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A Chimeric, Multivalent Assembly of Galectin-1 and Galectin-3 with Enhanced Extracellular Activity

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Abstract

Galectins are attractive therapeutic candidates to control aberrant immune system activation because they can alter the phenotype and function of various innate and adaptive immune cells. However, use of exogenous galectin-1 (“G1”) and galectin-3 (“G3”) as immunomodulators is challenged by their high dosing requirements and dynamic quaternary structures. Here we report a chimeric assembly of G1 and G3 with enhanced extracellular activity (“G1/G3 Zipper”), which was created by recombinant fusion of G1 and G3 via a peptide linker that forms a two-stranded α -helical coiled-coil. G1/G3 Zipper had higher apparent binding affinity for immobilized lactose and a lower concentration threshold for inducing soluble glycoprotein crosslinking than G1, a recombinant fusion of G1 and G3 with a flexible peptide linker (“G1/G3”), or a recently reported stable G1 dimer crosslinked by poly(ethylene glycol) diacrylate (“G1-PEG-G1”). As a result, G1/G3 Zipper was more effective at inducing Jurkat T cell apoptosis in media containing serum, and was the only variant that could induce apoptosis at low concentrations under serum-free conditions. The monomeric G1/G3 fusion protein lacked extracellular activity under all conditions tested, suggesting that the enhanced activity of G1/G3 Zipper was due to its quaternary structure and increased carbohydrate-recognition domain valency. Thus, combining G1 and G3 into a non-native chimeric assembly provides a new candidate therapeutic with greater immunomodulatory potency than the wild-type proteins and previously reported engineered variants.

Graphical Abstract

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Conflicts of interest

There are no conflicts to declare.

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Introduction

Although glycobiology research was first recognized in the early 19th century, researchers are just now beginning to understand the complex role of lectins and glycans within the immune system.^{1, 2} Galectins are a family of soluble, β -galactoside-binding lectins that regulate the phenotype and function of various immune cells during the initiation and resolution of immune responses.³ For example, galectin-1 (G1) and galectin-3 (G3) are mediators of tumor immunosuppression and fetal-maternal tolerance, regulate autoimmune disease progression, can enhance or inhibit viral infection, and induce pro-inflammatory responses during osteoarthritis.^{4–10}

Extracellular G1 and G3 can act on innate immune cells, including monocytes, macrophages, neutrophils, and dendritic cells, yet it is their influence on T cells that receives the most attention for immunotherapy.^{11–18} Extracellular G1 and G3 can induce T cell apoptosis, regulate antigen-specific T cell activation, and alter T cell cytokine secretion.¹⁹ Despite sharing binding affinity for β -galactoside glycans, though, G1 and G3 often evoke these changes in T cells by recognizing different cell surface glycoproteins, suggesting they function through different signalling pathways.^{19, 20} For example, although both G1 and G3 bind to CD45, only G1 induces CD45 clustering to trigger T cell apoptosis.²¹ Both G1 and G3 have been shown to interact with CD7, yet G3 binding to CD7 does not trigger T cell death.^{22–24} G1 can bind to CD2 and CD3, whereas G3 binding to these glycoproteins has not been observed.^{22, 24} G1 can also selectively induce death of T helper (Th)1 and Th17 cells, yet has no effect on Th2 and regulatory T cells, due to polarization-induced changes in the T cell surface glycosylation profile.²⁵ Similarly, G3 preferentially kills double-negative thymocytes, but not double-positive thymocytes, again due to alterations in the surface glycosylation profile of cells as they mature or polarize.²² Interestingly, a combination of G1 and G3 did not have an additive or synergistic effect on T cell apoptosis, but rather elicited a similar extent of cell death as either galectin alone, possibly due to competitive interactions with CD45 or other T cell surface glycoproteins.²² Beyond apoptosis, G1 has been shown to stimulate antigen-specific T cell responses while G3 antagonized these responses;²⁶ however, in some contexts G1 can also antagonize T cell receptor signalling.²⁷ Finally, G1 and G3 also have differing effects on T cell cytokine secretion, with low concentration G3 inducing IL-2 secretion, while G1 upregulates IL-10 expression by Th17 cells.^{28, 29}

In light of these diverse effects on T cells, exogenous galectins and engineered variants have been evaluated as therapeutics to regulate adaptive immunity in various contexts.⁴

Recombinant G1 has received the most attention to date, and has demonstrated efficacy in rodent models of Crohn's disease, multiple sclerosis, myasthenia gravis, rheumatoid arthritis, graft vs host disease, autoimmune uveitis, and T-cell mediated hepatitis.^{5-8, 30-33} A key aspect of G1 and G3 extracellular activity is their non-covalent self-association into quaternary structures with multiple carbohydrate-recognition domains (CRDs). For example, G1 associates into homodimers at relatively high (μM) concentrations.³⁴ To stabilize its activity in dilute conditions, we and others engineered G1 dimers formed via a polypeptide linker, a synthetic polymer, a leucine zipper coiled-coil forming peptide, or an IgG Fc domain.^{4, 35-38} Often, these engineered G1 variants demonstrate a minimum effective dose that is approximately 10-fold lower than that of the wild-type protein. In contrast, G3 is unique among galectins in that it assembles into higher-ordered oligomers (>2 G3 molecules) upon glycan binding via interactions involving its N-terminal domain.³⁹ To the best of our knowledge, no engineered G3 oligomers with improved activity have been reported. Rather, G3 inhibitors have been developed by treating the wild-type protein with collagenase, which cleaves the N-terminal domain without disrupting the CRD.⁴⁰ Likewise, we recently reported G3 fusion proteins that bind glycans yet lack activity for inducing T cell agglutination, apoptosis, and cytokine secretion because they cannot oligomerize.⁴¹ Together, these examples demonstrate the potential of protein engineering approaches to manipulate G1 and G3 immunomodulatory activity by altering their oligomerization.⁴² Despite these efforts, though, existing engineered G1 and G3 variants still have minimum effective doses that are in the low μM range, which is impractical for many therapeutic applications.

