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Galectin-9 binds to *O*-glycans on protein disulfide isomerase

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Abstract

Changes in the T cell surface redox environment regulate critical cell functions, such as cell migration, viral entry and cytokine production. Cell surface protein disulfide isomerase (PDI) contributes to the regulation of T cell surface redox status. Cell surface PDI can be released into the extracellular milieu or can be internalized by T cells. We have found that galectin-9, a soluble lectin expressed by T cells, endothelial cells and dendritic cells, binds to and retains PDI on the cell surface. While endogenous galectin-9 is not required for basal cell surface PDI expression, exogenous galectin-9 mediated retention of cell surface PDI shifted the disulfide/thiol equilibrium on the T cell surface. *O*-glycans on PDI are required for galectin-9 binding, and PDI recognition appears to be specific for galectin-9, as galectin-1 and galectin-3 do not bind PDI. Galectin-9 is widely expressed by immune and endothelial cells in inflamed tissues, suggesting that T cells would be exposed to abundant galectin-9, in *cis* and in *trans*, in infectious or autoimmune conditions.

Key words: galectin-9, *O*-glycans, protein disulfide isomerase

Introduction

The cell surface redox status of T cells is dynamic, and changes in T cell surface redox status influence critical functions, including T cell activation and proliferation induced by dendritic cells, T cell adhesion and migration via integrins, and T cell susceptibility to viral pathogens such as HIV (Markovic et al. 2004; Stantchev et al. 2012; Hahm et al. 2013; Cerutti et al. 2014). Redox status is regulated by redox buffer systems based on thiol/disulfide exchange (Hurd et al. 2012; Gostner et al. 2013), and is controlled by both cell-intrinsic and cell-extrinsic factors. For example, the reducing environment produced by dendritic cells that promotes T cell activation and proliferation results from dendritic cell secretion of cysteine into the extracellular microenvironment, a cell-extrinsic effect (Lanzavecchia and Sallusto 2001; Angelini et al. 2002; Yan et al. 2009). In contrast, oxidoreductases present on the plasma membrane of lymphocytes and platelets are a cell-intrinsic mechanism of regulating cell surface redox status.

Both thioredoxin (Trx) and protein disulfide isomerase (PDI), members of the PDI superfamily, have been detected on the plasma membrane (Yoshimori et al. 1990; Chen et al. 1995; Turano et al. 2002; Bi et al. 2011; Hahm et al. 2013; Soderberg et al. 2013; Lasecka and Baron 2014). While PDI in the endoplasmic reticulum

(ER) is an oxidase, creating disulfide bonds in nascent proteins, cell surface PDI facilitates cleavage of disulfide bonds by thiol/disulfide exchange (Appenzeller-Herzog and Ellgaard 2008; Schulman et al. 2016). Cleavage of disulfide bonds on cell surface proteins may alter the function of the target proteins. For example, the T cell surface glycoprotein CD4 functions as an immune co-receptor when dimerized via a disulfide bond, but in the reduced form promotes HIV entry after viral gp120 binding (Cerutti et al. 2014). Similarly, the ability of cell surface integrins to regulate leukocyte migration is regulated by redox status (Bi et al. 2011).

The C-terminus of PDI contains a KDEL retention motif mechanism that retains PDI in the ER. However, while PDI reaches the plasma membrane via both classical and non-classical secretion pathways, little is known about how PDI is retained at the plasma membrane, or the specific events that trigger cell surface PDI localization (Rubartelli et al. 1992; Yi and Khosla 2016; Araujo et al. 2017). The KDEL sequence is present on cell surface PDI but is not required for plasma membrane retention (Yoshimori et al. 1990; Terada et al. 1995).

We previously found that galectin-9 binding to the surface of specific T cell subsets increased the abundance of cell surface PDI and increased free thiols on plasma membrane proteins. The galectin-9 mediated increase in cell surface PDI facilitated integrin-mediated migration, as well as entry of HIV into T cells, consistent with effects of reducing disulfide bonds in CD61 and CD4, respectively (Bi et al. 2011). Galectin-9, produced by dendritic cells, macrophages, endothelial cells, B cells and T cells, can bind both *N*- and *O*-linked glycans on glycoproteins (Bi et al. 2008; John and Mishra

