-1 by leukocyte-mediated damage of viable tissue may therefore facilitate the containment of leukocytes to the area of original tissue injury. As Gal-1 undergoes oxidative inactivation in the absence of ligand [46, 51], Gal-1 released during the original necrotic event likely undergoes oxidative activation prior to significant leukocyte recruitment, enabling leukocytes to remove necrotic tissue and neutralize pathogens in an unimpeded fashion. However, Gal-1 release from viable tissue during inflammation likely remains active, engages leukocyte ligands, which stabilizes Gal-1 [46, 51], and induces leukocyte turnover [82]. As inflammation occurs in all tissue types, the ubiquitous expression of Gal-1 suggests that Gal-1 may serve as a common parenchymally derived factor regulating host immunity.

In addition to modulating immune function [4], several studies implicate Gal-1 in the regulation of muscle development and regeneration [164]. Gal-1 induces myoblast fusion *in vitro* and Gal-1 null mice experience reduced myofiber formation [163, 164]. Gal-1 also induces dermal fibroblasts to express muscle specific markers such as desmin and Gal-1 null mice experience impaired capacity to undergo muscle regeneration following injury *in vivo* [163, 164, 174, 175]. In addition to inducing muscle cell differentiation [164, 174, 175], the present results suggest that Gal-1 may contribute to muscle function and

development as a result of intracellular associations of the protein. Other studies suggest that Gal-1 may interact with other cellular signaling components, such as Ras [165], through protein-protein interactions. Similar interactions may be responsible for Gal-1 organization within striated muscle cells.

In conclusion, these results provide the first description of Gal-1 expression and localization in adult tissue using a well-defined monoclonal antibody. The ubiquitous expression of Gal-1 suggests a unique role in the regulation of leukocyte function. Furthermore, Gal-1 localization in striated tissue suggests an unprecedented intracellular function within muscle tissue. Future studies will explore these intriguing possibilities.

Figure 4-1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RFU X 10²
30
25
20
15
10
5
0
Gal-1
0 0.3 0.5 1 3 5 5
αGal-1 (μg/ml)
Gal-2
Gal-3
Gal-4
Gal-7
B
MW (Kd)
95
54
37
29
20
Gal-1
Gal-1
Gal-2
Gal-3
Gal-4
Gal-7
C
Absorbance (495 nm)
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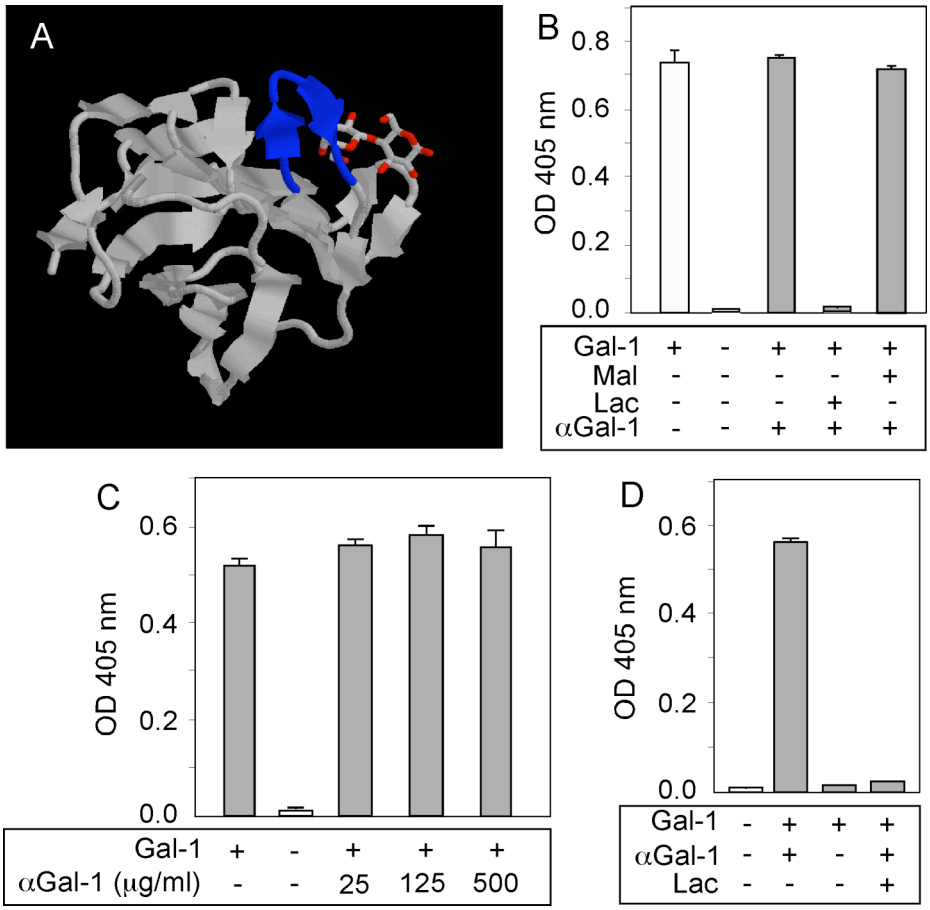
0.2
0.1
0.0
SKDGGAWG
Gal-1 octapeptides

Figure 4-2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B
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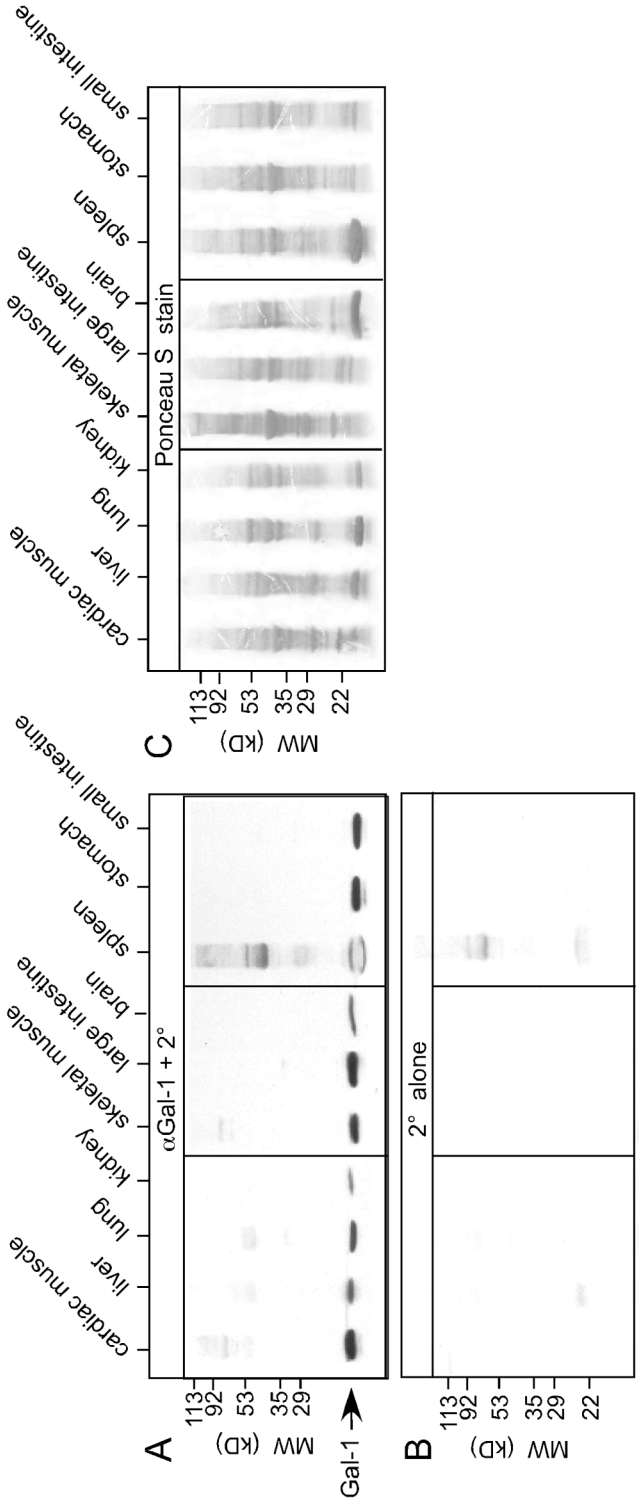
0.2
0.4
0.6
0.8
Gal-1 + - + + +
Mal - - - - +
Lac - - - + -
αGal-1 - - + + +
C
OD 405 nm
0.0
0.2
0.4
0.6
Gal-1 + - + + +
αGal-1 (μg/ml) - - 25 125 500
D
OD 405 nm
0.0
0.2
0.4
0.6
Gal-1 - + + +
αGal-1 - + - +
Lac - - - +

Figure 4-3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MW (kD)
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92
53
35
29
Gal-1
cardiac muscle
liver
lung
kidney
skeletal muscle
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brain
spleen
stomach
small intestine
B
2° alone
MW (kD)
113
92
53
35
29
22
C
Ponceau S stain
MW (kD)
113
92
53
35
29
22
cardiac muscle
liver
lung
kidney
skeletal muscle
large intestine
brain
spleen
stomach
small intestine

Figure 4-4

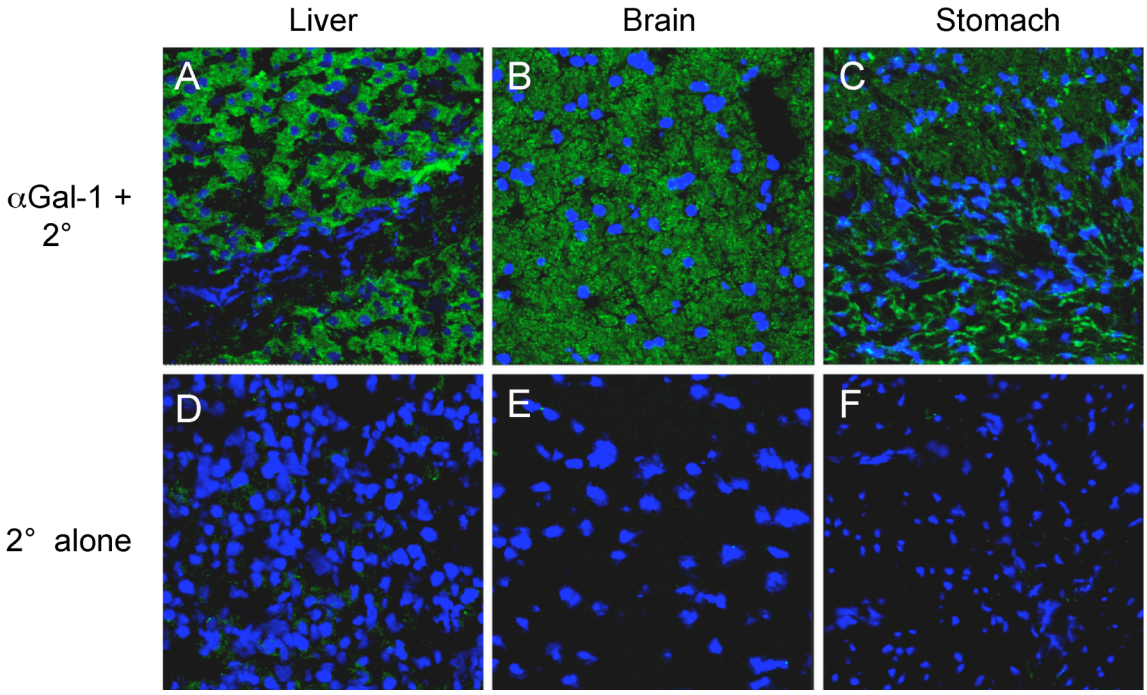

Figure 4-5

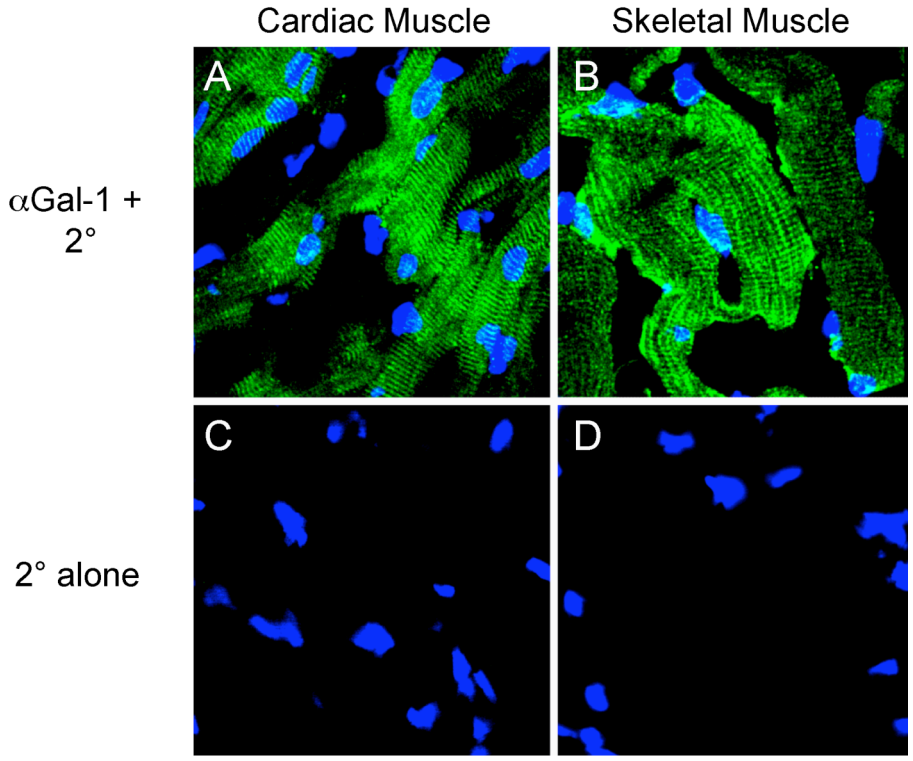

Figure 4-6

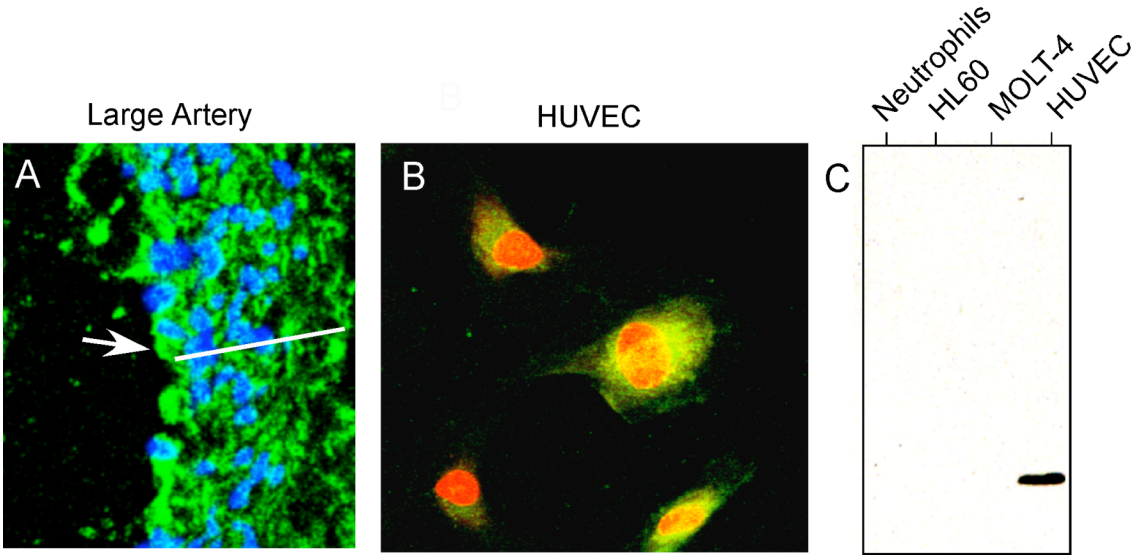

Figure 4-7

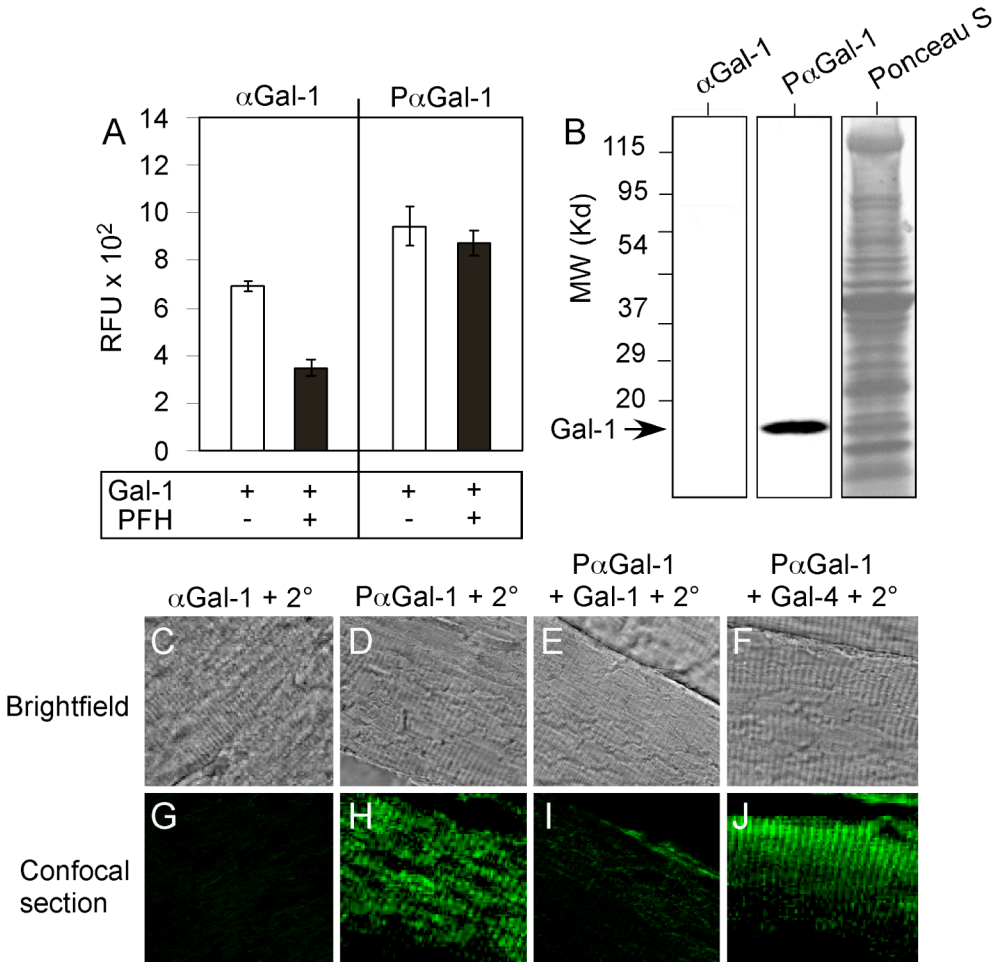
A
αGal-1
PαGal-1
RFU x 10²
14
12
10
8
6
4
2
0
Gal-1 + + + +
PFH - + - +
B
αGal-1
PαGal-1
Ponceau S
MW (Kd)
115
95
54
37
29
20
Gal-1
αGal-1 + 2°
PαGal-1 + 2°
PαGal-1 + Gal-1 + 2°
PαGal-1 + Gal-4 + 2°
Brightfield
Confocal section
C
D
E
F
G
H
I
J

FIGURE LEGENDS

Figure 4-1. αGal-1 specially recognizes recombinant human Gal-1. (A) Each well was incubated with the indicated concentrations of antibody against each of the indicated recombinant galectins followed by detection using an ALEXA 488 labeled secondary antibody. (B) Western blot analysis of recombinant human galectins using αGal-1**.** (C) Pin mapping of the αGal-1 epitope using synthetic octapeptides spanning the entire sequence of human Gal-1.

Figure 4-2. αGal-1 detects Gal-1 without interfering with Gal-1 binding. (A) Crystal structure of human Gal-1 with epitope region highlighted. (B) Pre-incubation of aGal-1 with Gal-1 followed by detection of Gal-1 binding to laminin with or without the inclusion of 20 mM lactose (Lac) or 20 mM maltose (Mal) as indicated. Pre-incubation of aGal-1 with Gal-1 followed by detection of Gal-1 binding with or without the inclusion of 20 mM lactose (Lac) or 20 mM maltose (Mal) as indicated. (C) Pre-incubation of the indicated concentration of antibody with Gal-1 followed by detection of Gal-1 binding to laminin. (D) Incubation of Gal-1 with laminin with or without 20 mM lactose followed by detection of Gal-1 with αGal-1.

Figure 4-3. Gal-1 detection in multiple tissues by western blot analysis using αGal-1 Western blot analysis of porcine tissue from each respective organ followed by SDS-PAGE and western blot analysis using (A) αGal-1 + secondary or (B) Secondary alone. (C) Ponceau S stain prior to analysis.

Figure 4-4. Confocal analysis of Gal-1 expression in liver, brain and stomach. Frozen sections of liver, brain and stomach were incubated with (A-C) αGal-1 + secondary or (D-E) Secondary alone.

Figure 4-5. Confocal analysis of Gal-1 expression in striated tissue. Frozen sections of cardiac and skeletal muscle were incubated with (A-B) αGal-1 + secondary or (C-D) Secondary alone.

Figure 4-6. Gal-1 expression in vascular tissue (A) Gal-1 expression in a large artery detected using αGal-1. (B) Gal-1 expression in HUVECs using αGal-1. (C) Western blot analysis of Gal-1 expression in neutropihl, HL60 cells, MOLT-4 cells and HUVECs using αGal-1.

Figure 4-7. Gal-1 displays organized localization in striated tissue. (A) Detection of Gal-1 before and after fixation with 2% paraformaldehyde with either αGal-1 or affinity purified polyclonal anti-Gal- (pαGal-1). (B) Western blot analysis of Gal-1 expression using αGal-1 or pαGal-1 of fixed tissue. (C) Confocal analysis of fixed skeletal muscle tissue using αGal-1 or pαGal-1. Pre-incubation with 10 μM Gal-1 or 10 μM Gal-4 occurred where indicated.

Chapter 5

Galectins-1, -2, and -4 Induce Surface Exposure of Phosphatidylserine in Activated Human Neutrophils but Not Activated Lymphocytes

Cellular turnover is associated with exposure of surface phosphatidylserine (PS) in apoptotic cells, leading to their phagocytic recognition and removal. But recent studies indicate that surface PS exposure is not always associated with apoptosis. Here we show that several members of the human galectin family of glycan binding proteins (galectins-1, -2, and -4) induce PS exposure in a carbohydrate-dependent fashion in activated, but not resting, human neutrophils and in several leukocyte cell lines. PS exposure is not associated with apoptosis in activated neutrophils. The exposure of PS in cell lines treated with these galectins is sustained and does not affect cell viability. Unexpectedly, these galectins bind well to activated T-lymphocytes, but do not induce either PS exposure or apoptosis, indicating that galectin effects are cell specific. These results suggest novel immunoregulatory contribution of galectins in regulating leukocyte turnover independently of apoptosis.

INTRODUCTION

The physiological causes of leukocyte turnover in homeostasis and during disease conditions are not well understood. It is believed that leukocytes are partly eliminated by programmed cell death or apoptosis[73, 74, 176, 177] through phagocytosis by macrophages, dendritic cells, or neighboring cells[2, 178]. The efficient removal of dying cells is important in homeostasis, since it limits accumulation of cellular debris that could be potentially immunogenic or toxic[179-181]. However, the role of apoptosis in removing large numbers of cells in inflammation and during the resolution phase remains uncertain. Although human neutrophils undergo apoptosis spontaneously when cultured *in vitro*, the role of apoptosis in regulating neutrophil turnover *in vivo* is unclear[2, 182-184]. Apoptosis impairs cellular functions and might impair proinflammatory functions of neutrophils[185]. However, excessive neutrophil influx with loss of membrane integrity during late apoptotic events could contribute to neutrophil-mediated injury of surrounding viable parenchymal tissue[173]. Exuberant apoptosis may therefore be pro-inflammatory instead of anti-inflammatory[108, 155, 156], presumably due to the release of cellular contents prior to phagocytic removal. In this regard non-apoptotic neutrophils can be cleared by phagocytosis *in vivo*[38]. Finally, factors known to induce or to block apoptosis, such as ligation of Fas/FasL and expression of bcl-2, respectively, do not alter neutrophil turnover in mouse models[3, 34, 109, 186]. These studies suggest that unidentified factors may be involved in the phagocytic removal of viable, rather than apoptotic, cells.

The removal of apoptotic cells partly occurs through tethering to phagocytic cells due to receptor-ligand interactions involving recognition of phosphatidylserine (PS) exposed on the surfaces of apoptotic cells[39]. Surface PS is recognized by a defined PS-receptor in

macrophages[29], and by other receptors[187-189]. However, PS exposure in leukocytes can occur independently of apoptosis[77, 112]. Thus, factors that induce PS exposure independently of apoptosis may be involved in leukocyte turnover.

Recently, we showed that galectin-1 (Gal-1), a prototypical homodimeric member (subunit ~14.5 kDa) of the galectin family, which has immunomodulatory functions[25, 190-193], can induce PS exposure in activated, but not resting, neutrophils, independently of cell death, while concomitantly rendering them sensitive to phagocytic recognition and removal[40]. We also found that the signaling pathway of Gal-1 in activated neutrophils is unique and involves elevations of cytosolic Ca^{2+} and mobilization of PS through the actions of Src kinases and phospholipase Cγ[42].

However, there are many conflicting reports about galectin effects on leukocytes. Several groups have reported that Gal-1 induces apoptosis along with PS exposure *in vitro* of activated T lymphocytes and several T leukemic cell lines[14, 194, 195]. It has also been reported that human Gal-2, a protein structurally related to Gal-1, induces T cell apoptosis *in vitro*[13]. By contrast, Gal-4, another member of the galectin family with two tandemly-repeated carbohydrate-recognition domains, stimulates IL-6 production in CD4+ T cells without accompanying apoptosis[196]. Thus, it is important to reinvestigate the activities of these galectins on activated human neutrophils and human T cells.

Here we report that human galectins-1, -2, and -4 induce PS exposure in activated, but not resting, human neutrophils in a carbohydrate-dependent fashion independently of apoptosis. By contrast, Gal-1 does not induce apoptotic cell death or PS exposure in activated T cells, although it does induce PS exposure in T leukemic MOLT-4 and CEM cell lines. Unexpectedly, we found that Gal-1 could induce PS exposure in T cells only

when the cells were pretreated with dithiothreitol (DTT), a common reducing agent utilized in many studies assessing the pro-apoptotic potential of Gal-1 toward T cells. These studies indicate that activated neutrophils, but not activated T lymphocytes, possess unique receptors and signaling pathways for some galectin family members, which trigger PS exposure independently of apoptosis and may be important in regulating neutrophil turnover.

RESULTS

Gal-2 and Gal-4 induce PS exposure in MOLT-4 and HL60 cells

MOLT-4 cells and HL60 cells were treated with either Gal-2 or Gal-4, followed by analysis for PS exposure by staining with FITC-Annexin V using flow cytometric analysis. Annexin V is the commonly used reagent to detect surface PS, since it specifically binds PS with high affinity[197, 198] and can interfere *in vivo* with recognition and uptake of PS-expressing apoptotic cells[199]. Both Gal-2 and Gal-4 induced surface PS exposure in both HL60 and MOLT-4 cells (Table 5-1). The inclusion of 20 mM lactose with Gal-2 or 30 mM TDG with Gal-4 abrogated the induction of PS exposure, whereas sucrose had no effect (Table 5-1). Importantly, the binding of Gal-2 (Fig. 5-1A) and Gal-4 (Fig. 5-1B) to leukocytes was also dependent on carbohydrate recognition. Pre-treatment of either cell line with neuraminidase significantly enhanced both the binding of Gal-2 (Fig. 5-1A) and Gal-4 (Fig. 5-1B) and the ability of Gal-2 (Fig 5-1C) and Gal-4 (Fig. 5-1D) to redistribute PS. PS exposure was maximal within ~2 h in HL60 cells treated with 10 μM Gal-2 or 3 μM Gal-4 (Fig. 5-2A). Galectin signaling in HL60 cells was dose dependent; Gal-4 was slightly more potent, inducing maximal effects around 2.5 μM, whereas at least 10 μM Gal-2 was required to obtain maximal effects (Fig. 5-2B). These results demonstrate that binding of Gal-2 and Gal-4 to these cells induces PS exposure in a carbohydrate- and dose-dependent fashion.

Leukocytes induced to expose PS by Gal-2 and Gal-4 fail to display other known features of programmed cell death

We next examined whether the induction of PS exposure by these galectins was associated with loss of cell viability or apoptosis. MOLT-4 or HL60 cells were treated for 18 h with either PBS (Fig. 5-3A,D, and E), 5 μM Gal-4 (Fig. 5-3D) or 10 μM Gal-2 (Fig. 5-

3B,E) followed by the TUNEL assay to detect DNA fragmentation. Known inducers of apoptosis, camptothecin and etoposide in HL60 and MOLT-4 cells, respectively, produced extensive DNA fragmentation over this time period (Fig. 5-3C,D, and E). By contrast, no significant DNA fragmentation was observed in galectin-treated cells (Fig. 5-3B,D, and E). Potential DNA fragmentation induced by galectins was also assessed using hypodiploid DNA content, as determined by PI staining. No increases in hypodiploid DNA content were observed following treatment of cells with either Gal-2 or Gal-4, although camptothecin- and etoposide-treated cells displayed extensive hypodiploid DNA content when treated over this same time period (data not shown).

To further determine whether cell viability might be affected following galectin treatment, MOLT-4 cells (Fig 5-3F) and HL60 cells (Fig 5-3G) were treated with 5 μM Gal-4 followed by analysis for the number of viable cells excluding trypan blue using a hemocytometer. Treatment of cells with Gal-4 caused no significant alterations in growth rate, whereas treatment of cells with either camptothecin or etoposide caused loss of viable cells within 24 h (Fig. 5-3F,G). Treatment of HL60 cells with Gal-2 produced similar results (data not shown). Another cellular feature associated with apoptosis is cell blebbing (zeosis) and cell shrinkage[80], which can be measured by changes in light scattering properties of the cell in flow cytometric analysis[80]. HL60 cells (Fig. 5-3H-J) were treated for 18 h with Gal-2, and although the cells continued to express surface PS (data not shown), there was no significant change in the light scatter properties of the cells (Fig. 5-3H,I). Treatment of both MOLT-4 cells and HL60 cells in the same manner with Gal-4 or MOLT-4 cells with Gal-2 produced similar results (data not shown). By contrast, treatments of cells with either camptothecin or etoposide caused significant changes in light scatter profiles, as shown for HL60 cells treated with camptothecin (Fig.

5-3J). These results demonstrate that both Gal-2 and Gal-4 induce PS exposure in these cell lines independently of cell death.

Activated neutrophils, but not activated T cells, are sensitive to galectin-induced PS exposure

To further explore the specificity of Gal-2- and Gal-4-induced PS exposure in blood leukocytes, we first examined responses of human neutrophils. Gal-4 induced significant PS exposure in fMLP-activated neutrophils, but had only a modest effect on resting neutrophils (Fig. 5-4A). Treatment of cells with Gal-2 and Gal-1 also induced PS exposure in fMLP-activated human neutrophils (Fig. 5-4B), but not in resting neutrophils (data not shown). As a control, cells were treated with anti-Fas, a known inducer of apoptosis[3], which also induced PS exposure (Fig. 5-4B). However, treatment of fMLP-activated neutrophils with Gal-1, Gal-2, or Gal-4 did not induce apoptosis, as measured by the TUNEL assay, whereas anti-Fas treatment induced apoptosis (Fig. 5-4C). In addition, galectins did not induce cellular shrinkage of fMLP-activated neutrophils, whereas anti-Fas induced cell shrinkage (Fig. 5-4D). These results demonstrate that Gal-1, Gal-2 and Gal-4 induce PS exposure in fMLP-activated, but not resting neutrophils, independently of cell death.

Since both Gal-2 and Gal-4 induced PS exposure independent of cell death in activated neutrophils, we next sought to determine whether Gal-2 and Gal-4 mobilize Ca^{2+} as previously observed during Gal-1 induced PS exposure[200]. Resting and fMLP-activated neutrophils were treated with Gal-1, Gal-2, or Gal-4 followed by detection for changes in intracellular Ca^{2+}. Similar to previous observations for Gal-1[200] (Fig. 5-5A,B), Gal-2 induced a rapid, yet transient, Ca^{2+} flux in both resting and fMLP-activated neutrophils (Fig. 5-5D,E). However, both resting and activated neutrophils showed no

changes in intracellular Ca^{2+} levels following Gal-4 treatment (Fig. 5-5G,H). In addition, both resting and activated neutrophils were responsive to Gal-1 following Gal-4 administration, indicating that the cells were sensitive to Gal-1 induced Ca^{2+} mobilization (Fig. 5-5G,H). In all cases, treatments of cells with Gal-1, Gal-2, or Gal-4 induced PS exposure (Fig. 5-5C,F, and I), respectively. These results demonstrate that Gal-4 induces PS exposure through separate proximal signaling pathways distinguishable from those for Gal-1 and Gal-2.

Previous reports indicated that Gal-1 induces PS exposure in activated, but not resting T cells, with accompanying apoptosis[14], which is clearly different from the responses of activated neutrophils to galectins. To further explore the basis for this apparent difference, resting and PHA-activated T cells (Fig. 5-6A,B) were incubated with Gal-1 for 9 h. Cell activation was confirmed by incubating cells with anti-IL-2R, anti-CD-3, anti-TCR-$\alpha\beta$ or an isotype control followed by flow cytometric analysis. Unexpectedly, Gal-1 treatment of activated T cells from 3 different donors had no effect on PS redistribution (Fig. 5-6C), although in parallel control assays Gal-1 induced PS exposure in MOLT-4 cells (Fig. 5-6C). Thus, contrary to previous reports, Gal-1 is unable to signal PS exposure or apoptosis in activated T cells.

In the presence of DTT galectin-1 induces PS exposure of activated T cells

In exploring the basis for these apparently conflicting results in comparison to other published studies, we noted that most previous studies included the reducing agent dithiothreitol (DTT) in Gal-1 preparations used to treat cells, presumably to maintain Gal-1 activity[14, 81]. Thus, we titrated increasing concentrations of DTT into activated T cells treated with a uniform amount of Gal-1. Consistent with previous results[14, 81], Gal-1 induced PS exposure in activated T cells, but only when DTT was included in a

concentration-dependent manner (Fig. 5-7A). Thus, while Gal-1 is able to induce PS exposure in fMLP-activated neutrophils, but not activated T-cells, it induces robust PS exposure in activated T cells only after inclusion of DTT.

To address the possibility that Gal-1 might be losing activity during these treatments in the absence of DTT, thus affecting its potential activity toward activated T cells, we generated a mutant form of Gal-1, termed C2S-Gal-1, in which a key Cys residue at position-2 is changed to Ser. The C2S mutant form of Gal-1 retains lectin activity for days in the absence of reducing conditions or hapten sugars such as lactose[46, 51]. When incubated at 37°C in the absence of DTT over 24 h, the C2S-Gal-1 showed no appreciable loss in activity compared to freshly prepared C2S-Gal-1 (Fig. 5-7B). The C2S-Gal-1 was comparable to wt Gal-1 in inducing PS exposure in MOLT-4 cells (Fig. 5-7C). Additionally, the C2S-Gal-1 retained all carbohydrate-binding properties of the wild-type lectin toward a wide variety of glycan ligands (data not shown).

When activated T cells were treated with either wt Gal-1 or C2S-Gal-1 there was no increase in surface PS exposure (Fig. 5-7C). However, when activated T cells were treated with wt Gal-1 or C2S-Gal-1 in the presence of DTT, they became responsive and PS exposure was apparent (Fig. 5-7C). Furthermore, inclusion of 20 mM TDG abrogated the Gal-1 effect (Fig. 5-7D). These results demonstrate that Gal-1 induces PS exposure in activated T cells only in the presence of DTT.

We next addressed whether Gal-2 or Gal-4 could induce PS exposure in T cells in the absence of DTT. Neither Gal-2 nor Gal-4 induced PS exposure in activated T cells (Fig. 5-8A), although upon inclusion of DTT Gal-2 induced PS exposure (data not shown), consistent with previous observations[13]. Gal-2 behaved similarly to Gal-1, requiring

reducing conditions to maintain complete activity over a 24 h period (data not shown). However, Gal-4 was much less unstable and in fact was more stable than C2S-Gal-1, as evidenced by its ability to induce PS exposure in HL60 cells following prolonged incubation in the absence of DTT (Fig. 5-8B). These results demonstrate that neither C2S-Gal-1, wt Gal-1, Gal-2, nor Gal-4 induce PS exposure in activated T cells unless DTT is included in the treatment.

We next tested whether Gal-1 can induce apoptosis in cell lines when co-incubated with DTT, as reported by others [14]. We incubated MOLT-4 cells with wt or C2S-Gal-1 with or without DTT, and measured PS exposure and hypodiploid content. wt Gal-1 and C2S-Gal-1, when incubated in the absence of DTT, induced PS exposure in MOLT-4 cells (Fig. 5-9A) independently of DNA fragmentation (Fig. 5-9B), cell shrinkage (Fig. 5-9C) or increases in late apoptosis as measured by PI staining (Fig. 5-9A). However, when DTT was included with either wt Gal-1 or C2S-Gal-1, MOLT-4 cells experienced significant increases in PI staining (Fig. 5-9A), DNA fragmentation (Fig. 5-9B), and cell shrinkage (Fig. 5-9C) in a dose dependent manner. To determine whether this DTT-dependent effect could also be observed in a different T-leukemic cell line, we used T-leukemic CEM cells. CEM cells responded in the same fashion as MOLT-4 cells, only becoming sensitive to Gal-1-induced apoptosis when co-incubated with DTT, as measured by both hypodiploid stain, cell shrinkage and PI staining (data not shown). These results demonstrate that Gal-1 induces PS exposure in activated T cells only when DTT is present and induces apoptosis in MOLT-4 and CEM cells only when primed by DTT treatment. Thus, it is likely that the deleterious effects of DTT on activated T cells make them sensitive to Gal-1 signaling, whereas Gal-1 induces signals in activated neutrophils independently of DTT.

We next sought to determine whether the sensitizing effects of DTT on cells resulted from reduction and therefore potential alterations in cells surface glycoproteins or receptors or whether DTT might exert its deleterious effects once it accumulates inside the cells. We treated cells with reduced glutathione (RG), a reducing agent that is impermeable to the plasma membrane[201]. As previously observed, cells treated with DTT became sensitive to Gal-1 induced cell death, experiencing both increases in late apoptosis as evidenced by increased PI staining (Fig. 5-10A) and cellular shrinkage (Fig. 5-10 C,F), when compared to controls (Fig. 5-10A,B, and E). However, cells treated with RG displayed no increase in sensitivity toward Gal-1 induced cell death (Fig. 5-10 A,D, and G). These results demonstrate that DTT sensitizes cells toward Gal-1-induced apoptosis through alterations in intracellular processes.

DISCUSSION

Our results demonstrate that Gal-1, Gal-2 and Gal-4 induce PS redistribution in activated, but not resting, human neutrophils, and in leukemic MOLT-4 and HL60 cell lines, and that these changes do not accompany cell death. Importantly, activated T cells are not induced to expose PS in response to Gal-1, Gal-2 and Gal-4. These results demonstrate a novel and specific regulatory effect of galectins on leukocytes and further support the possible role of these galectins in the physiological turnover of neutrophils.

A key membrane event in regulating neutrophil turnover is the externalization of PS, which commonly accompanies apoptosis and serves as a key recognition marker for the phagocytic recognition and removal of apoptotic cells[30]. However, the cellular processes and factors that cause PS exposure are not well understood. Our previous studies showed that human Gal-1 induces PS exposure in activated neutrophils independently of apoptosis, which is accompanied *in vitro* by their efficient recognition and phagocytosis by macrophages[40]. Our new studies show that Gal-2 and Gal-4 also induce PS exposure in activated neutrophils but not in activated T cells. Importantly, Gal-4 induced PS exposure in the absence of Ca^{2+} mobilization, although Gal-1 induced PS exposure requires changes in intracellular Ca^{2+} [200], thus suggesting that there are at least two separate proximal pathways whereby PS can be externalized in an apoptosis-independent manner. The increased potency of Gal-4 over Gal-1 and Gal-2 is likely due to the tandem repeat nature of the protein, since engineered tandem repeat forms of Gal-1 demonstrate significant increases in biological potency[202, 203].

The ability of galectin family members to signal apoptosis independently of PS exposure corroborates growing evidence for apoptosis-independent mechanisms of leukocyte

phagocytosis[30]. The ability of galectin family members to induce PS exposure may therefore provide an explanation for the ability of macrophages to recognize and remove neutrophils through non-apoptotic mechanisms[38]. Furthermore, the ability of galectins to induce changes enabling phagocytic removal without inducing concomitant apoptosis allows neutrophils to maintain membrane integrity, thus limiting the potential for the unregulated release of deleterious enzymes prior to phagocytic removal[108, 156],[155]. Consistent with this hypothesis, Gal-1 administration attenuates acute inflammation *in vivo* and protects parenchyma architecture during inflammatory episodes[57].

There is growing evidence that non-apoptotic processes contribute to regulating neutrophil homeostasis. For example, mice that over-express bcl-2 in myeloid cells have impaired neutrophil apoptosis, yet they have normal numbers of neutrophils that are sensitive to phagocytic removal[37]. It appears that mechanisms regulating neutrophil turnover are partially distinct from those regulating lymphocyte turnover. For example, patients in which bcl-2 is over-expressed, as occurs in follicular lymphoma, accumulate lymphocytes[204]. Similarly, patients with autoimmune lymphoproliferative syndrome due to inherited defects in Fas, a proapoptotic signaling receptor, exhibit lymphocytosis[35, 36]. However, these same patients often have normal or reduced neutrophil counts[35, 36]. Similarly, mice deficient in Fas or Fas L display normal levels of circulating neutrophils and show no change in acute inflammatory responses[109], further suggesting that the turnover of neutrophils and lymphocytes may be regulated by alternative pathways. This is also consistent with evidence that Kupffer cells phagocytose non-apoptotic neutrophils *in vivo* during inflammatory resolution[38]. Potential differences observed between lymphocyte and neutrophil turnover *in vivo* may partially explain why only activated neutrophils, but not activated T lymphocytes, are sensitive to galectin-induced effects.

Our finding that Gal-1, -2, and -4 induce PS exposure in T-leukemic MOLT-4 cells, but not in activated T cells except when DTT is included, is novel and potentially resolves some long-standing controversies. Many studies have reported the effects of Gal-1 on cells in the presence of DTT[80]; under these conditions Gal-1 was reported to induce apoptosis of activated T cells, MOLT-4 cells and CEM cells[14, 17, 80, 205]. The present study demonstrates that Gal-1-induced apoptosis in the presence of DTT results from alterations in cellular physiology and not a requirement to maintain Gal-1 activity. Thus, C2S-Gal-1, which is resistant to oxidative inactivation, does not induce apoptosis in the absence of DTT but only in its presence. It should be noted that the extracellular milieu is normally an oxidizing environment[206], suggesting that reduction of normally oxidized cell surface proteins may result in the increased sensitivity of cells to Gal-1-induced death. However, the inability of reduced glutathione, a cell impermeable reducing agent[201], to sensitize cells to Gal-1 induced apoptosis demonstrates that the deleterious effects of DTT result from non-physiologic changes induced by DTT inside cells. Although very low concentrations of DTT may be protective to cell stress[207], higher concentrations of DTT, similar to those used in this and previous studies[14, 17, 80, 205], have been used to induce the unfolded-protein response, cause changes in gene expression, inhibit protein synthesis, and generate free radicals, causing generalized cell stress[83, 84, 208, 209], which often results in apoptotic cell death[84, 85, 210]. Thus, the DTT priming effect on cells is likely to be complex and could involve numerous pathways. Although we have shown that Gal-1, Gal-2 and Gal-4 do not induce apoptosis in T cells, we have not excluded the possibility that other galectin family members may affect T cell viability. For example, Gal-3 has been shown to induce apoptosis in activated T cells in the conspicuous absence of DTT[12]. Future

studies should examine the roles of other galectin family members in leukocyte viability in the absence of reducing agents.

The sensitivity of MOLT-4 and CEM cells, but not T cells, to Gal-1, -2, and -4 may arise from changes in the cell lines that accompanied neoplastic transformation. It is important to note that although both HL60 cells and neutrophils respond to galectin treatment, neutrophils only respond once they are activated, whereas HL60 cells are sensitive without a prior priming event. The ability of these galectins to signal through unique pathways in transformed cells may reflect potential roles for galectin family members in neoplastic disease. Recent studies suggests that PS exposure on tumor cells may serve as a decoy to phagocytic cells and trigger their release of anti-inflammatory cytokines as a mechanism of establishing an immune-privileged environment[211, 212]. This could be especially important in light of evidence implicating various galectin family members in neoplastic disease[10]. Future studies are needed to address the roles of galectin family members in the regulation of innate immunity and in neoplastic cell survival *in vivo*.

