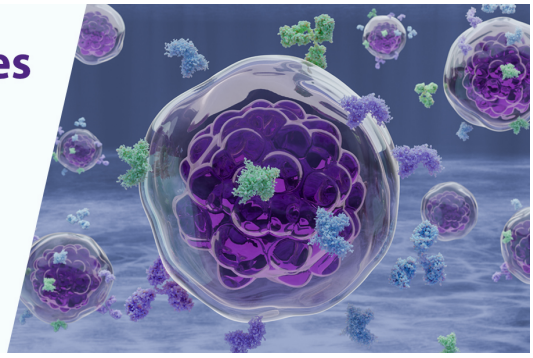

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Modulation of *O*-Glycans and *N*-Glycans on Murine CD8 T Cells Fails to Alter Annexin V Ligand Induction by Galectin 1[1]

Douglas A. Carlow,[2] Michael J. Williams, and Hermann J. Ziltener

Thymic negative selection and contraction of responding T cell oligoclones after infection represent important cell ablation processes required for maintaining T cell homeostasis. It has been proposed that galectin 1 contributes to these processes through interaction with lactosyl sequences principally on cell surface glycoproteins bearing core 2 (C2GnT1)-branched *O*-glycans. According to this model, specific T cell surface proteins cross-linked by galectin 1 induce signaling, ligand redistribution, and apoptosis in both immature thymocytes and activated T cells. The influence of lactosyl residues contained in branched *O*-glycans or complex *N*-glycans on galectin 1 binding and induction of annexin V ligand in murine CD8 T cells was assessed. Neither galectin binding nor galectin-induced expression of annexin V ligand was perturbed under conditions in which: 1) C2GnT1 activity was differentially induced by CD8 T cell activation/culture with IL-2 vs IL-4; 2) activated $CD8^+$ T cells lacked C2GnT1 expression; or 3) complex *N*-glycan formation was blocked by swainsonine. The maintenance of galectin 1 binding and induced annexin V expression under conditions that alter lactosamine abundance on *O*- or complex *N*-glycans suggest that galectin 1-mediated apoptosis is neither a simple function of fluctuating C2GnT1 activity nor a general C2GnT1-dependent mechanism underlying contraction of CD8 T cells subsequent to activation. ***The Journal of Immunology*, 2003, 171: 5100–5106.**

Galectin 1 is a member of a family of carbohydrate-binding lectins present in mammals (1, 2). Despite the discovery that galectins exhibit preferential carbohydrate specificity for lactosamine sequences, and the identification of a cadre of cell surface molecules carrying potential lactosamine targets or that exhibit demonstrable galectin 1 binding (3), the physiological role and specificity of this lectin remain a subject of inquiry and debate. The analysis of the physiological function of galectin is complicated by: 1) the diversity of potential lactosamine-bearing targets on *O*-linked glycoproteins, *N*-linked glycoproteins, and glycoplipids; 2) the potential impact of additional modifications within or adjacent to lactosamine sequences, such as sialylation or fucosylation that can affect galectin binding (4), but are difficult to track and manipulate in vivo; and 3) galectin binding has inherent instability inasmuch as maintenance of binding capacity requires either a reducing environment, such as exists within the cell, or that galectin be bound to a carbohydrate ligand (5–7). Indeed, free galectin 1 secreted into medium lacking high concentrations of reducing agents loses carbohydrate-binding activity (5), raising questions about where galectin binding occurs in vivo.

A focus of recent galectin reseach has suggested that galectin 1 may be used to regulate T cell immunity by inducing apoptosis in those activated T cells not destined for the memory pool. The hypothesis that galectin 1 is a physiological regulator of T cell death principally through binding core 2-branched lactosamine extensions on *O*-gylcans and thereby cross-linking specific cell surface glycoproteins was recently reviewed (3). This hypothesis is essentially based on: 1) the galectin 1-mediated killing of mitogen-activated, but not resting, human T cells (8); 2) the galectin 1-induced killing of a $\beta(1-6)$*N*-acetyl glucosaminyl transferase I (C2GnT1)-transfected T cell line (9); 3) increased galectin 1-induced death in thymocytes from C2GnT1 transgenic mice (9); and 4) guilt by association insofar as galectins are expressed in activated T cells and in the thymus, where negative selection of $CD4^+8^+$ T cells occurs, and C2GnT1 is expressed preferentially in both cortical thymocytes (10) and mature T cells after activation in vitro (11) or in vivo (12).

Despite the array of observations that galectin 1 can promote death of both immature thymocytes and activated human T cells, the generality of these observations, the involvement of C2GnT1, and the conclusive evidence that galectin 1 is involved in these processes under physiological conditions is lacking. One would predict that conditions altering C2GnT1 activity or galectin 1 expression in vivo would adversely affect T cell homeostasis. However, mice lacking either galectin 1 (13) or the C2GnT1 enzyme (14) show no such deregulation; redundancy in the galectin/C2GnT multigene families and compensatory lactosamine-bearing extensions of core 1 glycans may obscure relevant functions in these knockout mice. Furthermore, Blaser et al. (15) reported that murine galectin 1 inhibited proliferation of Ag-specific activated murine CD8 T cells but did not report cytolytic effects, and Rabinovich et al. (16) reported that chicken galectin 1 induced apoptosis when included during primary Con A stimulation of rat lymphocytes. Novelli et al. (17) reported that human galectin 1 inhibited proliferation of human PHA-stimulated and IL-2-expanded PBLs, but, contrary to observations cited above (8), apoptosis was not observed. Most recently, Amano et al. (18) reported that ST6 sialyltransferase modification of CD45 *N*-glycans inhibited galectin-induced death of T cell lines, whereas Fajka-Boja et al. (19) confirmed galectin 1 binding to CD45, but concluded that CD45 does not mediate the apoptotic signal initiated by galectin. Clearly, the role of C2GnT-branched *O*-glycans in galectin-induced T cell death is not resolved.

