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miR-24, miR-30b, and miR-142-3p Regulate Phagocytosis in Myeloid Inflammatory Cells

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Micro-RNAs (miRNAs) are small noncoding RNAs that regulate various biological pathways. As their role in phagocytosis remains poorly understood, we investigated their impact on phagocytosis in myeloid inflammatory cells. Seven miRNAs (miR-24, -30b, -101, -142-3p, -652-3p, -652-5p, and -1275) that were differentially expressed during monocyte to macrophage (Mφ) and monocyte to dendritic cell (DC) differentiation were screened for their potential role in phagocytosis. Among these, overexpression of miR-24, miR-30b, and miR-142-3p in human myeloid-derived Mφ, DC, monocytes, and PBMCs significantly attenuate phagocytosis of *Escherichia coli* and *Staphylococcus aureus*, as well as the secretion of inflammatory mediators, including TNF-α, IL-6, and IL-12p40. miRNA-mediated changes in cytokine profiles were observed at transcriptional and/or posttranscriptional levels and importantly exhibit miRNA-specific impact. To examine the underlying mechanism, we monitored the expression of phagocytosis pathway-associated genes and identified several genes that were altered in Mφ and DC transfected with miR-24, miR-30b, and miR-142-3p mimics. Some of these genes with altered expression also harbor putative miRNA binding sites. We show that miR-142-3p directly regulates protein kinase Cα (PKCα), a key gene involved in phagocytosis. Interestingly, miR-142-3p and PKCα exhibit antagonistic expression during Mφ and DC differentiation. Short interfering RNA-mediated knockdown of PKCα in Mφ leads to reduced bacterial uptake, further highlighting the role of the gene in phagocytosis. Overall, these results demonstrate that miR-24, miR-30b, and miR-142-3p regulate phagocytosis and through modulation of various genes involved in the pathway.

Monocytes, macrophages (Mφ), and dendritic cells (DC) are myeloid inflammatory cells capable of recognizing a plethora of microorganisms via conserved pathogen-recognition receptors. Ligation of these pathogen-recognition receptors results in a pattern of altered gene expression that enhances pathogen recognition, uptake, and removal (1, 2). This response is fine-tuned by various factors, including the class of pathogen present, the local cytokine milieu, concomitant signaling via damage-associated molecular patterns (3), as well as site-specific mechanisms of immune regulation.

Critical for innate immunity and host survival is the ability of myeloid inflammatory cells to engulf microorganisms, activate antimicrobial functions, and degrade, process, and present foreign Ag to the adaptive immune system. Each type of myeloid inflammatory cell has a role for which it is best evolved. For example, whereas Mφ are the most potent at killing engulfed microorganisms, DC are most effective at presenting Ag to lymphocytes (2, 4). In monocytes, phagocytosis induces autocrine and paracrine signaling that influences local monocyte differentiation, monocyte production in the bone marrow, and extravasation into tissues (5).

Micro-RNAs (miRNAs) represent a class of regulatory, non-protein-coding RNAs that bind to mRNA transcripts bearing complementary sequences leading to target degradation or translation suppression (6–8), and have recently emerged as potent regulators of diverse biological pathways, including cell differentiation, apoptosis, and immunity (9, 10). For instance, miR-146a and miR-155 regulate the function of various immune cells, including Mφ and DC, by targeting genes that comprise key components of immune function (11–13). Various miRNAs including cluster miR-17–92, miR-223, and miR-142-3p have been associated with the differentiation of myeloid and granulocytic cells, suggesting lineage-specific functions (14, 15).

Recent reports demonstrate that pathogens modulate host miRNA profiles to escape host responses. Mycobacterial infection of Mφ and DC affects phagocytosis and production of cytokines and results in differential expression of miRNAs (16, 17). Singh et al. (17) showed induced expression of miR-99b in both Mφ and DC infected with *Mycobacterium tuberculosis* strain H37Rv, which contributed toward impaired cytokine responses. However, miRNA involvement in phagocytosis is poorly studied. In this study, we identify miR-24, miR-30b, and miR-142-3p as regulators of phagocytosis in Mφ, DC, and monocytes. Overexpression of these miRNAs modulates secretion of TNF-α, IL-6, and IL-12p40 and expression of various genes involved in pathogen recognition and downstream signaling. We further show that miR-142-3p directly regulates protein kinase C (PKC)α in Mφ and DC, and depletion of PKCα had adverse impact on bacterial uptake. Taken together, our data demonstrate that miR-24, miR-30b, and miR-142-3p regulate phagocytosis and associated innate responses by targeting genes involved in the pathway.
Materials and Methods

Primary human monocyte isolation and differentiation

Freshly prepared buffy coats were collected from healthy donors (n ≥ 3; Sylvan N. Goldman Oklahoma Blood Institute, Oklahoma City, OK) by density gradient centrifugation, as described earlier (18). Briefly, PBMCs were purified using Ficoll Paque (GE Healthcare, Piscataway, NJ)–based density centrifugation. PBMCs were incubated with magnetic-labeled CD14 beads (Myltenyi Biotec, Cologne, Germany), according to manufacturer’s instructions. The purity of CD14+ cells was >95%, as determined by flow cytometry. For generation of M1 and M2 M
dmiRNA mimics or inhibitors at final concentration mentioned above, fields for each donor.

