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Galectin-3 and Galectin-1 Bind Distinct Cell Surface Glycoprotein Receptors to Induce T Cell Death

Brianna N. Stillman,* Daniel K. Hsu,† Mabel Pang,* C. Fred Brewer,‡ Pauline Johnson,§ Fu-Tong Liu,† and Linda G. Baum2*

Galectins are a family of mammalian β-galactoside-binding proteins that positively and negatively regulate T cell death. Extracellular galectin-1 directly induces death of T cells and thymocytes, while intracellular galectin-3 blocks T cell death. In contrast to the antiapoptotic function of intracellular galectin-3, we demonstrate that extracellular galectin-3 directly induces death of human thymocytes and T cells. However, events in galectin-3- and galectin-1-induced cell death differ in a number of ways. Thymocyte subsets demonstrate different susceptibility to the two galectins: whereas galectin-1 kills double-negative and double-positive human thymocytes with equal efficiency, galectin-3 preferentially kills double-negative thymocytes. Galectin-3 binds to a complement of T cell surface glycoprotein receptors distinct from that recognized by galectin-1. Of these glycoprotein receptors, CD45 and CD71, but not CD29 and CD43, appear to be involved in galectin-3-induced T cell death. In addition, CD7 that is required for galectin-1-induced death is not required for death triggered by galectin-3. Following galectin-3 binding, CD45 remains uniformly distributed on the cell surface, in contrast to the CD45 clustering induced by galectin-1. Thus, extracellular galectin-3 and galectin-1 induce death of T cells through distinct cell surface events. However, as galectin-3 and galectin-1 cell death are neither additive nor synergistic, the two death pathways may converge inside the cell. The Journal of Immunology, 2006, 176: 778–789.

Programmed lymphocyte cell death is critical for normal immune development, immune regulation, and prevention of lymphomagenesis (1). Given the critical functions of cell death in the immune system, several endogenous protein families participate in this process. In general, proteins that regulate cell death and survival can be classified into two main groups: those that function inside the cell such as Bcl-2 family members (2) and those that function outside the cell such as TNF family members (3).

The galectins are a family of mammalian proteins that positively and negatively regulate cell death (4–6). The galectins are unique in that different family members, including galectin-1, -2, -3, -7, -8, -9, and -12, can regulate cell death either inside or outside the cell (7–13). As galectins are carbohydrate-binding proteins, galectins that act extracellularly to induce cell death do so by binding specific glycoproteins in a saccharide-dependent manner. For example, galectin-1, which kills human thymocytes and activated peripheral T cells (14, 15), binds CD7, CD43, and CD45 on T cells (16). All three glycoproteins are involved in regulating susceptibility to galectin-1, and specific oligosaccharide modifications of CD43 and CD45 control galectin-1 binding and T cell susceptibility to death (17, 18).

Although galectin-1 regulates T cell death only via the extracellular route, and galectin-7 acts only intracellularly to promote epithelial cell apoptosis (10, 19), galectin-3 regulates cell death from both outside and inside the cell (9, 20). Moreover, galectin-3 is the only family member with both pro- and antiapoptotic activity (5). Cytoplasmic galectin-3 antagonizes apoptosis by associating with the mitochondrial membrane to maintain mitochondrial membrane integrity and antagonize the release of cytochrome c (9, 21, 22). In contrast, extracellular galectin-3 directly induces T cell death (20).

Galectin-1 and galectin-3 are the most ubiquitously expressed members of the galectin family. In some tissues, only galectin-1 or galectin-3 is expressed; galectin-1 is expressed in skeletal and cardiac myocytes, hepatocytes, and prostate stromal cells, while galectin-3 is expressed in epithelial cells of the digestive and respiratory tracts (23). In many immune cells and tissues, galectin-1 and galectin-3 are coexpressed, these include T cells, B cells, NK cells, macrophages, dendritic cells, endothelial cells, and fibroblasts, with high levels of both galectins in thymus and lymph nodes (5, 24–26).

Both galectin-1 and galectin-3 can bind to T cells and trigger T cell death (14, 20); however, these two lectins likely recognize discrete sets of oligosaccharide ligands expressed on distinct glycoprotein backbones bearing these ligands. Although both galectin-1 and galectin-3 recognize the disaccharide galactose-β1,4-N-acetylglucosamine as a minimal saccharide ligand, the two galectins differ in fine specificities for modified oligosaccharide ligands, due to structural differences in the carbohydrate-recognition domain (27–31).

As thymocytes and T cells will encounter galectin-1 and galectin-3 in normal and inflamed tissues, it is critical to identify similarities and differences between the galectin-1 and galectin-3 T cell death pathways, to understand the distinct roles of the
galectins in vivo and the effects of administering exogenous galectins as immunosuppressive agents. For example, while both galectin-3 and galectin-1 are immunosuppressive in models of autoimmune disease, administration of galectin-3 results in suppression of Th2-type cytokines, while administration of galectin-1 results in suppression of Th1-type cytokines (4, 32, 33). Understanding the mechanisms that regulate susceptibility of T cell populations to the different galectins will allow precise modulation of T cell responses by specific galectins.

