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Authors
Chen, I-Ju
Chen, Hung-Lin
Demetriou, Michael

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Lateral Compartmentalization of T Cell Receptor Versus CD45 by Galectin-N-Glycan Binding and Microfilaments Coordinate Basal and Activation Signaling

I-Ju Chen, Hung-Lin Chen, and Michael Demetriou

From the Department of Neurology and Department of Microbiology and Molecular Genetics, University of California, Irvine, California 92697

Lateral compartmentalization of membrane proteins into microdomains regulates signal transduction; however, structural determinants are incompletely understood. Membrane glycoproteins bind galectins in proportion to the number (i.e. N(X(S/T)) sites) and degree of GlcNAc branching within attached N-glycans, forming a molecular lattice that negatively regulates T cell function and autoimmunity. We find that in resting T cells, partition of CD45 inside and T cell receptor (TCR)/CD4-Lck/Zap-70 outside microdomains is positively and negatively regulated by the galectin lattice and actin cytoskeleton, respectively. In the absence of TCR ligands, the galectin lattice counteracts F-actin to retain CD45 in microdomains while concurrently blocking TCR/CD4-Lck/Zap-70 partition to microdomains by preventing a conformational change in the TCR that recruits Nck/Wiscott Aldrich Syndrome (WASp)/SLP76/F-actin/CD4 to TCR. The counterbalancing activities of the galectin lattice and actin cytoskeleton negatively and positively regulate Lck activity in resting cells and CD45 versus TCR clustering and signaling at the early immune synapse, respectively. Microdomain-localized CD45 inactivates Lck and inhibits TCR signaling at the early immune synapse. Thus, the galectin lattice and actin cytoskeleton interact on opposing sides of the plasma membrane to control microdomain structure and function, coupling basal growth signaling with thresholds to activation.

Separation of plasma membrane proteins into microdomains enriched in cholesterol and glycosphingolipids has been implicated in cell signaling and activation (1–6). However, the hypothesis that their formation is determined solely by “phase separation” of cholesterol and saturated phospholipids from unsaturated phospholipids has been challenged (1–6). An emerging model suggests other factors, such as protein-protein binding and the actin cytoskeleton, play important roles in the stability, size, and retention of proteins in membrane microdomains (1–5). The clearest example of this is the immunological synapse, where agonist-induced T cell receptor (TCR)2 signaling provokes actin cytoskeleton-dependent coalescence of membrane microdomains enriched in signaling molecules at the contact site between T cells and antigen presenting cells (7–12).

TCR clustering at the immune synapse is negatively regulated by multivalent cross-linking of cell surface glycoproteins with galectins, interactions that form a molecular lattice restricting glycoprotein movement in the plane of the membrane and endocytosis (13–17). Glycoproteins are differentially incorporated into the galectin lattice based on the number of associated N-glycan chains (i.e. N(X(S/T)) sites) as well as degree of GlcNAc branching (13, 15, 18). The latter is dynamically controlled by Golgi processing and metabolic production of UDP-GlcNAc (18, 19), the sugar nucleotide donor for medial Golgi GlcNAc-branching enzymes Mgt1, -2, -4, and -5. In naïve T cells, genetic and metabolic control of GlcNAc branched N-glycans sets thresholds for activation and T141 differentiation by negatively regulating agonist-induced TCR signaling (13, 19, 20). Once activation thresholds are exceeded (21), T cells undergo multiple rounds of cell division and arrest their growth after induction of CTLA-4 to the cell surface (22). Membrane turnover is high in T cell blasts, and Src kinases/phosphatidylinositol 3-kinase/extracellular signal-regulated kinase stimulate metabolic flux and Golgi processing to GlcNAc-branched N-glycans, promoting CTLA-4 surface retention via incorporation into the galectin lattice (18). In this manner GlcNAc branching negatively regulates naive T cell growth early by dampening activation signaling and late by promoting growth arrest. In vivo, genetic and metabolic control of GlcNAc branching negatively regulates delayed type hypersensitivity and autoimmunity (13, 19, 23).

Activation of the Src tyrosine kinase Lck via autophosphorylation at Tyr394 is required for agonist-induced TCR signaling and T cell activation (24, 25). In resting T cells, a proportion of Lck partitions to membrane microdomains via palmitylation

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1 To whom correspondence should be addressed: Depts. of Neurology and Microbiology and Molecular Genetics, University of California, 250 Sprague Hall, Irvine, CA 92697. Tel.: 949-824-9775; Fax: 949-824-9847; E-mail: mdemetri@uci.edu.

The abbreviations used are: TCR, T cell receptor; GEM, GM1-enriched microdomain; GM1, ganglioside M1; CTB, cholera toxin B; TRITC, tetramethylrhodamine isothiocyanate; Ab, antibody; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; TSA, bovine serum albumin; FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex.
and interactions with CD4 (26), a localization required for TCR signaling (27, 28). The highly glycosylated tyrosine phosphatase CD45 positively and negatively regulates Lck activity via dephosphorylation of inhibitory Tyr(505) and activating Tyr(394), respectively (29, 30). In resting T cells, a small proportion of CD45 partitions to membrane microdomains (31–34), where it binds Lck (33) and inhibits Src kinase activity and TCR signaling (35, 36), suggesting CD45 predominantly functions as a negative regulator of Lck within membrane microdomains. The large extracellular domain of CD45 lacks a non-carbohydrate binding ligand but is paradoxically required for localization to membrane microdomains (34). Similarly, CD45 clusters at the early immune synapse (3 min) by unclear mechanisms (37). CD45 is modified with Mga5-produced β1,6GlcNAc-branched N-glycans extended by poly-N-acetyllactosamine (38), the high affinity ligand for galectins. When added to T cells in vitro, exogenous galectin-1 and galectin-3 bind to CD45 at the cell surface and induce T cell death (39, 40). Exogenous galectin-1 clusters CD45, whereas the cell surface distribution of CD45 remains uniform after the addition of galectin-3. However, whether CD45 interacts with endogenous galectins and is regulated by the galectin lattice is unknown.

