Toll-like Receptor-4 (TLR4) Down-regulates MicroRNA-107, Increasing Macrophage Adhesion via Cyclin-dependent Kinase 6*

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Toll-like receptors (TLRs) modulate the expression of multiple microRNAs (miRNAs). Here, we report the down-regulation of miR-107 by TLR4 in multiple cell types. The miR-107 sequence occurs in an intron within the sequence encoding the gene for pantothenate kinase 1α (PanK1α), which is regulated by the transcription factor peroxisome proliferator-activating receptor α (PPAR-α). PanK1α is also decreased in response to lipopolysaccharide (LPS). The effect on both miR-107 and PanK1α is consistent with a decrease in PPAR-α expression. We have found that the putative miR-107 target cyclin-dependent kinase 6 (CDK6) expression is increased by TLR4 as a result of the decrease in miR-107. This effect is required for increased adhesion of macrophages in response to LPS, and CDK6-deficient mice are resistant to the lethal effect of LPS. We have therefore identified a mechanism for LPS signaling which involves a decrease in miR-107 leading to an increase in CDK6.

Toll-like receptors (TLRs) are among the first line of defense in the body where they recognize conserved structures from bacteria, viruses, or fungi (1, 2). They play a critical role in innate immunity, and their activation leads to the transcription of genes involved in the immune and inflammatory responses. MicroRNAs (miRNAs) are an important family of small, non-coding RNAs that act as regulators of gene expression in a tissue- and cell-specific manner by base-pairing to a target messenger RNA (mRNA) sequence called a seed sequence. This leads to the partial or full degradation of the mRNA by RNases and the inhibition of its translation into a functional protein (3–5). miRNAs were originally found to have a role in cellular development, differentiation, adhesion, and apoptosis and are now known to function in cancer and immunity (5–8). Stimulation of cells with the TLR4-specific ligand LPS from Gram-negative bacteria results in an increase in the expression of several miRNAs including miR-21, miR-146a, and miR-155. miR-21 has been linked to cell migration, invasion, and adhesion, with target genes that include tropomyosin, programmed cell death protein 4 (PDCD4), and phosphatase and tensin homolog (PTEN). It was recently shown to regulate the immune response negatively by promoting the anti-inflammatory response by eliminating PDCD4 and up-regulating IL-10 (9, 10). miR-146a acts as a negative regulator of TLR signaling and cytokine production. Two genes known to be involved in TLR4 signaling, those encoding TRAF6 and IRAK1, are targets of miR-146a, and it was shown that LPS-induced activation of miR-146a is NF-κB-dependent (11). miR-155 has been shown to target suppressor of cytokine signaling 1 (SOCS1) and SH2 (Src homology 2)-containing inositol phosphatase-1 (SHIP-1) directly. Mice lacking miR-155 have defects in B cell differentiation as well as having severe deficiencies in immune responses when exposed to pathogens (12–14). miRNAs have also been shown to be down-regulated in expression in response to LPS, including Let-7i and miR-125b, which have been shown to target TLR4 and TNF-α, respectively. This aspect of miRNA biology has so far been less explored (6, 15, 16).

Here, we demonstrate that miR-107 is down-regulated in response to LPS. The mechanism involves a decrease in the transcription factor peroxisome proliferator-activating receptor-α (PPAR-α) (17). We also found decreases in the expression of pantothenate kinase 1α (PanK1α), an enzyme required for coenzyme A (CoA) biosynthesis whose gene contains the miR-107 sequence in intron 5. We have found that CDK6 is an important target for miR-107. LPS causes an increase in CDK6 expression via the decrease in miR-107. We show that this increase is required for adhesion of macrophages. CDK6-deficient mice are also less susceptible to the lethal effect of LPS. We have therefore uncovered a loop activated by TLR4, which limits the expression of miR-107, leading to an increase in its target CDK6, thereby enhancing adhesion of macrophages.