Figure 5-1

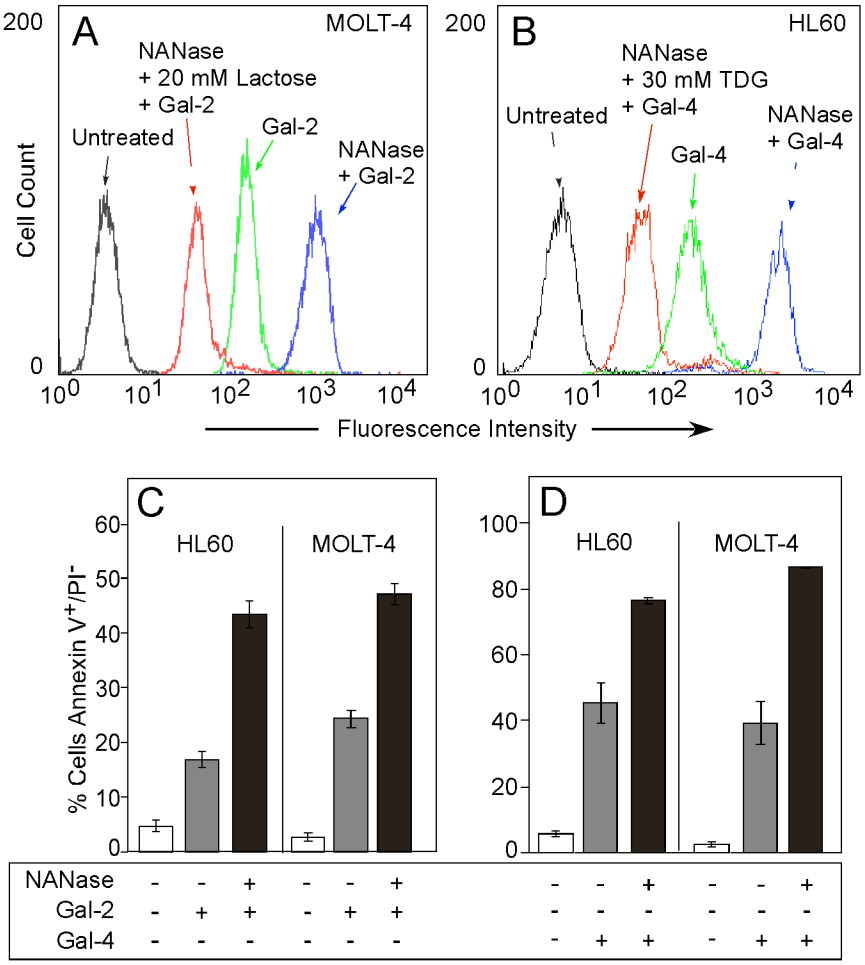
A
MOLT-4
NANase
+ 20 mM Lactose
+ Gal-2
Untreated
Gal-2
NANase
+ Gal-2
Cell Count
200
0
B
HL60
NANase
+ 30 mM TDG
+ Gal-4
Untreated
Gal-4
NANase
+ Gal-4
Fluorescence Intensity
C
HL60
MOLT-4
% Cells Annexin V+/PI-
D
NANase
Gal-2
Gal-4

Figure 5-2

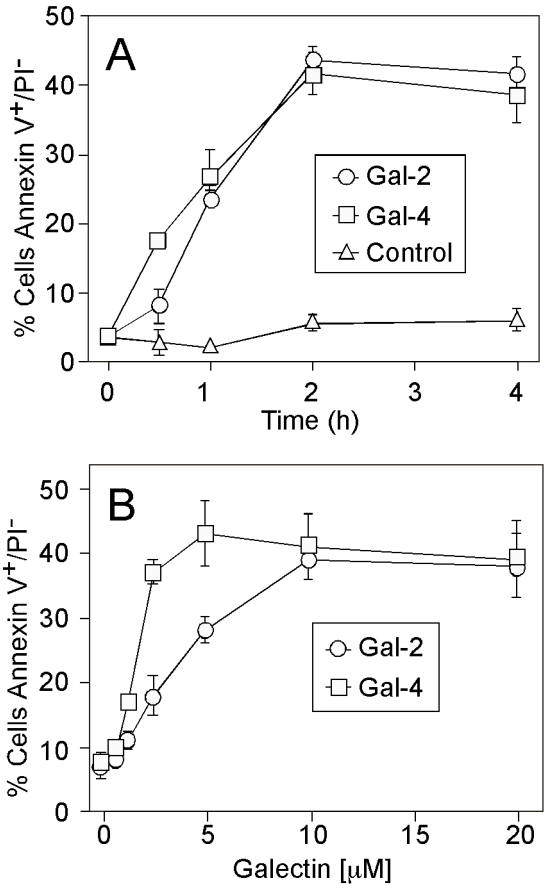
A
% Cells Annexin V+/PI-
Time (h)
Gal-2
Gal-4
Control
B
% Cells Annexin V+/PI-
Galectin [μM]
Gal-2
Gal-4

Figure 5-3

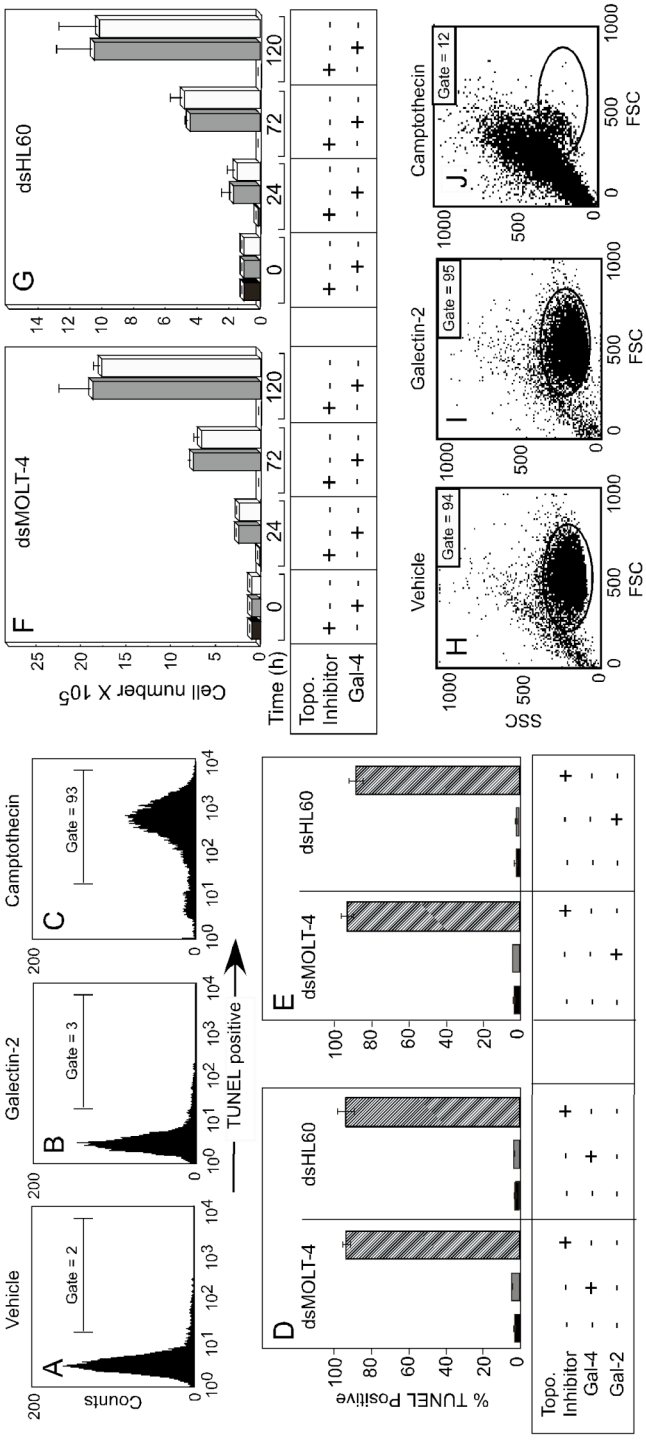
Vehicle
Galectin-2
Camptothecin
A
B
C
Gate = 2
Gate = 3
Gate = 93
Counts
TUNEL positive
D
dsMOLT-4
dsHL60
% TUNEL Positive
E
dsMOLT-4
dsHL60
Topo. Inhibitor
Gal-4
Gal-2
F
dsMOLT-4
G
dsHL60
Cell number X 10^5
Time (h)
Topo. Inhibitor
Gal-4
Vehicle
Galectin-2
Camptothecin
H
I
J.
Gate = 94
Gate = 95
Gate = 12
SSC
FSC

Figure 5-4

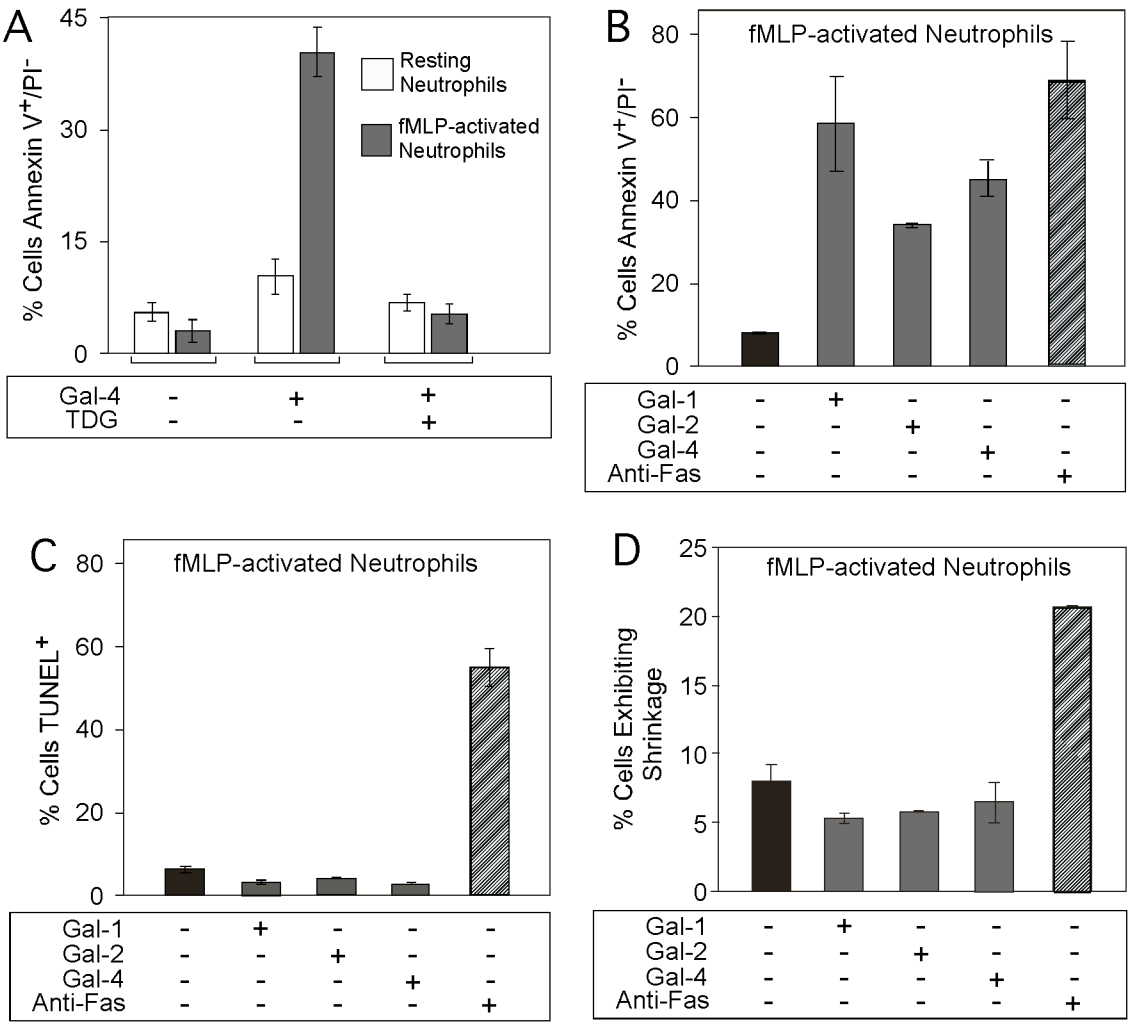
A
% Cells Annexin V+/PI-
45
30
15
0
Resting Neutrophils
fMLP-activated Neutrophils
Gal-4 - + +
TDG - - +
B
fMLP-activated Neutrophils
% Cells Annexin V+/PI-
80
60
40
20
0
Gal-1 - + - - -
Gal-2 - - + - -
Gal-4 - - - + -
Anti-Fas - - - - +
C
fMLP-activated Neutrophils
% Cells TUNEL+
80
60
40
20
0
Gal-1 - + - - -
Gal-2 - - + - -
Gal-4 - - - + -
Anti-Fas - - - - +
D
fMLP-activated Neutrophils
% Cells Exhibiting Shrinkage
25
20
15
10
5
0
Gal-1 - + - - -
Gal-2 - - + - -
Gal-4 - - - + -
Anti-Fas - - - - +

Figure 5-5

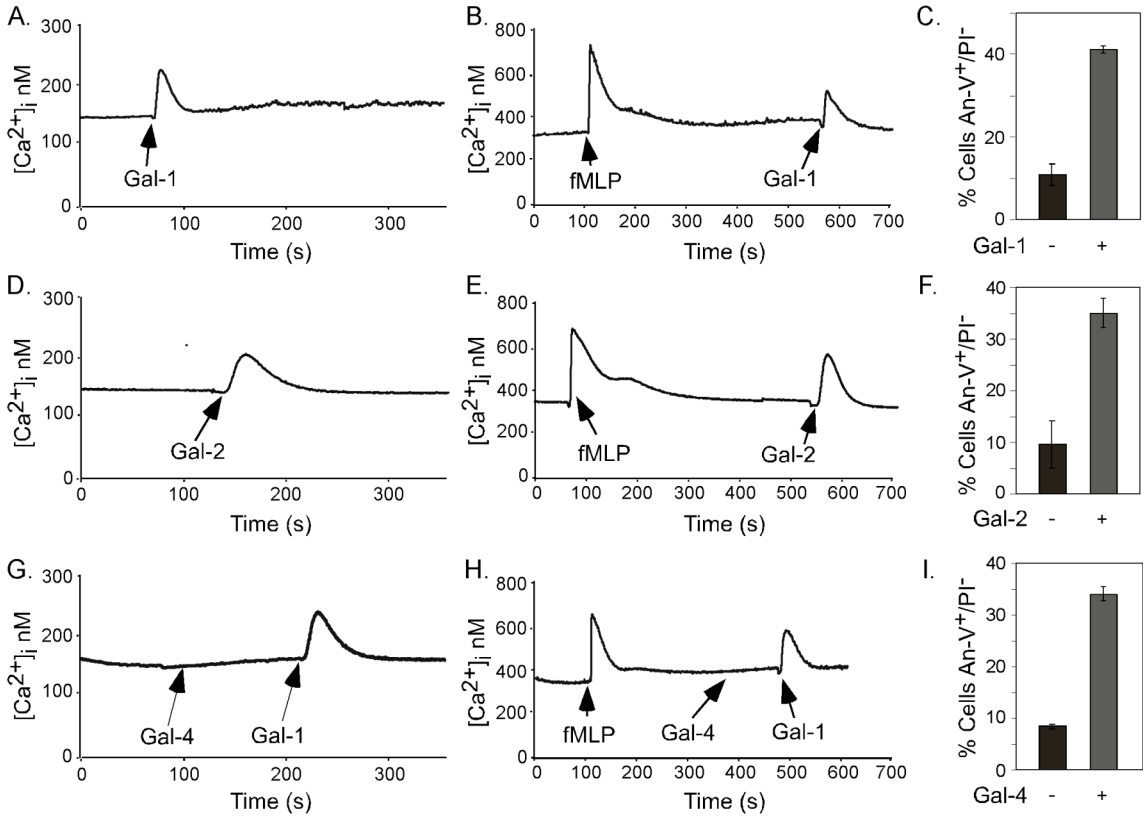

Figure 5-6

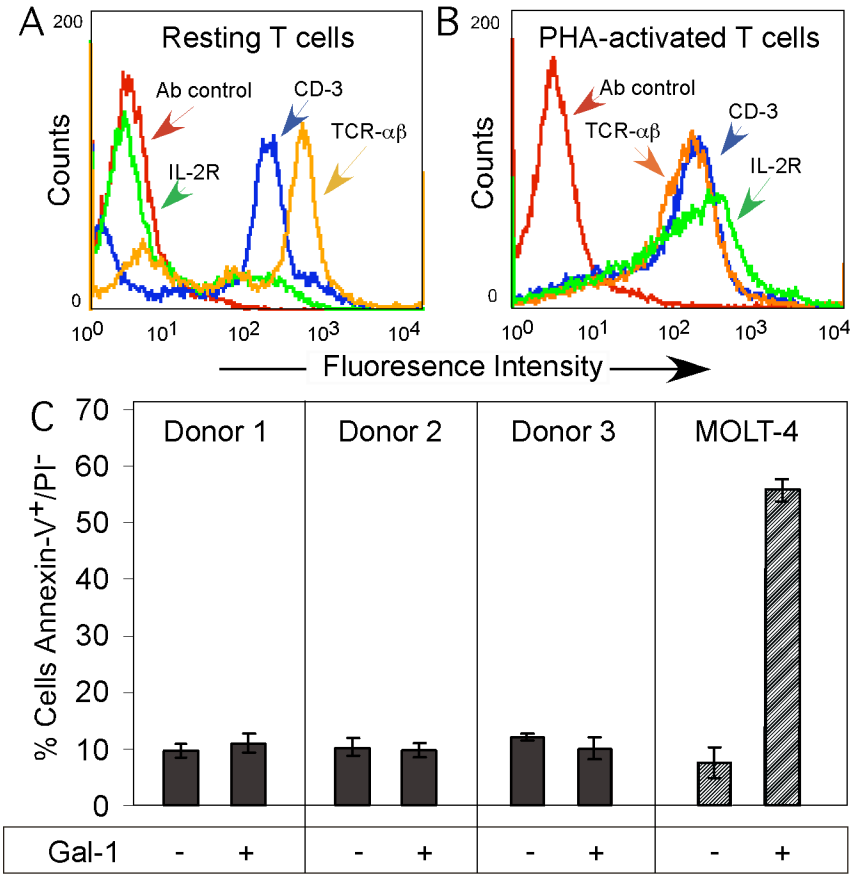
A
Resting T cells
Ab control
CD-3
TCR-αβ
IL-2R
Counts
200
0
10^0 10^1 10^2 10^3 10^4
B
PHA-activated T cells
Ab control
TCR-αβ
CD-3
IL-2R
Counts
200
0
10^0 10^1 10^2 10^3 10^4
Fluoresence Intensity
C
% Cells Annexin-V+/PI-
70
60
50
40
30
20
10
0
Donor 1
Donor 2
Donor 3
MOLT-4
Gal-1 - + - + - + - +

Figure 5-7

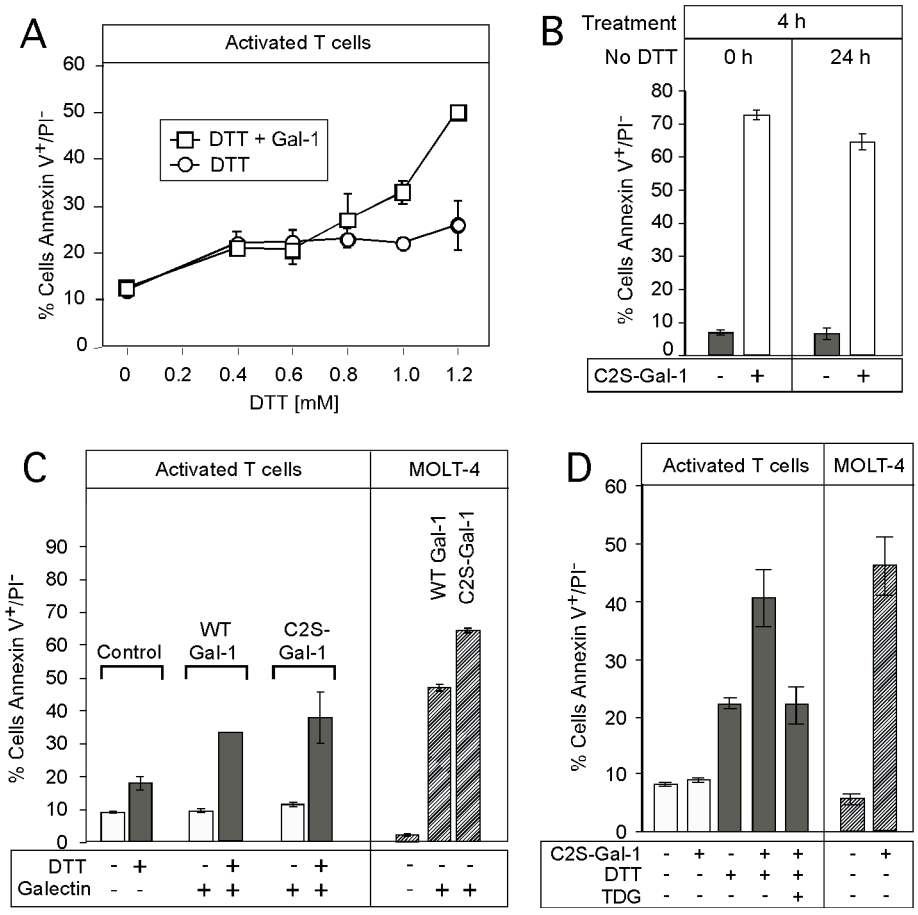
A
Activated T cells
DTT + Gal-1
DTT
% Cells Annexin V+/PI-
DTT [mM]
B
Treatment
4 h
No DTT
0 h
24 h
% Cells Annexin V+/PI-
C2S-Gal-1
C
Activated T cells
MOLT-4
Control
WT Gal-1
C2S-Gal-1
WT Gal-1
C2S-Gal-1
% Cells Annexin V+/PI-
DTT
Galectin
D
Activated T cells
MOLT-4
% Cells Annexin V+/PI-
C2S-Gal-1
DTT
TDG

Figure 5-8

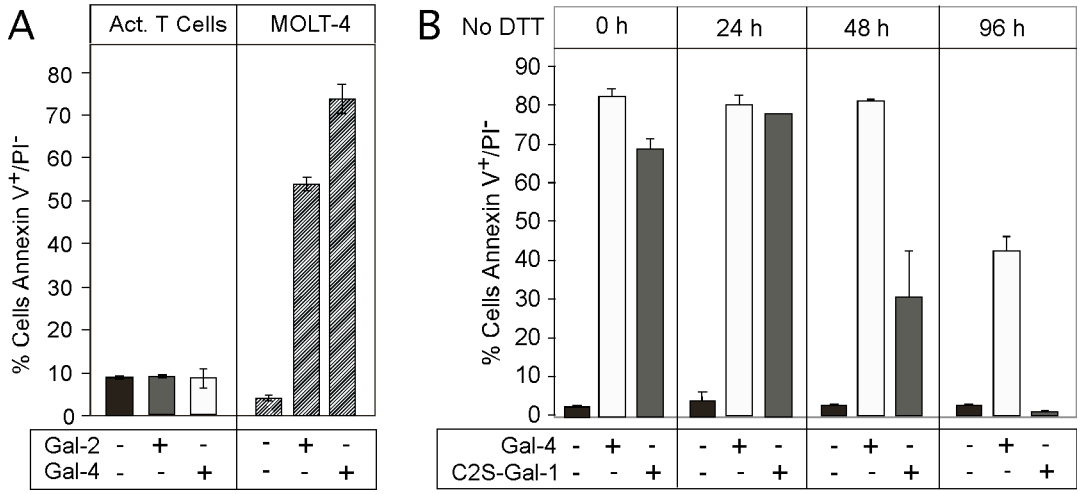
A
Act. T Cells
MOLT-4
% Cells Annexin V+/PI-
0
10
20
30
40
50
60
70
80
Gal-2 - + - - + -
Gal-4 - - + - - +
B
No DTT
0 h
24 h
48 h
96 h
% Cells Annexin V+/PI-
0
10
20
30
40
50
60
70
80
90
Gal-4 - + - - + - - + - - + -
C2S-Gal-1 - - + - - + - - + - - +

Figure 5-9

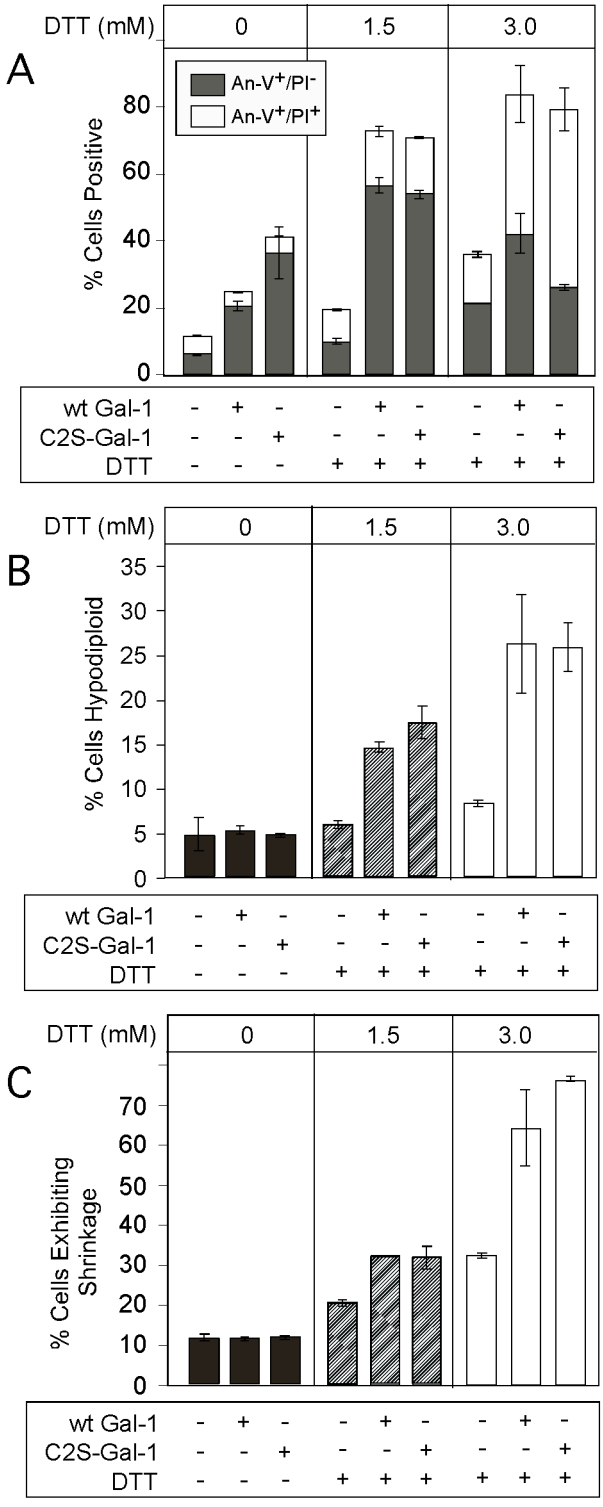
DTT (mM)
0
1.5
3.0
A
An-V+/PI-
An-V+/PI+
% Cells Positive
0
20
40
60
80
wt Gal-1 - + - - + - - + -
C2S-Gal-1 - - + - - + - - +
DTT - - - + + + + + +
B
% Cells Hypodiploid
0
5
10
15
20
25
30
35
C
% Cells Exhibiting Shrinkage
0
10
20
30
40
50
60
70

Figure 5-10

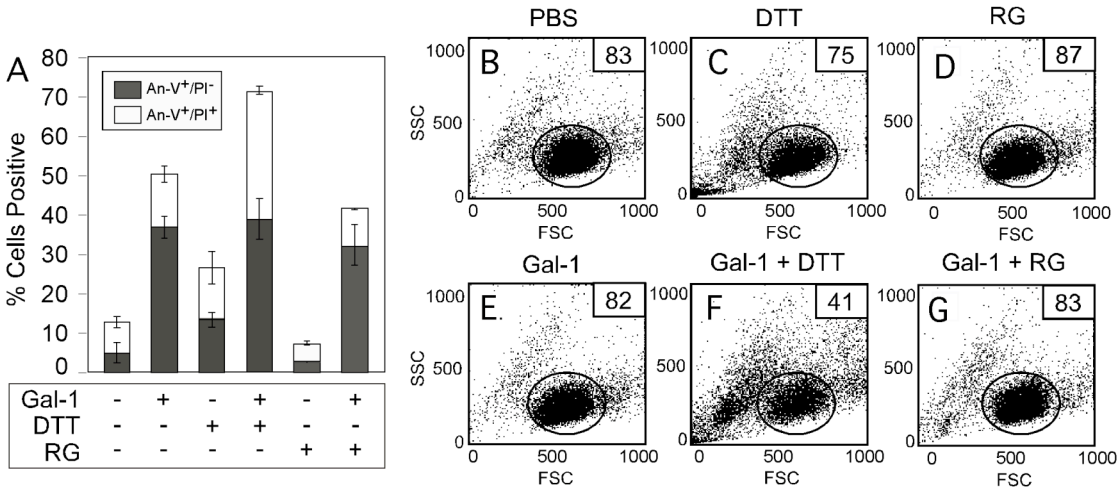
A
% Cells Positive
An-V+/PI-
An-V+/PI+
Gal-1
DTT
RG
PBS
DTT
RG
B
83
C
75
D
87
Gal-1
Gal-1 + DTT
Gal-1 + RG
E
82
F
41
G
83
SSC
FSC

Table 5-1. Gal-2 and Gal-4 induce PS exposure in MOLT-4 and HL-60 cells in a carbohydrate dependent fashion.

	Annexin V^+/PI^- (%)[a]	
Treatment	MOLT-4	HL60
Control	2	4
Gal-2	47	49
Gal-2 + lactose[b]	6	8
Gal-2 + sucrose	47	49
Gal-4	37	68
Gal-4 + TDG[c]	4	10
Gal-4 + sucrose	39	63

[a]MOLT-4 or HL60 cells were treated with 10 μM Gal-2 or 3 μM Gal-4 for 4 h followed by detection for PS exposure. The data shown represent averages of duplicate determinations where the S.E. was <10%.

[b]Gal-2 was co-incubated with either 20 mM lactose or 20 mM sucrose as indicated.

[c]Gal-4 was co-incubated with either 30 mM TDG or 30 mM sucrose as indicated.

FIGURE LEGENDS

Figure 5-1. Gal-2 and Gal-4 induced PS exposure in MOLT-4 and HL60 cells. (A) MOLT-4 cells were incubated with ALEXA 488-Gal-2 and (B) HL60 cells were incubated with ALEXA 488-Gal-4, followed by flow cytometric analysis. NANase indicates prior treatment of cells with *A. urefaciens* neuraminidase (100 mU) for 1 h at 37°C. (C-D) HL60 cells or MOLT-4 cells were incubated with either 10 μM Gal-2 (C) or 3 μM Gal-4 (D) for 4 h followed by flow cytometric analysis for PS externalization. NANase indicates prior treatment of cells with *A. urefaciens* neuraminidase (100 mU) for 1 h at 37°C.

Figure 5-2. Kinetics and concentration dependence of Gal-2 and Gal-4-induced PS exposure (A) Desialylated HL60 cells (dsHL60) cells were incubated with 3 μM Gal-4 or 10 μM Gal-2 for the indicated times followed by detection of PS externalization. Control indicates the inclusion of PBS alone. (B) dsHL60 cells were incubated with Gal-2 or Gal-4 at each indicated concentration for 4 h followed by detection for PS externalization. Results shown are the averages of duplicate analyses and are representative of at least two separate experiments.

Figure 5-3. Galectins induce PS exposure in cell lines without accompanying apoptosis. Representative histograms of dsHL60 cells treated with PBS (A),10 μM Gal-2 (B), or 20 μM camptothecin (C) for 18 h followed by flow cytometric analysis for DNA degradation (TUNEL assay). The dsMOLT-4 or dsHL60 cells were treated with 5 μM Gal-4 (D) 10 μM Gal-2 (E), or 20 μM topoisomerase inhibitors (etoposide for MOLT-4 cells or camptothecin for HL60 cells) for 18 h followed by flow cytometric analysis for DNA degradation (TUNEL assay). The dsMOLT-4 cells (F) or dsHL60 cells (G) were

treated with either 3 μM Gal-4 or 20 μM topoisomerase inhibitors (etoposide for MOLT-4 cells or camptothecin for HL60) for the indicated times followed by the determination of viable cell number by trypan blue exclusion using a hemocytometer. Results shown are the averages of duplicate analyses and are representative of at least two separate experiments. Representative dot plots for dsHL60 cells treated with PBS (H),10 μM Gal-2 (I), or 20 μM Camptothecin (J) for 18 h followed by flow cytometric analysis for changes in the light scattering properties of the cells. Gate values of cells experiencing no changes in forward and side scatter profile are shown.

Figure 5-4. Galectins induce PS exposure in fMLP-activated human neutrophils. (A) fMLP-activated or resting neutrophils were treated with either PBS, 3 μM Gal-4, or 3 μM Gal-4 plus 30 mM TDG for 4 h, as indicated, followed by analysis of PS exposure using Annexin V. (B) fMLP-activated neutrophils were incubated with either 10 μM Gal-1, 10 μM Gal-2, 3 μM Gal-4 or 100 ng/ml anti-Fas for 8 h followed by detection for PS exposure. (C) fMLP-activated neutrophils were incubated with either 10 μM Gal-1, 10 μM Gal-2, 3 μM Gal-4 or 100 ng/ml anti-Fas for 8 h followed by detection for DNA degradation (TUNEL assay). (D) fMLP-activated neutrophils were incubated with either 10 μM Gal-1, 10 μM Gal-2, 3 μM Gal-4 or 100 ng/ml anti-Fas for 8 h followed by flow cytometric analysis for the percentage of cells exhibiting shrinkage. Results shown are the averages of duplicate analyses and are representative of at least two separate experiments.

Figure 5-5. Gal-1 and Gal-2 but not Gal-4 induce Ca^{2+} flux in both resting and activated neutrophils. Resting neutrophils were treated with 10 μM Gal-1 (A), 10 μM Gal-2 (D) or 5 μM Gal-4 (G) followed by detection for changes in intracellular Ca^{2+} levels.

Resting neutrophils were activated with fMLP followed by treatment with either 10 μM Gal-1 (B), 10 μM Gal-2 (E) or 5 μM Gal-4 followed by 10 μM Gal-1 (H) followed by detection for changes in intracellular Ca^{2+} levels. fMLP-activated neutrophils in parallel assays were treated with 10 μM Gal-1 (C), 10 μM Gal-2 (F) or 5 μM Gal-4 (I) for 4 h followed by detection of PS exposure.

Figure 5-6. Gal-1 does not induce PS exposure in activated human T cells. (A-B) Resting T cells (A) or PHA-activated T cells (B) were incubated with anti-IL-2R, anti-CD-3, anti-TCR-αβ or isotype control followed by flow cytometric analysis. (C) PHA-activated T cells isolated from three separate donors as indicated or MOLT-4 cells were treated with 10 μM Gal-1 followed by detection for PS exposure.

Figure 5-7. DTT primes activated human T cells for Gal-1 induced PS exposure. (A) PHA-activated T cells were incubated with RPMI alone, 10 μM Gal-1 alone, or with the respective concentration of DTT with or without 10 μM Gal-1 for 9 h as indicated, followed by the detection for PS exposure. (B) HL60 cells were treated with either freshly prepared C2S-Gal-1 or C2S-Gal-1 incubated for 24 h in the absence of DTT. Following C2S-Gal-1 addition, cells were incubated for 4 h followed by analysis for PS exposure. (C) PHA-activated T cells or MOLT-4 cells were incubated with 10 μM Gal-1 or 10 μM C2S-Gal-1 with or without 1.2 mM DTT for 9 h, followed by detection for PS exposure. (D) PHA-activated T cells or MOLT-4 cells were incubated with 10 μM C2S Gal-1 with or without 30 mM TDG as indicated for 9 h followed by detection for PS exposure.

Figure 5-8. Gal-2 and Gal-4 do not induce PS exposure in activated T cells. (A) PHA-activated T cells or MOLT-4 cells were incubated with 10 μM Gal-2 or 3 μM Gal-4 for 9 h in the absence of DTT, followed by detection for PS exposure. (B) Gal-4 or C2S Gal-1 were incubated without DTT for the indicated time followed by treatment of HL60 cells for 4 h and detection for PS exposure.

Figure 5-9. DTT primes MOLT-4 cells for Gal-1 induced apoptosis. (A) MOLT-4 cells were incubated with either 10 μM wt Gal-1 or 10 μM C2S-Gal-1 with or without DTT at the concentrations indicated for 8 h, followed by analysis for PS exposure (Annexin-V^+/PI^-) and membrane integrity loss (Annexin-V^+/PI^+). (B) MOLT-4 cells were incubated with either 10 μM wt Gal-1 or 10 μM C2S-Gal-1 with or without DTT at the concentrations indicated for 8 h, followed by analysis for DNA degradation by assaying for hypodiploid DNA content by flow cytometry. (C) MOLT-4 cells were incubated with either 10 μM wt Gal-1 or 10 μM C2S-Gal-1 with or without DTT at the concentrations indicated for 8 h, followed by analysis for cellular shrinkage by flow cytometry.

Figure 5-10. DTT, but not reduced glutathione, primes MOLT-4 cells for Gal-1 induced apoptosis. (A) MOLT-4 cells were treated with 3 mM DTT or 3 mM reduced glutathione (RG) as indicated with or without 10 μM Gal-1 for 9 h followed by detection for PS exposure (Annexin-V^+/PI^-) and membrane integrity loss (Annexin-V^+/PI^+). MOLT-4 cells were treated with PBS (B), 3 mM DTT (C), 3 mM RG (D), 10 μM Gal-1 (E), 10 μM Gal-1 + 3 mM DTT (F), or 10 μM Gal-1 + 3 mM RG (G) for 9 h followed by detection for changes in the light scattering properties of the cell. Gate values of cells experiencing no changes in forward and side scatter profile are shown.

Chapter 6

Differential Roles of Galectin-1 and Galectin-3 in Regulating Leukocyte Viability and Cytokine Secretion

Galectin-1 (Gal-1) and galectin-3 (Gal-3) exhibit profound but unique immunomodulatory activities in animals but the molecular mechanisms are incompletely understood. Early studies suggested that Gal-1 inhibits leukocyte function by inducing apoptotic cell death and removal, but recent studies show that some galectins induce exposure of the common death signal phosphatidylserine (PS) independently of apoptosis. Here we report that Gal-3, but not Gal-1, induces both PS exposure and apoptosis in primary activated human T cells, whereas both Gal-1 and Gal-3 induce PS exposure in neutrophils in the absence of cell death. Gal-1 and Gal-3 bind differently to the surfaces of T cells and only Gal-3 mobilizes intracellular Ca^{2+} in these cells, although Gal-1 and Gal-3 bind their respective T cell ligands with similar affinities. While Gal-1 does not alter T cell viability, it induces IL-10 production and attenuates interferon gamma (IFN-γ) production in activated T cells, suggesting a mechanism for Gal-1-mediated immunosuppression *in vivo*. These studies demonstrate that Gal-1 and Gal-3 induce differential responses in T cells and neutrophils, and identify the first factor, Gal-3, capable of inducing PS exposure with or without accompanying apoptosis in different leukocytes, thus providing a possible mechanism for galectin-mediated immunomodulation *in vivo*.

INTRODUCTION

Effective immunological homeostasis relies on removal of activated leukocytes following inflammatory episodes [1, 213]. Disruption of various homeostatic mechanisms responsible for leukocyte turnover results in a wide variety of human diseases ranging from autoimmunity to acute inflammatory-mediated tissue damage [1, 68, 213, 214]. Many of these diseases remain refractory to current treatment options. In an effort to further understand regulatory mechanisms responsible for leukocyte homeostasis, we have searched for additional factors capable of regulating leukocyte turnover.

Leukocyte removal often requires the induction of apoptosis by effecter molecules such as Fas and tumor necrosis factor-alpha (TNFα) [1-3]. However, several studies suggest that different leukocyte populations may have alternative routes for removal. Mice genetically deficient in either Fas or Fas L exhibit lymphocytosis while maintaining normal neutrophil numbers [34]. Patients with autoimmune lymphoproliferative disease, who also possess mutations in either Fas or Fas L, likewise exhibit defects in lymphocyte removal while maintaining normal neutrophil numbers [35, 36]. Acute inflammatory challenge in Fas- or Fas L-null mice resolves normally [109], further suggesting that neutrophil removal at sites of active inflammation may occur through apoptosis-independent pathways. Consistent with this possibility, a significant percentage of phagocytosed neutrophils display no signs of apoptosis [38]. Furthermore, transgenic mice expressing the anti-apoptotic protein Bcl-2 in neutrophils display normal neutrophil numbers and exhibit no significant alterations in sensitivity toward phagocytosis [37]. These results suggest that neutrophils and T cells likely possess distinct pathways for turnover.

Many studies demonstrate that members of the galectin family of b-galactoside binding proteins are directly involved in regulating leukocyte function and turnover [19-21, 25, 53, 157-160], but the molecular mechanism and pathways involved are not clear. Previous studies suggested that galectin-1 (Gal-1) primarily modulates immunity by inducing apoptosis in activated T cells [4, 14]. However, these studies were confounded by the inclusion of the reducing agent dithiothreitol (DTT) in treatment conditions [80, 82, 117], leaving questions concerning the mechanisms of galectin-mediated immunosuppression unanswered. Gal-1, Gal-2, and Gal-4 induce externalization of the membrane lipid, phosphatidylserine (PS), a common ligand for macrophage-mediated phagocytosis of apoptotic cells [82, 215, 216]. The induction of PS in activated neutrophils by Gal-1, Gal-2, and Gal-4 occurs independently of apoptosis, but makes cells targets for phagocytosis [40, 82, 200], providing a possible mechanism of galectin-induced leukocyte turnover. These studies also provided the first pathway whereby neutrophils may be induced to undergo removal by apoptosis-independent pathways. However, Gal-1, Gal-2, and Gal-4 displayed no effect on T cell viability or PS exposure [82], further demonstrating the neutrophil-specific nature of non-apoptotic cell removal and also failing to explain at a mechanistic level the effects of galectins on T cells *in vivo* [19-21, 25, 53, 157-160]. Other studies have implicated other galectin family members in the regulation of T cell viability. Gal-3, one of the most well studied members of this family [217], is thought to induce apoptosis in T cells, although these studies primarily utilized T leukemic cell lines [12, 16]. In contrast to leukemic cell lines, several studies suggest that Gal-3 exhibits no effect on primary T cell viability [14, 218]. Gal-1, Gal-2, and Gal-4 also induce PS exposure in several T leukemic cell lines, although this occurs in the absence of apoptosis [82]. Since Gal-1, Gal-2, and Gal-4 fail to alter PS distribution or viability of primary activated T cells [82] and the effects of Gal-3 on

primary T cell viability remain unclear [12, 14, 218], we questioned whether Gal-3 modulates immune function by altering the viability of primary activated T cells.

In this study we explored the signaling responses of neutrophils and T cells to Gal-1 and Gal-3 and the potential consequences of this signaling on cellular turnover and immune function in an effort to understand at a mechanistic level the effects of these protein *in vivo* [19-21, 25, 53, 157-160]. Previous studies examined the effect of Gal-1 under treatment conditions that included DTT [14]. However, DTT can complicate these assays [14, 82-85, 208]. To eliminate artificial effects introduced by DTT inclusion [14, 82-85, 208], while controlling for potential loss of Gal-1 activity, we stabilized Gal-1 with iodoacetamide which was previously shown to protect Gal-1 from oxidative inactivation [94, 98, 127-129, 219]. In this study, iodoacetamide treated Gal-1 (iGal-1) retained key biological activities previously documented for the unmodified protein [14, 40, 42, 82, 202, 220, 221], demonstrating that iGal-1 retains function while resisting oxidative inactivation. Furthermore, these results demonstrate that the failure of unmodified Gal-1 to induce apoptotic death in previous studies [40, 82, 200] was not a reflection of activity loss. By contrast, Gal-3, which does not require reducing conditions to remain active, induced PS exposure and apoptosis in primary activated T cells, while it induced PS exposure without accompanying apoptosis in activated neutrophils and in T leukemic cells, suggesting pathways whereby Gal-3 modulates leukocyte turnover *in vivo*. Taken together, these results provide mechanisms of Gal-1 and Gal-3 mediated immunomodulation and describe the first example of a single effector molecule, Gal-3, capable of inducing PS exposure in two separate leukocyte types with or without accompanying apoptosis.

RESULTS

Gal-1 and Gal-3 induce PS exposure in activated neutrophils

We first sought to evaluate the potential action of Gal-3 with activated neutrophils and effects on viability. Because previous studies exploring the response of leukocytes to galectins utilized the membrane permeable reducing agent DTT to maintain Gal-1 activity during treatment [80], we defined the stability of Gal-1 and Gal-3 in the absence of DTT [51]. Gal-3 remained active over extended incubations (several days at 37°C) in the absence of DTT, although Gal-1, as observed previously [51], underwent oxidative inactivation in the absence of ligand within 24 h at 37°C (data not shown). However, inclusion of DTT induces the unfolded protein response in cells [83-85, 208] and artificially primes T cells to undergo apoptosis in response to Gal-1 [82]. To ascertain Gal-1 activity toward leukocytes, while still controlling for potential effects of Gal-1 inactivity during treatment conditions, we alkylated Gal-1 with iodoacetamide as done in many previous studies to stabilize Gal-1 [94, 98, 127-129, 219]. Importantly, not only does alkylation with iodoacetamide stabilize Gal-1 [94], but the alkylated protein retains quaternary structure [145] and exhibits the same structure as the non-alkylated protein, as ascertained by comparing the solution based structural studies using small angle neutron and x-ray scattering of the alkylated protein with crystal structures of the non-alkylated protein [98]. However, to additionally determine whether alkylation might alter the glycan-binding properties of Gal-1, we tested the binding of iGal-1 to a set of glycans used to previously define the relative affinity and specificity of Gal-1 [64, 65, 222]. Gal-1 and iGal-1 exhibited similar binding patterns (Fig. 1A,B), with no significant differences in glycan recognition ($p>0.05$). These results demonstrate that alkylation does not alter tertiary and quaternary structure [98, 145], as evidenced by unaltered glycan binding properties of Gal-1. We also wanted to confirm that iGal-1 signals PS exposure in

neutrophils, similar to Gal-1, and whether this PS exposure occurs in the presence or absence of cell death [40, 82]. Consistent with previous observations using Gal-1 [40, 82, 200], iGal-1 induced PS exposure in activated neutrophils (Table 6-1, Fig. 6-1C). Thiodigalactoside (TDG), a potent inhibitor of galectin-carbohydrate interactions, abrogated iGal-1-induced PS exposure (Table 6-1). When treated with Gal-3, activated neutrophils also displayed significant PS externalization, which was likewise blocked by TDG inclusion (Table 6-1). Activated neutrophils were also more sensitive then resting neutrophils to Gal-3 (data not shown), similar to previous results with Gal-1, Gal-2, and Gal-4 [40, 82, 200]. These results demonstrate that like Gal-1, iGal-1 and Gal-3 induce PS exposure in activated neutrophils by a mechanism that requires carbohydrate recognition.

Gal-3 induces PS exposure in activated T cells

To determine whether the induction of PS exposure by Gal-3 was specific to neutrophils, as previously observed for Gal-1, Gal-2, and Gal-4 [82], we explored the effect of Gal-3 on T cells. Unexpectedly, Gal-3 induced robust PS exposure in activated T cells (Table 6-1). We also evaluated the response of T cells to iGal-1. Similar to previous observations with Gal-1 [82], iGal-1 displayed no effect on PS redistribution in T cells in parallel experiments (Table 6-1). TDG inhibited Gal-3-induced PS exposure in T cells, indicating that the response requires carbohydrate recognition (Table 6-1). Interestingly, Gal-3 failed to induce PS exposure in resting T cells (data not shown), indicating that T cell activation is required to prime cells to become responsive to Gal-3. These results demonstrate that, unlike Gal-1, Gal-2, or Gal-4 [82], Gal-3 induces PS exposure in activated T cells.

iGal-1 and Gal-3 induce PS exposure in the absence of apoptosis in activated neutrophils

Previous studies demonstrated that Gal-1, Gal-2, and Gal-4 induce PS exposure but not apoptosis in activated neutrophils [82, 196]. Thus, we explored whether iGal-1 and Gal-3-induced PS exposure in activated neutrophils also occurred in the absence of apoptotic cell death. Both iGal-1 and Gal-3 induced maximal PS exposure in activated neutrophils following 4 h of treatment (Fig. 6-1D). Furthermore, neutrophils exhibited a similar dose response following iGal-1 and Gal-3 treatment (Fig. 6-1E), with neutrophils displaying a slightly more sensitive response to Gal-3 than iGal-1. Importantly, TDG, but not sucrose, inhibited both iGal-1 and Gal-3 binding to neutrophils, demonstrating that binding, like the signaling of PS exposure, required carbohydrate recognition (Fig. 6-1F,G).

With an understanding of the dose response and kinetics of iGal-1 and Gal-3 induced PS exposure, we directly examined whether PS exposure in neutrophils induced by Gal-3 was accompanied by apoptosis. Although both iGal-1 and Gal-3 induced robust PS exposure in activated neutrophils (Table 6-1), neutrophils treated with either iGal-1 or Gal-3 did not show DNA fragmentation as analyzed by both TUNEL assay (Fig. 6-2A,B) and hypodiploid DNA content (Fig. 6-2C). By contrast, parallel treatment of neutrophils with anti-Fas induced substantial apoptosis (Fig. 6-2A-C). iGal-1 and Gal-3 also failed to induce cell shrinkage (Fig. 6-2D) or increased membrane permeability as measured by propidium iodide (PI) staining (data not shown), which typically accompanies apoptosis in activated human neutrophils. Furthermore, iGal-1 and Gal-3 failed to accelerate the rate of spontaneous neutrophil apoptosis when evaluated at later time points (18 hours - data not shown) or lower concentrations (10 μM – data not shown). Treatment of cells with anti-Fas, however, caused significant increases in late apoptosis (data not shown)

and cell fragmentation (Fig. 6-2D) in parallel assays. These results demonstrate that Gal-3, like Gal-1, induces PS exposure but not apoptosis in activated neutrophils.