During analysis of C2GnT1 expression in short-term cultures of activated CD8 T cells, we recently reported that C2GnT1 could be effectively regulated by cytokines (20). Supplementing cultures with

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IL-2 efficiently induced high levels of C2GnT1 enzyme activity, whereas IL-4 supplements supported T cell expansion comparably without inducing C2GnT1. This differential C2GnT1 enzyme induction activity was paralleled by differential glycosylation of known C2GnT1 substrates in activated CD8 T cells. Notably, the effects of these cytokines on C2GnT1 expression were manifested relatively quickly within a 4-day activation/expansion of T cells isolated from spleen or lymph node.

The ability to modify C2GnT1 activity in parallel primary cultures supplemented with IL-2 vs IL-4 afforded a simple method to test whether lactosamines on branched *O*-glycans would alter galectin 1-mediated killing in T cells. Lymphocytes from normal or C2GnT1-deficient mice together with the *N*-glycan inhibitor swainsonine were applied with this simple culture system to explore galectin 1 killing in primary cultured murine T cells. Specifically, our goal was to assess the contribution of *O*- or *N*-linked lactosamine extensions on galectin 1-mediated killing of activated murine CD8 T cells.

Materials and Methods

Mice

Mice ages 9–16 wk were used for analyses. C57BL/6 mice were bred at the Biomedical Research Centre from founders obtained originally from The Jackson Laboratory (Bar Harbor, ME). $CD43^{-/-}$ mice (21) backcrossed for eight generations with C57BL/6 mice (22) were used. $C2GnT1^{null}$ mice were kindly provided by Drs. J. Marth and L. Ellies (Howard Hughes Medical Institute, University of California, San Diego, CA) (14).

Media

Cell suspensions were prepared in RPMI medium (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% FCS, 5×10^{-5} M 2-ME, 100 U/ml penicillin, 100 U/ml streptomycin (StemCell Technologies, Vancouver, BC, Canada), and 2 mM glutamine (Sigma-Aldrich, St. Louis, MO). Staining with biotinylated reagents for flow analysis was performed in DMEM (Life Technologies) supplemented with 2.5% FCS.

Staining reagents and flow cytometry

Abs were obtained from the following sources: 1B11-PE (BD PharMingen, San Diego, CA; 09695A) and CD8α FITC (BD PharMingen; 01044D). For cell surface staining, cells were suspended in DMEM containing 5% (v/v) FCS and incubated with Abs for 20–40 min on ice in Nunclon 96-well round-bottom plates (VWR, West Chester, PA). Cells were washed twice and analyzed on a FACScan IV flow cytometer (BD Biosciences, Mountain View, CA). Human IgG1-P-selectin fusion protein (BD PharMingen; 28111A) was detected with biotinylated anti-human IgG (Jackson ImmunoResearch, West Grove, PA; 109-065-098) and CyChrome-conjugated steptavidin (BD PharMingen; 554062). Galectin 1 was conjugated with either biotin using polyethylene oxide-maleimide reagent (21901; Pierce, Rockford, IL) or AlexaFluor 488 C5 maleimide (A-10254; Molecular Probes, Eugene, OR), according to supplier's instructions. For staining, 100 μl of 5–10 μM conjugated galectin was incubated with 10^5 cells in DMEM with 2 mM DTT (Sigma-Aldrich; D-5545), $\pm$ lactose where indicated, for 20 min at room temperature in a 96-well flat-bottom plate. When biotinylated galectin 1 was used, cells were resuspended to 300 μl, transferred to a 5-ml test tube, pelleted washed with 4 ml of DMEM + DTT, and incubated with SA-CyC in, DMEM + DTT for 5 min, followed by another 4-ml wash and flow analysis.

Lymphocyte cultures

Leukocyte cultures were performed, as previously described (20). Briefly, primary stimulations were conducted with spleen cells cultured at 10^6/ml in 4 μg/ml Con A (Sigma-Aldrich; C-0412) for 48 h at 37°C in 5% CO_2. Cultures (2 or 10 ml) were prepared in 24-well Falcon 3047 plates or 6-well Falcon 3046 plates, respectively (BD Biosciences). After 48 h, cells were harvested, washed, counted, and replated in secondary cultures at 0.25×10^6/ml with 2.5% IL-2 supernatants or at 0.05×10^6/ml with 2.5% IL-4 supernatants for optimum differential induction of C2GnT1. IL-2 and IL-4 were obtained as conditioned medium from the myeloma X.653 transfected with the cDNAs for murine IL-2 and IL-4, respectively (F. Melchers; Basel Institute of Immunology, Basel, Switzerland).

Galectin purification

The cDNA for rat galectin 1 was kindly provided by H. Leffler (Lund University, Lund, Sweden) in *Escherichia coli* BL21 in pET3 days. For galectin 1 preparation, an individual ampicillin-resistant bacterial colony was inoculated into 50 ml in Luria-Bertani medium supplemented with ampicillin (70 μg/ml) and grown to late log phase. This culture was then used to inoculate 2×500-ml Luria-Bertani cultures containing 70 μg/ml ampicillin and grown overnight at 37°C. The next morning, cells were harvested by centrifugation for 15 min at 4°C, washed once, and resuspended in 30–40 ml of cold MEPBS (PBS plus 2 mM EDTA + 4 mM 2-ME). The suspension was then frozen at −70°C to facilitate cell lysis. Samples were thawed and sonicated 5×30 s in 4×10 ml in small glass beakers. Lysates were centrifuged for 20 min at 4°C in polycarbonate tubes in a SS-34 Sorvall rotor. Cleared lysates were passed through a 0.45-μm filter, and EDTA (4 mM final) and 2-ME (8 mM final) were added. Galectin 1 was purified by column chromatography with a lactose agarose matrix (Sigma-Aldrich; L7634) at 4°C. The lactosyl-Sepharose columns were equilibrated with PBS containing 4 mM EDTA and 8 mM 2-ME, and samples were passed through the column once. The column was briefly washed with 2-column volumes of PBS plus 4 mM EDTA and 8 mM 2-ME. Galectin 1 was eluted with water in $10 \times$ 1-ml fractions. Columns were cleaned for reuse with MEPBS plus 100 mM lactose to elute residual bound material. Columns were then rinsed with water and re-equilibrated and stored in PBS plus 0.02% Na-azide. Protein concentration in fractions was measured by OD_{280}, and the purity of galectin was assessed by SDS-PAGE (15%) and silver staining. Fractions containing a heavy enrichment of galectin 1 with minimal evidence of contaminating protein were pooled and dialyzed overnight in serum-free RPMI containing 3 mM DTT using Spectra/Pore dialysis membrane (25218038, molecular weight exclusion 6000–8000). Working concentrations of galectin 1 were specified based on the molecular mass of the dimer (28 kDa) and were generally used at concentrations of 10–20 μM for induction of annexin V ligand.

