

Galectin-1 Specifically Modulates TCR Signals to Enhance TCR Apoptosis but Inhibit IL-2 Production and Proliferation¹

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Galectin-1 is an endogenous lectin expressed by thymic and lymph node stromal cells at sites of Ag presentation and T cell death during normal development. It is known to have immunomodulatory activity *in vivo* and can induce apoptosis in thymocytes and activated T cells (1–3). Here we demonstrate that galectin-1 stimulation cooperates with TCR engagement to induce apoptosis, but antagonizes TCR-induced IL-2 production and proliferation in a murine T cell hybridoma and freshly isolated mouse thymocytes, respectively. Although CD4⁺CD8⁺ double positive cells are the primary thymic subpopulation susceptible to galectin-1 treatment alone, concomitant CD3 engagement and galectin-1 stimulation broaden susceptible thymocyte subpopulations to include a subset of each CD4[−]CD8[−], CD4⁺CD8⁺, CD4[−]CD8⁺, and CD4⁺CD8[−] subpopulations. Furthermore, CD3 engagement cooperates with suboptimal galectin-1 stimulation to enhance cell death in the CD4⁺CD8⁺ subpopulation. Galectin-1 stimulation is shown to synergize with TCR engagement to dramatically and specifically enhance extracellular signal-regulated kinase-2 (ERK-2) activation, though it does not uniformly enhance TCR-induced tyrosine phosphorylation. Unlike TCR-induced IL-2 production, TCR/galectin-1-induced apoptosis is not modulated by the expression of kinase inactive or constitutively activated Lck. These data support a role for galectin-1 as a potent modulator of TCR signals and functions and indicate that individual TCR-induced signals can be independently modulated to specifically affect distinct TCR functions. *The Journal of Immunology*, 1999, 162: 799–806.

Engagement of the Ag-specific TCR on developing and mature T cells can result in a number of distinct biological functions. Indeed, TCR stimulation by Ag/MHC on thymic epithelial and APCs directs thymocyte cell fate during positive and negative selection in the thymus (4, 5). During positive selection, cells expressing TCRs with a minimal ability to interact with peptide/MHC are selected on thymic epithelial cells to undergo a number of activation and differentiation events including rescue from apoptosis and maturation into CD4 or CD8 single positive cells (4). During negative selection, thymocytes with TCRs highly reactive with self/MHC ligands presented on dendritic cells and/or thymic epithelial cells are purged from the repertoire through TCR-induced apoptosis to prevent autoreactivity (5). Peripheral T cells can respond to antigenic stimulation by producing lymphokines, proliferating, undergoing apoptosis, or becoming anergic. Relatively little is known regarding how signaling requirements for individual TCR-mediated functions differ and what controls the functional consequences of TCR engagement in T cells. However, T cells are known to discriminate between a variety of stimuli including the type of Ag, the magnitude of the TCR signal, the

coordinate engagement of accessory or costimulatory molecules (6–8), or the presence of cytokines.

Galectin-1 is a highly evolutionarily conserved β -galactoside binding protein demonstrated to have immunomodulatory and growth regulatory activities (1). Galectin-1 is expressed as a non-covalently linked homodimer with two ligand binding sites capable of mediating cell/cell interactions by binding to saccharide ligands on apposing cell surfaces. It has no transmembrane domain but remains cell associated by binding to glycoproteins on the surface of the cell producing it. It is expressed in a number of tissues including epithelial cells and APCs in the lymph node, spleen, and thymus (1–3). Galectin-1 can bind T cells and thymocytes and has been demonstrated to mediate interactions between T cells and galectin-1 expressing endothelial cells (3). Lactosamine is the minimal oligosaccharide structure recognized by galectin-1, though glycoproteins with multiple and complex sugar structures are likely the preferred counterreceptors for galectin-1. Indeed, CD43, CD45, CD4, CD3, and CD7 have been reported to be primary T cell glycoproteins bound by galectin-1 (1, 3). Furthermore, Abs directed against galectin-1 as well as CD45 and CD43 glycoproteins can disrupt thymic epithelial cell/T cell interactions (3). Although initial reports indicated that CD45/galectin-1 interactions may modulate galectin-1-induced apoptosis (9), subsequent studies indicate that this may not always be the case (J.T.N., M.C.M., L.G.B., unpublished observations).

In addition to mediating cell/cell interactions, galectin-1, binding to oligosaccharides containing multiple lactosamine units on T cell surface proteins, may facilitate multimerization or crosslinking and therefore induce signal transduction by these proteins (1). Recent models of TCR activation suggest that CD45 and CD43 must be excluded from the TCR/APC contact site at the same time CD4 and other accessory molecules are recruited in order for efficient T cell activation events to occur (10). Galectin-1 binding may additionally modify this T cell surface-molecular reorganization and thus influence the degree of TCR multimerization (11), inclusion of coreceptor or costimulatory

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molecules within TCR aggregates, and potentially the functional outcome of antigenic stimulation (10).

Because galectin-1 is expressed in the thymus (3) and induces apoptosis in thymocytes (12), galectin-1 binding may contribute to cell death during thymic selection. Galectin-1 is also expressed in peripheral immune organs including the lymph node and spleen, as well as at sites of immune privilege and on tumor cells, and has been demonstrated to induce apoptosis in activated peripheral T cells (1). Therefore, galectin-1 binding may also contribute to apoptosis of mature T cells in the periphery (9). Because of its abundant expression at sites of self and antigenic presentation, it is likely that a T cell might simultaneously encounter galectin-1 and TCR ligand. However, the consequences of galectin-1 stimulation in the context of coordinate TCR engagement have not yet been examined. Furthermore, most experiments examining galectin-1 T cell immunomodulatory activity have been performed using human T cells, T cell lines, or thymocytes. Here we determine the signal transduction and biological consequences of stimulating a mouse T cell hybridoma or freshly isolated mouse thymocytes with galectin-1 alone or coordinately with anti-TCR Abs. We demonstrate that TCR stimuli that otherwise lead to IL-2 production or proliferation in a T hybridoma and freshly isolated thymocytes, respectively, are converted into efficient stimuli for apoptosis in the context of galectin-1 stimulation. Examination of the TCR signals affected reveal that galectin-1 does not uniformly intensify or dampen all TCR signals. However, a subset of TCR signal transduction events are intensified when TCR is engaged in the presence of galectin-1.