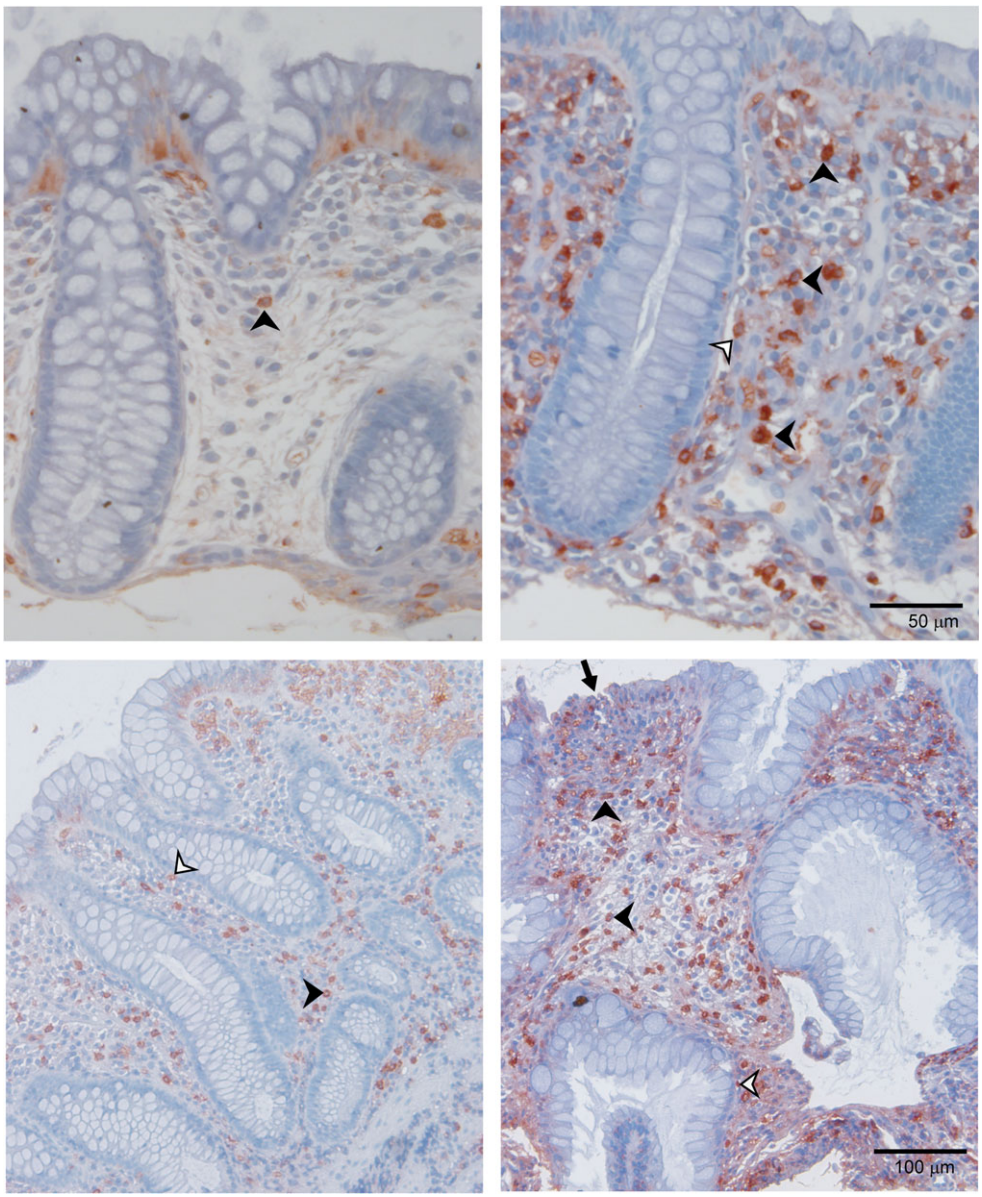

Fig. 1. Galectin-9 protein is increased in inflamed tissue, detected by immunohistochemistry. Minimal galectin-9 is seen in the lamina propria of normal colon (top left), while there are increased numbers of immune cells in the lamina propria in tissue from a patient infected with HIV (top left), many of which express galectin-9 (black arrowhead). Galectin-9 was also detected in endothelial cells lining small vessels (white arrowhead). A similar pattern was seen in tissue from a patient with UC, both in areas that were not grossly ulcerated (bottom left) as well as an ulcerated lesion (arrow) (bottom right). This figure is available in black and white in print and in color at *Glycobiology* online.

