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Galectin-1-Matured Human Monocyte-Derived Dendritic Cells Have Enhanced Migration through Extracellular Matrix[1]

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Dendritic cells (DCs) are potent mediators of the immune response, and can be activated by exogenous pathogen components. Galectin-1 is a member of the conserved β-galactoside-binding lectin family that binds galactoside residues on cell surface glycoconjugates. Galectin-1 is known to play a role in immune regulation via action on multiple immune cells. However, its effects on human DCs are unknown. In this study, we show that galectin-1 induces a phenotypic and functional maturation in human monocyte-derived DCs (MDDCs) similar to but distinct from the activity of the exogenous pathogen stimuli, LPS. Immature human MDDCs exposed to galectin-1 up-regulated cell surface markers characteristic of DC maturation (CD40, CD83, CD86, and HLA-DR), secreted high levels of IL-6 and TNF-α, stimulated T cell proliferation, and showed reduced endocytic capacity, similar to LPS-matured MDDCs. However, unlike LPS-matured DCs, galectin-1-treated MDDCs did not produce the Th1-polarizing cytokine IL-12. Microarray analysis revealed that in addition to modulating many of the same DC maturation genes as LPS, galectin-1 also uniquely up-regulated a significant subset of genes related to cell migration through the extracellular matrix (ECM). Indeed, compared with LPS, galectin-1-treated human MDDCs exhibited significantly better chemotactic migration through Matrigel, an in vitro ECM model. Our findings show that galectin-1 is a novel endogenous activator of human MDDCs that up-regulates a significant subset of genes distinct from those regulated by a model exogenous stimulus (LPS). One unique effect of galectin-1 is to increase DC migration through the ECM, suggesting that galectin-1 may be an important component in initiating an immune response. *The Journal of Immunology,* **2006, 177: 216–226.**

Dendritic cells (DCs)[4] play a pivotal role in both the innate and adaptive immune responses. Immature DCs monitor the periphery, and upon interaction with a pathogen, mature to become professional APCs. During the maturation process, DCs decrease their ability to internalize Ag, while up-regulating the expression of certain cell surface molecules and cytokines involved in immune responses as well as migration to lymphoid organs. DCs are the most potent APCs and are highly efficient at initiating T cell responses (1, 2). DC activation and migration to lymph nodes is crucial to mounting an effective immune response.

DC migration to and from sites of inflammation involves a diverse network of effector molecules. For cells to migrate, a series of coordinated events must occur. First, cells must undergo a morphological change involving polarization as well as membrane extension for locomotion. This involves cytoskeletal remodeling, using actin polymerization/depolymerization in cooperation with actin-binding proteins to reorganize the actin cytoskeleton (3, 4). Next, cell migration uses adhesion proteins on both the cell surface and in the extracellular matrix (ECM) to guide remodeling and cell movement through the environment. Integrins, expressed on cell surface, change membrane distribution upon interaction with an ECM receptor and this clustering induces signaling involved in cytoskeletal remodeling and actin binding (4). Other effector molecules such as proteases also play a significant role in cell trafficking. Matrix metalloproteinases (MMPs) degrade ECM components and regulate normal cell migration as well as tumor cell invasion and metastases (5, 6). MMPs, specifically MMP-2 and MMP-9, are involved in migration of DCs and Langerhans cells (7–9). As cells migrate, varying chemotactic signals direct cells to sites of action. Chemokines and chemokine receptors play crucial roles in targeting DCs both to sites of inflammation and back to the secondary lymphoid organs following maturation. Immature DCs express the chemokine receptor CCR6, which binds to CCL20 (MIP-3α) secreted in peripheral tissues and guides DC migration to the mucosa. Upon maturation, DCs decrease CCR6 expression and increase CCR7 expression, which binds CCL19 (MIP-3β) expressed in high endothelial venules and T cell zones of lymph nodes (1, 10, 11).

A variety of pathogen-derived products are capable of inducing DC maturation via binding to pathogen recognition receptors, such

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[4] Abbreviations used in this paper: DC, dendritic cell; ECM, extracellular matrix; MMP, matrix metalloproteinase; MDDC, monocyte-derived DC; MFI, mean fluorescent intensity; PMA, present, marginal, absent; HMVEC, human microvascular endothelial cell.

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as TLRs (12, 13). In the absence of pathogen interaction, DCs also respond to generalized inflammatory signals. Certain inflammatory cytokines present at sites of injury and infection, such as IL-1 and TNF-α, are able to induce maturation in an autocrine/paracrine manner (2). Additionally, endogenous molecules known to possess proinflammatory activity, such as oxidized low density lipoprotein and uric acid, also activate DCs as part of the inflammatory response (14, 15). Although DC maturation in response to exogenous stimuli, such as to TLR ligands, has been well-studied, less is known about the role of endogenous regulators in DC function and immune response.

Recent gene expression studies have found a remarkable plasticity with regard to DC responses to different stimuli. Although varying pathogens do indeed elicit a common core response, there are distinct differences in gene expression specific to different pathogens and their components (16, 17). While some studies have examined gene expression in relation to endogenous cytokines, no studies have compared gene expression between DCs activated with exogenous stimuli vs endogenous stimuli (18).

Galectins are a family of highly conserved β-galactoside-binding lectins that function in cell growth regulation, cell-cell adhesion, metastasis, inflammation, and immunomodulation (19). Galectin-1, one of the most widely studied galectins, has a broad range of immunomodulatory activities involving both innate and adaptive immune cells. Galectin-1 regulates inflammatory responses in macrophages and neutrophils (20, 21). In addition, galectin-1 induces apoptosis of thymocytes and peripheral T cells, and is also highly expressed in $CD4^+CD25^+$ regulatory T cells (22, 23). Galectin-1 also controls B cell development as a pre-BCR ligand (24). In animal studies, galectin-1 therapy ameliorated disease in models of arthritis, hepatitis, nephritis, and graft-vs-host disease. In several of these models, galectin-1 shifted the cytokine balance in these animals from a Th1-type profile toward a Th2-type profile (25).

Previously, we have shown that galectin-1 induces secretion of IL-6 and TNF-α from monocyte-derived DCs (MDDCs) and this may play a role in innate antiviral defense (26). In the present study, we show that galectin-1 binds to and activates human MDDCs to become phenotypically and functionally mature DCs. However, using microarray and functional analyses, we show that galectin-1 induces a similar but distinct set of genes as the classical pathogen stimulus, LPS. We establish a unique functional difference between galectin-1 and LPS-matured MDDCs, and therefore reveal a novel role for galectin-1 in the immunobiology of DCs.

Materials and Methods

DC differentiation and galectin-1 treatment

Human monocytes for in vitro differentiation of MDDCs were isolated from peripheral blood obtained from healthy donors through the Virology Core of the University of California Los Angeles (UCLA) AIDS Institute. Purified monocytes were obtained using RosetteSep Monocyte Enrichment Cocktail (StemCell Technologies) according to manufacturer's guidelines. Monocytes were plated in 12-well plates (3×10^5 cells/well) and differentiated in RPMI 1640 containing 10% FBS plus 50 ng/ml (>500 U/ml) GM-CSF and 100 ng/ml (>500 U/ml) IL-4 (PeproTech) for 5 days. At day 5, indicated treatments were added and cells were cultured for an additional 48 h unless otherwise noted. To control for possible endotoxin contamination, all galectin-1-treated cells were preincubated with 10 μg/ml polymyxin B (Sigma-Aldrich) at 37°C for 30 min.

