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Evolving Concepts in the Roles of Galectins in Innate Immunity

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Abstract for "Evolving Concepts in the Roles of Galectins in Innate Immunity"

By

Connie Maridith Arthur
University of South Carolina, Aiken, 2006

Advisor: Richard D. Cummings, Ph.D.

Abstract for dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry, Cell and Developmental Biology

Graduate Division of Biological and Biomedical Sciences

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ABSTRACT
Evolving Concepts in the Roles of Galectins in Innate Immunity
Connie Arthur

Multiple facets of an effective immune response require efficient communication between multiple immune factors. Much of this signaling occurs through decoding the signals of distinct carbohydrate patterns found on individual cells. Members of the galectin family of carbohydrate binding proteins play a significant role in this process. Indeed previous studies have implicated galectin family members in regulation of numerous immune functions, including immune cell turnover, immune cell proliferation, and induction of cytokine secretion. Additionally, several galectins have been implicated in the regulation of leukocyte turnover. Several members of the galectins family possess the unique ability to induce exposure of phosphatidylserine (PS) to the surface of non-apoptotic cells, thus allowing their clearance by macrophages, independent of apoptosis. Crosslinking of carbohydrate receptors by galectin family members facilitates galectin signaling. However, previous studies focused on galectins that crosslink receptors through oligomerization of a single carbohydrate recognition domain (CRD). It remained unclear however, whether tandem repeat galectin, Gal-8 which possesses two distinct CRDs, could also function in this role, and what effect multiple CRDs would have on the glycan recognition and signaling by this lectin. Additionally, recent studies have demonstrated that galectins may serve important roles in direct recognition of microbial invaders. However, given what is known about the preference of galectins for self antigens, it seems unlikely that they could serve in a traditional pattern recognition receptor (PRR) context. However, studies have shown that several bacterial species display self-like antigens on their surface that might serve to shield these bacteria from recognition by immune defenses. Given their proclivity for self antigens, galectins are uniquely poised to provide protection against these bacteria. As receptor-ligand interactions provide the fundamental basis for biological activity, *we first sought to explore the carbohydrate binding specificity of Gal-4 and Gal-8.* Using obtained information regarding ligand specificity, *we asked whether and how this ligand specificity might impact cellular signaling and specific immunity against microbes baring self-like antigens*. Taken together, these studies provide significant insight into the pivotal role of the galectin family of carbohydrate binding proteins as factors of immune protection.

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Chapter 1- Introduction

Glycoconjugate Diversity and Biosynthesis

Although early studies established the roles of carbohydrates as fundamental components of cellular metabolism, the biological activity of complex carbohydrates on the cell surface remained somewhat enigmatic. However, it has become increasingly clear that these highly complex structures provide a unique template for regulating a broad range of biological activities. Indeed, cell surface carbohydrates appear to impact a wide variety of fundamental processes, ranging from subcellular trafficking to leukocyte viability. Proteins and lipids destined for membrane localization or export are decorated with oligosaccharide side chains that form the glycocalyx, a distinct layer of carbohydrates covering each cell. The components of this glycocalyx serve as a means of mediating many biological activities, such as fertilization, development and leukocyte migration (1,2).

The potential variation of these carbohydrate structures is immense and extends beyond that of linear nucleic acids and proteins. Complex carbohydrates exist in a wide variety of compositions and structures dictated by the expression of various glycosyltransferases in a given cell. Each glycosyltransferase possesses unique ability to differentially attach distinct monosaccharides to growing structures allowing unique carbohydrates to be formed depending on the expression and localization of glycosyltransferases in a given cell (1). Indeed, the same glycoprotein can possess distinct glycoforms depending on the activation and differentiation state of a cell. Importantly, different glycoforms of the same glycoprotein can significantly impact their biological function (3). Furthermore,

carbohydrates can be further modified by sulfation, methylation, and phosphorylation (1,2), which enable additional structural complexity. In addition, the process through which carbohydrates are added to a cell has evolved over time. Such key differences in glycosylation patterns of vertebrate organisms during evolution likely provide key mechanisms by which immune defense is able to recognize non-self (1,2). In this way, carbohydrates provide an unprecedented level of post-translational regulation of glycoprotein function and cellular control. The glycome of a cell is considered to be the entire repertoire of all complex glycans made by a cell, and it is estimated that the glycome of any particular cell may represent many thousands of different glycan structures(4,5)

While terminal carbohydrate structures undergo modification in a cell specific manner, most carbohydrates are attached to proteins through specific linkages to amino acids, of which over forty different sugar-amino acid combinations are known(6). However, the most common among those in mammals are structures linked to asparagine (N-linked, N-glycan) or serine/threonine (O-linked, O-glycan) residues. This distinction no only dictates the amino acid to which the carbohydrate will be attached, but also reveals the subcellular localization of their biosynthesis. N-glycan biosynthesis begins in the endoplasmic reticulum by the addition of a presynthesized complex glycan with 14 monosaccharide residues *en bloc* to Asn residues during protein translation and translocation into the ER lumen. Depending on the cell, a series different glycosidases and glycosyltransferases then modify this structure in both the ER and Golgi apparatus. By contrast, O-glycan formation of the mucin-type (GalNAcα1-Ser/Thr) begins in the Golgi apparatus with the addition of single monosaccharide directly to the peptide backbone. This is then extended by a series of glycosyltransferases to form complex O-glycans. In addition, distinct carbohydrate

modifications of lipids facilitate similar structural diversity on the cell surface. While the diversity of terminal structures would be predicted to significantly influence glycan and glycoprotein function, the potential influence of the core glycan structure on glycoprotein behavior and recognition remains unknown.

Glycan-binding Proteins – Galectins

Although the enormous complexity of carbohydrate structures strongly suggests a role in regulating biological processes, the mechanisms whereby glycosylation regulates such diverse processes is still not fully understood. Recent studies suggest that carbohydrate- or glycan-binding proteins, or lectins, possess the unique capacity to decode cell surface glycans. One of the largest and most ancient families of mammalian lectins, galectins, provides a prototypical example of lectin regulation of cellular behavior (7). The galectin family has members represented in all metazoans, with additional relatives in plants and fungi. Each galectin possesses affinity for galactose containing carbohydrate structures (8-10), however, differential recognition of cell surface glycans appears to enable different galectin family members to induce distinct signals in a variety of cell types. Divided into prototypical, chimeric and tandem repeat subfamilies based on their quaternary organization (Fig. 1-1), the structure of individual galectin family members also likely impacts galectin function. Alterations in glycosylation following T cell activation and differentiation results in differential sensitivity to the apoptosis-inducing or cytokine-secreting activities of different galectin family members. In this way, galectins appear to behave much like extracellular adapter proteins, with alterations in glycosylation resulting in differential recognition and subsequent signaling much like phosphorylation serves as a master regulator of intracellular processes.

Numerous studies suggest that galectin family members possess distinct signaling properties to regulate immune activity. The earliest study known to implicate galectins in immune regulation investigated the role of galectin-1 (Gal-1) in the neuromuscular junction of rabbits with experimental myasthenia gravis. Administration of Gal-1 to these rabbits ameliorated disease symptoms by suppressing the autoimmunity that caused the disease (11,12). Numerous studies followed verifying the role of galectin family members, particularly Gal-1 and galectin-3 (Gal-3), in the regulation of cells involved in the adaptive immunity. These studies have postulated roles for galectin family members that include altered activation and killing of T cells, induction or suppression of cytokines, and involvement in development of adaptive immune cells (Fig. 1-2) (9,13-16). In particular, recent studies demonstrate that not only do distinct T cell subsets possess unique cellular functions, but also alterations in glycosylation appear to distinctly regulate cellular sensitivity to galectin-induced signaling. In this way, galectins possess the unique ability to differentially recognize a cells glycan signature with important consequences in cellular viability and function.

Galectins in Innate Immunity and Leukocyte Turnover

While the recent studies examined the role of galectins in the regulation of adaptive immunity, less is known about the potential roles of this family in regulating cells involved in the innate immune response. However, given the broad regulatory activities of similar families, such as the TNF family, potential roles of galectins in regulating neutrophil activity appeared likely. Indeed, recent studies demonstrated that galectins might also partly regulate neutrophil function and turnover (14,17,18). Following an immune reaction, accumulated neutrophils must be removed efficiently to prevent damage to surrounding tissues by these

destructive cells (19). Previous studies suggested that neutrophil removal during inflammatory resolution occurs through apoptotic cell death, similar to physiological cellular removal in many other organs. However, recent studies suggest that neutrophils undergo apoptosis-independent removal, strongly suggesting that other mechanisms of cellular turnover likely exist and galectin may play a critical role in this process (14,18). For example, neutrophils immortalized by transgenic expression of bcl-2 in mice, continue to turnover normally, suggesting that non-apoptotic processes must be involved in their removal (20). A common means of recognizing and removing dying cells is through macrophage recognition and removal of cells through a receptor that recognizes externalized phosphatidylserine (PS) on the outer membrane of cells undergoing programmed cell death (19). In contrast to factors that induce apoptosis, several prototypical galectins, including Gal-1 (Gal-1) and Gal-3 (Gal-3) induce PS exposure in neutrophils independent of canonical features of apoptosis, such as caspase cleavage, mitochondrial potential changes or DNA fragmentation (17). Interestingly, PS exposure can occur in the absence of cell death, but such cells are sensitive to phagocytosis, which represents the first example of *living* cells being signaled for removal, a process recently termed preaparesis, signifying the ability of galectins to prepare cells for phagocytic removal without causing traditional programmed cell death (Fig. 1-3) (21). However, while Gal-1, Gal-2, and Gal-3 appear to possess this unique activity, whether other galectin family members also induce preaparesis remains untested.

While studies have clearly demonstrated human and murine galectins' involvement in adaptive immunity, the evolutionarily ancient history of this protein family suggests that these lectins may also possess fundamental behaviors that predate adaptive immunity. Correspondingly, recent studies suggest that galectins in lower species, lacking an adaptive

response, directly impact pathogen infectivity and persistence (22,23). These studies suggest that galectin orthologs may serve as pathogen recognition receptors, however relatively few studies focus on direct interaction of murine and human galectins with microbes. Furthermore, unlike traditional pathogen recognition receptors, which bind unique pathogen associated molecular motifs (PAMPs) that identify microbes as "non-self" (24), mammalian galectins appear to exclusively recognize self-glycans, such as blood group-related structures (21,25-28). Importantly, recognition of self-glycan determinants serves as the fundamental basis for galectin-induced immune-regulation, suggesting that murine and human galectins may have lost the anti-microbial activities documented in other organisms.

Microbial Glycomes and Host-like Glycans

Although the unique antigenic determinants of pathogens serve as receptors for many innate immune proteins and a focus for the specificity of an adaptive immune response, recent studies suggest that several microbes uniquely express self-like antigens; the mechanism whereby individuals protect themselves from self-like antigen baring pathogens remains unknown. Deletion of self-reactive lymphocytes during central tolerance helps reduce the probability of autoimmunity, but it leaves a gap in the ability of adaptive immunity to protect an individual against pathogens with self-like antigens. For example, consider the human ABO(H) system, which is comprised of specific glycan structures whose expression are uniquely and genetically defined in people and are also strong antigens. Individuals of A blood group do not possess antibodies against A antigen, but may have antibodies to B blood group, thus precluding blood transfusion between such different blood types. While blood group B positive pathogens stimulate antibodies in blood group B negative individuals, and this may be the origin in people of antibodies to their opposite blood types,

similar responses fail to occur in blood group B positive individuals. Therefore, such B-expressing bacteria would be able to effectively "hide" from the protective forces of adaptive immunity, posing a constant risk of infection to an individual with the cognate blood type. Thus, additional mechanisms likely exist whereby blood group B individuals protect themselves against blood group B positive microbes, and group A individuals would have mechanisms to prevent their infection by A positive microbes, etc. Given the proclivity of mammalian galectins for self-antigens, in addition to their history as microbial regulators, galectins may be uniquely poised to provide this very unique form of innate immune activity. However, whether mammalian galectins possess the ability to recognize or alter the viability of microbes was unknown, prior to this thesis work.

Glycan Microarrays as Tools to Study Lectin Recognition of Glycans

While previous studies suggest that individual galectin family members likely possess intriguing activities toward both leukocytes and self-antigen baring pathogens, significant limitations in understanding these putative galectin functions results from severe limitations in the tools available to study protein-carbohydrate interactions. Unlike other biological macromolecules, carbohydrates are not readily synthesized using simple molecular techniques. As a result, development of large libraries of defined carbohydrates structures previously appeared improbable and significantly limited the ability of investigators to fully elucidate protein-carbohydrate interactions. However, recent advances in carbohydrate synthesis, labeling and microarray development provide a compelling tool to define the binding specificity of any carbohydrate binding protein. This has led to the development of glycan microarrays, in which specific glycan structures are immobilized on glass slides and other surfaces in nanograms quantities, thus permitting their evaluation as potential ligands

for different lectins. Such glycan microarrays developed in the past 10 years have revolutionized the field of glycoscience, permitting high-throughput analyses of glycan recognition by lectins (29-31). As a result, glycan microarrays provide a vast improvement over previous technologies and provide hope that examination individual galectin family members will provide key insights and ideas into the biological functions of these proteins.

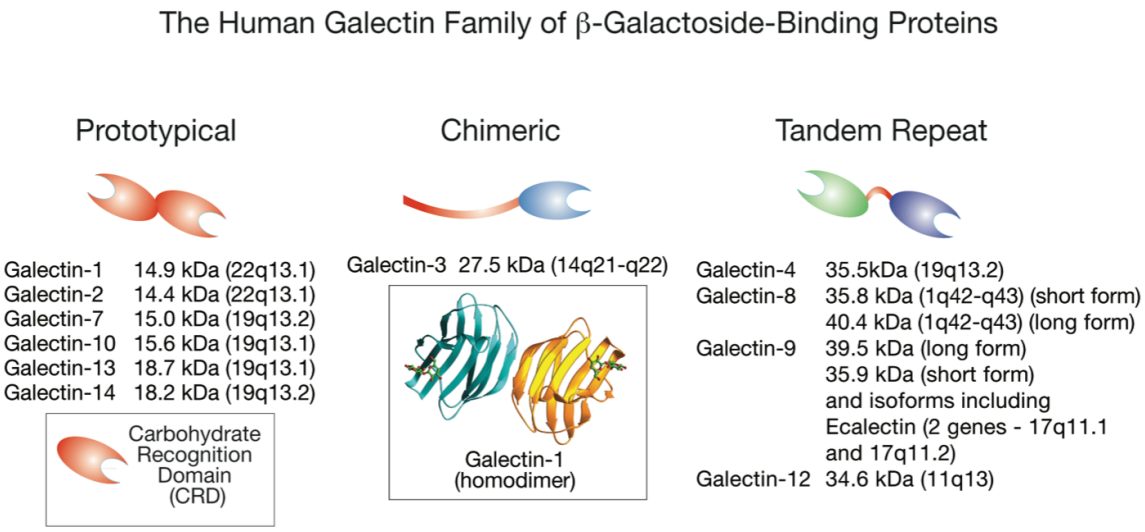
Focus on Tandem-Repeat Galectins in Innate Immune Responses

Given the evolutionarily ancient nature of the galectin family members and their genetically defined roles in regulating leukocyte function, additional studies are needed to further define their biological roles. There are 11 currently defined human galectins and 15 different galectins overall in mammals (7-9); thus, a detailed examination of every galectin family member is beyond the scope of this project. While many previous studies primarily focused on Gal-1 (Gal-1) and Gal-3 (3), homo-oligomeric proteins, very few studies examined the binding specificity and signaling potential of tandem repeat galectins, which possess two unique carbohydrate domains (CRD) in a single polypeptide with each CRD linked by a "bridging" peptide (8,9). Thus, it is possible that each of these two CRDs in such tandem-repeat galectins has different functions and recognizes different glycans. Given the unique structural organization of tandem repeat galectins and the relatively paucity of data concerning their biological activities, we decided to primarily focus our studies on the first two tandem repeat galectins described, galectin-4 (Gal-4) and galectin-8 (Gal-8). These proteins are highly expressed in intestinal epithelial cells, but can be found at some level in many different cell types (32). Indeed, the colon is the only site where Gal-8 was shown to be expressed based on the criteria of immunohistochemistry, Northern blot, RT-PCR, and based on data from the Cancer Genome Anatomy Project (CGAP library analysis) (33).

As receptor-ligand interactions provide the fundamental basis for biological activity, *we first sought to explore the carbohydrate binding specificity of Gal-4 and Gal-8*. Using that information as a clue in regard to their ligand specificity, *we then asked whether and how this ligand specificity might impact cellular signaling*. Finally, based on our discoveries about the unique ability of galectins to bind self-like antigens and the potential ability of evolutionarily ancient galectins to regulate microbial viability, we explored *the intriguing hypothesis that galectins provide specific immunity by targeting microbes baring self-like antigens*. **Overall, this thesis work was led by the overarching hypothesis that each domain of Gal-4 and Gal-8 has unique carbohydrate specificity, and that this carbohydrate specificity ultimately results in distinct immune signaling and antimicrobial activity**.

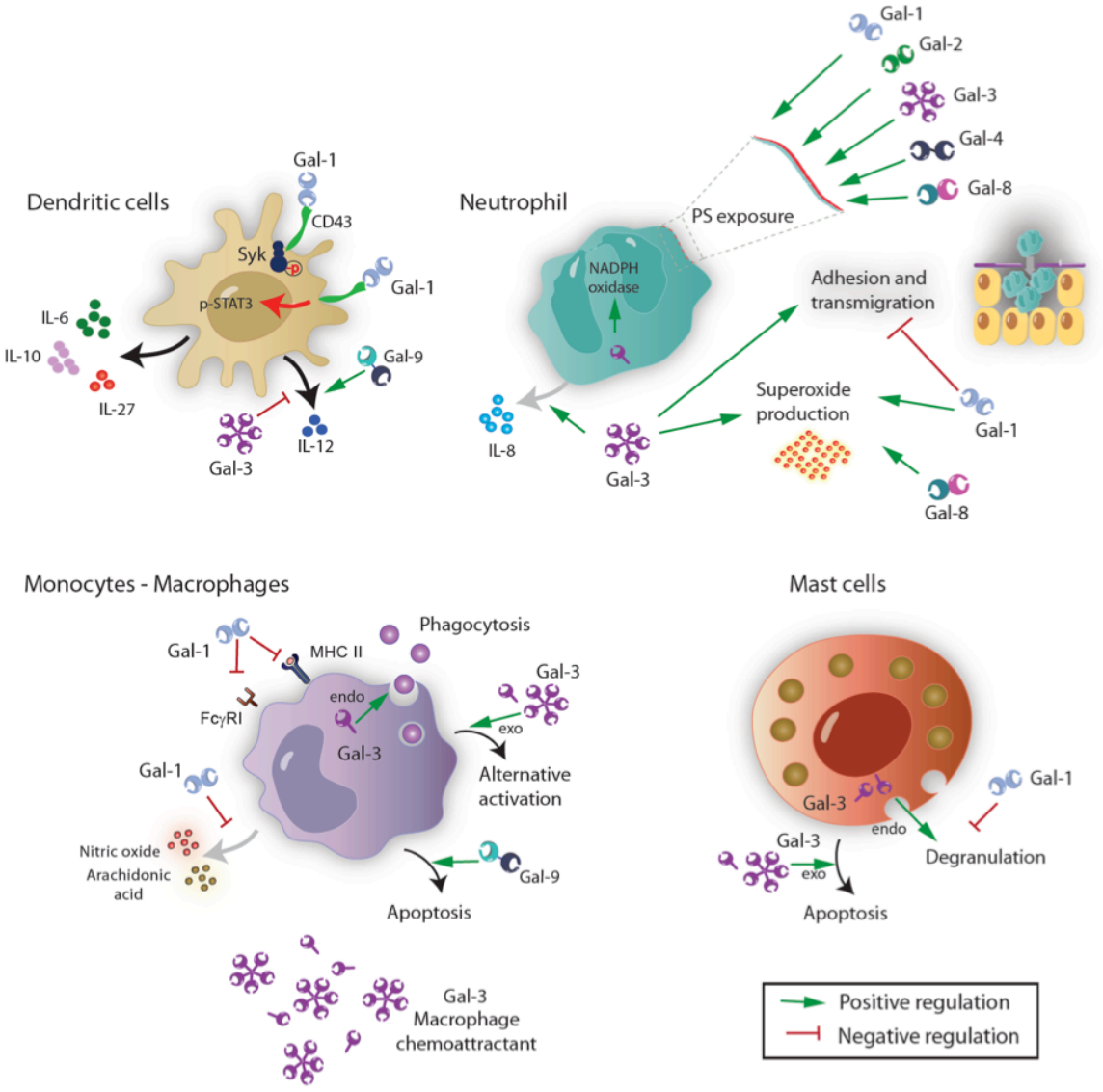
Figures

Figure 1-1



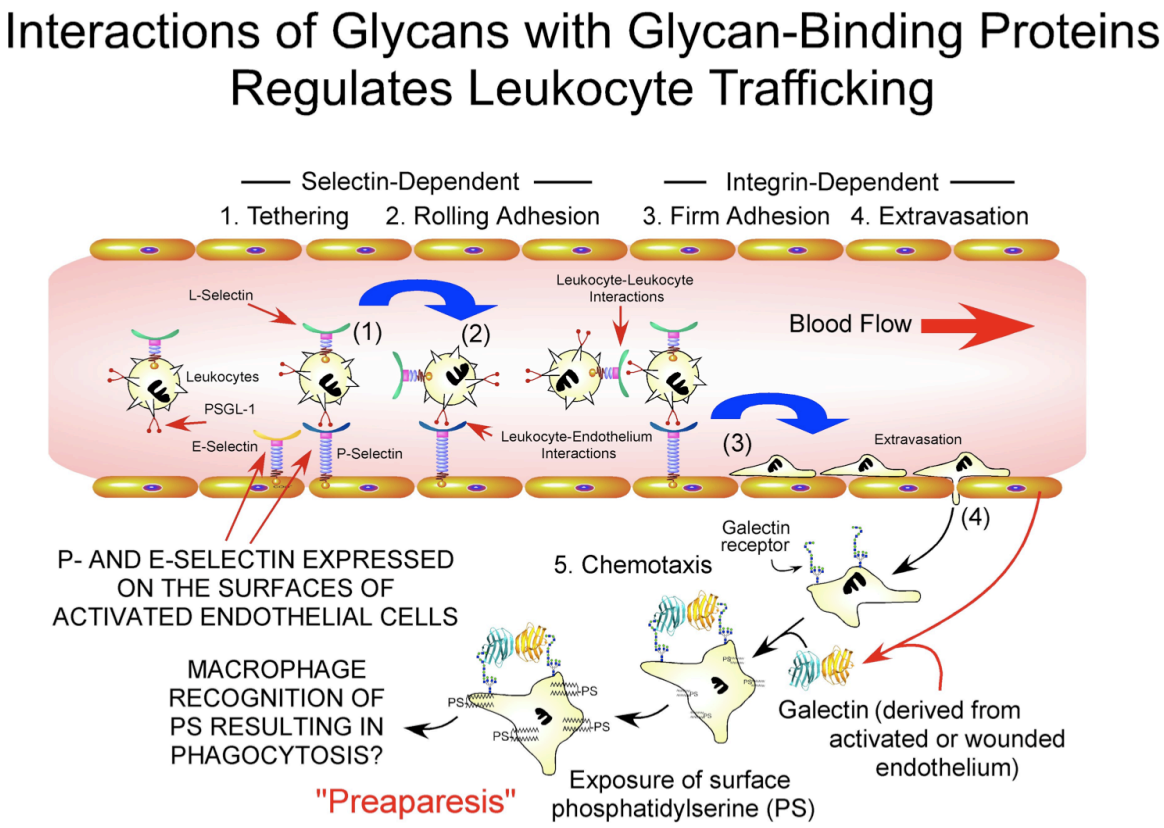
*R. D. Cummings

Figure 1-2



*Cerliani, Stowell, Mascanfroni, Arthur, Cummings & Rabinovich (2011) *J. Clin. Immunol.* **31**(1):10-21

Figure 1-3



*R.D. Cummings

Figure legends

Figure 1-1- The human galectin family of β-galactoside binding proteins - Schematic of known human galectins, separated into either prototype, chimera, or tandem repeat subfamilies based on differences in protein structure. (Figure by R. D. Cummings)

Figure 1-2- Putative functions of known mammalian galectin family members- Functions of known galectin family members are depicted. (Figure from Cerliani, Stowell, Mascanfroni, Arthur, Cummings & Rabinovich (2011) *J. Clin. Immunol.* **31**(1):10-21)

Figure 1-3- Role of galectins in inflammatory resolution- Simplified schematic of galectin signaling of PS exposure in the inflammatory process. Induction of PS exposure by galectin leads to phagocytic removal of live neutrophils by macrophages. (Figure by R.D. Cummings)

Chapter 2- Dimeric Galectin-8 induces phosphatidylserine exposure in leukocytes through polylactosamine recognition by the C-terminal domain.

Human galectins have distinct and overlapping biological roles in immunological homeostasis. However, the underlying differences among galectins in glycan binding specificity regulating these functions are unclear. Galectin-8 (Gal-8), a tandem repeat galectin, has two distinct carbohydrate recognition domains (CRDs) that may crosslink cell surface counter receptors. Here we report that each human Gal-8 CRD has differential glycan binding specificity and that cell signaling activity toward leukocytes resides in the C-terminal CRD. Both full-length forms of human Gal-8 and recombinant individual domains (Gal-8N, Gal-8C) bound to human HL60 cells, but only full-length Gal-8 signaled phosphatidylserine (PS) exposure in cells, which occurred independent of apoptosis. While desialylation of cells did not alter Gal-8 binding, it enhanced cellular sensitivity to Gal-8-induced PS exposure. By contrast, desialylation of HL60 cells increased their binding by Gal-8C, but reduced Gal-8N binding. Enzymatic reduction in surface poly-N-acetyllactosamine (polyLacNAc) glycans in HL60 cells reduced cell surface binding by Gal-8C, but did not alter Gal-8N binding. Cross-linking and light scattering studies showed that Gal-8 is dimeric, and studies on individual subunits indicate that dimerization occurs through the Gal-8N domain. Mutations of individual domains within full-length Gal-8 confirmed that signaling activity toward HL60 cells resides in the C-terminal domain. In glycan microarray analyses, each CRD of Gal-8 showed different binding, with Gal-8N recognizing sulfated and sialylated glycans, and Gal-8C recognizing blood group antigens and polyLacNAc glycans. These results demonstrate that Gal-8

dimerization promotes functional bivalency of each CRD, which allows the C-terminal domain of Gal-8 to signal PS exposure in leukocytes by recognition of their polyLacNAc glycans.

Introduction

Cellular turnover represents a key regulatory process in inflammation resolution. Many factors regulate leukocyte turnover, including members of the TNF and galectin families (18,34-38). TNF family members, such as FasL and TNFα, induce apoptotic cell death in target cells (35-37), which favors immunologically silent removal (39). However, some members of the galectin family of glycan binding proteins can induce two distinct pathways for leukocyte turnover (18). For example, galectin-3 (Gal-3) induces phosphatidylserine (PS) exposure and apoptotic cell death in T cells, while galectin-1 (Gal-1) likely alters T cell physiology predominately through pathways functioning largely independently of cell death (40,41). In contrast to Gal-3-induced PS exposure in T cells, Gal-3 induces PS exposure in the absence of cell death in neutrophils (40). Gal-1, Gal-2, and Gal-4 also induce PS exposure independently of apoptosis in neutrophils (18). Importantly, galectin-induced PS exposure in neutrophils results in phagocytic engulfment of target cells (38), allowing clearance of living cells. This distinct mode of cellular turnover, recently termed preaparesis (14), differs fundamentally from apoptosis and necrosis, as it prepares cells for phagocytic clearance without inducing apoptosis. This unique process appears to be specific to neutrophils thus far (18). Since neutrophils harbor significant destructive potential following activation and undergo apoptosis-independent removal in vivo (42-46), preaparesis may be

important in protecting viable tissue from neutrophil-mediated injury that can occur following exuberant neutrophil apoptosis (47-50).

In contrast to TNF family members, which induce trimerization of counter receptors through protein-protein interactions (35-37,51), galectins engage cell surface receptors through their binding to cell surface carbohydrate ligands (52-54). Unlike typical receptor-ligand interactions, which are restricted to specific sets of ligands, glycan ligands can be modulated by a variety of other sugar modifications following changes in leukocyte activation and/or differentiation (54-57). Such changes allow for an additional regulatory level controlling cellular sensitivity toward galectin family members. Understanding differences in glycan recognition by individual galectin family members will allow a more clear understanding of how changes in glycosylation might differentially impact cellular sensitivity toward various galectin family members.

Galectin-8 is a tandem repeat galectin of ~36 kDa with two carbohydrate-recognition domains (CRDs). It is expressed in a wide variety of organs and cells, including endothelial cells and human thymocytes (58,59), and its expression is up-regulated in several cancers (60). Similar to Gal-1, Gal-2, and Gal-3, Gal-8 signals key responses in different leukocyte populations (26,58,61,62). However, unlike Gal-1, Gal-2, and Gal-3, which form homo-oligomeric structures required for cellular signaling (38), the oligomeric structure of Gal-8 and the contributions of each CRD required for cell signaling toward leukocytes are not well understood. Gal-8 is currently thought to exist as a monomer with each CRD joined by a common linker region, providing functional bivalency (63-65). However, the two separate CRDs of Gal-8 share little sequence similarity (65), consistent with preliminary findings that

each domain likely recognizes distinct ligands (26,27). As previous studies suggest that galectins crosslink homotypic receptors (38,66), the mechanism whereby Gal-8 may signal similar responses remains enigmatic.

Here we describe the signaling activity and binding specificity of Gal-8 toward cell surface glycans and chemically defined glycans on a glycan microarray. Our results show each CRD of Gal-8 recognizes distinct glycans. More importantly, we found that Gal-8 exists as a dimer, thus functionally expressing four CRDs, allowing functional bivalency at each separate domain. While each domain recognizes cell surface glycans, only the C-terminal domain of Gal-8 recognizes polyLacNAc cell surface glycans (-3Galβ1-4GlcNAcβ1-)$_n$ and induces preaparesis in human promyelocytic HL60 cells. These results challenge the current paradigm concerning the mechanisms of tandem repeat galectin signaling, and strongly suggest complex biological roles for this subfamily of tandem repeat galectins.

Results

Gal-8 induces PS exposure in HL60 cells

To elucidate the binding specificity of Gal-8 toward leukocyte cell surface glycans, we first examined the ability of Gal-8 to induce PS exposure in HL60 cells. We have successfully used this approach to define the cell surface binding specificity for Gal-1, Gal-2, and Gal-3 (67). Gal-8 induced robust PS exposure in HL60 cells (Fig. 2-1B,E). Importantly, inclusion of thiodigalactoside (TDG) inhibited Gal-8-induced PS exposure (Fig. 2-1C,E), while

sucrose had no effect (Fig. 2-1D,E), indicating that Gal-8-induced PS exposure required recognition of cell surface glycans. Gal-8 also induced PS exposure in a dose-dependent manner (Fig. 2-1F) with similar kinetics as observed with other galectins (18,67) (Fig. 2-1G). This PS exposure occurred independently of apoptosis, since there was no enhancement of cell or DNA fragmentation (Fig. 2-2A-E) and cell viability was unaltered by Gal-8 treatment over 72 hours (Fig. 2-2F).

Desialylation of HL60 cells does not alter Gal-8 binding, yet enhances Gal-8-induced PS exposure

Since HL60 cells have functional leukocyte glycan ligands for Gal-8, we examined the glycan binding requirements of Gal-8 for functional receptors. Gal-8 bound to HL60 cells (Fig. 2-3A), and binding was inhibited by TDG but not by sucrose (Fig. 2-3A), which demonstrates that binding and signaling by Gal-8 require recognition of surface glycan ligands (Fig. 2-1E). A recent study demonstrated that sialylation results in differential effects on the binding and signaling of other galectin family members (67). Thus, we examined the effects of desialylation on Gal-8 recognition of cell surface glycans. Removal of sialic acids by treating cells with neuraminidase did not significantly alter Gal-8 binding (Fig. 2-3B,H); by contrast, the binding of treated cells to RCA, a plant lectin that recognizes terminal galactose residues (68,69) that would be exposed after removal of sialic acid, showed a significant increase in binding (Fig. 2-3C,H). We next determined whether desialylation of cells affected cellular sensitivity to Gal-8-induced PS exposure. Interestingly, desialylated HL60 cells were significantly more sensitive toward Gal-8-induced PS exposure (Fig. 2-3D-G, I). Taken

together, these results demonstrate that removal of cell surface sialic acid does not appreciably alter full-length Gal-8 binding, but it does enhance cellular sensitivity to Gal-8-induced PS exposure.