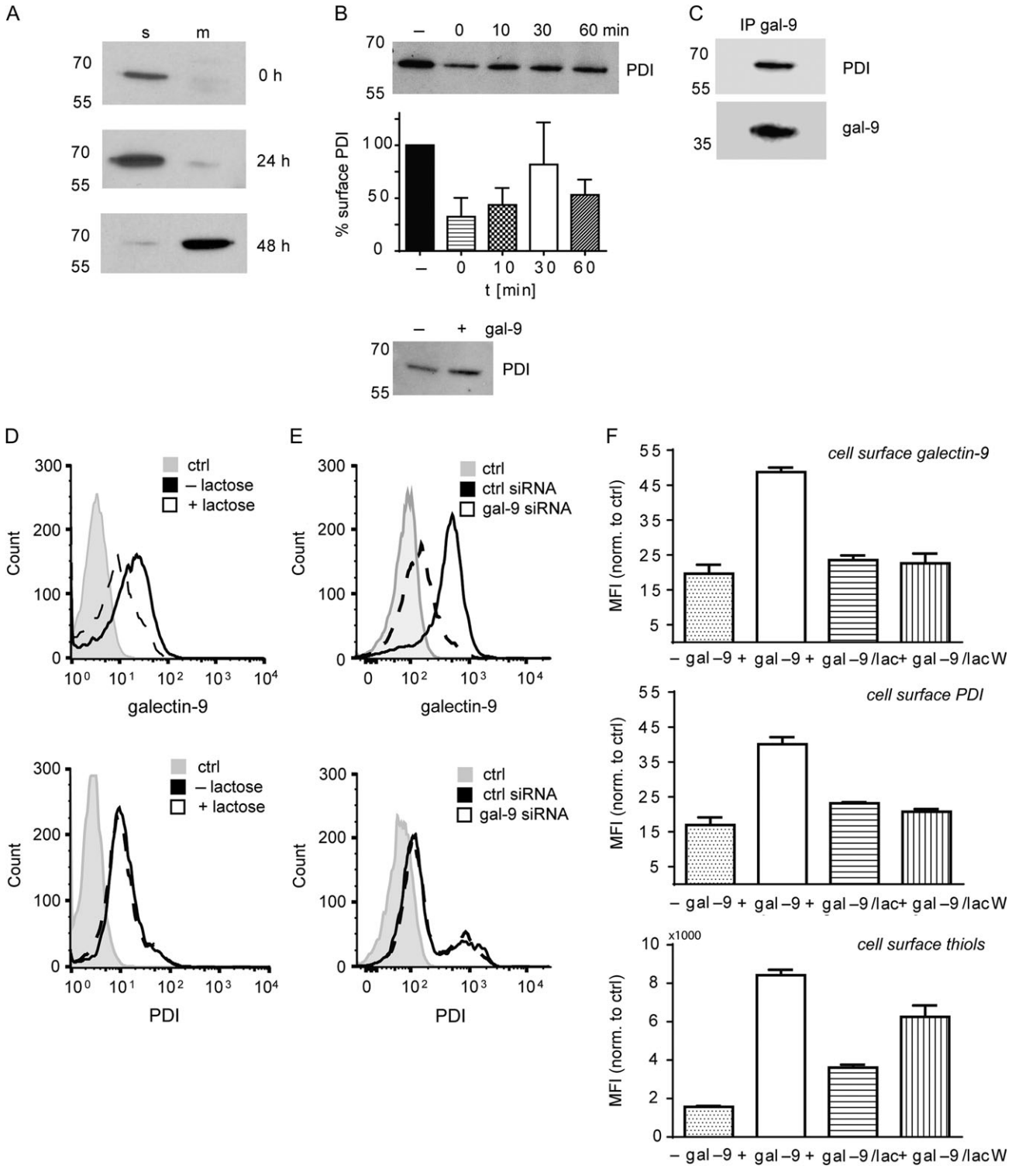

Fig. 2. Galectin-9 retains PDI on the cell surface. (**A**) PDI is shed from the T cell surface. T cells were biotinylated to label cell surface proteins and biotinylated PDI precipitated from whole cell lysate (surface, s) and from the culture media (m) at the indicated time points and detected by immunoblotting. (**B**) PDI is internalized from the T cell surface. T cells were biotinylated and cell surface biotin cleaved at the indicated points. Internalized biotin-PDI was precipitated from cell lysates and detected by immunoblotting (top panel). The mean ± SEM of the fraction of internalized PDI from three independent experiments is shown; total biotin-labeled cell surface PDI (–) (middle panel). Galectin-9 binding to biotin-labeled cells increases the amount of cell surface PDI in the cell lysate (bottom panel). (**C**) Exogenous galectin-9 binding to cell surface PDI. Galectin-9 was added to T cells and cell surface proteins cross-linked prior to lysis. Immunoprecipitation with anti-galectin-9 precipitated both galectin-9 and PDI. (**D**) Basal level of T cell surface PDI is carbohydrate independent. Top: MOLT-4 T cells were incubated at 37°C for 1 h with (+) or without (–) 100 mM lactose and cell surface galectin-9 and PDI detected by flow cytometry. Filled histograms are isotype controls. Lactose incubation reduced endogenous gal-9 on the cell surface, while basal PDI levels were unaffected (bottom). (**E**) Basal level of T cell surface PDI does not require endogenous galectin-9. siRNA targeting galectin-9 reduced cell surface galectin-9 (top) but had no effect on cell surface PDI (bottom).

2016; Thiemann and Baum 2016). Here we show that PDI bears *O*-glycans that are required for galectin-9 binding.

Results

Galectin-9 is increased in inflamed tissue

Prior work has identified galectin-9 expression in vitro in many cell types, including eosinophils, T lymphocytes, dendritic cells, macrophages, intestinal epithelial cells and vascular endothelial cells (John and Mishra 2016). Increased galectin-9 expression has been described in viral infection and autoimmune conditions such as ulcerative colitis (UC) (Nebbia et al. 2012; Shi et al. 2012; Chagan-Yasutan et al. 2013; Kared et al. 2013; Katoh et al. 2014; Tandon et al. 2014). To examine the distribution of galectin-9 expression in inflamed human tissue, we examined colon biopsies from patients infected with HIV, with UC or normal controls. As shown in Figure 1, minimal galectin-9 was detected in the lamina propria of normal human colon (top left). However, in an individual infected with HIV (top right), there was abundant galectin-9 present in immune cells infiltrating the lamina propria between the intestinal glands, as well as in endothelial cells lining small vessels. The amount of galectin-9 detected was qualitatively similar in all biopsies from the HIV-infected patient, indicating that galectin-9 expression was generally up-regulated. We also examined biopsies from a patient with UC. Galectin-9 was abundant in the tissue biopsied in a region of active ulcerative disease (bottom right) but was also abundant in distant tissue that was considered grossly normal by the endoscopist (bottom left). Again, this indicates general up-regulation of galectin-9 expression in inflamed tissues, and that, in vivo, immune cells will be exposed to both endogenous galectin-9 as well as exogenous galectin-9 released from neighboring cells in inflamed tissue.

Release and internalization of cell surface PDI

While PDI traffics to the T cell plasma membrane, little is known about the fate of cell surface PDI (Yoshimori et al. 1990; Chen et al. 1995; Terada et al. 1995; Bi et al. 2011; Wan et al. 2012; Hahm et al. 2013; Lasecka and Baron 2014). We labeled the surface of human T cells with biotin to determine if biotinylated PDI was released from the plasma membrane into the culture media, and/or was internalized into the cells. As shown in Figure 2A, we detected soluble biotinylated PDI in the culture medium between 24 h and 48 h after biotinylation, indicating that a fraction of cell surface PDI was shed from the cell surface, as has been shown for endothelial cells (Rubartelli et al. 1992; Araujo et al. 2017). Cell death did not appear to be involved in release of cell surface PDI, as we detected no increase in cell death over the time course of the experiment (data not shown).

To examine internalization, cell surface proteins were labeled with reversible EZ-link sulfo-NHS-SS-biotin. Cell surface biotin was cleaved with sodium 2-mercaptoethane sulfonate (MesNa) at the indicated times, and intracellular biotinylated PDI examined (Figure 2B, top and middle panels). We detected intracellular biotin-labeled PDI by 10 min after cleavage, and the amount of intracellular labeled PDI appeared to increase with time after cleavage, indicating that a fraction of cell surface PDI was internalized. In addition, when we added galectin-9 to the labeled cells and assessed total biotinylated PDI, galectin-9 increased the total amount of labeled PDI (Figure 2B, bottom panel). Thus, while PDI is present on the T cell plasma membrane, our findings in Figure 2A and B indicates that there is a turnover of cell surface PDI, both by release from the plasma membrane as well as internalization.

Galectin-9 binds to PDI on the cell surface

We previously found that exogenous galectin-9 binding to T cells increased cell surface PDI in a carbohydrate-dependent manner (Bi et al. 2011). To directly ask if galectin-9 binds to cell surface PDI, we added galectin-9 to cells for 1 h and cross-linked cell surface proteins prior to cell lysis. We immunoprecipitated galectin-9 and probed for PDI (Figure 2C), demonstrating that galectin-9 directly binds PDI on the T cell surface.

To ask if baseline retention of PDI on the T cells surface is carbohydrate dependent, cells were treated with lactose; this reduced the amount of endogenous galectin-9 on the cell surface but did not affect the basal level of PDI on the cell surface (Figure 2D). Similarly, siRNA-mediated reduction of endogenous galectin-9 expression in the T cells reduced the amount of cell surface galectin-9, but did not affect the basal level of cell surface PDI (Figure 2E). Thus, basal retention of PDI on the T cell surface does not appear to be carbohydrate dependent nor influenced by the level of endogenous galectin-9.

