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Inhibition of MicroRNA let-7i Depresses Maturation and Functional State of Dendritic Cells in Response to Lipopolysaccharide Stimulation via Targeting Suppressor of Cytokine Signaling 1

Maomao Zhang,*† Fang Liu,*† Haibo Jia,*† Qi Zhang,*† Li Yin,*† Wei Liu,* Hulun Li,*† Bo Yu,*† and Jian Wu*†

Dendritic cells (DCs) can initiate immune responses or confer immune tolerance depending on functional status. LPS-induced DC maturation is defined by enhanced surface expression of CD80 and CD86. MicroRNAs are critical for the regulation of DC function and immunity, and the microRNA let-7i was upregulated during LPS-induced DC maturation. Downregulation of let-7i significantly impeded DC maturation as evidenced by reduced CD80 and CD86 expression. DCs stimulated by LPS promoted T cell proliferation in coculture, whereas LPS-stimulated DCs with downregulated let-7i were not effective at stimulating T cell proliferation but promoted expansion of the regulatory T cell (Treg) population. There were two subpopulations of LPS-stimulated DCs with downregulated let-7i, CD86+ and CD86−, and it was the CD86− DCs that were more effective in inducing T cell hyporesponsiveness and enhancing Treg numbers, indicating that this DC population had tolerogenic properties. Furthermore, Tregs with upregulated IL-10 underscored the tolerogenic effect of CD86− DCs. Suppressor of cytokine signaling 1 (SOCS1), a crucial mediator of DC maturation, was confirmed as a target gene by luciferase construct assay. Suppression or overexpression of let-7i caused reciprocal alterations in SOCS1 protein expression, but had no significant effects on SOCS1 mRNA levels, indicating that let-7i regulated SOCS1 expression by translational suppression. The modulation of SOCS1 protein by let-7i was mainly restricted to CD86− DCs. Our study demonstrates that let-7i regulation of SOCS1 is critical for LPS-induced DC maturation and immune function. Dynamic regulation of let-7i may fine-tune immune responses by inducing Ag-specific immune tolerance. The Journal of Immunology, 2011, 187: 1674–1683.

Dendritic cells (DCs) are potent professional APCs that are critical for the initiation of primary immune responses. In response to inflammatory stimulation, DCs exhibit a remarkable pattern of differentiation and maturation that underlies specific mechanisms to control immunity (1). The functional activities of DCs are dependent on the states of activation and maturation (2). Mature DCs express high levels of MHC and costimulatory molecules on the plasma membrane that regulate T cell activation and Ag-specific immune responses. In contrast, immature DCs (imDCs) with low surface expression of MHC and costimulatory molecules cause immune deviation from the Th1 to Th2 cell subset, which evokes Ag-specific T cell hyporesponsiveness and Ag-specific tolerance (2, 3). Therefore, dynamic control of DC maturation acts to further specify Ag-specific immune responses.

MicroRNAs (miRNAs) are evolutionarily conserved, small noncoding RNAs that regulate gene expression by degrading specific target mRNA or by repressing translation (4, 5). A growing body of evidence has demonstrated the importance of miRNAs in the development and response of the immune system, including the development and differentiation of immune cells, Ag production, inflammatory mediator release, and innate and adaptive immune responses (3, 6–8). MiRNAs act as regulators to fine-tune the immune response, including DC function. The miRNA let-7 is the prototype of the miRNA family and is conserved from invertebrates to humans. In Caenorhabditis elegans, let-7 acts as a master temporal regulator of multiple genes required for cell cycle exit in stem cells and controls the timing of terminal differentiation (9, 10). Most of the studies involving let-7 have focused on cell cycle control of cancer cells, and several targets of let-7 have been confirmed, including RAS, HMGA2, and Cdc34 (11–14).

Suppressor of cytokine signaling (SOCS) is a family of genes that are induced by cytokines and act in a classic negative feedback loop to inhibit cytokine signal transduction. Pathogen recognition via TLRs or TLR ligands such as LPS can regulate the expression of SOCS proteins in host cells. Previous studies have demonstrated that SOCS1 plays a pivotal role in regulating the activation, development, and differentiation of macrophages, T cells, and DCs.
especially in response to TLR ligands (15-18). Several mechanisms have been proposed for the suppressive effect on cytokine production and macrophage activation by SOCS1 through the TLR/NF-κB signaling pathway (19). The inhibition of the secondary activated JAK/STAT pathway is another important mechanism for the suppressive effect of SOCS1 (20, 21). Moreover, SOCS1 can interact with phosphorylated Mal, an adaptor molecule required for TLR2 and TLR4 signaling, resulting in Mal polyubiquitination and subsequent degradation (22).

