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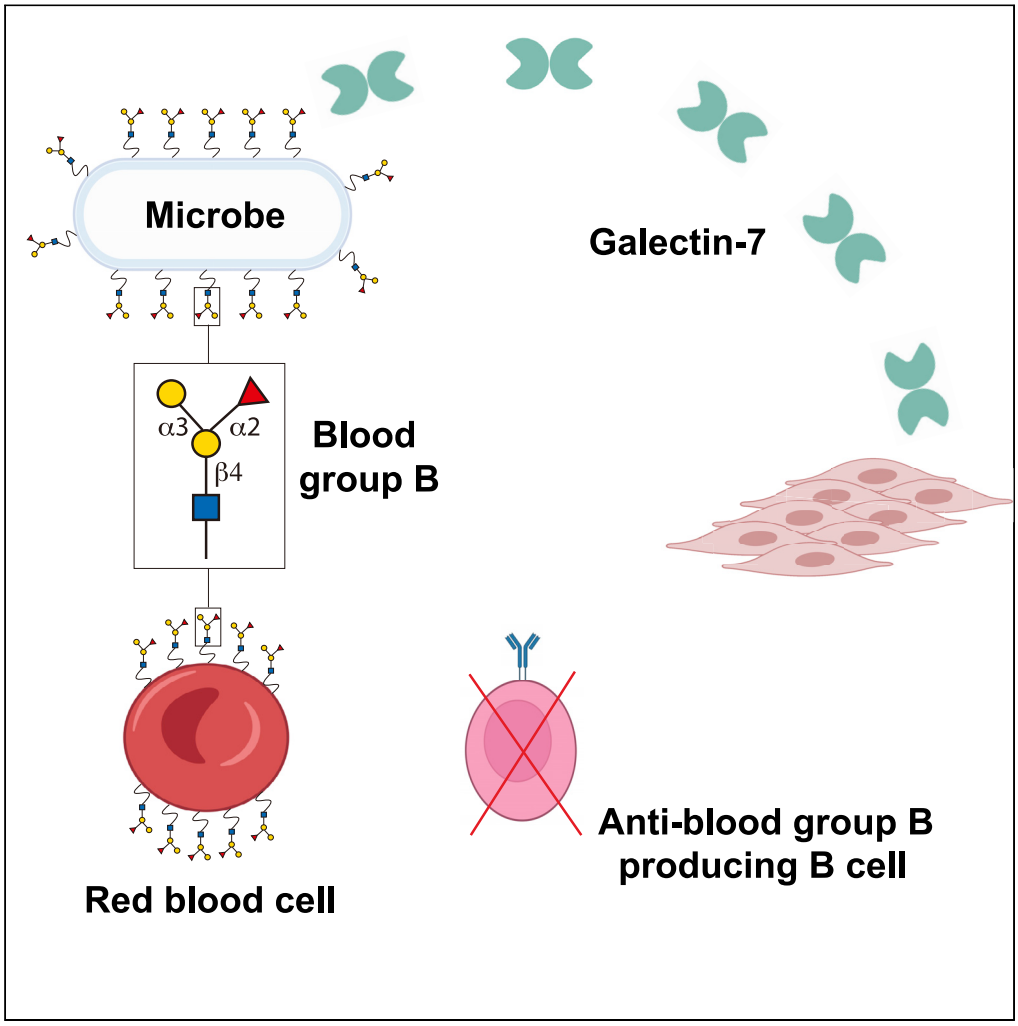

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Highlights

Galectin-7 exhibits high specificity for blood group containing antigens

Galectin-7 binds and kills microbes that express blood-group like antigens

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Innate immune Galectin-7 specifically targets microbes that decorate themselves in blood group-like antigens

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SUMMARY

Adaptive immunity can target a nearly infinite range of antigens, yet it is tempered by tolerogenic mechanisms that limit autoimmunity. Such immunological tolerance, however, creates a gap in adaptive immunity against microbes decorated with self-like antigens as a form of molecular mimicry. Our results demonstrate that the innate immune lectin galectin-7 (Gal-7) binds a variety of distinct microbes, all of which share features of blood group-like antigens. Gal-7 binding to each blood group expressing microbe, including strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Providencia alcalifaciens*, and *Streptococcus pneumoniae*, results in loss of microbial viability. Although Gal-7 also binds red blood cells (RBCs), this interaction does not alter RBC membrane integrity. These results demonstrate that Gal-7 recognizes a diverse range of microbes, each of which use molecular mimicry while failing to induce host cell injury, and thus may provide an innate form of immunity against molecular mimicry.

INTRODUCTION

ABO(H) blood group antigens were not only the first polymorphisms described in the human population, but the detection of ABO(H) antigens and corresponding anti-ABO(H) antibodies in blood donors and patients also provided the first method to accurately predict immune compatibility before transfusion and transplantation (Stowell and Stowell, 2019a, 2019b). Indeed, ABO(H) testing represents the earliest and most common example of personalized medicine. Despite the routine use of anti-A and anti-B antibody testing clinically, the consequences of ABO(H) expression on general host immunity remain incompletely defined (Cooling, 2015). Immunological tolerance against ABO(H) reduces the probability of autoimmunity while also allowing blood from blood group compatible individuals to be transfused in the absence of acute immune-mediated complications (Blank et al., 2007; Elkayam et al., 2007; Shoenfeld et al., 2008). However, the lack of anti-ABO(H) antibody formation in ABO(H) blood group positive individuals also creates a gap in adaptive immunity toward microbes that may decorate themselves in ABO(H)-like antigens (Arthur et al., 2015c). Tolerance toward ABO(H) antigens in blood group positive individuals, coupled with the presence of microbes that decorate themselves in blood group-related antigens, raises fundamental questions regarding host protection against microbes that utilize blood group molecular mimicry (Blank et al., 2007; Damian, 1964, 1989).

Unlike many other polymorphisms, ABO(H) antigens are posttranslational carbohydrate modifications generated by the inheritance of distinct glycosyltransferases responsible for their synthesis (Cummings, 2009; Stowell and Stowell, 2019a; Yamamoto et al., 1990). This type of modification operates in parallel to microbial mimics, as microbes can also synthesize carbohydrates to decorate their surfaces in the absence of significant changes to general cellular function (Arthur et al., 2015c). Consistent with this, within a single microbial species, distinct strains have been historically defined by the unique carbohydrate structures that decorate their surface (Cooling, 2015; Rasko et al., 2000; Shao et al., 2002). The most well defined microbial mimic is likely *Escherichia coli* O 86, which was previously shown to possess blood group B activity (Springer et al., 1961). *E. coli* O 86 O-polysaccharide biosynthesis pathway is initiated by glycosyltransferases located in the inner membrane, including WbnH, WbnJ, WbnK, and WbnI. These glycosyltransferases begin glycan

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assembly in the cytoplasm followed by translocation to the periplasmic site. The enzyme Wzy catalyzes the polymerization of O antigen repeats, whereas polysaccharide chain length is controlled by Wzz (Guo et al., 2005; Woodward et al., 2010; Yi et al., 2005, 2006a, 2006b). Thus, blood group antigen synthesis on microbes, while capable of producing similar overall structures, is distinct from the synthesis of blood group antigens on mammalian cells (Clausen and Hakomori, 1989; Clausen et al., 1986; Yamamoto et al., 1990).

Although some microbial strains possess unique biological traits within a given species, such as the ability to produce toxins, the distinct carbohydrate structures that cover a given microbial strain often have no obvious impact on the intrinsic biology of the microbe (Arthur et al., 2015c; Blank et al., 2007; Cooling, 2015; Elkayam et al., 2007). Although such structural diversification may have initially enhanced the ability of microbes to avoid innate immunity, the evolution of adaptive immunity may have in part been selected to allow hosts to more readily engage a diverse microbial glycan repertoire. However, the development of immunological tolerance within adaptive immunity limits its ability to directly combat microbes that decorate themselves with glycans that mimic self, including blood group-like structures. Given the inherent limitations of adaptive immunity in this regard, innate immune factors may exist that provide host immune protection against molecular mimicry (Arthur et al., 2015c; Runza et al., 2008).

As blood group antigens and microbial surface structures in general are carbohydrates, factors that may possess the ability to protect against blood group-based molecular mimicry would be predicted to have carbohydrate binding activity. However, defining the binding specificity of carbohydrate binding proteins was historically challenging because of a lack of suitable tools and sufficient glycan libraries. Using microarrays populated with hundreds of mammalian glycans, including blood group antigens, our recent studies demonstrated that several members of the galectin family, including galectin-3 (Gal-3), galectin-4 (Gal-4), and galectin-8 (Gal-8), possess the ability to bind blood group A and B with high affinity (Stowell et al., 2010, 2014; Wu et al., 2021b), strongly suggesting that these galectins may be uniquely poised to provide innate immunity against blood group molecular mimicry. Consistent with this, Gal-3, Gal-4, and Gal-8 not only engage blood group antigens on the glycan microarray but also specifically recognize and kill microbes that likewise express blood group-like determinants (Stowell et al., 2010, 2014; Wu et al., 2021b).

Although prior studies demonstrated that Gal-3, Gal-4, and Gal-8 are capable of binding and killing microbes that utilize blood group-like molecular mimicry, the extent to which other members of the galectin family likewise share the ability to provide innate immunity against molecular mimicry remains incompletely defined. Gal-3, Gal-4, Gal-8, and other galectin family members are expressed along the intestinal mucosa (Vasta, 2009; Wooters et al., 2005), where interactions with microbial communities would be predicted to favor target microbes that express blood group-like structures. However, whether other galectins, which are primarily expressed along other sites of host-microbial interfaces (Sato et al., 2009), likewise possess the ability to bind and kill microbes remains unknown (Verkerke et al., 2022). The binding specificity and possible antimicrobial of Gal-7 in particular, which is uniquely expressed along stratified epithelia (Magnaldo et al., 1995, 1998), remains incompletely defined. Here, we demonstrate that Gal-7 also possesses the ability to bind and kill microbes that utilize blood group molecular mimicry. However, unlike Gal-3, Gal-4, and Gal-8, Gal-7 exhibits very little affinity for mammalian A and B antigens. Despite this, Gal-7 exhibited high specificity toward multiple microbes that express glycans with blood group-like features. These results demonstrate that antimicrobial activity among galectin family members is not limited to Gal-3, Gal-4, and Gal-8, but that Gal-7 likewise possesses the ability to bind and kill microbes expressing blood group-like antigens.