However, as we have previously demonstrated, addition of exogenous galectin-9 increased the amount of PDI on the surface of T cells (Figure 2F). Concomitant addition of lactose inhibited binding of galectin-9 to the cells and the subsequent increase in PDI, and bound galectin-9 was reduced by addition of lactose after 1 h, which also resulted in return of cell surface PDI to the basal level. Addition of exogenous galectin-9 to T cells also increased the abundance of thiol groups on cell surface proteins. Importantly, while an increase in cell surface thiols was not observed when galectin-9 was added in the presence of lactose, the increased abundance of cell surface thiols remained when bound galectin-9 was removed by lactose washing after 1 h. Unlike the increase in cell surface PDI that required continuous galectin-9 binding, the increase in cell surface thiols persisted when exogenous galectin-9 was removed. In toto, the findings in Figure 2 suggest that exogenous galectin-9 retains cell surface PDI, and that the retained PDI increased the abundance of free thiol groups on cell surface proteins, an effect that persisted after the exogenous galectin-9 was removed.

Galectin-9 binds *O*-glycans on HA-PDI

Galectin-9 is a carbohydrate binding protein with a preference for extended LacNAc repeats on either *N*- and *O*-glycans (Hirabayashi et al. 2002; Nagae et al. 2006, 2008; Venkataraman et al. 2015). The amino acid sequence of PDI does not contain any canonical *N*-glycosylation sequons, implying that galectin-9 binding to PDI involves recognition of *O*-glycans. To directly ask if PDI is *O*-glycosylated, we expressed HA-tagged PDI in T cells and in *Escherichia coli*, that lack the machinery to glycosylate mammalian proteins, and purified

Isotype control (gray filled histograms), control siRNA (black), gal-9 siRNA (dotted). (**F**) Galectin-9 mediated increase in cell surface thiols persists after removal of galectin-9. Top: Exogenous galectin-9 binds T cells, but binding is reduced in the presence of lactose added at time 0 or after 1 h. Middle: Exogenous galectin-9 increases cell surface PDI, but the increase is reversed by addition of lactose at time 0 or at 1 h. Bottom: Exogenous galectin-9 increases the abundance of cell surface thiols. An increase in maleimide labeling is not seen if galectin-9 is added in the presence of lactose (+gal-9/lac), but persists when galectin-9 is removed by lactose wash after 2 h (+gal-9/lacW). Results are presented as mean fluorescence (MFI) normalized to isotype control.

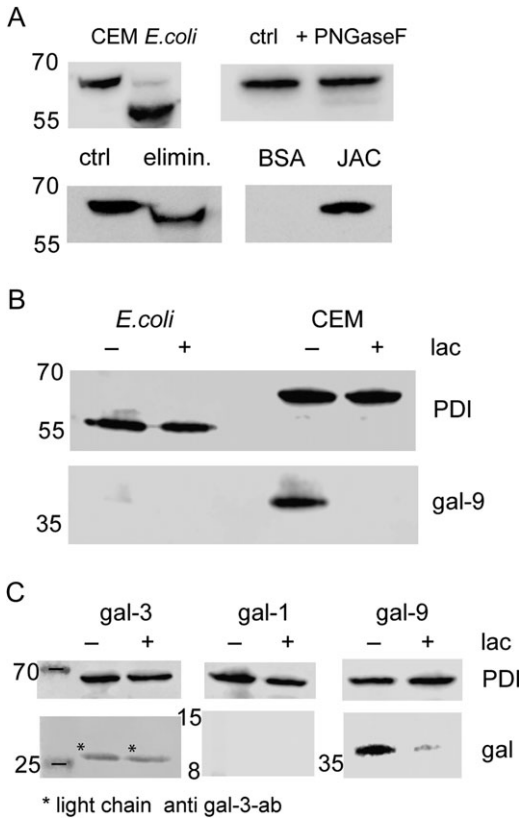

Fig. 3. HA-PDI bears *O*-glycans and is specifically bound by galectin-9. (**A**) Top left: HA-PDI expressed in T cells vs. HA-PDI expressed in *E. coli*. The Mr of HA-PDI expressed in *E. coli* is approximately equal to the predicted MW of the HA-PDI polypeptide, 57 kD. The increased Mr of HA-PDI expressed in T cells suggests addition of glycans. Top right: Treatment with PNGase F did not alter the Mr of HA-PDI expressed in T cells. Bottom left: β-elimination decreased the Mr of HA-PDI expressed in T cells, indicating at least partial loss of *O*-glycans. Bottom right: HA-PDI was precipitated by Jacalin (JAC), a lectin that recognized *O*-glycans, and detected by immunoblotting; BSA, control. (**B**) HA-PDI expressed in T cells or *E. coli* bound to HA-agarose was incubated with gal-9 (GalPharma) in the presence (+) or absence (–) of lactose and bound galectin-9 detected by immunoblotting. No binding to HA-PDI produced in *E. coli* was detected. (**C**) Galectin-9 specifically binds HA-PDI. HA-PDI expressed in T cells bound to anti-HA-agarose was incubated with gal-9 (GalPharma), gal-3, or gal-1 in the presence (+) or absence (–) of lactose. HA-PDI and bound galectin were identified by immunoblotting. Galectin-9, but not galectin-3 or galectin-1, bound HA-PDI. Only galectin-9 specifically binds HA-PDI.

HA-PDI on an anti-HA affinity resin (Figure 3A). HA-PDI produced in bacteria migrated on SDS-PAGE at the expected Mr of ~57 kD, based on amino acid sequence, while PDI produced in T cells migrated at ~68 kD, indicating significant post-translational modification. HA-PDI expressed in T cells was treated with PNGase F to remove high mannose, hybrid and complex *N*-glycans; however, PNGase F treatment did not alter the relative mobility of HA-PDI. In contrast, β-elimination of *O*-glycans increased the relative mobility of HA-PDI produced in T cells, although not to the degree seen with PDI produced in *E. coli*, suggesting incomplete *O*-glycan removal by β-elimination. Moreover, the plant lectin jacalin (JAC), that binds both sialylated and asialo core 1 *O*-glycans, bound to HA-PDI produced in T cells. Those glycans on PDI are required for galectin-9 binding was demonstrated by the lack of galectin-9 binding to PDI expressed in *E. coli* (Figure 3B).