Recombinant dimeric and monomeric galectin-1

Recombinant human galectin-1 was produced as previously described (27). Monomeric galectin-1 is the N-Gal-1 mutant (28) and was made as previously described, except that a 3 × 7-cm lactosyl-Sepharose affinity column was used to isolate the recombinant galectin-1.

Detection of galectin-1 cell surface binding

MDDCs were differentiated as described above, except cells were differentiated and treated on poly-D-lysine-coated coverslips (BD Biosciences). After differentiation, cells were washed and biotinylated galectin-1 (20 μM) was added in PBS with or without lactose (0.1 M) or sucrose (0.1 M) and allowed to bind for 1 h at 37°C. Cells were washed three times with PBS and bound galectin-1 was detected using streptavidin-conjugated Alexa 488 (Invitrogen Life Technologies) diluted 1/200 in PBS for 1 h at room temperature. Cells were washed three times with PBS and fixed in 2% paraformaldehyde. Slides were mounted with fluorescent mounting medium (DakoCytomation) and images were acquired on Nikon Eclipse TE300 fluorescent microscope using MetaMorph software (Universal Imaging).

FACS analysis of DC surface phenotype

Cells were harvested and washed twice with PBS with 2% FBS. Cells were stained for 30 min with the following PE-conjugated Abs: anti-CD83 (Immunotech), anti-CD40 (Caltag Laboratories) and with the following allophycocyanin-conjugated Abs: anti-CD209 (DC-SIGN) (R&D Systems), anti-CD86 (Caltag Laboratories). Murine IgG1-PE and IgG1-allophycocyanin were used as isotype controls (Caltag Laboratories). After staining, cells were washed an additional two times, and fixed in 2% paraformaldehyde. Samples were then analyzed using a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

Quantification of cytokine production

DC cytokine secretion was measured using cytometric bead array (Human Inflammation Kit; BD Biosciences). MDDCs were differentiated and treated as described above in 12-well plates (3×10^5 cells/well in 500 μl of RPMI 1640). Cell culture supernatants were collected 48 h following treatment with LPS (100 ng/ml), galectin-1 (20 μM), N-Gal-1 (20 μM), or no treatment and stored at −80°C until analysis.

FITC-dextran uptake assay

To measure endocytic capacity, MDDCs were treated as indicated for 48 h, harvested, and washed with ice-cold PBS. MDDCs (2.5×10^5) cells were incubated with 1 mg/ml FITC-dextran (Invitrogen Life Technologies) in 2 ml of culture medium for 30 min at 37°C and at 4°C (as control). Cells were washed three times with ice-cold PBS and analyzed by flow cytometry. Data are shown as Δ mean fluorescent intensity (MFI) = MFI at 37°C − MFI at 4°C (control).

DC-T cell cocultures

$CD4^+$ T cells were isolated from whole blood using the RosetteSep $CD4^+$ T Cell Enrichment kit (StemCell Technologies) according to the manufacturer's guidelines. Naive $CD4^+CD45RO^-$ T cells were isolated using negative selection with CD45RO microbeads (Miltenyi Biotec). MDDCs were differentiated and treated as described above. Treated MDDCs were irradiated with 3000 rad and cocultured with naive allogeneic $CD4^+$ T cells (1×10^5 cells/well) in 200 μl of RPMI 1640/10% FBS at DC:T cell ratios of 1:10, 1:50, and 1:250. To assay proliferation, 10 μM BrdU was added during the final 5 h of culture, and BrdU incorporation was measured using the chemiluminescent ELISA kit (Roche).

RNA preparation and microarray hybridization

MDDCs from three separate donors were differentiated as described above. At day 5, MDDCs were treated as follows: LPS (100 ng/ml); galectin-1 (20 μM) in the presence of 10 μg/ml polymyxin B as described above; vehicle control consisting of equivalent volume of 8 mM DTT (galectin-1 storage buffer) and 10 μg/ml polymyxin B; or untreated. At 18 h after treatment, total RNA was independently isolated for each donor and condition using the RNeasy mini kit (Qiagen). Total RNA was analyzed on both a spectrophotometer and an Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with a 28S:12S ratio of >1.5 and no evidence of ribosomal peak degradation were included. Probes were prepared using standard Affymetrix protocols and hybridized to Affymetrix HG-U133 plus 2.0 arrays (Affymetrix). Platforms were scanned using an Affymetrix GeneChip Scanner 3000. All of the microarrays were examined for surface defects, grid placement, background intensity, housekeeping gene expression, and a 3′:5′ ratio of probe sets from genes of various length (signal 3′:5′ ratio <3).

Microarray preprocessing and statistical analysis

Microarray Suite 5.0 was used to define absent/present calls and generate .cel files using the default settings. Scatter plots of present, marginal, absent (PMA) calls were created using R software, Bioconducter package, to view global trends in gene expression. dChip software was used for analysis because it has been shown to operate consistently well in comparison to other analysis software/algorithms (29). Data files (.cel) were uploaded into the dChip program and normalized to the median intensity array (30). Quantification was performed using model-based expression and the perfect match minus mismatch method in dChip (31). A total of 22,534 transcripts were called absent in all four conditions and eliminated from further data analysis. A total of 32,142 transcripts remained for further analysis. Control and vehicle samples were compared in dChip using a joint criteria of fold change >1.5 and $p < 0.05$, resulting in 17 differentially regulated genes. To assess the false positive rate of this set, we used 100 random data set permutations in dChip, which produced 17 or more false positive genes, or a 100% false discovery rate. Thus, because this is the same number of differentially regulated genes in this set, we can conclude that the vehicle samples did not alter any gene expression and these genes were excluded from further analysis. The same assessment of false positives was applied to all additional samples to verify that alterations in gene expression are indeed true positives. Control vs LPS-treated samples and control vs galectin-1-treated samples under joint criteria of fold change >1.5 and $p < 0.05$ resulted in 885 genes with a false discovery rate of 0.9% (8 genes) and 2412 genes with false discovery rate of 0.5% (13 genes), respectively. Hierarchical clustering was also performed in dChip.

Microarray functional analysis

All genes in the LPS vs galectin-1 sample comparison with a mean fold change >1.2 were considered for the functional analysis. Genes were uploaded to the GOstat website to find statistically overrepresented gene ontologies within the group of genes (32). We selected functional groups within a very strict $p < 0.00001$, to avoid false positives and assess for multiple comparisons. This p value indicates the probability that the observed number of counts could have resulted from randomly distributing the gene ontology term between the group of genes and the complete database of annotated genes. Using this strategy, we see the contribution of all genes to a functional group but pick only the most significantly differentially regulated functional groups between LPS and galectin-1-treated MDDCs for further investigation.

Real-time quantitative RT-PCR

MDDCs were differentiated and treated as described for microarray preparation. Total RNA was extracted with the RNeasy mini kit (Qiagen). The QuantiTect Probe RT-PCR kit was used for cDNA synthesis (Qiagen). Transcripts were quantified by real-time quantitative PCR on a DNA Engine Opticon 2 (MJ Research) with predesigned TaqMan gene expression assays and reagents according to the manufacturer's instructions (Applied Biosystems). For each sample, mRNA abundance was normalized to the amount of β-actin expressed. Full-length clones of each gene analyzed were used to create standard curves for quantification (Open Biosystems).

