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Galectin-1 Binds Different CD43 Glycoforms to Cluster CD43 and Regulate T Cell Death

Joseph D. Hernandez,* Julie T. Nguyen,* Jiale He,* Wei Wang,† Blair Ardman,§ Jonathan M. Green,¶ Minoru Fukuda,‖ and Linda G. Baum2*‡

Galectin-1 kills immature thymocytes and activated peripheral T cells by binding to glycans on T cell glycoproteins including CD7, CD45, and CD43. Although roles for CD7 and CD45 in regulating galectin-1-induced death have been described, the requirement for CD43 remains unknown. We describe a novel role for CD43 in galectin-1-induced death, and the effects of O-glycan modification on galectin-1 binding to CD43. Loss of CD43 expression reduced galectin-1 death of murine thymocytes and human T lymphoblastoid cells, indicating that CD43 is required for maximal T cell susceptibility to galectin-1. CD43, which is heavily O-glycosylated, contributes a significant fraction of galectin-1 binding sites on T cells, as T cells lacking CD43 bound ~50% less galectin-1 than T cells expressing CD43. Although core 2 modification of O-glycans on other glycoprotein receptors is critical for galectin-1-induced cross-linking and T cell death, galectin-1 bound to CD43 fusion proteins modified with either unbranched core 1 or branched core 2 O-glycans and expression of core 2 O-glycans did not enhance galectin-1 binding to CD43 on T cells. Moreover, galectin-1 binding clustered CD43 modified with either core 1 or core 2 O-glycans on the T cell surface. Thus, CD43 bearing either core 1 or core 2 O-glycans can positively regulate T cell susceptibility to galectin-1, identifying a novel function for CD43 in controlling cell death. In addition, these studies demonstrate that different T cell glycoproteins on the same cell have distinct requirements for glycan modifications that allow recognition and cross-linking by galectin-1. The Journal of Immunology, 2006, 177: 5328–5336.

Regulation of T cell death is critical for development of a functional immune repertoire and for resolution of an immune response (1). Several death triggers for T cells are known, including galectin-1, a lectin highly expressed by dendritic cells, endothelial cells, tumor cells, and activated T cells (2–4). Galectin-1 kills immature thymocytes and activated T cells through an unconventional death pathway that is mechanistically distinct from death pathways triggered by other signals such as Fas (5–7). Although T cells dying in response to galectin-1 display characteristics common to other death pathways, such as phosphatidylinerine externalization, membrane blebbing, and nuclear and DNA fragmentation, several features of galectin-1-induced cell death are unique (5, 8–10).

First, galectin-1 death does not require caspase activation or cytochrome c release from mitochondria (10). Second, although conventional death pathways such as Fas-Fas ligand act through a single ligand binding to a cognate receptor, galectin-1 has multiple receptors. The major T cell counterreceptors for galectin-1 include the cell surface glycoproteins CD7, CD43, and CD45 (8, 11, 12). These receptors have distinct functions after binding galectin-1. CD7 is required for galectin-1-induced death, and CD45 is not required but can positively or negatively regulate susceptibility to galectin-1 death. However, the role of CD43 in galectin-1 T cell death is not known (8, 11, 13). Third, galectin-1 binding to and signaling through glycoprotein receptors is controlled by addition of specific glycan sequences to these cell surface glycoproteins (13–15). Glycosylation of CD45 by core 2 N-acetylgalactosaminyltransferase (C2GnT),3 which modifies O-glycans, positively regulates susceptibility to galectin-1, whereas glycosylation of CD45 by β-galactoside α-2,6-sialyltransferase, which modifies N-glycans, negatively regulates susceptibility to galectin-1 (13–15). As galectin-1 kills T cells by a unique death pathway, galectin-1 could synergize with other agents to modulate T cell death in disease states such as autoimmunity and cancer (4, 6, 10); thus, it is critical to understand the specific structural features of glycoprotein receptors responsible for regulating T cell susceptibility to galectin-1.

CD43 is a T cell surface mucin, an extended glycoprotein that is heavily O-glycosylated (see Fig. 1A). CD43 is decorated with ~80 O-glycans and extends ~45 nm from the cell surface (16). As mentioned, the role of CD43 in galectin-1 T cell death is not yet understood. In addition to binding galectin-1, several other functions have been attributed to CD43, including regulating cell proliferation and cell survival (17–26). Specifically, CD43−/− mice

3 Abbreviations used in this paper: C2GnT, core 2 N-acetylgalactosaminyltransferase; CHO, Chinese hamster ovary; DP, double positive; PI, propidium iodide; Bmax, maximum binding capacity; PNA, peanut lectin agglutinin.
demonstrated decreased negative selection of thymocytes and increased proliferation of thymocytes and peripheral T cells in response to various stimuli (20, 24). Moreover, after viral infections, CD43−/- mice had increased accumulation and decreased apoptosis of peripheral CD8 effector T cells (20, 25). However, the physiologic mechanism responsible for reduced death of CD43−/- T cells in vivo is not known.