**Materials and Methods**

**Cells and reagents**

Human T cell lines MOLT-4, Jurkat E6-1, J45.01 (American Type Culture Collection), CEM, CEM.DKO (Ref. 34; a gift from Dr. B. Arman, New England Medical Center, Boston, MA), Jurkat A-11, and the CD29-deficient derivative (Ref. 35; a gift from Dr. Y. Shimizu, University of Minnesota, Minneapolis, MN) were maintained in complete RPMI 1640 (Invitrogen Life Technologies) supplemented with 2 mM glutamine, 10% FBS, and 10 mM HEPES) in 5% CO2 at 37°C. J45.01 cells reconstituted with murine CD45RABC were previously described (36). Murine T cell lines BW5147 and CD45~−~ (derivative (Ref. 17; a gift from Dr. K. Bottomly, Yale University, New Haven, CT) were maintained in Iscove’s modified DMEM (Invitrogen Life Technologies) supplemented with 10% FBS and 0.1% 2-ME in 10% CO2 at 37°C.

The following mAbs were obtained from the indicated suppliers: CD43 (DFT1), CD45 (leukocyte common Ag), CD4-PE (MT310), CD8-allophycocyanin (D123), and mouse IgG1- and IgG2b-negative control Abs from DakoCytomation; CD45 (F10-89-4) and CD29-FTTC (TDM29) from Southern Biotechnology Associates; CD7 (M-T701), CD71 (M-A712), and anti-mouse CD45 (50F11) from BD Pharmingen; CD29 (4B7R) from Serotech; anti-galectin-3 (Mac-2; M/38) from Boehringer Mannheim. Secondary detection reagents were obtained from the indicated supplier: goat anti-mouse IgG-FTTC (DakoCytomation); streptavidin (SA)-FITC (Jackson Immunoresearch Laboratories); SA-HRP and rabbit anti-mouse IgG-HRP (Zymed Laboratories); goat anti-rabbit IgG-HRP (Bio-Rad); Alexa Fluor 568 goat anti-mouse IgG and SA-Oregon green (Molecular Probes).

Recombinant human galectin-1 and galectin-3 were prepared as previously described (37, 38). Anti-galectin-1 and anti-galectin-3 polyclonal rabbit sera were previously described (39, 40).

**Tissue immunostaining**

Five-micrometer sections of paraffin-embedded human tonsil or thymus on glass slides were deparaffinized in xylene and ethanol, rinsed with dH2O, and fixed with 3% H2O2 for 5 min. Slides were blocked with 20% goat serum in PBS for 20 min before addition of 1/1000 dilution of anti-galectin-1 serum, anti-galectin-3 serum, or nonimmune rabbit serum in 2% goat serum/PBS. For immunohistochemistry, bound primary Ab was detected with mouse anti-rabbit IgG-HRP diluted 1/1000 in PBS and developed with 3-amin-9-ethylcarbazole (Biomedia) for 10 min at 37°C. Slides were counterstained with hematoxylin for 1–2 min, washed with PBS, and mounted with Faramount (DakoCytomation). For immunofluorescence studies, galectin-1 and galectin-3 were detected with rabbit polyclonal anti-galectin-1 and mouse monoclonal anti-galectin-3 Abs, followed by goat anti-rabbit IgG-Texas Red and goat anti-mouse IgG-FTTC.

Primary cultures of human thymic epithelial cells were cultured and cells were stained with anti-galectin-1 and anti-galectin-3 polyclonal antisera and bound Ab detected with goat anti-rabbit IgG-FTTC as previously described (39). Images were collected on an Olympus AX70 microscope for immunohistochemistry, an Axioskop 2 plus fluorescent microscope, or an Olympus IX70 confocal microscope, and analyzed using Spot Advanced Version 4.0.4 software (Diagnostic Instruments) or Fluoview 2.0 software (Olympus).

**Determination of galectin cell surface binding**

Binding of biotinylated galectin-1 or galectin-3 to T cells or thymocytes was performed as described (18). Briefly, 5 × 105 cells were incubated for 30 min at 4°C with increasing concentrations of biotinylated galectin-1 or galectin-3 to construct binding curves, and galectin concentrations that gave ~50% maximal binding (5.5 μg/mL galectin-1 and 0.67 μg/mL galectin-3) were used for inhibition-binding assays with 0.1 M β-lactose. Binding assays were performed at 4°C to minimize cell death. For galectin-3-galectin-1 competition-binding studies, cells were incubated with 10 μM biotinylated galectin-1 in the presence of 10 μM unlabeled galectin-3. Cells were washed two times with PBS and stained with 3 μg of SA-FITC and 0.6 μg of 7-aminononoximycin D (Molecular Probes) to detect galectin binding and dead cells, respectively. Flow cytometry data were acquired using a BD Biosciences BD-LSR I Analytic Flow Cytometer and analyzed with CellQuest software (BD Biosciences).

