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MicroRNA-146a and RBM4 form a negative feed-forward loop that disrupts cytokine mRNA translation following TLR4 responses in human THP-1 monocytes

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Abstract

Within hours after its initiation, the severe systemic inflammatory response of sepsis shifts to an adaptive anti-inflammatory state with coincident immunosuppression. This anti-inflammatory phenotype is characterized by diminished proinflammatory cytokine gene expression in response to toll-like receptor (TLR) stimulation with bacterial endotoxin/ LPS, also known as endotoxin tolerance/adaptation. Our and other studies have established that gene-specific reprogramming following TLR4 responses independently represses transcription and translation of proinflammatory genes such as TNFα. We also previously demonstrated that TNFα and IL-6 mRNA translation is repressed in endotoxin adapted THP-1 human monocytes by a miRNA-based mechanism involving the argonaute family protein Ago2. Here, we further define the molecular nature of reprogramming translation by showing that TLR4-induced microRNA-146 promotes a feed-forward loop that modifies the subcellular localization of the RNA-binding protein RBM4 and promotes its interaction with Ago2. This interaction results in assembly of a translation repressor complex that disrupts TNFα and IL-6 cytokine synthesis in endotoxin adapted THP-1 monocytes. This novel molecular path prevents phosphorylation of RBM4 on serine-309 by p38 MAPK, which leads to RBM4 accumulation in the cytosol and interaction with Ago2. We further find that microRNA-146a knockdown by antagomirs or inhibiting protein phosphatases by okadaic acid, increases p38 MAPK phosphorylation and results in RBM4 serine-309 phosphorylation and nuclear re-localization, which disrupts RBM4 and Ago2 interactions and restores TLR4-dependent synthesis of TNFα and IL-6. We conclude that miR-146a plays a diverse and critical role in limiting an excessive acute inflammatory reaction.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Keywords

Severe Systemic Inflammation; Sepsis; Endotoxin Tolerance; microRNA; RNA-binding Protein; Translational Repression

INTRODUCTION

The severe systemic inflammatory response to serious infection (e.g., sepsis) or non-infectious causes is initiated by toll-like receptor (TLR)-mediated activation of NF-kB-RelA/p65-dependent transcription and mitogen-activated protein kinases in innate immunity cells, mainly neutrophils, monocytes, and tissue macrophages[1–5]. These cells initiate the acute systemic inflammatory response by rapidly increasing production of proinflammatory mediators such as TNFα, IL-6, and IL-1β, which support early host defense against invading microbes[5–7]. However, if the initial inflammatory response is excessive, innate immunity cells will shift to an adaptive anti-inflammatory and immunosuppressive state by reprogramming gene expression[5,8,9]. While this shift may limit tissue damage caused by excessive inflammation, when sustained, it coincidentally induces profound immunosuppression, elevates the risk of recurring infection, and increases mortality; in contrast, its resolution correlates with improved immune responses and better survival[3–5]. The state of inflammation adaptation and immunosuppression affects innate and adaptive immunity and is typified by the phenomenon of endotoxin tolerance/adaptation[2,10,11]. The gold standard for defining endotoxin tolerance is diminished expression of proinflammatory genes following TLR4 re-exposure to bacterial endotoxin or LPS[12]. This inflammation phase-shift from responsive to adapted state occurs in cell models of TLR responses and in acute systemic inflammation of animals and humans with sepsis[4,10,12–14]. In healthy humans, intravenous administration of small doses of bacterial endotoxin generates tolerance to subsequent endotoxin challenge within 12 hours[15]. We have previously shown that mice administered with sublethal dose of LPS into the peritoneal cavity develop endotoxin tolerance within 3 days, as demonstrated by diminished circulating levels of proinflammatory cytokines upon subsequent intravenous challenge with LPS[16]. Peritoneal macrophages from these mice did not produce significant amounts of proinflammatory TNFα and IL-6 when challenged with LPS ex vivo.

Previous studies in adapted murine macrophages[14,17,18] and human monocytes[12] established that expression of proinflammatory cytokines like TNFα, IL-6, IL-1β, and chemokines CCL4 and CCL3 is repressed, while expression of anti-inflammatory genes like IL-10, IkBα, and IL-1Ra is sustained[19–22]. Thus, reprogramming of genes during development of tolerance/adaptation is gene specific. To study gene reprogramming, we developed a cell model of gene reprogramming using THP-1 human monocytes[23]; over the ensuing two decades, this cell model has correctly predicted reprogramming events occur in human sepsis. Through a series of studies that used this cell model and cells from human sepsis, we discovered that human systemic inflammation is repressed both at the transcriptional level by an epigenetic mechanism involving the transcription factor RelB and histone and DNA modifiers[24–27], and at the translational level by miRNA-mediated processes[18,28]. We also found that microRNA-146a (miR-146a), which is markedly induced in endotoxin adapted

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THP-1 cells, indirectly promotes assembly of a translation repressor protein complex of the argonaute family protein, Ago2[28]. This repressor complex mediates translation repression of TNFα and IL-1β[18,28]. Knockdown of miR-146a in endotoxin adapted THP-1 cells with antagomirs blocks repressor complex assembly and restores TNFα and IL-1β protein levels[28], suggesting that miRNA-based mechanisms directly or indirectly contribute to limiting translation during acute inflammation adaptation.

In this study, we sought to further elucidate the molecular events that underlie repressed translation of proinflammatory cytokines during the anti-inflammatory/adapted phase of acute inflammation. Using our THP-1 cell model of endotoxin tolerance/adaptation, we found that the RNA-binding protein RBM4 was required for translation repression of TNFα and IL-6 as it formed a translation repressor complex with Ago2. The RBM4 interactions with Ago2 are controlled by site-specific phosphorylation of RBM4 on serine-309, which also modifies the subcellular localization of RBM4. We also found that this path of disrupted translation depends on an indirect miR-146a-generated feed-forward loop that involves p38 MAPK and an unknown phosphatase.

METHODS

Cell culture

THP-1 human monocytes were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Hyclone Laboratories, Logan, UT), and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) at 37°C and 5% CO_2. Cells were made tolerant by overnight incubation with 1 μg/ml of lipopolysaccharide (LPS) from gram-negative bacteria (*E. coli*, strain 0111:B4; Sigma-Aldrich, St. Louis, MO). The LPS preparation used in these experiments is TLR4-specific and free of contaminating proteins that activate cells via a non-TLR4-dependent mechanism.

Transfections

Cells were seeded at 0.5×10^6 cells/ml one day prior to transfection. Cells were transfected by electroporation using the Bio-Rad GenePulser MXcell transfection system per the manufacturer's instructions (Bio-Rad, Hercules, CA).

For RBM4 and RelB knockdown, cells were transfected with pools of gene-specific or scrambled (control) siRNAs at 0.5 μM final concentration (Santa Cruz Biotechnology, Santa Cruz, CA).

For miR-146a knockdown, cells were transfected with negative control or miR-146a-specific antisense 2'-*O*-Me oligonucleotides (antagomirs; final concentration 100 nM) (Ambion, Austin, TX).

