

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 of the gene encoding for Pantothenate kinase-1 α (Pank1 α), which is regulated by the transcription factor PPAR- α . Pank1 α is also decreased in response to lipopolysaccharide (LPS). The effect on both miR-107 and Pank1 α 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.

TLRs are among the body's first line of defense 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. miRNAs are an important family of small, non-coding RNAs which 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 negatively regulate the immune response 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- κ B-dependent (11). miR-155 has been shown to directly target suppressor of cytokine signaling 1 (SOCS1) and SH2 (Src homology 2)-containing inositol phosphatase-1 (SHIP-1). 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 Pank1 α , 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. Anti-sense-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 (I-BMDM), MyD88-deficient and TRIF-deficient I-BMDM were a kind gift from Douglas Golenbock (University of Massachusetts), Wild-type and CDK6-deficient bone marrow was obtained from Dr. David Santamaria (Centro Nacional de Investigaciones Oncológicas, Madrid Spain). Marrow was isolated from the tibias and femurs of C57/Bl6 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, 1% penicillin/streptomycin solution (v/v).

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

RT-PCR Differentiated primary BMDM, PBMC or I-BMDM and were set up at 1×10^6 /ml, or $3 \times$

10^5 /ml respectively, in 12-well plates 1 day prior to stimulation. Cells were stimulated with LPS as indicated in the figure legend. 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 to 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 the following primers:

mmu-pre-107,

5'-GTGCTTTCAGCTTCTTTACAGTG -3',
forward, 5'- TCTCTGTGCTTTGATAGCCCTGT
-3', reverse;

glyceraldehyde-3-phosphate dehydrogenase
(GAPDH),

5'- GAACGGGAAGCTTGTTCATCAA -3',
forward, 5'- CTAAGCAGTTGGTGGTGCAG -3',
reverse;

mPanK1 α ,

5'- CTGCGGAGGAGGATGGACT -3', forward,
5'- CCACCGATATCCATACCAAACC -3',
reverse;

mPPAR- α

5'- GCAGCTCGTACAGGTCATCA -3', forward,
5'- CTCTTCATCCCAAGCGTAG -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 Delta Delta CT 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 Murine TNF- α expression was measured from the supernatants of stimulated cells using an enzyme-linked immunosorbent assay 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 3×10^5 /ml in 96-well plates were transfected using 2% Lipofectamine2000 (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 1×10^6 /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-PAGE gels and transferred onto polyvinylidene difluoride membrane before being immunoblotted with a mouse monoclonal anti-CDK6 antibody (Cell Signaling Technology Inc.), a rabbit polyclonal anti-PanK1 α 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 Inc.).

Cell Adhesion Assay The xCELLigence Real-Time Cell Analyzer (RTCA DP; Roche) was used in this work to measure adhesion of Raw264.7 macrophages in real time. E-plate 16 wells were incubated with 50 μ l of 10 μ g/ml fibronectin for 30 minutes at room temperature. Wells were then washed with PBS and 100 μ l of media 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 anti-sense or precursor miRNA oligonucleotides for 16 hours. Media was then changed to contain 0.1%BSA. Cells were stimulated with LPS as indicated in the figure legend and E-plates were placed within the plate station for monitoring of Cell Index (CI). The RTCA monitors the impedance of each distinct well of the E-plate and delivers CI values every five minutes. The CI 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 since we consistently observed a decrease in this miRNA in multiple cell types. Figure 1a shows that in primary bone marrow derived macrophages (BMDM) (Upper) and human peripheral blood mononuclear cells (PBMC) (Lower), miR-107 expression is decreased by approximately four fold from four hours 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 three fold over a 24 hour period in primary BMDM and five fold in PBMC over 24 hours 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 if 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 pantothenate kinase 1 α (PanK1 α) gene found on chromosome 19 of the murine genome (21). Figure 1c shows that the pri-miR-107 transcript (Left) is down-regulated four to eight fold in response to LPS in primary BMDM. The pre-miR-107 transcript (Right) also decreased four fold following eight hours of LPS stimulation leading to the conclusion that the down-regulation of mature miR-107 is transcriptionally regulated. We next examined if 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 hour and 24 hour treatments times as shown in Figure 1d Left. Trif-deficient cells still showed the decrease. To determine if the effect was NF- κ B-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 approximately four fold in the wild-type MEF but did not decrease in the p65-deficient cells.