The discordance between Gal-8 binding and cell signaling suggests a more complex relationship between the interaction of Gal-8 with cell surface glycans and subsequent signaling events than we anticipated. Previous studies demonstrated that the ability of Gal-3 to bind cell surface glycans independently of the sialylation status of the cells results from recognition of internal N-acetyllactosamine (LacNAc) motifs within long chain poly-N-acetyllactosamine (polyLacNAc) (-3Galβ1-4GlcNAcβ1-)$_n$ glycans (67). By contrast, Gal-1 and Gal-2, which also recognize cell surface polyLacNAc glycans (52,67,70), primarily recognize the terminal LacNAc unit, making modifications of this LacNAc relevant to glycan recognition (67). To examine whether Gal-8 displays similar binding preferences as Gal-3, we first treated cells with β-galactosidase, which removes the terminal galactose of terminal LacNAc containing glycans. Treatment of cells with β-galactosidase did not significantly alter Gal-8 binding (Fig. 2-3J), although RCA binding was significantly reduced (Fig. 2-3J), demonstrating the accessibility of cell surface glycans to β-galactosidase treatment. By contrast, treatment of cells with endo-β-galactosidase, which specifically cleaves polyLacNAc, significantly reduced Gal-8 binding in a manner comparable to that observed for LEA (Fig 2-3K), a plant lectin that strongly binds polyLacNAc glycans (71). These results suggest that Gal-8 recognizes cell surface polyLacNAc residues through internal LacNAc motif recognition rather than through the terminal LacNAc determinant.

Gal-8N and Gal-8C fail to induce PS exposure in HL60 cells

Unlike Gal-1, Gal-2 and Gal-3, Gal-8 possesses two unique CRDs, suggesting that a more complex interaction with cell surface glycans may occur. To test this, we first examined whether the C-terminal domain (Gal-8C) and/or the N-terminal domain (Gal-8N) can recognize leukocyte counter receptors as individual domains (Fig. 2-4A). Both CRDs bound HL60 cells (Fig. 2-4B and data not shown). Binding by each domain required glycan recognition and was inhibited by TDG but not sucrose (Fig. 2-4B and data not shown). Importantly, treatment of cells with neuraminidase significantly reduced cell surface binding by Gal-8N (Fig. 2-4C,J,L). By contrast, neuraminidase treatment significantly enhanced binding by Gal-8C (Fig. 2-4D,K,L), consistent with the enhanced cellular sensitivity toward Gal-8 induced PS exposure (Fig. 2-3I). Unexpectedly, although both domains bound HL60 cells, neither individual domain induced PS exposure (Fig. 2-4F,G,I). Furthermore, neither domain inhibited the ability of full-length Gal-8 to induce PS exposure (Fig. 2-4I). Since treatment of cells with neuraminidase enhanced Gal-8C binding and cellular sensitivity to Gal-8-induced PS exposure, we explored whether treatment of cells with neuraminidase might enhance cellular sensitivity toward either domain. However, pre-treatment of cells with neuraminidase failed to alter cellular sensitivity toward either independent domain (data not shown). Furthermore, co-incubation of both Gal-8N and Gal-8C failed to induce PS exposure in HL60 cells (Fig. 2-5A-D). Taken together, these results demonstrate that although each domain recognizes cell surface glycans, neither alone can induce PS exposure. Although treatment of cells with neuraminidase increased Gal-8C binding, similar to the enhancement of cellular sensitivity to Gal-8-induced PS exposure, treatment with neuraminidase failed to make cells sensitive to Gal-8C.

Gal-8 exists as a dimer

Previous studies with other galectins, especially Gal-1, demonstrated a requirement for galectin dimerization in galectin-induced signaling, most likely due to a need for galectin-induced crosslinking of cell surface receptors in the successful propagation of cellular signaling (38). The current paradigm concerning tandem repeat galectin signaling suggests that the linker region provides the necessary bivalency between the two separate CRDs, consistent with the inability of each independent CRD or co-incubation with both CRDs to signal on their own (Fig. 2-4I, Fig. 2-5D). However, to explore whether each domain indeed behaves as a monomer, we performed chemical cross-linking studies using BS3, a homobifunctional, water-soluble, non-cleavable crosslinker with a diameter of 11.4 Å. Incubation of Gal-8C with or without BS3 did not trap a homodimeric Gal-8C species (Fig. 2-5E). By contrast, incubation of Gal-8N with BS3, resulted in significant trapping of a dimeric Gal-8N species (Fig. 2-5E). These results suggest that the N-terminal domain of Gal-8 may be capable of dimerizing.

Since co-incubation of individual Gal-8C and Gal-8N with leukocytes failed to induce PS exposure, we next examined whether Gal-8C and Gal-8N may dimerize when added together. We fluorescently labeled Gal-8C to enable detection of Gal-8C migration following crosslinking in a mixture of Gal-8N and Gal-8C. Labeled Gal-8C, similar to Gal-8C alone, could not be significantly cross-linked (Fig. 2-5F). Furthermore, Gal-8C did not prevent crosslinking of Gal-8N when mixed together in parallel experiments (Fig. 2-5F). Finally, Gal-8C, when co-incubated with Gal-8N, did not enhance the amount of chemically crosslinked dimer (Fig. 2-5F). These results suggest that not only does Gal-8C fail to prevent

Gal-8N from dimerization, it does not likely form heterodimers with Gal-8N. As a control to ensure that labeling does not preclude chemical trapping, we labeled Gal-1, previously demonstrated to be dimeric (72,73), using the identical protocol. Incubation of BS3 with Gal-1 resulted in significant trapping of dimer (Fig. 2-5G). The ability of Gal-8N to dimerize suggested that full-length Gal-8 may also exist as a dimer. However, to rule out the possibility that Gal-8N only dimerizes as an independent domain and does not reflect a potential quaternary structure of the full-length protein, we incubated Gal-8 with BS3. Similar to Gal-8N, incubation of Gal-8 with chemical crosslinker trapped significant amount of dimeric species (Fig. 2-5H). In addition, analysis of Gal-8 using multiangle light scattering demonstrated that Gal-8 and Gal-8N exist as dimers, while Gal-8C is a monomer (data not shown). The identification of unmodified monomeric Gal-8C through light scattering studies also ruled out the possibility that fluorescent labeling might somehow prevent oligomerization. Although future studies will examine in detail the monomer-dimer equilibrium, including the extent to which ligand may regulate this equilibrium, these results demonstrate that Gal-8 exists as a dimer, likely through homodimeric interactions of the N-terminal domain.

Only Gal-8NM recognizes cell surface polyLacNAc glycans

The ability of Gal-8 to dimerize potentially changes the current paradigm concerning the nature of tandem repeat galectin crosslinking and signaling of functional cellular receptors. To test this, we examined the specificity of each CRD in the context of the full-length protein. We mutated the critical canonical arginine in each CRD to a histidine (R69H in N-terminal domain and R233H in the C-terminal domain), which generated Gal-8NM and Gal-

8CM, respectively (Fig. 2-6A). To confirm that this mutation eliminated glycan recognition by the respective mutated CRD, we tested whether Gal-8C and Gal-8N exhibit differential recognition of cell surface glycans in the context of the full-length protein. To test this, we first examined the effect of neuraminidase treatment on cell surface binding by Gal-8NM and Gal-8CM. Gal-8NM and Gal-8CM recognized cell surface glycans, and TDG, but not sucrose, inhibited recognition, which showed that binding was carbohydrate-dependent (Fig. 2-6B and data not shown). Similar to data with the Gal-8N domain alone, treatment of neuraminidase significantly reduced cell surface binding by Gal-8CM (Fig. 2-6E). By contrast, Gal-8NM showed enhanced binding toward cell surface glycans following removal of sialic acid, consistent with changes observed in binding of the Gal-8C domain alone (Fig. 2-6E). Interestingly, treatment of cells with endo-β-galactosidase resulted in a nearly complete loss of Gal-8NM binding (Fig. 2-6C,F), while the same treatment did not significantly alter Gal-8CM cell surface recognition (Fig. 2-6D,F). Furthermore, treatment of HL60 cells with endo-β-galactosidase resulted in an intermediate decrease in binding of Gal-8 (Fig. 2-6F), suggesting that the N-terminal domain, in the context of the full-length protein, retains the ability to recognize non-polyLacNAc containing cell surface glycans. Importantly, similar binding preferences also occurred following examination of the Gal-8N and Gal-8C domain constructs alone (Fig. 2-6G), confirming that this preference relies on the intrinsic binding properties of each individual domain. Taken together, these results demonstrate that specific mutations in each domain of the full-length protein resulted in binding profiles similar to each domain alone. Furthermore, sialylation differentially impacts the binding of each domain and recognition of cell surface polyLacNAc glycans by Gal-8 occurs through the C-terminal domain.

Gal-8 induces PS exposure entirely through C-terminal domain

Previous studies demonstrated that Gal-1, Gal-2, and Gal-3 signal PS exposure in leukocytes through recognition of cell surface polyLacNAc (67). The dependence of Gal-8 on the C-terminal domain for polyLacNAc recognition and the enhanced binding of Gal-8C following treatment of cells with neuraminidase suggests that the C-terminal domain may alone be responsible for signaling. To test this, we treated cells with Gal-8NM or Gal-8CM followed by examination for PS exposure. Gal-8NM, but not Gal-8CM, induced robust PS exposure in HL60 cells (Fig. 2-7A-C,G), and signaling was inhibited by TDG but not by sucrose (Fig. 2-7H). These results also suggested that the increased sensitivity of cells to Gal-8 following treatment with neuraminidase may reflect enhanced recognition of the functional receptor by Gal-8C, despite the failure of neuraminidase to alter Gal-8 binding. Consistent with this possibility, treatment of cells with neuraminidase significantly enhanced PS exposure induced by Gal-8NM (Fig. 2-7D-F,G), while failing to alter cellular sensitivity to Gal-8CM. Taken together, these results demonstrate that Gal-8 exists as a dimer and induces PS exposure entirely through C-terminal domain recognition of polyLacNAc glycans.

Gal-8 recognizes four primary classes of glycans

To further define the glycan recognition of Gal-8 and each CRD within the protein, we examined binding toward a chemically defined glycan microarray. Examination of Gal-8 at concentrations previously used to explore binding specificity (30,52,67,74) resulted in saturated binding of many glycans (Fig. 2-8A,D), suggesting that Gal-8 may accommodate many different glycan modifications. Yet these results are not consistent with the specific

types of glycans likely recognized by Gal-8 in concentration ranges over which Gal-8 binds to and signals leukocytes. Although Gal-8 induced PS exposure in the micromolar range, the apparent affinity of Gal-8 toward leukocyte counter-ligands is unknown. To examine the binding in more detail, we measured binding of Gal-8 toward HL60 cells. Gal-8 exhibited saturable binding to HL60 cells, with an apparent K_d of ~0.5 μM (Fig. 2-8B). Thus, we examined Gal-8 binding toward the glycan microarray at sub-micromolar concentrations. When binding studies of Gal-8 were performed at 0.3 μM, we observed four distinct classes of glycans recognized by Gal-8. These included sulfated glycans, sialylated glycans, polyLacNAc, and blood group antigens (Fig. 2-8C,E).

Each Gal-8 CRD binds distinct classes of glycans

Because each CRD of Gal-8 appeared to display unique glycan recognition properties toward cell surface glycans, we next sought to determine whether similar preferences occurred following examination of Gal-8N and Gal-8C binding on the glycan microarray. We first examined the individual domains at a higher concentration to determine whether any overlap in binding may occur. Interestingly, Gal-8N (Fig. 2-9A) and Gal-8C (Fig. 2-9B) displayed completely distinct binding even at high concentrations. When evaluated at lower concentrations, Gal-8N and the full-length Gal-8 recognized the same sulfated and sialylated glycans with a similar relative affinity (Fig 2-9E,F). By contrast, Gal-8N did not bind to polyLacNAc glycans or blood group antigens. Gal-8C recognized polyLacNAc and blood group antigens, while exhibiting no binding toward sulfated or sialylated glycans (Fig. 2-9G,H). Importantly, sialylation significantly inhibited binding of Gal-8C to polyLacNAc structures (data not shown), consistent with enhanced binding of Gal-8C toward cell surface

glycans following neuraminidase treatment. In contrast to Gal-8N, however, Gal-8C displayed much weaker binding toward these glycans (Fig. 2-9G,H) when compared to the full-length Gal-8.

To determine whether the mutations in each CRD effectively prevented glycan recognition by the respective mutated CRD, we first analyzed each mutant on the glycan microarray at higher concentrations. Gal-8 binding following mutation of the C-terminal domain (Gal-8CM), produced identical binding with similar relative affinity toward respective glycans as Gal-8N (Fig. 2-9C,E,F), which shows that this mutation precluded Gal-8C domain from recognizing glycan. Similarly, Gal-8 possessing the analogous mutation in the N-terminal domain (Gal-8NM) produced identical specificity as the Gal-8C domain (Fig. 2-9D). However, in contrast to Gal-8C domain, Gal-8NM exhibited higher binding toward these respective glycans (Fig. 2-9G,H), which suggested that although the C-terminal domain may not independently dimerize, it likely behaves as a functionally bivalent CRD in the context of the full-length dimeric protein.

Discussion

Using a combined approach of cell surface binding, cell signaling, and glycan recognition by glycan microarray analyses, our results demonstrate that Gal-8 signals PS exposure by dimeric binding through C-terminal CRD recognition of cell surface polyLacNAc glycans. These results provide new information about the mechanism of Gal-8 signaling and strongly suggest that differential recognition of polyLacNAc glycans may underscore key differences in the biological activities of galectin family members.

The dimeric state of Gal-8 enables functional bivalency at each independent CRD and provides an explanation for differences observed between binding and signaling of Gal-8 before and after treatment of cells with neuraminidase. Although neither domain alone induced PS exposure, the N-terminal domain, but not the C-terminal domain, can dimerize, suggesting that crosslinking of functional cell surface receptors must rely on recognition by a functionally bivalent C-terminal domain. Consistent with this, only Gal-8NM, which contains the active C-terminal domain within the context of the full-length Gal-8, induced PS exposure in HL60 cells. The ability of Gal-8N to dimerize may also partially explain the similar relative affinity for glycans on the microarray between Gal-8N, Gal-8CM, and Gal-8. By contrast, Gal-8C, which does not dimerize, showed significantly lower binding than Gal-8. Similarly, Patnick et al demonstrated that Gal-8N bound much better to CHO cell surface glycans than Gal-8C (27). In our study, only Gal-8NM appeared to possess similar binding toward polyLacNAc glycans as Gal-8, suggesting that dimerization may not only provide functional bivalency with two C-terminal domains, but also may enhance the affinity of binding to polyLacNAc glycans to successfully induce PS exposure.

The inability of Gal-8C to induce PS exposure, in contrast to Gal-8NM, suggests a general requirement for dimerization-induced crosslinking of functional counter receptors in galectin signaling. Previous studies demonstrated that mutations that inhibit Gal-1 dimerization also prevent signaling, although monomeric Gal-1 still appears to bind similar receptors (38). Importantly, monomeric Gal-1 has lower affinity for glycan ligands than dimeric Gal-1 (52), similar to what we observed for Gal-8C. Removal of the N-terminal domain of Gal-3, responsible for mediating Gal-3 oligomerization (75-81), also diminishes Gal-3-induced

signaling (66). In this way, regulation of monomer-dimer equilibrium appears to be an important mechanism of regulating galectin activity.

The unique structural organization of Gal-8 may reflect a general architecture of tandem repeat galectins. Consistent with this, a recent study on the N-terminal domain of Gal-9 (Gal-9N) demonstrated that it is also a dimer, as evidenced by studies of the crystal structure and solution-based experiments (82). Although we found that only the C-terminal domain of Gal-8 binds the functional signaling receptors on HL60 cells, the unique binding properties of each separate domain corroborates previous studies (25-27), and suggests that different cells may possess unique sensitivity to the potential signaling effects of each individual domain (60,83). Furthermore, because the N-terminal domain of Gal-8 intrinsically dimerizes, cleavage of the linker region between Gal-8N and Gal-8C may allow regulatory circuits to dissect potential signaling pathways initiated by each separate domain. Recent results demonstrate that the Gal-8 linker region displays sensitivity to cleavage by thrombin (84), providing at least one possible regulatory pathway capable of separating the potential functional consequences downstream of these two domains. By contrast, the N-terminal domain may also facilitate interactions with extracellular matrix (ECM) components, which often contain highly sulfated glycans similar to those recognized by the N-terminal domain. This would allow the C-terminal domain to freely signal leukocyte responses. In this way, the two separate binding domains Gal-8 have some analogy to many chemokines, with one domain responsible for signals leukocytes and the other allowing traction on the ECM (85). Interestingly, only the C-terminal domain of Gal-8 mediates cellular adhesion *in vitro* (62). Several galectins exhibit chemotaxis activity and recognize ECM components (62,73,86-89), which may reflect a certain level of convergent evolution between these two families.

The ability of Gal-8 to signal leukocytes through glycan recognition by the C-terminal domain provides insight into the functional glycans required for Gal-8 induced PS exposure in leukocytes. We found that the C-terminal domain primarily binds to blood group antigens and polyLacNAc glycans on the glycan microarray. However, HL60 cells do not express blood group antigens, which suggested that Gal-8 signals exclusively through cell surface recognition of polyLacNAc glycans. Indeed, treatment of cells with endo-β-galactosidase significantly reduced Gal-8C cell surface recognition. Specific elongation of polyLacNAc glycans on selective glycoproteins may direct galectin binding toward functional cell surface receptors. Consistent with this, previous studies showed that only a few glycoproteins in HL60 cells express significant amounts of polyLacNAc (90).

Although Gal-1, Gal-2, Gal-3, and Gal-8C display specificity toward polyLacNAc cell surface glycans, their fine binding preferences for polyLacNAc differ significantly. Previous results demonstrate that Gal-3 recognizes polyLacNAc glycans independently of the polyLacNAc sialylation state (67,91). In contrast, sialylation modulates binding of polyLacNAc glycans by Gal-1 and Gal-2 (67,91), similar to Gal-8C. However, the mechanism whereby differential sialylation of cell surface ligands may affect Gal-8 binding and signaling appears to differ from Gal-1 and Gal-2. While desialylation significantly enhanced Gal-8C recognition, similar treatment of cell surface glycans nearly eliminated binding by Gal-8N. As a result, Gal-8N may behave more like a member of the Siglec family of mammalian lectins, which require terminal sialic acid for ligand binding (92). In this way, differences in the sialylation state of functional ligands may alter cellular sensitivity to the

potential signaling abilities of either Gal-8N or Gal-8C in the context of the full-length protein.

The unique preferences of Gal-1, Gal-2, Gal-3 and Gal-8 for polyLacNAc may partially reflect differences in quaternary organization and suggest a model of how each of these galectins interacts with leukocytes (Fig. 2-10). In contrast to the relatively rigid homodimeric structures of Gal-1 and Gal-2 (72,73,93,94), Gal-3 exists as a flexible oligomer through N-terminal domain interactions (75-78) (Fig. 2-10). Similar flexibility may exits between the dimeric Gal-8N domain and linkers that attach the C-terminal domain (Fig. 2-10). Because Gal-1 and Gal-2 exist as rigid homodimers with glycans bound in opposite orientations (52,67), the preference for polyLacNAc exhibited by these two proteins likely reflects the conformational flexibility provided by polyLacNAc in exposing the terminal LacNAc, allowing concomitant docking of two terminal LacNAc motifs in each domain (Fig. 2-10). Importantly, the preference of Gal-1 and Gal-2 for polyLacNAc only occurs following polyLacNAc immobilization, as neither demonstrate preference for polyLacNAc in solution based assays where this type of conformational flexibility loses relevance (52,67). Furthermore, monomeric Gal-1 fails to share polyLacNAc preference (52), further suggesting that Gal-1 and Gal-2 exhibit polyLacNAc preference due to similarities in quaternary structure. By contrast, the quaternary organization of Gal-3 and Gal-8, which may be more flexible, may underscore the ability of these galectins to bind internal LacNAc motifs within polyLacNAc (Fig. 2-10). It is also possible that galectin interactions, as depicted in Fig. 2-10, may involve higher order cross-linking and lattice formation, as proposed for Gal-1 and Gal-3 (95,96).

Taken together, these studies provide new insights into Gal-8 quaternary structures, cell surface interactions, and signaling through each CRD that may generally reflect signaling mechanisms of other tandem repeat galectins. It will be important in the future to examine in detail the domain or domains responsible for dimerization, and the oligomeric nature of glycan recognition and signaling through the CRDs of the tandem repeat galectins.

Figures

Figure 2-1

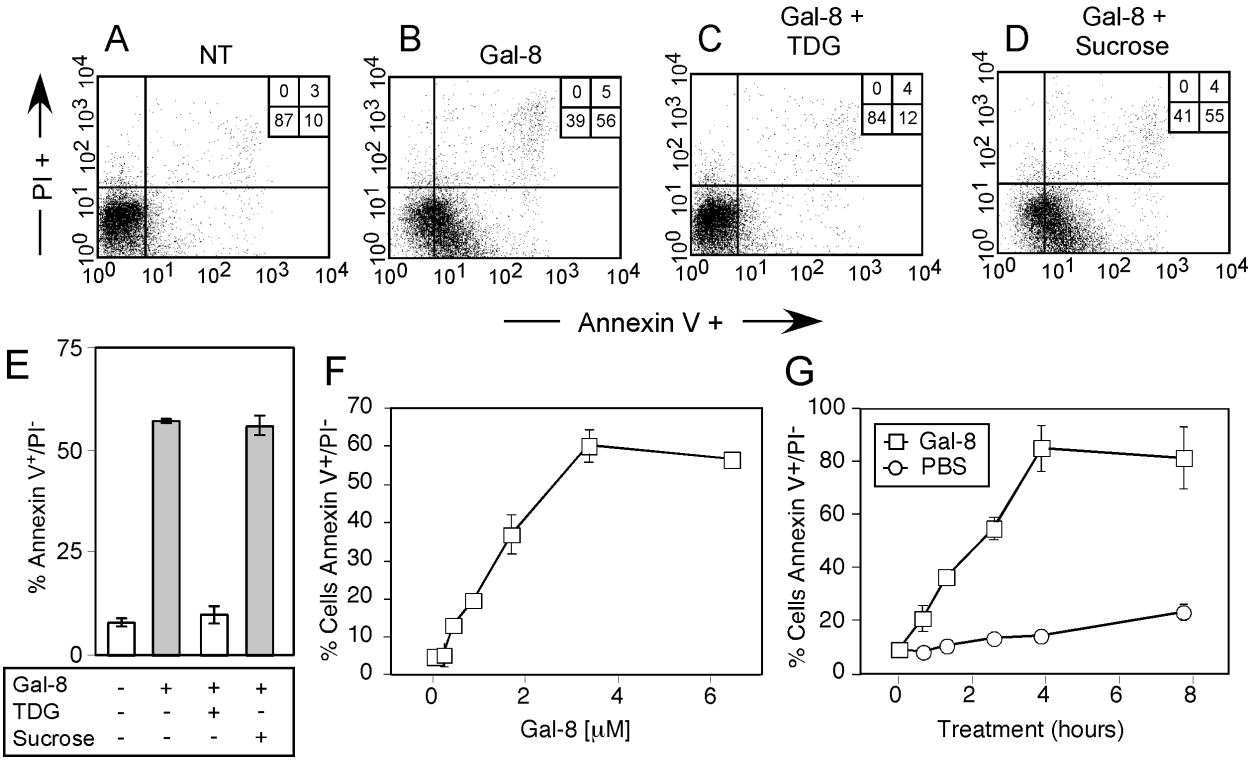
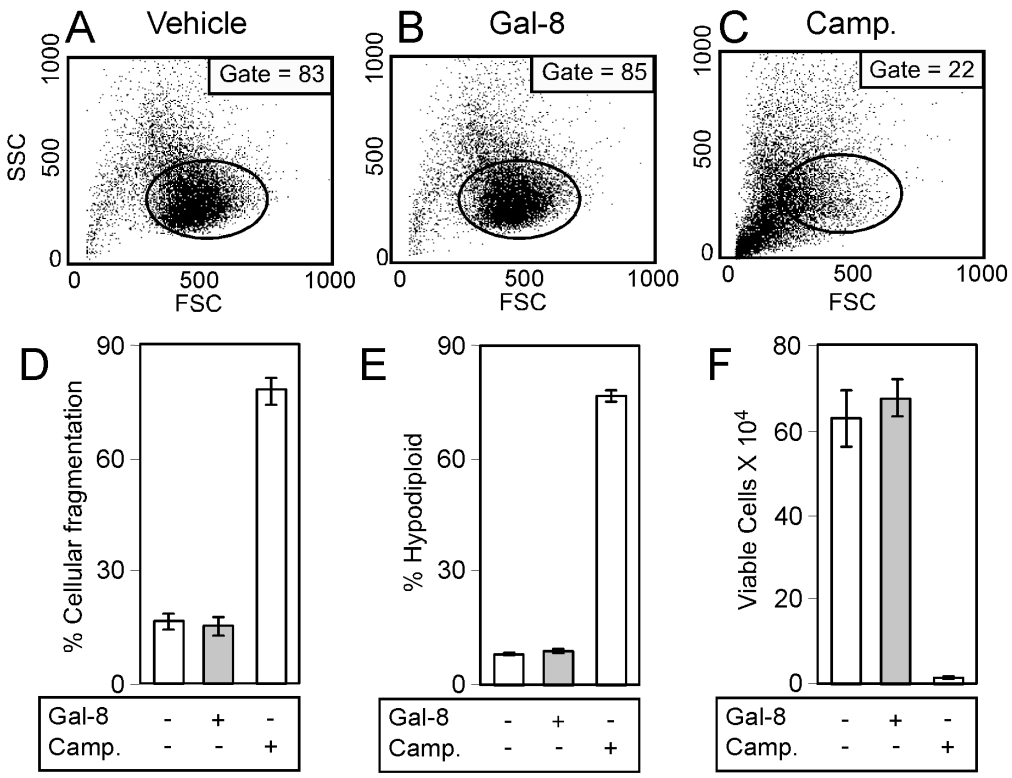


Figure 2-2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Vehicle
Gate = 83
B
Gal-8
Gate = 85
C
Camp.
Gate = 22
SSC
FSC
0
500
1000
D
% Cellular fragmentation
E
% Hypodiploid
F
Viable Cells X 10^4
Gal-8
Camp.
-
+

Figure 2-3

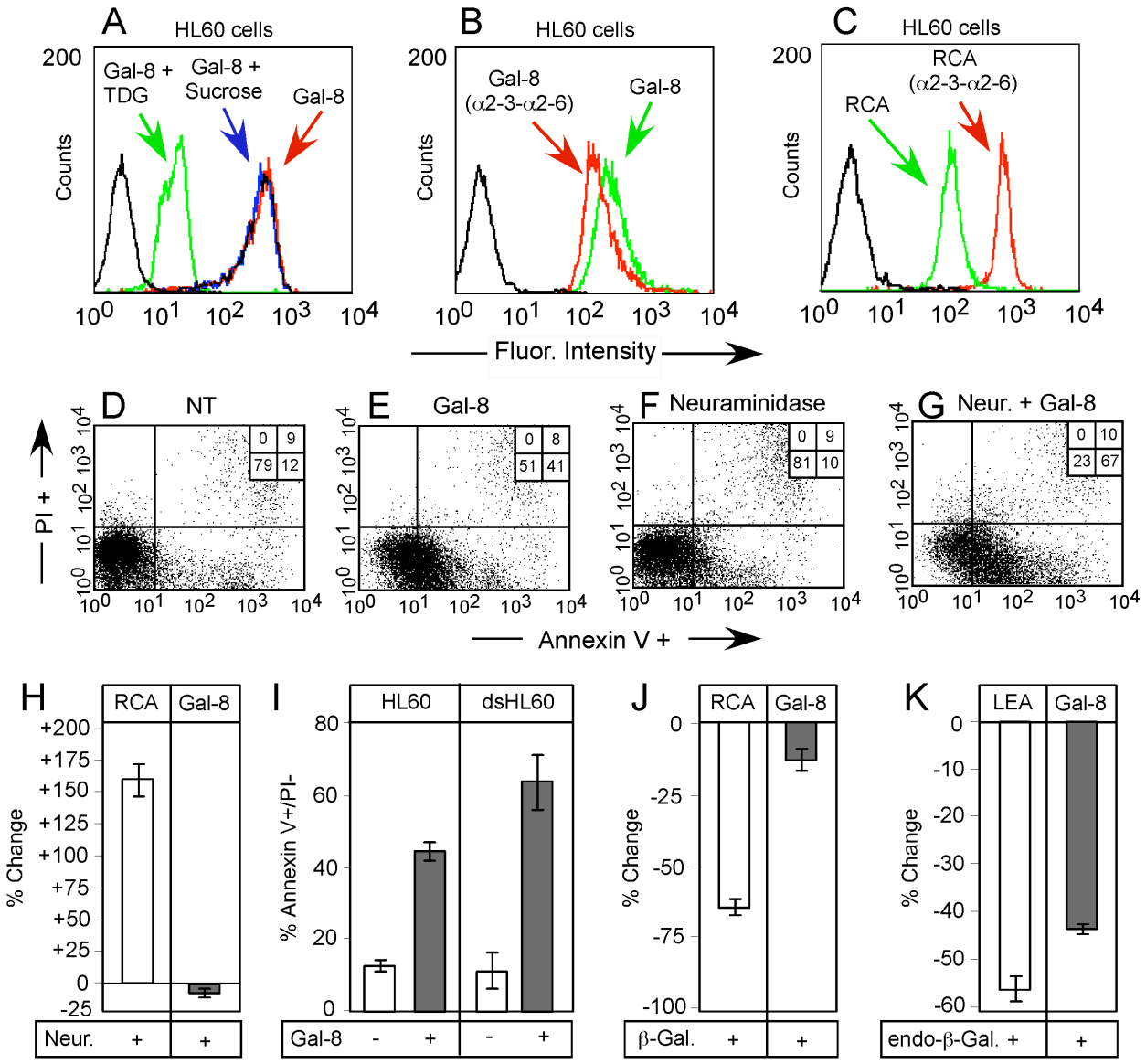
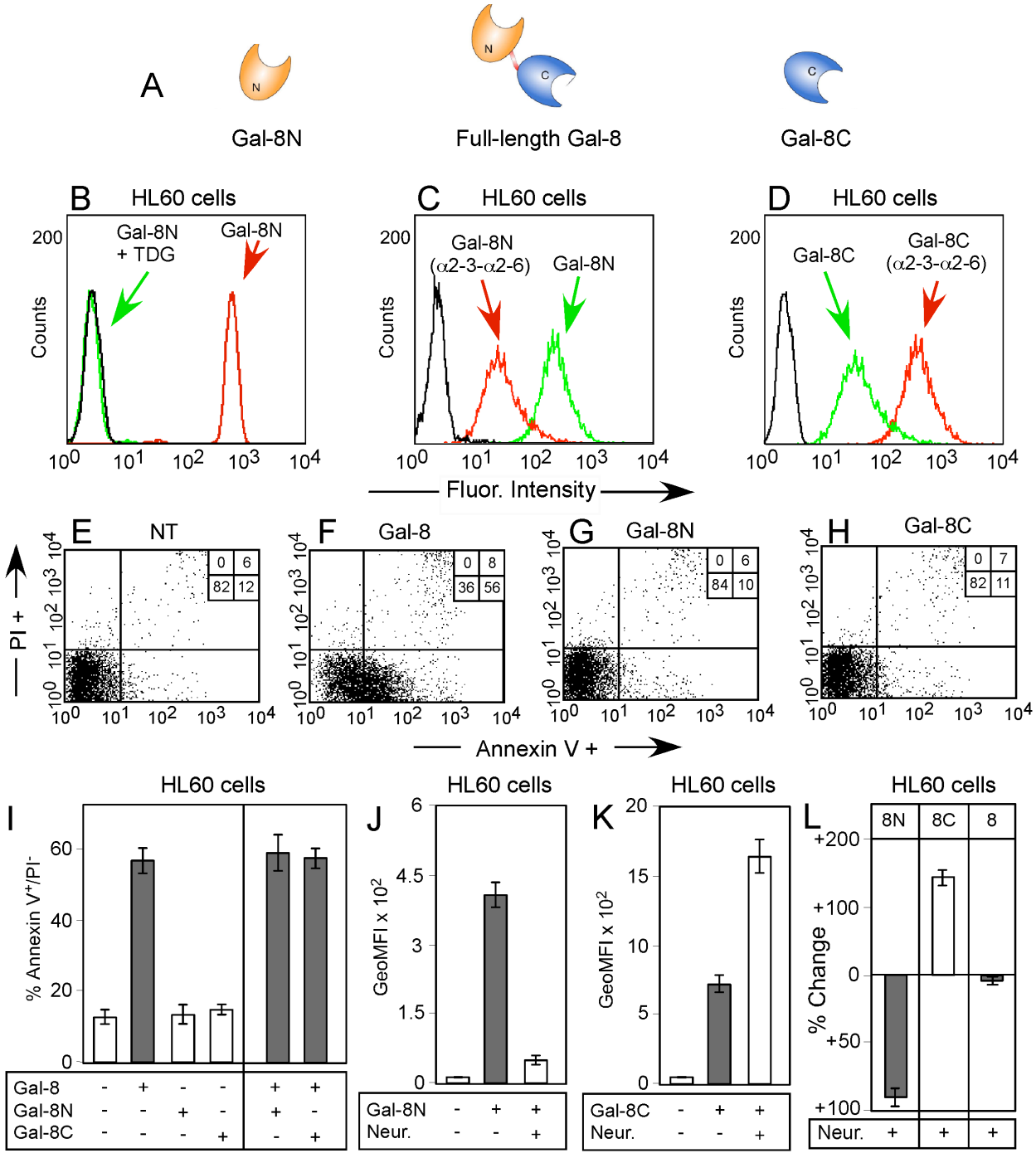

A
HL60 cells
Gal-8 + TDG
Gal-8 + Sucrose
Gal-8
Counts
B
HL60 cells
Gal-8 (α2-3-α2-6)
Gal-8
C
HL60 cells
RCA
RCA (α2-3-α2-6)
Fluor. Intensity
D
NT
E
Gal-8
F
Neuraminidase
G
Neur. + Gal-8
PI +
Annexin V +
H
RCA
Gal-8
% Change
Neur.
I
HL60
dsHL60
% Annexin V+/PI-
Gal-8
J
RCA
Gal-8
% Change
β-Gal.
K
LEA
Gal-8
% Change
endo-β-Gal.

