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Downregulating Galectin-3 Inhibits Proinflammatory Cytokine Production by Human Monocyte-Derived Dendritic Cells

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Abstract

Galectin-3 (Gal-3), a β-galactoside-binding lectin, serves as a pattern-recognition receptor (PRR) of dendritic cells (DCs) in regulating proinflammatory cytokine production. Galectin-3 (Gal-3) siRNA downregulates expression of IL-6, IL-1β and IL-23 p19, while upregulates IL-10 and IL-12 p35 in TLR/NLR stimulated human MoDCs. Furthermore, Gal-3 siRNA-treated MoDCs enhanced IFN-γ production in SEB-stimulated CD45RO CD4 T-cells, but attenuated IL-17A and IL-5 production by CD4 T-cells. Addition of neutralizing antibodies against Gal-3, or recombinant Gal-3 did not differentially modulate IL-23 p19 versus IL-12 p35. The data indicate that intracellular Gal-3 acts as cytokine hub of human DCs in responding to innate immunity signals. Gal-3 downregulation reprograms proinflammatory cytokine production by MoDCs that inhibit Th2/Th17 development.

Keywords

Galectin-3; Th17; Dendritic cells; Inflammation; Cytokines; Innate Immunity

Introduction

Galectin-3 (Gal-3) is one of more than 15 members of the β-galactoside-binding lectin superfamily. This multifunctional molecule is involved in pattern recognition and affects diverse cellular and molecular events via extracellular and intracellular actions in lymphoid and non-lymphoid organs [1, 2]. The functional complexity reflects the presence of a plethora of N- and O-linked cell surface glycoproteins, and Gal-3 carbohydrate recognition domain (CRD)-mediated recognition results in signal transduction in various cell types [3–

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Disclosures

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5]. In addition, the extensive and numerous carbohydrate-independent functions of Gal-3 in cytosolic organelles as well as the nucleus adds further complexity to this protein [1, 3]. Remarkably, Gal-3 is critically involved in diverse cellular processes, including immune activation, inflammation, allergic asthma, angiogenesis, cancer metastasis, atherosclerosis, obesity, idiopathic pulmonary fibrosis (IPF), and in particular its key role in the lung, kidney, liver, heart fibrosis and heart failure [2, 6, 7, 8, 9–11]. However, its role in controlling immunity and inflammation by lung dendritic cells is not yet known.

Monocyte-derived dendritic cells (MoDCs) present in nonlymphoid organs as well as lymph nodes mediate local inflammation, and in particular allergic asthma [12, 13]. DCs are sentinel antigen-presenting cells that express a wide spectrum of pattern recognition receptors (PRR), which recognize pathogen-associated molecular patterns (PAMP), including the families of toll-like receptors (TLR) and NOD-like receptors (NLR) [12, 14, 15] as well as C-type lectins (CLEC) recognizing surface mannans and β-glucans [16, 17]. Gal-3 can be considered yet another novel family of PRR that mediates immunological functions in multiple immune cells, e.g., macrophages, T cells, neutrophils, B-cells of germinal center follicles [1, 2, 18]. Gal-3 plays a key role in regulating allergic asthma [19], and is constitutively expressed in DCs [20]; however, it is not known whether Gal-3 may play a critical role in regulating proinflammatory cytokines by DCs and influence CD4 T-cell development. Herein we show that Gal-3 siRNA attenuates proinflammatory MoDCs by upregulating the secretion of IL-12 p35 and IL-10, while downregulating IL-23 p19, IL-6, IL-1β, which inhibit subsequent Th17 and Th2 development.

Methods

2.1. Reagents

Lipofectamine RNAiMAX and Opti-MEM (Invitrogen, Carlsbad, CA) to MoDC was optimized to over 85% by fluorescent RNA markers. *Recombinant cytokines:* recombinant human GM-CSF, IL-1β, IFN-γ, TNF-α (BioLegend, San Diego; PreproTech, Rocky Hill, NJ); recombinant human IL-6, human SCF, human TSLP (PreproTech); PGE2 (Sigma, St Louis, MO); recombinant human Gal-3; *ELISA kits for human cytokines*: IL-1β, TGF-β, p19 (IL-23), p35 (IL-12), IL-10, IL-17A, and were obtained from eBioscience and PreproTech; anti-actin (Sigma). *Cytokine neutralizing antibodies (used from 5–10 μg/ml):* anti-IL-12 (eBioscience); anti-human IL-12 p35, anti-IL-12/IL23 p40, anti-IL-23 p19, anti-IL-12 p70, anti-human IFN-γ, IL-β, IL-6, TGF-β1 (R&D); goat anti-Gal-3 (pAb, R&D), biotin mAb anti-Gal-3 (M3/38, BioLegend for western), mAb anti-Gal-3 (B2C10, prepared by this lab; and inhibition of radioactive Gal-3 binding to solid phase IgE by β-galactosides was previously described in the lab [21]. *FACS reagents:* FITC-anti-p35, PE-anti-p19, APC-anti-CD83, PE anti-CD205 (DEC-205) (BioLegend); PE-anti-CD8α, FITC anti-human CD11c, APC anti-human CD11c, Brefeldin A, eFluor 710 streptavidin, and appropriate fluorochrome-matched control antibodies (eBioscience); FITC anti-human Gal-3 (BioLegend), APC anti-human Gal-3 (R&D Systems). *TLR reagents:* TLR2, Pam3Csk4, synthetic triacylated lipoprotein, zymosan, and TLR7/8 ligand, R848 (InvivoGen, San Diego); house dust mite (HDM) extracts (LPS/dectin-1, 2) (Greer Lab, Lenoir, NC); TLR4 ligand, LPS (E. coli. 0111:B4, List Labs, Campbell, CA). Other reagents are Ficoll-Hypaque

(Amersham/GE, Piscataway, NJ); CYBR Green PCR Master Mix (AB Applied Biosystems/ Invitrogen); anti-α-tubulin (Thermo/Fisher, Waltham, MA); human AB serum (VWR, Radnor, PA), staphylococcal superantigen B (SEB, Sigma), human CD4+ T cells and CD45RO separation kits (Miltenyi Biotec, Gladbach, Germany).

2.2. Gal-3 siRNA and cytokine primers for qRT-PCR

Four cross-species siRNAs and scramble non-silencing RNA sequence (sc/snRNA) were designed by Invitrogen's BLOCK-iT™ RNAi Designer, and synthesized by Invitrogen: siRNA-1: 5'- GAACAACAGGAGAGUCAUU-3'; siRNA-2: 5'-CCCAAACCCUCAAGGAUAU-3'; siRNA-3: 5' GCUGACCACUUCAAGGUUG-3'; siRNA-4: 5′- UAAAGUGGAAGGCAACAUCAUUCCC-3′. Non-silencing (ns) sequence (Open Biosystem): 5'- ATCTCGCTTGGGCGAGAGTAAG-3'. Human MoDCs and mouse RAW264.7 cells for cross species (Supplemental Fig.1) were treated with Gal-3 siRNAs with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) or TransIT (Mirus LLC, Madison, WI), and analyzed by RT-PCR and western blots. MoDCs and RAW264.7 cells were transfected respectively with 4 siRNAs targeting Gal-3 or a non-targeting, scrambled control RNA (scRNA) control that does not target any human and mouse genes. Two days after transfection, cells were harvested and used for western blots or for FACS analysis by a FACSCalibur flow cytometer. The levels of Gal-3 protein and mRNA were measured by western blots and real-time RT-PCR respectively, normalized against α-tubulin and GAPDH (BioRad, Hercules, CA), respectively. The total RNAs were isolated via Trizol method (Invitrogen) and used for first-strand cDNA synthesis (ProtoScript® M-MuLV First Strand cDNA Synthesis Kit, NEB). The cDNAs were used for real time quantitative PCR with a pair of human LGALS3 specific primers, LGALS3-F (5'-GGAATGATGTTGCCTTCCAC-3') and LGALS3-R (5'-CTGCAACCTTGAAGTGGTCA- 3') (Applied Biosystems). The primers used for human p35: p35-F (5'- CTCCAGACCCAGGAATGTTC-3') and p35-R (5'-ATCTCTTCAGAAGTGCAAGGG-3'). Human p19: p19-F (5'-ATGTTCCCCATATCCAGTGTG-3') and p19-R (5'-GCTCCCCTGTGAAAATATCCG-3'). Human p40 are: p40-F (5'-CACATTCCTACTTCTCCCTGAC-3') and p40-R (5'-CTGAGGTCTTGTCCGTGAAG-3'). Human IL-10: IL-10-F (5'-GCCTAACATGCTTCGAGATC-3') and IL-10-R (5'-CTCATGGCTTTGTAGATGCC-3'). Human IL-1β: IL-1β-F (5'-ATGCACCTGTACGATCACTG-3') and IL-1β-R (5'-ACAAAGGACATGGAGAACACC-3'). **A**ctin and GAPDH mRNA was used as internal control for RT-PCR. Actin: actin-F (5'- GCGAGAAGATGACCCAGATC-3') and actin-R (5'-CCAGTGGTACGGCCAGAGG-3'); GAPDH: Tri-GAPDH-F (5'-CCCTTCATTGACCTCAACTA-3') and Tri-GAPDH-R (5'-CCTTCTCCATGGTGGTGAA-3'). SYBR Green qPCR Master Mixture (2X) (Applied Biosystems) was used for PCR reaction in a 96-well optical module for real-time PCR (CFX96™ optical reaction module 184-5096) includes CFX Manager™ software, qbase^{PLUS} software license for use with C1000 Touch™ thermal cycler chassis. The relative levels of mRNA of Gal-3 gene, LGALS3, IL-12 p35, IL-12 p19, IL-12 p40 and IL-10 were

normalized with the internal control of actin or GAPDH. The PCR products were analyzed on 1.5% agarose gel.

