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**IMMUNOPATHOLOGY AND INFECTIOUS DISEASES****Galectin-3 Modulates Th17 Responses by Regulating Dendritic Cell Cytokines**

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Galectin-3 is a β -galactoside-binding animal lectin with diverse functions, including regulation of T helper (Th) 1 and Th2 responses. Current data indicate that galectin-3 expressed in dendritic cells (DCs) may be contributory. Th17 cells have emerged as critical inducers of tissue inflammation in autoimmune disease and important mediators of host defense against fungal pathogens, although little is known about galectin-3 involvement in Th17 development. We investigated the role of galectin-3 in the induction of Th17 immunity in galectin-3-deficient ($gal3^{-/-}$) and $gal3^{+/+}$ mouse bone marrow-derived DCs. We demonstrate that intracellular galectin-3 negatively regulates Th17 polarization in response to the dectin-1 agonist curdlan (a β -glucan present on the cell wall of fungal species) and lipopolysaccharide, agents that prime DCs for Th17 differentiation. On activation of dectin-1, $gal3^{-/-}$ DCs secreted higher levels of the Th17-axis cytokine IL-23 compared with $gal3^{+/+}$ DCs and contained higher levels of activated c-Rel, an NF- κ B subunit that promotes IL-23 expression. Levels of active Raf-1, a kinase that participates in downstream inhibition of c-Rel binding to the *IL23A* promoter, were impaired in $gal3^{-/-}$ DCs. Modulation of Th17 by galectin-3 in DCs also occurred *in vivo* because adoptive transfer of $gal3^{-/-}$ DCs exposed to *Candida albicans* conferred higher Th17 responses and protection against fungal infection. We conclude that galectin-3 suppresses Th17 responses by regulating DC cytokine production. (*Am J Pathol* 2013, 183: 1209–1222; <http://dx.doi.org/10.1016/j.ajpath.2013.06.017>)

IL-17-producing CD4⁺ T helper 17 (Th17) cells play an essential role in the clearance of extracellular bacterial and fungal pathogens^{1,2} and promote inflammatory responses involved in autoimmune disease.^{3,4} The factors associated with Th17 cell development have been well characterized and include cytokines such as transforming growth factor- β (TGF- β) and IL-6, which promote Th17 differentiation in mice,^{5,6} and IL-23, which is required for Th17 cell expansion and effector functions.⁴ Dendritic cells (DCs) express germline-encoded pattern recognition receptors (PRRs) that recognize conserved molecular components expressed on microbial pathogens⁷ and are capable of generating the appropriate co-stimulatory molecules and cytokines that support Th17 development. Among the receptors known to induce Th17-promoting factors is dectin-1, a C-type lectin receptor that recognizes β -glucans expressed on cell walls of fungi.^{8,9} Engagement of dectin-1 with the β -glucan curdlan

or *Candida albicans* has been shown to stimulate IL-23 production in DCs and promote Th17 cell responses *in vitro* and *in vivo*.^{10,11} Members of the Toll-like receptor (TLR) family, specifically TLR2, TLR3, TLR4, and TLR9, have also been reported to elicit Th17 responses through the induction of Th17-promoting cytokines.^{6,12}

Galectin-3 is a β -galactoside-binding animal lectin, a pleiotropic protein capable of participating in a variety of cellular processes through intracellular and extracellular mechanisms.^{13,14} Extracellular galectin-3 has been shown to modulate cell adhesion, cell activation, and cell migration,¹⁵ whereas intracellular galectin-3 has been implicated in the regulation of cell survival,¹⁶ pre-mRNA splicing,¹⁷ and

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phagocytosis.¹⁸ We previously demonstrated that intracellular galectin-3 translocates to lipid raft microdomains in mouse bone marrow–derived DCs (BMDCs) on chemokine receptor activation and positively regulates DC migration.¹⁹ Increasing evidence suggests that galectin-3 may also play a role in the regulation of Th1/Th2 differentiation by affecting IL-12 production in DCs.^{20–22} However, the role of galectin-3 in Th17 development remains largely undefined. Regarding innate immunity, galectin-3 has been shown to be involved in the recognition of several microbial species and innate defense.²³ Its expression is essential for the recognition of *C. albicans* by macrophages,^{24,25} and it has fungicidal activity against *Candida* species,²⁶ indicating a direct role for the lectin in antifungal immunity. Whether galectin-3 plays a role in antifungal defense through the Th17 response has not been investigated.

Using an ovalbumin (OVA)-specific T-cell activation model, we compared antigen-specific Th17 polarization induced by *gal3*^{+/+} and *gal3*^{-/-} BMDCs. *Gal3*^{-/-} DCs primed with curdlan or high-dose lipopolysaccharide (LPS) produced higher levels of IL-17–axis cytokines and induced higher Th17 responses compared with *gal3*^{+/+} DCs. These findings were also observed in the presence of lactose in culture media, suggesting that galectin-3 affects DC functions through an intracellular mechanism. In addition to differences in cytokine secretion, *gal3*^{-/-} DCs treated with either curdlan or high-dose LPS exhibited impaired mitogen-activated protein kinase extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, suggesting that galectin-3 may regulate DC cytokine expression in response to dectin-1 and TLR4 stimulation through a common mechanism. Furthermore, curdlan-stimulated *gal3*^{-/-} DCs expressed higher levels of c-Rel, a critical transcription factor involved in IL-23 production. On closer examination of dectin-1 signaling pathways, we found that curdlan-stimulated *gal3*^{-/-} DCs expressed lower levels of phosphorylated Raf-1, a serine-threonine kinase that negatively regulates IL-23. In agreement with our *in vitro* studies, the adoptive transfer of *C. albicans*–exposed *gal3*^{-/-} DCs into mice induced higher Th17 responses and promoted greater fungal clearance than *gal3*^{+/+} DCs, indicating that galectin-3 in DCs plays a pivotal role in the induction of antifungal immunity *in vivo*. Taken together, these findings reveal a novel role for endogenous galectin-3 in the regulation of DC cytokine expression involved in Th17 induction. Thus, targeting galectin-3 in DCs may be a means to modulate adaptive immune responses to fungal infections and in autoimmune disease.

Materials and Methods

Mice

Gal3^{-/-} mice were developed as previously described²⁷ and were backcrossed to C57BL/6 mice for nine generations. *Gal3*^{+/+} and *gal3*^{-/-} littermates obtained from heterozygous

breeders were used to prepare BMDCs. OVA-TCR transgenic mice (OT-II) on the C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice used in adoptive transfer experiments were bred in-house or were purchased from The Jackson Laboratory. All the experiments were approved by the Institutional Animal Care and Use Committee of the University of California, Davis (Sacramento, CA) or the National Taiwan University College of Medicine (Taipei, Taiwan).

Reagents

LPS from *Escherichia coli* O111:B4 was from List Biological Laboratories (Campbell, CA). Heat-killed *C. albicans* (HKCA) was from InvivoGen (San Diego, CA). β -Lactose, sucrose, and curdlan were from Sigma-Aldrich (St. Louis, MO). Recombinant granulocyte-macrophage colony-stimulating factor, IL-1 β , IL-6, IL-10, IL-12, IL-17A, and interferon γ (IFN- γ) were from PeproTech (Rocky Hill, NJ) and recombinant IL-23 was from eBioscience Inc. (San Diego, CA). All capture and detection antibodies for enzyme-linked immunosorbent assays (ELISAs) and neutralizing antibodies against IL-6 (MP5-20F3), IL-23 p19 (G23-8), and IL-12/23 p40 (C17.8) were from eBioscience Inc. Anti-phospho-p44/p42 (ERK1/2) mitogen-activated protein kinase, total p44/p42, and anti-phospho-Raf-1 (Ser 338) (56A6) were from Cell Signaling Technology Inc. (Danvers, MA), and anti-phospho-Raf-1 (Tyr340/341) (44-506G) was from Invitrogen (Carlsbad, CA). Total Raf-1 antibody was from BD Transduction Laboratories (San Jose, CA).

Preparation of Endotoxin-Free OVA

Egg whites from organic eggs were extracted under sterile conditions and were lyophilized using a SpeedVac concentrator (Thermo Fisher Scientific Inc., Rockford, IL). Lyophilized egg whites were reconstituted in sterile PBS, and the protein concentrations were determined by bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL). Endotoxin levels were below the level of detection, as determined by Pyrogen gel clot limulus amoebocyte lysate assay (Lonza Inc., Walkersville, MD).

In Vitro Generation and Activation of BMDCs

BMDCs were generated from *gal3*^{+/+} and *gal3*^{-/-} mice by culturing bone marrow cells for 8 to 10 days as described previously²⁸ in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Thermo Fisher Scientific Inc.), penicillin/streptomycin (Invitrogen), 2ME (Gibco, Carlsbad, CA), HEPES (Mediatech Inc., a Corning subsidiary, Manassas, VA), minimal essential medium nonessential amino acids (Invitrogen), and 20 ng/mL of granulocyte-macrophage colony-stimulating factor (PeproTech). For measuring cytokine production, 2×10^5 DCs per well were stimulated with 0.1 or 100 ng/mL of LPS;

1, 10, or 100 $\mu\text{g/mL}$ of curdlan; or 10^5 , 10^6 , 10^7 cells/mL of HKCA in 96-well plates at 200 μL per well for 24 hours.

