Galectin-1 is a galactoside-binding lectin expressed in multiple tissues that has pleiotropic immunomodulatory functions. We previously showed that galectin-1 activates human monocytic-derived dendritic cells (MDDCs) and triggers a specific genetic program that up-regulates DC migration through the extracellular matrix, an integral property of mucosal DCs. Here, we identify the galectin-1 receptors on MDDCs and immediate downstream effectors of galectin-1-induced MDDC activation and migration. Galectin-1 binding to surface CD43 and CD45 on MDDCs induced an unusual unipolar co-clustering of these receptors and activates a dose-dependent calcium flux that is abrogated by lactose. Using a kinome screen and a systems biology approach, we identified Syk and protein kinase C tyrosine kinases as mediators of the DC activation effects of galectin-1. Galectin-1, but not lipopolysaccharide, stimulated Syk phosphorylation and recruitment of phosphorylated Syk to the CD43 and CD45 co-cluster on MDDCs. Inhibitors of Syk and protein kinase C signaling abrogated galectin-1-induced DC activation as monitored by interleukin-6 production; and MMP-1, -10, and -12 gene up-regulation; and enhanced migration through the extracellular matrix. The latter two are specific features of galectin-1-activated DCs. Interestingly, we also found that galectin-1 can prime DCs to respond more quickly to low dose lipopolysaccharide stimulation. Finally, we underscore the biological relevance of galectin-1-enhanced DC migration by showing that intradermal injection of galectin-1 in MRL-fas mice, which have a defect in skin DC emigration, increased the in vivo migration of dermal DCs to draining lymph nodes.

Dendritic cells (DCs) are critical regulators of immunity that sample and present antigen, initiate adaptive immune responses through T cell interactions, and maintain self-tolerance through T cell instruction (1, 2). To effectively mount an immune response, DCs must encounter antigen and receive a signal to initiate an activation program termed “maturation.” Both exogenous and endogenous signals can initiate DC maturation. Exogenous maturation signals include Toll-like receptor ligation via pathogen components such as bacterial proteins (e.g. LPS), bacterial DNA (through CpG-containing motifs), and viral double-stranded RNA (3, 4). In synergy with these pathogen signals, or alone, endogenous DC activators include inflammatory cytokines, prostaglandins, and other danger signals (5).

Recent work has also demonstrated that galectins, a family of endogenous β-galactoside binding lectins, can initiate DC maturation (6–8). The galectins have numerous known immunomodulatory activities involving T and B cells, but the role of these lectins in DC function is only beginning to be investigated. Galectin-9 matures DCs into IL-12-producing cells, which can elicit a Th1 response from T cells following co-culture (8). On the other hand, galectin-3 influences the type of adaptive immune response initiated by DCs but does not directly affect the maturation process (9). We and others have shown that galectin-1 matures DCs and further enhances the migratory capacity of these cells (6, 7). Furthermore, galectin-1 differentially regulates gene expression in maturing DCs, as compared with LPS stimulation, indicating that galectin-1 employs a distinct maturation pathway (7). In the current study we identify and characterize the immediate downstream effectors that preferentially mediate the effects of galectin-1 on DC maturation.

The downstream signaling events associated with classical DC maturation have been partially elucidated. For example, LPS induces activation of NF-κB and of MAPK pathways (particularly p38 and Erk1/2) (3). However, the persistence of the

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**Supplemental Figures and Text**

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5 Charles E. Culpepper Medical Scholar supported by the Rockefeller Brothers Fund. To whom correspondence should be addressed: 257 BSRB, 615 Charles E. Young Dr. East, UCLA, Los Angeles, CA 90095. Tel.: 310-794-2132; Fax: 310-267-2580; E-mail: bleeblh@ucla.edu.

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The downstream signaling events associated with classical DC maturation have been partially elucidated. For example, LPS induces activation of NF-κB and of MAPK pathways (particularly p38 and Erk1/2) (3). However, the persistence of the
maturation signal and the nature of stimulus can have disparate effects on the functional outcomes of pathways leading to DC maturation (10). For example, Erk1/2 can exert both positive and negative effects on LPS-induced activation (10–12) and can synergize with p38 to increase cytokine production but exert inhibitory effects on migration. This highlights the remarkable ability of DCs to integrate the extracellular environment, which dictates the variety and persistent quality of signals, into distinct outcomes.