We envisioned that one way to further decrease the effective dose of G1 and G3 could be to combine them into chimeric multivalent assemblies. In one design, we created a construct in which G1 was connected to the N-terminus of G3 via a flexible linker ("G1/G3") (Fig. 1).⁴³ This design was based on our recent report demonstrating that fusing enzymes to the N-terminus of G3 endowed them with carbohydrate-binding affinity, yet also abolished G3's activity for inducing T cell apoptosis.⁴¹ Here, we proposed that the G3 domain would primarily serve to anchor G1 at the T cell surface by increasing its affinity for membrane glycans. In a second design, we created a construct in which G1 was connected to G3 via a peptide domain derived from GCN4 that forms a two-stranded parallel α -helical coiled-coil ("G1/G3 Zipper") (Fig. 1).⁴¹ We expected that this design would yield a variant with a lower effective dose by stabilizing the active homodimeric structure of G1, yet would not restore G3 signalling activity based on our prior report demonstrating that a trimeric G3 assembly does not induce T cell apoptosis.⁴¹ In all designs, we employed a variant of G1 which has all four surface cysteine residues mutated to serine residues (C2S, C16S, C88S, C130S) to avoid oxidative inactivation.^{38, 44-46} We evaluated lactose binding affinity, solution-phase glycoprotein crosslinking, and extracellular activity of G1, G1/G3, G1/G3 Zipper, and a recently reported G1 dimer formed by reacting a single surface cysteine with poly(ethylene glycol) diacrylate ("G1-PEG-G1").³⁸ Our results demonstrate that G1/G3 was inactive as an extracellular signal, whereas G1/G3 Zipper had higher carbohydrate-binding affinity and a significantly lower minimum effective dose than G1 or G1-PEG-G1 for inducing Jurkat T cell apoptosis in vitro.

Results and Discussion

Design, Expression, and Characterization of G1/G3 variants

All Galectin-1 mutants and fusions were expressed in *E. coli* and recovered from the soluble fraction in > 90% purity and mg/L yields (Fig. 2A). G1/G3, G1/G3 Zipper, and G1 migrated to distances corresponding to their predicted molecular weights under denaturing gel electrophoresis conditions. Note that here G1/G3 Zipper migrates to a molecular weight consistent with one protein subunit of the dimer (i.e., G1-GCN4 peptide-G3) because the concentration of sodium dodecyl sulfate present in the sample loading buffer is sufficient to dissociate the two-stranded α -helical coiled-coil.⁴⁷ The slightly shorter migration distance for G1/G3 Zipper relative to G1/G3 was expected due to the higher molecular weight of G1-GCN4 peptide-G3, which is attributed to the addition of the GCN4 peptide domain between G1 and G3 (Fig 2A). G1-PEG-G1 was prepared as previously reported (Fig. S1).³⁸ We also attempted to prepare a PEG-crosslinked G1/G3 Zipper, however our established protocol did not yield a sufficient amount of product (Fig. S2).

We used a combination of size-exclusion chromatography (SEC) and dynamic light scattering (DLS) to compare the native size of G1/G3 Zipper to that of G1/G3. The equilibrium dissociation constant (K_D) of the GCN4 coiled-coil dimer (i.e., the Zipper) is 8 nM.⁴⁸ For DLS and SEC measurements, the concentration of G1/G3 Zipper and G1/G3 was 15 μ M which is three orders of magnitude higher than the Zipper K_D . This high protein concentration relative to the Zipper K_D was intended to favour α -helical coiled-coil dimer formation. SEC demonstrated that the hydrodynamic size of G1/G3 Zipper was significantly larger than that of G1/G3 under native conditions (Fig. 2B). The empirical molecular weights of G1/G3 Zipper and G1/G3, which were estimated by fitting their SEC elution volumes to a standard curve, were consistent with their predicted native molecular weights (Fig. 2B table). Here, the slightly lower observed molecular weight for G1/G3 Zipper under native conditions when compared its theoretical native molecular weight likely reflects differences in the shape of G1/G3 Zipper and the globular standards used for calibration. Further, G1/G3 Zipper had a significantly larger hydrodynamic diameter than G1/G3 when analysed via DLS number-weighted size distribution (Fig. 2C). These observations demonstrated that G1/G3 fusions can be expressed and purified in high yield and purity, and that a peptide that forms a two-stranded α -helical coiled-coil can be used to assemble a G1/G3 Zipper.

Carbohydrate Binding and Glycoprotein Crosslinking Properties of G1/G3 Fusion Proteins

We compared the carbohydrate-binding properties of G1/G3 and G1/G3 Zipper to G1 and G1-PEG-G1 using lactose affinity chromatography. G1, G1-PEG-G1, and G1/G3 eluted at similar concentrations of soluble lactose, whereas G1/G3 Zipper eluted at a higher soluble lactose concentration with a broader distribution profile (Fig. 3A). Collectively, these observations suggested that engineering G1 to have 2 CRDs did not establish avidity effects that significantly increased its apparent binding affinity for immobilized lactose, regardless of whether G1 was linked to another G1 molecule or to G3. Unexpectedly, these data also suggested that the G1 CRD of G1/G3 may preferentially interact with immobilized lactose, given that we have observed that G3 has higher affinity for immobilized lactose than G1

(Fig. S3). In contrast, G1/G3 Zipper had higher apparent binding affinity for immobilized lactose than G1, G1-PEG-G1, or G1/G3, suggesting that assembly of G1 and G3 into a structure having 4 CRDs established multivalent avidity effects not afforded by only having 2 CRDs.