**EXPERIMENTAL PROCEDURES**

**Reagents**—LPS from *Escherichia coli*, serotype 0111:B4, was from Alexis. WY14643 was from Sigma. Antisense miR-107 (anti-miR-107), precursor miR-107 (pre-miR-107), anti-miR-control (anti-control), and precursor-miR-control (pre-control) oligonucleotides, TaqMan mmu-miR-107 primer/probe expression assay, primary-miR-107 probe, and mCDK6 gene expression assay were obtained from Applied Biosystems.

**Cell Culture**—Immortalized wild-type bone marrow-derived macrophages (1-BMDM) and MyD88-deficient and Trif-defi-
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cient I-BMDM were a kind gift from Douglas Golenbock (University of Massachusetts). Wild-type and CDK6-deficient bone marrow was obtained from Dr. David Santamaría (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain). Marrow was isolated from the tibias and femurs of C57BL/6 mice, and primary BMDM were generated as described previously (9). Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood using a Ficoll gradient (18). HEK293T cells were obtained from the European collection of animal cell cultures. RAW264.7 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). In all cases, Dulbecco’s modified Eagle’s medium was supplemented with 10% fetal calf serum, 2 mm L-glutamine, and 1% penicillin/streptomycin solution (v/v).

**Mice**—CDK6−/− mice were generated as described previously (19). Mice were maintained according to the animal care standards established by the European Union.

**RT-PCR**—Differentially primary BMDM, PBMC, or I-BMDM and were set up at 1 × 10⁶/ml, or 3 × 10⁵/ml, respectively, in 12-well plates 1 day prior to stimulation. Cells were stimulated with LPS as indicated in the figure legends. Total RNA was extracted using the RNeasy kit (Qiagen), modified to obtain small RNA species. For miRNA analysis, miRNA TaqMan assays for miR-107 or miR-146a and RNU6B (Applied Biosystems) were used according to the manufacturer’s instructions where 5 ng/ml total RNA was used as starting material. For mRNA expression analysis, CDNA was prepared from 20–100 ng/ml total RNA using the High-Capacity cDNA archive kit (Applied Biosystems) according to the manufacturer’s instructions. mRNA expression was then monitored using either TaqMan gene expression assays with Fast Universal PCR Master Mix or SYBR Green-based chemistry (Invitrogen) using either TaqMan gene expression assays with Fast Universal PCR Master Mix or SYBR Green-based chemistry (Invitrogen) using the following primers: mmu-pre-107, 5′-GTGCTTTCAAGCTTCTTTACAGTGGTGCAG-3′, forward, 5′-TCTCTGTGGCTTTGATA-GCCCTGT-3′, reverse; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GACGGGAAGCTTGGCATCAA-3′, forward, 5′-CTAAGCAGTTGGTGCTGACG-3′, reverse; mPanK1α, 5′-CTGCGGAGGAGGATGGACT-3′, forward, 5′-CCACCGATATCCTACACAAAC-3′, reverse; mPPAR-α, 5′-GCCAGTCCTGTAAGTTCA-3′, forward, 5′-CTTCTCATTCCCAAGGCCTAG-3′, reverse. miRNA and mRNA expression were measured on the 7900 RT-PCR system (Applied Biosystems), and -fold changes in expression were calculated by the ΔΔCT method using RNU6b as an endogenous control for miRNA analysis and GAPDH as an endogenous control for mRNA expression. All -fold changes are expressed normalized to an unstimulated control for each cell type.

**Enzyme-linked Immunosorbent Assay (ELISA)**—Murine TNF-α expression was measured from the supernatants of stimulated cells using an ELISA DuoSet kit (R&D Biosystems) according to the manufacturer’s instructions.

**Luciferase Assays**—CDK6 3′-UTR luciferase plasmid was obtained from Labomics. HEK293T cells seeded at 3 × 10⁴/ml in 96-well plates were transfected using 2% Lipofectamine 2000 (Invitrogen) with wild-type or mutant plasmid and TK-Renilla and 50 nM murine anti-control-miR, pre-control-miR, or 50 nM anti-miR-107 or pre-miR-107 (Applied Biosystems). In all cases, cells were lysed in passive lysis buffer before being analyzed for both luciferase and TK-Renilla activity as described previously (20). Data were normalized to TK-Renilla activity.