Protein extraction

Cytoplasmic and nuclear proteins were extracted using the NE-PER nuclear and cytoplasmic extraction kit per the manufacturer's instructions (Pierce, Rockford, IL). Immediately after

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harvesting, cells were washed in PBS and resuspended in CER1 lysis buffer with protease inhibitor cocktail and incubated on ice for 1 min. CER2 buffer was added and the incubation continued for 5 min. Supernatants (cytoplasmic proteins) were recovered by centrifugation for 5 min at 4°C and 14,000 rpm. The nuclear pellets were resuspended in NER lysis buffer with protease inhibitor cocktail and incubated for 40 min on ice with occasional vortexing. The nuclear proteins were recovered by centrifugation for 10 min at 4°C and 14,000 rpm.

For preparation of whole cell lysate, cells were lysed in 1× RIPA buffer containing 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholic acid, and 1 mM EDTA (Millipore, Temecula, CA) plus 1× protease inhibitor cocktail. Protein concentations were determined by Bradford assay (Bio-Rad).

Immunoprecipitation and western blot

Ago2 or RBM4 protein complexes were immunoprecipitated as described previously[29] with some modifications. Briefly, cytoplasmic or nuclear extracts were pre-cleared by incubation with pre-blocked protein G-agarose beads for 1 h at 4°C. Beads were pre-blocked by incubation for 1 h with 100 μg/ml of bovine serum albumin. Pre-blocked beads were washed with buffer C (250 mM sucrose, 10 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 30 U/ml RNase inhibitor, and 1× protease inhibitor cocktail). Protein extract was then centrifuged at 2,000 rpm for 5 min and supernatant (900 μl) was added to 100 μl of pre-blocked protein G-agarose beads that were coated with 10 μl antibody against human Ago2 (clone #4G8; Wako, Richmond, VA), RBM4, or IgG isotype control (Santa Cruz Biotechnology). After overnight rotation at 4°C, the beads were centrifuged and washed three times with buffer C. Aliquots of bound protein complexes were used for protein analysis as described below. The RBM4 antibody against phospho-serine-309 was custom-generated by the Vanderbilt University Antibody and Protein Resources Center.

For western blot analysis, equal amounts of protein extracts, or Ago2- or RBM4-immunoprecipitated protein complexes were mixed with 5× Laemmeli sample buffer, resolved by gradient SDS- 10% polyacrylamide gel (Bio-Rad), transferred to nitrocellulose membranes (Pierce), and incubated with primary antibodies diluted in 10% skim milk in Tris-buffered saline/Tween 20. After washing, membranes were incubated with secondary antibody conjugated to horseradish peroxidase. Proteins were visualized with the ECL detection system (Pierce). Membranes were striped and reprobed with actin or nucleoporin antibody as a control.

Real-time qPCR

The mRNA levels of TNFα, IL-6 or RelB and RBM4 (for knockdown experiments) were measured using TaqMan gene-specific primer/probe sets (Qiagen, Valencia, CA) as described previously[10]. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). To detect levels of TNFα, IL-6, or IkBα mRNA enrichment in the RBM4 protein complex, RBM4 immunoprecipitates were treated with DNase at 37°C for 15 min and subsequently with proteinase K (35 μg/ml) for 15 min at 37°C before RNA was purified by TRIzol reagent and analyzed by PCR using primers specific to the 3' UTR.

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The MiR-146a levels were determined using first strand synthesis and the miScript II RT kit according to the manufacturer's instructions (Qiagen). The reverse transcription (RT) reaction consisted of 4 μl of polyadenylated miRNA, 3 μl of 25 μM universal RT primer and 1 μl annealing buffer. The reaction was incubated at 65°C for 5 min followed by the addition of 10 μl of 2× first-strand synthesis reaction mix containing dNTP, 2 μl of SuperScript III RT/RNase out enzyme mix. The reaction (20 μl) was then incubated at 50°C for 50 min, followed by 85°C for 5 min to stop the reaction. The real-time PCR reaction consisted of 5 μl of 1:10 dilution of the RT product, 1 μl of 10 μM universal reverse primer, 1 μl of 10 μM miR-146a-specific forward primer (an oligonucleotide identical to the entire mature miRNA sequence, in which U is replaced with T) and 25 μl of SYBR Green Fluor qPCR Mastermix (Qiagen). PCR was run in duplicate at 95°C for 10 min followed by 40 cycles at 95°C for 15s and 60°C for 1 min using iCycler iQ5 detection system (Bio-Rad). The relative expression of miR-146a was calculated using the $2^{-\ \ Ct}$ cycle threshold method after normalization to the endogenous U6 small RNA (as an internal control).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to determine the levels of TNFα and IL-6 proteins using commercially available kits according to the manufacturer`s instructions (eBioscience, San Diego, CA). Samples were assayed in duplicate.

Statistical analysis

Data were analyzed with Microsoft Excel. Differences between two groups were analyzed by unpaired student's t-test. Data are presented as mean (± s.d.). Significance is reported for values of p 0.05.

RESULTS

RBM4 is essential for assembling a translation repressor complex in endotoxin adapted cells and its knockdown disrupts the complex

We previously reported that the posttranscriptional regulation of TNFα in endotoxin adapted THP-1 cells is controlled by mRNA decay and translation repression[18]. We further demonstrated that the small RNA-binding proteins TTP, AUF1, and TIAR were detected at the TNFα 3' UTR in the cytosol of adapted cells after LPS stimulation[18]. These proteins coupled with miR-221, miR-579, and miR-125b, which specifically recognized TNFα 3' UTR sequence, promoted mRNA decay and repressed its translation. Significantly, TNFα mRNA, along with these small proteins and the specific miRNAs, were detected in the cytosol of adapted cells as part of a large protein complex immunoprecipitated with Ago2 antibody; herein referred to the "translation repressor complex"[28]. This protein complex also contained the RNA-binding motif protein 4 (RBM4), whose knockdown restored TNFα protein levels in adapted cells[28]. Of note, this translational repressor complex was not detected at the IkBα mRNA, which is expressed normally in adapted cells[28], suggesting that this complex assembles only at the translationally repressed mRNAs.

While Ago2 is ubiquitously expressed and considered a core component of the translation repressor complex (also known as miRNA-induced silencing complex or miRISC), but the

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protein co-factors in this repressor complex determine its role in decay and/or translational repression of target mRNAs[30,31]. Recruitment of this translation repressor complex to the targeted mRNA is mediated by specific miRNA(s) that recognize specific sequences in the 3' UTR of the targeted mRNA. In the case of TNFα, these included miR-221, miR-579, and miR-125b[18]. RBM4, on the other hand, was recently identified as a co-factor of the Ago2 protein complex in HEK293 cells[30]. We hypothesized that RBM4 may couple with Ago2 to form a translation repressor complex to repress proinflammatory cytokine synthesis in the endotoxin adapted cells. To further characterize this repressor complex, first we examined whether Ago2 or RBM4 associate directly with TTP, AUF1, and TIAR that we detected at the TNFα 3' UTR[18]. Cytoplasmic proteins from adapted cells were immunoprecipitated with Ago2 or RBM4 antibody, and then immunoblotted with the indicated antibodies (Fig. 1). TTP, AUF1, and TIAR were detected both in Ago2 and RBM4 immunoprecipitates at 1 hr after LPS stimulation, concurrent with Ago2-RBM4 interaction (Fig. 1B). Interestingly, these complexes were detected after LPS stimulation. These results support our hypothesis that Ago2 and RBM4 assemble the translation repressor complex that represses TNFα protein synthesis in endotoxin adapted THP-1 cells and that a TLR4-mediated signaling path controls assembly of this complex.