Different galectins specifically bind unique sets of glycoprotein counterreceptors (Pace et al. 1999; Daniels et al. 2002; Zhu et al. 2005; Stillman et al. 2006; Bi et al. 2008, 2011; Niki et al. 2009; Clark and Baum 2012; Wu et al. 2014). We had identified PDI as a T cell surface receptor for galectin-9, while PDI was not identified as a T cell membrane glycoprotein that bound either galectin-1 or galectin-3. To directly detect binding of PDI to galectin-9, we added galectin-9 to HA-PDI immobilized on agarose beads in the presence or absence of lactose. Galectin-9 bound to HA-PDI in the absence of lactose, while lactose inhibition demonstrated that galectin-9 binding was carbohydrate dependent. Furthermore, we detected no binding of galectin-1 or galectin-3 to immobilized HA-PDI, demonstrating the specific binding of galectin-9 (Figure 3C).

PDI is a 57 kDa soluble protein with two active thioredoxin-like domains, a and a', linked by two catalytically inactive domains, b and b' (Figure 4A) (Wang et al. 2013). PDI contains 35 Ser and Thr residues, distributed among the four domains. While recent work has shed light on the features of proteins that influence or control addition of *O*-glycans (Gill et al. 2011), it is not yet clear how generalizable these features will be, especially since much of this work has been done with extended or mucin-like polypeptides, rather than globular domains such as those of PDI (Kong et al. 2015; Revoredo et al. 2016). To further interrogate galectin-9 binding to PDI, we used surface plasmon resonance to examine the interaction of the two molecules, as well as the requirement for *O*-glycosylation. Galectin-9 was immobilized on a sensor chip and HA-PDI solutions at varying concentrations were applied to the chip at a flow rate of 10 μL/min. We first confirmed our qualitative finding in Figure 3B, demonstrating that HA-PDI produced in bacterial cells did not bind galectin-9, while we detected robust binding of HA-PDI expressed in T cells to immobilized galectin-9 (Figure 4).

An approach termed SimpleCells (Schjoldager et al. 2012) has been developed to identify sites of *O*-GalNAc addition to cellular proteins. Examining cells of neural and epithelial origin, this method identified a site of *O*-GalNAc addition on PDI at T442 (Steentoft et al. 2013). To ask if this site was important for galectin-9 binding to PDI, we mutated this threonine to alanine (T442A) and expressed the variant PDI in T cells. We compared binding of HA-PDI with and without the mutation at T442 to galectin-9 (Figure 4B and C). We detected no difference in galectin-9 binding to HA-PDI with or without a threonine residue at T442, even with quantitative surface plasmon resonance (SPR). This implied that, if this threonine residue were glycosylated, an *O*-glycan at this site would not contribute significantly to galectin-9 binding. Moreover, in examining the PDI model, T442 would be localized on domain a', close to C397 in the active site; this would likely not be a site of galectin-9 binding on the T cell surface that would promote PDI reductase activity.

Alternatively, we considered that galectin-9 might preferentially bind to *O*-glycans with extended LacNAc sequences on core 2 *O*-glycans, as these sequences are preferentially bound by galectin-1 (Galvan et al. 2000; Cabrera et al. 2006; Bi et al. 2008). To examine the potential role of core 2 *O*-glycans, we expressed HA-PDI in the human HH T cell line, that has a mutation in the C2GnT enzyme that initiates LacNAc addition to core 2 *O*-glycans (Galvan et al. 2000; Cabrera et al. 2006; Bi et al. 2008). We compared the binding of HA-PDI from HH cells to that of PDI from CEM cells that have abundant core 2 *O*-glycans (Figure 4B and C). Again, we saw no significant qualitative or quantitative differences in galectin-9 binding to HA-PDI from HH vs. CEM T cells, indicating that core 2 *O*-glycans on HA-PDI do not significantly contribute to binding.