In this report, we demonstrate that expression of miRNA let-7i was upregulated during LPS-induced maturation of DCs. We developed a method to manipulate intracellular let-7i levels by transfecting immature bone marrow-derived DCs with let-7i mimic or let-7i inhibitor and demonstrated that alteration of the let-7i level regulated LPS-induced DC maturation. By using a coculture system including DCs of variable function state (imDCs, LPS-treated DCs, let-7i regulator, and LPS-treated DCs) plus T cells, we demonstrate that let-7i reduction in DCs resulted in a significant downregulation of costimulatory molecule expression, a reduced capacity to stimulate T-cell proliferation, an enhanced ability to increase the numbers of regulatory T cells (Tregs), and a modulated cytokine profile with decreased proinflammatory cytokines and enhanced anti-inflammatory cytokines. Bioinformatic databases suggested SOCS1 as a possible target gene of let-7i, and this was confirmed by using a luciferase construct assay. In conclusion, our study revealed that let-7i regulated DC maturation and functional state in response to LPS stimulation by suppressing SOCS1 translation. A population of let-7i-deficient CD86+ DCs was the main effector of Ag-specific tolerogenic activity.

Materials and Methods

Generation of bone marrow-derived DCs and LPS stimulation

DCs were generated from bone marrow cells of Wistar rats as described previously (23). Briefly, bone marrow was flushed from femurs and passed through a 100-μm pore size mesh to remove fibrous tissue. The RBCs were lysed and the remaining cells were cultured at 1 × 10^6 cells/ml in RPMI 1640 medium (HyClone, Logan, UT) supplemented with cytokines (10 ng/ml each mouse GM-CSF and IL-4; PeproTech, Rocky Hill, NJ). To induce DCs, DCs were stimulated with other TLR ligands, including flagellin ligand LPS (10 ng/ml; Sigma-Aldrich, St. Louis, MO). In some experiments, DCs were stimulated with other TLR ligands, including flagellin (0.1 μg/ml; InvivoGen, San Diego), lipoteichoic acid (1 μg/ml; Sigma-Aldrich, St. Louis, MO). In some experiments, DCs were stimulated with other TLR ligands, including flagellin (0.1 μg/ml; InvivoGen, San Diego), lipoteichoic acid (1 μg/ml; Sigma-Aldrich), or polyinosinic-polycytidylic acid (2 μg/ml; Sigma-Aldrich). The resulting clusters of putatively mature DCs were harvested and a subpopulation was labeled with anti-OX62 and phenotypically analyzed by surface expression of specific costimulatory molecules and enhanced anti-inflammatory cytokines. Bioinformatic databases suggested SOCS1 as a possible target gene of let-7i, and this was confirmed by using a luciferase construct assay. In conclusion, our study revealed that let-7i regulated DC maturation and functional state in response to LPS stimulation by suppressing SOCS1 translation. A population of let-7i-deficient CD86+ DCs was the main effector of Ag-specific tolerogenic activity.

Flow cytometry

DC purity and phenotype were analyzed by surface expression of specific markers using flow cytometry. The following Abs were used: FITC-OKT4, PE-CD80, PE-CD86 (eBioscience, San Diego, CA), and FITC-TLR4 (Abcam, Cambridge, MA). For each staining protocol, the appropriate isotype-matched control was included. For Treg assays, the cells in MLRs were washed twice with PBS and then stained with primary mAbs for 30 min at 4˚C. The following fluorochrome-conjugated mAbs were used: CD4-FITC, CD25-allophycocyanin, and Foxp3-PE (all from eBioscience). After primary incubation, the cells were washed, fixed and permeabilized using fixation/permeabilization agents (supplied by eBioscience), and stained for intracellular Foxp3. All reagents were used at optimal concentrations, as determined in pilot experiments. Analysis was performed using FACSCount and analyzed using CellQuest Pro software.

Cell sorting by MACS

The let-7i inhibitor-treated DCs were separated into two populations, CD86+ DCs and CD86- DCs, by MACS. Briefly, DCs transfected with let-7i inhibitor and then treated with LPS were stained with PE-CD86 Ab and incubated with anti-PE magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). An LS MACS column (Miltenyi Biotec) was used to separate DCs. The purity of the DC populations was assessed by FACS analyses and was typically >90%.

The CD4+CD25+ Tregs were also isolated by cocultured MLRs by MACS (24, 25). First, CD4+ T cells were obtained by negative selection with anti-CD8 mouse IgG1 Ab and anti-mouse IgG1 microbeads (Miltenyi Biotec). Subsequently, the CD4+ T cells were incubated with anti-CD25 mouse IgG1 Ab and anti-mouse IgG1 microbeads, then separated into CD4+CD25+ and CD4+CD25- fractions by positive selection using an MS column (Miltenyi Biotec). The purity of CD4+CD25+ T cells was >80% as assessed by FACS.