Figure 2-4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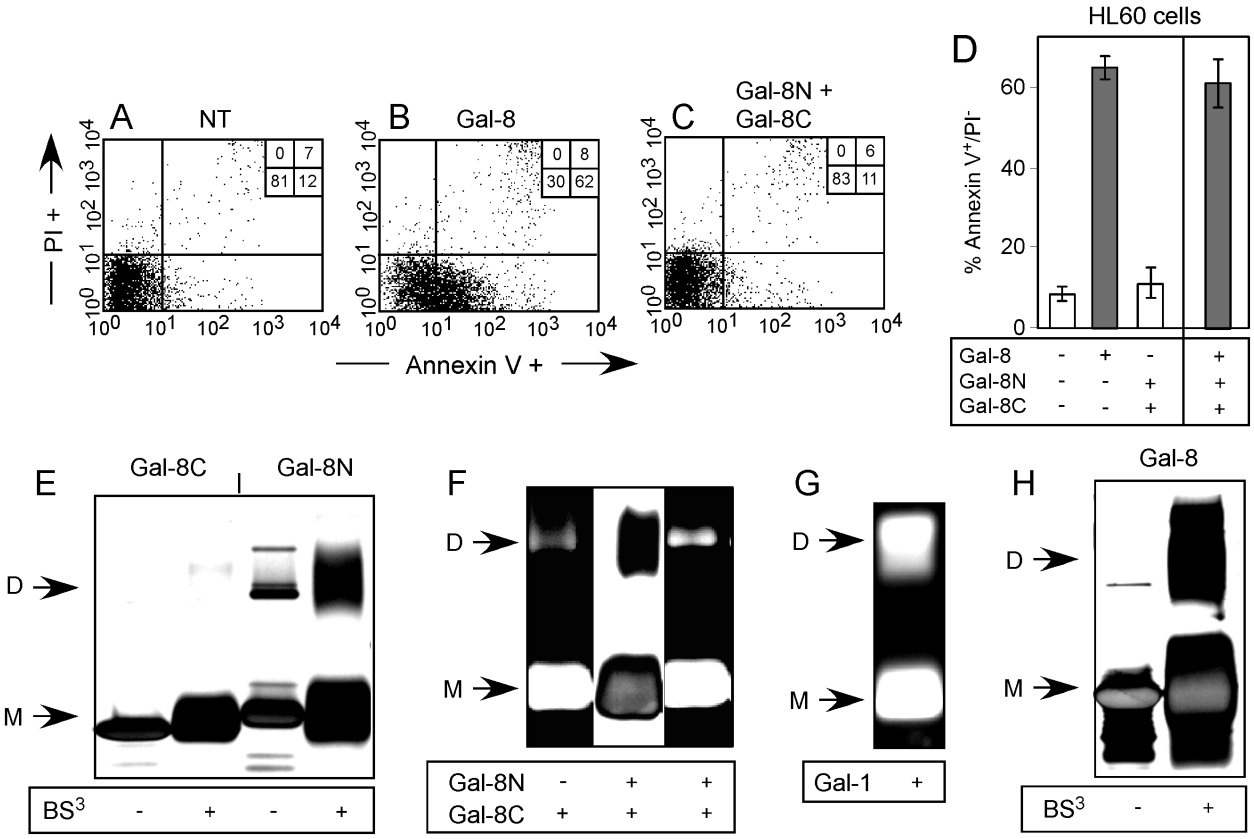
Gal-8N
N
C
Full-length Gal-8
C
Gal-8C
B HL60 cells
Gal-8N + TDG
Gal-8N
Counts
200
C HL60 cells
Gal-8N (α2-3-α2-6)
Gal-8N
D HL60 cells
Gal-8C
Gal-8C (α2-3-α2-6)
Fluor. Intensity
E NT
F Gal-8
G Gal-8N
H Gal-8C
PI +
Annexin V +
I HL60 cells
% Annexin V+/PI-
Gal-8
Gal-8N
Gal-8C
J HL60 cells
GeoMFI x 10²
Neur.
K HL60 cells
L HL60 cells
8N
8C
8
% Change

Figure 2-5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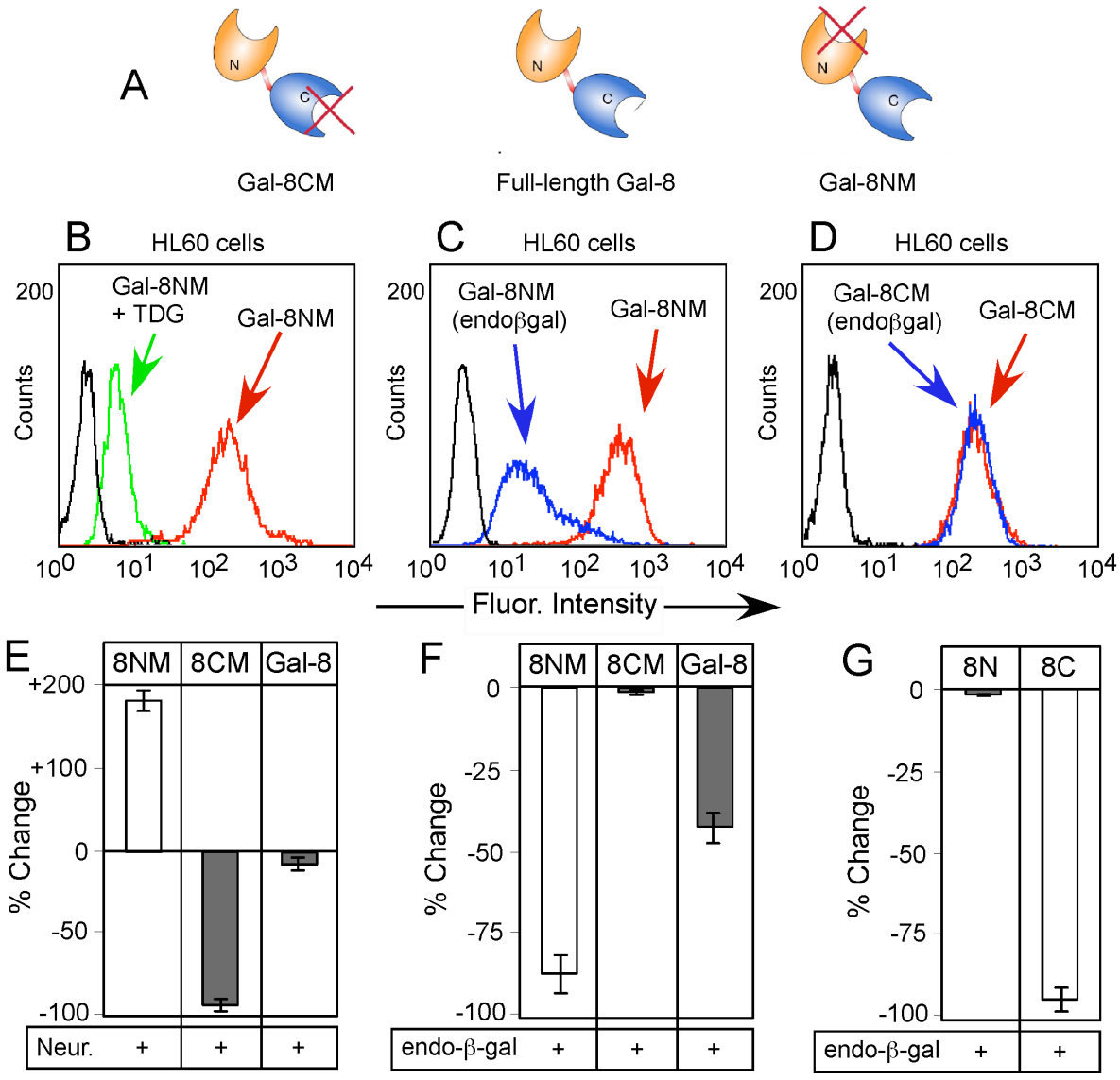
NT
B
Gal-8
C
Gal-8N +
Gal-8C
PI +
Annexin V +
0 7
81 12
0 8
30 62
0 6
83 11
D
HL60 cells
% Annexin V+/PI-
Gal-8 - + - +
Gal-8N - - + +
Gal-8C - - + +
E
Gal-8C
Gal-8N
D
M
BS3 - + - +
F
D
M
Gal-8N - + +
Gal-8C + + +
G
D
M
Gal-1 +
H
Gal-8
D
M
BS3 - +

Figure 2-6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N
C
Gal-8CM
Full-length Gal-8
Gal-8NM
B
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+ TDG
Gal-8NM
Counts
200
10^0 10^1 10^2 10^3 10^4
C
HL60 cells
Gal-8NM
(endoβgal)
Gal-8NM
D
HL60 cells
Gal-8CM
(endoβgal)
Gal-8CM
Fluor. Intensity
E
8NM
8CM
Gal-8
+200
+100
0
-50
-100
% Change
Neur. + + +
F
8NM
8CM
Gal-8
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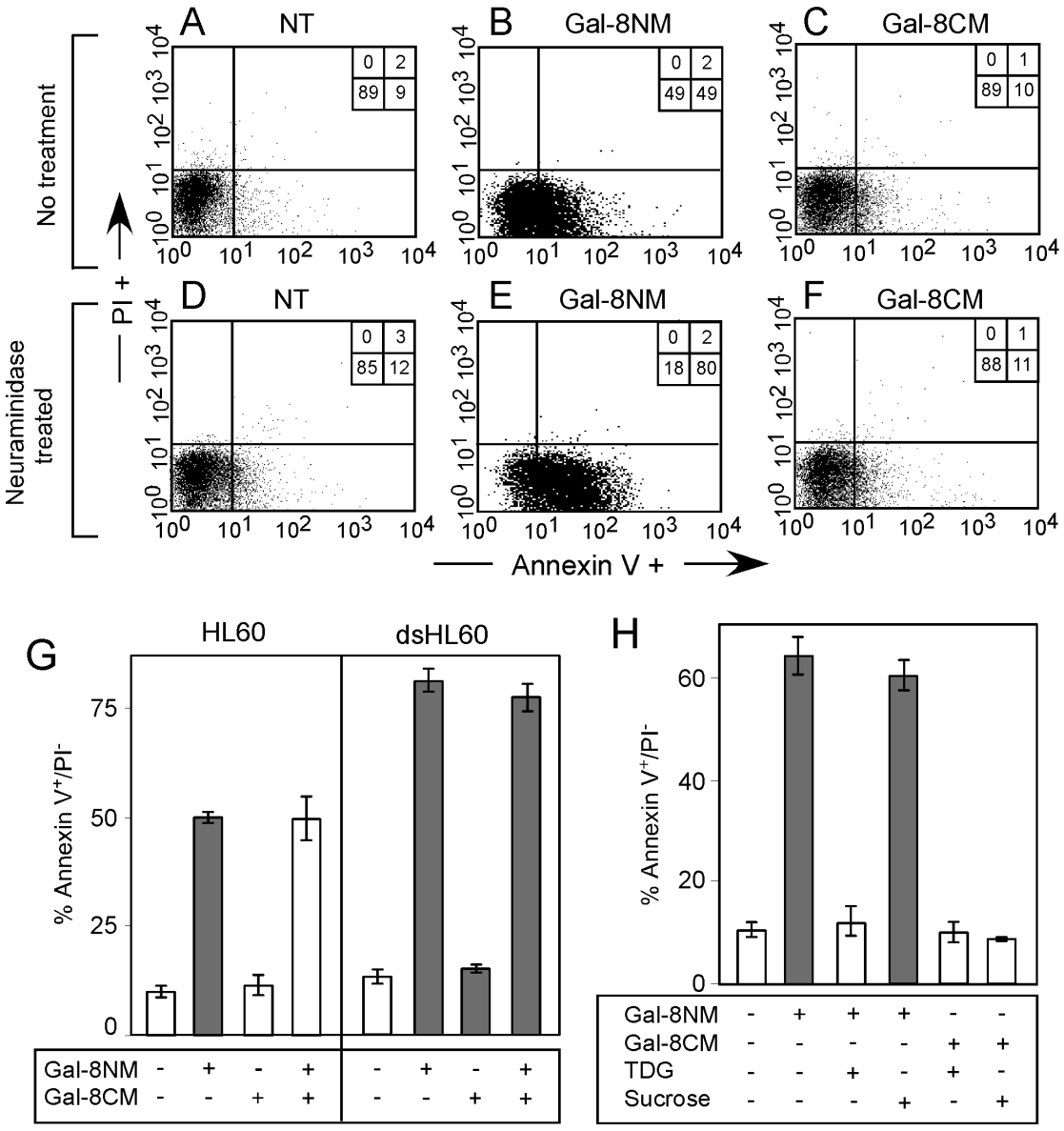
-25
-50
-75
-100
endo-β-gal + + +
G
8N
8C
endo-β-gal + +

Figure 2-7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NT
B
Gal-8NM
C
Gal-8CM
D
NT
E
Gal-8NM
F
Gal-8CM
No treatment
Neuraminidase treated
PI +
Annexin V +
0 2
89 9
0 2
49 49
0 1
89 10
0 3
85 12
0 2
18 80
0 1
88 11
G
HL60
dsHL60
% Annexin V+/PI-
Gal-8NM
Gal-8CM
H
% Annexin V+/PI-
Gal-8NM
Gal-8CM
TDG
Sucrose

Figure 2-8

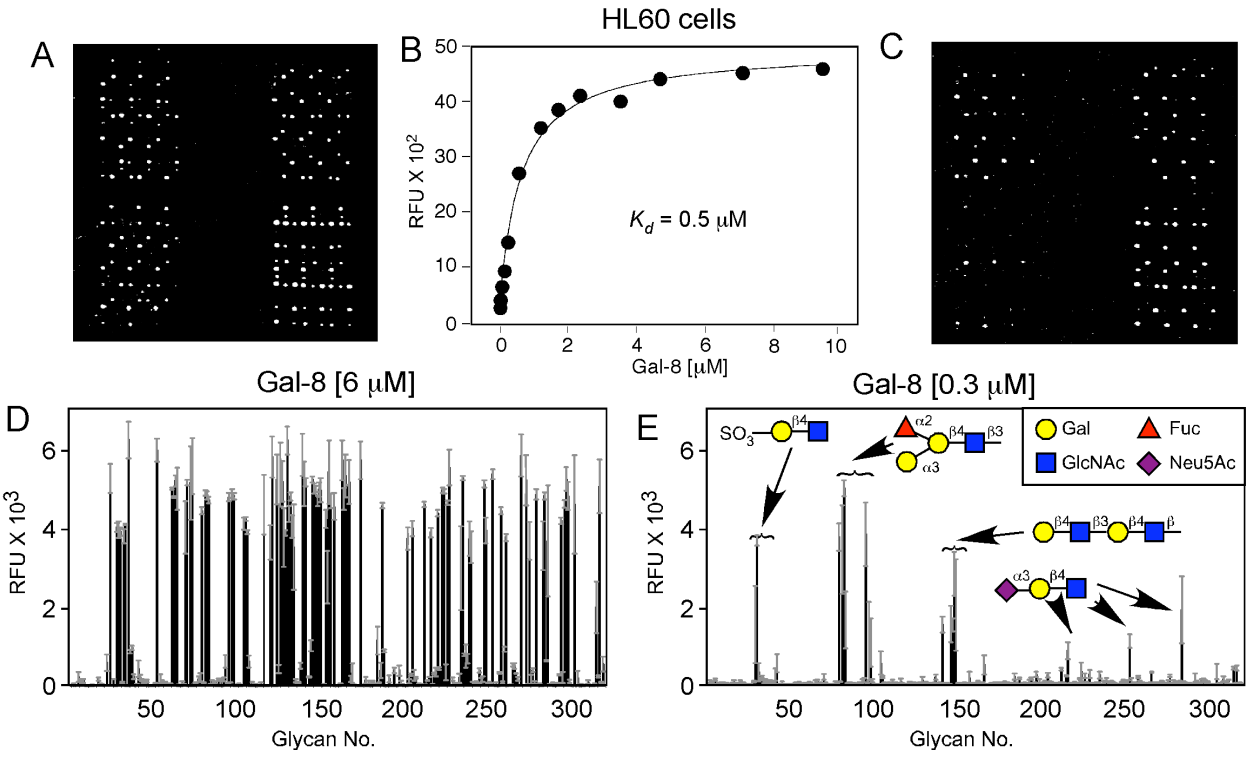
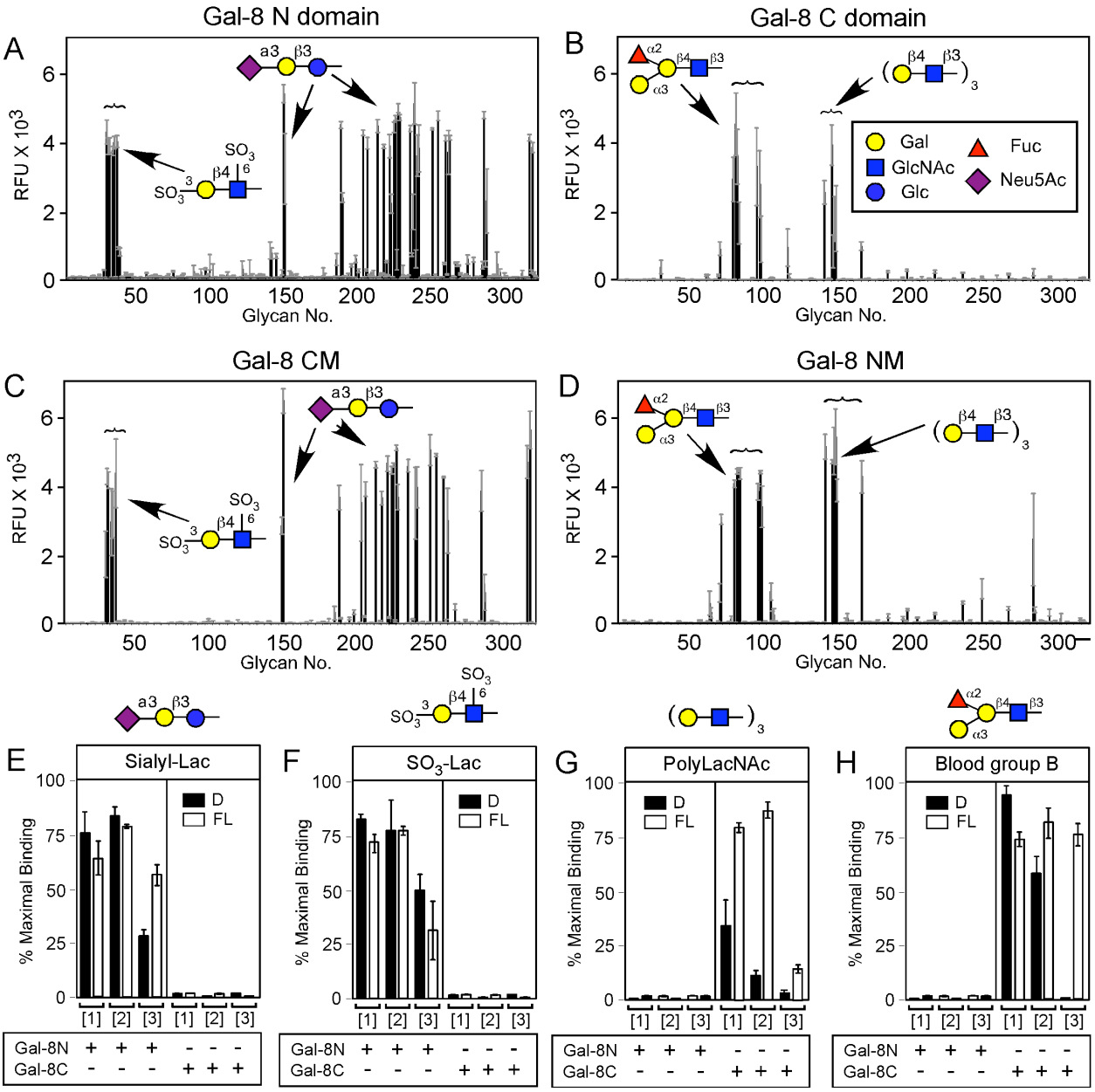

A
B
HL60 cells
RFU X 10²
K_d = 0.5 μM
Gal-8 [μM]
C
D
Gal-8 [6 μM]
RFU X 10³
Glycan No.
E
Gal-8 [0.3 μM]
RFU X 10³
Glycan No.
Gal
Fuc
GlcNAc
Neu5Ac

Figure 2-9


Gal-8 N domain
Gal-8 C domain
Gal-8 CM
Gal-8 NM
RFU X 10^3
Glycan No.
Gal
GlcNAc
Glc
Fuc
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PolyLacNAc
Blood group B
% Maximal Binding
D
FL
Gal-8N
Gal-8C

Figure 2-10

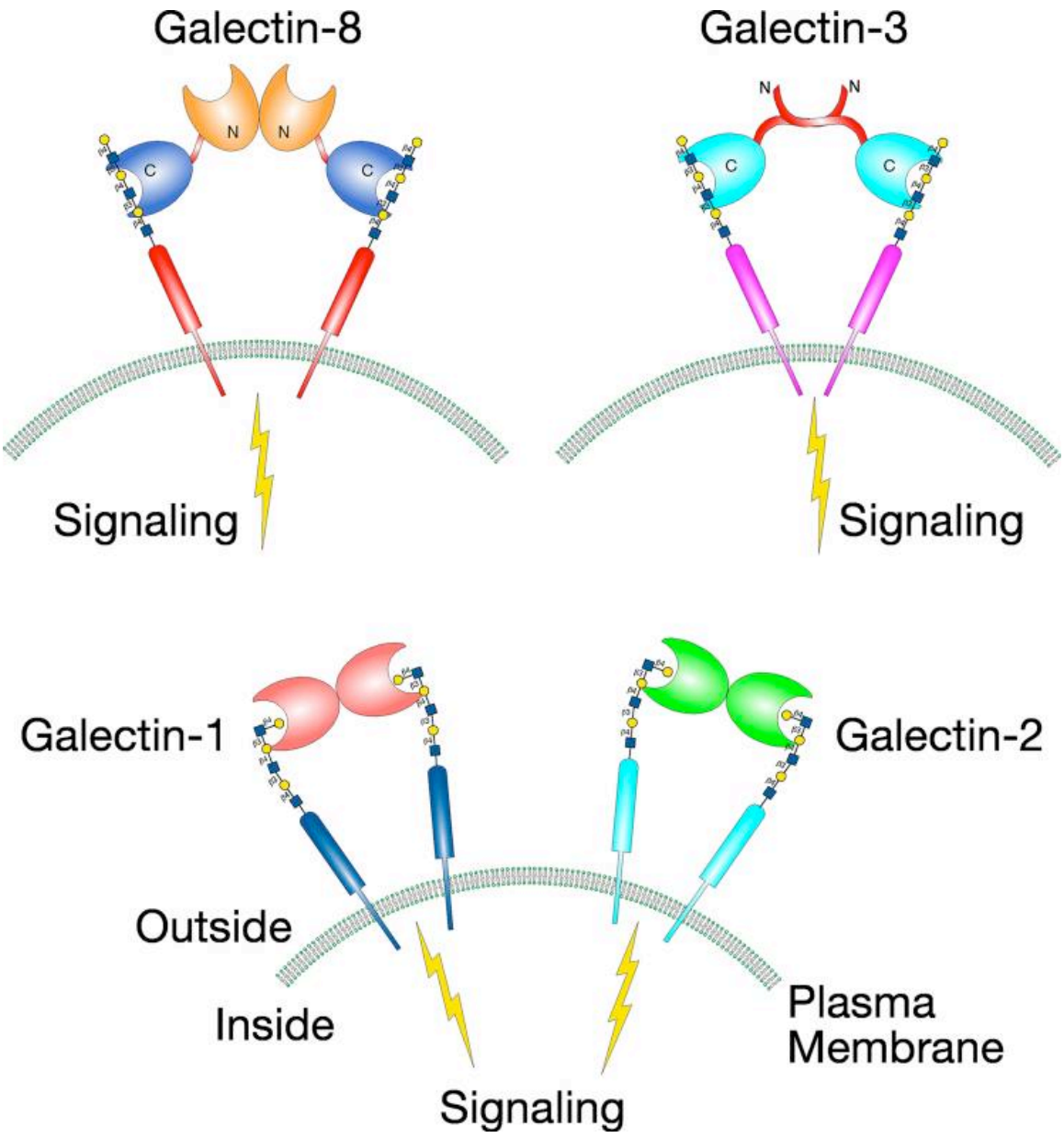

Galectin-8
N
N
C
C
Signaling
Galectin-3
N
N
C
C
Signaling
Galectin-1
Galectin-2
Outside
Inside
Plasma
Membrane
Signaling

Figure Legends

Figure 2-1- Gal-8 induces PS exposure in HL60 cells- (A-D) representative dot plots of HL60 cells treated with PBS (A), 3 μM Gal-8 (B), 3 μM Gal-8 plus 20 mm TDG (C), 3 μM Gal-8 plus 20 mm sucrose (D) for 8 h followed by detection for PS by Annexin V and propidium iodide (PI) staining. Cells that were Annexin V-positive and PI-negative were considered positive for PS. (E) quantification of PS exposure (Annexin-V+/PI-) of cells treated in A-D. (F) HL60 cells were treated with 5 μM Gal-8 for the time indicated followed by detection for PS by Annexin V and PI staining. (G) HL60 cells were treated with the indicated concentrations of Gal-8 for 8 h followed by detection for PS by Annexin V and PI staining.

Figure 2-2- Gal-8 induces PS exposure in HL60 cells in the absence of cell death- (*A-C)* representative dot plots of HL60 cells treated with PBS (*A*), 3 μM Gal-8 (*B*), or 3 μM camptothecin (*Camp*) (*C*) for 12 h followed by detection for cell fragmentation by analyzing changes in forward and side scatter profiles using flow cytometric analysis. *Gate* = percent of total cells not demonstrating fragmentation. (*D)* quantification of cell fragmentation following treatments outlined in *A-C*. (*E)* HL60 cells were treated with PBS, 3 μM Gal-8, 3 μM Camp for 12 h as indicated followed by detection for DNA degradation by hypodiploid analysis using flow cytometric analysis. (*F)* HL60 cells were treated with PBS, 3 μM Gal-8, 3 μM Camp for 72 h as indicated followed by determining the number of viable cells by trypan blue exclusion using a hemocytometer.

Figure 2-3- Treatment of cell with neuraminidase fails to alter Gal-8 cell surface binding yet enhances cellular sensitivity to Gal-8-induced PS exposure- (*A)* binding of Gal-8 to HL60 cells with or without 20 mm TDG or 20 mm sucrose as indicated. (*B)* binding of Gal-8 to HL60 cells treated with or without *A. ureafaciens* neuraminidase. (*C)* binding of RCA to HL60 cells treated with or without *A. ureafaciens* neuraminidase. (*D-G)* representative dot plots of HL60 cells first treated with or without *A. ureafaciens* neuraminidase for 1 h followed by treatment with 3 μM Gal-8 for 4 h as indicated. (*H)* quantification of RCA or Gal-8 binding to HL60 cells following treatment with *A. ureafaciens* neuraminidase. (*I)* quantification of PS exposure (Annexin-V^+/PI^-) of cells treated in *D-F.* (*J)* RCA or Gal-8 binding to HL60 cells following treatment with bovine testes β-galactosidase. (*K)* LEA or Gal-8 binding to HL60 cells following treatment with *E. freundii* endo-β-galactosidase. *Bars* represent the percent change in cell surface binding when compared with the mean fluorescent intensity of non-treated cells ± S.D.

Figure 2-4- Gal-8N and Gal-8C fail to induce PS exposure in HL60 cells- (*A)* schematic representation of full-length Gal-8 and individual domains. (*B)* binding of Gal-8N toward HL60 cells with or without incubation 20 mm TDG or 20 mm sucrose. (*C)* binding of Gal-8N toward HL60 cells treated with *A. ureafaciens* neuraminidase. (*D)* binding of Gal-8C toward HL60 cells treated with *A. ureafaciens* neuraminidase. (*E-H)* representative dot plots of HL60 cells treated with PBS (*E*), 3 μM Gal-8 (*F*), 6 μM Gal-8N (*G*), or 6 μM Gal-8C (*H*) for 8 h followed by detection for PS by Annexin V and PI staining. Cells that were Annexin V-positive and PI-negative were considered positive for PS. (*I)* treatment of HL60 cells with PBS, 3 μM Gal-8, 6 μM Gal-8N, 6 μM Gal-8C, 3 μM Gal-8 plus 6 μM Gal-8N, or

3 μM Gal-8 plus 6 μM Gal-8C for 8 h followed by detection for PS exposure by Annexin V and PI staining. (*J)* geometric mean fluorescent intensities (*GeoMFI*), a measure of mean fluorescent intensity on logarithmic scales, of Gal-8N binding before and after treatment of cells with *A. ureafaciens* neuraminidase. (*K)* GeoMFI of Gal-8C binding before and after treatment of cells with *A. ureafaciens* neuraminidase. (*L)* comparison of Gal-8, Gal-8N, or Gal-8C binding toward HL60 cells following treatment with *A. ureafaciens* neuraminidase. *Bars* represent the percent change in cell surface binding when compared with the mean fluorescent intensity of non-treated cells ± S.D.

Figure 2-5- Gal-8 exists as a dimer- (*A-C)* representative dot plots of HL60 cells treated with PBS (*A*), 3 μM Gal-8 (*B*), or 6 μM Gal-8N plus 6 μM Gal-8C (*C*) for 8 h followed by detection for PS by Annexin V and PI staining. Cells that were Annexin V-positive and PI-negative were considered positive for PS. (*D)* quantification of PS exposure (Annexin-V^+/PI^-) of cells treated in *A-C*. (*E)* silver stain of Gal-8C or Gal-8N following incubation with the chemical cross-linker BS^3 and SDS-PAGE analysis. (*F)* direct analysis of Gal-8C with or without co-incubation with Gal-8N or silver stain of Gal-8C or Gal-8N following incubation with BS^3 and SDS-PAGE analysis. (*G)* direct analysis of Gal-1 following incubation with BS^3 and SDS-PAGE analysis. (*H)* silver stain of Gal-8 following incubation with BS^3 and SDS-PAGE analysis.

Figure 2-6- Gal-8NM and Gal-8CM exhibit similar cell surface binding as Gal-8C and Gal-8N, respectively- (*A)* schematic representation of full-length Gal-8 and full-length Gal-8 with individually mutated CRDs. (*B)* binding of Gal-8NM toward HL60 cells with or

without incubation 20 mm TDG or 20 mm sucrose as indicated. (*C)* binding of Gal-8NM toward HL60 cells treated with *E. freundii* endo-β-galactosidase. (*D)* binding of Gal-8CM toward HL60 cells treated with *E. freundii* endo-β-galactosidase. (*E)* quantification of Gal-8, Gal-8NM, or Gal-8CM binding toward HL60 cells following treatment with *A. ureafaciens* neuraminidase. *Bars* represent the percent change in cell surface binding when compared with the mean fluorescent intensity of non-treated cells ± S.D. (*F)* quantification of Gal-8, Gal-8NM, or Gal-8CM binding toward HL60 cells following treatment with *E. freundii* endo-β-galactosidase. *Bars* represent the percent change in cell surface binding when compared with the mean fluorescent intensity of non-treated cells ± S.D. *G*, quantification of Gal-8N or Gal-8C binding toward HL60 cells following treatment with *E. freundii* endo-β-galactosidase. *Bars* represent the percent change in cell surface binding when compared with the mean fluorescent intensity of non-treated cells ± S.D.