Gal-3, but not Gal-1, induces apoptosis in activated T cells

Since the above results demonstrate that Gal-3 induced PS exposure in the absence of cell death in neutrophils, we asked whether PS exposure induced in T cells reflects a common Gal-3-induced pathway between T cells and neutrophils and therefore also occurs in the absence of cell death. This is an important issue in light of a recent report that PS exposure can occur independently of apoptosis during T cell activation [78]. To determine whether Gal-3 affects T cell viability, we assessed the dose response and kinetics of PS exposure in activated T cells. Unlike neutrophils, T cells treated with Gal-3 continued to display increased PS exposure over time, with significant PS exposure observed following 4 h of treatment (Fig. 6-3A). Similar to the effects of Gal-3 on neutrophils, however, maximal PS exposure occurred at a concentration of ~7 mM (Fig. 6-3B), demonstrating a similar optimal concentration for PS exposure in these two cell types. In control experiments, iGal-1 had no effect on T cells over any time period tested or concentration utilized (Fig. 6-3A,B), consistent with our earlier findings using Gal-1 [82]. Importantly, TDG inhibited iGal-1 and Gal-3 binding to T cells (Fig. 6-3C,D), demonstrating that T cells possessed ligands for both proteins, similar to previous studies on Gal-1 [14]. Furthermore, iGal-1 induced PS exposure in MOLT-4 cells in parallel assays in a dose- and time-dependent manner (Fig. 6-3E,F), as demonstrated previously for Gal-1 [40, 82], indicating that in these experiments iGal-1 was active and capable of signaling cells. Importantly, Gal-3 also induced PS exposure in MOLT-4 cells (Fig. 6-3E,F).

Having determined the optimal dose response and time required for Gal-3-induced PS exposure in T cells, we explored whether PS exposure induced by Gal-3 was accompanied by cell death. Gal-3-induced PS exposure in T cells was accompanied by DNA fragmentation as demonstrated by increased TUNEL positivity (Fig. 6-4A) and hypodiploid stain (Fig. 6-4B). Gal-3-induced DNA fragmentation, like PS exposure, required carbohydrate recognition of cell surface receptors, and TDG, but not sucrose, blocked this effect (Fig. 6-4A). Gal-3 also induced DNA fragmentation at lower concentrations, such as 10 μM (data not shown), consistent with ability of Gal-3 to induce PS exposure at 10 μM (Fig. 6-3B), while 10 mM iGal-1 had no effect on DNA fragmentation in parallel assays (data not shown). To further confirm that Gal-3-induced PS exposure and DNA fragmentation was accompanied by cell death, we directly examined cell viability using the MTT assay. Gal-3 treated cells demonstrated decreased conversion of the MTT substrate (Fig. 6-4C), an indicator of cell viability [223], while iGal-1 failed to alter MTT conversion (Fig. 6-4C). To determine whether PS exposure induced by Gal-3 was comparable to apoptosis induced by Fas, we treated cells with Gal-3 or Fas. Both Gal-3 and Fas induced apoptosis in activated T cells (Fig. 6-4D). These results demonstrate that while Gal-3 induced PS exposure in the conspicuous absence of apoptosis in neutrophils, Gal-3 induced both PS exposure and cell death in T cells.

Gal-3 induces PS exposure independently of apoptosis in T leukemic cell lines

Because both iGal-1 and Gal-3 induced PS exposure in T leukemic cells (Fig. 6-3E,F), yet only Gal-3 induced PS exposure in activated primary T cells, we next examined whether PS exposure induced in T leukemic cells by Gal-3 might also accompany cell death as observed in primary T cells. As a control, we also evaluated the effects of iGal-1 on MOLT-4 cells, as previous studies demonstrated that Gal-1 induces PS exposure in

the absence of apoptosis in MOLT-4 cells [40, 82]. Unlike the effects of Gal-3 on primary T cells, T leukemic MOLT-4 cells treated with Gal-3 did not show changes in viability as measured by increased PI stain (Fig. 6-4E), although PS exposure was induced (Fig. 6-3E,F; 6-4E). Gal-3 also failed to induce DNA fragmentation (Fig. 6-4F) or alter cell growth (data not shown), although etoposide, a pro-apoptotic agent in these cells [40], induced apoptosis in parallel assays (Fig. 6-4E,F). Similarly, iGal-1 also failed to induce apoptotic cell death in MOLT-4 cells as determined by the same indicators (Fig 6-4E,F). These results demonstrate that only Gal-3 induce PS exposure and apoptosis in activated primary T cells, while iGal-1 and Gal-3 induce PS exposure in activated neutrophils and a T leukemic MOLT-4 cells in the absence of cell death, similar to previous results for Gal-1 [40, 82].

iGal-1 does not modulate apoptosis induced by Gal-3 in activated T cells

Several studies have suggested that Gal-1 may modulate Gal-3-induced effects at the receptor level [218, 224], which led us to determine whether Gal-1 may modulate Gal-3-induced PS exposure in T cells. T cells treated with both iGal-1 and Gal-3 showed very little change in PS exposure when compared to Gal-3 alone (Fig. 6-5A). iGal-1 also failed to alter the Gal-3 induction of cell death in T cells as measured by increased staining with PI (Fig. 6-5B) or by DNA fragmentation assessed by the TUNEL assay (Fig. 6-5C). Gal-3 induced PS more rapidly in MOLT-4 cells than iGal-1 (Fig. 6-3E), suggesting, among several possibilities, faster kinetic on-rates for receptor engagement compared to iGal-1. To test this we preincubated T cells for 30 min with iGal-1 to allow iGal-1 to bind receptors prior to the addition of Gal-3. Pre-incubation with iGal-1 did not significantly alter the ability of Gal-3 to induce cell death in T cells (Fig. 6-5B). Similar results were obtained when incubating T cells with 10 μM iGal-1 and Gal-3 as outlined above (data not shown). These results suggest that Gal-1 and Gal-3 recognize distinct

signaling receptors on the T cell surface that likely account for the unique ability of Gal-3 to induce apoptosis in T cells.

Gal-3 induces mobilization of intracellular Ca^{2+} in activated T cells

The above results demonstrate that both iGal-1 and Gal-3 can induce PS exposure independently of apoptosis in neutrophils. Thus, we explored whether iGal-1 and Gal-3 signal through common or distinct pathways in neutrophils. This question is important in light of recent evidence that Gal-1 and Gal-4 signal PS exposure independently of apoptosis in neutrophils through two separate pathways [82]. To this end, we treated activated neutrophils with iGal-1 or Gal-3 and measured changes in intracellular Ca^{2+}, which we showed previously to be required for Gal-1-induced PS exposure in these cells [200]. iGal-1 and Gal-3 both induced Ca^{2+} flux in neutrophils (Fig. 6-6A,B), suggesting a common pathway. To determine whether iGal-1 and Gal-3 might signal Ca^{2+} mobilization through a common receptor, we determined whether iGal-1 might block Gal-3 induced Ca^{2+} flux. We first tested whether iGal-1 could cause further Ca^{2+} release following a second treatment of cells with iGal-1. We observed that while the first treatment of cells with iGal-1 induced transient Ca^{2+} mobilization, the second treatment had no effect on Ca^{2+} flux (data not shown). This result suggests that either the iGal-1 receptors become saturated following initial treatment or that the signaling pathway becomes refractory to further stimulation. Similarly, we found that an initial treatment of cells with Gal-3 induced a transient Ca^{2+} mobilization, but a second treatment had no effect (data not shown). Importantly, neutrophils treated first with iGal-1 were insensitive to further stimulation by Gal-3 (Fig. 6-6C). Because the responses of T cells and neutrophils to iGal-1 and Gal-3 were fundamentally different, we determined whether iGal-1 or Gal-3 mobilized Ca^{2+} in T cells. In contrast to the effects of iGal-1 on neutrophils, T cells treated with iGal-1 failed to mobilize Ca^{2+} (Fig. 6-6D), whereas Gal-3

induced significant Ca^{2+} flux in T cells in parallel assays, which was sustained in T cells (Fig. 6-6E). We also treated activated T cells with iGal-1, which was followed by treatment with Gal-3. However, iGal-1 failed to alter the sensitivity of T cells to Gal-3-induced Ca^{2+} mobilization (Fig. 6-6F), further demonstrating that iGal-1 and Gal-3 recognize distinct receptors on the T cell surface. These results demonstrate that Gal-1 and Gal-3 recognize different receptors on the T cell surface and that Gal-3 initiates a signaling pathway in activated T cells that is not shared by Gal-1.

iGal-1 and Gal-3 signal additive PS exposure in neutrophils

To further evaluate whether iGal-1 and Gal-3 signal through similar pathways in neutrophils, we treated neutrophils with iGal-1, Gal-3, or both galectins. Co-incubation of activated neutrophils with iGal-1and Gal-3 induced PS exposure to a level that was similar to that observed with iGal-1 or Gal-3 alone (Fig. 6-7A), further supporting the notion that iGal-1 and Gal-3 signal through a similar pathway in neutrophils.

Gal-1 and Gal-3 bind to distinct microdomains on activated T cells

Because the T cell response to iGal-1 and Gal-3 was fundamentally different and iGal-1 failed to attenuate Gal-3-induced T cell death, we examined the localization of iGal-1 and Gal-3 binding sites on the surface of activated T cells using confocal microscopy. Both iGal-1and Gal-3 bound to discrete microdomains on the cell surface (Fig. 6-7B). Interestingly, the binding was largely to separate domains, although there was some overlap (Fig. 6-7B). These studies were performed at 4°C, which limits the possibility of receptor reorganization. These results show that Gal-1 and Gal-3 recognize discrete receptors on T cells, and also recognize discrete microdomains on the T cell surface, further demonstrating that Gal-1 and Gal-3 have differential activities toward T cells.

Gal-1 and Gal-3 display similar affinities for T cell ligands

We next sought to examine the affinity of Gal-1 and Gal-3 toward T cell counter ligands. To accomplish this, we examined the binding of Gal-1 and Gal-3 toward T cells using a solid phase approach as described previously [65]. Gal-1 and Gal-3 recognized T cells and recognition was inhibited by lactose, demonstrating carbohydrate dependent recognition of T cell ligands (Fig. 6-8A,B). Binding isotherms of Gal-1 (Fig. 6-8C) or Gal-3 (Fig. 6-8D) toward T cells revealed a similar binding affinity toward T cell surface ligands, with Gal-1 exhibiting a K_d of approximately ~ 4 μM and Gal-3 displaying a K_d of ~ 2 μM (Fig. 6-8C,D). Importantly, iGal-1 exhibited a similar affinity toward T cells as Gal-1 (data not shown). These results demonstrate that Gal-1 and Gal-3 recognize their respective counter receptors with similar affinity and strongly suggest that the inability of Gal-1 to inhibit Gal-3 induced PS exposure in T cells likely reflects discrete receptor recognition.

iGal-1 retains biological activity toward T cells

Although iGal-1 bound to T cell ligands (Fig 6-3C, 6-8A,C), no changes in T cell viability occurred following treatment, similar to previous results with Gal-1 [82]. Previous studies demonstrated that Gal-1 induces apoptosis in T leukemic cell lines and primary activated T cells in the presence of DTT. Although iGal-1 retained all the previously documented effects toward neutrophils, T cells, and leukemic T cells (Figs. 6-1 through 6-4) [40, 82, 200], we sought to determine whether iGal-1 might induce additional previously described biological endpoints in T cells. Similar to previous results with Gal-1 [80, 82, 221, 225, 226], iGal-1 induced apoptotic cell death in T leukemic CEM cells (Fig. 9A,B) and MOLT-4 cells (data not shown) only in the presence of DTT in a dose dependent manner (Fig 6-6-9A,B). Similarly, iGal-1 induced apoptotic cell death in primary T cells only when incubated in the presence of DTT (Fig. 6-9C), consistent with

previous findings using Gal-1 [14, 82]. Few studies have examined the effects of Gal-1 in the absence of DTT. However, Gal-1 has been shown to increase IL-10 production and attenuate IFN-γ production in the absence of DTT [202]. We next sought to determine if iGal-1 induced the same effects. Similar to Gal-1 [202], iGal-1 induced increased IL-10 production while attenuating IFN-γ production (Fig 6-9D,E). Taken together these results demonstrate that iGal-1 retains the function of Gal-1 toward T cells and neutrophils. As the inability of iGal-1 to inhibit Gal-3-induced apoptosis was a novel finding in this study, as a final control we determined if Gal-1 also fails to inhibit Gal-3 induced apoptosis. Similar to iGal-1, Gal-1 failed to induce T cell death or inhibit Gal-3-induced PS exposure (Fig. 6-9F), DNA fragmentation or Ca^{2+} flux (data not shown) in T cells, although Gal-1 remained active over the treatment condition as indicated by its ability to induce PS exposure in MOLT-4 cells in parallel experiments (Fig. 6-9F) and agglutinate T cells in the absence of Gal-3 over the duration of the experiment (data not shown). Taken together, these results demonstrate that Gal-1 and Gal-3 induce disparate effects in T cells while retaining the common ability to induce PS exposure independent of apoptosis in neutrophils.

DISCUSSION

These results demonstrate that human neutrophils and T cells have distinct responses to Gal-1 and Gal-3, which may reflect differences between these leukocyte populations in their turnover and function. Gal-1 and Gal-3 exhibit robust and differential modulation of neutrophils and T cells, implicating this family as key regulators of both innate and adaptive immune responses. Furthermore, our data provide the first description of a single factor, Gal-3, capable of differentially inducing exposure of PS with or without apoptosis in two different leukocyte populations.

Gal-1 and Gal-3 are expressed in almost all tissues by many different types of cells [162, 217]. Gal-3 is the Mac-2 antigen that is expressed constitutively on the surface of macrophages [227]. Both galectins are expressed by fibroblasts, smooth muscle cells, skeletal muscle, neuronal cells, endothelial cells, and some lymphocytes, including T lymphocytes [162, 217]. Gal-1 and Gal-3 are also upregulated in response to inflammatory stimuli [162, 217]. Thus, migrating neutrophils and T cells are likely to encounter these galectins during their normal movement into inflamed tissues. As a result, the effects of these galectins on neutrophils and T cells are likely relevant in many aspects of leukocyte biology, including trafficking, turnover, and the modulation of leukocyte responses during the course of inflammation.

Increasing evidence suggests that galectin family members possess key regulatory activities toward leukocytes with members exerting both overlapping and unique effects. Although Gal-1, Gal-2, Gal-3, and Gal-4 all induce apoptosis-independent PS exposure in neutrophils [82], only Gal-3 induces apoptotic cell death in activated T cells. Furthermore, only Gal-4 induces non-apoptotic PS exposure independent of intracellular

Ca^{2+} mobilization in neutrophils [82], suggesting the existence of at least two separate pathways regulating non-apoptotic PS exposure. In contrast to the present study, previous results demonstrated that transient treatment of resting neutrophils with Gal-3 enhanced spontaneous apoptosis when analyzed following prolonged incubation [228]. However, since high levels of hapten are unlikely to efficiently disengage Gal-3 once bound to neutrophils, we felt that evaluating the effect of Gal-3 on neutrophil viability and PS exposure following continual treatment with Gal-3 would more appropriately reflect Gal-3 interactions with neutrophils *in vivo*. Indeed, previous studies demonstrated that cells actually require continual Gal-1-ligand engagement for full PS exposure to be realized [200]. Uninterrupted binding may not only be required to sustain PS exposure, but also serve to signal the maintenance of cell viability. Future studies will evaluate this intriguing possibility.

It is likely that Gal-1 and Gal-3 share common receptors or a convergent signaling pathway in neutrophils, since pre-incubation with Gal-1 prevented further Ca^{2+} flux following incubation with Gal-3. In addition, co-incubation of Gal-1 and Gal-3 caused additive, rather than synergistic, signaling responses in neutrophils. By contrast, activated T cells likely possess unique receptors and/or signaling pathways for Gal-1 and Gal-3, since Gal-1 did not attenuate the ability of Gal-3 to induce PS exposure and apoptosis in activated T cells. Furthermore, Gal-1 failed to block Gal-3-induced intracellular Ca^{2+} mobilization in activated T cells, which was sustained, unlike the transient Gal-1 and Gal-3 induced Ca^{2+} flux in neutrophils. The similar affinity of Gal-1 and Gal-3 toward their respective T cell counter ligands suggests that the inability of Gal-1 to alter Gal-3 likely results from recognition of different cell surface glycans. Gal-1 and Gal-3 also recognized discrete microdomains on activated T cells, further demonstrating differential recognition of T lymphocyte ligands. Consistent with this, recent studies

demonstrate that Gal-1 and Gal-3 exhibit differential binding to T cell surface ligands [16, 22].

Earlier studies demonstrated that Gal-1 exhibited potent adaptive immunosuppressive activity toward the *in vivo* [19-21, 157]. Subsequent studies suggested that Gal-1 may modulate the immune system by inducing cell death in activated T cells [14]. The results of the present study suggest that the *in vivo* effects of Gal-1 may result from altered expression of IL-10 and IFN-γ in T cells as opposed to directly altering T cell viability, consistent with previous results [202]. IL-10 exhibits potent immunosuppressive activity, inhibiting T cell activation and Th1 cytokine secretion and decreasing co-stimulatory receptors on antigen-presenting cells [229, 230]. Gal-1 administration *in vivo* reduces Th1 responses [19, 27], and Gal-1 null mice exhibit a Th2 biased responses [22]. Additional regulatory mechanisms may be involved in Gal-1-mediated suppression of adaptive immune responses. CD4+ CD25+ regulatory T cells (Tregs) from Gal-1-null mice exhibit significantly compromised suppressive behavior [159] and Gal-1 itself induces FoxP3 expression in naive T cells [231]. The ability of Gal-1 to induce IL-10 production, a key cytokine involved in regulatory T cell function [232], in both PBMCs and activated T cells may therefore reflect mechanisms whereby regulatory T cells suppress the immune system *in vivo*. Consistent with this, Blois *et. al.* recently demonstrated that Gal-1 requires IL-10 and Treg mediated pathways in the maintenance of immunological tolerance at the maternal-fetal interface during early gestation [161]. Interestingly, the first studies demonstrating that Gal-1 suppresses adaptive immunity suggested a role for suppressor T cells [23, 88, 157]. These studies therefore suggest that Gal-1 suppresses adaptive immune responses by altering cytokine production without directly affecting T cell viability. Gal-4 also shows no effect on T cell viability [82, 196] yet exacerbates chronic colitis through increasing IL-6 production [196], and Gal-3

directly induces apoptosis in T cells. Such results suggest that galectins exert similar effects on activated neutrophils but unique and potentially opposing effects on T lymphocytes.

The ability of Gal-1, Gal-2, Gal-3, and Gal-4 to induce PS exposure independently of apoptosis in neutrophils, but not T cells, may be related to the unique selective pressures on neutrophils within a harsh inflammatory environment. The inflammatory milieu in which neutrophils neutralize pathogens or remove necrotic tissue is distinct from the inflammatory setting of T cells and may preclude these cells from maintaining membrane integrity for prolonged time between the induction of apoptosis and successful phagocytic removal. This may be especially important when considering that the number of neutrophils usually far exceeds the number of phagocytes that can remove them, especially during the early stages of acute inflammation [233]. Indeed, neutrophil necrosis exacerbates the inflammatory response and may in part result from non-phagocytosed cells in late stages of apoptotic cell death [150, 151, 214, 234]. In this setting, galectins released during neutrophil-mediated tissue damage may prepare cells for removal by signaling exposure of PS without inducing cell death, thus enabling these cells to maintain membrane integrity until successfully phagocytosed. Because T cells act in a more direct fashion, exhibiting antigen specific immunity, similar selective pressures likely failed to convey the same avenue of removal for T cells [68].

The use of iGal-1 in this study allowed for the examination of Gal-1 function on leukocytes without introducing the deleterious effects of DTT inclusion in treatment media [83-85, 208] while controlling for the potential loss of Gal-1 activity as a result of oxidative inactivation. Many studies have utilized iGal-1 to control for loss of Gal-1 activity to oxidative inactivation [94, 98, 127-129, 219]. Previous studies demonstrated

that iGal-1 retains quaternary structure and tertiary structure as measured by solution based small angle neutron and x-ray scatting compared to the crystal of the non-alkylated protein [98, 145]. Furthermore, iGal-1 retained the carbohydrate binding specificity of Gal-1. More importantly, iGal-1 retained previously documented biological endpoints of Gal-1 activity on leukocytes, including the induction of PS exposure in activated neutrophils, MOLT-4 cells and CEM cells in the absence of cell death [40, 82, 200], the induction of Ca^{2+} flux in activated neutrophils [200], the induction of apoptosis in T cells, CEM cells and MOLT-4 cells in the presence of DTT [14, 80, 82, 221, 235] and the ability to alter T cell cytokine production [202]. Taken together, these results demonstrate that iGal-1 retains the biological activities of the Gal-1. Equally important, these results also rule out the possibility that the failure of Gal-1 to induce apoptosis in neutrophils in previous studies reflected loss of activity during treatment [40, 82, 200]. Importantly, Gal-1 failed to alter Gal-3-induced Ca^{2+} flux or cell death in T cells, similar to iGal-1, further demonstrating that alkylation fails to alter Gal-1 activity. Although DTT and iodoacetamide adduct formation are both artificial, the ability of iodoacetamide to stabilize Gal-1 will facilitate future studies of Gal-1 function while controlling for oxidative inactivation as done previously [94, 98, 127-129, 219] without introducing cells to an artificial reducing environment [83-85, 208].

The unique ability of galectins to signal PS exposure in living cells challenges current models of cell turnover and removal, which invoke only cellular apoptosis and necrosis. Galectin-induced cell removal could occur independently of cell death through the phagocytosis of living cells. We propose that this process be termed “preaparesis” [from the Latin *preaparare*], signifying that it prepares cells for phagocytic removal by causing PS exposure without accompanying apoptosis. We previously showed that Gal-1 induction of PS on activated human neutrophils, correlated with their phagocytosis by

activated macrophages [40]. Consistent with the possible unique role of galectins in this pathway, only neutrophils undergo apoptosis-independent removal *in vivo* [37]. Furthermore, genetic defects in classical apoptotic signaling pathways fail to alter neutrophil turnover *in vivo* [34, 109]. Future studies should examine the role of galectins in leukocyte turnover *in vivo* and determine whether apoptosis-independent phagocytic removal is specific to neutrophils.

Figure 6-1

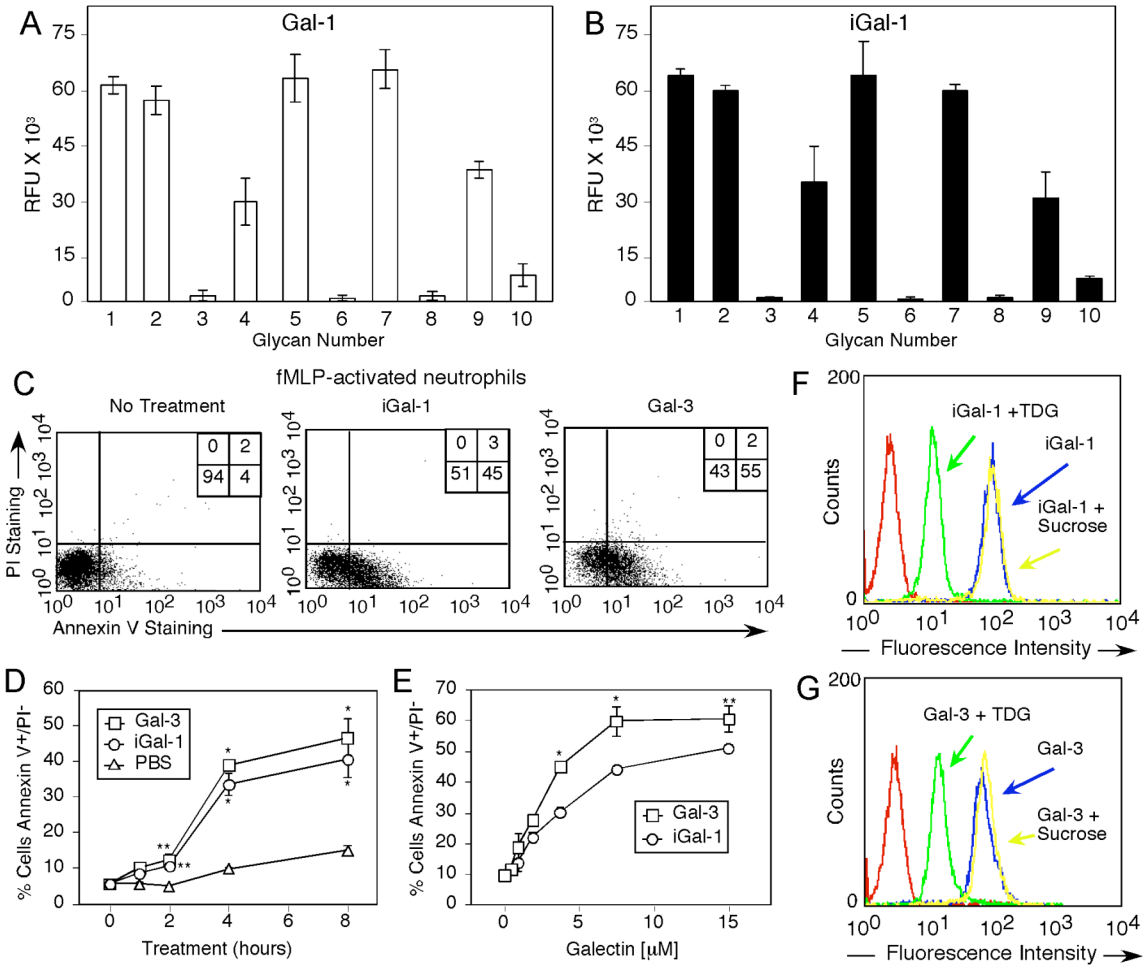
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Figure 6-2

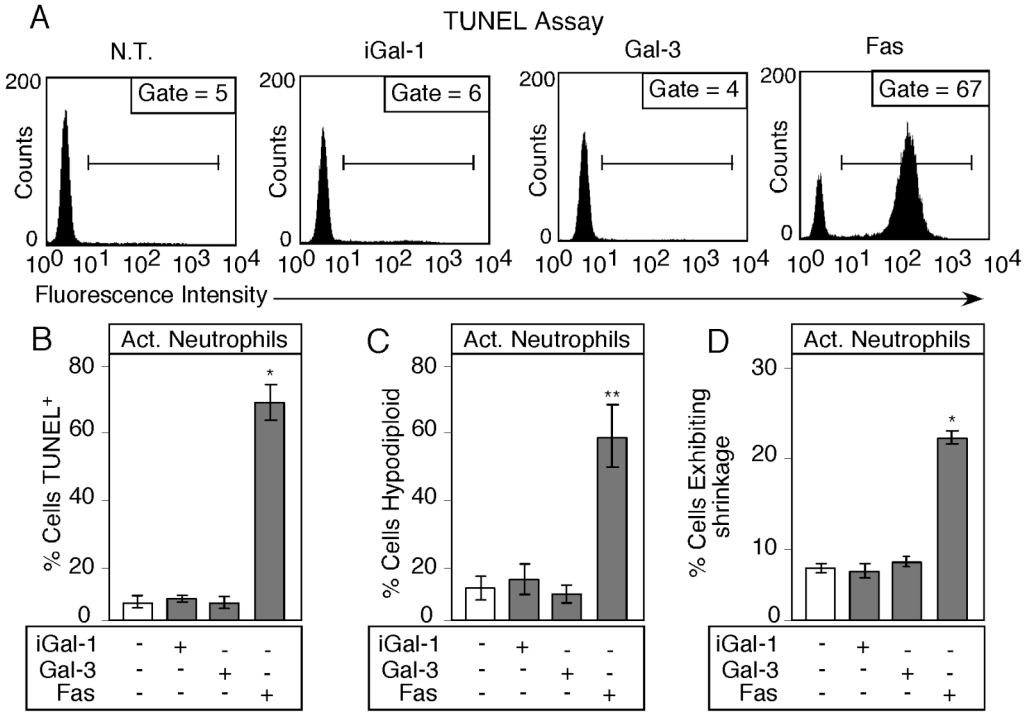
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Figure 6-3

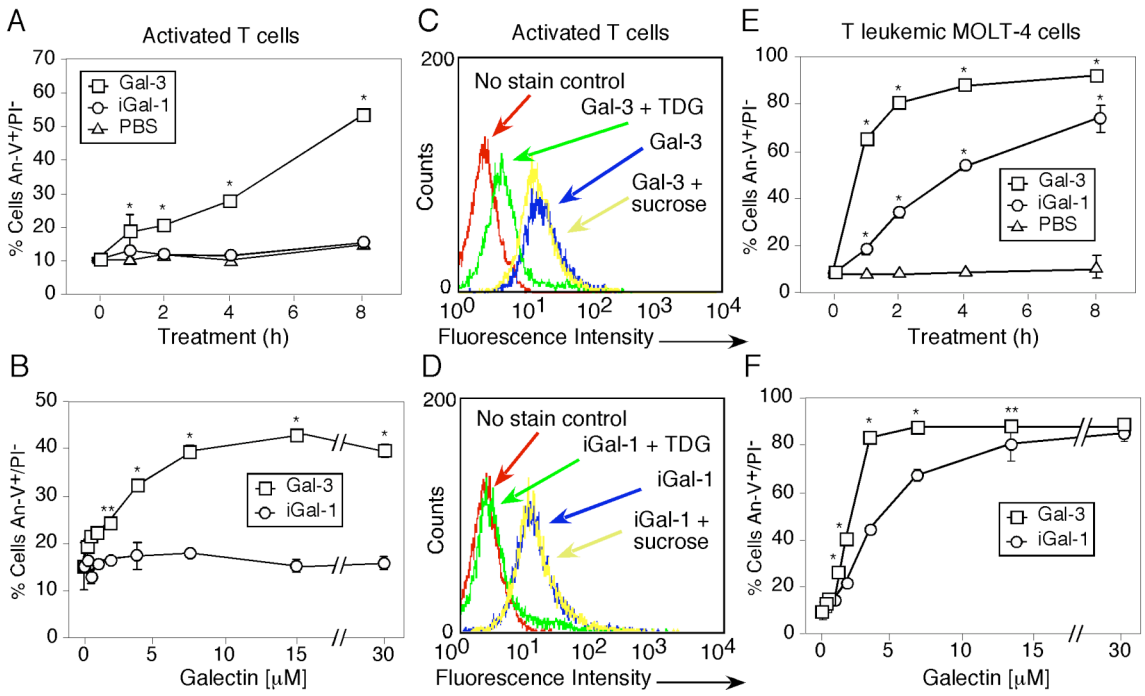
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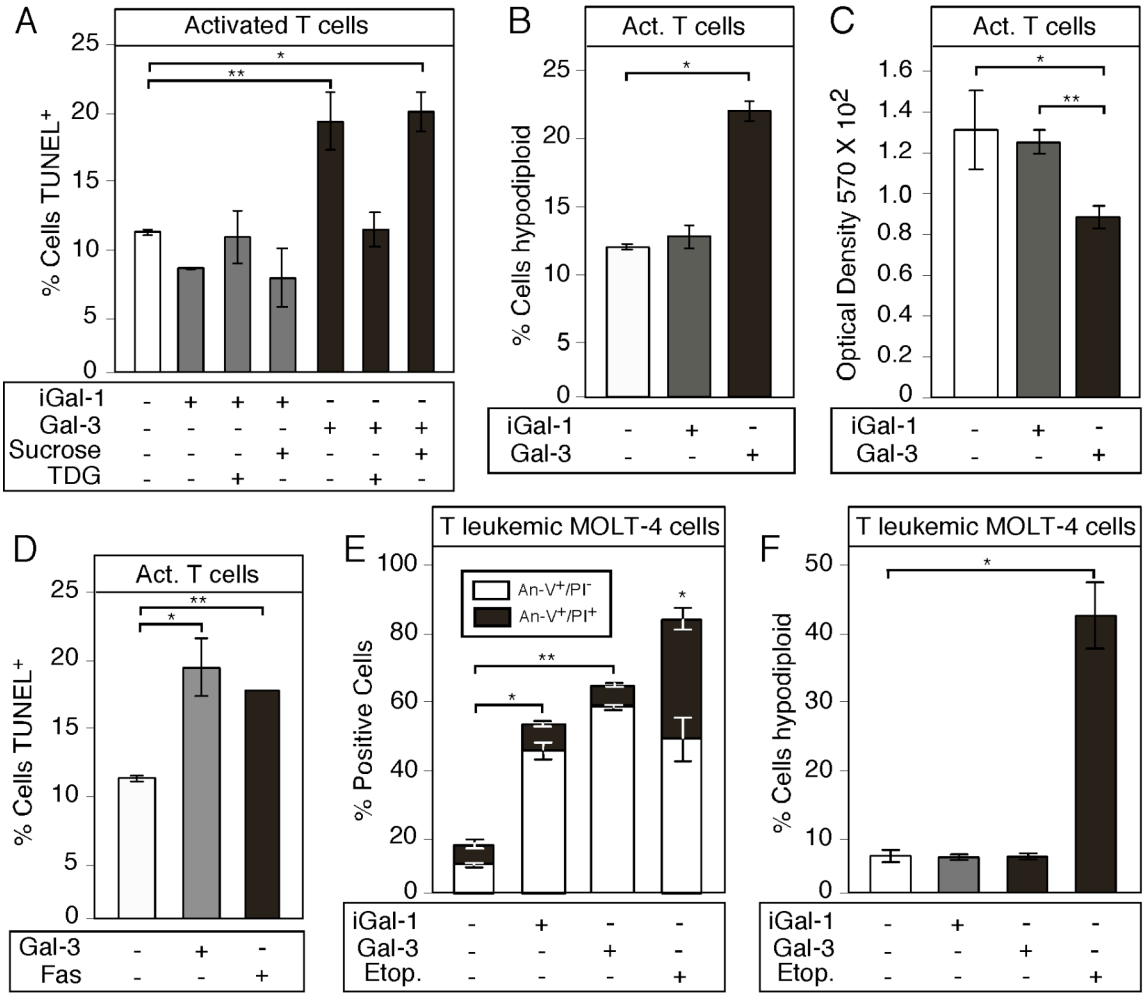
Figure 6-4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Optical Density 570 X 10-2
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Gal-3
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Gal-3
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Etop.
F
T leukemic MOLT-4 cells
% Cells hypodiploid
iGal-1
Gal-3
Etop.

Figure 6-5

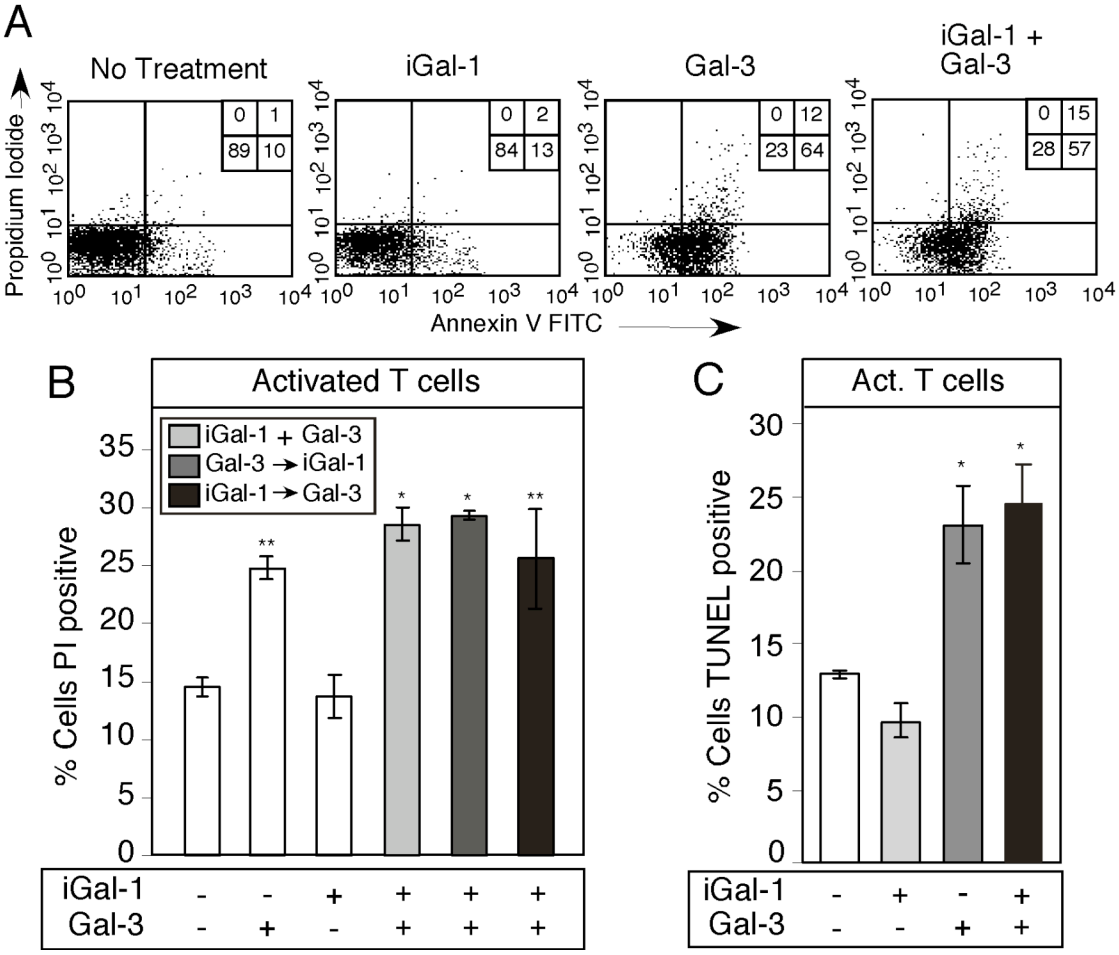

A
No Treatment
iGal-1
Gal-3
iGal-1 +
Gal-3
Propidium Iodide
Annexin V FITC
0 1
89 10
0 2
84 13
0 12
23 64
0 15
28 57
B
Activated T cells
iGal-1 + Gal-3
Gal-3 → iGal-1
iGal-1 → Gal-3
% Cells PI positive
iGal-1 - - + + + +
Gal-3 - + - + + +
C
Act. T cells
% Cells TUNEL positive
iGal-1 - + - +
Gal-3 - - + +

Figure 6-6

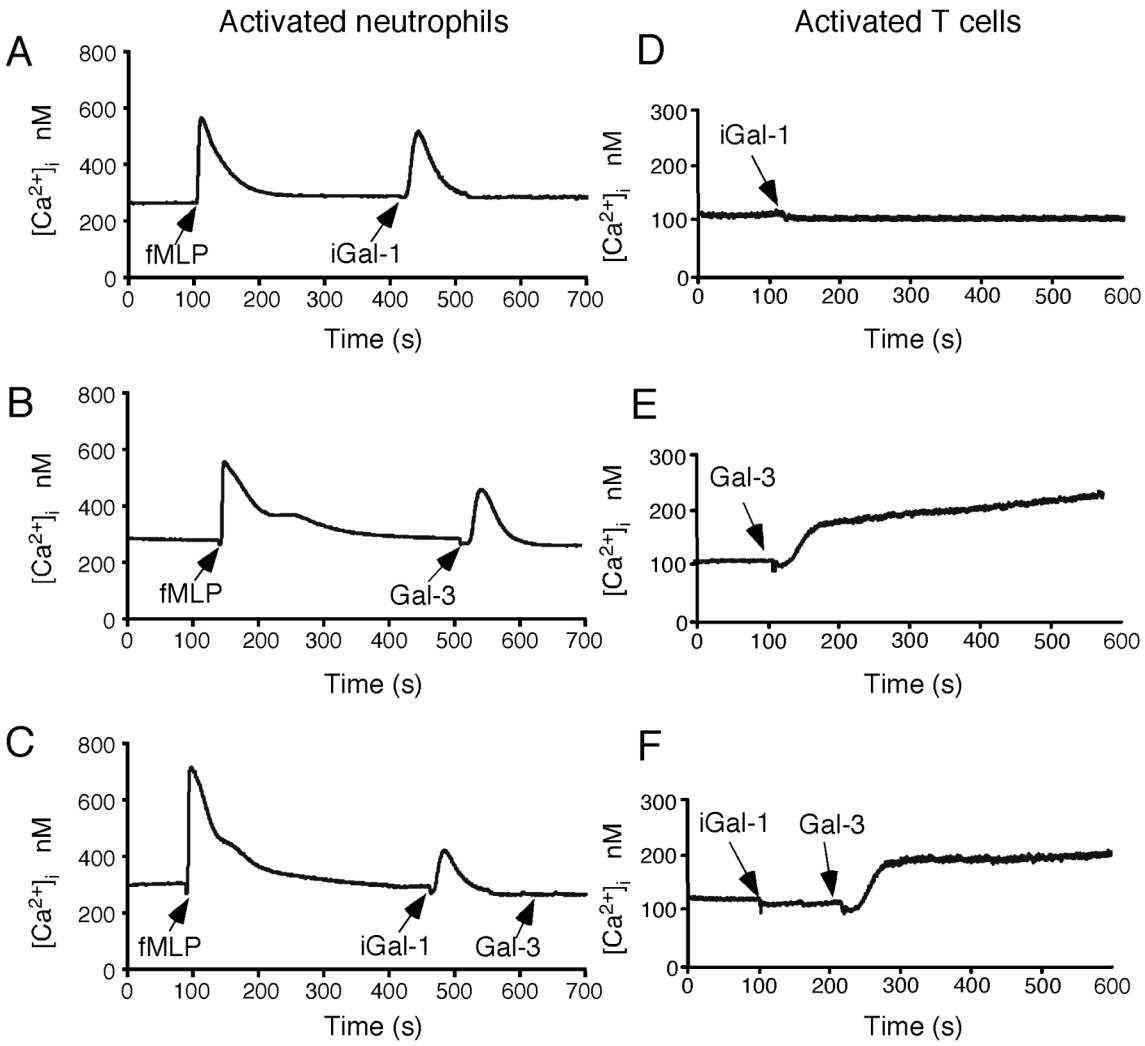

Activated neutrophils
Activated T cells
A
B
C
D
E
F
[Ca2+]i nM
Time (s)
fMLP
iGal-1
Gal-3

Figure 6-7

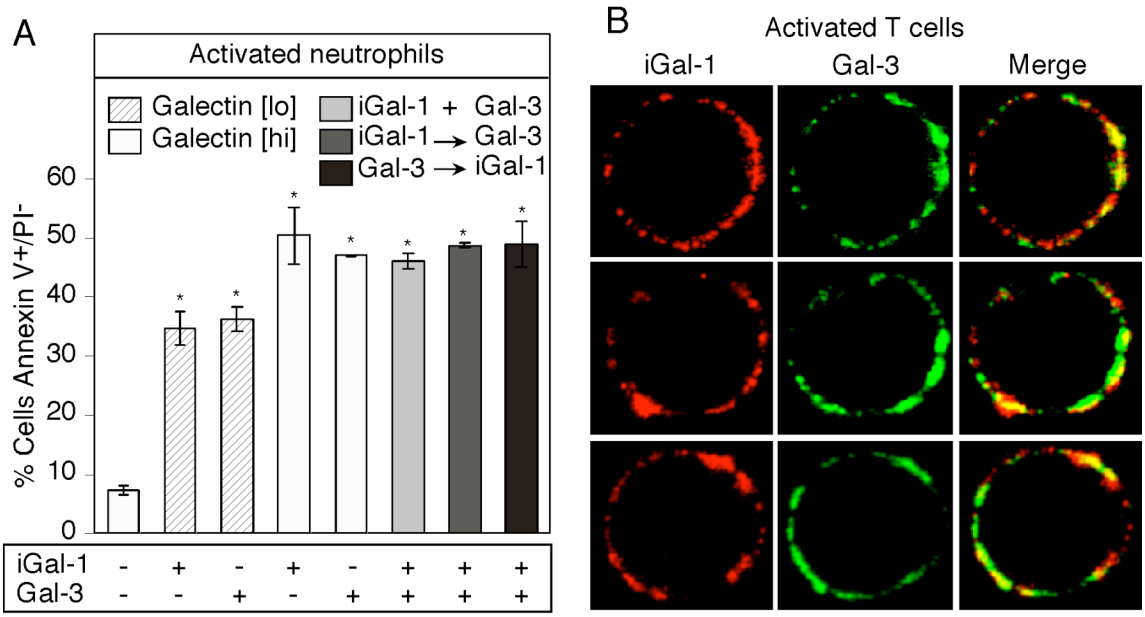

A
Activated neutrophils
Galectin [lo]
Galectin [hi]
iGal-1 + Gal-3
iGal-1 → Gal-3
Gal-3 → iGal-1
% Cells Annexin V+/PI-
0
10
20
30
40
50
60
iGal-1 - + - + - + + +
Gal-3 - - + - + + + +
B
Activated T cells
iGal-1
Gal-3
Merge

Figure 6-8

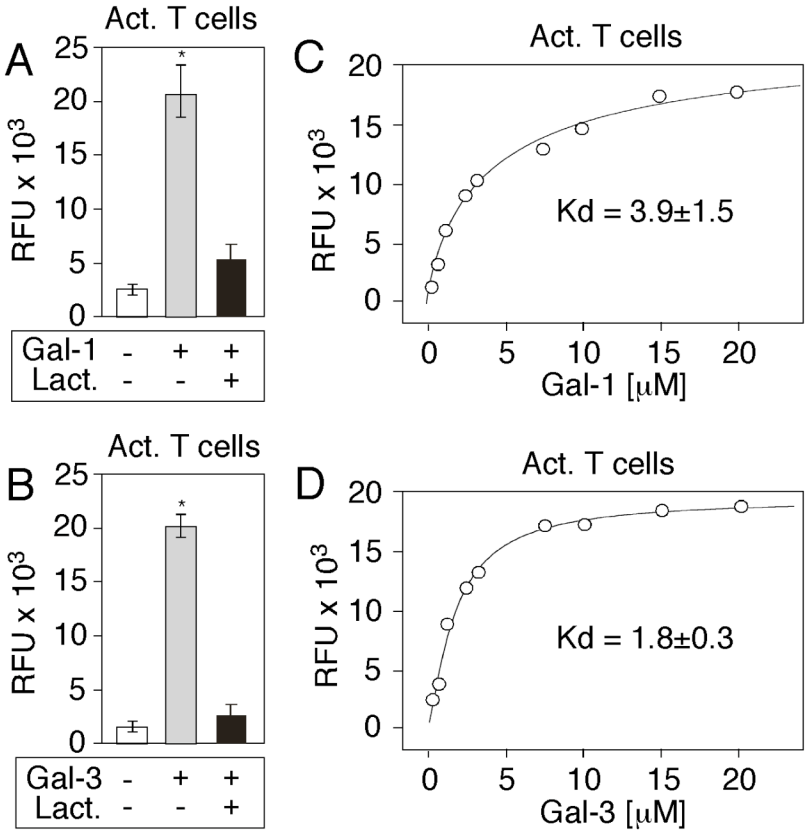

A
Act. T cells
RFU x 10^3
25
20
15
10
5
0
*
Gal-1 - + +
Lact. - - +
B
Act. T cells
RFU x 10^3
25
20
15
10
5
0
*
Gal-3 - + +
Lact. - - +
C
Act. T cells
RFU x 10^3
20
15
10
5
0
Kd = 3.9±1.5
0 5 10 15 20
Gal-1 [μM]
D
Act. T cells
RFU x 10^3
20
15
10
5
0
Kd = 1.8±0.3
0 5 10 15 20
Gal-3 [μM]

Figure 6-9

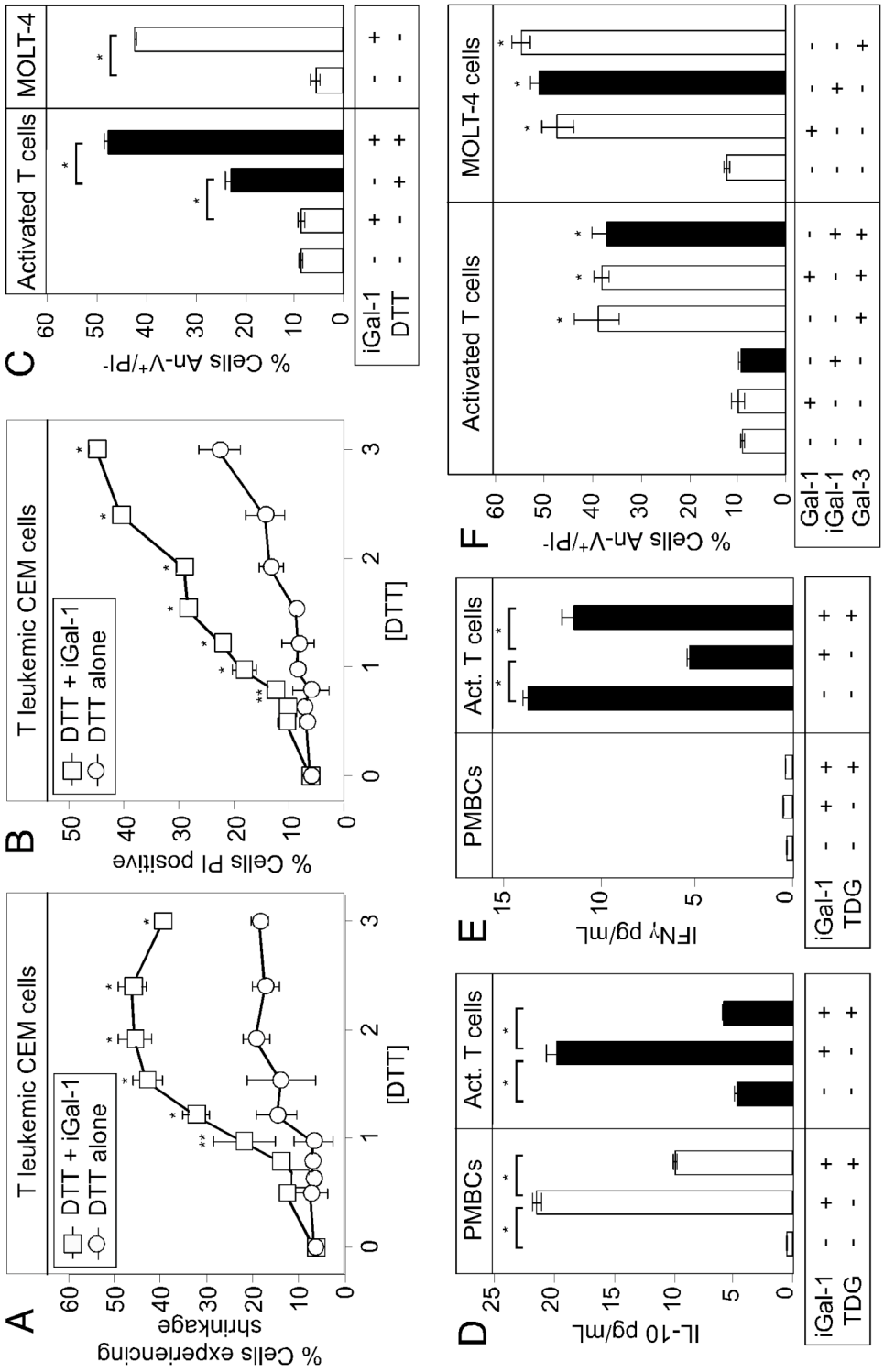

A
T leukemic CEM cells
DTT + iGal-1
DTT alone
% Cells experiencing shrinkage
[DTT]
B
T leukemic CEM cells
DTT + iGal-1
DTT alone
% Cells PI positive
[DTT]
C
Activated T cells
MOLT-4
% Cells An-V+/PI-
iGal-1
DTT
D
PMBCs
Act. T cells
IL-10 pg/mL
iGal-1
TDG
E
PMBCs
Act. T cells
IFNγ pg/mL
iGal-1
TDG
F
Activated T cells
MOLT-4 cells
% Cells An-V+/PI-
Gal-1
iGal-1
Gal-3

Table 6-1. Gal-3 induces PS exposure in neutrophils and T cells in a carbohydrate dependent fashion.