Materials and Methods

Preparation of recombinant galectin-1

Human galectin-1 was purified from *Escherichia coli* transformed with the expression vector pT7IML-1 as previously described (13). Galectin-1 was stored in 8 mM DTT at -70°C and used in all procedures in medium containing 1.0–1.2 mM of DTT.

Cells, Abs, and Annexin V

BI-141 is a CD4⁻CD8⁻ MHC class II-restricted murine T cell hybridoma that recognizes beef insulin in the context of IA $\alpha^b\beta^k$ (14). The BI-141 transfectants expressing F505Lck or R273F505Lck have been described (6). Thymocytes were obtained from C57Bl/6 females (4–8 wk) and single cell suspensions were made using standard procedures. Abs against the TCR β -chain (H57-597) (15), and CD3 ϵ (145-2C11) (16) were purified from hybridomas. Antisera against extracellular signal-regulated kinase-2 (ERK-2)³ (C-14) and 4G10 antiphosphotyrosine Ab were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY), respectively.

For analysis of thymic subpopulations, 4×10^5 thymocytes were incubated at 37°C with media containing 0.3, 0.6, or 1.2 mM DTT alone, together with 5, 10, or 20 μM galectin-1 and/or 1.25 $\mu\text{g}/\text{ml}$ plate-bound anti-CD3 ϵ for 5 h. Cells were resuspended in 0.1 M β -lactose for 1 min at room temperature, washed twice with PBS, and incubated with phycoerythrin conjugated anti-CD4 and biotinylated anti-CD8 (PharMingen, San Diego, CA) for 30 min at 4°C . Cells were then washed and incubated with streptavidin tricolor conjugate (Caltag, Burlingame, CA) for 30 min at 4°C , washed, and stained with 2.5 μl Annexin V-FITC for 15 min on ice as per the manufacturer's recommendations (PharMingen). Annexin V positive cells were electronically gated out using Cellquest software (Becton Dickinson, San Jose, CA), and cell death was calculated based on the number of live cells in the treated sample relative to the number in the media alone containing a comparable concentration of DTT using the following equation: $100 \times [1 - (\text{no. of Annexin V negative galectin-1 and/or anti-CD3 treated cells})/(\text{no. of Annexin V negative control media treated cells})]$. There were 50,000 total events analyzed in each sample.

Measurement of cell death by propidium iodide (PI) staining

BI-141 or thymocytes at concentration of 2×10^6 or 0.5×10^6 cells/ml, respectively, were incubated at 37°C in medium with 1.2 mM DTT and 20 μM of galectin-1 in the presence or absence of 1 $\mu\text{g}/\text{ml}$ of plate-bound or soluble anti-TCR mAbs. Samples were adjusted to 0.1 M lactose to dissociate galectin-1-agglutinated cell clumps and washed 12 h poststimulation. Cell death was measured by staining cells with 0.2 ml of 2 $\mu\text{g}/\text{ml}$ PI. PI staining was detected using a FACScan (Becton Dickinson), and data were analyzed using Cellquest software. The percentage of induced death was calculated relative to the percentage of live cells obtained in the unstimulated culture using the following equation: induced death = $100 \times [1 - (\% \text{ treated live cells}/\% \text{ untreated live cell})]$.

Measurement of IL-2 production

BI-141 (0.5×10^6) cells were stimulated as above. Supernatants were harvested 12 h poststimulation and assayed for IL-2 production by ELISA as per the manufacturer's recommendations (Endogen, Cambridge, MA).

Measurement of cell proliferation

Thymocytes at concentration of 1×10^6 cells/ml were cultured at 37°C in media containing 1.2 mM DTT and 20 μM of galectin-1 and/or 1 μg of 2C11. Cultures were pulsed for the last 16–18 h of a 30-h incubation with 1 μCi of [^3H]thymidine/well, DNA were harvested on glass filters, and counts incorporated were determined using a scintillation counter.

Antiphosphotyrosine Western blot analysis

T cells ($2.5 \times 10^6/100 \mu\text{l}$) were incubated with medium alone or with 5 μg anti-CD3 ϵ for 30 min on ice and then incubated for 5 min at 37°C with different stimuli (20 μM galectin-1, 1.2 mM DTT, or rabbit anti-hamster IgG) (Cappel, Durham, NC). Cells were washed in RPMI 1640 and lysed in 50 μl of TNE (50 mM Tris (pH 8.0), 1% Nonidet P-40, and 2 mM EDTA), supplemented with protease and phosphatase inhibitors for 30 min on ice. Lysates were boiled in Laemmli buffer containing 2-ME, resolved on 10% SDS-PAGE, and transferred to nitrocellulose. Immunoblot analysis was performed using antiphosphotyrosine mAb 4G10 at 1 $\mu\text{g}/\text{ml}$ and detected with 10 μCi [^{125}I] Protein A (ICN, Irvine, CA) and proteins were visualized by autoradiography.

Measurement of mitogen-activated protein (MAP) kinase/ERK activity

T cells ($5 \times 10^6/100 \mu\text{l}$) were incubated with complete medium alone or with 5 μg anti-CD3 ϵ for 30 min at 4°C and then incubated for 5 min at 37°C with various stimuli (20 μM galectin-1, 1.2 mM DTT, or rabbit anti-hamster IgG). Cells were pelleted and lysed with ERK lysis buffer (25 mM HEPES (pH 7.6), 0.1% Triton X-100, 20 mM β -glycerolphosphate, 10 mM *p*-nitrophenylphosphate, 150 mM NaCl, and 1 mM Na_2VO_3) supplemented with protease inhibitors for 30 min at 4°C . ERK-2 was immunoprecipitated and in vitro ERK kinase activity measured using myelin basic protein (MBP) as an exogenously added substrate essentially as described (17).