Galectin 1 treatment and annexin V assay and cell counting

Generally, 2×10^5 cells were plated per flat-bottom well in 96-well tissue culture plates with galectin for 8 h at 37°C and then subjected to staining with anti-CD8 Abs, galectin, and/or annexin V. Media in the 8-h culture included 2–3 mM DTT and 20–50 mM lactose, as indicated. Annexin V-PE staining (BD PharMingen; 65875X) was performed according to manufacturer's instructions. Briefly, after the 8-h incubation with galectin, cells were transferred to U-bottom plates, diluted with an equal volume of PBS containing 100 mM lactose, washed once in PBS plus 100 mM lactose, and resuspended in 30 μl of 100 mM lactose in DMEM with CD8 FITC for 15 min. Cells were then washed once in PBS lactose, once in PBS, and then stained with annexin V-PE 1:20 in 50 μl of annexin V-binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) plus 50 ng/ml propidium iodide (PI) for 20 min on ice. Annexin V staining was evaluated by FACS analysis. Where indicated, 100 μl of 10-μm latex beads (2-1000; Interfacial Dynamics, Portland, OR) diluted to 10^5/ml in annexin V-binding buffer was added to samples just before flow analysis to determine the ratio of events:beads. Because volumes were constant, this ratio was directly proportional to event count.

Swainsonine treatment and PHA-biotin staining

Where indicated, swainsonine (Sigma-Aldrich; S-9263) was included in the 48-h secondary culture medium at a concentration of 1 μg/ml from a sterile stock solution of 500 μg/ml in water. Staining with PHA-biotin was used to assess swainsonine efficacy. PHA-biotin (Sigma-Aldrich; L7019) was used for cell surface staining at 2 μg/ml in DMEM + BSA (1 mg/ml) and detected with CyChrome-conjugated steptavidin (BD PharMingen; 554062).

Results

Galectin 1 induces annexin V ligand in Con A-activated murine CD8 T cells

To demonstrate that galectin 1 induction of annexin V ligand proceeded as had been reported in activated human T cells, day 4 murine Con A-activated $CD8^+$ blasts were exposed to galectin 1 and assessed for annexin V staining. After 7.5- to 8-h incubation, the majority of $CD8^+$ T cells became annexin V^+ in the presence of both DTT and galectin 1, as shown in Fig. 1*A*. Annexin V ligand was not induced significantly by galectin in shorter 5-h assays. When lactose was included in the assay, both aggregation (data not shown) and formation of annexin V ligand were effectively blocked, demonstrating the carbohydrate dependence of ligand induction by galectin.

To further confirm that the induction of annexin V ligand occurred through the action of galectin 1, the reducing agent DTT included in the assay was titered out. The carbohydrate-binding

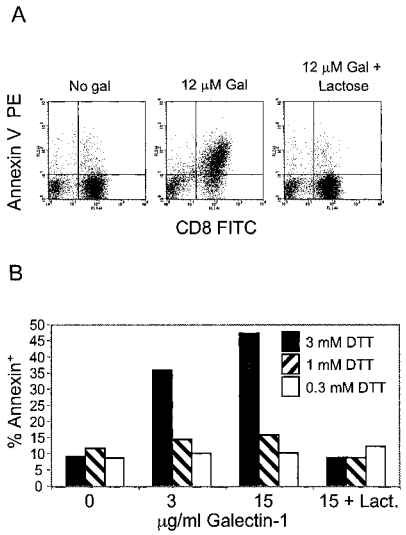

FIGURE 1. Galectin 1 induces annexin V ligand in Con A-activated murine CD8 T cells. *A*, Annexin V ligand induction by galectin 1 is inhibited with free lactose. C57BL/6 (B6) spleen cells were stimulated for 2 days with Con A and subcultured for an additional 2 days with medium supplemented with IL-2 (see *Materials and Methods*). Cells were harvested and exposed to 12 mM rat galectin 1 for 8 h ± 50 mM lactose and assessed for expression of annexin V ligand by flow cytometry. *Left panel*, No galectin 1; *middle panel*, 12 μM galectin 1; *right panel*, 12 μM galectin 1 + 50 mM lactose. *B*, Sufficient DTT concentrations are required for induction of annexin V ligand by galectin 1. Cells were exposed to galectin 1, and DTT was titrated into the culture medium for the duration of the 8-h galectin assay.

property of galectin 1 requires a strong reducing environment, and loss of binding capacity occurs rapidly in the absence of sufficient concentrations of reducing agent. As shown in Fig. 1*B*, galectin 1 induction of annexin V ligand was prevented in the absence of sufficient concentrations of DTT, as expected.

Cytokines modulate lactosamine-containing O*-glycans, but do not affect annexin V ligand induction by galectin 1*

Lactosamine sequences are considered to be the preferred ligands for galectin 1 and, when present in lymphocyte *O*-glycans, they reside in branched structures arising through the action of C2GnT1 (23). P-selectin (P-Sel) binding to activated T cells is dependent on C2GnT1 branching and subsequent modifications of P-selectin alycoprotein ligand-1 expressed on the T-cell surface. For example, C2GnT1^{null} lymphocytes lack branched *O*-glycans (14, 24), and therefore lack the lactosamine sequences required for formation of P-selectin ligand (P-SelL) on activated CD8 T cells (20). As shown in Fig. 2*A*, P-SelL expression was influenced by cytokine exposure. IL-2 promoted significantly higher levels of P-SelL staining relative to IL-4, as described previously (20).