MLR assay

DCs were used at different densities (0-15 × 10^6) as stimulators for allogeneic T cells (2 × 10^5) isolated from Sprague Dawley rat spleens by nonadherence to nylon wool. To assess the impact of DCs in different functional states on T cell proliferation and function, a coculture system was developed. Isolated rat T cells were cultured for 5 d in 96-well round-bottom microplates in the presence of 10 μg/ml mitomycin C-pretreated DCs at different DC/T cell ratios (1:10, 1:20, 1:50, or 1:100). These cocultures were incubated with 10 μM BrdU for 24 h, and BrdU incorporation by proliferating responder cells (T cells) was quantified using a Brdu ELISA according to the manufacturer’s instructions (Chemicon International, Temecula, CA). All proliferation assays were performed in triplicate. To confirm the immunosuppressive activity on T cell proliferation, CD4+CD25+ Tregs isolated from MLRs by MACS were added to the coculture of mitomycin C-treated DCs and T cells at a Treg/T cell/DC ratio of 10:10:1. The proliferation of T cells was again detected by BrdU-ELISA as indicated to assess the suppressive capacity of Tregs on T cell responses.

ELISA

To measure cytokine release by DCs, supernatants were collected from cultures after let-7i mimic or inhibitor transfection and LPS stimulation. The levels of IL-10, IL-12, IL-27, IFN-γ, TNF-α, and IL-6 were determined by ELISA according to the manufacturer’s instructions (BD Biosciences, San Jose, CA, USA). These assays were performed in triplicate.

Quantitative RT-PCR

A quantitative RT-PCR (qRT-PCR) approach was established by modification of a previous method (26). Briefly, total RNA was harvested from the cells, reverse-transcribed to cDNA with specific stem-loop primers, and amplified using SYBR Premix Ex Taq II (TaKaRa, DaLian, China). The reverse transcription reactions were incubated for 30 min at 16˚C, 42 min at 42˚C, and 5 min at 85˚C. The PCR protocol consisted of 40 cycles of 10 s at 95˚C and 1 min at 60˚C, followed by the thermal denaturation protocol. The highly conserved and universally expressed small nuclear RNA U6 was used as an endogenous control in the qRT-PCR. The qRT-PCR amplification products were analyzed by melting curve analysis and confirmed by agarose gel electrophoresis. The reverse primers specific for let-7i and U6 were as follows: let-7i, 5'-GTGCTATCCAGCGTGGTC-GGTA GTGCGCAATCGCTGAGTACGACAACGAC-3' and U6, 5'-CGCTTCAGAATTTGCAGCTTCG-3'. Primers specific for let-7i (forward, 5'-GGGGTGTAGGTAGTGGTGTG-3' and reverse, 5'-GTGCGTCGGTAGTCCAGCAG-3') and U6 (forward, 5'-GGTCCGGCGACCATACG-3' and reverse, 5'-CGCTTCAGAATTTGCAGCAG-3') were used to amplify the cDNA. All reactions were performed in triplicate. The expression of miRNA relative to U6 RNA was determined using the 2^-ΔCt method. Additionally, qRT-PCR analysis of SOCS1 and IL-10 mRNA was performed using the same protocol. The specific primer sequences were as follows: SOCS1, forward, 5'-CGGTCTGATCCATCGGTGATCC-3' and reverse, 5'-GAAACCCGTTTCCGAGGGA-3'; IL-10, forward, 5'-CCAGTGCGCGCCGAGAGA-3' and reverse, 5'-CAGATCTCTCTTCCAGG-3'.

Transfection with let-7i mimic and inhibitor

To manipulate let-7i in DCs before LPS stimulation, cultured DCs were transfected with let-7i mimic or inhibitor (60 nM; GenePharma, Shanghai, China) or with RPMI 1640 medium (control) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described previously. Most experiments used let-7i mimic or inhibitor at 60 nM for transfection. The cells were usually used for experiments 12 h after transfection.
Identification of miRNA let-7i binding sites

Based on experimental observations of miRNA binding to the 3'-untranslated region (UTR), several target prediction software programs have been developed. To avoid overprediction, we used a consensus approach employing four widely used software programs to perform the target prediction (DIANA MicroTest, miRanda, PicTar, and TargetScanS). Only those targets that were detected by all software programs to bind to the same location in the target 3'-UTR sequence were considered for subsequent expression analysis.

Luciferase reporter constructs and luciferase assay

Two oligonucleotides (5'-GAGCCAGGACCTGAACTCGCACCTCCTACCTCCTTTCTGTTTACGAGCCAGGACCTGAACTCGCACCTCCTACCTCCTTTCTGTTTAC-3' and 5'-GAGCCAGGACCTGAACTCGCACCTCCTACCTCCTTTCTGTTTACGAGCCA GGACCTGAACTCGCACCTCCTACCTCCTTTCTGTTTAC-3') corresponding to a region containing a putative let-7i target site at the 3'-UTR of murine SOCS1 mRNA were synthesized. The oligonucleotides were annealed and cloned into the XhoI and NotI sites of the luciferase reporter psiCHECK-2 control vector (Promega, Madison, WI). The sites are located downstream of the luciferase open reading frame. The orientation of the insertion was confirmed by sequencing. When expressed in DCs, this reporter construct will produce a luciferase reporter mRNA harboring a SOCS1-derived putative let-7i targeting sequence at the 3'-UTR. This reporter (0.2 μg) was cotransfected into DCs with different concentrations of let-7i mimic or inhibitor (usually 60 nM) together with a Renilla luciferase reporter (10 ng). Assays were performed 24 h after transfection using the dual luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity. The experiments were performed three times with six replicates each.