	Annexin V^+/PI^- (%)[a]	
Treatment	Neutrophils	T cells
Control	4	7
iGal-1	55	11
iGal-1 + TDG [b]	6	12
iGal-1 + sucrose	50	8
Gal-3	49	33
Gal-3 + TDG	7	13
Gal-3 + sucrose	45	38

[a] Activated neutrophils or T cells were treated with 20 μM iGal-1 or 20 μM Gal-3 for 8 h followed by detection for PS exposure. The data shown represent averages of duplicate determinations where the S.E. was <10%.

[b] iGal-1 and Gal-3 were co-incubated with either 20 mM TDG or 20 mM sucrose as indicated.

FIGURE LEGENDS

Figure 6-1. **Gal-3 induces PS exposure in activated neutrophils.** The binding of Gal-1 with βME (A) or iodoacetamide treated Gal-1 (B) to a representative panel of glycans is shown. Glycans are represented numerically as follows: 1) Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc; 2) Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ; 3) Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc; 4) Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc 5) Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc; 6) GlcNAcβ1-4Galβ1-4GlcNAc; 7) Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc; 8) Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc; 9) Galβ1-4GlcNAc; 10) Galβ1-4Glc No significant difference was observed between Gal-1 and iGal-1 ($P>0.05$) (C) A representative facsimile of activated neutrophils treated for 8 h with either PBS (Vehicle), 20 μM Gal-1 or 20 μM Gal-3 as indicated were analyzed for PS exposure by Annexin-V binding and propidium iodide (PI) exclusion. Percent of total cells in each quadrant are shown. (D) Activated neutrophils treated with PBS (vehicle), 20 μM Gal-1 or 20 μM Gal-3 for the indicated times were analyzed for PS exposure by Annexin-V binding and PI exclusion. Data are represented as mean values ± SD (*$P<0.001$ when comparing iGal-1 or Gal-3 to control). (E) Activated neutrophils treated with PBS (vehicle), iGal-1 or Gal-3 at the indicated concentrations for 8 h were analyzed for PS exposure by Annexin-V binding and PI exclusion. Data are represented as mean values ± SD (*$P<0.001$ when comparing iGal-1 or Gal-3 to control; **$P<0.01$ when comparing Gal-3 to iGal-1). (F) Activated neutrophils were incubated with iGal-1-ALEXA, iGal-1-ALEXA + 25 mM TDG, iGal-1-ALEXA + 25 mM sucrose followed by flow

cytometric analysis for binding. (G) Activated neutrophils were incubated with Gal-3-ALEXA, Gal-3-ALEXA + 25 mM TDG, Gal-3-ALEXA + 25 mM sucrose followed by flow cytometric analysis for binding.

Figure 6-2. **Gal-3 induces PS exposure in activated neutrophils in the absence of apoptosis.** (A) A representative facsimile of activated neutrophils treated with PBS (Vehicle), 20 μM iGal-1 or 20 μM Gal-3 for 8 h were analyzed for DNA fragmentation using the TUNEL assay. (B) Quantitative analysis of DNA fragmentation in activated neutrophils treated with iGal-1 and Gal-3 using the TUNEL assay. Data are represented as mean values ± SD (*P<0.01). No significant difference was observed between iGal-1 or Gal-3 and control (P>0.05). (C) Activated neutrophils treated with PBS (Vehicle), 20 μM iGal-1 or 20 μM Gal-3 for 8 h were analyzed for DNA fragmentation using hypodiploid analysis. Data are represented as mean values ± SD (*P<0.01). No significant difference was observed between iGal-1 or Gal-3 and control (P>0.05). (D) Activated neutrophils treated with PBS (Vehicle), 20 μM iGal-1 or 20 μM Gal-3 for 8 h were analyzed for cellular shrinkage. Data are represented as mean values ± SD (*P<0.01). No significant difference was observed between iGal-1 or Gal-3 and control (P>0.05).

Figure 6-3. **Gal-3 induces PS exposure in activated T cells.** (A) Activated T cells treated with PBS (vehicle), 20 μM iGal-1 or 20 μM Gal-3 for the indicated times were analyzed for PS exposure by Annexin-V binding and PI exclusion. Data are represented as mean values ± SD (*P<0.001 between Gal-3 and control). No significant difference was observed between iGal-1 and control (P>0.05). (B) Activated T cells treated with PBS (vehicle), iGal-1 or Gal-3 at the indicated concentrations for 9 h were analyzed for

PS exposure by Annexin-V binding and PI exclusion. Data are represented as mean values ± SD (*P<0.001; **P<0.05). No significant difference was observed between iGal-1 and control (P>0.05). (C) Activated T cells were incubated with Gal-3-ALEXA, Gal-3-ALEXA + 25 mM TDG, Gal-3-ALEXA + 25 mM sucrose followed by flow cytometric analysis for binding. (D) Activated T cells were incubated with iGal-1-ALEXA, iGal-1-ALEXA + 25 mM TDG, iGal-1-ALEXA + 25 mM sucrose followed by flow cytometric analysis for binding. (E) T leukemic MOLT-4 cells treated with PBS (vehicle), 20 μM iGal-1 or 20 μM Gal-3 for the indicated times were analyzed for PS exposure by Annexin-V binding and PI exclusion. Data are represented as mean values ± SD (*P<0.001 between Gal-3 and control and also between Gal-3 and iGal-1; **P<0.001 between Gal-1 and control and also between iGal-1 and Gal-3). (F) T leukemic MOLT-4 cells treated with PBS (vehicle), Gal-1 or Gal-3 at the indicated concentrations for 8 h were analyzed for PS exposure by Annexin-V binding and PI exclusion. Data are represented as mean values ± SD (*P<0.001 between iGal-1 or Gal-3 and control; **P<0.001 between Gal-3 and iGal-1).

Figure 6-4. **Gal-3 induces apoptotic PS exposure in activated T cells and non-apoptotic PS exposure in T leukemic MOLT-4 cells.** (A) Activated T cells treated with PBS (Vehicle), 20 μM Gal-1, 20 μM iGal-1 + 25 mM TDG, 20 μM iGal-1 + 25 mM sucrose, 20 μM Gal-3, 20 μM iGal-1+ 25 mM TDG or 20 μM Gal-3 + 25 mM sucrose for 9 h were analyzed for DNA fragmentation using the TUNEL assay. Data are represented as mean values ± SD (*P<0.05). No significant difference was observed between all iGal-1 treated samples, Gal-3 + 25 mM TDG and control (P>0.05). (B) Activated T cells treated with PBS (Vehicle), 20 μM iGal-1 or 20 μM Gal-3 for 9 h were analyzed for DNA fragmentation using hypodiploid analysis. Data are represented as mean values ± SD

(*P<0.01). No significant difference was observed between iGal-1 and control (P>0.05). (C) Activated T cells treated with PBS (Vehicle), 20 μM Gal-1 or 20 μmM Gal-3 for 18 h were analyzed for cell viability utilizing the MTT assay. Data are represented as mean values ± SD (*P<0.05). No significant difference was observed between iGal-1 and control (P>0.05). (D) Activated T cells treated with PBS (Vehicle), 20 μM Gal-3 or 100 ng/mL a-Fas for 9 h were analyzed for DNA fragmentation using the TUNEL assay. Data are represented as mean values ± SD (*P<0.01). (E) T leukemic MOLT-4 cells treated with PBS (Vehicle), 20 μM iGal-1, 20 μM Gal-3 or 20 μM Etoposide (Etop.) for 10 h were analyzed for PS exposure by Annexin-V binding and PI exclusion and cell death by Annexin-V binding and PI staining. Data are represented as mean values ± SD (*P<0.01 between iGal-1, Gal-3 and Etop for PS exposure compared to control; **P<0.01 between Etop treated cells and iGal-1, Gal-3 and control for PI staining). No significant difference was observed between iGal-1, Gal-3 and control for PI staining (P>0.05). (F) T leukemic MOLT-4 cells treated with PBS (Vehicle), 20 μM iGal-1, 20 μM Gal-3 or 20 μmM Etop. for 10 h were analyzed for DNA fragmentation using hypodiploid analysis. Data are represented as mean values ± SD. (*P<0.01). No significant difference was observed between iGal-1, Gal-3 and control (P>0.05).

Figure 6-5. **Gal-1 fails to alter Gal-3 mediated T cell apoptosis.** (A) A representative facsimile of activated T cells treated with 20 μM Gal-1, 20 μM Gal-3 or 20 μM Gal-1 + 20 μM Gal-3 for 9 h were analyzed for PS exposure by Annexin-V binding and PI exclusion. Percent of total cells in each quadrant are shown. (B) Activated T cells treated with 20 μM Gal-1, 20 μM Gal-3, 20 μM Gal-1 + 20 μM Gal-3 (Gal-1 + Gal-3), 20 μM Gal-1 for 30 min followed by 20 μM Gal-3 (Gal-1 → Gal-3) or 20 μM Gal-3 for 30 min followed by 20 μM Gal-1 (Gal-3 → Gal-1) for 9 h were analyzed for apoptotic cell death by staining with

PI. Data are represented as mean values ± SD. (*P<0.05). No significant difference was observed between iGal-1 co-incubated with Gal-3 compared to Gal-3 alone (P>0.05) or between Gal-1 and control (P>0.05). (C) Activated T cells treated with 20 μM Gal-1, 20 μM Gal-3 or 20 μM Gal-1 + 20 μM Gal-3 for 9 h were analyzed for DNA degradation using the TUNEL assay. Data are represented as mean values ± SD. (*P<0.05). No significant difference was observed between iGal-1 co-incubated with Gal-3 compared to Gal-3 alone (P>0.05) or between Gal-1 and control (P>0.05).

Figure 6-6. **Gal-3 induces Ca^{2+} mobilization in both activated neutrophils and activated T cells.** Activated neutrophils treated with (A) 20 μM iGal-1, (B) 20 μM Gal-3 or (C) 20 μM iGal-1 followed by 20 μM Gal-3 were analyzed for changes in intracellular [Ca^{2+}]. Activated T cells treated with (D) 20 μM iGal-1, (E) 20 μM Gal-3, or (F) 20 μM iGal-1 followed by 20 μM Gal-3 were analyzed for changes in intracellular [Ca^{2+}].

Figure 6-7. Gal-3 and Gal-1 recognize separate ligands on the T cell surface. (A) Activated neutrophils treated with 20 μM Gal-1 [lo], 40 μM Gal-1 [hi], 20 μM Gal-3 [lo], 40 μM Gal-3 [hi], 20 μM Gal-1 + 20 μM Gal-3 (Gal-1 + Gal-3), 20 μM Gal-1 for 30 min followed by 20 μM Gal-3 (Gal- 1 Gal-3) or 20 μM Gal-3 for 30 min followed by 20 μM Gal-1 (Gal-3 Gal-1) were analyzed for PS exposure by Annexin-V binding and PI exclusion. Data are represented as mean values ± SD. (*P<0.001 between iGal-1, Gal-3 or iGal-1 and Gal-3 treated and control). No significant difference was observed between iGal-1 and Gal-3 high alone and co-incubation of iGal-1 with Gal-3 (P>0.05). (B) Activated T cells were stained with Gal-1 and Gal-3 followed by detection for ligand localization by confocal analysis.

Figure 6-8. Gal-1 and Gal-3 recognize T cell ligands with similar affinity. (A) T cells were incubated with 10 μM ALEXA-flour 488 labeled Gal-1 with or without 0.1 M lactose as indicated followed by detection of bound Gal-1 using a fluorimeter. Data are represented as mean values ± SD. (*P<0.01). No significant difference between control and Gal-1 + lactose (P>0.05) (B) T cells were incubated with 10 μM ALEXA-flour 488 labeled Gal-3 with or without 0.1 M lactose as indicated followed by detection of bound Gal-3 using a fluorimeter. Data are represented as mean values ± SD. (*P<0.01). No significant difference between control and Gal-3 + lactose (P>0.05). Binding isotherms of (C) ALEXA-Gal-1 and (D) ALEXA-Gal-3 toward activated T cells.

Figure 6-9. iGal-1 retains biological activities toward T cells. (A) T leukemic CEM cells were treated with 20 μM unmodified Gal-1 (Gal-1) or iGal-1 with as indicated for 9 h following by assessing cell shrinkage by flow cytometric analysis. Data are represented as mean values ± SD. (*P<0.01). No significant difference occurred between Gal-1 and control (P>0.05). (B) T leukemic CEM cells were treated with 20 μM unmodified Gal-1 or iGal-1 with DTT as indicated for 9 h following by assessing cell death by propidium iodide staining. Data are represented as mean values ± SD. (*P<0.01). No significant difference occurred between Gal-1 and control (P>0.05). (C) Activated T cells or MOLT-4 cells were treated with 20 μM Gal-1 with or without 1.2 mM DTT as indicated. Data are represented as mean values ± SD. (*P<0.001). No significant difference occurred between Gal-1 and control for activated T cells (P>0.05). (D) PMBCs or PBMCs treated with anti-CD3 and anti-CD28 (Act. T cells) were treated with 20 μM Gal-1 with or without 20 mM TDG as indicated followed by detection for IL- 10. Data are represented as mean values ± SD. (*P<0.01). (E) PMBCs or PBMCs treated with anti-CD3 and anti-CD28 (Act. T cells) were treated with 20 μM Gal-1 with or without 20 mM TDG as indicated followed by detection for IFN-γ. Data are represented as mean values ± SD. (*P<0.01).

(F) Activated T cells or MOLT-4 cells were treated 20 μM Gal-1, 20 μM iGal-1, 20 μM Gal-3, 20 μM Gal-1 + 20 μM Gal-3 or 20 μM iGal-1 + 20 μM Gal-3 for 9 h as indicated followed by analysis for PS exposure by Annexin-V binding and PI exclusion. Data are represented as mean values ± SD. (*P<0.01). No significant difference occurred between Gal-1 + Gal-3 or iGal-1 + Gal-3 when compared to Gal-3 alone (P>0.05) or between Gal-1 or iGal-1 and control (P>0.05).

Chapter 7

Human Galectin-1 Recognition of Poly-N-Acetyllactosamine and Chimeric Polysaccharides

Human galectin-1 is a 14.5 kD dimeric carbohydrate binding protein (dGal-1) widely expressed by many cells, but whose carbohydrate binding specificity is not well understood. Because of conflicting evidence regarding the ability of galectins in general to recognize N-acetyllactosamine (LN – Galβ4GlcNAc) and poly-N-acetyllactosamine sequences (PL – [-3Galβ4GlcNAcβ1-]$_n$), we synthesized a number of neoglycoproteins containing galactose-, N-acetylgalactosamine, fucose, LN-, and PL conjugated to bovine serum albumin (BSA). All neoglycoproteins were characterized by matrix-assisted laser desorption/ionization time-of-flight. Binding was determined in ELISA-type assays with immobilized neoglycoproteins and apparent binding affinities were estimated. For comparison, we also tested the binding of these neoglycoconjugates to *Ricinus communis* Agglutinin I, (RCA-I, a galactose binding lectin) and *Lycopersicon esculentum* agglutinin (LEA or tomato lectin), a PL-binding lectin. dGal-1 bound to immobilized Galβ4GlcNAcβ3Galβ4Glc-BSA with an apparent K_d of ~43 μM, but bound better to BSA conjugates with long PL sequences (K_d's ranging from 23.7 ± 5.8 μM to 41.7 ±10.1 μM). By contrast, dGal-1 did not bind glycans containing non-reducing terminal GalNAcβ4GlcNAcβ3Galβ1-R (the lacdiNAc or LDN antigen), Galβ4(Fucα3)GlcNAcβ̃R (Lewis X antigen), GlcNAcβ3Galβ4GlcNAcβb3Galb1-R (terminal GlcNAc), and also bound very poorly to lactosyl-BSA (Galβ4Glc-BSA). By contrast, RCA bound equally well to all glycans containing terminal, non-

reducing Galβ1-R independently of the presence of PL, including lactosyl-BSA. LEA bound with increasing affinity to unmodified PL proportional to chain length. Thus, dGal-1 binds terminal β4Gal residues and its binding affinity is enhanced significantly by the presence of this determinant on long-chain PL.

INTRODUCTION

Galectins are a family of related carbohydrate binding proteins found in all metazoans, including sponges, invertebrates, fungi, and mammals [236]. This wide distribution among species, coupled with a highly evolutionary conserved sequence within the carbohydrate recognition domain, suggests that galectins are involved in conserved biological processes [237-240]. In mammalian systems, galectins have been implicated in numerous processes including neutrophil turnover and phagocytosis [241], cell migration [242], immunomodulation [157, 191, 243], growth regulation [87, 244-246], apoptosis [11, 14], cell adhesion [247], embryogenesis [6, 248] tumor spreading [249] and pre-mRNA splicing [250]. However, the *in vivo* biological functions of galectins in mammals remain enigmatic and are being actively investigated in many laboratories.

There is also uncertainty surrounding the carbohydrate binding requirements of the galectins, particularly in regard to galectin-1, the first galectin family member identified [139, 251, 252]. Galectin-1 homologs occur as homodimers of ~14.5 kDa subunits [171, 238]. Dimeric bovine galectin-1 was reported to bind N-acetyllactosamine (Galβ4GlcNAc) but preferentially bound to poly-N-acetyllactosamine sequences (PL – [-3Galβ4GlcNAcβ1-]$_n$) containing multiple, linear LN units[166, 253, 254], suggesting that LN motifs within the PL chains were possibly recognized by bovine galectin-1. These results were later partly confirmed using short chain synthetic PL to assess the binding of hamster galectin-1 [255]. However, crystallographic data suggests that bovine galectin-1 recognizes the terminal non-reducing β4Gal residue and binds weakly to the penultimate GlcNAc residue within the terminal LN sequence[141, 256]. Other recent results have also shed doubt upon the ability of bovine galectin-1 to recognize internal LN motifs within a PL structure [257]. Recent studies on the dimeric human galectin-1

(dGal-1) indicate that it binds better to N-glycans containing terminal LN units than to PL units[64]. These differences in the apparent specificity of galectin-1 for saccharide ligands has prompted us to further investigate the binding characteristics of this protein. It is hoped that a better understanding of these requirements will prove helpful in the advent of using dGal-1 as a target for therapeutic intervention, as has been previously suggested [258].

To assess the requirements for saccharide recognition by human dGal-1, we prepared a series of neoglycoproteins in which glycans were covalently attached to bovine serum albumin (BSA). Some of these glycans contained the PL sequence, whereas others contained PL-related sequences with internal modifications, such as the presence of internal -GalNAcβ4GlcNAcβ1- sequences lacdiNAc or LDN antigen), or Galβ4[Fucα3]GlcNAcb̃R (Lewis X antigen) in what we have termed “chimeric-PL” sequences. These neoglycoproteins were then tested in ELISA-type formats using fluorescently-labeled dGal-1 and a number of plant lectins and monoclonal antibodies previously shown to be able to discriminate LN- and PL-containing glycans. Our results demonstrate that human dGal-1 requires the presence of a terminal, non-reducing LN for high affinity binding and that terminal, non-reducing LN expressed on long chain PL sequences or chimeric-PL sequences enhance recognition.

RESULTS

Preparation of neoglycoproteins

The oligosaccharides LN-LN-LN-L, LN-LN-L, LN-Lex-Lex-L, LN-LDN-L, LNnT, LDNT, Lex-L, triose, and lactose (Fig. 7-1) were prepared by enzymatic synthesis and coupled to BSA by reductive amination as described in the Materials and Methods. We attempted to derivatize the glycans to relatively equal densities. To test the density of covalent coupling each conjugate was analyzed by MALDI-TOF (Fig. 7-2). Each neoglycoprotein demonstrated relative uniform glycan average density ranging from 1 to approximately 27 mol of glycan per mol BSA (Fig. 7-2). Interestingly, some glycans were coupled to very high density (e.g. lactosyl-BSA containing ~27 mol/mol sugar), others were coupled to lower densities (e.g. LNnT-BSA containing 1.2-7.0 mol/mol sugar), while some neoglycoproteins were relatively homogeneous in coupling density (e.g. LDNT-BSA containing 10.3 mol/mol sugar). Although we were concerned that high coupling densities for some neoglycoproteins might confound interpretations of binding data for human dGal-1, our studies below demonstrated that for LDNT-BSA, Triose-BSA and Lactosyl-BSA their high coupling densities did not significantly enhance dGal-1 binding.

dGal-1 displays higher binding toward poly-N-acetyllactosamine than N-acetyllactosamine

For the study of dGal-1 binding to these neoglycoconjugates we utilized the lectin directly fluorescently labeled with the stable fluorescent dye ALEXA-488. The ALEXA-dGal-1 quantitative rebound to a column of lactosyl-Sepharose (data not shown), indicating that fluorescence-derivatization did not affect the stability of the protein or its

ability to bind to the original affinity column used in purification of the recombinant protein.

Microtiter wells were directly coated with neoglycoproteins as described in Materials and Methods, and the binding of ALEXA-dGal-1 was measured toward lactosyl-BSA, LNnT-BSA, LN-LN-L-BSA and LN-LN-LN-L-BSA (Fig. 7-3). ALEXA-dGal-1 demonstrated the highest apparent binding affinity toward LN-LN-LN-L-BSA (K_d = 23.7 mM) (Fig. 7-3A,B). LN-LN-L-BSA and LNnT-BSA bound with similar affinity to dGal-1 at K_d values equaling 41.7 mM and 42.9 mM respectively (Fig. 7-3A,B). Interestingly ALEXA-dGal-1 exhibited no appreciable binding to Lactosyl-BSA (Fig. 7-3B). These results suggest that dGal-1 affinity for PL-containing glycans was dependent not only on the presence of the LN unit but was significantly enhanced by extension of LN repeats within the glycans. The results indicate that dGal-1 requires LN within long chain PL sequences for high affinity recognition.

To control for the accessibility of the BSA-glycan conjugates, we explored their binding to plant lectins with defined specificities toward either PL sequences of terminal b4Gal residues. To this end we utilized tomato lectin (Lycopersicon esculentum agglutinin - LEA) labeled with fluorescein-isothiocynate - FITC-LEA) to explore the accessibility of PL sequences. This lectin has been shown to demonstrate high affinity binding to glycoconjugates containing repeating LN sequences in PL[259]. FITC-LEA showed high affinity binding to LN-LN-LN-L-BSA (Fig. 7-3C) and generally demonstrated increased affinity toward those ligands with increasing number of LN units (Table 7-1). However, LEA binding was different from dGal-1 in the respect that LEA appeared to recognize LN sequences when positioned within the LN containing glycan, regardless of the presence of a terminal b4Gal residue, as has been previously reported [259]. As a result, LEA

demonstrated no binding to LNnT-BSA and also showed no binding to Lactosyl-BSA (Table 7-1).

To explore binding to neoglycoproteins containing terminal β4Gal residues, we employed Ricinus communis agglutinin-I (RCA-I), a plant lectin that recognizes terminal β4Gal residues [260-264]. FITC-RCA bound tightly to LN-LN-LN-L-BSA (Fig. 7-3D). However, FITC-RCA-I displayed relatively uniform affinity for each neoglycoprotein containing terminal β4Gal residues, including Lactosyl-BSA, but did not bind to those lacking this residue (Table 7-1). The results of using the control lectins FITC-LEA and FITC-RCA demonstrate that all glycans within the neoglycoproteins are accessible and provide appropriate and predictable expression as neoglycoproteins immobilized in microtiter plates. In addition, the relatively similar binding affinities of FITC-RCA-I indicate that different coupling densities of glycans to the BSA do not appreciable alter the binding affinity of the lectin.

dGal-1 displays similar affinity to poly-N-acetyllactosamine regardless of internal N-acetyllactosamine modification

To address the importance of terminal, non-reducing β-linked galactosyl residues and the potential role of the internal LN units in the PL chains for recognition by dGal-1, we generated several novel neoglycoproteins containing chimeric PL sequences, i.e. either LN or lactose disaccharides coupled with other repeating motifs. These included LN-Lex-Lex-L-BSA, containing internal LN residues modified by an α3-linked fucose residue, Lex-L-BSA, containing fucose linked α3 to the penultimate GlcNAc residue, and LDNT-BSA in which a terminal, nonreducing GalNAc residue replaced the terminal Gal residue linked to lactose (Fig. 7-1). Interestingly, dGal-1 bound well to LN-Lex-Lex-L-BSA and LN-LDN-L-BSA (Fig. 4A,B), but did not bind to Lex-L-BSA, LDNT-BSA, or

Triose-BSA (Fig. 7-4A-C and Table 7-1). These data demonstrate that dGal-1 requires an unmodified terminal, Gal residue on a non-reducing LN for recognition.

Interestingly, FITC-RCA-I bound well to LN-Lex-Lex-L-BSA, LN-LDN-L-BSA, and LDNT-BSA (Fig. 7-4D-F), but did not bind Lex-L-BSA (Fig. 7-4D). These results indicate that RCA-I requires terminal non-reducing and unmodified LN sequences for high affinity binding, although the lectin can recognize LDNT-BSA, which contains terminal, non-reducing β4GalNAc residue (Fig. 7-4E). This latter finding is consistent with a previous study documenting the ability of RCA-I to bind such terminal sequences[265]. By contrast, FITC-LEA bound to LN-LDN-L-BSA, although less well than it did to LN-LN-L-BSA, but did not bind either LDNT-BSA or Lex-L-BSA (Fig. 7-4G and Table 7-1), indicating that LEA requires repeating LN sequences for recognition and that the presence of an internal LDN motif depresses LEA recognition. Because neither dGal-1, RCA-I or LEA bound these lectins bound Lex-L-BSA and only FITC-RCA recognized LDNT-BSA, the possibility arose that these glycan determinants on the neoglycoproteins might not be fully accessible to external reagents. To control for this possibility, we utilized monoclonal antibodies previously prepared toward the Lewis X and LDN antigens [266-268]. These monoclonal antibodies were able to bind to the appropriate neoglycoprotein conjugate, as shown in Fig. 7-4H. These results demonstrate that neither Lex-L-BSA nor LDNT-BSA is recognized by dGal-1, although both glycan conjugates can be recognized by appropriate monoclonal antibodies to each determinant. Thus, the inability of dGal-1 to bind to these determinants is due to its lack of recognition, rather than lack of exposure of the determinants within the neoglycoconjugates.

dGal-1 does not independently recognize internal LN units within PL chains

To determine whether dGal-1 required terminal LN units or if recognition of PL sequences would occur when only internal unmodified LN units where present, we treated LN-LN-LN-L-BSA with β-galactosidase from bovine testes to specifically remove terminal, nonreducing β4-galactose residues to generate GlcNAc-LN-LN-L-BSA. To control for the degree of terminal galactose removal we used FITC-RCA-I. FITC-RCA binding dropped over ninety percent when compared to pretreatment (Fig. 7-5A). Following FITC-RCA-I binding, the bound FITC-RCA was removed by incubating wells with buffer containing 200 mM lactose to remove all fluorescently-labeled RCA-I, and subsequently tested binding by ALEXA-dGal-1. Interestingly, dGal-1 was reduced to approximately the same extent demonstrated by FITC-RCA (Fig. 7-5B). Residual dGal-1 and RCA binding was most likely due to the incomplete removal of galactose from some terminal LN units following β-galactosidase treatment. To control for the integrity of the remaining glycan subsequent to β-galactosidase treatment, FITC-LEA binding affinity was determined subsequent to dGal-1 removal by treatment of plates with 200 mM lactose. LEA demonstrated similar binding to both LN-LN-LN-L-BSA and GlcNAc-LN-LN-LN-L-BSA (data not shown). These results demonstrate that dGal-1 requires terminal non reducing unmodified LN units and will not independently bind LN units located within an LN-containing glycan.

DISCUSSION

Our study shows that human dGal-1 requires the presence of terminal, non-reducing b4-linked galactosyl residues in PL sequences for high affinity binding and that the lectin recognizes long chain PL sequences even when these sequences have internal substitutions. However, human dGal-1 does not recognize certain terminal sequences, such as those containing terminal LN with α3-linked fucose in the Lex antigen structure or a terminal β4-linked GalNAc residue. In addition, our results indicate that dGal-1 does not independently recognize LN sequences located within the glycan sequence in the absence of an intact, terminal, non-reducing LN unit. These observations extend those of others on the recognition determinants for human dGal-1 and allow predictions to be made concerning the types of biological ligands likely to be recognized by the lectin.

There have only been a few studies to date on the carbohydrate binding specificity of human dGal-1 [64, 269, 270] and the results have been somewhat conflicting. For example, an early study concluded that human dGal-1 (termed galaptin from human spleens) binds branched glycans containing multiple, terminal β4-galactosyl residues in LN sequences and that its binding affinity was not particularly enhanced by clustering the number of terminal β4-galactosyl residues [269]. However, a recent study found some increased binding affinity to N-glycans with increased branching and numbers of terminal LN units [64]. One study identified the ganglioside GM1 as a human dGal-1 ligand [270], but a more recent study found no binding of the lectin to a free glycan containing the structure found in GM1 [64]. Importantly, none of these studies utilized the types of chimeric PL sequences used in our study or addressed the issue of internal modifications of PL sequences.

Some of the conflicting information to date about human dGal-1 binding specificity may arise due to different methods used for the analysis. For example, frontal affinity chromatography approaches of Hirabayashi et al [64] requires immobilized human dGal-1 coupled to an affinity support and the use of fluorescently-labeled free glycans. This is an equilibrium method, but utilizes free glycans, rather than glycoconjugates, and also allows for the possibility that cross-linking of the free glycans to immobilized glycan could occur, thereby enhancing the apparent affinity of multibranched glycans. The approach of Kopitz et al [270] relied on GM1-coated beads to cells and indirect, correlative evidence that human dGal-1 was involved in the recognition. Finally, the study of Lee et al [269] utilized a variety of synthetic clustered glycosides and glycopeptides in addition to neoglycoproteins containing glycans linked covalently to BSA, with measurements involving inhibition of binding (I_{50} values). However, we decided to take a more direct approach using glycoconjugates that could allow a closer approximation to the native state, i.e. glycans linked to a carrier protein, and allow direct measurements of their binding to fluorescently-labeled recombinant human dGal-1.

Taken together, our results are generally consistent with those of others [64, 269] in that terminal LN sequences were found to be recognized by human dGal-1. However, our studies demonstrate that dGal-1 binds long chain PL sequences and that internal modifications of these sequences, as seen in the chimeric PL structures, do not appreciably diminish binding of the lectin. Importantly, dGal-1 did not bind PL sequences terminating in either a β3-linked GlcNAc residue or a β4-linked GalNAc residue, indicating a requirement for a terminal, non-reducing β4-Gal residue. The inability of human dGal-1 to bind long chain PL sequences terminating in GlcNAc residues suggests that it is different from bovine dGal-1. Earlier results indicated that the bovine dGal-1 recognizes PL-containing N-glycans [254] even when they contained

a terminal, non-reducing GlcNAc residue [166]. Other studies on bovine dGal-1 also indicated that it recognizes terminal LN sequences [253, 255, 271] as well as weakly binding internal Gal residues within PL chains [255]. A recent study on bovine dGal-1 shows that the presence of a terminal, non-reducing α3-linked galactosyl residue in the sequence Galα3Galβ4GlcNAc-R actually enhanced recognition by that lectin [257]. Thus, the results indicate that both human and bovine dGal-1 bind long chain PL sequences, although the mode and fine structural determinants required for recognition may differ.

The preference of human dGal-1 for the terminal LN motif is interesting. The terminal sequences of O-glycans and many complex-type N-glycans in most mammalian glycoproteins contain LN, but this sequence is often further modified by sialylation, fucosylation, galactosylation, sulfation, and other types of modifications [272]. Such modifications of the LN unit may be important in regulating the affinity and thereby the degree of biological activity of dGal-1 toward these LN containing ligands. For example, it has been reported that both human and bovine dGal-1 bind weakly to LN sequences modified by α6-linked, but not by α3-linked, sialic acid [64, 253, 271, 273, 274] and that the effects of human dGal-1 on human T cells is blocked by α6-, but not α3-sialylation [275]. We recently found that activation of human neutrophils enhances their binding to human dGal-1, resulting in a signaling event leading to exposure of phosphatidylserine on the cell surface and subsequent phagocytic recognition of the cells by macrophages [241]. We also found that enzymatic desialylation of resting neutrophils did not enhance their sensitivity to human dGal-1 effects, although it did significantly enhance binding of the lectin [241]. In the case of cell lines, such as human HL-60 and MOLT-4 cells, enzymatic desialylation enhanced both their binding and sensitivity to human dGal-1

[241]. Such results may relate to other studies indicating that activation of neutrophils is accompanied by a concomitant mobilization of an endogenous sialidase to the plasma membrane [276, 277].

The ability of dGal-1 to recognize long chain PL sequences may be related to its biological activity. Although there are no direct experimental results indicating that cell surface PL sequences are required for human dGal-1 binding or activity, there is indirect evidence implicating cell surface PL chains in human dGal-1 [4, 220] and murine dGal-1 activity [278]. PL sequences are known to occur on basement membrane proteins, such as laminin, and selected cell surface glycoproteins [14, 166, 205, 279, 280]. The nature and length of long chain PL extending above the glycocalyx may promote recognition by dGal-1 and increase the ability of dGal-1 to bind and cross-link biological ligands. Recent studies using a mutated form of human dGal-1 to generate a monomeric protein, demonstrated that dimerization is required for biological activity toward activated human neutrophils [241]. The presence of other modifications of the PL sequences, such as fucosylation or internal LDN motifs, may not interfere with galectin binding and allow the chains to be multifunctional in that they can also serve as ligands to other carbohydrate binding proteins. Future detailed biochemical and structural studies on cell surface macromolecular ligands for human dGal-1 will be required to identify the predicted role of PL chains in recognition and function of this interesting lectin.

Figure 7-1

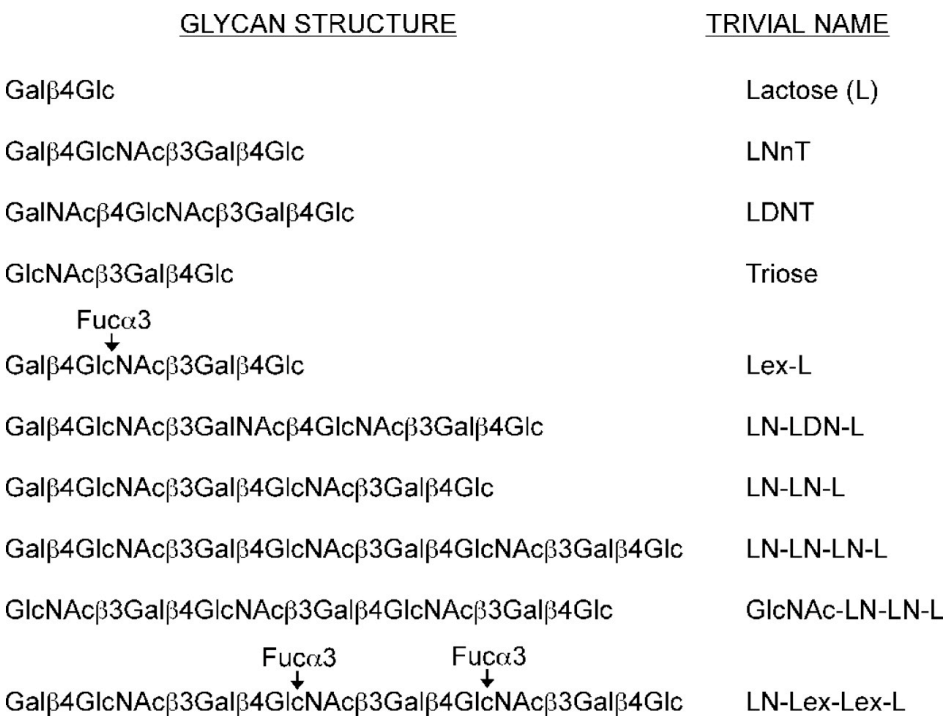
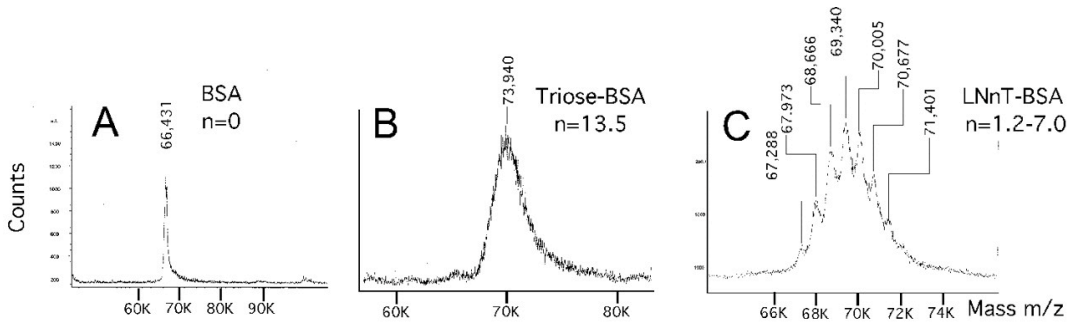

GLYCAN STRUCTURE
TRIVIAL NAME
Galβ4Glc
Lactose (L)
Galβ4GlcNAcβ3Galβ4Glc
LNnT
GalNAcβ4GlcNAcβ3Galβ4Glc
LDNT
GlcNAcβ3Galβ4Glc
Triose
Fucα3
Galβ4GlcNAcβ3Galβ4Glc
Lex-L
Galβ4GlcNAcβ3GalNAcβ4GlcNAcβ3Galβ4Glc
LN-LDN-L
Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc
LN-LN-L
Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc
LN-LN-LN-L
GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc
GlcNAc-LN-LN-L
Fucα3
Fucα3
Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc
LN-Lex-Lex-L

Figure 7-2



D

Neoglycoprotein	Observed Mass (Da)	Mol Glycan/Mol BSA
BSA (unconjugated)	66,431	-
Lactosyl-BSA	76,294	27.4
Triose-BSA	73,940	13.5
LNnT-BSA	69,340	1.2-7.0
Lex-L-BSA	72,108	1.1-8.1
LN-LDN-L-BSA	73,940	8.1
LN-LN-L-BSA	73,387	6.6
LN-LN-LN-L-BSA	72,334	2.3-5.1
LN-Lex-Lex-L-BSA	75,224	2.1-7.9

Figure 7-3

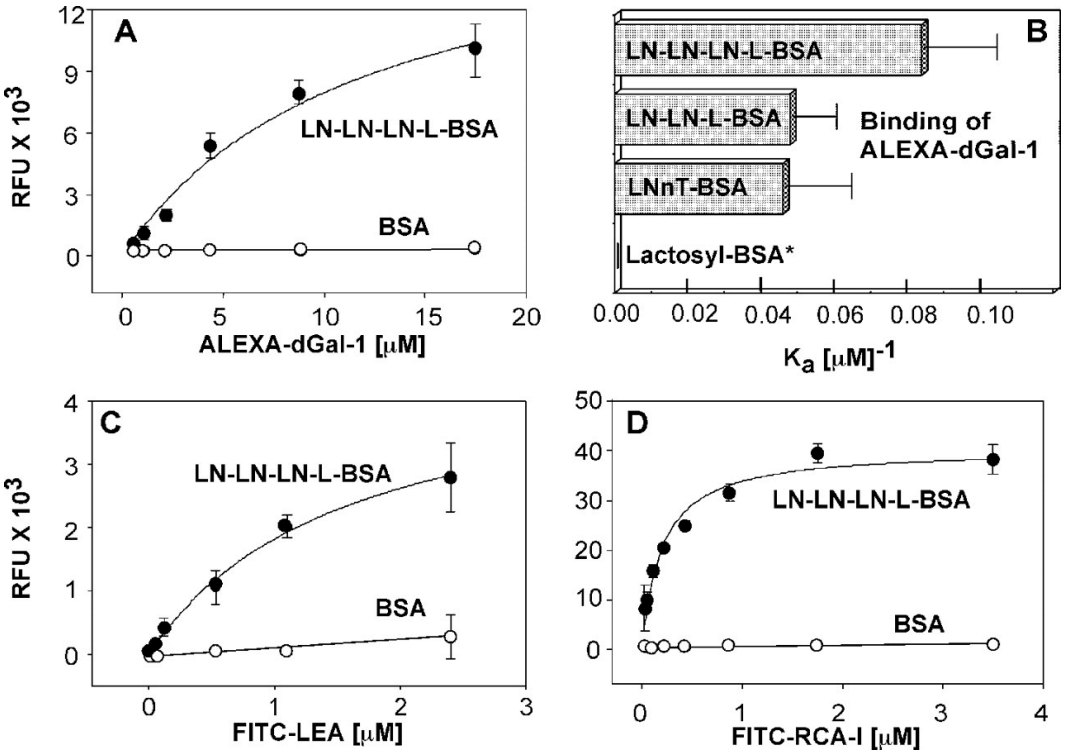

A
RFU X 10^3
LN-LN-LN-L-BSA
BSA
ALEXA-dGal-1 [μM]
B
LN-LN-LN-L-BSA
LN-LN-L-BSA
LNnT-BSA
Lactosyl-BSA*
Binding of ALEXA-dGal-1
Ka [μM]^-1
C
RFU X 10^3
LN-LN-LN-L-BSA
BSA
FITC-LEA [μM]
D
LN-LN-LN-L-BSA
BSA
FITC-RCA-I [μM]

Figure 7-4

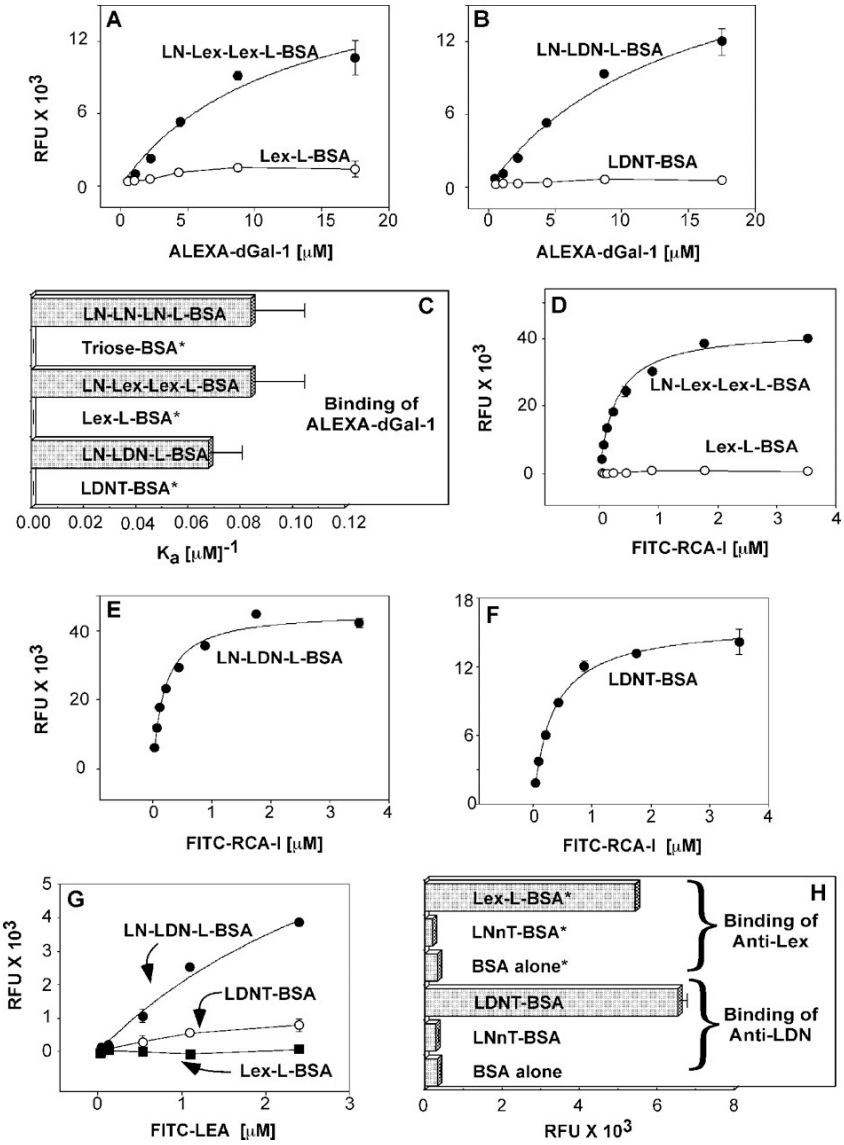

A
LN-Lex-Lex-L-BSA
Lex-L-BSA
RFU X 10^3
ALEXA-dGal-1 [μM]
B
LN-LDN-L-BSA
LDNT-BSA
ALEXA-dGal-1 [μM]
C
LN-LN-LN-L-BSA
Triose-BSA*
LN-Lex-Lex-L-BSA
Lex-L-BSA*
LN-LDN-L-BSA
LDNT-BSA*
Binding of ALEXA-dGal-1
Ka [μM]-1
D
LN-Lex-Lex-L-BSA
Lex-L-BSA
RFU X 10^3
FITC-RCA-I [μM]
E
LN-LDN-L-BSA
RFU X 10^3
FITC-RCA-I [μM]
F
LDNT-BSA
FITC-RCA-I [μM]
G
LN-LDN-L-BSA
LDNT-BSA
Lex-L-BSA
RFU X 10^3
FITC-LEA [μM]
H
Lex-L-BSA*
LNnT-BSA*
BSA alone*
Binding of Anti-Lex
LDNT-BSA
LNnT-BSA
BSA alone
Binding of Anti-LDN
RFU X 10^3

Figure 7-5

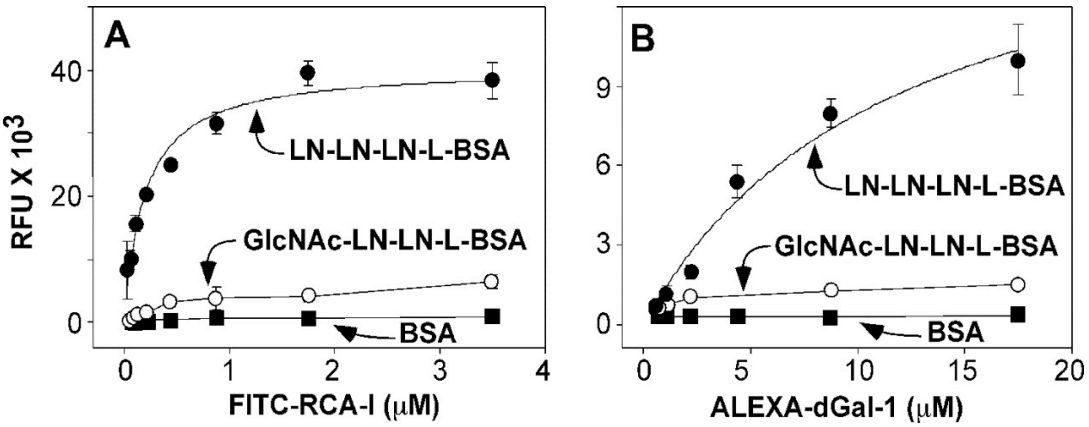

A
RFU X 10^3
40
20
0
LN-LN-LN-L-BSA
GlcNAc-LN-LN-L-BSA
BSA
0
1
2
3
4
FITC-RCA-I (μM)
B
9
6
3
0
LN-LN-LN-L-BSA
GlcNAc-LN-LN-L-BSA
BSA
0
5
10
15
20
ALEXA-dGal-1 (μM)

Table 7-1. Galectin-1 binding to neoglycoproteins requires a terminal N-acetyllactosamine unit.