In Vitro Activation of CD4⁺ T Cells by DCs

CD4⁺ cells were purified from OT-II splenocytes using biotinylated anti-mouse CD4⁺ and a biotin selection kit (STEMCELL Technologies Inc., Vancouver, BC, Canada) according to the manufacturer's instructions. For OT-II activation, DCs were pulsed overnight (approximately 16 hours) with 100 $\mu\text{g/mL}$ of endotoxin-free OVA alone or in the presence of 0.1 ng/mL of LPS, 100 ng/mL of LPS, or 10 $\mu\text{g/mL}$ of curdlan. DCs were washed and co-cultured with CD4⁺ OT-II cells (100,000 CD4⁺ cells per well and 20,000 DCs per well) in 96-well round-bottomed plates. In some conditions, neutralizing antibodies were added at 10 $\mu\text{g/mL}$ on day 0. Cell culture supernatants were harvested after 3 days, and cytokines were measured by ELISA.

Cell Signaling

DCs were plated in media without fetal bovine serum at 2×10^6 cells per well in 12-well plates for 2 hours and then were left untreated or stimulated with curdlan or LPS for 5, 10, 30, or 60 minutes. Cell lysates were prepared by resuspending cell pellets in lysis buffer [20 mmol/L Tris (pH 7.4), 137 mmol/L NaCl, 2 mmol/L EDTA (pH 7.4), 1% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany), 25 mmol/L β -glycerophosphate, 2 mmol/L sodium pyrophosphate, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na_3VO_4 , 5 mmol/L NaF, and $1 \times$ protease inhibitor cocktail] before centrifugation at 12,000 rpm, 4°C for 20 minutes. Twenty micrograms of protein were loaded per lane onto a 10% SDS-PAGE gel, and the separated proteins were transferred onto polyvinylidene difluoride membranes. Membranes were incubated with the indicated antibodies, and bands were detected by using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific Inc.).

Flow Cytometry

For cell surface staining of co-stimulatory molecules, DCs were incubated with anti-CD80-PE, anti-CD86-PE, and anti-major histocompatibility complex II-fluorescein isothiocyanate (eBiosciences Inc.) in PBS containing 1% fetal bovine serum and 0.09% sodium azide for 30 minutes and were analyzed by flow cytometry (BD FACScan; BD Biosciences, San Jose, CA). To compare cell viability in DCs after activation, cells were first stained with anti-CD11c-fluorescein isothiocyanate (eBiosciences Inc.) and were subsequently stained with 7-aminoactinomycin D (Sigma-Aldrich) in PBS on ice for 30 minutes. After washing, cells were fixed in 1% paraformaldehyde supplemented with 50 $\mu\text{g/mL}$ of actinomycin D before flow cytometry analysis. To compare regulatory T cell (Treg) induction by

curdlan-OVA-stimulated DCs, T-cell-DC co-cultures were harvested after 3 days and were stained using anti-CD4-PE-Cy5 (eBioscience Inc.) and anti-CD25-PE (BD Pharmingen, San Diego, CA). Cells were fixed and permeabilized using a BD Cytfix/Cytoperm kit before intracellular staining with anti-Foxp3-fluorescein isothiocyanate (BD Pharmingen).

Detection of Raf-1 Phosphorylation

DCs were stimulated with curdlan for 15 minutes and then were lysed with buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Nonidet P-40 (Caledon Laboratories Ltd., Georgetown, ON, Canada), 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na_3VO_4 , 5 mmol/L NaF, and protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were immunoprecipitated with mouse anti-Raf-1 and recombinant Protein G-Sepharose 4B (Zymed-Invitrogen, Carlsbad, CA). Phosphorylated Raf-1 was detected by immunoblotting with rabbit anti-phospho-Raf-1 (Ser338) and rabbit anti-phospho-Raf-1 (Tyr340/341).

Transcription Factor Activation Assays

1×10^6 DCs per milliliter were stimulated with curdlan for 2 hours. Nuclear extracts from DCs were prepared using an NE-PER nuclear and cytoplasmic extraction reagents kit (Thermo Fisher Scientific Inc.). NF- κB DNA binding was performed by using a TransAM NF- κB family kit (Active Motif, Carlsbad, CA).

Immunization with Antigen-Pulsed DCs

The protocol for immunization with antigen-pulsed DCs was adapted from that of d'Ostiani et al²⁹ with slight modifications. DCs were stimulated with 5×10^5 HKCA cells/mL overnight. Cells were washed in sterile PBS and were s.c. injected twice, a week apart, into C57BL/6 mice at a concentration of 5×10^5 cells per mouse in 50 μL of PBS. Seven days after the last injection of DCs, splenocytes were harvested and restimulated with HKCA at 1×10^5 cells/mL. CD4⁺ cells were also purified from splenocytes and were restimulated with HKCA-treated antigen-presenting cells. After 72 hours, supernatants were harvested for cytokines.

Adoptive Transfer of DCs and Fungal Infection

The following protocol for adoptive transfer of DCs was followed: 1×10^6 DCs/mL were co-cultured with 1×10^6 viable *C. albicans* cells/mL in RPMI 1640 medium at room temperature for 1 hour. The mixture was subsequently i.v. injected into wild-type (*gal3^{+/+}*) mice (500 μL per mouse). Brain and kidney were harvested 3 and 6 days after infection. To determine the cytokine levels, brain and kidney tissues were homogenized in lysis buffer (RayBiotech Inc., Norcross, GA). Homogenate supernatants were collected after

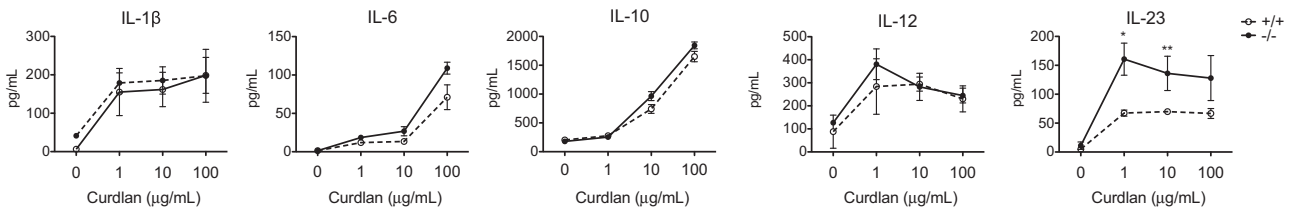


Figure 1 Curdlan-stimulated *gal3*^{-/-} DCs produced higher levels of IL-23 compared with *gal3*^{+/+} DCs. ELISA of cytokines produced by *gal3*^{+/+} and *gal3*^{-/-} DCs after stimulation with various amounts of curdlan overnight. Data are given as means ± SD and are representative of at least four independent experiments. **P* < 0.05, ***P* < 0.01.

centrifugation, and cytokine concentrations were measured by ELISA. For fungal burden determination, tissues were homogenized in RPMI 1640 medium using a tissue grinder. Homogenates were serially diluted and plated on YPD agar. Yeast colonies were counted 3 days after incubation at 30°C. CD4⁺ T cells from the spleen were purified using an EasySep mouse CD4⁺ selection kit (STEMCELL Technologies Inc.).

Real-Time Quantitative PCR

Total RNA was extracted from purified CD4⁺ T cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized with Moloney Murine Leukemia Virus High-Performance Reverse Transcriptase (Epicentre Biotechnologies, Madison, WI) according to the manufacturer’s instructions. Real-time quantitative PCR (qPCR) was performed by using the iCycler real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The primers (forward and reverse) used for qPCR are as follows: *mll17a*: 5'-AGGCAGCAGCGATCATCC-3' and 5'-TGGAACGGTTGAGGTAGTCTG-3'; *mll-17f*: 5'-CTG-GAGGATAACACTGTGAGAGT-3' and 5'-TGCTGAAT-GGCGACGGAGTTC-3'; *RORγt*: 5'-CGCCTCACCTGA CCTACC-3' and 5'-TTGCCTCGTTCTGGACTAT-AC-3'; *RORα*: 5'-TCTCCCTGCGCTCTCCGCAC-3' and 5'-TCC-ACAGATCTTGCATGGA-3'; and *mβ-actin*: 5'-TGTAT-GAAGGCTTTGGTCTCCCT-3' and 5'-AGGTGTGCACT TTTATTGGTCTCAA-3'. Each sample was analyzed in triplicate. The relative cytokine mRNA expression level of each sample was normalized against β-actin expression.