Results

Galectin-1 cooperates with anti-TCR Abs to induce apoptosis, but antagonizes anti-TCR-induced IL-2 production in a T cell hybridoma

Because APCs and stromal cells at sites of T cell antigenic stimulation express galectin-1 (1), it is likely that an Ag-specific T cell might encounter both Ag and galectin-1. Therefore, we were interested in determining the effects of galectin-1 stimulation in the context of TCR engagement. BI-141 T hybridoma cells were stimulated with a suboptimal concentration of anti-TCR β -chain, H57-597, or with a more optimal concentration of 145-2C11 anti-CD3 ϵ alone or together with purified recombinant galectin-1. We have previously reported that the BI-141 T hybridoma both secretes IL-2 and undergoes apoptosis in response to TCR stimulation (6). Here we demonstrate that this murine T hybridoma also undergoes apoptosis in response to galectin-1 (Fig. 1A). Furthermore, suboptimal stimulation of plate-bound H57-597, unable to induce apoptosis by itself, enhances the ability of galectin-1 to induced apoptosis in the BI-141 T cell hybridoma (Fig. 1A). The ability of galectin-1 and

³ Abbreviations used in this paper: ERK-2, extracellular signal-regulated kinase-2; PI, propidium iodide; MBP, myelin basic protein; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling; GAH, goat anti-hamster; MAP, mitogen-activated protein.

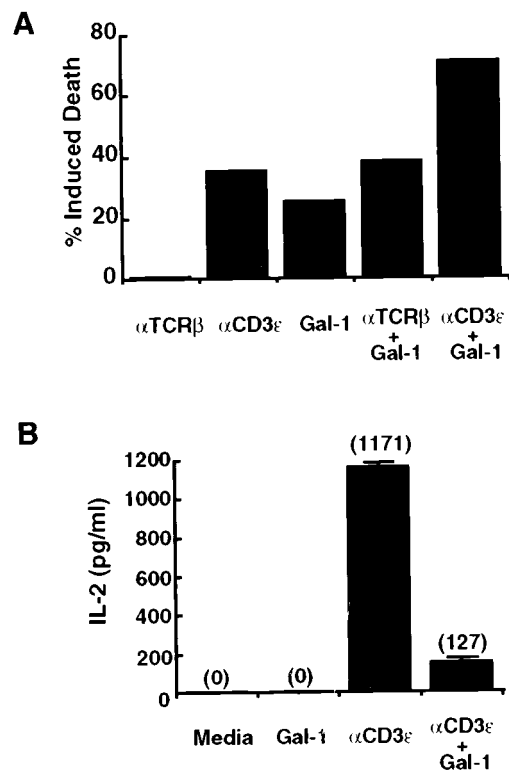


FIGURE 1. Galectin-1 cooperates with anti-TCR Abs to induce apoptosis, but antagonizes anti-TCR-induced IL-2 production. BI-141 cells at 2×10^6 cells/ml (A) or 0.5×10^6 cells/ml (B) were stimulated with $20 \mu\text{M}$ galectin-1 (Gal-1) or anti-TCR mAbs, H57-597 (αTCR β -chain), or 145-2C11 ($\alpha\text{CD3}\epsilon$) individually or together. A, 12 h after addition of galectin-1, cells were harvested, stained with PI, and analyzed by flow cytometry. Percent of induced cell death was calculated relative to live cells in unstimulated controls. B, Supernatants were assayed for IL-2 production 12 h poststimulation by ELISA.

TCR engagement to cooperate in signaling apoptosis is also evident when optimal concentrations of plate-bound anti-CD3 ϵ (145-2C11) are used in combination with galectin-1 (Fig. 1A). Differences between results obtained with 2C11 and H57-597 relate to the efficacy of the concentration of Ab used rather than the TCR/CD3 epitope against which they are directed (data not shown). Similar results are seen when cells are incubated with plate-bound anti-TCR Ab 12 h before the addition of galectin-1 (results not shown).

To determine the effects of galectin-1 stimulation on CD3-mediated IL-2 production, supernatants were collected from cells stimulated with plate-bound anti-CD3 ϵ alone or in combination with galectin-1. As shown in Fig. 1B, simultaneous addition of galectin-1 impairs the ability of anti-CD3 to produce IL-2. These results are not the effect of galectin-1 interfering with IL-2 detection, because similar inhibition of detection was neither observed when rIL-2 was directly diluted in an equivalent concentration of galectin-1 nor was IL-2 activity recovered by preabsorbing galectin-1 from supernatants using a lactosyl Sepharose column before IL-2 assay (data not shown). Inhibition of IL-2 production is not likely the result of killing IL-2-producing cells before they are able to produce sufficient quantities of IL-2, because maximal levels of IL-2 can be produced by BI-141 under conditions in which death due to 2C11 alone is as high as 80% (results not shown). Therefore, significant levels of IL-2 can be produced under conditions leading to death of most of the cells.