PanK1 α and PPAR- α are down-regulated in response to LPS over time, similar to miR-107. PanK1 α expression was measured next to establish if miR-107 was co-transcribed with PanK1 α since the miR-107 sequence occurs in intron 5 of the PanK1 α gene. Figure 2a (Upper) shows that PanK1 α mRNA decreased in primary BMDM, a four-fold decrease being evident after eight hours of LPS stimulation. PanK1 α 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, PanK1 α 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, PanK1 α 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 PanK1 α . The PanK1 α promoter region contains four putative peroxisome proliferator response elements (PPREs) for the transcription factor PPAR- α (17). We found that PPAR- α mRNA (Fig 2c Upper) and protein expression (Fig 2c Lower) decreased four fold following four to eight hours of LPS stimulation. Figure 2d (Upper) shows PPAR- α mRNA in the adaptor deficient BMDM and similar to PanK1 α 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 PanK1 α and miR-107.

Through the use of the chemical ligand WY14643 which acts as a specific agonist towards PPAR- α , we found that miR-107 and PanK1 α expression in macrophages increased in the same manner as PPAR- α in response to treatment with this compound for 24 hours (Fig 2e). Macrophages

that were pre-treated with the compound prior to LPS stimulation exhibited an inhibition in TNF- α cytokine secretion (Fig 2f) and an increase in miR-107, PanK1 α and PPAR- α levels. This is in agreement with the known anti-inflammatory role of PPAR- α (22).

CDK6, Dicer1 and HIF-1 α 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 four hours and declining at 24 hours (Fig. 3a). Figure 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-1 α , two other identified target genes of miR-107 to determine if their mRNA also increased in response to LPS (Figure 3c). The expression of both of these genes increased more than two fold following four to eight hours 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 knockdown or over-express miR-107 in immortalized BMDM (I-BMDM) respectively and then measured CDK6 mRNA and protein levels. We first examined the effect of using anti-sense to miR-107 on CDK6 mRNA and protein expression. We found that cells transfected with the anti-miR-107 exhibited at least a three-fold increase in CDK6 mRNA and protein following eight hours of LPS stimulation. This compared to cells transfected with a non-targeting anti-miR-control molecule where CDK6 increased only two fold. These results are shown in Figure 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 to the I-BMDM (Fig. 4a) in response to LPS. We also transfected I-BMDM with pre-miR-107 to observe the effect of over-expressing miR-107 on CDK6

mRNA and protein. We detected less CDK6 mRNA and protein following eight hours of LPS stimulation compared to pre-miR-control transfected cells where CDK6 mRNA expression increased two fold in response to LPS as shown in figure 4b Left and right.

In order 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. Figure 4c shows that transfection of cells with anti-miR-107 does not have an affect on the luciferase activity because it is constitutive regardless of targeting miR-107. 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 over-expressing 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 examined if over-expression of miR-107 would also have an effect on TNF- α . Figure 4e illustrates that transfection of macrophages with pre-miR-107 resulted in less secreted TNF- α compared with pre-miR-control following 24 hours of LPS stimulation. Transfection with anti-miR-107 resulted in enhanced TNF- α secretion compared to 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. Figure 4d (Upper) illustrates adhesion of

macrophages transfected with anti-miR-107 in response to LPS over a 40 hour time period. LPS treatment doubled the rate of macrophage adhesion over 40 hours 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. Figure 4d (Lower) illustrates adhesion of macrophages transfected with pre-miR-107 in response to LPS over a 40 hour time period. Again, a doubling of adhesion was evident over 40 hours 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 over-expressing miR-107 also exhibited significantly less adhesion in response to LPS as shown in figure 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 hour after LPS injection showed that TNF- α concentrations were lower in CDK6-deficient mice treated with LPS (Fig. 5c), consistent with lower susceptibility. These data indicate that CDK6 has a pro-inflammatory 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 PanK1 α are down-regulated in a MyD88/NF- κ B/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 PanK1 α via PPAR- α leading to increased CDK6 expression and subsequent adhesion of macrophages in response to LPS. Both PanK1 α 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. PanK1 α is involved in the first and most highly regulated step in the synthesis of CoA an essential cofactor 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 PanK1 α is decreased in response to LPS and this could be part of the mechanism for the decrease observed 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- κ B and AP-1 to their target gene sequences possibly by preventing the phosphorylation of p65 and inhibiting the degradation of I κ B α (36). In our study, the down-regulation of PPAR- α was maximal at 4 hours 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- κ B. A glutathione S-transferase pull-down experiment demonstrated that PPAR- α physically interacts with c-Jun and the NF- κ B subunit p65 and that there is a bidirectional antagonism between PPAR- α , c-Jun and p65 (37). It is known that PPAR- α can positively auto-regulate its own expression