DC Matrigel migration assays

RPMI 1640 medium containing 10% FBS plus 200 ng/ml CCL19 (MIP-3β) was placed in the bottom of a 24-well plate. MDDCs (2.5×10^4) were differentiated and treated as described above and were added to the top of a Matrigel-coated insert or uncoated control insert with pore size of 8.0 μm (BD Biosciences) in serum-free RPMI 1640. Cells were allowed to migrate at 37°C for 22 h. Inserts were removed, nonmigrated cells were washed off, and the membrane was stained using Diff-Quik stain set (Dade Behring). For transendothelial migration assays, human microvascular endothelial cells (HMVECs) (5×10^5) were added to the top of an uncoated control insert with a pore size of 8.0 μm (BD Biosciences) in a 6-well plate and allowed to grow to confluency for 48 h. HMVECs immortalized with the human telomerase catalytic protein (hTERT) were a gift from R. Shao (University of Massachusetts, Amherst, MA). HMVEC-coated inserts were transferred to a new 6-well plate with EBM-2 medium containing 10% FBS plus 200 ng/ml MIP-3β in the bottom chamber and serum-free EBM-2 medium in the top chamber. MDDCs (5×10^4) were labeled with 10 μM CFSE (Invitrogen Life Technologies) and added to the top chamber. Migration took place at 37°C for 22 h. Following migration, inserts were removed, nonmigrated cells were washed off, and the membrane was fixed in 2% paraformaldehyde for 15 min. Insert membranes were then mounted on slides using fluorescent mounting medium (DakoCytomation). In all assays, the number of cells migrating through the Matrigel to the bottom membrane was counted at $\times 40$ magnification. The number of cells migrating through the HMVECs to the bottom membrane was counted at $\times 40$ magnification using a fluorescent microscope. Each experiment was counted by two independent counters, one blinded to the sample identity, and at least 50 fields were counted. Cells/field were normalized to 50 fields and data were expressed as relative migration compared with control cells (immature MDDCs).

Results

Galectin-1 binds to glycoconjugates on the surface of MDDCs

Galectin-1 specifically binds oligosaccharide ligands on many cell surface glycoconjugates (19). As DCs express many glycoproteins on their cell surface that are known galectin-1 ligands, such as CD2, CD43, and CD45, we used immunofluorescent microscopy to examine galectin-1 binding capabilities on the surface of human MDDCs. Fig. 1*A* shows clear binding of biotinylated galectin-1 to the surface of MDDCs. To determine whether binding was carbohydrate dependent, we used lactose, a cognate ligand for galectin-1, and sucrose, which does not bind galectin-1, to compete for cell surface binding. Although lactose blocked galectin-1 binding, sucrose did not block binding, indicating that galectin-1 binds to DC cell surface glycoconjugates in a lactose-dependent manner (Fig. 1*A*). The morphology of MDDCs in the presence or absence galectin-1 was indistinguishable and exhibited dendritic extensions and fine veils upon higher magnification that was characteristic of MDDCs.

Galectin-1 induces phenotypic maturation in human MDDCs

As DCs undergo maturation, the cells alter expression of certain cell surface proteins involved in Ag presentation and T cell stimulation. Changes in expression of these cell surface proteins can be used to monitor the maturation state of DCs. To investigate the effect of galectin-1 on the maturation state of DCs, we incubated immature MDDCs with 20 μM galectin-1 then assessed cell surface phenotype by flow cytometry after 48 h. Initial time course experiments monitoring cell surface phenotype showed peak expression of cell surface markers at 48 h, with a decrease beginning at 72 h (data not shown), and all subsequent experiments were done at a 48-h time point unless otherwise noted. For purpose of comparison, we also matured MDDCs with LPS, which is known to induce phenotypic and functionally mature DCs. As shown in Fig. 1*B*, galectin-1-treated DCs express CD83, a DC-specific maturation marker, as well as high levels of the costimulatory molecule CD86. Galectin-1-treated MDDCs significantly increased both CD83 and CD86 cell surface expression compared with untreated MDDCs ($p < 0.02$ using paired t test from eight independent experiments represented in Fig. 1, *B* and *C*). Furthermore, galectin-1 induced both CD83 and CD86 expression in a dose-dependent manner (Fig. 1*D*). Additional maturation markers such as DC-SIGN, CD40, CD80, and the MHC class II molecule HLA-DR were also examined and expressed at expected levels. Specifically, CD40, CD80, and HLA-DR expression increased while DC-SIGN expression decreased following treatment with galectin-1 as well as LPS (data not shown). As galectin-1 has unpaired cysteines in the binding pocket that, in the absence of saccharide ligand, can form intramolecular disulfide bonds and prevent saccharide ligand binding, we prepare and store galectin-1 in a DTT buffer as previously described (27). Treatment with DTT buffer control resulted in no up-regulation of DC maturation markers (data not shown).

Galectin-1 effects can involve both carbohydrate binding as well as protein-protein interactions (19). Although we have shown that galectin-1 binding to MDDCs appears to involve specific carbohydrate binding (Fig. 1*A*), to determine whether galectin-1 maturation of MDDCs requires carbohydrate binding we used lactose to block maturation effects. Indeed, galectin-1 maturation was