Ago2 is constitutively present in the nucleus and cytosol in adapted cells, whereas RBM4 is restricted to the cytosol and interacts with Ago2 after TLR4 stimulation

To delineate the kinetic of the Ago2-RBM4 complex assembly during the transition of THP-1 cells from the responsive to the adapted state, we measured Ago2 and RBM4 nucleo-cytoplasmic localization and interactions in responsive and adapted cells before and after TLR4 stimulation. Western blot analysis showed that Ago2 was equally distributed between the nucleus and cytosol in both responsive and adapted cells, and that LPS stimulation did not affect this pattern (Fig. 2A). Interestingly, we detected RBM4 in the nucleus and cytosol in responsive cells after LPS stimulation but RBM4 was only in the cytosol in adapted cells (Fig. 2A). Co-immunoprecipitation followed by immunoblotting showed that Ago2 and RBM4 interacted in the cytosol in adapted cells only after TLR4 simulation (Fig. 2B). This Ago2-RBM4 protein complex was observed, albeit at much lower level, as early as 30 min after LPS stimulation (data not shown). We also found that translationally repressed TNFα and IL-6 but not the translationally active IkBα mRNAs were enriched in RBM4 protein complex (Fig. 2C). These results demonstrate that Ago2 and RBM4 interact and form a translation repressor complex at the proinflammatory mRNAs in endotoxin adapted THP-1 cells and that formation of this complex is induced by TLR4-mediated signaling.

RBM4 phosphorylation status controls its localization and interaction with Ago2

A previous study showed that RBM4 is phosphorylated on serine-309 by p38 MAPK in Hela cell during cell stress induced by arsenite exposure treatment, and that this phosphorylation promoted RBM4 shuttling between the nucleus and cytosol[32]. We hypothesized that the signal that promotes RBM4 translocation from the nucleus to the cytosol after LPS stimulation in responsive THP-1 cells may be disrupted in adapted cells and thus be responsible for its retention in the cytosol and interaction with Ago2. In addition, p38 MAPK activation (phosphorylation) is inhibited within 1 hr of LPS stimulation in murine macrophage[33]. MAPKs, including p38, are inactativated (dephosphorylated) by

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dual specificity protein phosphatases[34]. To determine if retention of RBM4 in the cytosol of adapted cells is mediated by a phosphatase activity, we measured RBM4 levels in the nucleus and cytosol after pretreatment with the general protein phosphatase inhibitor okadaic acid. As shown in Fig. 3A, upon LPS stimulation in adapted cells pre-treated with okadaic acid, the level of unphosphorylated RBM4 was markedly reduced in the cytosol. Simultaneously, Ago2-RBM4 protein interaction in the cytosol was diminished, as shown by co-immunoprecipitation using cytoplsmic extract from adapted cells (Fig. 3B). Next, we examined whether protein phosphatase inhibition induced RBM4 relocalization by changing its phosphorylation status. Using an RBM4 antibody that recognizes phospho serine-309, we found that the RBM4 retained in the cytosol of adapted cells was mainly an unphosphorylated form (Fig. 3C). Significantly, protein phosphatase inhibition promoted RBM4 phosphorylation after LPS stimulation, and this phosphorylated form was localized equally in the nucleus and cytosol. Most important, LPS stimulation after protein phosphatase inhibition restored TNFα protein near to its normal levels in responsive cells (FIG. 3D). Together, these results clearly demonstrate that RBM4 phosphorylation regulates its nucleo-cytoplasmic localization and that its dephosphorylation is responsible for its retention in the cytosol in adapted cells and thus its interaction with Ago2 and repression of TNFα protein synthesis.

MiR-146a knockdown promotes RBM4 phosphorylation and restores proinflammatory protein synthesis

We[28] and others[35,36] have reported that miR-146a expression is induced and sustained in THP-1 cells following TLR4 stimulation by LPS. MiR-146a directly targets and inhibits expression of the signaling proteins IRAK1 and TRAF6 downstream of TLR activation[36]. In addition, we have shown that miR-146a knock down in adapted cells, similar to the protein phosphatase inhibition by okadaic acid (Fig. 3), cells restores TNFα protein levels[28]. Given that RBM4 phosphorylation is induced by p38 MAPK signal[32], we hypothesized that miR-146a may disrupt RBM4 phosphorylation in adapted cells, resulting in its retention in the cytosol and interaction with Ago2. To test this hypothesis, we knocked down miR-146a in adapted cells simultaneously with RelB, which restores proinflammatory gene transcription (mRNA) but not protein synthesis[28]. Note that, in the presence of miR-146a, the TNFα mRNA transcribed after RelB knock down is rapidly degraded[28]. Levels of RelB and miR-146a were reduced by more than 80% after their knockdown (Fig. 5A and data not shown). In the absence of miR-146a, LPS stimulation for 1 hr induced RBM4 phosphorylation on serine-309, and this resulted in RBM4 re-shuttling between the nucleus and cytosol (Fig. 4A). In a separate co-immunoprecipitation experiment, we found that anti-RBM4 antibody did not cross-react with the phosphorylated RBM4 and, reciprocally, anti-phospho serine-309 RBM4 antibody did not interact with RBM4 (data not shown). This result suggests that the RBM4 detected in the nucleus and cytosol of adapted cells after miR-146a knockdown is phosphorylated RBM4. Concurrent with promoting RBM4 phosphorylation, miR-146a knockdown also restored TNFα and IL-6 protein levels after LPS stimulation (Fig. 4B). Together, these results demonstrate that miR-146a disrupts RBM4 phosphorylation in adapted cells and thus prevents the translation repressor complex assembly.

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MiR-146a inhibits p38 activation and its knockdown restores p38 activation and disrupts Ago2-RBM4 protein interaction

Since miR-146a inhibits signaling proteins leading to MAPK activation[36], we examined whether miR-146a prevents RBM4 phosphorylation by inhibiting p38 MAPK activation. Western blot, using protein extracts from adapted cells with scrambled anti-miRNA (control) knockdown, detected total but not active (phosphorylated) p38, even after LPS stimulation (Fig. 5A). With miR-146a knockdown, phosphorylated p38 was detected 1 hr after LPS stimulation. Importantly, miR-146a knockdown resulted in disruption of Ago2-RBM4 protein complex in the cytosol of adapted cells (Fig. 5B). The disruption of this complex was not due to absence of RBM4, because RBM4 was detected in the cytosol after miR-146a knockdown; but was in the phosphorylated form (Fig. 4A). In addition, western blot analysis revealed that IRAK1 and TRAF6, which are required for p38 MAPK activation and are inhibited by miR-146a[35,36], protein levels were restored after LPS stimulation in adapted cells lacking miR-146a (Fig. 5C). Taken together, the results presented in Figs. 4 and 5 demonstrate that miR-146a prevents RBM4 phosphorylation in endotoxin adapted THP-1 cells through inhibiting p38 MAPK activation, which promotes RBM4 retention in the cytosol and its interaction with Ago2 and thus assembling the translation repressor complex.