**Protein Expression**—Primary BMDM seeded at 1 × 10⁶/ml in 6-well plates were stimulated with LPS as indicated in the figure legends. Cells were lysed in low stringency lysis buffer complete with protease inhibitors, and protein concentration was determined using the Coomassie Bradford reagent (Pierce). Lysates were resolved on 12% SDS-polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane before being immunoblotted with a mouse monoclonal anti-CDK6 antibody (Cell Signaling Technology), a rabbit polyclonal anti-PanK1α antibody (a kind gift from Dr. Suzanne Jackowski from St. Jude Children’s Research Hospital, Memphis, TN), a rabbit polyclonal anti-PPAR-α antibody (BioVision), or a mouse anti-β-actin (AC-15; Sigma). Blots were developed by enhanced chemiluminescence (ECL) (Cell Signaling Technology).

**Cell Adhesion Assay**—The xCELLigence real-time cell analyzer (Roche Applied Science) was used in this work to measure adhesion of RAW264.7 macrophages in real time. E-plate 16 wells were incubated with 50 μl of 10 μg/ml fibronectin for 30 min at room temperature. Wells were then washed with PBS, and 100 μl of medium was added to the wells, and the E-plate was placed into the incubator for a background measurement. 50,000 cells were plated in each well. Cells were allowed to settle for 30 min at room temperature before they were transfected with 50 nm antisense or precursor miRNA oligonucleotides for 16 h. The medium was then changed to contain 0.1% BSA. Cells were stimulated with LPS as indicated in the figure legends, and E-plates were placed within the plate station for monitoring of the cell index. The real-time cell analyzer monitors the impedance of each distinct well of the E-plate and delivers cell index values every 5 min. The cell index is calculated as a dimensionless parameter, which increases with higher cell numbers and decreases with lower cell numbers.

**RESULTS**

miR-107 Is Down-regulated in Response to LPS Over Time in a MyD88- and p65-dependent Manner—In a screen of miRNAs we found that as well as increasing a range of miRNAs (notably miR-146a, miR-155, and miR-21), LPS can down-regulate miRNAs, notably miR-149 and miR-107. We focused on miR-107 because we consistently observed a decrease in this miRNA in multiple cell types. Fig. 1a shows that in primary BMDM (upper) and human PBMC (lower), miR-107 expression is decreased by ~4-fold from 4 h post-LPS stimulation relative to the unstimulated samples. miR-146a is known to be up-regulated in response to LPS so it was used as a positive control for miRNA expression, and it increased 3-fold over a 24-h period in primary BMDM and 5-fold in PBMC over 24 h of stimulation with LPS (Fig. 1b, upper and lower).

We next examined the primary (pri-miR) and precursor (pre-miR) transcripts of miR-107 to determine whether the down-regulation of mature miR-107 in response to LPS was transcriptionally regulated. miR-107 is transcribed as a primary (pri-miR-107) transcript from the fifth intron of the PanK1α gene found on chromosome 19 of the murine genome (21). Fig. 1c shows that the pri-miR-107 transcript (left) is down-regulated 4–8-fold in response to LPS in primary BMDM. The pre-
miR-107 transcript (right) also decreased 4-fold following 8 h of LPS stimulation, leading to the conclusion that the down-regulation of mature miR-107 is transcriptionally regulated. We next examined whether the effect of LPS on miR-107 was dependent on the MyD88 or Trif signaling pathways. We determined that the down-regulation of miR-107 was MyD88-dependent because there was no decrease in MyD88-deficient cells at both the 8-h and 24-h treatments times as shown in Fig. 1d, left. Trif-deficient cells still showed the decrease. To determine whether the effect was NF-κB-dependent, we used p65-deficient mouse embryonic fibroblasts (MEF), and we established that the decrease in miR-107 is dependent on p65 (Fig. 1d, right). miR-107 expression decreased ~4-fold in the wild-type MEF but did not decrease in the p65-deficient cells.