Histologic Staining

H&E and PAS staining of tissue sections was performed in the Pathology Core Laboratory at the Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan. Enumeration of infiltrated neutrophils in H&E slides was performed by a certified veterinary pathologist at the Institute of Biomedical Sciences Pathology Core Laboratory based on the presence of polymorphic nuclei and appropriate cell size.

Statistical Analysis

Statistical analysis of experimental groups was performed by unpaired Student’s *t*-tests using GraphPad Prism 5.0

software (GraphPad Software Inc., San Diego, CA). *P* < 0.05 was considered significant.

Results

Galectin-3 in DCs Negatively Regulates Th17 Polarization in Response to Dectin-1 Agonists

β-Glucans present on the cell wall of fungal species activate dectin-1 and induce the production of Th1- and Th17-polarizing cytokines in DCs.¹⁰ To study the effect of galectin-3 on DC cytokine production in response to dectin-1 activation, we compared cytokine profiles in *gal3*^{-/-} and *gal3*^{+/+} DCs after stimulation with the β-glucan curdlan. Curdlan-stimulated *gal3*^{-/-} DCs secreted higher levels of IL-23 compared with *gal3*^{+/+} DCs. In contrast, levels of IL-1β, IL-6, IL-10, and IL-12 were comparable (Figure 1), and levels of TGF-β were undetectable (data not shown). Differences in cytokine levels were not due to differences in cell death after stimulation (Supplemental Figure S1A). Furthermore, curdlan induced surface expression of co-stimulatory molecules on *gal3*^{-/-} and *gal3*^{+/+} DCs to a similar extent (Supplemental Figure S1, B and C).

To investigate the ability of galectin-3 to influence DC-mediated Th17 polarization in response to β-glucan, we pulsed *gal3*^{-/-} or *gal3*^{+/+} DCs with endotoxin-free OVA in the presence of curdlan, co-cultured the cells with OT-II CD4⁺ cells, and measured T-cell cytokines in co-culture supernatants. *Gal3*^{-/-} DCs induced more antigen-specific IL-17 production relative to *gal3*^{+/+} DCs after curdlan treatment (Figure 2A). Apart from Th17 cells, curdlan-stimulated DCs also promote the induction of Th1 cells.¹⁰ Curdlan-primed *gal3*^{-/-} DCs induced higher IFN-γ secretion in T cells relative to *gal3*^{+/+} DCs; however, the differences were not statistically significant. As expected, DCs pulsed with endotoxin-free OVA alone failed to activate OT-II cells, presumably owing to relatively low levels of co-stimulatory molecule expression.³⁰ Using a different approach, we cultured *gal3*^{-/-} and *gal3*^{+/+} DCs with curdlan for 16 hours and tested Th17-polarizing activity in the presence of the superantigen *Staphylococcus Enterotoxin B*. In agreement with the findings from the OVA-specific T-cell activation model described in Figure 2A, curdlan-primed *gal3*^{-/-} DCs induced higher IL-17 production in CD4⁺ cells compared with *gal3*^{+/+} DCs (Figure 2B).

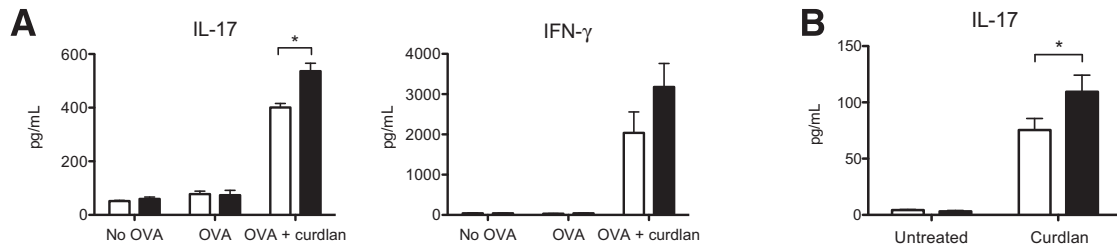


Figure 2 Galectin-3 in DCs negatively regulated Th17 responses. **A:** ELISA of cytokines in supernatants of CD4⁺ OT-II T cells that had been cultured with OVA-pulsed *gal3*^{+/+} (white bars) or *gal3*^{-/-} (black bars) DCs in the presence or absence of curdlan for 3 days. **B:** ELISA of IL-17 in supernatants of CD4⁺ cells cultured with curdlan-primed *gal3*^{+/+} (white bars) or *gal3*^{-/-} (black bars) DCs for 3 days in the presence of the superantigen *Staphylococcus enterotoxin B*. Data are given as means \pm SD. Each experiment was performed at least two times. **P* < 0.05.

Tregs have been reported to act as a source of TGF- β to drive Th17 differentiation.⁶ To determine whether galectin-3 in DCs indirectly affected Th17 responses by altering Treg development, we compared the number of CD4⁺CD25⁺Foxp3⁺ cells induced by curdlan-primed *gal3*^{-/-} and *gal3*^{+/+} DCs. The frequencies of Foxp3⁺ Treg cells induced by both DC genotypes were comparable (Supplemental Figure S2), suggesting that the higher Th17-polarized responses by *gal3*^{-/-} DCs were independent of Treg induction.

Differential IL-23 Production Accounts for Enhanced Th17 Induction by *gal3*^{-/-} DCs

Curdlan-induced Th17 cell differentiation was shown to depend on IL-23,¹⁰ suggesting that the enhanced Th17 induction by *gal3*^{-/-} DCs in this model may be due to differential IL-23 secretion by *gal3*^{-/-} and *gal3*^{+/+} DCs (Figure 1). To confirm the importance of IL-23 in Th17 induction by curdlan-primed DCs, we performed the OVA-specific T-cell activation experiment in the presence of IL-23-neutralizing antibodies. Neutralization of IL-23 p19 decreased Th17 differentiation induced by *gal3*^{-/-} and *gal3*^{+/+} DCs. Unexpectedly, neutralization of IL-23 p40 did not have a significant effect (Figure 3). On the other hand, it reduced Th1 development induced by *gal3*^{-/-} and *gal3*^{+/+} DCs, likely due to the targeting of IL-12 by antibody, which shares the p40 chain with IL-23. As expected, blocking p19 had no effect on Th1-polarized responses.

Galectin-3 in DCs Negatively Regulates Th17 Polarization in Response to High-Dose LPS

To determine whether galectin-3 in DCs also influences Th17 responses to other stimuli, we tested other Th17-priming agents on DC-mediated Th17 induction. LPS derived from Gram-negative bacteria activates TLR4 and is commonly associated with Th1 cell differentiation; however, recent studies indicate that high concentrations of LPS can also induce Th17 responses.³⁰ After stimulation with LPS at high concentrations, *gal3*^{-/-} DCs produced higher levels of the Th17-associated cytokines IL-6 and IL-23 compared with *gal3*^{+/+} DCs (Figure 4). In contrast, *gal3*^{-/-} and *gal3*^{+/+} DCs secreted similar cytokine levels after low-dose LPS treatment (Figure 4).

To determine the effect of galectin-3 on LPS-mediated Th17 priming, we studied Th17 induction by *gal3*^{-/-} and *gal3*^{+/+} DCs after treatment with LPS at different concentrations. Compared with *gal3*^{+/+} DCs, *gal3*^{-/-} DCs primed with high-dose LPS induced significantly more IL-17 production in OT-II CD4⁺ cells (Figure 5A). In accordance with previous reports that galectin-3 deficiency promotes Th1 responses,^{21,22} we also observed higher IFN- γ production in T cells cultured with low-dose LPS-primed *gal3*^{-/-} DCs.

Neutralization of either IL-6 or IL-23 subunits significantly reduced Th17-polarized responses induced by *gal3*^{-/-} DCs, supporting the notion that higher production of these

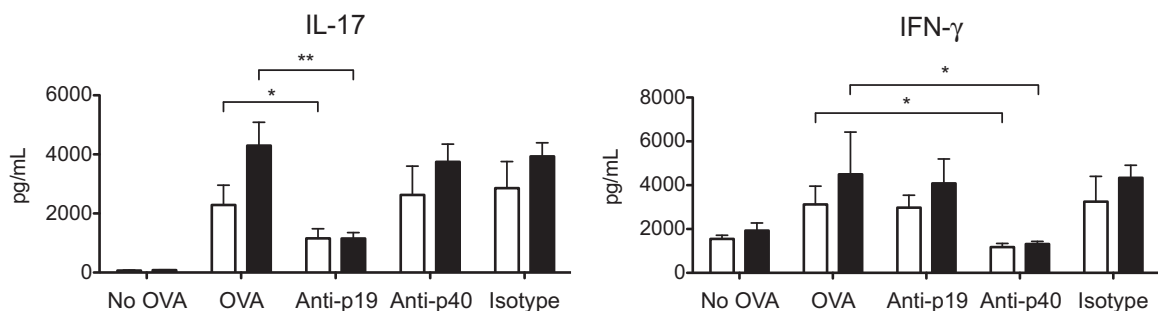


Figure 3 Differential IL-23 production by *gal3*^{-/-} (black bars) and *gal3*^{+/+} (white bars) DCs contributed to differences in Th17 induction. ELISA of cytokines in OVA-curdlan DC-T-cell (OT-II) co-cultures after the addition of neutralizing anti-IL-23p19, anti-IL-12/23p40, or isotype control antibodies on day 0. The co-cultures were maintained for 3 days. Data are given as means \pm SD and are representative of at least four independent experiments. **P* < 0.05, ***P* < 0.01.