DISCUSSION

Both transcriptional and translational processes are differentially regulated by TLR4-dependent mechanisms, which reprogram and silence inflammatory gene expression during the severe inflammation phase shift from initiation to adaptation and immunosuppression[8,25,28]. Reactivation of transcription and translation in the endotoxin adapted THP-1 cell model of severe systemic inflammation restores endotoxin responsiveness and reconstitutes immune reactivity[28]; which correlates with inflammation resolution and survival[4,8]. This study shows that RNA-binding proteins couple with TLR4-dependent signaling to assemble a multiprotein repressor complex and specifically repress translation of inflammatory gene mRNAs in the endotoxin adapted THP-1 cells. We further show that RNA-binding protein RBM4 plays a central role in assembling the repressor complex that disrupts translation of proinflammatory TNFα and IL-6 mRNAs. To further define the molecular pathway, we show that miR-146a modifies RBM4 protein to promote the repressor complex assembly by a path that inhibits TLR4 signaling to p38 MAPK activation. Based on these results, we propose a model (Fig. 6) of miR-146a and RBM4 as critical components of the negative feedback mechanism modulating TLR4 signaling to proinflammatory protein synthesis during inflammation adaptation. In this model, prolonged stimulation of TLR4 in responsive/normal monocytes/macrophages with bacterial endotoxin induces miR-146a which by directly targeting IRAK1 and TRAF6 limits p38 MAPK activation. Results with okadaic acid support contribution of a MAPK phosphatase. In these now adapted cells, RBM4 is not phosphorylated by its regulator, p38 MAPK, and thus is retained in the cytosol where it binds to Ago2 to assemble the translation repressor complex at the proinflammatory gene mRNAs. This inhibits their protein synthesis. During inflammation initiation, i.e., in responsive cells, RBM4 is phosphorylated and shuttles between the nucleus and cytosol. This phosphorylated form is not able to interact with

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Ago2, and thus cannot assemble the translation repressor complex. We conclude that the TLR4-dependent miRNA-based translation repression mechanism, in addition to transcription silencing by RelB, promotes inflammation adaptation and thus immunosuppression during the late phase of severe systemic inflammation. Importantly, we show that this process is reversible by blocking the signal that modifies RBM4 protein.

Assembly of the translation repressor complex (or miRISC) by Ago2 and protein co-factors is the first and most critical step in the pathway of miRNA-guided translation repression[30,31,37]. Although there are four Ago proteins implicated in mRNA metabolism, only human Ago2 possesses endoribonuclease activity[37] and has been detected in most translation repressor complexes as a key component of the translation repression machinery[30,31]. Ago2 provides a platform for RNA binding of gene-specific miRNAs and small RNA-binding proteins as well as protein co-factors[30,31,37]. The first two components are specific to each target mRNA and help guide the translation repressor complex to that target, whereas the latter regulates the complex activity and its recruitment/deposition into cytoplasmic granules known as processing bodies (p-bodies) where translation repression takes place[31,38,39]. A recent study has reported that phosphorylation of Ago2 on serine-387, induced by treatment with sodium arsenite, increases Ago2 localization in the cytosol of HEK-293 cells[40]. We found that Ago2 was ubiquitously expressed and equally distributed in the nucleus and cytosol of endotoxin responsive and adapted THP-1 cells. In addition, we did not detect changes in Ago2 localization in adapted cells after okadaic acid treatment or miR-146a knockdown (data not shown) These results indicate that Ago2, while required for the repressor complex assembly, is not directly modified during transition from inflammation initiation to adaptation. Thus, the Ago2 partnership with RBM4 may determine when and how the complex is assembled and recruited (with the targeted mRNA) to p-bodies.

In endotoxin responsive cells, RBM4 localization was restricted and appeared in the cytosol after LPS stimulation, yet did not interact with Ago2 (Fig. 1B). Interestingly, in adapted cells RBM4 was restricted to the cytosol and interacted with Ago2 after LPS stimulation. This supports the hypothesis that RBM4 localization and assembling of the translation repressor complex is modulated by TLR4-dependent signaling. Our previous study showed that RBM4 co-immunoprecipitates with the small RNA-binding proteins TTP, AUF1 and TIAR as well as with the trio of miRNAs that specifically repress TNFα protein synthesis in adapted cells[18]. Importantly, association of RBM4 with these TNFα mRNA-specific proteins and miRNAs as well as with Ago2 and their presence at the TNFα 3' UTR was detected only after LPS stimulation in adapted cells. This association was not observed after RBM4 knockdown (Fig. 1), which also restores TNFα protein levels[28]. In addition to TNFα, IL-6 but not the translationally active IkBα was detected in RBM4 complex. Thus, RBM4 is critical for assembling the translation repressor complex that represses proinflammatory protein synthesis during inflammation adaptation.

RBM4, along with RNA-binding proteins implicated in mRNA metabolism, has been detected with Ago2 protein in human cells by co-immunoprecipitation assays[30]. Recently, Lin et al[32] have shown that RBM4 is phosphorylated on serine-309 by a p38 MAPK signal in Hela cells following exposure to sodium arsenite. They further showed that although

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RBM4 phosphorylation induced its relocalization to the cytosol, its interaction with Ago2 was independent of RBM4 phosphorylation and that RBM4 facilitated recruitment of Ago2 protein with the associated target mRNA to cytoplasmic granules for translation repression during mouse C2C12 myoblast differentiation[41]. We detected RBM4 both in the nucleus and cytosol following LPS stimulation in adapted cells pre-treated with okadaic acid. Under this condition, RBM4 in both compartments was phosphorylated on serine-309. In contrast, cells without prior treatment with okadaic acid retained RBM4 in the cytosol in the unphosphorylated form (Fig. 3C). Thus, a protein phosphatase activity in adapted cells blocks RBM4 phosphorylation. Inhibiting this phosphatase activity promotes RBM4 phosphorylation and shuttling between the nucleus and cytosol, as observed in responsive cells (Fig. 2). RBM4 phosphorylation also disrupted the translation repressor complex assembly, because phospho serine-309 RBM4 could not interact with Ago2 despite the presence of both proteins in the cytosol. Importantly, RBM4 phosphorylation and disruption of its interaction with Ago2 restored TNFα protein levels. Based on these results, we conclude that RBM4 phosphorylation prevents translation repression of proinflammatory genes in response to TLR4 simulation. This process is dysregulated in adapted cells through induction of a protein phosphatse activity downstream of TLR4.

