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Galectin-1 Induces Partial TCR ζ-Chain Phosphorylation and Antagonizes Processive TCR Signal Transduction

Chan D. Chung,* Viresh P. Patel,† Miriana Moran,‡ Linda A. Lewis,† and M. Carrie Miceli2†‡

Galectin-1 is an endogenous lectin with known T cell immunoregulatory activity, though the molecular basis by which galectin-1 influences Ag specific T cell responses has not been elucidated. Here, we characterize the ability of galectin-1 to modulate TCR signals and responses by T cells with well defined hierarchies of threshold requirements for signaling distinct functional responses. We demonstrate that galectin-1 antagonizes TCR responses known to require costimulation and processive protein tyrosine phosphorylation, such as IL-2 production, but is permissive for TCR responses that only require partial TCR signals, such as IFN-γ production, CD69 up-regulation, and apoptosis. Galectin-1 binding alone or together with Ag stimulation induces partial phosphorylation of TCR-ζ and the generation of inhibitory pp21ζ. Galectin-1 antagonizes Ag induced signals and TCR/costimulator dependent lipid raft clustering at the TCR contact site. We propose that galectin-1 functions as a T cell “counterstimulator” to limit required protein segregation and lipid raft reorganization at the TCR contact site and, thus, processive and sustained TCR signal transduction. These findings support the concept that TCR antagonism can arise from the generation of an inhibitory pp21ζ-based TCR signaling complex. Moreover, they demonstrate that TCR antagonism can result from T cell interactions with a ligand other than peptide/MHC. The Journal of Immunology, 2000, 165: 3722–3729.

T he activation and inactivation of Ag-specific T cells are central to the development of an immune response and the maintenance of self-tolerance and peripheral T cell homeostasis. At the center of this process is TCR recognition of peptide/MHC complexes on the surface of APCs. TCR engagement can result in a number of distinct functions, including those regulating both T cell activation and inactivation. How the TCR discriminates subtleties in Ag presentation and instructs the cell to respond appropriately by inducing anergy, apoptosis, lymphokine production, or proliferation is just beginning to be elucidated. Both the quality of peptide/MHC TCR ligands and the availability of counterligands for T cell costimulators are crucial in determining the functional outcome of TCR engagement.

Only agonist peptide/MHC TCR ligands capable of dwelling at the TCR for sufficient length of time to induce processive TCR ζ-chain phosphorylation (pp23ζ) can induce immune synapses and activate T cells to produce IL-2 and proliferate (1–3). Substituted peptide/MHC complexes with faster dissociation rates (partial agonists and antagonist TCR ligands) only induce partial ζ-chain phosphorylation (pp21ζ) and cannot initiate downstream protein tyrosine phosphorylation, IL-2 production, or T cell proliferation (2, 3). Rather, partial agonist ligands induce T cell anergy and apoptosis (4, 5). When presented together with agonist peptide, partial agonist/antagonist peptides inhibit agonist-induced IL-2 production and proliferation (6). This TCR antagonism is proposed to result from the generation of an inhibitory pp21ζ-based TCR complex, which antagonizes processive protein tyrosine phosphorylation and T cell activation (7, 8). These and other recent studies suggest that the functional outcome of TCR engagement can be modulated by regulating the degree to which TCR signals are allowed to progress and that the accumulation of pp21ζ is inhibitory to complete T cell activation.

Costimulator engagement also regulates the functional outcome of TCR stimulation. Two types of surface receptors need to be engaged for T cell activation: the TCR and a costimulator. When both TCR and costimulator are engaged, the T cell is activated. When the TCR is engaged in the absence of a costimulator, the T cell is inactivated, either through the induction of anergy or apoptosis (9, 10). These findings led to suggestions that two independent signals are required for T cell activation. An alternate emerging view is that costimulators function to fine tune the TCR signal by organizing the T cell/APC contact site for optimal TCR engagement and processive signal transduction (11). This idea has gained support with recent findings that Ag recognition induces the formation of an immunological synapse at the T cell/APC junction in which T cell costimulators and signal transducers are spatially segregated into distinct domains and from which other proteins are specifically excluded (1, 11–13). How costimulators function to organize the TCR contact site and how synapse formation facilitates TCR signal transduction are still not well understood. However, recent studies indicate that costimulator recruitment of lipid rafts to the TCR contact site may help organize the immune synapse, sustain TCR induced protein tyrosine phosphorylation, and modulate the functional outcome of TCR signal transduction (11, 13–15).