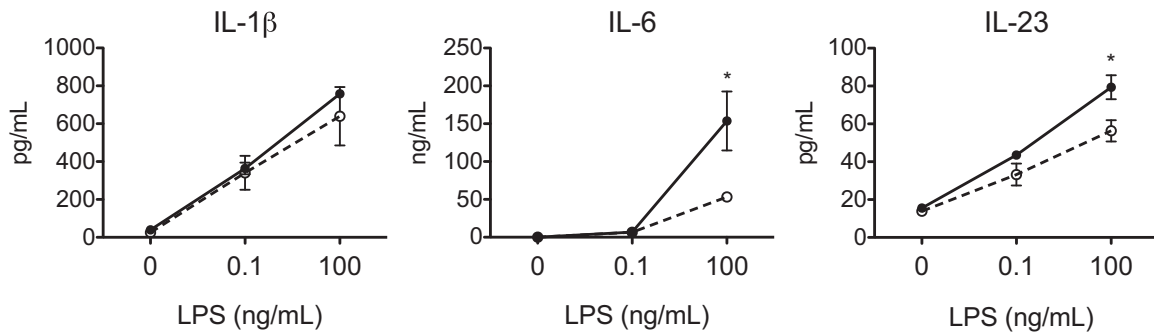


Figure 4 *gal3*^{-/-} (filled circles) DCs produced higher levels of the Th17-axis cytokines IL-6 and IL-23 compared with *gal3*^{+/+} (open circles) DCs after high-dose LPS stimulation. ELISA of cytokines produced by *gal3*^{+/+} and *gal3*^{-/-} DCs after stimulation with low- or high-dose LPS overnight. Data are given as means ± SD and are representative of at least three independent experiments. **P* < 0.05.

cytokines contributed to differences in Th17 induction (Figure 5B).

Galectin-3 Negatively Regulates Activation of c-Rel in DCs after Curdlan Stimulation

These data suggest that galectin-3 negatively regulates IL-23 production in DCs after dectin-1 or TLR4 stimulation. Dectin-1 signals through a spleen tyrosine-kinase and caspase recruitment domain protein 9 (Syk-CARD9)-dependent pathway and leads to the activation of NF-κB subunits involved in cytokine production.¹¹ Regarding IL-23 expression, several groups have shown that c-Rel is a critical NF-κB subunit that promotes IL-23 p19 transcription.^{11,31} To investigate whether galectin-3 influenced IL-23 by affecting c-Rel activation, we compared nuclear translocation and DNA binding of c-Rel in curdlan-stimulated *gal3*^{-/-} and *gal3*^{+/+} DCs. Consistent with higher IL-23 production, *gal3*^{-/-} DCs also expressed higher levels of active c-Rel relative to *gal3*^{+/+} DCs after curdlan treatment (Figure 6A). Significantly higher levels of active p50 and p52 were also observed in *gal3*^{-/-} DCs.

Galectin-3 Promotes Raf-1 Activation in Dectin-1-Activated DCs

Galectin-3 had no influence on cell surface expression of dectin-1, as we observed no differences in receptor levels

between *gal3*^{-/-} and *gal3*^{+/+} DCs (data not shown). In addition to the Syk-CARD9 pathway, dectin-1 also activates a second signaling cascade mediated by the serine-threonine kinase Raf-1. Raf-1 modulates Syk-induced signaling at the point of NF-κB activation by inducing the phosphorylation and acetylation of p65, the latter of which increases its DNA binding affinity.¹¹ The enhanced DNA-binding affinity of acetylated p65 enables it to compete with c-Rel for binding to the *IL-23A* promoter, thereby decreasing IL-23 expression.

We compared Syk and Raf-1 activation in curdlan-stimulated *gal3*^{-/-} or *gal3*^{+/+} DCs. Syk activation was comparable in curdlan-treated *gal3*^{-/-} and *gal3*^{+/+} DCs (data not shown), indicating that galectin-3 does not affect c-Rel activation through Syk-induced signaling. We then compared Raf-1 activation in *gal3*^{-/-} and *gal3*^{+/+} DCs after curdlan treatment. A requirement for Raf-1 activation is phosphorylation at Ser338 by p21-activated kinases and at Tyr340/341 by Src kinases.^{32,33} Cell lysates from curdlan-stimulated *gal3*^{-/-} or *gal3*^{+/+} DCs were immunoprecipitated with anti-Raf-1, and Raf-1 phosphorylation was determined by immunoblotting with anti-phospho-Raf-1 Ser338 and anti-phospho-Raf-1 Tyr340/341. Phosphorylation of Raf-1 at Ser338 in *gal3*^{-/-} DCs after curdlan stimulation was twofold to threefold less compared with *gal3*^{+/+} DCs, suggesting that galectin-3 promotes Raf-1 activation (Figure 6, B and C).

In agreement with lower Raf-1 activation, *gal3*^{-/-} DCs stimulated with curdlan had impaired activation of ERK,

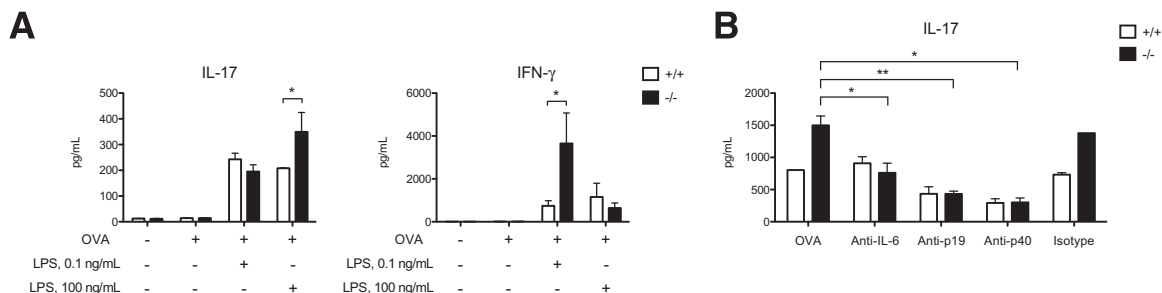


Figure 5 *gal3*^{-/-} DCs primed with high-dose LPS induced higher Th17 responses compared with *gal3*^{+/+} DCs. **A:** ELISA of cytokines in supernatants of CD4⁺ OT-II T cells that had been cultured with OVA-pulsed *gal3*^{+/+} or *gal3*^{-/-} DCs for 3 days in the presence of low-dose (0.1 ng/mL) or high-dose (100 ng/mL) LPS. **B:** ELISA of IL-17 in OVA-LPS (100 ng/mL) DC-T-cell (OT-II) co-cultures after the addition of anti-IL-6, anti-IL-23p19, anti-IL-12/23p40, or isotype control antibodies on day 0. The co-cultures were maintained for 3 days. Data are given as means ± SD and are representative of at least three independent experiments. ***P* < 0.01, ****P* < 0.001.