We have previously shown that miR-146a plays a critical role in translation repression of proinflammatory genes in endotoxin adapted THP-1 cells, where miR-146a knockdown restored TNFα and IL-6 protein levels[28]. The current study shows that miR-146a knockdown in endotoxin adapted cells, as with okadaic acid pre-treatment, promotes RBM4 phosphorylation, disrupts Ago2-RBM4 interaction, and importantly restores TNFα and IL-6 protein levels. MiR-146a induction upon TLR4 activation downregulates TLR4-dependent signaling leading to NF-kB and MAP kinase activation by downregulating the signaling proteins IRAK1 and TRAF6, and this effect is considered to be a negative feedback mechanism limiting overactivation of innate immunity cells and thus minimizing tissue damage caused by excess inflammation[36]. We found IRAK1 and TRAF6 protein levels were markedly increased after LPS stimulation in adapted cells lacking miR-146a. It should be noted that transcription of proinflammatory genes is also repressed in endotoxin adapted cells by a RelB-dependent mechanism[28]. Therefore, transcription must be restored first by RelB knockdown. However, restored mRNAs are rapidly degraded due to the translational repression mechanism[28]. So, in our experiments RelB and miR-146a were knocked down simultaneously, to restore transcription and translation, respectively.

MAP kinases, including p38 MAPK, are activated downstream of IRAK1 and TRAF6 following TLR stimulation with various ligands and induce inflammatory protein synthesis[33,42]. P38 MAPK activation/phosphorylation is induced shortly after LPS stimulation in monocytes/macrophages, followed by its inactivation within 1 hour[33]. As expected, we did not detected phospho (active) p38 MAPK in endotoxin adapted cells. Interestingly, miR-146a knockdown resulted in p38 MAPK re-activation after LPS stimulation. Of note, p38 MAPK activation resulted in RBM4 phosphorylation and shuttling between the nucleus and cytosol and disruption of the Ago2-RBM4 interaction. A previous study reported that p38 MAPK induces RBM4 phosphorylation at serine-309 and relocalization from the nucleus to the cytosol in Hela cells exposed to sodium arsenite[32]. Another study has shown that RBM4 is phosphorylated during muscle cell differentiation[41].

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In that study, phosphorylated RBM4 accumulated in the cytosol and co-localized with Ago2 during the differentiation of mouse C2C12 myoblasts, and Ago2-RBM4 interaction was independent of RBM4 phosphorylation. Thus, our results suggest that inactivation of p38 MAPK downstream of miR-146a plays leads to the accumulation of unphosphorylated RBM4 in the cytosol of adapted cells, which interacts with Ago2. We observed a similar pattern of RBM4 phosphorylation and localization in adapted cells both after protein phosphatase inhibition and miR-146a knockdown. It is unclear from these experiments, however, whether lack of RBM4 phosphorylation in adapted cells is due to inactivation of p38 MAPK by a protein phosphatase or is caused by direct dephosphorylation of RBM4 by a protein phosphatase. MAPKs are inactivated/dephosphorylated by a group of dual specificity MAPK protein phosphatases that dephosphorylate tyrosine and threonine residues critical for MAPK activation[34]. Of note, the dual specificity MAPK phosphatase-1 (MKP-1) is a negative regulator of innate immune response[33], where it restrains proinflammatory cytokine protein synthesis in LPS-stimulated macrophages by inactivating p38 MAPK[42]. Our results showed that RBM4 was phosphorylated on serine-309. Given that MAPK phosphatases dephosphorylate tyrosine and threonine residues[34], it is possible that a MAPK phosphatase like MKP-1 does not directly dephosphorylate RBM4 in adapted cells but instead prevents RBM4 phosphorylation by inactivating/dephosphorylating p38 MAPK on tyrosine and/or threonine. We observed that pretreatment of adapted cells with triptolide, which inhibits MKP-1 expression[42], restored TNFα protein levels after LPS stimulation (data not shown). MKP-1 may be induced in adapted cells due to disruption of the signaling proteins IRAK1 and TRAF6 by miR-146a.

In summary, TLR4-mediated translational repression in endotoxin adapted cells represses proinflammatory protein synthesis, and thus promotes inflammation adaptation with immunosuppression. This mechanism is triggered and sustained by TLR4-mediated events. Our study provides the first evidence that RBM4 is a critical regulator of inflammation adaptation through its interaction with Ago2 and the assembling of a translation repressor complex. This action by RBM4 is function-specific as it targets only proinflammatory gene products. Our study also supports the role of miR-146a as a negative feedback regulator of TLR4-dependent inflammatory response. By disrupting TLR4 signaling and inactivating p38 MAPK, miR-146a promotes Ago2-RBM4 interactions and thus disrupts proinflammatory protein synthesis. Importantly, our study shows that this process is reversible. These findings extend the regulatory role of miRNA-dependent translational repression and suggest miR-146a as an attractive therapeutic target for the treatment of endotoxin tolerance and inflammation adaptation with immunosupression; a prominent feature during sepsis.

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ABBREVIATIONS

TLR	toll-like receptor

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LPS	lipopolysaccharide
TNFα	tumor necrosis factor alpha
IL	interleukin
miR	microRNA
miRISC	miRNA-induced silencing complex
Ago2	argonaute 2
RBM4	RNA-binding motif protein 4
MAPK	mitogen-activated protein kinase
siRNA	small interfering RNA

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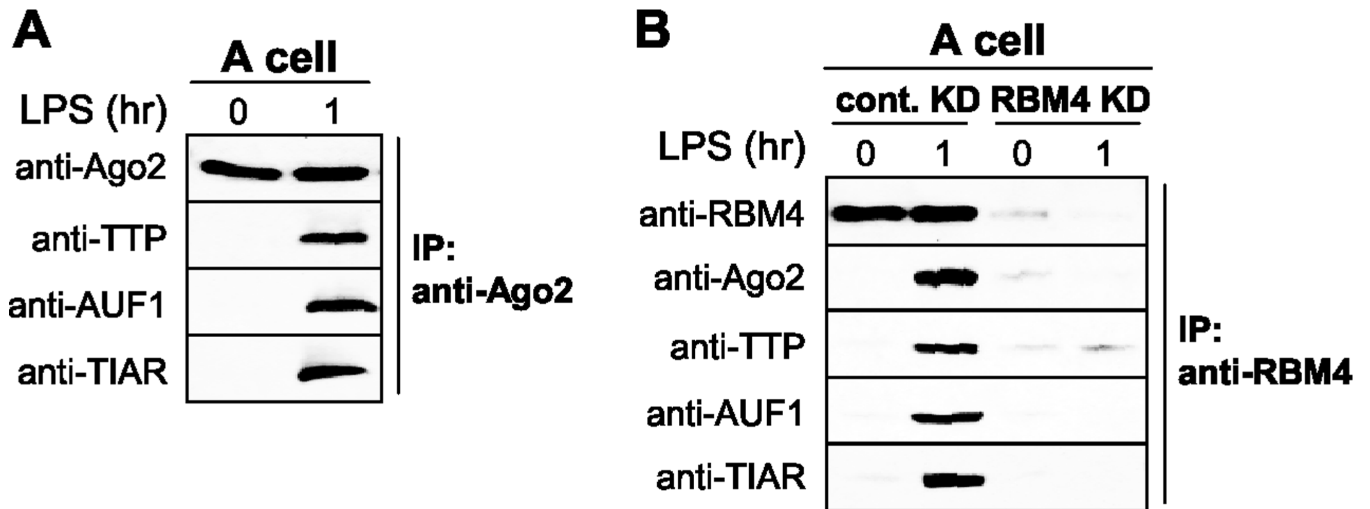

