Structural Features of Galectin-9 and Galectin-1 That Determine Distinct T Cell Death Pathways*

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The galectin family of lectins regulates multiple biologic functions, such as development, inflammation, immunity, and cancer. One common function of several galectins is the ability to trigger T cell death. However, differences among the death pathways triggered by various galectins with regard to glycoprotein receptors, intracellular death pathways, and target cell specificity are not well understood. Specifically, galectin-9 and galectin-1 both kill thymocytes, peripheral T cells, and T cell lines; however, we have found that galectin-9 and galectin-1 require different glycan ligands and glycoprotein receptors to trigger T cell death. The two galectins also utilize different intracellular death pathways, as galectin-9, but not galectin-1, T cell death was blocked by intracellular Bcl-2, whereas galectin-1, but not galectin-9, T cell death was blocked by intracellular galectin-3. Target cell susceptibility also differed between the two galectins, as galectin-9 and galectin-1 killed different subsets of murine thymocytes. To define structural features responsible for distinct activities of the tandem repeat galectin-9 and dimeric galectin-1, we created a series of bivalent constructs with galectin-9 and galectin-1 carbohydrate recognition domains connected by different peptide linkers. We found that the N-terminal carbohydrate recognition domain and linker peptide contributed to the potency of these constructs. However, we found that the C-terminal carbohydrate recognition domain was the primary determinant of receptor recognition, death pathway signaling, and target cell susceptibility. Thus, carbohydrate recognition domain specificity, presentation, and valency make distinct contributions to the specific effects of different galectins in initiating T cell death.

Cell death is an essential factor in T cell development, which regulates selection of functional T cells during development in the thymus, as well as elimination of activated T cells after microbial infection or other exposure to antigen (1, 2). A number of distinct T cell death pathways have been described, including those triggered by members of the galectin family of vertebrate lectins (3–5). Galectin-1 was the first family member described to induce death of developing thymocytes and activated peripheral T cells, and the galectin-1 T cell death pathway is the best characterized to date. Specific glycoprotein receptors involved in galectin-1 death and specific types of O- and N-glycan ligands required for galectin-1 death have been identified, and galectin-1 has been shown to trigger a novel intracellular death pathway (6–14). Other galectins have also been reported to trigger death of T cell lines and various T cell subsets, including galectin-2, galectin-3, galectin-8, and galectin-9 (15–19). Relatively little is known about the glycoprotein receptors, glycan ligands, and intracellular death pathways used by these galectins. However, our laboratory has found that galectin-1 and galectin-3 kill different subsets of thymocytes, and use distinct sets of glycoprotein receptors and glycan ligands to kill T cells (16); this suggests that, in vivo, different galectins expressed at different times and in different anatomical sites may target specific T cell subsets for death.

Galectin-1 and galectin-9 are highly expressed in many immune organs, including bone marrow, lymph nodes, spleen, and thymus (7, 20–23). Whereas galectin-1 and galectin-9 have the canonical carbohydrate recognition domains (CRDs)2 that define the galectin family, the two galectins have distinct structural features and belong to different subfamilies. Galectin-1 belongs to the dimeric galectin subfamily; galectin-1 is synthesized as a monomer with one CRD that non-covalently homodimerizes ($K_d \sim 7 \mu M$) (24). The dimeric form appears to be required for induction of T cell death (6). The galectin-1 homodimer is a relatively rigid structure, with the two identical CRDs oriented in an anti-parallel orientation (25). In contrast, galectin-9 belongs to the tandem repeat subfamily, with two CRDs joined by a flexible peptide linker. Three galectin-9 isoforms with peptide linkers of different lengths have been described in different tissues and cell lines (20, 26, 27). Unlike galectin-1, where the two CRDs are identical, the N-terminal and C-terminal CRDs of galectin-9 are different; the two CRDs share an amino acid sequence homology of only 39%, and bind distinct sets of saccharide ligands (26). The differences in the N- and C-terminal CRD sequences and respective ligand specificities suggest that the two CRDs of galectin-9 may play different roles in mediating the functions of galectin-9, e.g. triggering cell death. Given the length of the galectin-9 linker peptide, the N- and C-terminal CRDs of galectin-9 would also be predicted to

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2 The abbreviations used are: CRD, carbohydrate recognition domain; 7-AAD, 7-aminoactinomycin D; C2GnT, core 2 β1,6-N-acetylgalactosaminyltransferase; DMNJ, deoxyxymannojirimycin; DN, double negative; DP, double positive; FITC, fluorescein isothiocyanate; PI, propidium iodide; RT, reverse transcript; PBS, phosphate-buffered saline; Th1, T helper 1.
have greater rotational flexibility and greater flexibility in the spacing between the CRDs, compared with the two CRDs of the galectin-1 dimer.