RESULTS

Galectin-7 (Gal-7) specifically interacts with blood group containing structures

To define the specificity of Gal-7 toward blood group antigens, we first examined Gal-7 binding toward mammalian glycans, including ABO(H) blood group antigens. To accomplish this, we employed the consortium for functional glycomics (CFG) glycan microarray. Prior studies, including our own, primarily analyzed glycan binding specificity using the CFG platform by employing a single concentration of the carbohydrate binding protein on the CFG microarray, followed by rank order analysis of preferred glycans. Using this approach, we initially analyzed Gal-7 binding at 0.04 μM in an effort to identify the highest affinity ligands present in this array format. At 0.04 μM, Gal-7 engaged only three glycan structures out of nearly 600, a type 2 (Galβ1-4GlcNAc) blood group A structure, and two polylactosamine (polyLacNAc, i blood group) containing structures capped with the H antigen (iH) (Table S1; Figure 1A). The supplemental table

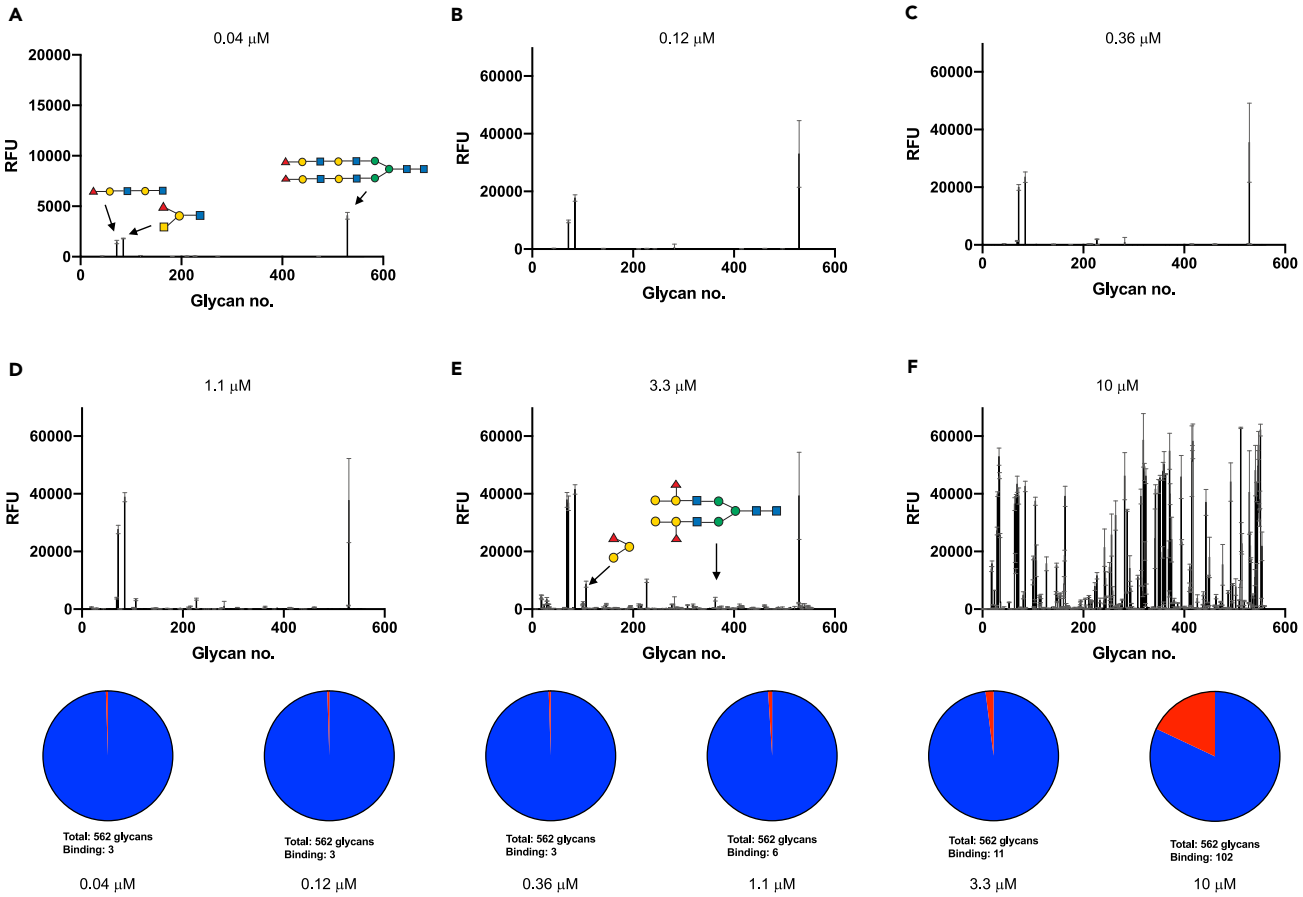

Figure 1. Galectin-7 (Gal-7) displays specific interactions with blood group containing structures over a range of concentrations

(A–F), Consortium for functional glycomics (CFG) glycan microarray data obtained after incubation at the following Gal-7 concentrations: 0.04 (A), 0.12 (B), 0.36 (C), 1.1 (D), 3.3 (E), and 10 μM Gal-7 (F). RFU, relative fluorescence units. Error bars represent mean ± SD. Circle chart shown in red is the number of glycans bound by Gal-7 at the indicated concentration, whereas the remaining glycans are shown in blue.

was designed to provide an overview of the K_D values obtained. In addition to displaying the K_D values, we have also shown binding events where saturation does not occur over the concentration range tested, but where binding did occur to differentiate these glycans from those where no glycan binding was observed. Binding toward these later glycans is shown as a percent maximum binding, similar to the rank analysis typically used for glycan array result evaluation (Heimburg-Molinaro et al., 2011) to provide some information regarding the relative binding preferences shown toward these weaker Gal-7 ligands (Table S1). Although these results suggested that type 2 blood group A and iH represent high affinity interactions for Gal-7, prior glycan microarray analysis of other galectin family members suggested a broader binding profile toward blood group antigens. As a result, we next examined Gal-7 binding profiles at higher concentrations. Evaluation of Gal-7 at 0.12, 0.36 and 1.1 μM resulted in higher binding levels, while retaining binding only to the same three glycans (Figures 1B–1D). To determine if this same specificity was retained at even higher Gal-7 concentrations, we examined Gal-7 at 3.3 and 10 μM (Figures 1E and 1F). Gal-7 at 3.3 μM exhibited binding toward additional glycans, including several glycans containing the blood group B antigen. At 10 μM, Gal-7 is bound to a common non-blood group containing glycans recognized by other galectin family members, including biantennary N-glycans and those with poly-N-acetyllactosamine (polyLacNAc) structures. The selective binding specificity of Gal-7 over a broad range of concentrations distinguishes it from prior reports on other galectins and suggests that unlike Gal-3, Gal-4, and Gal-8 (Carlsson et al., 2007; Stowell et al., 2008a, 2010, 2014), Gal-7 may not readily engage blood group positive microbes.

Although the rank order approach of glycan array analysis can provide a rapid overview of preferred glycan partners for carbohydrate binding proteins, examination of Gal-7 over a range of concentrations can also

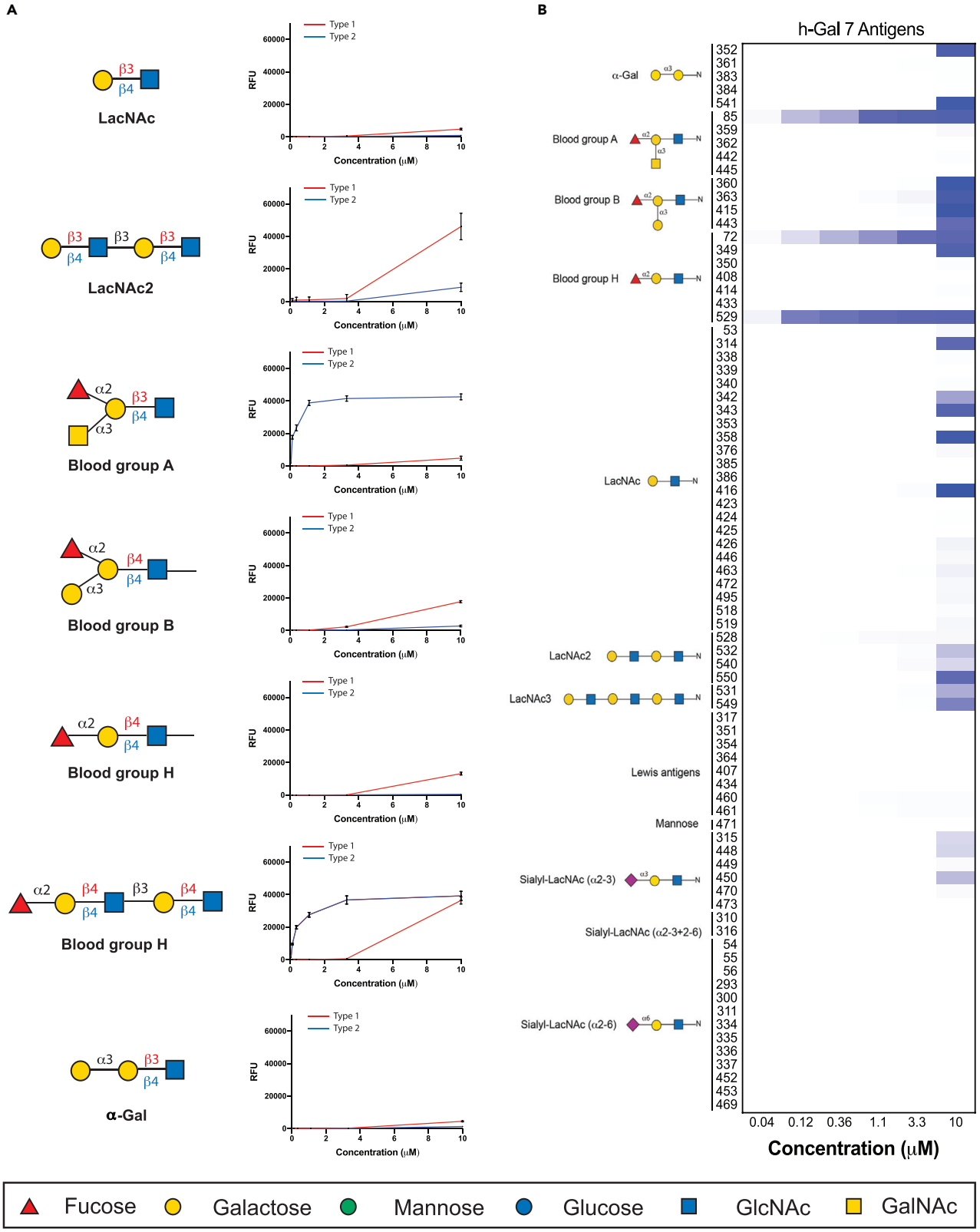
A
LacNAc
LacNAc2
Blood group A
Blood group B
Blood group H
Blood group H
α-Gal
Type 1
Type 2
RFU
Concentration (μM)
B
h-Gal 7 Antigens
α-Gal
Blood group A
Blood group B
Blood group H
LacNAc
LacNAc2
LacNAc3
Lewis antigens
Mannose
Sialyl-LacNAc (α2-3)
Sialyl-LacNAc (α2-3+2-6)
Sialyl-LacNAc (α2-6)
0.04 0.12 0.36 1.1 3.3 10
Concentration (μM)
Fucose
Galactose
Mannose
Glucose
GlcNAc
GalNAc

Figure 2. Galectin-7 (Gal-7) binding to selected core lactosamine and blood group A, B, and O(H) containing glycans

(A) Binding isotherms generated following incubation of Gal-7 on the CFG glycan microarray are shown. Type 1 glycans are shown in red and type 2 glycans are shown in blue. Error bars represent mean ± SD Detailed symbol nomenclature for glycan structures are shown at the bottom. *N*-Acetylgalactosamine (GalNAc) and *N*-Acetylglucosamine (GlcNAc).