software. Images were captured for three independent, randomly selected and cultured with 1% (v/v) FBS and 0.08% sodium azide. Cellular debris and autofluorescence was excluded based on SSC versus FSC and SSC versus pulse width measurements. Fluorescence minus-one samples constituted controls for fluorescence minus one.

miRNA transfections

miScript miRNA mimics (miR-24, -30b, -101, 142-3p, -652-3p, -652-5p, and -1275) and inhibitors were purchased from Qiagen (Germantown, MD). For control, all star-negative mimics (Qiagen) were used. For PKCα knockdown, gene–specific and control short interfering RNA (siRNA) were purchased from Sigma-Aldrich (St. Louis, MO). Transient transfections were performed using Lipofectamine 2000 (Life Technologies), according to manufacturer’s instructions. 

Waltham, MA) were used to determine transfection efficiency.

Flow cytometry

Cells were harvested after treatments and washed in ice-cold PBS supplemented with 1% (v/v) FBS and 0.08% sodium azide. Cellular debris and detritus were excluded based on size (forward scatter [FSC]) and granularity (side scatter [SSC]). The FSC/SSC gate for M2, DC, and monocytes comprised ~60%, ~30–90%, and ~90% of total events, respectively. Couples were excluded based on SSC versus FSC and SSC versus pulse width measurements. Fluorescence minus-one samples constituted controls for cells treated with bioparticles. Samples were analyzed using a FACScan or BD Cyan flow cytometer using CellQuest software (BD Biosciences, San Jose, CA). Further analysis was performed using FlowJo software (Tree Star, Ashland, OR).

Cell viability assay

Cell viability was determined using the CellTiter 96 AQueous Cell Proliferation Assay Kit (Promega, Madison, WI). Briefly, 4 × 10⁴ cells (M2, DC, and monocytes) grown in 96-well plates were transfected with miRNA mimics or inhibitors at final concentration mentioned above, and assays were performed after 24 h, according to manufacturer’s instructions.

Phagocytosis assay and imaging

For M2 (M1 and M2) and DC, cells at a density of 400,000/well (96-well plate) were transfected on day 7 with miScript miRNA mimics, inhibitors, or control miRNA mimics (Qiagen). Monocytes and PBMCs were transfected at a final concentration of 100 nM. Red SGO oligos (ThermoScientific, Waltham, MA) were used to determine transfection efficiency.

miR-24-challenged M2 and DC, and M1 and M2 M2M and DC, respectively. At day 7, cells were harvested, and surface expression of CD14, CD163, and HLA-DR was examined by flow cytometry. For DC, monocytes were cultured in RPMI 1640 supplemented with 10% FBS and rh-GM-CSF (1000 U/ml) (both from PeproTech). Media was replaced with resuspended labeled bioparticles. The cells were challenged with E. coli–challenged M2 and DC, and the cell lysates were diluted (1:10) before use. Absorption was measured at 450 nm on a SpectraMax M2 (Molecular Devices, Sunnyvale, CA) plate reader.

Cytokine analysis

Supernatants were collected after a 4- and 18-hour challenge and analyzed for levels of IL-6, IL-10, IL-12p40, and TNF-α by ELISA (Cytoset; Invitrogen). Cellular levels of cytokines were measured in E. coli–challenged M2 (M1 and M2), DC, and monocytes after 4 or 18 h. Supernatants and cell lysates were diluted (1:10) before use. Absorption was measured at 450 nm on a SpectraMax M2 (Molecular Devices, Sunnyvale, CA) plate reader.

Intracellular levels of TNF-α were monitored in miRNA inhibitor, or control-transfected M2M challenged with E. coli for 4 h. Cells were fixed in 4% paraformaldehyde for 10 min and incubated in 10% sheep serum in 0.1% PBS–Tween for 2 h. Staining was performed with TNF-α Ab (1:100 dilution; ab1793; Abcam, Cambridge, U.K.) overnight at 4°C in PBS containing 0.1% BSA and 0.1% Tween 20. Cells were washed five times before incubation with Dylight-488 goat anti-mouse Ab (BioLegend, San Diego, CA) at 1:250 dilution for 1 h. After washing five times, cells were counterstained with Hoechst nuclear staining dye. Images were captured on the EVOS microscope at original magnification ×20 and processed on Adobe CS3 Photoshop (Adobe Systems, San Jose, CA).

Total RNA isolation, cDNA synthesis, and PCR array

M2 and DC transfected with miRNA mimic or inhibitor were harvested after 36 h. Total RNA was isolated using the miRNeasy Micro Kit (Qiagen), according to manufacturer’s instructions. First-strand cDNA was synthesized using 500 ng total RNA in an Eppendorf Thermal Cycler (Eppendorf). A custom PCR array plate (96 well) containing 88 different genes involved in phagocytosis was used to assess gene expression (Qiagen). One microgram of cDNA was aliquoted onto each well, and real-time PCR was performed using a StepOne 7500 thermocycler (Applied Biosystems, Carlsbad, CA). Expression levels were normalized with respect to ß2– microglobulin as the denominator and the most consistent levels across all transfections. Next, the fold change was calculated with respect to the negative miRNA control. Finally, the ratio of miRNA inhibitor and miRNA mimic was calculated. Real-time PCR for three randomly selected genes was also carried out in two independent donors.