Agonist clustering of the TCR complex induces a conformational change required for full TCR activation and binding of the adaptor protein Nck to the cytoplasmic tail of CD3ε (41, 42). Nck binds WASp, a protein that induces actin microfilament re-arrangement and is required for reforming the immune synapse in naïve T cells after breaks in symmetry (12). The adaptor protein SLP76 has also been proposed to bring Nck/WASp to TCR (43, 44). Here we demonstrate that in the absence of TCR engagement by ligand, the galectin lattice opposes F-actin to lateral compartmentalization of CD45 in GM1-enriched microdomains (GEM) and concurrently prevents Nck/WASp/SLP76 and CD4 binding to the TCR complex, F-actin targeting of TCR/CD4/Lck to GEMs, Lck autophosphorylation at Tyr(394), and Zap70 recruitment. Upon TCR stimulation, microdomains re-structured a priori by opposing actions of the galectin lattice and Nck/WASp/SLP76/F-actin cluster at the early immune synapse to control CD45 versus TCR content and signaling. Galectin lattice-mediated partition of CD45 to microdomains (34) and the early synapse (37) negatively regulate Lck-Tyr(P)(394) and TCR signaling. Thus, lateral compartmentalization of CD45 versus TCR/CD4-Lck in resting T cells via galectin lattice and F-actin competition regulates homeostatic growth signaling via Lck as well as the structure and signaling activity of the early immune synapse. We propose a general mechanism for controlling membrane microdomain structure and function, namely, competition between galectin binding to GlcNAc branched N-glycans attached to extracellular domains and adaptor proteins/polymerized F actin via cytoplasmic domains.

**EXPERIMENTAL PROCEDURES**

**Mice and Cell Lines**—T cells isolated from C57BL/6 Mga5+/+ and Mga5−/− mice were used for experiments and were congenic by backcross from 129/sv Mga5−/− mice (45) for six generations. All mice used were age-matched at 8–10 weeks. Human Jurkat cell line E6-1 and its derivative cell lines with deficiency in CD45 (J45.01), TCRβ (J.RT3-T3.5), CD4 (D1.1), and Lck (J.CaM1.6) were purchased from ATCC. Cells were maintained in RPMI 1640 medium with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), 50 μM β-mercaptoethanol at 37 °C in a 5% CO2-humidified atmosphere.

**TCR Signaling and Western Blotting**—Polyethylene beads (6 μm, Polysciences) were coated with 0.5 μg/ml anti-CD3ε antibody (2C11 for mouse, OKT3 for Jurkat, ebioscience) at 4 °C overnight. 1 × 10⁶ purified splenic CD3+ T cells isolated by negative selection (R&D Systems) and/or Jurkat T cells treated with or without 5 μM swainsonine (SW) (Sigma) for 3 days were preincubated for 20 min at 37 °C with or without 50 mM sucrose (Fisher), 50 mM β-lactose (Fisher), 1 μM latrunculin-A (Sigma), 20 μM 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (protein-tyrosine kinase inhibitor (PP2)) (Calbiochem), or N-(9,10-dixo-9,10-dihydrophenanthren-2-yl)-2,2-dimethyl-propionamide (protein-tyrosine phosphate CD45 inhibitor) (EMD) and then mixed with or without anti-CD3ε Ab-coated beads, pelleted at 5000 rpm for 15 s, and incubated at 37 °C for the indicated times. Cells were solubilized with ice-cold 50 mM Tris, pH 7.2, 300 mM NaCl, 1.0% Triton X-100, a panel of protease inhibitor mixture, and 2 mM sodium orthovanadate for 20 min. Cell lysates were separated on NuPAGE 10% Bis-Tris gels (Invitrogen) or NuPAGE 7% Tris acetate gels (Invitrogen) under reducing conditions, transferred to polyvinylidene difluoride membranes, and immunoblotted with anti-phospho-Lck Tyr(505) Ab (Cell Signaling Technology), anti-phospho-Src family Tyr 416 Ab (Cell Signaling Technology), which cross-reacts with phospho-Lck Tyr(394), anti-phospho-Zap70 Ab (Cell Signaling Technology), anti-phospho-LAT Ab (Upstate), anti-actin Ab (Santa Cruz), anti-CD45 Ab (35-Z6 and M20, Santa Cruz), anti-Lck Ab (3A5, Santa Cruz), anti-Zap70 Ab (IE7.2, ebioscience), anti-CD3ε Ab (M20, Santa Cruz), anti-CD4 Ab (C18, Santa Cruz), anti-TCRβ Ab (H197, Santa Cruz), and anti-galectin-3 Ab (H160, Santa Cruz). The blots were developed with the ECL system (Pierce). To identify the GEM-enriched fractions in sucrose gradient fractions, aliquots of each fraction were spotted onto a nitrocellulose membrane, which was subsequently hybridized with horseradish peroxidase-conjugated cholaer toxin B (CTB) (List Biological Laboratory) to label endogenous GM1 ganglioside.