Both galectin-1 and galectin-9 can kill thymocytes, peripheral T cells, and T cell lines (6, 7, 20, 28, 29), and administration of both galectin-1 and galectin-9 have been shown to be therapeutically effective in a murine nephritis model (30). However, the few studies that have examined the mechanism of galectin-9 T cell death suggest that there are significant differences between the galectin-1 and galectin-9 death pathways. First, galectin-9 is much more potent than galectin-1 in inducing T cell death; even when galectin-1 is made as a leucine zipper dimer or a bivalent single chain molecule, minimal cell death is observed in vitro at concentrations <1 μM (31, 32). In contrast, 0.1 μM galectin-9 is sufficient to induce significant apoptosis of MOLT-4 and Jurkat T cells, thymocytes, and peripheral blood T cells (19, 20, 28). Second, galectin-9 and galectin-1 appear to recognize different T cell surface glycoprotein receptors; Tim-3 and CD44 have been identified as glycoprotein receptors for galectin-9 (33, 34), whereas galectin-1 binds to several T cell surface glycoproteins including CD2, CD3, CD7, CD43, and CD45 (8, 10, 13, 35). Finally, galectin-1-induced death of MOLT-4 T cells does not result in activation of caspases or release of cytochrome c from mitochondria (12). In contrast, galectin-9-induced death of MOLT-4 T cells results in cytochrome c release and is dependent on caspase activation (28).

The structural features of different galectins that determine differential recognition of target cells and distinct biologic effects remain unknown. In this study, we directly compared galectin-1- and galectin-9-induced T cell death with regard to requirements for glycan ligands and glycoprotein receptors, intracellular death mediators, and target cell susceptibility using murine thymocytes. To understand the structural features of galectin-1 and galectin-9 that contribute to differences in receptor requirements for death and susceptibility of target cell populations, we designed a series of hybrid proteins that combine various structural features of galectin-1 and galectin-9. Using these constructs, we determined that both CRD specificity and CRD presentation contribute to galectin potency in triggering cell death; however, the primary determinant of the T cell death pathway triggered by a specific galectin is the specificity of the C-terminal CRD. Elucidating the different T cell death pathways used by different galectins and determining the structural features of galectins required to trigger these distinct death pathways is a critical step in understanding how this family of molecules coordinately regulates T cell survival.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Reagents were purchased from indicated suppliers: TRIzol reagent, one-step RT-PCR kit, annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI), Dulbecco’s modified Eagle’s medium, RPMI 1640 medium, HEPES, Glutamax, penicillin-streptomycin, 7-aminoactinomycin D (7-AAD), phycoerythrin-conjugated monoclonal rat anti-mouse CD4, FITC-conjugated monoclonal rat anti-mouse CD8 antibodies, Texas Red-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rat IgG, CountBright absolute counting beads (Invitrogen); fetal bovine serum (HyClone, Logan, UT); dithiothreitol (Fisher Scientific, Chino, CA); ampicillin, gentamicin, G418, β-lactose, 10× PBS, minimal essential medium/sodium pyruvate solution (Sigma); polyclonal rabbit anti-human galectin-1 antibody (36); polyclonal mouse anti-human galectin-9 (Abnova, Taiwan); horseradish peroxidase-conjugated goat anti-mouse, anti-rabbit IgG (Bio-Rad); Ficoll-Paque (GE Healthcare); deoxyxymannosirinimycin (DMN), aqacide II (Calbiochem, San Diego, CA); 3-amino-9-ethylcarbazole, hematoxylin (Biomeria, Foster City, CA); monoclonal rat anti-human Tim-3 (R & D Systems, Minneapolis, MN).

**Cell Lines**—Human T cell lines CEM, HUT78, HH, Jurkat E6-1, J45.01 (American Type Culture Collection, Manassas, VA), and CEM.DKO (13), Jurkat A11, and the CD29-deficient derivative (gift of Dr. Yoji Shimizu, University of Minnesota, Minneapolis, MN) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.25% glucose, 1 mM sodium pyruvate, 2 mM Glutamax, 10 mM HEPES, and 50 units/ml penicillin/streptomycin. Jurkat E6-1 cells transfected with vector alone (E6 Neo) or with cDNA encoding galectin-3 (E6 Gal3) (12, 17) were maintained in the same medium supplemented with 10 μg/ml geneticin. Jurkat E6-1 cells transfected with vector alone (E6 Mock), or with cDNA encoding Bcl-2 (E6 Bcl-2) (37) (gift of Dr. J. C. Reed, The Burnham Institute, La Jolla, CA) were maintained in the same medium supplemented with 0.9 mg/ml G418. Murine T cell lines BW5147.3 (BW5147) and BW5147PhaR2.1 (PhaR2.1) (9) were maintained in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, 2 mM Glutamax, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 1 mM sodium pyruvate solution. PhaR2.1 ST6Gal I (PhaRST6) (11) was maintained in the same medium supplemented with 0.8 mg/ml geneticin.