2.3. Preparation of human MoDCs

Peripheral blood mononuclear cells (PBMCs) were purified from blood buffy coat of normal human donors (San Diego Blood Bank, San Diego, CA) via Ficoll-Hypaque density gradient centrifugation (Use of human PBMCs has been reviewed and approved by the IRB Committee). PBMC were adsorbed onto the plastic Petri plates for 2 h, decanted, and the adherent monocytes on the plates were then differentiated to immature dendritic cells (iDCs) by co-culturing with GM-CSF (100 U/ml) and IL-4 (200 U/ml) for 5 days, and were further stimulated to mature MoDCs by overnight culturing with different maturing media, containing (a) TNF-α (10 ng/ml), or (b) TNF-α/prostaglandin E_2 (1 mM PGE_2) or (c) TNF-α/ PGE_2, IL-1β and IL-6. All the cytokines were purchased from PeproTech. MoDCs were transfected with 1 nmol siRNA-3 and -4 targeting Gal-3 in 20 μl lipofectamine RNAiMAX to 1×10^6 MoDC in 0.5 ml cytokine cocktail media. Alternatively, The control MoDCs were transfected with scramble, non-targeting siRNA (scRNA/snRNA). MoDC were titrated with the optimal concentrations: R848 (5 μg/ml), LPS (250 ng/ml), Pam3Csk4 (1μg/ml), muramyl dipeptide (MDP, 2 μg/ml), zymosan (25 μg/ml) and house dust mite extract (25 μg/ml). Numerous pilot experiments were first performed in titrating, optimizing the dosage range of the respective candidate stimulants alone or in combinations using MoDCs in different maturation media (a–c) as described above. Key experiments using optimal dosages in mature MoDCs from maturation medium (c) were then repeated at least three times. The representative experiment was presented. For cytokine staining, cells were restimulated on day of harvest with phorbol myristate acetate (PMA, 0.1 μg/ml) and ionomycin (1 μM) for 5 hr with Brefeldin A (10 μg/ml) added for four hr. Levels of human cytokines measured in MoDCs-T cell cocultures: IL-12, IL-10, IL-23, IL-5, and IFN-γ were quantified by ELISAs according to manufacturers. More than seven experiments were performed and the representative experiments were shown. CD45RO T cells were prepared with by positive selection with CD45RO magnetic beads of the CD4-T cells selected by the CD4 MACS multisort kit (Miltenyi Biotec). CD45RO CD4 T-cells traditionally regarded as a more memory-prone cell type, are prompt in cytokine secretion such as IFN-γ, IL-4 and IL-5 [22]. Co-cultures of T cells and MoDCs were initiated at 1:3 ratios, and supernatants harvested on day 5, 7, 14 for cytokine assays, and representative data on day 7 were presented [23].

2.4. SDS-PAGE and western blot

MoDCs treated under different conditions were lysed with 1% SDS and boiled in DTT containing sample treatment buffer and loaded on 10% Lammeli gel, and transfer was performed in 0.1% SDS and 1% methanol, and probed with HRP-labeled antibodies followed by chromogenic or chemoluminescent substrates, and densitometer reading was performed with ImageJ software (NIMH, NIH).

2.5. Flow Cytometry

After Fc block, MoDCs were stained for 30 mins with various combinations of fluorochrome-conjugated antibodies. For intracellular staining, cells were resuspeneded in

fixation/permeabilization solution (BDCytofix/Cytoperm kit, BD Biosciences), and performed in parallel with isoptype control antibodies according to the manufacturer's protocol. The samples were analyzed with a FACSCalibur flow cytometer (BD Immunocytometry Systems) with 10,000 to 50,000 events collected. Further analyses were performed with Flow Jo software (Tree Star).

2.6 Statistical analysis

Experiments were repeated at least three times from different donors and showed consistent results. The results of the representative experiments in triplicate as means +/− SE were presented. The p value was computed between siRNA versus scRNA treated cultures stimulated by the same type of innate immunity ligand with a Mann-Whitney U test (GraphPad software, Inc., San Diego, CA) with a p value <0.05 (*) or <0.01 (**). Two-tailed Student's t-test was also used for comparison.

Results

3.1. Efficient knockdown of Gal-3 expression in human MoDC by RNA interference

Four human Gal-3 siRNA, sharing identical sequences with the mouse Gal-3 gene are tested for knocking out Gal-3 expression in human and murine cell lines. Two of the four Gal-3 siRNAs inhibited Gal-3 protein in human MoDCs (siRNA-3, -4; Fig. 1A), indicating the specificity of the Gal-3 siRNA treatment effect by two siRNAs on different targeting sequences of Gal-3 gene, and Gal-3 siRNA4 was employed for subsequent studies. The targeting specificity was also confirmed by testing fourteen short hairpin RNA (shRNA) constructs including the overlapping cross-species siRNA-3, and -4, expressed in a lentiviral vector, inhibiting Gal-3 messages in human HT-29 cell lines (Supplemental Fig. 3). Fig. 1B showed that Gal-3 is constitutively expressed in MoDCs, and Gal-3 messages were elevated 2 to 3-fold in MoDCs stimulated with LPS, R848 and LPS/R848. Approximately, 80% to 90% Gal-3 mRNA were reduced in Gal-3 siRNA-treated, LPS and/or R848 by quantitative real-time (qRT)-PCR. Fig. 1C showed that approximately 90% Gal-3 protein was diminished by staining with FITC-anti-Gal-3 in the CD205 (+) and CD205 (−) MoDCs sustained at 48 h following Gal-3 siRNA treatment. Expression of CD205 on MoDCs requires 2 d treatment in the maturation media [24] in contrast to overnight maturation reported herein [25]. Thus the observation of Gal-3 expression in the residual 10% CD205 (−) MoDCs, suggesting MoDCs undergoing prolonged maturation may be more prone to transfection by Gal-3 siRNA.

3.2. Differential IL-23 p19 and IL-12 p35 cytokine production is affected in Gal-3 siRNA-treated human MoDCs

MoDCs produce an array of cytokines, e.g., IL-12, IL-27, IFN-β, IL-23, IL-1β, IL-6, TNF-α, TGF-β and IL-10 but not IL-4. We therefore evaluate whether Gal-3 siRNA modulates critical cytokine gene expression in LPS and/or R848-stimulated MoDCs. Fig. 2A showed that Gal-3 siRNA treatment reduced levels of IL-23 p19 mRNA approximately up to 3.5-fold in R848 and/or LPS-stimulated MoDCs. In contrast, IL-12 p35 (Fig. 2B) and IL-10, an important immuno-regulatory cytokine (Fig. 2C) were upregulated in Gal-3 siRNA-treated MoDCs up to 2.5-fold to 3.3-fold, respectively. Noticeably, the common subunit of p40

shared by IL-12 and IL-23 remained unperturbed following Gal-3 siRNA treatment (Fig. 2D). Taken together, the observation that Gal-3 siRNA differentially modulates IL-23 p19 versus IL-12 p35 but does not affect the common IL-12 p40 strongly suggests that Gal-3 mRNA activated by innate immunity ligands and its downregulation may play an important role in deviating CD4 T-cells.

Next, we proceed to confirm that levels of cytokine production and secretion are also concordant with the message levels in Gal-3 siRNA-treated MoDCs. MoDCs were cultured in maturation cocktails without externally added IL-1β and IL-6, known to play a key role in Th17 differentiation. Gal-3 siRNA significantly downregulates production/secretion of IL-1β (up to 2.3 fold) as well as IL-6 (up to about 4 fold) in LPS and/or R848-stimulated MoDCs measured by ELISA as shown in Fig. 3A and Fig. 3B respectively.