**T cell death assays**

T cell death assays were performed as previously described (37). Briefly, 2 × 105 cells were incubated in 200 μl of complete RPMI with the indicated final concentration of galectin-1, galectin-3, both galectin-1 and galectin-3, or buffer control with or without 0.1 M β-lactose for 4 h at 37°C. Galectin was dissociated with 0.1 M β-lactose and cell death detected by labeling with Annexin V<sup>FITC</sup> and propidium iodide (PI, Molecular Probes). For detection of cells containing subdiploid DNA, fixed permeabilized cells were stained with PI and DNA content was determined as previously described (15, 37). All death assays were performed in triplicate and data was acquired using a BD Biosciences BD-LSR I Analytic Flow Cytometer and analyzed with CellQuest software (BD Biosciences). The percentage of cell death of T cell lines was determined as: (percent annexin V<sup>+</sup> cells in control-treated sample) − (percent annexin V<sup>+</sup> cells in galectin-treated sample)/percent annexin V<sup>+</sup> cells in control-treated sample). The percentage of cell loss of T cell lines was determined as described below for thymocytes.

**Thymocyte cell loss**

Human thymocytes were isolated from surgical specimens as previously described and cultured overnight in serum-free medium (AT-IMDM) consisting of IMDM (Omega Scientific) supplemented with 1100 μg/ml de-digalactosylated BSA (Sigma-Aldrich), 85 μg/ml transferrin (Sigma-Aldrich), 2 mM glutamine, and 25 U/25 μg/ml penicillin/streptomycin (15). The following day, 105 cells in 200 μl were incubated with the indicated concentration of galectin-1, galectin-3, or buffer control (DTT or PBS/glycerol) in AT-IMDM for 3–5 h at 37°C. Galectin was dissociated with 0.1 M β-lactose and cells were washed with PBS before staining with CD4-PE and CD8-allophycocyanin for 45 min at 4°C. Cells were analyzed on a BD Biosciences FACScan Flow Cytometer using CellQuest software. As we have previously described that there is high baseline binding of annexin V binding to thymocytes isolated in this manner, thymocytes were assessed for viability based on forward vs. side scatter and absence of 7-aminononoximycin D permeability, as we have previously described (17, 37). Loss of viable thymocytes from each population was determined as: percent cell loss = 100 × (number of viable control-treated cells) − (number of viable galectin-treated cells)/(number of viable control-treated cells).

**Isolation of galectin-3-binding proteins by affinity chromatography**

Galectin-3 was conjugated to cyanogen bromide-activated Sepharose 4B (Sigma-Aldrich). Cell surface proteins on 3 × 10⁵ cells were labeled with Sulfo-NHS-biotin (Pierce), plasma membrane preparations were isolated and solubilized in 0.1% Tween 20, and the extract was applied to the galectin-3 column as previously described (16).

After washing extensively with PBS, 0.1% Triton X-100, 0.02% sodium azide (wash buffer), bound proteins were eluted with 0.1 M β-lactose in wash buffer (elute buffer). Fractions were collected, resolved by reducing SDS-PAGE, transferred to nitrocellulose, and detected with SA-HRP. Proteins were visualized by ECL (Amersham).

**Identification of galectin-3-binding proteins**

Glycoproteins eluted from the galectin-3 affinity column were concentrated on a YM-3 Centricron (Millipore) and resolved by electrophoresis on 10% SDS-PAGE. Separated proteins were visualized by Coomassie Brilliant Blue (Sigma-Aldrich) and the relevant portions of the gel were excised for polypeptide sequence analysis. Sequence analysis was performed at the Harvard Microchemistry Facility (Cambridge, MA) by microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry on a Finnigan LCQ DECA XP Plus quadruple ion trap mass spectrometer (Thermo Electron). To confirm the identity of glycoproteins in the sample eluted from the galectin-3 column, candidate Abs were used for immunoprecipitation or immunoblotting. For immunoprecipitation with anti-CD29, biotinylated T cell glycoproteins eluted from the galectin-3 column, or total biotinylated membrane proteins as a control, were immunoprecipitated with anti-CD29, separated by 10% SDS-PAGE, and electroblotted onto nitrocellulose membrane (Hybond C-extra; Amersham). The membrane was stained with SA-HRP.
Cells were scanned by dual excitation of Oregon green and Alexa Fluor channels, respectively. Fluorescently labeled cells were analyzed using the control for nonspecific staining and compensation between fluorescent ular Probes). Isotype control-stained and single-stained cells were used to glass microscope slides using Prolong Anti-fade mounting media (Molec
goat anti-mouse IgG for 1 h at 4°C. Cells were washed and mounted on 

Characterization of receptor localization following galectin-3 treatment

A total of 10^5 CEM cells were incubated with 5 μM galectin-3 in PBS with 10% glycerc, or PBS/glycerol alone, for 2 h at 37°C with 5% CO2. Cells were cooled to 4°C, and bound galectin-3 dissociated with ice-cold 0.1 M β-lactose. Cells were stained with biotin-annexin V (Molecular Probes) for 30 min on ice and fixed with 2% paraformaldehyde for 30 min on ice. The reaction was quenched with 0.2 M glycine for 5 min, followed by staining with mouse anti-human CD45 leukocyte common Ag or mouse anti-human CD71, for 1 h on ice. After washing, bound annexin V was detected with SA-Oregon green, and bound Ab was detected with Texas Red-conjugated goat anti-mouse IgG for 1 h at 4°C. Cells were washed and mounted on glass microscope slide using Prolong Anti-fade mounting media (Molec