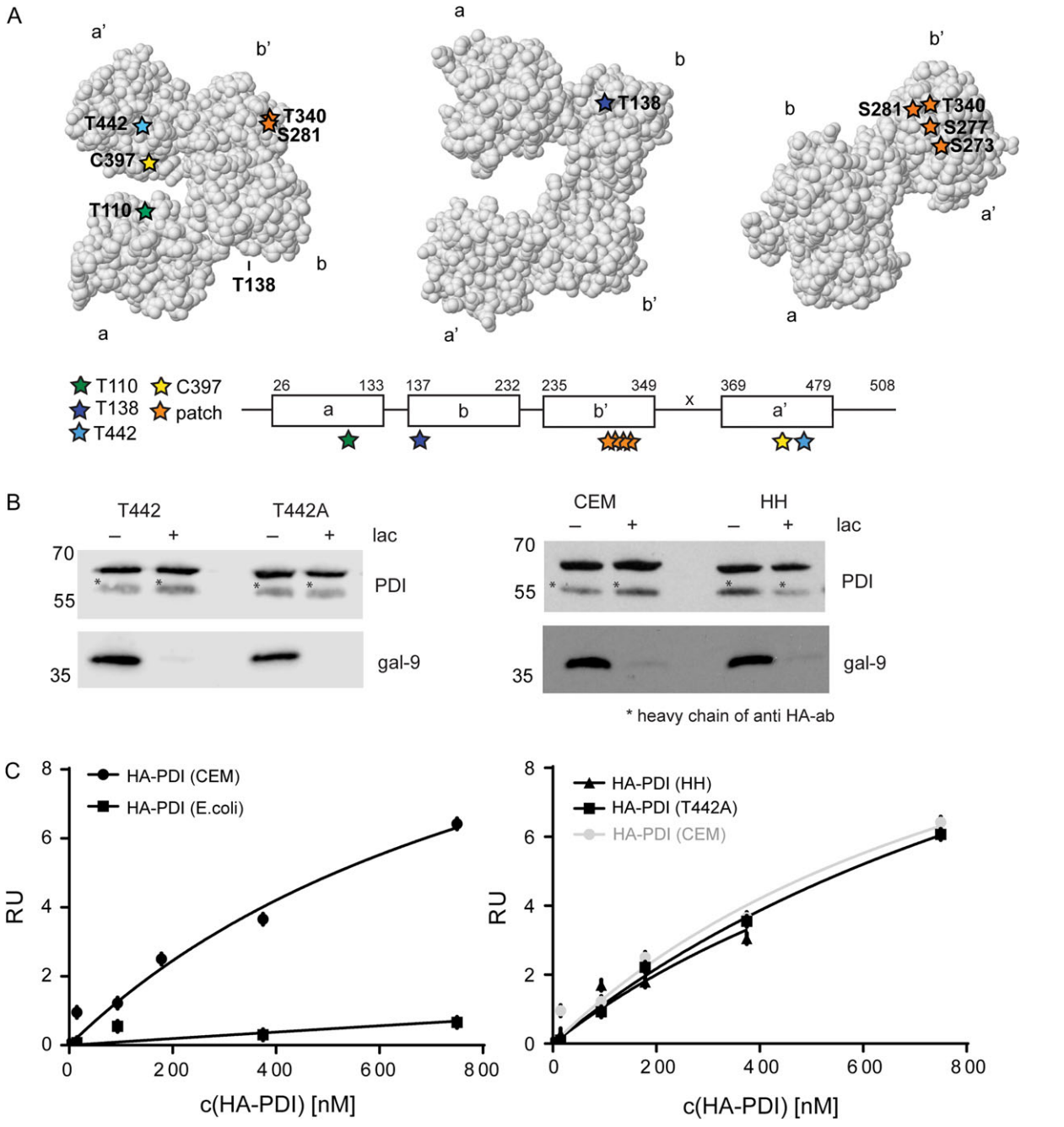

Fig. 4. *O*-glycans on PDI. (**A**) Mapping of potential O-glycosylation sites on PDI, identified in SimpleCells and predicted by Isoglyp and NEtOGlyc. While PDI was found to be O-glycosylated on T442 (turquoise) in the SimpleCell model, both prediction algorithms for mucin type *O*-glycans indicated potential O-glycan addition sites at T110 (green) and T138 (blue). T442 and T110 are located close to the active site, while T138 is in a cleft between a and b domains (180° rotation of PDI structure, middle). A patch of 4 serines and threonines (S273, S277, S281, T340, orange) on the backside of the b' domain is shown (right). Amino acids are mapped onto the reduced form of human PDI (pdb 4EKZ, visualized in JSmol). (**B**) Effect of *O*-glycans on galectin-9 binding to HA-PDI. Left: Mutation of T442 to T442A did not affect binding of galectin-9. Right: HA-PDI binding by galectin-9 does not require core 2 *O*-glycans. HH T cells have a mutation in C2GnT1 and produce reduced amounts of core 2 *O*-glycans compared to CEM cells. Galectin-9 bound to HA-PDI expressed in HH and CEM T cells in a carbohydrate dependent manner. (**C**) Binding of galectin-9 to HA-PDI analyzed by SPR. Galectin-9 was immobilized and HA-PDI binding, at the indicated concentrations, determined at a flow rate of 10 μL/min. Binding is depicted in response units (RU). Left: HA-PDI produced in T cells is bound by galectin-9, while binding to HA-PDI expressed in *E. coli* is not detected. Right: Binding of galectin-9 to HA-PDI made in HH cells or bearing the T442A mutation were identical to binding to HA-PDI made in CEM T cells (gray). Analysis by the one-site binding model gave similar K_D values for galectin-9 binding to HA-PDI (K_D = 1.0 μM (CEM), K_D = 1.4 μM (T442A), K_D = 1.1 μM (HH)). This figure is available in black and white in print and in color at *Glycobiology* online.

Discussion

The cell surface redox status is affected by several features, including the rate of turnover of different proteins at the cell surface, and these features may be influenced by the cell surface milieu. This dynamic quality allows cells to react to specific environmental changes. The amount of soluble PDI on the T cell surface at any given time is a balance of delivery of intracellular PDI to the plasma membrane with loss of cell surface PDI by release into the extracellular milieu or internalization (Figure 2). We found that T cell surface PDI increased with addition of exogenous galectin-9, suggesting that galectin-9 binds to and retains PDI that otherwise would have been lost from the cell surface. While we have previously shown that exogenous galectin-9 increases the amount of PDI on the T cell surface, we now demonstrate that the effect of retaining PDI, i.e., increased thiols on cell surface proteins, persists even after removal of exogenous galectin-9 (Figure 2). Thus, exposure of T cells to galectin-9 in vivo, in inflamed tissue (Figure 1), could have both immediate effects on cell migration or viral entry, as well as prolonged effects on T cell function, depending on the turnover of the modified cell surface proteins. While we have not identified other T cell surface reductases as receptors for galectin-9, we cannot exclude the possibility that galectin-9 may also be influencing the concentration or activity of other cell surface reductases, in addition to PDI.

Several studies have demonstrated the ability of galectins to retain glycoproteins on the cell surface. Galectin-9 retains glucose transporter 2 on the surface of pancreatic beta cells and clusters the glucagon receptor on hepatocytes (Ohtsubo et al. 2005; Johswich et al. 2014). Galectin-9 also clusters the T cell surface glycoprotein CD137, a member of the TNFR superfamily, on T cells to increase CD137 signaling and subsequent IFN-γ production (Imaizumi et al. 2007; Thijssen et al. 2008; Madireddi et al. 2014). Because galectin–glycan interactions are typically multivalent interactions, i.e., low affinity binding of individual carbohydrate recognition domains to glycan ligands with overall high avidity, galectin–glycan interactions can be dynamic and tunable (Thiemann and Baum 2016), and can be scaled up or down by changing cell surface glycans or by addition of glycan inhibitors. The changes in cell surface glycosylation seen during T cell development in the thymus, and during activation and proliferation of naïve T cells in the periphery, can result in changes in galectin binding and signaling (Gillespie et al. 1993; Galvan et al. 1998; Hernandez et al. 2007). Thus, galectin–glycan interactions may be well suited to regulate the dynamic nature of T cell surface redox status.