(B) Heatmaps for the RFU values of Gal-7 over a broad range of concentrations starting from 0.04 μM to 10 μM.

establish binding isotherms that can provide relative affinities toward distinct glycan determinants, including blood group antigens. To explore the binding specificity of Gal-7 in more detail, we examined the individual binding isotherms toward distinct ABO(H) antigens present on the glycan microarray. Gal-7 displayed very little detectable binding toward lactose, Galβ1-4GlcNAc (LacNAc), polyLacNAc or biantennary N-glycans (Figure 2A). The lack of binding toward these glycans stands in stark contrast to previous results examining the binding specificity of Gal-1, Gal-2, Gal-3, Gal-4, and Gal-8, all of which exhibited high binding toward either polyLacNAc-glycans or biantennary N-glycans (Carlsson et al., 2007; Leppanen et al., 2005; Stowell et al., 2004, 2008a, 2010). Although Gal-7 did not display high affinity toward common mammalian glycans recognized by other galectins, as mentioned earlier, it did exhibit high binding toward glycans capped with the H antigen (Figure 2A). However, the enhanced binding observed toward H antigen bearing glycans was only observed on polyLacNAc structures; similar binding was not observed toward the H antigen alone (Fucα1-2Gal) or H antigen attached to various LacNAc configurations (Fucα1-2Galβ1-3/4GlcNAc). Other than the type 2 blood group A structure, there was little binding toward other blood group A configurations regardless of the concentration, with the exception of some binding noted toward polyLacNAc-capped with blood group A (). Similarly, no significant binding toward blood group B was observed at lower concentrations, whereas a variety of blood group B containing glycans were engaged by Gal-7 at the highest concentrations tested (Figure 2B). Gal-7 also displayed very little detectable binding toward type 1 α-Gal capped structures. However, Gal-7 did bind to type 2 α-Gal on the N-glycan with better binding affinity in comparison to type 1 α-Gal on the N-glycan (Table S1). These results indicate that at higher concentrations Gal-7 can recognize various blood group structures, but the binding toward the vast majority of such glycans reflects lower affinity interactions.

Galectin-7 (Gal-7) recognizes and kills blood group (BG) B+ *E. coli*

Given the differences in glycan recognition observed between Gal-7 and previous results examining the glycan binding specificity of other galectin family members, these results suggest that unlike other galectins, Gal-7 may not possess the ability to effectively engage blood group B positive microbes. To formally test this, we examined whether Gal-7 could bind to blood group B positive microbes. In contrast to CFG microarray findings, Gal-7 readily engaged blood group B positive *E. coli* (BG B^+ *E. coli*). This binding was inhibited by thiodigalactoside (TDG), a non-metabolizable inhibitor of galectin-glycan interactions, demonstrating that this interaction depended on carbohydrate recognition (Figure 3A).

Given the ability of other galectins not only to bind BG B^+ *E. coli*, but also induce microbial death, we next sought to determine whether Gal-7 likewise possesses the ability to impact BG B^+ *E. coli* viability. Incubation of BG B^+ *E. coli* with Gal-7 over a range of concentrations resulted in significant microbial death, with changes in microbial viability detected in the low μM range (Figure 3B). As Gal-7 failed to recognize blood group B on CFG array over the same concentration range with which microbial killing activity toward BG B^+ *E. coli* was observed, we next examined whether killing, like binding, relied on carbohydrate recognition. Similar to binding, inclusion of lactose prevented killing of BG B^+ *E. coli*, strongly suggesting that Gal-7-mediated killing activity is carbohydrate dependent (Figure 3C). To further define the consequence of Gal-7 interactions with BG B^+ *E. coli*, we next examined the impact of Gal-7 on the BG B^+ *E. coli* using scanning electron microscopy (SEM). Similar to prior results with Gal-4 and Gal-8 (Stowell et al., 2010), Gal-7 induced significant morphological changes to the membrane of BG B^+ *E. coli*. These results strongly suggest that Gal-7 may alter membrane integrity. Consist with this, incubation of BG B^+ *E. coli* with Gal-7 resulted in propidium iodide positivity, an indication of compromised membrane integrity (Figure 3D).

The ability of Gal-7 to readily bind and kill BG B^+ *E. coli*, despite little binding toward blood group B antigens on the CFG glycan microarray, strongly suggested that despite recognizing BG B^+ *E. coli* through a carbohydrate-dependent process, Gal-7 interactions likely reflect engagement of other blood group B-independent glycan determinants. To test this, we examined Gal-7 binding toward a mutant of BG B^+ *E. coli* lacking O antigen lipopolysaccharide (LPS), and thus lacking the BG B antigen (Δ*WaaL*) (Figure 4A) (Sperandeo et al., 2009; Yi et al., 2006a). To confirm loss of the blood group B antigen on the Δ*WaaL* mutant, we evaluated the intact microbe for cell surface glycan composition using on a lectin microarray,

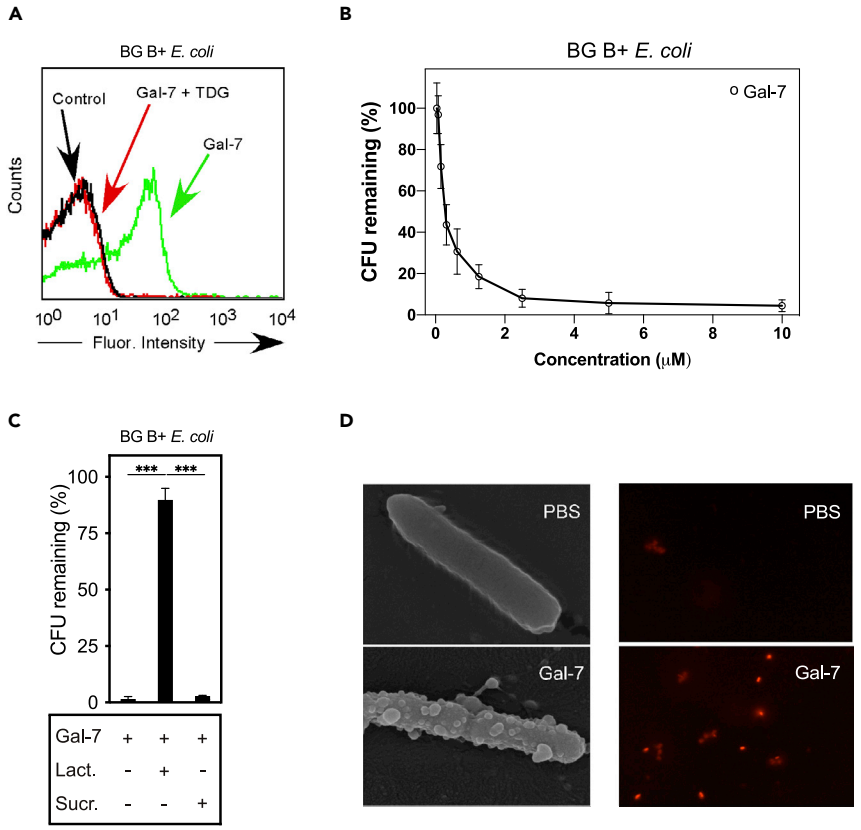

Figure 3. Galectin-7 (Gal-7) recognizes and kills blood group (BG) B + *E. coli*

(A) Flow cytometric analysis of Gal-7 binding toward BG B + *E. coli* with or without inclusion of 20 mM TDG as indicated.

(B) Colony forming units (CFUs) remaining after incubation with Gal-7 at the indicated concentration.

(C) CFUs after incubation of BG B+ *E. coli* with 5 μM Gal-7 with or without 20 mM lactose (Lact) or 20 mM sucrose (Sucr). Data are represented as mean values ±SD ***, $p < 0.001$ and statistical analysis was performed using one-way ANOVA with Tukey's test.

(D) Scanning electron microscopy and PI staining images of BG B + *E. coli* following incubation with 5 μM Gal-7 or PBS.

as done previously to evaluate other glycan determinants (Hsu and Mahal, 2006; Kuno et al., 2005). Interactions between BG B^+ *E. coli* and the lectin microarray could be readily detected using this approach, with significant binding by the 25-EEL lectin, which binds the blood group B antigen and 44-GSL, which prefers αGal. In contrast, no binding was observed toward the Δ*WaaL* mutant (Figure 4B). To test whether Gal-7 can recognize the Δ*WaaL* mutant, we examined Gal-7 binding by flow cytometry. Unlike the binding observed toward BG B^+ *E. coli*, no significant binding was observed toward BG B^- *E. coli* (Figure 4C). To determine whether the lack of recognition may impact the ability of Gal-7 to kill the Δ*WaaL* mutant, Gal-7 was incubated with BG B^+ *E. coli* or the Δ*WaaL* mutant. Although Gal-7 readily impacted the viability of BG B^+ *E. coli*, it failed to kill Δ*WaaL* (Figure 4D). Similarly, incubation of Gal-7 with a completely distinct strain of *E. coli* that does not express the blood group B antigen or other microbes altogether that likewise do not express blood group-like determinants, such as strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, failed to result in any change in microbial viability (Figure 4E). Taken together, these results suggest that, in contrast to the inability of Gal-7 to engage blood group B on the CFG glycan microarray, it can readily bind and kill BG B^+ *E. coli*.