Galectins can crosslink soluble glycoproteins into microscopic aggregates via their multiple CRDs^{49–51}, and galectin-mediated crosslinking of cell surface glycoconjugates has been proposed as the mechanism by which galectins regulate outside-in signaling.^{52–55} Here we used turbidity as a model to characterize crosslinking of the soluble glycoprotein, asialofetuin (ASF), into aggregates via G1, G1-PEG-G1, G1/G3, or G1/G3 Zipper. At the concentration tested, neither G1, G1-PEG-G1, nor G1/G3 crosslinked ASF into insoluble aggregates (Fig. 3B–C). In contrast, G1/G3 Zipper rapidly crosslinked ASF into aggregates, as demonstrated by a significant increase in solution turbidity (Fig. 3C). In previous reports, G1 was able to crosslink ASF at higher concentrations than those used here.⁵⁶ Likewise, previous reports demonstrated that wild-type G3 can also crosslink ASF into aggregates; however, we previously reported that G3 lost its ability to crosslink ASF when a non-galectin protein was fused to its N-terminus.⁴¹ Taken together, these data demonstrated that G1 variants having CRD valency < 2 (i.e., G1, G1/G3, and G1-PEG-G1) cannot crosslink ASF at the conditions tested, whereas G1/G3 Zipper having a CRD valency of 4 can. A previous study reported that G1 and G3 have comparable affinity for ASF, and in all experiments the total G1 and G3 CRD concentration was maintained constant, which suggested that the lower threshold for crosslinking soluble glycoproteins was not due to an affinity advantage afforded by having G1 versus G3 present. Rather, these observations suggested that assembling G1 and G3 into structures having higher CRD valency decreased the threshold for glycoprotein crosslinking by establishing avidity effects that favoured protein-glycan interactions, such as statistical rebinding. Based on these observations, we reasoned that G1/G3 Zipper would have a lower effective dose as an extracellular signal when compared to G1, G1/G3, or G1-PEG-G1.

Extracellular Activity of G1/G3 Fusion Proteins

We compared the relative activity of G1, G1-PEG-G1, G1/G3, and G1/G3 Zipper to induce Jurkat T cell agglutination and apoptosis *in vitro*. After 18 hours, high concentration G1 and G1-PEG-G1 induced Jurkat T cell agglutination, consistent with prior reports (Fig. 4A).³⁸ Interestingly, G1/G3 did not agglutinate Jurkat T cells after 18 hours, while high concentration G1/G3 Zipper did; however, many micron-sized protein aggregates were also visible in the culture media of samples treated with high concentration G1/G3 Zipper, which were not present in cultures treated with G1, G1-PEG-G1, or G1/G3 (Fig. 4A, No Cells).

To understand the dose dependence of agglutination, we treated cells with a lower concentration of G1, G1-PEG-G1, G1/G3, and G1/G3 Zipper ([G1] = 0.5 μ M) (Fig. 4B). Both agglutinated and single cells were observed in cultures treated with 0.5 μ M G1, while low concentration G1-PEG-G1 induced significant cell agglutination, consistent with a prior report.³⁸ Low concentration G1/G3 did not induce agglutination, which was not surprising since this protein also failed to induce agglutination at a 10-fold higher concentration. Low concentration G1/G3 Zipper induced cell agglutination, although some single cells were also

observed. Notably, much fewer protein aggregates were observed in cultures treated with low concentration G1/G3 Zipper when compared to cultures treated with high concentration G1/G3 Zipper, as demonstrated by the clear background that was similar to that of cultures treated with G1, G1-PEG-G1, or G1/G3. This suggested that the protein aggregation observed in cultures treated with high concentration G1/G3 Zipper (Fig. 4A, No Cells) was dependent on G1/G3 Zipper concentration.

In addition to inducing agglutination, G1 and G3 can also alter T cell metabolism. Here we compared changes in Jurkat T cell NADH content mediated by G1, G1-PEG-G1, G1/G3, or G1/G3 Zipper using the CellTiter-Blue® assay (Fig. 4C). G1 only decreased Jurkat T cell metabolism at a high concentration, whereas G1-PEG-G1 decreased Jurkat T cell metabolic activity at both high and low concentration, consistent with prior reports.^{34, 38} G1/G3 failed to alter Jurkat T cell metabolic activity at any concentration tested, whereas G1/G3 Zipper decreased Jurkat T cell metabolic activity at both high and low concentrations to a significantly greater extent than G1 or G1-PEG-G1. Taken together with agglutination data, these observations suggested that G1/G3 generally lacks activity for inducing T cell death. This observation contrasts with a recent report which demonstrated that fusions of G1 and G3 can negatively regulate neuroblastoma cell growth.⁵⁷ Here, the loss of activity of G1/G3 could be due to many factors, such as perturbation of G1 homodimerization when fused to G3; however, G1 fused to GFP induced Jurkat T cell agglutination and apoptosis (Fig. S4), so any such effect seems to be limited to cases in which G1 is linked to G3. Alternatively, G1/G3 may recognize different T cell surface glycoproteins than G1 or G3 that are not linked. Given that G1 and G3 induce apoptosis by binding and crosslinking different T cell surface glycoproteins, altering their binding properties by fusing them together could perturb G1- or G3-specific glycoprotein clustering patterns that are required to activate pro-apoptotic signalling pathways. Notably, though, inducing dimerization of G1/G3 by inserting a coiled-coil forming peptide into the linker domain not only restored extracellular galectin-like activity but amplified it compared to a G1 dimer (G1-PEG-G1). This suggests that G1 and G3 can have additive or synergistic effects when physically linked in a context that supports a dimeric G1 conformation, which contrasts with a prior report demonstrating that a combination of wild-type G1 and G3 did not have additive or synergistic effects on T cell death.²² We postulate that the G3 domains in G1/G3 Zipper are inactive as biological signals, instead serving to increase G1 binding affinity for T cell surface glycoproteins, based on our recent report demonstrating that a trimeric G3-luciferase assembly bound Jurkat T cell glycans with higher apparent affinity than a monomeric G3-luciferase fusion yet did not induce agglutination or metabolic activity loss.⁴¹ Thus, here G3-mediated anchoring could serve to increase the concentration of G1 at the T cell surface, thereby significantly decreasing its minimum effective dose for inducing apoptosis.