Figure 1. RBM4 protein colocalizes with Ago2 and the RNA-binding protein TTP, AUF1 and TIAR in adapted cells

Responsive (normal) THP-1 cells were made tolerant (adapted) by pretreatment overnight with 1 μg/ml of the gram-negative bacterial LPS. (**a**) Ago2 associates with TTP, AUF1, and TIAR. Adapted cells were left unstimulated or stimulated for 1 hr with 1 μg/ml of LPS. Cytoplasmic proteins were extracted and immunoprecipitated (IP) with antibody against Ago2 or IgG isotype. The Ago2 complexes were resolved by SDS- 10% PAGE and then immunoblotted with antibody against Ago2, TTP, AUF1, or TIAR. (**b**) RBM4 forms the translation repressor complex with Ago2 and associated proteins and its knock down disrupts the repressor assembly. Adapted cells were transfected with a pool of RBM4-specific siRNAs or scrambled siRNAs (control KD). After 36 hr, cells were left unstimulated or stimulated for 1 hr with 1 μg/ml of LPS. Cytoplasmic proteins were extracted and immunoprecipitated with antibody against RBM4 or IgG isotype. The immunoprecipitated complexes were resolved and immunoblotted with antibody against RBM4, Ago2, TTP, AUF1, or TIAR. IgG IP sample showed background bindings (not shown). Note the protein complexes assemble only after LPS stimulation. The results represent three independent experiments. A, adapted/tolerant cell; KD, knockdown; IP, immunoprecipitated.

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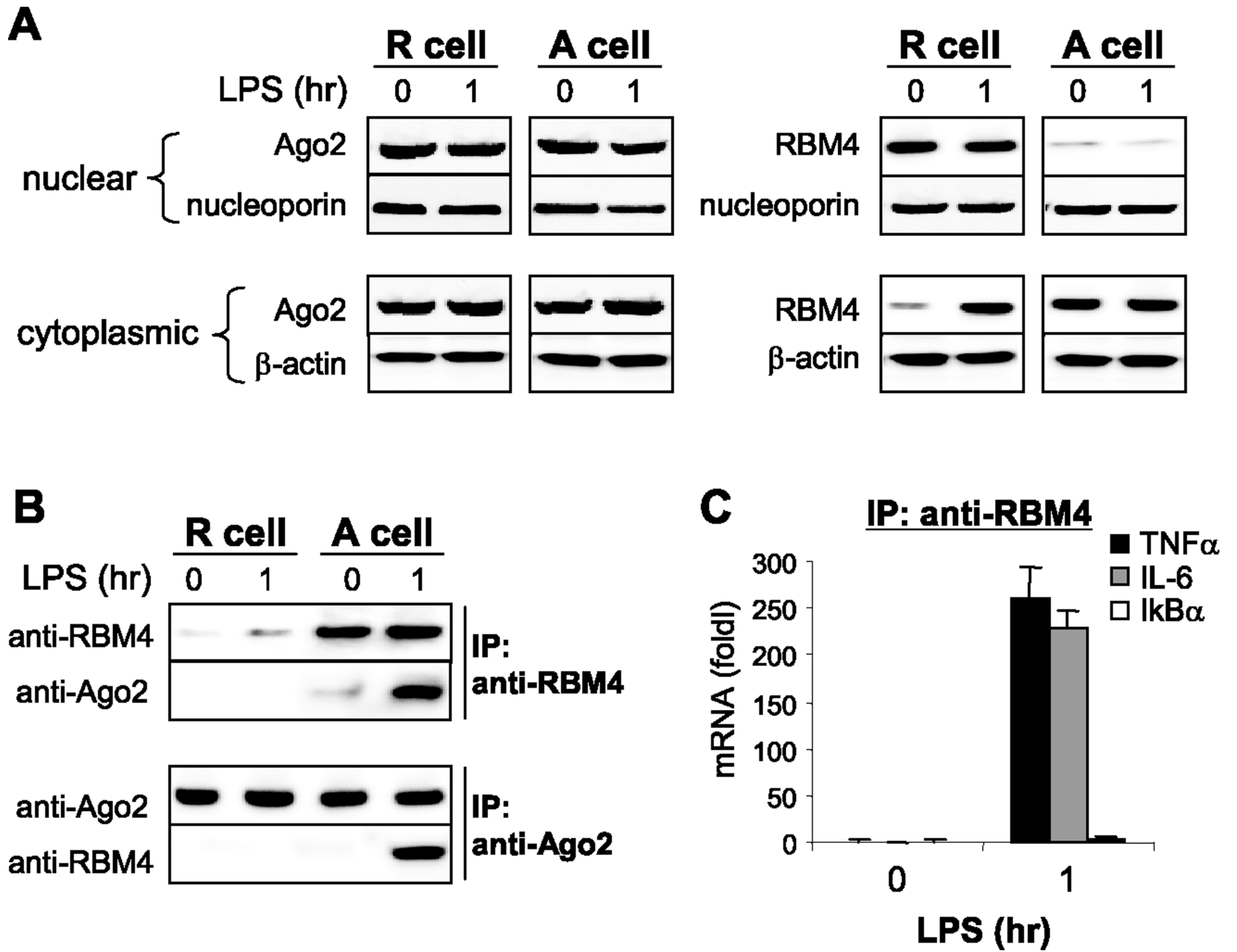

Figure 2. Ago2 is constitutively present in the nucleus and cytosol both in responsive and adapted cells, whereas RBM4 is restricted to the cytosol in adapted cells

(**a**) Adapted THP-1 cells were made by pre-treatment overnight with 1 μg/ml of LPS. Adapted and responsive (normal) cells were stimulated for 1 hr with 1 μg/ml of LPS. Nuclear and cytoplasmic proteins were extracted and immunoblotted with Ago2 or RBM4 antibody. Membranes were re-probed with nucleoporin or actin antibody as a control. (**b**) RBM4 is restricted to the cytosol in adapted but not responsive cells and forms a complex with Ago2 after LPS stimulation. Cytoplasmic proteins were immunoprecipitated with antibody against RBM4 or IgG isotype, resolved by SDS- 10% PAGE and then immunoblotted with RBM4 or Ago2 antibody. (**c**) TNFα and IL-6, but not IkBα, mRNAs are enriched in RBM4 immunoprecipitate after LPS stimulation in adapted cells. RNA was extracted from RBM4 IP and analyzed by RT-PCR using primers specific for the 3' UTRs. Sample data were normalized to GAPDH RNA (extracted before immunoprecipitation) and are presented as mean ± s.d. relative to cells without stimulation (set at 1-fold). The results represent three independent experiments. R, responsive; A, adapted; IP, immunoprecipitated.