O antigen polymerization enhances galectin-7 (Gal-7) binding and killing

Given the differences in Gal-7 binding toward the blood group B antigen on the CFG glycan microarray and its ability to bind and kill BG B^+ *E. coli*, we next sought to define Gal-7 interactions with BG B^+ *E. coli* in more detail. Blood group B on RBCs exists primarily as a single terminal modification on glycoproteins and glycolipids. In contrast, although terminating blood group structures are present on microbes, these antigens are expressed as polymerized units with many repeats of the same blood group-like motif. The high

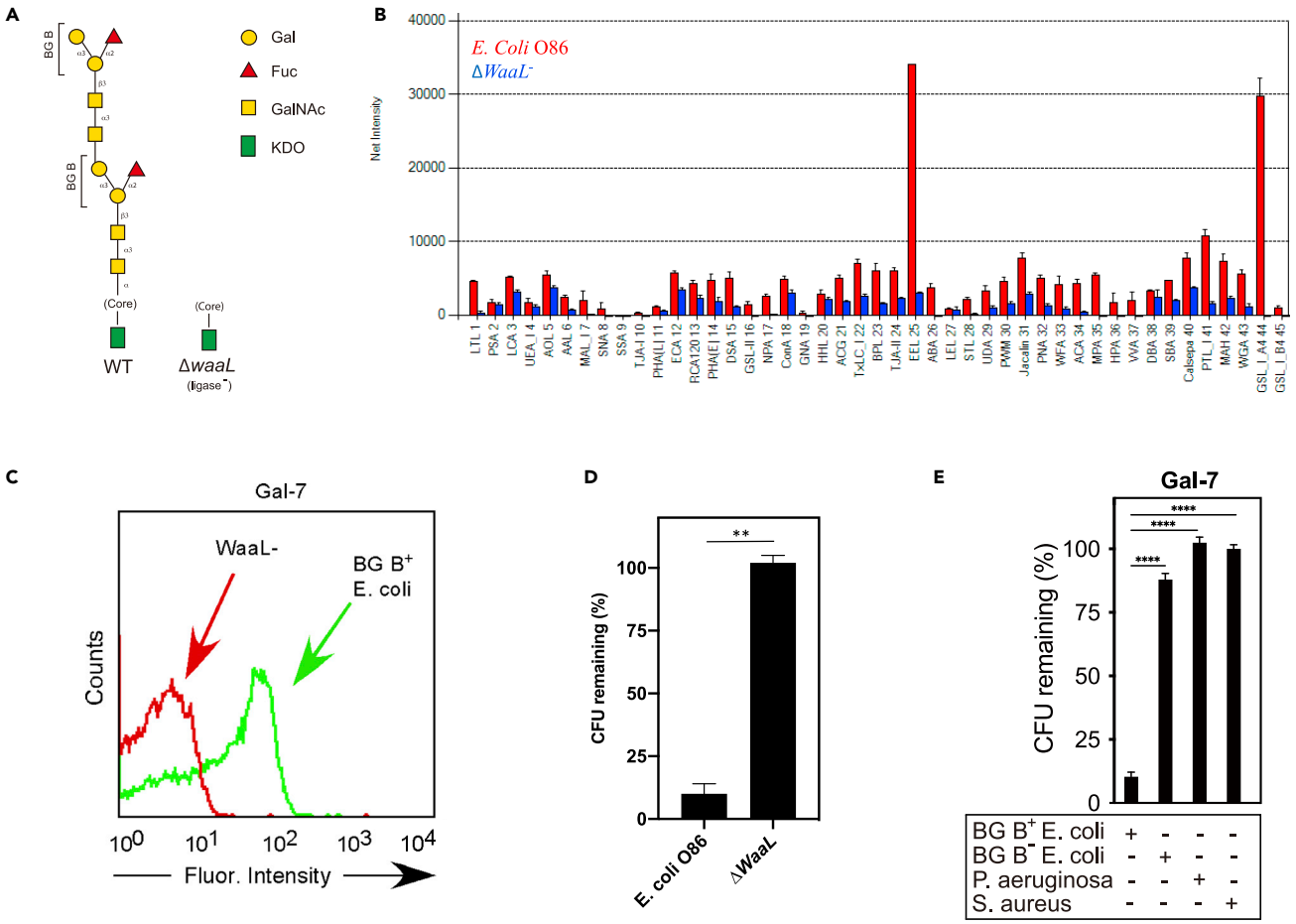

Figure 4. Galectin-7 (Gal-7) requires recognition of blood group B to bind and kill of blood group (BG) B + *E. coli*

(A) Schematic of O antigen structures on wild type BG B+ *E. coli* and the Δ*WaaL* mutant of BG B+ *E. coli*.

(B) Lectin microarray analysis of whole bacterial surface glycans. BG B+ *E. coli* shown in red and Δ*WaaL* mutant in blue. Data are the averages ±SD of triplicate determinations.

(C) Flow cytometric analysis of Gal-7 binding after incubation with WT BG B + *E. coli* or the Δ*WaaL* mutant.

(D) Colony forming units (CFUs) remaining after incubation of Gal-7 with WT BG B+ *E. coli* and the Δ*WaaL* mutant. Data are represented as mean values ±SD **, $p < 0.01$.

(E) CFUs remaining after incubation of Gal-7 with BG B + *E. coli*, BG B$^-$ *E. coli*, and *P. aeruginosa* or *S. aureus*. Data are represented as mean values ±SD ****, $p < 0.0001$.

preference for the iH antigen of the CFG array suggests that Gal-7 may prefer blood group antigens in the context of longer glycan polymers. To test this, we examined the binding of Gal-7 toward the mutant of BG B$^+$ *E coli* lacking polymerase activity (the Δ*Wzy* mutant), which allows one blood group modification to cap the core LPS structure but prevents further polymerization (Woodward et al., 2010) of the core B antigen generated by additional glycosyltransferases (Guo et al., 2005; Yi et al., 2005) (Figure 5A). Unlike incubation with the Δ*WaaL* mutant, incubation of Gal-7 with the Δ*Wzy* mutant did result in binding. However, the binding observed toward the Δ*Wzy* mutant was reduced when compared to BG B$^+$ *E coli* (Figure 5B). To determine whether these differences in binding reflect actual difference in glycan recognition, we isolated the LPS from BG B$^+$ *E coli* and each mutant (Δ*Wzy* and Δ*WaaL*) and printed these glycans in a microarray format (Figure 5C). Using this approach, we observed appreciable binding toward the isolated LPS of BG B$^+$ *E coli*, whereas significantly less binding toward the LPS of the Δ*Wzy* mutant was observed and virtually no binding to the LPS of Δ*WaaL* was apparent (Figure 5D). These results suggest that differences observed by flow cytometric examination likely reflect different binding preferences to the actual glycan determinant. To determine whether differences in binding impact the ability of Gal-7 to kill the Δ*Wzy* mutant, we compared the killing activity of Gal-7 toward BG B$^+$ *E coli* or the Δ*Wzy* mutant. Unlike the killing activity observed

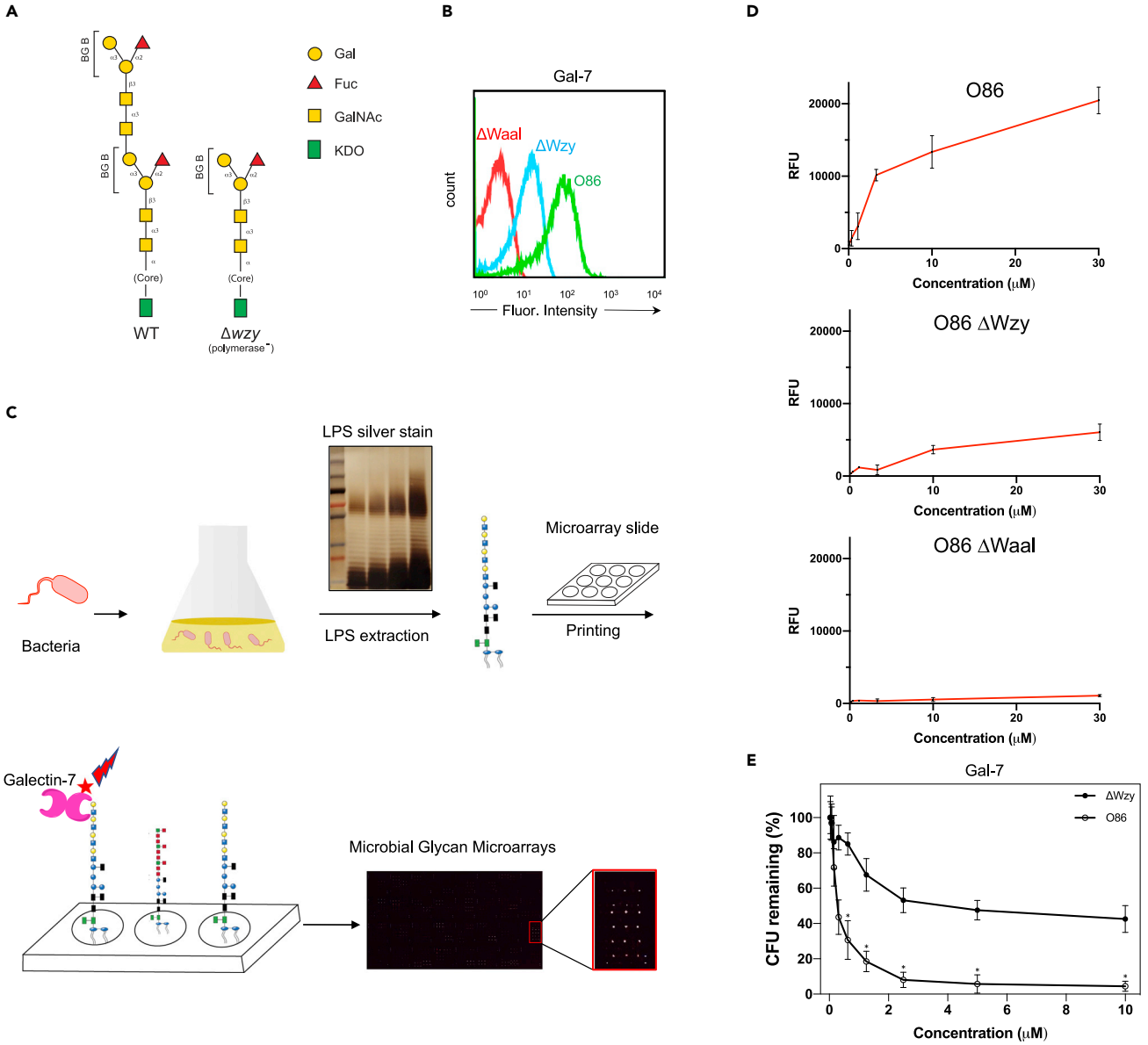

Figure 5. O antigen polymerization enhances galectin-7 (Gal-7) binding and killing

(A) Schematic of extended blood group structure and O antigen structures on WT BG B + *E. coli* and the Δ*Wzy* mutant of BG B + *E. coli* with only a single BG B structure.