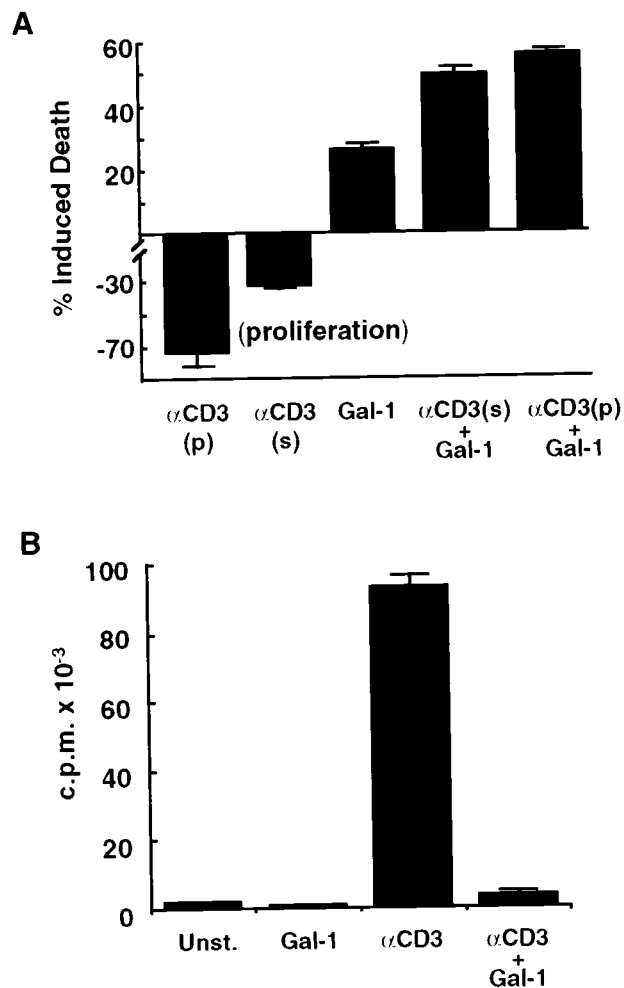


FIGURE 2. Galectin-1 cooperates with anti-TCR to induce cell death, but antagonizes its ability to induce proliferation in thymocytes. A, Thymocytes (2×10^6 cells/ml) from C57Bl/6 were stimulated with $20 \mu\text{M}$ galectin-1 in the presence or absence of soluble (s) or plate-bound (p) $\alpha\text{CD3}\epsilon$. After 9 h, cells were harvested, stained with PI, and analyzed by flow cytometry. Percent of induced cell death was calculated relative to live cells in unstimulated controls. B, Thymocytes (1×10^6 cells/ml) from C57Bl/6 were stimulated with $20 \mu\text{M}$ of galectin-1 (Gal-1) in the presence of plate-bound anti-CD3 ϵ . Cultures were pulsed for the last 16–18 h of a 30-h incubation with $1 \mu\text{Ci}$ of [^3H]thymidine per well. Cells were harvested on glass filters and assayed in a scintillation counter. Data are reported as cpm of [^3H]thymidine incorporated.

Galectin-1 cooperates with anti-TCR Abs to induce apoptosis but antagonizes the ability of anti-TCR Abs to induce proliferation in freshly isolated thymocytes

The thymus is a primary site of T cell apoptosis. During positive and negative selection, TCR engagement cues the apoptotic elimination of autoreactive and nonfunctional thymocytes and the rescue and development of T cells bearing TCRs likely to be useful in the peripheral immune system. Because galectin-1 is expressed by thymic epithelial and some APCs (1, 2), and has been demonstrated to mediate thymic epithelial/T cell interactions (9), we were interested in determining the effects of stimulating freshly isolated mouse thymocytes with galectin-1 alone or in coordinately with anti-CD3 ϵ Ab. Mouse thymocytes were isolated from 6 wk C57Bl/6 mice and stimulated with galectin-1 and anti-CD3 ϵ individually or in combination. The effects of stimulation with both plate-bound and soluble anti-CD3 ϵ were assessed. Cells were

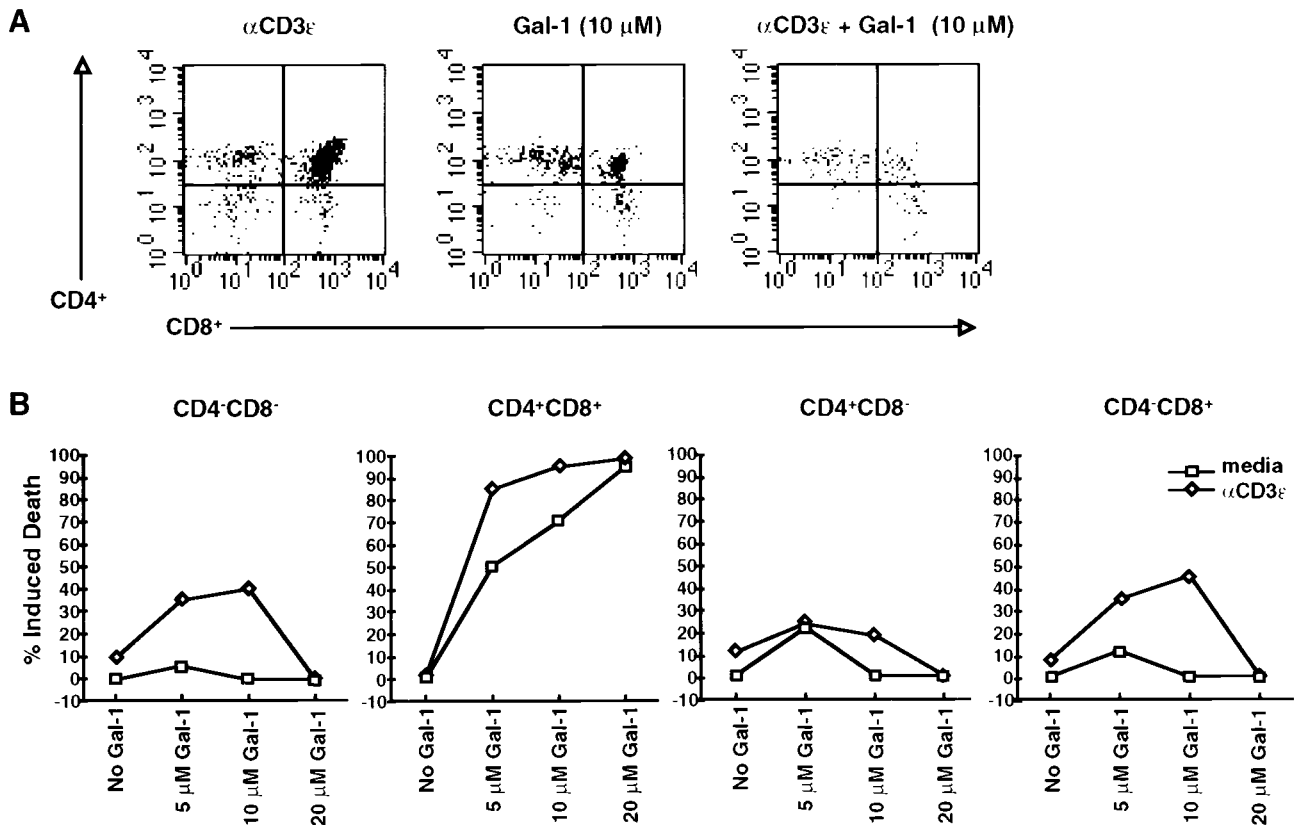


FIGURE 3. CD4⁺CD8⁺ thymocytes undergo apoptosis in response to galectin-1 treatment and treatment with anti-CD3 cooperates with galectin-1 to induce death in CD4⁻CD8⁻, CD4⁺CD8⁺, CD4⁺CD8⁻, and CD4⁻CD8⁺ subpopulations. To determine which thymic subpopulations undergo apoptosis in response to anti-CD3/galectin-1, 4×10^5 thymocytes were incubated at 37°C with control media or media containing varying concentrations of galectin-1 alone or together with 1.25 μ g/ml plate-bound anti-CD3 ϵ . After a 5-h incubation at 37°C, cells were resuspended in 0.1 M β -lactose and washed. Samples were incubated with phycoerythrin-conjugated anti-CD4 and biotinylated anti-CD8. Streptavidin tricolor conjugate was subsequently added to detect surface expression of CD8. For detection of dead cells, samples were stained with Annexin V. Annexin V positive cells were considered dead and electronically gated out. **A**, FACS plots and the quadrants used to define thymic subpopulations are shown for the 10 μ M galectin concentration. Similar quadrants were used to define subpopulations in samples treated with varying concentrations of galectin-1. **B**, The ability of varying concentrations of galectin-1 alone or in the context of CD3 coengagement to induce cell death were calculated for each CD4⁻CD8⁻, CD4⁺CD8⁺, CD4⁺CD8⁻, and CD4⁻CD8⁺ thymic subpopulations. Induced cell death was calculated based on the number of live cells in the treated population relative to the number of live cells in the unstimulated control population. For each sample, FACS analysis was performed on 50,000 events.