because the PPAR- α /RXR 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 cofactor resulting in the inhibition of either the formation of the PPAR- α /RXR complex leading to a decrease in PPAR- α , PanK1 α and miR-107 expression.

This study is the first to directly link any of the cyclin-dependent kinase 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 over-expression of miR-107 or deletion of CDK6 blocked this process.

We have also found that CDK6 is important for LPS lethality since 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-1 α 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-1 α levels (41). miR-107 has been shown to mediate p53 regulated hypoxic signaling by targeting HIF-1 β (21). We also found that Dicer1 mRNA expression increased 2 fold following 1 to 2 hours 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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FOOTNOTES

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FIGURE LEGENDS

Fig 1. miR-107 expression in response to LPS over time in primary and MyD88 and p65 deficient cells. (a) (Upper) Primary BMDM and (Lower) PBMC were stimulated with LPS (100 ng/ml), for the times indicated. Expression of miR-107 was measured by RT-PCR. Results were normalized to the endogenous control RNU6b, a ribosomal RNA and represented as fold stimulation over the unstimulated control. Results in all cases are shown as relative expression (R.E.). (b) (Upper) Primary BMDM and (Lower)

PBMC were stimulated with LPS (100 ng/ml), for the times indicated. Expression of miR-146a was measured by RT-PCR in the same manner as for miR-107. (c) Primary BMDM were stimulated with LPS (100 ng/ml), for the times indicated and (Left) pri-miR-107 and (Right) pre-miR-107 were measured by RT-PCR. Results were normalized to the endogenous control GAPDH and represented as fold stimulation over the non-stimulated control. (d) (Left) WT I-BMDM, MyD88 deficient I-BMDM and Trif deficient I-BMDM were stimulated with LPS (100 ng/ml) for 8 or 24 h. (Right) WT MEF or p65 deficient MEF were stimulated with LPS (100 ng/ml) for the times indicated. The horizontal axis in all graphs represents time in hours of stimulation. In all cases, results are expressed as mean \pm S.E.M. of triplicate determinations from three separate experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 2. PanK1 α and PPAR- α expression in response to LPS over time. (a) (Upper) Primary BMDM were stimulated with LPS (100 ng/ml), for the times indicated. Expression of PanK1 α was measured by RT-PCR. Results were normalized to the endogenous control GAPDH and represented as fold stimulation over the non-stimulated control. (Lower) Primary BMDM were stimulated with LPS (100 ng/ml), for the times indicated and assessed for PanK1 α and β -actin. Relative intensity (R.I) is shown to compare intensity of the western blot bands relative to time 0. (b) (Upper) WT I-BMDM, MyD88 deficient I-BMDM and Trif deficient I-BMDM were stimulated with LPS (100 ng/ml) for 8 or 24 h. (Lower) WT MEF or p65 deficient MEF were stimulated with LPS (100 ng/ml) for the times indicated. (c) (Upper) Primary BMDM were stimulated with LPS (100 ng/ml), for the times indicated. Expression of PPAR- α was measured by RT-PCR in the same manner as PanK1 α . (Lower) Primary BMDM were stimulated with LPS (100 ng/ml), for the times indicated and assessed for PPAR- α and β -actin, R.I. is shown below. (d) (Upper) WT I-BMDM, MyD88 deficient I-BMDM and Trif deficient I-BMDM were stimulated with LPS (100 ng/ml) for 8 or 24 h. (Lower) WT MEF or p65 deficient MEF were stimulated with LPS (100 ng/ml) for the times indicated. (e) I-BMDM were pre-treated with 10 μ M WY14643 for 30 minutes before the addition of LPS (100ng/ml) for 24 hours. Expression of miR-107, PanK1 α and PPAR- α were measured by RT-PCR. Results were normalized to the endogenous controls RNU6b (miR-107) or GAPDH (PanK1 α and PPAR- α) and represented as fold stimulation over the DMSO control. (f) Enzyme-linked immunosorbent assay (ELISA) of mouse TNF- α was measured in supernatants of samples pre-treated with WY14643 before LPS stimulation. In all cases, results are expressed as mean \pm S.E.M. of triplicate determinations from three separate experiments. *, $p < 0.05$; **, $p < 0.01$.