**Messenger RNA Isolation and RT-PCR**—Total RNA from Jurkat E6-1 T cells was isolated using TRIzol reagent according to the manufacturer’s protocol. Galectin-9 and galectin-1 cDNA were obtained using a one-step RT-PCR kit (Invitrogen). For galectin-9, primer sequences were: Gal-9FNde, 5′-tgcaacatta-ttgctctaggcagtccccag-3′, and Gal-9RXho, 5′-cctctgcatctgtg-gcacatgggt-3′. For galectin-1, primers were: Gal1FNde, 5′-gga-gatagatagatagcttgcttggctgc-3′, and Gal1RXho, 5′-cctctgcatctgtg-gcacatgggt-3′. cDNAs encoding gal-9-9-1, galectin-9 cDNA was used as PCR template and the primers were: Gal1FNde (described above); Gal1RDR3, 5′-ctgcactcttggtgacgtgcttgggtgccacacattttgat-3′; Gal1FDR3, 5′-ctgctcgtgtgtgacgtgcttgggtgccacacattttgat-3′; Gal1RDR3, 5′-ctgctcgtgtgtgacgtgcttgggtgccacacattttgat-3′; Gal1FDR3, 5′-acctgactctgtgacgtgcttgggtgccacacattttgat-3′; Gal1RXho, described above. The PCR products of both reactions were purified and mixed to use as a PCR template for generating gal-1-9-1, using primers Gal1FNd, Gal1FDR3 or Gal1RDR3, Gal1RXho. To construct cDNA encoding gal-9-9-1, galectin-9 cDNA was used as a template to obtain the N-terminal CRD sequence, and galectin-1 cDNA was used as a template for the C-terminal CRD. The primers were: Gal9FNde (described above), Gal9NCRDLNKR, 5′-gacgctctgcttggtgacgtgcttgggtgccacagcttgag-3′; Gal1LN9K9, 5′-cacagctgcttggtgacgtgcttgggtgccacag-3′; and Gal1RXho (described above). The PCR prod-

**Galectin Structure Dictates T Cell Death Pathways**

have greater rotational flexibility and greater flexibility in the spacing between the CRDs, compared with the two CRDs of the galectin-1 dimer.

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ucts of both reactions were purified and mixed to use as a PCR template for generating gal-9-9-1, using Gal9FNde, Gal9NCRD/LNKR or Gal11LNK9F, and Gal1RXho primers. To construct cDNA encoding gal-1-9-9, galectin-1 cDNA was used as the template for the N-terminal CRD, and galectin-9 cDNA was used as the template for the C-terminal CRD. The primers were: Gal1FNde (described above), Gal1–9R, 5'-tgtctgggtgctaaagggcaca-3'; Gal9SF, 5'-acctcagactcatccaca-3'; and Gal9RXho, 5'-cctctgagactgtgtgcatgg-3'. The PCR products of both reactions were purified and mixed to use as PCR template for generating gal-1-9-9, using Gal1FNde, Gal1–9R or Gal9SF, and Gal1RXho primers.

To construct cDNA encoding single chain bivalent galectin-1 (gal-1 GG), overlapping PCR as described by Battig et al. (31) was used with the following modification. The first domain of galectin-1 cDNA was amplified from a modified pGEMEX-1 vector (Promega) using primer F1 (5'-ggacccgaacagtggcctttgacggcggtatggcttgtggtctggtc-3') which anneals to the T7 promoter region of the pGEMEX vector and primer R2 (5'-acactcagactcatccaca-3'). The second domain of galectin-1 cDNA was amplified with primer F2 (5'-gtggcctttgacggcggtatggcttgtggtctggtc-3') and primer R1 (5'-acactcagactcatccaca-3'), which anneals to the pGEMEX vector 20 bp after the galectin-1 stop codon. The resulting PCR products were amplified together for 5 cycles followed by amplification with primers F1 and R2 for 18 cycles. The final PCR products of gal-9, gal-1-9-1, and gal-1-9-9 were cloned into TOPO-TA-PCR vector (Invitrogen), and gal-1 GG was cloned into TOPO-XL-PCR vector from Invitrogen.

Construction of Galectin Expression Vectors—For galectin-9, gal-1-9-1, gal-9-9-1, and gal-1-9-9, cDNA was cut from the TOPO-TA-PCR vectors containing the sequence of interest with NdeI and Xhol (New England Biolabs, Ipswich, MA). DIGested cDNA was purified and ligated into pET vector 3.1 (Novagen, EMD Biosciences, San Diego, CA) linearized by the same two restriction enzymes. Gal-1 GG cDNA was cut from the TOPO-XL-PCR vector with Xbal and BamHI (New England Biolabs) and ligated to modified pGEMEX-1 vector linearized by the same two restriction enzymes. The ligated products were transformed into chemically competent Escherichia coli DH5α (Novagen) to amplify the expression vectors. Plasmids (pET/galectin vectors and modified pGEMEX-1/gal-1 GG) were isolated and the inserts verified by restriction enzyme analysis and DNA sequencing.

Expression of Galectins—Recombinant galectin-1, gal-1 GG, gal-1-9-1, gal-9-9-1, and gal-1-9-9, and galectin-9 were purified as previously described (38), except that E. coli Rossetta (DE3)/pLysS cells (Novagen), that have enhanced capability to express mammalian proteins with rare codons for E. coli, were used. All galectins were eluted from a lactose-agarose affinity column using 200 mM lactose, followed by further purification on a sizing column (38). After concentration, galectin-1, gal-1 GG, and gal-1-9-1 were dialyzed against 1× PBS with 8 mM dithiothreitol, whereas gal-9-9-1, gal-1-9-9, and galectin-9 were dialyzed against 1× PBS.