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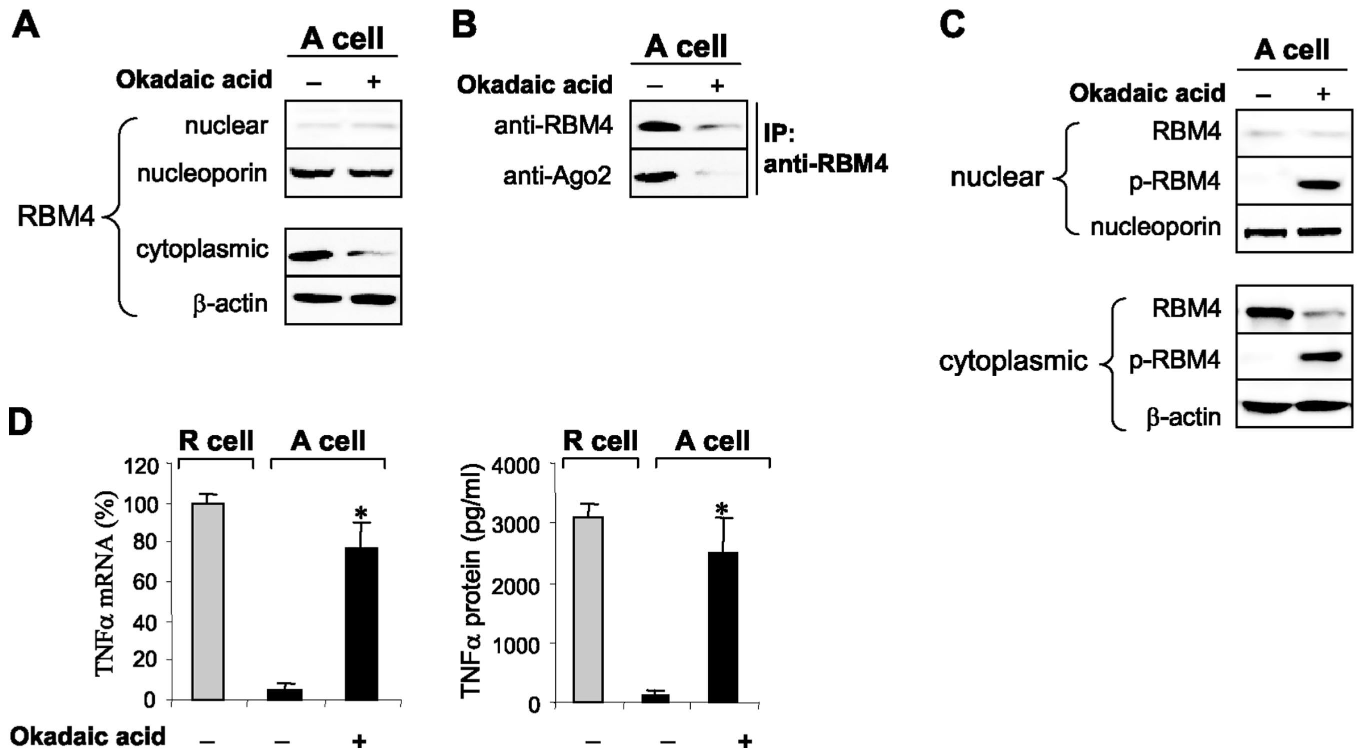

Figure 3. Protein phosphatase inhibitor disrupts RBM4-Ago2 interaction and restores TNFα protein levels in adapted cells

(**a**) Okadaic acid treatment re-localizes RBM4 from the cytosol to the nucleus of adapted cells after LPS stimulation. Adapted THP-1 cells were made by pre-treatment overnight with 1 μg/ml of LPS. Adapted cells were treated with okadaic acid for 6 hr and then stimulated for 1 hr with 1 μg/ml of LPS. RBM4 protein levels in the nuclear and cytoplasmic extracts were analyzed by immunoblotting. (**b**) Okadaic acid treatment disrupts RBM4-Ago2 protein complex. Adapted cells were treated as in *a*. Cytoplasmic proteins were extracted, immunoprecipitated with antibody against RBM4, resolved by SDS- 10% PAGE, and then immunoblotted with RBM4 or Ago2 antibody. (**c**) RBM4 is phosphorylated after okadaic acid treatment and shuttles between the nucleus and cytosol. Cells were treated as in *A*. Levels of un-phosphorylated and phosphorylated RBM4 in the nucleus and cytopsol were measured by immunoblotting. (**d**) Okadaic acid treatment restores TNFα protein levels in adapted cells. Adapted ***c***ells were transfected with control or RelB siRNA (to reverse transcription silencing). After 36 hr, adapted cells were incubated for 6 hr without or with 100 nM okadaic acid (which inhibits protein phosphatase activity) or DMSO (–), followed by adding 1 μg/ml of LPS. TNFα mRNA and protein levels were analyzed after 1 hr and 4 hr in LPS, respectively. TNFα mRNA levels were normalized to GAPDH expression and are presented relative to responsive cells, as a reference (set at 100%). Data are mean ± s.d. of three assays. *, $p < 0.05$ compared with A cell without okadaic acid treatment. The results represent three independent experiments. R, responsive; A, adapted; IP, immunoprecipitated.

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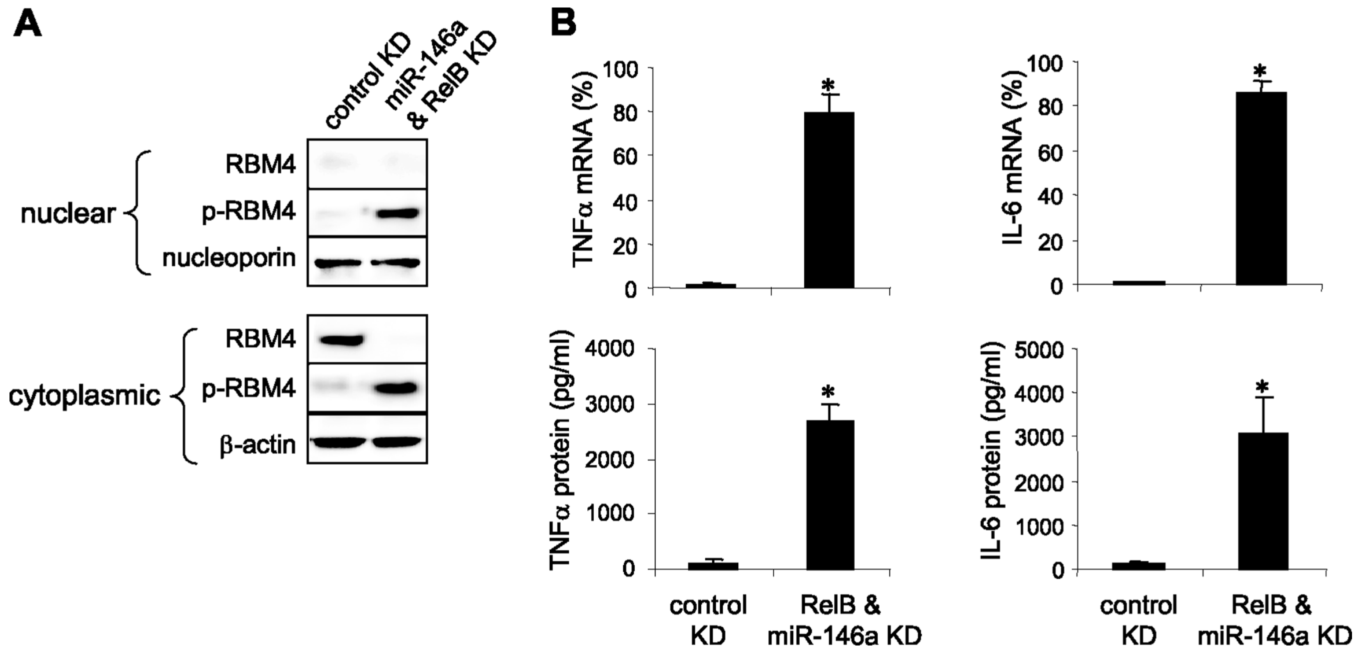