Total RNA was isolated from E. coli–challenged M2 and DC, and the expression of proinflammatory cytokine mRNA was analyzed by RT-PCR. To monitor PKCα mRNA levels, total RNA was also isolated from monocytes, differentiating M2 and DC at various time points. The data were presented as normalized fold change with SD.

Reporter constructs and dual luciferase assays

The cloning of PKCα 3′ untranslated region (UTR) was performed, as described previously (19). Briefly, PKCα 3′UTR was amplified (forward primer, 5′–GCACTCGGAGAAGGGGCGGATTACCTA–3′; reverse primer, 5′–ATGCGGGCAGACACAGCGGACGGGAG–3′) using genomic DNA isolated from freshly prepared PBMCs. The amplicons were digested with XhoI and NotI and cloned into the psiCHECK-2 vector (Promega). The 3′UTR of PKCα was PCR amplified using Phusion Taq polymerase (New England Biolabs, Ipswich, MA). Dual-luciferase assays were carried out in a 96-well format. In brief, HEK293 cells were seeded at the density of 2 × 10⁴ in DMEM supplemented with 10% FBS. All the transfections were performed in quadruplicate using 0.3 μL Lipofectamine 2000 (Life Technologies), 60 ng dual luciferase reporter plasmids, and a final concentration of 2 or 5 pmol synthetic miRNA mimics or scramble (Qiagen). After 36 h posttransfection, cells were lysed in passive lysis buffer (Promega), and dual luciferase assays (Promega) were performed using the Lumat plate reader (Turner BioSystems, Sunnyvale, CA). For each reporter 3′UTR construct, the Rlu/Fluc value obtained was normalized to the value obtained for control psiCHECK-2 empty vector cotransfected with the same miRNA mimic. The values obtained were plotted as histograms, in which empty vector is set at 1.

Western blotting

M2 or DC transfected with miRNA mimics, inhibitors, or control mimics were harvested after 36 h and lysed in cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease inhibitors (Roche, Basel, Switzerland). Lysates were incubated on ice for 30 min and were clarified using centrifugation at 14,000 rpm for 15 min at 4°C, and protein content was estimated using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were resolved in 10% Mini-PROTEAN TGX (Bio-Rad) gels and electrotransferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked with 5% skimmed milk for 2 h and incubated with primary Abs against PKCα or GAPDH.
were transiently transfected with miRNA mimics on phagocytosis by cotransfecting M1 or M2 Mφ with E. coli, indicating an inhibitory role of these miRNAs on phagocytosis. Using labeled oligonucleotides, we achieved transfection efficiency in M1 and DC, respectively (Supplemental Fig. 1A, 1B). Primary monocyte-derived Mφ were transiently transfected with miRNA mimics, corresponding inhibitors, or control mimic. Rhodamine-labeled E. coli bioparticles were added after 24 h, and their uptake was observed by fluorescent microscopy and quantified by flow cytometry. Overexpression of miR-24, -30b, and -142-3p mimics, but not others (miR-101, -652-3p, -652-5p, and -1275), significantly decreased the uptake of bacteria, compared with either miRNA inhibitors or control mimic (Fig. 1A, Supplemental Fig. 2A), indicating an inhibitory role of these miRNAs on phagocytosis. Cells treated with cytochalasin D, an inhibitor of actin polymerization, blocked the uptake of labeled bacteria and served as negative controls (Supplemental Fig. 2A). Importantly, this miRNA-mediated effect on phagocytosis was not specific to E. coli (Gram negative), as similar results were observed with Staphylococcus aureus (Gram-positive) bioparticles (Supplemental Fig. 2B), suggesting that these miRNAs regulate phagocytosis independent of the pathogen. We further examined possible cooperative effects of miRNA mimics on phagocytosis by cotransferring Mφ with the three miRNA mimics. Compared with the individual miRNA mimics, no further reduction in phagocytosis was observed (Supplemental Fig. 2C). Therefore, subsequent experiments were focused on individual miRNA transfections.

Flow cytometric analysis of Mφ showed ~20–30% reduction in the number of cells positive for bioparticle uptake in the presence of miR-24, miR-30b, and miR-142-3p mimics, but not with inhibitors, nor control mimic (p < 0.01; Fig. 1B). Furthermore, comparing the geometric mean fluorescent intensity (geo. MFI) of the cells, being semi-quantitative for the number of bioparticles taken up on a per cell basis, indicated a larger degree of inhibition (~40–60%) than the percentage uptake alone, and similar to that observed by microscopy (Fig. 1C). Bacterial challenge to miRNA mimic-transfected cells had no effect on viability, as assessed by cell viability assay (Supplemental Fig. 1C).