Western blots

Western blotting was used to assess SOCS1 expression in DCs. Briefly, total cell lysates were obtained after exposure to LPS and blotted for SOCS1 and β-actin (the loading control) using anti-SOCS1 and anti–β-actin (both from Santa Cruz Biotechnology, Santa Cruz, CA). The SOCS1 protein levels were expressed as the densitometric ratio of SOCS1 to β-actin.

Immunofluorescence

Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 30 min at room temperature, blocked with goat serum, and incubated with primary Abs against SOCS1 (1:100) overnight at 4°C. After washing, cells

FIGURE 1. Upregulation of let-7i expression in DCs by LPS. Immature DCs were stimulated with 10 ng/ml LPS (A) or other TLRs agonists (B) (1 μg/ml TLR2, 2 μg/ml TLR3, or 0.1 μg/ml TLR5 for the indicated times) and let-7i expression was determined by relative qRT-PCR and normalized to U6 RNA levels. The data indicate the means (±SD) of a triplicate quantitative PCR, representing three independent experiments, each derived from a different DC preparation. *p < 0.05, compared with the control group.

FIGURE 2. Suppression of LPS-stimulated CD80 and CD86 expression on DCs by let-7i inhibition. DCs were first transfected with let-7i mimic (60 nM), inhibitor (60 nM), or RPMI 1640 medium to modulate let-7i expression for 12 h before LPS stimulation. Expression of let-7i was assessed by qRT-PCR (A). Then, the LPS-induced CD80 and CD86 expressions were assessed by flow cytometry. The let-7i inhibitor depressed the phenotype expression of LPS-stimulated DCs (B) with a lower percentage (C) and decreased the mean fluorescence intensity (MFI) (D). Data are shown as the means ± SD of three independent experiments (A, C, D). Similar results were obtained in three independent experiments (B). *p < 0.05, compared with imDCs; *p < 0.05, compared with LPS-stimulated DCs.
were incubated with FITC-conjugated goat anti-mouse Ab (1:200; Santa Cruz Biotechnology) for 1 h at 37°C. Nuclei were counterstained with DAPI (0.1 g/ml; Sigma-Aldrich). Fluorescent images were acquired with a confocal laser-scanning microscope (FluoView v5.0, Olympus, Tokyo, Japan).

**Results**

**MiRNA let-7i expression was upregulated in LPS-induced DC maturation**

Immature DCs from rat bone marrow can be induced to mature by LPS stimulation after 6 d in vitro. The purity of imDCs was analyzed by flow cytometry using surface expression of the specific marker OX62. Only those cultures with purity >85% were used for subsequent experiments. LPS-induced DC maturation was accompanied by elevated surface expression of CD80 and CD86, as well as an increased capacity to stimulate native T cells. The expression of the miRNA let-7i was assessed by qRT-PCR during LPS-induced DC maturation. After 24 h LPS stimulation, let-7i expression was upregulated 2-fold in DCs compared with untreated imDCs (Fig. 1A), suggesting that let-7i might be required for maturation of DCs.

To confirm whether let-7i was induced only by the TLR4 ligand LPS, we investigated the effect of the TLR2 ligand lipoteichoic acid, the TLR3 ligand polyinosinic-polycytidylic acid, and the TLR5 ligand flagellin (Fig. 1B). There was no significant increase in let-7i expression following TLR2, TLR3, and TLR5 agonist stimulation for 24 h compared with untreated imDCs, indicating that the dynamic changes in let-7i expression were mediated through TLR4.

**let-7i inhibition functionally depressed LPS-induced DC maturation**

We tested the potential role of let-7i in the process of DC maturation by regulating the basal let-7i level prior to LPS-induced maturation. DCs were transfected with let-7i mimic, inhibitor, or RPMI 1640 medium (negative control) for 12 h before LPS stimulation. As expected, transfection with the let-7i mimic caused let-7i overexpression whereas the let-7i inhibitor suppressed expression of let-7i in DCs as determined by qRT-PCR (Fig. 2A). The effect of let-7i expression levels on DC maturation was then assessed by measuring the expression patterns of maturation-specific proteins. After 12 h LPS stimulation, the levels of CD80 and CD86 were significantly higher in LPS-treated DCs compared with imDCs. DCs transfected with let-7i inhibitor before LPS stimulation, however, expressed lower levels of CD80 and CD86 compared with DCs stimulated by LPS (LPS-DCs), whereas DCs transfected with the let-7i mimic before LPS stimulation displayed a slight elevation of CD80 and CD86 expression that did not reach statistical significance (Figs. 2B–D). Therefore, inhibition of let-7i impeded LPS-stimulated DC maturation as defined by surface CD80 and CD86 expression.