	ALEXA Galectin-1 K_d* [μM]	FITC RCA K_d* [μM]	FITC LEA K_d* [μM]
LNLNLNL-BSA	23.7 ± 5.7	0.20 ± 0.03	2.22 ± 0.7
LNLNL-BSA	41.7 ±10.1	0.17 ± 0.08	3.01 ± 1.5
LNnT-BSA	42.9 ±16.2	0.14 ± 0.02	*N.D.*
Lactosyl-BSA	*N.D.*	0.30 ± 0.02	*N.D.*
LDNT-BSA	*N.D.*	0.34 ± 0.02	*N.D.*
LewL-BSA	*N.D.*	*N.D.*	*N.D.*
LNLewLewL-BSA	23.7 ± 5.8	0.28 ± 0.02	*N.D.*
LNLDNL-BSA	29.0 ± 5.3	0.19 ± 0.01	8.31 ± 6.7
Triose-BSA	*N.D.*	*N.D.*	*N.D.*

*The apparent K_d values, taken as the concentration of lectin that gave one-half maximal saturation of binding (1/2Max values) expressed in μM, are shown for each lectin binding to neoglycoproteins in the solid-phase binding assay using fluorescently labeled lectins as indicated. Each value was achieved using nonlinear curve fitting to a rectangular hyperbola. ND indicates those neoglycoproteins to which respective lectins displayed no detectable binding. Neoglycoprotein syntheses and lectin binding analyses were accomplished as outlined in the Materials and Methods.

FIGURE LEGENDS

Figure 7-1. Structures of glycans.

Figure 7-2. MALDI-TOF analysis of neoglycoproteins. Glycans from Fig. 1 were covalently linked to lysine residues of BSA through reductive amination, and the neoglycoproteins were analyzed by MALDI-TOF, as described in Materials and Methods. The peak molecular weights are indicated for several examples in (A) unconjugated BSA; (B) Triose-BSA ; and (C) LNnT-BSA. The observed mid-range peak masses of all neoglycoproteins and the range of conjugation in mol glycan/mol BSA are listed.

Figure 7-3. Binding of ALEXA-dGal-1, FITC-RCA-I and FITC-LEA to poly-N-acetyllactosamine neoglycoproteins. Solid phase fluorometric binding assays were accomplished as outlined in the Materials and Methods. (A) Binding of ALEXA-dGal-1 to LN-LN-LN-L-BSA and unconjugated BSA. (B) Apparent association constants (K_a in mM^{-1}) (($K_a = 1/K_d$)) for binding of ALEXA-dGal-1 to indicated neoglycoproteins. (C) Binding of FITC-LEA to LN-LN-LN-L-BSA and unconjugated BSA. (D) Binding of FITC-RCA-I to LN-LN-LN-L-BSA and unconjugated BSA. All assays were performed in triplicate and the average values are shown. Apparent affinity constants were calculated using a rectangular hyperbola equation to derive the nonlinear curve-fitting.

Figure 7-4. Binding of ALEXA-dGal-1, FITC-RCA-I, and FITC-LEA to chimeric neoglycoproteins. Solid phase fluorometric binding assays were accomplished as outlined in the Materials and Methods. (A) ALEXA-dGal-1 binding to LN-Lex-Lex-L-BSA and Lex-L-BSA. (B) Binding of ALEXA-dGal-1 binding to LN-LDN-L-BSA and LDNT-BSA. (C) Apparent association constants (K_a in mM^{-1}) (($K_a = 1/K_d$) for binding of

ALEXA-dGal-1 to indicated neoglycoproteins. (D) Binding of FITC-RCA-I to LN-Lex-Lex-L-BSA and Lex-L-BSA. (E) Binding of FITC-RCA to LN-LDN-L-BSA. (F) Binding of FITC-RCA-I to LDNT-BSA. (G) Binding of FITC-LEA to indicated neoglycoproteins. (H) Binding of monoclonal anti-Lex and anti-LDN to indicated neoglycoproteins. Binding of the primary antibody was detected by fluorescently-labeled goat-anti-mouse. Secondary antibody alone in the absence of primary antibody showed no difference in binding from that observed toward unconjugated BSA (data not shown). All assays were performed in triplicate and the average values are shown.

Figure 7-5. Lack of binding of ALEXA-dGal-1 and FITC-RCA-I to GlcNAc-LN-LN-L-BSA. (A) Binding of FITC-RCA-I to LN-LN-LN-L-BSA and to GlcNAc-LN-LN-L-BSA generated by treatment of the former neoglycoprotein with β-galactosidase, and to unconjugated BSA. (B) Binding of ALEXA-dGal-1 to indicated neoglycoproteins and unconjugated BSA and in (A). All assays were performed in triplicate and the average values are shown.

Chapter 8

Galectin-1, -2, and -3 Exhibit Differential Recognition of Sialylated Glycans and Blood Group Antigens

Human galectins have functionally divergent roles, although most of the members of the galectin family bind weakly to the simple disaccharide lactose (Galβ1-4Glc). To assess the specificity of galectin-glycan interactions in more detail, we explored the binding of several important galectins (Gal-1, Gal-2, and Gal-3) using a dose-response approach toward a glycan microarray containing hundreds of structurally diverse glycans, and compared these results to binding determinants on cells. All three galectins exhibited differences in glycan binding characteristics. On both the microarray and on cells, Gal-2 and Gal-3 exhibited higher binding than Gal-1 to fucose-containing A and B blood group antigens. Gal-2 exhibited significantly reduced binding to all sialylated glycans, whereas Gal-1 bound α2-3, but not α2-6, sialylated glycans and Gal-3 bound to some glycans terminating in either α2-3 or α2-6 sialic acid. The effects of sialylation on Gal-1, Gal-2, and Gal-3 binding to cells also reflected differences in cellular sensitivity to Gal-1, Gal-2, and Gal-3-induced phosphatidylserine (PS) exposure. Each galectin exhibited higher binding for glycans with poly-N-acetyllactosamine (polyLacNAc) sequences (Galβ1-4GlcNAc)$_n$ when compared to N-acetyllactosamine (LacNAc) glycans (Galβ1-4GlcNAc). However, only Gal-3 bound internal LacNAc within polyLacNAc. These results demonstrate that each of these galectins mechanistically differ in their binding to glycans on the microarrays, and that these differences are reflected in the determinants required for cell binding

and signaling. The specific glycan recognition by each galectin underscores the basis for differences in their biological activities.

INTRODUCION

The galectin family of β-galactoside-binding proteins has over a dozen human members and each galectin may have different biological roles and recognize different glycan receptors [162, 217, 281, 282]. These conclusions are supported by recent studies on the first three vertebrate galectins identified, termed galectin-1 (Gal-1), galectin-2 (Gal-2), and galectin-3 (Gal-3). Gal-1 inhibits mast cell degranulation [25], whereas Gal-3 induces degranulation in mast cells independently of IgE-mediated antigen stimulation [52]. Gal-1 blocks leukocyte chemotaxis [53], whereas Gal-3 has the opposite effect, inducing leukocyte chemotaxis [54] and the release of pre-formed IL-8 from neutrophils [55], which further augments chemotaxis of leukocytes [56]. In addition, while Gal-1 inhibits acute inflammatory responses through various mechanisms, including suppression of PLA-2-induced edema [57] and inhibition of neutrophil extravasation [53], Gal-3 enhances the extravasation of neutrophils and Gal-3 null mice also exhibit attenuated leukocyte infiltration following challenge [58]. Interestingly, patients with reduced Gal-2 expression were found to have reduced risk for myocardial infarction, suggesting that Gal-2 may also have pro-inflammatory roles [59]. Furthermore, Gal-1, Gal-2, and Gal-3 have all been reported to signal T cells through different receptors [13, 16, 221]. These types of studies suggest that Gal-1, Gal-2, and Gal-3 recognize distinct receptors on leukocytes.

There is compelling evidence, however, that different galectins may also recognize related receptors. For example, Gal-3 attenuates Gal-1 inhibition of growth in neuroblastoma cells at the receptor level, and both Gal-1 and Gal-3 induce superoxide production in human neutrophils [283-285]. Gal-1 and Gal-2 both induce surface exposure of phosphatidylserine (PS) in activated human neutrophils in the absence of

apoptosis through a Ca^{2+}-dependent pathway [200]. Therefore, while Gal-1, Gal-2, and Gal-3 may recognize discrete glycoconjugates, they may also recognize some common receptors. In this way, galectins likely exhibit unique versatility in a wide range of biological functions [281, 282].

Although some differences have been reported in glycan recognition by these galectins [63, 64], most studies do not show striking differences in glycan specificity [63, 64, 274, 286-290], leaving many questions remaining about their glycan recognition and subsequent effects. It has been suggested that differences in the biological effects of Gal-1 and Gal-3 result from differences in tertiary structure, rather than ligand binding properties [14, 218], since Gal-3 was thought to behave primarily as a monomer [291]. Tertiary structure differences may contribute to differences in cellular responses to different galectins [124, 218]; however, Gal-3 can form homo-oligomeric structures [124], which supports the likelihood that the major differences in biological functions by these lectins are due to differences in glycan recognition.

Previous studies on glycan recognition by galectins and most other glycan binding proteins (GBPs) have been limited due to the availability and diversity of glycans tested [63]. This limitation arises from the difficulty of synthesizing a large diverse library of glycan structures [292]. In addition, the methods of analysis may have also hindered the identification of differential specificity. For example, we recently found that the specificity of Gal-1 for glycans depends not only on the structure of glycans, but also the mode of their presentation [65], either in solid-phase or in solution. In equilibrium gel filtration assays, similar to other solution based assays [63, 64], Gal-1 binds glycans with a single N-acetyllactosamine (LacNAc) unit (Galβ1-4GlcNAc) equivalently to those with poly-N-acetyllactosamine (polyLacNAc) sequences $(Gal\beta 1\text{-}4GlcNAc)_n$; however, the dimeric

form of Gal-1 showed a significant preference for the polyLacNAc-containing glycans in solid-phase assays [65, 222]. Significantly, Gal-1 failed to recognize internal LacNAc units within polyLacNAc [65, 222], suggesting that this preference likely reflects favorable polyLacNAc conformational constraints of the terminal LacNAc unit that are enhanced by immobilization [65]. Gal-1 also recognized polyLacNAc-containing glycans on leukocyte surfaces with a similar affinity as observed for immobilized polyLacNAc glycans [65], corroborating the reliability of the solid-phase binding studies. Similarly, analyses of Gal-1, Gal-2, and Gal-3 utilizing frontal affinity chromatography or isothermal calorimetry, in which the glycans are free in solution, also do not reveal profound differences in carbohydrate recognition [63, 64, 293], further suggesting that galectin-glycan interactions may be most usefully tested in the context of immobilized glycan presentation. However, whether Gal-2 and Gal-3 behave like Gal-1 in showing a different glycan preference when glycans are immobilized has not been studied, nor has the *in vitro* binding data been correlated to binding determinants on cell surfaces

These issues prompted us to evaluate Gal-1, Gal-2, and Gal-3 interactions using immobilized glycans in a glycan microarray format that includes several hundred structurally diverse glycans [65, 292, 294], along with parallel studies of binding to a variety of human cells. To more accurately determine the binding specificity of Gal-1, Gal-2, and Gal-3 using the glycan microarray format, we evaluated binding of each galectin over a broad concentration range. This allowed us to extrapolate a binding isotherm for each galectin toward each respective glycan. To determine if the specificity obtained using this method reflected similar binding patterns toward cells, we tested the binding toward promyelocytic HL60 cells, which respond to signals by these galectins resulting in exposure of surface PS [12, 82], and tested binding to human erythrocytes. Our results provide novel insights into differential glycan recognition by each of these

galectins, and provide support for using glycan microarrays in conjunction with cell-binding studies to explore glycan recognition by glycan binding proteins [292].

RESULTS

Gal-1, Gal-2, and Gal-3 differentially recognize O- and N-glycans

For analyses on the glycan microarrays, we characterized the binding of Gal-1, Gal-2, and Gal-3 over a wide range of concentrations (~8 – ~0.2 mM). This allowed us to extrapolate a binding isotherm in an effort to more accurately estimate the glycan preference and specificity of each galectin. Such dose-dependency is important to define. Historically, studies using the glycan array display are done at saturating binding conditions and high lectin concentrations [295], making it difficult to distinguish subtle differences in specificity that may occur.

We first compared Gal-1, Gal-2, and Gal-3 in parallel to evaluate recognition of O- and N-glycans. Following biotinylation, each galectin was re-chromatographed over lactosyl-Sepharose to insure that homogenous preparations of active proteins were examined on the array. Gal-1 did not bind to the various core O-glycan structures, whereas it did bind to some core structures that were extended to contain a terminal LacNAc unit, as demonstrated for binding to extended core 2 and core 4 (Fig. 8-1A). Although Gal-2 showed binding toward extended core 4, Gal-1 showed significantly more binding than Gal-2 (Fig. 8-1A,B). In striking contrast, Gal-3 displayed very little binding toward all O-glycans tested (Fig. 8-1C).

Gal-1 exhibited the strongest binding toward the biantennary N-glycan (LacNAc2 NG) (Fig. 8-1D). In our studies the term “strong binding” refers to those glycans still recognized by the galectin at very low concentrations (≤mM). Gal-2 also exhibited strong binding toward LacNAc2 NG (Fig. 8-1E). By comparison, Gal-3 bound weakly to LacNAc2 NG (Fig. 8-1F). Interestingly, the presence of α2,3 sialic acid on the terminal

LacNAc unit of LacNAc2 NG completely blocked LacNAc2 NG recognition by Gal-2 (Fig. 8-1E) and reduced binding by Gal-1 (Fig. 8-1D), while it had no significant effect on recognition of LacNAc2 NG by Gal-3 (Fig. 8-1F). The presence of α2,6 sialic acid on LacNAc2 NG blocked glycan recognition by all three galectins (data not shown). These results demonstrate that Gal-1, Gal-2 and Gal-3 differ significantly in their recognition of N- and O-glycans. Interestingly, *Ricinus communis* agglutinin-I (RCA-I), a plant lectin that recognizes terminal Gal residues [260, 261], bound strongly to all N- and O-glycans terminating in LacNAc, demonstrating the presence and accessibility of these glycans (data not shown). Peanut agglutinin (PNA) bound to the core 1 structure (data not shown), consistent with previous results [296, 297]. Thiodigalactoside (TDG) blocked all galectin-glycan recognition, although sucrose had no effect on galectin binding (data not shown), further demonstrating the carbohydrate dependency of these interactions.

Gal-1, Gal-2, and Gal-3 exhibit differential recognition of LacNAc derivatives

To examine galectin interactions with LacNAc in more detail, we specifically evaluated galectin interactions with LacNAc-containing glycans. Unexpectedly, Gal-2 and Gal-3 showed very weak binding to LacNAc (Fig. 8-2B,C), although both bound more strongly to some derivatives of LacNAc (Fig. 8-2E,F). By contrast, Gal-1 bound LacNAc (Fig. 8-2A), although binding was weak relative to LacNAc2 NG (Fig. 8-1A). Gal-1, Gal-2, and Gal-3 failed to recognize LacDiNAc (GalNAcβ1-4GlcNAc) or Galα3Gal (Fig 2A-C).

Modifications of LacNAc, as commonly occurs *in vivo* [272], resulted in significant differences in glycan recognition by these three galectins. For example, α1-2-fucosylation of LacNAc did not alter Gal-1 recognition (Fig. 8-2D), although the same modification significantly increased recognition by Gal-2 and Gal-3 (Fig. 8-2E,F).

Similarly, the addition of a Galα1-3 terminal sequence to LacNAc also increased Gal-2 and Gal-3 binding (Fig. 8-2E,F), while it had no effect on Gal-1 binding (Fig. 8-2D). Interestingly, the addition of both Fucα1,2 and Galα1-3 to LacNAc (representing the blood group B antigen), significantly improved binding by both Gal-2 and Gal-3 (Fig. 8-2E,F), while significantly reducing recognition by Gal-1 (Fig. 8-2D). Similar results were obtained with the blood group A antigen (Fig. 8-3D-F). Although Gal-1 and Gal-2 exhibited no preference for type 1 or type 2 LacNAc (Galβ1-3GlcNAc versus Galβ1-4GlcNAc, respectively) either alone or in the context of modification (Fig. 8-2A,B,D,E), Gal-3 displayed higher binding toward type 2 LacNAc, compared to type 1 LacNAc, following modification (Fig. 8-2F). These results demonstrate that common modifications of LacNAc cause significant changes in glycan recognition by Gal-1, Gal-2, and Gal-3.

To further define the effect of substitutions of LacNAc on galectin binding, we examined the effects of both sulfation and sialylation of glycans. Gal-1 recognized α2,3 sialylated LacNAc and nonsialylated LacNAc equally (Fig. 8-2A; 8-3A), while α2,6 sialylation eliminated recognition (Fig. 8-3A). Interestingly, although α2,3 sialylation of LacNAc blocked recognition by RCA-I, α2,6 sialylation had no affect on RCA-I recognition (data not shown). *Sambucus nigra* agglutinin (SNA), previously demonstrated to prefer α2,6 sialylated LacNAc [298], also exhibited recognition of α2,6 sialylated LacNAc glycans (data not shown). These results show that the failure of Gal-1, Gal-2, and Gal-3 to recognize these glycans was not a reflection of their lack of accessibility on the microarray. Sialylation of LacNAc-containing glycans by either α2,3 or α2,6 linkage completely blocked recognition by both Gal-2 and Gal-3 (Fig. 8-3B,C). Furthermore, all three galectins failed to recognize GM1. This ganglioside was previously identified as a

potential ligand for Gal-1 (data not shown) [270, 299]. As a control for this finding, we found that cholera toxin subunit B (CT-B), previously demonstrated to recognize GM1 [300], bound tightly to GM1 in this solid phase format (data not shown). Since Gal-2 failed to recognize any sialylated compounds, we sought to partly test whether this simply reflected a lack of tolerance by Gal-2 for charge modification at the 3-OH of galactose. Interestingly, 3-O-sulfation of Gal residues in LacNAc significantly increased glycan recognition by Gal-2 (Fig. 8-3B), whereas 4-O- or 6-O-sulfation of Gal blocked binding (Fig. 8-3B and data not shown). Gal-3 also showed an increase in LacNAc recognition following specific sulfation of Gal (Fig 8-3C). Gal-1 exhibited the most significant increase in LacNAc recognition following sulfation (Fig. 8-3A), consistent with previous results [301]. These results demonstrate that all three galectins demonstrate preference for sulfated versus unsulfated glycans. This contrasts strongly in regard to sialylated glycans, since only Gal-1 bound to sialylated LacNAc.

Gal-1, Gal-2, and Gal-3 recognize polyLacNAc structures

Previous studies implicated polyLacNAc as a common determinant for galectin binding [65, 248, 302, 303]. We examined the binding of Gal-1, Gal-2, and Gal-3 toward polyLacNAc-containing glycans in the microarray format. Interestingly, all three galectins preferred longer polyLacNAc structures compared to the single LacNAc unit (Fig. 8-4A-C, Fig. 8-2A-C); Gal-1 and Gal-3 exhibited the most significant preference in binding (Fig. 8-4A,C). Importantly, tomato lectin (LEA), which primarily recognizes internal LacNAc within polyLacNAc [259], also showed higher binding toward polyLacNAc glycans in the microarray (data not shown). Furthermore, modification of polyLacNAc with terminal sialic acid or substitutions of the terminal Gal residues with a Fucα1,2 residue had no effect on Gal-1 or Gal-3 recognition of polyLacNAc (Fig 8-4A,C). By

contrast, α1,2-fucosylation significantly increased Gal-2 recognition of polyLacNAc, while α2,3-sialylation of polyLacNAc completely eliminated recognition by Gal-2 (Fig. 8-4B).

Gal-1, Gal-2, and Gal-3 display differential recognition of polyLacNAc glycans and chimera polyLacNAc glycans

Previous studies demonstrated that Gal-1 prefers polyLacNAc over LacNAc when they are immobilized on a solid-phase compared to solution binding, and that Gal-1 primarily recognizes the terminal LacNAc unit in polyLacNAc containing glycans [65, 222]. To further explore whether Gal-2 or Gal-3 might recognize terminal or internal LacNAc motifs within polyLacNAc, we analyzed galectin interactions with lactose, $(LacNAc)_2$ and $(LacNAc)_3$ using surface plasmon resonance (SPR) to measure binding in a solution-based equilibrium format. Gal-1, Gal-2, and Gal-3 exhibited rapid on and off rates for each immobilized glycan tested (data not shown), demonstrating that each lectin bound each glycan, that recognition was dose dependent, and that these interactions were readily reversible. Importantly, Gal-3 bound to lactose using this solution based assay, although it failed to recognize lactose or LacNAc when immobilized in the glycan array (Fig 8-3C). This demonstrates that the context of glycan presentation can significantly influence glycan recognition by Gal-3, similar to the earlier findings with Gal-1 [65]. Binding isotherms generated from the SPR data provided K_d values for each galectin-glycan interaction (Table 8-1). Gal-1 bound equivalently to lactose, $(LacNAc)_2$, and $(LacNAc)_3$ (Fig. 8-5A, D, G, Table 8-1), corroborating our earlier findings, and further demonstrating that Gal-1 principally recognizes the terminal LacNAc unit [65, 222]. Conversely, Gal-2 showed lower binding toward $(LacNAc)_2$ and $(LacNAc)_3$ in solution (Fig 5 B, E, H, Table 8-1), which demonstrates that Gal-2 does not recognize internal LacNAc motifs. By contrast, Gal-3 exhibited significant increases in affinity with each LacNAc extension (Fig 5 C, F, I, Table 8-1).

To determine whether similar recognition patterns dictate polyLacNAc recognition following immobilization, we examined the binding of Gal-1, Gal-2 and Gal-3 toward polyLacNAc and modified polyLacNAc glycans, as was previously partly done for Gal-1 [222]. Gal-1, Gal-2, and Gal-3 all recognized extended polyLacNAc $(LacNAc)_3$, although all three galectins did not bind to glycans terminating in Le^x (Le^x-Le^x-Le^x) (data not shown). Similarly, RCA-I and LEA did not bind Le^x-Le^x-Le^x (data not shown), consistent with previous results [65]. Importantly, Gal-1 showed significant binding toward polyLacNAc with internally fucosylated LacNAc units (LacNAc-Le^x-Le^x) (Fig. 8-5J). This result demonstrates that Gal-1 recognizes the terminal LacNAc unit in polyLacNAc chains, similar to the results obtained in solution-based assays. Furthermore, Gal-3 did not bind LacNAc-Le^x-Le^x (Fig. 8-5L), which also shows that it primarily recognizes internal LacNAc units within polyLacNAc. Importantly, RCA-I, like Gal-1, also recognized LacNAc-Le^x-Le^x, while LEA failed to demonstrate polyLacNAc recognition following internal LacNAc modification (data not shown). Furthermore, only Gal-3 recognized α2,6 sialylated polyLacNAc (Fig. 8-5L).

To complete our studies on the potential requirements of terminal LacNAc recognition within polyLacNAc on the solid phase array, we examined Gal-1, Gal-2, and Gal-3 recognition of polyLacNAc lacking a terminal galactose residue. The absence of the terminal galactose on polyLacNAc chains significantly reduced binding by Gal-1 and Gal-2 (Fig. 8-5J,K), although this did not affect recognition by Gal-3 (Fig. 8-5L). Control experiments corroborated these results, since we found that RCA did not bind polyLacNAc lacking a terminal galactose residue, while LEA binding was unaltered and robust (data not shown). These results demonstrate that Gal-3 prefers glycans containing polyLacNAc due to recognition of internal LacNAc sequences, while the

preference of polyLacNAc for Gal-1 and Gal-2 likely represents conformational constraints on the glycan that are enhanced when the glycans are immobilized on a surface. Taken together, these results demonstrate that Gal-1, Gal-2, and Gal-3 all bind polyLacNAc, but that this recognition arises from fundamentally different mechanisms of interaction.

Gal-1, Gal-2, and Gal-3 differentially recognize sialylated cell surface glycans

We next sought to determine whether the differences seen in glycan specificity for each galectin reflected their binding to cell surface glycans. To test this we examined the binding of Gal-1, Gal-2, and Gal-3 toward promyelocytic leukemia HL60 cells prior to and following enzymatic digestion of specific glycan structures. Leukocytes exhibit signaling responses following exposure to galectins [12, 82]. We used this response to ascertain whether the differential specificity observed on the array also occurs in the context of cell surface glcyans, and to give information about the nature and composition of glycans recognized on endogenous functional receptors. Furthermore, enzymatic modification of endogenous receptors allowed us to explore the recognition of glycans by galectins on cell surfaces, without using genetically-engineered modification, which can alter glycan expression in unpredictable ways. The importance of this is illustrated when considering that global alterations in glycosylation can have profound effects on glycoprotein trafficking, affecting surface half-life and total expression [304-306], making it difficult to easily translate differences in binding to possible differences in specificity as opposed to differences in cell surface receptor numbers. Furthermore, previous results suggest that specific modification of select ligands may be critical in conveying receptor specificity for Gal-1 [65], raising the question whether similar requirements exist for Gal-2 and Gal-3.

To examine binding of Gal-1, Gal-2 and Gal-3 toward HL60 cells, we first sought to determine whether binding of each galectin could be readily detected and whether this binding was carbohydrate dependent. Gal-1, Gal-2, and Gal-3 each bound HL60 cells (Fig. 8-6A,D). Importantly, binding was inhibited by lactose (Fig. 8-6A and data not shown), indicating that cell surface recognition was carbohydrate-dependent. On the glycan microarray, Gal-1 displayed no binding to α2,6 sialylated glycans, although it exhibited similar binding to either α2,3 sialylated or non-sialylated glycans. To test whether similar binding behavior might occur on the cell surface, cells were treated with either *Salmonella typhimurium* (ST) neuraminidase, an α2,3 specific neuraminidase, or *Clostridium perfringens* (CP), an α2,3-2,6 neuraminidase. Treatment of HL60 cells with either α2,3 neuraminidase or α2,3-2,6 neuraminidase resulted in a comparable reduction in MAL binding (Fig. 8-6B), indicating removal of α2,3 sialylated linkages following each treatment. Importantly, Gal-1 displayed a significantly greater increase in cell surface binding following treatment of cells with α2,3-2,6 neuraminidase, compared to that with α2,3 neuraminidase alone (Fig. 8-6D). These results suggest that Gal-1 has a greater tolerance for the α2,3 sialyl LacNAc modification than α2,6 sialylation on cell surface glycans. By contrast, Gal-2 bound significantly better to cells treated with either neuraminidase (Fig. 8-6C,D), suggesting that both α2,3 and α2,6 sialylation of endogenous ligands significantly inhibit binding. Unlike Gal-1 or Gal-2, Gal-3 demonstrated a less significant increase in binding following either treatment (Fig. 8-6D).

Although the binding of Gal-1 and Gal-3 toward CHO cells and mutant CHO cell derivatives (Lec mutants) has been extensively studied [307], the binding of Gal-2 toward CHO cells, which only generate α2-3 sialylated glycans, and Lec mutants has not been evaluated. Previous results demonstrated that Gal-1 and Gal-3 fail to increase

binding toward Lec 2 CHO cell mutants, which fail to generate α2-3 sialylated glycans [307], consistent with the present findings. Although the functional consequence of galectin binding to CHO cells is unknown, we next sought to determine whether similar binding preferences observed toward HL60 cells by Gal-2 also occurred on CHO cells. Importantly, Gal-2 exhibited a significant increase in binding toward Lec 2 cells, which fail to generate α2-3 sialylated glycans, when compared to wt CHO (Fig. 8-6E,F), consistent with HL60 cells (Fig. 8-6C,D), while it exhibited significantly reduced binding toward Lec 8 cells and Lec 1 cells (Fig. 8-6F), both of which fail to generate terminal LacNAc [307]. These results strongly suggest that sialylation of either linkage reduces Gal-2 glycan recognition. Taken together, these results demonstrate that sialylation uniquely modulates the recognition of cell surface glycans by Gal-1, Gal-2, and Gal-3.

Although these general changes in cell surface binding corroborate the binding observed toward immobilized synthetic glycans, it is not clear whether these changes occur on the actual receptors through which Gal-1, Gal-2, and Gal-3 signal cellular responses, or whether these changes simply reflect arbitrary glycan recognition following enzymatic manipulation or genetic alterations in cell surface glycans. To test this, we removed terminal sialic acid on HL60 cells with *Arthrobacter ureafaciens* (AU) α2,3-2,6 neuraminidase, which allows removal of sialic acid at physiological pH. Treatment of HL60 cells with AU neuraminidase resulted in similar alterations in cell surface glycan recognition by Gal-1, Gal-2, and Gal-3 (Fig. 8-7I,J), as observed following treatment with CP neuraminidase (Fig. 8-6D). To test the relationship of binding of galectins to their biological signaling activity, we examined their ability to induce surface exposure of phosphatidylserine (PS). We previously showed that some galectins induce PS exposure independently of apoptosis, by Src-kinase-mediated pathways [200]. We found that neuraminidase treatment of HL60 cells significantly enhanced their responses

to Gal-1 and Gal-2-induced PS exposure (Fig. 8-7B,C,F,G,K). By contrast, treatment of HL60 cells with neuraminidase failed to enhance sensitivity to Gal-3-induced PS exposure (Fig. 8-7D,H,K). Importantly, binding of Gal-1, Gal-2, and Gal-3 to HL60 cells before and after treatment with neuraminidase correlated with HL60 sensitivity to each galectin (Fig. 8-7J,K), demonstrating that these changes occur on the functional galectin counter receptors. To determine whether this altered sensitivity toward Gal-1 and Gal-2 occurred over a wide range of concentrations, we examined a dose response of HL60 toward Gal-1, Gal-2, and Gal-3 with or without pre-treatment with neuraminidase. HL60 cells experienced the greatest enhancement in sensitivity toward Gal-2-induced PS exposure following treatment with neuraminidase, with ~20 μM Gal-2 required to induce substantial PS exposure in non-treated and around ~2 μM to induce similar responses in neuraminidase treated cells (Fig. 8-8B). Neuraminidase-treated HL60 cells also showed enhanced PS exposure in response to Gal-1, although not to the same extent as Gal-2 (Fig. 8-8A). Interestingly, unlike Gal-1 and Gal-2, HL60 sensitivity toward Gal-3 was unaltered over all the concentrations tested (Fig. 8-8C). Gal-1, Gal-2, and Gal-3-induced agglutination paralleled the induction of PS exposure (data not shown), demonstrating that changes in cell surface recognition underscored alterations in sensitivity. Taken together, these results demonstrate that cell surface sialylation distinctly alters cellular signaling by Gal-1, Gal-2, and Gal-3.

Gal-1, Gal-2, and Gal-3 display differential recognition of cell surface polyLacNAc

The unique effects of sialylation on cell surface glycan recognition strongly suggested that Gal-1, Gal-2, and Gal-3 possess fundamentally different mechanisms of LacNAc recognition on the cell surface. Since Gal-1, Gal-2, and Gal-3 exhibited distinct modes of polyLacNAc interaction on the array, we next sought to determine whether distinct polyLacNAc interaction might also occur with cell surface ligands. We first treated HL60

cells with jack bean β-galactosidase to remove terminal galactose residues. Treatment of cells with β-galactosidase resulted in reduced RCA binding (Fig. 8-9A), indicating that many cell surface galactose residues were accessible to the enzyme. Importantly, a similar reduction in cell surface recognition by Gal-1 and Gal-2 occurred following β-galactosidase treatment (Fig. 8-9B,E). However, the binding of Gal-3 to cells was less affected by β-galactosidase treatment (Fig. 8-9B). These results suggest that Gal-3 does not share the same requirement for terminal galactose residues on the cell surface as seen for Gal-1 and Gal-2.

To determine the extent to which polyLacNAc recognition may be important for galectin binding, we next treated cells with either *Bacteroides fragilis* (BF) or *Escherichia freundii* (EF) endo-β-galactosidase; both can potentially cleave polyLacNAc glycans, yet exhibit different preferences of cleavage on the cell surface. BF endo-β-galactosidase substantially cleaves polyLacNAc on tetraantennary N glycans, while it has little activity toward polyLacNAc on triantennary N glycans; by contrast, EF endo-β-galactosidase efficiently cleaves polyLacNAc on N-glycans with fewer branches [308]. Treatment of cells with EF, but not BF, endo-β-galactosidase, resulted in reduced LEA binding (Fig. 8-9C,F). LEA is a plant lectin that has strong specificity for polyLacNAc chains [259]. Importantly, a similar reduction in glycan recognition by Gal-1, Gal-2, and Gal-3 also occurred following EF treatment (Fig. 8-9D,F). Similar results occurred if cells were pre-treated with neuraminidase (data not shown). Taken together, these results demonstrate that although the requirement for terminal galactose residues may differ between Gal-1, Gal-2 and Gal-3, each of these galectins recognize polyLacNAc-containing glycans on HL60 cells.

We were curious as to whether the ligands for these three galectins were co-localized with LEA-binding sites. Dual staining and confocal microscopy revealed that Gal-1, Gal-2, and Gal-3 all colocalized with LEA binding on the cell surface (Fig. 8-9C). Unlike Gal-1, Gal-2 and Gal-3, RCA-1 displayed a uniform cell surface binding to HL60 cells (Fig. 8-9D), suggesting that Gal-1, Gal-2 and Gal-3 bind specific ligands that may reside within membrane microdomains. Since treatment of cells with neuraminidase enhanced binding and signaling by Gal-1 and Gal-2, we next sought to determine whether treatment of cells with neuraminidase might alter the discrete binding pattern exhibited by Gal-1, Gal-2 and Gal-3. Although RCA-I continued to exhibit a uniform staining pattern following neuraminidase treatment (Fig. 8-9D), Gal-1, Gal-2 and Gal-3 staining appeared unaltered (Fig. 8-9E). To determine whether Gal-1, Gal-2 and Gal-3 remained co-localized with LEA following neuraminidase treatment, cells were co-stained with LEA. Importantly, even after treatment with neuraminidase, Gal-2 and LEA remained predominately co-localized (Fig. 8-9F), demonstrating that neuraminidase treatment likely exposes additional polyLacNAc-containing glycans, as opposed to simply increasing non-specific binding to LacNAc-containing glycans over the entire cell surface. Similar results were observed for Gal-1 and Gal-3 (data not shown). Taken together, these results strongly suggest that polyLacNAc-containing glycans are components of the receptors for Gal-1, Gal-2, and Gal-3 on the cell surface.

In contrast to Gal-1, both Gal-2 and Gal-3 displayed significant increases in binding toward H antigen (blood group O), blood group A and blood group B glycans, when compared to LacNAc on the solid phase array (Fig 8-2A-F). To further explore whether the predicted glycan specificity for Gal-1, Gal-2, and Gal-3 using the solid phase format represents cell surface glycan recognition, we examined the binding of these three galectins toward erythrocytes expressing different blood group glycans. Attempts to

analyze Gal-1, Gal-2 and Gal-3 erythrocyte cell surface binding directly was impeded by the fragile nature of erythrocytes and the high propensity of galectins to agglutinate these cells, which caused massive fragmentation upon flow cytometric analysis (data not shown). Therefore, we evaluated the agglutination potential of Gal-1, Gal-2 and Gal-3 toward erythrocytes and compared this to binding toward HL60 cells. Although agglutination requires both binding and dimerization of each galectin, comparison of agglutination between several cell types for a particular galectin should give a relative correlation for binding between cell types decorated with different glycans. Using this approach, Gal-2 and Gal-3 showed more potent agglutination of erythrocytes when compared to binding to HL60 cells, and demonstrated a preference for blood group A and blood group B erythrocytes (Table 8-2). By contrast, Gal-1 exhibited similar agglutination toward erythrocytes as toward binding to HL60 cells, confirming that Gal-1 has no blood group preference (Table 8-2). These results demonstrate that Gal-2 and Gal-3 display preference for blood group cell surface glycans, corroborating the predicted specificity obtained from the glycan microarray and further demonstrating that Gal-1, Gal-2, and Gal-3 exhibit unique glycan recognition.

DISCUSSION

In our studies we have explored the glycan specificity of the galectins Gal-1, Gal-2, and Gal-3 using a combined approach of a solid-phase assay system with glycan microarrays compared to cell surface binding experiments. Defining the dose response of Gal-1, Gal-2 and Gal-3 allowed a greater understanding of differences in glycan recognition and specificity between the galectins. These results demonstrate that each galectin exhibits significant differences in glycan binding specificity, but also show overlapping recognition of some glycans (Fig. 8-10, Table 8-3). These results provide new insights into both the overlapping and unique biological effects induced by these proteins. Taken together, our results, which provide relative apparent affinities for a wide variety of glycans (Table 8-3), suggest a new functional map of the carbohydrate recognition domain (CRD) for each galectin. This map illustrates the impact of modification of the basic LacNAc core on glycan recognition by Gal-1, Gal-2, and Gal-3 and suggests that unique subsites exist with each CRD. Modification of the LacNAc core can enhance, permit, or preclude modified LacNAc recognition by each respective galectin (Fig. 8-10). The binding modes of the different glycans suggest that each galectin may be viewed to have within its carbohydrate recognition domain a number of subsites, which we have indicated as A/B,C,D,D', and E.

The combined use of the glycan microarray coupled with the evaluation of cell surface binding provided a useful strategy to predict the nature of endogenously expressed glycans recognized by Gal-1, Gal-2, and Gal-3 on functional receptors. We chose to use glycan modification of fixed cells rather than manipulating the entire glycome of the cell; such manipulations could alter receptor trafficking and surface half-life in unpredictable ways [304-306]. Using this approach, one of the most striking examples of differential

glycan recognition, both on the array and on the cell surface, was the differential recognition of sialylated glycans by Gal-1, Gal-2, and Gal-3. For example, the presence of terminal sialic acid on glycans in either α2,3 or α2,6 linkage to galactose blocked their recognition by Gal-2 on the microarray. Similarly, Gal-2 binding to HL60 cells was significantly enhanced following treatment of cells with either α2,3 or α2,3-2,6 neuraminidase. By contrast, Gal-1 bound well to α2,3, but not α2,6, sialylated glycans on the microarray, which was consistent with our finding that treatment of HL-60 cells with α2,3-2,6 neuraminidase enhanced recognition of HL60 cells much more than a2,3 neuraminidase alone. Although Gal-3 failed to recognize α2,6 sialylated N-glycans lacking polyLacNAc, it did bind to α2,6 sialylated polyLacNAc on the microarray, suggesting that sialylation of polyLacNAc does not alter its binding by Gal-1. Similarly, Gal-3 exhibited little change in cell surface binding following treatment with either neuraminidase, suggesting that sialylation is not a key regulator of cellular sensitivity toward Gal-3. This finding is consistent with previous results demonstrating that α2,3 cell surface sialylation fails to significantly alter either Gal-1 or Gal-3 binding to CHO cells [307], whereas a reduction in α2,3 sialylation significantly enhanced Gal-2 binding toward CHO cells.

Changes in cell binding following neuraminidase treatment correlated with altered cellular sensitivity to each galectin, indicating that sialylation can regulate the functional receptors utilized by Gal-1, Gal-2, and Gal-3. Consistent with this, recent results demonstrate that α2,3 and α2,6 sialylation fails to alter T cell sensitivity toward Gal-3; by contrast, α2,6, but not α2,3, sialylation blocks T cells from responding to Gal-1 [22]. These results demonstrate that receptor sialylation can significantly and uniquely alter cellular sensitivity toward Gal-1, Gal-2, or Gal-3.

Differential recognition of polyLacNAc likely underscores the disparate effects of sialylation on glycan recognition and signaling by Gal-1, Gal-2, and Gal-3. Unlike Gal-3, Gal-1 and Gal-2 require the terminal LacNAc unit for polyLacNAc recognition, making modifications of the terminal Gal relevant in glycan recognition. By contrast, although Gal-3 did not bind to the α2,6 sialylated LacNAc2 N-glycan, which contains single terminal LacNAc units on each branch, Gal-3 bound well to α2,6 sialyl polyLacNAc. The ability of Gal-3 to bind polyLacNAc in the absence of a terminal, non-reducing and available Gal residue strongly suggests that Gal-3 binds α2,6 sialyl polyLacNAc as a result of recognizing internal, as opposed to terminal, LacNAc units.

The ability of Gal-1, Gal-2, and Gal-3 to recognize polyLacNAc is consistent with several previous studies [65, 222, 248, 290, 302, 303], and suggests that polyLacNAc may serve as the key glycan ligand for galectin-mediated effects. Indeed, previous studies demonstrated that HL60 cells possess few polyLacNAc glycans sensitive to *E. freundii* endo-β-galactosidase [309], suggesting that a specific polyLacNAc modification may serve as a functional glycan ligand to convey receptor specificity for Gal-1, Gal-2, and Gal-3 among many possible cell surface glycans containing LacNAc. Consistent with this, Gal-1, Gal-2, and Gal-3 co-localized with LEA, in restricted membrane microdomains that were identified by their binding of cholera toxin to the glycosphingolipid GM1 (Sean R. Stowell and Richard D. Cummings, unpublished data). Neuraminidase, which exposed binding sites for RCA, failed to alter Gal-1, Gal-2, and Gal-3 cell surface localization or co-localization with LEA, suggesting that polyLacNAc glycans promote specific interactions with Gal-1, Gal-2, and Gal-3 within the functional receptor(s).

Although biochemical approaches to assessing glycan binding properties of glycan binding proteins (GBPs) can provide useful information, the presentation of cell surface glycans may differ sufficiently from presentation in these artificial formats such that binding may not reflect actual binding specificity toward cells surface glycans. For example, although Gal-1 exhibits high binding toward polyLacNAc glycans on cell surface glycans, many studies using solution-based platforms fail to demonstrate any preference of polyLacNAc in solution [64, 65], demonstrating that cell surface presentation uniquely promotes preferential binding. This may result from conformational constrains only relevant following immobilization of the non-reducing ends of the glycans. Similarly, although the present platform provided specificity information that was largely corroborated by cell surface binding in this study and in previous studies, such as the high affinity of Gal-1 for sulfated LacNAc [301, 310] and polyLacNAc [65, 222] and Gal-3 for blood group antigens and polyLacNAc [63, 64, 274, 286-289], the presentation of many glycans on the microarray, including polyLacNAc, only reflect terminal glycan modifications in the absence of their context as extensions and modifications of N-glycans, O-glycans or glycolipids where they are normally presented on the cell surface. Therefore, the reduced binding of Gal-2 to polyLacNAc on the microarray, in comparison to the binding of Gal-2 to polyLacNAc on HL60 cells, may reflect a more specific requirement of Gal-2 for polyLacNAc presentation in the context of cell surface glycans. Such results demonstrate that although the current glycan microarray provides unique insights into the specificity of GBPs, the development of glycan microarrays from glycans harvested from natural sources will greatly facilitate the full elucidation of the binding requirements of GBPs. Furthermore, with a greater understanding of the carbohydrate binding requirements and specificity of Gal-1, Gal-2, and Gal-3 toward HL60 cell ligands, the identification of the functional glycoprotein receptors through which these galectins signal will be greatly facilitated. Studies of the

functional receptor will likely provide key insight into the functional glycans recognized by Gal-1, Gal-2, and Gal-3 and the mechanisms by which they preferentially recognized these glycans.

The ability of Gal-2 and Gal-3 to recognize blood group antigens corroborates some earlier findings for Gal-3 [64, 287-290], and raises important questions concerning the functional consequence of this interaction. Interestingly, we did not detect the expression of any blood group antigens in HL60 cells using blood-group specific monoclonal antibodies (Connie Arthur, Sean R. Stowell and Richard D. Cummings, unpublished data), and our results suggest that polyLacNAc-containing ligands, rather than blood group antigens, comprise the major ligands on these cells for Gal-1, Gal-2, and Gal-3. Furthermore, although Gal-1 exhibited similar agglutination potential toward HL60 cells and erythrocytes, Gal-2 and Gal-3 exhibited much higher agglutination toward erythrocytes. These results suggest that Gal-2 and Gal-3 recognize erythrocyte glycans containing blood group antigens compared to non-blood group ligands on HL60 cells. Blood group antigens are thought to elicit immune responses which underlie the formation of anti-blood group antigen antibodies [60, 311-314]. Thus, Gal-2 and Gal-3 may be important in innate immune mechanisms in recognizing blood group-related glycans. Consistent with this, recent studies suggest a role for Gal-3 in innate immune recognition of several pathogens, including *Candida albicans* [60], *Leishmania major* [62, 313] and *Schistosoma mansoni* [61, 315]. Given the relationship between galectins and the innate immunity [316], future studies will continue to examine the effect and modulation galectins impose on the innate immune system.