Depending on the activation status, Mφ phenotype can be broadly classified into two categories, as follows: classically (M1) or alternatively (M2) activated. The M1 Mφ are primarily involved in Th1 responses, lymphokine production, and degradation of intracellular pathogens, whereas M2 Mφ trigger Th2 responses, immunotolerance, and tissue remodeling. Incubation of Mφ with different cytokines can alter their activation status (20). In the presence of M-CSF and GM-CSF, monocytes differentiate into Mφ with M2 and M1 phenotype, respectively. In our hands, M1 Mφ were HLA-DR high, CD163 low, and CD14 low compared with donor-matched M2 Mφ and correlate with reported pheno-typing (Supplemental Fig. 3A). To investigate whether miR-24, miR-30b, and miR-142-3p impact bacterial uptake by M1 Mφ, cells were transfected with miRNA mimics and assessed for phagocytic potential. Overexpression of miRNA mimics inhibited uptake of bacteria with respect to control mimics (Fig. 1C, Supplemental Fig. 3B). We also noticed that M1 Mφ were less efficient in phagocytosis compared with M2 Mφ, as expected with the former phenotype (Supplemental Fig. 3B). Nonetheless, flow cytometry analysis show that percentage of mean fluorescent intensity values were similarly reduced by miRNA mimics in M1 and M2 Mφ (~40–60%; Fig. 1C). Together, these results demonstrate that overexpression of miR-24, miR-30b, and miR-142-3p attenuates phagocytosis by primary M1 or M2 Mφ.

Having demonstrated a role of miR-24, miR-30b, or miR-142-3p in regulating phagocytosis in Mφ, we investigated whether these miRNAs regulate this process in other myeloid inflammatory phagocytes, namely, DC and monocytes. Cells were transiently transfected with labeled oligonucleotides, and transfection efficiency was assessed by microscopy and flow cytometry. We were able to achieve ~85% and ~60% transfection efficiency in DC and monocytes, respectively (Supplemental Fig. 1A, 1B). DC transfected with mimics, but not with inhibitors or control mimic, showed reduced E. coli uptake as observed under microscopy (Fig. 2A, Supplemental Fig. 3D). Quantitative analysis on flow cytometry showed ~4 to 6-fold decrease in bacterial uptake by mimic-transfected DC (Fig. 2B). The geo. MFI data further confirmed reduced uptake (Fig. 2C). In monocytes, overexpression of miRNA mimics significantly reduced phagocytosis of bioparticles (Fig. 2D), whereas miRNA inhibitors or control mimic had no adverse effect (data not shown). Flow cytometric analysis showed 5- to 8-fold less bacterial uptake in miRNA mimic-transfected monocytes (Fig. 2E). However, compared with Mφ and DC, we noticed fewer uptake of bioparticles during the assay incubation (1 h), which increased over time (data not shown). Nonetheless, miRNA mimic-mediated effects were still evident, as determined by the geo. MFI data that reflect reduced E. coli uptake (Fig. 2F).

Finally, we examined the regulatory role of these miRNAs in PBMCs. The composition of PBMCs isolated from buffy coats is ~70–90% lymphocytes, 10–30% monocytes, and 1–2% DC (our unpublished observations). We show that PBMCs transfected with miRNA mimics also exhibit significant decrease in phagocytosis (Fig. 2G, 2H). Overall, these results unequivocally show that miR-24, miR-30b, and miR-142-3p regulate phagocytosis in myeloid cells.

miRNA mimics decrease TNF-α production

Secretion of TNF-α is associated with active phagocytosis (21). Mφ, DC, and monocytes were transiently transfected with miRNA mimics, inhibitors, or control mimic and incubated with E. coli. Supernatant levels of TNF-α were significantly lower in cells transfected with miR-24, miR-30b, and miR-142-3p mimics compared with inhibitors or control mimics (Fig. 3). In M2 Mφ, miRNA mimic-mediated reduction of TNF-α secretion was more pronounced at 18 h, whereas in DC and monocytes the secretion of TNF-α was affected more significantly at 4 h (Fig. 3A, 3C, 3D).
Moreover, time-dependent increase in TNF-α secretion was observed for M2 Mφ and monocytes, but not DC, suggesting cell-specific innate responses. In addition, we compared the impact of miRNA mimics on the release of TNF-α in M1 and M2 Mφ. We noted that transfection of M1 Mφ with miR-30b and miR-142-3p resulted in a greater inhibition of secreted TNF-α levels compared with M2 Mφ at 4 h. Conversely, M2 Mφ transfected with miR-24 mimic had a greater impact on M2 Mφ, indicating subtle differences most likely associated with the respective phenotype (Fig. 3B). Taken together, TNF-α secretion profiles correlated with the uptake of bacteria, indicating that miRNA mimics also affect phagocytosis-associated innate immune responses.