The range of CD43 functions may relate to the biochemical heterogeneity of CD43. Two different glycoforms of CD43 are expressed on thymocytes and T cells (see Fig. 1B). CD43−/ CD48− mature thymocytes and resting peripheral T cells express the 115-kDa CD43 glycoform that bears core 1 O-glycans. In contrast, CD43−/CD48− immature thymocytes and activated peripheral T cells express both the 115-kDa glycoform and the 130-kDa glycoform that bear branched core 2 O-glycans (25, 27–29). Changes in CD43 glycoform expression have been correlated with differences in T cell function. For example, increased expression of the 130-kDa CD43 glycoform is found on T cells from patients with T cell leukemia, AIDS, and Wiskott-Aldrich syndrome (30–33), and viral-specific CD8 effector T cells express increased levels of the 130-kDa CD43 glycoform compared with naive or memory CD8 T cells (25, 34). However, the biologic consequences of differential CD43 glycosylation have not been directly examined.

Binding studies using purified or synthetic oligosaccharides indicate that galectin-1 has relatively high affinity for core 2 O-glycans compared with core 1 O-glycans; solution binding assays demonstrated that galectin-1 affinity for Gaβ1,3GalNAc sequences in core 1 O-glycans was 125-fold lower than for Gaβ1,4GlcNAc sequences in core 2 O-glycans (35, 36). Indeed, we have previously shown that, for CD45, core 2 O-glycan modification of CD45 is essential for galectin-1 binding and clustering (13). This response would predict that galectin-1 would preferentially bind the 130-kDa CD43 glycoform-bearing core 2 O-glycans, but not the 115-kDa CD43 glycoform that bears only core 1 O-glycans. However, recent work has shown that galectin-1 binds with high avidity to some mucins bearing only core 1 O-glycans, such as IgA and the cancer Ag CA125 (37, 38), due to the multivalent presentation of low-affinity glycan ligands on extended mucin polypeptide backbones. This finding raised the possibility that galectin-1 could bind CD43 bearing either core 1 or core 2 O-glycans.

Although galectin-1 preferentially kills thymocyte and peripheral T cells that express core 2 O-glycans and overexpression of the C2GnT enzyme enhanced thymocyte susceptibility to galectin-1 death (14), it is not known whether core 2 O-glycan modification of CD43 is specifically required for galectin-1 recognition of CD43. In addition, the role of CD43 in triggering cell death after galectin-1 binding has not been examined. In the present study, we address these two questions: we define the types of CD43 O-glycosylation that permit galectin-1 binding; and we determine the requirement for CD43 in delivering the galectin-1 death signal. These studies identify a novel function for CD43 in regulating cell survival, and demonstrate that modification of different T cell glycoproteins by specific glycans creates structures that have unique biologic functions.

Materials and Methods

Reagents and Methods

Recombinant human galectin-1 was prepared as previously described (39). CEM and the derivative 43K0 cell lines (19) were maintained in RPMI 1640, 10% FBS; with 2 mM GlutaMAX (Invitrogen Life Technologies), and 10 mM HEPES. BWS5147, CD45+, and transfected cell lines were maintained as previously described (13). To inhibit O-glycan elongation, cells were grown in the presence of 2 mM benzyl-α-GalNAc (EMD Biosciences) or vehicle alone for 3 days before further analysis. Chinese hamster ovary (CHO) cells producing FcCD43 fusion proteins have been previously described (40). Fusion proteins were collected in serum-free, protein-free chemically defined CHO-A medium (Invitrogen Life Technologies). CD43−/ mice have been previously described (20), and protocols were reviewed and approved by the University of California, Los Angeles Animal Research Committee. The L10 mAb that recognizes human CD43 was from CalTag Laboratories, the ID4 mAb that recognizes human CD43 bearing core 2 O-glycans was from MBL, and the 57 and 1B11 mAbs that recognize murine CD43 bearing core 1 and core 2 O-glycans, respectively, were from BD Biosciences.

Galectin-1 binding assay

Galectin-1 binding assays were done as previously described (15). Briefly, 5 × 105 cells were incubated with the indicated amount of biotinylated galectin-1 (5), followed by detection with streptavidin-FITC (Jackson Immunoresearch Laboratories) and flow cytometric analysis on a FACSscan or FACScalibur (BD Biosciences). Relative binding was determined by the mean fluorescent intensity of the samples.

Immunoprecipitation and immunoblotting

Nonidet P-40 was added to tissue culture supernatants to a final concentration of 1% in a volume of 1 ml. Fusion proteins were incubated with pan-CD43 mAb L10, mAb ID4 that recognizes the high m.w. glycoform of CD43, or isotype control. Immunoprecipitates were separated by 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and probed with polyclonal anti-human Fc (Jackson Immunoresearch Laboratories). Blots were visualized using SuperSignal West Pico Chemiluminescent substrate (Pierce).