Less is known about immediate early activation events in DC maturation. In particular, what pathways or adaptor molecules link receptor engagement to these late activation events? Additionally, do early events differ between an endogenous stimulus (galectin-1) and exogenous stimulus (LPS), even though both stimuli result in DC maturation? Many immune cell signals employ adaptor proteins and kinases to integrate signals from different receptors into downstream events. Primarily, these upstream mediators include protein-tyrosine kinases (PTKs), such as Src family kinases. Phosphorylated PTKs in turn recruit upstream mediators including protein-tyrosine kinases (PTKs), small GTPases (Rac1 and Cdc42), and transcription factors (13). The recruitment of distinct PTKs and/or adaptor proteins could be one mechanism by which DCs coordinate and regulate distinct and multiple stimuli into a specific outcome.

Galectin-1 exists primarily as a noncovalent homodimer that recognizes the N-acetyl-lactosamine residues present on various glycoproteins and glycolipids. Thus, galectin-1 can cross-link multiple receptors on the cell surface, thereby eliciting different cell functions via formation of membrane microdomains (14). We have shown that dimeric galectin-1 is required for DC maturation (7), implying that cross-linking of receptors is involved in transducing the maturation signal. Here, we identify the cognate galectin-1 receptors on MDDCs and further characterize the immediate downstream effectors that mediate the novel effects of galectin-1 on these cells. Specifically, we show that galectin-1 binds to two DC surface glycoproteins, CD43 and CD45, and induces unipolar co-clustering of these two receptors on the DC surface. Additionally, using a kinome array and a systems biology approach, we identify multiple phosphorylated signaling mediators preferentially induced by galectin-1 over LPS and implicate Syk and PKC as unique PTKs that mediate galectin-1 specific effects on DCs. Furthermore, we show that galectin-1 can prime DCs to respond more quickly to low dose LPS stimulation, suggesting a synergism between endogenous and exogenous DC activation signals that has been heretofore unappreciated. Finally, we also show that galectin-1 can enhance the in vivo migration of dermal DCs to draining lymph nodes in lupus-prone MRL-fas mice, which have a defect in skin DC emigration, underscoring the biological relevance of our results.

**EXPERIMENTAL PROCEDURES**

**Mice**—MRL-fas mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were housed under guidelines set by the National Institutes of Health, and experiments were conducted in accordance with the University of California Los Angeles Chancellor’s Animal Research Committee and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

**MDDC Differentiation and Galectin-1 Treatment**—MDDCs were differentiated with granulocyte macrophage-colony-stimulating factor and IL-4 from purified human monocytes as previously described (7). Mouse bone marrow-derived dendritic cells (BMDCs) were differentiated from mononuclear cells harvested from the marrow of femurs and tibias of 6–10-week-old mice as previously described (15). The indicated treatments were added to day 5 immature MDDCs and day 7 BMDCs, and the cells were cultured for an additional 48 h unless otherwise noted. To control for possible endotoxin contamination, all of the galectin-1 treated cells were preincubated with 10 μg/ml polymyxin B (Sigma) at 37 °C for 30 min.

**Reagents**—Recombinant human galectin-1 was produced as previously described (16). The following reagents were purchased from the indicated suppliers: lipopolysaccharide from *Escherichia coli* 0127:B8 (Sigma), bisindolylmaleimide 1 (Calbiochem), Syk inhibitor (Calbiochem 574711), U73122 (Calbiochem), LY294002 (Calbiochem), and polymyxin B and polymyxin B-agarose beads (Sigma).

**Co-immunoprecipitation and Western Blots**—5 × 10^6 MDDCs were used per condition for co-immunoprecipitation. The cells were allowed to bind with galectin-1 (20 μM) for 60 min at 4 °C, and then galectin-1 was cross-linked to cognate cell surface receptors using a non-membrane-permeable reversible cross-linker, 3,3’-dithiobis(sulfosuccinimidyl propionate) (Pierce). MDDCs were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM Na3VO4, 1× complete protease inhibitor mix; Roche Applied Science), and clarified lysate was then subjected to immunoprecipitation overnight at 4 °C using anti-galectin-1 rabbit polyclona (1:100), which was cross-linked to protein G beads (Pierce) using dimethyl pimelimidate (Pierce). Immunoprecipitates were reduced in SDS buffer, separated on a 10% polyacrylamide gel, and then transferred to polyvinylidene difluoride membrane. Western blotting was performed using the antibodies indicated in the supplemental Methods.