What regulates initiation of *O*-glycosylation and distribution of *O*-glycans? Unlike *N*-glycosylation initiation, there are no canonical *O*-glycosylation sequons; moreover, the existence of over twenty different polypeptide GalNAc transferases (GalNAc-Ts) that initiate *O*-GalNAc addition in a tissue-specific manner adds yet another layer of complexity (Gill et al. 2011; Gerken et al. 2013; Kong et al. 2015; Revoredo et al. 2016). Attempts to predict sites of *O*-glycosylation have largely used algorithms based on extended peptides, unlike the globular domains of PDI. We used the prediction programs ISOGlyP (http://isoglyp.utep.edu/index.php) and NEtOGlyc (http://www.cbs.dtu.dk/services/NetOGlyc/), which predicted *O*-glycosylation sites on PDI at T110 and T138. However, T138 is localized in a cleft between the a and b domains (Figure 4A), while T110, like T442, is found on the domain a close to the active site; *O*-glycans at these sites would likely not contribute to galectin-9 mediated retention of catalytically active PDI on the T cell surface.

Galectin-9 has been shown to bind either *N*-glycans, *O*-glycans, or both *N*- and *O*-glycans on counterreceptors (Ohtsubo et al. 2005; Oomizu et al. 2012; Madireddi et al. 2014). As we have clearly identified *O*-glycans on PDI and demonstrated that these *O*-glycans are essential for galectin-9 binding (Figures 3 and 4), where might the *O*-glycans that participate in galectin-9 binding and retention of the enzyme be localized? On folded proteins, *O*-glycans can be localized in patches (Cohen and Varki 2014). An *O*-glycan patch could provide multivalent ligands for galectin-9, resulting in high avidity binding. In fact, that core 2 *O*-glycans are not essential for galectin-9 binding of PDI (Figure 4) suggests that clustered short core 1 *O*-glycans may be sufficient for binding, as we have previously observed for galectin-1. Interestingly, the folded b' domain of PDI contains a patch of 4 serines and threonines on the back face that would likely not interfere with the catalytic activity (Figure 4A). This type of patch may provide the avidity needed for galectin-9 to bind.

Both PDI and galectin-9 have a wide range of functions, all of which are context-dependent. PDI in the ER makes disulfide bonds, while PDI on the cell surface reduces disulfide bonds (Jordan and Gibbins 2006; Appenzeller-Herzog and Ellgaard 2008; Wang et al. 2013). The outcomes of PDI activity, whether in the ER or on the cell surface, are pleiotropic and determined by the function of the protein PDI modifies. Even when PDI modifies the same protein in the same cell, e.g., CD4 in T cells, dimerization in the ER favors CD4 recognition of Class II major histocompatibility molecules and thus an effective immune response, while reduction of disulfide bonds on cell surface CD4 promotes HIV infection (Cerutti et al. 2014; Yi and Khosla 2016). Similarly, galectin-9 promotes expansion of regulatory T cells but contraction, i.e., death, of effector T cells (Golden-Mason and Rosen 2017). Galectin-9 has been characterized as a checkpoint inhibitor, turning off T cell responses to tumors, but loss of galectin-9 expression in hepatocellular carcinoma is associated with disease progression (An et al. 2016). Like PDI, galectin-9 promotes HIV entry into T cells, but also reduces viral replication in infected cells (Elahi et al. 2012; Abdel-Mohsen et al. 2016). Moreover, galectins are secreted molecules that can bind back to the cell after release or can bind to adjacent cells in a tissue. Thus, the galectin effects on a particular cell type will be influenced by galectins made by that cell and by galectins made by other cells in the vicinity, as well as by the dynamic glycosylation of the cell.

Materials and methods

Cell lines and reagents

Human CEM, MOLT-4 and HH T cell lines were cultured as described (Cabrera et al. 2006). Monoclonal antibodies: PDI, PDIRL90 (flow cytometry) and PDIRL77 (immunoblotting), Novus Biologicals; galectin-9, clone ECA42, (flow cytometry), MBL; galectin-1, clone 6C8.4–1, Thermo Fisher; galectin-3, clone M3/38, Biolegend; HA-antibody clone 2-2.2.14 and anti-HA-agarose, Pierce. Polyclonal antibodies: galectin-9, biotinylated (immunoblot), R&D; (immunohistochemistry), Novus Biologicals. Galectin-9 was purchased at R&D, if not noted otherwise.

PDI secretion and internalization

To assay secretion of endogenous PDI, 5×10^7 CEM cells were washed 3× with PBS. Freshly prepared EZ-link sulfo-NHS-biotin (Fisher Scientific) in PBS (0.75 mg/mL; 1 mL per 1.25×10^7 cells)

was added for 30 min at r.t. (Hausmann et al. 1995; Mendis et al. 2001). Cells were washed 2× with PBS and 1× with culture media. Biotinylated cells were suspended in 10 mL culture media and cell viability assayed by trypan blue exclusion to ensure that PDI release was not the result of cell lysis; cell viability was >97%. Cells (3×10^5 cells/mL) were incubated at 37°C for indicated times. Conditioned media was collected and cells lysed at the indicated time point and biotinylated proteins precipitated with 25 µg of SA-agarose for 2 h at 4°C.

Internalization of cell surface PDI was performed essentially as described, with cleavable EZ-Link-Sulfo-NHS-SS-biotin (Fisher) and sodium 2-mercaptoethanesulfonic acid (MesNA) (Altschuler et al. 2000; Hasenauer et al. 2013). About 1×10^7 cells per time point were biotinylated at pH8.0 for 30 min at 4°C, washed 3× with PBS and stored on ice. Cell surface biotin on the cell surface was stripped 3× with 1 mL ice-cold 100 mM MesNA in 50 mM Trizma-HCl buffer (100 mM NaCl, 1 mM EDTA, 0.2% BSA, pH8.6) for 20 min on ice. Excess MesNA was quenched with 1 mL ice-cold 120 mM iodoacetamide in PBS for 10 min on ice. Cells were lysed in 1% NP-40 lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM PMSF, 1 µg/mL aprotinin, 1 µg/mL leupeptin, pH7.4) and biotinylated proteins were precipitated with SA-agarose.