Fig. 3. CDK6, Dicer1 and HIF-1 α expression in response to LPS over time and the effect of miR-107 levels on CDK6. (a) (Upper) Primary BMDM were stimulated with LPS (100 ng/ml), for the times indicated. Expression of CDK6 was measured by RT-PCR in the same manner as for PanK1 α . (Lower) Cells were also assessed for CDK6 and β -actin protein expression. R.I. is shown below. (b) (Upper) WT I-BMDM, MyD88 deficient I-BMDM and Trif deficient I-BMDM were stimulated with LPS (100 ng/ml) for 8 or 24 h and analyzed for CDK6 expression by RT-PCR. (Lower) WT MEF or p65 deficient MEF were stimulated with LPS (100 ng/ml) for the times indicated. (c) I-BMDM were stimulated with LPS (100 ng/ml), for the times indicated. Expression of (Left) Dicer1 and (Right) HIF-1 α were measured by RT-PCR. In all cases, results are expressed as mean \pm S.E.M. of triplicate determinations from three separate experiments. *, $p < 0.05$; **, $p < 0.01$.

Fig. 4. The effect of miR-107 expression on adhesion of macrophages in response to LPS. (a) (Left) I-BMDM were transfected with 50 nM anti-sense miRNA oligonucleotides and stimulated with LPS (100 ng/ml), for the times indicated. Expression of CDK6 was measured as before using RT-PCR. (Right) Cells were also assessed for CDK6 and β -actin protein expression in response to LPS for the times indicated. R.I. is shown below. (b) (Left) I-BMDM were transfected with 50 nM antisense miR-107, pre-miR-107 or control oligonucleotides and stimulated with LPS (100 ng/ml), for the times indicated. Expression of CDK6 was measured as before. (Right) Cells were also assessed for CDK6 and β -actin protein expression in response to LPS for the times indicated. R.I. is shown below. (c) CDK6 3'UTR

luciferase plasmid and CDK6 mutant 3'UTR luciferase plasmid were co-transfected with 50 nM anti-miR-107, anti-miR-control, pre-miR-107 or pre-miR-control in HEK293T cells. Luciferase activity was measured and results were normalized for TK-Renilla activity, data is shown as percent luciferase relative to control. Results are expressed as mean \pm S.E.M. of triplicate determinations from three separate experiments. (d) (Upper) Adhesion of macrophages transfected with anti-miR-107 or pre-miR-107 was assessed in response to LPS. 5×10^5 Raw264.7 were transfected with 50 nM anti-miR-107 or anti-miR-control and stimulated with LPS (100 ng/ml), for the times indicated and assessed for adhesion in real-time using the xCELLigence system. (Lower) 5×10^5 Raw264.7 were transfected with 50 nM pre-miR-107 or pre-miR-control and stimulated with LPS (100 ng/ml) and assessed for adhesion in real-time using the xCELLigence system. Data is plotted as fold change cell index, or the fold change in cell index values between LPS stimulated cells and the untreated control cells. (e) I-BMDM were transfected with 50 nM anti-sense miRNA oligonucleotides and stimulated with LPS (100 ng/ml), for 24 hours. Enzyme-linked immunosorbent assay (ELISA) of mouse TNF- α was measured. In all cases, results are expressed as mean \pm S.E.M. of quadruplicate determinations from three separate experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 5. The effect of CDK6 expression on adhesion of macrophages in response to LPS and the effect of LPS on CDK6 knockout mice. (a) Adhesion of WT and CDK6 deficient BMDM in response was assessed using the xCELLigence system. 5×10^5 CDK6 deficient BMDM or wild-type BMDM were plated in the E-plate and stimulated with LPS (100 ng/ml). (b) Wild-type (WT) control mice and CDK6-deficient (Cdk6 $-/-$) mice 8 weeks of age were injected intraperitoneally with LPS (30 mg/ml), and monitored over a period of 3 days; results are plotted as a percentage of total numbers ($n = 14$ mice per group). Kaplan-Meier analysis demonstrated a significant difference in survival between Cdk6 $-/-$ mice and WT (log-rank test Cdk6 $-/-$ versus WT; $P = 0.01$). (c) Enzyme-linked immunosorbent assay (ELISA) of mouse TNF- α in blood samples from wild-type and CDK6-deficient mice ($n = 14$ mice per group), 1 h and 4 h after LPS injection. **, $p < 0.01$; ***, $p < 0.001$. (d) Schematic illustrating the possible role of miR-107 in TLR4 signaling and on macrophage cell adhesion in response to LPS. In response to LPS, MyD88 adaptor molecules initiate a signaling cascade, which leads to the activation of the NF- κ B subunit p65 and the repression of PPAR- α . This repression leads to the decrease in PanK1 α expression and the subsequent decrease in miR-107 because of its intronic location in PanK1 α . This decrease in miR-107 releases its hold on CDK6 leading to an increase in its expression and an increase in macrophage cell adhesion.