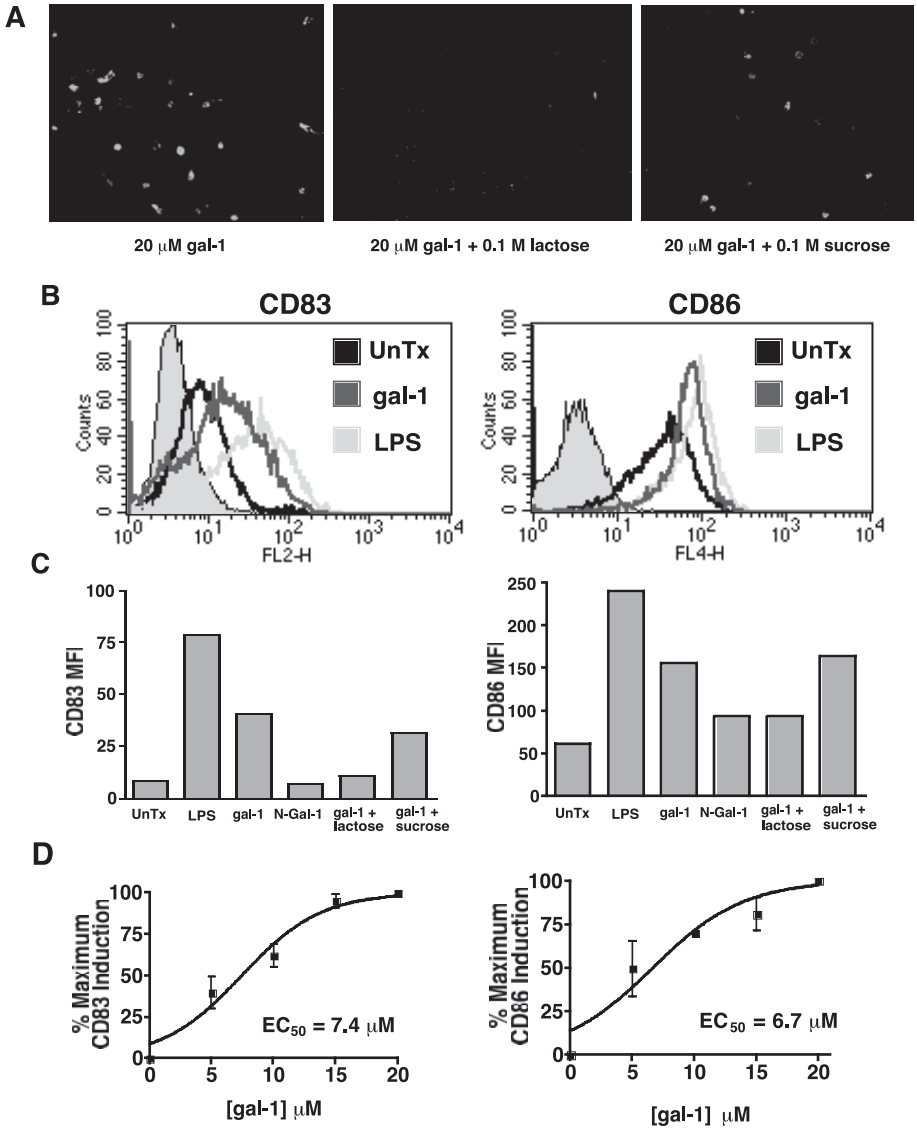


FIGURE 1. Galectin-1 binds to and matures human MDDCs. *A*, Biotinylated galectin-1 (20 μM), with or without 0.1 M lactose or sucrose, was added to immature MDDCs and bound galectin-1 was detected with streptavidin-conjugated Alexa-488. Images were acquired using fluorescent microscopy at $\times$40 magnification. *B* and *C*, CD83 and CD86 expression on MDDCs (3×10^5) cultured with LPS (100 ng/ml), galectin-1 (20 μM), N-Gal-1 (20 μM), galectin-1 (20 μM) + lactose (0.1 M) or sucrose (0.1 M). All wells using galectin-1 or N-Gal-1 were pretreated by incubation with 10 μg/ml polymyxin B. FACS histograms are shown in *B* and a graphical representation of the MFI for each marker is shown in *C*. The filled histogram is the isotype control. Data shown is representative of eight independent experiments. *D*, MDDCs were treated with increasing amounts of galectin-1 and cell surface marker expression was analyzed by flow cytometry. Data are normalized to maximal CD83 or CD86 expression (set at 100%) from three independent experiments. Nonlinear regression and EC_{50} was determined using GraphPad Prism. UnTx, untreated; gal-1, galectin-1.

blocked by 0.1 M lactose but not 0.1 M sucrose, indicating that galectin-1 binding to saccharide ligands on DCs is necessary for maturation (Fig. 1*C*). Galectin-1 exists as a noncovalent homodimer, and most extracellular effects of galectin-1 require the dimeric form (19). The EC_{50} of galectin-1 in inducing DC maturation is in the 6–8 μM range with respect to CD83 and CD86 expression (Fig. 1*D*), which is the approximate concentration at which galectin-1 forms noncovalent homodimers (22, 33). This suggests that galectin-1 dimerization is necessary to induce DC maturation. We confirmed this by using a monomeric mutant form of galectin-1 (N-Gal-1), that binds saccharide ligands but did not induce up-regulation of CD83 or any other maturation markers tested (Fig. 1*C*). This N-Gal-1 mutant has three amino acid replacements in the N terminus that disrupts the hydrophobic interface required for galectin-1 dimerization, and thus exists as a monomer at concentrations $<$250 μM yet still retains carbohydrate-binding capacity (28). We have previously shown that N-Gal-1 is indeed monomeric at our 20 μM working concentration (26). The dependence on carbohydrate binding and requirement for dimeric galectin-1 also suggest that this effect is specific to galectin-1, and not due to any contaminant in the preparations. To further ensure that the galectin-1 maturation is not due to potential endotoxin contamination, we preincubated cells with 10 μg/ml polymyxin B which blocked maturation in LPS-treated cells but not in galectin-1-treated cells (data not shown and Fig. 1, *B–D*, respectively).

Galectin-1-treated DCs exhibit functions characteristic of mature DCs

Immature DCs are very efficient at Ag uptake to enable sampling of the environment, and upon maturation the endocytic capacity decreases as mature DCs become focused on presenting Ag. As shown in Fig. 2, *A* and *B*, both LPS-treated DCs and galectin-1-treated DCs show limited uptake of FITC-dextran as compared with untreated DCs. The N-Gal-1 monomeric galectin-1 does not decrease the ability of the cells to uptake FITC-dextran, again indicating that dimeric galectin-1 is required to induce DC maturation (Fig. 2*B*). A hallmark of mature DCs is their ability to potently stimulate T cells to initiate an immune response. To determine whether galectin-1-treated DCs are truly mature functional DCs, we compared the ability of LPS-treated DCs and galectin-1-treated DCs to stimulate naive $CD4^+$ T cell proliferation in an allogeneic mixed lymphocyte reaction. Both LPS-treated DCs and galectin-1-treated DCs stimulate T cell proliferation at comparable efficiencies (Fig. 2*C*), suggesting that galectin-1 stimulates functionally mature DCs similar to those activated by LPS.

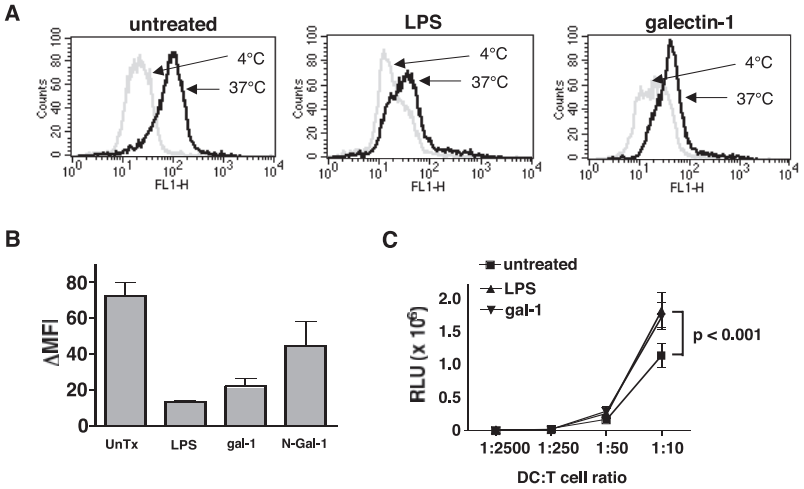


FIGURE 2. Galectin-1 functionally matures human MDDCs. *A* and *B*, MDDCs were left untreated or treated as described and FITC-dextran uptake was measured by flow cytometry. Data is shown as (*A*) representative histograms of FITC-dextran uptake (FL1-H) at both 37°C and 4°C (control) and (*B*) as mean ΔMFI (37°C MFI − 4°C MFI) ± SEM for three independent experiments. *C*, Allogeneic naive $CD4^+$ T cells (1×10^5) were incubated with untreated, LPS-treated, or galectin-1-treated MDDCs at the indicated ratios for 5 days. BrdU (10 μM) was added for the final 5 h of culture and BrdU incorporation was detected by chemiluminescent ELISA. Data are shown as mean ± SEM for three independent experiments. Values of *p* were calculated using paired *t* test. UnTx, untreated; gal-1, galectin-1.