T Cell Death Assays—1 × 10⁶ cells were incubated with the indicated concentration of the various galectins or buffer control for 6 (mouse cell lines) or 5 h (human cell lines), in 24-well tissue culture plates (Sarstedt Inc., Newton, NC) at 37 °C in 5% CO₂. Buffer control for galectin-1, gal-1 GG, and gal-1-9-1 was 0.2 mM dithiothreitol in 1× PBS, to match the final dilution of the galectin stock in 1× PBS. Buffer control for gal-1-9-9, gal-9-9-1, and galectin-9 was 1× PBS. 100 mM β-Lactose was added to dissociate the cells and the cells were washed with ice-cold 1× PBS. Cell death was determined by annexin V-FITC binding and PI uptake using a Becton Dickinson FACScan (BD Biosciences, San Diego, CA) and CellQuest software as described (6). 10,000 events were acquired per sample, and percent cell death determined as: (1 − (viable annexin V⁻ (galectin)/viable annexin V⁻ (control))) × 100.

Thymocyte Death Assays—Thymus tissues were harvested from 6-week-old C57BL/6 mice. Thymocytes were harvested as previously described (13). Cell viability was >95%. 1 × 10⁶ cells were incubated with galectins at the indicated concentrations or buffer control for 4 h at 37 °C. Cells were dissociated with 100 mM lactose and washed with 1× PBS before phenotypic analysis using CD4-phycocerythrin and CD8-FITC at 0.2 μg/ml. Isotype-matched controls were included for all reagents. Staining was performed at 4 °C for 30 min. After washing, cells were resuspended in HEPES buffer with 0.2 μg/ml 7-AAD. To quantitate percent cell death in each thymocyte subset, 10,000 total events were collected on a FACScan flow cytometer and analyzed with CellQuest software, and live cells were determined by gating for forward versus side scatter profiles and absence of 7-AAD (13, 16). To quantitate absolute cell death in each thymocyte subset, CountBright absolute counting beads (Invitrogen) were used and analyzed according to the manufacturer’s protocol. Briefly, 25 μl of beads were added to 100 μl of thymocytes (1 × 10⁶ cells) and mixed well prior to flow cytometry analysis; 3,000 bead events were collected per sample. Thymocyte viability was assessed based on forward versus side scatter and absence of 7-AAD uptake. The absolute number of viable cells in each subset was obtained by reference to the number of beads per sample.

Immunochemistry and Immunofluorescence—Consecutive 0.5-μm sections of human thymus were prepared as previously described (7, 36). Slides were blocked with 20% goat serum in 1× PBS for 30 min before addition of 1:1000 rabbit anti-human galectin-1 antibody, 1:200 mouse anti-human galectin-9 antibody, or non-immune serum control in 10% goat serum, bound galectin-1 antibody, 1:200 mouse anti-human galectin-9 antibody, or non-immune serum control in 10% goat serum, bound to dissociate the cells and the cells were washed with ice-cold 1× PBS. Cell death was determined by annexin V-FITC binding and PI uptake using a Becton Dickinson FACScan (BD Biosciences, San Diego, CA) and CellQuest software as described (6). 10,000 events were acquired per sample, and percent cell death determined as: (1 − (viable annexin V⁻ (galectin)/viable annexin V⁻ (control))) × 100.

RESULTS

Galectin-9-induced T Cell Death Requires Different Glycan Ligands and Glycoprotein Receptors, Compared with Galectin-1—Galectin-9, like galectin-1 and galectin-3, has been reported to kill T cell lines, thymocytes, and peripheral T cells. However, the mechanism of galectin-9 cell death is not yet elucidated in detail, nor is it understood how galectins trigger these different death pathways (16, 19, 28). We thus examined the requirement...
Galectin Structure Dictates T Cell Death Pathways

A, complex N-glycans are essential for both galectin-9- and galectin-1-induced T cell death. PhaR2.1 cells were grown in DMNJ or medium for 72 h, and treated with 1 μM galectin-9, 10 μM galectin-1, or buffer control. B, expression of ST6Gal1 and C2GnT regulate susceptibility to galectin-9. PhaR2.1, PhaRST6, and BW5147 cells expressing the indicated glycosyltransferases were treated with 1 μM galectin-9, 10 μM galectin-1 dimer, or buffer control. Cell death was determined by annexin V binding and PI uptake. Results are mean ± S.D. of triplicate measurements.

for specific glycan ligands and cell surface glycoprotein receptors in galectin-9 death. It is well established that galectin-9 is significantly more potent than galectin-1 in killing T cells (19, 28). We confirmed these observations and found significant death of Jurkat T cells at 0.3 μM galectin-9, whereas >5 μM dimeric galectin-1 was required to trigger death of these cells; we obtained similar dose-response curves for galectin-9 and galectin-1 using human T cell lines MOLT-4 and CEM and the murine T cell line PhaR2.1 (data not shown). Thus, to examine glycan ligands and glycoprotein receptors, we used concentrations of galectin-9 (1 μM) and galectin-1 (10 μM dimer) that yielded comparable levels of cell death of the various target cells.

Galectins recognize galactose-containing saccharide sequences displayed on both N- and O-glycans on cell surface glycoconjugates (39). Both N-glycans and O-glycans are involved in galectin-1-induced T cell death (6, 8–10, 13). To investigate the requirement for complex N-glycans in galectin-9-mediated T cell death, murine PhaR2.1 T cells were treated with mannosidase I inhibitor DMNJ (11), which prevents the formation of complex N-glycans. As shown in Fig. 1A, treatment with DMNJ reduced susceptibility of PhaR2.1 T cells to both galectin-1 and galectin-9; thus, N-glycans are essential ligands in both cell death pathways. We also observed that DMNJ treatment dramatically reduced galectin-9-induced death of human CEM and MOLT4 T cells (data not shown).