Results

Galectin-1 and galectin-3 are expressed in human lymphoid tissue

Both galectin-1 and galectin-3 are expressed in lymphoid tissues such as thymus and lymph node, so that T cells could encounter either or both galectin-1 and galectin-3 at different anatomic sites (4, 5). To demonstrate the coexpression of galectin-1 and galec

Galectin-3 receptors and T cell death

Like galectin-1, galectin-3 has been reported to kill T cell lines, although the mechanism of galectin-3-induced death is not well understood (20). We compared the ability of galectin-3 and galec

FIGURE 1. Galectin-1 and galectin-3 are expressed in human thymus. Consecutive 0.5-μm sections of human thymus were incubated with nonimmune rabbit serum (A), or rabbit polyclonal antiserum to galectin-1 (B), or galectin-3 (C). Bound Ab was detected by HRP-conjugated goat anti-rabbit IgG. Positive staining is indicated by the brown reaction product. Sections were counterstained with hematoxylin (blue). Magnification, ×50. The same rabbit polyclonal antiserum were used to stain primary cultures of fixed, nonpermeabilized human thymic epithelial cells, shown beneath respective sections of stained thymus. Bound Ab was detected by FITC-conjugated goat anti-rabbit IgG and cells imaged by fluorescence microscopy. Abundant cell surface staining was observed with the galectin-1 and galectin-3 reagents, with staining visible over the epithelial cell surface including the plasma membrane region over the nucleus, while no reactivity with control rabbit serum was observed. Magnification, ×100.
To examine the potency of galectin-3 in triggering T cell death, increasing concentrations of galectin-3 were added to T cell lines and cell death was determined by labeling with annexin V and PI. As a positive control, cells were treated with 20 μM galectin-1. Galectin-3 induced significant death of T cell lines as demonstrated by annexin V binding and PI uptake (Fig. 3B). We also observed an increase in the fraction of cells with subdiploid DNA in galectin-3-treated samples compared with control, as previously described (20), and loss of viable cells was determined by forward vs side scatter (data not shown). Moreover, we observed galectin-3-induced T cell death at lower concentrations compared with galectin-1. A total of 1–5 μM galectin-3 induced significant T cell death (Fig. 3B), while 5 μM galectin-1 induced negligible cell death (data not shown) and comparable levels of cell death required 20 μM galectin-1. Lactose abrogated both galectin-3- and galectin-1-induced cell death, demonstrating that galectin-3, like galectin-1, induces death of T cells in a carbohydrate-dependent manner (Fig. 3C).

Although galectin-3 is expressed in human thymus, the susceptibility of human thymocytes to galectin-3-induced death is unknown. We added galectin-3 or galectin-1 to total human thymocytes and analyzed loss of viable cells from different thymocyte subsets. As shown in Fig. 4, galectin-3 induced loss of both CD4CD8 double-negative (DN) and double-positive (DP) thymocytes, with negligible loss of CD4 or CD8 single-positive thymocytes (data not shown). Increasing concentrations of galectin-3 resulted in increased loss of cells in both DN and DP subsets. However, DN thymocytes were more susceptible to galectin-3, as shown in Fig. 4A. Treatment with 5 μM galectin-3 resulted in an ~25% loss of viable DN thymocytes and treatment with 20 μM galectin-3 resulted in a loss of >60% of DN thymocytes. In contrast, we observed a <10% cell loss of DP thymocytes treated with 5 μM galectin-3, and maximal loss of DP thymocytes with 20 μM galectin-3 was <30%. The relative selectivity of galectin-3 for DN thymocytes is very different from the effects of galectin-1 on thymocyte subpopulations. As we have previously shown (21), human DN and DP thymocytes were both susceptible to galectin-1 (Fig. 4B). Thus, while thymocytes would encounter both galectin-3 and galectin-1 in the thymic stroma, the two galectins may have distinct effects on different thymic subsets in vivo.

Although galectin-3 and galectin-1 had distinct effects on different thymocyte subsets, it was not clear whether differential susceptibility to the two galectins resulted from differences in cell surface receptor recognition, differences in intracellular pathways, or both. To examine whether the intracellular death pathways differ between galectin-3 and galectin-1, T cells were treated with increasing concentrations of galectin-3 alone or in combination with 10 or 20 μM galectin-1 (Fig. 5). As previously described, 10 μM galectin-1 induced variable levels of T cell death, while 20 μM galectin-1 consistently induced significant T cell death. As we observed in Fig. 3B, treatment with 1–5 μM galectin-3 resulted in significant levels of T cell death, with maximal cell death observed at 5 μM galectin-3. Importantly, no additive or synergistic effects of galectin-1 and galectin-3 were observed. In no case did addition of either galectin-1 or galectin-3 increase the level of T cell death above that observed for either galectin alone. These results suggested that the intracellular death pathways triggered by galectin-3 and galectin-1 intersect at some point. Thus, we reasoned that differential susceptibility to galectin-3 vs galectin-1 would likely be determined by binding to different sets of cell surface glycoprotein receptors.
Galectin-3 and galectin-1 bind to distinct complements of glycoprotein receptors