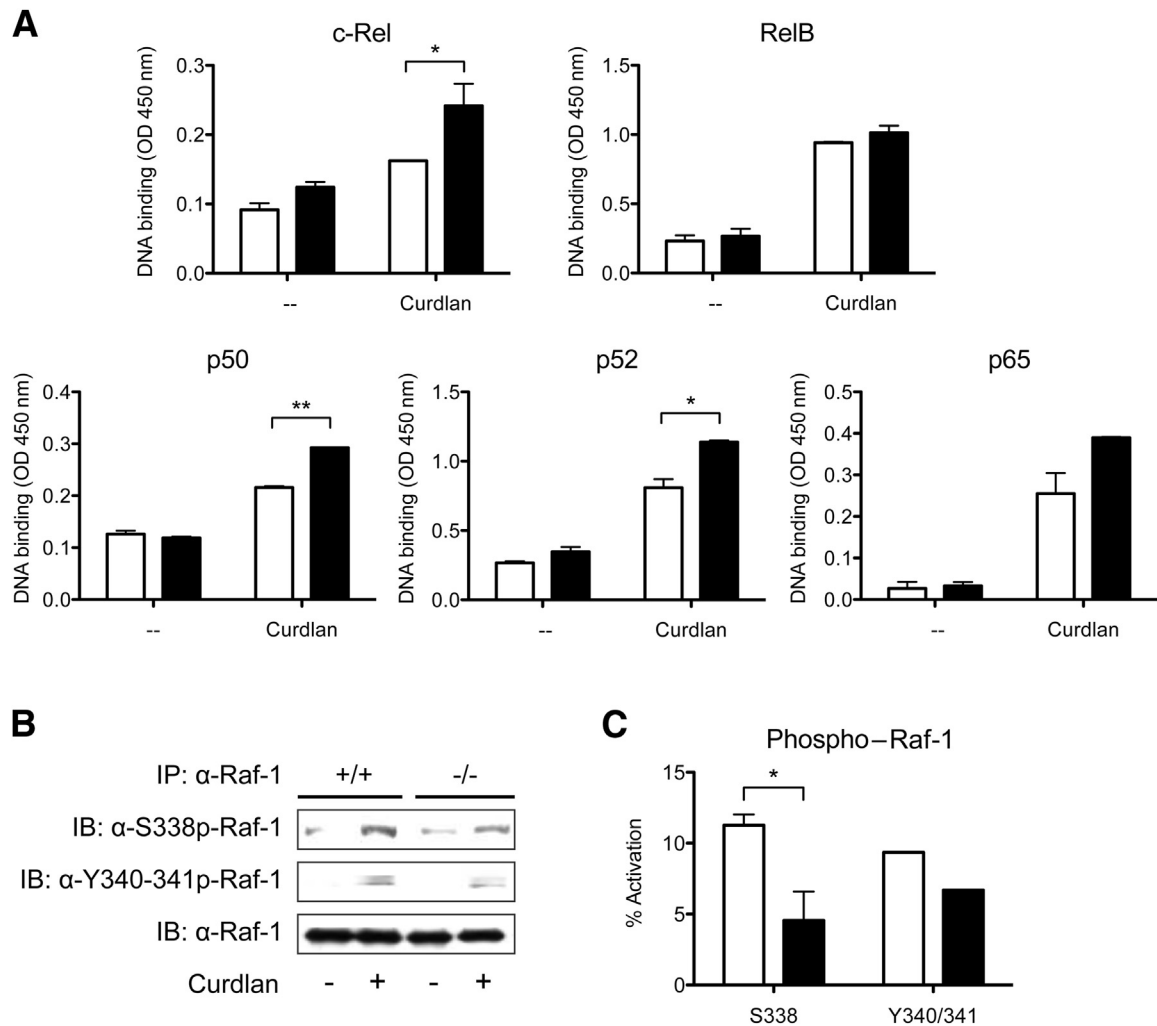


Figure 6 Galectin-3 negatively regulates c-Rel transcription factor activation through the Raf-1 signaling pathway. **A:** Nuclear extracts from unstimulated or curdlan-stimulated *gal3*^{+/+} (white bars) and *gal3*^{-/-} (black bars) DCs were added to plates coated with oligonucleotide containing an NF-κB consensus-binding site. Binding of activated NF-κB subunits was detected using antibodies against c-Rel, RelB, p50, p52, and p65. **B:** Raf-1 phosphorylation at Ser338 and Tyr340/341 as determined by immunoblot (IB) analysis in unstimulated and curdlan-stimulated *gal3*^{+/+} and *gal3*^{-/-} DCs. Data are representative of at least two independent experiments. IP, immunoprecipitate. **C:** Densitometry analysis of phosphorylated Raf-1. Percentage of activation was calculated as the ratio of phosphorylated Raf-1 to total Raf-1. Data are given as means ± SD and are representative of two independent experiments. **P* < 0.05, ***P* < 0.01.

a downstream signaling molecule of the Raf-1 signaling pathway, 5 and 10 minutes after stimulation but recovered to normal levels at later time points (Figure 7, A and B). *gal3*^{-/-} DCs stimulated with LPS also exhibited impaired ERK activation as early as 10 minutes after stimulation and failed to sustain activation after 1 hour (Figure 7, C and D). The trend in defective ERK signaling by *gal3*^{-/-} DCs after dectin-1 and TLR4 stimulation suggests that galectin-3 may modulate a common factor shared by the signaling pathways initiated by these two distinct receptors.

C. albicans—Exposed *gal3*^{-/-} DCs Adoptively Transferred into Mice Induce Higher Th17 Responses *in Vivo*

β-Glucans are expressed on the inner wall of fungal species. However, they can become exposed on the cell surface in

budding or heat-killed yeasts and subsequently activate dectin-1.^{34,35} To study the effect of endogenous galectin-3 on DC cytokine expression in response to fungi, we stimulated DCs with HKCA and compared cytokine profiles in *gal3*^{-/-} and *gal3*^{+/+} DCs. *Gal3*^{-/-} DCs produced significantly higher levels of IL-23 but lower levels of IL-12 after stimulation with HKCA at 10⁶ and 10⁷ cells/mL compared with their wild-type counterparts (Figure 8A). Although there were no differences in IL-1β production, *gal3*^{-/-} DCs produced higher levels of IL-6 in response to HKCA at 10⁷ cells/mL. Thus, galectin-3 also affects DC cytokine production in response to *C. albicans*.

To assess the ability of galectin-3 in DCs to influence CD4⁺ priming *in vivo*, we immunized mice with HKCA-pulsed *gal3*^{-/-} or *gal3*^{+/+} DCs by s.c. injection twice at 1-week intervals. A week after the last injection, we measured cytokine production in culture supernatants from purified splenic

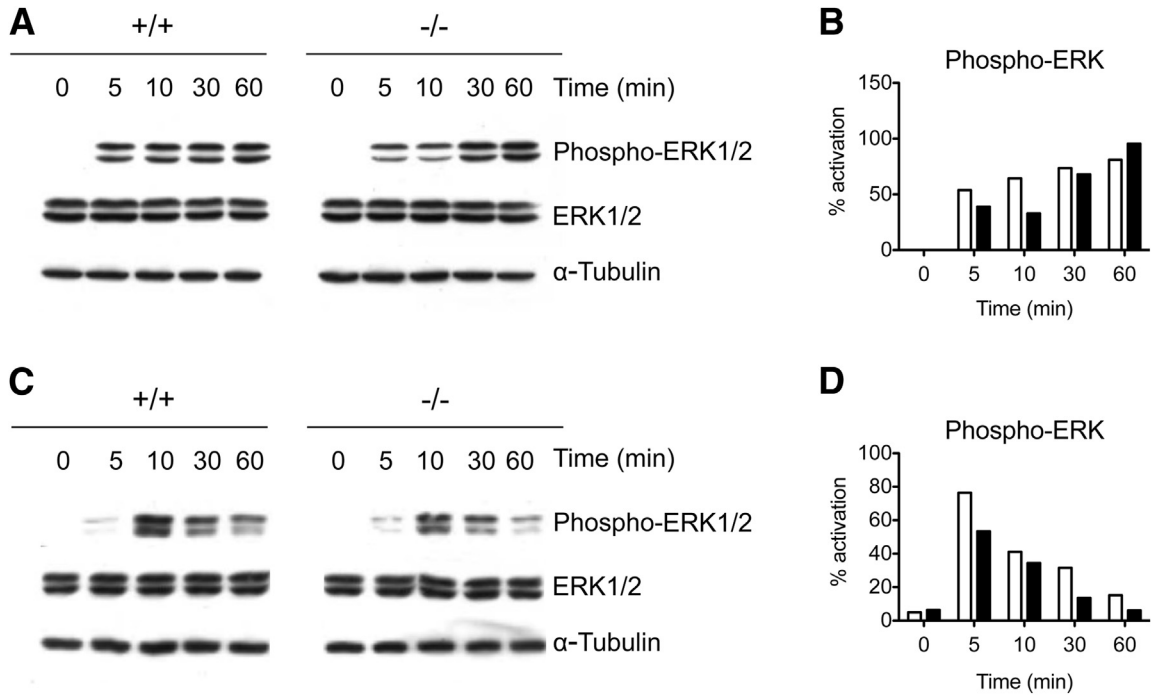


Figure 7 Defective ERK signaling in *gal3*^{-/-} DCs stimulated with curdlan or LPS. **A:** Immunoblot analysis of ERK activation in *gal3*^{-/-} (black bars) and *gal3*^{+/+} (white bars) DCs stimulated with 10 μg/mL of curdlan. **B:** Densitometric analysis of ERK activation in curdlan-stimulated DCs. Data are representative of three experiments performed. **C:** Immunoblot analysis of ERK activation in *gal3*^{-/-} and *gal3*^{+/+} DCs stimulated with 100 ng/mL of LPS. **D:** Densitometric analysis of ERK activation in LPS-stimulated DCs. Data from one of two experiments are represented. Densitometric analyses were performed using ImageJ software version 1.45s (NIH, Bethesda, MD). Values shown are levels of phospho-ERK divided by total ERK.