**DCs treated with let-7i inhibitor induced T cell hyporesponsiveness and expanded Tregs in MLRs**

The functional effects of DCs on allogeneic T cells were investigated by measuring the proliferative responses of T cells cocultured with DCs of variable maturity and functional status. The ability of DCs to activate T cell proliferation was markedly enhanced by LPS stimulation compared with imDCs, suggesting that CD80 and CD86 surface expression might act to enhance T cell proliferation. In contrast, T cells cocultured with LPS-stimulated DCs transfected with let-7i inhibitor exhibited reduced proliferation compared with T cells stimulated with untransfected LPS-DCs (Fig. 3A).

To determine whether these unresponsive T cells had the characteristic cell surface phenotype of Tregs, the expression of CD4, CD25, and Foxp3 was examined after 2 d coculture. We observed that the population of Tregs expressing CD4, CD25, and Foxp3 was significantly higher when T cells were cocultured with DCs transfected with let-7i inhibitor before LPS stimulation (mean, 18%; Fig. 3C) than when T cells were cocultured with untransfected LPS-DCs (mean, 8%; Fig. 3C). Thus, DCs

![FIGURE 3](http://www.jimmunol.org/correction/3.jpg)

**FIGURE 3.** DCs transfected with let-7i inhibitor induced T cell hyporesponsiveness and favored Treg expansion. DCs were transfected with let-7i mimic (60 nM), inhibitor (60 nM), or RPMI 1640 medium for 12 h, then treated with 10 ng/ml LPS (12 h) and 10 μg/ml mitomycin C (2 h). The allogeneic T cells were cocultured with these DCs at ratios of 1:10, 1:20, 1:50, and 1:100 (DC/T cell) for 48 h and then incubated with BrdU (10 μM, 24 h) to quantify proliferation. The T cell proliferation was assessed by BrdU ELISA (A). CD4+CD25+Foxp3+ Tregs in cocultures were also assessed by flow cytometry (B) and the percentage of Tregs is shown in C. Data are shown as the means ± SD of three independent experiments (A, C). Similar results were obtained in three independent experiments (B). *p < 0.05, compared with LPS-stimulated DCs.
transfected with let-7i inhibitor were more potent inducers of Tregs. DCs transfected with let-7i mimic did not show significant effects on Treg induction. These data revealed that inhibition of let-7i impeded aspects of DC maturation, particularly surface expression of CD80 and CD86, and that these DCs failed to induce T cell proliferation (T cell hyporesponsiveness) and instead supported the expansion of Tregs.

DCs transfected with let-7i inhibitor that had tolerogenic properties were mainly CD86+. As shown in Fig. 2B, DCs transfected with let-7i inhibitor and then treated with LPS could be divided into two subpopulations (CD86+ DCs and CD86- DCs). To determine the main effector cell mediating the induction of Tregs, these DCs were separated into populations of CD86+ DCs and CD86- DCs by MACS before T cell coculture (shown in Supplemental Fig. 1). In keeping with previous observations, imDCs induced a T cell hyporesponsiveness and Treg expansion compared with LPS-stimulated DCs. Unexpectedly, purified CD86+ DCs that were transfected with the let-7i inhibitor did not suppress T cell proliferation compared with LPS-DCs. Conversely, coculture with purified CD86+ DCs led to a marked reduction in T cell proliferation compared with LPS-induced DCs, and this reduction was comparable to that induced by coculture with imDCs (Fig. 4A). Thus, surface expression of CD86 may be an important determinant of T cell immunogenicity. We also investigated the capacity of DC subpopulations to induce Tregs in MLRs to further confirm the activity of this functional state. As shown in Fig. 4B and 4C, CD86+ DCs exerted no effect into populations of CD86+ DCs and CD86- DCs by MACS before T cell coculture (shown in Supplemental Fig. 1). In keeping with previous observations, imDCs induced a T cell hyporesponsiveness and Treg expansion compared with LPS-stimulated DCs. Unexpectedly, purified CD86+ DCs that were transfected with the let-7i inhibitor did not suppress T cell proliferation compared with LPS-DCs. Conversely, coculture with purified CD86+ DCs led to a marked reduction in T cell proliferation compared with LPS-induced DCs, and this reduction was comparable to that induced by coculture with imDCs (Fig. 4A). Thus, surface expression of CD86 may be an important determinant of T cell immunogenicity. We also investigated the capacity of DC subpopulations to induce Tregs in MLRs to further confirm the activity of this functional state. As shown in Fig. 4B and 4C, CD86+ DCs exerted no effect...
on the induction of Tregs compared with LPS-DCs, whereas the expansion of Tregs was significantly promoted by CD86-DCs compared with both LPS-DCs and imDCs.