Concordant with upregulated IL-10 messages (Fig. 2), IL-10 secretion was significantly augmented (up to 4-fold) in Gal-3 siRNA-treated, LPS and/or R848-stimulated MoDCs (Fig. 3C). TGF-β is another important Th17 differentiating cytokine for most rodent CD4 T-cells and naïve human CD4 T-cells [23, 26–28]. Nevertheless, Gal-3 siRNA treatment did not affect secreted levels of TGF-β produced by MoDCs (Fig. 3D).

Thus the above observations indicate a dichotomy that Gal-3 downregulation inhibits proinflammatory IL-1β and IL-6 (required for Th17 differentiation) by MoDCs, while augments immuno-regulatory IL-10. Interestingly, TGF-β levels (required for Treg or Th17 differentiation) is not affected.

Next, we evaluate effect of Gal-3 siRNA on IL-23 p19 versus IL-12 p35 secretion in MoDCs stimulated with a variety of innate immunity ligands, e.g., TLR, NLR, c-type lectin as well as house dust mites (HDM). Fig. 4 showed that secreted levels of IL-23 p19 were significantly suppressed up to 4.5-fold in Gal-3 siRNA-treated MoDCs stimulated with LPS, R848, Pam3CSK4 (TLR2 ligand), muramyl-dipeptide, MDP (NOD-2 ligand), zymosan (c-type lectin β-glucans for dectin-1 ligand) and HDM (β-glucans, LPS and proteases) (Fig. 4A, p value was computed between the two groups of siRNA-treated MoDCs versus snRNA-treated MoDCs, stimulated with the respective innate immunity ligand) [29–32]. In contrast, levels of secreted IL-12 p35 were significantly elevated in MoDCs, stimulated with LPS, R848, zymosan as well as HDM up to about 3-fold but interestingly not in Pam3Csk4 and MDP stimulated MoDCs (Fig. 4B). Noticeably, Fig. 3C showed that levels of IL-12 p40 subunit like that of mRNA levels (Fig. 2C) was not affected by Gal-3 siRNA treatment. In contrast, production of IL-12 hetero-dimer, e. g., IL-12 p70 in Gal-3 siRNA treated MoDCs was upregulated (Fig. 3D) as a result of differential upregulation of IL-12 p35 (Fig. 3C). Thus, importantly, differential modulation of secreted IL-23 p19 versus IL-12 p35 by Gal-3 siRNA but not IL-12 p40 is concordant with that of differential mRNA levels of IL-23 p19 versus IL-12 p35 but non-perturbed IL-12 p40 in LPS and/or R848 stimulated MoDCs (Fig. 2).

3.3 Immune deviation of Th1, Th2, and Th17 by Gal-3 siRNA-treated MoDCs

We next evaluated whether cytokine reprogramming in Gal-3 siRNA-treated MoDCs may regulate trichotomous CD4 T cell development by promoting Th1, while inhibiting Th2 and

Th17 development. Human MoDCs/T-cell cocultures were initiated. Staphylococcal enterotoxin B (SEB) was previously shown to stimulate the production of IFN-γ, IL-5, IL-13, and IL-17A in human DCs-CD4 T-cell cocultures [33–35]. Circulating CD45RO CD4 T-cells regarded as a memory cell type, readily respond to innate immunity ligands and antigenic stimulation to secrete IL-4, IL-5, IL-17A and IFN-γ in contrast to CD45RA CD4 T-cells [22, 23]. Therefore CD45RO CD4 T-cells isolated from PBMCs were cocultured with SEB-pulsed, Gal-3 siRNA-treated MoDCs [33], and levels of IL-12, IL-5, and IL-17A were harvested from day 7 supernatants. As shown in Fig. 5A, IFN-γ production by CD45RO CD4 T-cells was significantly enhanced by coincubation with Gal-3 siRNA-treated, LPS and/or R848-stimulated MoDCs, pulsed with SEB (Gr. 2 vs. Gr.1; Fig 5A). Addition of anti-p35 IL-12 and IL-12 p40 abrogated enhanced levels of IFN-γ (Gr. 3, 4 vs. Gr. 2; Fig 5A). In contrast, Gal-3 siRNA treated, Pam3CSK4-stimulated MoDCs did not elevate levels of IFN-γ in cocultures (Gr. 2 vs. Gr. 1; Fig. 5A), consistent with the observation of lack of IL-12 p35 upregulation in Gal-3 siRNA-treated, Pam3Csk4- or MDP-stimulated MoDCs (Fig. 4B).

Fig. 5B showed that production of IL-17A by CD4 T-cells was inhibited by coincubation with Gal-3 siRNA-treated MoDCs stimulated with LPS, R848, LPS/R848, Pam3Csk4 or MDP-stimulated, but not with scRNA-treated control MoDCs (Gr. 2 vs. Gr. 1; Fig 5B). Addition of anti-IL-12 p35 antibodies partially reversed Th17 production (Gr. 3 vs. Gr. 2; Fig 5B). In contrast, adding anti-IL-23 p19, anti-IL-1β and anti-IL-6 antibodies profoundly inhibited IL-17A levels in cocultures (Gr. 4 vs. Gr. 1, 2, 3; Fig. 5B).

Fig. 5C showed that elevated IL-5 production by CD4 T-cells co-cultured with control LPS and/or R848-stimulated MoDCs (Gr. 1, Fig. 5C). In contrast, IL-5 levels were diminished in cocultures with Gal-3 siRNA-treated MoDCs (Gr. 2 vs. Gr. 1; Fig. 5C). In contrast, neutralizing anti-IL-12 p35 addition reversed suppression of IL-5 (Gr. 3 vs. Gr. 2; Fig. 5C). Taken together, these observations indicate that Gal-3 downregulation in MoDCs inhibits IL-5 production by CD4 T-cells via upregulating IL-12 secretion by MoDCs and/or CD4 T-cells. Taken together, we propose that Gal-3 as a cytokine hub in MoDCs reprograms inflammatory-prone MoDCs to anti-inflammatory or regulatory-prone MoDCs for immune homeostasis.

3.4. Intracellular action of Gal-3

Next, we evaluate whether mechanisms of Gal-3 siRNA are mediated by inhibiting levels of extracellular and/or intracellular Gal-3. Extracellularly secreted Gal-3 mediates its action via the CRD domain, which can be blocked by anti-Gal-3 (B2C10) mAb made in the lab [21], or polyclonal anti-Gal-3 antibodies. Adding B2C6 or polyclonal antibodies did not augment IL-12 p35 nor suppress IL-23 p19 production in LPS and/or R848 stimulated MoDCs as shown in Fig. 6A and Fig. 6B, respectively. Fig. 6C ascertained that the dosage of B2C10 used in the above studies effectively abrogated radioactive Gal-3 binding to human IgE, and the potency of blocking CRD by B2C6 completely at 10 μg/ml is similar to high dose of NAC-lactosamine [36]. The observations strongly suggest that differential IL-23 p19 versus IL-12 p35 is not mediated by elevated extracellularly secreted Gal-3.

The lack of an extracellular role of secreted Gal-3 is also supported by reconstituting Gal-3 siRNA treated MoDCs with externally added recombinant Gal-3. Fig. 6D showed that addition of recombinant Gal-3 to Gal-3 siRNA-treated MoDCs did not restore IL-23 p19 production nor suppress the enhanced levels of IL-12p35 due to downregulating Gal-3 mRNA. Taken together, these observations strongly suggest that intracellular pools of Gal-3 but not extracellularly secreted Gal-3 play a key role in differential regulation of IL12 p35 versus IL-23 p19 production. The molecular signal transduction governing the endogenous Gal-3-mediated events are being investigated.

Discussion

Data herein show that Gal-3 in MoDCs, prepared form adherent monocytes of human PBMC plays a key role in immune homeostasis of Th1 versus Th2 and Th17 development. Gal-3 siRNA differentially upregulates IL-12 p35 versus IL-23 p19 unique subunit in MoDCs stimulated by innate immunity ligands without affecting IL-12/IL-23 p40 common subunit. In addition, IL-1β and IL-6 are also downregulated, while IL-10 production is augmented. Moreover, Gal-3 siRNA treated MoDCs redirect Th1, Th2 and Th17 development. Since fungal dectins and β-glucans present in HDM are known asthmogenic triggers, diminished IL-23 p19 versus elevated IL-12 p35 in β-glucans-stimulated MoDCs suggest a role of Gal-3 siRNA in treating MoDCs-mediated allergic asthma. Data presented herein prompt the hypothesis that Gal-3 acting as a cytokine hub reprograms proinflammatory versus anti-inflammatory cytokines in innate ligands-stimulated MoDCs. CD205 has been reported variably expressed in human MoDCs as well as expressed in multiple leukocyte lineages [24]. Gal-3 expression in both CD205+ and CD205 (−) MoDCs subsets, cultured from enriched adherent monocytes via maturation cocktails, were suppressed by Gal-3 siRNA treatment. It will be important to also extend the above observations to conventional DCs (cDCs) e.g., CD1c+/CD11b+ cDCs and CD1c+/CD103 or CD141+ cDCs [13, 37–39]. MoDCs play a critical role in house dust mite (HDM) induced allergic asthma in the rodent model [13], and siRNAs (without nanoparticles) against pertinent target genes in lung DCs and macrophages were shown to alleviate allergic asthma [40–43]. Future studies will be extended to targeting lung DCs and other cell types *in vivo* in the rodent asthma model [19].