Although both galectin-3 and galectin-1 bind to T cells in a carbohydrate-dependent manner (Fig. 3A), the saccharide ligands preferentially recognized by the two galectins are expressed on numerous T cell surface glycoproteins. As galectin-3 and galectin-1 have very distinct structural features, with galectin-3 (which is primarily a monomer in solution) forming a pentamer, joined by flexible N termini, when the C-terminal-binding domains bind to clustered saccharide ligands, and galectin-1 forming a noncovalent
rigid homodimer (28, 41, 42), galectin-3 and galectin-1 may recognize similar saccharides on distinct glycoprotein backbones. To investigate whether galectin-3 and galectin-1 bind to different glycoprotein receptors, we asked whether galectin-3 and galectin-1 compete for binding to receptors on the T cell surface. Biotinylated galectin-1 (10 nM) was bound to T cells in the absence or presence of increasing concentrations of unlabeled galectin-3 at 4°C, and bound galectin-1 detected with SA-FITC. Galectin-3 only partially inhibited galectin-1 binding to the T cell surface; maximal inhibition of galectin-1 binding by galectin-3 was 50% (Fig. 6). These results indicated that galectin-3 and galectin-1 preferentially recognize distinct repertoires of T cell surface glycoprotein receptors.

To isolate specific T cell glycoproteins that bind galectin-3, we performed galectin-3 affinity chromatography, as we have previously done to isolate galectin-1 receptors (16). Cell surface proteins were labeled with biotin, and cell membrane extracts were applied to a galectin-3 affinity column. After extensive washing, bound glycoproteins were eluted with lactose, and the eluate was analyzed by blotting with SA-HRP to detect cell surface proteins. As shown in Fig. 7, several major protein bands were visualized in the lactose eluate. The pattern of protein bands eluted from the galectin-3 affinity column differed from the pattern we had previously observed for T cell glycoproteins bound to a galectin-1 affinity column (16), indicating that the set of T cell surface glycoproteins that preferentially bind to galectin-3 is different from the set of receptors bound by galectin-1.

To identify specific galectin-3 T cell glycoprotein receptors, we obtained peptide sequence information for proteins eluted from the galectin-3 column. Several T cell membrane glycoproteins were identified in the eluate; those previously implicated in apoptosis or binding to galectin-1 or galectin-3 are presented in Table I. These included the previously identified galectin-3 receptors Mac-2-binding protein, CD29, and CD98 (5, 43, 44), as well as the galectin-1 receptors CD43 and CD45 (16). This approach also identified several novel galectin-3-binding proteins, including CD71 and the TCRβ chain. Notably, CD7, an important TCR for galectin-1 (7), was not identified in the material eluted from the galectin-3 column. To examine T cell glycoprotein receptors that could participate in galectin-3-induced death, we focused on CD29, CD43, CD45, and CD71, all of which have been previously implicated in T cell death (20, 45–49). We confirmed the presence of these four glycoproteins in the eluate from the galectin-3 affinity column by immunoprecipitation or Western blotting. As shown in Fig. 7, CD29, CD43, CD45, and CD71 glycoproteins were four of the predominant bands isolated from the galectin-3 affinity column.

CD45, but not CD29 nor CD43, contributes to galectin-3-induced T cell death

Although identification of T cell surface glycoproteins that bound galectin-3 was a critical step in defining the glycoproteins involved in galectin-3-mediated death, it is important to note that T cell death induced by galectin-3 is not triggered by CD45, CD29, or CD43 binding. We found that CD45, but not CD29 nor CD43, contributes to galectin-3-induced T cell death.
Glycoproteins modified with the appropriate glycans may bind to galectin-3, yet not be required for T cell death. This has previously been demonstrated for galectin-1; while galectin-1 binds CD2 and CD3, neither of these glycoproteins are required to trigger galectin-1-induced T cell death (7, 14).

To determine the roles of CD29, CD43, and CD45 in galectin-3-induced T cell death, we examined a panel of cell lines with pairs of cells expressing or lacking the indicated glycoprotein. Cells were treated with 5 μM galectin-3 and cell death was determined by annexin V. As shown in Fig. 8A, CD29null Jurkat cells were susceptible to galectin-3-induced death. Similarly, CD43null CEM cells were susceptible to galectin-3-induced death (Fig. 8A). Thus, neither CD29 nor CD43 are required for galectin-3-induced T cell death.

However, CD45 appears to participate in galectin-3-induced cell death. This is in contrast to galectin-1, which can kill CD45null T cell lines (17). Jurkat E6–1 cells were susceptible to galectin-3-induced death, while the CD45null derivative J45.01 was resistant to galectin-3; addition of galectin-3 to J45.01 cells resulted in a negligible shift in annexin V binding (Fig. 8A). Moreover, reconstitution of CD45RABC expression in J45.01 cells rendered the cells susceptible to galectin-3, while J45.01 cells transfected with vector alone remained resistant to galectin-3, indicating that CD45 has a critical role in galectin-3-induced T cell death (Fig. 8B). Similarly, we found that galectin-3 induced death of murine BW5147 cells that express CD45 (17), while CD45−/− BW5147 cells were resistant to galectin-3 death (data not shown).