PanK1α and PPAR-α Are Down-regulated in Response to LPS over Time, Similar to miR-107—PanK1α expression was measured next to establish whether miR-107 was co-transcribed with PanK1α because the miR-107 sequence occurs in intron 5 of the PanK1α gene. Fig. 2a (upper) shows that PanK1α mRNA decreased in primary BMDM, a 4-fold decrease being evident after 8 h of LPS stimulation. PanK1α protein level also decreased in primary cells over time (Fig. 2a, lower). mRNA expression in the adaptor-deficient and p65-deficient cells was also examined, and similar to miR-107, PanK1α decreased in the wild-type and Trif-deficient cells but failed to decrease in the MyD88-deficient cells (Fig. 2b, upper) and in p65-deficient cells (Fig. 2b, lower). Similar to miR-107, therefore, PanK1α expression is inhibited by LPS treatment in a MyD88- and p65-dependent fashion.
We next examined the expression of the transcription factor PPAR-α which is known to regulate the expression of PanK1α. The PanK1α promoter region contains four putative peroxisome proliferator response elements for the transcription factor PPAR-α (17). We found that PPAR-α mRNA (Fig. 2c, upper) and protein expression (Fig. 2c, lower) decreased 4-fold follow-
ing 4–8 h of LPS stimulation. Fig. 2d (upper) shows PPAR-α mRNA in the adaptor-deficient BMDM, and similar to PanK1α and miR-107, PPAR-α decreased in the wild-type and Trif-deficient cells but failed to decrease in the MyD88-deficient cells. Similarly in the MEF, PPAR-α decreased in the wild-type cells but failed to decrease in the p65-deficient cells (Fig. 2d, lower). These results suggest that all three transcripts are under the same mechanism leading to their down-regulation and that the decrease in PPAR-α was likely to be mediating the inhibition of both PanK1α and miR-107.

Through the use of the chemical ligand WY14643, which acts as a specific agonist toward PPAR-α, we found that miR-107 and PanK1α expression in macrophages increased in the same manner as PPAR-α in response to treatment with this compound for 24 h (Fig. 2e). Macrophages that were pretreated with the compound prior to LPS stimulation exhibited an inhibition in TNF-α cytokine secretion (Fig. 2f) and an increase in miR-107, PanK1α, and PPAR-α levels possibly signifying two competing pathways of signaling. This is in agreement with the known anti-inflammatory role of PPAR-α (22).

**CDK6, Dicer1, and HIF-1α Are Up-regulated in Response to LPS over Time**—Having clearly demonstrated a decrease in miR-107 expression by LPS, we next investigated a functional outcome for this effect. CDK6 has emerged as a potentially important miR-107 target (23, 24). The seed sequence match between miR-107 and CDK6 is found at position 308–314 in the 3’-UTR of the CDK6 transcript. CDK6 expression was up-regulated by LPS in BMDM at both the mRNA and protein levels with induction evident at 4 h and declining at 24 h (Fig. 3a). Fig. 3b demonstrates that the up-regulation of CDK6 in response to LPS is both MyD88- (upper) and p65-dependent (lower). We also examined Dicer1 and HIF-1α, two other identified target genes of miR-107 to determine whether their mRNA also increased in response to LPS (Fig. 3c). The expression of both of these genes increased more than 2-fold following 4–8 h of LPS stimulation.

**CDK6 mRNA and Protein Expression Are Affected by miR-107 Levels**—To verify that miR-107 was targeting CDK6, we transfected antisense miR-107 (anti-miR-107) or precursor miR-107 (pre-miR-107) molecules to knock down or overexpress miR-107 in I-BMDM, respectively, and then measured CDK6 mRNA and protein levels. We first examined the effect of using antisense to miR-107 on CDK6 mRNA and protein expression. We found that cells transfected with the anti-miR-107 exhibited at least a 3-fold increase in CDK6 mRNA and protein following 8 h of LPS stimulation. These results are shown in Fig. 4a, left and right. It is interesting to note the difference in response to LPS between cell types. CDK6 mRNA
expression increased more in the primary BMDM (Fig. 3a) compared with the I-BMDM (Fig. 4a) in response to LPS. We also transfected I-BMDM with pre-miR-107 to observe the effect of overexpressing miR-107 on CDK6 mRNA and protein. We detected less CDK6 mRNA and protein following 8 h of LPS stimulation compared with pre-miR-control transfected cells where CDK6 mRNA expression increased 2-fold in response to LPS as shown in Fig. 4b, left and right.