Just as there are ligands that engage T cell surface proteins and facilitate reorganization events promoting T cell activation, we propose that there are likely ligands that function to prevent optimal reorganization and processive signal transduction (counterstimulators). Such counterstimulators might play a role in T cell tolerance induction or down-regulation of activated T cells following an immune response. We have been examining a role for galectin-1 as such a ligand.
Galectin-1 is an endogenous lectin expressed at sites of T cell Ag presentation and immune privilege and by Ag-activated T cells (16). Galectin-1 is secreted, but becomes cell associated by binding to lactosamines on glycoproteins expressed by the cell secret- ing it or by neighboring cells. CD45, CD43, CD4, CD2, CD3, CD7, and the glycolipid GM1 have been identified as potential T cell galectin-1 counterligands (17–20). In cells expressing galectin-1, it has been estimated to represent ~1% of total cellular protein. Because the $K_d$ for galectin-1 homodimerization is between 1–7 μM, it is likely that cell surface bound galectin-1 exists as homodimers capable of cross-linking glycoproteins on the same or adjacent cells (21, 22).

Several reports indicate that galectin-1 has potent T cell growth and immunoregulatory activity, though the cellular and molecular basis of its activity is not well understood. Administration of galectin-1 or cells engineered to secrete galectin-1 abrogates induction of experimental autoimmune encephalitis (23), collagen-induced arthritis (24), or myasthenia gravis (25) autoimmune diseases in animal models. Similarly, galectin-1 treatment ameliorates Con A-induced hepatitis and graft vs host disease in mouse disease model systems (26, 27). Because galectin-1 induces apoptosis in thymocytes, a subset of activated but not resting peripheral T cells, and some transformed T cell lines, it has been sug- gested that galectin-1 immunomodulatory capacity may be related to its ability to induce T cell death (22, 28, 29). In an arthritis model, galectin-1 treatment skews the Th1 response to a Th2 re- sponse, implicating galectin-1 in immune deviation (24). Galec- tin-1 may additionally regulate T cell responses by selectively antag- onizing TCR-mediated functions. Indeed, galectin-1 cooperates with TCR engagement to induce apoptosis in T hybridoma cells and freshly isolated double-positive and single-positive thymocytes (30). Furthermore, galectin-1 has been demonstrated to antagonize TCR- induced IL-2 production and proliferation in T hybridoma cells and freshly isolated peripheral T cells (24, 29–31). However, a molecular basis for galectin-1 TCR antagonism has not been elucidated.

Similarities between TCR antagonism induced by galectin-1 and peptide antagonists led us to consider the possibility that, like an- tagonist peptides, galectin-1 might function to limit processive TCR engagement to induce apoptosis in T hybridoma cells and implicating galectin-1 in immune deviation (24). Galect- in-1 may additionally regulate T cell responses by selectively antag- onizing TCR-mediated functions. Indeed, galectin-1 cooperates with TCR engagement to induce apoptosis in T hybridoma cells and freshly isolated double-positive and single-positive thymocytes (30). Furthermore, galectin-1 has been demonstrated to antagonize TCR- induced IL-2 production and proliferation in T hybridoma cells and freshly isolated peripheral T cells (24, 29–31). However, a molecular basis for galectin-1 TCR antagonism has not been elucidated.

Materials and Methods

**Cells and peptides**

Bi-141 is a CD4+ CD8– MHC class II-restricted murine T cell hybridoma that recognizes a beef insulin-derived peptide in the context of IAα/β2 (35). Splenocytes were obtained from 4- to 8-wk-old mice, and single cell suspensions were made using standard procedures. A.E7 is a CD4+ Th1 T cell clone specific for pigeon cytochrome c 88–104/I-Eα and was main- tained as described (3). The P13.9 L cell transfectants expressing I-Eα, ICAM-1, and B7.1 (CD80) were used as APC to stimulate A.E7 T cells. The amino acid sequence of pigeon cytochrome c 88–104 is KAERA DLAYLKQATAK and, thus, is referred to as K99. The Y99 peptide has the same sequence, except that K at a primary TCR contact residue 99, is a tyrosine. Peptides were synthesized by Research Genetics (Huntsville, AL).