**Confocal Microscopy**—MDDCs were differentiated on polylsine-coated coverslips (BD Biosciences) in 12-well plates. The MDDCs were treated with galectin-1 in the presence of polymyxin B (10 μg/ml) at the indicated concentrations for 1 h at 37 °C. All of the immunofluorescence staining was conducted on ice. The cells were washed twice with PBS and then fixed with 2% paraformaldehyde/PBS for 20 min. After fixing, the cells were allowed to bind with galectin-1 (20 μg/ml) at 37 °C for 30 min. To control for possible endotoxin contamination, all of the galectin-1 treated cells were preincubated with 10 μg/ml polymyxin B (Sigma) at 37 °C for 30 min. If necessary, the cells were incubated with blocking solution (1:100), which was cross-linked to protein G beads (Pierce) using dimethyl pimelimidate (Pierce). Immunoprecipitates were reduced in SDS buffer, separated on a 10% polyacrylamide gel, and then transferred to polyvinylidene difluoride membrane. Western blotting was performed using the antibodies indicated in the supplemental Methods.

**Mechanisms of Galectin-1-induced DC Activation**
Mechanisms of Galectin-1-induced DC Activation

FIGURE 1. Galectin-1 specifically binds to CD43 and CD45 and co-cluster these glycoconjugates on human MDDC surface. A, MDDCs were cell surface biotinylated and then incubated with 20 μM of galectin-1, as described under “Experimental Procedures.” After cross-linking with cell-impermeant reversible cross-linker, anti-galectin-1 serum was used to immunoprecipitate (IP) galectin-1 and any bound cell surface receptors. Western blotting (WB) with streptavidin-horseradish peroxidase revealed putative galectin-1 counter receptors on MDDC surfaces. Note that band 1 and band 2 correspond to CD45 and CD43 identified as indicated below. B, CD45 and CD43 were identified as specific receptors in the co-immunoprecipitate by Western blotting with specific anti-CD45 or -CD43 antibodies (lanes 3 and 7, respectively). Galectin-1 binding to CD43 and CD45 was carbohydrate-dependent because it could be competed off with 0.1 M lactose (lanes 4 and 8). Note that the endogenous amount of galectin-1 produced by DCs was sufficient to mediate co-immunoprecipitation of CD45 (lanes 1 and 2) but not CD43 (lanes 5 and 6). C and D, MDDCs grown on polylysine-coated coverslips were exposed to galectin-1 (at indicated concentrations) for 1 h at 37 °C. The cells were then fixed and processed for confocal microscopy as detailed under “Experimental Procedures” using antibodies against CD45, CD43, and CCR5. 60× images are shown. D, the graph indicates the percentage of cells with CD43 and CD45 clustering (250 cells counted per condition). The buffer-, LPS-, and lactose-treated MDDCs served as additional specificity controls. Note that CD43 and CD45 co-clustering occurred in >50% of cells only above 10 μM concentration of galectin-1.

Quantification of Cytokine Production—DC cytokine secretion was measured using cytokine-specific ELISA (eBioscience and BD). Human MDDCs and murine BMDCs were plated in 96-well plates (2 × 10⁶ cells/well in 200 μl of RPMI). MDDCs were preincubated with the indicated lactose or chemical inhibitors for 30 min or 2 h, respectively, at 37 °C. Additionally, galectin-1-treated wells were preincubated with 10 μg/ml polymyxin B at 37 °C for 30 min. In conditions where MDDCs were co-stimulated with galectin-1 and LPS, galectin-1 was preincubated with polymyxin B-agarose beads (Sigma) for 30 min rotating at 4 °C to remove endotoxin contamination (17). The polymyxin B-agarose beads were subsequently removed to prevent inhibition of LPS activity. Cell culture supernatants were collected at the indicated times following treatment with the indicated doses of LPS, galectin-1, buffer control, or no treatment and stored at −80 °C until analysis.