Surface plasmon resonance analysis of galectin-1 interaction with FcCD43 fusion proteins

Experiments were performed using a BIAcore 2000 biosensor (Pharmacia Biosensor) at 25°C in HBSE buffer (150 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM EDTA and 0.005% surfactant). Recombinant protein A (Pierce) was covalently immobilized to the three flow cells (Fc2, Fc3, and Fc4) of a CM5 sensor chip. Fc1 was used as sensor chip blank, and Fc3 was used as protein A blank. FcCD43 core 1 fusion protein was coupled to protein A on Fc2 and FcCD43 core 2 fusion protein was coupled to protein A on Fc4, by injecting serum-free tissue culture supernatants from CHO cells expressing the fusion proteins. Injections of galectin-1 were followed by injection with 100 mM lactose, which was used as regeneration buffer. Two injections for each galectin-1 concentration were performed. Responses at increasing concentrations of galectin-1 were used to perform saturation binding analysis. Nonlinear regression analysis for saturation binding and Scatchard transformations were performed using Prism (GraphPad).

Core 1 FcCD43 fusion proteins were deglycosylated before BIAcore analysis as follows. Tissue culture supernatant pH was adjusted to 5.5 with HCl. One hundred milliliters of neuraminidase from Vibrio cholerae (Sigma-Aldrich), which removes both α2,3 and α2,6 sialic acids, and 25 μl of endo-α-acetylgalactosaminidase (O-glycanase) from Alcaligenes species (Seikagaku) were added to 1 ml of tissue culture supernatant and incubated overnight at 37°C. The mock-treated sample was pH adjusted and incubated with enzymes. Binding of peanut lectin agglutinin (PNA; 100 μg/ml) and galectin-1 (25–100 μg/ml) were analyzed as described earlier.

Confocal microscopy analysis of CD43 clustering

Cells were treated with galectin-1 in 1.2 mM DTTRP/PMI 1640/10% FBS, or 1.2 mM DTTRP/PMI 1640/10% FBS alone as buffer control for 3 h at 37°C. Galectin-1 was eluted with cold 0.1 M β-lactose, and cells were washed with PBS. Cells were fixed with 2% paraformaldehyde and washed again with PBS. Cells were blocked with 10% goat serum and subsequently stained with annexin V–Alexa Fluor 594 (Molecular Probes) and 57 anti-CD43–FITC for BWS5147 cells or 1B1 anti-CD43–FITC for BW295rT cells. Cells were analyzed using a Zeiss LSM 510 confocal microscope using the ×63 objective. Dual emission fluorescent images were collected in separate channels. The images were processed with Zeiss LSM 510 META image analysis software. Microscope settings were adjusted to eliminate nonspecific fluorescence and cross-channel bleed-through. Areas of red and green overlapping fluorescence were represented with a yellow signal. Images were collected from 5 to 10 fields (~100–150 cells) for each experimental point. The percentage of cells with clustered CD43 was calculated as the number of cells demonstrating clustered CD43 divided by the total number of cells examined. The percentage of annexin V-positive
cells was calculated as the number of cells demonstrating red annexin V binding divided by the total number of cells examined.

**Galectin-1 cell death assays**

Death assays of cell lines were performed as previously described, using concentrations of galectin-1 that we have demonstrated are optimal for cell death (39). Briefly, cells were treated with 20 μM galectin-1 in 1.2 mM DTT/RPMI 1640/10% FBS, or 1.2 mM DTT/RPMI 1640/10% FBS alone as buffer control for 3 h at 37°C. Galectin-1 was dissociated with 0.1 M β-lactose and cells were washed with PBS. Cells were resuspended in annexin V binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂) with annexin V-FITC (Molecular Probes) or annexin V-GFP (BD Clontech) and propidium iodide (PI) (Molecular Probes). Five thousand or 10,000 events were collected on a FACSscan or FACSCalibur (BD Biosciences). Cell death was calculated as the percentage of cell death shown as 100 × [(number of annexin V−PI−events buffer treated) − (number of annexin V−PI−events galectin treated)]/(number of annexin V−PI−events buffer treated).

For death assays of thymocytes, single cell suspensions of thymocytes were prepared from 6- to 8-old-wk wild-type and CD43−/− mice. A total of 2 × 10⁶ cells was treated with 20 μM galectin-1 in 1.2 mM DTT/DMEM/10% FBS or 1.2 mM DTT/DMEM/10% FBS alone as buffer control for 3 h at 37°C. We have found this concentration of galectin-1 to be optimal for triggering thymocyte cell death (6, 14, 41). Cells were dissociated with 0.1 M β-lactose and washed with PBS. Cells were stained with anti-mouse CD4 R-PE (clone H129.19; BD Biosciences) and anti-mouse CD8α-allophycocyanin (clone 53-6.7; BD Biosciences), and resuspended in HEPES buffer with 7-aminoactinomycin D (Molecular Probes). Ten thousand total events were collected on a FACScalibur, and live cells were determined by gating for forward vs side scatter profiles and absence of 7-aminoactinomycin D uptake.