We have previously shown that addition of terminal α2,6-linked sialic acids to N-glycans by the ST6Gal1 and C2GnT modulates susceptibility to galectin-1 (8, 10, 13). To ask if CD7, CD43, and CD45 are required for galectin-9-induced T cell death, we used T cell lines that lack expression of each glycoprotein (Fig. 2). We reproductively observed enhanced death of CEM.DKO cells to galectin-9, compared with parental CEM cells (13). In contrast, both CEM and CEM.DKO cells were sensitive to galectin-9-induced death (Fig. 2B). In fact, we reproducibly observed enhanced death of CEM.DKO cells to galectin-9, compared with parental CEM cells, suggesting that loss of expression of the large, heavily glycosylated CD43 molecule from the T cell surface exposes other galectin-9 receptors that trigger death, or makes more galectin-9 available to bind other receptors. The Jurkat derivative J45.01 lacks CD45 expression. Parental Jurkat E6-1 cells and J45.01 cells were both susceptible to galectin-9; thus, although CD45 expression regulates susceptibility to galectin-1 (10) and galectin-3 (16), CD45 expression is not required for death to galectin-9 (Fig. 2B). We also examined a Jurkat A11 derivative that lacks CD29, as CD29 has been implicated in galectin-3 cell death (16, 41), but saw no difference in cell death between parental and CD29-null cells (Fig. 2B). In summary, the data in Fig. 2 demonstrate that galectin-9 uses distinct complements of T cell glycoproteins to trigger T cell death compared with parental CEM cells (13). HUT78 cells are susceptible to galectin-9-induced death, as BW5147 T cells that lack C2GnT expression are susceptible to galectin-9 (data not shown). Thus, unlike galectin-1, galectin-9-induced death does not require expression of core 2 O-glycans on cell surface glycoprotein receptors.

Specific T cell surface glycoproteins are involved in delivering the galectin-1 death signal to T cells. CD7 is essential for galectin-1 death, whereas CD43 enhances susceptibility of T cells to galectin-1, and CD45 can positively and negatively regulate susceptibility to galectin-1, depending on the glycosylation status of CD45 (8, 10, 13). To ask if CD7, CD43, and CD45 are required for galectin-9-induced T cell death, we used T cell lines that lack expression of each glycoprotein (Fig. 2). HUT78 cells do not express CD7 and are resistant to galectin-1 death (8). Although HUT78 cells were resistant to galectin-1 death, the cells were very sensitive to galectin-9, indicating that CD7 is not required for galectin-9 death (Fig. 2A). The CEM.DKO cell line is a CD43-negative derivative of CEM that shows reduced susceptibility to galectin-1 death, compared with parental CEM cells (13). In contrast, both CEM and CEM.DKO cells were sensitive to galectin-9-induced death (Fig. 2B). In fact, we reproducibly observed enhanced death of CEM.DKO cells to galectin-9, compared with parental CEM cells, suggesting that loss of expression of the large, heavily glycosylated CD43 molecule from the T cell surface exposes other galectin-9 receptors that trigger death, or makes more galectin-9 available to bind other receptors. The Jurkat derivative J45.01 lacks CD45 expression. Parental Jurkat E6-1 cells and J45.01 cells were both susceptible to galectin-9; thus, although CD45 expression modulates susceptibility to galectin-1 (10) and galectin-3 (16), CD45 expression is not required for death to galectin-9 (Fig. 2B). We also examined a Jurkat A11 derivative that lacks CD29, as CD29 has been implicated in galectin-3 cell death (16, 41), but saw no difference in cell death between parental and CD29-null cells (Fig. 2B). In summary, the data in Fig. 2 demonstrate that galectin-9 uses distinct complements of T cell glycoproteins to trigger T cell death compared with parental CEM cells (13). HUT78 cells are susceptible to galectin-9-induced death, as BW5147 T cells that lack C2GnT expression are susceptible to galectin-9 (data not shown). Thus, unlike galectin-1, galectin-9-induced death does not require expression of core 2 O-glycans on cell surface glycoprotein receptors.

Recent work has identified two other T cell surface glycoproteins as receptors for galectin-9. Tim-3, expressed on T helper 1 (Th1) CD4 T cells, was identified as an essential receptor for galectin-9-induced death of Th1 cells in vitro and in vivo (33). We analyzed Tim-3 protein expression on Jurkat E6-1, MOLT4, and CEM T cells by flow cytometry, but detected negligible Tim-3 expression on all three cell lines over a range of antibody concentrations (data not shown). Moreover, addition of a blocking anti-Tim-3 antibody to T cells prior to the addition of galectin-9 did not reduce T cell death (data not shown). Thus, whereas Tim-3 may be required for galectin-9 death of Th1 cells, Tim-3 expression is not universally required for galectin-9 death of T cells. In addition, CD44 has been identi-
fied as a T cell glycoprotein receptor for galectin-9 that is important for adhesion of T cells to hyaluronic acid (34). However, as Jurkat T cells do not express CD44 (Ref. 42 and data not shown), yet die in response to galectin-9, CD44 is also not required for T cell susceptibility to galectin-9.