To confirm that miR-107 is directly targeting the 3′-UTR of CDK6, a luciferase construct containing the 3′-UTR of CDK6 with the wild-type miR-107 seed sequence located at position 308–314 or a mutated form at a single nucleotide was used. Because the luciferase construct contains an SV40 promoter and enhancer sequences, there is constitutive expression of the luciferase activity. Fig. 4c shows that transfection of cells with anti-miR-107 does not have an affect on the luciferase activity.
because it is constitutive. However, cells transfected with the wild-type CDK6 construct and pre-miR-107 exhibited a 75% decrease in luciferase indicating that miR-107 directly binds to the seed sequence. Cells transfected with the mutant CDK6 luciferase and pre-miR-107 exhibited a 30% decrease in response to transfection with pre-miR-107, but importantly this is less than in the wild-type construct. This 30% decrease is most likely due to a second seed sequence in the CDK6 3'-UTR at position 1815–1821.

Inhibition of TNF-α Secretion and Adhesion by Cells Overexpressing miR-107—We next examined the functional relevance of miR-107 and CDK6 expression. Based on the observation of decreased TNF-α production by cells treated with the PPAR-α-specific agonist we wanted to see whether overexpression of miR-107 would also have an effect on TNF-α. Fig. 4e illustrates that transfection of macrophages with pre-miR-107 resulted in less secreted TNF-α compared with pre-miR-control following 24 h of LPS stimulation. Transfection with anti-miR-107 resulted in enhanced TNF-α secretion compared with cells transfected with anti-miR-control.

CDK6 has been shown to play a role in the cell cycle but also cellular adhesion (25–27). LPS is a weak inducer of cellular proliferation, but it promotes cell adhesion. We therefore chose the latter as a response to measure. A quantitative assay of macrophage adhesion to fibronectin was used. Fig. 4d (upper) illustrates adhesion of macrophages transfected with anti-miR-107 in response to LPS over a 40-h time period. LPS treatment doubled the rate of macrophage adhesion over 40 h in cells transfected with the control oligonucleotide. This was enhanced by 50% in cells transfected with anti-miR-107. The subtle magnitude of this effect is typical for miRNA modulation by antisense. Fig. 4d (lower) illustrates adhesion of macrophages transfected with pre-miR-107 in response to LPS over a 40-h time period. Again, a doubling of adhesion was evident over 40 h in cells transfected with the control oligonucleotide. Transfection with pre-miR-107, however, completely blocked the increased adhesion in response to LPS.

Inhibition of Adhesion of Cells Lacking CDK6 and CDK6-deficient Mice Are Protected from the Lethality of LPS—We obtained bone marrow from CDK6-deficient mice, and BMDM isolated from the bone marrow, like macrophages overexpressing miR-107, also exhibited significantly less adhesion in response to LPS as shown in Fig. 5a. To examine the role of CDK6 in TLR signaling and inflammation further, we injected CDK6-deficient and wild-type control mice with LPS and monitored their survival. CDK6-deficient mice were less susceptible to LPS, with lower mortality than wild-type mice (Fig. 5b). Analysis of circulating cytokine concentrations 1 h after LPS injection showed that TNF-α concentrations were lower in CDK6-deficient mice treated with
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LPS (Fig. 5c), consistent with lower susceptibility. These data indicate that CDK6 has a proinflammatory role in LPS signaling. Overall, therefore, our results indicate that a decrease in miR-107 induced by LPS leads to an increase in CDK6 expression, which in turn leads to macrophage adhesion with CDK6 also having a role in LPS lethality in vivo.