CD4CD8 double-positive (DP) cell loss was calculated as the percentage of DP cell loss = 100 × (1 − (number live DP cells in galectin-1 treated)/(number live DP cells in buffer treated)).

**Results**

**CD43 on T cells contributes almost 50% of total galectin-1 binding sites**

We have previously shown that CD43 extracted from T cell membranes binds galectin-1 (8). However, the contribution of cell surface CD43 to galectin-1 binding to intact T cells has never been examined. As mentioned, CD43 is an extended mucin glycoprotein decorated with ~80 O-glycans, each of which bears one or more disaccharides that could be ligands recognized by galectin-1 (Fig. 1). To determine the contribution of CD43 to total galectin-1 binding of galectin-1, we compared galectin-1 binding to CD43+ CEM cells and the 43KO derivative of this cell line that lacks CD43 expression (Fig. 2A). As shown in Fig. 2, B and C, galectin-1 binding to 43KO cells was substantially reduced compared with CD43+ CEM cells. At a galectin-1 concentration of 7 μM, 43KO cells bound ~50% less galectin-1 than CEM cells. Binding to both cell lines was completely inhibited in the presence of lactose, demonstrating that binding was saccharide-dependent. These results demonstrate that CD43 contributes significantly to total T cell binding of galectin-1.

**O-glycans contribute to T cell binding of galectin-1**

Although galectin-1 appears to bind to both N-glycans and O-glycans on CD45 (13, 15), CD43 bears ~80 O-glycans and only a single N-glycan. This finding implies that O-glycans would be primarily responsible for galectin-1 binding to CD43. To evaluate the contribution of total T cell O-glycans to galectin-1 binding, we inhibited O-glycan elongation on CEM cells with benzyl-α-GalNAc. Inhibition of O-glycan elongation on benzyl-α-GalNAc-treated CEM cells was confirmed by loss of binding of the 1D4 mAb that recognizes core 2 O-glycan epitopes on human CD43 (42) (Fig. 2D). Treatment of CEM cells with benzyl-α-GalNAc diminished galectin-1 binding by ~50% (Fig. 2, E and F), a decrease in binding similar to that observed for galectin-1 binding in the absence of CD43 (Fig. 2, B and C). To confirm that decreased binding of galectin-1 to benzyl-α-GalNAc-treated cells was due solely to decreased O-glycan elongation and not to loss of cell surface glycoproteins that bear the glycans, we evaluated expression of CD7, CD43, and CD45 on these cells. The level of CD7, CD43, and CD45 expression was equivalent on control and benzyl-α-GalNAc-treated cells (data not shown), demonstrating that decreased galectin-1 binding was not due to loss of the glycoproteins from the cell surface. In summary, these data indicate that, on intact T cells, CD43 contributes the major proportion of O-glycan binding sites for galectin-1 on T cells.

**CD43 bearing either core 1 or core 2 O-glycans binds galectin-1**

The CEM T cells we examined express C2GnT glycosyltransferase, so all O-linked glycans on these cells could bear core 2 structures. To directly compare the ability of galectin-1 to bind to T cells bearing core 1 vs core 2 O-glycans, we used the murine T cell line BW5147, which we have previously shown does not express C2GnT glycosyltransferase that initiates core 2 O-glycan branching (14). BW5147 cells were transfected with cDNA encoding C2GnT-1 or with plasmid alone, and stable transfectants were screened with 1B11 mAb that recognizes a core 2 O-glycan-dependent epitope on murine CD43 (14). BW5147 T cells expressing C2GnT-1 bound high levels of 1B11 mAb compared with BW5147 cells transfected with plasmid alone (Fig. 3A).

We compared the level of galectin-1 binding to BW5147 cells with and without core 2 O-glycans. As shown in Fig. 3, B and C, expression of C2GnT-1 in BW5147 cells increased galectin-1 binding compared with control cells transfected with plasmid alone. However, there was significant galectin-1 binding to the control BW5147 cells bearing only core 1 O-glycans. The mean
fluorescence intensity for galectin-1 binding to control BW5147 cells was 35, compared with a mean fluorescence intensity of 4 in the presence of lactose (Fig. 3B) or 3 for secondary alone (data not shown). However, CD43 and CD45 on these cells could be modified by core 2 O-glycans. To interrogate the contribution of core 2 O-glycans on CD43 to binding of galectin-1, we used a CD45/H11002 derivative of BW5147 (13). These cells were transfected with C2GnT-1 or plasmid alone, as described for BW5147 cells (Fig. 3D). In contrast to CD45+ cells, expression of core 2 O-glycans in CD45- cells did not increase the total binding of galectin-1 to the cells compared with CD45- cells transfected with plasmid alone (Fig. 3, E and F). Thus, the increased galectin-1 binding to BW5147 cells expressing C2GnT (Fig. 3, B and C) appears to result primarily from core 2 O-glycan addition to CD45, as no