To examine the role of CD71 in galectin-3 cell death, we attempted to reduce CD71 expression in Jurkat E6–1 and CEM T cells by small-interfering RNA. Although we were able to decrease CD71 expression in Jurkat E6–1 cells by ~50%, as detected by mean fluorescent intensity of CD71 staining, we observed no difference in cell death induced by galectin-3 in small-interfering RNA-treated cells compared with control transfected cells (data not shown). The fact that transferrin is a survival factor for many cell types (50) may explain why we were unable to reduce CD71 expression by >50%, and the partial reduction in CD71 expression that we could achieve may not have been sufficient to affect T cell susceptibility to galectin-3.

Galectin-3 and galectin-1 induce death via different cell surface receptors

Our laboratory previously demonstrated a requirement for CD7 in galectin-1-induced cell death (7), and previous work using blocking Abs suggested a role for CD7 in galectin-3-induced T cell death (20). However, we did not detect CD7 among the T cell membrane glycoproteins that specifically bound to galectin-3 (Table I). To specifically address the role of CD7 in galectin-3 cell death, we examined the effect of galectin-3 on CD7null Hut78 T cells. Hut78 cells are resistant to galectin-1-induced death, while expression of CD7 on Hut78 cells rendered the cells susceptible to galectin-1 (7). As shown in Fig. 8C, while Hut78 cells were resistant to cell death induced by galectin-1, we observed significant death of Hut78 cells after addition of galectin-3. These data demonstrate that galectin-3 and galectin-1 induce T cell death via binding to different cell surface glycoproteins; galectin-1 T cell death requires CD7, while galectin-3 T cell death does not, and CD45 is not required for galectin-1-induced death, while CD45 appears to be required for galectin-3-induced death (Fig. 8).

Galectin-3 induces clustering of CD71, but not CD45, on the T cell surface

Our laboratory has previously shown that galectin-1 binding results in clustering of CD45 and colocalization of CD45 with phosphatidylinerine on apoptotic blebs during galectin-1-induced T cell death (16). As shown above, galectin-3, like galectin-1, binds to CD45, and CD45 may contribute to galectin-3 induced cell death (Figs. 7 and 8). However, as galectin-3 and galectin-1 have distinct structural features (5, 27, 28), the two galectins may have different effects on glycoprotein localization after binding. Thus, we determined the effect of galectin-3 binding on CD45 localization on the surface of CEM T cells. Before galectin-3 treatment, CD45 was diffusely distributed on the T cell surface, as demonstrated by immunofluorescence (data not shown). After galectin-3 binding, CD45 remained diffusely distributed on the T cell surface (Fig. 9A). To confirm that the T cells exposed to galectin-3 were undergoing cell death, cells were also labeled with annexin V. As shown in Fig. 9A, annexin V binding was concentrated in patches on apoptotic blebs of dying cells, as we observed during galectin-1 T cell death (16). However, unlike galectin-1, galectin-3 did not induce redistribution of CD45 to apoptotic blebs on dying cells.

As CD71 also bound to galectin-3, we investigated the cell surface distribution of CD71 following treatment with galectin-3. Before galectin-3 binding, and on cells treated with buffer control, CD71 was uniformly distributed on the surface of CEM T cells. However, as shown in Fig. 9A, binding of galectin-3 resulted in CD71 localization into distinct clusters on the T cell surface. Approximately 60% of galectin-3-treated CEM cells demonstrated clustering of CD71, while no clustering of CD45 was observed following galectin-3 treatment (Fig. 9B). Of the cells with clustered CD71, 90% also bound annexin V. Moreover, numerous cells had clustered CD71 colocalized with annexin V on membrane blebs. Importantly, none of the cells that retained uniform distribution of CD71 bound annexin V, suggesting that CD71 redistribution is involved in galectin-3 death.

Discussion

Galectin-3 and galectin-1 both bind to T cells to induce cell death in a carbohydrate-dependent manner. At first glance, it may appear that galectin-3 and galectin-1 have redundant functions, suggesting that one galectin could substitute for the other. Indeed, several other galectins, including galectin-2, -7, and -9, also kill T cells (8, 10, 13). However, the data presented here indicate that galectin-3...
and galectin-1 kill T cells by binding to different cell surface glycoproteins to initiate cell death. Similarly, the little that is known about galectin-mediated T cell death pathways supports the hypothesis that different galectins do not use identical mechanisms to kill T cells. For example, during galectin-1 cell death, endonuclease G is translocated from the mitochondria to the nucleus without release of cytochrome c (51). In contrast, during galectin-2-induced T cell death there is loss of mitochondrial membrane potential and release of cytochrome c (8). In vivo, different galectins may act at different times in development, at different anatomic sites, or on different cell populations, to regulate cell death. As the patterns of cell and tissue expression of many galectins overlap, we need to understand the different mechanisms used by different galectins to regulate T cell death.