Figure 2-7- Gal-8 induces PS exposure through glycan recognition by C-terminal domain- (*A-C)* representative dot plots of HL60 cells treated with PBS (*A*), 3 μM Gal-8NM (*B*), or 3 μM Gal-8CM (*C*) for 8 h followed by detection for PS by Annexin V and PI staining. Cells that were Annexin V-positive and PI-negative were considered positive for PS. *D-F*, representative dot plots of HL60 cells pretreated with *A. ureafaciens* neuraminidase followed by PBS (*D*), 3 μM Gal-8NM (*E*), or 3 μM Gal-8CM (*F*) for 8 h followed by detection for PS by Annexin V and PI staining. *G*, quantification of PS exposure (Annexin-V^+/PI^-) of cells treated in (*A-F).* (*G)* HL60 cells were treated with 5 μM Gal-8 for the time indicated followed by detection for PS by Annexin V and PI staining. (*H)* HL60 cells were treated with 3 μM Gal-8NM or 3 μM Gal-8CM with or without 20 mm TDG or 20 mm sucrose as indicated for 8 h followed by detection for PS by Annexin V and PI staining.

Figure 2-8- Each domain of Gal-8 recognizes distinct classes of glycans- (*A*) the glycan microarray followed incubation of the glycan microarray with 6 μM Gal-8. (*B)* incubation of Gal-8 with HL60 cells at the indicated concentrations. (*C)* glycan microarray followed incubation of the glycan microarray with 0.3 μM Gal-8. (*D)* glycan microarray data obtained following incubation with 6 μM Gal-8. (*E)* glycan microarray data obtained following incubation with 0.3 μM Gal-8.

Figure 2-9- Gal-8NM and Gal-8CM exhibit similar specificity as Gal-8C and Gal-8N, respectively- (*A)* glycan microarray data obtained following incubation with 12 μM Gal-8N. (*B)* glycan microarray data obtained following incubation with 12 μM Gal-8C. (*C)* glycan microarray data obtained following incubation with 6 μM Gal-8CM. (*D)* glycan microarray data obtained following incubation with 6 μM Gal-8NM. (*E-H)* binding of Gal-8N, Gal-8C, Gal-8NM, or Gal-8CM over a range of concentrations with *1* = 3 μM, *2* = 1.5 μM, and *3* = 0.3 μM for Gal-8CM and Gal-8NM and *1* = 6 μM, *2* = 3 μM, and *3* = 0.6 μM for Gal-8N and Gal-8C to sialyllactose (*E*), SO_3-lactose (*F*), polyLacNAc (*G*), and blood group B (*H*).

Figure 2-10- Schematic representation of Gal-1, Gal-2, Gal-3, and Gal-8 interacting with cell surface polyLacNAc glycans- Gal-1 and Gal-2 primarily recognize the terminal LacNAc of polyLacNAc with preference for polyLacNAc displayed by Gal-1 and Gal-2 reflecting favorable conformational flexibility of polyLacNAc only relevant following immobilization. In contrast, Gal-3 and Gal-8 recognize internal LacNAc within polyLacNAc.

Chapter 3- Signaling of PS Exposure by Gal-8 Requires the Presence of Complex-N-glycans

Galectins have diverse biological activities toward leukocytes, but the endogenous glycans responsible for galectin binding and signaling in are not well understood. We previously found that human galectin-8 (Gal-8) has signaling activity to human neutrophils and the promyelocytic cell line HL60 and induces phosphatidylserine exposure on the surface independent of apoptosis in a process termed preaparesis. Gal-8 was also found to bind select glycan determinants potentially present in many classes of glycans. To define the types of glycans involved in cell signaling by Gal-8, HL60 cells were systematically treated with specific inhibitors to block formation of complex-type N-glycans, complex O-glycans, or glycolipids at the cell surface. While treatments of cells with inhibitors to block formation of either complex O-glycans or glycolipids did not affect binding or signaling by Gal-8, decreased expression of complex-type N-glycans eliminated both. These results indicate that recognition of complex-type N-glycans by Gal-8 is required for binding and signaling of PS exposure in HL60 cells.

Introduction

Leukocyte turnover represents one of the most fundamental regulatory processes of immune homeostasis. Failure to properly remove leukocytes following an inflammatory episode often results in significant tissue damage and may contribute to the development of a variety

of diseases, including autoimmunity and neoplastic transformation. Several factors, including members of the TNF and galectin families regulate leukocyte turnover. However, the mechanisms of signaling and the modes of leukocyte turnover induced by these family members fundamentally differ. TNF family members, such as TNFα, Fas and TRAIL induce receptor trimerization and subsequent engagement of apoptotic programs through specific protein-protein interactions (19,97). In contrast, galectin family members signal through recognition of highly modifiable cell surface glycans, enabling engagement of a wide variety of counter receptors depending on the cell type and activation state. Furthermore, in contrast to TNF family member-induced apoptosis, several galectin family members induce the turnover of neutrophils independent of the apoptotic program, a process recently termed preaparesis (14,17,18,67).

The ability of galectins to recognize cell surface glycans provides an additional regulatory mechanism governing cellular sensitivity toward galectin-induced turnover. For example, simple alterations in cell surface sialylation can enhance or eliminate glycan recognition depending on the distinct carbohydrate recognition properties of different galectins (67). In this way, changes in glycosylation provide a mechanism of modulating signaling similar to intracellular processes, such as phosphorylation. Indeed, the addition or subtraction of single monosaccharides can significantly alter cellular sensitivity to a variety of galectin-induced signaling events.

Although previous studies clearly demonstrate the impact of terminal glycan modification on galectin signaling (67), the role of core glycan structures on galectin recognition and signaling remains less clear. Many terminal glycan determinants reside on a variety of core structures,

primarily divided into complex O-glycans, complex N-glycans or glycolipids depending on the site of initial glycan attachment and mode of synthesis. These differences generate distinct core structures upon which common terminal glycan determinants reside (98-100). Although previous studies suggest that galectins primarily recognize terminal glycan structures, several studies also suggest that core glycans may significantly influence carbohydrate recognition and therefore subsequent signaling by galectin family members (101).

Although several studies recently demonstrated that different galectin family members possess distinct modes of glycan recognition, galecin-8 (Gal-8) displays a unique mode of cell surface carbohydrate interaction and signaling. Unlike other members of the family, such as galectin-1, galectin-2 and galectin-3 (Gal-1, Gal-2 and Gal-3), Gal-8 possesses two unique carbohydrate recognition domains (CRD) with distinct glycan specificity (62). The N-terminal CRD of Gal-8 (Gal-8N) preferentially binds sialylated glycans, while the C-terminal domain (Gal-8C) displays significant specificity for polylactosamine. Importantly, although both domains recognize cell surface glycans, Gal-8 signals leukocytes entirely through glycan recognition by the C-terminal domain. While differences in terminal glycan structure may partially account for differences in cell surface recognition between Gal-8N and Gal-8C, the potential influence of complex core glycan structures on recognition by Gal-8 remains unknown. In this study, we examined Gal-8 binding toward chemically defined complex core glycans and compared these results to cell surface binding following inhibition of complex O-, complex N- and glycolipid cell surface glycans. Our results demonstrate that Gal-8N and Gal-8C display a distinct preference for complex N-glycans and that Gal-8 signals entirely through recognition of complex N-glycans.

Gal-8 recognition of complex glycan structures

We independently evaluated two isoforms of Gal-8, the short isoform Gal-8S and the long isoform Gal-8L, along with each independent carbohydrate-binding domain (CRD), Gal-8N and Gal-8C. The shot and long isoforms differ in the length of the polypeptide linker between the two CRDs. Binding studies toward a defined glycan microarray were conducted over a wide range of concentrations (~10 – ~0.1 mM) to extrapolate a binding isotherm in an effort to more accurately ascertain the apparent affinity and therefore specificity of each galectin for unique carbohydrate structures. This is important, considering that many studies using the glycan array display saturated binding, making it difficult to distinguish subtle differences in specificity. Furthermore, each galectin was re-chromatographed over lactosyl sepharose following labeling to ensure that homogenous active protein was examined on the array. We first compared Gal-8S, Gal-8L, Gal-8N and Gal-8C in parallel to evaluate recognition of O- and N-glycans. Gal-8S and Gal-8L demonstrated some binding to Core 1 and more significant binding to Core 2 O-glycans, which was increased following extension. Neither isoform recognized Core 4 O-glycan without the galactose extension. Interestingly, neither domain alone recognized any of these O-glycans (Fig. 3-1A). Gal-8S did exhibit a slightly higher binding capacity than Gal-8L, suggesting that the intrinsic dimeric character of the full-length isoforms may be required for binding these glycans using this solid phase format. (Fig. 3-1A). Gal-8S and Gal-8L exhibited the highest binding toward the biantennary N-glycan (LN2 NG). Sialylation of this glycan with sialic acid in either α2,3 or α2,6 Neu5Ac significantly reduced binding to the LN2 NG by both full-length proteins. Similar to the binding profiles toward O-glycans, each independent domain failed to recognize any of these structures alone (Fig. 3-1B). Taken together these results

demonstrate that each full-length protein recognizes some N- and O-glycans, although each independent domain fails to bind well to these glycans as independent CRDs. .

We next sought to examine the binding of each of the Gal-8 binding to glycans commonly presented on glycosphingolipids. Both Gal-8S and Gal-8L exhibited significant binding toward highly sialylated glycolipids. However, the addition of b1-3 GalNAc to the Gal, which allows elongation of these glycolipids, significantly blocked glycan recognition by full-length Gal-8. Importantly, Gal-8N bound each of the sialylated glycolipids lacking the GalNAc extension with similar affinity as the full-length protein, suggesting that the inability of Gal-8N to recognize N and O-glycans may not result from differences in tertiary structure. Gal-8C failed to recognize any of these glycans, suggesting that high binding of the full-length protein may be attributable to the N-terminal domain (Fig. 3-1C,D).

Gal-8 cell surface recognition of complex O-glycans and glycolipids

The ability of full length Gal-8 to recognize core O-glycans suggests that these core structures may influence cell surface carbohydrate recognition. To test this, we incubated cells with benzyl-GalNAc, an inhibitor of complex O-glycan biosynthesis (102) Exposure of cells to benzyl-GallNAc significantly reduced cell surface recognition by CHO131, a monoclonal antibody that specially recognizes Core 2 complex O-glycans (103), consistent with a reduction in complex O-glycans on the cell surface (Fig. 3-2A). However, treatment of cells with benzyl-GalNAc did not significantly alter binding by Gal-8 or Gal-8C, although it resulted a moderate reduction in glycan recognition by Gal-8N (Fig. 3-2B-D). Similarly, inhibition of O-glycan biosynthesis failed to alter cellular sensitivity to Gal-8 signaling (Fig.

3-2E,F). Taken together, these results suggest that while inhibition of O-glycan biosynthesis does not inhibit Gal-8 signaling and C-terminal domain recognition of cell surface glycans, it may influence glycan recognition by the N-terminal domain.

The preference of the N-terminal domain for glycolipids, in contrast to the C-terminal domain, suggests that the N-terminal domain may uniquely recognize cell surface glycolipid structures. Treatment of cells with 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), an inhibitor of glycolipid biosynthesis (104), reduced binding by cholera toxin, which binds to the glycolipid GM1 (105). However, this treatment did not significantly alter binding by Gal-8, Gal-8C and Gal-8N or signaling by the full-length protein (Fig. 3-3). These results indicate that neither complex O-glycans nor glycolipids contribute significantly to the signaling functions of Gal-8 toward HL60 cells.

Gal-8 signals through recognition of complex N-glycans

The ability of Gal-8 to recognize complex N-glycans, coupled with significant evidence suggesting a role for these core structures in carbohydrate recognition by other galectin family members (101), strongly suggested that Gal-8 might signal leukocytes through recognition of complex N-glycans. To test this, we incubated cells with an inhibitor of complex N-glycan biosynthesis, kifunensine, which inhibits alpha-mannosidase-I in the Golgi apparatus. This leads to a blockage in the complex-type N-glycan biosynthesis pathway that results in expression of only high mannose-type N-glycans (106). . Treatment with kifunensine significantly reduced cell surface recognition by PHA, a plant lectin that requires complex N-glycans for significant binding (107) (Fig. 3-4A). Importantly,

kifunensine significantly reduced binding by Gal-8 and Gal-8C, while having an intermediate effect the binding on the Gal-8N. These results strongly suggest that Gal-8C displays significant specificity for cell surface complex N-glycans (Fig. 3-4B,C). Consistent with this, kifunensine completely eliminated cellular sensitivity toward Gal-8 induced signaling (Fig. 3-4D).

Complex N-glycan significantly influence glycan recognition by Gal-8C

The ability of kifunensine to inhibit signaling by Gal-8 and block cell surface recognition by Gal-8C, suggests that Gal-8C preferentially recognizes complex N-glycans. Furthermore, the ability of kifunensine to more significantly inhibit binding by Gal-8C when compared to Gal-8N, also suggests that complex N-glycans differentially influence glycan recognition by these two domains (Fig. 3-4C,D). Although the glycan array provides a small series of well-defined complex N-glycans to distinguish whether this preference exists using characterized glycan structures, we sought to evaluate a more exhaustive set of structures in order to evaluate potential preference for complex N-glycans exhibited by these two domains. However, complex N-glycans require significant material and time to generate using traditional chemoenzymatic approaches. To overcome this, we employed a recently developed strategy utilizing glycans harvested from natural sources followed by separation and printing on a glycan microarray. This approach allows for analysis of hundreds of structurally diverse N-glycans. Similar to cell surface recognition, Gal-8C displayed higher binding to a wider range of N-glycans than Gal-8N, suggesting an underlying preference for complex N-glycan core structures (Fig. 3-4F-H).

Gal-8N inhibits Gal-8 signaling through recruitment away from Gal-8C signaling domains

The ability of Gal-8 to signal entirely through glycan recognition by the C-terminal domain, despite the ability of the N-terminal domain to recognize cell surface glycans, strongly suggests that, the N- and C-terminal domain to may recognize discrete microdomains, and each domain may compete for binding to distinct counter receptors. If each domain of Gal-8 competes for its respective binding site, then removal of N-terminal glycan binding should eliminate competition with the C-terminal domain and therefore facilitate Gal-8C binding and signaling. Consistent with this, mutation of the N-terminal domain significantly enhanced signaling by Gal-8, strongly suggesting that Gal-8N actually competes with Gal-8C for receptor sites on the cell surface at distinct locations (Fig. 3-4E).

Discussion

The ability of galectins to recognize highly modifiable cell surface glycans provides an additional level of complexity in cellular signaling networks responsible for regulating cellular turnover. Terminal glycan modifications can significantly and differentially impact binding and signaling by a variety of galectins (67). However, the potential influence of core glycan structure on terminal glycan recognition remains under characterized. Our results demonstrate that in addition to terminal glycan modification, core complex glycan structure can significantly and differentially influence recognition by Gal-8. Furthermore, differential glycan recognition by Gal-8N and Gal-8C enables intrinsic regulation of Gal-8 signaling by Gal-8N, a process also likely influenced by differential recognition of core glycan structures.

Many of the determinants recognized by galectin family members represent common structures that terminate distinct core glycan structures. The Galb1-4GlcNac motif can be found on N-glycans, O-glycans and glycolipids. Modification of this common galectin-binding motif can significantly alter galectin recognition. For example, previous studies demonstrated that Gal-2 fails to recognize Galb1-4GlcNAc following a2-3 or a2-6 sialylation, while Gal-1 accommodates a2-3, but not a2-6 (102). In contrast, Gal-3 and the C-terminal domain of Gal-8 display significant preference for internal Galb1-4GlcNAc regardless of the sialylation state, while Gal-8N displays little preference of Galb1-4GlcNAc unless sialylated (102). However, while galectins may recognize these modifications irrespective of core glycan structures, previous studies demonstrated that galectins may prefer these modifications when present on particular core glycan structures. Prevention of complex N-glycan biosynthesis by genetic alterations in chinese hamster ovary (CHO) cells or by inhibition of biosynthesis in leukocytes demonstrated that while Gal-1 can recognize complex O-glycan structures, Gal-1 preferentially recognizes and signals leukocytes through complex N-glycans, similar to the C-terminal domain of Gal-8 in the present study (101).

While the present studies demonstrate a preference of N-glycans in a particular leukocyte, different leukocytes and different receptors may convey preferences for terminal modifications in a variety of core complex glycan settings. For example, while several studies demonstrate a role for complex N-glycans in Gal-1-induced leukocyte signaling, other studies suggest a key role for complex O-glycans. In addition, several studies suggest that Gal-1 may signal through recognition of glycolipids. The ability of Gal-8 to accommodate glycan recognition when presented a variety of core glycan formats, suggest

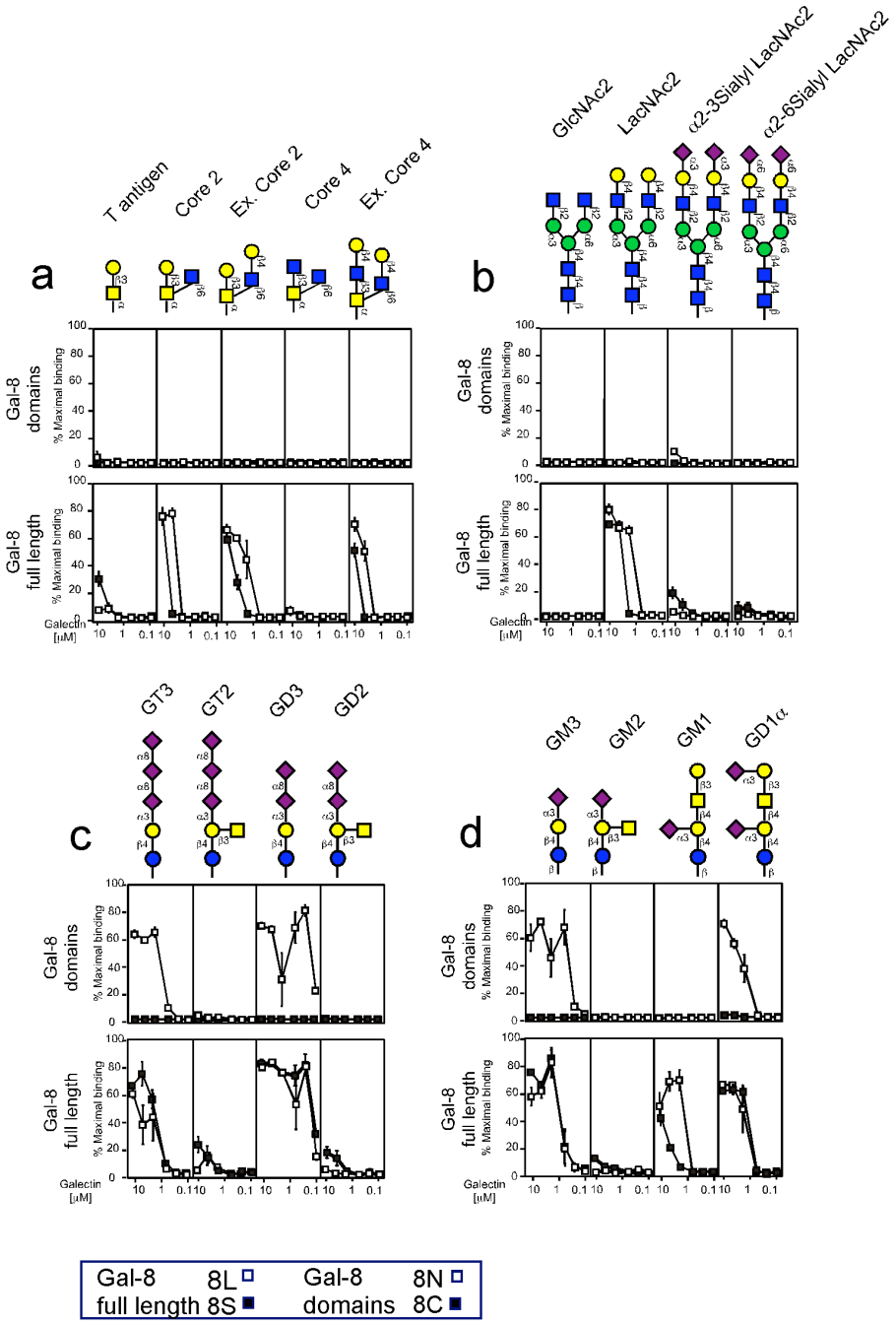
that Gal-8 may recognize terminal modifications of other core glycan structures depending on the cell type and the glycoproteins or glycolipids present on the cell surface.

Previous studies demonstrated that Gal-8N and Gal-8C display distinct glycan binding profiles. Indeed, less overlap in binding specificity occurs between Gal-8N and Gal-8C than between any other galectin family members analyzed to date. Earlier studies suggested that Gal-8 signaled leukocytes through crosslinking of functional receptors through a linker peptide that connects each separate Gal-8 CRD. However, recent studies demonstrated that Gal-8 actually exists as a dimer and signals leukocytes entirely through glycan recognition by its C-terminal domain. The unique binding properties of the C- and N-terminal domain not only enable discrete glycan recognition of glycan determinants and core glycan structures, but also appear to provide distinct binding to different microdomains on the cell surface. Although recognition of cognate glycans on different leukocytes may induce important signaling events, this distinct recognition of counter ligands provides some degree of competition between glycan binding by each individual domain in the context of the full-length protein. Consistent with this, elimination of the carbohydrate binding significantly increased the ability of Gal-8 to signal PS exposure in leukocytes, suggesting that cell surface recognition by the N-terminal domain actually serves as a regulator of Gal-8 signaling. Consistent with this, removal of sialylic acid which prevents Gal-8N binding also resulted in increased Gal-8 signaling, providing an example of how changes in the glycosylation may mask or unmask decoy receptors as an additional mechanism of regulating cellular sensitivity to Gal-8 irrespective of the glycosylation state of the functional receptor. In contrast, increased cell surface sialylation in cells may also increase Gal-8N engagement and therefore signaling pathways unrelated to cell turnover. In this way, Gal-8 is uniquely poised to deliver

a multitude of signaling events depending on the type and sialylation state of an individual cell.

Figures

Figure 3-1



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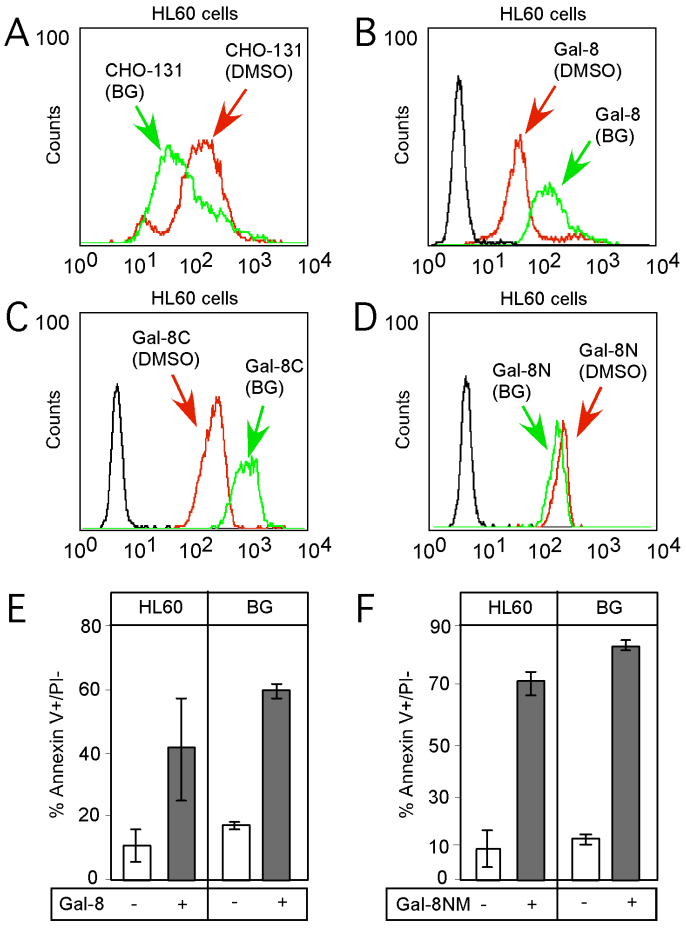
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Figure 3-3

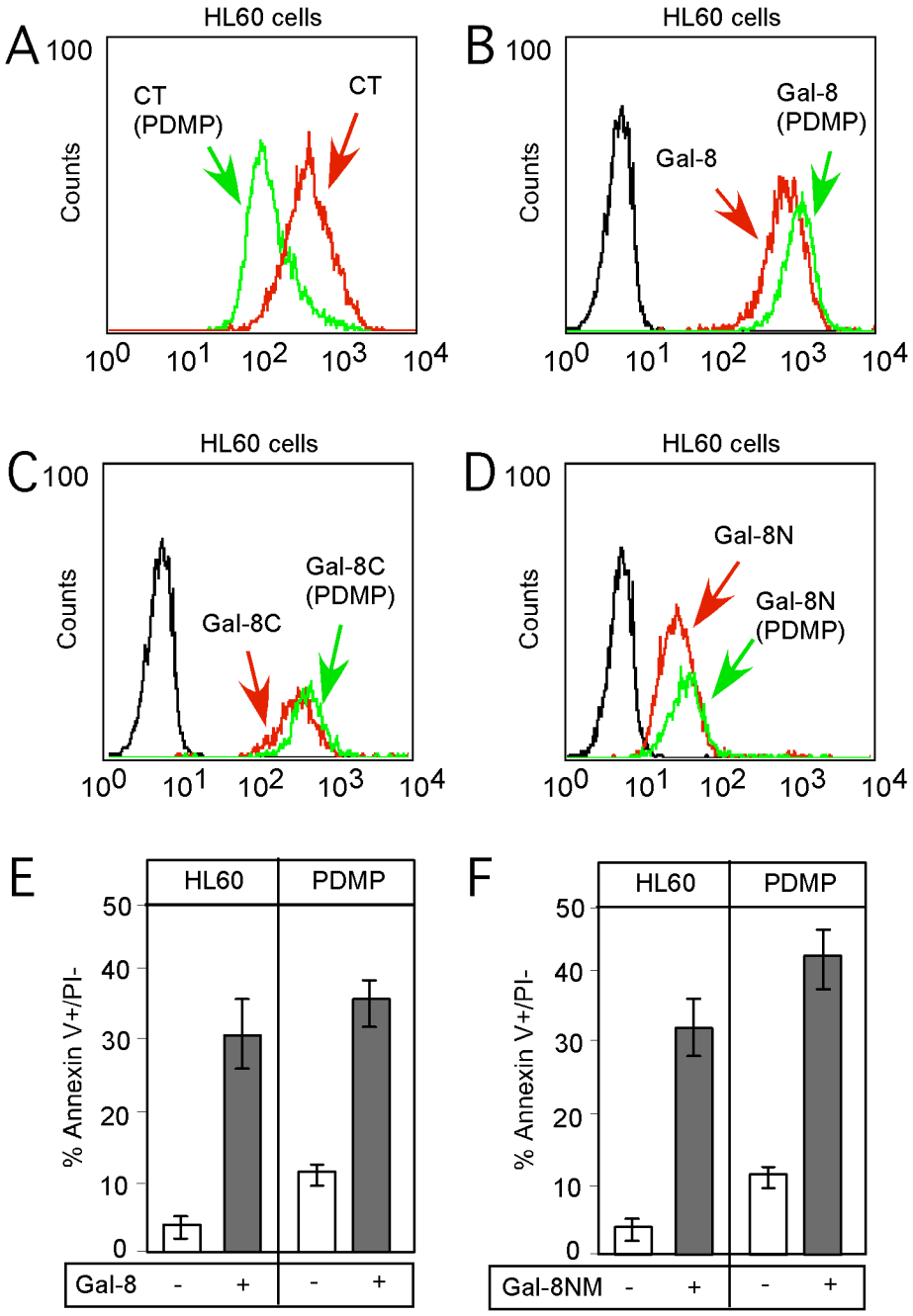
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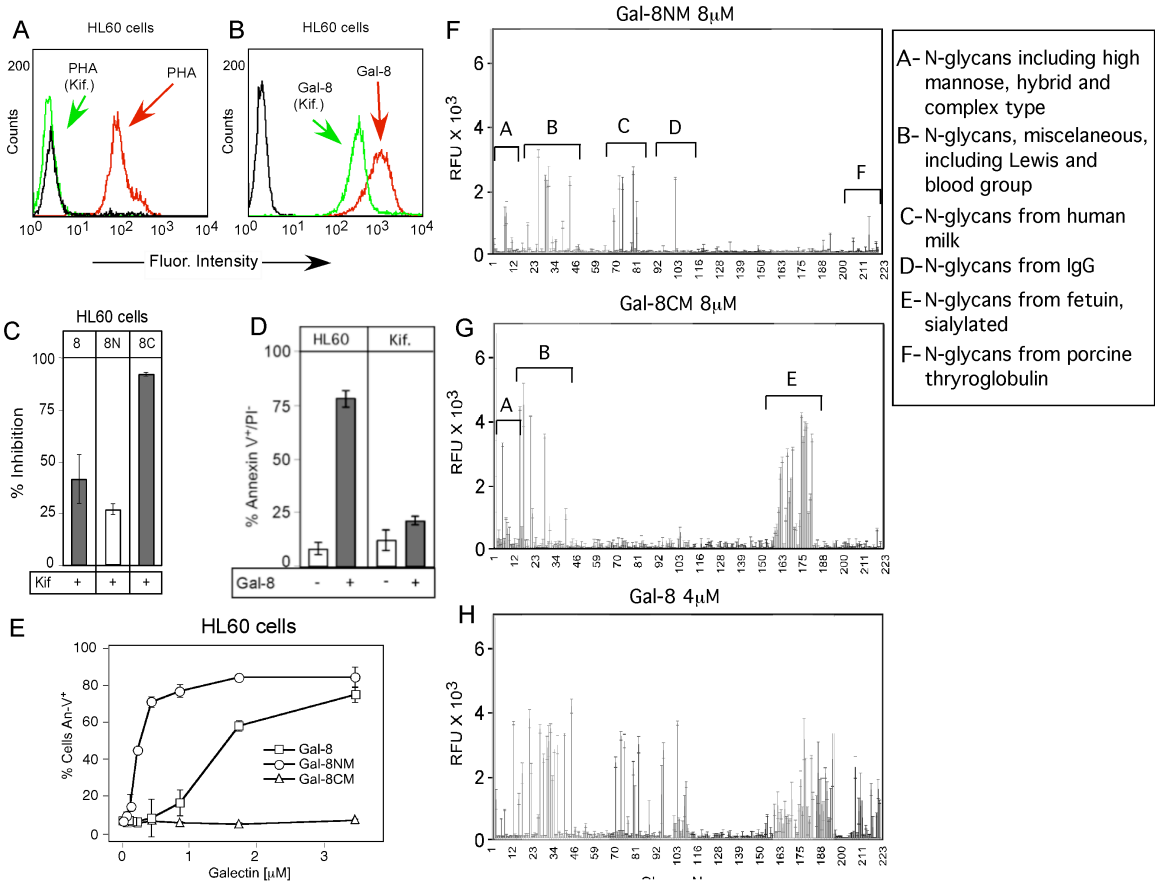
Figure 3-4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8C
% Inhibition
Kif
D
HL60
Kif.
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Gal-8
E
HL60 cells
% Cells An-V+
Gal-8
Gal-8NM
Gal-8CM
Galectin [μM]
F
Gal-8NM 8μM
RFU X 10^3
G
Gal-8CM 8μM
H
Gal-8 4μM
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B- N-glycans, miscelaneous, including Lewis and blood group
C- N-glycans from human milk
D- N-glycans from IgG
E- N-glycans from fetuin, sialylated
F- N-glycans from porcine thryroglobulin

Figure legends

Figure 3-1- Gal-8 recognition of complex glycan structures- Dose response of binding of represented complex O-glycan (A), complex N-glycan (B), or glycolipid (C,D) structures by Gal-8L, Gal-8S, Gal-8N domain, or Gal-8C domain as indicated. Recognition of each representative glycan is displayed as the percent bound when compared with the highest bound ligand at each concentration tested by Gal-8 as indicated.

Figure 3-2- Gal-8 does not signal through recognition of cell surface complex O-glycan structures- (A-D) Flow cytometric analysis of binding of HL60 cells treated with or without benzyl-GalNAc as indicated by CHO-131 antibody (A), Gal-8S (B), Gal-8C (C), or Gal-8N (D). (E-F) Quantification of the percent of HL60 cells treated with or without benzyl-GalNAc as indicated that exhibit exposed PS, as indicated by Annexin V staining, after treatment for 4 hours with Gal-8S (E) or Gal-8NM (F).