The presence of core 2-branched sequences in *O*-glycans was also monitored indirectly by the expression of the CD43-dependent and CD45RB-dependent epitopes of mAb 1B11. When assessed on a CD43^{null} background, 1B11-CD45RB expression on activated CD8 T cells is lost when C2GnT1-branching activity is present, whereas 1B11-CD43 epitopes require both C2GnT1-branched *O*-glycans and sialic acid residues (25). As shown in Fig. 2*B*, varying culture density and cytokine exposure resulted in large differences in 1B11-CD45RB epitope expression on Con A-activated CD8$^+$ T cells. Previous observations have established that this variation in 1B11-CD45RB expression corresponds with differential C2GnT1 enzyme activity and consequent glycosylation of physiological substrates CD43 and CD45RB in vivo (20, 25).

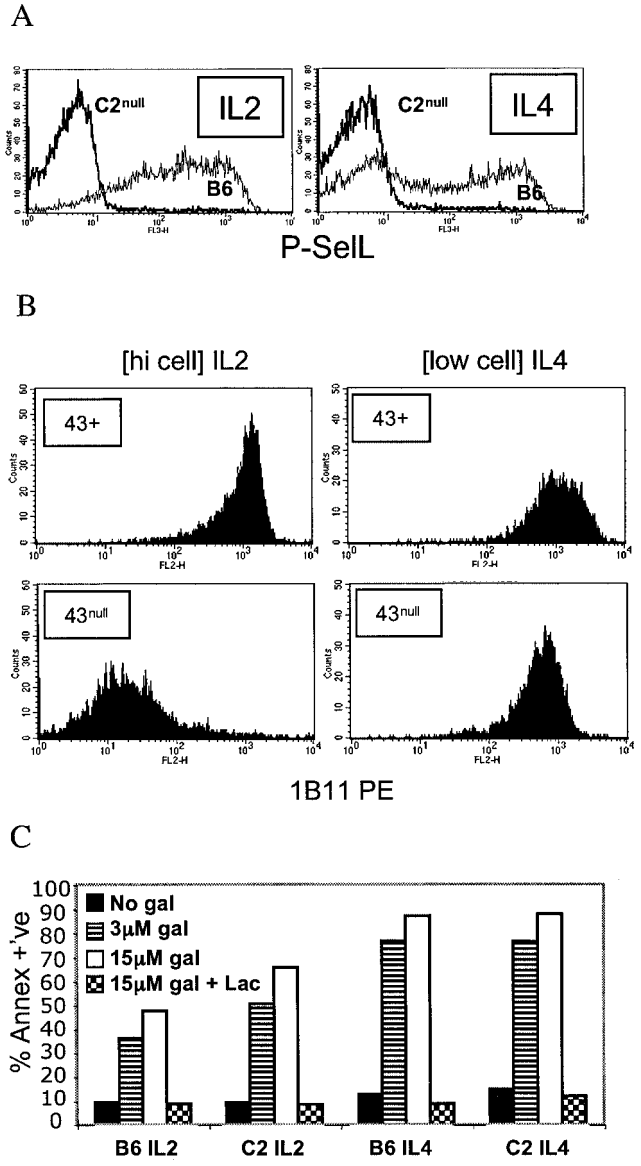

FIGURE 2. Cytokines modulate lactosamine-containing *O*-glycans, but fail to affect annexin V ligand induction by galectin 1. *A*, Cytokines influence the extent of P-SelL induction in Con A-activated CD8 T cells. A total of 0.4 × 10^6 day 2 Con A-stimulated spleen cells from B6 (thin line) or C2GnT1^{null} (bold line) mice was subcultured for an additional 2 days in 2-ml cultures supplemented with either IL-2 (*left panel*) or IL-4 (*right panel*). Expression of P-SelL was assessed on day 4 by gated analysis of CD8$^+$ cells by flow cytometry. *B*, Cytokine impact on C2GnT1 activity assessed by staining with mAb 1B11. B6 (CD43$^+$, *top panels*) and CD43^{null} (*bottom panels*) Con A-activated splenocytes were seeded at 4 × 10^5 (high density + IL-2, *left panels*) or 10^5 (low density + IL-4, *right panels*) cells/2 ml well for an additional 2-day culture. The effects of cytokines on C2GnT1 activity can be easily tracked on the CD43^{null} background, where 1B11-CD45 staining of activated CD8 cells occurs when C2GnT1 activity is low (i.e., IL-4 supplemented), and this staining is reduced/lost when C2GnT1 activity is high (i.e., IL-2 supplemented) (20). *C*, Conditions associated with high C2GnT1 activity are not associated with greater annexin V induction by galectin. Cells prepared as described in *b* were exposed to galectin 1, as indicated, for 8 h.

If galectin 1 induction of annexin V ligand on CD8 T cells occurs primarily through its affinity for lactosamine residues within branched *O*-glycans generated by C2GnT1, then activated CD8 T cells, cultured as described in Fig. 2*B*, should exhibit relative susceptibility to galectin 1. CD8 T cells cultured at high

density for 4 days with IL-2 should be more susceptible to galectin 1 action than CD8 cells grown at low density in IL-4. As shown in Fig. 2*C*, cells grown in IL-4 and IL-2 exhibited comparable susceptibility to induction of annexin V ligand. Furthermore, C2GnT1^{null} CD8 T cells from parallel IL-2-supplemented cultures retained comparable galectin 1 susceptibility. Thus, galectin 1 induction of annexin V ligand in activated murine CD8 T cells was not a simple correlate of C2GnT1 activity.