FIGURE 2. CD43 and O-glycans contribute to galectin-1 binding to T cells. A, CEM T cells (solid line histogram) express CD43, detected by 1D4 mAb that recognizes human CD43 modified with core 2 O-glycans, whereas 43KO cells (dashed line histogram) do not express CD43. B, CEM cells (solid line histogram) bound more biotinylated galectin-1 than 43KO cells (dashed line histogram), as detected with streptavidin-FITC. Galectin-1 binding was inhibited by 0.1 M β-lactose (filled histogram). C, CEM or 43KO cell lines were incubated with indicated concentrations of biotinylated galectin-1 in the absence or presence of 0.1 M β-lactose. Values are mean fluorescence of triplicate samples ± SEM. D, Benzyl-α-GalNAc-treated CEM cells (dashed line histogram) bound less biotinylated galectin-1 than vehicle-treated cells (solid line histogram). Inhibition of O-glycan elongation with benzyl-α-GalNAc did not increase background binding of streptavidin-FITC (filled histogram). E, CEM cells treated with or without benzyl-α-GalNAc were incubated with indicated amounts of biotinylated galectin-1. Values are mean fluorescence of triplicate samples ± SEM.

FIGURE 3. Core 2 O-glycan modification of CD43 does not contribute to galectin-1 binding to T cells. A, Transfection of murine BW5147 T cells with C2GnT-1 (solid line histogram) increased expression of core 2 O-glycans as detected by the 1B11 mAb that recognizes murine CD43 bearing core 2 O-glycans, compared with transfection with vector alone (dashed line histogram). B, BW5147 cells transfected with C2GnT-1 (solid line histogram) bound more biotinylated galectin-1, as detected with streptavidin-FITC, than BW5147 cells transfected with vector alone (dashed line histogram). Binding was inhibited by 0.1 M β-lactose (filled histogram). C, Galectin-1 binding to BW5147 cells transfected with C2GnT-1 or vector alone at the indicated concentrations. Values are the mean fluorescence of triplicate samples ± SEM. D, The CD45-deficient derivative of BW5147 (CD45-) was transfected with C2GnT-1 (solid line histogram) or vector alone (dashed line histogram). Expression of core 2 O-glycans was detected with 1B11 mAb, as shown in A. E, C2GnT expression in CD45- cells (solid line histogram) did not result in increased galectin-1 binding compared with cells transfected with vector alone (dashed line histogram). Binding was inhibited by 0.1 M β-lactose (filled histogram). F, Galectin-1 binding to CD45- cells transfected with C2GnT-1 or vector alone at the indicated concentrations. Values are mean fluorescence of triplicate samples ± SEM.
precipitated with pan-specific anti-human CD43 mAb L10, isotype control (FcCD43 core 2). Glycans. Soluble human FcCD43 fusion proteins were expressed in CHO FIGURE 4. Galectin-1 binds to CD43 modified with core 1 and core 2 O-glycans. Tissue culture supernatants or immunoprecipitates were separated by SDS-PAGE and blotted with anti-human Fc antisera, followed by anti-goat IgG-HRP. Although both fusion proteins were precipitated by the pan anti-CD43 L10, only CD43 fusion proteins expressed in CHO cells expressing C2GnT-1 (FcCD43 core 2) were precipitated with I4D, demonstrating addition of core 2 O-glycans. B, FcCD43 core 1 (dashed line) and FcCD43 core 2 (solid line) were immobilized on BIAcore sensor chips. At an approximate galectin-1 concentration of 20 µM, both glycoforms bound appreciable amounts of galectin-1. Sensograms were corrected for relative amounts of FcCD43 glycoforms immobilized on the chip. C, Steady-state binding analyses at various galectin-1 concentrations were used to construct saturation binding curves. Each point is the average of duplicate injections ± SEM. Nonlinear regression analysis followed by increase in galectin-1 binding was seen when C2GnT-transfected cells did not express CD45. These results indicated that core 2 O-glycans are not essential for galectin-1 binding to CD43 on T cells.