Figure 3-3- Gal-8 does not signal through recognition of cell surface glycolipids- (A-D) Flow cytometric analysis of binding of HL60 cells treated with or without PDMP as indicated by Cholera Toxin (A), Gal-8S (B), Gal-8C (C), or Gal-8N (D). (E-F) Quantification of the percent of HL60 cells treated with or without PDMP as indicated that exhibit exposed PS, as indicated by Annexin V staining, after treatment for 4 hours with Gal-8S (E) or Gal-8NM (F).

Figure 3-4- Gal-8 signals through C-terminal domain recognition of cell surface complex N-glycans- (A-B) Flow cytometric analysis of binding of HL60 cells treated with

or without PDMP as indicated by PHA (A) or Gal-8S (B). (C) Quantification of percent inhibition of binding of HL60 cells by Gal-8S, Gal-8N, or Gal-8C, as indicated after treatment with kifunensine *Bars* represent the percent change in cell surface binding when compared with the mean fluorescent intensity of non-treated cells ± S.D. (D) Quantification of the percent of HL60 cells treated with or without kifunensine as indicated that exhibit exposed PS, as indicated by Annexin V staining, after treatment for 4 hours with Gal-8S. (E) Quantification of the percent of HL60 cells that exhibit exposed PS, as indicated by Annexin V staining, after treatment for 4 hours with Gal-8S, Gal-8NM, or Gal-8CM, at indicated concentrations. (F-H) Glycan microarray data obtained after incubation with 8 μM Gal-8CM (F), 8 μM Gal-8NM (G), or 4 μM Gal-8 (H). RFU, relative fluorescence units. Error bars represent means ± s.e.m.

Chapter 4- Innate Immune Lectins kill pathogens expressing blood group antigens

The expression of ABO(H) blood group antigens causes deletion of cells that generate self-specific antibodies to these antigens but this deletion limits adaptive immunity toward pathogens bearing cognate blood group antigens. To explore potential defense mechanisms against such pathogens, given these limitations in adaptive immunity, we screened for innate proteins that could recognize human blood group antigens. Here we report that two innate immune lectins, galectin-4 (Gal-4) and Gal-8, which are expressed in the intestinal tract, recognize and kill human blood group antigen–expressing *Escherichia coli* while failing to alter the viability of other *E. coli* strains or other Gram-negative or Gram-positive organisms both *in vitro* and *in vivo*. The killing activity of both Gal-4 and Gal-8 is mediated by their C-terminal domains, occurs rapidly and independently of complement and is accompanied by disruption of membrane integrity. These results demonstrate that innate defense lectins can provide immunity against pathogens that express blood group–like antigens on their surface.

Introduction

Recent studies suggest that blood group antigen diversity may provide a mechanism of pathogen evasion whereby distinct ABO(H) antigen structures may reduce pathogen attachment and therefore infectivity (108). However, expression of ABO(H) blood group antigens causes deletion of cells that would produce antibodies to these antigens, which limits adaptive immunity toward pathogens bearing blood group–like structures. Because

ABO(H) antigens are composed of carbohydrate structures that only differ by distinct monosaccharides on the terminal structures of glycans (109), factors that might be responsible for providing innate immunity toward pathogens expressing blood group antigens must recognize carbohydrates. A growing list of glycan-binding proteins, including galectins and C-type lectins, recognize carbohydrate determinants on pathogens and participate in innate immune responses (110-112). Notably, previous studies suggest that several galectins may recognize blood group antigens (67) along with various other carbohydrate ligands. Given the ability of innate immune lectins to recognize cell surface carbohydrates, we explored the carbohydrate binding of several innate immune lectins for potential blood group binding specificity and subsequent activity.

Galectins recognize blood group-positive bacteria

We analyzed publicly available data from the screening of nearly 100 different lectins from the Consortium for Functional Glycomics, many of which are mammalian lectins with documented immunological activity, including members of the galectin family. Members of the galectin family had some of the most specific interactions observed among the lectins tested after screening of over 300 structurally diverse glycans. Human Gal-3, Gal-4 and Gal-8, which recognize multiple glycan structures at relatively high concentrations (67), showed specificity for human blood group A and B antigens at submicromolar concentrations and did not bind blood group O(H) at these concentrations, whereas human Gal-1, a related galectin family member, did not recognize blood group antigens (Fig. 4-1a-d). This specificity was not as striking in our previous studies concerning members of this protein family, where we tested binding at high protein concentrations and found that the lectins

recognized multiple carbohydrate ligands along with blood group antigens (67).

Bacteria generate a wide variety of glycan- based antigenic structures, many of which possess blood group antigen activity (113,114). 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 (115). Notably, whereas individuals of blood group A or O produce antibodies that kill *E. coli* O86, individuals with blood group B do not generate antibodies capable of altering *E. coli* O86 viability (115,116), providing a specific example of the immunological limitation in adaptive immunity toward a blood group antigen–bearing pathogen. The ability of human Gal-3, Gal-4 and Gal-8 to specifically recognize blood group A and B antigens suggests that they may be uniquely poised to provide innate immunity toward blood group–bearing pathogens regardless of the blood group antigen status of an individual. However, although *E. coli* O86 generates an identical blood group B epitope (Fig. 4-1E) to that of humans (117), the context of this epitope may differ from the common human presentations found on the glycan microarray. Therefore, we examined whether Gal-3, Gal-4, and Gal-8 recognize *E. coli* O86. Consistent with their ability to specifically recognize blood group antigens on the microarray, Gal-3, Gal-4, 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. 4-1F-I). Binding of all galectins to bacteria was inhibited by lactose, an inhibitor of galectin-carbohydrate interactions, indicating that galectin binding was toward glycan determinants on the surface of BG B+ *E. coli*.

Gal-4 and Gal-8 kill blood group positive-bacteria

Previous studies demonstrated high levels of galectin expression in the intestinal mucosa where the galectins may serve as pathogen recognition proteins (118,119), suggesting Gal-3, Gal-4, and Gal-8 may facilitate innate immunity toward BG B+ pathogens. While previous studies have shown that several innate immune lectins can directly affect pathogen viability (110,120,121), indicating potential roles for galectins in pathogen adhesion, recognition and killing (119), there is no evidence regarding whether galectins possess the ability to alter prokaryote viability. Thus, we asked whether Gal-3, Gal-4, and Gal-8 might confer intrinsic immunity by directly killing BG B+ *E. coli*. Incubation with both Gal-4 and Gal-8 caused direct killing of BG B+ *E. coli,* whereas Gal-3, which also binds BG B+ *E. coli* did not affect viability, and Gal-1, which does not bind BG B+ *E. coli*, had no effect (Fig. 4-2A). As expected, lactose completely inhibited both Gal-4– and Gal-8–induced death, whereas sucrose, a disaccharide unable to inhibit galectin-carbohydrate interactions, failed to alter killing of BGB^+ *E. coli* (Fig. 4-2b,c). Gal-4 and Gal-8 displayed similarly potent concentration-dependent killing of BG B+ *E. coli* with a half maximal lethal dose of 0.1 μM (LD50 ~0.1 μM) (Fig. 2D), a concentration similar to that observed *in vivo* (61) and used to evaluate glycan binding specificity on the microarray. In addition, effects of Gal-8 treatment appeared to be rapid, since treated BG B+ *E. coli* lost all motility compared to untreated nearly immediately following the addition of Gal-8 (Fig. 4-2E). BG B+ *E. coli* positively stained for propidium iodide (PI) following 30 min incubation with Gal-8 (Fig. 4-2F), and showed considerable disruption of membrane morphology (Fig. 4-2G-L). These results show that Gal-8 kills BG B+ *E. coli* through directly altering membrane integrity. Comparable effects were observed following incubation with Gal-4 (data not shown). Taken

together, these results demonstrate that both Gal-4 and Gal-8 directly kill BG B+ *E. coli* through recognition of bacterial surface carbohydrates via a mechanism that drastically alters membrane integrity and bacterial viability. Killing of BG B+ *E. coli* by Gal-8 did not require complement (Fig. 4-2), 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 (121).

Unlike Gal-1 and Gal-3, which contain a single carbohydrate recognition domain (CRD), Gal-4 and Gal-8 possess two distinct CRDs (32), suggesting that these galectins may utilize one domain for target recognition and the other domain for killing the target once bound, similar to many prokaryotic AB toxins (122). To test this, we mutated each CRD of Gal-8, in the context of the whole protein, to determine which domain recognizes BG B+ *E. coli.* Inactivation of the C-terminal CRD (Arg223→His) (Gal-8R223H) eliminated recognition of blood group antigens on both the glycan microarray and BG B+ *E. coli* (data not shown), while the analogous mutation in the N-terminal CRD (Arg69→His) (Gal-8R69H) failed to alter blood group antigen recognition in either context (data not shown). To determine whether the N-terminal domain is required for Gal-8 killing independently of glycan recognition, we expressed the individual domains of Gal-8. Whereas the N-terminal domain (Gal-8N) failed to bind blood group antigens on either the glycan microarray or BGB^+ *E. coli* (Fig. 4-3b and data not shown), the C-terminal domain of Gal-8 (Gal-8C) independently recognized blood group antigens and killed BGB^+ *E. coli* (Fig. 4-3b,c). These results show that recognition and killing of BGB^+ *E. coli* by Gal-8 resides entirely within its blood group–binding domain. By contrast, both domains of Gal-4 showed specific recognition of BGB^+ *E. coli* (Fig. 3d). Thus, we asked whether Gal-4N and Gal-4C might independently kill BGB^+

E. coli. However, similar to Gal-3, Gal-4N showed substantial recognition of BGB^+ *E. coli* yet failed to alter BGB^+ *E. coli* viability (Fig. 4-2a and Fig. 4-3e). By contrast, Gal-4C had substantial killing activity toward BGB^+ *E. coli* (Fig. 4-3e). Notably, the Gal-4C and Gal-8C domains show phylogenetic similarities not shared by Gal-3 and the Gal-4N domain (123), which suggests a conserved mechanism shared between these two protein domains.

Galectin killing requires blood group antigen recognition

The ability of the blood group–binding domain of Gal-4 and Gal-8 to independently kill BGB^+ *E. coli* (Fig. 4-3a,c,e) suggested that Gal-4 and Gal-8 might specifically kill BGB^+ *E. coli*. To test this, we examined whether Gal-4 and Gal-8 recognize strains of *E. coli* that fail to express the blood group B–related antigen. Although both Gal-4 and Gal-8 recognize BGB^+ *E. coli*, they did not substantially bind or affect the viability of BGB^- *E. coli* (Fig. 4-4a-c and data not shown). In addition, Gal-4 and Gal-8 did not recognize or kill the Gram-negative, BGB^- species *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, and they neither bound nor altered the viability of Gram-positive *Staphylococcus aureus* (Fig. 4-4d-g and data not shown). We next asked whether Gal-8 specifically kills BGB^+ *E. coli* in a mixed population of BGB^+ and BGB^- bacteria. We incubated GFP^+ BGB^- *P. aeruginosa* with Gal-8 to determine whether Gal-8 altered GFP expression or viability. Gal-8 failed to alter GFP expression (Fig. 4-4h) or viability (data not shown), allowing us to discriminate between GFP^+ *P. aeruginosa* and BGB^+ *E. coli* within a mixed population. To examine whether Gal-8 specifically kills BGB^+ *E. coli*, we incubated various ratios of BGB^+ *E. coli* to GFP^+ *P. aeruginosa* with or without Gal-8. Even at a 4:1 ratio of BGB^+ *E. coli*:GFP^+ *P. aeruginosa*, Gal-8 selectively eliminated the GFP^- BGB^+ *E. coli* (Fig. 4-4i,j). Furthermore, defined mutations that prevent synthesis of the blood

group antigen formation on BGB$^+$ *E. coli* (Δ*waaL*) prevented recognition and killing by Gal-4 and Gal-8, whereas bacteria carrying mutations that allow formation of at least one repeat of the blood group antigen (Δ*wzy*) remained sensitive to Gal-4 and Gal-8 (Fig. 4-5a-d), further illustrating the specificity of Gal-4 and Gal-8 for the blood group B antigen (124). Of note, lactose, but not sucrose, prevented Gal-4 and Gal-8 killing (data not shown). Notably, although both Gal-4 and Gal-8 recognized BGB$^+$ human erythrocytes, neither affected the membrane integrity of these cells (data not shown), which indicates that the killing activity of Gal-4 and Gal-8 not only shows antigen specificity but also uniquely targets prokaryotes. Furthermore, Gal-4– and Gal-8–induced killing of BGB$^+$ *E. coli* did not represent a simple agglutination-associated reduction in colony-forming unit (CFU) counts, as Gal-4 and Gal-8 bound BGB$^+$ *E. coli* at 4 °C but did not alter viability (data not shown). In addition, both Gal-1 and human BGB-specific antibodies recognized and agglutinated BGB$^+$ *E. coli* at high concentrations, yet failed to affect CFU counts of BGB$^+$ *E. coli* after incubation with the bacteria (data not shown).

Whereas these results show that Gal-4 and Gal-8 kill BGB$^+$ *E. coli in vitro*, we used mice to test whether similar activities occur *in vivo*. We first examined whether the mouse galectin-4 (Gal-4) possesses a similar ability to bind and kill BGB$^+$ *E. coli* to human Gal-4. Recombinant mouse Gal-4 recognized BGB$^+$ *E. coli*, and the recognition was inhibited by both lactose (Fig. 4-6a) and thiodigalactoside, a non-metabolizable inhibitor of galectins (data not shown). Furthermore, mouse Gal-4 recognition of BGB$^+$ *E. coli* seemed to be specific to the BGB antigen, as mouse Gal-4 failed to recognize the Δ*waaL* mutant (Fig. 4-6b), similar to human Gal-4 (data not shown). Mouse Gal-4 also showed high binding of blood group antigens on the glycan microarray (Fig. 4-6c). Of note, mouse Gal-4 recognition

of BGB$^+$ *E. coli* resulted in a substantial reduction in viability, which seemed to be specific to BGB antigen binding, as mouse Gal-4 failed to alter the viability of the Δ*waaL* mutant (Fig. 4-6d), and Gal-4–mediated killing was inhibited by thiodigalactoside (data not shown). However, mouse Gal-4–mediated killing was less potent when compared to human Gal-4, possibly owing to the reduced affinity of mouse Gal-4 for BGB when compared to BGA (Fig. 4-6c,d).

Galectins specifically kill BGB$^+$ bacteria *in vivo*

The selective killing of BGB$^+$ *E. coli* by mouse Gal-4 suggests that the Δ*waaL* mutant should show better growth *in vivo* as a result of the inability of endogenous galectins to bind and kill these bacteria, whereas BGB$^+$ *E. coli* should be limited in their growth owing to killing by endogenous Gal-4 and Gal-8. It has been shown previously that Gal-4 and Gal-8 are the only intestinal proteins that detectably bind β-galactosides (125), but Gal-4 and Gal-8 double-knockout mice are not available, and such mice may not be viable. Thus, to specifically test the physiological functions of these intestinal galectins, we fed wild-type (WT) mice with BGB$^+$ *E. coli* or Δ*waaL* mutant *E. coli*. In this *in vivo* model, we first treated mice with streptomycin to deplete endogenous bacteria followed by feeding the mice with the WT and Δ*waaL* mutant strains of bacteria. The number of WT bacteria detected was significantly lower *in vivo* compared to the Δ*waaL* mutant (Fig. 4-6e), although both types of bacteria showed equal growth kinetics *in vitro* (data not shown), which implicated a possible galectin-mediated process *in vivo*. The few bacteria isolated from mice inoculated with WT bacteria were positive for BGB antigen. Similarly, bacteria isolated after introduction of the Δ*waaL* mutant were negative for the BGB antigen, indicating that the bacteria examined

reflected those used during the inoculation (data not shown). To test the potential role of galectins in the observed difference in growth of the two types of bacteria, we incubated BGB^+ *E. coli* or Δ*waaL E. coli* with or without the inclusion of thiodigalactoside *in vivo*. Although thiodigalactoside failed to alter the growth of BGB^+ *E. coli* or Δ*waaL E. coli* in the absence of mGal-4 (data not shown), thiodigalactoside significantly increased BGB^+ *E. coli* viability *in vivo* while failing to alter Δ*waaL E. coli* viability (Fig. 4-6f). These results strongly suggest that endogenous galectins specifically alter the viability of BGB^+ *E. coli in vivo*. Although blood group antigens are expressed to some extent in glycosphingolipids and mucins of the gastrointestinal tract (126), it has been found that they are susceptible to degradation by bacterial-derived glycosidases (127,128), and in infants this bacterial-induced degradation of blood group antigens is observed soon after weaning (129). Thus, it is not likely that host blood group antigens, which are expressed in low amounts, can bind all of the galectins present, as Gal-4 and Gal-8 are highly expressed in the intestinal tract (130,131).

Although Gal-4 and Gal-8 seem to kill specifically BGB^+ *E. coli*, whether Gal-4 or Gal-8 possess the ability to recognize and kill bacteria expressing other types of blood group antigens remained unknown. To test this, we examined whether Gal-4 and Gal-8 could recognize and kill bacteria expressing the α1-3Gal epitope (α-Gal *E. coli*), a common glycan moiety found in many mammalian species (Fig. 4-6g). Similar to BGB^+ *E. coli*, α-Gal *E. coli* were recognized by Gal-4 and Gal-8 (Fig. 4-6h), and recognition was inhibited by thiodigalactoside (data not shown). Furthermore, Gal-4 and Gal-8 recognition of α-Gal *E. coli* resulted in a considerable decrease in viability (Fig. 4-6i), although killing of α-Gal *E. coli* by Gal-4 and Gal-8 was reduced when compared to Gal-4– and Gal-8–mediated killing of BGB^+ *E. coli*, suggesting a possible reduced binding affinity toward this glycan epitope.

Consistent with this, Gal-4 and Gal-8 only recognized α-Gal epitopes on the glycan array when incubated at higher concentrations (data not shown). Taken together, these results demonstrate that Gal-4 and Gal-8 possess the ability to specifically kill bacteria expressing common blood group–associated mammalian-like antigens.

Discussion

Many human pathogens decorate their surfaces with diverse carbohydrate structures, and many of these structures have similarities to human antigens, a common mechanism used by both commensal and pathogenic organisms to render themselves immunologically inert. However, mechanisms must also be in place to prevent the overgrowth of any potential pathogens that are shielded from normal adaptive immune responses. Thus, the ability of Gal-4 and Gal-8 to specifically kill BGB^+ *E. coli* extends previous observations suggesting crucial roles for galectins in innate immunity (119) and may reflect a common but unrealized feature of other innate immune lectins to provide direct protection against pathogens expressing particular self-like antigens, where adaptive immunity cannot.

Similar to many innate immune factors, the galectins represent an ancient family of proteins present in a wide variety of species (123). As galectins evolved long before the selection of adaptive immunity, it is intriguing to speculate that the types of carbohydrate modifications on some self-antigens, such as blood group antigens, may reflect the binding specificity of preexisting innate immune factors such as the galectins. The generation of ABO(H) antigen diversity in the human population has been proposed to facilitate pathogen evasion during human evolution (108). For example, differential expression of blood group ABO(H) antigens in host tissues can differentially affect pathogen adhesion and infection, as recently

shown for *Helicobacter pylori* (132). However, this diversity might have arisen with a considerable fitness cost, as development of these antigens precludes adaptive immune responses against blood group antigen–bearing pathogens. The ability of galectins to recognize blood group antigen–bearing pathogens may have facilitated the selection of ABO(H) expression on human erythrocytes rather than alternative antigens that did not have the same preexisting innate immune protection. In contrast, the ability of Gal-4 and Gal-8 to also kill α-Gal–expressing bacteria shows that galectin-mediated killing is not limited to human blood group antigen–expressing bacteria and suggests that galectins may affect the composition of multiple populations of intestinal bacteria, thereby modulating the intestinal microbiome. Future studies will examine these possibilities.

Figures

Figure 4-1

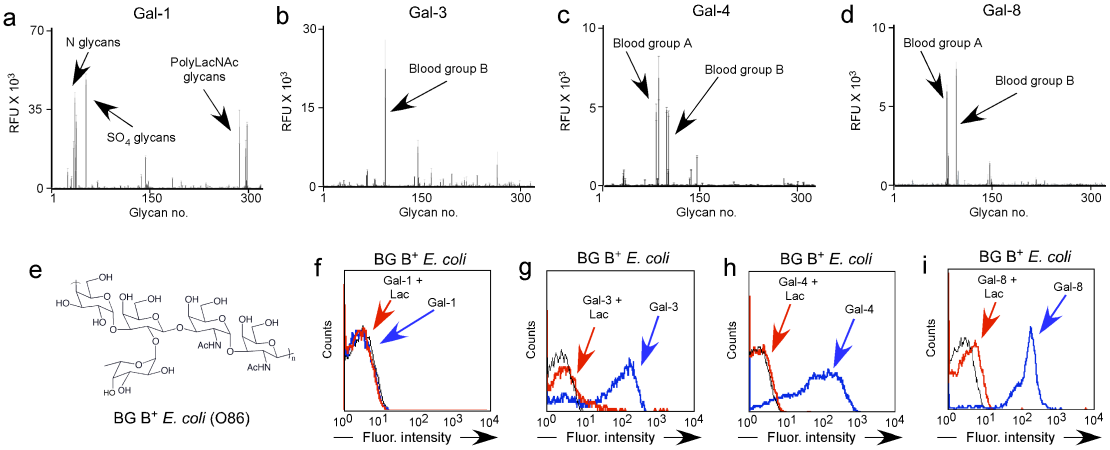

Figure 4-2

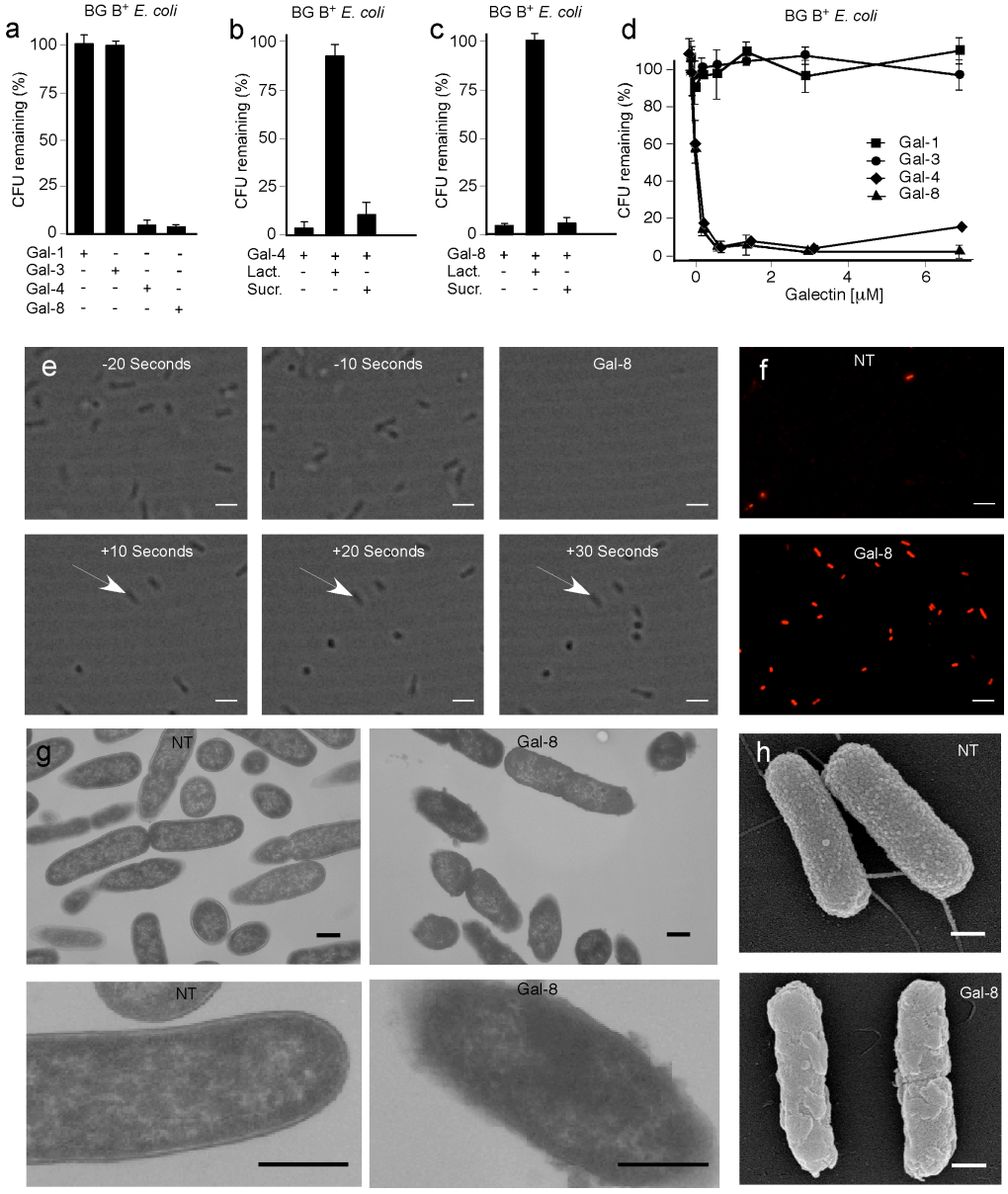
a
BG B+ E. coli
CFU remaining (%)
100
75
50
25
0
Gal-1 + - - -
Gal-3 - + - -
Gal-4 - - + -
Gal-8 - - - +
b
BG B+ E. coli
CFU remaining (%)
Gal-4 + + +
Lact. - + -
Sucr. - - +
c
BG B+ E. coli
CFU remaining (%)
Gal-8 + + +
Lact. - + -
Sucr. - - +
d
BG B+ E. coli
CFU remaining (%)
100
80
60
40
20
0
0
2
4
6
Galectin [μM]
Gal-1
Gal-3
Gal-4
Gal-8
e
-20 Seconds
-10 Seconds
Gal-8
+10 Seconds
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Figure 4-3

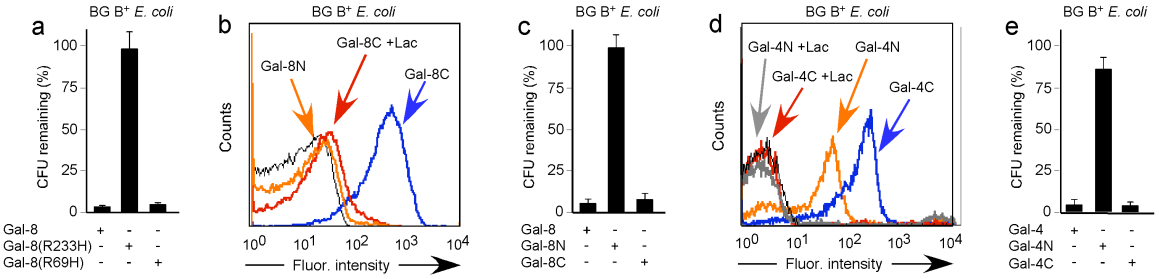
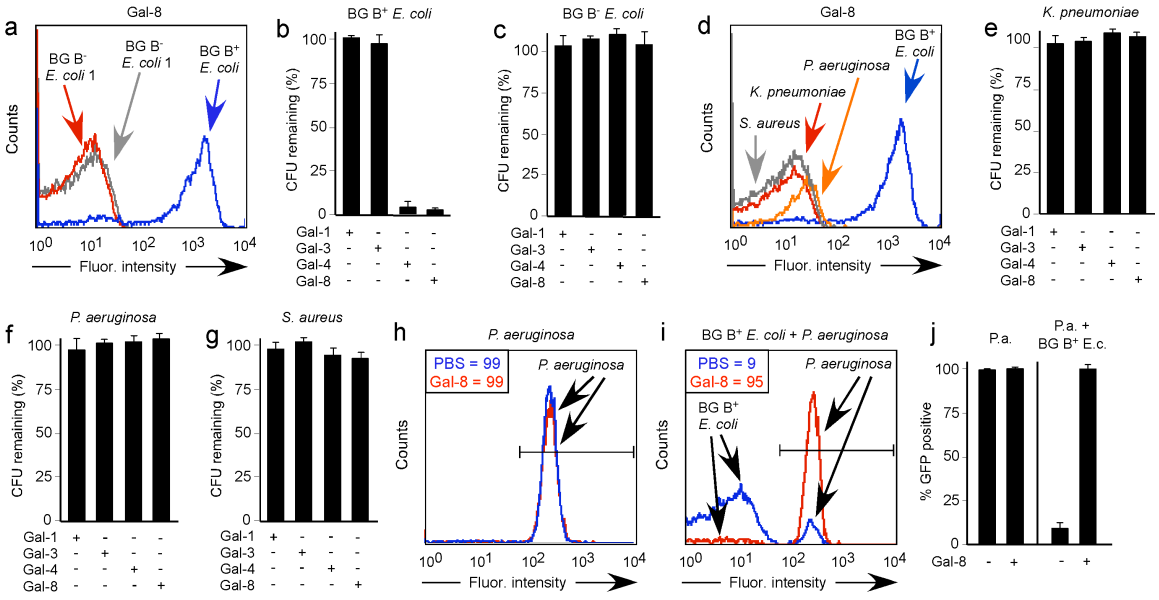


Figure 4-4



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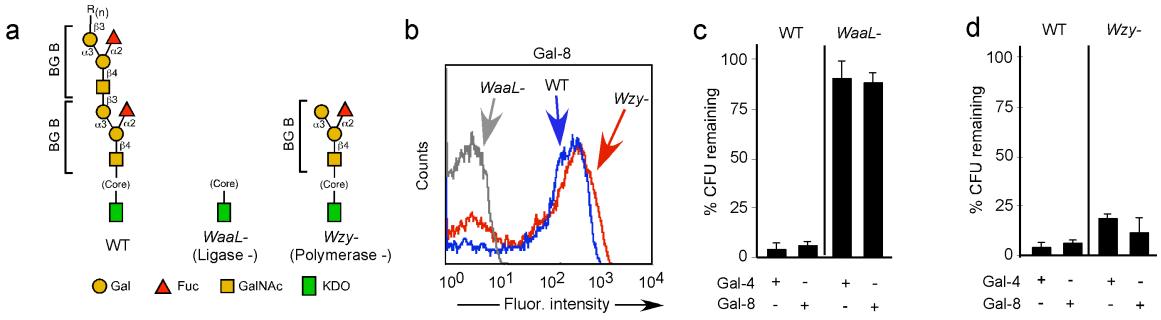


Figure 4-6

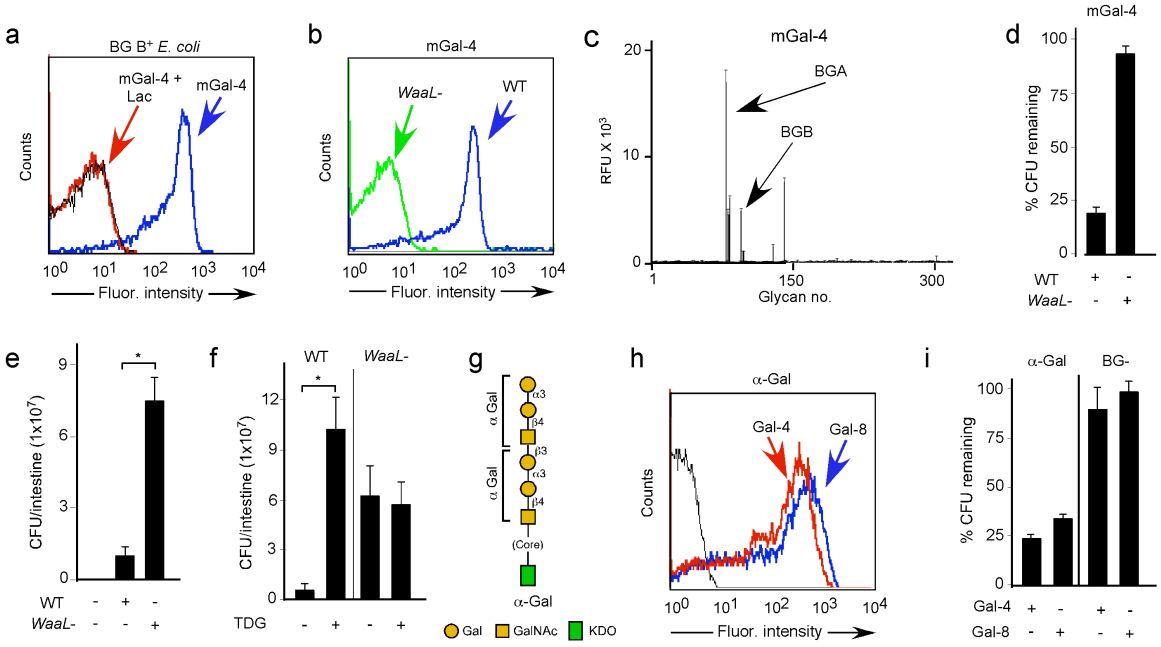


Figure Legends

Figure 4-1- Gal-3, Gal-4, and Gal-8 recognize blood group B positive *E. coli*. (a–d) Glycan microarray data obtained following incubation with (a) 0.2 μM Gal-1, (b) 0.2 μM Gal-3, (c) 0.5 μM Gal-4, and (d) 0.02 μM Gal-8. RFU = relative fluorescence units represented on the y-axis. Error bars = +/- 1 SEM. See Supplementary Table 1 for complete list of glycans represented on the x-axis. (e) Structure of *E. coli* O86 O antigen. (f-i) Flow cytometric analysis following incubation of *E. coli* O86 with (f) Gal-1, (g) Gal-3, (h) Gal-4, and (i) Gal-8 all tested at 0.1 μM with or without inclusion of 20 mM lactose (Lac) where indicated.