**T cell functional assays**

A.E7 cells were stimulated for functional assays as described (5). Briefly, 5 × 10^5 cycling A.E7 cells were incubated for 24 h with galectin-1 alone or together with 5 × 10^5 P13.9 APCs pulsed with 100 μM agonist peptide. P13.9 cells were pretreated with 100 μM mitomycin C for 30 min at 37°C and washed before using in the assay. A.E7 cells were preloaded with 0.1 μM fluorescein isothiocyanate-labeled peptide (Molecular Probes, Eugene, OR) to allow gating on live A.E7 cells after stimulation. All samples were adjusted to 0.1 M β-lactose to dissociate galectin-1-agglutinated cell clumps and washed 24 h poststimulation. Cell death was measured by staining cells with 0.2 ml of 2 μg/ml propidium iodide and FACS analysis using a FACScaliber (Becton Dickinson, Mountain View, CA), as de- scribed (32). For the determination of T cell CD69 expression, T cells were stained with CD69-biotin (1 μg/ml) followed by streptavidin-tricolor (1 μg/ml) and CD3ε-ITTC (1 μg/ml) (PharMingen, San Diego, CA). CD3+ T cells were examined for CD69 expression by FACS analysis. Supernatants were harvested 24 h poststimulation and assayed for IL-2 or IFN-γ production by ELISA (PharMingen).

**Protein isolation, subcellular fractionation, and immunoblotting**

Human galectin-1 was purified from Escherichia coli transformed with the expression vector pET17ML-1 as described (36). Galectin-1 was stored in 8 mM DTT/PBS at −70°C and used in all procedures in medium containing 0.25–1.2 mM DTT.

A.E7 cells were stimulated by APCs essentially as described (5). A total of 5 × 10^5 P13.9 APCs were incubated for 3 h at 37°C with 5% CO2 in 1 ml alone or with peptide Ag (K99 or Y99) at 100 μM in a six-well plate. Pulsed APCs were washed once with 1 ml ice-cold PBS. A total of 5 × 10^3 resting A.E7 T cells were then centrifuged together with Ag pulsed APCs alone or together with galectin-1 and warmed to 37°C for 15 min. Alter- natively, 1 × 10^6 T cells were incubated with 5 μg/ml of anti-CD3ε (2C11) for 30 min at 4°C and cross-linked with goat anti-hamster (GAH) alone or in the presence of galectin-1 at 37°C for 5 min. After stimulation, cells were lysed for 30 min at 4°C in TNE buffer (50 mM Tris, pH 8, 1% Nonidet P-40, and 2 mM EDTA), containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF and 1 mM Na_3 VO_4 . Phospho-extracellular signal-related kinase (ERK)1 and -ERK2 Abs used for immunoblotting were purchased from New England Biolabs (Beverly, MA). Briefly, TNE lysates were centrifuged at 13,000 _g_ for 10 min at 4°C and the supernatant designated the soluble fraction. The insoluble pellet was resuspended in 20 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, and 2 mM EDTA, containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1 mM Na_3 VO_4. For cellular fractionation into raft and nonraft associated proteins, 5 × 10^3 cells were resuspended in Buffer A (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA) containing phosphatase and protease inhibitors and sonicated briefly. Lysates were centrifuged at 800 _g_ for 10 min and the supernatants incubated in 1% Brij-58 at 4°C. An equal volume of 80% sucrose in Buffer A was added to the Brij-58 lysates and samples were placed in “Ultra-Clear” centrifuge tubes (Beckman Instruments, Fullerton, CA). Samples were then overlaid with 2 vol 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, with protease and phosphatase inhibitors. Insoluble proteins were extracted from the pel- let by sonication and mechanical disruption, incubated for 30 min at 4°C, and centrifuged at 13,000 × g for 10 min at 4°C. A.E7 cells were harvested 24 h poststimulation and assayed for IL-2 or IFN-γ production by ELISA (PharMingen).

**Cells were fractionated into cytosol/membrane (soluble) and cytoskel- etally associated (insoluble) fractions as we have described in detail (15).** Briefly, TNE lysates were centrifuged at 13,000 × g for 10 min at 4°C and the supernatant designated the soluble fraction. The insoluble pellet was resuspended in 20 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, and 2 mM EDTA, containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1 mM Na_3 VO_4. Phospho-extracellular signal-related kinase (ERK)1 and -ERK2 Abs used for immunoblotting were purchased from New England Biolabs (Beverly, MA).