Annexin V induction by galectin 1 is paralleled by reduced viable cell recovery

To further characterize galectin impact on cell survival, we enumerated CD8$^+$ events detected by flow cytometry that were distinguished by light scatter properties or staining with annexin V and PI. CD8$^+$ T cells distinguished by these criteria were enumerated by a flow-counting assay whereby a known quantity of latex beads is combined with samples such that the ratio of events:beads was proportional to event concentration in the initial suspension (see *Materials and Methods*). As shown in Fig. 3, *A* and *C*, there was a significant increase in CD8$^+$ annexin V$^+$ PI$^-$ T cells after galectin 1 treatment for 8 h. There was also an increase in PI$^+$ events, but this increase was less dramatic and less consistent when all cell types were considered, as summarized in Fig. 3*H*. Annexin V$^+$ PI$^-$ events predominantly fell into the forward light scatter (FSC)^{high} region shared with viable annexin$^-$ PI$^-$ events, while PI$^+$ events exhibited reduced FSC properties, as expected (Fig. 3, *C*, *E*, *F*, and *G*). Thus, induction of annexin V ligand could be measured by applying gating based on either FSC^{high} or FSC^{high} + FSC^{low} light scatter properties.

At the 8-h time point, the quantitative contribution of debris detected after staining/processing of samples was minor (Fig. 3, *E*, *F*, and *G*). Although debris was not grossly evident by microscopic examination after galectin treatment, debris generated by cell loss during galectin treatment could be lost during sample processing and was therefore not regarded as a reliable gauge of cell destruction. As shown in Fig. 3*I*, recovery of viable cells after 24-h exposure to galectin was also depressed consistent with either a cytotoxic and/or cytostatic effect of galectin treatment. It was clear from the data in Fig. 3*H* that galectin treatment resulted in a significant loss of annexin V$^-$ PI$^-$ FSC^{high} (viable) CD8$^+$ events in all cell types regardless of whether C2GnT1 was expressed or not.