Figure 4-2- Gal-4 and Gal-8 kill blood group B positive *E. coli*. *E. coli* O86 (BG B^+ *E. coli*) were mixed with (a) 5 μM Gal-1, Gal-3, Gal-4, or Gal-8, (b) 5 μM Gal-4 with or without 20 mM lactose (Lac) or 20 mM sucrose (Suc), (c) 5 μM Gal-8 with or without 20 mM lactose (Lac) or 20 mM sucrose (Suc), or (d) the indicated concentrations of Gal-1, Gal-3, Gal-4, or Gal-8. Viable bacteria were quantified by dilution plating, n=3, 1 representative experiment in duplicate over 2 dilutions shown (a–c), error bars=SD. (e) Still-frame images from real-time video microscopy demonstrating bacterial mobility at 10-s intervals before and after addition of 5 μM Gal-8 as indicated. Arrows indicate one group of immobilized bacteria. Scale bars = 100 mm. (f) *E. coli* O86 (BG B^+ *E. coli*) were grown to mid-log phase followed by addition of 5 μM Gal-8. Untreated and Gal-8 treated bacteria were stained with propidium iodide (red) and visualized by fluorescence microscopy. Scale bars = 100 mm. (g) Transmission electron microscopy images of *E. coli* O86 (BG B^+ *E. coli*) followed by addition of PBS (NT) or 5 μM Gal-8. Lower panels show close up view of single bacterium. Scale

bars = 500 nm. (h) Scanning electron microscopy images of *E. coli* O86 (BG B^+ *E. coli*) followed by addition of PBS (NT) or Gal-8. Scale bars = 500 nm.

Figure 4-3- Gal-4 and Gal-8 kill blood group B positive *E. coli* entirely through the C-terminal domain. (a) 5 μM Gal-8, Gal-8R233H, or Gal-8R69H were added to mid-log phase *E. coli* O86 (BG B^+ *E. coli*). Viable bacteria were quantified by dilution plating, n=3, 1 representative experiment in duplicate over 2 dilutions shown, error bars=SD. (b) Flow cytometric analysis following incubation of *E. coli* O86 (BG B^+ *E. coli*) with Gal-8N or Gal-8C at 0.1 mM with or without inclusion of 20 mM lactose (Lac) where indicated. (c) 5 μM Gal-8, Gal-8N, or Gal-8C were added to mid-log phase *E. coli* O86 (BG B^+ *E. coli*). Viable bacteria were quantified by dilution plating, n=3, 1 representative experiment in duplicate over 2 dilutions shown, error bars=SD. (d) Flow cytometric analysis following incubation of *E. coli* O86 (BG B^+ *E. coli*) with Gal-4N or Gal-4C at 0.1 mM with or without inclusion of 20 mM lactose (Lac) where indicated. (e) 5 μM Gal-4, Gal-4N, or Gal-4C were added to mid-log phase *E. coli* O86 (BG B^+ *E. coli*). Viable bacteria were quantified by dilution plating, n=3, 1 representative experiment in duplicate over 2 dilutions shown, error bars=SD.

Figure 4-4- Gal-4 and Gal-8 specifically kill blood group B positive *E. coli*. (a) Flow cytometric analysis following incubation of blood group B positive *E. coli* O86 (BG B^+ *E. coli*) and clinical BG B negative *E. coli* (BG B^- *E. coli*) reference strains with ~0.1 μM Gal-8. (b–c) Incubation of (b) BG B^+ *E. coli* or (c) BG B^- *E. coli* strain 1 with 5 μM Gal-1, Gal-3, Gal-4, or Gal-8 as indicated. Viable bacteria were quantified by dilution plating, n=3, representative experiment in duplicate over 2 dilutions shown, error bars=SD. (d) Flow cytometric analysis following incubation of BG B^+ *E. coli*, *K. pneumoniae*, *P. aeruginosa,* and *S.*

aureus with ~0.1 μM Gal-8. (e–g) Incubation of (e) *K. pneumoniae*, (f) *P. aeruginosa,* or (g) *S. aureus* with 5 μM Gal-1, Gal-3, Gal-4, or Gal-8 as indicated. Viable bacteria were quantified by dilution plating, n=3, representative experiment in duplicate over 2 dilutions shown, error bars=SD. (h–i) Incubation with or without 5 μM Gal-8 with (h) GFP^+ *P. aeruginosa* alone or (i) GFP^+ *P. aeruginosa* mixed with BG B^+ *E. coli* followed by determination of percent GFP^+ *P. aeruginosa* by flow cytometric analysis in a mixing experiment. Gated values of GFP^+ bacteria treated with PBS (blue) or Gal-8 (red) are shown. (j) Quantification of percent GFP^+ bacteria utilizing flow cytometric analysis obtained following incubation of Gal-8 with either GFP^+ *P. aeruginosa* alone (P.a.) or GFP^+ *P. aeruginosa* mixed with BG B^+ *E. coli* (P.a. + BG B^+ E.c.).

Figure 4-5- Gal-4 and Gal-8 specifically recognize blood group B antigen on blood group B positive *E. coli.* (a) Schematic of O antigen structures on wild type (WT) BG B^+ *E. coli* and mutants of BG B^+ *E. coli* $WaaL^-$ (Ligase-) and Wzy^- (Polymerase-) lacking a complete BG antigen. (b) Flow cytometric analysis following incubation of BG B^+ *E. coli* and mutants $WaaL^-$ and Wzy^- with ~0.1 μM Gal-8. (c–d) Incubation of (c) WT and $WaaL^-$ mutant BG B^+ *E. coli* or (d) WT and Wzy^- mutant BG B^+ *E. coli* with 5 μM Gal-4 or Gal-8 as indicated. Viable bacteria were quantified by dilution plating, n=3, representative experiment in duplicate over 2 dilutions shown, error bars=SD.

Figure 4-6- Gal-4 and Gal-8 specifically kill blood group B positive *E. coli in vivo.* (a) Flow cytometric analysis following incubation of BG B^+ *E. coli* with ~0.1 μM mGal-4 with or without lactose. (b) Flow cytometric analysis following incubation of BG B^+ *E. coli* and mutants $WaaL^-$ with ~0.1 μM mGal-4. (c) mGal-4 binding to the CFG glycan microarray at

20 μg/ml (0.5 μM). (d) ~0.1 μM mGal-4 incubation with WT and *WaaL*⁻ mutant BG B^+ *E. coli*. Viable bacteria were quantified by dilution plating, n=3, representative experiment in duplicate over 2 dilutions shown, error bars=SD. (e) Live antibiotic-treated mice were fed PBS, Wild type (WT), or *WaaL*⁻ mutant BG B+ *E. coli*. The number of viable bacteria in the intestine of mice sacrificed 24 h after feeding was quantified by dilution plating. * = p value 0.049. (f) Growth of WT and *WaaL*⁻ mutant BG B^+ *E. coli* in the presence and absence of TDG. * = p value 0.008. (g) Schematic of O antigen structures on α-Gal *E. coli*. (h) Flow cytometric analysis following incubation of α-Gal expressing bacteria and BG^- bacteria with ~0.1 mM human Gal-4 and Gal-8. (i) Bar graph showing percent α-Gal expressing bacteria remaining following incubation with 5 mM Gal-4 and Gal-8 as compared to PBS treated control bacteria. Viable bacteria were quantified by dilution plating, n=3, representative experiment in duplicate over 2 dilutions shown, error bars=SD.

Chapter 5: Microbial microarray reveals complementary defense against potential pathogens

While genomic approaches provide unprecedented insight into the microbiome, concurrent interactions of host immunity with one's microbiota remain difficult to study. To overcome these limitations, we generated a microbial microarray (MGM) containing unique antigenic determinants from a diverse range of microbial flora. Serological examination on the MGM demonstrated distinct patterns of reactivity between different mammalian species, while exposure of animals to different microbes resulted in significant alterations in serological recognition. While unique serological patterns on the MGM illustrated the plasticity of adaptive immunity, two innate immune factors, galectin-4 and galectin-8, exclusively recognized microbes that expressed self-like antigens. Although deletion of self-reactive cells limits adaptive immunity toward these microbes, galectin-4 and galectin-8 not only recognized, but also killed a broad range of microbes baring self-like antigens. These results demonstrate that host immunity likely represents a balanced approach between adaptive and innate immunity, providing protection against evolving antigenic determinants while maintaining host defense against molecular mimicry.

Introduction

While infectious disease typically represents a breach in host immunity by an individual organism, hosts wage a quiet but continual battle with resident microbiota (133,134). Indeed, the microbiome represents a previously underappreciated entity that influences numerous biological processes, including subsequent immunity to newly acquired microbes.

Although genomic approaches underscore the diversity of the microbiome, questions remain regarding host immunity to the microbiome itself (135-137). However, no platform currently enables simultaneous examination of host interactions with multiple microbes, making it difficult to assess global host-microbiota interactions.

Generation of a Microbial Glycan Microarray (MGM)

In order to overcome this limitation, we sought to generate a novel array representing unique antigenic determinants from nearly 50 distinct microbial species. To accomplish this, we isolated, derivatized and attached unique carbohydrate antigens from a wide range of microbes to a microarray, hereafter referred to the microbial microarray (MGM). As serological analysis represents a common method of examining host exposure to microorganisms, we first examined sera isolated from five healthy human volunteers. Human sera displayed a unique pattern of reactivity on the array (Fig. 5-1A). These results not only demonstrated that antigenic determinants on the array retained accessibility to antibody recognition, but also demonstrated a distinct pattern of exposure by these individuals to particular microbial species that would be difficult to appreciate without the MGM format.

MGM analysis reveals distinct serological responses

As exposure to different microbiota would be predicted to induce distinct seroreactivity on the MGM, and recent studies suggest that distinct hosts often become inhabited by unique microflora, we next examined serum samples from a different host species (138). Pooled

sera isolated from mice exhibited a strikingly distinct pattern to that of human sera (Fig. 5-1B). To determine whether human sera represented an idiosyncratic response to microflora within the donor community, intravenous immunoglobulin (IVIG), isolated from over 10,000 healthy human volunteers, was analyzed on the MGM. Amazingly, IVIG provided a very similar pattern to that observed following analysis of pooled human sera (Fig. 5-1C). Taken together, these results suggest that humans and mice display very distinct serological responses to microflora.

Enhanced immunity following pathogen exposure often results in significant increases in antibody levels (133,139). To determine whether alterations in seroreactivity could be detected following specific microbial exposure, we next examined sera isolated from rabbits following inoculation with various microbes on the array. Importantly, high titer seroreactivity toward the inoculated microbe could be detected following exposure, whereas sera analyzed following exposure to unrelated antigens failed to display similar reactivity (Fig. 5-1D-F and data not shown). These results demonstrate that serological alterations during immunological responses can be detected using the MGM and that seroreactivity following inoculation by an individual microbe displays a high degree of specificity.

While adaptive immunity provides critical host protection, innate immunity represents the first line of defense against potential pathogens (140). As a result, we next examined potential interactions of innate immune factors with the MGM. Most innate immune factors recognize canonical molecular motifs within potential pathogens. Indeed, pathogen associated molecular patterns (PAMPs) typically represent common molecular structures recognized by innate immune proteins, such as toll-like receptors, a critical process for

initiating immunity (141,142). However, the antigenic determinants exposed on the array represent unique structures, strongly suggesting that innate immune factors may not recognize these antigens. Indeed, toll-like receptor 4, which recognizes the lipid portion of LPS, failed to recognize any of the antigens on the array, strongly suggesting that the antigenic determinants on the array represent unique molecular structures and that specific antigen recognition is likely confined to adaptive immunity (data not shown).

The ability of adaptive immunity to recognize a broad range of antigenic structures results from genomic combinatorial diversity required for successful antibody generation. As a result, antibody specificity is not hard-wired into an individual's genome. However, in order to protect an individual from autoimmunity, self-reactive B cells undergo deletion. Although this removal provides protection against autoimmunity, pathogens that decorate themselves with self-like antigens would in theory experience a selective advantage. Recent studies suggest that innate immune lectins, in particular galectin-4 (Gal-4) and galectin-8 (Gal-8), evolved to protect against blood group B expressing bacteria, suggesting a mechanism whereby blood group B positive individuals protect themselves against these microbes. Although expression of self-like antigens is not limited to the blood group B antigen, whether Gal-4 and Gal-8 provide protection against other microbes remained unknown.

MGM reveals potential targets for galectin-mediated immunity

As Gal-4 and Gal-8 appear to represent unique innate immune factors capable of recognizing distinct antigenic determinants on microbes, we next sought to determine whether Gal-4 and Gal-8 might recognize other microbes on the MGM (143). Gal-4 and

Gal-8 displayed specific recognition of only one structure, the antigen of *Providencia alcalifaciens O5* (*PAO5*) (Fig. 2A,B). Importantly, PAO5 represented the only microbe on the MGM with self-like antigenic structure, the common mammalian α-galactose antigen (see Supplemental Table 1), strongly suggesting that Gal-4 and Gal-8-mediated immunity may not be limited to blood group B. Equally important, these results demonstrate that Gal-4 and Gal-8 display antibody-like specificity for microbially derived self-like antigenic determinants. Although Gal-4 and Gal-8 appear to specifically interact with PAO5, it remained possible that artificial presentation on the pathogen array might erroneously inflate this interaction. To test this, we determined whether Gal-4 and Gal-8 might recognize the PAO5 bacterium. Gal-4 and Gal-8 not only recognized the PAO5 bacterium, but also failed to recognize *Providencia alcalifaciens O21* (PAO21), a related strain also printed on the pathogen array that possesses an antigen of similar composition but different configuration than PAO5. Furthermore, inclusion of TDG, an inhibitor of galectin-carbohydrate interactions, prevented recognition, strongly suggesting that Gal-4 and Gal-8-recognition required specific antigen binding (Fig. 2C-F). Importantly, Gal-4 and Gal-8 recognition resulted in significant loss of PAO5 viability, providing a mechanism whereby Gal-4 and Gal-8 may provide specific immunity against this microbe (Fig. 2G,H,J). Consistent with this, Gal-4 and Gal-8 failed to alter the viability of PAO21 (Fig. 2I,J). Similar to recognition, inclusion of TDG completely prevented Gal-4 or Gal-8 killing of PAO5 (Fig. 2G,H). Taken together, these results demonstrate that Gal-4 and Gal-8-medaited immunity is not limited to blood group B expressing pathogens and suggests that these innate immune lectins may provide more generalized protection against molecular mimicry.

While these results suggest that Gal-4 and Gal-8-mediated immunity extends beyond blood group B positive bacteria, previous results suggested that these innate immune lectins possessed a remarkable specificity toward blood group antigens. Indeed, when analyzed on an expanded array of mammalian glycans not available previously, Gal-4 and Gal-8 maintained a distinct specificity for blood group antigens (Fig. 3A). However, despite the lack of α-galactose recognition on the glycan array, Gal-4 and Gal-8 not only killed PAO5, but also displayed a killing potency similar to that previously shown for blood group positive bacteria (143). These results suggested that unique microbial presentation of the α-Gal antigen might enhance galectin recognition and killing. To determine whether Gal-4 and Gal-8 can recognize other self-like antigens, we examined these innate immune lectins at higher concentrations on the mammalian glycan array. Not only did Gal-4 and Gal-8 recognize the α-galactose antigen at higher concentrations, but they also bound additional classes of self-like antigens typically found on the surface of mammalian cells (Fig. 3B,C) (144). These results suggest that while Gal-4 and Gal-8 possess lower affinity for a broad range of self-like antigens, microbial presentation of these antigens may provide the required affinity for these innate immune lectins to recognize a broad range of microbes that express self-like antigens.

In silico approach identifies novel targets of galectin-mediated immunity

With the unique insight provided by the MGM and the mammalian glycan array, we next turned to an *in silico* approach to determine whether Gal-4 and Gal-8 may recognize a broader range of microbes baring self-like antigens. To accomplish this, we utilized a relatively new searchable database of antigenic structures from a diverse range of pathogenic

species (Bacteria Carbohydrate Structure Database, BCSD). We first sought to determine whether this approach would enable the identification of a similar self-like antigen expressing pathogen as obtained following analysis using the MGM. Using this analysis, we identified *Klebsiella pneumoniae* O1 (KPO1), which expresses a nearly identical antigenic structure as expressed by PAO5(145) (Fig. 3D). To determine whether *in silico* predictions of Gal-4 and Gal-8- antigen interactions reflect actual recognition, we examined the potential binding of Gal-4 and Gal-8 toward KPO1. Gal-4 and Gal-8 recognized KPO1 and inclusion of TDG inhibited recognition, strongly suggesting that binding reflected recognition of the unique KPO1 antigen (Fig. 3F, data not shown). Importantly, similar to their inability to recognize PAO21, Gal-4 and Gal-8 failed to recognize a related strain of KPO1, *Klebsiella pneumoniae O4* (KPO4), which possesses a antigen of similar composition as KPO1, yet fails to generate a self-like antigen (145) (Fig. 3E, data not shown). Similar to the effect of these innate immune lectins on PAO5, Gal-4 and Gal-8 induced significant loss of KPO1 cell viability, while each failed to alter the viability of KPO4 (Fig. 3G-I). Inclusion of TDG prevented Gal-4 and Gal-8-induced killing (Fig. 3G). These results demonstrate that this *in silico* approach can identify additional targets for Gal-4 and Gal-8-mediated immunity of pathogens baring self-like antigens and further demonstrates that these innate immune lectins can bind and kill self-like antigens when expressed on a broad range of microbial species.

As this *in silico* approach appeared to predict Gal-4 and Gal-8-pathogen interactions, we next sought to determine whether Gal-4 or Gal-8 might provide immunity against pathogens which bare an alternative self-like antigen. To accomplish this, we searched for pathogens that generate the common lactosamine antigen, the most common terminal antigenic

structure on the surface of mammalian cells. *In silico* analysis identified nontypeable *Haemophilus influenzae* (NTHi) 2019 as a pathogen that expresses the lactosamine antigen (146) (Fig. 3D). Consistent with the predicted recognition based on the carbohydrate binding properties of Gal-4 and Gal-8 on the mammalian glycan array and *in silico* analysis, Gal-4 and Gal-8 displayed significant recognition of NTHi 2019 (data not shown). Furthermore, incubation of Gal-4 and Gal-8 with NTHi 2019 resulted in significant loss of viability. Inclusion of TDG not only prevented Gal-4 and Gal-8 recognition of NTHi 2019, but also inhibited Gal-4 and Gal-8-induced killing (Fig. 3J). Importantly, Gal-4 and Gal-8 failed to recognize or kill strains of NTHi that fail to express the lactosamine antigen (Fig. 3K). These results importantly demonstrate that Gal-4 and Gal-8 possess the ability to recognize and kill a terminal lactosamine baring pathogen.

Gal-4 and Gal-8 specifically kill bacteria

Most innate immune lectins and effecter molecules of the adaptive immune system recognize unique determinants that appear to specifically reside on the surface of a targeted pathogen (140). Indeed, discrimination of self from non-self at the level of ligand recognition represents a fundamental paradigm within immunology concerning immune factor specificity for target pathogens (140). However, the ability of Gal-4 and Gal-8 to not only recognize, but to apparently exclusively recognize self-like antigens stands in stark contrast to other innate and adaptive immune factors and suggests that Gal-4 and Gal-8 may actually induce similar changes, such as loss of membrane integrity, in mammalian cells (Fig. 4A). However, while Gal-4 and Gal-8 induced significant loss in viability of PAO5, KPO1, and NTHi 2019, incubation of Gal-4 or Gal-8 with murine erythrocytes or chinese hamster

ovary cells, which express α-Gal or lactosamine terminal glycans respectively, failed to induce any detectable changes in membrane integrity despite their ability to recognize each of these mammalian cells in a carbohydrate dependent fashion (Fig 4B-D). Importantly, significant changes in membrane architecture accompanied Gal-4 and Gal-8-induced loss of bacterial viability, further demonstrating that Gal-4 and Gal-8 evolved an unprecedented ability to discriminate pathogens from self while recognizing very similar antigenic structures (Fig. 4E-H).

Discussion

The MGM provided unprecedented findings concerning the seroreactivity of different species toward a broad range of microbiota and provided unique insight into the specificity of innate immune lectins, Gal-4 and Gal-8. These results suggest that antibodies and innate immune factors work in a complementary fashion to provide overall host protection against microbiota. As pathogens began to evolve elaborate antigenic structures to avoid innate immunity, vertebrates evolved an equally impressive mechanism of combating antigenic diversity among pathogens. Indeed, adaptive immunity appears to possess the capacity to respond to an infinite number of antigenic structures, as evidence by the broad seroreactivity on the MGM. However, in order for adaptive immunity to retain plasticity while avoiding self-reactivity, the removal of self-reactive cells and selection of peripheral tolerance mechanisms lead to critical tolerance that allow immunological distinction of self from non-self (147). While elimination of self-reactive immune cells reduces the probability of autoimmunity, a fitness cost would be anticipated due to the reduced ability of an individual to respond to self-like antigens on pathogens. However, compensatory mechanisms at the

level of innate immunity may exist whereby individuals protect themselves against self-like antigens. The targeted innate immune activity of Gal-4 and Gal-8 stands in stark contrast to all other previously described innate or adaptive immune factors. The ability of these innate immune lectins to specifically target pathogens that bare self-like antigens provides innate immunity against molecular mimicry and complements an important limitation in adaptive immunity.

Figures

Figure 5-1

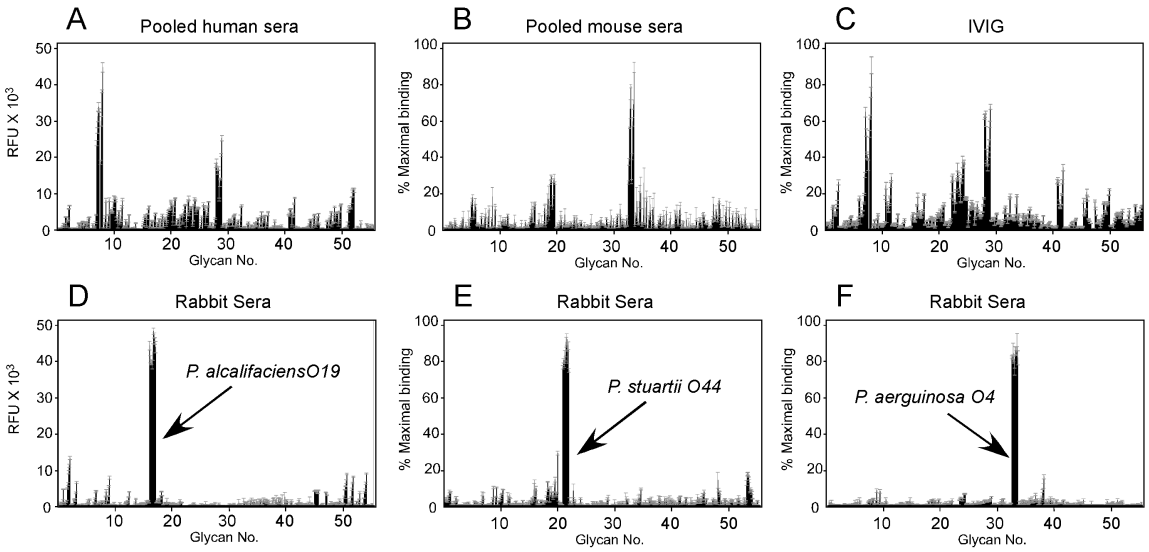


Figure 5-2

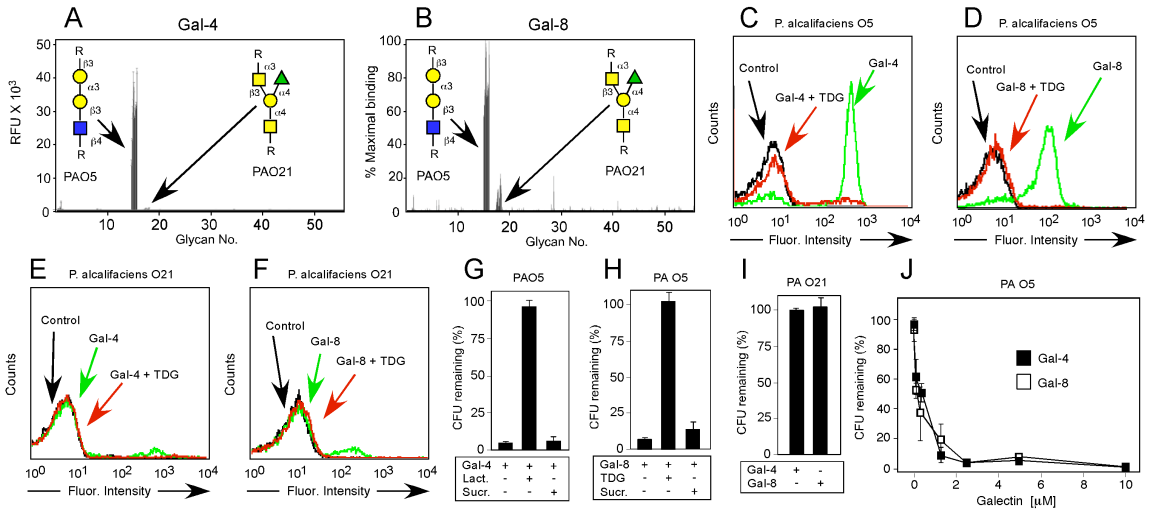
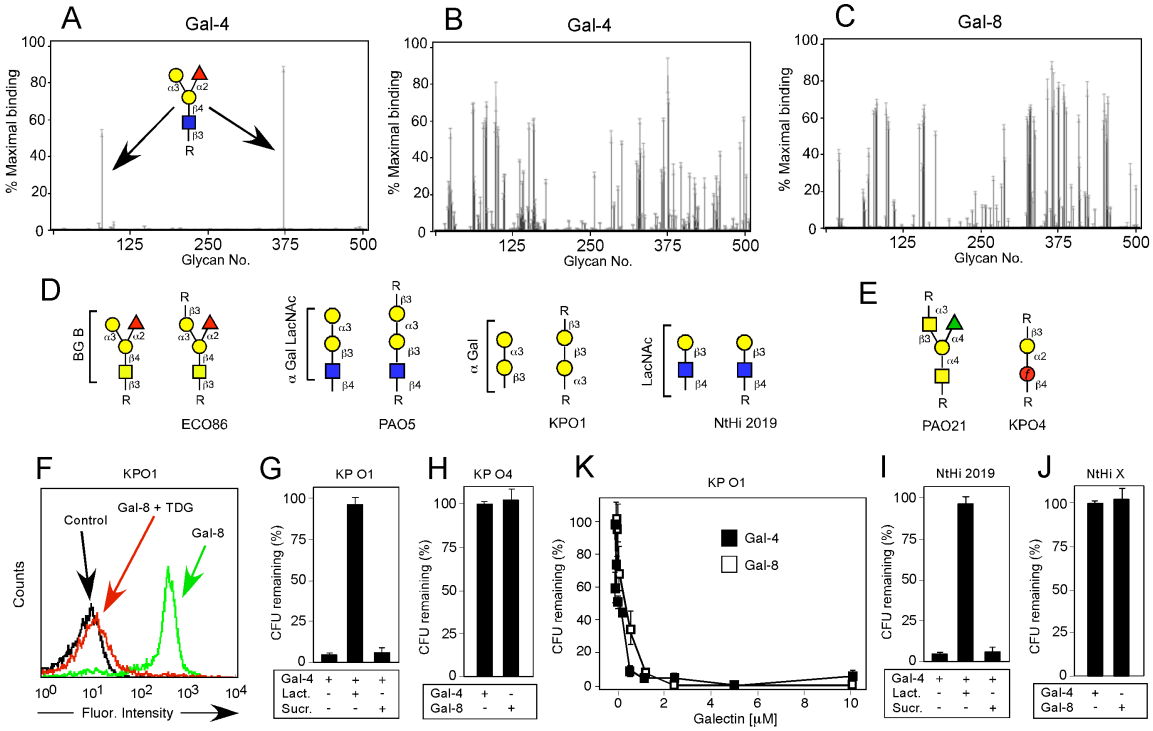


Figure 5-3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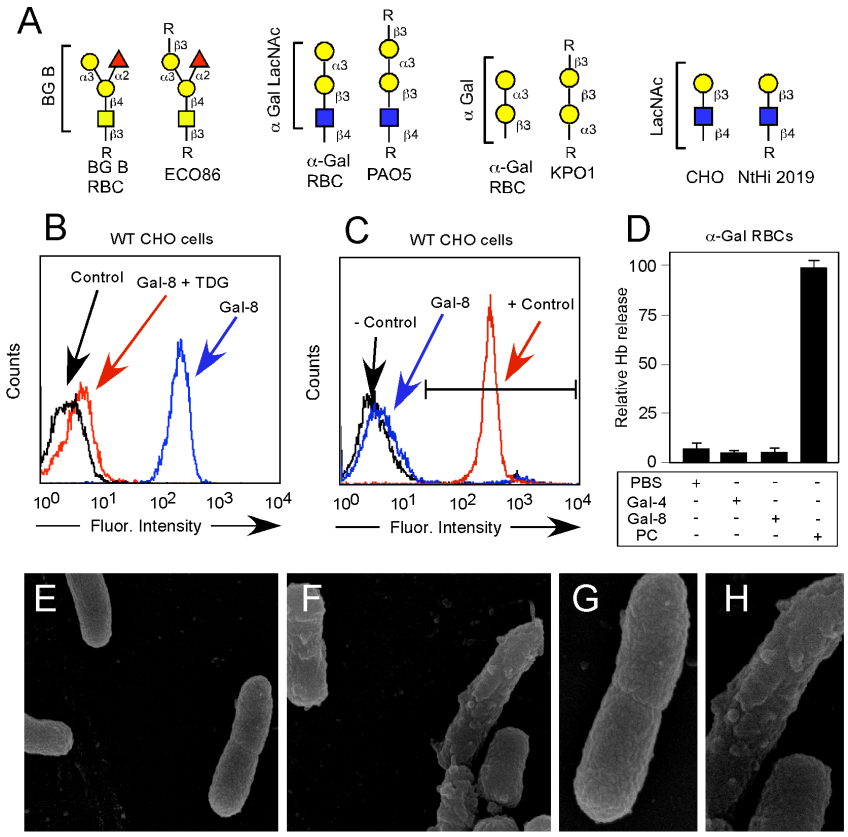
Glycan No.
B
Gal-4
C
Gal-8
D
BG B
ECO86
α Gal LacNAc
PAO5
α Gal
KPO1
LacNAc
NtHi 2019
E
PAO21
KPO4
F
KPO1
Control
Gal-8 + TDG
Gal-8
Counts
Fluor. Intensity
G
KP O1
CFU remaining (%)
Gal-4
Lact.
Sucr.
H
KP O4
Gal-8
K
KP O1
Galectin [μM]
I
NtHi 2019
J
NtHi X

Figure 5-4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BG B
BG B RBC
ECO86
α Gal LacNAc
α-Gal RBC
PAO5
α Gal
α-Gal RBC
KPO1
LacNAc
CHO
NtHi 2019
B
WT CHO cells
Control
Gal-8 + TDG
Gal-8
Counts
Fluor. Intensity
C
WT CHO cells
- Control
Gal-8
+ Control
Counts
Fluor. Intensity
D
α-Gal RBCs
Relative Hb release
PBS
Gal-4
Gal-8
PC
E
F
G
H

Figure legends

Figure 5-1- Recognition of microbial glycan structures by sera. (A-C) Pathogen array data obtained after incubation with either normal human sera (NHS) at 1:1000 (A), normal mouse sera (NMS) at 1:1000 (B), or 200μM IVIG pooled from 10,000 human donors. (D-F) Pathogen array data obtained after incubation with a 1:5000 dilution of sera taken from rabbit donors challenged with indicated bacterial species.