We show that Gal-3 siRNA suppress IL-23 p19 production in MoDCs stimulated by dectins, e.g., β-glucans of zymosan and HDM. On the other hand, IL-23 p19 production was shown upregulated in a recent study using Gal-3 deficient, bone marrow derived dendritic cells (BMDC) stimulated by *Histoplasma capulatum* or fungal dectins [44, 45]. The disparity of these two observations may be attributed to species difference, source of DCs and/or culture conditions. The diametrical outcome, however, is likely to be attributed to using DCs with temporary phenotypic knockdown of the Gal-3 gene versus those with permanent genotypic knockout of the gene. Gal-3 gene deficiency has been routinely used in analyzing its function in various cell types and animal models. It may be pointed out that genotypic deficiency leads to a permanent loss of not only the Gal-3 gene but also gene products that interact with Gal-3 during its up- and downregulation. The losses of Gal-3 gene and Gal-3 dependent physiologic networks are also accompanied with molecular and cellular compensatory responses, which may not be elicited in Gal-3 competent cells. In contrast,

phenotypic knockdown by Gal-3 siRNA in MoDCs maintains a normal homeostatic function of the Gal-3 gene and its molecular network of the gene within the appropriate spatial-temporal constraint, which can re-establish homeostasis following a transient knockdown [17]. In this vein the endogenous Gal-3 dependent network, present in Gal-3 knocked-down DCs but missing in Gal-3 knocked-out DCs underlies the discrepancy of the two observations.

The above explanations may in part also reconcile some un-anticipated observations *in vivo*. Gal-3 gene deficiency paradoxically leads to an un-anticipated poor prognosis in kidney and cardiac fibrotic inflammation; moreover, the deficiency also causes increased insulin resistance and weight gain in other animal models of diseases [10, 46, 47]. It is possible that basal/low levels of Gal-3 expression are required in order to maintain low levels of inflammation in Gal-3 sensitive target organs, while the permanent loss of Gal-3 dependent molecular regulatory networks leads to accelerated, severe, unregulated inflammatory responses in the target organs. Thus drug development using Gal-3 siRNA in assessing changes in pathophysiological outcomes in a transient knockdown in an animal model appears more patho-physiologically pertinent than that of using Gal-3 gene knockout mice.

We address two potential mechanisms of Gal-3 siRNA-mediated cytokine reprogramming in MoDCs. First Gal-3 siRNA is proposed to reduce cell-bound and/or secreted Gal-3 that binds to surface glycoproteins and/or glycans via CRD and polarize cytokines by MoDCs. Indeed, Gal-3 polarized alternative or M2 macrophages by binding to surface CD98 on monocytes via the PI-3K pathway [48]. Moreover, this modality of extracellular binding to EGF receptors and cytokine receptors via Mgat5-modified glycans also account for altering cytokine-mediated signaling in different cell types [4]. Herein we showed that the extracellular CRD-dependent signaling is unlikely for our model since neutralizing antibodies against Gal-3 did not affect differential regulation of IL-23p19 versus IL-12 p35 in LPS and/or R848-stimulated MoDCs (Fig. 5D).

We therefore propose that Gal-3 siRNA reprograms cytokine production by affecting intracellular Gal-3 dependent kinases and transcription factor independent of extracellular carbohydrate recognition [2, 49, 50]. Intracellular Gal-3 is known to mediate diverse biological effects via its binding to cytoplasmic and/or nuclear N-Ras, Bcl2, p53, β-catenin, integrin/ITK, Fak, and XBP-1 upregulation and UPR-mediated oxidative stress recently reported by our laboratory [49–55]. Cellular and molecular mechanisms of Gal-3-mediated signal transduction leading to cytokine reprogramming in MoDCs and subsequent CD4 T-cell immune deviations are currently being studied in the laboratory. Recently, augmented IL-27 was shown in human MoDCs treated with Gal-3 antagonists, and augmented CD4 Treg induction was also shown in another study using Gal-3 deficient mice [56, 57]. Thus the future studies in the laboratory will also address whether Gal-3 siRNA treated MoDCs also favor Treg/Tr1 deviation (in addition to Th2/Th17) by producing IL-27, IFN-β in addition to IL-10 reported herein [49, 58, 59].

In summary, the results herein suggest a role of Gal-3 to differentially regulates or reprograms cytokine production in favor of anti-inflammatory DCs in immune deviation of Th1/Th2/Th17. Unlike anti-cytokine antibody-based therapy tailored for a single

proinflammatory cytokine, targeting Gal-3 cytokine hub downregulates multiple proinflammatory cytokines at once in DCs inhibiting Th2/Th17 CD4 T-cells, and therefore mitigates PAMP-induced proinflammatory damage to the target organs [60].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

cDCs	conventional dendritic cells
Gal-3	galectin-3
Gal-3 siRNA	galectin-3 small interfering RNA
MoDCs	monocyte-derived dendritic cells
NLR	NOD-like receptors
PAMP	pattern-associated molecular pattern
PRR	pattern recognition receptors
TLR	Toll-like receptors

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Highlights

- Galectin-3 (Gal-3), a β-galactoside-binding lectin, reprograms proinflammatory cytokine production in human MoDCs.
- Gal-3 siRNA upregulates IL-12 p35, IL-10, while downregulates IL-23 p19, IL-6, IL-1β in TLR/NLR-stimulated MoDCs.
- Gal-3 siRNA-treated human MoDCs inhibit Th2 and Th17 development.
- Intracellular Gal-3 acts as a cytokine hub of human DCs, independent of carbohydrate recognition domain (CRD).