Real Time Quantitative RT-PCR—Total RNA from MDDCs was extracted with the RNeasy mini kit (Qiagen) at 18 h after the indicated treatment. QuantiTect Probe RT-PCR kit was used for cDNA synthesis (Qiagen). Transcripts were quantified by real time quantitative PCR on an iQ5 system (Bio-Rad) with predesigned TaqMan gene expression assays and reagents washed twice in ice-cold PBS and then sonicated in Kinex lysis buffer (20 mM MOPS, 1% Triton X-100, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 5 μM pepstatin A, 1X complete protease inhibitor mixture (Roche Applied Science), 1 mM DTT). 100 μg of cleared lysate protein combined from three donors were shipped to Kinexus Bioinformatics Corporation (Vancouver, Canada) for Kinex antibody microarray services.

Kinex Microarray Data Analysis—On receiving the Kinex protein microarray data, we created a corrected fold change to account for error in the replicates and stringently selected candidate proteins with a true increase that was outside the error limits of replicates. Using the data provided by Kinexus, we calculated our corrected fold change = [(average intensity of treated samples − % error of replicates)/(average intensity of control samples + % error of replicates)]. This corrected fold change was used to rank the candidate proteins, and those with a corrected fold change of >1.3 was used for pathway analysis with Ingenuity Pathway Analysis (Ingenuity Systems). See legend to supplemental Fig. S1 for details on further bioinformatic parsing.
according to the manufacturer’s instructions (Applied Biosystems). For each sample, mRNA abundance was normalized to the amount of β-actin expressed.

**MDDC Matrigel Migration Assays**—MDDCs matured with LPS or galectin-1 were treated with the indicated chemical inhibitors or not and assayed for migration through Matrigel in a transwell assay as described (7). In all assays, the number of cells migrating through the Matrigel to the bottom membrane was counted at 40× magnification. Each experiment was counted by two independent counters, one blinded to the sample identity, and at least 50 fields were counted. Cells/field was normalized to 50 fields, and the data are expressed as relative migration compared with control cells (immature MDDCs).

**In Vivo Skin DC Migration Assays**—MRL-fas mice were treated with an 10-μl intradermal injection of galectin-1 (dose scale from 20–100 μM in injected bolus) or buffer control (equivalent volume of PBS/80 μM DTT) (n = 4 animals/group) into the dorsum of the ear. At 24 h post-injection, 25 μl of a skin sensitizing fluorochrome, 1% FITC (Sigma) in dibuytphthalate:acetone (1:1), or vehicle alone (without FITC) was applied on the dorsal half of each ear. At 48 h after sensitization, the animals were sacrificed, and the draining cervical lymph nodes were harvested, separated into single cell suspensions, and analyzed by flow cytometry for the presence of FITC+ (migrated) cells. The migrant (FITC+) cells in the draining lymph nodes were further defined for different populations of DCs. Antibodies used include anti-mouse CD11c-biotin (clone N418), anti-mouse CD40-PE (clone 1C10), anti-mouse major histocompatibility complex II IA/IE-APC (clone M5), and anti-mouse CD207-APC (e-Bioscience).

**RESULTS**

**Galectin-1 Specifically Binds to CD43 and CD45 and Co-clusters These Glycoconjugates on Human MDDC Surface**—To identify galectin-1 receptors on MDDCs, total cell surface proteins were biotinylated prior to incubation with galectin-1. Figure 2A shows that galectin-1 induces calcium flux and differential tyrosine-phosphorylation patterns in MDDCs. A, immature MDDCs were loaded with Indo-1AM (3 μM) + 0.02% pluronic and then stimulated with galectin-1 at indicated times (†) during acquisition on a BD LSR flow cytometer. The data are shown as numbers of cells with calcium bound Indo-1/unbound Indo-1 (FL5/FL4) ratio above base line versus time. B, MDDCs were treated with galectin-1 (20 μM) or LPS (500 ng/ml) for the indicated times and then lysed, and equivalent amounts of cell lysate (30 μg/lane) were Western blotted using the 4G10 monoclonal anti-phosphotyrosine antibody. Galectin-1 was added to cells in the presence of polymyxin B (10 μg/ml) to eliminate any confounding effects of endotoxin contamination.