CD4⁺ cells, and splenocytes restimulated with HKCA. CD4⁺ cells and unfractionated splenocytes from mice that received *C. albicans*-pulsed *gal3*^{-/-} DCs produced higher levels of IL-17 compared with those from mice that received *C. albicans*-pulsed *gal3*^{+/+} DCs or untreated DCs (Figure 8, B and C). Furthermore, we adoptively transferred *gal3*^{-/-} or *gal3*^{+/+}

DCs mixed with live *C. albicans* into wild-type mice by i.v. injection. Three and 6 days after DC transfer, we measured cytokine levels in the kidneys of infected mice and evaluated IL-17 expression in splenic CD4⁺ cells. Levels of IL-6, TGF-β, IL-23, and IL-17 in mice receiving *gal3*^{-/-} DCs were significantly higher than in mice receiving *gal3*^{+/+} DCs on day 3 but

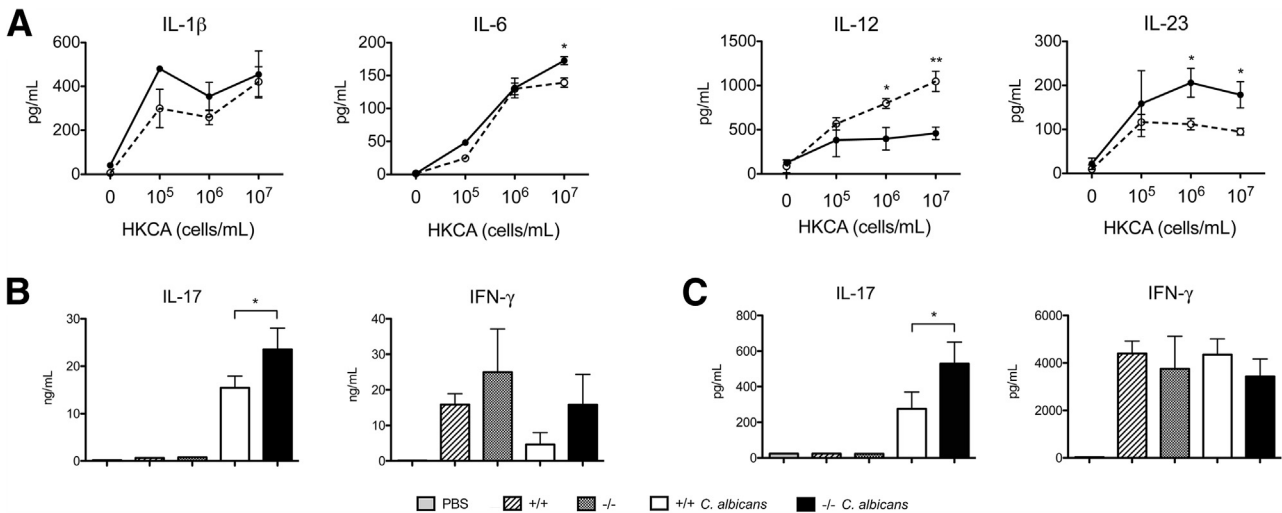


Figure 8 Adoptive transfer of *C. albicans*-treated *gal3*^{-/-} DCs conferred higher Th17 responses. **A:** ELISA of cytokines produced by *gal3*^{+/+} (open circles) and *gal3*^{-/-} (closed circles) DCs after stimulation with HKCA overnight at various concentrations. Data are representative of at least three independent experiments. **B:** Untreated or HKCA-treated *gal3*^{-/-} or *gal3*^{+/+} DCs were s.c. injected into mice twice, 1 week apart. A week after the last DC transfer, splenocytes were harvested and evaluated for cytokine expression. Cytokines in culture supernatants of splenic CD4⁺ cells restimulated *ex vivo* with HKCA-treated DCs. **C:** Cytokines in culture supernatants of unfractionated splenocytes restimulated *ex vivo* with HKCA. All culture supernatants were harvested after 3 days. Data are representative of at least two independent experiments. Data are given as means ± SD. **P* < 0.05, ***P* < 0.01.

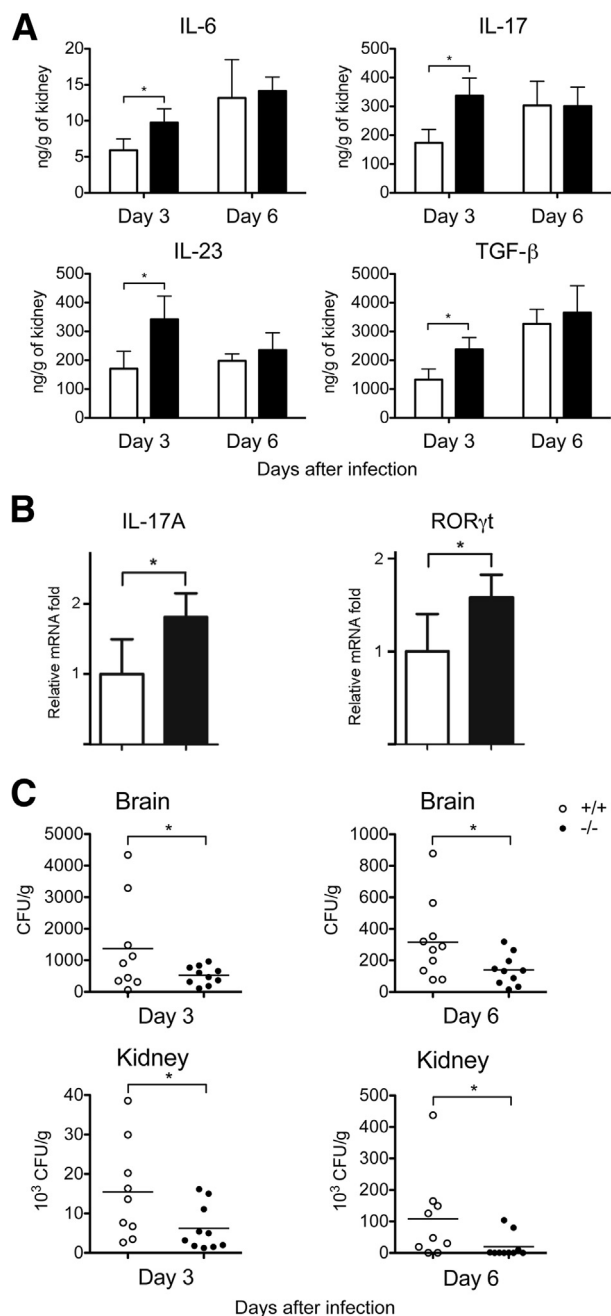


Figure 9 Galectin-3 in DCs regulated antifungal immunity *in vivo*. *Gal3*^{-/-} (black bars) or *gal3*^{+/+} (white bars) DCs mixed with live *C. albicans* were adoptively transferred into wild-type mice by i.v. injection. **A:** Kidneys of *C. albicans*-infected mice were collected on days 3 and 6 after adoptive transfer of *gal3*^{+/+} and *gal3*^{-/-} DCs and infection. Kidneys were homogenized, and cytokine levels in the supernatants of the homogenates were determined. The experiment was performed three times, with two to four mice included per group in each experiment. **B:** Real-time qPCR for indicated mRNAs in splenic CD4⁺ cells from mice that received *C. albicans*-exposed *gal3*^{-/-} or *gal3*^{+/+} DCs. The relative mRNA fold induction was calculated based on the fold induction of nontreated samples. Three to four mice were included in each group. The experiment was repeated three times. **C:** Fungal burdens [colony-forming units (CFUs) per gram of tissue] in brains and kidneys of mice that received DCs were collected on days 3 and 6 after infection. Nine to 10 mice were included in each group. Data are given as means ± SD. **P* < 0.05.

not on day 6 after infection (Figure 9A). Purified CD4⁺ cells from *C. albicans*-infected mice receiving *gal3*^{-/-} DCs expressed higher levels of IL-17A and the Th17-specific transcription factor RORγt compared with those from mice receiving *gal3*^{+/+} DCs on day 6 after infection (Figure 9B). Moreover, fungal burdens in the kidneys and brains of mice receiving *gal3*^{-/-} DCs were lower compared with those of mice receiving *gal3*^{+/+} DCs on both days 3 and 6 after infection (Figure 9C).

In agreement with higher IL-17A production, renal histologic analysis revealed higher cellular infiltrates in mice that received *C. albicans*-exposed *gal3*^{-/-} DCs (Figure 10A). Consistent with the fact that cellular infiltration is essential for fungal clearance and survival,³⁶ the number of pathogenic *C. albicans* hyphae was lower in kidneys of mice that received adoptively transferred *gal3*^{-/-} DCs (Figure 10B). Furthermore, mice that received *C. albicans*-exposed *gal3*^{-/-} DCs had significantly higher levels of infiltrating neutrophils, which may be explained by the higher levels of IL-17-axis cytokines that promote neutrophil recruitment (Figure 10C).³⁷ Thus, galectin-3 expression in DCs is associated with suppression of cellular infiltration and ineffective fungal clearance after *Candida* infection. Because Th17 cells play a critical role in antifungal immunity, these results suggest that the ability of *gal3*^{-/-} DCs to produce higher levels of IL-17-axis cytokines promotes differentiation of CD4⁺ T cells and maintenance of Th17 cells and protects mice against *C. albicans* dissemination to kidneys and brain. Collectively, these findings indicate that galectin-3 negatively regulates Th17 responses after *C. albicans* infection through a mechanism involving DC cytokine regulation.