Figure 1

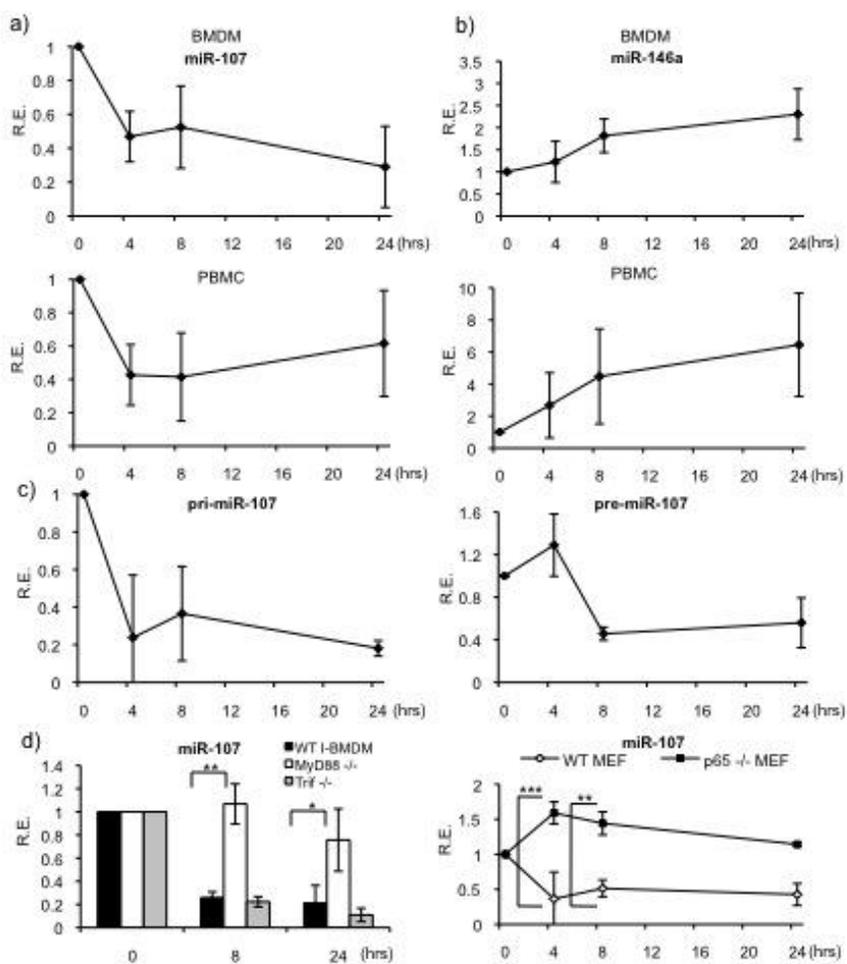


Figure 2