3 Abbreviations used in this paper: GAH, goat anti-hamster; MCD, methyl-b-fuco- dextrin; ERK, extracellular signal-related kinase.
Visualization of lipid raft dynamics

BI-141 T cells expressing lipid raft targeted GFP have been recently described in our laboratory (M. Moran and M. C. Miceli, manuscript in preparation). Briefly, nucleotides encoding the first 10 amino acid residues of Lck were fused to a humanized GFP gene (Clontech Laboratories, Palo Alto, CA; no. 6085-1) and cloned into the MSCV-IRES retroviral vector and BI-141 T cells were infected with the recombinant virus. Lck(1–10)-GFP expression was selected with puromycin. Lck(1–10)-GFP expression has been demonstrated to be primarily restricted to raft membranes and not to inhibit T cell IL-2 production (M. Moran and M. C. Miceli, manuscript in preparation). To visualize raft dynamics, $5 \times 10^4$ T cells were stimulated with $5 \times 10^4$ Ab-coated microspheres (50 μg/ml anti-CD3ε 200 μg/ml anti-CD48 or 6 200 μg/ml anti-CD28) in 35 μl for 20 min at 37°C in the presence or absence of 20 μM galectin-1. Cells were washed once with 0.1 M β-lactose/PBS to dissociate galectin-1-agglutinated cell clumps. Cells were fixed in 4% formaldehyde for 10 min, mounted onto slides, and analyzed by confocal microscopy (Bio-Rad MRC-1024ES, Hercules, CA) using a 100× objective. The images shown are 0.5-μm single sections of T cells. Conjugates in which $50\%$ of Lck(1–10)-GFP localized toward the site of TCR engagement as determined by visual inspection were scored as clustered.

Results

Galectin-1 permits TCR-induced functions that require partial TCR signals, but antagonizes functions requiring processive and sustained TCR signal transduction

Previous studies have characterized a hierarchy of TCR threshold requirements for signaling distinct functions in A.E7 and BI-141 T cells (5, 32, 33). Therefore, these Ag-specific T cells provide excellent systems to directly examine the influence of galectin-1 on T cell functions known to require partial or complete TCR signal transduction. IL-2 production by the A.E7 T cell clone requires high agonist (K99) Ag concentrations, is dependent on costimulation and processive $\zeta$ phosphorylation (pp23$\zeta$), and cannot be induced by partial agonist or antagonist Ags (5, 33). IFN-γ production requires lower agonist Ag concentrations and is less dependent on costimulation (33). Furthermore, partial agonist peptide Y99 is unable to induce IFN-γ production. Finally, either agonist (K99) or partial agonist (Y99) peptide Ags are able to induce apoptosis in A.E7 T cells, indicating that Ag induced apoptosis does not require significant processive $\zeta$ phosphorylation (5). Therefore, in A.E7 T cells, IL-2 has the most stringent TCR signal transduction requirements; IFN-γ has intermediate requirements; and apoptosis has the least stringent requirements. A similar hierarchy of signaling has been reported for other T cell clones, BI-141 T hybridoma cells, and some primary T cells (5, 10, 32, 36, 37). Although not previously characterized for the A.E7 T cell clone or BI-141 T cells, CD69 expression has been demonstrated only to require partial TCR signals and does not rely heavily on costimulation (38, 39).

To further characterize the effects of galectin-1 on TCR-induced signals and functions, we evaluated the ability of galectin-1 to...
modulate TCR induction of IL-2 production, IFN-γ production, CD69 expression, and apoptosis in A.E7 T cells. As shown in Fig. 1, galectin-1 antagonizes Ag-induced IL-2 production, but is permissive for Ag-induced IFN-γ production, CD69 expression, or apoptosis in A.E7 T cells. That galectin-1 does not interfere with Ag-induced CD69 expression, IFN-γ production, or cell death by A.E7 T cells, indicates that galectin-1 does not equally antagonize all TCR-induced functions (Fig. 1, B–D). Furthermore, galectin-1 stimulation by itself does not induce apoptosis in A.E7 T cells, demonstrating antagonism of IL-2 production is not secondary to galectin-1-induced cell death. In instances in which Ag-induced apoptosis is suboptimal, galectin-1 cooperates with TCR engagement to enhance cell death in A.E7 T cells (Fig. 1D). These data are in keeping with our previous findings that: 1) TCR engagement and galectin-1 stimulation cooperate to induce cell death in BI-141 T cells and single-positive thymocytes (29), and 2) TCR-induced apoptosis has less stringent signal transduction requirements than does IL-2 production in BI-141 T cells (32, 40). However, in our previous studies, T cells were stimulated with anti-CD3 Abs rather than Ag. We now show that galectin-1 also antagonizes Ag-induced IL-2 production and cooperates with Ag-induced cell death in A.E7 cells. Taken together, these data support our hypothesis that galectin-1 permits TCR-induced functions that require partial TCR signals, but antagonizes functions requiring processive and sustained TCR signal transduction. Furthermore, that galectin-1 enhances TCR-induced death indicates galectin-1 may cooperate with TCR engagement to enhance partial TCR signal transduction.