**Co-immunoprecipitation**—Purified splenic CD3+ T cells isolated by negative selection (R&D Systems) or Jurkat T cells treated with or without 5 μM SW (Sigma) for 3 days were pre-treated with or without anti-CD3 antibody (5 min at 37 °C, 10 μg/ml UCHT1, ebioscience), β-lactose (50 mM, Fisher), the control disaccharide sucrose (50 mM, Fisher), or PP2 (20 μM, Calbiochem) for 20 min at 37 °C and then incubated with or without the homobifunctional cross-linker dithiobis(sulfo-succinimidylpropionate) (Pierce) at 0.1 μg/ml with 10⁷ cells/ml in phosphate-buffered saline, pH 8.0, for 10 min at room temperature in the presence of the same. The cross-linking reaction was quenched by adding Tris, pH 7.2, to a final concentration of 50 mM and incubating for 15 min. Cells were then lysed and immunoprecipitated at 4 °C by incubation with anti-CD45 Ab (F10–89-4, SouthernBiotech), anti-galectin-3 Ab (H160, Santa Cruz), or anti-Nck Ab (Chemicon) for 2 h followed by protein G PLUS-agarose beads (Santa Cruz) overnight. After washing six
times, eluted proteins were blotted with anti-CD45 Ab (35-Z6 and M20, Santa Cruz), leukoagglutinin-biotin (EY Laboratories), anti-Nck Ab (Millipore), anti-TCRβ Ab (Jovi1, Abcam), anti-CD4 Ab (C18, Santa Cruz), anti-SLP-76 Ab (Cell Signaling Technology), and anti-WASp Ab (Cell Signaling Technology). Quantification of Western Blots by Densitometry—Bands detected on film after exposure of blots to enhanced chemiluminescence (Pierce) were digitally scanned and then analyzed by densitometry using the single band analysis function in Gel Pro Analyzer software. In this software changes in the amount of material concentrated in each band is measured by comparing the intensity of a band with that of an assigned band. For Figs. 1C and 5B, each band was first compared with the band in the non-stimulated lane to give a -fold difference and then normalized for differences in actin. For comparison of relative amounts of protein in GEM versus non-GEM fractions, the following was done. 1) Background was subtracted from all bands/lanes, 2) densitometry values were summed in lanes 1–6 for GEMs, lanes 7–10 for non-GEMs, and lanes 1–10 for the total, 3) the GEM fraction was the sum of lanes 1–6 divided by the sum of lanes 1–10, and the non-GEM fraction was the sum of lanes 7–10 divided by the sum of lanes 1–10.

Immunofluorescence Microscopy—Purified splenic CD3+ T cells isolated by negative selection (R&D Systems) and/or Jurkat T cells treated with or without 5 μM swainsonine (Sigma) for 3 days were preincubated for 20 min at 37 °C with or without 50 mM sucrose (Fisher), 50 mM β-lactose (Fisher), 1 μM latrunculin-A (Sigma), or 20 μM PP2 (Calbiochem). Cells were then patched for GEMs by incubation with CTB-TRITC at 10 μg/ml in 0.1% BSA/TBS for 45 min on ice, washed, and cross-linked by incubation with anti-CTB Ab (1/250 dilution in 0.1%BSA/TBS, Calbiochem) for 30 min on ice and then for 20 min at 37 °C. Patched cells were attached to poly-L-lysine-coated slides, fixed with 10% formalin at room temperature for 30 min, and then blocked with 0.5% bovine serum albumin in TBS for 1 h. Cells were then incubated with one or more of CTB-TRITC (non-patched cells, List Biology Laboratory), anti-CD45-FITC (30-F11, eBioscience), anti-CD4-PE-Cy5 (RPA-T4, eBioscience), anti-CD3-PE (OKT3, eBioscience), and leukoagglutinin-FITC (Vector Labs). Mouse T cells were stained with anti-CD45-FITC (30-F11, eBioscience), anti-CD4-PE-Cy5 (GK1.5, eBioscience), and anti-CD8a-PE (53-6.7, Pharmingen). All incubations were for 50 min on ice. Analyses were done with a FACScan flow cytometer using the CellQuest program (BD Biosciences).

RESULTS

The Galectin Lattice Negatively Regulates Lck Activation in Resting T Cells—At rest, T cell endocytosis rates are minimal, and disruption of the galectin lattice through genetic loss of β1,6GlcNAc-branched N-glycans (i.e. Mgat5-deficient cells) or inhibition of GlcNAc branching with the mannosidase II inhibitor SW (19, 20, 46) does not reduce surface levels of the TCR-CD3 complex or CD4, CD8, CD28, or CD45 in mouse or Jurkat T cells, respectively (Ref. 13 and supplemental Fig. 1, A and B). Strikingly, we noted that Lck-Tyr394 phosphorylation in unstimulated Mgat5−/− T cells is markedly increased relative to Mgat5+/+ T cells (Fig. 1A), not significantly enhanced by TCR stimulation (Fig. 1B), and similar to the maximal induction observed in TCR stimulated Mgat5−/− T cells (Fig. 1B). Active Lck phosphorylates Zap70, which in turn phosphorylates LAT, and both are also enhanced in resting Mgat5−/− T cells (Fig. 1A), although at much lower levels than present in TCR-stimulated cells (Fig. 1B). Inhibiting GlcNAc branching in resting Jurkat T cells with SW (19, 20, 46) similarly enhances phosphorylation of Lck-Tyr394, Zap70, and LAT in the absence of TCR stimulation (Fig. 1C and supplemental Fig. 2A). These data suggest the galectin lattice negatively regulates Lck activation and associated downstream basal growth signaling in the absence of TCR engagement. Indeed, disrupting the lattice in resting Mgat5−/− mouse T cells via 20 min of co-incubation with lactose (which binds galectins and disrupts the lattice (13)), but not control disaccharide sucrose, markedly enhanced Lck-Tyr394 phosphorylation (Fig. 1D). In contrast, Lck phosphorylation at inhibitory Tyr505 is not significantly altered by GlcNAc branching deficiency in resting or activated mouse (Fig. 1, A and B) and Jurkat T cells (supplemental Fig. 2A),
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A

| Mgat5   | Rest       |
|---------|------------|
| +/+     | WB: Lck-pY505 |
| +/-     | WB: Lck-pY394 |
| -/-     | WB: pZap70  |
| -/-     | WB: pLAT    |
| -/-     | WB: Actin   |

B

| Mgat5   | Anti-CD3 (min.) |
|---------|-----------------|
| +/+     | 0               |
| +/-     | 3               |
| -/-     | 10              |
| -/-     | 30              |

C

| Jurkat | CD45 deficient | TCRβ deficient | CD45 deficient | Lck deficient |
|--------|---------------|---------------|---------------|--------------|
| SW     | -             | -             | -             | -            |
| WB: Lck-pY394 | 1.00   | 2.75 | 1.71 | 1.24 |
| WB: pLAT | 0.65 | 0.75 | 0.37 | 0.48 |
| WB: Actin | 0.08 | 0.02 |

D

| Mgat5 | Rest       |
|-------|------------|
| +/+   | Sucrose    |
| -/+   | Lactose    |
| +/+   | Latrunculin-A |
| -/+   | WB: Lck-pY394 |
| -/+   | WB: Actin  |