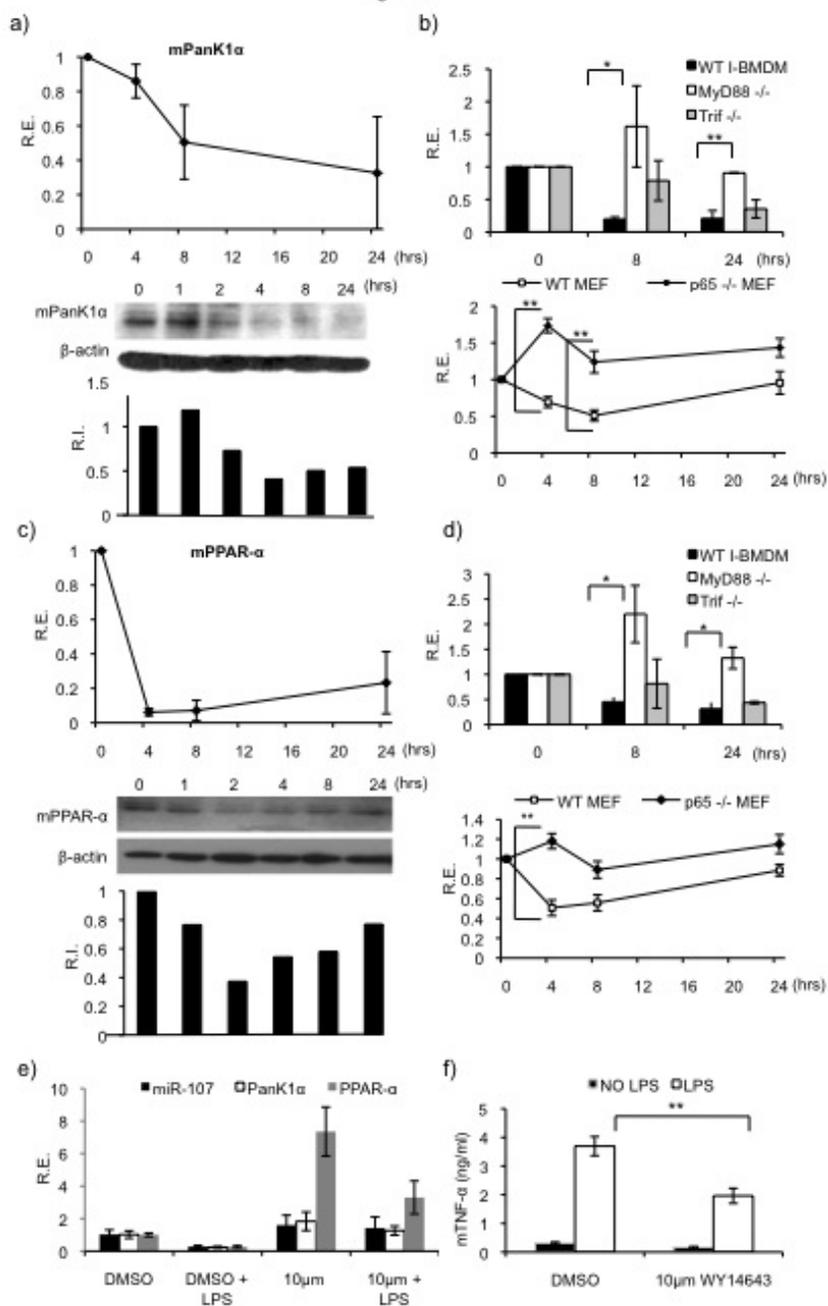


Figure 3

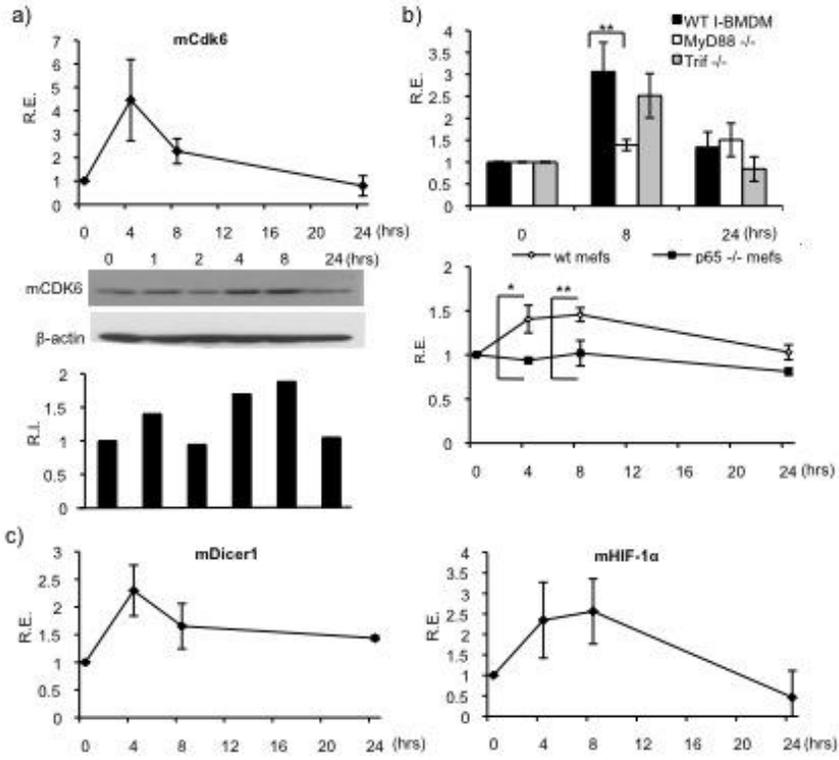