Galectin-1 T cell stimulation induces the partial phosphorylation of the TCR ζ-chain (pp21ζ)

To investigate the molecular mechanism by which galectin-1 selectively antagonizes TCR signals and functions, we analyzed proximal TCR signals in response to galectin-1 stimulation alone and in the context of TCR engagement. Because TCR engagement induces the translocation of phosphorylated ζ to the cytoskeletal detergent-insoluble fraction (41), we examined ζ-chain tyrosine phosphorylation in both soluble and insoluble fractions by anti-phosphotyrosine immunoblotting. Remarkably, galectin-1 stimulation alone induces the generation of partially phosphorylated pp21ζ in A.E7 T cells, BI-141 T cells, and primary T cells (Figs. 2 and 3D). Indeed, exposure of A.E7 T cells to galectin-1 induces pp21ζ in both soluble and insoluble fractions (Fig. 2A). Even at high concentrations, galectin-1 disproportionately enhances partial ζ phosphorylation (pp21ζ) relative to complete ζ phosphorylation (pp23ζ) (Fig. 2A). In contrast to agonist TCR engagement, which leads to processive ζ phosphorylation and high pp23ζ/pp21ζ ratios ranging from 0.4 to 1.5 (Figs. 2 and 3), galectin-1 engagement induces low pp23ζ/pp21ζ ratios (0.1) more characteristic of antagonist peptides that function in TCR antagonism (Figs. 2A and 3) (2, 3). Galectin-1 also induces partially phosphorylated pp21ζ, but not significant levels of processively phosphorylated pp23ζ in BI-141 T cells (Fig. 3D). Similarly, galectin-1 induces partial ζ phosphorylation in primary T cells (Fig. 2B). However, induced pp21ζ levels are not as easily appreciated in resting primary T cells due to high levels of constitutively phosphorylated pp21ζ (42).

**Galectin-1 converts TCR-ζ phosphorylation patterns from those characteristic of T cell activation to those characteristic of inactivation and TCR antagonism**

Next, we examined ζ tyrosine phosphorylation patterns in T cells stimulated with galectin-1 in the context of otherwise productive TCR engagement. As expected, in A.E7 T cells, agonist Ag (K99) stimulation in the absence of galectin-1 results in processive TCR-ζ phosphorylation, translocation to the detergent-insoluble fraction, and high pp23ζ/pp21ζ ratios in both soluble and insoluble fractions (0.9 and 1.5, respectively) (Fig. 3A). Galectin-1 stimulation of A.E7 alone primarily induces pp21ζ in both soluble and insoluble fractions. In the context of antigenic stimulation, galectin-1 induces disproportionate pp21ζ phosphorylation, resulting in a low pp23ζ/pp21ζ ratio in the soluble fraction and only pp21ζ in the insoluble fraction (Fig. 3A). Similar patterns are observed when ζ is immunoprecipitated directly or indirectly by coprecipitation with CD3ε (Fig. 3B), further identifying galectin-1-induced pp21ζ as TCR-associated tyrosine phosphorylated ζ-chain. Similarly, galectin-1/TCR costimulation decreases pp23ζ/pp21ζ ratios in TCR-stimulated BI-141 T cells (Fig. 3D). In some instances, pp23ζ phosphorylation levels are slightly diminished in galectin-1/TCR-treated cells relative to those stimulated through their TCR alone (Fig. 3D). Therefore, in the context of TCR engagement, galectin-1 converts TCR-ζ phosphorylation patterns from those characteristic of T cell activation to those characteristic of inactivation and TCR antagonism.

To determine whether galectin-1-induced ζ phosphorylation requires the integrity of lipid raft microdomains, we examined the
effects of disrupting lipid rafts on galectin-1-induced pp21ζ phosphorylation in A.E7 T cells using the cholesterol chelator, methyl-
β-cyclo-dextrin (MCD). As shown in Fig. 3C, MCD treatment dramatically reduces the pp21ζ phosphorylation induced by agonist (K99), partial agonist (Y99), and/or galectin-1. These findings demonstrate that, like TCR agonist-induced ζ-chain tyrosine phosphorylation, both partial agonist and galectin-1-treated cells, respectively. The galectin-1-treated sample demonstrates a 1.1-fold increase in pp23ζ and a 12-fold increase in pp21ζ relative to the K99-treated sample.