**Galectin-9 and Galectin-1 Use Different Intracellular Death Pathways**—Previous studies have indicated that the intracellular death pathways triggered by galectin-9 and galectin-1 differ, as galectin-9 treatment of MOLT4 T cells results in rapid cytochrome c release from mitochondria, whereas this is not observed in galectin-1-treated MOLT4 cells (12, 28). Bcl-2 family proteins regulate mitochondrial release of apoptotic factors such as cytochrome c (37). To determine whether Bcl-2 regulates galectin-9-induced death, Jurkat E6-1 cells expressing Bcl-2 (E6 Bcl2) or transfected with vector alone (E6 Mock) were treated with 1 μM galectin-9, 10 μM galectin-1, or buffer control. E6 Bcl2 cells were resistant to galectin-9 but not galectin-1 cell death, compared with E6 mock control cells. If intracellular galectin-3 expression does not protect T cells from galectin-9-induced cell death. Jurkat E6-1 cells transfected with vector control (E6 Neo) or with cDNA encoding galectin-3 (E6 Gal3) were treated with 1 μM galectin-9, 10 μM galectin-1 dimer, or buffer control. E6 Gal3 cells were resistant to galectin-1 but not galectin-9 cell death. Cell death was calculated by annexin V binding and PI uptake. Results are mean ± S.D. of triplicate measurements.

**Galectin-9 and Galectin-1 Are Expressed in Distinct but Overlapping Subsets of Stromal Cells in Human Thymus and Kill Different Thymocyte Subsets**—Galectin-9 and galectin-1 are both expressed in a variety of immune tissues, including thymus, spleen, and lymph nodes (16, 20, 23, 36). However, the sites of galectin-9 expression in lymphoid tissue have not been well characterized. We compared patterns of expression of these two galectins in human thymus, using polyclonal rabbit anti-galectin-1 or polyclonal mouse anti-galectin-9 alone or in combination. As shown in Fig. 4, intracellular galectin-3 expression significantly reduced galectin-9-induced T cell death but had little effect on susceptibility of the cells to galectin-1. Conversely, overexpression of anti-apoptotic galectin-3 in Jurkat cells reduced susceptibility to galectin-1-induced death (12). In contrast, overexpression of galectin-3 had no measurable effect on the susceptibility of the cells to galectin-9, as we observed equivalent galectin-9-induced death of cells transfected with galectin-3 cDNA (E6 Gal3) or vector alone (E6 Neo) (Fig. 3B). Thus, distinct intracellular regulatory mechanisms govern susceptibility to galectin-1 and galectin-9 cell death.

**Galectin Structure Dictates T Cell Death Pathways**
abundant as galectin-1. Fig. 4 (bottom panel) showed thymus tissue double-labeled with anti-galectin-9 and anti-galectin-1, detected with secondary antibodies conjugated to FITC and Texas Red, respectively. We identified thymic stromal cells that expressed only galectin-9 or only galectin-1, as well as cells that expressed both galectins, but overall staining for galectin-9 was reduced compared with galectin-1. Thus, galectin-9 and galectin-1 have different patterns of expression within the anatomical subdomains of the thymus, and, within a given region, individual stromal cells may express one or both galectins. These data suggest that different subsets of developing thymocytes will encounter different galectins at discrete stages during maturation and migration through the thymus.

Developing thymocytes can be divided into subsets, identified by expression of specific cell surface glycoproteins that correspond to discrete stages of thymocyte maturation. The most immature thymocytes that have recently emigrated from bone marrow express neither CD4 nor CD8 (CD4−CD8−) and are termed double negative (DN) cells. During the next stage of thymocyte development, cells express both CD4 and CD8 and are termed double positive (DP). Mature thymocytes express either CD4 or CD8 and are termed single positive. Galectin-1 preferentially kills CD4+CD8− DN and CD4+CD8+ DP thymocytes, whereas galectin-3 preferentially kills CD4−CD8− DN thymocytes (7, 16, 29). Although Kanwar and colleagues (20) demonstrated that galectin-9 induced apoptosis of murine thymocytes, that study did not define specific thymocyte subsets that are sensitive to galectin-9 death.

To define thymocyte subsets that are susceptible to galectin-9 and to identify differences between galectin-1 and galectin-9 with regard to target cell subsets, galectin-1 or galectin-9 were added to total murine thymocytes. Thymocyte cell death was determined by phenotyping viable cells (10). Fig. 5 shows scatterplots indicating the relative loss of viable cells from each of the four thymocyte subsets, and the absolute number of viable cells remaining in each subset, by normalization with counting beads. As shown in Fig. 5, 1 μM galectin-9 was much more potent than 10 μM galectin-1 homodimer in killing CD4+CD8+ thymocytes, with loss of cells from all four subpopulations. There was almost complete death (95%) of the DP population after galectin-9 treatment, whereas galectin-1 killed only ~50% of this subset. Galectin-9 was also more potent than galectin-1 in inducing death of CD4−CD8− DN thymocytes, as galectin-9 killed ~80%, whereas galectin-1 killed ~50% of this subset. Surprisingly, galectin-9 killed ~50% of mature single positive thymocytes, both CD4+ and CD8+ cells, whereas galectin-1 killed only 20–30% CD4+ and CD8+ single positive cells. In all assays, thymocyte cell death was completely abrogated by addition of 100 mM lactose (data not shown), demonstrating that cell death required galectin recognition of saccharide ligands on the thymocytes.