stained with PI and induced cell death was calculated relative to the percentage of live cells in unstimulated controls 9 h after stimulation (Fig. 2A). To assess the affects of galectin-1 on anti-CD3 ϵ -induced proliferation, tritiated thymidine uptake was used to measure proliferation of cultures within 30 h of stimulation (Fig. 2B). Fig. 2A demonstrates that galectin-1 induces cell death in freshly isolated mouse thymocytes. Furthermore, whereas stimulation of thymocytes with anti-CD3 alone results in proliferation, coordinate stimulation with anti-CD3 and galectin-1 inhibits proliferation and induces even greater apoptosis than treatment with galectin-1 alone (Fig. 2, A and B).

Although CD4⁺CD8⁺ double positive cells are the primary thymic subpopulation susceptible to galectin-1 stimulation, coordinate CD3 engagement broadens susceptible thymocyte subpopulations to include a subset of CD4⁻CD8⁻, CD4⁻CD8⁺, and CD4⁺CD8⁻ subpopulation and most of the CD4⁺CD8⁺ subpopulation

To determine which mouse thymic subpopulations undergo apoptosis in response to anti-CD3/galectin-1 treatment, thymocytes were coordinately stimulated with varying concentrations of galectin-1 and anti-CD3 for 5 h. Cells were stained for expression of CD4 and CD8 and for apoptotic cells using Annexin V and sub-

jected to FACS analysis. To avoid potential complications due to the nonspecific uptake of Abs by dead cells, Annexin V positive apoptotic and preapoptotic cells were electronically gated out and CD4/CD8 expression on the galectin-1/CD3 insensitive subpopulation examined. Annexin V negative thymocytes treated with control media show typical relative proportions of CD4⁺, CD8⁺ single positive, and CD4⁺CD8⁺ double positive thymocyte subpopulations (data not shown). Consistent with data reporting that human CD4⁺CD8⁺ double positive thymocytes undergo apoptosis in response to galectin-1 alone (12), we observed that galectin-1 treatment also induced apoptosis in CD4⁺CD8⁺ mouse thymocytes (Fig. 3). However, unlike findings with human thymocytes in which galectin-1 only induced apoptosis in ~5–15% of thymocytes (12), we found that the majority of double positive mouse thymocytes were susceptible to galectin-1 treatment, particularly at the higher concentrations of galectin-1 tested. Coordinate engagement of CD3 enhanced the ability of galectin-1 to induce apoptosis in double positive thymocytes, especially at suboptimal concentrations of galectin-1 (Fig. 3A). Furthermore, concomitant CD3 engagement and galectin-1 stimulation resulted in apoptosis of a subset of the CD4⁻CD8⁻, CD4⁻CD8⁺, and CD4⁺CD8⁻ subpopulations not susceptible to galectin-1 treatment alone (Fig. 3A).

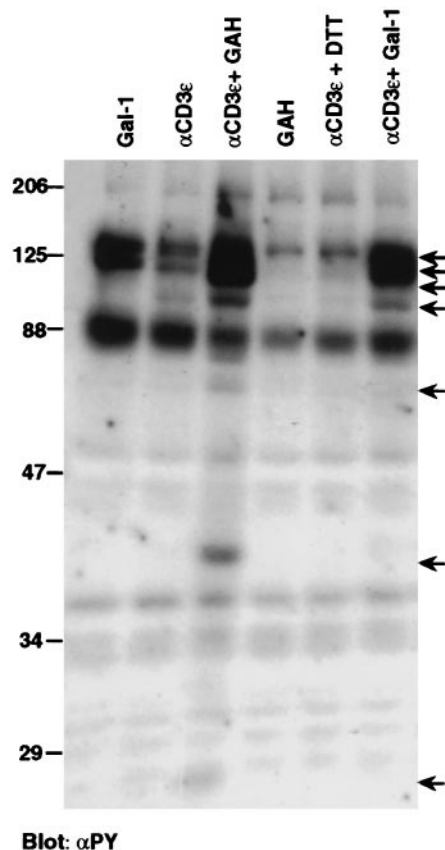


FIGURE 4. Galectin-1 stimulation induces tyrosine phosphorylation of proteins, but does not uniformly amplify the protein tyrosine phosphorylation pattern induced by TCR engagement. BI-141 were stimulated with 20 μ M of galectin-1 (Gal-1) and/or 5 μ g 2C11 for 5 min at 37°C. Parallel samples were treated with 2C11 + GAH (goat anti-hamster) as a positive control or with GAH as a negative control. Postnuclear cell lysates were separated by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using antiphosphotyrosine (α PY) mAb (4G10). The positions of proteins differentially affected by galectin-1, CD3, galectin-1/CD3, and CD3/GAH stimulations are indicated with arrows. Because galectins require the presence of a reduced-thiol group to maintain binding activity, recombinant galectin-1 is maintained in 8 mM DTT. Therefore, samples containing galectin-1 also contain diluted concentrations of DTT. The effects of stimulating 2C11 in the presence of comparable levels of DTT was included as a control (1.2 mM).

In Figs. 2 and 3, cell death was determined by the ability of T cells to exclude PI or bind Annexin V because these assays allowed for the most accurate quantitation of the total number of cells dying in response to various stimuli. However, in experiments in which Annexin V was used in combination with PI staining, an Annexin V⁺/PI negative population appeared before the appearance of the PI positive population (results not shown). These data demonstrate that galectin-1/TCR stimulation results in the initiation of an apoptotic program rather than necrosis and are consistent with previously published reports demonstrating that galectin-1 stimulation of T cells and TCR stimulation of the BI-141 hybridoma result in apoptotic-specific events including altered morphology and DNA fragmentation (6, 11, 12). Similarly, we have detected DNA fragmentation in response to galectin-1 and galectin-1/anti-CD3 treatment using a TUNEL-based assay (data not shown).