Galectin-1 antagonizes TCR-induced processive protein tyrosine phosphorylation

Recent data indicate that TCR antagonism results from the generation of a pp21ζ-based inhibitory TCR complex capable of antagonizing processive TCR tyrosine phosphorylation (7, 8). To determine whether galectin-1 induction of pp21ζ is correlated with the antagonism of processive protein tyrosine phosphorylation, we next examined Ag-induced protein tyrosine phosphorylation in A.E7 T cells stimulated with agonist peptide (K99) pulsed APC alone or in the presence of galectin-1. Because proper T cell activation requires lipid raft membrane compartmentalization, T cell membranes were separated into raft and nonraft membrane microdomains by sucrose density centrifugation (15, 43). A.E7 T cells stimulated with partial agonist peptide (Y99), which is known to induce pp21ζ, were included for comparison.

Agonist peptide (K99) induces efficient phosphorylation of proteins in both raft and nonraft fractions (Fig. 4A). Because lipid rafts only represent ~5% of the plasma membrane, enrichment of phosphorylated proteins within the raft fraction represents their dramatic concentration within lipid raft microdomains. Partial agonist peptide (Y99) only induces a subset of the phosphorylated proteins induced by agonist (K99) peptide in both raft and nonraft fractions (Fig. 4). Agonist peptide (K99) stimulation leads to the accumulation of phospho-proteins migrating at 36, 65–75, and 94–102

FIGURE 3. Galectin-1 decreases the pp23ζ/pp21ζ ratio generated in response to agonist Ag or anti-CD3 induced TCR stimulation. A and B, A.E7 were stimulated for 15 min at 37°C with 10 μM galectin-1 and agonist (K99) or partial; agonist (Y99) pulsed P13.9 APCs, alone or in combination. TCR ζ-chain was immunoprecipitated from soluble (membrane) or insoluble (cytoskeletal) fractions using anti-ζ (A) or anti-CD3ε (B). Abs. ζ immunoprecipitates from untreated T cells and APCs were included as controls. C, Same as A except in samples run in the last four lanes, A.E7 cells were pre-treated with 30 mM MCD for 30 min. D, BI-141 T cells were treated with GAH, anti-CD3 + GAH, and galectin-1 alone or in combination for 5 min at 37°C. Cell lysates from the soluble fraction was immunoprecipitated with anti-ε. Immunoprecipitates were resolved on 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine Ab. In lanes where pp21ζ and pp23ζ are present, the ratios of pp23ζ/pp21ζ were calculated and are shown beneath their respective lanes. The relative total (soluble and insoluble fractions) intensities for pp23ζ and pp21ζ in A as determined by densitometry are as follows: pp23ζ, 185 and 211; and pp21ζ, 179 and 2125 for agonist and galectin-1-treated cells, respectively. The galectin-1-treated sample demonstrates a 1.1-fold increase in pp23ζ and a 12-fold increase in pp21ζ relative to the K99-treated sample.
kDa within lipid rafts. However, partial agonist (Y99) is unable to induce pp36 and only leads to the accumulation of phosphoproteins migrating at 65–75 and 94–102 kDa within raft microdomains. Phosphoprotein pp36 most likely represents the raft adapter protein LAT, required for initiation of RAS/ERK and phospholipase C γ-mediated signal transduction cascades (44). Indeed, stripping and reprobing the same filter with anti-LAT antisera demonstrated that pp36 comigrates with LAT and that LAT partitions only within the raft fraction (not shown). Galectin-1 alone fails to induce detectable protein tyrosine phosphorylation in the raft fraction. When present together with agonist (K99) peptide, galectin-1 antagonizes the induction of raft associated pp36 (Fig. 4A). In nonraft fractions, galectin-1, partial agonist (K99), and agonist (Y99) peptides each induce phosphoproteins migrating at 102 and 141 kDa (Fig. 4A). Agonist peptide (K99) efficiently induces additional phosphoproteins at 94 and 44 kDa within the nonraft fraction, whereas galectin-1 antagonizes K99 agonist-induced phosphorylation of these proteins.

To determine the effects of galectin-1 TCR antagonism on a known tyrosine phosphorylated TCR substrate and to identify the 44-kDa protein(s) antagonized in the nonraft fraction, we examined levels of phosphorylated ERK-1 and ERK-2 using phosphospecific anti-ERK Abs (Fig. 4B). Tyrosine phosphorylation of ERK is essential for its activation and, thus, to its role as a mediator of TCR signal transduction (45). As shown in Fig. 4B, phosphorylated ERK-1 and ERK-2 partition within nonraft membrane of A.E7 T cells stimulated with agonist peptide, but not in cells stimulated with partial agonist peptide, galectin-1, or galectin-1 and agonist peptide together. Taken together, our findings demonstrate that galectin-1 can induce pp21ζ and antagonize downstream TCR signals and functions.