Discussion

In the present study, we identified a novel role for endogenous galectin-3 as a regulator of cytokine expression in DCs. We demonstrate that endogenous galectin-3 negatively regulates the production of IL-17-axis cytokines in DCs stimulated with dectin-1 and TLR4 agonists. *Gal3*^{-/-} DCs primed with either curdlan or LPS produced higher levels of IL-23 and promoted higher Th17 responses compared with *gal3*^{+/+} DCs. Neither the presence of lactose nor the addition of recombinant galectin-3 to DC cultures affected cytokine production (data not shown), supporting the notion that galectin-3 functions intracellularly to modulate DC cytokine expression.

Neutralization of the IL-23 p19 subunit in DC-T-cell cocultures abrogated differential cytokine responses between the two genotypes, thereby confirming the importance of IL-23. Neutralization of IL-23 p40 decreased Th17 induction by LPS-primed DCs but failed to suppress Th17 induction by curdlan-primed DCs. This discrepancy may be due to the neutralization of IL-12, which shares the p40 subunit in common with IL-23. Neutralization by anti-p40 antibodies and subsequent blockade of Th1 cells may also account for the retained production of IL-17, as Th1 cells have been reported

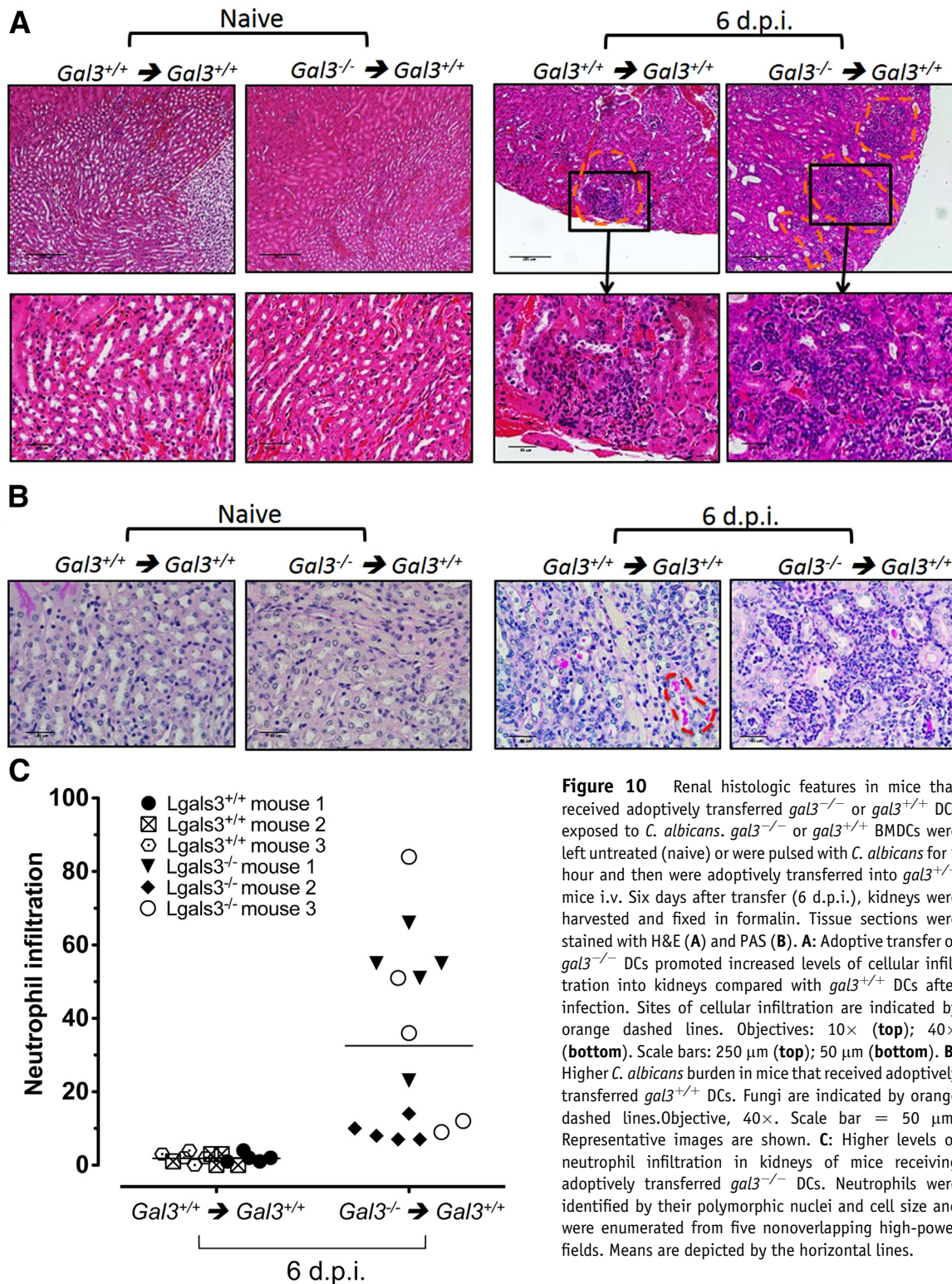


Figure 10 Renal histologic features in mice that received adoptively transferred $gal3^{-/-}$ or $gal3^{+/+}$ DCs exposed to *C. albicans*. $gal3^{-/-}$ or $gal3^{+/+}$ BMDCs were left untreated (naive) or were pulsed with *C. albicans* for 1 hour and then were adoptively transferred into $gal3^{+/+}$ mice i.v. Six days after transfer (6 d.p.i.), kidneys were harvested and fixed in formalin. Tissue sections were stained with H&E (A) and PAS (B). A: Adoptive transfer of $gal3^{-/-}$ DCs promoted increased levels of cellular infiltration into kidneys compared with $gal3^{+/+}$ DCs after infection. Sites of cellular infiltration are indicated by orange dashed lines. Objectives: 10× (top); 40× (bottom). Scale bars: 250 μm (top); 50 μm (bottom). B: Higher *C. albicans* burden in mice that received adoptively transferred $gal3^{+/+}$ DCs. Fungi are indicated by orange dashed lines. Objective, 40×. Scale bar = 50 μm. Representative images are shown. C: Higher levels of neutrophil infiltration in kidneys of mice receiving adoptively transferred $gal3^{-/-}$ DCs. Neutrophils were identified by their polymorphic nuclei and cell size and were enumerated from five nonoverlapping high-power fields. Means are depicted by the horizontal lines.

to negatively regulate differentiation of Th17 cells.³⁸ Because curdlan is a stronger inducer of IL-23 production than is LPS,¹⁰ it is also feasible that binding of p40 antibodies to IL-12 and IL-23 caused a depletion of antibody such that amounts remaining were insufficient to neutralize the persistent production of IL-23 by curdlan-stimulated DCs.

Although IL-23 is not required for Th17 cell differentiation from naive T cells, it induces Th17 cell expansion, is required for effector functions, and is also implicated in the terminal differentiation of Th17 cells *in vivo*.^{4,6,39} On stimulation by curdlan, $gal3^{-/-}$ and $gal3^{+/+}$ DCs mainly differed in IL-23 production, suggesting that galectin-3 in DCs may

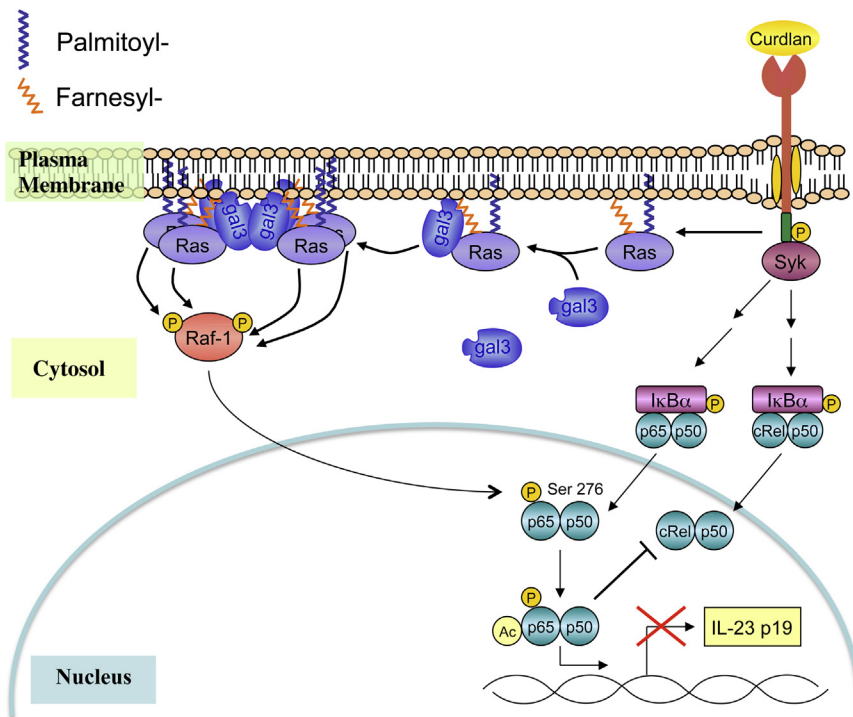


Figure 11 Proposed model for galectin-3 in the regulation of cytokine expression in DCs. On ligand-mediated DC activation, galectin-3 in the cell cytosol may be recruited to membrane components, as previously demonstrated with Ras-GTP.^{45,47} At the membrane, galectin-3 may participate in the reorganization of membrane proteins or may act as a protein scaffold and subsequently influence downstream signal transduction. In association with Ras-GTP, galectin-3 aids in the formation of Ras nanoclusters, which promotes the efficient activation of Raf-1. By means of p65 phosphorylation, Raf-1 negatively regulates IL-23 expression.