**TABLE 1**

| Protein name                        | Phosphorylation site(s) | Corrected fold change | Known signaling pathways
|-------------------------------------|------------------------|-----------------------|---------------------------|
| Caveolin 2                          | Ser23                  | 14.73                 | Scaffolding protein       |
| Myosin regulatory light chain       | Ser20                  | 2.79                  | Integran signaling, Rho-dependent signaling |
| ζ-chain (TCR)-associated kinase, 70 kDa (ZAP-70/spleen tyrosine kinase (Syk)) | Tyr292                 | 1.78                  | MAPK signaling, TCR signaling |
| Protein kinase N1 (PKN1)            | Thr774                 | 1.62                  | Inositol phosphate signaling, PKC signaling |
| Phosphatase and tensin homolog (PTEN), pseudogene 1 (PTENP1) | Ser380, Ser382, and Ser385 | 1.55                  | Inositol phosphate signaling, integrin signaling |
| Insulin receptor substrate 1 (IRS-1) | Tyr612                 | 1.49                  | Insulin receptor signaling, inositol phosphate signaling |
| LIM domain kinase 1/2               | Tyr206/Thr205          | 1.43                  | Rho-dependent signaling, inositol phosphate signaling |
| Myristoylated alanine-rich protein kinase C substrate (Marcks) | Ser122 and Ser162     | 1.42                  | PKC signaling             |
| MAPK-interacting protein-serine kinase 1 (MNK1) | Thr206 and Thr214 | 1.38                  | MAPK signaling, Cell cycle |
| Retinoblastoma-associated protein 1 | Thr607                 | 1.37                  | cAMP signaling, MAPK signaling, Calcium signaling |
| Ret receptor-tyrosine kinase         | Ser606                 | 1.31                  | MAPK signaling, inositol phosphate signaling |

*a Known signaling pathways obtained from ingenuity pathway analysis and EntrezGene data bases.

*b TCR, T-cell receptor.
Mechanisms of Galectin-1-induced DC Activation

Galectin-1 binding to CD43 and CD45 was completely abolished in the presence of 0.1 mM lactose, indicating that galectin-1 specifically bound to glycans on both CD43 and CD45 on the MDDC surface.

To determine whether galectin-1 binding results in qualitative changes in membrane distribution of specific cell surface receptors, we used confocal microscopy to visualize the location of CD43 and CD45 before and after galectin-1 binding. Following 1 h of incubation with galectin-1, both CD43 and CD45 showed dose-dependent clustering on the MDDC surface (Fig. 1C). In contrast to the phenotype seen on T cells where CD43 and CD45 are clustered but segregated in different domains (14), both CD43 and CD45 co-clustered in a unipolar fashion on the cell membrane in MDDCs. LPS or a buffer control did not induce co-clustering of CD43 and CD45, and preincubation with 0.1 M lactose inhibited redistribution of these markers (Fig. 1C), underscoring the saccharide specificity of the effects of galectin-1. Furthermore, clustering was only seen in the majority of cells at galectin-1 concentrations at or above 10 nM (quantified in Fig. 1D). Because the $K_d$ of dimerization for galectin-1 is in the 6–8 nM range (19), this suggested that cross-linking of receptors was involved in galectin-1 membrane reorganization. Additionally, galectin-1 did not cause global membrane reorganization, as evidenced by a lack of redistribution of CCR5, a highly expressed cell surface protein on MDDCs that is not known to bind galectin-1 (Fig. 1E).

Galectin-1 Induces Calcium Flux and Differential Tyrosine Phosphorylation Patterns in MDDCs—Many intracellular signals involve calcium mobilization through release of intracellular calcium stores or an influx of extracellular calcium. Antibody cross-linking of CD43 causes calcium flux in DCs (20), but it is not known whether endogenous ligands can initiate calcium flux. We found that galectin-1, but not LPS or the buffer control, specifically induced calcium flux in MDDCs (Fig. 2A). Galectin-1-induced calcium flux was dose-dependent and inhibited by 0.1 M lactose, underscoring the specificity of the galectin-1 effects. Next, to determine whether galectin-1 ligation of DC cell surface receptors activates a differential signaling cascade compared with LPS, we immunoblotted MDDC

FIGURE 3. Syk and PKC have significant roles in galectin-1-induced cytokine secretion from MDDCs. A, MDDCs were treated with LPS (250 ng/ml), buffer control (80 μM DTT), or galectin-1 (20 μM). 10 μg/ml of polymyxin B (PMB) and 0.1 M lactose were added 30 min prior to stimulation where indicated. Our galectin-1 stocks had minimal LPS contamination of 1–4 endotoxin units/ml as measured by the Limulus amebocyte lysate assay. 250 ng/ml of the LPS stock used had 750 endotoxin units/ml. Clearly, 10 μg/ml of polymyxin B was more than sufficient to eliminate any confounding effects of endotoxin contamination. B–E, MDDCs were preincubated with specific PTK inhibitors for PKC (bisindolylmaleimide I) (B), Syk (Calbiochem 574711) (C), PLCγ (U73122) (D), and PI3K (LY294002) (E) for 2 h prior to galectin-1 (20 μM) or LPS (250 ng/ml) stimulation. At 24 h, IL-6 secretion in the supernatant was assayed by ELISA. The data are shown as IL-6 levels normalized to the no inhibitor condition (0 μM) ± S.E. of five independent experiments in at least four human donors.
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by the ability of 0.1 M lactose to abrogate galectin-1-induced IL-6 secretion.