**Galectin-1 antagonizes costimulator-dependent lipid raft migration to the TCR contact cap**

Recent data indicate that costimulators function to organize the TCR contact cap by inducing the active redistribution of lipid rafts toward the site of TCR engagement (11). Indeed, when Ab-coated microspheres are used as surrogate APCs, only beads coated with Abs directed against both a costimulator (CD48 or CD28) and the TCR induce optimal raft migration to the T cell:bead interface (M. Moran and M. C. Miceli, manuscript in preparation) (14). We have observed similar costimulator-induced raft dynamics when raft redistribution is visualized using FITC-cholera toxin (which binds to the outer leaflet raft glycolipid GM1) or by tracking the distribution of an expressed fusion protein GFP (Lck-(1–10)-GFP), which is specifically targeted to raft inner leaflets (M. Moran and M. C. Miceli, manuscript in preparation).

To determine whether galectin-1 affects TCR/costimulator-induced raft dynamics, we measured the effect of galectin-1 engagement on raft coalescence in response to microspheres coated with anti-CD3, anti-CD48, or anti-CD28 alone or in combination. To avoid potential complications of galectin-1/GM1 interactions inherent in visualizing lipid rafts with FITC-cholera toxin, we visualized lipid raft migration by tracking Lck-(1–10)-GFP dynamics in BI-141 T cells. As shown in Fig. 5, and consistent with previous reports (14), in the absence of galectin-1, anti-CD3/anti-CD48-coated microspheres or anti-CD3/anti-CD28-coated microspheres induce the redistribution of lipid rafts to the bead proximal half of the T cell in 62% and 54% of the conjugates, respectively. However, when T cells are stimulated with anti-CD48/anti-CD3 or anti-CD3/anti-CD28-coated microspheres in the presence of galectin-1, raft redistribution is inhibited (15% and 10%, respectively). These data indicate that galectin-1 limits TCR/costimulator-induced raft migration to the contact site.

**Discussion**

Using two different T cell lines, BI-141 and A.E7, which have been well characterized with regard to their hierarchical requirements for signaling distinct functional responses (5, 32, 33), we demonstrate that galectin-1 selectively antagonizes signals and functions requiring processive TCR signal transduction and costimulation, but is permissive for or enhances those requiring only partial TCR signal transduction. We further demonstrate that galectin-1 stimulation leads to the accumulation of partially phosphorylated pp21ζ. Together with recent data indicating that partial ζ phosphorylation results in the generation of a pp21ζ-based inhibitory TCR complex responsible for TCR antagonism (7, 8), galectin-1 pp21ζ induction provides an explanation for the ability of galectin-1 to selectively antagonize TCR signals and functions.

How galectin-1 induces partial ζ phosphorylation has yet to be fully elucidated. Galectin-1 counterligands CD45, CD43, CD2, and CD4 have each been reported to associate with Fyn or Lck tyrosine kinases, and thus represent potential mediators of galectin-1-induced ζ phosphorylation (45–48). Alternatively, or additionally, galectin-1 could induce Lck or Fyn activity by binding to and clustering CD3, CD4, or GM1 and associated lipid rafts. This idea is consistent with our finding that MCD raft disruption inhibits galectin-1 ζ-chain phosphorylation (Fig. 3) and the recent identification of the raft enriched glycosphingolipid GM1 as a potential galectin-1 counterligand (20). However, because we do not
Galectin-1 antagonizes TCR/costimulator-induced relocalization of lipid rafts toward the TCR contact site. BI-141 T cells expressing LCK(1–10)-GFP were stimulated with beads coated with the indicated Abs in the presence or absence of 20 μM galectin-1. Cells were washed once with 0.1 M β-lactose/PBS to dissociate galectin-1-agglutinated cell clumps. Cells were fixed with 4% formaldehyde and mounted and analyzed by confocal microscopy. A. T cell: microsphere conjugates in which more than 50% of total cellular Lck(1–10)-GFP localized toward the bead proximal half of the cell were scored as clustered. At least 50 conjugates were scored for each condition. B. Representative images are shown for each condition. Confocal images of a single 0.5-μm cell section are shown in the left panels. Corresponding phase contrast images showing the position of the T cell: Ab coated bead contact site are shown in the right panels. A representative of three experiments is shown.