Galectin-1-treated DCs secrete cytokines similar to LPS-treated DCs, yet do not produce the Th1-polarizing cytokine IL-12

Mature DCs secrete cytokines involved in priming immune responses as well as differentiation of naive T cells into effector T cells. Therefore, we analyzed MDDC secretion of seven different cytokines following treatment with LPS and with galectin-1. Both LPS and galectin-1-treated MDDCs produced high levels of IL-6 and TNF-α, cytokines that are involved in a generalized inflammatory response (Fig. 3). Additionally, LPS-treated MDDCs produced IL-12p70, whereas galectin-1-treated MDDCs produced no detectable IL-12p70. IL-12 has been reported to induce Th1 differentiation in T cells, and its absence in galectin-1-treated DCs suggests that galectin-1 is not involved in stimulating Th1 polarization. An equivalent concentration of N-Gal-1, the monomeric mutant form of galectin-1, induced 10-fold less IL-6 secretion than dimeric galectin-1 (Fig. 3), and also stimulated no IL-10 or TNF-α production. This demonstrates again that dimeric galectin-1 is required for DC maturation, suggesting that cross-linking of receptors is critical to induce maturation signals and augment cytokine production. Cytokine secretion was also decreased by >10-fold in the presence of 0.1 M lactose, again indicating that carbohydrate binding is involved in galectin-1-induced functional maturation.

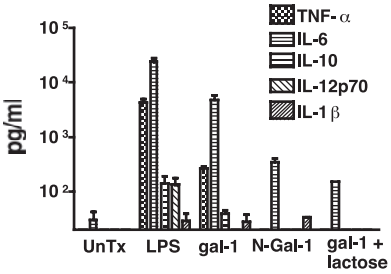


FIGURE 3. Galectin-1 induces unique cytokine expression by human MDDCs. Cytokine secretion by MDDCs measured by cytometric bead array using culture supernatant following 48 h culture of 3×10^5 cells/well with LPS (100 ng/ml), galectin-1 (20 μM) with or without lactose (0.1 M), or N-Gal-1 (20 μM). Data are shown as mean ± SEM for four independent experiments. Galectin-1-treated MDDCs show significantly increased secretion of IL-6, TNF-, and IL-10 ($p < 0.01$, calculated using Student's *t* test). UnTx, untreated; gal-1, galectin-1.

Galectin-1 uniquely regulates a subset of genes in addition to inducing expression of known DC maturation genes

Although the data in Figs. 1 and 2 demonstrate that both galectin-1 and LPS mature MDDCs, we noted that galectin-1-treated MDDCs have a unique cytokine expression profile distinct from LPS-treated MDDCs (Fig. 3). Given that cytokines are important determinants of distinct DC functions, we hypothesized that galectin-1-treated MDDCs may have unique functions that would not be revealed by our previous assays. In an effort to understand the distinct role of galectin-1 as an endogenous activator of DCs, we used HG-U133 plus 2.0 Affymetrix platforms to compare gene expression between MDDCs treated with LPS and galectin-1. To capture the most representative genetic picture, we evaluated gene expression 18 h after treatment, as a previous study has shown that this time point best encompasses early, middle, and late transcripts during DC maturation (16). Scatter plots (Fig. 4, *B* and *C*) show differential gene regulation in both LPS-treated and galectin-1-treated MDDCs as compared with control untreated MDDCs, as expected. More importantly, a comparison between galectin-1-treated and LPS-treated MDDCs also shows differential gene regulation (Fig. 4*D*). To ensure that DTT and polymyxin B, both present in our galectin-1 conditions, were not contributing to the differential gene expression, we also compared gene expression with MDDCs treated only with DTT and polymyxin B (vehicle control). As shown in Fig. 4*A*, there is no significant differential regulation between untreated and vehicle control MDDCs. To statistically assure that the vehicle conditions did not affect gene expression, the false discovery rate was assessed for the untreated vs vehicle-treated MDDCs using 100 permutations in dChip, resulting in 17 genes with a 100% false discovery rate (see *Materials and Methods*). This 100% false discovery rate assures that galectin-1-induced differential gene regulation is not due to the buffer components.

For our primary analysis, we compared gene expression between control untreated MDDCs and LPS-treated or galectin-1-treated MDDCs using a joint criteria of fold change >1.5 and $p <$

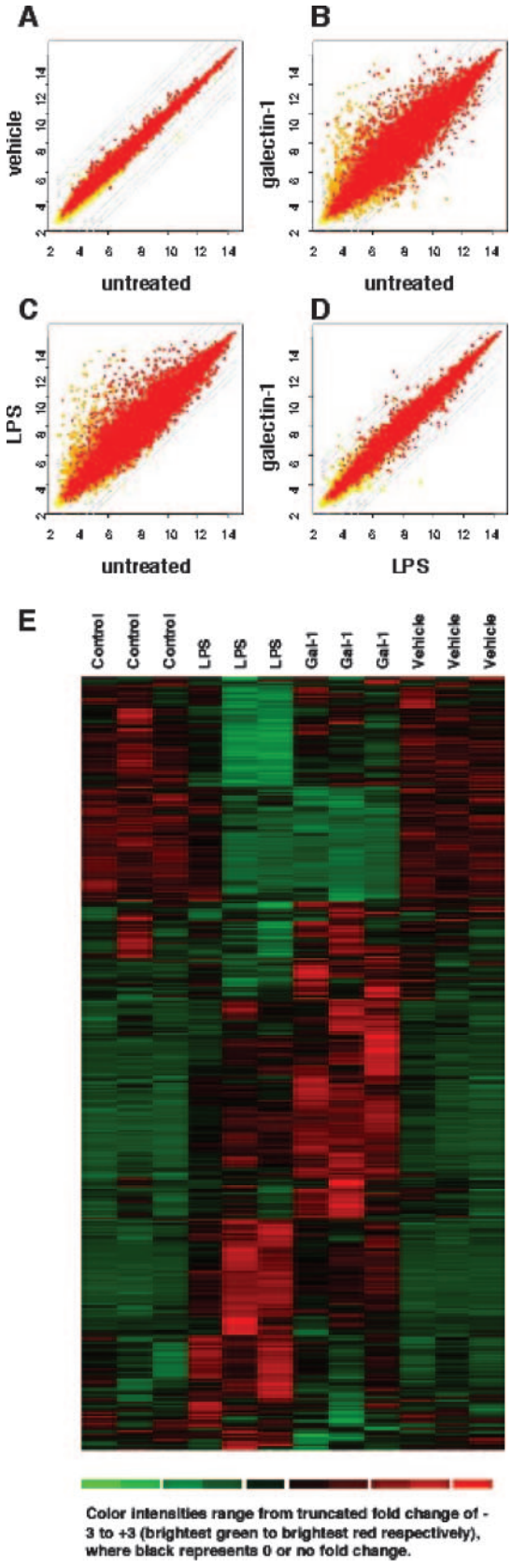


FIGURE 4. Galectin-1 induces unique gene expression in human MDDCs compared with LPS. Scatter plots of present, marginal, or absent (PMA) calls of different experimental conditions (created in R software). Dots represent genes: red is present in both conditions, orange is present in one condition and absent in the other, and yellow is absent in both conditions. Gray diagonal lines show fold change. Numbers on each axis show signal intensity from the chip (not log fold). Note that yellow dots are necessarily at the *lower left quadrant* of each scatter plot due to their low signal intensities which is at or below the background threshold. Each condition represents average of the three donors in that sample. *A*, Untreated MDDCs compared with vehicle (DTT and polymyxin B) treated MDDCs shows little gene differences. *B*, Untreated MDDCs compared with galectin-1-treated MDDCs shows differential gene up- and down-regulation. *C*, Untreated MDDCs compared with LPS-treated MDDCs shows differential gene up and down-regulation, and (*D*) direct comparison of LPS-treated MDDCs compared with galectin-1-treated MDDCs shows differential gene up- and down-regulation beyond 8-fold. *E*, Hierarchical clustering of all differentially regulated genes with mean fold change >1.2 in galectin-1 compared with LPS-treated MDDCs. A total of 1521 transcripts are shown in this cluster. Galectin-1 and LPS-treated MDDCs show clear differences in gene regulation, while vehicle and control MDDCs show similar patterns of gene expression.