In addition to providing further understanding of galectin recognition of glycans, our study also potentially resolves some questions concerning previously suggested glycan

ligands for Gal-1 and Gal-3. For example, several reports suggested that Gal-1 is a negative growth regulator of neuroblastoma cells, possibly through interactions with the ganglioside GM1 [224, 270]. Follow-up studies demonstrated that Gal-1 exhibited binding to GM1 in solution at very high concentrations [299]. However, in the present study, Gal-1 failed to recognize GM1 at any concentration on the glycan microarray, although CT-B readily bound GM1 in the same assay system, corroborating an earlier study [64]. Thus, in the cells studied it is unlikely that GM1 is a functional receptor for Gal-1. Additional studies implicate Gal-1 and Gal-3 in mechanisms of neoplastic metastasis [317, 318], possibly through interactions with the disaccharide T antigen which is the basic unit of core 1 O-glycans [257, 319]. However, in our study Gal-1 and Gal-3 did not bind significantly to core 1 O- glycan, suggesting that other neoplastic glycans may be responsible for mediating these effects. However, unique presentation of these glycan *in vivo* may be required for proper galectin recognition. Future studies will continue to evaluate the endogenous glycans responsible for galectin mediating functions *in vivo.*

In summary, our results provide significant clarification and additional insight into the specificity of Gal-1, Gal-2, and Gal-3 for glycan ligands. The glycan array largely predicted the binding preferences of Gal-1, Gal-2, and Gal-3 toward cell surface glycans, illustrating the utility of the array in elucidating the carbohydrate binding preferences of Gal-1, Gal-2, and Gal-3. These results also provide additional biochemical understanding for the overlapping functions of each of these proteins, while also providing an explanation for the functionally unique and often opposing roles of these galectin family members *in vivo* [281, 282].

Figure 8-1

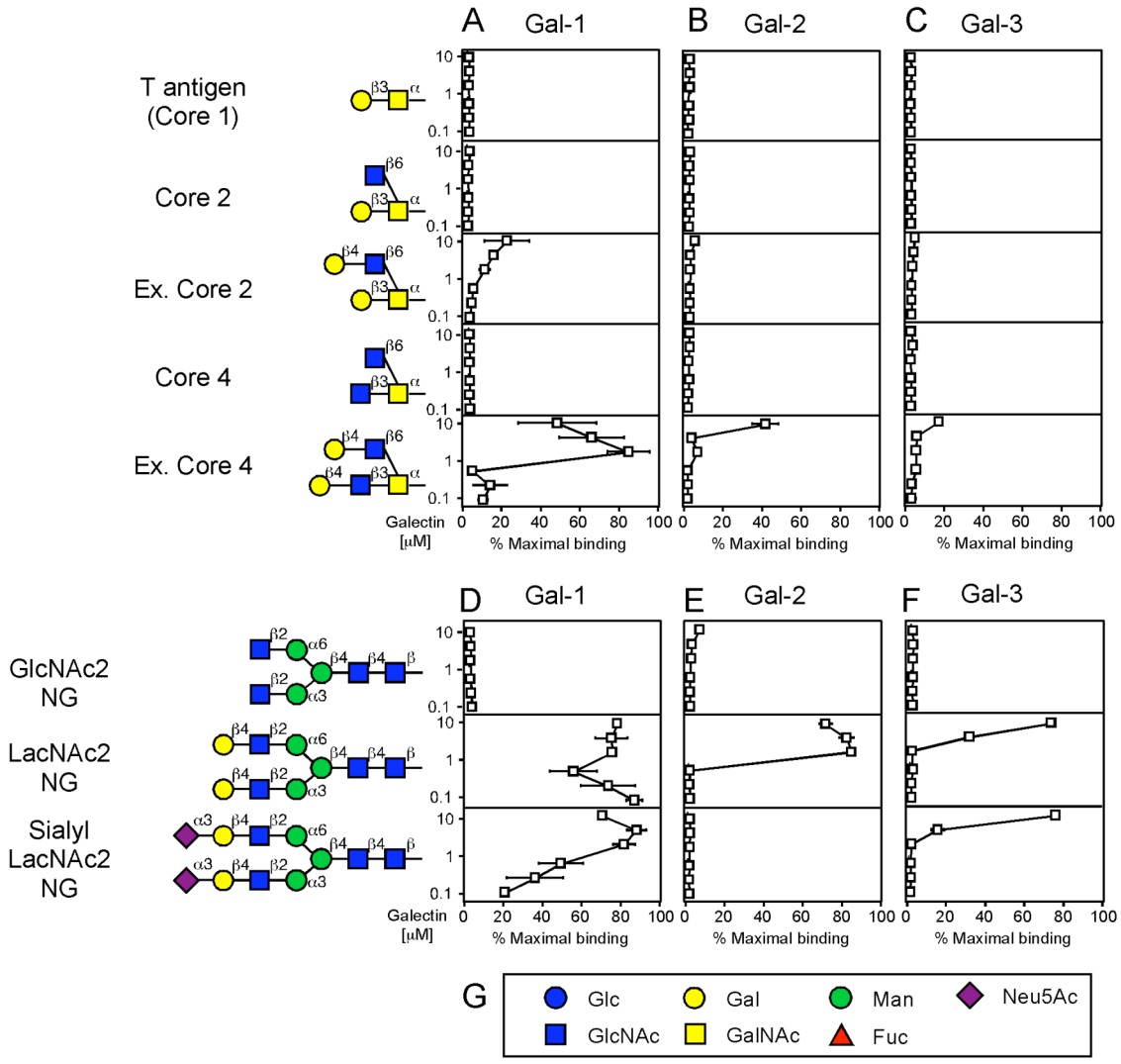

A
Gal-1
B
Gal-2
C
Gal-3
T antigen
(Core 1)
Core 2
Ex. Core 2
Core 4
Ex. Core 4
Galectin
[μM]
% Maximal binding
D
Gal-1
E
Gal-2
F
Gal-3
GlcNAc2
NG
LacNAc2
NG
Sialyl
LacNAc2
NG
G
Glc
Gal
Man
Neu5Ac
GlcNAc
GalNAc
Fuc

Figure 8-2

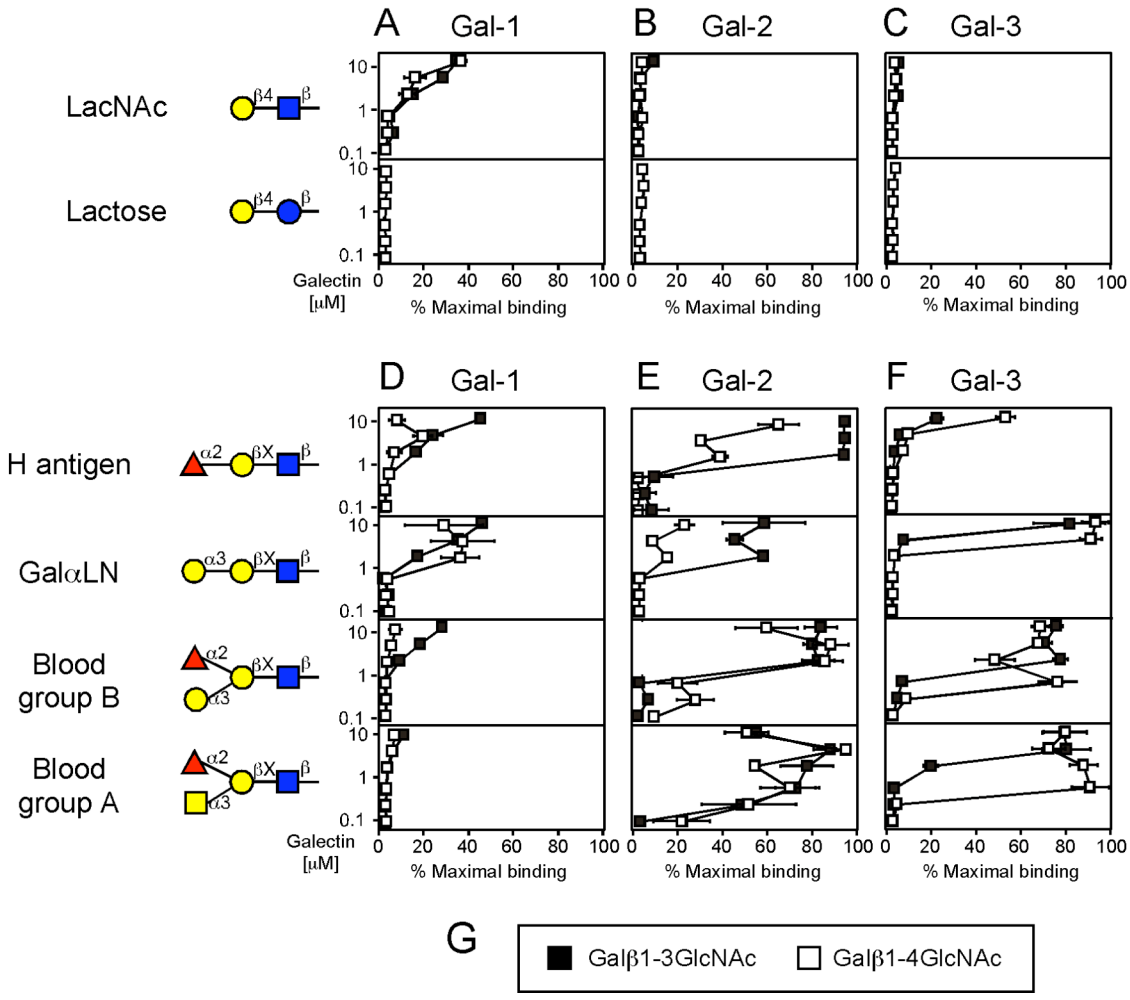

A
Gal-1
B
Gal-2
C
Gal-3
LacNAc
Lactose
β4
β
10
1
0.1
Galectin
[μM]
0
20
40
60
80
100
% Maximal binding
D
Gal-1
E
Gal-2
F
Gal-3
H antigen
GalαLN
Blood
group B
Blood
group A
α2
α3
βX
G
Galβ1-3GlcNAc
Galβ1-4GlcNAc

Figure 8-3

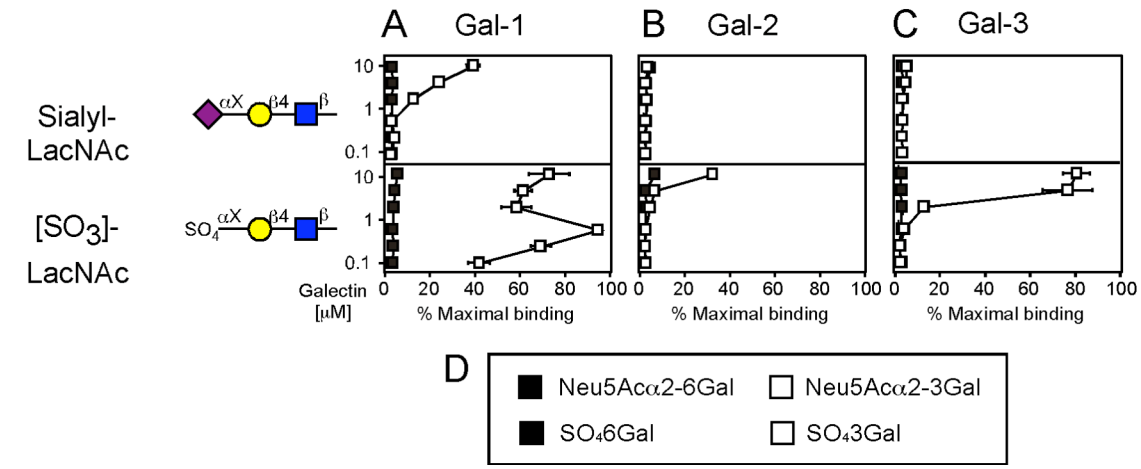

A
Gal-1
B
Gal-2
C
Gal-3
Sialyl-
LacNAc
[SO3]-
LacNAc
αX
β4
β
SO4
10
1
0.1
Galectin
[μM]
0
20
40
60
80
100
% Maximal binding
D
Neu5Acα2-6Gal
Neu5Acα2-3Gal
SO4 6Gal
SO4 3Gal

Figure 8-4

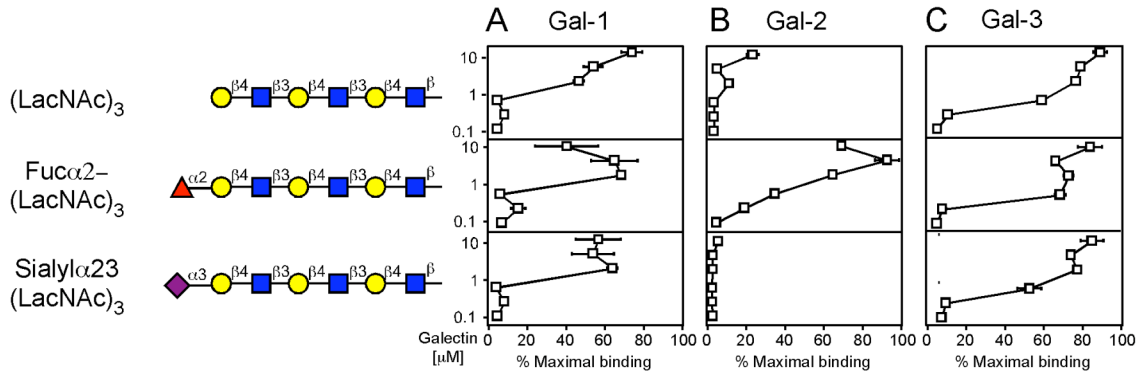

A Gal-1
B Gal-2
C Gal-3
(LacNAc)3
Fucα2–
(LacNAc)3
Sialylα23
(LacNAc)3
β4 β3 β4 β3 β4 β
α2 β4 β3 β4 β3 β4 β
α3 β4 β3 β4 β3 β4 β
10
1
0.1
Galectin
[μM]
0 20 40 60 80 100
% Maximal binding

Figure 8-5

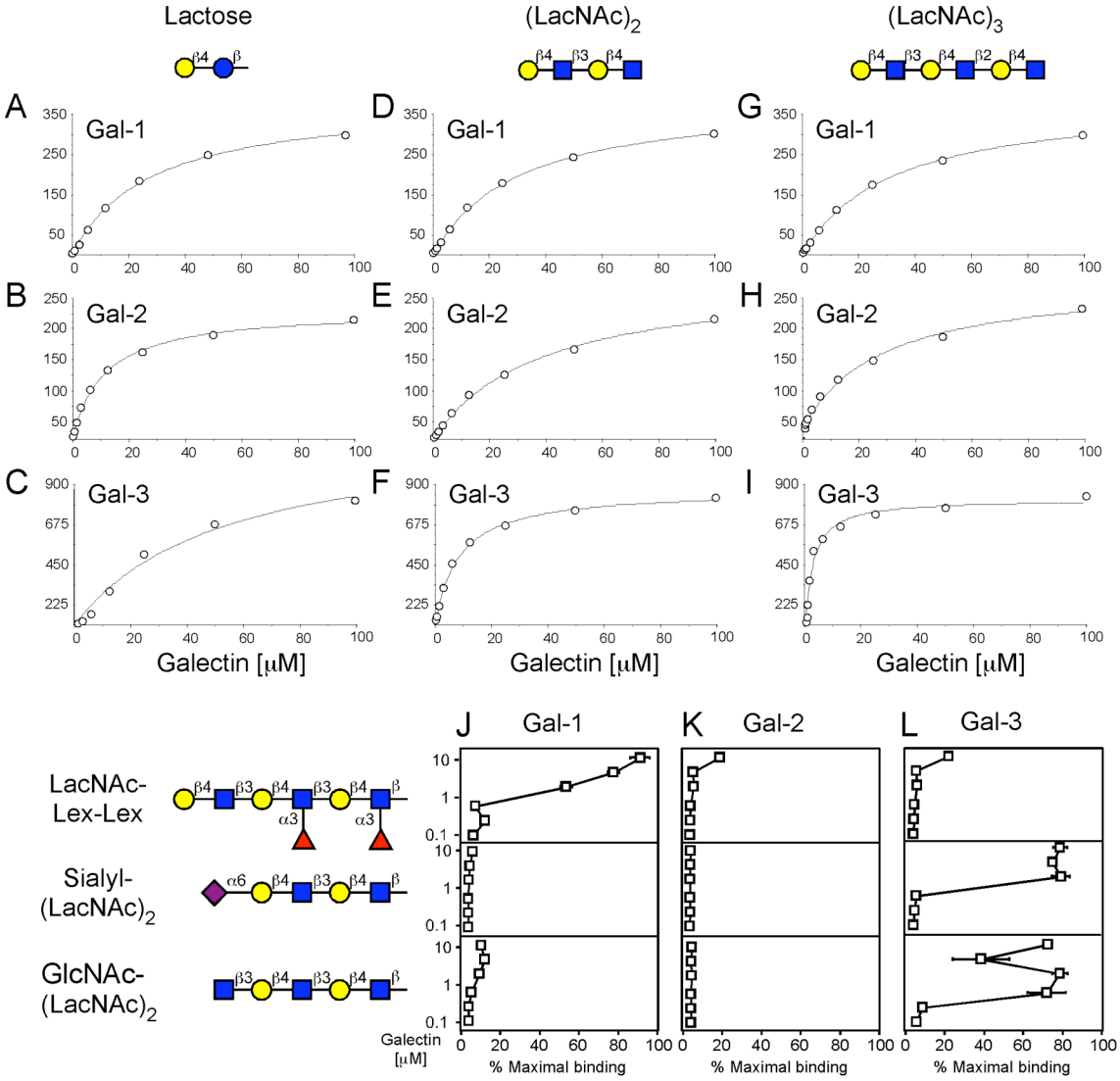

Lactose
(LacNAc)2
(LacNAc)3
A
Gal-1
B
Gal-2
C
Gal-3
D
E
F
G
H
I
Galectin [μM]
LacNAc-Lex-Lex
Sialyl-(LacNAc)2
GlcNAc-(LacNAc)2
J
K
L
% Maximal binding

Figure 8-6

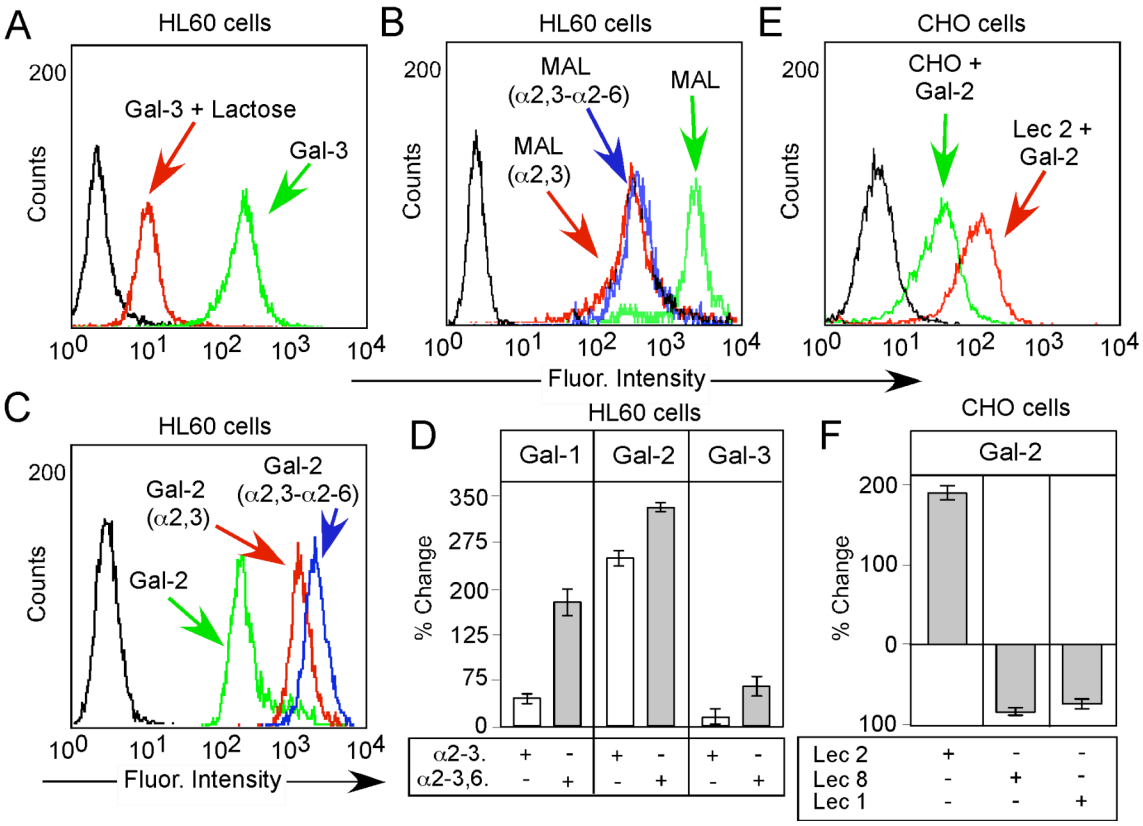

A
HL60 cells
Gal-3 + Lactose
Gal-3
Counts
200
B
HL60 cells
MAL
(α2,3-α2-6)
MAL
MAL
(α2,3)
E
CHO cells
CHO +
Gal-2
Lec 2 +
Gal-2
Fluor. Intensity
C
HL60 cells
Gal-2
(α2,3)
Gal-2
(α2,3-α2-6)
Gal-2
D
HL60 cells
Gal-1
Gal-2
Gal-3
% Change
α2-3.
α2-3,6.
F
CHO cells
Gal-2
Lec 2
Lec 8
Lec 1

Figure 8-7

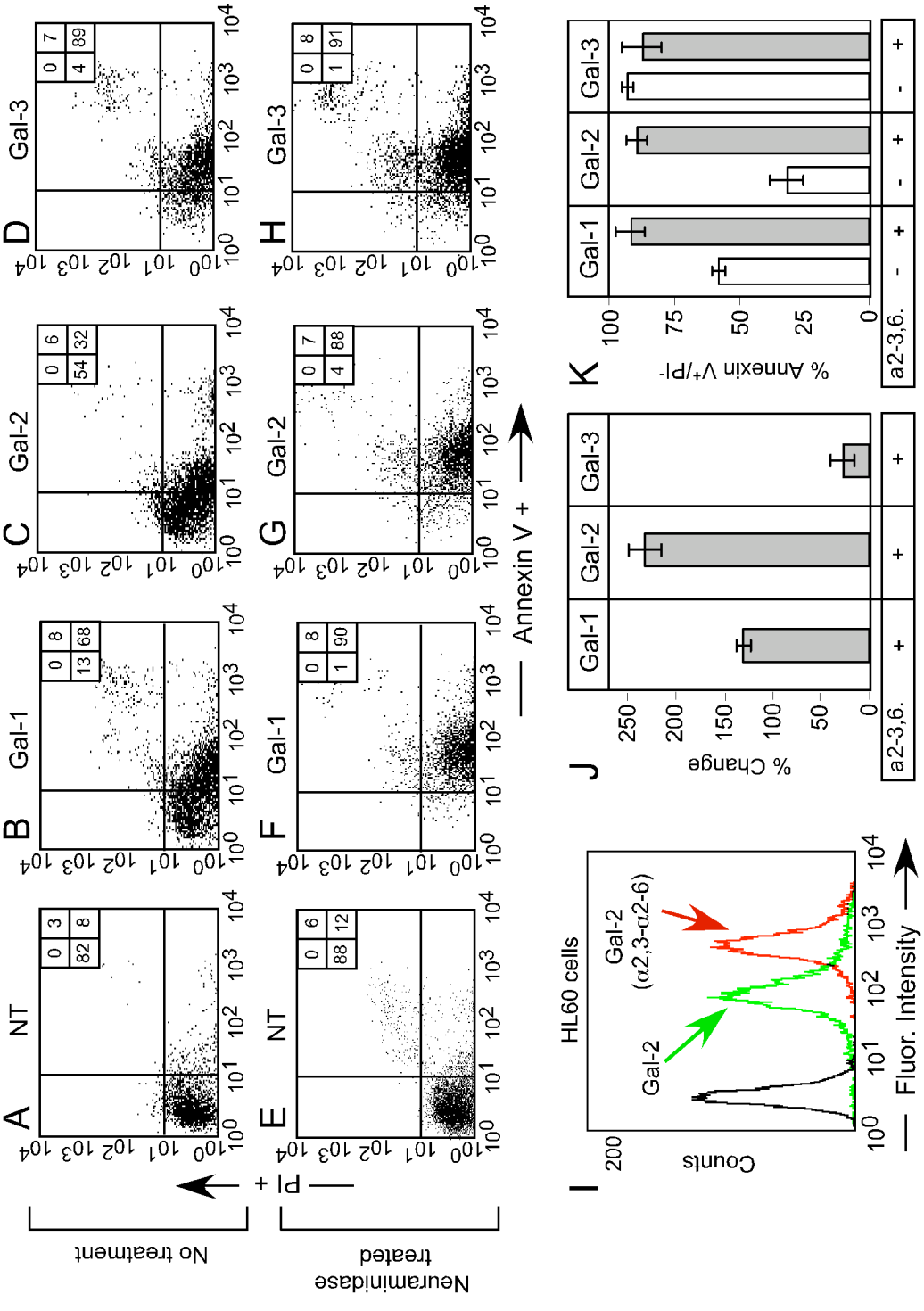

A
NT
B
Gal-1
C
Gal-2
D
Gal-3
E
NT
F
Gal-1
G
Gal-2
H
Gal-3
No treatment
Neuraminidase treated
PI +
Annexin V +
I
HL60 cells
Gal-2
Gal-2 (α2,3-α2-6)
Counts
Fluor. Intensity
J
% Change
Gal-1
Gal-2
Gal-3
a2-3,6.
K
% Annexin V+/PI-
Gal-1
Gal-2
Gal-3
a2-3,6.

Figure 8-8

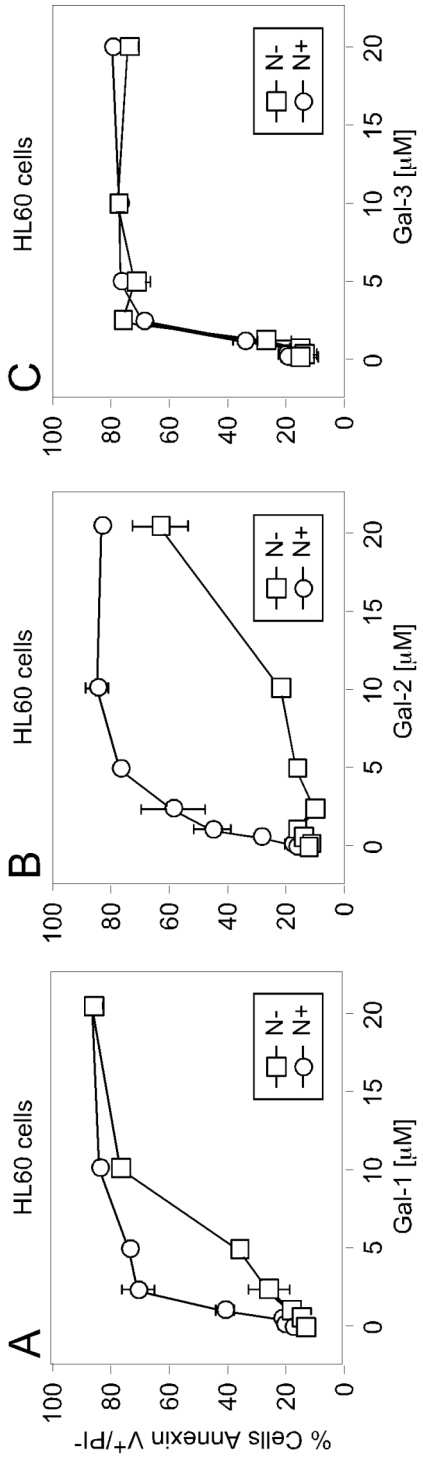
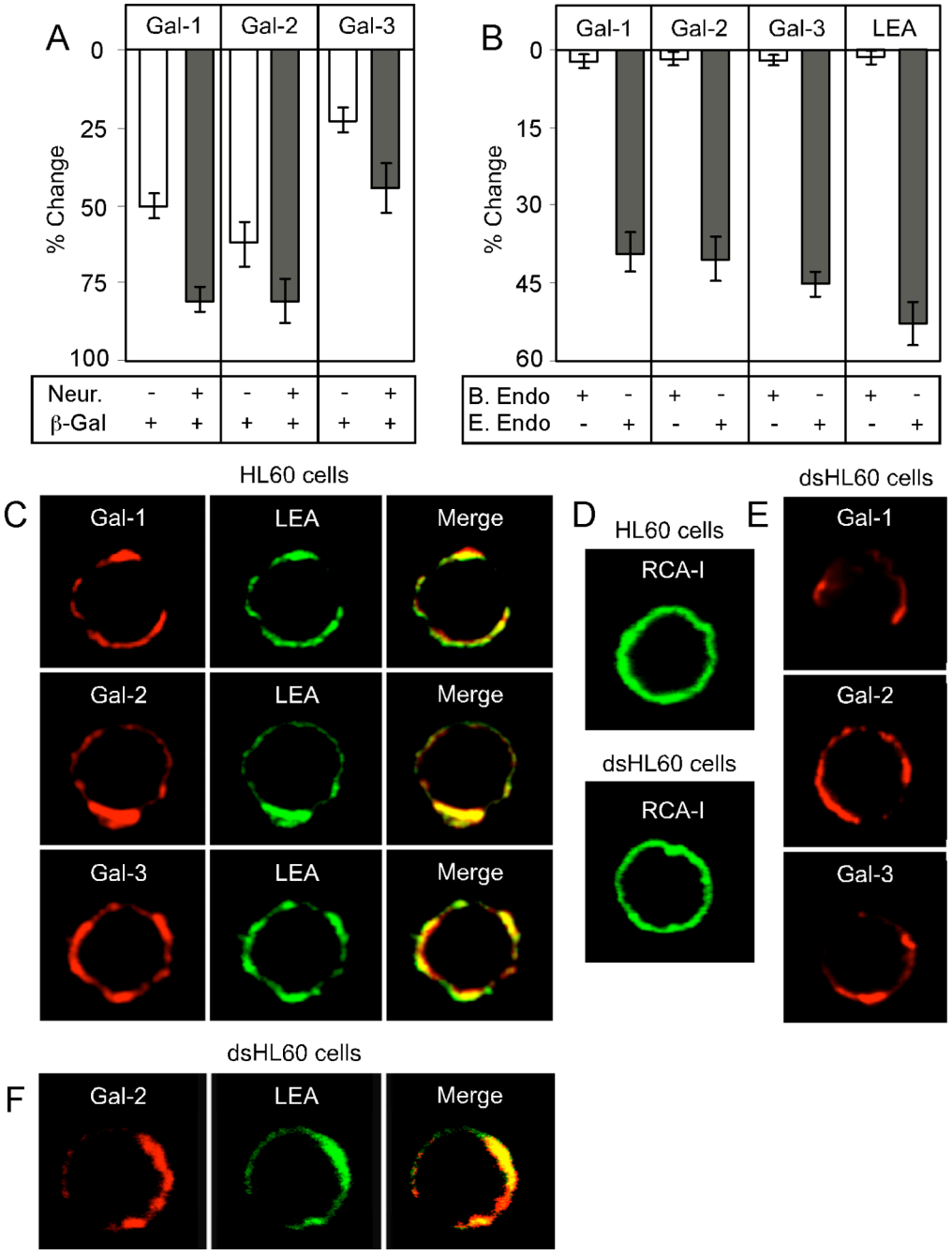
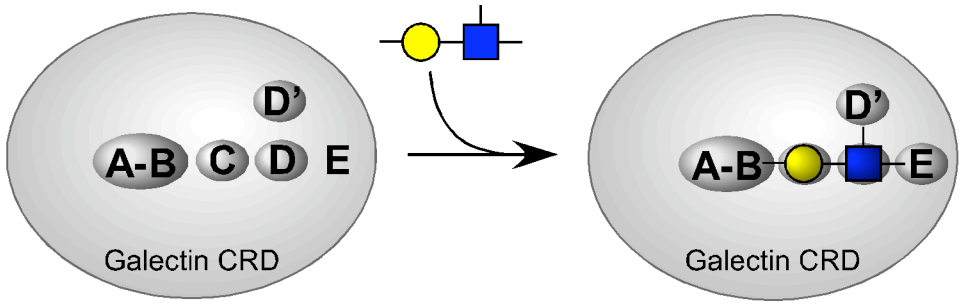

A
B
C
HL60 cells
HL60 cells
HL60 cells
% Cells Annexin V+/PI-
Gal-1 [μM]
Gal-2 [μM]
Gal-3 [μM]
N-
N+
0
20
40
60
80
100
0
5
10
15
20

Figure 8-9


A
Gal-1
Gal-2
Gal-3
% Change
0
25
50
75
100
Neur. - + - + - +
β-Gal + + + + + +
B
Gal-1
Gal-2
Gal-3
LEA
% Change
0
15
30
45
60
B. Endo + - + - + - + -
E. Endo - + - + - + - +
C
HL60 cells
Gal-1
LEA
Merge
Gal-2
LEA
Merge
Gal-3
LEA
Merge
D
HL60 cells
RCA-I
dsHL60 cells
RCA-I
E
dsHL60 cells
Gal-1
Gal-2
Gal-3
F
dsHL60 cells
Gal-2
LEA
Merge

Effects on Binding Relative to Galβ4GlcNAc

Red = reduces
Black = neutral
Blue = increases



Gal-1	Gal-2	Gal-3
D': Fucα3, Siaα6, 6-SO_3	D': Fucα3, Siaα6, 6-SO_3	D': Fucα3, Siaα6, 6-SO_3
A-B: GlcNAcβ4, Galα4, Galα3 + Fucα2, Galα4 + Fucα2, GalNAcα3 + Fucα2, GalNAcα4 + Fucα2, 3-SO_3, 4-SO_3, 6-SO_3, Fucα2, Galα3, Sialylα3, Siaα6, KDNα3	A-B: GlcNAcβ4, Galα4, Galα3 + Fucα2, Galα4 + Fucα2, GalNAcα3 + Fucα2, GalNAcα4 + Fucα2, 3-SO_3, 4-SO_3, 6-SO_3, Fucα2, Galα3, Sialylα3, Sialylα6, KDNα3	A-B: Galβ4GlcNAc, Galα4, Galα3 + Fucα2, Galα4 + Fucα2, GalNAcα3 + Fucα2, GalNAcα4 + Fucα2, 3-SO_3, 4-SO_3, 6-SO_3, Fucα2, Galα3, Sialylα3, Sialylα6 (LacNAc), Sialylα6(polyLacNAc), KDNα3
C: GalNAc, Galα6	C: GalNAc, Galα6	C: GalNAc
D: GalNAc, Gal, Glc	D: GalNAc, Gal, Glc	D: GalNAc, Gal, Glc
E: Gal, Man	E: Gal, Man	E: Gal, Man

Figure 8-10

Table 8-1 Analysis of Gal-1, Gal-2, and Gal-3 interactions with glycans utilizing SPR.

	Lactose (K_d [μM])	LacNAc-LacNAc (K_d [μM])	LacNAc- LacNAc-LacNAc (K_d [μM])
Gal-1 S1*	13.7	15.1	16.7
Gal-1 S2	14.4	16.9	19.2
Gal-1 S3	9.0	11.0	12.1
Gal-2 S1	24.9	60.5	62.6
Gal-2 S2	28.3	77.6	66.7
Gal-2 S3	32.3	58.4	54.5
Gal-3 S1	53.5	8.4	2.7
Gal-3 S2	60.7	9.5	3.2
Gal-3 S3	51.4	9.5	3.9

*S1, S2, and S3 represent separate analyses of each respective galectin under identical conditions.

Table 8-2 Cellular Agglutination by Gal-1, Gal-2, and Gal-3.

	O-RBCs*	A-RBCs	B-RBCs	HL60	dsHL60
Gal-1	1	1	1	1	0.25
Gal-2	0.13	0.06	0.06	20	0.5
Gal-3	0.5	0.25	0.25	1	1

*Cells were incubated with galectins in round bottom wells, as described in Materials and Methods. Each concentration in μM shown represents the lowest tested concentration in a serial dilution at which cell agglutination was vivo.

Table 8-3 Half-maximal binding of Gal-1, Gal-2, and Gal-3 to glycans on the microarray, derived from the dose-response curves

	>10 μM*	1 μM	<0.1 μM
Gal-1	Extended core 2 LacNAc BGB H antigen	Extended core 4 Sialylα3LacNAc2 NG	LacNAc2 NG SO_3LacNAc
Gal-2		LacNAc2 NG H antigen GalαLacNAc BGB	
Gal-3		BGB BGA $(LacNAc)_3$	

*Trivial names of each glycan as shown in Figs. 1, 2, 3, 4 and 5 are listed in the columns to which each respective galectin bound that glycan at 1/2Bmax.

FIGURE LEGENDS

Figure 8-1. Gal-1, Gal-2, and Gal-3 recognition of O-glycans and N-glycans. 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 to the highest bound ligand at each concentration tested by 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 used in this study.

Figure 8-2. Gal-1, Gal-2, and Gal-3 recognition of LacNAc and LacNAc-derivative glycans. 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 to the highest bound ligand by each respective galectin tested in this study. Glycan recognition is shown for Gal-1 (A, D), Gal-2 (B, E) and Gal-3 (C, F). (G) Legend for type 1 and type 2 structures. Black squares = type 1 LacNAc, white squares = type 2 LacNAc.

Figure 8-3. Gal-1, Gal-2, and Gal-3 recognition of sulfated LacNAc and sialylated LacNAc. 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 to the highest bound ligand by each respective galectin tested in this study. Glycan recognition is shown for Gal-1 (A), Gal-2 (B), and Gal-3 (C). (D) Legend describing linkages of sulfate and sialic acid. Black squares represent binding toward the glycan with attachment of sialic acid or sulfate to the 6 OH of galactose. White

squares represent binding toward the glycan with attachment of sialic acid or sulfate to the 3 OH of galactose.

Figure 8-4. Gal-1, Gal-2, and Gal-3 recognition of polyLacNAc. 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 to the highest bound ligand by each respective galectin tested in this study. Glycan recognition is shown for Gal-1 (A), Gal-2 (B), and Gal-3 (C).

Figure 8-5. Binding isotherms representing Gal-1, Gal-2, and Gal-3 recognition of lactose, $(LacNAc)_2$ and $(LacNAc)_3$ glycans using SPR. The binding isotherms and Kd values are shown for lactose with Gal-1 (A), Gal-2 (B), and Gal-3 (C), for $(LacNAc)_2$ with Gal-1 (D), Gal-2 (E), and Gal-3 (F), and for $(LacNAc)_3$ with Gal-1 (G), Gal-2 (H), and Gal-3 (I). (J-L) 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 to the highest bound ligand by each respective galectin tested in this study. Glycan recognition is shown for Gal-1 (J), Gal-2 (K), and Gal-3 (L).

Figure 8-6. Gal-1, Gal-2, and Gal-3 recognition of sialylated LacNAc on HL60 cells. (A) HL60 cells were incubated with 10 mg/ml Gal-3 with or without 50 mM lactose as indicated followed by flow cytometric analysis. (B) HL60 cells were treated with *Salmonella typhimurium* neuraminidase, an α2,3 specific neuraminidase or *Clostridium perfringens* α2,3-α2,6 neuraminidase for 12 h followed by staining with 10 mg/ml *Macckia amurensis* (MAL) as indicated followed by flow cytometric analysis. (C) HL60 treated as in B were stained with Gal-2 followed by analysis using flow cytometry. (D) Quantification of flow cytometic data. Bars represent the percent change in cell surface

binding when compared to the mean fluorescent intensity of non-treated cells. (E) A representative histogram of Gal-2 binding to CHO cells and Lec 2 cells as indicated. (F) Quantification of flow cytometric data of Gal-2 binding toward CHO cells. Bars represent the percent change in cell surface binding when compared to the mean fluorescent intensity wt CHO cells ± SD.

Figure 8-7. Desialylation differentially alters cellular sensitivity toward galectin-induced PS exposure. (A-H) HL60 cells were either incubated with buffer control (A-D) or 100 mU *Arthrobacter ureafaciens* neuraminidase (E-H) for 1 h followed by treatment of cells with 20 mM Gal-1, Gal-2, or Gal-3. Cells were washed in 50 mM lactose, stained with Annexin-V FITC (An-V) and propidium iodide (PI) followed by flow cytometric analysis. Cells that were An-V positive and PI negative were considered positive for PS exposure. Numbers represent the percent of total cells found in each quadrant. (I) HL60 cells were treated with 100 mU *Arthrobacter ureafaciens* neuraminidase for 1 h. followed by staining with 10 mg/ml Gal-2 and analysis by flow cytometry. (J) Quantification of Gal-1, Gal-2, and Gal-3 binding toward HL60 cells following treatment with *Arthrobacter ureafaciens* neuraminidase. Bars represent the percent change in cell surface binding when compared to the mean fluorescent intensity of non-treated cells ± SD. (K) Quantification of PS exposure ($An\text{-}V^+/PI^-$) on neuraminidase treated or untreated cells following treatment with Gal-1, Gal-2 or Gal-3 as outlined in A as mean percentage ± SD.

Figure 8-8. Dose response of desialylated HL60 cells to Gal-1, Gal-2 and Gal-3. HL60 cells were either incubated 100 mU *Arthrobacter ureafaciens* neuraminidase (circles) or buffer control (squares) for 1 h followed by treatment of cells with the indicated concentrations of Gal-1, Gal-2, or Gal-3 for 4 h. Cells were disengaged with

50 mM lactose and stained for PS exposure with Annexin-V-FITC. The percent cells Annexin V^+/Propidium $Iodide^-$ are shown ± SD.

Figure 8-9. Gal-1, Gal-2, and Gal-3 recognize polyLacNAc glycans on HL60 cells. (A) Gal-1, Gal-2 and Gal-3 binding toward HL60 cells following treatment with Jack bean β-galactosidase with or without pre-treatment of cells with *Arthrobacter ureafaciens* neuraminidase. (B) Gal-1, Gal-2 and Gal-3 binding toward HL60 cells following treatment with either *Bacteroides fragilis* or *Escherichia freundii* endo-β-galactosidase. Bars represent the percent change in cell surface binding when compared to the mean fluorescent intensity of non-treated cells ± SD. (C) Confocal analysis of Gal-1, Gal-2, Gal-3, and LEA binding toward cell surface glycans on HL60 cells. (D) Confocal analysis of RCA-I binding toward cell surface glycans on HL60 cells or HL60 cells treated with 100 mU *Arthrobacter ureafaciens* neuraminidase (dsHL60). (E) Confocal analysis of Gal-1, Gal-2, and Gal-3 binding toward cell surface glycans on HL60 cells treated with 100 mU *Arthrobacter ureafaciens* neuraminidase (dsHL60). (F) Confocal analysis of Gal-2 and LEA binding toward cell surface glycans on HL60 cells treated with 100 mU *Arthrobacter ureafaciens* neuraminidase (dsHL60).

Figure 8-10. Representative model of the CRD for Gal-1, Gal-2, and Gal-3 to illustrate the effect of LacNAc substitution on glycan recognition. Red indicates that the specific addition of the specified structure and linkage at the respective site reduces or abolishes recognition by the indicated galectin. Black refers to those modifications that had no effect on glycan recognition. Blue represents those modifications that produced more favorable binding than LacNAc alone.

Chapter 9

Innate Immune Lectin Provides Defense Against Molecular Mimicry

Over a century ago, Landsteiner demonstrated that blood group antigen ABO(H) expression can significantly differ between individuals within the human population [320]. Expression of these blood group antigens results in the deletion of cells that generate anti-blood group antibodies, allowing successful transfer of blood products between immunologically compatible individuals. However, this cellular deletion also generates a gap in the adaptive immune response toward blood group antigen bearing pathogens in blood group positive individuals, a response that normally occurs following exposure to these pathogens in blood group negative individuals within the first few years of life. The mechanism whereby this defect in adaptive immunity is overcome in blood group positive individuals remains unknown. Here we report that human galectin-8, a member of the galectin family of innate immune lectins, specifically recognizes and kills human blood group B antigen expressing *E. coli*, while it fails to alter the viability of other strains of *E. coli* or other gram-negative or gram-positive organisms. Galectin-8 killing resides entirely within its blood group B binding domain and occurs independent of complement, providing specific innate immune defense against blood group B positive pathogens regardless of the blood group status of an individual. These results demonstrate that specific innate defense lectins exist which provide immunity against pathogens that attempt to evade adaptive immunity through molecular mimicry.

INTRODUCTION

The major immunological barrier to tissue transplantation results from the diverse expression of carbohydrate-based blood group antigens ABO(H) within the human population, originally discovered by Landsteiner in 1900[320]. This discovery eventually enabled reliable transfer of life saving blood products from one individual to another. Although the identification of ABO(H) antigens significantly advanced our understanding of immunological barriers to transplantation, several fundamental questions remain unanswered concerning the presence of blood group antigen diversity. The persistence of ABO(H) antigens suggests some degree of selective advantage, although these antigens are not required for normal development or physiology. Recent studies suggest that blood group antigen diversity may provide a mechanism of pathogen evasion, where distinct ABO(H) antigen structures may reduce pathogen attachment and therefore infection[321]. In addition, although heterogeneity of blood group antigens may preclude transfer of tissue between certain individuals, this same diversity may provide a unique form of naturally occurring herd immunity within the human population against pathogens that may acquire these antigens during infection[322].

Although these recent studies suggest a role for the selection of blood group antigen diversity within the human *population*[321, 322], the consequence of blood group antigen expression on the immunological competence of an *individual* remains enigmatic. The development of blood group antibodies in blood group negative individuals results from exposure within the first few years of life to pathogens that generate these antigens as a form of molecular mimicry[311, 323]. However, blood group positive individuals delete cells that would normally be responsible for generating an adaptive immune response against blood group antigens[324, 325]. Although this reduces the probability of

autoimmunity, it also generates an obvious defect in the adaptive immune response, raising important questions regarding the ability of blood group positive individuals to defend themselves against blood group positive pathogens[323, 326]. The inability of blood group positive individuals to generate anti-blood group antibodies suggests that innate immune factors, which occur independent of adaptive immunity, likely offset this defect in acquired immunity.

RESULTS

Gal-8 and Gal-3 specifically recognize blood group B positive *E. coli.*

As ABO(H) antigens are composed of carbohydrate structures that only differ by the addition of distinct monosaccharide structures to terminal oligosaccharides [327], potential factors responsible for providing innate immunity toward blood group antigens must possess carbohydrate recognition or lectin activity. A growing list of carbohydrate binding proteins, including galectins and C-type lectins, recognize carbohydrate determinants on pathogens and participate in innate immune responses[96, 328]. However the actual carbohydrate structures recognized by many of these lectins remain unknown[292]. Previously, lack of diverse carbohydrate test libraries significantly inhibited our ability to elucidate the carbohydrate determinates recognized by these lectins [292]. However, recent advances in carbohydrate (glycan) microarrays enabled unprecedented insight into lectin binding specificity[292]. After screening nearly 100 different mammalian lectins with documented immunological activity, we discovered that human galectin-3 (Gal-3) and galectin-8 (Gal-8) displayed unprecedented specificity for human blood group antigens (Fig. 9-1A-C) at physiological (submicromolar) lectin levels. In contrast, galectin-1 (Gal-1), a related galectin family member, did not recognize blood group antigens. These results suggested that Gal-3 and Gal-8 might be uniquely poised to provide innate immunity toward blood group A- or B-bearing pathogens regardless of the blood group antigen status of an individual.