Next, we used fluorescence microscopy to visualize Jurkat T cell phosphatidylserine exposure and membrane permeability, two markers typically associated with early and late apoptosis, respectively (Figs. 5 and S5). Because galectins induce changes in these markers within a few hours, here we visualized outer leaflet phosphatidylserine via Annexin V staining and membrane permeability via propidium iodide (PI) staining 4 h after treatment with high or low concentration G1, G1-PEG-G1, G1/G3, or G1/G3 Zipper.^{58, 59} In most instances, extent of cell agglutination after 4 h was consistent with observations made at 18

h (Fig. 4A–B), with the exception of cells treated with G1/G3 Zipper, which were not appreciably agglutinated at either concentration tested at 4 h (Fig. 5B). Prior to Annexin V and PI staining, all agglutinated cell aggregates were dissociated with β -lactose. Both G1 and G1-PEG-G1 induced Jurkat T cell phosphatidylserine exposure and increased PI permeability. In contrast, G1/G3 induced weak phosphatidylserine exposure and PI permeability at high and low concentration, again suggesting this fusion lacked the extracellular activity of G1 and G3. In contrast, G1/G3 Zipper induced PI permeability in a high percentage of cells at low and high concentration; however, the total number of cells recovered from samples treated with high concentration G1/G3 Zipper was significantly lower than that recovered from cultures treated with high concentration G1, G1-PEG-G1, or G1/G3. These observations suggested that many cells treated with high concentration G1/G3 Zipper may already be in a very late stage of apoptosis by 4 hours. Notably, significant protein aggregation was again only observed in cultures treated with high concentration G1/G3 Zipper, and it is plausible that these aggregates could contribute to cell death, and in turn the lower number of cells recovered in cultures treated with high concentration G1/G3 Zipper. Interestingly, the induction of phosphatidylserine exposure and PI permeability by low concentration G1/G3 Zipper in the absence of agglutination suggests that these processes may not be causally linked in Jurkat T cell response to extracellular G1 and G3. Rather, at low concentrations, the G1/G3 Zipper may preferentially crosslink glycoproteins on the cell surface leading to activation of pro-apoptotic signalling pathways without mediating the cell-cell interactions that lead to agglutination.

Formation of Micron-sized Serum Protein Aggregates via G1/G3 Zipper

We observed formation of many micron-sized aggregates in cell culture media containing high concentration G1/G3 Zipper regardless of whether cells were present or not, which were absent in cultures treated with other proteins (Figs. 4A and 5A). We previously reported that a trimeric assembly of G3 can form micron-sized aggregates in cell cultures by crosslinking G3-binding serum glycoproteins.⁴¹ Here, we characterized serum glycoprotein crosslinking via G1/G3 Zipper in the absence of cells using bright-field microscopy and turbidity. No change in turbidity was observed for solutions containing G1/G3 Zipper alone, whereas turbidity of solutions containing G1/G3 Zipper and 10% fetal bovine serum increased rapidly (Fig. 6A), similar to solutions containing G1/G3 Zipper and ASF (Fig. 3C). When visualized with bright-field microscopy, solutions of G1/G3 Zipper in media supplemented with 10% and 1% serum contained micron-sized aggregates similar to those observed in Jurkat T cell cultures (Fig. 6B).

To determine if these aggregates formed due to specific interactions between G1/G3 Zipper and serum glycoproteins, we compared turbidity of samples with and without β -lactose, a known inhibitor of G1 and G3 glycan binding. Solutions containing 10% serum, G1/G3 Zipper, and β -lactose had significantly lower turbidity than solutions containing 10% serum and G1/G3 Zipper, which was comparable to control solutions without G1/G3 Zipper or with 10% FBS alone (Fig. 6C). This suggested that the G1/G3 Zipper aggregates observed in Jurkat T cell cultures formed via specific interactions between serum glycoproteins and the CRDs of G1 and G3.

To characterize the relationship between aggregate formation and serum glycoprotein concentration, we first measured the turbidity of samples containing G1/G3 zipper and 0.1–100% serum. Turbidity increased as serum concentration increased from 0.1–1%, and this increase in turbidity was dependent on the presence of G1/G3 Zipper in the sample (Fig. 6D). Turbidity approached a maximum between 1–10%, and then decreased with increasing serum concentration until a plateau was reached at about 60% serum (Fig. 6D–E). This decrease in turbidity with increasing serum concentration likely resulted from having an excess of serum glycans relative to G1 and G3 CRDs, which saturates G1/G3 Zipper glycan binding sites thereby limiting the extent of crosslinking.