To evaluate the importance of each of the candidate pathways identified by the combination of our Kinex screen (Table 1) and Ingenuity Pathway Analysis (supplemental Fig. S1A), we preincubated MDDCs with chemical inhibitors of Syk/ZAP70, PKC, PI3K, and PLCγ. Although it is likely that all of these signaling mediators are involved in DC activation via various stimuli, our goal was to identify a pathway that is preferentially utilized by galectin-1.

Indeed, both PLCγ and PI3K appeared to be necessary for both galectin-1- and LPS-induced IL-6 secretion, because specific PLCγ and PI3K inhibitors equivalently abrogated IL-6 production regardless of stimulus (Fig. 3D and E). A PKC inhibitor also inhibited both galectin-1 and LPS-induced IL-6 secretion at high concentrations (>10 μM), although galectin-1-induced IL-6 secretion appeared more sensitive to PKC inhibition at low doses (1 and 5 μM) of the inhibitor (Fig. 3B), suggesting that LPS and galectin-1 were differentially dependent on the PKC pathway at some level.

Galectin-1-stimulated IL-6 production was consistently more susceptible to Syk inhibition than LPS-induced IL-6 secretion (Fig. 3C). Treatment with high concentrations of Syk inhibitor (50 μM) completely blocked IL-6 production by galectin-1-activated MDDCs but only partially decreased IL-6 production by LPS activated MDDCs. This difference in sensitivity
indicated that galectin-1-induced DC maturation may be more dependent on Syk than LPS-induced DC maturation.

Galectin-1 but Not LPS Induces Increased Syk Phosphorylation in Human MDDCs—To confirm the specificity of our chemical inhibition experiments, we sought to determine whether galectin-1 physically activates Syk tyrosine kinase. We incubated MDDCs with galectin-1 for varying periods of time, collected whole cell lysate, and detected the levels of total and phosphorylated Syk by Western blot. Untreated MDDCs had a basal level of phosphorylated Syk (pSyk) expression. At 5 min post-galectin-1 stimulation, the pSyk/total Syk ratio increased by almost 3-fold (Fig. 4, A and B) and then declined back to basal levels by 60 min. Total Syk protein levels did not change throughout the time course (Fig. 4A). Notably, neither pSyk nor total Syk levels changed following LPS stimulation (Fig. 4, C and D), suggesting that Syk signaling was not an immediate effector of LPS signaling under the conditions examined. Cross-linking CD43 receptors with anti-CD43 monoclonal antibodies is known to trigger Syk phosphorylation (21), similar to the effects of galectin-1 stimulation (Fig. 4, E and F), and served as a positive control throughout this experiment. Intriguingly, cross-linking CD45 receptors on MDDCs with anti-CD45 monoclonal antibodies also induced Syk phosphorylation. Nonspecific IgGs did not affect Syk phosphorylation levels, underscoring the specificity of the CD43 and CD45 cross-linking effects (Fig. 4, E and F). Because galectin-1 binds to and clusters CD43 and CD45 on MDDCs (Fig. 1), this suggests that galectin-1 may cross-link CD43 and CD45 to induce Syk signaling.