Gal-8 kills blood group B positive *E. coli.*

Bacteria generate a wide variety of carbohydrate based antigenic structures, many of which possess blood group antigen activity[311, 329]. The most well characterized of these, *E. coli* O86, cross-reacts with human anti-blood group B antibodies and induces

significant blood group B antibodies in previously unexposed individuals[311]. Although *E. coli* O86 generates an identical blood group B epitope as occurs in humans[314] , the context of this epitope may differ from common human presentations as occur on the glycan microarray. Therefore, we examined whether Gal-3 and Gal-8 recognize *E. coli* O86. Consistent with their ability to specifically recognize blood group antigens on the microarray, Gal-3 and Gal-8, but not Gal-1, bound to *E. coli* O86, hereafter referred to as blood group B positive *E. coli* (BG B^+ *E. coli*) (Fig. 9-1E-G). Binding of galectin to bacteria was inhibited by lactose, an inhibitor of galectin-carbohydrate interactions, which demonstrated that recognition of BG B^+ *E. coli* by Gal-3 and Gal-8 occurs through binding of bacterial cell surface carbohydrates.

Previous studies exploring galectin-pathogen interactions demonstrated that galectins serve as pathogen recognition proteins[96], suggesting Gal-3 and Gal-8 may facilitate immune activation in response to BG B^+ pathogens. However, several innate immune lectins directly alter pathogen viability[328]. Although galectins possess no known effect on prokaryote viability, we asked whether Gal-3 and Gal-8 might confer intrinsic immunity by directly killing BG B^+ *E. coli*. Incubation with Gal-8 caused direct killing of BG B^+ *E. coli,* while Gal-1 and Gal-3 failed to alter BG B^+ *E. coli* viability (Fig. 9-2A). Gal-8 displayed potent concentration-dependent killing of BG B^+ *E. coli* (LD_{50} ~0.1 μM) at concentrations similar to those used to evaluate Gal-8 carbohydrate specificity on the microarray (Fig. 9-2C). In addition, Gal-8-treated BG B^+ *E. coli* lost all motility compared to untreated BG B^+ *E. coli* when viewed after 2 hours of treatment, further demonstrating a prominent loss of viability (data not shown). Similar to Gal-8 binding to BG B^+ *E. coli*, lactose completely inhibited Gal-8-induced death, while sucrose, a disaccharide unable to inhibit Gal-8-carbohydrate interactions, failed to alter Gal-8 killing of BG B^+ *E. coli* (Fig. 9-2B). Taken together, these results demonstrated that Gal-8 directly kills BG B^+ *E. coli*

through recognition of cell surface carbohydrates. Killing of BG B^+ *E. coli* by Gal-8 did not require complement, demonstrating that this lectin fundamentally differs from other innate immune lectins, such as mannan binding proteins (MBP), which do not directly alter viability but activate complement following pathogen recognition[330].

Gal-8 kills blood group B positive *E. Coli* entirely through its C-terminal blood group binding domain.

Unlike Gal-1 and Gal-3, which contain a single carbohydrate recognition domain, Gal-8 possesses two distinct carbohydrate recognition domains[331], suggesting that Gal-8 may utilize one domain for target recognition and the other domain for killing the target once bound, similar to many prokaryotic AB toxins[332]. To test this, we mutated each carbohydrate recognition domain to determine which domain recognizes BG B^+ *E. coli.* Inactivation of the C terminal CRD (Arg223→His) (Gal-8CM) eliminated recognition of blood group antigens on both the glycan microarray and BG B^+ *E. coli* (Fig. 9-3A,C), while the analogous mutation in the N terminal CRD (Arg69→His) (Gal-8NM) failed to alter blood group antigen recognition in either context (Fig. 9-3B,C). Importantly, Gal-8NM, but not Gal-8CM, also retained the ability to kill BG B^+ *E. coli* (Fig. 9-3D,E), which demonstrated that Gal-8-mediated killing required carbohydrate recognition only by the blood group binding domain of Gal-8. To determine whether the N terminal domain may be required for Gal-8 killing independent of carbohydrate recognition, we expressed the individual domains of Gal-8. While the N terminal domain (Gal-8N) failed to bind to blood group antigens on either the array or BG B^+ *E. coli* (Fig, 9-3F,H), the C terminal domain of Gal-8 (Gal-8C) retained the ability to independently recognize blood group antigens and kill BG B^+ *E. coli* (Fig. 9-3G,H-J), which demonstrated that recognition and killing of BG B^+ *E. coli* by Gal-8 resides entirely within its blood group binding domain.

Gal-8 specifically kills blood group B positive *E. Coli.*

The ability of the blood group binding domain of Gal-8 to independently kill BG B^+ *E. coli* (Fig. 9-3G) suggested that Gal-8 might be specifically bactericidal for BG B^+ *E. coli*. To test this, we examined whether Gal-8 recognizes strains of *E. coli* that fail to express the blood group B antigen. Although Gal-8 recognizes BG B^+ *E. coli*, it did not significantly bind to or affect the viability of BG B^- *E. coli* (Fig. 9-4A,B). In addition, Gal-8 failed to recognize or kill other gram-negative BG B^- species *K. pneumoniae* and *P. aeruginosa* and failed to bind or alter the viability of gram-positive *S. aureus* (Fig. 9-4E-H). Furthermore, Gal-8 specifically killed BG B^+ *E. coli* within a mixed population of BG B^+ and BG B^- bacteria (Fig. 9-4I-L). Taken together, these results demonstrate that Gal-8 specifically kills BG B^+ *E. coli*.

DISCUSSION

The generation of ABO(H) antigen diversity in the human population likely facilitated pathogen evasion during human evolution[321]. However, the generation of this diversity could have come at a significant fitness cost, as development of these antigens precludes adaptive immune responses against blood group-bearing pathogens. The ability of Gal-8 to specifically kill BG B^+ *E. coli* is distinct from other innate immune defenses[328] and provides a unique example of a very specific innate immune factor that allows for *individual* protection against blood group positive pathogens regardless of the blood group status of that individual. As many human pathogens decorate their surfaces with diverse carbohydrate structures and many of these structures have similarities to human antigens, the ability of Gal-8 to specifically kill BG B^+ *E. coli* may reflect a common feature of other innate immune lectins. Through recognition of pathogens that generate structures similar to human antigens, these innate immune factors likely protect against attempts by pathogens to evade adaptive immunity through molecular mimicry, filling an important gap generated following selection of cells involved in adaptive immunity[325]. Although galectins may normally provide adequate protection in blood group positive individuals against blood group positive pathogens, consumption of high quanties of naturally occurring galectin-ligand inhibitors, such as lactose, may distrupt galectin-induced immunity. Indeed, that ability of lactose in inhibit the ability of Gal-8 to kill blood group B postive bacteria not only demonstrates the critical involvement of carbohydrate recognition in Gal-8 killing, but it also suggests that individuals with blood group antigen expression with blood group B positive flora may experience a transient inhibition of the innate immune activities of galectins following ingestion of lactose. As a result, aquired lactose intolerance may actually reflect inhibition of natural immunity, allowing overgrowth of blood group positive flora that

generates the classic constilation of symptoms that characterize aquired lactose intolerance. Future studies will address these intriguing questions. In contrast to galectin-mediated innate immunity, adaptive immunity continues to protect individuals against the nearly infinite and unpredictable diversification of pathogen antigens unrelated to human antigens.

Figure 9-1

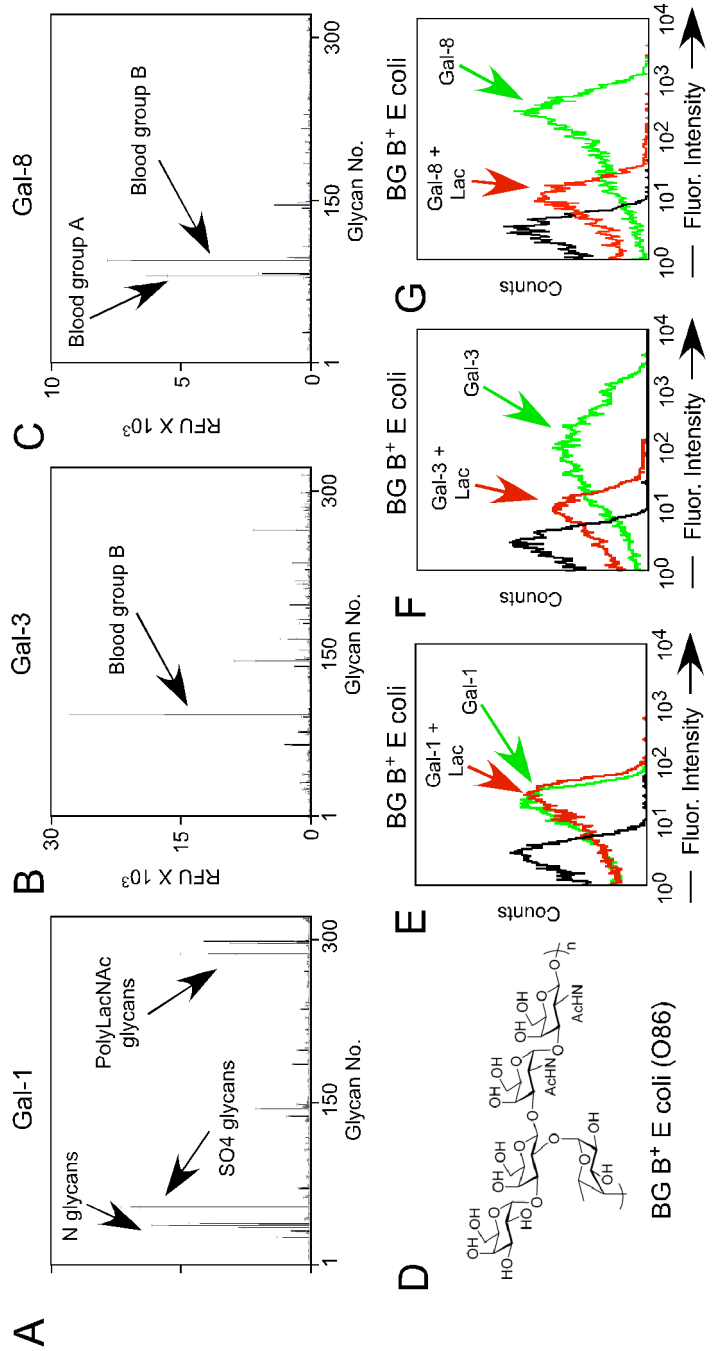

A
Gal-1
N glycans
PolyLacNAc glycans
SO4 glycans
RFU X 10³
Glycan No.
B
Gal-3
Blood group B
C
Gal-8
Blood group A
Blood group B
D
BG B⁺ E coli (O86)
E
BG B⁺ E coli
Gal-1 + Lac
Gal-1
F
Gal-3 + Lac
Gal-3
G
Gal-8 + Lac
Gal-8
Counts
Fluor. Intensity

Figure 9-2

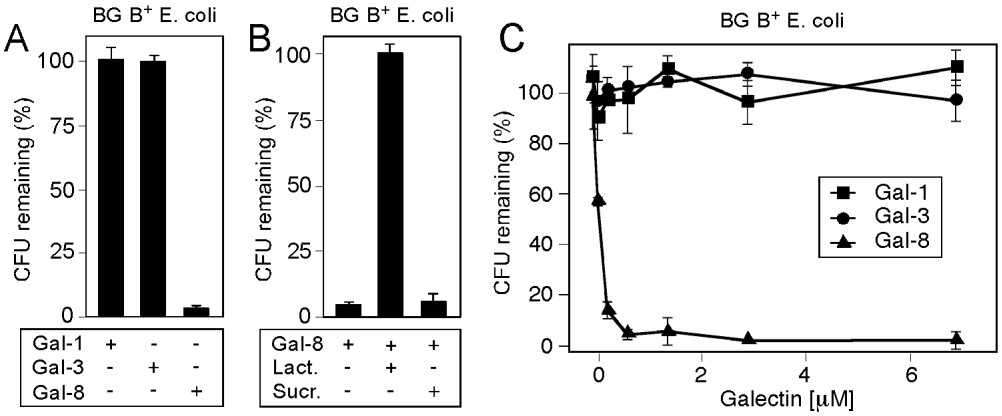

A
BG B+ E. coli
CFU remaining (%)
100
75
50
25
0
Gal-1 + - -
Gal-3 - + -
Gal-8 - - +
B
BG B+ E. coli
CFU remaining (%)
100
75
50
25
0
Gal-8 + + +
Lact. - + -
Sucr. - - +
C
BG B+ E. coli
CFU remaining (%)
100
80
60
40
20
0
0
2
4
6
Galectin [μM]
Gal-1
Gal-3
Gal-8

Figure 9-3

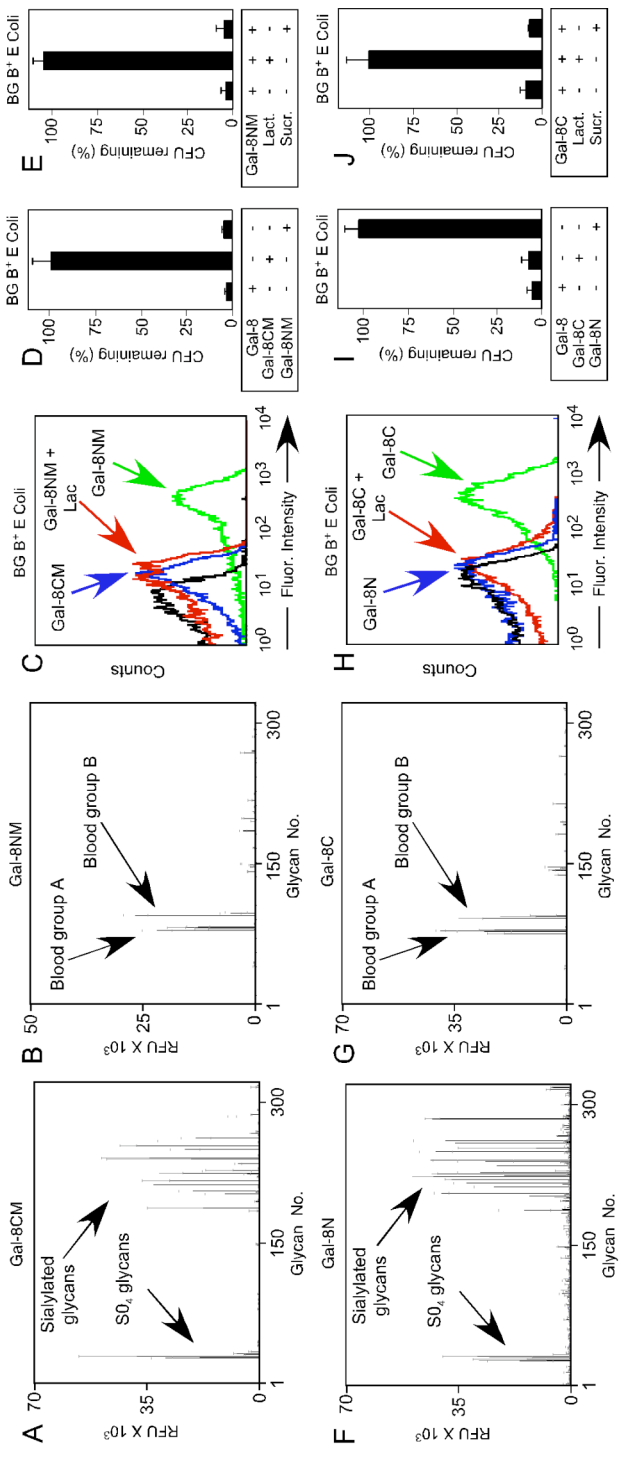

A
Gal-8CM
Sialylated glycans
SO_4 glycans
RFU X 10^3
Glycan No.
B
Gal-8NM
Blood group A
Blood group B
C
BG B^+ E Coli
Gal-8CM
Gal-8NM + Lac
Gal-8NM
Counts
Fluor. Intensity
D
BG B^+ E Coli
CFU remaining (%)
Gal-8
Gal-8CM
Gal-8NM
E
BG B^+ E Coli
CFU remaining (%)
Gal-8NM
Lact.
Sucr.
F
Gal-8N
Sialylated glycans
SO_4 glycans
G
Gal-8C
Blood group A
Blood group B
H
BG B^+ E Coli
Gal-8N
Gal-8C + Lac
Gal-8C
I
BG B^+ E Coli
CFU remaining (%)
Gal-8
Gal-8C
Gal-8N
J
BG B^+ E Coli
CFU remaining (%)
Gal-8C
Lact.
Sucr.

Figure 9-4

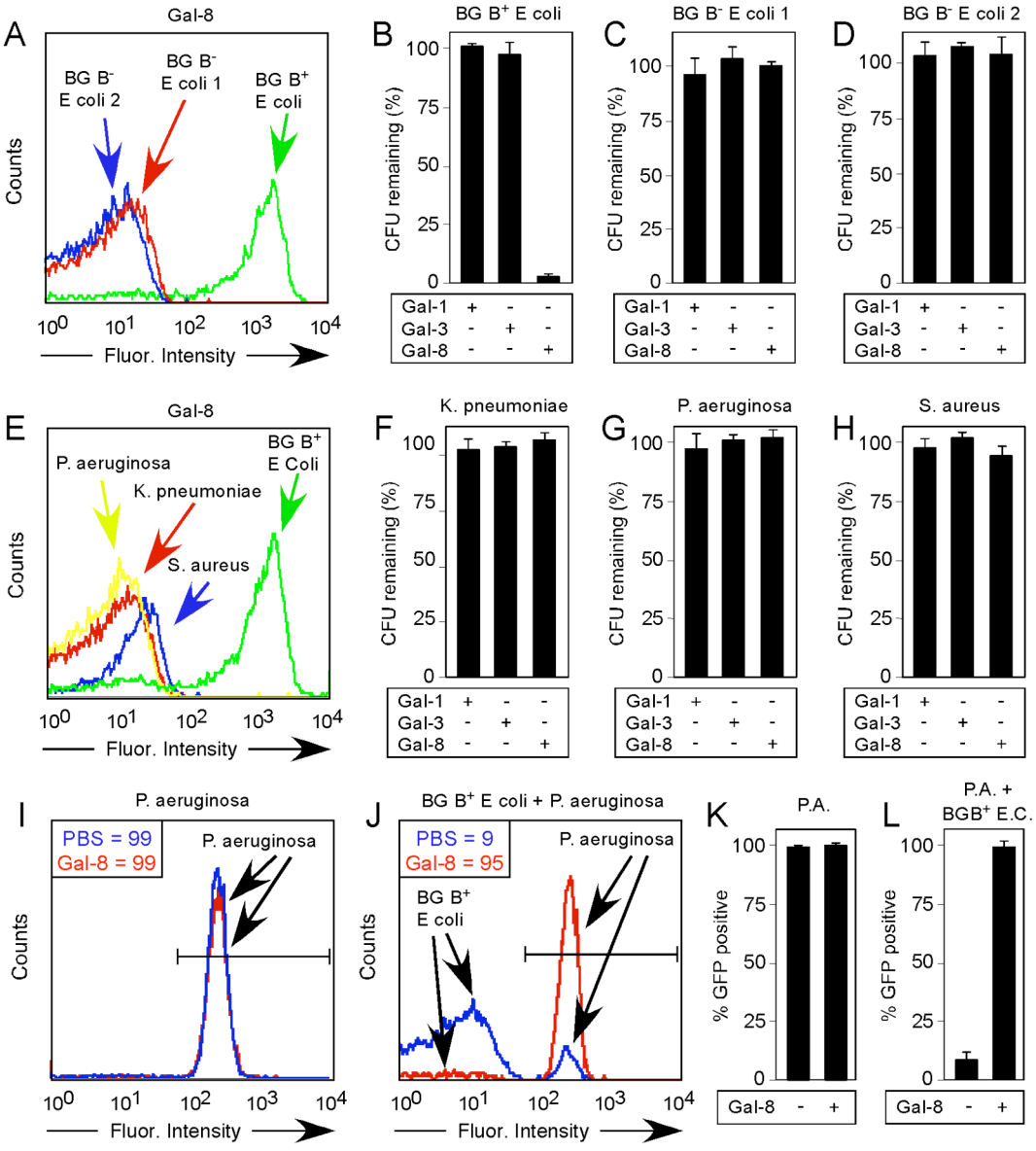

A
Gal-8
BG B⁻ E coli 2
BG B⁻ E coli 1
BG B⁺ E coli
Counts
10⁰ 10¹ 10² 10³ 10⁴
Fluor. Intensity
B
BG B⁺ E coli
C
BG B⁻ E coli 1
D
BG B⁻ E coli 2
CFU remaining (%)
100
75
50
25
0
Gal-1 + - -
Gal-3 - + -
Gal-8 - - +
E
Gal-8
P. aeruginosa
K. pneumoniae
S. aureus
BG B⁺ E Coli
F
K. pneumoniae
G
P. aeruginosa
H
S. aureus
I
P. aeruginosa
PBS = 99
Gal-8 = 99
J
BG B⁺ E coli + P. aeruginosa
PBS = 9
Gal-8 = 95
BG B⁺ E coli
K
P.A.
% GFP positive
Gal-8 - +
L
P.A. + BGB⁺ E.C.

FIGURE LEGENDS

Figure 9-1. Gal-3 and Gal-8 recognize blood group B positive *E. coli*. (A-C) Glycan microarray data obtained following incubation with (A) 0.1 mM Gal-1, (B) 0.1 mM Gal-3, or (C) 0.05 mM Gal-8. (D). Structure of *E. coli* O86 O antigen. (E-G) Flow cytometric analysis following incubation of *E. coli* O86 with (E) Gal-1, (F) Gal-3, or (G) Gal-8 with or without inclusion of 20 mM lactose (Lac) where indicated.

Figure 9-2. Gal-8 kills blood group B positive *E. coli*. *E. coli* O86 (BG B^+ *E. coli*) were grown to mid-log phase followed by addition of (A) 5 μM Gal-1, Gal-3 or Gal-8 or (B) 5 μM Gal-8 with or without 20 mM lactose (Lact.) or 20 mM sucrose (Sucr.) or (C) the indicated concentrations of Gal-1, Gal-3 or Gal-8 for 2 hours. Viable bacteria were quantified by dilution plating.

Figure 9-3. Gal-8 kills blood group B positive *E. coli* entirely through its C-terminal blood group binding domain. (A-B) Glycan microarray data obtained following incubation with (A) 0.5 mM Gal-8CM or (B) 0.5 mM Gal-8NM. (C) Flow cytometric analysis following incubation of *E. coli* O86 (BG B^+ *E. coli*) with Gal-8CM or Gal-8NM with or without inclusion of 20 mM lactose (Lac) where indicated. (D-E) *E. coli* O86 (BG B^+ *E. coli*) were grown to mid-log phase followed by the addition of (D) 5 μM Gal-8, Gal-8CM or Gal-8NM or (E) 5 μM Gal-8NM with or without 20 mM lactose (Lact.) or 20 mM sucrose (Sucr.) as indicated for 2 hours. Viable bacteria were quantified by dilution plating. (F-G) Glycan microarray data obtained following incubation with (F) 2 mM Gal-8N or (G) 2 mM Gal-8C with or without inclusion of 20 mM lactose (Lac) where indicated. (H) Flow cytometric analysis following incubation of *E. coli* O86 (BG B^+ *E. coli*)

with Gal-8C or Gal-8N with or without inclusion of 20 mM lactose (Lac) where indicated. (I-J) *E. coli* O86 (BG B^+ *E. coli*) were grown to mid-log phase followed by addition of (I) 5 μM Gal-8, Gal-8C, or Gal-8N or (J) 5 μM Gal-8C with or without 20 mM lactose (Lact.) or 20 mM sucrose (Sucr.) as indicated for 2 hours. Viable bacteria were quantified by dilution plating.

Figure 9-4. Gal-8 specifically kills blood group B positive *E. coli*. (A) Flow cytometric analysis following incubation of *E. coli* O86 (BG B^+ *E. coli*), or two clinical blood group B negative reference strains of *E. coli* with Gal-8. (B-D) Incubation of (B) blood group positive *E. coli* (O86), (C) blood group B negative *E. coli* strain 1 or (D) blood group B negative *E. coli* strain 2 grown to mid-log phase followed by addition of 5 μM Gal-8 as indicated for 2 hours. Viable bacteria were quantified by dilution plating. (E) Flow cytometric analysis following incubation of *E. coli* O86 (BG B^+ *E. coli*), *K. pneumoniae, P. aeruginosa,* or *S. aureus* with Gal-8. (F-H) Incubation of (F) *K. pneumoniae*, (G) P*. aeruginosa* or (H) *S. aureus* grown to mid-log phase followed by addition of 5 μM Gal-8 as indicated for 2 hours. Viable bacteria were quantified by dilution plating. (I-L) Incubation with (blue) or without (red) 5 μM Gal-8 with either (I) GFP expressing *P. aeruginosa* alone or (J) GFP expressing *P. aeruginosa* mixed with *E. coli* O86 (BG B^+ *E. coli*) followed by determination of the percent of *P. aeruginosa* by flow cytometric analysis. Gated values of GFP positive bacteria treated with PBS (blue) or Gal-8 (red) are shown. Quantification of the percent of GFP positive bacteria utilizing flow cytometric analysis obtained following incubation of Gal-8 with either (K) GFP expressing *P. aeruginosa* alone or (L) GFP expressing *P. aeruginosa* mixed with *E. coli* O86 (BG B^+ *E. coli*).

Chapter 10

Summary and future directions

Although the importance of cell surface carbohydrates in normal cellular physiology remained elusive for many years, recent advances demonstrate clear roles for these highly complex macromolecules in many cellular processes[333]. In addition to directly altering glycoconjugate function, carbohydrate modifications also impact signaling by a series of proteins that recognize cell surface carbohydrates, termed carbohydrate-binding proteins (CBPs), or lectins[334]. The importance of CBP-cell surface carbohydrate interactions recently received significant attention as a key interface in the regulation of immunity, where immune cell plasticity not only enables significant differentiation of cellular function, but also results in distinct alterations in cell surface glycosylation[96].

Among CBPs with immunoregulatory activities, galectin family members appear to regulate a wide variety a processes[23, 157], although the mechanisms whereby galectins impact immune cell function remain enigmatic. Early studies suggested that galectins, in particular galectin-1 (Gal-1), regulate T cell function by directly inducing apoptosis in activated, but not resting, T cells[14]. However, recent studies suggest that Gal-1 fails to alter T cell viability, yet induces profound alterations in cytokine secretion and T cell activity consistent with the immunological activities of this protein *in vivo*[161]. Furthermore, although many studies sought to determine the receptors and carbohydrate determinates responsible for galectin-mediated immunoregulation, no study to date provides definitive genetic data demonstrating the counter receptor(s) through which galectins may mediate their effects. In this review, we will examine the

original descriptions concerning the immunological activities for galectins as well as the evolution of mechanistic insight into these regulatory activities, including the putative receptors and carbohydrate moieties through which galectins may mediate their effects.

Discovery of galectins

Prior to the emergence of modern day glycobiology, the existence of mammalian CBPs remained highly questionable. However, the discovery of diverse and unique carbohydrate structures on the cell surface strongly suggested that proteins likely exist capable of decoding these complex macromolecules [333]. Indeed, in 1974 Gilbert Ashwell discovered the first mammalian CBP, the asialoglycoprotein or Ashwell receptor[335]. Although the physiological function of the asialoglycoprotein receptor only recently became apparent[334], these early studies provided sufficient evidence to spur further investigation of the possibility of other mammalian CBPs. In 1975, Teichburg and colleagues isolated the first vertebrate galectin followed shortly thereafter by the laboratories of Sam Barondes and Stuart Kornfeld who isolated similar proteins from avian and mammalian sources respectively[139, 251].

Galectin-1 regulation of T cell turnover

Although the discovery of Gal-1 by Teichburg importantly demonstrated the existence of additional CBPs, the function of the newly discovered galectins remained enigmatic. Several early studies suggested that Gal-1 might regulate muscle development, the first mammalian organ from which the protein was isolated[251], including maintenance of the neuromuscular junction[336]. To determine whether Gal-1 may regulate neuromuscular junction function *in vivo*, Teichburg and colleagues examined whether administration of Gal-1 might affect the pathological sequelae associated with neuromuscular junction pathology. To accomplish this, 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, administration of Gal-1 resulted in what appeared to be significant enhancement of muscle function. However, careful examination demonstrated that amelioration of disease did not reflect favorable Gal-1 induced alterations in muscle function. Instead, Gal-1 suppressed the autoimmunity needed to generate a myasthenia gravis model. As a result, Teichburg and colleagues not only provided the first evidence of the existence of galectins, but also suggested what continues to be one if its more intriguing properties, its ability to significantly suppress immune function[157].

Although this and other early studies provided significant evidence that Gal-1 can modulate the immune system[23], the mechanism whereby Gal-1 mediated these effects remained enigmatic. In addition to inducing lymphocyte proliferation, which suggested to the authors at the time that Gal-1 may enhance the development of suppressor T cells[157]. Gal-1 also appeared to mediate adhesion of thymocytes to epithelial cells, suggesting a role for Gal-1 in regulating T cell development[14, 157]. However, a seminal paper by Baum and colleagues which suggested that Gal-1 might alter T cell viability provided convincing mechanistic insight into the immunomodulatory activities of Gal-1 at that time[14]. This study demonstrated that Gal-1, but not a related galectin family member, Gal-3, induced apoptotic cell death in primary activated T cells and several T cell lines, including MOLT-4 and ARR, while having no effect on other T leukemic cell lines including Jurkat and CEM cells, which suggested that Gal-1 likely regulates adaptive immunity through directly inducing apoptotic death of effector T cells[14].

Following the original description of the pro-apoptotic activity of Gal-1 toward activated T cells[14], scores of papers followed describing the ability of Gal-1 to kill T cells. During this time, phosphatidylserine (PS) expression, a common feature of cells undergoing apoptotic cell death, became a uniform marker of cells undergoing cell death by coupling the use of FITC labeled Annexin-V, a protein with high specificity toward PS and flow cytometric analysis[197]. As a result, subsequent studies demonstrated that Gal-1 induced not just cell death, but PS exposure in a variety of contexts. For example, in addition to potentially regulating adaptive immunity by inducing apoptotic cell death in peripheral activated T cells, subsequent studies also suggested that Gal-1 might regulate fundamental processes associated with central tolerance. Gal-1 appeared to induced cell death in $CD4^{+}CD8^{+}$ thymocytes, although all populations exhibited a certain degree of susceptibility, through direct interactions with the T cell receptor (TCR)[337].

Examination of ligands through which Gal-1 may regulate T cell apoptosis suggested that Gal-1 might engage several cell surface receptors. Early studies utilizing primary T cells demonstrated that functional blocking antibodies against CD45 and CD43 inhibited both Gal-1 binding and signaling[14]. Subsequent studies turned to T leukemic cell lines, where CD45, CD43, and CD7 appeared to play a direct role in Gal-1 induced signaling events[205, 338]. Signaling appears to occur through Gal-1 induced segregation of CD45 into separate membrane microdomains [205, 339], which appeared to either physically separate CD45 from constitutive signaling events normally inhibited by CD45, [205, 339] or intrinsically reduce CD45 phosphatase activity[340]. Gal-1 induced segregation of CD45 may occur through direct lectin mediated lattice formation, although Gal-1-induced signaling events may actually be responsible for receptor redistribution[235, 339].

Recent studies raise questions concerning the involvement of CD45 in Gal-1-mediated signaling. For example, several studies demonstrated that elimination of CD45 failed to alter cellular sensitivity to Gal-1-induced cell death[339]. In contrast, elimination of CD7 significantly reduces cellular sensitivity to Gal-1 induced cell death[235, 341, 342], suggesting CD7 may be involved in Gal-1-induced T cell signaling. In some cellular contexts, engagement of the Gal-1 receptor appears to reduce levels of anti-apoptotic factor bcl-2[343], although conflicting data exists concerning the involvement of classical apoptotic signaling pathways such as caspase activation in Gal-1-induced apoptosis[221]. Differences in the genetic background of many of these transformed T leukemic cell lines likely accounts for many of these differences.

As Gal-1 induces T cell apoptosis through recognition of carbohydrate ligands, several studies also sought to examine the carbohydrate determinates responsible for these interactions. Glycoprotein glycosylation relevant in galectin signaling occurs in two principle varieties, commonly referred to as N-glycans and O-glycans. N-glycosylation begins in the ER, where an oligosaccharyltransferase attaches a preformed N-glycan to Asn residues, followed by N-glycan modification in the ER and Golgi apparatus[333]. In contrast, O-glycosylation begins in the Golgi apparatus in a step wise fashion following addition of a single GalNAc to Ser residues to form a series of complex core O-glycan structures[333]. Early studies suggested that Core 2 O-glycans may mediate Gal-1 induced signaling, as expression of Core 2 GnT, a key enzyme in O-glycan formation not expressed in the BW5147 cell line, made the cells susceptible to Gal-1-induced apoptosis[220, 339]. HIV infection of T cells increases Core 2 GnT expression and susceptibility to Gal-1 [344]. Furthermore, haploinsufficiency of Core 2 GnT-I also decreases the ability of Gal-1 to induce apoptosis[345].

In contrast, several other studies suggested that Gal-1 might actually signal through N-glycans. For example, while treatment of primary activated human T cells with benzyl-GalNAc, an inhibitor of O-glycan synthesis, increased susceptibility to Gal-1, incubation of cells with swainsonine, which inhibits complex N glycan synthesis[14], inhibited Gal-1 signaling. Additional studies on murine $CD8^+$ T cells suggested that Gal-1-induced apoptosis occurred independently of Core 2 GnT expression[81]. More importantly, T leukemic Jurkat cells, originally thought to be resistant to the signaling events of Gal-1[14, 17], lack O-glycan elongation beyond the Tn antigen GalNAca-Ser/Thr [346], consistent with an apparent need for further O-glycan modification for Gal-1 signaling. However, recent studies demonstrated that Jurkat T cells not only retain susceptibility to Gal-1[221, 347], but require N-glycans for galectin-1-induced apoptosis[347, 348], once again suggesting that the utilization of T leukemic cell lines likely underscores the apparent differences in the receptors, glycans and signaling induced by Gal-1. Importantly, no genetic data to date *in vivo* provide a receptor phenocopy for Gal-1 null mice.

Galectin-1 regulation of neutrophil turnover

The 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 viability, similar to members of the TNF family. Consistent with this, subsequent studies demonstrated that Gal-1 also induced PS exposure in activated neutrophils and the promyelocytic cell line, HL60[40, 82]. However, in contrast to the effects of Gal-1 on T cells, Gal-1 induced PS exposure in neutrophils occurred in the conspicuous absence of cell death[40, 82]. For example, although Gal-1 induced PS exposure, Gal-1 failed to induce DNA or cellular fragmentation, alter caspase activation, affect mitochondrial potential and in the case of

HL60 cells, alter cell growth. Gal-1-induced PS exposure appeared to fundamentally differ from PS exposure occurring during apoptotic cell death[40, 82]. While cells undergoing apoptotic cell death display uniform PS exposure over time, Gal-1-induced PS exposure remained in punctate microdomains over days of continuous incubation, without altering cell division. Full mobilization of PS in cells undergoing apoptosis ultimately reflects functional loss of an ATP driven aminophospholipid translocase due to elevated Ca^{2+} levels and a gradual loss of cellular capacity to generate ATP[103]. As Gal-1 fails to alter cellular viability, loss of ATP does not likely occur, preventing full inactivation of aminophospholipid translocase that in turn likely prevents full realization of PS externalization. In addition to inducing sustained PS exposure, Gal-1-induced PS exposure readily reverses following Gal-1 removal, without affecting cellular viability. Gal-1 induced PS exposure occurs through engagement of Src kinases, lyn and hck, which in turn activate PLC-γ and PKC[42]. Several studies suggest that PKC can directly activate scramblase, the enzyme responsible for cell surface PS mobilization, suggesting at least one possible pathway whereby Gal-1 may induce PS exposure without engaging irreversible processes such as caspase activation[107]. Indeed cells remain sensitive to Gal-1-induced re-stimulation of PS following reversion, further suggesting the involvement of reversible pathways in these signaling events. Taken together, these studies demonstrate that Gal-1 induces the externalization of PS independent of alterations in cell viability.

Although Gal-1 induced PS exposure in neutrophils independent of apoptosis, the functional consequence of this phospholipid redistribution remained unknown. Parallel studies conducted by Fadok and colleagues demonstrated that PS might serve as the primary ligand used by macrophages to recognize and engulf cells undergoing apoptotic cell death[29, 30]. Although Gal-1 failed to alter cell viability, the ability of PS to sensitize

cells to phagocytic removal suggested that Gal-1 might induce the phagocytic removal of living cells. Consistent with this, several studies demonstrated that neutrophils possess a unique apoptosis-independent pathway of cellular removal. Unlike T cells, which accumulate following perturbation of pathways involved in leukocyte apoptosis[34, 109], inhibition of neutrophil apoptosis fails to alter neutrophil turnover *in vivo*, demonstrating that neutrophils possess an apoptosis-independent pathway of removal, although the mechanism whereby viable neutrophil become targeted for removal remained unknown[34, 37, 38, 109]. As Gal-1 induced the expression a phagocyte recognition ligand, the possibility that Gal-1 may in part be responsible for this mode of removal became apparent. Indeed, Gal-1 not only induced PS exposure in neutrophils independent of apoptosis, but also sensitized neutrophils for phagocytic removal[40]. This form of removal, recently termed preaparesis, appears to prepare cells for phagocytic removal without directly altering cell viability[72]. Preaparesis may reflect a unique need for neutrophils to remain viable in order to maintain membrane integrity in the harsh environment of the acute inflammatory milieu until successfully phagocytosed[68]. This becomes especially apparent when considering that the number of neutrophils often far outweighs the number of phagocytes responsible for removing them[233]. Although these studies clearly demonstrate that Gal-1 can induce the phagocytic removal of living neutrophil *in vitro*, whether Gal-1 induces a similar phenomenon *in vivo* remains to be tested.

Galectin-1: S-type lectin

The ability of Gal-1 to induce PS exposure independent of apoptosis in neutrophils fundamentally differed from the effect of Gal-1 on T cells, which suggested that neutrophils and T cells simply possess fundamentally distinct modes of removal. However, early studies examining the potential effects of Gal-1 on neutrophils also

examined the effect of Gal-1 on T leukemic cell viability in parallel as a control[40]. However, unlike previous studies that suggested that Gal-1 induced apoptotic cell death in T leukemic cell lines[14, 339], Gal-1 failed to alter MOLT-4 viability, although Gal-1 did induce PS exposure in these cells similar to Gal-1-induced PS exposure in neutrophils[40].

The inability of Gal-1 to induce apoptotic cell death in MOLT-4 cells stood in stark contrast to many previous studies which demonstrated that Gal-1 induced significant cell death in MOLT-4 cells, primary activated T cells, and several other T leukemic cell lines[14, 339]. Careful examination of the literature indicated that unlike studies examining the effect of Gal-1 on neutrophil viability, previous studies seeking to elucidate the potential effects of Gal-1 on T cell viability utilized mM concentrations of dithiothreitol (DTT), a potent reducing agent, in treatment conditions[14, 339]. Early studies conducted by Teichburg and colleagues demonstrated that oxidation of Gal-1 results in profound conformational changes that preclude dimerization and recognition of carbohydrate ligands, both required for signaling leukocytes, resulting in the original nomenclature given to galectins, S-type, or thiol-dependent, lectins[46, 51]. As a result, early studies designed to examine the effect of Gal-1 on T cell viability included DTT in incubation media in an effort to reduce Gal-1 activity loss, although biochemical evidence that significant Gal-1 activity loss occurred in the absence of DTT during these assay conditions had never been demonstrated[14]. However, biochemical studies, in contrast to these early T cell viability studies, later demonstrated that Gal-1 oxidation and inactivation requires many hours and in the presence of ligand can take weeks to occur[46, 51]. Furthermore, many studies demonstrated significant galectin-mediated effects that required similar incubation periods and carbohydrate recognition, yet occurred in the absence of DTT[40]. Indeed, the only studies that included DTT when

examining potential activities of Gal-1 towards distinct cell populations were those that examined the effect of Gal-1 on T cell viability.

In addition to potentially stabilizing Gal-1, DTT can also induce profound changes in cellular physiology. DTT, along with tunicamycin and thapsigarin, serves as a common tool to induce the unfolded protein response in cells. DTT generates an artificial reducing environment within the ER which prevents efficient formation of disulfide bond formation critical to the proper folding and stability of many proteins targeted for cell surface expression or secretion[83]. As a result of inducing the unfolded protein response, DTT can actually directly induce apoptotic cell death[82, 85]. Since the inclusion of DTT not only provides potential stabilization of Gal-1 but also induces significant alterations in cell behavior, more recent studies sought to separate these potential DTT effects by generating stable forms of Gal-1 which do not require DTT to retain activity, either by mutating Cys residues to Ser or by alkylating Cys residues with iodoacetamide[72, 82], both previously documented procedures for stabilizing Gal-1[51, 94]. If the DTT requirement simply represented a need to retain Gal-1 activity, then stabilized versions of Gal-1 would be predicted to induce apoptosis in T cells in the absence DTT inclusion. However, while stabilized Gal-1 maintained the ability of Gal-1 to induce PS exposure in MOLT-4 cells, this PS exposure, similar to PS exposure induced by Gal-1 in neutrophils, did not accompany apoptotic cell death. Importantly, although both Gal-1 and stabilized Gal-1 induced PS exposure in MOLT-4 cells, neither protein altered PS redistribution or viability in primary activated T cells, although both proteins recognized T cell surface glycans[82]. However, co-incubation of Gal-1 or stabilized Gal-1 with DTT resulted in significant PS exposure and death in primary activated T cells and also induced MOLT-4 cells to not only externalize PS but also to undergo apoptotic cell death[82]. Taken together, these results clearly indicate that DTT

can significantly impact cellular sensitivity to the signaling effects of Gal-1 on cell viability.

Recent studies suggest that peculiar inflammatory environments, such as those surrounding neoplastic lesions, may actually be reducing, raising the possibility that the DTT dependent effects of Gal-1-induced apoptosis may be relevant in these unique settings[349]. Although these reducing environments may in fact alter cellular behavior, DTT unfortunately does not likely provide an accurate recapitulation of these environments in vitro. Not only does DTT provide a much more potent reducing environment than endogenous reducing agents, such as reduced glutathione, DTT, unlike endogenous reducing agents, artificially crosses biological membranes, a process that short circuits important compartmentalization of distinct reducing and non-reducing environments which occur within distinct organelles as well as within different cells[83, 85]. The likelihood that DTT exerts its priming effect on T cells through such alterations becomes apparent when considering that high concentrations of reduced glutathione fail to sensitize T cells to Gal-1-induced apoptosis[82]. However, it remains possible that, although DTT was originally utilized experimentally to maintain Gal-1 activity *in vitro*, it may somehow recapitulate the effects of an unknown factor or factors that then sensitize cells to Gal-1. However, as Gal-1 exerts significant effects on T cell in the absence of DTT[72, 161], as will be discussed below, future studies should continue to explore DTT independent activities of Gal-1 *in vivo*.

Although conflicting data exist concerning the effect of Gal-1 to the Jurkat T leukemic cell line[14, 221], recent studies demonstrate that stable mutants or alkylated versions of Gal-1 can induce apoptotic cell death in these cells (data not shown) [350]. However, Jurkat cells, similar to many T leukemic cells, possess many mutations that directly

affect cellular signaling, including mutations which adversely affect O glycan formation, making it difficult to interpret these results in the context of Gal-1 effects on primary T cells[346, 351]. Mutations in Jurkat cells may likely facilitate the ability of Gal-1 to engage pathways normally initiated by other galectins in primary T cells, such as galectin-3 (Gal-3), which can induce apoptosis in primary T cells in the absence of DTT[72]. Importantly, similar to Gal-1, Gal-3 also fails to induce apoptosis in MOLT-4 cells, although it induces apoptosis in primary T cells, further suggesting that cellular transformation in these cells results in the loss of the differential sensitivity of primary T cells to distinct signaling effects of different galectin family members[72]. These results provide further evidence demonstrating importance of evaluating primary non-transformed T cells when seeking to evaluate the potential impact of galectins on T cell biology.

In addition to recent results utilizing T leukemic Jurkat cells, others reported that secreted Gal-1 might induce apoptosis in the absence of DTT, although co-incubation with DTT always accompanied analysis of the recombinant protein[195, 352]. These effects appeared to be specific to Gal-1, as polyclonal anti-Gal-1 antibody preparations blocked the ability of media secreted by these cells to induce PS exposure and cell death in target cells[195]. Unfortunately, the specificity of this antibody preparation toward Gal-1 remains untested, strongly suggesting that other galectin family members, such as Gal-3, Gal-8, and Gal-9, which do induce apoptosis in T cells in the absence of DTT [11, 12, 353], may actually be responsible for these galectin-dependent effects. Consistent with this, media derived from Lec-8 cells, a mutant cell line of CHO cells that possess a genetic lesion that specifically prevents the secretion of active Gal-1[45], also displayed sensitivity to inhibition by the anti-Gal-1 blocking antibody preparations [195], strongly suggesting that other galectins may be responsible for these effects. Similarly,

conditioned media obtained from decidual NK cells, which also appears to possess killing activity blocked by a similar polyclonal antibody preparation, also likely possess other galectins[136]. Consistent with this, the concentration of Gal-1 in the media only approached 1 μM, over 10 fold below the concentration needed to examine the effect of the recombinant protein on the same target cells[136]. Similarly, previous studies demonstrated 10 μM as the minimum concentration required for Gal-1-induced apoptosis[14], although it remains possible that unidentified factors within the media sensitize cells to Gal-1-induced effects at much lower concentrations.

Gal-1: regulator of adaptive immunity

Although these recent studies suggested that Gal-1 failed to alter T cell fate without the inclusion of DTT[82], early studies predicted a significant effect of Gal-1 on cells involved in adaptive immunity[23, 157], which raised new questions concerning the mechanisms responsible for Gal-1 inhibition of autoimmune responses in various animal models. In addition to regulating immunity in the context of experimentally induced myasthenia gravis, subsequent studies demonstrated that injection of Gal-1 reduced the pathological and clinical sequelea associated with several other models of immune-mediated pathology, including Con-A-induced hepatitis, graft-vs-host disease, collagen-induced arthritis, experimental autoimmune encephalitis and uveitis[19-23, 27, 157, 354]. Furthermore, Gal-1 null mice display an enhanced susceptibility toward experimental autoimmune encephalomyelitis and impaired maternal-fetal immunological tolerance during gestation, which importantly demonstrated a role for Gal-1 in the regulation of adaptive immunity at the genetic level[22, 355].

Key findings obtained in the absence of DTT provide some insight into how Gal-1 may mediate these potent immunological effects. In vitro, 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[72, 202]. As Gal-1 displays significant agglutination properties[356], injected Gal-1 likely exerts its primary affects on resident leukocyte trafficking through the site of injection. Indeed, injection of Gal-1 into the vasculature results in immediate and nearly fatal vascular stasis due to Gal-1-induced agglutination of vascular contents, which suggests that Gal-1 likely induces its primary effects locally. Engagement of local leukocytes by Gal-1 may alter cytokine production of T cells as observed *in vitro*, which then traffic to distant sites where they may exert immunomodulatory effects. 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[27]. In addition, injection of Gal-1 into IL-10 null mice fails to convey the immunoprotective properties of Gal-1, strongly suggesting a role for IL-10 and possibly other cytokines, in mediating the immunosuppressive activities of this protein[161]. Importantly, although several studies report alterations in T cell viability following injection of Gal-1 *in vivo*, perturbation of the cytokine milieu can significantly impact the viability of distinct T cell populations that often rely on specific cytokines to maintain viability *in vivo*[357, 358]. Thus, Gal-1-induced alterations in the cytokine environment may directly or indirectly affect the viability of T cells. Recent studies suggest that 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 [159]. In addition to directly altering T cell cytokine production, several studies suggest that Gal-1 might also inhibit T cell activation, providing an additional mechanism whereby Gal-1 might inhibit adaptive immunity[359]. Consistent

with this, most studies reporting an immunosuppressive effect of Gal-1 *in vivo* actually injected Gal-1 during the initiation phase of the T cell response, shortly after exposure to antigen, where alterations in T cell activation and the cytokine environment can significantly impact T cell differentiation[19-23, 27, 157, 354]. In addition to altering peripheral T cell responses, recent studies 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 CD8$\alpha\alpha$ T cells, among other alterations in T cell behavior, providing further evidence that Gal-1 affects multiple aspects of T cell biology[360]. Finally, in contrast to the previous results suggesting that Gal-1 evokes death in activated T cells, recent studies demonstrate that Gal-1 might actually sustain naive T cell survival in peripheral tissue[361].