miRNA mimics modulate IL-6 and IL-12p40 production

During phagocytosis, Ag is processed for presentation to lymphocytes, and the cytokines produced influence the type of adaptive response that is mounted, for example bacterial responses include cytokines that promote Th1 and Th17 differentiation. We assayed the levels of the Th1/Th17-promoting cytokines IL-6 and IL-12p40, being a shared component of (Th1-promoting) IL-12 and (Th17-promoting) IL-23. Transfected Mφ and DC were incubated with E. coli, and supernatant levels of IL-6 and IL-12p40 were assayed by ELISA. After 4 and 18 h, IL-6 and IL-12p40 were reduced in the presence of miRNA mimics, but not inhibitors or control mimic (Fig. 4A, 4B). We also compared the levels of IL-6 in M1 and M2 Mφ after 4-h time point. The miRNA mimics downregulated the IL-6 secretion in supernatants in both M1 and M2 Mφ (Fig. 4C). However, we noticed relatively less impact of miR-24 on IL-6 secretion by M1 so observed with TNF-α earlier. Compared with DC, secretion of IL-12p40 in M2 Mφ showed marked reduction at both time points, further highlighting the differences in miRNA-mediated effects on cell function. Concurrently, secretion of the anti-inflammatory/immunosuppressive cytokine IL-10 by both M2 Mφ and DC was detectable at 18 h, but not 4 h, and was comparable in miRNA mimics, inhibitors, and control-transfected cells (Fig. 4D; Mφ, data shown). These results show that the effects of miRNA mimics are not only confined to the local and immediate innate response, but also extend to the release of factors that orchestrate adaptive immunity.

Differential regulation of proinflammatory cytokine expression by miRNA mimics

Because we performed miRNA profiling in M2 Mφ and over-expression of miR-24, miR-30, and miR-142-3p shows similar impact on the phagocytosis and associated innate responses by
both M1 and M2 Mφ, subsequent experiments were conducted on M2 Mφ alone. Unless otherwise mentioned, the Mφ in context will be M2 phenotype. To determine whether the reduced secretion of proinflammatory cytokines is a consequence of transcriptional regulation, we monitored mRNA levels of IL-6, IL-12p40, and TNF-α in Mφ and DC transfected with miRNA mimics and inhibitors. We observed that IL-12p40 mRNA levels were remarkably reduced (2- to 8-fold) in the presence of miRNA mimics and correlated with the ELISA data. Mφ transfected with miRNA inhibitors exhibited relatively decreased expression of TNF-α compared with miRNA or control mimics (Fig. 5A). Similarly, expression of IL-6 was significantly downregulated in Mφ transfected with miR-24 and miR-30b inhibitors, but not miR-142-3p. However, comparable expression of TNF-α and IL-6 transcript was observed in mimic- and inhibitor-transfected DC (Fig. 5A). Of note, comparing the expression profiles with the unchallenged controls clearly shows that enhanced expression of miRNA mimics does not compromise the signaling leading to induction of cytokines (data not shown). These observations suggest that enhanced expression of miRNA mimics attenuates innate responses, but does not compromise the signaling leading to induction of cytokines examined.

These observations tempted us to investigate whether miRNA mimics interfere with the production of proinflammatory cytokines. Intracellular levels of TNF-α, IL-6, and IL-12p40 were examined in cell lysates prepared from transfected Mφ and DC challenged with E. coli for 4 h. We observed a significant decrease in cellular TNF-α, IL-6, and IL-12p40 in Mφ and DC transfected with miRNA mimics compared with controls, which corroborates with the secretory profiles (Fig. 5B). These results show that whereas transcript levels of TNF-α and IL-6 are increased to a degree, in the presence of mimics, the corresponding proteins are downregulated, indicating possible posttranscriptional interference by these miRNAs.

To further confirm these findings, we examined the cellular levels of TNF-α by immunostaining. E. coli–challenged Mφ transfected with miRNA inhibitors or control mimics exhibit sharp punctate structures; however, diffuse and relatively reduced staining was observed with corresponding miRNA mimics (Fig. 5C). This corroborates with the intracellular cytokine profiles (Fig. 5B). Furthermore, we observed marked differences in the staining pattern of TNF-α in the presence of mimics. Unlike inhibitor or control-transfected cells, miRNA mimics do not show a consistent tapering pattern as expected for secreted proteins, from the nucleus toward the cell periphery. Interestingly, we also noticed

FIGURE 2. Overexpression of miRNA mimics attenuates phagocytosis in human DC, monocytes, and PBMCs. Monocyte-derived DC and monocytes were transfected with miR-24, miR-30b, and miR-142-3p miRNA mimic, inhibitors, or control mimics for 24 h. Phagocytosis assays were performed with pHrodo E. coli bioparticles. Representative confocal images of corresponding (A) DC and (D) monocytes with internalized E. coli are shown. Images were captured using settings mentioned above. Scale bars, 20 μm. Cells were harvested and analyzed by flow cytometry. Histograms show percentage of E. coli–positive (B) DC, (E) monocytes, and (H) PBMCs. The geo. MFI was assessed by FACS in (C) DC, (F) monocytes, and (H) PBMCs. Data are presented as ±SEM from four donors. *p < 0.01 is considered significant.
accumulation of TNF-α localized to the cell periphery in miR-142-3p mimic-transfected cells (Fig. 5C, yellow arrows). These results suggest that protein translation and/or secretory pathways are rendered less functional by these miRNAs.