Galectin-1 does not uniformly amplify all TCR signals

The TCR initiates signal transduction through the rapid activation of intracellular tyrosine kinases including Lck, Fyn, ZAP70, and

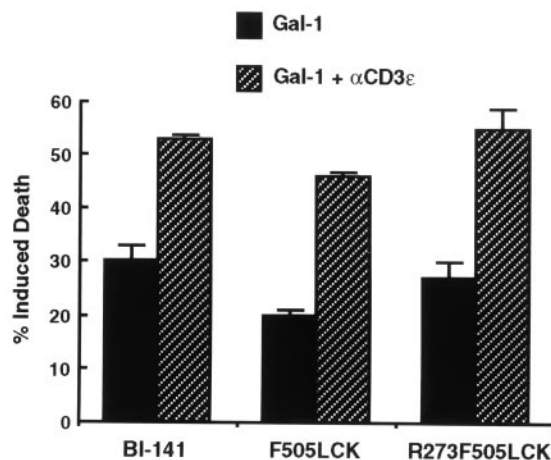


FIGURE 5. Expression of constitutively activated or kinase inactive Lck does not affect the ability of BI-141 to die in response to galectin-1 or anti-CD3 and galectin-1. BI-141 or transfectants at 0.5×10^6 cells/ml were stimulated with 20 μ M galectin-1 alone or together with anti-CD3 ϵ 2C11 (1 μ g/ml). After 12 h, cells were harvested, stained with PI, and analyzed on a FACSscan. The average percent that induced cell death of triplicate cultures was calculated relative to the number of live cells in unstimulated controls.

the subsequent tyrosine phosphorylation of signal transduction proteins (18). Because T cell surface galectin-1 counterreceptors CD45, CD43, CD3, and CD4 associate with Lck and/or Fyn (19, 20), we examined whether stimulation with galectin-1 directly modifies protein tyrosine phosphorylation or affects TCR-induced protein tyrosine phosphorylation.

As shown in Fig. 4A, galectin-1 stimulation of BI-141 T cells resulted in the rapid tyrosine phosphorylation of proteins migrating within at least two bands in the 120- to 130-kDa range. Stimulation with anti-CD3 resulted in less efficient protein tyrosine phosphorylation of proteins within the 120- to 130-kDa range and in significant phosphorylation of a 105-kDa protein (Fig. 4). TCR-induced protein tyrosine phosphorylation of each of these bands is intensified when goat anti-hamster (GAH) Ig is included as a crosslinking reagent. Furthermore, phosphorylation of p115, p70, p42, and p23 kDa (phosphorylated ζ -chain) proteins is additionally detected upon CD3 crosslinking. Coordinate stimulation with anti-CD3 ϵ and galectin-1 results in the tyrosine phosphorylation of the 120- to 130-kDa proteins to levels comparable with those seen with galectin-1 alone in addition to inducing the tyrosine phosphorylation of proteins phosphorylated by anti-CD3 ϵ alone (i.e., p105) (Fig. 4). However, the phosphorylation pattern observed with coordinate galectin-1 and anti-CD3 ϵ stimulation is qualitatively different from that seen with anti-CD3 ϵ and crosslinking GAH, indicating that galectin-1 is not simply acting to crosslink the TCR or uniformly amplify all TCR signals. Of note the anti-CD3/GAH-induced p70, p36, and p23 (corresponding to the m.w. of ZAP70, LAT, and ζ , respectively) are not detected in lysates from cells coordinately stimulated with galectin-1 and CD3 stimulation, whereas the 120- to 130-kDa galectin-1-induced bands as well as the p105 band seen with anti-CD3/GAH crosslinking are readily detectable (Fig. 4).

Expression of constitutively activated (F505Lck) or kinase inactive (R273F505Lck) mutants of Lck in BI-141 hybridoma does not affect galectin-1- or galectin-1/TCR-induced death

Because Lck has been reported to associate with the galectin-1 counterligands CD45 (18, 21), CD43 (20, 22), and CD4 (19), and

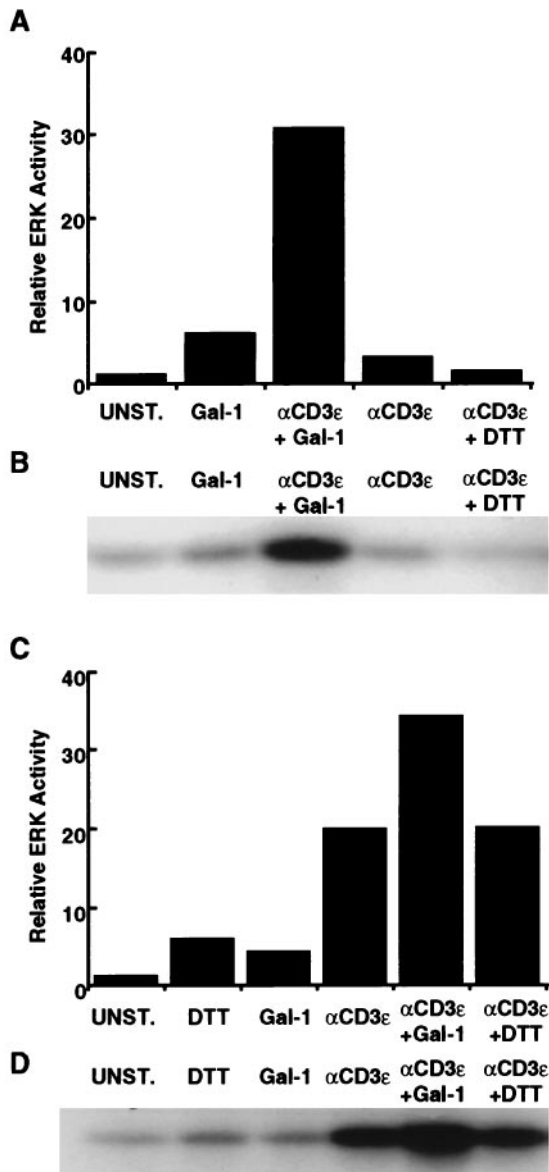


FIGURE 6. Galectin-1 and anti-CD3 synergize to induce ERK activation. BI-141 (A and B) or thymocytes (C and D) 5×10^6 were stimulated with $20 \mu\text{M}$ galectin-1 and/or $5 \mu\text{g}$ anti-CD3 ϵ at 37°C for 5 min. Cells were stimulated in parallel with medium containing 1.2 mM DTT or with media alone, which acted as a negative control. Cells were lysed, immunoprecipitated with anti-ERK2 anti-sera, and immunoprecipitates assayed for in vitro ERK kinase activity using MBP as a substrate. Phosphorylated MBP was separated from other proteins using 15% SDS-PAGE. Gels were exposed to film and bands were quantitated using a densitometer. Data are expressed as fold increase relative to ERK activity in unstimulated samples.