Co-immunprecipitation of galectin-9 and PDI

About 2×10^7 MOLT-4 T cells were washed 2× with PBS, pH7.4 and incubated in 2 mL 0.1 µM galectin-9 in PBS for 1 h at 37°C. Cell surface receptors were fixed with 2 mM DTSSP (end concentration) for 30 min at r.t., and the reaction neutralized with 1 M Tris pH7.5 for 15 min at r.t. Cells were washed with PBS pH7.4, and lysed in 1% NP-40 lysis buffer. Galectin-9 complexes were precipitated with 25 µg of anti-galectin-9 agarose prepared with SA-agarose and biotinylated anti-galectin-9 antibody.

Flow cytometry

About 3×10^5 cells were incubated in the presence or absence of 0.03 µM galectin-9, with or without 100 mM lactose, for 1 h at 37°C. Bound galectin-9 was removed at 1 h with 100 mM lactose for 10 min. Cells were washed with PBS pH7.4 and were fixed with 4% *p*-formaldehyde. Fixed cells were stained for cell surface galectin-9 and PDI with antibodies at concentrations recommended by the manufacturer. Thiol levels were determined after 2 h with 5 µM AlexaFluor-594 C5 maleimide as previously described (Sahaf et al. 2003). Flow cytometry was performed on a BD Biosciences LSR II and BD Scan cytometer and data analyzed with BD FACSDiva and FlowJo software.

siRNA knockdown of galectin-9

1 µM galectin-9 siRNA or control siRNA-A (Santa Cruz Biotechnology) were added to 2×10^6 MOLT-4 T cells in 100 µL nucleofector solution L and electroporated using AMAXA nucleofector (Lonza) with program C-005, per the manufacturer's protocol. After 24 h, loss of cell surface galectin-9 and PDI determined by flow cytometry.

Expression and isolation of HA-tagged PDI in human T cell lines and *E. coli*

About 2×10^6 cells T cells were transfected with 2 µg HA-PDI plasmid cDNA (kind gift of LW Ruddock) using AMAXA nucleofector device nucleofector kit C with program X-001 (CEM) and kit V with program O-012 (HH) following the manufacturer's protocol (Lonza). Cells were incubated at 37°C for 48 h. To generate HA-PDI in *E. coli*, the HA-PDI construct was expressed in BL21(DE3) adapted from Pace et al. (2003). Briefly, a single colony of transformed BL21(DE3) was cultured overnight at 37°C in LB-ampicillin, and protein expression was induced with 10 mM IPTG at OD_{600} = 0.6. Bacteria were cultured at 37°C for 4 h, and lysed in B-PER lysis buffer. HA-PDI for glycan analysis by mass spectrometry and for SPR experiments was immunoprecipitated with anti-HA-agarose for overnight at 4°C. HA-PDI was eluted with 3 M NaSCN on spin columns (Pierce) and dialyzed (MWCO 10,000) into 20 mM Tris, pH7.2 for MS analysis, or into PBS pH7.2 for SPR experiments, and stored at −80°C.

PNGase F digest, β-elimination and Jacalin precipitation of HA-PDI

1 µg isolated HA-PDI in PBS was denatured at 100°C for 10 min and incubated with PNGase F (New England Biolabs) for 1 h at 37°C following the manufacturer's protocol. Potential *O*-glycans on 2 µg HA-PDI were removed using a β-elimination kit (Sigma) following the manufacturer's protocol. HA-PDI was precipitated with biotinylated Jacalin (biotinylated BSA as control, Vector labs) bound to 20 µL SA-agarose at 4°C overnight.

Galectin binding assay

HA-PDI was transfected into T cells. Cells were lysed into 1% NP-40 lysis buffer, and 200 µg of whole cell lysate proteins was incubated with 30 µL anti-HA-agarose at 4°C for 2 h. After washing, the indicated galectin was added in excess (500 µM) in the presence or absence of (50 mM) lactose and incubated overnight at 4°C.

HA-PDI mutation

A point mutation changing T442 to A442 was introduced with: 5′-ctt aaa acc atc cag cgc gcg ttc ccc gtt gta at-3′ and 5′-att aca acg ggg aac gcg cgc tgg atg gtt ttt aag-3′, using QuikChange Site-directed Mutagenesis kit (Agilent). The mutation was verified by DNA sequencing on a 3730 Capillary DNA Analyzer (Applied Biosystems) using the primer: 5′-aac gtc ttt gtg gag ttc tat gc-3′.

Surface plasmon resonance

Binding of HA-PDI to immobilized galectin-9 was performed on a BIAcore T100 (GE Healthcare). Of the case, 2833 RU of galectin-9 (R&D) was immobilized on a CM5 sensor chip at 5 µL/min at 25°C. About 0–750 nM HA-PDI (made in CEM, T442A CEM, or *E. coli*) or 0–375 nM HA-PDI (made in HH) in 10 mM HEPES (150 mM NaCl, 0.05% Tween 20, pH7.4) were flowed over the surface at 10 µL/min for 3 min. The chip surface was regenerated with 10 mM glycine HCl, pH2.5/3 M NaCl for 30 s. Data were analyzed using BIAcore evaluation software, K_D determined using a one-site binding model and graphs plotted in Prism.

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Conflict of interest statement

None declared.

Abbreviations

gal, galectin; GalNac, *N*-acetylgalactosamine; HA-PDI, HA-tagged protein disulfide isomerase; HIV, human immunodeficiency virus; IFN-γ, interferon gamma; lac, lactose; LacNAc, *N*-acteyl-D-lactosamine; MesNa, sodium methanethiolate; MFI, mean fluorescence index; PDI, protein disulfide isomerase; PMSF, phenylmethylsulfonyl fluoride; SPR, surface plasmon resonance; TNFR, tumor necrosis factor receptor; Trx, thioredoxin

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