To directly determine the ability of galectin-1 to bind to CD43 with and without core 2 O-glycans, we used soluble FcCD43 fusion proteins produced in wild-type CHO cells or CHO cells stably transfected with human C2GnT-1 (40). Immunoprecipitation of tissue culture supernatants with the I4D mAb confirmed addition of core 2 O-glycans to FcCD43 fusion proteins expressed in CHO cells expressing C2GnT-1 but not in control CHO cells (Fig. 4A). Surface plasmon resonance analysis was used as a sensitive and quantitative approach to compare galectin-1 binding to core 1 and core 2 O-glycan-modified CD43 glycoforms. Soluble FcCD43 with either core 1 or core 2 O-glycans was bound to protein A immobilized on a sensor chip, and increasing concentrations of galectin-1 were flowed over the chip. At an ~20 µM galectin-1, both core 1 and core 2 O-glycan CD43 bound appreciable amounts of galectin-1 (Fig. 4B). Binding to both CD43 glycoforms showed rapid on/off rates (Fig. 4B), consistent with previous observations for galectin-1 binding to CD45 (43).

Steady-state binding analysis at multiple galectin-1 concentrations was used to construct saturation binding curves for galectin-1 binding to both CD43 glycoforms (Fig. 4C). The maximum binding capacity (Bmax) of core 2 O-glycan-modified CD43 was ~10-fold higher than that for core 1 O-glycan-modified CD43. Despite the higher galectin-1 binding capacity of core 2 O-glycan-modified CD43, the apparent binding constants (Kd) for galectin-1 binding to both glycoforms were similar. Scatchard transformation of binding data for both CD43 glycoforms revealed concave deviation from Scatchard lines derived from Bmax and Kd (Fig. 4D). This deviation is consistent with negative cooperativity of galectin-1 binding to both core 1 and core 2 O-glycan forms of CD43, possibly due to increased site occupancy at higher concentrations (44).

To confirm that O-glycans on CD43 were responsible for galectin-1 binding, soluble core 1 FcCD43 was treated with neuraminidase and O-glycanase before analysis of galectin-1 binding. After neuraminidase and O-glycanase treatment, there was negligible binding of galectin-1 to core 1 CD43 glycoform compared with mock-treated samples (Fig. 4E). The efficacy of enzymatic deglycosylation was confirmed by assays using the binding of the PNA that recognizes O-glycans; as shown in Fig. 4E, enzyme treatment abolished PNA binding, demonstrating complete loss of detectable O-glycans, compared with high PNA binding to mock-treated samples.

This analysis directly demonstrated that O-glycans are responsible for galectin-1 binding to CD43, in support of the observations made with intact cells (Figs. 2 and 3). In addition, although core 1 and core 2 glycoforms of CD43 have different binding capacities for galectin-1, both CD43 glycoforms bind galectin-1 with similar correction for relative levels of CD43 immobilized on the chip yielded Bmax = 130, Kd = 8 µM for core 1 modified CD43, and Bmax = 1380, Kd = 14 µM for core 2 modified CD43. One of three replicate analyses is shown. D, Scatchard transformations of binding data for galectin-1 to FcCD43 core 1 and FcCD43 core 2 are shown. E and F, FcCD43 core 1 fusion protein was deglycosylated using Vibrio cholerae neuraminidase and O-glycanase (dashed line) or mock-treated (solid line). Binding of galectin-1 (E) to FcCD43 core 1 was abolished by deglycosylation. Loss of PNA binding (F) confirmed removal of O-glycans.
characteristics. That the increase in binding to soluble, immobi-
ized FcCD43 bearing core 2 vs core 1 O-glycans detected by 
BIAcore analysis was not observed when we examined galectin-1 
binding to CD45− cells bearing core 2 vs core 1 O-glycans (Fig. 
3, E and F) suggests that other factors, such as abundance and 
accessibility of core 2 branches on the cell surface, contribute 
to galectin-1 binding to intact cells. For example, both core 1 and 
core 2 disaccharides might be accessible to galectin-1 on the BIA-
core chip (Fig. 1B), whereas the more extended core 2 disaccharide 
might sterically hinder galectin-1 access to core 1 disaccharides on 
cell surface CD43 (36).

Galectin-1 clusters CD43 with and without core 2 O-glycans on 
T cells

A hallmark of galectin-1 binding to T cell surface glycoproteins is 
clustering of the glycoproteins on the cell surface (8, 13, 15, 45). 
Galectin-1 binding to susceptible T cells results in clustering of 
CD45 and CD43, with the two glycoproteins segregated on 
different regions of the plasma membrane (8). Galectin-1-induced 
CD45 clustering requires expression of core 2 O-glycans, as we 
observed no clustering of CD45 on BW5147 cells that lack C2GnT 
expression, whereas expression of C2GnT allowed galectin-1-in-
duced CD45 clustering and cell death (13).

However, the observation that galectin-1 bound CD43 with ei-
ther core 1 or core 2 O-glycans (Fig. 4) suggested that, in contrast 
to what we had observed for CD45, core 2 O-glycan modification 
would not be essential for galectin-1 binding and clustering of 
CD43. Indeed, we recently examined migration of BW5147 cells, 
lacking core 2 O-glycans, through extracellular matrix coated with 
galectin-1 (46). In these assays, we observed abundant CD43 lo-
calized at the interface between T cells and the galectin-1 coated 
matrix, suggesting that galectin-1 can bind and cluster CD43 on 
these T cells.