Figure 4

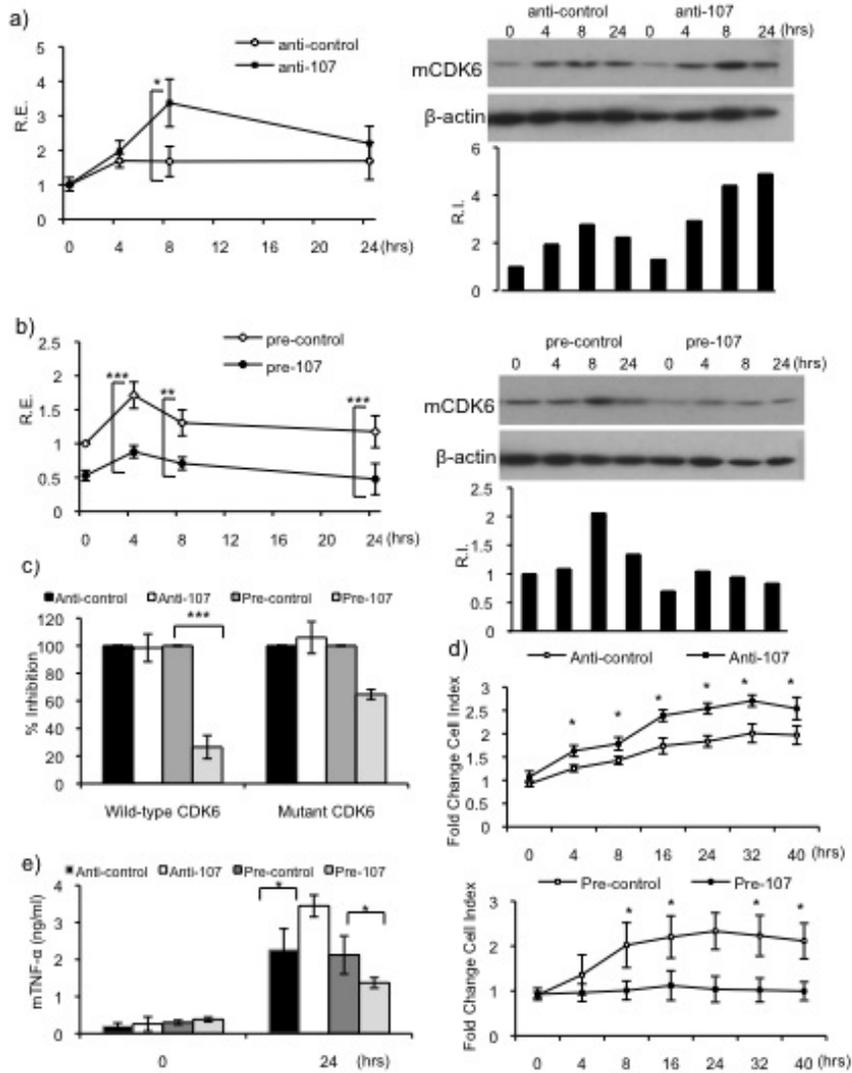


Figure 5