Figure 5-2- Pathogen array provides new pathogen targets for galectin binding and killing. (A-B) Pathogen array data obtained after incubation with 200μM galectin-4 (A) and 200μM galectin-8 (B). (C-F) Flow cytometric analysis of *P. alcalifaciens O5* counts (C,D) or *P. alcalifaciens O21* counts (E,F) after incubation with Gal-4 (C,E) or Gal-8 (D,F) at ~0.1 μM with or without inclusion of 20 mM TDG where indicated. (G-J) Quantification of *P. alcalifaciens O5* (G,H) or *P. alcalifaciens O21* (I) after addition of 5 μM Gal-4 or Gal-8 at mid-log phase with or without addition of 20mM TDG or Suc as indicated, or with the indicated concentrations of Gal-4 and Gal-8 (J). Viable bacteria were quantified by dilution plating, n = 3 experiments; one representative experiment in duplicate over two dilutions shown; error bars represent means ± s.d. of duplicates.

Figure 5-3- Additional targets of galectin killing. (A-C) Glycan microarray data obtained after incubation with 0.2 μM Gal-4 (A) 5 μM Gal-4 (B), and 5 μM Gal-8 (C). RFU, relative fluorescence units. Error bars represent means ± s.e.m. (D,E) Schematic representation of glycan structures found on the glycan array paired with similar structures found on various strains of bacteria as indicated. (F) Flow cytometric analysis of *K. pneumoniae O1* counts after

incubation with Gal-8 at ~0.1 μM with or without inclusion of 20 mM TDG where indicated. (G-K) Quantification of *K. pneumoniae O1* (G,I), *K. pneumoniae O4* (H), NTHi 2019 (J), or NTHi (K) after addition of 5 μM Gal-4 or Gal-8 as indicated at mid-log phase with or without addition of 20mM TDG or Suc as indicated, or *K. pneumoniae O1* with the indicated concentrations of Gal-4 and Gal-8. Viable bacteria were quantified by dilution plating, n = 3 experiments; one representative experiment in duplicate over two dilutions shown; error bars represent means ± s.d. of duplicates.

Figure 5-4- Galectin binding to prokaryotic, but not to eukaryotic cells results in rapid direct killing. (A) Schematic representation of glycan structures found on various eukaryotic cells as indicated. (B,C) Flow cytometric analysis of WT CHO cell counts (B) or Lec2 cell counts (C) after incubation with Gal-8 at ~0.1 μM with or without inclusion of 20 mM TDG where indicated. (D,E) Quantification of percent PI positive WT CHO cells (D) and Lec2 cells (E) after 2-hour incubation with 5μM Gal-8 or 1% Triton X (+ control) as indicated. (F) Quantification of hemoglobin release from murine erythrocytes after incubation with 5μM Gal-4, 5μM Gal-8, or 1% Triton X as indicated. (G,H) Scanning electron microscopy images of *K. pneumoniae O1* after incubation with PBS (G) or Gal-8 (H) for 25 minutes. (I,J) Increased magnification of panels F (I) and G (J) respectively.

Chapter 6- Galectin-7 possesses innate killing ability towards pathogens baring self-like antigen

The adaptive immune response continually protects against an infinite number of potential pathogens that are recognized by their non-self markers. However, since adaptive immunity maintains strict tolerance towards cells that possess "self" structures, pathogens that display these antigens on their surface may escape protective measures of adaptive immunity. Recent studies demonstrate that innate immune lectins, Gal-4 and Gal-8 are able to provide protective immunity against potential pathogens that display self-like antigens within the gastro-intestinal tract. However, as other locations of host-pathogen interaction exist, it remained unclear whether similar protective mechanisms exist elsewhere in the body. Our studies demonstrate that Gal-7, a prototypical galectin specifically expressed in keratinized epithelia protects the host from pathogens bearing self-like antigens, similar to Gal-4 and Gal-8. Gal-7 binds directly to bacteria bearing self-like carbohydrate antigen structures and binding results in disruption of membrane integrity and death of these bacteria.

Introduction

Pathogens that express self-like antigens pose a particular challenge to the immune system which is designed to recognize and eliminate foreign invaders while maintaining strict tolerance toward the antigenic determinants displayed by "self." The concept that

microorganisms may possess structures that resemble antigenic determinants of their host, thereby preventing development of an adaptive immune response to the microorganism, was first put forward in 1964 by R. Damian, who coined the term "molecular mimicry" to explain this concept(148-150). In more recent years, the term "molecular mimicry" is often used instead to refer to antigenic determinants of a microorganism that can elicit an autoimmune response(150). This is the case with several human pathogens, which induce antibodies that act against both the foreign organism and the body's own tissues, making the body react against itself. Mycobacterium tuberculosis, *Streptococcus pyogenes*, and *Haemophilus influenzae*, are all organisms associated with autoimmunity in humans (150,151). Often the onset of autoimmune disorders caused by these microorganisms occurs following acute infection that overcame innate defenses thereby triggering adaptive defenses which in turn act against one's self (97,150,151). These examples indicate the extreme importance of maintaining tolerance toward self, or self-like antigens, normally maintained through cellular removal or development of cellular anergy in immune cells with the potential for self-reaction. This reduces auto-reactivity thereby reducing or eliminating the potential for autoimmunity. However, while reduction of adaptive immunity may limit autoimmunity, it concomitantly diminishes adaptive immune response toward pathogens that may decorate themselves with self-like molecules.

Interestingly, recent results suggest that some members of the innate immune lectin family of galectins possess antibody-like specificity for blood group antigens, self-like antigens found on several bacterial species (143). These lectins, galectin-4 (Gal-4) and galectin-8 (Gal-8), display exquisite specificity for blood group antigens and appear to provide very specific immunity against blood group baring pathogens as evidenced by their ability to specifically

bind and kill bacteria expressing the blood group B antigen. Both are expressed in intestinal mucosa regardless of the blood group status of an individual, where they likely provide critical innate immune defense against blood group baring pathogens along the intestinal mucosa (143). These data provide a new model of innate immune lectin killing and indicate a likely mechanism for protection against pathogens that attempt to evade host immunity by displaying self-like antigens on their surface. However, pathogens commonly penetrate many other epithelial surfaces, strongly suggesting that innate immune factors expressed at other key pathogen-host interfaces likely possess similarly protective capacity. One such key interface is that of the keratinized epithelia, which has been shown to express high levels of the prototypical galectin-7 (Gal-7) (152,153). Not only is this innate immune lectin expressed in all keratinized epithelia, but also up-regulation of Gal-7 has been demonstrated in models of wounding or other epithelial damage (152). We therefore sought to determine whether Gal-7 might serve a similar protective role to that of Gal-4 and Gal-8.

Results

Gal-7 recognizes and kills blood group positive bacteria

The development of large libraries of test carbohydrates provided an unprecedented platform in the evaluation of carbohydrate binding proteins as demonstrated by several previous studies. These studies demonstrated that examination of lectins at high concentrations provides considerable information concerning potential ligands for carbohydrate binding proteins, yet fails to identify the high affinity ligands. We therefore examined Gal-7 recognition over a wide range of concentrations, high to low, in an effort to

elucidate all potential ligands while also identifying possible high affinity interactions. While Gal-7 did accommodate blood group antigens, it failed to display enhanced binding toward blood group antigens (Fig. 6-1a,b). Earlier studies demonstrated that modification of the common galectin binding motif, Galb1-4GlcNAc (LacNAc) results in significant and often distinct effects on individual galectin recognition of glycan ligands (152). As a result, the inability of blood group modification to alter Gal-7 recognition of the core LacNAc suggested that Gal-7-LacNAc interactions may not be significantly influenced following modification, unlike previously studied galectins, including Gal-1, Gal-2, Gal-3, Gal-4 and Gal-8, for which common modifications of LacNAc greatly enhance or decrease galectin binding (152).

These results contrast those observed for Gal-4 and -8, for which blood group modifications significantly enhanced recognition on the glycan array. This suggested that Gal-7 may not be positioned to adequately provide innate immunity against blood group expressing bacteria. However, unlike some other galectin family members , such as prototypical Gal-1, blood group modification did not eliminate Gal-7 binding which suggested that Gal-7 might retain some capacity to recognize blood group antigens when presented on a pathogen surface. To test this we incubated Gal-7 with *E. coli O86* (blood group positive) bacteria to determine whether Gal-7 could recognize BG B^+ E coli. Gal-7 not only recognized BG B^+ *E. coli*, but inclusion of thiodigalactoside (TDG), an inhibitor of galectin-ligand interactions, inhibited this recognition (Fig. 6-1c). This suggested that despite differences observed on the array, binding relied upon Gal-7-carbohydrate interactions similar to binding by Gal-4 and -8.

These results clearly demonstrate that Gal-7 can recognize carbohydrate structures on BG B^+ *E. coli*, however, previous studies demonstrated that recognition of BG B^+ *E. coli* does not necessarily result in loss of viability. For example, Gal-3 also recognized BG B^+ *E. coli*, but unlike Gal-4 and Gal-8, failed to alter BG B^+ *E. coli* viability (143). In addition, while Gal-4 and Gal-8 belong to the tandem repeat sub-family of galectins, Gal-7 exists as a homodimer and belongs to the prototypical sub-group, further suggesting that differences in quaternary structure may convey key properties to Gal-4 and Gal-8 responsible for killing not shared by other subgroups of galectin (65,153). However, incubation of Gal-7 with BG B^+ *E. coli* resulted in significant killing and co-incubation with TDG prevented Gal-7-induced killing, while co-incubation with Suc, a similar sugar that does not interact with galectin, did not (Fig. 6-1d,e). Interestingly, despite the inability of Gal-7 to display enhanced binding of blood group antigens on the glycan array, Gal-7 displayed a dose response of killing very similar to that observed for Gal-4, a galectin with considerable blood group antigen affinity when analyzed using the same glycan array format (Fig. 6-1f). Also Gal-7 produced similar gross membrane morphology changes within 30 minutes, again similar to effects observed with Gal-4 and Gal-8 (Fig. 6-1g,h).

Gal-7 killing requires blood group antigen recognition

The ability and potency with which Gal-7 killed BG B^+ *E. coli* contrasted its lack of preference for blood group antigens and suggested that Gal-7 may actually recognize an invariant carbohydrate of gram negative bacteria irrespective of blood group antigen expression, as previously described for other lectins with documented innate immune activity (140,154,155). To test this, we determined whether Gal-7 can recognize blood group

negative *E. coli*, or other gram negative or gram-positive bacteria. Similar to previous results found with Gal-4 and Gal-8, Gal-7 failed to either recognize blood group negative bacteria, or to alter the viability of bacteria that failed to express the blood group antigen. (Fig. 6-2a,b) In addition, BG B^+ *E. coli* specifically engineered to express an LPS structure lacking the BG B O antigen is no longer recognized or killed by Gal-7 (Fig. 6-2c-e). Taken together, these results demonstrate that Gal-7 possesses innate immune lectin activity specifically directed toward BG B^+ *E. coli*.

The discrepancy between Gal-7 recognition of blood group antigens on the glycan array and the potency with which it killed BG B^+ *E. coli* strongly suggested that Gal-7 interactions with BG B^+ *E. coli* O glycan may be more complicated than previously demonstrated for Gal-4 and Gal-8. O antigen carbohydrates expressed on bacterial surfaces differ significantly from the presentation and synthesis of similar structures found on mammalian cells. Perhaps most importantly, the synthesis of these antigens on the surface of bacteria often occurs as a repeating unit mediated by a polymerase that transfers in block distinct antigenic units to a growing carbohydrate chain O antigen (156). In contrast, blood group antigen on mammalian cells simply represents the terminal modification of a wide variety of glycoproteins and glycolipids without evidence of repeating unit structure.

Gal-7 recognizes extended polymerizing O antigen preferentially

Previous studies demonstrated that some galectins actually possess extended carbohydrate binding pockets, which likely allow recognition of multiple carbohydrate binding motifs concomitantly (81). The O antigen of BG B^+ *E coli* has distinct repeats of a LacNAc type

structure within the blood group antigen polymer (117). Therefore, Gal-7 may recognize more than the terminal glycan modification, as described for several previously studied galectins (67). Indeed, combined modification of polyLacNAc with the terminal H blood group antigen generated a very high affinity interaction when assayed with Gal-7 on the glycan array. In fact, examination of Gal-7 at the lowest detectable concentration for binding on the glycan array demonstrated that this presentation of the H blood group antigen, a critical component of blood group B, is the highest affinity ligand out of over nearly 300 ligands on the glycan microarray (Fig. 6-3a). These results suggest that Gal-7 prefers blood group antigen presentation in the context of a polymerizing structure, similar to the presentation on the surface of BG B^+ *E coli*.

To determine whether Gal-7 requires an extended polymerizing O antigen for high affinity interaction and killing, we examined Gal-7 recognition and potential killing of BG B^+ *E. coli* following elimination of the polymerase gene. While removal of polymerase activity prevents polymerization, it does not prevent the addition of a single blood group B antigen to the O antigen of BG B^+ *E. coli* (117). Although elimination of blood group B polymerization failed to significantly alter Gal-4 recognition, which displays high affinity for blood group antigen regardless of polymerization, it might result in a significant reduction in BG B^+ *E. coli* recognition by Gal-7. Both Gal-4 and Gal-7 still showed recognition of non-polymerized BG B^+ *E. coli*. However, while polymerase deficient BG B^+ *E. coli* remained sensitive to the killing activity of Gal-4, removal of the extended repeating structure significantly diminished BG B^+ *E. coli* sensitivity toward Gal-7-induced killing as observed by a comparative dose response of killing (Fig. 3b,c,d). These results strongly suggest that Gal-7 requires the presence of a polymerizing LacNAc presenting the blood group antigen for

killing of BG B^+ *E. coli*. Future studies will continue to assess effects that polymerization may have on Gal-7 binding affinity, and how this might contribute to efficiency of killing.

Gal-2 binds but does not kill blood group-positive bacteria

The ability of Gal-7 to kill BG B^+ E coli contrasts the inability of Gal-1 and Gal-3 to kill BG B^+ *E coli*. However, as Gal-3 belongs to the chimeric galectin subfamily, it remains possible that alterations in quaternary structure shared by prototypical and tandem repeat galectins no longer resides within the structure of Gal-3, despite its high affinity for blood group antigens. In addition, Gal-1 fails to significantly recognize blood group antigens, suggesting that it might possess the ability to kill but lack the carbohydrate recognition properties necessary to provide the first critical binding event. To test this, we examined Gal-2, a prototypical galectin previous shown to display higher affinity interactions with blood group antigens. Indeed, examination of the last detectable interaction at the lowest concentration examined demonstrated that Gal-2 exhibited high affinity interactions with blood group antigens. Importantly, Gal-2 interactions with blood group antigens on the glycan microarray correlated with Gal-2 interactions on the surface of BG B^+ *E. coli* (Fig. 3e). Consistent with this, Gal-2 recognized BG B^+ *E coli* and this recognition was blocked by TDG (Fig. 3f). Additionally, Gal-2 failed to recognize ligase deficient BG B^+ E coli, which completely prevents the formation of the blood group B antigen, also suggesting that Gal-2 interactions with BG B^+ *E. coli* completely rely upon blood group B antigen recognition (data not shown). However, similar to pervious results with Gal-3, the ability of Gal-2 to recognize BG B^+ *E. coli* failed to translate to Gal-2 killing (Fig. 3g), suggesting that the ability of Gal-7 to kill may represent a unique property among prototypical galectins.

Microbial Glycan Microarray (MGM) reveals novel targets for Gal-7-mediated immunity

As Gal-7 recognized other simple glycan structures on the array, such as the α-gal motif, which serves in place of a blood group structure in lower mammals, we asked the question of whether presentation of these structures in the context of the prokaryote surface would allow for recognition of other self-like structures. To test this we utilized the microbial glycan microarray that had proved useful for the identification of putative bacterial targets of Gal-4 and Gal-8. This array includes O antigen structures obtained from 48 species of bacteria to allow for assay of these structures in their natural presentation. Gal-7 displayed recognition of three distinct bacterial species, including *Providencia alcalifaciens O5*, which terminates in the self-like α-gal structure previously known to be recognized by other galectin family members (Fig. 4a). To verify these results we obtained *P. alcalifaciens O5* (PAO5) and tested binding and killing of this strain directly. Gal-7 recognized and killed PAO5 while failing to recognize or kill *Providencia alcalifaciens O21* (PAO21), a similar bacterial strain not recognized in the array format. Recognition and killing were both inhibited by co-incubation with TDG, indicating that both were a result of carbohydrate recognition (Fig. 4b,c,f). Based on this data we utilized an in silico approach to identify other bacterial strain with similar carbohydrate motifs. Using this method we identified *Klebsiella pneumonia O1* (KPO1), and found that Gal-7 could also recognize and kill this strain, but not a similar strain *Klebsiella pneumonia O4* (KPO4), which does not present the α-gal structure (Fig.

4d,e,f). Taken together, these results demonstrate a much wider range of possible targets for Gal-7 killing.

Discussion

While adaptive immunity provides immunity against a nearly infinite range of potential antigens, a limitation in this diversity occurs as a result of mechanisms designed to eliminate cells with high likelihood of causing autoimmunity. While elimination of these cells reduces the probability of autoimmunity, it also attenuates the ability of an individual to completely respond to pathogens that bare similar antigens on their surface. It appears that innate immune lectins, such as galectins, provide very specific and unique protection that fills this potential defect in adaptive immune responses. Taken together these results demonstrate that Gal-7, like Gal-4 and Gal-8, directly binds and kills bacteria expressing self-like antigens, including Blood group B antigen and the α-gal epitope. Gal-7 is expressed in all keratinized epithelia, including skin, surface of the eye, oral cavity, esophagus, and the anorectal surface (153,157). All of these are prime sites for host pathogen interactions to occur. While Gal-4 and Gal-8 are poised to provide protection against pathogens that come into contact with the epithelial cells of the GI track, there are multiple surfaces at the host-pathogen interface that these proteins are not present to protect against. Therefore, Gal-7's very distinct expression pattern allows for protection at major points of host-pathogen interaction where Gal-4 and -8 are not present. Likewise, Gal-4 and gal-8 are expressed in locations that allow them to protect against pathogens along the GI tract, where gal-7 protein is not found. Therefore, the multiple members of the galectin family can compensate for the lack of expression of other galectins in certain places.

The evolution of innate immune factors capable of recognizing and eliminating potential pathogens likely represents a very early event in host-pathogen interactions. Prior to selection of adaptive immune responses, organisms relied heavily on cellular and soluble factors capable of discriminating between host and pathogen and effectively destroying pathogenic organisms. Most of these factors discriminated between host and pathogen through the recognition of distinct structural motifs very unique to pathogens. Many of these molecular motifs, commonly referred to as pathogen associated molecular patterns, or PAMPs, are shared between different species of common pathogenic organisms, such as gram-negative bacteria. In this way, utilization of these factors enabled organisms to target a broad array of potentially pathogenic organisms.

Figures

Figure 6-1

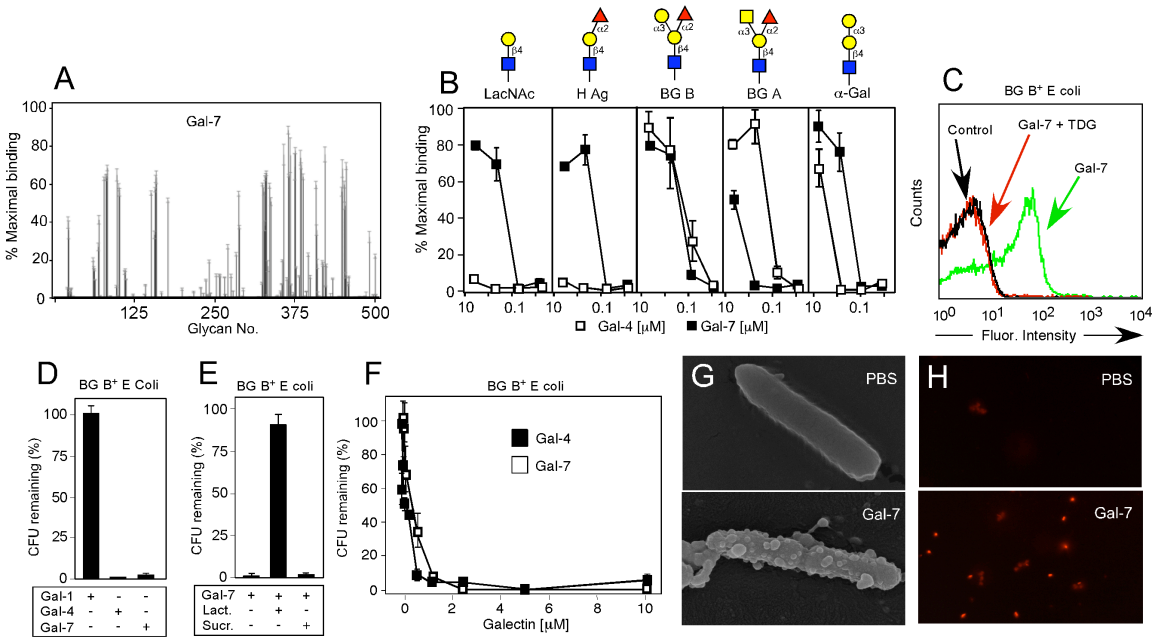

Figure 6-2

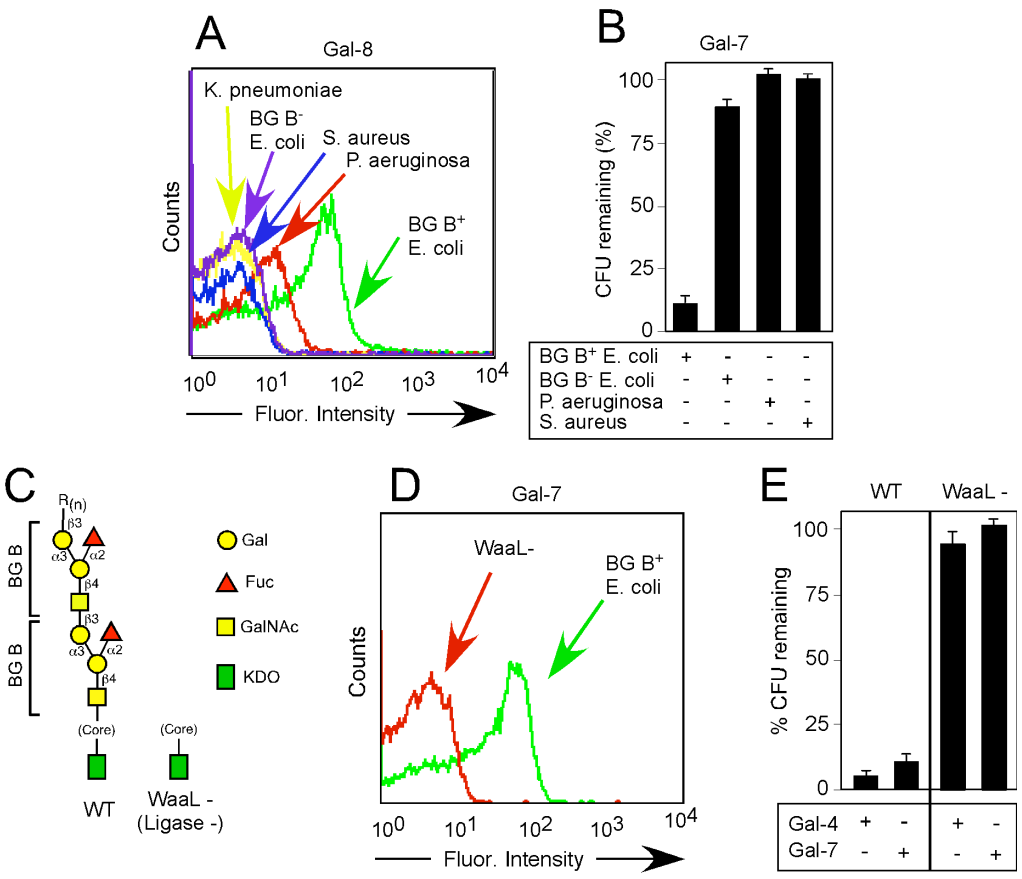
A
Gal-8
K. pneumoniae
BG B-
E. coli
S. aureus
P. aeruginosa
BG B+
E. coli
Counts
10^0 10^1 10^2 10^3 10^4
Fluor. Intensity
B
Gal-7
CFU remaining (%)
100
75
50
25
0
BG B+ E. coli + - - -
BG B- E. coli - + - -
P. aeruginosa - - + -
S. aureus - - - +
C
R(n)
β3
α3
α2
β4
BG B
β3
α3
α2
β4
BG B
(Core)
(Core)
WT
WaaL -
(Ligase -)
Gal
Fuc
GalNAc
KDO
D
Gal-7
WaaL-
BG B+
E. coli
Counts
10^0 10^1 10^2 10^3 10^4
Fluor. Intensity
E
WT
WaaL -
% CFU remaining
100
75
50
25
0
Gal-4 + - + -
Gal-7 - + - +

Figure 6-3

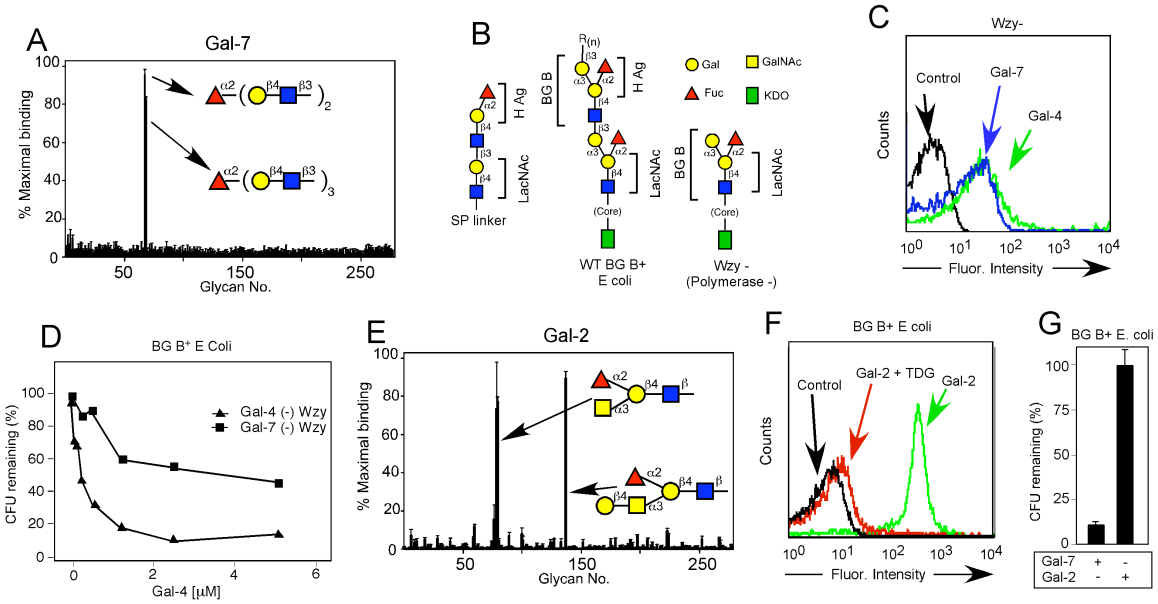
A
Gal-7
% Maximal binding
Glycan No.
B
H Ag
LacNAc
SP linker
BG B
WT BG B+
E coli
Wzy -
(Polymerase -)
Gal
GalNAc
Fuc
KDO
C
Wzy-
Control
Gal-7
Gal-4
Counts
Fluor. Intensity
D
BG B+ E Coli
Gal-4 (-) Wzy
Gal-7 (-) Wzy
CFU remaining (%)
Gal-4 [μM]
E
Gal-2
% Maximal binding
Glycan No.
F
BG B+ E coli
Control
Gal-2 + TDG
Gal-2
Counts
Fluor. Intensity
G
BG B+ E. coli
CFU remaining (%)
Gal-7 + -
Gal-2 - +

Figure 6-4

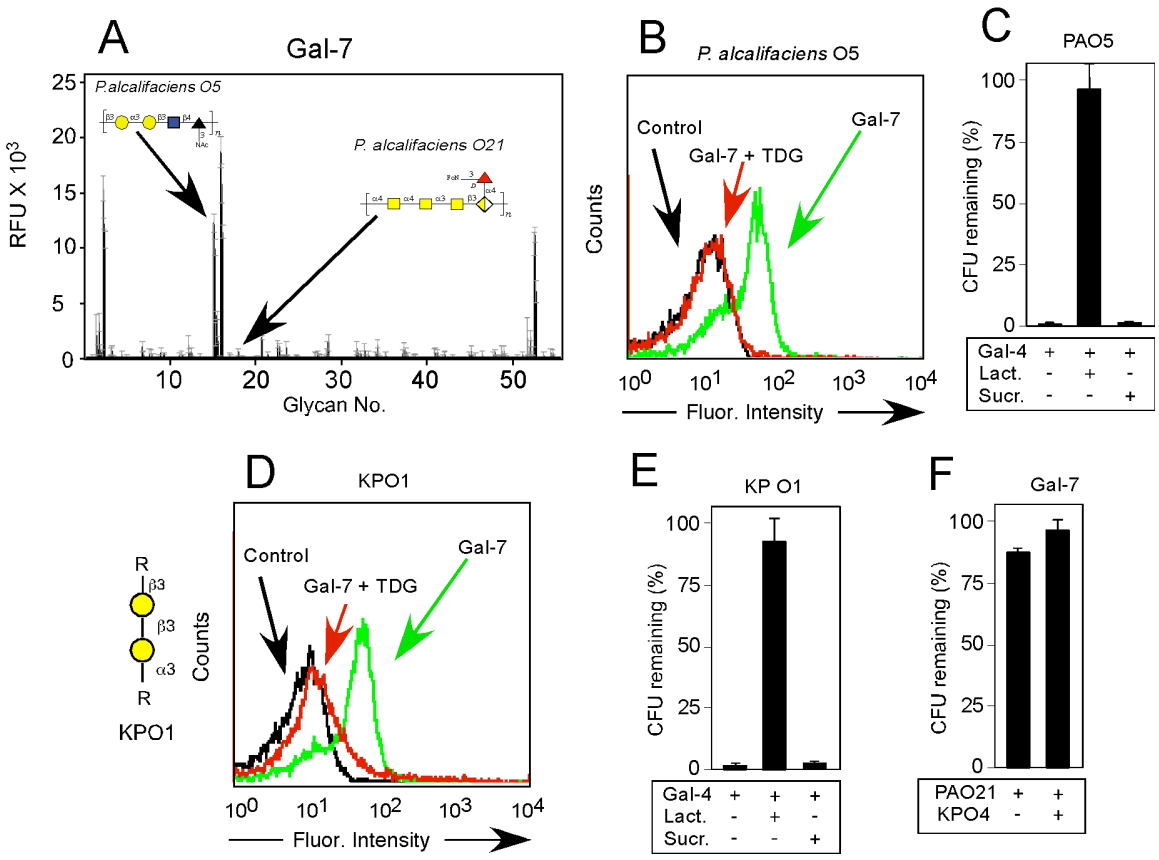
A
Gal-7
P.alcalifaciens O5
P. alcalifaciens O21
RFU X 10^3
Glycan No.
B
P. alcalifaciens O5
Control
Gal-7 + TDG
Gal-7
Counts
Fluor. Intensity
C
PAO5
CFU remaining (%)
Gal-4 + + +
Lact. - + -
Sucr. - - +
D
KPO1
Control
Gal-7 + TDG
Gal-7
Counts
Fluor. Intensity
KPO1
E
KP O1
CFU remaining (%)
Gal-4 + + +
Lact. - + -
Sucr. - - +
F
Gal-7
CFU remaining (%)
PAO21 + +
KPO4 - +

Figure Legends

Figure 6-1- Gal-7 recognizes and kills BG B+ E. coli. (A) Glycan microarray data obtained after incubation with 4.5 μM Gal-7. RFU, relative fluorescence units. Error bars represent means ± s.e.m. (B) Recognition of each representative glycan is displayed as the percent bound when compared with the highest bound ligand at each concentration tested by Gal-7 as indicated. (C) Flow cytometric analysis of BGB+ E. coli counts after incubation of BGB+ E. coli with Gal-7 at ~0.1 μM with or without inclusion of 20 mM TDG where indicated. (D-F) Quantification of viable bacteria after BGB+ E. coli were mixed with 5 μM Gal-1, Gal-4 or Gal-7 (D), 5 μM Gal-7 with or without 20 mM TDG or 20 mM sucrose (Suc) (E), or the indicated concentrations of Gal-4 and Gal-7 (F). (G) Scanning electron microscopy images of BGB+ *E. coli* followed by addition of PBS (NT) or Gal-7. (H) Images of PI staining following addition of PBS of Gal-7 to BG B+ E. coli.