We directly assessed CD43 clustering following galectin-1 
binding using BW5147 cells expressing C2GnT or control cells 
transfected with plasmid alone (Fig. 3). Galectin-1 or buffer con-
trol was added to the cells, CD43 localization on the cell surface 
was examined by confocal microscopy, and the percentage of cells 
with clustered CD43 on the cell surface was determined (Fig. 5, A 
and B). We observed clustered CD43 on both control BW5147 
cells and BW5147 cells expressing C2GnT; for both cell lines, 
binding of galectin-1 resulted in a ~2-fold increase in the fraction 
of cells with clustered CD43, compared with cells treated with 
buffer control. Thus, the presence of core 2 O-glycans is not re-
quired for galectin-1-mediated clustering of CD43 on the T cell 
surface.

In addition, cells were simultaneously stained with annexin V to 
detect cell death. We have previously demonstrated that clustering 
of CD45 is required for galectin-1-induced T cell death (13, 15). 
As shown in Fig. 5C, only BW5147 cells expressing C2GnT were 
susceptible to galectin-1 death, as we have previously described 
(14), with negligible death of BW5147 cells that did not express 
C2GnT. However, increased CD43 clustering was observed on 
both cell lines after binding galectin-1, indicating that clustering of 
CD43 on BW5147 cells occurred in the presence or absence of 
core 2 O-glycans, but was not sufficient to trigger galectin-1 death.

CD43 positively regulates T cell and thymocyte susceptibility to 
.galectin-1 death

To directly assess the role of CD43 in galectin-1-induced T cell 
death, we compared the susceptibility of CEM T cells with the 
43KO derivative of CEM that lacks CD43 (Fig. 2). Cell death was 
assessed by annexin V binding and PI uptake. As shown in Fig. 6, 
A and B, 43KO cells were markedly less susceptible to galectin-
1-induced death, compared with CEM cells that express CD43. We 
observed a decrease in both the mean annexin V binding to 43KO 
cells compared with CEM cells (Fig. 6A), as well as the fraction 
of annexin V-positive 43KO cells compared with CEM cells (Fig. 
6B). Despite assay-to-assay variation, we observed on average 
33% less death of the 43KO cells compared with the CD43+ par-
ental CEM cells, when data for ~20 independent determinations 
were examined (p = 0.0056) (Fig. 2B).

We also compared susceptibility of thymocytes from wild-type 
and CD43−/− mice (20) to galectin-1. We and others (6, 7, 14, 41)
have previously shown that CD4⁺CD8⁺ DP thymocytes are the population that is most susceptible to galectin-1 death. Thus, we quantified loss of viable DP thymocytes from CD43⁺/⁺ or CD43⁻/⁻ mice following incubation with galectin-1 or buffer control, as we have done previously (6, 14, 41).

CD43⁻/⁻ DP thymocytes were significantly less susceptible to galectin-1 cell death compared with wild-type DP thymocytes. Fig. 6C shows a representative sample, in which galectin-1 treatment of CD43⁻/⁻ thymocytes resulted in loss of 21% DP cells, while galectin-1 treatment of CD43⁺/⁺ thymocytes resulted in loss of 33% DP cells. On average, we observed loss of 20% of DP thymocytes from CD43⁻/⁻ mice after galectin-1 treatment, compared with loss of 31% of DP thymocytes from wild-type mice (p = 0.025) (Fig. 6D). These data indicate that loss of CD43 expression reduces DP thymocyte susceptibility to galectin-1. As we observed for CD43⁺ and CD43⁻ CEM T cells, loss of CD43 expression resulted in approximately one-third less cell death of CD43⁻/⁻ thymocytes compared with wild-type thymocytes. Together, these data demonstrate that CD43 expression is essential for optimal T cell susceptibility to galectin-1-induced death.

**Discussion**

CD43 has a complex array of functions in T cell biology, including regulation of susceptibility to cell death. CD43 cross-linking by mAbs can directly trigger T cell death (17, 21, 47), and CD43⁻/⁻ mice demonstrated decreased negative selection of thymocytes and decreased apoptosis of effector peripheral CD8 T cells after viral infection (24, 25). However, the physiologic ligand that triggers T cell death through CD43 has not been identified.

Our present studies demonstrate that CD43 expression is required for optimal T cell susceptibility to galectin-1-induced cell death. We observed decreased susceptibility to galectin-1 of CD43⁻/⁻ murine thymocytes and CD43⁻ human T lymphoblastoid cells (Fig. 6). As described, CD7, CD45, and CD43 are major glycoprotein receptors for galectin-1 on T cells; CD7 is essential for galectin-1-induced T cell death, whereas CD45 is a negative regulator of galectin-1-induced T cell death unless CD45 is modified by core 2 O-glycans (11, 13). In contrast, CD43 appears to positively regulate galectin-1-induced T cell death, in the presence or absence of core 2 O-glycans.