(B) Flow cytometric analysis of Gal-7 binding after incubation of Δ*WaaL*, Δ*Wzy* and WT BG B + *E. coli* as indicated.

(C) Schematic of a microbial glycan microarray design and utility.

(D) Microarray data obtained after incubation with the indicated concentrations of Gal-7.

(E) Colony forming units (CFUs) remaining after incubation of Gal-7 with BG B + *E. coli* and Δ*Wzy* mutant. Data are represented as mean values ±SD *, $p < 0.05$.

toward BG B$^+$ *E coli*, Gal-7 exhibited less potency and overall ability to kill the Δ*Wzy* mutant, strongly suggesting that the reduced binding ability influenced antimicrobial activity (Figure 5E).

Gal-7 recognizes and kills multiple microbes that express blood group-like antigens

Given the apparent discrepancy between glycan binding activity observed on the CFG glycan microarray and actual interactions with BG B$^+$ *E coli*, we sought to determine whether Gal-7 possesses the ability to recognize other microbes decorated in blood group-like antigens. To this end, we next examined Gal-7

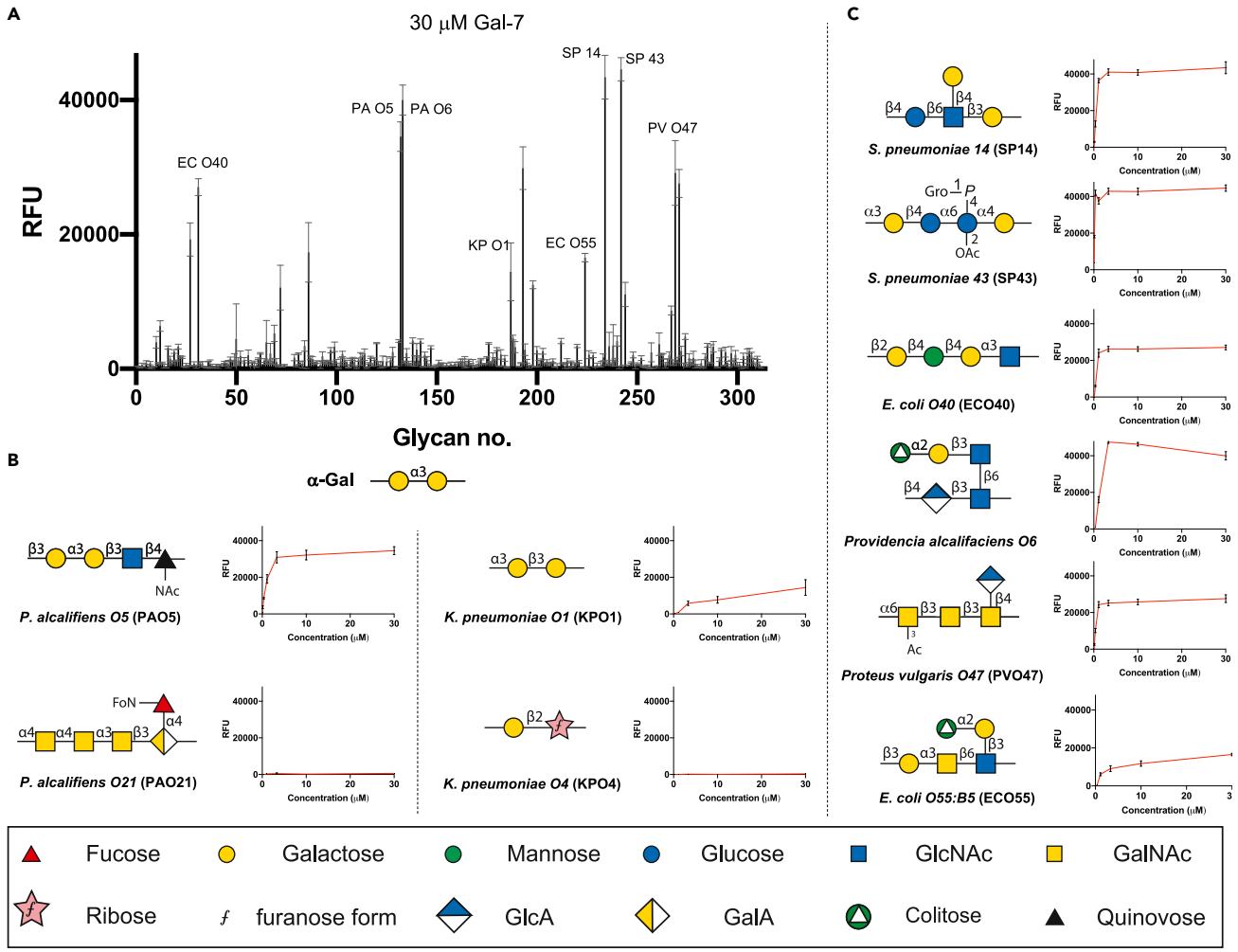

Figure 6. Galectin-7 (Gal-7) recognizes the microbial glycans of multiple bacterial strains that express self-like antigens

(A and B) Microbial glycan microarray data obtained after incubation with Gal-7. Error bars represent means ± SD (B) Binding isotherms obtained following incubation of Gal-7 with select microbial glycans represented on the microbial glycan microarray (microbial glycan structures for each respective microbe are shown). Symbols of monosaccharides are shown.

on the microbial glycan microarray (MGM) populated with several hundred unique glycan determinants isolated from distinct microbial strains across a variety of genera and species detailed in the supplementary data (Table S2). Examination of glycan binding on the MGM demonstrated that Gal-7 exhibited high binding toward several microbial glycans, including *Providencia alcalifaciens* O5 (PA O5), *Klebsiella pneumoniae* O 1 (KP O1) and *Streptococcus pneumoniae* type 14 (SP 14) (Figure 6A). Gal-7 engagement of microbial glycans appeared to be specific, as many glycan determinants were not recognized by Gal-7 on the MGM. To define in more detail the relative binding affinity of Gal-7 toward microbial glycans represented by the MGM, we next examined Gal-7 binding over a range of concentrations as done for the CFG array. Gal-7 exhibited similar affinity for a variety of structures. Although none of the structures were identical, many shared common features of blood group antigens (Figures 6B and 6C). Importantly, the observed affinity of Gal-7 for microbial glycans exceeded that observed for most of the mammalian blood group determinants present on the CFG array. These results suggest that although Gal-7 may not fully engage blood group antigens presented as mammalian glycans, Gal-7 can bind to a wide variety of blood group-like antigens that are expressed by different microbes.

To determine whether binding activity on the MGM accurately predicts actual interactions with intact microbes, we next examined possible Gal-7 engagement of *P. alcalifaciens* O5, *K. pneumonia* O 1, and *S. pneumoniae* type 14. Similar to BG B$^+$ *E coli,* Gal-7 readily bound each microbe, whereas inclusion of

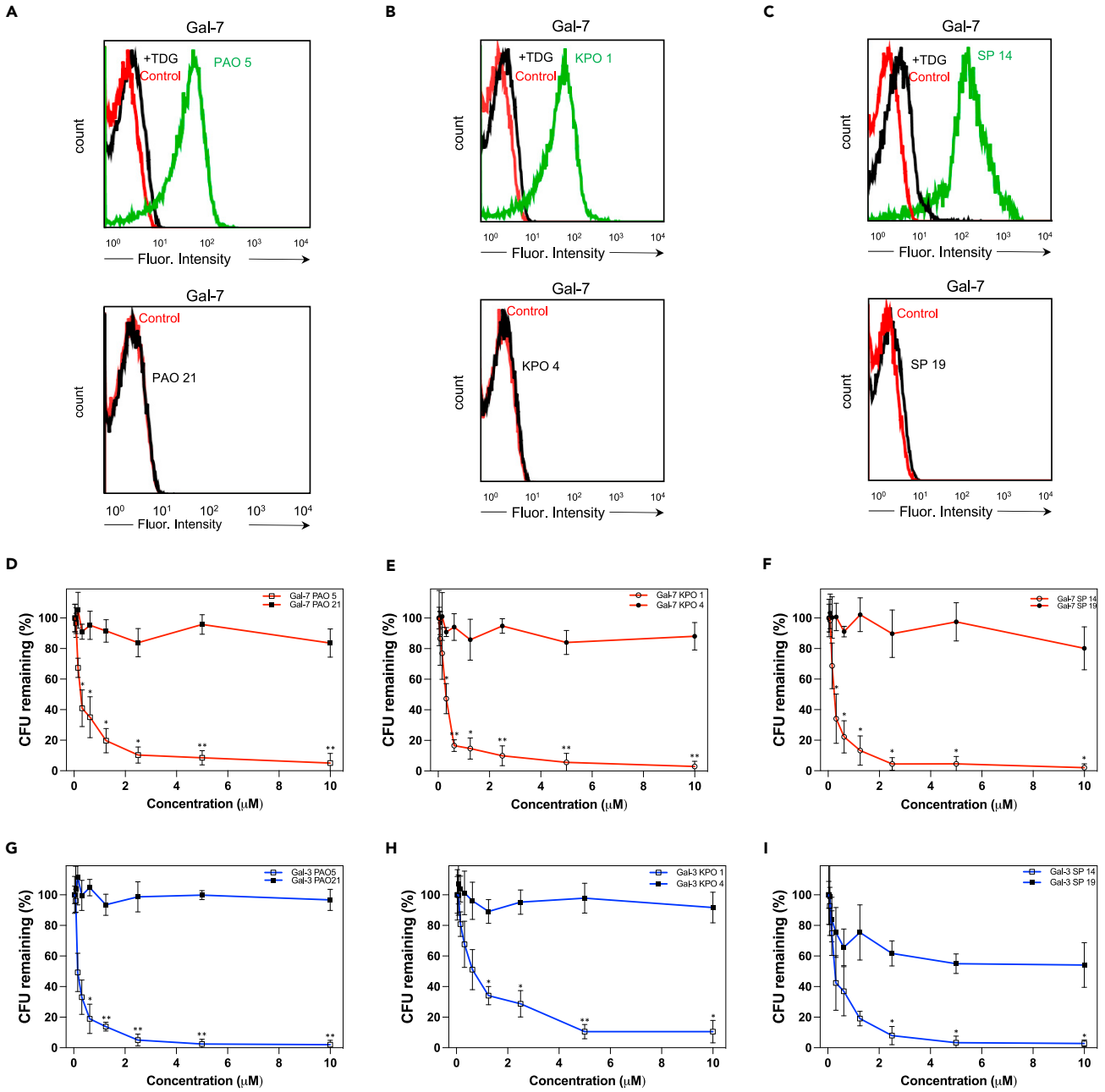

Figure 7. Galectin-7 (Gal-7) binds and kills multiple bacterial targets that express blood group-like antigens

(A–C) Flow cytometric analysis of Gal-7 binding after incubation of *P. alcalifaciens O5*, *P. alcalifaciens O21*, *K. pneumoniae O1*, *K. pneumoniae O 4*, *S. pneumoniae* 14, or *S. pneumoniae* 19 with or without inclusion of 20 mM TDG as indicated.