Next, we probed G1/G3 Zipper aggregate formation in the presence of specific serum glycoproteins that are known to bind to galectins. In particular, we characterized G1/G3 Zipper aggregate formation in the presence of haptoglobin and α -2-macroglobulin, which are known to interact with G3, as well as human serum albumin II (HSA II), which is reported to contain few glycoprotein contaminants and has no measurable interaction with G3.⁶⁰ Turbidity increased in samples containing G1/G3 Zipper and haptoglobin or α -2-macroglobulin with increasing glycoprotein concentration, whereas turbidity of samples containing G1/G3 Zipper and HSA II did not change (Fig. 7A). Likewise, micron-sized aggregates were observed in samples containing G1/G3 Zipper and haptoglobin or α -2-macroglobulin, but not G1/G3 Zipper plus HSA II (Fig. 7B). Collectively, these data demonstrate that the formation of micron-sized aggregates in cultures of Jurkat T cells treated with G1/G3 Zipper requires the presence of serum glycoproteins. These aggregates likely contain haptoglobin and/or α -2-macroglobulin, although detailed characterization of their composition is unknown and beyond the scope of this report. Nonetheless, given that G3 binds to a broader assortment of serum glycoproteins with higher affinity than G1,⁶⁰ it is likely that G3 is the primary mediator of these interactions, although it is plausible that G1 presented in this multivalent configuration may also be involved in serum glycoprotein crosslinking.

G1/G3 Zipper Activity in Serum-Free Conditions

CRD-dependent binding of G1/G3 Zipper to serum glycoproteins could alter its biological activity, for example by inhibiting its binding to cell surface glycoproteins, similar to competitive interactions reported previously between G3, serum glycoproteins, and a candidate multivalent G3 inhibitor.⁶¹ Here we compared agglutination and metabolic activity of Jurkat T cells treated with G1, G1-PEG-G1, or G1/G3 Zipper in serum-free culture media. Neither G1, nor G1-PEG-G1, nor G1/G3 Zipper induced significant agglutination of Jurkat T cells at either high or low concentration in serum-free media, strongly suggesting that serum proteins or glycoproteins contribute directly to galectin-mediated T cell agglutination (Fig. 8A–B). However, cells treated with high concentration G1/G3 Zipper were generally smaller than cells treated with low concentration G1/G3 Zipper, high and low concentration G1, or high and low concentration G1-PEG-G1. Cell shrinkage is a hallmark of apoptosis attributed to the breakdown of cells into smaller apoptotic bodies⁶² and these observations suggested that high concentration G1/G3 Zipper induced apoptosis in serum-free media to a greater extent than G1 or G1-PEG-G1. Consistent with this, G1 and G1-PEG-G1 had weakened activity for decreasing metabolic activity of Jurkat T cells cultured

in serum-free media when compared to their activity in media supplemented with serum (Fig. 4C and Fig. 8C). Conversely, G1/G3 Zipper had increased activity for suppressing metabolic activity of Jurkat T cells cultured in serum-free media when compared to its activity in media supplemented with serum (Fig. 4C and Fig. 8C). Taken together, these data suggest that specific interactions with serum glycoproteins can diminish the extracellular activity of G1/G3 Zipper by sequestering it within micron-sized aggregates that cannot bind to cell surface glycans as effectively as free protein.

Conclusions

This work demonstrates a chimeric, multivalent assembly of G1 and G3 with improved extracellular activity when compared to G1, G1-PEG-G1, or a monomeric G1/G3 fusion protein. G1/G3 Zipper has higher apparent binding affinity for lactose, as shown via immobilized lactose affinity chromatography, and crosslinked glycoproteins at a lower concentration than G1, G1-PEG-G1, or a G1/G3 fusion protein, as shown with ASF turbidity measurements. G1/G3 Zipper decreased T cell metabolism at both high and low concentrations to a greater extent than G1 and G1-PEG-G1, and was the only galectin variant that was effective at inducing T cell death at low concentrations under serum-free conditions. In contrast, G1/G3 failed to induce T cell death in any condition tested. G1/G3 may be inactive because G1 and G3 mediate T cell apoptosis via binding to different cell surface glycoproteins and reconfiguring these interactions by fusing G1 to G3 may preclude receptor clustering or lattice formation that are necessary to activate pro-apoptotic signalling pathways or induce changes in cell metabolism. The CellTiter-Blue® assay used here to determine metabolic activity indirectly measures NADH concentration via NADH-dependent reduction of resazurin to resorufin by mitochondrial enzymes.⁶³ Thus, our observations suggest that G1/G3 Zipper may more effectively suppress NADH-dependent metabolic processes related to T cell viability, activation, and division, such as the citric acid cycle, ATP generation, and nucleotide synthesis, although future research will be needed to identify the specific metabolic pathways that are perturbed. Interestingly, G1/G3 Zipper did not cause Jurkat T cell agglutination at 4 h, a trait commonly associated with galectin-like activity, yet did induce changes in markers associated with apoptosis, suggesting that these processes may not be operatively linked in Jurkat T cell response to extracellular galectins. Rather, assembling G1 and G3 into a multimeric configuration may result in a lower effective dose because CRD domains are preferentially involved in crosslinking cell surface glycoproteins (i.e. intra-cellular interactions) and, in turn, activating pro-apoptotic signalling cascades rather than mediating cell-cell adhesion (i.e. inter-cellular interactions). Future studies will move toward evaluating G1/G3 Zipper as an immunotherapeutic, where we envision that coupling G1 dimerization with the use of G3 as an anchoring domain will yield lower effective doses and may also extend local half-life as seen previously with enzymes fused to G3.⁴¹ One practical consideration in these efforts will be the observed interactions between G1/G3 Zipper and serum glycoproteins. Binding to serum glycoproteins modestly decreased G1/G3 Zipper activity in vitro, and it is not unreasonable to expect that partitioning of G1/G3 Zipper into aggregates with serum glycoproteins would decrease the amount of G1/G3 Zipper available to modulate immune cells in vivo. However, larger glycoprotein-galectin complexes may serve to extend the half-life of G1/G3 Zipper infused

intravenously. Further studies are required to assess the efficacy of G1/G3 Zipper as an immunomodulatory protein therapeutic.