**Structural Features Important for Functional Differences between Galectin-9 and Galectin-1**—Figs. 1–5 demonstrated that compared with galectin-1, galectin-9 requires different glycan ligands and glycoprotein receptors to trigger cell death, and that galectin-9 cell death occurs by a distinct intracellular path-
way compared with galectin-1 cell death, and that galectin-9 and galectin-1 kill different subsets of thymocytes. These differences between galectin-9 and galectin-1 may result from a number of factors, including differences in CRD specificity for glycan ligands, spacing, and flexibility of the CRDs contributed by linker peptides, and lectin valency. To identify features of galectin-9 and galectin-1 that are important for the biological differences we observed, we created a series of constructs with features of the two galectins (Fig. 6). Native galectin-1 is a monomer with a single CRD that forms non-covalent homodimers (24). Gal-1 GG has two galectin-1 CRDs linked by a glycine-glycine linker, so that gal-1 GG is a single chain bivalent galectin (31). Native galectin-9 is a bivalent tandem repeat galectin with two different CRDs that recognize different saccharide ligands, connected by a random coil linker (43). To understand the contributions of CRD specificity, linker peptide, and CRD presentation, we made three additional constructs. Gal-1-9-1 has two galectin-1 CRDs connected by the flexible 14-amino acid galectin-9 linker peptide. Gal-9-9-1 has the galectin-9 N-terminal CRD and the galectin-9 linker with the galectin-1 CRD at the C terminus. Gal-1-9-9 has an N-terminal galectin-1 CRD and the C-terminal galectin-9 CRD connected by the galectin-9 linker.

To confirm that the various bivalent galectin constructs could bind to and cross-link glycan ligands on the T cell surface, we performed agglutination assays. Agglutination of Jurkat T cells occurred with all galectins; all bivalent single chain galectins agglutinated Jurkat E6-1 T cells at lower concentrations than observed for native galectin-1 (data not shown). All T cell agglutination was reversed by addition of 100 mM lactose.

Previous studies (6, 7, 19, 20, 28) and Fig. 5 demonstrated that galectin-1 was much less potent than galectin-9 in inducing T cell and thymocyte cell death, respectively. We compared wild type galectin-1 and galectin-9 with the various constructs to determine whether the difference in potency was solely attributable to dissociation of dimeric galectin-1 at lower concentrations, or if other structural features of galectin-9 contribute to potency. As shown in Fig. 6B, bivalency was not the only feature that contributed to potency of galectins. The bivalent gal-1 GG was more potent than native galectin-1; however, when the two galectin-1 CRDs were connected by the flexible galectin-9 peptide linker (gal-1-9-1) rather than the two amino acid GG linker, we observed an even greater enhancement of potency, with significant cell death at 1 μM gal-1-9-1 (Fig. 6B). This implies that the flexibility of the 14-amino acid galectin-9 linker peptide further contributed to the ability of galectin-1 CRDs in gal-1-9-1 to bind T cell surface glycans to trigger cell death.

However, gal-1-9-1 was still less potent than native galectin-9 in inducing cell death (Fig. 6B), indicating that CRD specificity also contributes to potency. When we compared constructs with galectin-9 CRDs at the N or C terminus, we found that the gal-1-9-9 construct was as potent as native galectin-9, whereas the gal-9-9-1 construct had a dose-response curve virtually identical to that of gal-1-9-1 (Fig. 6B). Thus, the CRDs also significantly contribute to potency. Importantly, these results also indicated that the C-terminal CRD, but not the N-terminal CRD, determined the potency of these bivalent galectin constructs in inducing T cell death.

The C-terminal CRD of Galectins Determines the Death Pathway—The unexpected observation in Fig. 6B that the C-terminal CRD determined the potency of the neo-galectins prompted us to compare these constructs with regard to roles
Galectin Structure Dictates T Cell Death Pathways

We also examined the effects of these constructs on murine thymocytes (Fig. 8). Just as we observed with the cell lines in Fig. 7, all the constructs with a galectin-1 C-terminal CRD, i.e. gal-1 GG, gal-1-9-1, and gal-9-9-1, preferentially killed the same thymocyte subsets as galectin-1. In contrast, gal-1-9-9, which has a galectin-9 C-terminal CRD, killed all four thymocyte subsets to a degree comparable with native galectin-9. In particular, comparison of both scatterplot analyses and the absolute loss of viable thymocytes treated with either galectin-9 or gal-1-9-9 demonstrates almost identical profiles (Fig. 8). Thus, as we observed with T cell lines, the C-terminal CRD of the galectin constructs determined the activity against distinct thymocyte subsets.