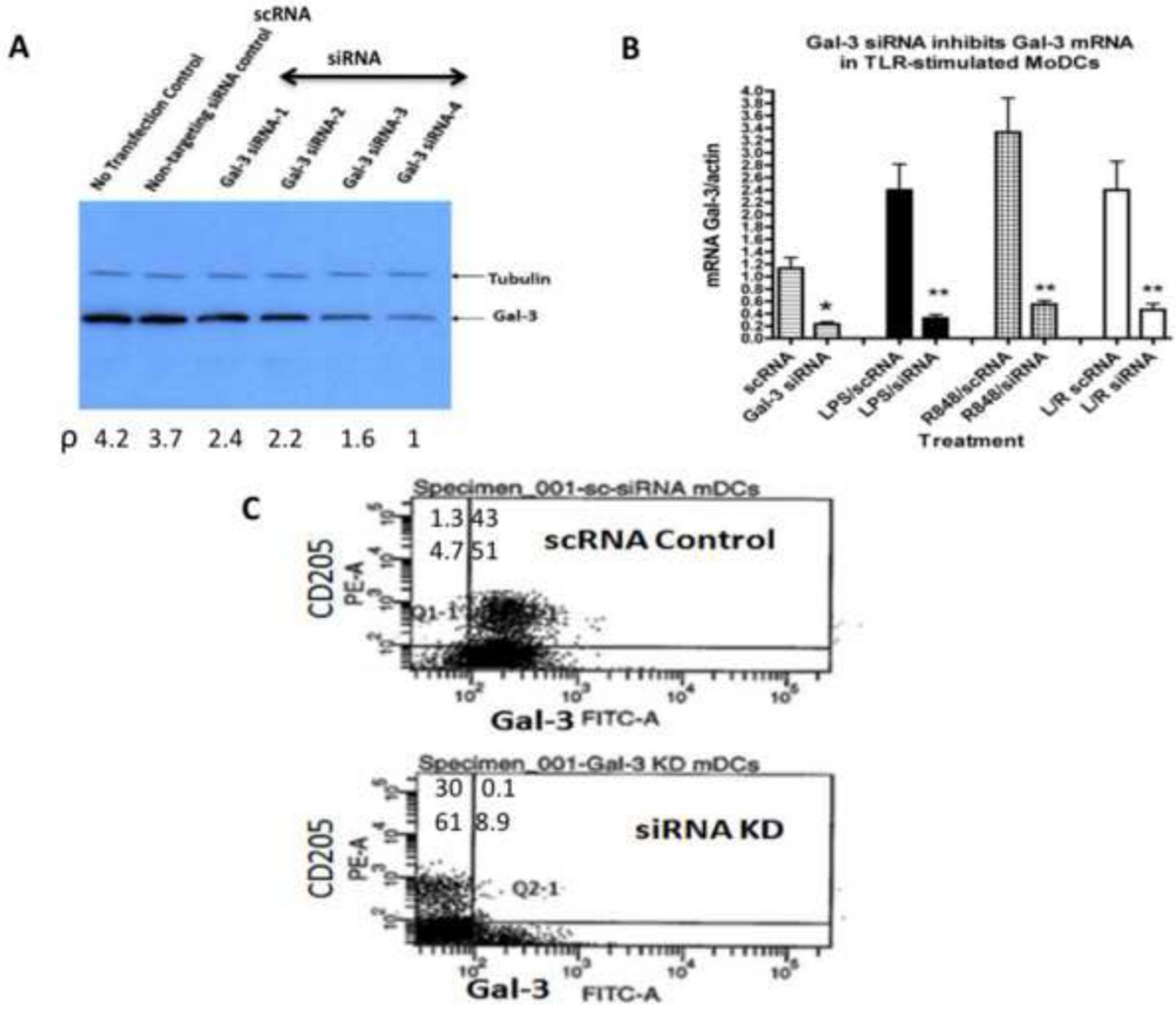


Fig. 1.

Gal-3 knockdown in MoDCs by four Gal-3 siRNA constructs. Adherent monocytes from PBMC were cultured in GM-CSF/IL-4 for 7 days, and then incubated in maturation cocktail containing TNF-α, PGE2, IL-1β, and IL-6 for 24 h. MoDCs were transfected at 1 nmol with 4 respective siRNAs targeting Gal-3 or with a non-targeting scramble control RNA (scRNA) in lipofectamine for 2 h, washed and then incubated in media for 72 h. Western blot was then performed in Gal-3 siRNA-transfected MoDCs. The experiment was independently confirmed (**A**). Gal-3 expression siRNA-4 transfected MoDCs stimulated with LPS (250 ng/ml) and/or R848 (5 μg/ml) for 72 h was evaluated by qRT-PCR with arbitrary units or fold against endogenous actin. Experiments were repeated three times from different donors with consistent results. The results of the representative experiments in triplicate determination as means and SE. The p value was computed with a Mann-Whitney U test with a p value <0.05 (*) or <0.01 (**). (**B**). MoDCs treated with Gal-3 siRNA-4 were

stained at 72 h with PE-anti-human CD205 (BioLegend) in ice-cold PBS, washed, fixed in 2% paraformaldehyde, then stained with FITC-anti-Gal-3 and evaluated by FACS analysis. The repeated experiment was independently confirmed (**C**).

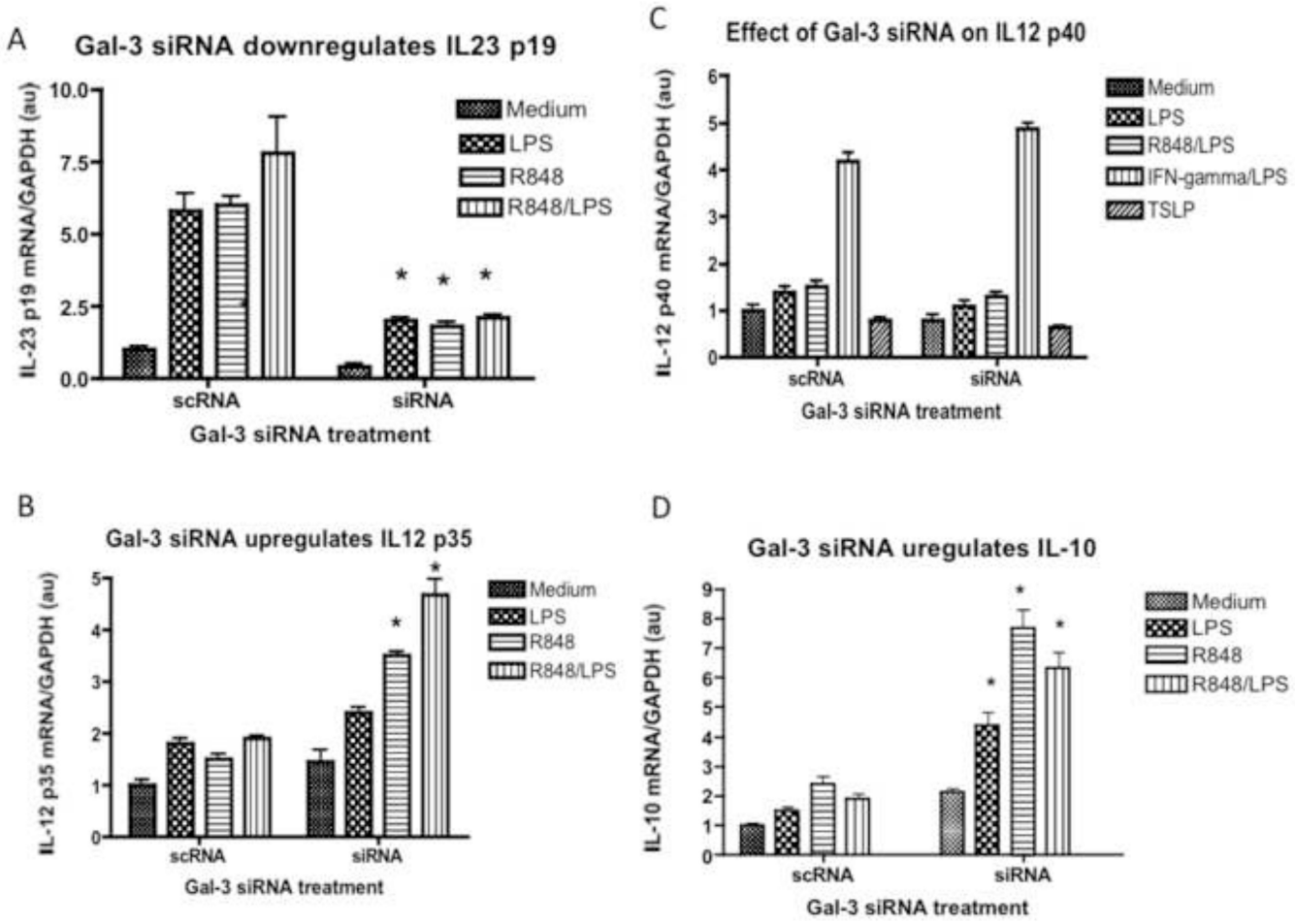


Fig. 2.

Altered cytokine messages in Gal-3 siRNA-treated MoDCs. MoDCs, matured in a full cocktail (TNF-α, PGE2, IL-1β, and IL-6) were transfected with 1 nmol siRNA-4 or treated with scramble control, non-targeting RNA control (sc/snRNA) for 2 h and then stimulated with LPS (250 ng/ml) and/or R848 (5 μg/ml) for 48 h. Messages for IL-12 p19 (A), IL-12 p35 (B), IL-10 (C), IL-12 p40 (D) and OX40L (E) in Gal-3 siRNA-4 and scRNA-treated MoDCs were evaluated by qRT-PCR as described. Primers have been titrated and optimized for reliable performance. Experiments were repeated three times from different donors. The results of the representative experiments in triplicates as means+/−SE, and the p value computed.