Numerous examples of similarities and differences in galectin-3 and galectin-1 functions have been described. Although both galectin-3 and galectin-1 participate in RNA splicing in the nucleus (52), only intracellular galectin-3 prevents apoptosis triggered by a variety of agents, by localizing to the mitochondria and preventing cytochrome c release (22, 53). Although both galectin-3 and galectin-1 can interact with the Fas-mediated death pathway, extracellular galectin-1 augments Fas-induced death (54), while intracellular galectin-3 directs the death pathway triggered by Fas binding (55). Although both galectin-3 and galectin-1 can mediate binding of pathogens to host cells, only galectin-1 promotes attachment of HIV-1 to target cells, while galectin-3 inhibits galectin-1-facilitated HIV attachment (56, 57). Although both galectin-3 and galectin-1 bind to T cells to trigger T cell death, we show here that the galectins differ with respect to cell surface receptors, receptor localization after binding, and requirements for specific receptors to trigger T cell death. Although administration of both galectin-3 and galectin-1 were immunosuppressive in several animal models of inflammatory disease, and both galectin-3 and galectin-1 influence the threshold of TCR signaling (58, 59), galectin-1 treatment reduces Th1-type cytokine production (5, 32, 60), while galectin-3 treatment reduces Th2-type cytokine production (33, 61). Moreover, galectin-3 and galectin-1 knockout mice have distinct phenotypes. Galectin-3-deficient mice have attenuated neutrophil recruitment, while galectin-1-deficient mice have increased neutrophil recruitment compared with wild-type mice in a model of peritoneal inflammation (J. C. Paulson, Consortium for Functional Glycomics: (www.teleimpac.com/glycomics/publicdata/phenotyping.jsp) and Ref. 62), consistent with observations that galectin-1 antagonizes neutrophil adhesion to endothelium, while galectin-3 promotes neutrophil adhesion to endothelium (63, 64). Thus, there is ample evidence that galectin-3 and galectin-1 have overlapping but distinct functions in immune regulation.

Our data clearly demonstrate that galectin-3 and galectin-1 require distinct cell surface glycoprotein receptors to trigger T cell death. Galectin-1 binds to and requires CD7 expression to trigger T cell death (7), but galectin-3 does not bind CD7 nor require CD7 to trigger T cell death. Furthermore, the major T cell glycoproteins recognized by galectin-3 differ from those recognized by galectin-1. As galectin-3 and galectin-1 bind distinct sets of oligosaccharide ligands (29–31), these ligands may be differentially displayed on specific T cell glycoproteins. Both galectin-1 and galectin-3 bind CD29, CD43, and CD71, but only galectin-3 binds CD71, and only galectin-1 binds CD7 and CD2 (7, 16, 65). Not only do galectin-3 and galectin-1 bind distinct complements of glycoprotein receptors, galectin-3 and galectin-1 also induce distinct patterns of clustering of these receptors on the T cell surface. Although both galectin-3 and galectin-1 bind CD45, CD45 clustering occurs after galectin-1 binding (16), while CD45 clustering was not observed during galectin-3-induced cell death. However, galectin-3 did induce clustering of CD71 on dying cells (Fig. 9), and we observed no death of cells that did not cluster CD71 after galectin-3 binding. Thus, CD71 clustering may be involved in the galectin-3 death pathway. Intriguingly, both DN and DP thymocyte subsets express CD45 and CD71, yet we observed differential susceptibility to galectin-3 between these two subsets (Fig. 4). Additional factors, such as differences in glycosylation between these thymocyte subsets (4), may determine the relative susceptibility of the cells to galectin-3. For example, different isoforms of CD45 are differentially glycosylated, and specific CD45 isoforms can be modified by glycosyltransferases expressed in different thymocyte subsets (17, 18); while the present work only determined that the CD45RABC isoform was sufficient to render Jurkat cells susceptible to galectin-3, ongoing studies in our laboratory are examining the roles of different CD45 isoforms and differential CD45 glycosylation in regulating cell susceptibility to galectin-3.

The precise oligosaccharide ligands that regulate galectin-3-induced T cell death remain undefined. However, our observation that galectin-3 kills BW5147 T cells indicates that galectin-3 and galectin-1 also have different requirements for cell surface glycosylation. BW5147 cells lack expression of the core 2 N-acetylglucosaminyltransferase (C2GnT) that is required for galectin-1 cell death (17). As BW5147 cells died in response to galectin-3, the specific glycans made by C2GnT are not required for galectin-3.
cell death. Differences in recognition of or requirement for specific oligosaccharide ligands may reflect differences in the carbohydrate recognition domains of the two galectins or may result from structural differences between galectin-3 and galectin-1 that dictate how the different galectins can interact with glycan ligands on the cell surface.

Galectin-3 and galectin-1 have several structural features that may contribute to differential receptor recognition and clustering of cell surface glycoproteins. Galectin-1 is a rigid noncovalent homodimer, with a $K_d$ in the low micromolar range (42). We have demonstrated that the dimeric form of galectin-1 is required for cell death, and the rigid homodimer may be critical for the galectin-1 pattern of receptor binding and homogeneous receptor clustering on the T cell surface (16). In contrast, galectin-3, that is a monomer in solution, forms pentamers via the flexible N-terminal domain after binding to saccharide ligands on an immobilized monomer in solution, forms pentamers via the flexible N-terminal domain after binding to saccharide ligands on an immobilized monomer in solution. Galectin-3 induces cell death at sites where the concentrations of the two galectins are equivalent.