Figure 6-2- Gal-7 killing requires BG B expression. (A) Flow cytometric analysis of galectin binding after incubation of BGB+ *E. coli*, BGB− *E. coli*, *K. pneumoniae*, *P. aeruginosa* or *S. aureus* reference strains obtained from a clinical laboratory with ~0.1 μM Gal-7. (B) Quantification of BGB^+ *E. coli*, BGB^- *E. coli*, P. aeruginosa or S. aureus following 2 hours incubation with 5 μM Gal-1, Gal-3, Gal-4 or Gal-8, as indicated. Viable bacteria were quantified by dilution plating; n = 3 experiments; one representative experiment in duplicate over two dilutions is shown; error bars represent means ± s.d. (C) Schematic of O antigen

structures on WT BGB$^+$ *E. coli* and the ΔwaaL mutant of BGB$^+$ *E. coli* lacking the complete O antigen. (D) Flow cytometric analysis of galectin binding after incubation of WT BGB$^+$ *E. coli* and the ΔwaaL mutant of BGB$^+$ *E. coli* with ~0.1 μM Gal-7. (E) Quantification of WT BGB$^+$ *E. coli* and the ΔwaaL mutant of BGB$^+$ *E. coli* following 2 hours incubation with 5 μM Gal-4 or Gal-7, as indicated. Viable bacteria were quantified by dilution plating; n = 3 experiments; one representative experiment in duplicate over two dilutions is shown; error bars represent means ± s.d.

Figure 6-3- O antigen polymerization enhances killing by Gal-7. (A-B) Glycan microarray data obtained after incubation with 0.01 μM Gal-7 (A) or 0.5 μM Gal-2 (B). RFU, relative fluorescence units. Error bars represent means ± s.e.m. (C) Flow cytometric analysis of galectin binding after incubation of BGB$^+$ *E. coli* with ~0.1 μM Gal-2 with or without inclusion of 20mM TDG where indicated. (D) Quantification of BGB$^+$ *E. coli* following 2 hours incubation with 5 μM Gal-7 or Gal-2 as indicated. (E) Schematic of extended Blood group H structure as displayed on the consortium glycan array and of O antigen structures on WT BGB$^+$ *E. coli* and the ΔWzy mutant of BGB$^+$ *E. coli* with only a single BGB structure repeat. (F) Flow cytometric analysis of galectin binding after incubation of the ΔWzy mutant of BGB$^+$ *E. coli* with ~0.1 μM Gal-4 or Gal-7 as indicated. (H) Quantification of the ΔWzy mutant of BGB$^+$ *E. coli* following 2 hours incubation with indicated the concentration of Gal-4 or Gal-7. Viable bacteria were quantified by dilution plating; n = 3 experiments; one representative experiment in duplicate over two dilutions is shown; error bars represent means ± s.d. (H) Glycan microarray data obtained after incubation with 2 μM Gal-2. RFU, relative fluorescence units. Error bars represent means ± s.e.m. (I) Flow cytometric analysis of BGB+ E. coli counts after incubation of BGB+ E. coli with Gal-2 at ~0.1 μM with or

without inclusion of 20 mM TDG where indicated. (J) Quantification of viable bacteria after BGB+ E. coli were mixed with 5 μM Gal-2. Viable bacteria were quantified by dilution plating; n = 3 experiments; one representative experiment in duplicate over two dilutions is shown; error bars represent means ± s.d.

Figure 6-4- Gal-7 targets multiple bacterial strains with self-like O antigen. (A) Microbial Glycan microarray data obtained after incubation with ~10 μM Gal-7.). RFU, relative fluorescence units. Error bars represent means ± s.e.m. (B) Flow cytometric analysis of galectin binding after incubation of *P. alcalifaciens O5* with ~0.1 μM Gal-7 with or without inclusion of 20mM TDG where indicated. (C) Quantification of viable bacteria after *P. alcalifaciens O5* were incubated with 5 μM Gal-7 for 2 hours with or without 20 mM TDG or 20 mM sucrose (Suc). (D) Flow cytometric analysis of galectin binding after incubation of *K. pneumoniae O1* with ~0.1 μM Gal-7 with or without inclusion of 20mM TDG where indicated. (E) Quantification of viable bacteria after *K. pneumoniae O1* were incubated with 5 μM Gal-7 for 2 hours with or without 20 mM TDG or 20 mM sucrose (Suc). (F) Quantification of viable bacteria after *P. alcalifaciens O5* or *K. pneumoniae O1* were incubated with 5 μM Gal-7 for 2 hours. Viable bacteria were quantified by dilution plating; n = 3 experiments; one representative experiment in duplicate over two dilutions is shown; error bars represent means ± s.d.

Chapter 7- Summary and Future Directions

The studies presented here elucidate vital functions of the tandem repeat galectins galectin-4 (Gal-4) and galectin-8 (Gal-8), as well as prototypical galectin-7 (Gal-7). We showed here that similar to previously studied galectins-1, -2, and -3, Gal-8 plays an important role in the regulation of neutrophil turnover in addition to previously described roles in regulation of other cellular immune processes (16,158,159). We also demonstrated a novel function of galectin family members in the direct binding and killing of bacterial species that express specific self-like antigens on their surface. Taken together, these findings demonstrate that unique carbohydrate specificity of galectin family members leads to distinct immune signaling and antimicrobial activity.

Specific recognition of cell surface glycans by galectin family members plays an important role in the regulation of signaling. Since two unique CRDs are linked together in tandem repeat galectins, an additional layer of complexity is added to ligand binding and cellular regulation by these proteins. For example, while Gal-8-induced signaling of PS exposure occurs through the C-terminal CRD, the N-terminal domain likely plays an important role in regulation of this signaling process as well. For instance, regulation of sialylated and sulfated glycans, which are specifically recognized by the N-terminal, but not the C-terminal domain, could serve to regulate the overall signaling of Gal-8, since competition for binding of the individual domains of Gal-8 for specific target glycans of the two domains likely occurs.

Indeed, studies have shown that neutrophils are capable of rapidly modulating sialic acid on their cell surface during recruitment to and activity in an inflammatory environment (160) and some studies have indicated a possible role for galectin family members in the recruitment of neutrophils to sites of inflammation (161-164). As sialylation of neutrophils is altered throughout inflammation, it is possible that the N- and C-terminal domains of Gal-8 possess complementary roles and that signaling by each domain is dominant at different stages of inflammation. However, it also possible that each of these domains recognize distinct glycoprotein ligands in cells, and that this differential recognition and signaling might have different roles during different stages of leukocyte trafficking and turnover. This would also be influenced by the site of expression of Gal-8 and the level of expression. For example, in the intestinal track where Gal-8 is highly expressed, it may have a greater influence on leukocyte trafficking than in skeletal muscle, where Gal-8 is less expressed (83).

Given the proposed importance of neutrophil turnover in maintaining homeostasis, its not surprising that some level of functional redundancy exists within this family of proteins. Indeed, numerous immune factors possess notable pleiotropic and redundant activities (165). Notably, expression of galectin family members varies considerably and redundancy could also reflect requirement galectin mediated inflammatory regulation in various locations (130,157,166,167). Specifically, Gal-8 is ideally located to regulate inflammatory resolution through induction of neutrophil turnover in several tissues including colon, liver, kidney, cardiac muscle, lung, and brain (32). Various galectin family members, including Gal-8, may also have different biological functions on other cell types, including T cells and antigen presenting cells (16,158,159,168). This variability of function may also play a role in the regulation of galectin expression and secretion, enhancing the need for redundancy in

mechanisms of neutrophil turnover. Additionally, it is worth noting that galectins likely signal multiple mechanisms of preaparesis induction depending on the family member. It was shown previous to this work that Gal-1 induces PS exposure in neutrophils through a calcium dependent pathway (101), however recent work in our lab has demonstrated at least one additional, *calcium-independent* pathway of signaling by other galectin family members (Stowell, Cummings, unpublished data). Interestingly, this demonstrates not only redundancy of galectin-induced PS exposure (preaparesis), but also suggests both overlapping and unique pathways through which this signaling occurs. Additionally, galectin-induced preaparesis only occurs in activated neutrophils or leukocytic cell lines, such as HL60, but not in resting neutrophils. This indicates that galectin signaling of preaparesis requires preceding pathways to be primed for galectin-induced preaparesis to occur (18). However, it is not clear whether multiple pathways exist for leukocyte priming, depending of the signaling requirements of individual galectins. Future work will address the importance of various putative pathways of priming for and signaling of PS exposure by galectins.

In addition to the role of Gal-8 in regulation of cellular turnover, this work explored the role of Gal-8, as well as various other galectin family members, in functions as innate immune regulators involving direct protection against pathogenic invaders. Interaction between host and microbe often occurs through interaction of host immune factors with carbohydrate structures found at the surface of a microbe. These carbohydrate structures on gram-negative bacteria are in the form of lipopolysaccharides (LPS). LPS is one of the most potent activators of immune activation, and the most common structure on bacteria to be specifically recognized by antibodies (113). Additionally, it is well established that dendritic cells and other innate immune cells contain pattern recognition receptors (PRRs), such as the

Toll-like receptors (TLRs) and Nod-like receptors (NLRs). These PRRs recognize pathogen-associated molecular patterns (PAMPs), which may be expressed by viral and bacterial pathogens, such as lipopolysaccharide (LPS), lipoproteins, and peptidoglycans (169). However, while these PRRs are very important in serving to shape the host innate immune response, they are not known to be important in recognition of pathogens that display self-mimicking antigens. For example, when LPS structures are sufficiently similar in structure to an antigen that is also found on the host's own cells, then one of two outcomes could occur. Such bacteria may be rendered invisible to the host immune system, or such bacteria may incite an immune response that could lead to deleterious effects via autoimmune reactions (170,171). Since neither of these possibilities is ideal for host survival, the host would seem to require an additional mechanism of protection against pathogens bearing self-like molecules. We have shown in this work that several members of the galectin family of innate immune lectins, including Gal-4, Gal-8, and Gal-7 possess the ability to specifically recognize and kill bacteria that display galectin specific self-like ligands on their surface.

All organisms predating vertebrates lack an adaptive immune response involving antibodies, and instead rely completely on the protection provided by mechanisms of innate immunity (172). Lectins are key players in innate immunity of invertebrate species, functioning as identifiers of foreign invaders that stimulate downstream activation and release secondary antimicrobial factors or assist in phagocytic engulfment of the invader (154,173). Likewise, the typical mechanism of protection by many innate immune lectins in mammalian species involves a role as PRRs, which recognize general, non-self, carbohydrate moieties on the surface of bacterial species in order to activate secondary defenses such as complement(174).

Galectins differ from the PRR class of innate immune lectins by virtue of their distinct difference in glycan recognition properties. Extensive analysis of galectin binding preferences has indicated that these lectins each possess specific binding preferences to glycan structures that would commonly be found on the cells of the organism that is expressing the galectin (26,27,70,82,168,175). Recent studies have identified one example of a PRR, the C-type lectin RegIIIγ, which has the ability to directly affect viability of targeted bacteria. This lectin targets all gram-positive bacteria by recognition of the peptidoglycan found on its surface (110,155). However, this too contrasts the highly specific recognition that results in direct killing of specific bacterial species, demonstrated by members of the galectin family. To date galectins are the only family of innate immune lectins that are known to bind specific target species through recognition of the O antigen structure, similar to recognition by bacteria specific immunoglobulin (113,176). In addition, they are only example of genomically encoded immune factors that exhibit this level of specific pathogen recognition. The killing activity of galectins we have observed is independent of complement and other exogenous factors. However, it will be interesting in future studies to further elucidate how many of the galectin family members possess this ability, and also to determine whether galectins contribute to other innate immune activities, such as complement fixation and opsonization.

It is important to note that although each member of the galectin family of proteins shares affinity for certain self-like antigens containing galactose, each also has distinct variance in carbohydrate binding preferences (26,27,70,82,168,175). For example, Gal-7 possesses its highest affinity binding towards the human H antigen blood group structure. Gal-7, which is specifically highly expressed by skin keratinocytes, would be perfectly poised to target

pathogens decorated with this antigen. Gal-7 can still recognize and kill pathogens with H-related structures, but may require oligomerization to kill efficiently. By contrast, the preferential glycan ligand for Gal-8 is the blood group B antigen present on *E. coli* O86, therefore Gal-8 is able to bind and kill these bacteria even more readily than Gal-7. Thus, this variation in binding by various galectin family members may allow for preferential recognition of certain bacterial species by different galectins. This is potentially biologically important, since together the members of the galectin family are likely able to compensate for small modifications bacteria make to their O antigen structure. Otherwise, alterations in polymer length or terminal modifications might allow bacteria to bypass galectin immunity as well. For example, *H. influenzae* is known to have the ability to alter its cell surface carbohydrate by the addition of sialic acid, but only when it is available from an outside source (177). While addition of sialic acid would deter recognition by some galectin family members it would enhance binding by others. Therefore the *H. influenzae* would still be recognized with or with out addition of sialic acid to its surface. Future studies will hopefully further elucidate which members of the galectin family that participate in direct bacterial killing, and also help to clarify how variations in carbohydrate binding preferences allows for complementary effects of galectins in protection against various pathogens.

In addition to distinct binding preferences, each galectin has a distinct pattern of expression. Differential expression, like differential binding, is likely important for compensatory protection by various galectins; this time against pathogens that take advantage of varying points of entry into a perspective host. This is illustrated by the fact that Gal-7 is specifically expressed in keratinized epithelia, whereas Gal-4 and Gal-8 are expressed in the alimentary tract (130,131,157). Both of these localities would require protection against pathogens.

Certain opportunistic pathogens are known to target host entry towards certain areas. For example, many strains of *E. coli* are known for their propensity to invade within the gastro-intestinal tract, whereas opportunistic pathogens from the genus *Providencia* can often cause health concerns for burn victims with damaged integrity of their keratinized epithelium (178,179). Also, opportunistic pathogens often can take advantage of multiple points of entry, as they are available (178,180,181). Studies have demonstrated galectin expression in every possible site of bacterial invasion (125,130,131,157), therefore, given the relative redundancy in binding and killing activities among galectin family members, the broad localization of this lectin family places it in a unique position to provide protection against pathogens with various points of entry. However, whether any preference by a galectin for a pathogen at a particular point of entry is unknown. It will be interesting in future studies to assess whether pathogens known to infect in certain common areas are recognized more readily by distinct galectins.

While this is the first example of precisely targeted innate immune lectin killing activity of this type, future work will also focus on the possibility of other lectin families with this type of activity. Also, since galectins are expressed in all metazoans (8), it will important to examine whether galectin homologs (orthologs) found in lower, non-chordate species, possessed direct killing ability, or if this was an aspect of galectin activity acquired later in evolution. If this is indeed a conserved function then it may have had significant evolutionary impact. The ability of galectins to specifically kill pathogens that express antigens that would otherwise shield them from immune recognition is distinct from other innate immune defenses and may have provided protection against pathogens displaying these antigens prior to their selection for expression on mammalian cells. Indeed, the theory

of co-evolution of pathogens and host species dictates that this arms race would have had a direct effect on evolutionary selection (182). Bacteria are able to generate a nearly infinite variation of antigen to coat their surface, but the adaptive immune system in turn is able to generate resistance to a nearly infinite variety of antigen presenting pathogens. A limitation is that the adaptive immune system displays a reduced capacity to produce an immune response to antigens that would also be found on the body's own cells. To do so would lead to a similar range of deleterious affects as occurs during the infectious process that host immunity evolved to avoid (133,171). Therefore, the types of carbohydrate modifications mammals evolved as self-antigens, to avoid pathogen infiltration, would need to reflect the pre-existing binding specificity of innate immune factors such as the galectins. This would reduce the negative impact of altering self cell surface glycosylation by filling an important gap generated following negative selection of cells involved in adaptive immunity normally directed against these antigens. As many human pathogens do indeed decorate their surfaces with diverse carbohydrate structures and many of these structures have similarities to mammalian antigens, the ability of galectins to specifically kill pathogens bearing particular antigens likely reflects an ancient function of this protein family that has been conserved throughout evolution.

While numerous examples exist of factors capable of recognizing common non-self motifs in order to eliminate bacterial invaders (183,184), whether galectins share a common mechanism with any of these remains thus far unknown. It is clear that after initial binding to its high affinity carbohydrate ligand a secondary event is required for killing to occur. It is likely that galectin encounters a secondary site of action whereby it disrupts membrane integrity of the bacteria. Some change in confirmation, proteolytic cleavage, or just an

increase of apparent affinity upon the initial binding of galectin may promote this second binding event. This was confirmed by our findings that specific binding to LPS alone is not sufficient for bacterial killing, since both Gal-2 and Gal-3 are able to bind blood group positive bacteria, but do not display killing activity, at least toward *E. coli* 086 strains. It is possible that variation of the mechanism of galectin killing occurs depending of the strain of bacteria targeted and that Gal-2 and Gal-3 display killing to other, possibly gram-positive bacteria. Future studies will continue to focus on the roles of various galectins in a wide variety of bacterial species.

It is intriguing that galectins bind both eukaryotic and prokaryotic cells, but only exert their killing effects on the latter. This indicates that galectins, like many other antimicrobial factors, likely exploit a fundamental difference in microbe versus multicellular lipid membrane structures. Bacterial outer membrane lipid layers are organized in such a way that they are negatively charged at their external face, while animal cell membranes are composed principally of lipids with little net charge (183). This allows some antimicrobial agents such as cationic peptides to bind tightly to the charged membrane and cause membrane distortion and eventually insertion of themselves into the bacterial membrane, effectively disrupting membrane activity and killing the bacteria. Interestingly galectins share some structural similarities with a subclass of cationic peptides that are folded into beta sheets stabilized by disulfide bonds (183). Alternatively galectins may disrupt LPS turnover upon binding, or induce a secondary signaling cascade within the bacteria. Also, the site of formation of LPS and exposure of LPS on the outer membrane involves a close connection between inner and outer membrane and multiple transmembrane components (185). This complex domain may be first recognized by galectin due to binding to the LPS, and this might promote a

secondary interaction with membrane components, leading to loss of membrane integrity. The uniqueness of the microbial membrane versus the mammalian membrane architecture and composition may also underlie the specific killing ability of galectins toward microbes compared to eukaryotic outer membranes; since in regard to the latter galectins have no known effects on membrane integrity. More information regarding kinetics and requirements of killing will help to determine which of these possibilities is most likely. Future studies should explore the specific domains within galectins important for killing versus binding LPS, and whether such protein domains or amino acid residues are shared between galectins with killing activities.

In addition to the intriguing findings regarding the newly discovered role of galectins in innate immunity, this work demonstrated the vast importance of exploratory studies identifying putative protein ligand interactions in a non-biased format. Through use of the recently developed glycan microarray technology, this work was able to identify the binding specificities of various galectin family members. These initial studies served as hypothesis generating platforms that enabled additional research. Future work will continue to develop platforms, particularly platforms for the investigation of lectin binding to pathogens, which will hopefully facilitate further understanding of the interaction between host and pathogen. Given the power of this format in the elucidation of protein-glycan interactions, it will be necessary to expand these arrays in the future to include all of the major LPS and outer polysaccharides of major human and animal pathogens, so that galectin interactions with a broader range of microbes could be explored. In addition, there may be other proteins, besides galectins, that might recognize microbial glycans and play important roles in specific microbial recognition and killing.

Taken together the results presented here provide evidence of the importance of galectin family members in the regulation of immune protection. Multiple facets of galectin-mediated immune protection work together to regulate a wide variety of immune effector functions, enabling this protein family to impact fundamental aspects of immunity. Given the evolutionary ancient nature of this family, these unique activities likely evolved over the course of host immunity and future studies will likely continue to uncover fundamental ways in which galectins regulate host immunity.

Materials and Methods

Expression and isolation of recombinant galectin

The expression of recombinant forms of human galectins-1, -2, -3, -4, -7 and -8 was accomplished using established procedures (38,186,187). Each recombinant galectin was purified by affinity chromatography on lactosyl-Sepharose and bound lectin was eluted with 100 mM lactose in PBS, 14 mM 2-mercaptoethanol (2-ME).

Generation of Gal-8 mutants

The human galectin-8 construct was a kind gift from Dr. Haakon Leffler. Gal-8 was prepared as outlined previously (27,62). Site directed mutagenesis was accomplished largely as outlined previously with slight primer modification (62). For generation of Gal-8NM (R69H) the following primers were used, forward primer 5'-GTGGCCTTTCATTTCAATCCTCATTTCAAAAGGGCCGGCTGCATG-3' and reverse primer 5'-CAATGCAGCCGGCCCTTTTGAAATGAGGATTGAATGAAAGGCCAC-3'. For generation of Gal-8CM (R233H) the following primers were used, forward primer 5'-GCTCTACACTTGAACCCACACCTGAATATTAAAGCATTTG-3' and reverse primer

5'CAAATGCTTTAATATTCAGGTGTGGGTTCAAGTGTAGAGC-3'. Mutated forms of Gal-8 were expressed and purified as outlined previously (38,186,187).

Derivatization of recombinant galectin

Prior to derivatization, 2-ME was removed from galectin samples by utilizing a PD10 gel filtration column (GE Healthcare, Piscataway, NJ), followed by the addition of lactose (100 mM final concentration) to help maintain the stability of each galectin and reduce the likelihood of adduct formation at or near the CRD. Gal-1, Gal-2, Gal-3, Gal-4, Gal-7, Gal-8, Gal-8 domains, or Gal-8 mutants were biotinylated by incubating 3-5 mg/mL of each galectin with 2 mM EZ-linkTM Sulfo-NHS-LC-Biotin (Sulfosuccinimidyl-6-biotinamido) hexanoate) (Pierce) for 2 h at 4ºC. Unconjugated EZ-linkTM Sulfo-NHS-LC-Biotin, Alexa Fluor 488 and free lactose were separated from derivatized galectin using a PD-10 gel filtration column. Galectins were re-chromatographed over lactosyl-Sepharose to remove any inactive material following labeling. Bound galectin was eluted with 100mM lactose, then applied to a PD-10 gel filtration column to remove lactose, and stored at 4°C in 14mM 2-ME in PBS until further use. Control lectin, Ulex E Agglutinin (UEA I) was purchased from Vector labs.

Binding of Galectin to Amino-alkyl Glycosides Immobilized on N-hydroxysuccinimidyl Activated Glass Surface

Glycan microarrays were prepared as described previously (30,74). For galectin recognition of glycans on the printed glycan microarray, biotinylated, Gal-2, Gal-3, Gal-4, murineGal-4, Gal-7, Gal-8, Gal-8 domains, or Gal-8 mutants were incubated in a solution of PBS containing 0.005% Tween 20 and 14 mM 2-ME for 1 h at 25°C. The slide was 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).

Cell Culture

Promyelocytic leukemia HL60 cells were obtained from ATCC and maintained at 37°C and 5% CO2 in complete RPMI (RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 100 mud/ml penicillin, 100 μg/ml streptomycin).

Enzymatic Deglycosylation

Prior to enzymatic deglycosylation, HL60 cells were fixed by washing three times in PBS at 4°C, followed by resuspension in 2% paraformaldehyde (PFH) 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 two times in the appropriate buffer as recommended by the

manufacturer. For treatment of cells with neuraminidase, cells were washed in PBS followed by incubation with 100 mU *Arthrobacter ureafaciens* neuraminidase for 1 h at 37°C. For treatment of cells with bovine testes b-galactosidase, cells treated with A. ureafaciens neuraminidase were washed in PBS (pH 5.0) followed by incubation with 100 mU bovine testes β-galactosidase for 12 h at 37°C. For treatment of cell with *Escherichia freundii* endo-β-galactosidase, cells were washed in 50 mM sodium acetate pH 5.8 and incubated with 200 mU *E. freundii* endo-β-galactosidase (Seikagaku Kogyo) for 24 h at 37°C. Following enzymatic deglycosylation, cells were washed in PBS. Buffer control treatments lacking enzymes were used for each individual condition.

Galectin Binding to Cells

For lectin binding, cells were washed twice in PBS at 4°C and incubated with biotinylated Gal-8, Gal-8 domains or Gal-8 mutants or the indicated plant lectins (LEA, RCA-I– 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 three times and incubated with Alexa Fluor 488 streptavidin or Alexa Fluor 633 streptavidin (Molecular Probes) at 4°C for 1 h. Cells were 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. Error bars in each graph represent standard deviation of duplicate analysis.

Chemical crosslinking of Gal-8 and Gal-8 CRDs

Gal-8, Gal-8N or Gal-8C (5 μM each) were incubated with 50-fold molar excess bis[sulfo-succinimidyl]suberate (BS3) according to the manufacturer's instructions for 30 min at RT. Unreacted BS3 was quenched with 1 M Tris-HCl. Following quenching, samples were boiled in SDS containing 14 mM 2-ME, followed by SDS-PAGE. For protein visualization, gels were incubated in a 50% methanol/10% acetic acid solution overnight, followed by incubation in 50% ethanol for 1 h. Gels were pretreated with 0.04g sodium thiosulfate in 200 mL deionized water (dH2O) for 1 min. Pretreated gels were washed with deionized water (three times), followed by impregnation with 0.4g silver nitrate in 200 ml dH2O containing 150 mL of 37% HCHO for 20 min. Following impregnation, gels were washed with dH2O (three times) and protein was detected following incubation with 12 g Na2CO3 in 200 ml dH2O, plus 100 μL of 37% HCHO and 4 mL of pretreatment solution. For co-incubation with Gal-8C and Gal-8N, Alexa-labeled Gal-8C was incubated alone or with Gal-8N followed by crosslinking as outlined above and subjected to SDS-PAGE. As a control, Alexa-labeled Gal-1 was cross-linked as outlined above. Protein was detected by silver stain as outlined above or by detection of fluorescence using a fluorochem imaging system.

Determining Gal-8 binding affinity toward HL60 cells

Cell binding experiments were performed as outlined previously (52). HL60 cells were biotinylated with NHS-LC-sulfo biotin (Pierce) according to the manufacturer's protocol. Biotinylated cells were fixed in 2% 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. Cells were incubated with the indicated concentrations of Alexa Fluor 488 Gal-8 followed by washing three times and detection of binding using a Perkin Elmer Victor2 fluorimeter. Analysis of binding isotherms and curve fittings was accomplished using Sigma Plot software.

Measuring Galectin-induced PS Exposure

For Annexin-V staining, cells were treated for 1 h with 100 mU A. ureafaciens neuraminidase or buffer control (RMPI 1640 media or HBSS). Cells were then washed in complete RPMI, followed by resuspension in complete RMPI at 106 cells/ml. Cells were treated with the indicated concentrations of Gal-8, Gal-8 domains or Gal-8 mutants at 37°C and 5% CO2 for the time indicated followed by disengagement with 50 mM lactose and staining with Annexin-V (CalTag/Invitrogen, Carlsbad, CA) as outlined previously (18). Galectin binding toward cells treated with A. ureafaciens neuraminidase was performed as outlined above.

Galectin binding to bacteria

Biotinylated galectins, domains, or mutants were incubated with bacteria on ice for approximately 20 minutes. Samples were washed and secondary detection was accomplished by incubation with Alexa-488 or Alexa-693 labeled streptavidin. Binding was quantified by flow cytometry. As controls, cells were incubated with 50mM lactose or 50mM sucrose along with the galectins.

Assay of anti-microbial effects of galectins

Each strain was grown to mid-log phase, followed by incubation with the indicated concentrations of galectin for 2 h at 37°C. The number of viable bacteria was determined by dilution plating and CFU enumeration. Alternatively, BG B+ *E. coli* was grown to mid-log phase, followed by incubation with indicated concentrations of galectin for 30 min and analyzed by electron microscopy for morphological changes or by fluorescence microscopy for uptake of propidium iodide. For mixing experiments, GFP expressing *P. aeruginosa* were incubated with or without BG B+ *E. coli* for 2 h with Gal-8 followed by determination of the percent *P. aeruginosa* by flow cytometric analysis.

Generating GFP expressing P. aeruginosa

We obtained *P. aeruginosa* strain 8830 from Dr. Ananda Chakrabaty (University of Illinois College of Medicine). To generate a GFP expressing *P. aeruginosa*, we grew bacteria in trypticase soy broth at 37 °C until the OD 600 nm was between 0.8 and 0.9. We pelleted bacteria by centrifugation at 5,000 rpm for 15 min at 4 °C and washed them first in PBS at 4 °C, then water at 4 °C and finally resuspended them in 10% glycerol. We added approximately 1 mg of plasmid pSMC21 encoding GFP, provided by Dr. O'Toole (Dartmouth Medical School), to 50 mL of the bacteria, then electroporated the bacteria using a 2-mm-gap electroporation cuvette (Bio-Rad Laboratories) at 18 kV with a Transporator^{TM} plus (BTX). We diluted the electroporated cells into 1 ml LB medium and grew them for 1 h followed by plating on LB agar supplemented with 500 mg mL^{-1} ampicillin. We identified positive colonies by fluorescence microscopy.

Preparation of samples for SEM

We incubated blood group B positive *E. coli* O86 (BG B+ *E. coli*), grown to mid-log phase, for 30 min with PBS control or 5 μM Gal-8 at 37 °C then added 20 mM lactose to halt treatment and reduce agglutination. We washed bacteria 2x with PBS to remove debris. Droplets containing either untreated or Gal-8-treated bacteria were placed onto poly-L-Lysine treated silicon chips and allowed to settle, then fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. We washed samples with distilled water; post fixed them in 1% osmium tetroxide, then washed with distilled water and dehydrated them through an ascending ethanol series to three changes of 100% ethanol. The ethanol was exchanged with Hexamethyldisilizane (HMDS) via three changes of HMDS then allowed the HMDS level to drop and the samples allowed to dry completely overnight. We mounted samples onto SEM stubs and sputter coated with either gold or chromium and viewed them in the DS130 SEM (ISI-TOPCON) using in-lens imaging. We viewed the displayed images at 20,000x magnification.

Preparation of samples for TEM

We treated and washed BG B+ *E. coli* as described for SEM preparation then centrifuged bacteria in a table-top centrifuge and fixed them overnight with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. We washed the bacterial pellets 2x in distilled water and post fixed them in 1% osmium tetroxide, then washed them in distilled water and dehydrated them through an ascending ethanol series to two changes of 100% ethanol. At the 50%

ethanol step we en bloc stained the cells with 2.5% Uranyl Acetate in 50% ethanol. We accomplished resin infiltration via two steps of ethanol and resin mixtures (1:1 and 1:2) and finally two exchanges of 100% fresh resin and then embedded cells in fresh resin and polymerized them for three days. We performed microtomy using glass knives and an MT-7000 (RMC) ultra-microtome, and cut thin sections to about 70 to 90 nm thickness and collected onto 200 mesh copper grids. We post stained thin sections with 5% Uranyl acetate and Reynold's Lead Citrate and documented results using a JEOL JEM 1210 TEM at 100KV. We viewed the displayed images at 8,000x magnification and also show a close up view of a single bacterium from the same field.

Animal studies

We obtained C57BL/6 Specific Pathogens Free (SPF) mice from the animal facilities of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil and maintained them at 20 °C in an isolator rack (Alesco, Monte Mor-SP, Brazil) with an autoclaved water and chow diet *ad libitum*, on 12 h light cycles. We conducted all experiments in accordance with the guidelines of the Animal Care Committee from this University (process # 09.1.543.53.5)

In vivo bacterial killing assay

We plated *E. coli* strains (BG B^+ *E. coli* and *WaaL*$^-$ BG B^- *E. coli*) for isolation on LB agar (Difco Laboratories) and selected colonies to inoculate 50 mL of LB broth (Difco Laboratories), to grow overnight at 37 °C. To eliminate resident facultative bacteria and

optimize intestinal colonization with *E. coli* strains, we treated 5 week old male mice for 48 h with streptomycin sulfate (5 g L^{-1}) (Sigma-Aldrich) in sterile drinking water followed by sterile water for 24 h prior to ingestion of bacterial suspension(188,189). We inoculated mice (four per group) by oral gavage with 10^6 colony-forming units (CFU) per mL of BG B^+ *E. coli* or *WaaL*$^-$ *E. coli* in 100 μL (10^5 CFU) of sterile PBS using a 22-gauge stainless steel animal feeding needle. Mice also received by oral gavage 300 μL of TDG (50 mM – 5.38 mg per animal) or 300 μL of PBS divided into three injections of 100 μL administered immediately before, 6 h after and 12 h after inoculation. Control mice, treated with streptomycin, received PBS alone. To recover bacteria, we sacrificed mice 24 h after bacteria inoculation, using CO_2 inhalation. From each mouse we aseptically excised, weighed, and homogenized the entire intestine in 6 mL sterile PBS using a homogenizer T10 (IKA®-Works, Inc.). We determined the number of viable bacteria in each intestine homogenate by plating serial dilutions (1000-fold) onto lactose MacConkey agar plates. After incubation at 37 °C for 20 h, we enumerated CFU and expressed per intestine ± SEM. We applied student's t-test for statistical analysis of data. P-values were <0.05, which was considered statistically significant. Results represent two independent experiments.

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