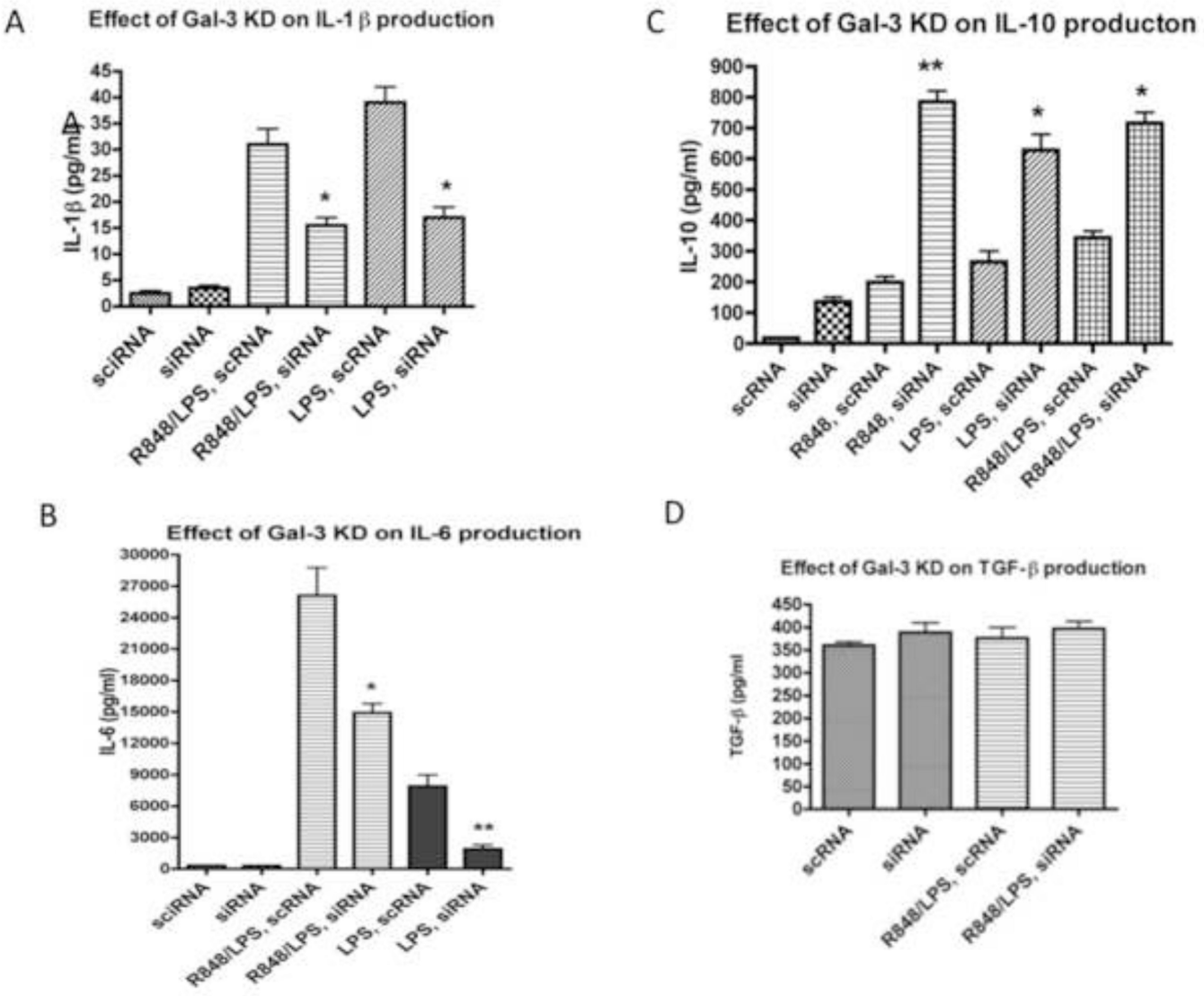


Fig. 3.

Production of IL-1β, IL-6, TGF-β and IL-10 in Gal-3 siRNA-treated MoDCs. MoDCs, matured in TNF-α/PGE2 were treated with 1 nmol Gal-3 siRNA-4 or scRNA for 2 h, and then stimulated for 48 h with LPS (250 ng/ml) and/or R848 (5 μg/ml). Levels of cytokines that were secreted in the supernatants, were evaluated by ELISAs (Panel A-D). Experiments were repeated three times from different donors. The results of the representative experiments in triplicates as means+/−SE and the p value computed.