E

| IP: CD45 | SW       |
|----------|----------|
| -        | +        |
| WB: L-PHA-biotin |  |
| WB: anti-CD45  |  |

F

| Mgcat5 | IP: Gal-3 |
|--------|-----------|
| +/-    | Lactose   |
| +/-    | Crosslinker |
| +/-    | WB: CD45  |

FIGURE 1. Negative regulation of Lck-Tyr394 phosphorylation in resting T cells by the galectin-glycoprotein lattice. A–C, purified CD3+ mouse T cells of the indicated genotypes or Jurkat T cells and its derivative cell lines J45.01 (CD45-deficient), JRT3-T3.5 (TCRβ-deficient), D1.1 (CD4-deficient), and JCaM1.6 (Lck-deficient) grown in the presence or absence of SW were treated with or without anti-CD3-coated beads, lysed, and Western-blotted (WB) as indicated. SW is a specific inhibitor of Golgi mannosidase II that blocks N-glycan GlcNAc branching beyond mono-antennary. Bands in C were quantified using Gel Pro Analyzer software analysis of digitally scanned blots and compared initially to the resting non-stimulated lane to calculate a -fold difference and then normalized to actin levels (see “Experimental Procedures”). Numbers are shown below each band. D, resting mouse Mgat5+/+ and Mgat5−/− T cells were preincubated with or without lactose (competitive inhibitor of galectin binding), sucrose (control disaccharide), or latrunculin-A (F-actin-disrupting agent) for 20 min at 37 °C, lysed, and Western blotted for pLck-Tyr394. E, Jurkat T cells pretreated with or without SW were lysed, immunoprecipitated for CD45, and blotted with CD45 and leukoagglutinin (L-PHA), an animal lectin specific to Mgat5-modified N-glycans (13). F, Mgat5+/+ and Mgat5−/− T cells were pretreated for 20 min at 37 °C with or without lactose, chemically cross-linked with diithobis(sulfosuccinimidylpropionate) (Crosslinker), immunoprecipitated (IP), and Western-blotted as indicated.

indicating the galectin lattice specifically regulates Tyr394 phosphorylation.

Activation of Lck via autophosphorylation at Tyr394 is critical for initiation of TCR signaling; however, the molecular mechanism inducing activation is not well understood. Galectin lattice disruption promotes Lck phosphorylation at Tyr394 in the absence of TCR ligation, suggesting hypoactivity of a negative regulator and/or hyperactivity of a positive regulator. The galectin lattice promotes surface retention of full-length CTLA-4 in T cell blasts, a phenotype that inhibits TCR signaling and induces growth arrest (18). However, full-length CTLA-4 is not expressed at the cell surface of resting naïve mouse or Jurkat T cells and does not regulate activation thresholds in naïve T cells (22), and background surface staining as well as intracellular levels are unchanged by Mgat5 deficiency (18). This indicates CTLA-4 does not contribute to Lck-Tyr(P)394 hyperphosphorylation after galectin lattice disruption in resting and TCR-stimulated mouse or Jurkat T cells.

CD45 promotes and inhibits Lck activity by dephosphorylating Tyr505 and Tyr394, respectively. CD45 contains Phaseolus vulgaris leukoagglutinin (L-PHA) reactive β1,6GlcNAc-branched N-glycans (Fig. 1E and Ref. 38) and binds endogenous galectin-3 at the cell surface, an interaction reduced by Mgat5 deficiency and co-incubation with lactose (Fig. 1E). CD45 deficiency prevents SW from increasing Lck-Tyr(P)394 phosphorylation in resting Jurkat T cells (Fig. 1C). The TCR complex binds galectin-3 via GlcNAc-branched N-glycans (13) and, when engaged by agonists, activates Lck. TCR complex deficiency in resting Jurkat T cells blocks SW-induced Lck-Tyr(P)394 hyperphosphorylation (Fig. 1C). The majority of Lck is bound to CD4, and deficiency of CD4 also inhibits enhancement of Lck-Tyr(P)394 phosphorylation by SW (Fig. 1C). These data indicate CD45, CD4, and the TCR complex are all required for galectin lattice-mediated negative regulation of Lck Tyr394 phosphorylation in resting T cells.

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To confirm these microscopy results using an independent method, we utilized cell lysis with cold Triton X-100 and sucrose density gradient separation of detergent-resistant membrane fractions. Although this technique may not necessarily reflect membrane microdomains present in live cells, it is a reasonable approach to confirm the relative redistribution of individual proteins in or out of microdomains after experimental manipulation of intact cells (47, 48).

Indeed, this analysis was consistent with our microscopy data and confirmed that disruption of the galectin lattice via Mgat5 deficiency in mouse T cells or SW treatment of resting Jurkat T cells promotes removal of CD45 while also increasing TCR-CD3 and CD4 partition to GEMs (Fig. 3, A–C, supplemental Figs. 4, A and B, and 5). A small proportion of galectin-3 partitions to GEMs in Mgat5+/+ but not Mgat5−/− resting T cells, suggesting β1,6GlcNAc branched N-glycans promote galectin-3 localization to GEMs (Fig. 3D). SW-induced changes in TCR-CD3 and CD45 localization to GEMs in Jurkat T cells is unaltered by deficiency of the TCR complex, CD45 or CD4, indicating galectin lattice-mediated maintenance of TCR outside and CD45 inside GEMs are independent of each other and upstream of CD4 (Fig. 3, A–C). In contrast, deficiency of the TCR complex, but not CD45, inhibits CD4 translocation to GEMs after SW treatment of resting Jurkat T cells (Fig. 3C). This indicates CD4 targeting to GEMs after lattice disruption requires the TCR complex.