is known to be pivotal in initiating TCR-induced signals for IL-2 production, we investigated the role of Lck kinase activity in mediating galectin-1 signals. To this end, we assessed the ability of galectin-1 to stimulate apoptosis in BI-141 transfectants expressing constitutively active (F505Lck) and kinase inactive (R273F505Lck) mutant forms of Lck. We have previously demonstrated that these transfectants are dramatically modified in their ability to mediate Lck-dependent protein tyrosine phosphorylation and IL-2 production (6, 23). Although the expression of F505 enhances Lck-mediated TCR activation events, the expression of R273F505 leads to the inhibition of Lck-mediated TCR activation

events. We have also reported that TCR-mediated apoptosis in BI-141 is relatively Lck kinase activity independent in so far as it is unaffected by the expression of either F505Lck or R273F505Lck (6). As shown in Fig. 5, the parental BI-141 cells and both F505Lck and R273F505 transfectants respond similarly to galectin-1 or coordinate galectin-1/TCR stimulation. These data demonstrate that unlike T cell activation events known to require Lck kinase activity, the ability of galectin-1 to induce apoptosis or cooperate with the TCR to induce apoptosis is not dramatically affected by the manipulation of Lck kinase activity and thus indicate that galectin-1 is not highly dependent on Lck kinase activity to mediate these functions.

Galectin-1 and anti-TCR Abs synergize to activate ERK in BI-141 and thymocytes

MAP kinases are evolutionarily conserved mediators of a wide variety of signal transduction pathways contributing to biological processes including induction of differentiation, apoptosis, and proliferation (24). TCR-mediated tyrosine phosphorylation events are known to result in the activation of the ERK/MAP kinase cascade (18). To determine whether galectin-1 stimulation induces ERK activation by itself or whether galectin-1 influences the ability of the TCR to induce ERK activity, BI-141 and thymocytes were stimulated with galectin-1 alone or in combination with anti-CD3 ϵ . ERK was immunoprecipitated using anti-ERK-2 mAb and immunoprecipitates were subjected to in vitro kinase assay with MBP as an exogenous substrate. As shown in Fig. 6, A and B, galectin-1 and anti-CD3 ϵ signals synergize to dramatically up-regulate ERK activity in both the BI-141 T cell hybridoma (Fig. 6, A and B) and thymocytes (Fig. 6, C and D). Although galectin-1 and TCR each stimulate low levels of ERK activity, coordinate stimulation with both galectin-1 and anti-CD3 ϵ induces significantly more ERK activity than either reagent alone. ERK activation in response to anti-CD3/galectin-1 is quite rapid, occurring within 1 min of stimulation, peaking at five minutes, and significantly diminishing by 20 min poststimulation (data not shown). Control stimulations with media containing DTT alone (Fig. 6, C and D) or in combination with anti-CD3 ϵ (Fig. 6, A–D) demonstrate that the effects observed are due to galectin-1 rather than DTT present in the stimulating media.

Discussion

Galectin-1 is an endogenous lectin expressed at sites of immune function which can potentially modulate specific TCR signals and functions. Here we demonstrate that murine thymocytes and a T cell hybridoma undergo apoptosis in response to galectin-1 stimulation in keeping with reports describing its apoptotic activity on human thymocytes and some human transformed T cell lines (9, 12). However, unlike a previous report indicating that only a small subset (15%) of CD4 $^+$ CD8 $^+$ and some CD4 $^-$ CD8 $^-$ human thymocytes are susceptible to $20 \mu\text{M}$ galectin-1 (12), we find that the majority of CD4 $^+$ CD8 $^+$ murine thymocytes undergo apoptosis in response to ($20 \mu\text{M}$) galectin-1 treatment. Based on findings that indicate that many of the galectin-1-sensitive apoptotic human thymocytes have a DNA content $>2 \text{ N}$, Perillo et al. (12) suggested that cycling CD4 $^+$ CD8 $^+$ cells are the primary thymic subpopulation affected by galectin-1 treatment. In our studies, as many as 95% of murine CD4 $^+$ CD8 $^+$ thymocytes can be susceptible to galectin-1-induced apoptosis. Because only 5–15% of CD4 $^+$ CD8 $^+$ thymocytes have been reported to be cycling (25), our data indicate that noncycling, as well as cycling CD4 $^+$ CD8 $^+$, murine thymocytes can undergo apoptosis in response to galectin-1. Differences between our findings and those previously reported (12) may relate

to differences between mouse and human immune systems or between the sensitivity of the techniques used to measure apoptosis. Because we have not directly analyzed the cell cycle status of the apoptotic cells in our study, we cannot rule out the possibility that cycling cells are hypersensitive to galectin-1 treatment relative to noncycling cells.

We further demonstrate that coordinate galectin-1 stimulation and TCR engagement result in enhanced apoptosis in the CD4⁺CD8⁺ population, especially at suboptimal concentrations of galectin-1. Because galectin-1 is expressed by APCs and thymic epithelial cells, our finding that galectin-1 stimulation cooperates with TCR engagement to induce apoptosis in CD4⁺CD8⁺ thymocytes may implicate a role for galectin-1 in TCR-directed apoptosis during negative selection. The electronic gates defining CD4⁺CD8⁺ double positive cells in our FACS analysis include transitional subpopulations reported to be sensitive to negative selection (5). Indeed, it is possible that galectin-1 is the thymic costimulatory ligand reportedly required for efficient TCR-induced negative selection (26).