Genes involved in phagocytosis are downregulated by miRNA mimics

To examine the underlying mechanism of miRNA-mediated impact on phagocytosis, we next focused on identifying genes altered by overexpression of miRNA mimics. To this end, we selected 88 genes associated with phagocytosis and analyzed their levels by quantitative RT-PCR. Mφ and DC were transfected with miRNA mimics, inhibitors, or control mimic, and gene expression levels were monitored after 36 h. PCR array profiling showed that mRNA levels were modulated in the presence of miRNA mimics compared with control mimic. Dramatic modulation of gene expression was observed in DC, but only a few genes were affected in Mφ. In DC, a total of 29, 25, and 40 genes were altered in presence of miR-24, miR-30b, and miR-142-3p, respectively (Supplemental Table I). In contrast, altered expression of one, six, and four genes was noted in Mφ transfected with miR-24, miR-30b, and miR-142-3p, respectively (Supplemental Table I). Venn diagram shows that most of the altered genes were common to these miRNAs, whereas only few genes were specific to each miRNA (Fig. 6A). Intriguingly, the majority of the deregulated genes that were common targets of the miRNAs are receptors for pathogen recognition; however, miR-142-3p uniquely impacted signaling-associated genes in DC. The PCR array results were confirmed by an independent RT-PCR analysis of three genes altered by miRNA mimics on two additional donors (data not shown).

We next focused on identifying genes with altered expression that are direct targets of miRNAs. For this, 3′ untranslated regions (UTRs) were scanned for potential miRNA binding sites using miRWalk (22). We noted that, among the genes exhibiting altered expression, only a few possess corresponding miRNA binding sites (data not shown). Furthermore, a small fraction of predicted targets exhibited altered expression, indicating that these genes may be subject to posttranscriptional regulation by miRNA. Taken together, these results show that miR-24, miR-30b, and miR-142-3p modulate phagocytosis by directly and indirectly targeting key genes linked to phagocytosis.

**MiR-142-3p targets PKCa**

PKCa was among the list of altered genes with a potential miR-142-3p binding site. The 3′UTR region comprising 829–851 nt harbors miR-142-3p-interacting sequences that are highly conserved in mammals (Fig. 6B). To validate the predicted miRNA-
target interaction, we performed dual luciferase assays. HEK293 cells transfected with miR-142-3p (2 pmol) showed reduced (∼50%) luciferase activity when cotransfected with plasmids containing 3’UTR of PKCα, but not with control mimic (p < 0.001; Fig. 6C). Increasing the concentration of miRNA mimic to 5 pmol, however, had no further significant effect on luciferase activity. This suggests that miR-142-3p can bind target sites with high affinity, leading to efficient silencing of the reporter gene. We then investigated the functional consequence of this miRNA–target interaction in primary Mφ and DC. Overexpression of miR-142-3p decreased PKCα levels by ∼30% compared with control mimic in both the primary cells (Fig. 6D, 6E). However, more pronounced differences (∼42% decrease) in the levels of PKCα were observed, comparing miRNA mimic and corresponding in-

FIGURE 4. miRNA mimics modulate secretion of IL-6 and IL-12p40, but not IL-10. Mφ and DC were transfected at day 7 of culture with miR-24, miR-30b, and miR-142-3p mimics, inhibitors, or control mimic and stimulated by E. coli pHrodo bioparticles 24 h later. Supernatant levels of (A) IL-12p40, (B) IL-6, and (D) IL-10 were assayed after 4 and 18 h in supernatants of Mφ and DC by ELISA. (C) Comparison of supernatants IL-6 levels in M1 and M2 Mφ challenged with E. coli for 4 h. n = 3–6 biological replicates. Mean and SD are shown. *p < 0.01 is considered significant.
FIGURE 5. miRNA mimics differentially modulate transcription and intracellular accumulation of cytokines. miRNA mimics, inhibitors, or control-transfected cells were challenged with E. coli, and cytokine mRNA levels were examined by quantitative RT-PCR after 4 h. (A) Expression levels of TNF-α, IL-6, and IL-12p40 are shown for Mφ and DC. (B) ELISA showing intracellular cytokine expression in Mφ and DC. (C) Immunostaining shows decreased intracellular TNF-α in miRNA mimic overexpressing Mφ. Images were captured using a fluorescent microscope and a 20× objective lens. Green signals indicate intracellular TNF-α. Cells were counterstained with Hoechst nuclear dye. Scale bar, 200 μm. The yellow arrows point to accumulation of TNF-α localized to the cell periphery. The data represent three different fields captured from each well from at least three independent experiments. Student t test was conducted to calculate p values. *p < 0.01 was considered significant.
FIGURE 6. PKCα is a novel target of miR-142-3p. (A) Venn diagram showing the distribution of unique and overlapping genes downregulated by miR-24, miR-30b, and miR-142-3p mimics in DC and Mφ. (B) Sequence alignment of predicted miR-142-3p binding site in the 3′UTR of PKCα of various mammals. (C) HEK293 cells were cotransfected with PKCα 3′UTR construct and with either miR-142-3p or control mimic. Renilla activity was normalized to firefly activity, and the ratios subsequently normalized to empty vector transfected with miR-142-3p mimic set as 1. Data are expressed as ±SEM of four independent transfections. Western blot analysis of PKCα levels in miR-142-3p overexpressing (D) Mφ and (E) DC. Day 7 differentiated cells were transfected with miR-142-3p mimic, inhibitor, or control mimics. Cell lysates were prepared after 36 h, and PKCα levels were detected by Western blotting. GAPDH was used as internal control. Densitometric analysis of bands was performed with ImageJ. The normalized PKCα in control-transfected cells was set as 1. (F) Expression kinetics of PKCα in Mφ and DC. Expression levels of PKCα transcript were monitored at indicated time points during the monocyte to Mφ and monocyte to DC differentiation. (G) siRNA-mediated knockdown of PKCα as observed by RT-PCR and immunoblotting in Mφ. GAPDH served as endogenous control. Phagocytosis assays were performed with labeled E. coli in Mφ transfected with PKCα or scramble siRNA and untreated cells. (H) Representative images were captured using a fluorescence microscope and (I) geo. MFI values obtained from flow cytometry values. Scale bar, 200 μm. The data presented are representative of three independent experiments in each cell type. Student t-test was conducted to calculate p values. *p < 0.01 was considered significant.
hibitor as the later blocks the miRNA function leading to increased target gene translation. Overall, these results validate that PKCα is a bona-fide target of miR-142-3p.