Actin microfilaments and the galectin lattice act on opposing sides of the plasma membrane to affect lateral mobility of glycoproteins, suggesting these may interact to differentially partition TCR/CD4 versus CD45 within GEMs. The cytoplasmic domain of CD45 binds ankyrin, an interaction proposed to maintain CD45 outside GEMs via interaction of the membrane-associated ankyrin/spectrin(fodrin) scaffold with actin microfilaments (49). Actin reorganization is required for clustering of TCR and GEMs at the immune synapse (11, 44, 50), suggesting F-actin may differentially target TCR inside and CD45 outside GEMs. Indeed, blocking F-actin polymerization with latrunculin-A in resting mouse Mgat5+/+ T cells reduces

naïve mouse T cells via Mgat5 deficiency or co-incubation of wild type cells with lactose (20 min) significantly reduced the co-localization of CD45 with GEMs (Fig. 2A). Similar results were obtained in Jurkat T cells treated with SW (supplemental Fig. 3). Remarkably, Mgat5 deficiency and lactose treatment had the opposite effect on TCR and CD4, significantly enhancing their co-localization to GEMs in resting mouse T cells (Fig. 2A). These data suggest that in live cells the galectin lattice promotes partition of CD45 within GEMs and TCR/CD4 outside GEMs.

FIGURE 2. The galectin lattice counteracts actin microfilaments to partition CD45 inside and TCR and CD4 outside GEMs. A, GEMs were patched using CTB-TRITC (CTB) and anti-CTB antibody in resting mouse T cells pretreated for 20 min with or without lactose, sucrose, and/or latrunculin-A. After patching, T cells were fixed and then stained with anti-CD45-FITC (a pan CD45 antibody), anti-CD4-FITC, or anti-TCR-FITC. Quantification of the co-localization coefficient (i.e. R²) between CD45-GM1, CD4-GM1, and TCR-GM1 were determined after image deconvolution and analysis with Metamorph and ImageJ software. Statistical analyses were carried out using analysis of variance followed by Bonferroni’s multiple comparison test with five or greater replicates per condition. p values were generated by comparison to untreated Mgat5+/+ cells. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Error bars are S.E. of five or more replicates. Wt, wild type. B, Jurkat T cells pretreated as indicated were lysed, immunoprecipitated (IP) for Nck, and Western blotted (WB) as indicated.
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TCR/CD4 and increases CD45 partition within GEMs (Fig. 2A), an effect opposite to galectin lattice disruption. Co-incubation of latrunculin-A with lactose reversed lactose-induced changes in TCR/CD4 versus CD45 partitioning within GEMs (Fig. 2A). These data indicate F-actin polymerization, rather than a non-specific physiochemical change that alters lipid solubility, mediates the redistribution of TCR/CD4 and CD45 after disruption of the galectin lattice.

A critical regulator of actin re-organization in T cells is WASp, which binds the Arp2/3 complex to initiate F-actin polymerization (12, 51, 52). The adaptor protein Nck binds WASp, which recruits actin to TCR during T cell activation (44). Nck directly binds the cytoplasmic tail of CD3e after a conformational change induced by agonist-mediated clustering of two TCR complexes, a phenotype independent of tyrosine phosphorylation (41, 42). We hypothesized that in the absence of TCR agonists, the galectin lattice may actively prevent clustering of TCR complexes, the conformational change and recruitment of Nck/WASp to TCR. Indeed, co-immunoprecipitation demonstrates that disrupting galectin binding in resting Jurkat T cells via SW or lactose treatment induced Nck binding to the TCR complex with the same magnitude as 5 min of stimulation with anti-CD3 antibody (Fig. 2B). WASp association with Nck was also induced by galectin lattice disruption in the absence of TCR ligand (Fig. 2B). SLP76, an adaptor protein implicated in targeting Nck to TCR (43, 44), also co-immunoprecipitated with Nck after galectin lattice disruption. Conformational changes in TCR have been suggested to induce binding of CD4 in the absence of MHC (53–55). Although CD4-TCR interactions are believed to require the MHC, these data suggest that the conformational change associated with Nck-CD3e binding may also induce CD4-TCR binding. Indeed, CD4 co-immunoprecipitated with Nck after SW or lactose treatment, indicating binding of CD4 to the TCR-CD3e-Nck complex (Fig. 2B). Importantly, the effects of galectin lattice disruption were independent of Src kinase activity, as coinubation with 20 μM PP2 did not alter the association of Nck with the TCR complex, CD4, WASp, or SLP76. PP2 is a potent inhibitor of all Src tyrosine kinases and, at 20 μM, markedly reduces Lck-Tyr539 phosphorylation in resting T cells (supplemental Fig. 2B) and prevents >90% of TCR agonist-induced Lck-Tyr539 phosphorylation at the immune synapse (50). Taken together, these data indicate that in resting T cells, the galectin lattice opposes F-actin-dependent exclusion of CD45 from GEMs, and concurrently, Nck/WASP/SLP76/F-actin mediated TCR/CD4 recruitment to GEMs. Moreover, the data demonstrate that the earliest steps in ligand-induced TCR activation are re-capitulated by simply disrupting TCR- galectin binding. Many other proteins have been implicated in actin re-organization in T cells (e.g. vav, WAVE), and our data do not exclude their participation.

The Galectin Lattice Prevents Lck Activation in Resting T Cells by Regulating Membrane Microdomain Structure—We next confirmed that galectin lattice mediated re-structuring of GEMs is upstream of Lck Tyr539 hyperphosphorylation. Incubation of resting mouse T cells with 20 μM PP2 did not alter the distribution of TCR, CD4, and CD45 within GEMs (Fig. 4). Coincubation of PP2 with lactose did not prevent re-structur-

### Figure 3: Galectin lattice partition of TCR outside and CD45 inside GEMs are independent of each other and upstream of CD4 re-distribution.