0.05. This resulted in 885 transcripts differentially regulated between LPS-treated and control untreated cells, while 2412 transcripts were differentially regulated between galectin-1-treated and control untreated cells. As galectin-1-treated MDDCs regulate nearly 3-fold more genes than LPS-treated MDDCs, this might imply that galectin-1 modulates additional DC functions distinct from LPS. To further investigate differences between galectin-1 and LPS, we selected a subset of differentially regulated genes for functional analysis. Because only a fractional increase in the abundance of a protein may indeed have biologically important consequences (34), we used a liberal mean fold change >1.2 to obtain our subset of 1521 genes differentially regulated between LPS and galectin-1-treated MDDCs (Supplemental Table I).[5] Hierarchical clustering revealed clear differential regulation between LPS and galectin-1-treated samples within that fold change criteria (Fig. 4*E*), again suggesting that galectin-1 may regulate unique DC functions compared with LPS.

To validate our microarray data with the in vitro assays shown in Figs. 1 and 2, we compared patterns of gene regulation by both LPS-treated and galectin-1-treated MDDCs by hierarchical clustering of all samples using the list of genes involved in DC activation from the GEArray S Series Human Dendritic and Ag-Presenting Cell Gene Array (SuperArray) (data not shown). In addition to being functionally defined, many of these genes are characteristically expressed in mature DCs. Although the intensities of the gene regulation may differ slightly between LPS and galectin-1-treated samples, the overall direction and trend of expression is the same, including up-regulation of CD40, CD80, CD83, CD86, CCR7, ICAM1/CD54, and DCLAMP DC maturation markers (data not shown). There were also no significant differences in the level of CD14 expression between untreated, galectin-1 or LPS-treated MDDCs (data not shown), consistent with current concepts regarding the lack of CD14 expression in immature or mature MDDCs. This transcriptional data confirms our findings that galectin-1 induces a mature DC phenotype.

Functional analysis reveals significant differences in functional groups up-regulated by galectin-1

Previous studies have shown that certain transcription factors and other signaling proteins require only a small shift in expression to create a large difference in function. Therefore, it was important to include all differentially regulated genes regardless of magnitude to see whether an entire functional group is regulated (34, 35). For functional analysis, we used our subset of 1521 transcripts differentially regulated between galectin-1 and LPS-treated MDDCs (Fig.

[5] The online version of this article contains supplemental material.

4*E*). To compensate for the liberal fold change, a strict $p < 0.00001$ was used in the GOstat functional analysis. GOstat assigns a significance to overrepresented functional groups within a defined set of genes (32). GOstat functional analysis of overrepresented genes from this subset revealed a total of 26 groups in galectin-1-treated MDDCs with significant up-regulation as opposed to LPS-treated MDDCs (data not shown). Functional groups related to known galectin-1 activities, such as cell death, cell proliferation, carbohydrate binding and many more were also a part of this list, confirming the reliability of the analysis. Additional groups including chemotaxis ($p = 2.11 \times 10^{-7}$), taxis ($p = 2.11 \times 10^{-7}$), additional receptor binding ($p = 3.62 \times 10^{-10}$), and cytokine activity ($p = 7.42 \times 10^{-10}$) also were significantly up-regulated over LPS treatment, suggesting that galectin-1, as an endogenous activator, may regulate additional functions of DCs. No functional groups were found to be significantly down-regulated by galectin-1 in comparison to LPS-treated MDDCs, using the same criteria.

Strict fold change analysis reveals genes more specific to results of functional grouping

Due to inevitable donor-to-donor variance, the power of statistical tests cannot be relied upon exclusively to identify candidate genes

Table I. *Up-regulated genes in galectin-1-treated MDDCs relative to LPS-treated MDDCs*[a]

Genes with Mean Fold Change >7				
Gene Name	Accession Number	Gene ID	Fold Change	Function
Tenascin C (hexabrachlon)	NM_002160	TNC	7.51	Cell adhesion, protein binding, ECM
Plasminogen activator, tissue	NM_000930	PLAT	8.16	Plasmin-mediated proteolysis, cell migration
G protein-coupled receptor 109B	NM_006018	GPR109B	8.75	Orphan receptor, putative chemokine receptor
Transcribed locus	D59900		9.66	Unknown
Aldo-keto reductase family 1, member C2	U05598	AKR1C2	11.3	Member of the aldo/keto reductase superfamily
Matrix metalloproteinase 1 (interstitial collagenase)	NM_002421	MMP1	13.05	Processing and degrading ECM proteins, tissue remodeling, angiogenesis, cell migration, invasion, and metastasis
Matrix metalloproteinase 10 (stromelysin 2)	NM_002425	MMP10	14.99	Processing and degrading ECM proteins, tissue remodeling, angiogenesis, cell migration, invasion, and metastasis
Matrix metalloproteinase 12 (macrophage elastase)	NM_002426	MMP12	17.93	Processing and degrading ECM proteins, tissue remodeling, angiogenesis, cell migration, invasion, and metastasis
Genes with p Value <0.01 and Mean Fold Change >1.5				
Gene Name	Accession Number	Gene ID	Fold Change	Function
UDP-Gal:β GlcNAc β 1,3-galactosyl-transferase, polypeptide 3	AB050856	B3GALT3	1.92	Member of the β-1,3-galactosyltransferase gene family
Tenascin C (hexabrachlion)	NM_002160	TNC	7.51	Cell adhesion, protein binding, ECM
TRAF3-interacting JNK-activating modulator	NM_025228	TRAF3IP3	2.62	Kinase activity
Actinin, α1	BC003576	ACTN1	1.59	Binding actin to the membrane, cytoskeletal reorganization
KIAA1671 protein	AB051458		2.36	Unknown
MOB1, Mps one binder kinase activator-like 2B	NM_024761	MOBKL2B	1.92	Protein kinase essential for spindle pole body duplication, mitotic checkpoint regulation
CDNA clone IMAGE:4514712, partial cds	AK026181		1.75	Unknown

[a] Gene functions obtained from GeneCards and EntrezGene Databases of gene ontologies and functions.

(36). To find candidate genes that we could specifically test for function, we used a combined comparison based on fold change or *p* value. We ranked our initial subset of 32,142 transcripts according to: 1) fold change and 2) lowest *p* value. In this specific analysis, we wanted to identify genes with the strongest functional implication. Because we found no significant functional groups were down-regulated in galectin-1-treated MDDCs, we focused our ranking only on genes up-regulated in galectin-1-treated MDDCs vs LPS-treated MDDCs. Identifying genes according to fold change revealed eight genes with mean fold change >7 in galectin-1-treated MDDCs compared with LPS-treated MDDCs (Table I). The second ranking method, based on statistical *p* values, identified seven genes differentially regulated between LPS and galectin-1-treated MDDCs with a minimum mean fold change >1.5 and $p < 0.01$ (Table I). Upon examining the known functions in these two subsets of genes, we noted that many of these genes encode proteins that participate in cell migration, such as actin binding and remodeling proteins (*α-actinin 1*), adhesion molecules (*tenascin-C*), and ECM degrading proteinases (*MMP-1*, *MMP-10*, *MMP-12*, and *tissue plasminogen activator*). This gene list, identified using statistical methods as well as greatest mean fold change, coupled with the functional grouping results previously mentioned, suggests that enabling DC migration could be one unique and additional function of galectin-1.