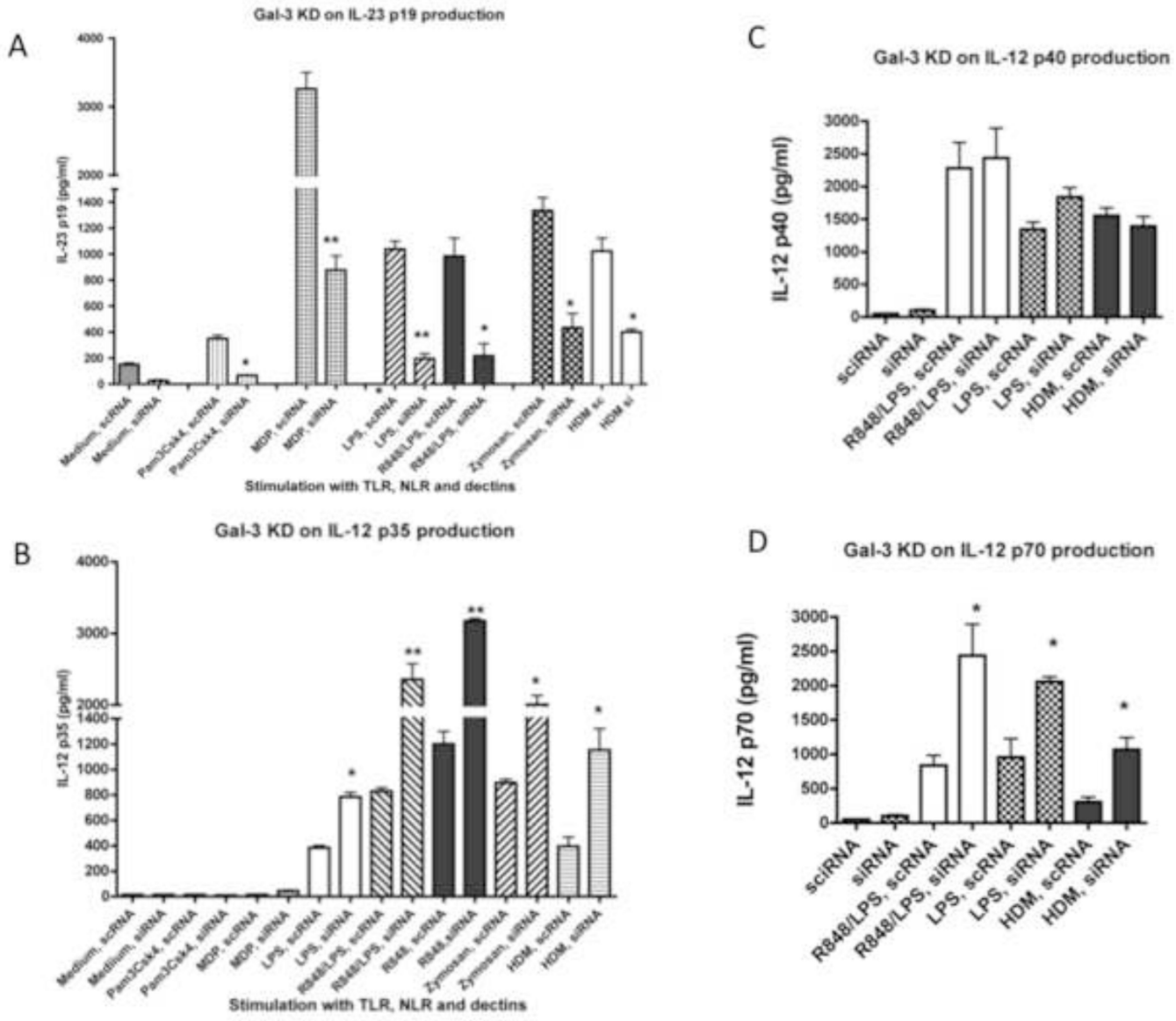
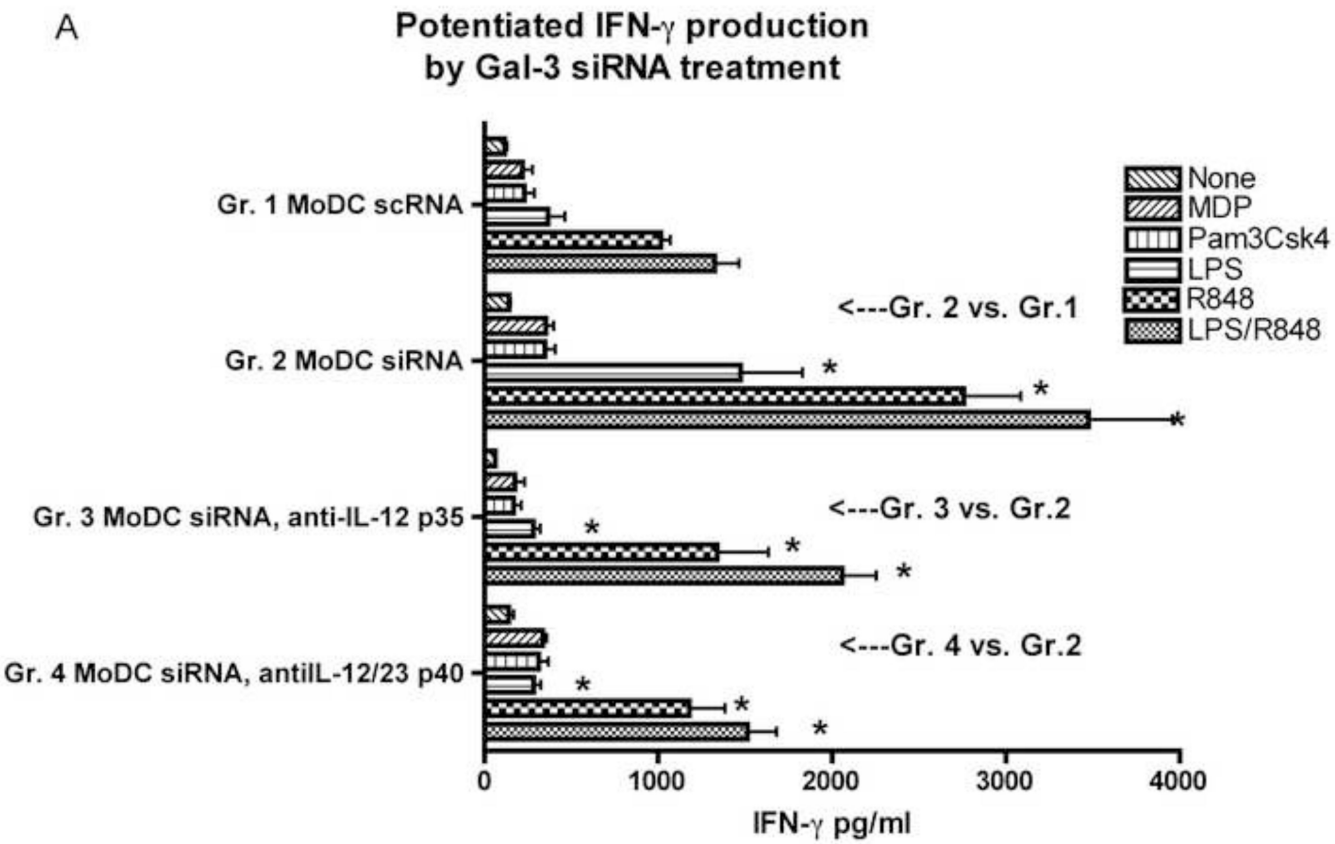
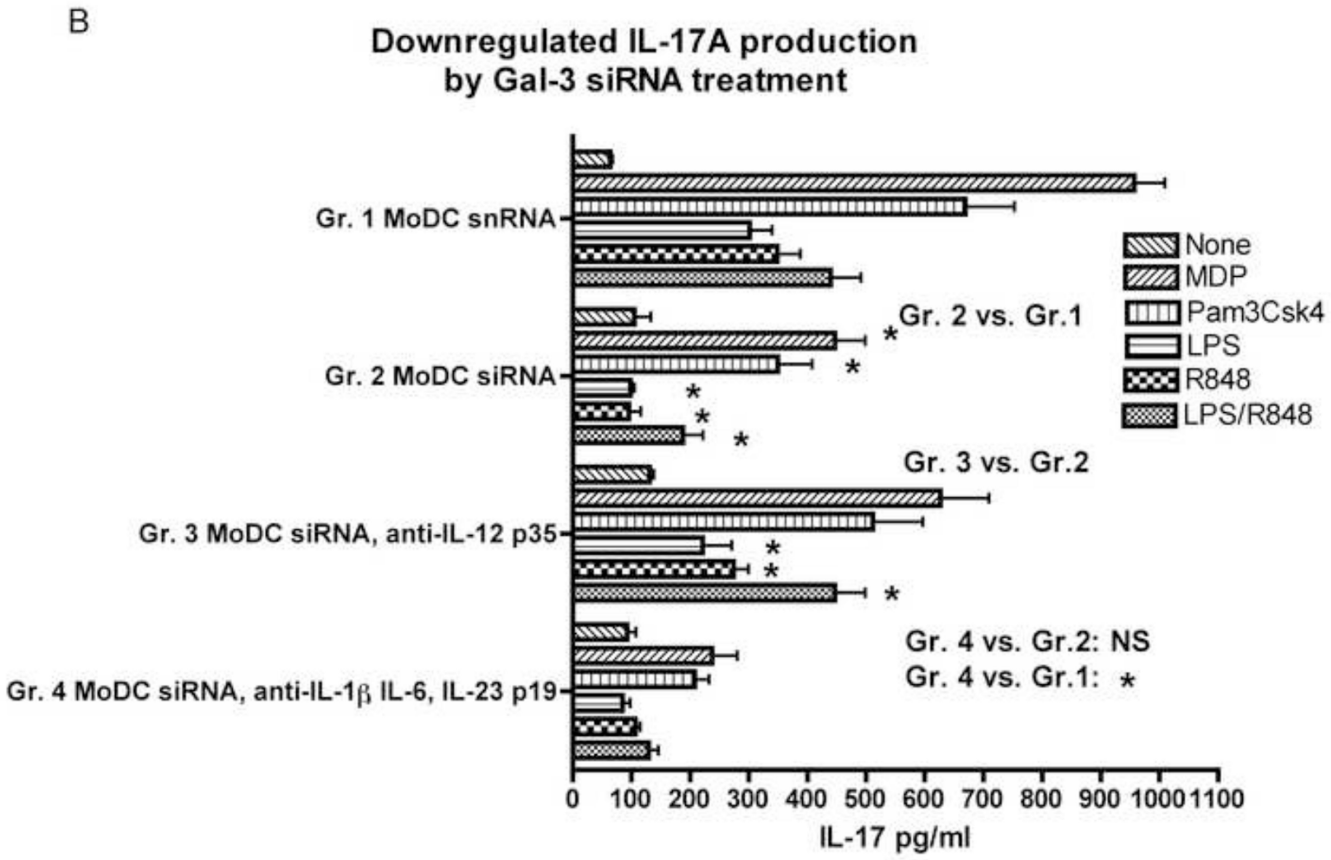


Fig. 4.

Differential IL-23 p19 versus IL-12 p35 production in Gal-3 siRNA-treated MoDCs stimulated with TLR/NOD and innate immunity ligands. GM-CSF/IL-4 cultured MoDCs were matured with a full cocktail (TNF-α, PGE2, IL-1β, and IL-6). MoDCs were treated with Gal-3 siRNA-4 or scRNA for 2 h and then stimulated for 48 h with different innate immunity ligands, e.g., LPS (250 ng/ml) and/or R848 (5 μg/ml), Pam3CSK4 (10 μg/ml), MDP (2 μg/ml), zymosan (25 μg/ml), or house dust mites (HDM, 25 μg/ml). Levels of secreted IL-23 p19 (Panel A) and IL-12 p35 (Panel B) were evaluated by ELISAs. Experiments were repeated three times. The results of the representative experiments in triplicates as means+/−SE, and the p value computed.