As shown in Table I, several T cell glycoproteins were eluted from the galectin-3 affinity column (Table I), galectin-3 interaction with TCR/CD3 complex may also contribute to the inhibitory effect of galectin-3 on TCR signaling. Alternatively, galectin-3 may form a lattice that cross-links CD43, CD45, or CD71 cross-linking by the Yersinia protein Invasin all induce T cell apoptosis (45–49). However, while CD29 Abs blocked galectin-3-induced T cell apoptosis (20), our data indicate that CD29 expression is not absolutely required for cell death (Fig. 8). Similarly, while CD43 contributes to galectin-1-induced cell death (J. Hernandez and L. G. Baum, unpublished data), CD43 expression was not required for galectin-3-induced cell death (Fig. 8). As we have also observed that galectin-3 kills murine BW5147 cells that do not express the TCR complex, this implies that the TCRβ chain that was isolated on the galectin-3 affinity matrix (Table I) is also not required for galectin-3-induced cell death. Instead, our data suggest that CD45 is involved in triggering galectin-3-mediated cell death, as cells that lack CD45 expression were not susceptible to galectin-3, and restoration of CD45 expression restored susceptibility to galectin-3 (Fig. 8). Moreover, we observed galectin-3-induced clustering of CD71 (Fig. 9), suggesting that galectin-3 binding to CD71 is also involved in sending a death signal. Further work is required to elucidate the precise roles of CD45 and CD71 in galectin-3-induced death.

Identification of galectin-3 TCRs and characterization of receptor clustering not only have important implications for the galectin-3 death pathway, but also suggest a mechanism by which galectin-3 may restrict TCR recruitment to the immunological synapse (58). As we also isolated TCRβ from the galectin-3 affinity column (Table I), galectin-3 interaction with TCRβ may contribute to setting the threshold for TCR response to Ag. Similarly, as localization of CD71 to the peripheral ring of the immunological synapse occurs during T cell activation (66), galectin-3 clustering of CD71, preventing CD71 recruitment to the synapse, may also contribute to the inhibitory effect of galectin-3 on TCR signaling. Alternatively, galectin-3 may form a lattice that cross-links and restricts movement of many different receptors, including CD29, CD43, CD45, and CD71, to antigenic formation of the immune synapse.

Although upstream glycoprotein receptors for galectin-3 and galectin-1 are distinct, downstream mechanisms of cell death may be common, as we observed that T cell death induced by galectin-3 and galectin-1 was neither additive nor synergistic (Fig. 4). This is in contrast to the additive or synergistic effects of galectin-1 in cell
death induced by CD3 or Fas cross-linking (15, 54). Commonality of galectin-3 and galectin-1 intracellular functions following distinct upstream events is not unusual. Similar findings were reported for the induction of neutrophil respiratory burst by galectin-3 and galectin-1. Although both galectins induce NADPH oxidase activity of primed neutrophils with similar kinetics, galectin-1 and galectin-3 bound distinct subsets of receptors on neutrophils to trigger a respiratory burst (67).

FIGURE 8. Identification of T cell glycoproteins that participate in galectin-3-induced T cell death. A, Parental T cell lines and derivatives lacking CD29, CD43, or CD45 were treated with galectin-3 (solid line) or buffer control (dotted line) for 4 h and cell death determined by annexin V binding. B, Reconstitution of murine CD45RABC expression in CD45− Jurkat 45.01 cells restored susceptibility to galectin-3 death. Top panel, CD45 expression on cells transfected with vector alone (45.0 vector) or with murine CD45RABC (dotted line, isotype control; solid line, anti-CD45, clone 30-F11). Jurkat cells expressing CD45RABC were susceptible to galectin-3 death, shown by annexin V binding (dotted line, buffer control; solid line, galectin-3) and by cell loss (□, buffer control; ■, galectin-3). Cells transfected with vector alone (45.0 vector) remained resistant to galectin-3. C, Hut78, a T cell line lacking CD7, was treated with galectin-1 or galectin-3 and cell death determined by annexin V binding. Galectin-3, but not galectin-1, induced death of Hut78 T cells. Dotted and black lines represent annexin V binding to cells treated with buffer control and galectin, respectively. Data shown are from one representative of three independent experiments.
Why do galectin-1 and galectin-3 both kill T cells? Both galectin-1 and galectin-3 are abundantly expressed in several tissues, including thymus and lymph nodes (4, 24, 39, 57, 68, 69). Although a recent report noted that the amount of galectin-3 protein in total lymph node tissue is much lower than the amount of galectin-1 and galectin-3 are abundantly expressed in several tissues, including thymus and lymph nodes (4, 24, 39, 57, 68, 69). Although a recent report noted that the amount of galectin-3 protein in total lymph node tissue is much lower than the amount of galectin-1 and galectin-3 are abundantly expressed in several tissues, including thymus and lymph nodes (4, 24, 39, 57, 68, 69).

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Disclosures

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References