Galectin-1-treated DCs migrate through Matrigel more efficiently than LPS-treated DCs

A critical function of DCs is migration both to sites of inflammation and from tissues to secondary lymphoid organs. This involves movement through the ECM, which uses MMPs to degrade matrix proteins and facilitate migration (5). To confirm our microarray data regarding MMP expression in galectin-1-treated MDDCs, we used real-time quantitative RT-PCR on three separate human donors. We found that despite donor-to-donor variance in fold change, galectin-1 consistently induced the highest mRNA expression in all three tested MMP genes (*MMP-1*, *MMP-10*, and *MMP-12*) previously identified from our microarray data (Fig. 5). Given that three MMPs are highly expressed in galectin-1-treated MDDCs, and the subset of genes uniquely up-regulated in galectin-1-treated DCs share a common function in cell-ECM interactions, we wanted to compare DC migration through ECM between galectin-1-treated and LPS-treated DCs. To do this, we tested MDDC migration through in vitro Transwell assays using Matrigel, a biologically active basement membrane matrix, following treatment with LPS or galectin-1. We used a physiologically relevant chemoattractant, CCL19 (MIP-3β), a lymph node homing chemokine for DCs that is the cognate ligand for CCR7, a chemokine receptor highly expressed on mature DCs. Our initial experiments showed that CCR7 cell surface expression and transcript levels were comparable between galectin-1 and LPS-matured MDDCs (data not shown), implying that differences in migration were not be due to differences in receptor expression. Galectin-1-treated MDDCs migrated through Matrigel toward a MIP-3β gradient significantly better than LPS-treated DCs (Fig. 6*A*, $p = 0.002$). However, chemotaxis through Transwells without Matrigel coating was similar between LPS and galectin-1-treated DCs (Fig. 6*B*), indicating that galectin-1-matured DCs preferentially migrate through ECM compared with LPS-matured DCs. To test whether galectin-1 also enhanced transendothelial migration of MDDCs, we repeated the migration assays using Transwells coated with a monolayer of HMVECs. In this assay, transendothelial migration was similar between LPS and galectin-1-treated DCs across an HMVEC monolayer (Fig. 6*C*). This suggests that galectin-1-mediated enhancement of MDDC migration is specific for invasive-type ECM migration, and does not affect migration across the endothelium.

Discussion

DC responses to pathogen and inflammatory stimuli are marked by phenotypic changes and acquired functional ability. We have shown that galectin-1, an endogenous lectin present constitutively and increased with inflammation, is able to induce cell surface marker expression in MDDCs consistent with a mature

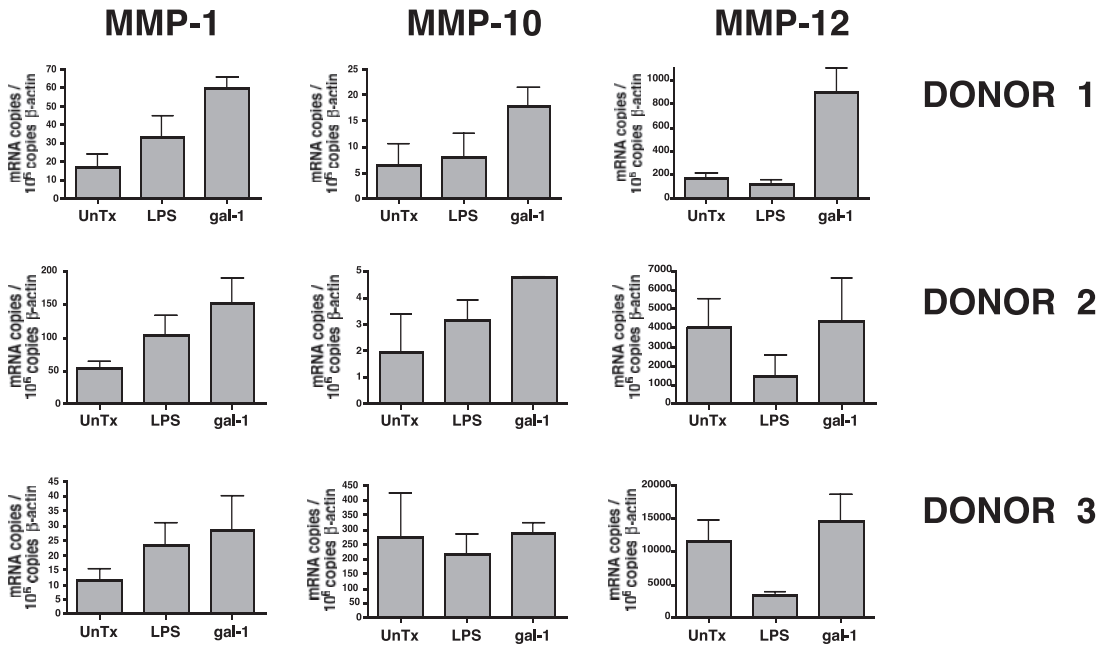
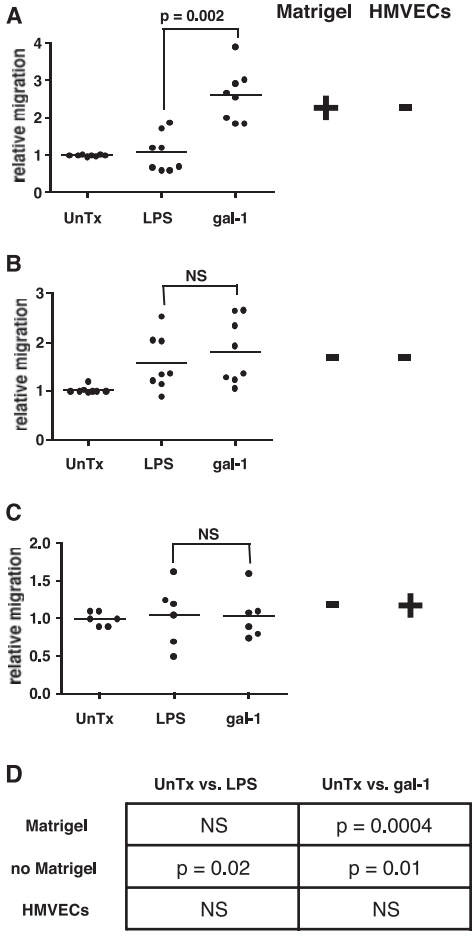


FIGURE 5. Quantitative RT-PCR of MMP mRNA expression shows higher expression in galectin-1-treated MDDCs. Total RNA from human MDDCs untreated or treated with LPS (100 ng/ml) or galectin-1 (20 μM) was isolated and used for real-time quantitative RT-PCR. Data are shown as mRNA copies of each gene normalized to the housekeeping gene, β-actin ± SD from three replicates.