DISCUSSION

miR-107 and PanK1α are down-regulated in a MyD88/NF-κB/PPAR-α-dependent fashion in response to LPS most likely via a decrease in PPAR-α expression. Following the modulation of miR-107 levels we found that the decrease in miR-107 leads to an increase in CDK6 thereby promoting adhesion of macrophages, confirming a role for CDK6 in this process. We have therefore found a regulatory loop requiring a decrease in miR-107 and PanK1α via PPAR-α leading to increased CDK6 expression and subsequent adhesion of macrophages in response to LPS. Both PanK1α and PPAR-α have known roles in lipid metabolism in the cell, but little is known about their possible roles in TLR and immune signaling. PanK1α is involved in the first and most highly regulated step in the synthesis of CoA, an essential co-factor in fatty acid oxidation and gluconeogenesis. Intracellular CoA levels decrease in response to glucose and insulin. LPS stimulation induces hypoglycemia and decreases liver gluconeogenesis in rats and decreases fatty acid oxidation during sepsis (28–33). LPS may decrease gluconeogenesis and fatty acid oxidation as a way to conserve glucose during infection, but this decrease can be detrimental in some tissues and beneficial in other tissues like the heart (34). We have shown that PanK1α is decreased in response to LPS, and this could be part of the mechanism for the decrease in gluconeogenesis and fatty acid oxidation seen in the previous studies.

We have found that LPS stimulation leads to a decrease in PPAR-α mRNA and protein expression. Previous studies have shown that recombinant TNF-α or LPS stimulation decreases PPAR-α mRNA expression in rat liver hepatocytes. In mice exposed to TNF-α or LPS, PPAR-α mRNA in the lungs was decreased by 50–60% (35). PPAR-α has anti-inflammatory capabilities and prevents the binding of NF-κB and AP-1 to their target gene sequences possibly by preventing the phosphorylation of p65 and inhibiting the degradation of IκBα (36).

In our study, the down-regulation of PPAR-α was maximal at 4 h post-LPS challenge. The precise mechanism of PPAR-α down-regulation by LPS is not known. LPS stimulates the expression of c-Jun, a component of the AP-1 signaling pathway, and also activates NF-κB. A glutathione S-transferase pull-down experiment demonstrated that PPAR-α physically interacts with c-Jun and the NF-κB subunit p65 and that there is a bidirectional antagonism among PPAR-α, c-Jun, and p65 (37). It is known that PPAR-α can positively autoregulate its own expression because the PPAR-α/ΔRXR complex can directly bind to the PPAR-α promoter (38). Through the use of p65-deficient cells we have shown that the decrease in PPAR-α mRNA in response to LPS is p65-dependent. This suggests that either p65 is directly interacting with PPAR-α at the PPAR-α promoter preventing its transcription or there is an unknown factor involved, and p65 is affecting this co-factor, resulting in the inhibition of the formation of the PPAR-α/ΔRXR complex leading to a decrease in PPAR-α, PanK1α, and miR-107 expression.

This study is the first to directly link any of the CDK-encoding genes as LPS-responsive. CDK6 is required to progress cells from G1 to S phases of the cell cycle. CDK expression has been linked to changes in the actin cytoskeleton and increased adhesion of cells (27, 39, 40). We have found that the deletion of miR-107 boosted adhesion, and the overexpression of miR-107 or deletion of CDK6 blocked this process.

We have also found that CDK6 is important for LPS lethality because CDK6-deficient mice were resistant to LPS. This is likely due to a macrophage defect as was indicated from decreased TNF-α production in CDK6-deficient mice.

Apart from CDK6, we also found two other potential miR-107 targets to be up-regulated by LPS: HIF-1α and Dicer1. LPS is a known inducer of hypoxia signaling in a time- and dose-dependent manner, and this could be promoted by decreased miR-107 leading to increased HIF-1α levels (41). miR-107 has been shown to mediate p53-regulated hypoxic signaling by targeting HIF-1α (21). We also found that Dicer1 mRNA expression increased 2-fold following 1–2 h of LPS stimulation. Elevated levels of miR-107 expression have been found in human breast cancer, and these cells were shown to target Dicer1 mRNA, resulting in overall miRNA down-regulation (42). The effect of LPS on Dicer1 here may therefore regulate other LPS-regulated miRNAs. Overall therefore, our data indicate a role for miR-107 in the regulation of TLR signaling. The decrease in miR-107 impacts on CDK6 levels, which in turn promotes macrophage adhesion.

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