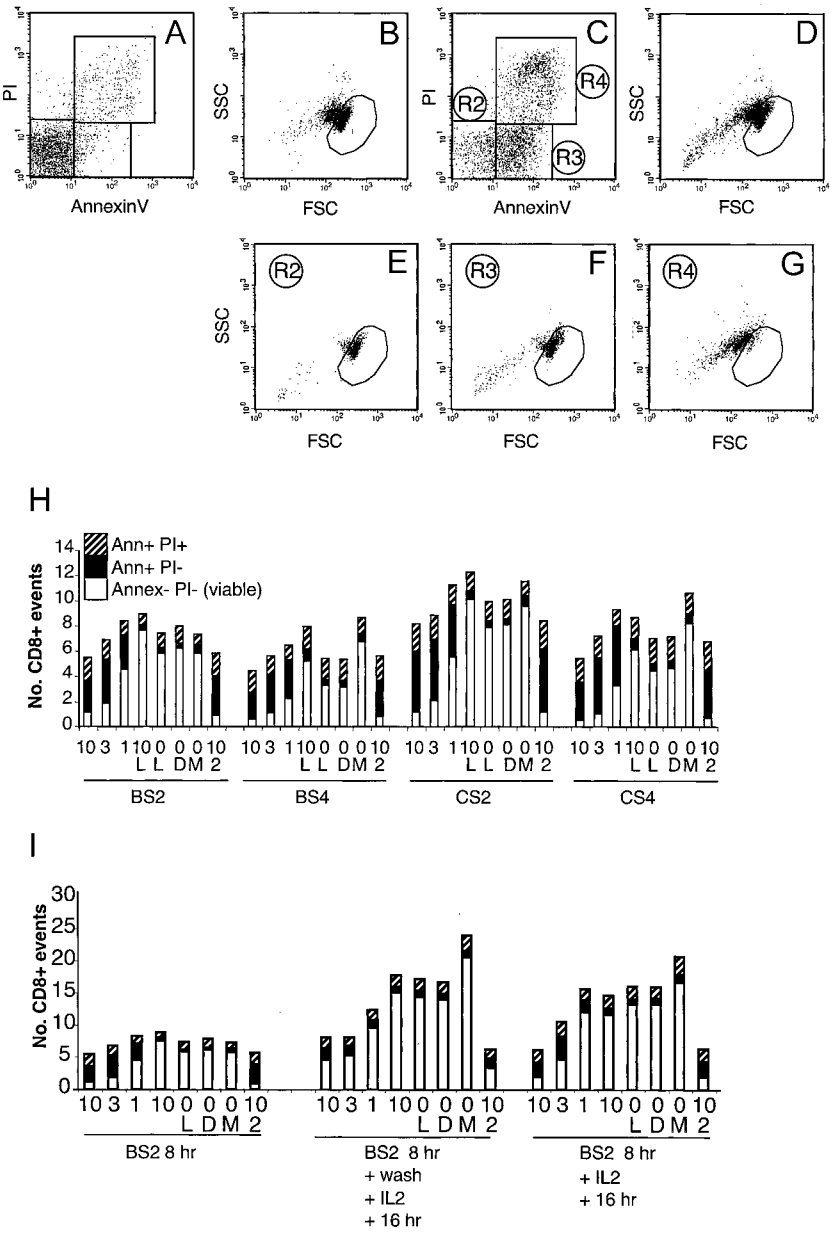

FIGURE 3. Annexin V induction by galectin 1 is paralleled by reduced viable cell recovery. Day 4 Con A-activated T cells from IL-2- or IL-4-supplemented secondary cultures of B6 or C2GnT1^{null} spleen cells (BS2, BS4, CS2, CS4, respectively) were stained with annexin V and PI for flow cytometry after 8-h galectin 1 treatment. All data shown were CD8 gated and include events exhibiting both high and low FSC properties. All samples were spiked with latex microbeads for simultaneous counting, fluorescence, and light scatter analysis, as described in *Materials and Methods*. Log FSC and log side scatter (SSC) settings were used to monitor contribution of debris. Annexin V and PI staining of BS2 cells after 8 h with medium (*A*) or 10 μM galectin (*C*) with corresponding FSC $\times$ SSC profiles (*B*) and (*D*), respectively. FSC $\times$ SSC profiles for region 2 (R2)-, R3-, and R4-gated events are shown in *E*, *F*, and *G*, respectively. Quantification of CD8$^+$ events at 8 h (h) falling into annexin V$^-$ PI$^-$ region (R2), annexin V$^+$ PI$^-$ (R3), or annexin V$^+$ PI$^+$ (R4) reported as No. of CD8$^+$ events (i.e., event:bead ratio, see *Materials and Methods*). *I*, Eight- and 24-h comparison of BS2 yields of events gated as in *C*. Parallel BS2 experiments were harvested at 8 h for analysis (*left series* in *I*), washed once with 100 mM lactose and once with medium, and recultured overnight in medium with IL-2 (*middle series* in *I*), or simply supplemented with IL-2 after the first 8-h incubation and cultured overnight for similar analysis at 24 h (*right series* in *I*). Culture supplements included 10 μM galectin (10), 3 μM galectin (3), 1 μM galectin (1), and 20 mM lactose with 2 mM DTT (L); 2 mM DTT was included in all cultures, except M, and was the only supplement in columns marked D; medium alone (M), 2.5% IL-2 supernatant supplement (2) during the first 8-h incubation period.

Blocking formation of N*-glycan lactosamine extensions fails to alter galectin 1-induced annexin V ligand*

In view of our failure to observe altered galectin-induced annexinV ligand expression in activated murine CD8 T cells when C2GnT1 activity was reduced or eliminated, we investigated whether inhibitors of lactosamine formation on *N*-glycans would affect relevant galectin ligands. Galectin ligands on *N*-glycans had been previously implicated in galectin-induced apoptosis of PHA-activated human T cells through use of the α mannosidase II inhibitor swainsonine (8). Swainsonine partially blocks glycan extension from the mannose core structure such that cells treated with swainsonine express predominantly high mannose and hybrid *N*-glycans, but lack complex *N*-glycans (26). Cultures were supplemented with swainsonine throughout the 2-day secondary culture with either IL-2 or IL-4 and, as shown in Fig. 4*A*, the efficacy of swainsonine treatment was monitored by loss of PHA binding to cell surface complex *N*-glycans on day 4. Despite its efficacy in eliminating PHA staining, swainsonine had no significant impact on galectin 1-induced annexin V ligand expression by activated CD8 T cells, as shown in Fig. 4*B*.

Galectin 1 binding correlates with induction of annexin V ligand

Annexin V staining after galectin treatment suggested that CD8 T cells cultured in IL-4 exhibited marginally enhanced susceptibility to apoptosis relative to cells cultured in IL-2. This result was unexpected because previous studies demonstrated that IL-2 strongly promoted C2GnT1 expression and branched *O*-glycan formation on prototype cell surface mucins CD43, CD45, as well as P-selectin glycoprotein ligand 1; IL-4 supported C2GnT1 enzyme activity and function weakly, if at all. The high level of annexin V staining observed in IL-4-cultured cells after galectin treatment

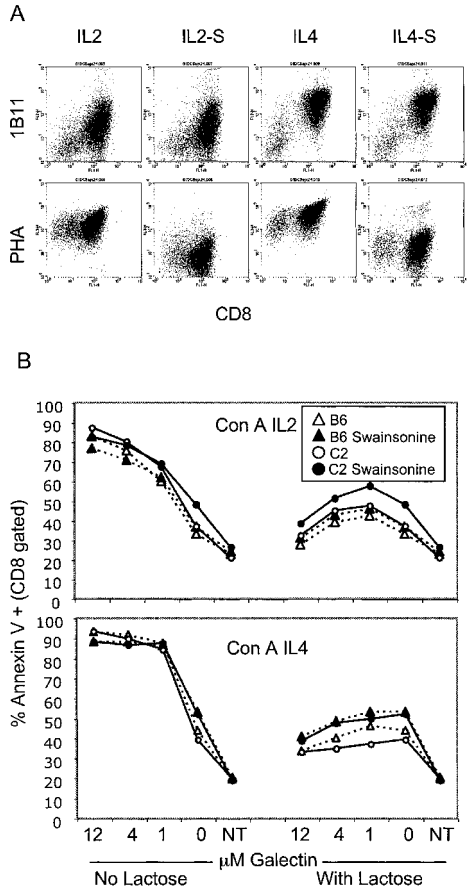

FIGURE 4. Blocking formation of *N*-glycan lactosamine extensions fails to alter galectin 1-induced annexin V ligand. *A*, Swainsonine inhibits formation of complex *N*-glycans. Day 2 Con A-activated CD43^{null} spleen cells were subcultured for 2 more days with cytokine supplements (IL-2 or IL-4, as indicated) and swainsonine (S). mAb 1B11 and biotinylated PHA were used to monitor changes in *O*- and *N*-glycans, respectively, on CD8$^+$ T cells. *B*, Induction of annexin V ligand by galectin 1 is unaffected by changes in *O*-glycan branching or inhibition of complex *N*-glycan formation. Day 2 Con A-activated spleen cells from B6 or C2GnT1^{null} mice were subcultured with IL-2 (*top panel*) or IL-4 (*bottom panel*) for 2 more days and subjected to an 8-h galectin assay with (*right graphs*) or without (*left graphs*) 50 mM lactose, followed by annexin V and CD8 staining for flow cytometric analysis. CD8 gating was applied to the data shown (NT = neither galectin nor DTT included in culture).