In addition to regulating T cell behavior directly, several studies suggest that Gal-1 can exert significant effects on other cell types that may directly or indirectly alter adaptive immune responses. For example, Gal-1 also appears to inhibit leukocyte extravasation and impairs chemotaxis[53], although the direct impact on T cell trafficking remains unknown. Gal-1 also facilitates repair of injured tissue, which may also directly and indirectly reduce production of pro-inflammatory cytokines[146, 164]. Depending on the inflammatory milieu, Gal-1 may also positively or negatively regulate DC migration and activation[362-365].

In addition to possessing lectin activity, with putative roles exerted once outside the cell, recent studies clearly demonstrate that Gal-1 also possesses significant biological activity within the cell[366-368], likely through lectin-independent mechanisms. For example, Gal-1 appears to interact with H-Ras-GTP, influencing a variety of fundamental cellular processes[368]. This intracellular bioactivity does not appear to be specific to

Gal-1 among galectin family members[369]. As a result, phenotypes observed in galectin null mice must be carefully interpreted, as each of these proteins appear to play critical roles in fundamental intracellular processes which likely affect the behavior of many cells, including those involved in immunity.

The other galectins

In the wake of early studies demonstrating that Gal-1 possessed potent immunoregulatory effects, many studies began to examine the potential immunological properties of other galectin family members, although it should be noted that the first studies demonstrating a potential effect of Gal-3 on mast cells paralleled early studies on Gal-1 function. Subsequent studies demonstrate that Gal-3, Gal-8, and Gal-9 regulate thymocyte and T cell viability in the absence of DTT[12, 26, 353, 370]. Similar to Gal-1, utilization of different leukemic T cell lines yields conflicting results on the functional receptors through which galectins may signal apoptosis. For example, early studies suggested that CD29 and CD7 mediate Gal-3 induced T cell apoptosis, while subsequent studies suggest that Gal-3 engages CD45 and CD 71, but not CD29 to invoke cell death[12, 16]. Similarly, recent work demonstrated that Gal-9 specifically induces apoptosis in CD4+ T_H1 cells through engagement of Tim-3[26], although Tim-3 fails to relay Gal-9 signaling in several T leukemic cell lines[371]. Regardless of the specific receptors utilized, 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[72], demonstrating that T cells possess distinct functional receptors for these two galectins.

In addition to regulating T cell viability, Gal-3 appears to also 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[123]. Similar to Gal-1, Gal-2 also induces IL-10 production, along with IL-5 and TGF-β, all of which can exhibit anti-inflammatory properties, while also decreasing IFN-γ and IL-2[13]. Gal-10, originally known as Charcot Leyden protein, appears to also be a key player in proper Treg function[160]. In contrast, several studies suggest that Gal-4 may actually exhibit pro-inflammatory activity, inducing IL-6 production in CD4+ T cells[196]. Similarly, Gal-3 may also inhibit T_H2 immunodeviation by reducing IL-5 secretion[372]. In contrast to the pro-apoptotic effects of extracellular Gal-3, intracellular Gal-3 appears to inhibit cell death through intracellular interactions with key players involved the regulation of the apoptotic program[369, 373].

In addition to regulating adaptive immunity, early studies also demonstrated that galectins, in particular Gal-3, might be key regulators of cells involved in innate immunity. In contrast to the ability of Gal-3 to induce apoptosis in activated T cells, recent studies demonstrate that Gal-3 induces PS exposure in the absence of cell death in neutrophils, similar to Gal-1[72], providing an interesting example of a factor capable of inducing turnover of two distinct cell types through either apoptotic or apoptosis-independent pathways. Earlier studies also demonstrated that Gal-3, first identified as the epsilon binding protein (EBP)[374], recognized IgE and the IgE receptor and induced mast cell degranulation independent of IgE mediated antigen stimulation[375, 376]. Recent studies in Gal-3 null mice corroborate these earlier studies[377]. Gal-3 also appears to recognize LPS[378], which may modulate immunological sensitivity to endotoxin[379]. In addition to modulating LPS interactions, Gal-3 appears to directly induce changes in endothelial and macrophage cytokine production and facilitate macrophage-mediated phagocytosis[55, 158, 380]. In contrast to the inhibitory role of Gal-1 in leukocyte extravasation, Gal-3 also appears to facilitate both neutrophil and

monocyte extravasation in several inflammatory conditions[381, 382]. Several galectins also appear to possess chemotaxic activity, with Gal-3 and Gal-9 inducing the chemotaxis of monocytes and eosinophils respectively[158, 383, 384], in contrast to the ability of Gal-1 to inhibit leukocyte chemotaxis[53]. In addition to regulating immune cell function, recent studies suggest that galectin may also provide direct innate immune activity[62, 313, 316]. In contrast, galectins, in particular Gal-1, may facilitate pathogen attachment and invasion[385, 386].

Galectins as lectins

Perhaps the most unique feature of galectins lies in their ability to regulate cell behavior through the recognition of highly modifiable carbohydrate structures[96]. Early studies failed to demonstrate significant differences in the carbohydrate specificity of different galectin family members, although galectins appeared to possess distinct biological activities[63]. As a result, several studies attributed the differential activities of different galectin family members to differences in quaternary structure[14]. Limitations in the availability and diversity of glycan test libraries made elucidating the binding specificities of CBPs difficult[292]. However, recent advances in glycan microarrays and other large glycan library formats significantly facilitated the determination of carbohydrate binding specificity of CBPs[64, 292].

Recent studies utilizing a combined approach of glycan microarrays and cell surface binding indicated that distinct interactions of galectin family members with cell surface polylactosamine glycans can convey a significant and differential impact on galectin recognition of sialylated glycans[134]. For example, while Gal-1 and Gal-2 only recognize the terminal LacNAc motifs of glycans[222], it appears that these prototypical galectins prefer extended glycans, either N-glycans or polylactosamine (polyLacNAc)

glycans baring this the terminal LacNAc motif[134, 222, 387]**.** However, in solution-based assays Gal-1 and Gal-2 fail to display a preference for extended ligands[134, 387]. 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 likely make crosslinking interactions more favorable.

Not only may homodimerization influence carbohydrate binding specificity, but also binding of ligand itself actually enhances Gal-1 dimerization. Enhanced dimerization facilitates Gal-1 signaling and reduces Gal-1 sensitivity to oxidation, suggesting that dimerization likely serves as a key regulator of Gal-1 function**.** In contrast to Gal-1 and Gal-2, Gal-3 and Gal-8, which do not exist as rigid homodimers, actually recognize internal LacNAc motifs within extended polyLacNAc[134, 222]. As these two galectin family members possess oligomeric carbohydrate recognition domains organized through flexible linker peptides, conformational constraints that provide 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[133].

Differential recognition of galectins for 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 occurs in two different linkages with distinct impacts on galectin binding and signaling. 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. For example, although Gal-1

recognizes α2-3 sialylated glycans, it fails to recognize glycans following α2-6 sialylation[22, 134]. In contrast, Gal-2 fails to recognize glycans following sialylation with either linkage[133]. Gal-3 binds internal LacNAc within polyLacNAc, and sialylation of polyLacNAc with either linkage fails to significantly alter Gal-3 binding and signaling[22, 134], although in different cellular contexts sialylation may also impact Gal-3 binding and signaling[388]. Unlike Gal-1, Gal-2, and Gal-3, Gal-8 possesses two unique carbohydrate recognition domains, the N terminal domain, which recognizes sialic acid and the C terminal domain which prefers non-sialylated glycans[295]. Recent studies demonstrated that Gal-8 exists as a dimer, and actually signals PS exposure in leukocytes entirely through the C terminal domain[95]. Similar results suggest that other tandem repeat galectins may also possess a unique mode of signaling. In this way, simple modifications of carbohydrate structures serve a similar role as protein phosphorylation; the addition or subtraction of a single monosaccharide can significantly alter cellular sensitivity toward different galectins.

Summary

Although the discovery of galectins over 30 years ago stemmed from interest in understanding the roles of carbohydrates in fundamental biological process, this discovery ultimately uncovered an entire family of potent immunological regulatory proteins [139, 251]. Recent studies corroborate earlier results and demonstrate that galectins clearly regulate immunity *in vivo*, although the mechanism whereby this regulation occurs is only beginning to be understood[22, 159, 161]. The combined studies provide promising results and suggest that these novel factors may regulate a variety of aspects of immunity. Futures studies will continue to examine the many roles of galectins in immune function *in vivo*, while continuing to identify the specific carbohydrate moieties responsible for mediating their effects.

MATERIALS AND METHODS

Expression and isolation of recombinant galectin family members

The expression of recombinant forms of human galectins-1, -2, -3, -4, -7 and -8 was accomplished using established procedures [40, 389, 390]. The C2S-Gal-1 and C2SV5D galectin-1 (mGal-1) mutants were generated from human Gal-1 by site directed mutagenesis as outlined previously [51, 391]. The Gal-8NM (R69H) and Gal-8CM (R233H) galectin-8 were generated using the following primers: Gal-8NM-forward primer 5'-GTGGCCTTTCATTTCAATCCTCATTTCAAAAGGGCCGGCTGCATG-3' and reverse primer 5'-CAATGCAGCCGGCCCTTTTGAAATGAGGATTGAATGAAAGGCCAC-3'; and Gal-8CM (R233H)-forward primer 5'-GCTCTACACTTGAACCCACACCTGAATATTAAA-GCATTTG-3' and reverse primer 5'CAAATGCTTTAATATTCAGGTGTGGGTTCAAGTG-TAGAGC-3'. Briefly, a 1-Liter culture of transformant positive *E. coli* of each galectin was grown until the absorbance (600 nm) reached approximately 0.5, at which point cultures were induced with 1.17 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C except that Gal-2 transformant positive *E. coli* were induced at 0.25–0.3 optical density at 600 nm with 0.5 mM IPTG for 1-1.25 h at 30°C. Cultures were then centrifuged at 4200 x g for 30 min., followed by discarding the supernatant and freezing the pellets overnight at – 80°C. Cell pellets were thawed on ice and resuspended in 10 ml lysis buffer (MEPBS (0.01 M Na_2HPO_4, 0.01 M Na_2HPO_4, 0.85% NaCl, pH 7.4,14 mM 2-ME), 1 Complete Mini EDTA-Free Protease Inhibitor Cocktail Tablet (Roche), 0.1 mg/ml lysozyme, 10 μg/ml DNase, 10 μg/ml RNase) and allowed to incubate at R.T. for 30 min. Cells were further lysed by sonication (Branson Cell Disruptor 185), and debris was pelleted by centrifugation at 13,000 x g for 30 min. Supernatant was applied to 30-ml lactosyl-Sepharose pre-equilibrated in MEPBS. The column was then washed extensively with MEPBS. Each bound galectin was eluted with MEPBS containing 0.1 M

lactose. To confirm that the galectin quantitatively retained carbohydrate-binding activity, the protein was re-chromatographed on lactosyl-Sepharose. The elution profile of each re-purified galectin revealed a single peak that was quantitatively eluted with lactose (data not shown). SDS-PAGE analysis of each elution profile demonstrated a single band of the predicted molecular weight. Both lactose and β-ME were removed from galectin samples prior to use using gel filtration chromatography [222]. To ensure that galectin samples were endotoxin-free, Detoxi-Gel Endotoxin removing gel (Pierce Biotechnology, Rockford, IL) was employed. Endotoxin levels measured using the Limulus Amebocyte Lysate endotoxin detection kit (Cambrex Corp., Rutherford, NJ) demonstrated that the final preparations of galectins utilized in experiments contained less than 0.04 ng of LPS/mL.

Agglutination assays and separation of monomers and dimers of Gal-1 on size exclusion HPLC

For agglutination assays, cells were incubated at RT until cells achieved an equilibrium of agglutination and graded for the degree of agglutination based on the percent of cells remaining agglutinated. For serial dilution agglutination of erythrocytes, cells were plated in round bottom 96 well plates, mixed with serial dilutions of each galectin and allowed to agglutinate. The last concentration at which agglutination occurred was defined as the agglutination endpoint. Erythrocytes of different blood group antigen specificity were obtained from Immucor. Cells were plated in round bottom 96 well plates, mixed with serial dilutions of each galectin and allowed to agglutinate. The last concentration at which agglutination occurred was defined as the agglutination endpoint.

For size exclusion HPLC, galectins were stored at 4°C in described concentrations and subjected to size-exclusion chromatography. Separation of monomeric and dimeric

forms of human Gal-1 and mutated forms of Gal-1 was accomplished by size-exclusion HPLC as described [46], using a TSK-GEL SW 2000 column (Beckman) (7.5mm x 30 cm) on a Beckman System Gold HPLC. The column was calibrated using bovine γ-globulin, 158 kDa; bovine serum albumin, 67 kDa; chicken ovalbumin, 44 kDa; equine myoglobin, 25 kDa; chymotrypsinogen, 17 kDa; and ribonuclease A, 13.7 kDa, as described previously [46].

Determination of Gal-1 activity by soluble protein fraction detection and affinity chromatography

dGal-1 or mGal-1 were incubated for the indicated times in a humidified incubator at 37°C followed by centrifugation at 16,000 x g to pellet the principle precipitated matter. The fraction of soluble protein was detected by dividing the absorbance of the soluble material at 280 nm by the absorbance of the starting material at the same wavelength. To determine the amount of active protein at a given time point, each galectin was incubated at 37°C for the indicated time followed by subjecting the soluble fraction to affinity chromatography utilizing lactosyl sepharose. Fraction bound was calculated by dividing the material eluted with lactose over the total material passed over the column.

Chemical cross-linking proteins

Both 2-ME and lactose were removed from ALEXA labeled or non-labeled galectin-1 by gel filtration. In the case of ALEXA labeled galectin-1, prior to cross-linking, free cysteines were quenched with iodoacetamide to reduce activity loss and potential erroneous Cys-Cys bond formation during the cross-linking experiment in the absence of βME. Samples were incubated overnight at RT in PBS to allow each sample at each respective concentration to achieve equilibrium prior to cross-linking. Each sample was

then incubated with 50-fold excess BS^3 (Pierce) for 30 min at RT. Excess cross-linker was quenched using 50 mM Tris for 15 min, followed by analysis of the extent of covalently cross-linked dimer by SDS-PAGE in reducing conditions.

Iodoacetamide treatment of galectin

Both 2-ME and lactose were removed from galectin-1 samples by gel filtration. Galectin-1, at approximately 2-5 mg/ml, was then re-suspended in 100 mM lactose in PBS containing 100 mM iodoacetamide and allowed to incubate at 4°C overnight. Free iodoacetamide was removed following treatment by gel filtration using a PD-10 column as previously described [222]. Activity iodoacetamide treated Gal-1 (iGal-1) was assessed by incubating iGal-1 for 24 hours at 37°C followed by addition to HL60 cells at 20 mM for 4 hours followed by detection for PS exposure using flow cytometric analysis or subjection to affinity chromatography over lactosyl-sepharose.

Mass spec analysis of iodoacetamide galectin

Galectin-1 (1 mg) in PBS was divided into two equal aliquots, half of which was alkylated with iodoacetamide as outlined above. The other half was allowed to incubate with PBS alone. Either sample of protein was reduced by the addition of dithiothreitol (DTT) (10 mM) and incubated for 2 h at room temperature. The galectin samples were then digested into peptides using TPCK (L-1-tosylamide-2-phenylethyl choloromethyl ketone)-treated trypsin added in a 1:50 (w/w) trypsin-to-protein ratio and incubated at 37°C for 24 h. The digest was stopped by adding TLCK (Na-Tosyl-L-lysine chloromethyl ketone) in slight molar excess of the trypsin. Tryptic galectin-1 peptide mixtures were separated by gradient elution from a Vydac C_{18} column (4.6 mm × 250 mm, 218TP54) on a Beckman Gold Chromatography System (Beckman Instruments, San Ramon, CA). The C_{18} column was equilibrated using 100% mobile phase A (0.1 % TFA in water) at a flow

rate of 1 mL/min for at least four column volumes (CV). Peptide mixtures (150 μg total protein) were injected at a flow rate of 1 mL/min in a linear gradient ranging over 60 min from 100% mobile phase A to 60% mobile phase B (99.9% ACN with 0.1% TFA). The gradient was then ramped from 60% mobile phase B to 100% mobile phase B over 10 min. Throughout the analysis, an on-line UV detector set at 215 nm was used to monitor separation of the peptide mixtures. Galectin peptides were collected as they eluted from the chromatography system in 1 mL fractions. MALDI-TOF mass spectrometry was performed using a Voyager DE-RP BioSpectrometry Workstation (PE Biosystems, Framingham, MA). Peptides were prepared by mixing a 1μl aliquot of a fraction with 1μl of matrix solution. The matrix solution was a 10 mg/ml 2,5 dihydroxy-benzoic acid (DHB) in 50% water – 49.9% ACN with 0.1% TFA. The mixture was spotted onto a well of the MALDI target plate and allowed to air-dry before being placed in the mass spectrometer. All peptides were analyzed in the reflective, positive ion mode by delayed extraction. ESI mass spectrometry was performed using a MSD Trap (Bruker Daltonics, Billerica, MA) in the positive ion mode. Reversed phase separated peptide fractions were reduced to a uniform volume of 100 μL and an equal volume of MeOH was added to all fractions. These peptide solutions were electrosprayed into the MSD system at 5 μL/min and the initial MS scan utilized *m/z* range of 400 to 2,000 the most abundant ions were selected for MS/MS analysis.

Generation of monoclonal αGal-1 antibody

BALB/c mice were given initial intraperitoneal injections with 20 μg of recombinant human galectin-1 emulsified in Freund's complete adjuvant. Seven and fourteen days later, booster injections were given intraperitoneally with 10 μg of the same antigen emulsified with Freund's incomplete adjuvant. A final injection with 20 μg recombinant

human galectin-1 (without adjuvant) was given intraperitoneally and intravenously 3 days before spleens were removed. The spleen cells were fused with SP2/O mouse myeloma cells according to standard protocols [392]. Fused cells were plated into eight 96-well plates and maintained in Iscove's medium containing 20% fetal bovine serum, 10 ng/ml recombinant interleukin-6, 2X HAT and OPI for 14 days. After 14 days, the media was changed to Iscove's medium containing HT instead of HAT. Hybridomas secreting antibodies to recombinant galectin-1 were selected on day 14 by ELISA using 50 μl of culture supernatants. Single cell clones secreting anti-galectin-1 antibodies were generated by limited dilution. Positive clones were further grown in Iscove's medium containing 20% fetal bovine serum, and further screened for specificity toward galectin-1 among other galectin family members using ELISA.

Detection of galectin-1 using αGal-1 in microtiter wells

50 μL aliquots of a 0.5 μg/ml solution of either galectin-1, -2, -3, -4, or -7 in PBS (0.01 M Na_2HPO_4, 0.01 M Na_2HPO_4, 0.85% NaCl, pH 7.4) were used to coat each well followed by blocking with a solution of 5% BSA in PBS. Triplicate analyses of each coating density were preformed. Antibody dilutions were carried out in PBS-Tween 20 (0.3% Tween 20 in PBS) containing 1% BSA. A 50 μL volume of αGal-1 at the indicated concentrations was allowed to incubate in each respective well for 1 h, followed by 5 PBS-Tween 20 washes, incubation with ALEXA 488 goat anti-mouse secondary antibody (Molecular Probes), 5 X wash and fluorescence detection using the Perkin Elmer fluorimeter. Alternatively, galectin-1 was biotinylated by using EZ-Link™ sulfo-NHS-Biotin (Pierce) as outlined by the manufactures protocol. ELISAs were preformed to detect biotinylated galectin-1 when bound to laminin. This was accomplished by coating microtiter wells with 50 μl of a 1 mg/ml laminin (Roche) solution in PBS followed

by blocking with 5% BSA in PBS. Biotinylated galectin-1 (10 μg/ml) pre-incubated in the presence or absence of the indicated concentrations of αGal-1, 20 mM lactose or 20 mM maltose for 1 h, incubated in a total volume of 50 μl, washed 3 X with PBS-Tween 20. Bound biotinylated galectin-1 was then detected using peroxidase labeled streptavidin diluted 1:5000 in PBS, followed by a 3X PBS-Tween 20 wash. To detect bound streptavidin, each well was then incubated with 100 μl of ABTS/peroxidase substrate for 10 or 20 min, followed by absorbance detection at 405 nm using a microtiter plate reader (Molecular Devices). Each assay was preformed in triplicate.

Western blot analysis of recombinant galectins and tissue using αGal-1

Equal wet weights of each porcine tissue were minced in MEPBS with protease inhibitors followed by further homogenization using a dounce homogenizer. Homogenized tissue extracts were then directly subjected to SDS denaturation at 100°C for 1 h. To determine the protein concentration, tissue homogenate treated with SDS and μME was diluted 1:20 with dH_20, followed by protein precipitation using TCA. The TCA pellet was then washed with acetone and resuspended in modified Lowery reagent according to the manufactures protocol (Pierce). BSA, treated under the same conditions, served as a standard. Tissue samples or recombinant galectins were resolved by SDS-PAGE (4-20% Tris-glycine gels, Invitrogen) under reducing conditions and transferred to nitrocellulose membranes (Biorad). Pre-stained SDS-PAGE standards, broad range (Biorad), were used as molecular weight markers. Blots were blocked with 5% nonfat dried milk in TBS (20 mM Tris-HCl, ph 7.5, 0.3 M NaCl) overnight and then incubated with 10 μg/mL αGal-1 or polyclonal antibody preparation as indicated. After washing three times with TBS, the blots were incubated with peroxidase-labeled secondary antibody (goat anti-mouse diluted 1:5000) in TBS, with

1% bovine serum albumin for 1 h at R.T. After washing 3X in TBS, immunoreactive bands were detected by enhanced chemiluminescence on BioMax film.

Immunohistochemistry and confocal analysis

Immunohistochemical analysis was accomplished using previously established procedures [393, 394]. Briefly, 5 micron sections of frozen tissue were incubated with 10 μg/ml αGal-1 or polyclonal antibody (pαGal-1) prepared by affinity purification over an immobilized Gal-1 column as outlined previously [6] for 30 min at RT, followed by blocking with 5 % goat serum and detection using a ALEXA labeled goat anti mouse secondary antibody, secondary antibody (Molecular Probes) alone for 30 min, or isotype primary control followed by secondary as indicated. For detection of galectin-1 using the pαGal-1 antibody, tissues fixed in 2% paraformaldehyde were incubated for 30 min with pαGal-1 antibody, followed by blocking with 5% goat serum and detection using an ALEXA labeled goat anti-rabbit secondary antibody. Polyclonal Gal-1 was preincubated with Gal-1 or Gal-4 as indicated for 1 h. Confocal analysis was accomplished using a Lieca TCS NT confocal microscope.

For cells in suspension, cells were incubated with 2 μg/mL of biotinylated Gal-3 and ALEXA-488-labeled Gal-1 or with 2 μg/mL cholrea toxin subunit B (Molecluar Probes) as indicated for 1 h at 4ºC. After washing, cells were incubated with streptavidin ALEXA Fluor 568 (Molecular Probes) for 1 h at 4ºC. Cells were then plated on cover slips pretreated with Poly-L-Lysine (Sigma) and allowed to adhere for 30 min at 4ºC. Cells were then fixed with 2% paraformaldehyde buffered in PBS at 4ºC for 2 h. For confocal analysis of PS exposure, cells were treated with 10 μM Gal-1 or 10 μM Camptothecin followed by staining in a 100-μl final volume in HEPES buffer (10 mM HEPES, 140 mM

NaCl2, 5mM CaCl2, pH 7.4) with biotin-conjugated Annexin V (Molecular Probes, 2 µl of conjugate per 100 µl of resuspended cells, as described by the manufacturer) for 15 min at 4°C. Cells were then washed 3X in HEPES buffer at 4°C followed by staining with 2 μg/ml ALEXA 633 Streptavidin alone or with 2 μg/ml ALEXA 488 Gal-1 or 2 μg/ml ALEXA 488 Cholera Toxin subunit B. Cells were then plated on cover slips pretreated with Poly-L-Lysine (Sigma) and allowed to adhere for 30 min at 4ºC and fixed with 2% paraformaldehyde buffered in PBS at 4ºC for 2 h. For cytospin assays, 200 ml of 5 X 10^5 cells/ml were spun onto a slide at 800 rpm followed by incubation with 10 mM Gal-1 for 4 hours and staining for PS exposure by Annexin V as outlined above. Cells were analyzed using a Leica TCS NT confocal microscope and Leica TCS software.

Scanning electronic micrograph analysis

For SEM analysis, neutrophils were treated with Gal-1, PBS or anti-Fas for 8 hours followed by removal of galectin in lactose and fixation in 2% glutaraldehyde in Hanks' balanced salt. Following fixation, cells were dehydrated by sequential incubation with inceasing concentrations of methanol solutions and then dried in liquid carbon dioxide by using a critical point dryer (Autosamdri 814). These fixed dehydrated cells were then mounted on bulk specimen holders (JEOL) and coated with 60:40 gold/palladium utilizing a Hummer VI sputter coater, and viewed with a JEOL JSM880 scanning electron microscope under 15-kV accelerating voltage (OU Norman, OK).

Isolation, activation and treatment of human cells

The isolation of neutrophils and T cells was in accordance with a protocol approved by the Emory institutional review board. More than 10 separate healthy donors were used to isolate neutrophils and T cells. Results shown in each experiment are representative of at least three independent experiments utilizing at least three separate donors. Cells

were isolated and activated as outlined previously [14, 82, 395]. Briefly, for neutrophil isolation, heparinized blood obtained from normal donors was subjected to dextran sedimentation followed by hypotonic lysis and density gradient centrifugation using Histopague-1077 (Sigma). For activation, neutrophils were treated with 1 μM fMet-Leu-Phe (fMLP) in HBSS/HSA for 10 min at 37°C. For T cells, fresh heparinized blood isolated as described for neutrophils was mixed in equal volume with HBSS (without Ca^{2+} or Mg^{2+}) and subjected to density gradient centrifugation using Ficoll-Hypaque. Plasma and platelets were removed and lymphocytes were washed three times in HBSS, followed by resuspension at 1 x 10^6 cells/ml in complete RPMI (RPMI 1640, 10% fetal bovine serum, glutamine [2 mM], penicillin [100 mU/ml], and streptomycin [100 μg/mL]) and activated with 8 μg/mL PHA (Sigma) for approximately 4 days (Sigma) as outlined previously [14, 82]. T leukemic MOLT-4 cells, T leukemic CEM cells and promyelocytic HL60 cells were obtained from ATCC and also maintained in complete RPMI. Leukocytes were treated with the indicated galectins, IgM anti-Fas (200 ng/mL, Upstate Biotechnology Inc.) or 10 μM camptothecin or 10 μM etoposide for the length of time and concentrations indicated in figure legends and analyzed for Annexin V staining and cell fragmentation as outlined previously [80, 82].

Flow cytometric analysis

Flowing treatment with the indicated concentrations of galectin, anti-Fas, camptothecin or etoposide, cells were incubated in 20 mM lactose to disengage galectin treated cells. Where indicated cells were pretreated with 6 mM MβCD for 1 hour followed by Gal-1 treatment. Incomplete disengagment can results in errouneous apoptosis appearing data following flow cytometric analysis due to mechanical fragmentation of cells during assay (data not shown). Following disengagement, cells were stained with sterile PBS

and then incubated in a 100-µl final volume in HEPES buffer (10 mM HEPES, 140 mM NaCl2,5mM CaCl2, pH 7.4) with a mixture of FITC-conjugated Annexin V (Roche Applied Science, 2 µl of conjugate per 100 µl of resuspended cells, as described by the manufacturer) and PI (Molecular Probes, 1 µg/ml final concentration) at 4°C for 15 min. Into this tube was pipetted 300 µl of HEPES buffer at 4°C.

Cellular DNA fragmentation was assessed using the TUNEL reaction (In Situ Cell Death Detection Kit, Roche Applied Science) or hypodiploid analysis as outlined previously [82, 396]. For TUNEL assay, cells were fixed in 1% paraformaldehyde buffered with PBS, and permeabilized with 70% ethanol on ice. The TUNEL reaction (In Situ Cell Death Detection Kit, Roche Applied Science, Indianapolis, IN) was conducted by incubating the cells for 1 h at 37 °C with 50 µl of TUNEL reaction mixture. The cells were then washed and analyzed by flow cytometry. For hypodiploid analysis, cells were fixed with 80% ethanol at -20°C for 30 min followed by staining overnight with 50 µg/mL propidium iodide in 0.1% Triton, 50 µg/mL RNase A, 0.1 mM EDTA buffered in PBS at 4°C for 3 hours or overnight. Cells were directly examined for hypodiploid content.

For lectin binding using flow cytometry, cells were washed twice in PBS at 4°C and incubated with biotinylated Gal-1, Gal-2, Gal-3 or the indicated plant lectins (LEA, RCA-I and *Macckia amurensis* Lectin II (MAL II) – Vector Labs) at a concentration of between 5-10 µg/ml at 4°C for 1 h. As controls, cells were incubated with 50 mM lactose along with the galectins. Cells were washed 3 times and then incubated with ALEXA fluor 488 streptavidin or ALEXA fluor 633 streptavidin (Molecular Probes) at 4°C for 1 h. Cells were then washed twice, followed by resuspension in 400 µL PBS for analysis by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). The bars in each

graph represent the % change in binding when compared to the binding of control buffer treated cells from each enzymatic pair.

For galectin binding to bacteria, cells were washed twice in PBS at 4°C and incubated with biotinylated Gal-1, Gal-3, Gal-4, Gal-4 domains, Gal-8, or Gal-8 domains at concentrations between 1-5 µg/ml at 4°C for 30 min. As controls, cells were incubated with 20 mM lactose along with the galectins. Following incubation, cells were washed three times and incubated with Alexa Fluor 488 streptavidin or Alexa Fluor 633 streptavidin (Molecular Probes) at 4°C for 30 min. Cells were washed twice, followed by resuspension in 400 µL PBS for analysis by flow cytometry.

All samples were assayed using a FACSCalibur flow cytometer (BD biosciences) with a minimum of 10,000 counts/sample. Data was analyzed using Cell Quest software.

Cell sorting

Cells were treated with Gal-1 for 12 hours followed by staining for PS using Annexin-V FITC as described above. Following staining, cells were subjected to a 70 mM mesh filter to remove cell aggregates, mounted in a water cooled jacket to maintain a temperature of 4°C and analyzed utilizing a MoStar cell sorter. Cells were then physically sorted into Annexin V positive and negative based on gates obtained from untreated stained cells. Cells were then washed in Ca2+ free buffer to remove Annexin V staining, examined by flow cytometry and shown to display no Annexin V positivity regardless of fraction. Cells were then re-stained with Annexin V FITC and examined by flow cytometry. Cells sorted for Annexin V positivity consistently displayed over 90% positivity while negative sorted cells displayed less than 10% positivity. Cells were then treated with Gal-1 as indicated and analyzed for Annexin V staining or DNA

fragmentation as outlined above or stained with biotinylated Gal-1 followed by detection with witih ALEXA 633 strepatvidin. For cell sorting based on strength of Gal-1 binding, cells were incubated with biotinylated Gal-1 followed by detection with ALEXA 633 strepatvidin. Cells were then sorted into higher and lower binding fractions, followed by removal of Gal-1 by lactose and reanalysis by flow cytometry to insure complete removal. Cells were then incubated with 10 μM Gal-1 for 4 hours followed by detection of PS exposure by Annexin V staining.

Ca^{++} flux measurements

HL60 cells, neutrophils or T cells were loaded with 3 mM Fluor AM at 37° C for 30 min in the presence of 4 mM probenecid, and inhibitor of anion transport, to minimize dye leakage. The cells were washed with HBSS, incubated for 30 min at RT to allow the Fluo-4 AM dye to completely de-esterify, washed twice more and resuspended at 107 cells/ml HBSS with 0.5 human serum albumin. Fluorescence readings were obtained in a stiring cell fluorometer (PerkinElmer Life Sciences LS-50) equipped with a water-jacketed cuvette holder. After obtaining the basal signal, fluorescence intensities were acquired at 0.1 s intervals for 10-15 in with continuous stirring of the cell suspension. The cells were lysed with 0.1% Triton X-100 to determine the maximum fluorescence. The minimum fluorescence was determined by adding EGTA to the lysed cells. These fluorescence measurements were converted to molar concentrations as previously described [397].

Cell viability measurements using the MTT assay

Relative viable cell number using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was also determined as outlined previously [223]. Briefly, cells were treated with PBS, Gal-1 or Gal-3 as indicated followed by the addition of MTT at a

final concentration of 0.5 mg/mL. Cells were then allowed to incubate for an additional 4 hours at 37°C. Cells were then incubated in a final concentratation of 2.5% SDS 0.0 1N HCl overnight at 37°C. Viable cell number was then assessed by determining the absorbance at 540 nm for each condition.

Cytokine detection

For stimulation of peripheral blood mononuclear cells (PBMC), 96-well plates (Costar) were pre-coated overnight at 37°C with anti-human CD3 (clone UCHT1, at 5 µg/mL) and anti-human CD28 (clone 28.2, at 1 µg/mL) (BD Pharmingen) in a volume of 50 µl/well. PBMCs obtained from three healthy volunteers were separately plated at 5 x 10^5 cells per well (50 µl) in the presence or absence of Gal-1 (20 µM) and cultured for 24 h in 5% humidified CO_2. TDG (20 mM) was added during the incubations to inhibit galectin binding. The following day, supernatants were harvested and submitted to cytokine analysis. IL-10 and INF-γ levels were determined simultaneously by the human cytometric bead array (CBA) kit (BD Biosciences Pharmingen), using a FACScan flow cytometer and CBA software (BD Biosciences).

Enzymatic Synthesis of Glycans

LNnT was degalactosylated by treatment with β-galactosidase to generate the trisaccharide, GlcNAcβ3Galβ1-4Glc (Triose). LDNT was synthesized from triose by addition of a terminal β4 linked GalNAc, as previously described[266] with some modifications, using bovine milk β4-galactosyltransferase and UDP-GalNAc as the donor. The reaction mixture consisted of 5 mmol of triose, 20 mmol UDP-GalNAc, and 5 U of β4-galactosyltransferase in 100 ml of 50 mM sodium cacodylate buffer, pH 7.4, containing 20 mM $MnCl_2$, 0.02% NaN_3, and 10mg/ml α-lactalbumin. The reaction was

carried out at 37˚C and aliquots were analyzed daily by HPAE-PAD chromatography to monitor conversion of the triose to the tetrasaccharide GalNAcβ4GlcNAcβ3Galβ1-4Glc (LDNT). The reaction was stopped after 3 days when it had proceeded to ~95% completion. The LDNT product was purified from the reaction mixture by chromatography on a Bio Gel P-2 column (1.5 cm X 160 cm) in H_20 and 3 ml fractions were collected. The chromatographic profile was monitored by measuring the absorbance of the fractions at 214 nm. Fractions containing the tetrasaccharide product were pooled and the yield was determined by absorbance at 214 nm using GlcNAc as standard. The reaction yielded 4.8 mmol of LDNT. Aliquots of the tetrasaccharide product were hydrolyzed by strong acid and analyzed by HPAE-PAD chromatography to confirm its monosaccharide composition.

The pentasaccharide Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (Lex-L) was synthesized from LNnT using GDP-Fuc as donor and recombinant human α3-fucosyltransferase VI (Calbiochem). The reaction was carried out in eight aliquots at 37˚C in a total reaction volume 200 ml/aliquot. Each reaction mixture contained 740 nmol of LNnT, 1 mmol GDP-Fuc, and 5 mU of enzyme in 50 mM sodium cacodylate, pH 7.5, containing 20 mM $MnCl_2$, 0.02% NaN_3, and 0.5 U alkaline phosphatase. (Aliquots of the reaction mixture were analyzed daily, as described above, to monitor conversion of LNnT to Lex-L.) The reaction was stopped after 72 h and a 2 nmol sample of the reaction mixture was analyzed by HPAE-PAD chromatography, which showed that all acceptor was converted to product. The product was purified by chromatography on a Bio Gel P-2 column, as described above. The total yield of Lex-L, as determined by absorbance at 214 nm, was 5.4 mmol per reaction. The remaining saccharides, LN-LN-LN-L, LN-Lex-Lex-L, LN-LDN-L, and LN-LN-L were prepared as described [398].

Neoglycoproteins

The oligosaccharides LN-LN-LN-L, LN-LN-L, LN-Lex-Lex-L, LN-LDN-L, LNnT, LDNT, Lex-L, Triose, and lactose were derivatized to BSA by reductive amination [399]. Briefly, 1.0 mg of each oligosaccharide was mixed with 670 mg BSA and 520 mg $NaBH_3CN$ in a total volume of 50 ml of 0.2M KH_2PO_4 buffer, pH 7.0. The mixture was incubated at room temperature in the dark for 14 days and the reaction was stopped by addition of water (2 ml). The mixture was dialyzed against water and the protein content was determined by BCA assay (Pierce, Rockford, IL). The mol of sugar derivatized per mol of BSA was determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). The molecular weights and numbers of glycans conjugated in each of the conjugates are described in Chapter 7 results Figure 1.

Preparation of ALEXA-dGal-1 and ALEXA Gal-3

dGal-1 was derivitized using either C5 maleimide or carboxylic acid, succinimidyl ester, dilithium salt reactive dyes while Gal-3 was derivatized using only carboxylic acid, succinimidyl ester, dilithium salt reactive dyes. A PD-10 column (Amersham Pharmacia Biotech) equilibrated in PBS was used to desalt the dGal-1 prior to mixing with the ALEXA-488 reactive dye. An aliquot of 400 μL desalted dGal-1 (3-5 mg/ml) was mixed with 50 μL of 1M lactose and 50 μL of 1 M NaH_2CO_3 to yield a 500 μL 3-5 mg/ml dGal-1, 100 mM lactose 100 mM NaH_2CO_3 solution. (Lactose was included in the labeling to block possible ALEXA-488 attachment at or near the CRD). This mixture was then mixed with the ALEXA-488 carboxylic acid, succinimidyl ester, dilithium salt reactive dye (Molecular Probes), and allowed to incubate on a microstir plate for 2h. This mixture was then applied to a Sephadex G25 column equilibrated in PBS containing 0.2 mM NaN_3. Protein quantification was performed as outlined in the protocol accompanying the kit (Molecular Probes protein labeling kit #A-10235). The elution profile of the re-

purified dGal-1 revealed a single peak of protein that was quantitatively eluted with lactose (data not shown).

β-Galactosidase treatment of LN-LN-LN-L-BSA

Generation of GlcNAc-LN-LN-L-BSA was accomplished by removal of the terminal non-reducing galactose residue from LN-LN-LN-L-BSA by incubating the immobilized conjugate in wells in 60 mL of 1U/ml Bovine testes β-galactosidase (Glyko) for 12 hr at 37ºC. Enzyme incubation was followed by PBS wash 5X and lectin binding detection.

Monospecific antibody detection of LDNT-BSA and Lex-L-BSA

Microtiter wells were coated with 50 μl of 0.3 mg/ml neoglycoprotein solution in a PBS buffer followed by a 5% BSA blocking solution. An IgM monoclonal antibody (SMLDN1.1) generated from the spleens of *S. mansoni* infected mice [266] was employed for detection of terminal LDN containing glycans. An IgG monoclonal antibody (F9A1.1.2), previously prepared [267, 268], was used to detect Le^x containing glycans. Bound antibodies were detected by incubation with 50 μl of either 10-20 mg/ml ALEXA-488 rabbit anti mouse IgG or ALEXA-488 goat anti mouse IgM for 1 h followed by PBS-Tween 20 (0.5% Tween 20) wash 5X and fluorescent detection using a PerkinElmer Wallac Victor^2 1420 Multilabel counter. Antibodies were diluted in a PBS, .05% Tween, 1% BSA solution.

Solid phase binding assays

A 50 μl aliquot of 0.3 mg/ml neoglycoprotein solution in a PBS buffer coated each well followed by a 5% BSA in PBS blocking solution. Triplicate analyses of each coating density were performed. Lectin dilutions were carried out in PBS-Tween containing 1%

BSA. A 50 μl volume of each lectin concentration was then allowed to incubate for 1 h. followed by PBS-Tween 20 (0.5% Tween 20) wash 5X and fluorescent detection using the PerkinElmer counter. Following RCA and dGal-1 fluorescent detection, each lectin was removed by incubating with a 200 mM lactose solution for 2 h. This allowed for each of the lectin binding determinations to be accomplished repeatedly on the same wells containing immobilized neoglycoproteins.

For cell binding assays, T cells were isolated and activated as outlined above with cells binding experiments conducted largely as outlined previously [65]. Following cellular activation, cells were biotinylated with NHS-LC-sulfo biotin (Pierce) according to the manufactures protocol. Biotinylated cells were fixed in 2% paraformaldehyde (PFH) buffered in PBS pH 7.4 at 4°C, followed by washing three times in PBS. Cells were incubated in streptavidin coated 96 microtiter wells (Pierce) at 50 μl per well (2 x 10^6 cells/ml). Cells were then incubated with ALEXA 488 Gal-1 or ALEXA 488 Gal-3, followed by washing three times and detection of binding using a Perkin Elmer Victor^2 flourimeter with an excitation/emission pair of 488/535 nm. Analysis of binding isotherms and curve fittings was accomplished using Sigma Plot software.

Binding of Galectin to Aminoalkyl Glycosides Immobilized on Activated (N-hydroxysuccinimidyl) Glass Surface

Glycan microarrays were prepared as described previously [292, 294] and obtained from the NIH/NIGMS-funded Consortium for Functional Glycomics [see http://www.functionalglycomics.org/static/index.shtml]. For galectin recognition of glycans on the printed glycan microarray, a solution of between 0.1 to 10 μM galectin in PBS containing 0.005% Tween 20 and 14 mM βME was incubated for 1 h at 25°C. The

slide was then immersed in PBS containing 0.005% Tween 20, drained, and then overlaid with FITC-Streptavidin. After 1 h at room temperature in a dark humid chamber, the slide was washed by successive immersion in PBS/0.01% Tween 20 (three times) and water/0.1% Tween 20 (twice). The slide was briefly rinsed with distilled water and dried under microfiltered air. An image of bound fluorescence was obtained using a microarray scanner (Scan Array Express, PerkinElmer Lifer Sciences). The integrated spot intensities were determined using Metamorph software (Universal Imaging, Downingtown, PA).

Measurement of Galectin Binding Affinity Using Surface Plasmon Resonance (SPR)

All surface plasmon resonance (SPR) experiments were preformed at 25°C on a Biacore 3000 instrument (Biacore AB (part of GE Healthcare), Uppsala, Sweden) largely as outlined previously [292, 294, 400, 401]. Biotinylated glycosides were captured on research grade streptavidin-coated sensor chips (Sensor Chip SA, Biacore Inc.) that were pretreated according to the manufacturer's instructions. A solution of each biotinylated glycoside (10 fmol/ml) was injected at 2 ml/min in PBS, pH 7 containing 0.005% Tween 20 (running buffer) for varying lengths of time (3-7 min) until an optimal amount of glycan was captured on each independent surface. Three related glycosides were studied using one streptavidin sensor chip. A control (non-binding) glycan, arabinose, was also captured on the same sensor chip, and the specific binding of non-derivatized recombinant Gal-1, Gal-2, or Gal-3 for the test glycans was measured using the in-line reference subtraction feature of the Biacore 3000 instrument. Increasing concentrations of Gal-1, Gal-2, or Gal-3 (0.1-100 μM) were injected at a flow rate of 60 ml/min over all four surfaces of the sensor chip. Bound Gal-1, Gal-2, or Gal-3 were eluted with the running buffer after the injection was complete. The equilibrium binding

data of Gal-1, Gal-2, or Gal-3 were analyzed by non-linear curve fitting using the BIAevaluation software (Biacore Inc.).

Enzymatic cell surface deglycosylation

Enzymatic cell surface deglycosylation was accomplished as outlined [133]. Prior to enzymatic deglycosylation, HL60 cells were fixed by washing three times in PBS at 4°C, followed by resuspension in 2% paraformaldehyde buffered in PBS (pH 7.4) at 4°C. Cells were allowed to fix overnight on a shaker at 4°C. Following fixation, cells were washed three times in PBS and then two times in the appropriate buffer as recommended by the manufacturer. For enzymatic digestion of cell surface glycans, fixed cells were washed in the following buffers. Cells were washed in 50 mM sodium citrate pH 6.0 and incubated with 250 mU *Salmonella typhimurium* α2,3 neuraminidase (New England Biolabs) at 10^7 cells/ml for 12 h at 37°C. Cells were washed in 50 mM sodium citrate with 100 mM sodium chloride pH 6.0 and incubated with 250 mU *Clostridium perfringens* α2,3- α2,6 neuraminidase (New England Biolabs) at 10^7 cells/ml for 12 h at 37°C. Cells were washed in 50 mM sodium acetate pH 5.8 and incubated with 200 mU *Escherichia freundii* endo-β-galactosidase (Seikagaku Kogyo) at 10^7 cells/ml for 24 h at 37°C. Cells were washed with 50 mM sodium phosphate pH 5.8 and incubated with 200 mU *Bacteroides fragilis* endo-β-galactosidase (Calbiochem; QA labs) at 10^7 cells/ml for 24 h at 37°C. Cells were washed in 50 mM sodium phosphate pH 5.0 and incubated with 100 mU Jack bean β-galactosidase (Glyko) at 10^7 cells/ml for 12 h. Buffer control treatments lacking enzymes were used for each individual condition.

Data analysis and curve fitting

Dissociation constants for each lectin with neoglycoprotein were calculated using a rectangular hyperbola equation to derive the nonlinear curve fitting (Sigma Plot software). Standard deviations were derived using the same software. For comparison of data obtained following flow cytometric analysis, results are expressed as mean values ± SD. The statistical analyses were preformed using one or two-way analysis of variance (ANOVA), as indicated in the figure legends. Pos hoc comparisons were performed using Bonferroni's test. All data were analyzed using Prism computer software (Graph-Pad). Differences were considered significant when $P<0.05$.

Assaying microbicidal activity

Blood group B positive *E. coli*, *E. coli* O86, originally identified by Springer and colleagues as a blood group B reactive *E. coli* strain isolated from human blood, was a kind gift from Dr. George Wang (The Ohio State University). The two blood group negative strains of *E. coli* (ATCC# 25922, ATCC# 35218), *K. pneumoniae* (ATCC#700603), *P. aeruginosa* (ATCC# 27853), and *S. aureus* (ATCC# 29213) were clinical reference strains obtained from the Emory University Clinical Microbiology lab. To examine potential binding by each galectin, bacteria were grown to mid-log phase, followed by resuspension in PBS and incubation with the indicated galectin as outlined above. When assaying potential anti-microbial effects of galectins, each strain was grown to mid-log phase, followed by incubation with the indicated concentrations of each galectin for 2 h at 37°C. Following incubation with each respective galectin, the number of viable bacteria was determined by dilution plating and CFU enumeration. Twenty mM lactose or sucrose was incubated with the galectin when indicated for 10 min prior to incubation with the bacteria.

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