We assessed the function of Tregs induced by CD86-DCs in MLRs by measuring their immunosuppressive capacity and production of the anti-inflammatory cytokine IL-10. In light of the known suppressive effect of CD4+CD25+ Treg cells on T cell proliferation, we separated CD4+CD25+ Tregs from the culture mixture of conditioned DCs and T cells by MACS, and then cocultured purified CD4+CD25+ Tregs with T cells (responders) and LPS-induced DCs (APCs) at a ratio of 10:10:1. Fig. 4D shows that the proliferative response of T cells was drastically inhibited in the presence of the CD4+CD25+ Tregs isolated from cocultures with imDCs or CD86-DCs transfected with the let-7i inhibitor. In contrast, CD4+CD25+ Tregs induced by LPS-DCs or the CD86+ population did not exhibit these suppressive activities, which may be due to the low percentage of Foxp3 Tregs, as shown in Fig. 4B. Tregs may have the potential to produce the IL-10, so we measured IL-10 mRNA expression in MACS-sorted CD4+CD25+ Tregs by qRT-PCR. As shown in Fig. 4E, IL-10 expression was significantly increased in Tregs induced by the CD86+ DC population compared with Tregs isolated from cocultures with imDCs or LPS-DCs. This result is consistent with previous data and confirms that CD86-DCs can induce the expansion of immunosuppressive Tregs. Moreover, these results demonstrated that DC cells with reduced let-7i expression adopt a functional phenotype (CD86+) that induces T cell hyporesponsiveness and supports the expansion of Tregs with immunosuppressive capacity.

let-7i reduces SOCS1 expression in DCs by translational suppression

Because we implicated the important role of let-7i in LPS-induced DC maturation, we sought to confirm the target genes of let-7i in DCs. Using bioinformatic databases, let-7i was predicted to target murine SOCS1. The core binding sequence (seeding region) for this miRNA in the 3'-UTR of SOCS1 had perfect seven base target–microRNA matches (Fig. 5). Previous studies showed that SOCS1 negatively regulated LPS-induced DC maturation. Therefore, we further explored the direct link between let-7i and SOCS1 in the process of DC maturation.

To directly address whether let-7i binds to the 3'-UTR of SOCS1 mRNA, we generated a psiCHECK-2 luciferase construct containing the SOCS1 mRNA 3'-UTR with the putative let-7i binding site. Additionally, another psiCHECK-2 luciferase construct containing the SOCS1 mRNA 3'-UTR with a mutation at the putative let-7i binding site (CTACCTC to ATGAGATC) was generated as a control. We then transfected cultured DCs with each reporter construct, as well as let-7i mimic or inhibitor. Luciferase activity was assessed at 24 h after transfection. As shown in Fig. 5, the translational level of the luciferase reporter was significantly decreased by let-7i mimic, but markedly increased by let-7i inhibitor. No changes in luciferase were observed in cells transfected with the mutant SOCS1 3'-UTR construct when combined with let-7i mimic or inhibitor. This suggested that the seed region for let-7i binding within the SOCS1 3'-UTR was critical for SOCS1 translational regulation in DCs and that SOCS1 was indeed a direct target of let-7i.

We tested whether alteration of let-7i levels affected SOCS1 protein expression in mixed DCs and the two CD86-defined populations. As in previous experiments, DCs were transfected with the let-7i mimic or inhibitor for 12 h and then stimulated with LPS for 12 h. The level of SOCS1 protein was measured by Western blotting in LPS-DCs and in the CD86-defined DC populations after separation. As shown in Fig. 6A, overexpression of let-7i inhibited SOCS1 protein expression. In contrast, downregulation of let-7i using let-7i inhibitor (0, 20, or 60 nM) resulted in a dose-dependent increase in SOCS1 protein. The elevation of SOCS1 protein was significant in CD86-DCs, but modest in the CD86+DCs population compared with let-7i mimic or let-7i inhibitor. Luciferase activities were measured and normalized to the control Renilla luciferase level. Bars represented the means ± SD from three independent experiments. *p < 0.05, compared with cells transfected with the reporter construct.

Targeting of let-7i to the 3'-UTR region of SOCS1 mRNA. A reporter construct with the potential binding site for let-7i in the 3'-UTR region of SOCS1 was generated. DCs were transiently cotransfected for 24 h with the reporter construct and let-7i mimic or let-7i inhibitor. Luciferase activities were measured and normalized to the control Renilla luciferase level. Bars represented the means ± SD from three independent experiments. *p < 0.05, compared with cells transfected with the reporter construct.

To test the effect of let-7i on SOCS1 mRNA, we measured the SOCS1 mRNA level in cultured DCs by qRT-PCR. No significant changes in SOCS1 mRNA were found in cells transfected with let-7i mimic or let-7i inhibitor compared with LPS-stimulated DCs, or between the CD86+ and CD86- subpopulations of inhibitor-treated DCs (Fig. 6E). Therefore, we conclude that let-7i-mediated regulation of SOCS1 protein expression is mediated by posttranscriptional effects, mainly in CD86-DCs population.