**A**—D, Mgat5+/+ and Mgat5−/− T cells as well as SW-treated or non-treated Jurkat T cells and its derivative cell lines J45.01 (CD45-deficient), JRT3.T5 (TCR-β-deficient), D1.1 (CD4-deficient), and JCam1.6 (Lck-deficient) were lysed in cold 0.5% Triton X-100 followed by separation of detergent-resistant membrane fractions by sucrose density gradient. Each partner cell line (Jurkat or Jurkat + SW) and blotting of each protein (e.g. CD45 for Mgat5+/+ and Mgat5−/−) was done in parallel with blots exposed for the same time and normalized by total cell number. The two numbers below each blot represent the relative amount of protein, as determined by quantification of digitally scanned blots using Gel Pro Analyzer software, within GEMs (fractions 1–6) and non-GEM (fractions 7–10) as a fraction of the total (see “Experimental Procedures”). GEM fractions were defined by dot blotting for GM1 with CTB (supplemental Fig. 4, A and B). Blots are overexposed in the non-GEM fractions to best display levels within GEMs. Comparison of lighter and darker exposures gave the same results, indicating exposure time of the blots is within an appropriate range for densitometry comparisons (supplemental Fig. 5). WB: Western blot.
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Co-localization of CD45 to GEMs is required for TCR agonist-induced Lck-Tyr(P)394 hyperphosphorylation. In CD4+ T cells, ~75–95% of Lck binds to CD4, an interaction that promotes partition to GEMs (26). Disruption of the galectin lattice increases Lck content in GEMs of resting mouse and Jurkat T cells (Fig. 5A, supplemental Fig. 3, A and B). Deficiency of CD4, but not CD45, blocks this phenotype in Jurkat T cells (Fig. 5A). The TCR complex is required for partition of CD4 to GEMs (Fig. 3C), and its deficiency also blocks SW-induced enhancement of Lck activity in GEMs after galectin lattice disruption (Fig. 4). Similarly, deficiency of Lck in Jurkat T cells, which also reduces surface CD4 expression via enhanced endocytosis (supplemental Fig. 1B and Ref. 56), did not prevent SW-induced partitioning of CD45 outside and TCR inside GEMs of resting cells (Fig. 3, A and B). These data demonstrate that galectin lattice regulation of TCR/CD4 versus CD45 partitioning to GEMs is independent of Lck and Src kinase activity and precedes Lck Tyr394 hyperphosphorylation.

In CD4+ T cells, ~75–95% of Lck binds to CD4, an interaction that promotes partition to GEMs (26). Disruption of the galectin lattice increases Lck content in GEMs of resting mouse and Jurkat T cells (Fig. 5A, supplemental Fig. 3, A and B). Deficiency of CD4, but not CD45, blocks this phenotype in Jurkat T cells (Fig. 5A). The TCR complex is required for partition of CD4 to GEMs (Fig. 3C), and its deficiency also blocks SW-induced enhancement of Lck within GEMs (Fig. 5A). This suggests that relative levels of TCR/Lck-Tyr(P)394 and CD45 at the early immune synapse (9, 10, 37, 58). Increased Lck activity and partition of the TCR complex into GEMs should induce TCRζ immunoreceptor activation motif hyperphosphorylation and recruitment of Zap70. Indeed, Mgat5 deficiency and SW treatment enhance Zap70 expression in GEMs of resting cells, a phenotype blocked by Lck deficiency (Fig. 5D). We conclude that the galectin lattice prevents Lck activation in the absence of TCR agonists by promoting CD45 retention within GEMs as well as preventing TCR/CD4-Lck recruitment to GEMs.

Galectin Lattice-mediated Recruitment of CD45 to the Early Immune Synapse Inhibits TCR Signaling—TCR binding to peptide-MHC induces clustering of GEMs, the TCR complex, Lck-Tyr(P)394, and CD45 at the early immune synapse (9, 10, 37, 58). This suggests that relative levels of TCR/CD4-Lck-Tyr(P)394 versus CD45 present in the early immune synapse may in part be determined before TCR engagement via galectin lattice regulation of GEM structure. Because GEM clustering at the immune synapse is independent of CD28 in mouse T cells (10), we tested this hypothesis by stimulating Mgat5+/+ and Mgat5−/− T cells with anti-CD3 coated microbeads. Mgat5 deficiency and/or coinubcation of wild type T cells with lactose increases TCR (13) and Lck-Tyr(P)394 levels while reducing CD45 clustering at the contact site with anti-CD3-coated microbeads (Fig. 6A). Importantly, the amount of clustered GEMs is not significantly altered by Mgat5 deficiency, consistent with only a change in TCR versus CD45 content. Lysis of mouse and Jurkat T cells stimulated with anti-CD3-coated microbeads indicates the greatest difference in phosphorylation of Lck-Tyr394, Zap70, and LAT produced by galectin lattice disruption occurs 3 min post-stimulation (Fig. 1B, supplemental Fig. 2A), consistent with CD45 negatively regulating TCR signaling at the early immune synapse. Defi-
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FIGURE 5. The galectin lattice prevents Lck activation and Zap70 recruitment to GEMs in the absence of TCR agonist. A, C, and D, sucrose density gradient separation of detergent-resistant membrane fractions and Western blotting (WB) were performed with the indicated cells and analyzed as described in Fig. 3. B, resting Mgat5+/+ mouse T cells were treated with latrunculin-A and/or N-Ddpdp as indicated for 20 min at 37 °C, lysed, and Western-blotted for Lck-Tyr(P)394. N-Ddpdp is a cell-permeable and reversible inhibitor of clathrin-mediated endocytosis (68). C, Mgat5+/+ Jurkat T cells were co-spun for 15 s with microbeads coated as indicated, incubated at 37 °C for 3 min, fixed, stained with CD45-FITC and CTB-TRITC, permeabilized, and then stained with anti-Lck-Tyr(P)394 antibody followed by aminomethylcoumarin acetic acid conjugated secondary antibody. *, 20 T cell-bead conjugates scored for presence or absence of Lck-Tyr(P)394 in GEMs and/or GEMs at the cell-microbead contact. #, 20 T cell-bead conjugates scored for presence or absence of Lck-Tyr(P)394 in CD45 and/or GEMs at the cell-microbead contact. p values are by Fisher’s exact test. B, Mgat5+/+ mouse T cells were conjugated with or without antibody-coated microbeads via a 15-s spin, incubated at 37 °C for various times, lysed, and Western-blotted as indicated.