Our previous miRNA profiling data of differentiating M₆ and DC showed that the expression of miR-142-3p continually decreases from day 3 onward (Gene Expression Omnibus submission GSE60839). We monitored the expression of its novel target PKCα during the course of differentiation. Indeed, in both M₆ and DC, a significant increase (~3-fold) in the expression levels of PKCα was noted after day 1, which peaked at approximately day 3 in M₆ and day 5 in DC (Fig. 6F). These results establish the antagonistic expression of miR-142-3p and its novel target, PKCα. We further examined the role of PKCα in bacterial phagocytosis. M₆ were transiently transfected with PKCα-targeting siRNA. Fig. 6F shows significant knockdown of PKCα at the mRNA and protein level. Moreover, cells transfected with PKCα siRNA exhibit reduced phagocytosis of labeled E. coli, as observed by florescent microscopy (Fig. 6H). Flow cytometry analysis shows ~20% reduction in mean fluorescent intensity in PKCα-transfected cells compared with control siRNA (p < 0.05; Fig. 6I). These results further support direct involvement of PKCα in bacterial phagocytosis.

Discussion

The importance of miRNA for the function of myeloid inflammatory cells has been revealed in recent years, particularly in the area of pathogen recognition and innate signaling; however, very little has been reported linking miRNA expression with phagocytosis. In this study, we show that overexpression of miR-24, miR-30b, and miR-142-3p, which are downregulated during M₆ and DC differentiation, attenuates phagocytosis by myeloid inflammatory cells. Moreover, both M₁ and M₂ M₆ show similar defects in bacterial uptake upon forced expression of the tested miRs. These results demonstrate a conserved role of miRNA in regulating the key myeloid inflammatory cell function that bridges innate and adaptive arms of immunity.

Recent studies highlight the role of miRNA in phagocytosis. For instance, mice M₆ J774.A1 challenged with nonviral strain, Mycobacterium smegmatis, show induced expression of miR-142-3p (23), lending support to our data that overexpression of miR-142-3p inhibits uptake of bacteria in myeloid cells. miR-142-3p regulates expression of actin-binding protein N-wasp, thereby disturbing actin dynamics, which is required for efficient phagocytosis (23). Our PCR array results also show reduced N-wasp levels in miR-142-3p-overexpressing DC (Supplemental Table I). In another report, induced expression of miR-615-3p in hyper-splenic M₆ was shown to promote phagocytosis by targeting ligand-dependent nuclear receptor corepressor expression, a negative regulator of peroxisome proliferator-activated receptor γ (24). Taken together, these findings indicate that dysregulation of miRNAs may render cells susceptible to pathogen invasion and disease.

All three miRNAs (miR-24, miR-30b, and miR-142-3p) identified in this study as regulator of phagocytosis were downregulated during differentiation. A growing body of evidence indicates that expression patterns of miRNAs permit cells to enter a new functional state, and it is possible that dysregulation at any point can result in aberrant phagocytic function. For instance, Ma et al. (25) demonstrated that downregulation of miR-29 in T cells allows for enhanced expression of IFN-γ, which promotes classical M₆ activation and Th1-dominant adaptive responses appropriate to bacterial infection. Similarly, we have previously reported that LPS-mediated reduction of miR-29b regulates expression of its target, IL-6R, a key component of innate immune responses (19). These findings signify a role of miRNA in shaping immune responses. Indeed, during phagocytosis it was observed that cells overexpressing miRNA mimics secrete significantly less IL-6, IL-12p40, and TNF-α compared with inhibitors or control, whereas the anti-inflammatory cytokine IL-10 was unaffected. Interestingly, miRNA mimic-transfected cells show modulation of cytokines with the decrease in IL-12p40 and increase, albeit less, in IL-6 and TNF-α mRNA levels.