Furthermore, we demonstrate that engagement of the TCR during galectin-1 stimulation induces apoptosis in CD4⁻CD8⁻, CD4⁻CD8⁺, and to a lesser extent CD4⁺CD8⁻ subpopulations that are insensitive to galectin-1 stimulation alone. The fact that the CD4⁻CD8⁻ subpopulation is affected by CD3 stimulation at some concentrations of galectin-1 may implicate a role for galectin-1 in modulating pre-TCR signals. Moreover, that CD4⁺CD8⁻ and CD4⁻CD8⁺ subpopulations can be induced to undergo apoptosis in response to galectin-1 treatment in the context of TCR engagement indicate that galectin-1 can modulate TCR-mediated responses in mature T cells and may be relevant to the ability of galectin-1 to modulate TCR-mediated responses in the periphery. In keeping with this idea, we demonstrate that TCR engagement and galectin-1 stimulation cooperate to induce apoptosis in the BI-141 T cell hybridoma.

A previous study reports that 16 h of pretreatment with anti-CD3 increases human thymocyte susceptibility to subsequent 5 h of galectin-1-induced apoptosis (12). However, whether increased susceptibility correlated with the number of cycling cells and which thymic subpopulation was effected were not determined. These data were interpreted to suggest that anti-CD3 treatment may induce intracellular transcription of apoptotic machinery, which is subsequently activated by galectin-1 (12). Assuming that similar phenomena occur in mouse and human thymocytes, our data, demonstrating that enhanced apoptosis is observed within 5 h of CD3/galectin-1 costimulation, indicate that extensive pretreatment with anti-CD3 is not necessary. If anti-CD3 does prime thymocytes for subsequent galectin-1-induced death, such a priming event would have to occur within 1–2 h of TCR engagement. Alternatively, signals resulting from CD3 and galectin-1 coordinate engagement may cooperate to enhance apoptosis.

In this manuscript, we additionally provide evidence that galectin-1 modulates TCR-mediated IL-2 production and proliferation. Although galectin-1 cooperates with TCR engagement to induce apoptosis, it antagonizes TCR-induced proliferation and IL-2 production in thymocytes and T hybridoma cells, respectively. Additional experiments are required to determine which T cell subpopulation is susceptible to the inhibition of TCR-induced proliferation and to determine whether this effect is secondary to the ability of galectin-1 to inhibit TCR-induced IL-2 production. However, the fact that individual TCR-mediated functions are differentially affected by galectin-1 costimulation in the BI-141 T cell hybridoma indicates that galectin-1 independently modulates TCR-mediated signals and functions.

To determine whether galectin-1 stimulation modifies early TCR signal transduction events, we examined the abilities of galectin-1, anti-CD3, and galectin-1/anti-CD3 to affect protein tyrosine phosphorylation and ERK activity. We demonstrate the galectin-1 induces protein tyrosine phosphorylation but does not uniformly amplify all TCR-induced tyrosine phosphorylation. Furthermore, galectin-1/TCR-mediated cell death is resistant to the dominant negative expression of impaired forms of Lck that are capable of modulating TCR-induced IL-2 production. We also demonstrate that galectin-1 synergizes with anti-CD3 to activate ERK. These data indicate that galectin-1 differentially influences particular TCR-induced signals. The fact that galectin-1 differentially modulates CD3-induced protein tyrosine phosphorylation, ERK-2 activity, and downstream functions may account for galectin-1 immunomodulatory properties.

The fact that galectin-1 and TCR cooperate to induce both apoptosis and ERK activation in T cells implicates a potential role for ERK in affecting apoptosis. Experiments are underway to determine whether ERK activity is required for galectin-1/TCR-induced apoptosis or inhibition of IL-2 production and which thymic subpopulation is responsible for the synergistic TCR/galectin-1 ERK activation observed in lysates from total thymocytes. The role of ERK activity in positive and negative selection has been indirectly assessed through the expression of dominant negative upstream regulators of ERK activity, dominant negative Ras, and dominant negative MAP kinase kinase-1 (MEK-1) (27). Interestingly, mice expressing dominant negative forms of Ras and MEK-1 are severely impaired in TCR-mediated positive selection, though negative selection remains unaffected. Interpretation of the effects of disrupting Ras- and MEK-1-mediated ERK activation with regard to the role for galectin-1-induced ERK activity in negative and/or positive selection requires additional characterization of the signaling mechanism through which galectin-1 induces ERK activation. Furthermore, studies comparing thymocyte development in TCR transgenics on wild-type and galectin-1 null (28) backgrounds should lend additional insight as to whether galectin-1 contributes to thymocyte development and which TCR signal transduction pathways are affected by galectin-1 stimulation.

The ability of galectin-1 stimulation to cooperate with TCR signals to mediate apoptosis and inhibit IL-2 production may also be relevant to TCR-directed apoptosis involved in peripheral tolerance induction, immune privilege, or tumor escape from immune surveillance (1). Indeed, galectin-1 is expressed in a number of immune-privileged sites including testes, cornea, brain, placenta, and prostate and by malignancies including ovarian and colon carcinomas (1). If peripheral T cells, like single positive thymocytes and hybridoma cells, respond to coordinate TCR and galectin-1 signals by inducing apoptosis and/or inhibiting IL-2 production and proliferation, cells expressing TCRs with specificities for Ag expressed in immune privileged tissues or tumors would be particularly susceptible to galectin-1 immunomodulatory activity. The ability of galectin-1 to enhance TCR-induced cell death and inhibit IL-2 production may provide a molecular basis for the finding that galectin-1 can prevent disease induction in rat experimental autoimmune encephalomyelitis (EAE) model (29). Indeed, galectin-1 modulation of EAE and down-regulation of lymphokine production is reminiscent of the immunomodulatory capacity of some antagonist peptides that have been demonstrated to interfere with the onset of EAE by shifting a Th1 response to a Th2 response (30). That galectin-1 is an endogenous protein with the potential to independently modulate particular antigenic responses without requiring prior knowledge of the Ag peptide recognized or homogeneity of the specificity of the T cell response makes galectin-1 an

intriguing candidate for modulating TCR-mediated T cell responses in vivo. Our demonstration that galectin-1 affects apoptosis and differentially affects TCR signals and functions in mouse T cells and thymocytes lays the groundwork for studies that capitalize on the availability of genetic variants and the ability to manipulate immune responses in the murine system to begin to address these issues.

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