The galectin lattice prevents Lck activation and Zap70 recruitment to GEMs in the absence of TCR agonist. A, C, and D, sucrose density gradient separation of detergent-resistant membrane fractions and Western blotting (WB) were performed with the indicated cells and analyzed as described in Fig. 3. B, resting Mgat5+/+ mouse T cells were treated with latrunculin-A and/or N-Ddpdp as indicated for 20 min at 37 °C, lysed, and Western-blotted for Lck-Tyr(P)394. N-Ddpdp is a cell-permeable and reversible protein-tyrosine phosphatase (PTP) inhibitor with selectivity for CD45 (57). The IC<sub>50</sub> for plck peptide is 3.8 μM, compared with IC<sub>50</sub> > 30 μM for protein-tyrosine phosphatase 1B (protein-tyrosine phosphatase N1) and FAP (protein-tyrosine phosphatase N13). Bands were quantified using Gel Pro Analyzer software analysis of digitally scanned blots and compared initially to the resting non-stimulated lane to calculate a -fold difference and then normalized to actin levels (see “Experimental Procedures”). Numbers are shown below each band.

FIGURE 6. The galectin lattice clusters CD45 at the early immune synapse to inhibit TCR signaling. A, Mgat5+/+ and Mgat5−/− T cells were co-spun for 15 s with microbeads coated as indicated, incubated at 37 °C for 3 min, fixed, stained with CD45-FITC and CTB-TRITC, permeabilized, and then stained with anti-Lck-Tyr(P)394 antibody followed by aminomethylcoumarin acetic acid conjugated secondary antibody. *, 20 T cell-bead conjugates scored for presence or absence of Lck-Tyr(P)394 in GEMs and/or GEMs at the cell-microbead contact. #, 20 T cell-bead conjugates scored for presence or absence of Lck-Tyr(P)394 in CD45 and/or GEMs at the cell-microbead contact. p values are by Fisher’s exact test. B, Mgat5+/+ mouse T cells were conjugated with or without antibody-coated microbeads via a 15-s spin, incubated at 37 °C for various times, lysed, and Western-blotted as indicated.

2A). Conjugating Mgat5−/− T cells with anti-CD3/anti-CD45-coated microbeads restores expression of CD45 in GEMs at the cell-microbead contact site and markedly reduces Lck-Tyr(P)394 phosphorylation (Fig. 6A). Lysis of Mgat5−/− T cells stimulated with anti-CD3/anti-CD45-coated microbeads demonstrates reduced phosphorylation of Zap70 and LAT relative to controls (Fig. 6B). In the mature immune synapse, CD45 is excluded from peripheral TCR microclusters that are actively signaling but co-localizes with central TCR microclusters that have terminated their signaling, consistent with a negative regulatory role for CD45 (59). Taken together these data indicate galectin lattice regulation of the GEM structure at rest pre-sets T cell activation thresholds by controlling Lck activity and TCR versus CD45 clustering at the early immune synapse and indicates a negative regulatory role for CD45 in TCR signaling at the immune synapse.

DISCUSSION

Lateral compartmentalization of transmembrane proteins into functional microdomains plays critical roles in cell signaling; however, their structural determinants are controversial...
FIGURE 7. Regulation of microdomains and Lck activity in resting T cells via galectin lattice and actin microfilament interactions. A, schematic model for the opposing actions of the galectin lattice and actin cytoskeleton in regulating TCR and CD45 partition within GEMs of resting T cells. On the extracellular side of the plasma membrane, the galectin lattice cross-links TCR, CD45, and other glycoproteins. Inside the cell the cytoplasmic domains of the same molecules interact with the actin cytoskeleton via adaptor molecules. Interactions of CD45 with F-actin, presumably via ankyrin-spectrin (A-S) (49) or other adaptors, promotes removal from GEMs. In contrast, F-actin interaction with TCR, via Nck, SLP-76, and WASp, promotes TCR/CD4 partition to GEMs. In both cases, the galectin lattice negatively regulates these movements to balance the amount of CD45 and TCR/CD4 within GEMs.

B, disrupting galectin binding promotes the following; 1) Nck/WASp/SLP76/CD4 recruitment to TCR, presumably after TCR clustering and the CD3ε conformational change (41, 42), 2) F-actin-dependent partition of CD45 outside GEMs concurrent with and independent of Nck/WASp/SLP76/F-actin-mediated TCR/CD4-lck partition to GEMs, 3) Lck autophosphorylation at activating Tyr394, 4) Lck-dependent targeting of Zap-70 to GEMs and after encounter with antigen-presenting cell (APC), 5) coalescence of TCR-enriched and CD45-deficient GEMs at the early immune synapse.
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and not well understood (1–6). Our data demonstrate that partition of TCR versus CD45 within GEMs in resting T cells is dependent on two countering mechanisms regulating lateral movement, notably multivalent galectin binding to GlcNAc-branched N-glycans on extracellular domains and cytoplasmic domain tethering to actin microfilaments (Fig. 7A). This provides a molecular mechanism for coupling microdomain organization on the outer and inner leaflets of the cell membrane.

Galectins bind surface glycoproteins in proportion to the number of associated N-glycans, an encoded feature of protein sequence (i.e. NX(S/T)), as well as degree of GlcNAc branching within N-glycans (13, 15, 18). The latter is sensitive to metabolic flux through the hexosamine pathway to UDP-GlcNAc as well as genetic determinants of Golgi activity. Galectins are noncovalently associated with the cell surface. Galectin-1 and -3 surface levels are reduced by in vitro washing of ex vivo T cells3 and enhanced by the addition of recombinant galectins (40). This indicates the galectin lattice is highly dynamic, under metabolic and genetic control, and varies with in vitro manipulation of the cell, factors that may contribute to microdomain heterogeneity and inconsistencies in their structural definition. For example, over-washing of cells, which is strictly avoided in our experiments, would weaken the galectin lattice and alter membrane microdomain structure and function. Current estimates suggest membrane microdomains are very small (~5–20 nm) and short-lived (half-life ~100 ns) (2), measurements that should be re-evaluated in the presence and absence of the galectin lattice.

Many details of microdomain regulation by galectin lattice-actin cytoskeleton competition remain to be defined, including identification of other glycoproteins and lectins on the outer membrane and cytoskeletal adaptor proteins, protein-tyrosine kinases, and phosphatases on the inner membrane. For example, the galectin family has 15 members, and their relative roles require examination. Additional molecular details for F-actin-mediated exclusion of CD45 from microdomains is also required, although others have proposed a role for ankyrin, which binds the cytoplasmic tail of CD45 and tethers it to F-actin via the ankyrin-spectrin/iodrin scaffold (49).