Galectin-1 Stimulates Redistribution of pSyk to Co-localize with the CD43 and CD45 Co-cluster on MDDCs—Many receptor-associated cell signaling events occur at the cell membrane because signaling molecules are recruited to form a complex termed a “signosome.” To determine whether galectin-1 induces the formation of such a signaling complex, we used confocal microscopy to visualize the respective cellular locations of CD43, CD45, and pSyk following galectin-1 stimulation in human MDDCs. Before galectin-1 treatment, CD43 and CD45, and basal levels of pSyk were uniformly distributed around the cell membrane (Fig. 5, 0 min). At subsequent time points, galectin-1 began to cluster CD43 and CD45 until the 60-min time point where both CD43 and CD45 appeared unipolarly co-clustered. There was moderate base-line co-localization of pSyk with CD43 and CD45 (Fig. 5, CD43+pSyk (magenta) and CD45+pSyk (yellow) at time 0 min). However, with the increasing time of galectin-1 binding, pSyk appeared to follow the redistribution of these receptors because CD43 and CD45 began to show galectin-1-induced co-clustering on the cell membrane (Fig. 5). Galectin-1 binding was necessary to cluster these three molecules together in a putative “signosome,” because the unipolar co-clustering was blocked by lactose, which inhibits galectin-1 binding and galectin-1-induced MDDC maturation (7). This suggests that the formation of the CD43-CD45-pSyk cluster may be critical for the galectin-1-induced effects of MDDCs.

Next, we sought to determine whether Syk and PKC inhibition would also decrease galectin-1 enhancement of MDDC migration through extracellular matrix. This enhanced migratory activity is a novel function of galectin-1-activated MDDCs and requires MMPs, the expression of which was severely hampered by Syk and PKC inhibitors (Fig. 6A). To do this, we tested...
Galectin-1 Synergizes with LPS for Faster Activation of MDDCs—Because galectin-1 and LPS can activate DCs via overlapping by distinct pathways and galectin-1 is an endoge-
Mechanisms of Galectin-1-induced DC Activation

**A**

![Graph A](image1)

**B**

![Graph B](image2)

**C**

![Graph C](image3)

**FIGURE 7.** Galectin-1 enhances the inflammatory response of MDDCs to LPS. A, MDDCs were stimulated with increasing doses of LPS (10\(^{-4}\) to 10\(^{3}\) ng/ml) or galectin-1 (0–40 \(\mu\)M). Supernatants were collected at 24 h, and IL-6 was measured by ELISA. Cells treated with galectin-1 were preincubated with polymyxin B (10 \(\mu\)g/ml) for 30 min to eliminate any contaminating endotoxin effects. B, MDDCs were treated with 250 ng/ml LPS (black squares), 20 \(\mu\)M galectin-1 (gray triangles), or buffer control (80 \(\mu\)g/ml DTT + 10 \(\mu\)g/ml polymyxin B) (open circles), and IL-6 secretion was measured by ELISA of the culture supernatants at the indicated time points. The data are represented as percentages of maximal IL-6 production. C, MDDCs were stimulated with LPS (0.1 ng/ml) alone or were preincubated with galectin-1 (20 \(\mu\)M) for 8 h prior to LPS stimulation. Supernatant was collected after the indicated time periods, and IL-6 was measured by ELISA. The data are presented as percentages of maximal IL-6 secretion. The time to 50% maximal response is 3.8 h for LPS treatment alone (filled circle, solid line) and 2.5 h for LPS and galectin-1 co-stimulus (open circle, dotted line) (p < 0.0001, two-way analysis of variance). Galectin-1 was preincubated with polymyxin B-agarose beads for 30 min to eliminate endotoxin contamination from the galectin-1 preparations.

(FITC^+ CD40^+ cells) in the draining lymph nodes were further defined for different populations of DCs, including epidermal DC (CD11c<sub>hi</sub>-int Langerin^+<sub>) and dermal DC (CD11c<sub>hi</sub>-int Langerin^-<sub>) (Fig. 8B) (33). The results show a dose-dependent increase in skin DC migrants in animals injected with galectin-1 (Fig. 8C), of which 35–45% of the migrant FITC-positive cells were also positive for the Langerin marker (Fig. 7B). Thus, in addition to our in vitro observations regarding the enhanced migratory activity of galectin-1-activated MDDCs, galectin-1 also enhanced the in vivo skin emigration that is defective in lupus-prone MRL-fas mice.

**DISCUSSION**

Although galectin-1 and LPS can both induce MDDC maturation, there are differences that distinguish the two stimuli. For example, galectin-1 specifically increases expression of genes related to cell motility and migration and enhances MDDC migration through the extracellular matrix (7).