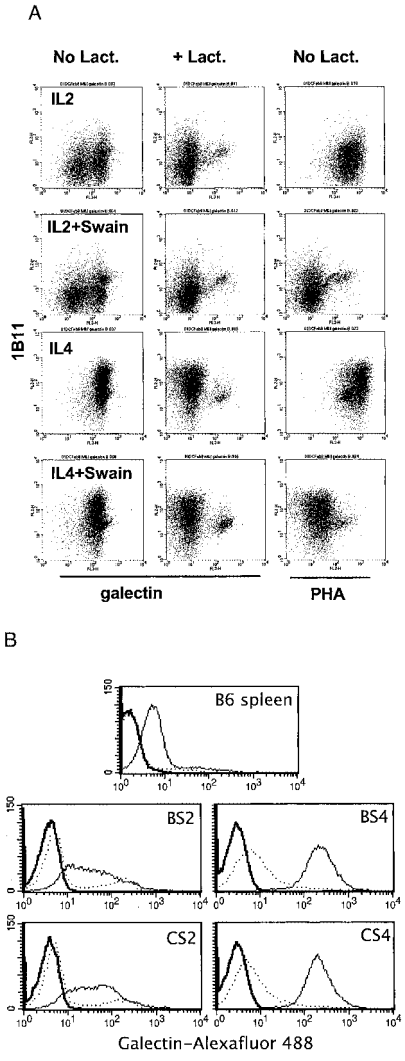

FIGURE 5. Galectin 1 binding coorelates with induction of annexin V ligand. *A*, Staining with biotinylated galectin 1 is inhibited with free lactose, but is not altered significantly by modifications in C2GnT1 activity or inhibition of complex *N*-gylcan formation. Day 2 CD43^{null} Con A-activated spleen cells were subcultured with cytokines and swainsonine, as indicated, for an additional 2 days and stained with 1B11-PE, biotinylated galectin 1 ± lactose, or biotinylated PHA for flow cytometric analysis. *B*, B6 C2GnT$^+$ and C2GnT1^{null} activated CD8$^+$ T cells bind galectin comparably. Five micromolar galectin conjugated to Alexafluor 488 was used to stain naive CD8$^+$ B6 spleen cells or day 4 Con A-activated CD8 T cells from secondary B6 spleen cultures grown in IL-2 (BS2) or IL-4 (BS4) or corresponding C2GnT1^{null} spleen cultures (CS2, CS4). Unstained cells (heavy line), galectin stained (light line), and staining with galectin in the presence of 100 mM lactose (dotted line) are shown.

suggested that either these cells expressed comparable (or even increased) levels of total cell surface galectin ligand relative to cells cultured with IL-2, or that only the relevant galectin ligand involved in apoptosis was maintained, while total cell surface galectin ligand expression was reduced in parallel with changes induced in *O*- or *N*-glycans by cytokines or swainsonine. To determine which of these possibilities applied, direct assessment of galectin binding was used to measure relative quantities of ligand on cells cultured with cytokines or swainsonine. The results of galectin and PHA binding shown in Fig. 5*A* indicated that CD8 T cells maintained in IL-4 and exhibited high susceptibility to apoptosis also expressed uniformly high levels of galectin ligand. Swainsonine had no significant impact on galectin binding consistent with its failure to alter susceptibility to apoptosis. Thus, galectin 1 ligand expression appeared to be essentially independent both of *O*-glycan lactosamines substituted on branched *O*-glycans and of complex *N*-glycans.

Finally, to confirm that galectin binding paralleled annexin V induction in C2GnT^{null} cells, the binding of galectin to activated CD8^{+} T cells lacking C2GnT1 and P-SelL was assessed, as shown in Fig. 5*B*. Lactose-sensitive galectin binding was essentially unaffected when activated cells lacked C2GnT1. This observation was consistent with the effective induction of annexin V ligand on cells deficient in C2GnT1 shown in Fig. 2*C* and confirms that the predominant biochemistry supporting galectin interaction with the cell surface is C2GnT1 independent.

Discussion

We have examined the contribution of branched *O*-glycans during galectin 1 induction of apoptosis-associated annexin V ligand in primary activated murine CD8 T cells. Our effort was motivated by 1) a proposed hypothesis that galectin 1 normally contributes to contraction of T cell populations after clonal expansion in response to Ag stimulation in vivo 2) primarily through C2GnT1-modified *O*-glycans (3) and, that IL-2, which has been widely implicated in promoting activation-induced cell death, also up-regulates C2GnT1 (20). Given the major importance of homeostatic mechanisms regulating the T cell pool and the paucity of published data using primary T cell populations to evaluate galectin function (murine or human), we sought to generalize galectin 1 action in short-term cultured murine cells and verify the participation of branched *O*-glycans as galectin ligands in the process of annexin V ligand induction.

We observed that galectin 1 did indeed induce expression of annexin V ligand in activated murine CD8 T cells. This process was dependent on sufficient concentrations of DTT and was inhibited by lactose, thereby implicating the carbohydrate-binding specificity of galectin. However, neither in vitro modulation of C2GnT1 *O*-glycan-branching activity with cytokines nor genetic elimination of C2GnT1-branching activity altered the efficiency of annexin V ligand induction by galectin 1. These observations were consistent with the ability of galectin 1 to induce annexin V ligand on activated human T cells (8), but were not consistent with the general concept that C2GnT1-branched *O*-glycans constitute the relevant target of galectin 1, at least with respect to murine CD8 T cells. Our observations instead suggest that ligands other than C2GnT1-branched *O*-glycans are most relevant in action of galectin 1 on activated murine CD8 cells.

What then, are the relevant ligands for galectin on activated murine CD8 cells? The inhibition of galectin binding, the inhibition of annexin V ligand expression, and inhibition of aggregation (aggregation data not shown) that we observed with 20–100 mM lactose were consistent with the recognized specificity of galectin for lactose and lactosamine sequences (4). Another disaccharide, sucrose, failed to block galectin binding at similar 100 mM concentrations, thereby confirming that residues of lactose or some related structures are indeed relevant in galectin binding to the cell surface (data not shown). Although other unidentified carbohydrate ligands may be involved, these observations are most consistent with a simple model, whereby galectin bound lactose/lactosamine moieties residing on cell surface molecules and initiated signaling events leading to expression of annexin V ligand. The question of galectin ligand identity thus becomes focused on those structures known to carry lactosyl moieties.