Agonist-mediated clustering of two TCR complexes induces Nck binding to the cytoplasmic tail of CD3ε via a tyrosine phosphorylation-independent conformational change (41, 42). Nck binding to CD3ε is not essential for agonist-induced proliferation (60) but serves as a marker of a more general conformational change required for T cell activation (42). Remarkably, we find that in the absence of TCR ligand and Src kinase activity, disruption of the galectin lattice induces Nck/WASp/SLP76 and CD4 binding to the TCR complex and F-actin-mediated targeting of TCR/CD4-Lck to GEMs (Fig. 7B). This phenocopies many of the earliest steps in ligand-induced TCR activation and indicates that separation of TCR molecules by the galectin lattice actively prevents TCR clustering/conformational change in the absence of ligand. Affinity of TCR for peptide-MHC is similar to that of galectin for N-acetyllactosamine in N-glycans (i.e. ~10⁻⁵ M) (13). Thus, only peptide-MHC complexes with affinities for TCR above those of TCR-galectin binding are predicted to overcome negative regulation by the galectin lattice and induce TCR clustering, the conformational change and activation signaling. Reductions in N-glycan GlcNAc branching reduce avidity of TCR for galectin and, therefore, should lower thresholds for ligand-induced TCR dimerization/conformational change. Thus, a nonstimulatory/antagonistic peptide-MHC such as self-peptide-MHC may be converted to an activating complex simply by lowering N-glycan GlcNAc branching. In this manner, GlcNAc branching deficiency may induce loss of tolerance to self-peptides and thereby promote autoimmunity. Indeed, Mgtα⁻/⁻ mice develop spontaneous autoimmune and susceptibility of inbred mouse strains to T cell-mediated autoimmunity is regulated by strain specific differences in GlcNAc branching (13, 23).

Microclusters of ~140 TCR are observed within ~30 s after attachment of preactivated T cells to MHC-peptide-containing planar bilayers (50). Galectin lattice regulation of TCR partition to GEMs, the ligand-induced CD3ε conformational change, and TCR microcluster formation are all prevented by blocking F-actin polymerization with latrunculin-A but not by Src kinase inhibition with PP2. We speculate that these three phenomena are observations of the same molecular process, namely loss of galectin binding to TCR, allowing clustering, the conformational change in CD3ε, and finally, Nck/WASp/SLP76/F-actin transfer of TCR/CD4-Lck clusters to GEMs. These data also suggest that the first critical step in TCR signaling is removal of galectin, a phenotype achieved by TCR agonists or GlcNAc branching deficiency.

Lck phosphorylation at Tyr394 is required for agonist-induced TCR signaling and T cell activation. Our data indicate that in the absence of TCR agonists, the galectin lattice actively prevents Lck activation by antagonizing F-actin-mediated partition of CD45 outside and TCR/CD4-Lck inside GEMs (Fig. 7B). Our data also indicate activation thresholds are regulated by this same mechanism, as Lck activity and TCR versus CD45 clustering at the early immune synapse are in part determined by restructuring membrane microdomains before encounter with antigen (Fig. 7B). These data provide a novel mechanism coupling basal and activation signaling and demonstrate that N-glycan GlcNAc branching regulates Lck activation independent of TCR engagement by ligand. miR-18 has recently been shown to positively regulate multiple TCR signaling molecules, including Lck, via down-regulation of multiple non-receptor tyrosine phosphatases (61). Thus, TCR sensitivity is regulated post-transcriptionally by miR-18 and posttranslationally by the galectin lattice.

Multiple protein-tyrosine phosphatases regulate Lck Tyr394 phosphorylation and proximal TCR signaling in addition to CD45, such as LYP/PEP (protein-tyrosine phosphatase non-receptor type 22 (PTPN22)) and possibly PTPH1 (62). However, a critical negative regulatory role for CD45 in galectin lattice-mediated control of Lck activity is confirmed by 1) negative regulation of Lck-Tyr(P)394 and TCR signaling by the galectin lattice in the presence but not absence of CD45 (Fig. 1C, supplemental Fig. 2A), 2) partition of CD45 to GEMs after F-actin depolymerization is associated with reduced Lck-Tyr394 phosphorylation in the absence but not presence of a

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Selective CD45 phosphatase inhibitor (Figs. 2, 1D, and 5B), and 3) forced localization of CD45 to the early immune synapse inhibits Lck-Tyr(P)394, Zap-70, and LAT phosphorylation (Fig. 6). These data are also consistent with earlier observations that 1) average Src kinase activity is increased in CD45-deficient cell lines despite hyperphosphorylation at Tyr(P)505 (63, 64), 2) expression of LckY505F in CD45−/− thymocytes results in hyperphosphorylation of Tyr(P)394 (65), 3) overexpression of CD45 within membrane microdomains inhibits Src kinase activity and TCR signaling, whereas CD45 localization outside membrane microdomains positively regulates T cell activation (35, 36), and 4) in the mature immune synapse CD45 co-localizes with central TCR microclusters that have terminated their signaling (59). Although deficiency of CD45 induces Lck hyperphosphorylation at inhibitory Tyr(P)505 and reduces TCR signaling (29, 30), our data indicate that the galectin lattice does not significantly alter Lck phosphorylation at Tyr(P)394. Therefore, we conclude that within microdomains, maintenance of Lck-Tyr394 after phosphorylation of Tyr394 (65), 3) overexpression of CD45 inhibits Lck-Tyr394, Zap-70, and LAT phosphorylation (Fig. 6). 3) forced localization of CD45 to the early immune synapse inhibits basal growth signaling and TCR agonist thresholds to activate. However, TCR signaling markedly increases N-glycan GlcNAc branching promote loss of self-tolerance and autoimmunity. With greater insight into the functional interplay of the galectin lattice and the actin cytoskeleton, an improved understanding of membrane microdomain structure and its regulation of T cell function should be achieved.

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