Materials and Methods

Protein Expression and Purification

Origami B (DE3) *E. coli* (Novagen) were transformed with pET-21d(+)-C2S/C16S/C88S/C130S Galectin1, pET-21d-C2S/C16S/C88S/C130S Galectin1-Galectin3, and pET-21d-C2S/C16S/C88S/C130S Galectin1-Galectin3Dimer vectors and selected on 100 µg/mL ampicillin- and 50 µg/mL kanamycin A-doped LB/agar plates overnight at 37 °C. Positive clones were selected to inoculate 5 mL 100 µg/mL ampicillin- and 50 µg/mL kanamycin A-containing LB broth. Cultures were grown overnight at 37 °C, 220 rpm on an orbital shaker. Cultures of positive clones were then subcultured into 1 L 100 µg/mL ampicillin- and 50 µg/mL kanamycin A-containing 2xTY broth (10 g/L yeast extract, 16 g/L tryptone, and 5 g/L NaCl) and grown at 37 °C, 225 rpm on an orbital shaker until an optical density of 0.6–0.8 ($\lambda = 600$ nm) was reached. Protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and incubated for 18 h at 18 °C, 225 rpm in an orbital shaker. Bacteria were pelleted by centrifugation and washed with phosphate buffered saline (PBS). Bacteria were lysed with B-PER (ThermoFisher), a protease inhibitor tablet (ThermoFisher), 300 units DNase I from bovine pancreas (Sigma), and 100 µg lysozyme (Sigma) for 20 min. Lysed bacteria was cleared by centrifugation, and supernatant containing recombinant proteins was loaded onto columns containing HisPur™ Cobalt Resin (ThermoFisher) equilibrated with PBS. Columns were washed with 20–30 column volumes of PBS and bound galectin was eluted with a gradient of imidazole in PBS. Imidazole was removed by centrifugation using Amicon filter tubes (MWCO 10 kDa) (Millipore). Protein molecular weight and purity were analysed with SDS-PAGE. Protein concentrations were determined using the 660 nm assay (ThermoFisher) calibrated with bovine serum albumin. Proteins were endotoxin removed using Detoxi-Gel Endotoxin Removing Columns (ThermoFisher) and final endotoxin content was determined using Pierce LAL Chromogenic Endotoxin Quantitation kit (ThermoFisher), according to the manufacturer's instructions.

Characterization of Fusion Protein Size and Molecular Weight

Size-exclusion chromatography (SEC) was used to determine fusion protein molecular weight of under native conditions. A SuperDex-200 10/30 GL column (GE Healthcare) connected to an ÄKTA pure FPLC system was used. Protein was injected at ~15 µM and eluted protein was detected at A280 nm, which was normalized based on maximum signal intensity. Protein molecular weight was calculated by fitting protein elution volume to a curve prepared using protein standard markers (Bio-Rad, GE Healthcare). Hydrodynamic diameter of fusion proteins was approximated with 15 µM protein via DLS on a NanoBrook 90Plus Particle Size Analyzer using BIC Particle Sizing Software (Brookhaven Instruments). Hydrodynamic diameter $\pm$ standard deviation by number-, volume-, and intensity-weighted size distribution was determined from ten 30 s runs in triplicate or more.

Lactose Affinity Chromatography

Lactose binding affinity of G1, G1-PEG-G1, G1/G3, and G1/G3 Zipper was determined using affinity chromatography on an AKTA Pure chromatography system (GE Life Sciences) equipped with an α -lactose:agarose column (Sigma-Aldrich). 500 μ L of 15 μ M protein was eluted with a linear gradient of β -lactose (Sigma-Aldrich) in PBS. Proteins were detected via absorbance at 280 nm. Binding affinity experiments were repeated in triplicate and representative traces were plotted.

Turbidity

Turbidity was measured using absorbance at 420 nm, similar to previously reported methods.⁴¹ 50 μ L of 7 μ M ASF and 50 μ L of 10 μ M protein was pipetted into a 96 well plate. Immediately after pipetting the ASF and protein samples, kinetics reads over 10 minutes at 30 second intervals were collected.