influence Th17 cell expansion and/or effector functions after dectin-1 activation but have little effect on naive T-cell differentiation. In contrast to dectin-1 agonists, stimulation of DCs with high-dose LPS led to enhanced production of IL-6 and IL-23 in *gal3*^{-/-} DCs, suggesting that galectin-3 in DCs negatively regulates Th17 cell differentiation and development in response to TLR4 agonists. It remains unclear why galectin-3 in DCs affects different stages of Th17 cell development in response to dectin-1 and TLR4 stimulation. A possible explanation may lie in the distinct signaling pathways mediated by dectin-1 and TLR4. As dectin-1 signals through Syk-dependent and Syk-independent (Raf-1) pathways, we show that galectin-3 promoted Raf-1 activation but seemed to have little effect on the Syk signaling pathway. Thus, galectin-3 seems to affect the Raf-1 arm of the dectin-1 signaling pathway in DCs.

The recognition of fungal pathogens by DCs is mediated by several PRRs. In addition to TLR4 and dectin-1, *C. albicans* activates TLR2, dectin-2, mannose receptor, and DC-SIGN.³⁴ The adoptive transfer of *gal3*^{-/-} DCs in systemic infection with *C. albicans* resulted in higher production of IL-6, IL-23, and TGF-β, suggesting that PRRs other than TLR4 and dectin-1 may also be involved in the negative regulation of IL-17–axis cytokines by galectin-3 *in vivo*. A potential PRR may be TLR2, as other studies have shown that galectin-3 in association with TLR2 or dectin-1 is required for the recognition of *C. albicans* in macrophages.^{24,25} Galectin-3 was not required for the endocytosis of yeasts but was found in macrophage phagocytic cups and phagosomes containing *C. albicans*.²⁴ Thus, galectin-3 may not be necessary for the phagocytosis of yeasts but instead plays a role in modulating signaling pathways in response to fungal pathogens.

In contrast to the studies on galectin-3 in Th1/Th2 polarization, the literature on galectin-3 regarding Th17 regulation is scarce. Recently, Radosavljevic et al⁴⁰ reported high serum levels of IFN-γ and IL-17 in *gal3*^{-/-} mice inoculated with B16F1 melanoma cells. Although the role of IL-17 in pro-tumor and anti-tumor responses remains controversial, their findings suggest that galectin-3 may negatively regulate Th1 and Th17 responses in tumor models. In animal models of autoimmune disease, where Th17 cells are known to contribute to inflammation, galectin-3 was shown to promote IL-17 production. Using an experimental autoimmune encephalomyelitis mouse model, Jiang and colleagues⁴¹ observed attenuated experimental autoimmune encephalomyelitis symptoms in *gal3*^{-/-} mice accompanied by lower levels of IFN-γ and IL-17. They attributed Th1 and Th17 impairment in *gal3*^{-/-} mice to an increased frequency of Foxp3⁺ Tregs present in the central nervous system and enhanced IL-10 production by *gal3*^{-/-} DCs. We, however, observed higher Th1 and Th17 differentiation induced by *gal3*^{-/-} DCs and no difference in Treg induction or IL-10 secretion. Such discrepancies may be due to differences in cell culture conditions or the experimental disease model.

We demonstrate that endogenous galectin-3 affects cytokine expression in DCs after stimulation of dectin-1 or TLR4, two different classes of PRRs. Although these receptors signal through different pathways, we observed impaired ERK mitogen-activated protein kinase signaling in *gal3*^{-/-} DCs in response to both stimulants. Similar defects were reported in *gal3*^{-/-} DCs after chemokine stimulation.¹⁹ Collectively, these studies indicate a common factor in receptor-mediated signal transduction that may be regulated by galectin-3. Dectin-1 and TLR4 have been reported

to translocate to lipid raft microdomains on ligand stimulation,^{42,43} an event that initiates signal transduction. Disruption of lipid rafts using chemical agents resulted in the selective attenuation of ERK activation in LPS-stimulated macrophages⁴⁴ and dectin-1-activated DCs,⁴² illustrating the importance of lipid raft integrity for ERK signaling. We previously showed that galectin-3 positively regulates DC migration by functioning in membrane structures. Compared with *gal3*^{+/+} DCs, *gal3*^{-/-} DCs exhibited structural differences in membrane ruffles along with impaired chemotaxis and defective signal transduction in response to chemokine stimulation.¹⁹ Regarding the present study, impaired ERK activation in *gal3*^{-/-} DCs suggests that galectin-3 may modulate the stability of lipid raft microdomains and contribute to efficient ERK signaling.

The association of galectin-3 with membrane domains may also be the basis for impaired Raf-1 activation in *gal3*^{-/-} DCs after curdlan stimulation. Galectin-3 was previously identified as a binding partner of K-Ras,⁴⁵ a member of the Ras family of guanine nucleotide-binding proteins present on the inner leaflet of the plasma membrane that also serves as an upstream activator for Raf-1.^{46–48} Interactions between galectin-3 and Ras promote Ras organization into nanoclusters. Although its role in DC signaling is yet to be fully delineated, galectin-3 may function as a scaffold for K-Ras-GTP nanocluster formation, providing a platform for Raf-1 recruitment to the plasma membrane and subsequent activation by phosphorylation.⁴⁹ Because Raf-1 functions upstream of the mitogen-activated protein kinase/ERK signaling cascade,⁴⁶ impaired Raf-1 activation in curdlan-stimulated *gal3*^{-/-} DCs may correlate with our observations of defective ERK signaling.

A consequence of impaired Raf-1 activation in DCs is altered cytokine production. Recent studies have shown that Raf-1 inhibition increases IL-23 p19 mRNA expression in curdlan-stimulated DCs,¹¹ indicating that Raf-1 negatively regulates production of this cytokine. The mechanism for Raf-1-mediated regulation of IL-23 is largely due to acetylated p65, which prevents c-Rel binding to the *IL23A* promoter. We show that galectin-3 promotes Raf-1 activation after dectin-1 stimulation, which may lead to increased levels of acetylated p65 and consequently less c-Rel activation. As c-Rel is a stronger activator of *IL23A* transcription than acetylated p65,¹¹ enhanced Raf-1 activation induced by galectin-3 would, therefore, decrease IL-23 production. In support of this hypothesis, we show higher c-Rel nuclear translocation and DNA binding in curdlan-stimulated *gal3*^{-/-} DCs compared with similarly treated *gal3*^{+/+} DCs. Significantly elevated levels of p50 and p52 were also observed in *gal3*^{-/-} DCs, which may also contribute to *IL23A* promoter activation through heterodimer formation with NF- κ B family members.⁵⁰ In addition to IL-23, Raf-1-mediated acetylation of p65 regulates the expression of IL-6, IL-10, and IL-12.¹¹ Although levels of p65 were slightly higher in curdlan-stimulated *gal3*^{-/-} DCs, these levels did not reach statistical significance. In addition, the negative regulation

of acetylated p65 on *IL23A* promoter activation may be offset by higher activation of c-Rel, p50, and p52 in curdlan-stimulated *gal3*^{-/-} DCs.

In summary, these data suggest that endogenous galectin-3 regulates DC cytokine expression and signal transduction in response to dectin-1 and TLR4 agonists. Based on published findings and the present observations, we propose that galectin-3 may be recruited from the cytosol to the plasma membrane in dectin-1- and TLR4-activated DCs and may participate in the organization of membrane components by acting as a protein scaffold or affecting lipid raft stability. Regarding dectin-1 signaling, galectin-3 may function as a scaffold for proteins such as Ras-GTP and subsequently promote efficient activation of Raf-1 (Figure 11). In turn, this may modulate downstream signaling events and, ultimately, influence cytokine expression.

The finding that galectin-3 negatively regulates Th17-polarizing cytokines in DCs suggests that targeting this lectin in DCs may be useful in the development of new strategies to modulate Th17 responses. This could be applicable to antifungal immunity and autoimmune diseases.

Acknowledgments

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.06.017>.

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