FIGURE 5. Galectin-1 antagonizes TCR/costimulator-induced relocalization of lipid rafts toward the TCR contact site. BI-141 T cells expressing LCK(1–10)-GFP were stimulated with beads coated with the indicated Abs in the presence or absence of 20 μM galectin-1. Cells were washed once with 0.1 M β-lactose/PBS to dissociate galectin-1-agglutinated cell clumps. Cells were fixed with 4% formaldehyde and mounted and analyzed by confocal microscopy. A. T cell: microsphere conjugates in which more than 50% of total cellular Lck(1–10)-GFP localized toward the bead proximal half of the cell were scored as clustered. At least 50 conjugates were scored for each condition. B. Representative images are shown for each condition. Confocal images of a single 0.5-μm cell section are shown in the left panels. Corresponding phase contrast images showing the position of the T cell: Ab coated bead contact site are shown in the right panels. A representative of three experiments is shown.

Observe extensive raft clustering in response to galectin-1 after 20 min of stimulation (Fig. 5). Galectin-1 induced raft clusters are likely submicroscopic or highly transient.

It is also unclear why galectin-1 stimulation does not lead to processive Tyr phosphorylation and significant generation of pp21ζ. One recent report demonstrates that pp21ζ phosphorylation and TCR antagonism is associated with the generation of pp21ζ: SHP-1 phosphatase complexes (8). We have, as yet, been unable to detect such complexes in galectin-1-stimulated cells. This may reflect a fundamental difference between galectin-1 and antagonist peptide-mediated antagonism or alternatively may be related to the limit of detection of our immunoblots. Recent models of T cell activation require the lipid raft mediated segregation of the CD45 phosphatase from CD3/TCR complex and Lck and Fyn Tyr kinases to enable processive and sustained protein Tyr phosphorylation (15). In the absence of CD45 segregation, substrates phosphorylated by TCR activated kinases, including TCR-ζ, might be continually dephosphorylated, thus limiting sustained or processive protein Tyr phosphorylation.

We propose that galectin-1 limits the extent of membrane reorganization and sustained and processive TCR signal transduction by cross-linking lipid raft (GM1, CD4), raft translocatable (CD3), and nonraft (CD43, CD45) microdomain constituents. Such receptor cross-linking events would limit the size of lipid raft clusters at the TCR contact site and thus the efficiency of processive protein Tyr phosphorylation. In this manner, functions relying on partial TCR signal transduction are permitted, whereas complete TCR signals are inhibited by galectin-1. Our observations that TCR/costimulator-induced raft relocalization and raft-associated protein Tyr phosphorylation are antagonized by galectin-1 are consistent with this hypothesis. In further support of this suggestion, galectin-1 binding has recently been shown to segregate T cell plasma membrane proteins into large patches containing either CD3:CD45 or CD43:CD7 clusters (18). However, it has yet to be determined whether galectin-1 induces CD3:CD45 clustering in BI-141 or A.E7 T cells or whether galectin-1 induces such receptor patterning in the context of TCR engagement.

Our findings that galectin-1 induces pp21ζ and antagonizes T cell activation support the concept that TCR antagonism can arise from the generation of an inhibitory signal within the TCR complex. Moreover, by demonstrating that pp21ζ can result from T cell stimulation by a ligand other than peptide/MHC, our findings introduce the concept the TCR antagonism may result from nonpolymorphic T cell ligand binding. Galectin-1 antagonism of TCR/costimulator raft reorganization events corroborate other reports correlating lack of raft coalescence at the TCR contact cap with partial TCR signal transduction (14), thus further validating a role for raft reorganization in modulating TCR signal transduction and functional outcome. Together, these data point to the existence of “counterstimulators” which function to operationally oppose costimulatory ligands by: 1) limiting reorganization of the TCR contact site; 2) preventing processive and sustained TCR signal transduction; and 3) skewing TCR functional responses from those promoting T cell activation to those promoting T cell inactivation and tolerance induction.

In general, endogenous galectins are ideally suited for such counterstimulatory activity because galactoside binding allows for specific binding to carbohydrate epitopes which can be shared by several T cell surface proteins and regulated developmentally through the expression of specific glycosyltransferases (49–52). Reported changes in glycosyltransferase and galectin-1 expression patterns in activated and memory T cells support a potential role for endogenous galectin-1 as an autocrine factor that inhibits proliferation and induces apoptosis in T cells during the resolution phase of an Ag-specific immune response in vivo (30, 49–52). Studies addressing the role of endogenous galectin-1 in regulating TCR-mediated responses in vivo using galectin-1 null mice (53) are underway in our laboratory. Together with recent studies demonstrating the efficacy of galectin-1 treatment in autoimmune and graft vs host disease models (27), our studies highlight the potential of galectin-1 as an immunotherapeutic that facilitates Ag-specific tolerance induction and blocks TCR activation.

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