Annexin V and Propidium Iodide Staining of Jurkat T Cells

Extracellular activity of G1, G1-PEG-G1, G1/G3 and G1/G3 Zipper was characterized using Jurkat T cells, similar to previously reported methods.^{38, 41, 44} For all experiments, cells were first expanded in complete Jurkat T cell media (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin–streptomycin, L-glutamine 200 mM, 1% HEPES buffer) at 37 °C, 5% CO₂. Cells were then aliquoted (~200,000 cells/well) into a sterile, clear, tissue cultured coated 24-well plate. To evaluate agglutination, cells were incubated with G1, G1-PEG-G1, G1/G3 or G1/G3 Zipper (final [G3] = 5 μ M or 0.5 μ M) and then imaged at 4 hours using a Zeiss Axio Observer inverted microscope. Following imaging, cells were harvested from the wells and washed with 10 mL of cold 100 mM β -lactose to remove bound galectins followed by centrifugation at 413 $\times$ g for 7 min on a Centrifuge 5804R (Eppendorf). Supernatant was removed thereafter and cells were resuspended in 200 μ L 1 $\times$ Annexin V binding buffer. 100 μ L of each sample (~100,000 cells) was transferred into a 96 well plate, and then 1 μ L of FITC–Annexin V and 1 μ L of PI (BD556547, ThermoFisher) were added to each well. Samples were incubated for 15 minutes protected from light, and then both brightfield and fluorescence images were collected. Images were taken on a Zeiss Axio Observer inverted microscope with FITC (excitation = 470/40 nm and emission = 525/50 nm) and rhodamine (excitation = 546/12 nm and emission = 575–640 nm) filter sets.

Jurkat T Cell Metabolic Activity

Jurkat T cells were aliquoted into sterile, clear, non-tissue culture coated 96-well microplates (20,000 cells/well). In some experiments, cells were cultured in complete media, while in other experiments cells were cultured in complete serum-free media. Cells were incubated with G1, G1-PEG-G1, G1/G3 or G1/G3 Zipper (final [G3] = 5 μ M or 0.5 μ M) for 18 h and then imaged using a Zeiss Axio Observer inverted microscope. Following imaging, cells were treated with 20 μ L of CellTiter-Blue® reagent (PR-G8080, Promega) for 2 hours. Fluorescence emission of each well was measured using a SpectraMax M3 plate reader (excitation = 560 nm, emission = 590 nm).

Micron-sized G1/G3 Zipper Aggregate Analysis

Micron-sized aggregates were observed when G1/G3 Zipper was mixed with serum containing media. To characterize the involvement of serum glycoproteins, 50 μ L of complete media or complete serum-free media (RPMI 1640 supplemented with 1% penicillin–streptomycin, L-glutamine 200 mM, 1% HEPES buffer) was mixed with 50 μ L of a PBS solution containing 10 μ M of G1/G3 Zipper. Immediately after pipetting the media and protein samples, kinetic turbidity measurements were collected as described above. Brightfield images of samples allowed to settle for 15 minutes were then collected using a Zeiss Axio Observer inverted microscope.

To determine if turbidity changes were due to specific glycoprotein-galectin interactions, 50 μ L PBS with or without 10 μ M G1/G3 Zipper was mixed 50 μ L of either serum-free complete media or complete media and incubated for 10 minutes. Then, samples were treated with an additional 50 μ L of PBS or PBS containing 100 mM β -lactose before sample turbidity was measured via absorbance at 420 nm.

To determine the dependence of aggregation on serum glycoprotein concentration, complete media was diluted with complete serum-free media to 80%, 60%, 40%, 20%, 10%, 1%, 0.5%, and 0.1% serum. 50 μ L of each media type mixed with 50 μ L of PBS containing 10 μ M of G1/G3 protein was allowed to incubate for 10 minutes, after which absorbance at 420 nm was measured.

To observe if specific serum glycoproteins interacted with G1/G3 Zipper, haptoglobin, α -2-macroglobulin, or human serum albumin were diluted in PBS (0.2 and 0.4 mg/mL) and mixed with 50 μ L of 10 μ M protein and measured as described above.

Statistical Analysis

All experimental and control groups had at least $n = 4$ for binding affinity, Jurkat T cell apoptosis studies, and Jurkat T cell metabolic activity studies. A representative graph was shown for binding affinity studies. Data was analysed for statistically significant differences using one-way ANOVA with Tukey's post hoc ($p = 0.05$) or two-tailed t test ($p = 0.05$) in GraphPad Prism software.

Protein sequences

G1—MASGLVASNLNLKPGESLRVRGEVAPDAKSFVLNLGKD
SNNLCLHFNPRFNAHGDANTIVCNSKDGGAWGTEQREAVF
PFQPGSVAEVSITFDQANLTVKLDPGYEFKFPNRLNLEAINY
MAVDGDFKIKSVAFD

G1/G3—MASGLVASNLNLKPGESLRVRGEVAPDAKSFVLNLG
KDSNNLCLHFNPRFNAHGDANTIVCNSKDGGAWGTEQREA
VFPPQPGSVAEVSITFDQANLTVKLDPGYEFKFPNRLNLEAI
NYMAADGDFKIKSVAFDGSGGGSGGSGGGSGGEFADNFS
LH
DALSGSGNPXQGWPGAWGNQPAGAGGYPGASYPGAYPG
QAPPGAYPGQAPPGAYPGAPGAYPGAPAGVYPGPPSGPGA

YPSSGQPSAPGAYPATGPYGAPAGPLIVPYNLPLPGGVVPR
MLITILGTVKPNANRIALDFQRGNDVAFHFNPRFNENRRRV
IVCNTKLDNNWGREERQSVFPFESGKPFKIQLVEPDHFKV
AVNDAHLLQYNHRVKKLNEISKLGISGDIDLTSASYNMILE
HHHHHH