How could CD43 act as a positive regulator of T cell death? Although the cytoplasmic domain of CD43 associates with a putative serine/threonine kinase (48), pharmacologic inhibition of serine/threonine kinases did not decrease T cell susceptibility to galectin-1 (J.T. Nguyen and L.G. Baum, unpublished observation), implying that CD43 associated serine/threonine kinases are not required for T cell susceptibility to galectin-1. Thus, a role for the intracellular domain of CD43 in regulating T cell susceptibility remains to be established. The extracellular domain of CD43 is highly glycosylated, with ~80 O-linked glycans that can bind galectin-1 (Fig. 4). After galectin-1 binding, CD43 associates with CD7 on the T cell surface (8), and CD7 bears fewer glycan ligands.

**FIGURE 6.** CD43 enhances T cell susceptibility to galectin-1-induced death. A, CEM human T cells or CD43-deficient 43KO were treated with galectin-1 (solid line histogram) or buffer control (dashed line histogram). Representative histograms of annexin V binding to CEM and 43KO cells indicate that galectin-1 (solid line histogram) or buffer control (dashed line histogram). Representative histograms of annexin V binding to CEM and 43KO cells are shown. B, Cell death was calculated by annexin V binding and PI uptake as described in Materials and Methods. Analysis of multiple experiments (n = 23) for CEM cells (■) demonstrated an average of 50% cell death, whereas analysis (n = 19) of 43KO cells (▲) demonstrated an average of 36% cell death (p = 0.006). Each point represents the mean of triplicate samples from an individual experiment. C, Thymocytes from CD43⁺/⁺ and CD43⁻/⁻ mice were treated with galectin-1 or buffer for 3 h and viable cells, determined by gating for forward vs side scatter and 7-aminoactinomycin D exclusion, were analyzed for CD4 and CD8 expression to determine changes in different thymocyte populations. Representative density plots are shown. Values in each quadrant represent viable cells of 10,000 total events collected. D, Loss of DP thymocytes from CD43⁺/⁺ (n = 10) and CD43⁻/⁻ (n = 7) mice was calculated as described in Materials and Methods. CD43⁻/⁻ DP thymocytes (▲) were less susceptible to galectin-1-induced death than CD43⁺/⁺ DP thymocytes (■) (p = 0.025). Each point represents the mean DP cell loss of triplicate samples from a single mouse.
for galectin-1 than CD43; CD7 has only two potential N-glycosylation sites and no large mucin-like domain (49). In fact, loss of CD7 expression, or elimination of either or both putative N-glycosylation sites on CD7, does not appreciably reduce total T cell binding of galectin-1 detected by flow cytometry (M. Amano and L.G. Baum, unpublished observation). In contrast, loss of CD43 expression significantly reduced galectin-1 binding by ~50% (Fig. 2). These findings imply that CD43 may concentrate galectin-1 on the T cell surface, to facilitate CD7 accessibility to, binding of, or cross-linking by galectin-1 (8).

Surprisingly, we found that modification of CD43 with only core 1 O-glycans was sufficient for galectin-1 binding. Previous work has shown that the preferred minimal saccharide ligand for galectin-1 is Galβ1,4GlcNAc, a sequence found in branched core 2 O-glycans but not core 1 O-glycans (Fig. 1B) (35, 36) and abundant on N-linked glycans (50). In fact, the affinity of galectin-1 for isolated Galβ1,3GlcNAc sequences found in core 1 O-glycans was 125-fold lower than for Galβ1,4GlcNAc sequences (35). However, our data show that galectin-1 can bind the extracellular domain of CD7 expression, or elimination of either or both putative O-glycans may also enhance galectin-1 avidity (45, 55). Moreover, the ordered display of saccharide ligands on extended core 2 glycosylated glycans on CD45 to negatively regulate galectin-1 binding of galectin-1 detected by flow cytometry (M. Amano and L.G. Baum, unpublished observation). In contrast, loss of CD43 may influence T cell processes other than death. CD43 ligation and galectin-1 binding to T cells both influence T cell signaling and set a threshold for T cell activation (18, 26, 57). CD43 ligation and galectin-1 binding both affect T cell cytokine production (26, 57–59). CD43 ligation and galectin-1 binding both regulate T cell adhesion and trafficking (25, 46, 59–61). A detailed understanding of how galectin-1 interacts with distinct glycoprotein counterreceptors on T cells, including CD43, is critical for elucidating how galectin-1 modulates such a broad range of critical T cell functions.

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**Disclosures**

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**References**