(D–F) Colony forming units (CFUs) remaining after incubation of *P. alcalifaciens O5*, *P. alcalifaciens O21*, *K. pneumoniae O1*, *K. pneumoniae O4*, *S pneumoniae* 14, or *S pneumoniae* 19 with Gal-7.

(G–I) Colony forming units (CFUs) remaining after incubation of the same bacteria tested with Gal-3 as a control. Data are represented as mean values ±SD *, $p < 0.05$, **, $p < 0.01$.

TDG inhibited these interactions, demonstrating that binding is carbohydrate dependent (Figures 7A–7C). To determine whether the interactions observed toward *P. alcalifaciens* O5, *K. pneumonia* O 1 and *S. pneumoniae* type 14 are specific, we examined Gal-7 binding toward related strains of each microbe that do not express blood group-like antigens. In contrast to the binding observed toward *P. alcalifaciens* O5, *K. pneumonia* O 1 and *S. pneumoniae* type 14, Gal-7 failed to exhibit appreciable

binding toward *P. alcalifaciens* O21, *K. pneumonia* O 4, and *S. pneumoniae* type 19 (Figures 7A–7C). Taken together, these results suggest that Gal-7 binding exhibits strain specificity toward microbes that express blood group-like antigens.

To test whether Gal-7 interactions with *P. alcalifaciens* O5, *K. pneumonia* O 1, and *S. pneumoniae* type 14 result in microbial death, similar to that observed following Gal-7 incubation with BG B^+ *E coli*, we next examined the impact of Gal-7 on microbial viability and used Gal-3 as a control for potency comparison. This was accomplished by incubating Gal-7 over a range of concentrations first with *P. alcalifaciens* O5. Similar to the impact of Gal-7 on BG B^+ *E coli*, incubation with *P. alcalifaciens* O5 resulted in microbial death, whereas similar incubation with *P. alcalifaciens* O21 failed to result in a significant change in microbial viability (Figure 7D). Despite being a completely distinct genus and species than *P. alcalifaciens* O5, *K. pneumonia* O 1 expresses a similar overall glycan determinant. To determine whether similar killing activity would be observed following incubation with *K. pneumonia* O 1, we next examined Gal-7 antimicrobial activity toward *K. pneumonia* O 1. Similar to incubation of Gal-7 with *P. alcalifaciens* O5, Gal-7 induced significant microbial death of *K. pneumoniae* O 1, whereas Gal-7 failed to similarly induce loss of *K. pneumoniae* O 4 viability (Figure 7E). To determine whether Gal-7 possesses the ability to kill a Gram-positive microbe that was likewise bound on the MGM, we examined Gal-7 activity toward *S. pneumoniae* type 14. Similar to its ability to bind and kill *P. alcalifaciens* O5 and *K. pneumonia* O 1, Gal-7 likewise possessed the ability to bind and kill *S. pneumoniae* type 14. The killing activity observed toward *S. pneumoniae* type 14 appeared to likewise be specific, as binding or antimicrobial activity was not observed toward *S. pneumoniae* type 19 (Figure 7F). We compared the ability of Gal-3 to kill the same microbes tested. Using this approach, Gal-3 and Gal-7 exhibited similar levels of potency toward *P. alcalifaciens* O5, *K. pneumonia* O 1 and *S. pneumoniae* type 14 (Figures 7G–7I).

Most host immune factors target features of microbes that are unique to pathogens, thereby allowing host immunity to specifically engage possible pathogens while reducing injury to self. However, the ability of Gal-7 to bind host mammalian glycans, albeit with reduced breadth compared to other galectin family members, raises important questions regarding the impact of Gal-7 binding toward mammalian cells on overall membrane integrity. To determine whether Gal-7 possesses the ability to bind host cells without inducing loss of membrane integrity, we first examined whether Gal-7 can bind red blood cells (RBCs) and used Gal-3 as a control. Gal-7 readily bound RBCs, with binding toward blood group O RBCs exceeding the binding observed for A and B RBCs (Figure 8A). In addition, Gal-3 also bound RBCs, with binding toward blood group A and B RBCs exceeding the binding observed for O RBCs (Figure 8B). We next examined the consequence of Gal-3 and Gal-7 binding to each population on membrane integrity. Despite readily engaging blood group A, B, or O RBCs, Gal-3 and Gal-7 failed to alter membrane integrity (Figures 8C and 8D). To determine whether Gal-3 and Gal-7 possess the ability to change the level of RBD ROS production, we performed the cellular ROS assay. No change in ROS production was observed following incubation with Gal-3 or Gal-7 (Figure 8E). These results demonstrate that while Gal-3 and Gal-7 possess the ability to bind microbes and RBCs, they only appear to impact microbial viability.

DISCUSSION

Although adaptive immunity provides host detection of a nearly infinite range of potential antigens, a limitation in the breadth of adaptive immunity results from tolerance mechanisms that eliminate or render unresponsive cells that target self. Although immunological tolerance reduces the probability of autoimmunity, it creates a gap in adaptive immunity toward microbes that decorate themselves in blood group-like antigens. Our prior results demonstrated that Gal-3, Gal-4, and Gal-8 possess the ability to bind and kill microbes that exhibit key features of mammalian blood-group antigens, suggesting that these galectins may fill this gap by targeting microbes that utilize molecular mimicry (Stowell et al., 2010, 2014; Wu et al., 2021b). However, the extent to which other galectins likewise possess the ability to bind and kill microbes that express blood group-like antigens remained relatively unknown. The present results demonstrate that in addition to Gal-3, Gal-4, and Gal-8, Gal-7 also possesses the ability to bind and kill distinct microbial strains that express blood group-like antigens and thus provide innate immunity against molecular mimicry.

Gal-7 is a homodimeric galectin belonging to the prototypical subgroup within the galectin family, which is unlike Gal-4 and Gal-8, that are tandem repeat galectins consisting of two separate carbohydrate recognition domains tethered with a linker peptide (Hadari et al., 1995; Levy et al., 2001; Rustiguel et al., 2016)

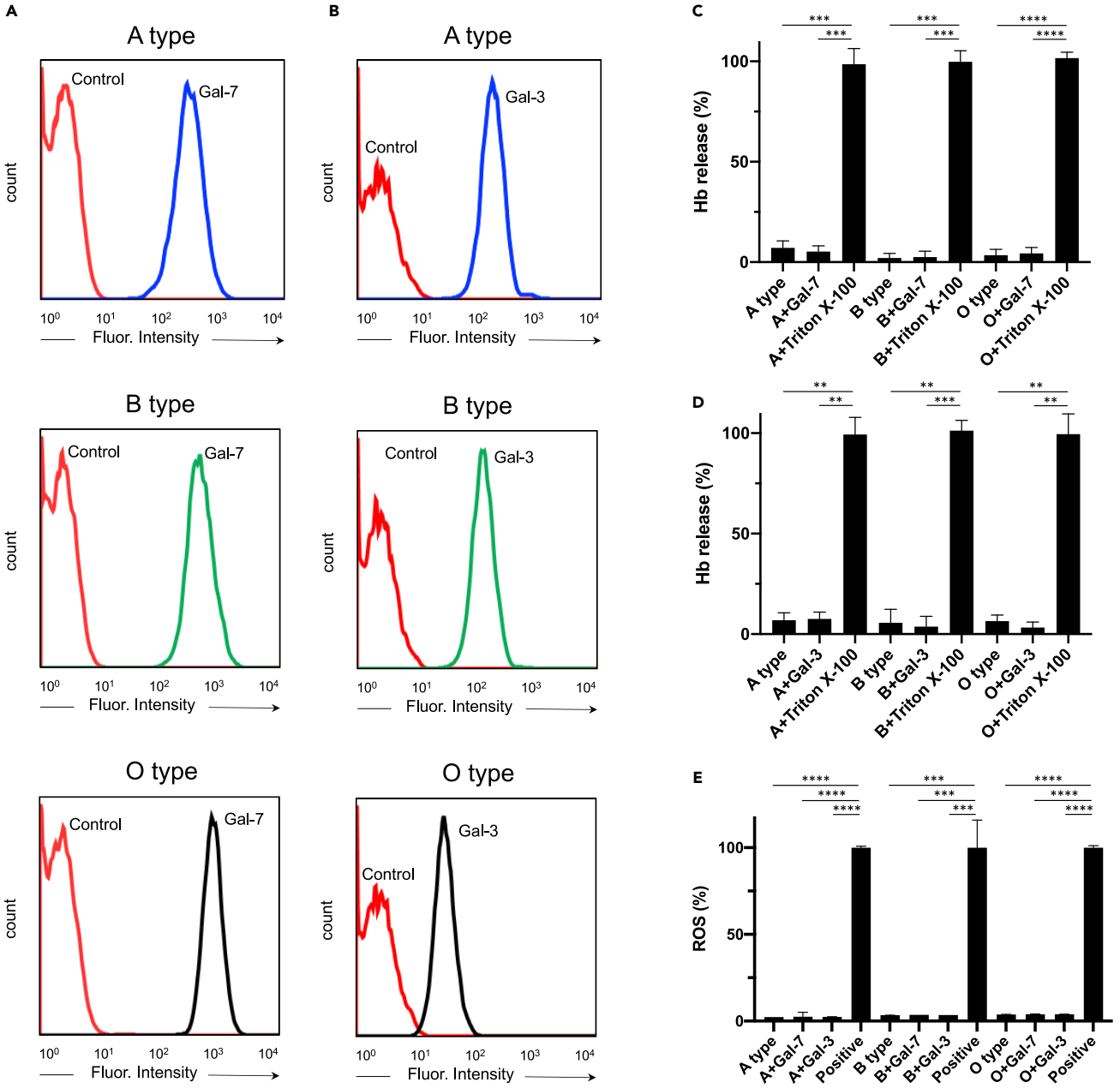

Figure 8. Galectin-7 (Gal-7) binds but does not induce loss in membrane integrity of human RBCs

(A) Flow cytometric analysis of Gal-7 binding to blood group A, B, or O human RBCs.

(B) Flow cytometric analysis of Gal-3 binding to blood group A, B, or O human RBCs.

(C) Quantification of hemoglobin (Hb) release from human RBCs after incubation with Gal-7 or Triton X-100 as indicated.

(D) Quantification of hemoglobin (Hb) release from human RBCs after incubation with Gal-3.