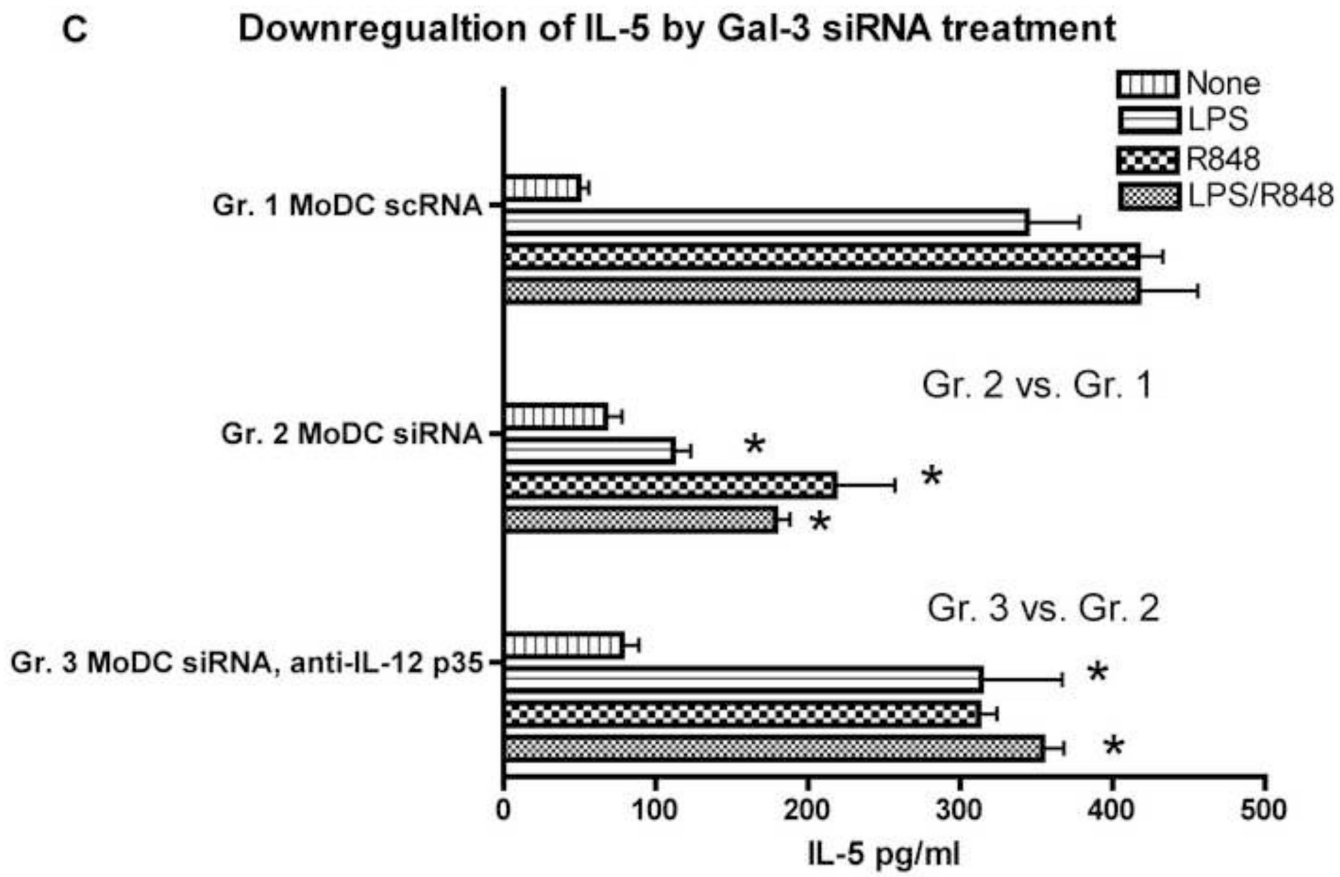


Fig. 5.

Effect of Gal-3 siRNA-treated MoDCs on CD4 T cell differentiation. CD45RO T cells were prepared with by positive selection with CD45RO magnetic beads of the CD4-T cells selected by the CD4 MACS multisort kit. Gal-3 siRNA-4 treated MoDCs were stimulated with LPS (250 ng/ml) and/or R848 (5 μg/ml), Pam3CSK4 (10 μg/ml) or MDP (2 μg/ml) for 48 hr in the presence of SEB (100 ng/ml). To assess IL-5 production, siRNA or scRNA treated MoDCs were also coincubated with CD40L (10 μg/ml) along with innate immunity ligand. MoDCs (3.3×10^5) were then washed and added to CD45RO (1×10^6) at a ratio of 1:3, and cocultures were incubated continually for seven days. Supernatants were then collected and evaluated for IFN-γ (**A**), IL-17A (**B**), and IL-5 (**C**) by ELISAs. The experiments were repeated five times with the optimal conditions obtained and the representative experiment is presented. Comparator between the two selected groups are specified in the arrowed legend placed within the respective drawing (Panel A to C). Thus a siRNA treatment group will be compared to the snRNA control group (e.g. siRNA vs. snRNA) as well as compared to another treatment group with added mAbs (e.g., siRNA vs. siRNA+ mAbs) to yield pertinent information. Experiments were repeated three times from different donors. The results of the representative experiments in triplicates as means+/−SE, and the p value computed.

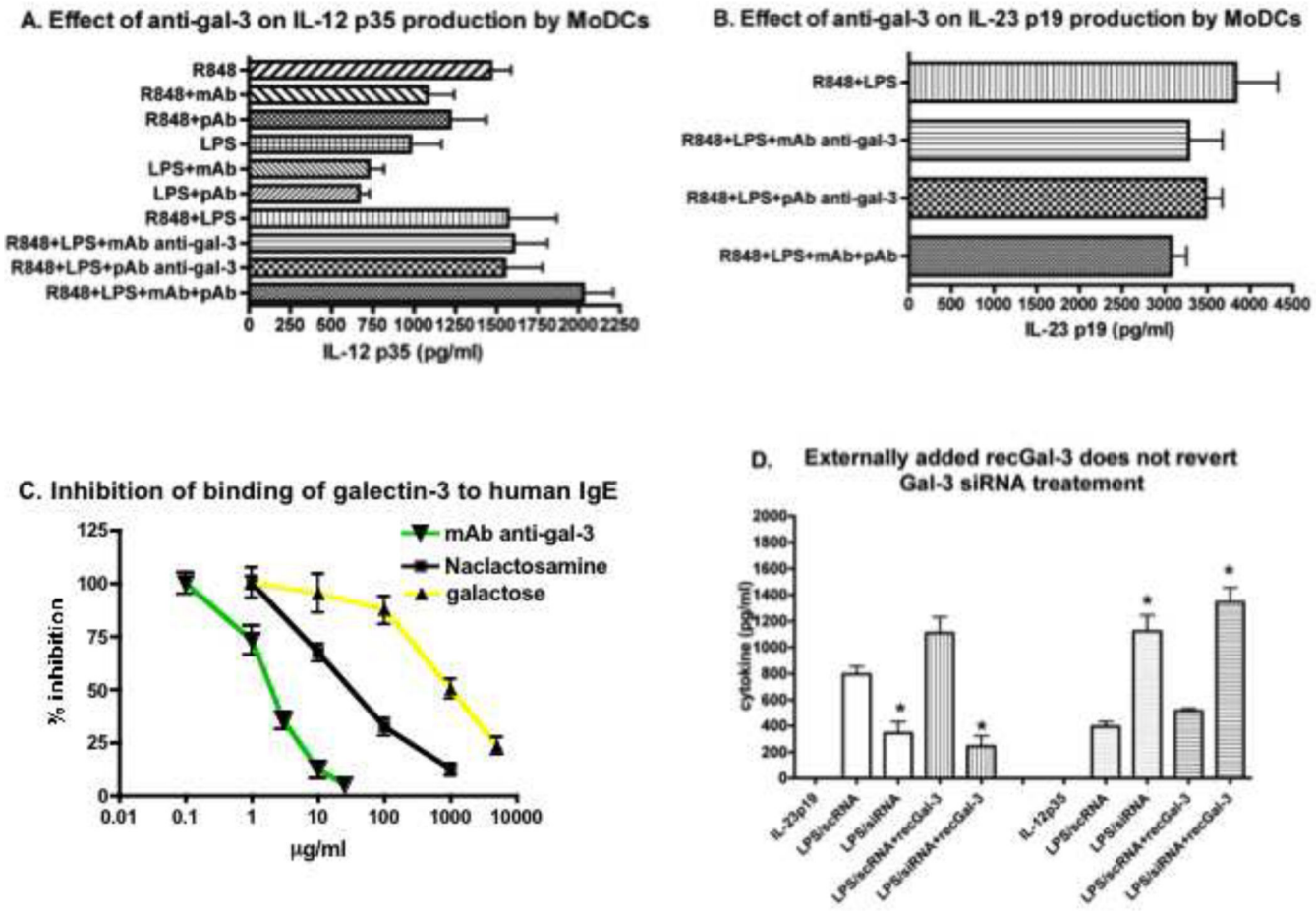


Fig. 6.

Effect of anti-Gal-3 antibodies and Gal-3 antagonists on IL-23 p19 versus IL-12 p35 production by MoDCs. MoDCs were stimulated with LPS (250 ng/ml) and/or R848 (5 µg/ml) in the presence of anti-Gal-3 B2C10 (25 µg/ml), polyclonal anti-Gal-3 (20 µg/ml), Gal-3 antagonists, e.g., NAC lactosamine, galactose. Supernatants were harvested 2 days later and evaluated for levels of IL-12 p35 (**A**) and IL-23 p19 (**B**) by ELISAs. I^{125}-Gal-3 (1.2×10^6 cpm) was preincubated with B2C10 (0.1 to 25 µg/ml) at rt for 2 h or β-galactosides, NAC lactosamine (1–1000 ug/ml) or galactose (10 µg to 10 mg/ml) at rt for 2 h, and then added to NP-specific IgE coated on NP-BSA solid phase for 2 h. The radiolabeled Gal-3 bound to IgE on the solid phase was then evaluated by a γ-counter (**C**). MoDCs treated with Gal-3 siRNA or control scRNA were stimulated with LPS with recombinant human Gal-3 added back to cultures at 20 µg/ml for 48 h. Supernatants were then harvested and evaluated for IL-23 p19 and IL-12 p35 by ELISAs (**D**). Experiments were repeated three times from different donors. The results of the representative experiments in triplicates as means+/−SE, and the p value computed.