In terms of *O*-glycans, activated murine CD8 T cells subcultured with IL-4 were marginally more susceptible to galectin binding and annexin V induction than parallel cultures supplemented with IL-2. Previous investigations have demonstrated that activated CD8 T cells cultured with IL-4 express much lower C2GnT1 enzyme activity and predominantly unbranched *O*-glycans on physiological substrates CD43 and CD45 in vivo (20). The only other potential sources of lactosamine residues in *O*-glycans would be those contained within core 1 extensions or in *O*-glycan branches generated by other C2GnT isoenzymes (C2GnT2 and C2GnT3). However, significant contributions of extended core 1 structures have not been detected in activated lymphocytes (23), although they have been observed in high endothelial venules of C2GnT1^{null} mice (24), where they contribute to the L-selectin ligand formation and thereby support homing of naive T cells to lymphoid organs. To our knowledge, no evidence of residual C2GnT activity in mature C2GnT1^{null} lymphocytes has been reported. Furthermore, C2GnT2 and C2GnT3 do not replace C2GnT1 to a detectable extent in either HEV (24) or splenocytes (14). Therefore, the failure of C2GnT1 to affect galectin 1 induction of annexin V in activated murine CD8 T cells is most easily explained by the irrelevance of core 2-branched *O*-glycans in this process.

One explanation to account for the discrepancy between this conclusion and previous observations (8) is that galectin 1 induction of apoptosis in activated human T cells is reported to be primarily determined through interactions with human CD7 (hCD7)[3] (27). Comparing sites of predicted *O*-glycan substitution using Net-*O*-Glycan (28), one finds that the extracellular portion of hCD7 may be extensively substituted at Ser/Thr^{36}, Ser/Thr^{150}, Ser/Thr^{157}, Ser/Thr^{159}, Ser/Thr^{168}, Ser/Thr^{166}, and Ser/Thr^{177} (transmembrane domain aa 178:200), whereas murine CD7 (mCD7) has a single juxtamembrane-predicted site of *O*-glycan modification at Ser^{139} (transmembrane domain aa 149:171). If *O*-glycans on CD7 are indeed relevant targets of galectin 1 in activated human T cells that are absent in mCD7, and these predicted sites of *O*-glycan modification in hCD7 and mCD7 reflect true glycosylation differences, this could account for both our failure to implicate *O*-glycans in annexin V induction by galectin 1 and the frank absence of data implicating *O*-glycans in galectin 1-induced apoptosis in primary activated murine T cells. To our knowledge, the only evidence that *O*-glycans support galectin 1-induced apoptosis in peripheral murine T cells is based upon C2GnT1 overexpression studies in murine T cell lines (9, 29).

Inhibition of complex *N*-glycans had no effect on ability of galectin to induce annexin V in our analysis. The failure of swainsonine to detectably alter susceptibility to annexin V induction in activated murine CD8 T cells described in this work contrasts with results reported for activated human T cells (8). The rationale for using swainsonine in both previous studies and our current study is that swainsonine is tolerated relatively well by cells and the drug is highly effective at blocking expression of complex *N*-glycans.

[3] Abbreviations used in this paper: hCD7, human CD7; Ctx, cholera toxin; FSC, forward light scatter; mCD7, murine CD7; P-SelL, ●●●; PI, propidium iodide; SSC, side scatter; C_2GnT_1, β(1-6)*N*-acetyl glucosaminyl transferase I; P-SelL, P-selectin ligand.

However, interpretation of swainsonine inhibition data is compromised, as clear evidence exists that formation of hybrid *N*-glycans containing polylactosamine sequences is not impeded by this drug (30, 31). Hence, swainsonine in fact only partially prevents expression of lactosamine-containing *N*-glycans. Other drugs such as deoxymannojirimycin can fully prevent extensions off the mannose core structure, but its application may be limited by toxicity and interference with surface expression/secretion of particular proteins (31). The fact that no reduction in galectin 1 labeling was evident after treatment with swainsonine is consistent with its failure to affect annexin V induction and strongly suggests that those *N*-glycans blocked by swainsonine are not ligands for galectin 1 in murine CD8 T cells. Whether remaining *N*-glycan extensions represent relevant galectin ligands was not addressed in this study, but has found some support in recent observations by others (18).

Another potential ligand for galectin 1 on CD8 T cells is the glycolipid GM1, a ganglioside bearing the α 2,3 sialylated lactosyl sequence and a known ligand of galectin 1 in other cell systems (32). GM1 cross-linking with B subunits of either *E. coli* enterotoxin (EtxB) or cholera toxin (Ctx) induces GM1 capping, and ETxB reportedly induces apoptosis in activated murine CD8 T cells (33). Importantly, Ctx binding of GM1 interferes with lectin-mediated stimulation of lymphocytes (34). We attempted to block galectin 1 (7 μM dimer) binding to CD8 T cells with Ctx (25 μM monomer, 5 μM pentamer) in efforts to implicate GM1 in the process of annexin V ligand induction. Ctx did not alter lactose-dependent galectin binding to activated CD8 T cells detectably (data not shown).

In summary, we have found that C2GnT1 modifications of *O*-glycans do not appear to affect galectin 1 binding or annexin V ligand induction in activated murine CD8 T cells. These results are difficult to reconcile with the proposal that contraction of the post-stimulation T cell pool depends on apoptosis induced by endogenous galectin 1 binding to lactosyl sequences forming on branched *O*- or *N*-linked gylcans, at least in the murine model. Although our data demonstrate that galectin 1 can effectively promote annexin V ligand expression in activated murine CD8 T cells and that this process relies on lactosyl specificity of galectin, we have not identified the relevant lactosyl-bearing targets. Specifically, our data suggest that *O*-glycans are in fact not relevant in galectin 1 action because potential galectin ligands arising through formation of core 1 extensions are highly unlikely and C2GnT branching in activated murine T cells is apparently dispensable.

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