(E) ROS release from human RBCs after incubation with Gal-3 or Gal-7. TBHP (*tert*-Butyl hydroperoxide) used as a positive control. Data are represented as mean values ±SD **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$.

or Gal-3, which exists as a chimeric galectin composed of a carbohydrate recognition domain and an N terminal collagen-like domain (Arthur et al., 2015a; Liu and Rabinovich, 2010). Given that Gal-7 is a prototypical galectin, unlike Gal-3, Gal-4, and Gal-8, whether antimicrobial activity was confined to tandem-repeat or chimeric galectins or whether a prototypical galectin, such as Gal-7, possesses antimicrobial activity remained unknown. The results of the present study demonstrate that antimicrobial activity within the galectin family is not limited to tandem repeat or chimeric galectins, but that prototypical galectins also possess

the ability to bind and kill blood group positive microbes. These results are especially important when considering that previous results demonstrated that galectin-1 (Gal-1), a prototypical galectin and the first galectin described, fails to bind or kill blood group positive microbes (Stowell et al., 2010). These prior results demonstrated that antimicrobial activity may not be a universal feature of galectin family members and perhaps is an activity confined to tandem repeat and chimeric family members. Gal-1, similar to Gal-7, does not display a high preference for blood group antigens. However, unlike Gal-7, Gal-1 does exhibit high binding toward polyLacNAc glycans and biantennary N-glycans in general (Stowell et al., 2004, 2008a). Indeed, these glycan structures are thought to mediate many of the immune regulatory activities attributed to Gal-1 and other galectins (Stowell et al., 2007). However, among galectin family members in general, much less is known regarding the host cell receptors engaged by Gal-7, including the specific glycan determinants that may mediate these interactions. As H antigen expression is higher on blood group O RBCs when compared to RBCs isolated from blood group B or A individuals, the enhanced binding of Gal-7 toward O cells may reflect a preference for the H antigen on the RBC surface, similar to that observed on the CFG array. However, the overall consequences of these interactions, including the actual functional glycans that are responsible for mediating possible extracellular interactions between Gal-7 and target cells, remain largely unknown. Although such studies are beyond the scope of the present study, the present findings do raise important questions regarding the binding specificity of Gal-7 toward mammalian glycans in general.

The apparent discrepancy between Gal-7 binding on the CFG glycan microarray and actual interactions with microbial glycans, including those observed on the microbial glycan microarray, is unique. Prior studies examining Gal-1, Gal-3, Gal-4, and Gal-8 demonstrated that CFG microarray findings largely predicted actual interactions with similar glycan determinants when expressed on microbes (Stowell et al., 2010, 2014; Wu et al., 2021b). Although the glycan motifs on microbes certainly possess the ability to mimic mammalian blood group antigens, they are distinct. Subtle differences in the presentation of blood group antigens between microbes and mammals may account for differences observed between the CFG glycan microarray and actual interactions with intact microbes. Of note, the MGM, which is populated with microbial glycan determinants, did predict actual interactions, suggesting that the array formats can predict actual interactions with microbes. As blood group recognition more likely reflects a role for galectins in providing antimicrobial immunity, the higher apparent binding affinity toward microbial blood group antigens may account for selective pressures that maintained this binding affinity above that observed for mammalian blood group antigens. Differences in the residues that surround critical amino acids with the carbohydrate recognition domain required for core glycan recognition may not only drive distinct glycan binding preferences toward mammalian glycans among different galectin family members (Carlsson et al., 2007; Stowell et al., 2008a) but may likewise be responsible for distinct glycan interactions observed toward microbial and mammalian glycans.

Subtle, but important, differences in the mode of glycan binding may in part be responsible for differences in the recognition of microbial and mammalian blood group antigens by different galectin family members. For example, Gal-3, Gal-4, and Gal-8 display high affinity for mammalian blood group antigens when presented as a single terminal blood group unit, suggesting that intrinsic affinity for the blood group modification on a LacNAc backbone may support substantial binding affinity (Stowell et al., 2010; Wu et al., 2021b). In contrast, Gal-7 not only failed to bind blood group antigens with high affinity in general, but only bound the H antigen when presented on an extended polyLacNAc structure (iH). These results suggest that Gal-7-glycan interactions may reflect combined interactions with the blood group determinant and extended glycan structures. Consistent with this, Gal-7 displayed reduced affinity toward the intact Δ*Wzy* mutant and its isolated LPS structure, which contains only one blood group terminating structure. As blood group-like antigens on microbes exist as polymerizing structures, it is likely that Gal-7 evolved to recognize these repeating units in the context of microbial evolution and not as a possible ligand responsible for engaging host glycans. This is especially likely given that ABO(H) antigens are polymorphic and are therefore not likely to be responsible for mediating fundamental cellular processes within human biology driven by Gal-7 or other galectins.

The ability of Gal-7 to specifically recognize the iH antigen and type 2 blood group A over a broad range of concentrations has never been observed for a galectin family member. Indeed, this level of specificity is unique even among most other lectins examined, including plant lectins and other vertebrate lectins (Blixt et al., 2004; Gao et al., 2019). As already noted, the likelihood of iH or type 2 blood group A serving as a key

ligand for Gal-7 to regulate mammalian cell function is unlikely given the polymorphic nature of these antigens and the relatively restricted expression of these glycans to distinct types of tissues. These results may therefore be more revealing regarding unique features of Gal-7 interactions with glycans in general. Several prior studies demonstrated that Gal-7 can exhibit subtle but important differences in glycan binding. Gal-7, for example, exhibits higher affinity for type 1 LacNAc when compared to type 2 LacNAc, which contrasts the binding preference observed for Gal-1 and Gal-3 (Hsieh et al., 2015). Similar to the present results, prior results suggested that Gal-7 can exhibit relatively little binding with blood group A and blood group B glycans (Hirabayashi et al., 2002). Similar to the preference of Gal-7 toward the H antigen when present on extended polyLacNAc glycans, a slight preference for glycans containing LacNAc repeats when compared to one LacNAc glycan was also previously noted (Brewer, 2002). Enhanced binding toward extended LacNAc containing glycans was also observed when examining Gal-7 binding toward human milk oligosaccharides (Gao et al., 2019; Noll et al., 2016). These collective data suggest that extended glycans bearing LacNAc determinants may be preferred glycan ligands for Gal-7 among mammalian carbohydrates. This intrinsic preference may also underlie the ability of Gal-7 to engage microbial glycans, where the actual affinity for individual glycan motifs may be low, but the collective binding interactions toward repeating structures may convey a higher overall binding affinity. It should be noted that although appreciable Gal-7 binding could be detected toward other glycan ligands at the highest concentrations tested, these concentrations were only employed to establish binding isotherms. Ligands only bound at the highest concentrations tested are less likely to reflect physiological interactions.

The ability of Gal-7, in addition to previous reports on Gal-3, Gal-4, and Gal-8 to kill microbes that utilize molecular mimicry suggests that the antimicrobial activity of Gal-7 may be a more common feature of galectin family members. The ability of galectins in general to target microbes that express distinct glycans is unique among innate immune proteins, which generally target molecular features shared among many different microbial species that are distinct from the host as a mechanism of detecting and eliminating microbes while avoiding injury to self (Ganz, 2003; Levy, 2004; Otte et al., 2003). However, for immune factors to bind and kill microbes that utilize molecular mimicry, the ability to recognize structures that resemble the host would be predicted to result in some level of cross reactivity. As noted, Gal-7 appears to be unique among galectins in its ability to more specifically engage microbial glycans when comparing the breadth of glycan binding observed toward mammalian glycans on the CFG glycan microarray observed for other galectins. However, Gal-7 certainly recognizes host glycans, as evidenced by binding to RBCs and in prior studies examining Gal-7 binding to other glycan determinants (Brewer, 2002; Gao et al., 2019; Hirabayashi et al., 2002; Hsieh et al., 2015; Noll et al., 2016). Despite these interactions, Gal-7 fails to induce detectable changes in membrane integrity of RBCs.

The ability of Gal-7 and other galectins to recognize host cells and microbes but only kill microbes is unique. The phospholipid asymmetry of prokaryotes differs from eukaryotes, suggesting that although galectins require initial glycan recognition, additional interactions with the prokaryotic membrane may be necessary for microbial death to occur. Consistent with this, antimicrobial peptides in general are often selective for microbes because of distinct binding microbial membrane preferences when compared to mammalian membranes (Grant et al., 1992; Malmsten et al., 2011; Sigurdardottir et al., 2006). However, for galectins to be able to effectively disrupt membrane integrity, a sufficient effective concentration that may only be achieved following initial glycan binding may be required for efficient engagement of the microbial membrane. However, it is certainly possible that galectins kill microbes through mechanisms that are completely distinct from direct interactions with the microbial membrane. These potential mechanisms await additional exploration.

Regardless of the mechanism whereby Gal-7 and other galectins kill microbes, the results of this study suggest that an ancient carbohydrate binding protein family expressed in mammals exists that can target a variety of microbes, each of which utilize features of blood-group molecular mimicry. Such an ability may have allowed blood groups to more freely evolve because the negative consequences of possible increases in susceptibility to microbes that utilize molecular mimicry may have not been as apparent. Although the selective pressures that facilitate blood group diversity remain to be fully defined, differential binding to blood group host glycans in addition to the development of naturally occurring antibodies that target viruses that may incorporate blood group-like antigens reflect some of the possible selective pressures that resulted in the selection of ABO(H) polymorphisms (Barnkob et al., 2020; Harris et al., 2005; Hutson et al., 2002; Wu et al., 2021a). Thus, blood group evolution, which is of fundamental consequence to

allogeneic transfusion and transplantation, likely reflects complex interplay between microbes and host immune factors at a variety of levels that have sustained the diversity of ABO(H) antigens within the human population.

Limitations of the study

Our study is largely focused on providing a thorough analysis of the glycan binding specificity of Gal-7 toward a variety of distinct mammalian and microbial glycans, in addition to determining whether interactions observed on these array platforms predict actual interactions with intact microbes. Future studies will be needed to expand upon these initial findings, including an examination of the impact of Gal-7 on microbial flora using *in vivo* approaches. Additional studies will also be needed to examine in detail the overall structural features of each galectin that dictate the distinct and overlapping binding preferences displayed and the mechanisms whereby Gal-7 induces microbial death. However, the present results do demonstrate that Gal-7 can bind and kill a diverse range of microbes, and thus suggest that Gal-7, along with other galectin family members, may provide an innate form of immunity against molecular mimicry.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104482.