A previous study reported that the TLR4 protein was also a target of let-7i in human cholangiocytes (27). In rats, however, the 3'-UTR of TLR4, especially the seeding region, does not match well with let-7i according to bioinformatic databases. Nonetheless, we evaluated the effect of the let-7i level on TLR4 expression in rat DCs. As shown in Fig. 6F, the modulation of let-7i did not show a significant effect on TLR4 expression in DCs. Therefore, TLR4 was not an actual target of let-7i in DCs in our study.
let-7i modulated the cytokine profile secreted by DCs upon LPS stimulation

Different cytokines can act as proinflammatory or anti-inflammatory mediators, so the cytokine expression profile of DCs is one of the ultimate determinants of functional status. To further investigate the effect of let-7i on cytokine secretion by DCs, the cytokine levels in culture supernatants were measured using specific ELISAs. The production of the IL-12 family (IL-12, IL-23, and IL-27) in response to LPS with and without manipulation of let-7i was analyzed by ELISA. The imDCs released these cytokines, and let-7i modulation showed no effect on TLR4 expression in DCs.
cytokines at low levels. Upon LPS stimulation, the levels of IL-12, IL-23, and IL-27 significantly increased compared with imDCs. However, downregulation of let-7i caused a significant depression of IL-12 and IL-27 production and a mild inhibitory effect on IL-23 expression in the total inhibitor-transfected (and LPS-treated) DC population and in the CD86<sup>−</sup> DC subpopulation, but not in CD86<sup>+</sup> DCs or let-7i mimic transfected DCs. We also analyzed the levels of other proinflammatory cytokines, such as IL-6, TNF-α, and IFN-γ in the culture supernatants of LPS-stimulated DCs, as these signaling molecules are thought to be important for DC maturation. We demonstrated that in response to LPS stimulation, the culture supernatants of LPS-DCs contained more IFN-γ, TNF-α, and IL-6 than did imDCs. The production of IFN-γ and TNF-α was reduced in DCs transfected with the let-7i inhibitor and in the CD86<sup>−</sup> DC subpopulation compared with LPS-DCs, but not in the CD86<sup>+</sup> DC population or mimic-treated DCs. However, IL-6 production was not significantly affected by modulation of let-7i expression.

Finally, we investigated the production of IL-10 by DCs transfected with let-7i mimic or inhibitor. The results showed that DCs transfected with let-7i inhibitor produced more IL-10 than did LPS-DCs, especially in the population of CD86<sup>−</sup> DCs, but not in CD86<sup>+</sup> DCs. However, transfection with let-7i mimic did not show a significant modulatory effect on IL-10 levels in DCs (Fig. 7).

**Discussion**

As potent APCs, DCs are central to the maintenance of immunologic tolerance and the initiation and control of immunity (2). DCs possess a high capacity for Ag capture and processing, migration to lymphoid organs, and expression of various costimulatory molecules for Ag-specific lymphocyte activation. The maturation state of DCs may be a key factor influencing the induction of T cell tolerance by promotion of regulatory T cell differentiation (28, 29). Mature DCs are potent APCs that enhance T cell immunity, but immature DCs are involved in the induction of peripheral T cell tolerance. Therefore, the DC functional state is partially determined by DC maturation. The regulators of DC maturation thus determine the balance between T cell immunity and tolerance. We demonstrated that the miRNA let-7i was a regulator of DC maturation and functional state, which help to balance the immune response. Recently, the role of miRNAs in the control of the immune system and the regulation of DC function has come under more intense scrutiny (7, 8). It was reported that several miRNAs, such as miRNA-155, miRNA-146a, miRNA-148/152, and miRNA-29a, act as regulators for acquired and innate functions of DCs by targeting different signaling molecules (30–35). Transcriptional and translational regulation by miRNAs provide a new mechanism to fine-tune DC function and the immune response. Indeed, we found that let-7i expression was upregulated during LPS-induced DC maturation, but not by other TLR ligands. Several studies have confirmed the functional role of let-7i in innate immunity, especially in the LPS response. In cholangiocytes, let-7i can directly regulate TLR4 expression or act synergistically with miRNA-98 to regulate expression of the SOCS family member CIS through translational suppression, thus contributing to immune responses against LPS. In our study, let-7i expression was upregulated during the LPS-induced DC maturation. The preponderance of evidence suggests that let-7i might be involved in DC maturation and function.

To elucidate the functional significance of let-7i expression in LPS-induced DC maturation, we tested whether alteration of basal let-7i levels affect LPS-induced DC maturation. As we expected, underexpression of let-7i not only depressed DC maturation, as evidenced by lower surface expression of costimulatory molecules, but it also induced T cell hyporesponsiveness and promoted Treg expansion in MLRs. The DC lineage is markedly heterogeneous, but it has an array of subpopulations characterized by different surface Ags, phenotypes, and tissue distributions (36). To identify which of these subpopulations let-7i actually targeted, we separated DCs transfected with the let-7i inhibitor and then
stimulated with LPS into CD86^+ DCs and CD86^- DCs subpopulations and demonstrated that expansion of Tregs relative to T cells was mediated mainly by CD86^- DCs. These results not only supported our initial hypothesis that let-7i was critical for determining the functional state of DCs, but they also revealed a potential specific function of CD86^- DCs in promoting tolerogenic activity concomitant with let-7i downregulation.