D

	UnTx vs. LPS	UnTx vs. gal-1
Matrigel	NS	p = 0.0004
no Matrigel	p = 0.02	p = 0.01
HMVECs	NS	NS

FIGURE 6. Galectin-1 augments human MDDC migration through Matrigel. Migration of MDDCs untreated or treated with LPS (100 ng/ml) or galectin-1 (20 μM) was measured using Transwell assays with (*A*) Matrigel-coated inserts (8.0-μm pore) or (*B*) inserts without Matrigel coating (8.0-μm pore). For transendothelial migration assays (*C*), human microvascular endothelial cells (HMVECs) were grown to confluency on Transwell inserts (8.0-μm pore). Bottom chamber contained 200 ng/ml CCL19 (MIP-3β) chemoattractant. Each experiment consists of at least two independent counts. Data are shown as relative migration compared with immature MDDCs in which the average from all counts in each experiment, represented by a bar, is set at 1.0. *D*, Values of *p* were calculated using paired *t* test. NS, No statistical difference; UnTx, untreated; gal-1, galectin-1.

DC phenotype. Additionally, galectin-1 matures DCs to functional APCs exhibiting decreased uptake of FITC-dextran, potent T cell-stimulating capacity, and production of inflammatory cytokines IL-6, TNF-α, and IL-10 similar to LPS. However, in contrast to LPS, galectin-1-treated DCs do not produce the Th1-polarizing cytokine IL-12. Galectin-1 similarly induces expression of genes known to be involved in DC activation and maturation, but also uniquely regulates expression of a significant subset of genes belonging to distinct functional gene ontologies. Namely, galectin-1-treated DCs highly express many genes related to cell migration, and in an in vitro model of ECM migration, galectin-1-treated MDDCs migrate significantly better than LPS-treated MDDCs.

Galectin-1 is expressed in multiple anatomic sites, and accumulates most highly in ECM and lymphoid stroma (37, 38). Galectin-1 expression and export in endothelial cells is increased by inflammatory cytokines, and increased endothelial expression has been shown in inflamed lymph nodes (39). Furthermore, we and others have detected galectin-1 expression in DCs (40), and proteomic analysis has shown that galectin-1 expression increased upon DC maturation (41). As we have now shown that galectin-1 activates DCs, it is possible that this increase in galectin-1 expression by both endothelial cells and DCs serves, in part, as an endogenous feedback mechanism to amplify the immune response at the initial site of inflammation and infection. Galectin-1-treated DCs express cytokines that act as autocrine regulators, particularly IL-6 and TNF-α, that induce DC maturation and enhance immunostimulatory capacity (42). Galectin-1 has been repeatedly demonstrated to possess anti-inflammatory properties for other immune cells as well as models of immune-mediated disease (19). It may seem paradoxical that we now show that galectin-1 has immune-stimulating activity in DCs. Our study shows that galectin-1-mediated DC maturation requires a relatively high concentration of galectin-1, 20 μM, to induce a maximal maturation phenotype (Fig. 1*D*). This concentration, while physiologically plausible, is higher than the concentrations used in previous studies. Such a high concentration can accumulate in stromal sites, and possibly endothelial cells, during inflammatory states when galectin-1 expression is increased (37). Thus, we postulate a bifunctional role for galectin-1 in immune regulation. During initial inflammation, when galectin-1 concentrations are high, galectin-1 functions to enhance immune activation by maturing DCs and augmenting their migration into and out of tissues. As infection and inflammation wane and the local galectin-1 concentration decreases, the anti-inflammatory and immune-suppressing effects prevail. We acknowledge that this study represents in vitro data only, and we cannot formally exclude potential confounding factors due to prolonged in vitro culture conditions. However, further studies are warranted to determine whether galectin-1 may indeed have opposing functions in regulating inflammation and immunity.

Critical to DC function in initiating an immune response is the ability to migrate across endothelial barriers and into and out of tissue; however, regulation of DC migratory behavior is still relatively unexplored. Although the role of chemokines and various adhesion molecules in lymph node homing has been well-studied, the entry and exit from peripheral tissues is not well-understood. Our microarray analysis revealed significant up-regulation in galectin-1-treated DCs of genes potentially involved in different aspects of cell migration. We found that the relative migration of galectin-1-treated DCs through Matrigel was significantly higher than LPS-treated DCs (Fig. 6*A*). Cells must undergo cytoskeletal rearrangements to permit motility, and some of our most significantly up-regulated genes are involved in actin binding and stabilization to the cell membrane and extracellular adhesion molecules (e.g., *α-actinin-1*, Table I) (3, 4). However, up-regulation of these particular genes is likely not sufficient for the observed functional difference between galectin-1 and LPS-treated MDDCs because enhanced migration was only observed through Matrigel. It is possible that these cytoskeletal genes act in conjunction with some of the other up-regulated genes in Table I known to be involved in migration through ECM (e.g., *MMPs* and *tissue plasminogen activator*) (7, 43). Significantly increased expression of all these genes together may suggest a functional cooperativity in aiding DC migration. If galectin-1 acts as an endogenous enhancer of DC activation and function, enabling invasive-type migration into and out of tissues would allow DCs to more efficiently encounter Ag and travel to lymphoid organs to initiate an adaptive immune response. Perhaps galectin-1, present at inflammatory sites, serves to regulate this tissue migration and aids in the ability of mature DCs to respond to chemotactic signals.

MMPs, specifically MMP-9 and MMP-2, play a role in DC migration (8, 9). We found both MMP-9 and MMP-2 transcripts are expressed in galectin-1-treated MDDCs at levels similar to, or slightly higher than, LPS-treated MDDCs (data not shown). In addition, galectin-1-treated MDDCs highly express other MMPs (MMP-1, MMP-10, and MMP-12), which currently have no known role in DC migration but likely share similar functions. These most highly up-regulated genes, *MMP-1*, *MMP-10*, and *MMP-12*, were confirmed using quantitative RT-PCR (Fig. 5). However, MMPs undergo extensive posttranslational regulation (5), and in this study based on mRNA expression we can only postulate a link between expression of these genes and enhanced ECM migration. Further functional studies are needed to assess the role of galectin-1 in modulating MMP activity in DCs. Interestingly, two of our highly up-regulated genes, *tenascin-C* and *tissue plasminogen activator*, in addition to roles in migration, have been reported to modulate MMP expression in other cell types (44–46). Whether these genes actually affect MMP activity in galectin-1-treated DCs, and the extent to which they actually contribute to the increased migratory behavior of galectin-1-treated DCs through ECM, is a subject of ongoing studies. Altogether, these genes may represent a small portion of an intricate network, modulated in part by galectin-1, to regulate DC migration in response to inflammation.

Clinical trials recently have had some success using therapeutic vaccines based upon ex vivo-matured DCs (47). Administration of these DC-based vaccines typically involves intradermal injection, and the efficacy of such vaccine is therefore dependent on the ability of the DCs to successfully migrate out of the skin and reach the lymph nodes (48). As we have shown that galectin-1 maturation of DCs induces gene expression that may enhance DC migration through ECM, and presumably out of tissues, it may be useful to consider the mechanism of DC maturation in studies optimizing ex vivo maturation of DCs for more effective vaccines. In this study, we have shown that not only does galectin-1 mature human DCs to functional, immune-stimulating cells, but it also enhances the migratory capacity of DCs, making galectin-1 an attractive candidate as an adjuvant for cell-based vaccines. Further understanding of the role of galectin-1-treated DCs in the context of the immune response will allow greater understanding of endogenous immune regulation, and provide targets for novel immune-based therapeutics.

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Disclosures

The authors have no financial conflict of interest.

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