G1/G3 Zipper—MASGLVASNLNLKPGESLRVRGEVAPDAKSFV
LNLGKDSNNLCLHFNPRFNNAHGDANTIVCNSKDGGAWGTE
QREAVFPFQPGSVAEVSITFDQANLTVKLPDGYEFKFPNRL
NLEAINYMAADGDFKIKSVAFDGSGGGSGGGSGGSGGMARM
KQLEDKVEELLSKNYHLENEVARLKKLVGERGGGGSGGGSGG
GGSGGGSGGEFADNFSLHDAALSGSGNPNXQGWPGAWGNQPA
GAGGYPGASYPGAYPGQAPPGAYPGQAPPGAYPGAPGAYP
GAPAPGVYPGPPSGPGAYPSSGQPSAPGAYPATGPYGAPAG
PLIVPYNLPLPGGVVPRMLITILGTVKPNANRIALDFQRGND
VAFHFNPRFNENRRRVIVCNTKLDNNWGREERQSVFPFESG
KPFKIQLVEPDHFKVAVNDAHLLQYNHRVKKLNEISKLGI
SGDIDLTSASYNMILEHHHHHH

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Schematic representation of the wild-type reversible galectin-1 homodimer (“G1”), a stable G1 homodimer formed by chemical crosslinking with poly(ethylene glycol) (“G1-PEG-G1”), a recombinant fusion of G1 linked to the N-terminus of G3 (“G1/G3”), and a recombinant fusion of G1 linked to G3 via a peptide that forms a two-stranded α -helical coiled-coil (“G1/G3 Zipper”).

Fig. 2.

Characterization of the physical properties of G1/G3 and G1/G3 Zipper. (A) SDS-PAGE gel of G1/G3, G1/G3 Zipper, and G1. (B) SEC traces of G1/G3 and G1/G3 Zipper. Table: approximate molecular weights estimated from SEC elution volume. MW_r is theoretical molecular weight and MW_o is the observed molecular weight. (C) DLS histograms for G1/G3 and G1/G3 Zipper (number-weighted size distribution).

Fig. 3. Carbohydrate binding and glycoprotein crosslinking properties of G1 variants and G1/G3 fusions. (A) Lactose affinity chromatography traces for G1, G1-PEG-G1, G1/G3, and G1/G3 Zipper. Kinetic turbidity measurements of the formation of insoluble asialofetuin (ASF) aggregates via (B) G1 or G1-PEG-G1 and (C) G1/G3 or G1/G3 Zipper.

Fig. 4. Jurkat T cell agglutination and metabolic activity. Brightfield micrographs of Jurkat T cells after 18 h treatment with (A) 5 μ M or (B) 0.5 μ M G1, G1-PEG-G1, G1/G3, or G1/G3 Zipper. * in the micrographs denotes cell agglutination. (C) Metabolic activity (normalized to untreated cells) of Jurkat T cells treated with 5 or 0.5 μ M G1, G1-PEG-G1, G1/G3, or G1/G3 Zipper for 18 h. * denotes significant difference between concentrations, & denotes significant difference compared to G1/G3 Zipper, ^ denotes significant difference compared to untreated control (dashed line). ns= no significance, one symbol = $p < 0.05$, two symbols = $p < 0.01$, three symbols = $p < 0.001$, four symbols = $p < 0.0001$. Scale bars = 50 μ m in (A) and (B).

Fig. 5. Phosphatidylserine exposure determined via Annexin V-FITC staining (green) and membrane permeability determined via propidium iodide (PI) (red) after Jurkat T cells were treated with (A) 5 μ M or (B) 0.5 μ M G1, G1-PEG-G1, G1/G3, or G1/G3 Zipper for 4 h. Brightfield images in top row are representative of extent of agglutination before treatment with β -lactose. * in the micrographs denotes cell agglutination. Single channel fluorescence images are available in Supplementary Information Fig. 5. Scale bars = 50 μ m.

Fig. 6. Micron-sized particulates formed via G1/G3 Zipper in serum-containing culture media. (A) Kinetic turbidity measurements of the formation of insoluble aggregates in mixtures of media and G1/G3 Zipper with or without serum (FBS). (B) Brightfield micrographs of aggregates formed in mixtures of 5 μ M G1/G3 Zipper and media with 10% or 1% serum. (C) Turbidity end-point measurements of solutions containing 5 μ M G1/G3 Zipper with or without serum and with or without the addition of β -lactose as a binding inhibitor. (D) Turbidity end-point measurements of solutions containing decreasing amounts of FBS with or without 5 μ M G1/G3 Zipper. (E) Turbidity end-point measurements of solutions containing 5 μ M G1/G3 Zipper with or without FBS from 0–100%. In (C), **** denotes $p < 0.0001$ compared to all other groups. In (D) ns= no significance, **** denotes $p < 0.0001$ compared to indicated group. Scale bars = 50 μ m.

Fig. 7. G1/G3 Zipper interaction with various serum proteins. (A) Turbidity end-point measurements of solutions containing 5 μM G1/G3 Zipper and varying concentrations of haptoglobin, α-2-macroglobulin, or human serum albumin II (HSA II). Brightfield micrographs of 5 μM G1/G3 Zipper and 0.4 mg/mL serum protein. ns= no significance, *** = $p < 0.001$, **** = $p < 0.0001$. Scale bars = 50 μm in (B).

Fig. 8. Extracellular activity of G1/G3 Zipper in serum-free media. Brightfield micrographs of Jurkat T cells after 18 h treatment with (A) 5 μ M or (B) 0.5 μ M G1, G1-PEG-G1, or G1/G3 Zipper. (C) Metabolic activity (normalized to untreated cells) of Jurkat T cells treated with 5 or 0.5 μ M G1, G1-PEG-G1, or G1/G3 Zipper for 18 h. * denotes significant difference between concentrations, & denotes significant difference compared to G1/G3 Zipper, ^ denotes significant difference compared to untreated control (dashed line). One symbol = $p < 0.05$, two symbols = $p < 0.01$, three symbols = $p < 0.001$, four symbols = $p < 0.0001$. Scale bars = 50 μ m in (A) and (B).