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AUTHOR CONTRIBUTIONS

S.C.W. and N.A.K. designed and conducted key experiments. M.F.R. generated key reagents. M.D.B., C.D.J., M.Y.Y., W.J.L., J.W., H.M.J., S.R.N., R.D.C., and C.M.A. provided critical oversight in the design and execution of these studies. S.C.W., N.A.K., S.R.S., and C.M.A. wrote the manuscript, which was additionally commented on and edited by the remaining authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli O86:B7	P.G. Wang (Georgia State University)	N/A
P. alcalifaciens O5 and *P. alcalifaciens* O19	Y. Knirel (ND Zelinsky Institute of Organic Chemistry, Moscow, Russia)	N/A
K. pneumoniae O 1 and *K. pneumoniae* O4	C. Whitfield (University of Guelph)	N/A
S. pneumoniae 14 and *S. pneumoniae* 19	M. Nahm (University of Alabama at Birmingham)	N/A
One Shot™ BL21(DE3) Chemically Competent *E. coli*	Thermo Fisher	Cat#C600003
One Shot™ TOP10 Chemically Competent *E. coli*	Thermo Fisher	Cat#C404006
Chemicals, peptides, and recombinant proteins		
Human galectin-7 protein	This study	Plasmid from Emory Cloning Center
Human galectin-3 protein	This study	Plasmid from Emory Cloning Center
Alexa Fluor™ 647 NHS Ester (Succinimidyl Ester)	Thermo Fisher	Cat#A20006
D-Lactose monohydrate, ACS Certified Grade, Fisher Chemical	Thermo Fisher	Cat#64044-51-5
2-Mercaptoethanol	Sigma	Cat# 60-24-2
Propidium Iodide	Thermo Fisher	Cat#P3566
SYTOX™ Orange Nucleic Acid Stain	Thermo Fisher	Cat#S11368
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Thermo Fisher	Cat#BP1755100
Ampicillin	Sigma	Cat#A9518
Lb Broth Powder	Thermo Fisher	Cat#501977020
Triton X-100	Sigma	Cat#9036-19-5
Thiodigalactoside	Sigma	Cat#51555-87-4
Critical commercial assays		
DCFDA / H2DCFDA - Cellular ROS Assay Kit	Abcam	Cat#ab113851
Recombinant DNA		
Human galectin-7 expression plasmid	This study	Plasmid from Emory Cloning Center
Human galectin-3 expression plasmid	This study	Plasmid from Emory Cloning Center
Software and algorithms		
Prism - GraphPad	GraphPad by Dotmatics	Prism 8.4.2
GenePix® Pro 7 Microarray Acquisition & Analysis Software	Molecular Devices	GenePix Pro 7
GlycoStation ToolsPro	GlycoTechnica	GlycoStation®ToolsPro Ver.3.0
FlowJo	BD Biosciences	Version 10
Other		
CFG Glycan microarray	National Center for Functional Glycomics (NCFG)	Version 3
Microbial glycan microarray (MGM array)	National Center for Functional Glycomics (NCFG)	PMCID: PMC4158828

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be fulfilled by lead contact Connie M. Arthur (cmarthur@bwh.harvard.edu).

Materials availability

Some plasmids are available for distribution.

Data and code availability

This paper does not report original code. All data are reported in the main text or in the supplemental information of this work. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

E. coli strains for protein production

TOP10 Chemically Competent *E. coli* was used to make Human Gal-3 and Gal-7 plasmids. The *E. coli* strain (BL21) was used to express Human Gal-3 and Gal-7.

METHOD DETAILS

Protein expression and purification of human Gal-3 and Gal-7

Human Gal-3 and Gal-7 were expressed as outlined previously (Paul et al., 2022; Stowell et al., 2010). Briefly, Gal-3 or Gal-7 transformant positive *E. coli* BL21 (DE3) were cultured in LB broth containing 100 μg/mL ampicillin with agitation (250 rpm) at 37°C (Paul et al., 2022; Wu et al., 2021c). When bacteria were grown to the mid-log phase, protein expression was induced by addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG, 1.5 mM). After 20 h induction in 16°C, bacteria pellets were harvested by centrifugation. Pellets were lysed and Gal-3 or Gal-7 was purified by affinity chromatography. Gal-3 or Gal-7 were labeled with Alexa Fluor™ 647 NHS Ester (Succinimidyl Ester) by incubating 2 mg mL^{-1} Gal-3 or Gal-7 with 1 mg Alexa Fluor™ 647 for 1 h at room temperature as outlined previously (Leppanen et al., 2022; Stowell et al., 2004). Unconjugated Alexa Fluor™ 647 and free lactose were separated using a PD-10 gel filtration column (GE Healthcare). Labeled Gal-3 or Gal-7 were purified again by lactosyl-sepharose column to remove possible inactive protein that may have formed during labeling process. Bound Gal-7 was eluted with 100 mM lactose in PBS plus 2-mercaptoethanol (2-ME) and then a PD-10 gel filtration column was used to remove 2-ME and lactose prior to the use of Gal-7 for experiments (Blenda et al., 2022; Stowell et al., 2007, 2008b; Wu et al., 2021b, 2021c). The molecular weight of monomeric Gal-7 is 15 kDa. As the protein has been shown to form stable dimers in the solution (Morris et al., 2004), molecular weights were calculated based on the dimeric molecular weight (10 μM is ~0.3 mg/mL).

Glycan array analysis

For Gal-7 recognition of glycans on the printed glycan microarray (Blixt et al., 2004; Stowell et al., 2008a, 2014), we incubated fluorescent labeled Gal-7 on the array at the concentrations indicated in Tris buffer/Salts/Metal ions (TSM) Binding Buffer (20 mM Tris-HCl, 150 mM sodium chloride, 2 mM calcium chloride, 2 mM magnesium chloride, pH 7.4, 1% BSA, and 0.05 % Tween 20, with 14 mM 2-ME) for 1 h at room temperature in a dark humid chamber. Slides were washed by successive immersion in TSM wash buffer (Stowell et al., 2022). An image of bound fluorescence was then obtained using a microarray scanner (GenePix 4000 B, Molecular devices). Integrated spot intensities were acquired using Imagene software (GenePix Pro 7) (Arthur et al., 2022).

Lectin array analysis of blood group B+ *E. coli*

Blood group + *E. coli* or the Δ*WaaL* mutant (1×10^9-2×10^{10}) were washed in PBS three times, followed by labeling with 10 μM SYTOX Orange nucleic acid stain dye (ThermoFisher) in 4 mL PBS for 5 min. Labeled bacteria were then diluted to 0.5×10^9-5×10^9 per well and incubated with lectin chip for 1 h at 4°C. Unbound bacteria were removed by washing with PBS. The fluorescently labeled blood group + *E. coli* or Δ*WaaL* bound to the microarray was determined using Glycostation (TM) Reader 1200 (GP Biosciences) and image processed by LecChip software using an approach similar that outlined previously (Yasuda et al., 2011).

Measuring the impact of Gal-3 or Gal-7 on bacterial viability

E. coli O 86:B7 (Blood group B+ *E. coli*) was kindly provided by P.G. Wang (Georgia State University). *P. alcalifaciens* O5 and *P. alcalifaciens* O19 were provided by Y. Knirel (ND Zelinsky Institute of Organic Chemistry, Moscow, Russia). *K. pneumoniae* O 1 and *K. pneumoniae* O4 were provided by C. Whitfield

(University of Guelph). The *Streptococcus pneumoniae* 14 and *Streptococcus pneumoniae* 19 were kindly provided by M. Nahm (University of Alabama at Birmingham). Each strain was grown and maintained as outlined previously (Wu et al., 2021b). When assaying potential antimicrobial effects of galectins, each strain was used in the mid-logarithmic growth phase (OD_{600} of ~0.1). Bacterial cells were suspended with the indicated concentrations of Gal-3 or Gal-7 at 37°C for 2 h. Colony forming unit (CFU) determination was then assessed by limited dilution analysis (Arthur et al., 2015b; Kamili et al., 2022). For assessment of membrane permeability, *E. coli O86* (Blood group B+ *E. coli*) was incubated with Gal-7, followed by incubation with 0.5 μL propidium iodide (PI, Invitrogen) and visualization by confocal microscopy.

Scanning electron microscopy (SEM) of bacteria following Gal-7 exposure

SEM analysis of bacteria was accomplished as outlined previously (Stowell et al., 2010). Briefly, BG B+ *E. coli* O86 was incubated with Gal-7 or PBS at 37°C for 30 min. The bacteria were then washed, fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and exposed to 1% osmium tetroxide. The sample was then dehydrated with ethanol. Following hexamethyldisilizane (HMDS) ethanol displacement, the samples were mounted with either gold or chromium and observed using DS130 SEM (ISI-TOPCON) in-lens imaging. The displayed images were viewed at 20,000× magnification.

Red blood cell viability test and flow cytometry analysis

Loss of cell viability was measured by quantifying hemoglobin (Hb) release from human red blood cells following incubation with 5 μM Gal-3, 5 μM Gal-7 or 1% Triton X-100. RBC supernatants were examined for hemolysis by measuring absorbance at 540 nm. To examine potential binding by Gal-3 or Gal-7, RBCs were resuspended and washed twice in PBS at 4°C and then 1 μL packed RBCs incubated with 0.1 μM Alexa Fluor™ 647 labeled Gal-3 or Gal-7 at 4°C for 20 min. After incubation, cells were washed twice and resuspended in 400 μl FACS buffer (PBS with 2% BSA) for flow cytometry analysis with a FACSCalibur flow cytometer (BD Biosciences).

Red blood cell cellular reactive oxygen species (ROS) detection assay

ROS detection followed the manufacturer's instructions of the cellular ROS assay kit (Abcam, ab113851). Briefly, human red blood cells at a density of 2×10^6 cells/mL were resuspended in 20 μM 2′, 7′-dichlorodihydrofluorescein diacetate (DCFDA) in the assay buffer provided by the kit. Cells were then incubated at 37°C for 30 min in the dark. After washing with PBS, cells were resuspended with 200 μL assay buffer with 10% FBS and transferred to a 96 well microplate. Gal-3 and Gal-7 were added into each well at a final concentration of 5 μM. TBHP (*tert*-Butyl hydroperoxide) was used for positive control according to manufacturer's instruction. The ROS was detected by fluorescence intensities at 535 nm using 485 nm as excitation wavelength by UV-Vis spectroscopy.

Flow cytometry analysis for bacteria

To evaluate the potential binding by Gal-7 to bacteria, bacteria were resuspended, washed twice in PBS at 4°C and then incubated with Alexa Fluor™ 647 labeled Gal-7 with or without TDG. After incubation, cells were washed twice and then resuspended in 400 μL FACS buffer (PBS with 2% BSA) for flow cytometry analysis using a FACSCalibur flow cytometer (BD Biosciences).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

GraphPad Prism 8.4.2 software was used for statistical analysis. Two groups were compared using Student *t* test. Three or more groups were analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. A p value ≤ 0.05 was defined as statistically significant.