Transcription of TNF-α and IL-6 expression is predominantly regulated by NF-κB and MAPK (in particular p38) in LPS-treated M₆ and other cells (26–28). Our PCR array data did not show significant differences in the expression of NF-κB and p38 transcripts. IL-12p40 expression, besides being regulated by NF-κB and Jun, is also regulated by transcription factor NFAT, which in turn is activated by CLEC7A/Dectin-1 (29). MiR-24, miR-30b, and miR-142-3p mimics significantly decreased (>15-fold) the levels of CLEC7A and most likely block IL-12p40 transcription. However, other factors and posttranscriptional mechanisms, including miRNA synthesis, transcript stability, translation, and protein secretion, could also control production of these cytokines. This is supported by the observation that, despite the differences in mRNA profiles, cellular and secreted levels of both TNF-α and IL-6 were downregulated. TNF-α immunostaining in E. coli–challenged M₆ also shows reduced cellular staining in the presence of miRNA mimic, further validating the cellular cytokine profiles. Interestingly, remarkable differences in the TNF-α staining pattern were observed among the miRNA mimics with miR-142-3p–transfected M₆ exhibiting accumulation of TNF-α localized to the cell periphery. These findings suggest that, besides modulating shared pathways, each miRNA mimic also modulates unique pathways that direct immune responses.

Phagocytosis can be broadly categorized into three steps, as follows: ligand binding, signal transduction, and internalization. Dysregulation at any point can result in aberrant phagocytic function. TLRs are the largest family involved in pathogen recognition. Our PCR array data showed miRNA mimic mediated downregulation of various TLRs, including TLR1, 2, 3, 4, 5, 6, and 9. Although TLR1, 2, 4, 5, and 6 are present on the cell surface, TLR3 and 9 are located on endosomal membranes (30). It is thus likely that these miRNAs can regulate recognition and removal of both extracellular and intracellular pathogens. In addition to recognition, these TLRs are intimately involved in phagocytosis, the production of inflammatory mediators, activation of the adaptive immune system, and enhancement of microbicidal mechanisms (31, 32). Besides TLRs, opsonic receptors, which include receptors for IgG and complement components, also participate in pathogen recognition. We noticed modulation of FcγR genes, specifically FcγRI, FcγRII, FcγRII, and FcγRIII, and complement component 3. Opsonins circulate in the bloodstream, detecting and binding to foreign Ags, increasing phagocytic uptake via FcγRs that include FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) (33, 34). Interestingly, we also found that, similar to E. coli and S. aureus uptake, overexpression of miR-24, miR-30b, and miR-142-3p mimics has profound effect on opsonin-mediated phagocytosis (A.R. Naqvi, J.B. Fordham, B. Ganesh, and S. Nares, manuscript in preparation). The miRNA-mediated impact on phagocytosis appears independent of the receptors involved and signifies their key role in the process.

PKCα belongs to the family of serine/threonine kinases that regulate various cellular functions, including cell differentiation, apoptosis, and LPS signaling (35, 36). Employing dual-luciferase assays, we show PKCα is a novel target of miR-142-3p. Enforced expression of miR-142-3p downregulated PKCα expression in M₆.
and DC. The siRNA-mediated knockdown of PKCα led to reduced uptake of bacteria, supporting its key role in the process. This observation is in agreement with previous studies demonstrating a requirement of PKCα during phagocytosis (37–39). However, we noted that siRNA knockdown of PKCα alone had relatively less impact (~20%) on phagocytosis compared with miRNA overexpression (~70%). This is most likely attributed to the capability of miRNAs to simultaneously regulate a wide range of targets and/or functional redundancy among PKC isoforms.

Initial reports on PKCα showed that inhibiting its activity blocked secretion of various proinflammatory cytokines in LPS-stimulated Mφ and was associated with defects in phagocytosis (40). Consistent with these findings, we observed significant reduction in IL-6, IL-12p40, and TNF-α release in miR-142-3p-transfected cells. Interestingly, PKCα is also reported to regulate TLR signaling by interacting with TLRs. Unlike other conventional PKCs, however, PKCα can trigger both Myd-dependent (TLR2) and -independent (TLR3) signaling (41, 42). TLR2 signaling terminates with the activation of NF-κB and MAPK, whereas TLR3 activation leads to IFN regulatory factor 3-mediated IFN-β expression; thus, PKCα can affect both early and late innate responses. Our studies demonstrate that miR-142-3p expression is downregulated during myeloid cell differentiation, whereas its novel target PKCα level increases. The role of PKC isoforms in differentiation of monocyte-derived Mφ is well established. In line with earlier reports, we support the significance of PKCs in both Mφ and DC differentiation. Importantly, this functional miRNA-target interaction highlights the regulatory potential of miRNA in both differentiation and function of immune cells.

In conclusion, we present miRNAs that significantly inhibit phagocytosis and the production of inflammatory mediators in myeloid inflammatory cells, including monocytes, M1/M2 Mφ, and DC. These miRNAs target pathways associated with phagocytosis, cytokine production, and inflammation and the productive pairing of the innate to the adaptive immune system. This study opens new avenues to explore novel roles of miRNAs identified in this study in diseases with impaired phagocytosis and related inflammatory disorders.

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Disclosures

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