Tregs are a key component of the immune response. The number and function of Tregs are tightly regulated through the local cytokine milieu. Tregs potently regulate adaptive responses mediated by effector T cells and also suppress innate immunity. The Treg marker Foxp3 is thought to be essential for this regulatory activity. IL-10 is a potent anti-inflammatory cytokine that has been shown to suppress alloimmunity and autoimmunity. Some studies have also shown that IL-10 is a key differentiation factor for the selection and effector function of Tregs (25, 28). Within the isolated CD4^+ CD25^+ T cell population primed by CD86^- DCs underexpressing let-7i, a larger fraction expressed Foxp3^+ and elevated IL-10 mRNA compared with Tregs primed with imDCs or LPS-DCs. These cells exhibited more potent immunosuppressive activity, further supporting the tolerogenic function of the CD86^- population derived from DCs underexpressing let-7i.

It is well documented that once activated, DCs secrete a wide array of cytokines, including proinflammatory and anti-inflammatory cytokines, through which DCs signal to other DCs and other immune cells to coordinate the global immune response. To further explore the functional state of DCs overexpressing or underexpressing let-7i, we evaluated the secreted cytokine profile after let-7i modulator transfection and LPS stimulation. Consistent with a previous study, the production of proinflammatory cytokines (IL-12, IFN-γ, TNF-α, and IL-6) were all increased in DCs following LPS stimulation. Downregulation of let-7i, however, depressed LPS-induced proinflammatory cytokine (IL-12, IL-27, TNF-α, and IFN-γ) secretion in the total DC population, as well as in the CD86^- subpopulation, but without significant effects on IL-23 and IL-6 production. Moreover, an increased level of the anti-inflammatory cytokine IL-10 was also observed in these cells. Suppression of proinflammatory cytokines and induction of anti-inflammatory cytokines by let-7i underexpression (and concomitant higher SOCS1 expression) in the CD86^- DC population were consistent with the functional state of the DCs and accounted, at least in part, for the tolerogenic properties of these cells.

To further explore the mechanism of let-7i action on DC maturation and function, we used four widely used software programs to predict the putative let-7i targets, especially those that could potentially regulate DC maturation. SOCS1 mRNA was selected by all programs and subsequently shown to be a target of let-7i in DCs by a luciferase assay. Furthermore, the actual effects of let-7i on SOCS1 were detected at the protein level. Suppression or induction of intracellular let-7i levels caused reciprocal alterations in SOCS1 protein as assessed by Western blot and immunofluorescence analysis. In response to let-7i downregulation, elevation of SOCS1 protein was detected in CD86^- DCs, but there was no significant change in SOCS1 mRNA in DCs under any treatment conditions. The most parsimonious molecular pathway consistent with these results is that a reduction in let-7i led to enhanced SOCS1 protein expression, mainly in CD86^- DCs, and that SOCS1 expression may have in turn influenced the cytokine expression profile.

The SOCS1 protein is a key negative regulator of cytokine signaling and so acts to maintain balance in the immune system (15). SOCS1 expression can be induced by various other stimuli, such as LPS, and can also negatively regulate LPS-induced DC maturation (37, 38). It has been reported that SOCS1-deficient DCs expressed higher levels of MHC class II and costimulatory molecules, and secreted larger amounts of IFN-γ, IL-6, IL-12, and TNF in response to LPS compared with wild-type cells (39). In our study, let-7i inhibitor-transfected DCs exhibited a low expression of costimulatory molecules and decreased production of proinflammatory cytokines (IL-12, IL-27, IFN-γ, and TNF-α), which might be partially due to the increased SOCS1 protein level. The production of IL-6 and IL-23 was not significantly affected by the let-7i level, however, suggesting that the cytokine expression profile is under complex regulation by several signaling factors in addition to SOCS1. Chen and colleagues (40) reported that silencing of SOCS1 in DCs strongly enhanced Ag-specific anti-tumor immunity, indicating that SOCS1 is an essential negative regulator for T cell activation by DCs and helps to promote immunological tolerance. By transfecting DCs with SOCS1-expressing adenovirus vector, however, DCs overexpressing SOCS1 expressed lower levels of costimulatory and MHC molecules and were resistant to maturation and activation stimulation. They induced allogeneic T cell hyporesponsiveness and promoted the generation of regulatory-like T cells in vitro. Furthermore, treatment of DCs overexpressing SOCS1 also significantly prolonged the survival of allografts and led to a substantial increase in the generation of Tregs (41). Collectively, these studies indicated that the SOCS1 protein participates in the regulation of DC maturation and function. It is thus a promising target for genetic engineering studies to examine the influences of DCs in different functional states in the immune response. Moreover, these studies are consistent with our results that DCs transfected with let-7i inhibitor had an impaired ability to simulate the T cell response, while instead promoting Treg differentiation and shifting the cytokine secretion profile away from the inflammatory state. Alternatively, by suppressing SOCS1 expression, let-7i promotes T cell proliferation and inflammation.

In conclusion, our study revealed that let-7i at least partially mediates the maturation and functional state of DCs induced by LPS by targeting SOCS1. In a tolerogenic state, DCs underexpressing let-7i, especially the CD86^- population, would exhibit low surface expression of costimulatory molecules, impair T cells proliferation, and promote induction of Tregs, as well as shift the cytokine profile away from proinflammatory mediators.

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Disclosures

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References