In the current study, we identified galectin-1 receptors on MDDCs and characterized the immediate downstream effectors that mediate the effects of galectin-1 on MDDCs. Galectin-1 bound to glycans on CD43 and CD45 and co-clustered these glycoproteins on the DC surface (Fig. 1). Galectin-1 induced a calcium flux and activated overlapping as well as distinct signaling pathways compared with LPS (Figs. 2–4). Specifically, we identified Syk and PKC protein-tyrosine kinases as critical mediators of the effects of galectin-1 on MDDCs, because Syk and PKC inhibitors abrogated the effects of galectin-1 on MDDCs (Figs. 3 and 6). Galectin-1 stimulated Syk phosphorylation (Fig. 4), which then clustered with both galectin-1 receptors CD43 and CD45 on membranes of MDDCs (Fig. 5). Syk and PKC activities were also necessary for galectin-1-induced MMP gene expression and enhanced migration through Matrigel (Fig. 6). Finally, although galectin-1-induced maturation of DCs has been independently demonstrated in vivo using various murine models (6, 34), we now also provide in vivo evidence for a distinguishing feature of galectin-1-activated DCs: enhanced migration through the extracellular matrix. Fig. 8 shows that a single intradermal injection of recombinant galectin-1 could partially rectify the defect in skin DC emigration seen in lupus-prone MRL-fas mice and induced migration of various types of skin DCs into draining lymph nodes.

Although we have shown that galectin-1 clearly bound and clustered CD43 and CD45 on MDDCs, we acknowledge that galectin-1 may have additional counter-receptors on the DC plasma membrane, and the role of other receptors in galectin-1-mediated signaling on MDDCs remains to be determined. For example, Syk has essential roles in integrin signaling (35–37), and integrin \(\beta1\) is another known galectin-1 receptor (18). Further, data from the kinome array shows that galectin-1 affected multiple phosphorylated proteins that have known roles in integrin signaling, and integrin \(\beta1\) itself was also phosphorylated (Table 1). Galectin-1-mediated DC maturation likely involves coordinated binding of multiple receptors and utilization of unique upstream mediators to link common signaling pathways. Thus, future studies will test the contributory role of each galectin-1 receptor to fully delineate the complex mechanism of galectin-1 signaling in DCs.

The “danger signal model” proposes that immune responses can also be driven by self-signals that signal danger in the absence of a pathogen, i.e., by signals sent from dying or damaged cells (38–40). Because galectin-1 is present at high concentrations (up to 48 mg/kg) in extracellular matrix and in multiple anatomic sites (31) and its secretion by endothelium and immune cells is increased during inflammation (30) or can be concentrated in stromal sites to...
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potentiates its activity (41), galectin-1 may function as an endogenous inducible danger signal (42) that enhances the inflammatory response to exogenous pathogen signals. Indeed, we found that priming the DCs with pre-exposure to galectin-1 enhances the inflammatory response time of DCs to suboptimal LPS stimulus (Fig. 7C). Thus, endogenous cues, such as galectin-1, which can be highly up-regulated in inflammatory situations, can potentially synergize with pathogen-derived signals (like LPS) and activate dendritic cells in a manner that has not been heretofore appreciated. Our results also suggest that, at some level, LPS and galectin-1 signaling pathways converge. Thus, although our data show that the Syk and PKC pathways can preferentially mediate galectin-1 effects on DCs, we do not imply that LPS does not activate these pathways at all. Indeed, LPS is known to activate the Syk pathway in neutrophils (43), and our inhibitor, time course, and phosphorylation studies indicate that LPS may simply activate the Syk pathway in DCs in a distinct but overlapping manner.

Our data also show that the role of galectin-1 as an endogenous activator of DCs can induce functionally different responses from a classic exogenous stimulus like LPS. Galectin-1 is well known as a fine-tuner of immune responses (44–46), and a multitude of studies have shown that exogenously administered galectin-1 can dampen a host of autoimmune and inflammatory responses in animal models (47). However, the vast majority of studies have focused on the ability of galectin-1 to modulate T cell responses. Galectin-1 clearly matures dendritic cells, as has been independently demonstrated by Morelli and co-workers (6), but even this work focused on the ability of transgenic galectin-1-matured DCs to modulate T cell responses in vivo. Here, we showed that our in vitro observation that galectin-1-activated DCs have enhanced migratory capacity through the extracellular matrix is also seen in vivo. Indeed, galectin-1 rescued the defect in skin DC emigration seen in lupus-prone MRL-fas mice. This raises the question of whether galectin-1 also affects DCs, a cell that is the nexus of innate and adaptive immunity, is called for.

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