DISCUSSION

The galectin family of lectins has a remarkably diverse range of functions in many cell types and at different points in development (4, 21, 44). Some of these functions are unique to a given galectin in a particular cell type, e.g. galectin-3 bundling of cell surface glycoproteins contributes to polarized glycoprotein localization in renal epithelial cells (45). In other cases, many galectins regulate the same function in a variety of cell types, e.g. galectins-1, -2, -3, -7, -8, -9, and -12 all regulate cell death (6, 15, 17, 28, 46–48). However, even in the same cell type, different galectins trigger distinct intracellular death pathways, e.g. galectin-1, galectin-2, galectin-3, and galectin-9 all trigger T cell death, but differ in the requirement for caspase activation (6, 12, 15, 17). Similarly, we have found that galectin-1, galectin-3, and galectin-9 kill different subsets of thymocytes and peripheral CD4 T cells (Fig. 5 and Refs. 16 and 20). As different galectins, which may be expressed in overlapping distributions within the same tissues, do not trigger the same cell death pathways nor kill the same T cell subpopulations, these galectins may act in concert within a tissue to coordinately control and finely regulate the critical event of cell death. This model is supported by the finding that galectin-1 null mice and galectin-3 null mice have distinct types of T cell-mediated immune defects, indicating that these two galectins control T cell death at different points during T cell development and peripheral immune responses (49–51). Similarly, administration of recombinant galectin-1, -3, or -9 has distinct therapeutic effects in mouse models of autoimmune disease such as asthma and nephritis (30, 34, 52).

The mechanisms by which galectins trigger these distinct effects are not well understood. As there are different patterns of expression and localization of various galectins within lymphoid tissues, T cells may encounter gradients of galectins as the cells migrate through the tissues. Structural differences...
Galectin Structure Dictates T Cell Death Pathways

Orientation, rotational flexibility, and spacing of the CRDs presented in bivalent or multimeric galectins will also contribute to recognition of specific glycan ligands on different cell types. In contrast to the relatively rigid homodimer structure of native galectin-1 (25), the two galectin-9 CRDs are on either end of a flexible peptide linker, so that the tandem repeat galectin-9 CRDs would be predicted to have greater rotational flexibility than the galectin-1 CRDs in the homodimer. Thus, galectin-9 may be able to cross-link glycoproteins that are more sparsely distributed or spatially distant on the cell surface, compared with galectin-1. CRD valency will also contribute to the recognition of specific glycan ligands on different cell types; whereas galectin-1 forms homodimers, a recent report has shown that murine galectin-9 can multimerize via protein-protein interactions of the N-terminal CRDs (54), so that higher order multimers of galectin-9 could bind to the surface of the cell.

Although addition of the galectin-9 linker peptide to galectin-1 CRDs in the gal-1-9-1 construct increased potency compared with native galectin-1, this construct was still less potent compared with galectin-9 in killing T cells (Fig. 6B), indicating that the increased spacing and rotational flexibility of the CRDs were not the only features determining potency and target cell killing of the two galectins. In addition, a galectin-9 construct with most of the linker peptide removed was shown to retain potency in T cell death assays (55). The higher potency of galectin-9 could be due to the higher valency of multimeric galectin-9 compared with dimeric galectin-1, if galectin-9 oligomerizes via the N-terminal CRDs (54, 56). However, increased valency alone would not account for the difference in potency between galectin-1 and galectin-9, as the gal-9-9-1 construct and native galectin-9 both have the galectin-9 N-terminal CRD, yet gal-9-9-1 behaved identically to gal-1-9-1 with regard to potency, receptor recognition, death pathway effectors, and thymocyte subset susceptibility, and both constructs were less potent than gal-1-9-9 or native galectin-9 in inducing T cell death (Figs. 6–8). As multimerization of galectin-1 CRDs has not been reported, it was surprising that gal-1-9-9 and native galectin-9 were almost equally potent; however, we have not yet examined the ability of any of these constructs to form higher order oligomers.

Remarkably, the C-terminal CRD of all the galectin constructs appeared to have the most dramatic effect on all aspects of T cell death, including potency, requirement for glycan ligands, recognition of critical glycoprotein receptors, triggering of intracellular death pathways, and specificity for different thymocyte subsets (Figs. 6–8). Although the potencies of gal-1-9-1 and gal-9-9-1 were greater than native galectin-1 or gal-1 GG, the requirement for glycan ligands, requirement for CD7 to trigger death, and cell death inhibition by intracellular galectin-3 were identical to what we observed for galectin-1, demonstrating that the C-terminal CRD dictated the behavior of the galectins in these assays. Similarly, gal-1-9-9 was identical to native galectin-9 with regard to requirement for glycan ligands and glycoprotein receptors, mediators of the intracellular death pathway and thymocyte subset susceptibility. The N- and C-terminal CRDs of galectin-9 are distinct, with only 39% amino acid homology, and the two CRDs bind distinct sets of...
saccharide ligands (26, 53). Thus, the dominant effect of the galectin-9 C-terminal CRD may relate to the structure or ligand specificity of this CRD. Although the specific glycan ligands required for galectin-9-induced T cell death are not yet known, complex N-glycans are important for galectin-9-induced T cell death (Fig. 1). Isolation and identification of the T cell glycoprotein receptors that participate in galectin-9-induced death are currently underway in our laboratory.

The patterns of thymocyte cell death (Fig. 8) were strikingly similar between gal-1-9-9 and native galectin-9, with massive cell death from all four thymocyte subsets. Importantly, this is the first study that has specifically investigated the susceptibility of all thymocyte subsets to galectin-9 death is similar between gal-1-9-9 and native galectin-9, with massive death (Fig. 1). Isolation and identification of the T cell glycoprotein receptors that participate in galectin-9-induced death are not yet known, over, understanding structural features important for different types, it is essential to understand the structural features that confer these effector functions on the various galectins. Moreover, understanding structural features important for different activities of galectins will allow targeting of desired effects in different disease states.

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