Figure 4. MiR-146a knock down in adapted cells leads to RBM4 phosphorylation and restores TNFα and IL-6 protein levels

Adapted cells were made by pre-treatment for overnight with 1 μg/ml of LPS. Adapted cells were transfected with a pool of RelB-specific siRNAs plus anti-miR-146a-specific oligonucleotides (antagomirs) or scrambled siRNAs plus anti-miRNA oligonucleotides (control KD). After 36 hr, cells were washed and stimulated for 0–4 hr with 1 μg/ml of LPS. (**a**) Nuclear and cytoplasmic proteins were extracted after 1 hr and immunoblotted with antibody against total or phospho serine-309 RBM4. (**b**) After 1 hr in LPS, RNA was extracted and analyzed by PCR. TNFα and IL-6 mRNA levels were normalized to GAPDH expression and are presented relative to the control KD (set at 1%). After 4 hr in LPS, supernatants and cells were harvested and protein levels were measured by ELISA. Samples were run in duplicate. Note, RelB knockdown alone restores mRNA level but not protein synthesis (Ref # 28). Data are the mean ± s.d. of three experiments. *, $p < 0.05$. R, responsive; A, adapted; KD, knock down.

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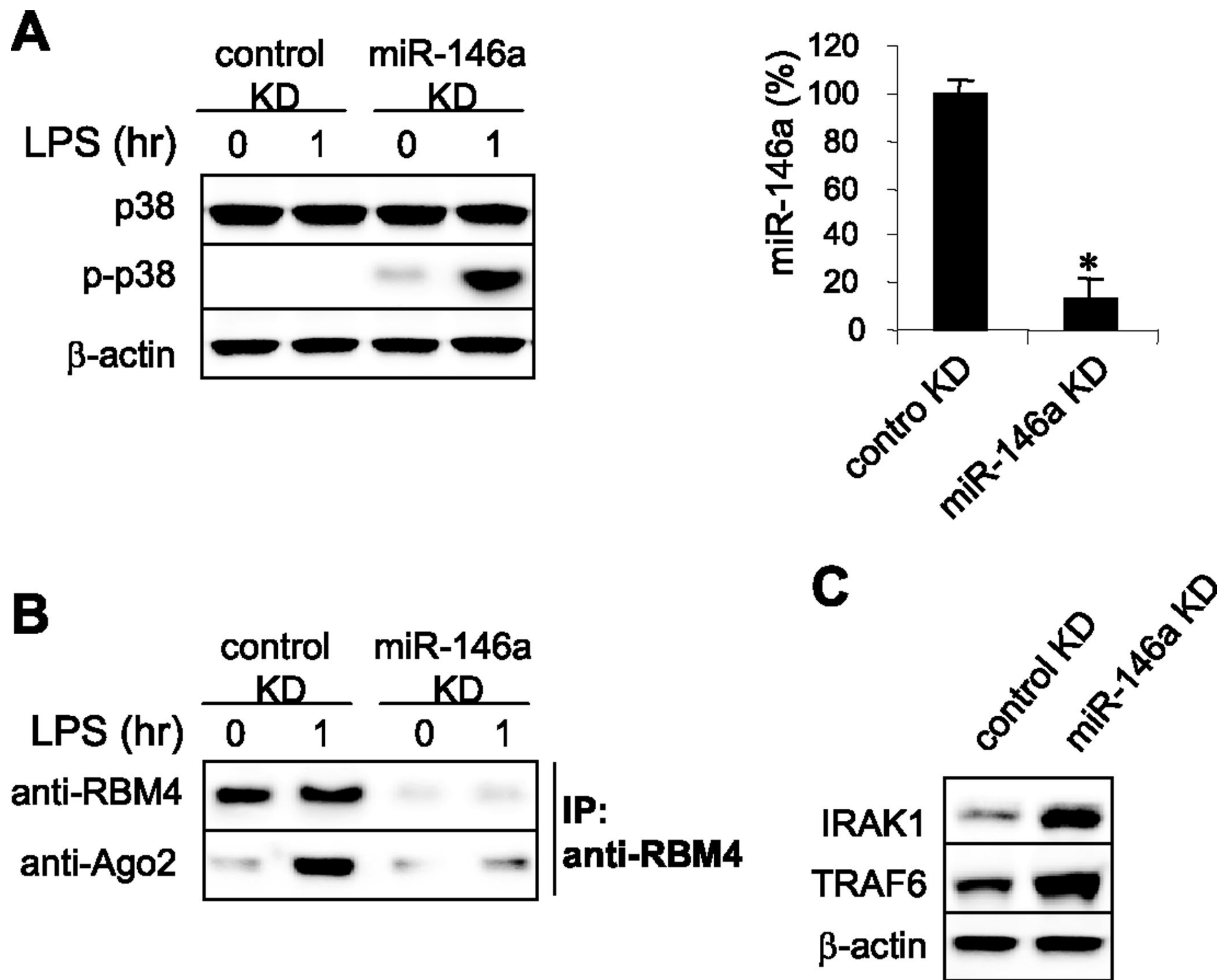

Figure 5. MiR-146a knockdown in adapted cells promotes p38 phosphorylation and disrupts RBM4-Ago2 interaction

(**a**) Adapted cells were induced by pre-treatment overnight with 1 μg/ml of LPS. Adapted cells then were transfected with anti-miR-146a-specific oligonucleotides (antagomirs) or scrambled anti-miRNA oligonucleotides (control KD). After 36 hr, cells were washed and stimulated for 1 hr with 1 μg/ml of LPS. Whole cell extract was analyzed for total and phospho p38 MAPK by immunoblotting. MiR-146a levels in adapted cells were analyzed by PCR before (control KD) and after its knockdown. MiR-146a levels were normalized to U6 small RNA expression (as an internal control) and are presented relative to the control KD (set at 100%). Data are the mean ± s.d. of three assays from two knock down experiments. *, $p < 0.05$. (**b**) Cytoplasmic proteins were extracted, immunoprecipitated with antibody against RBM4, resolved by SDS- 10 PAGE, and then immunoblotted with RBM4 or Ago2 antibody. (**c**) Adapted cells without or with miR-146a knockdown were stimulated for 1 hr with 1 μg/ml of LPS and whole cell extract was analyzed for IRAK1 and TRAF6 proteins by immunoblotting. R, responsive; A, adapted; KD, knockdown; IP, immunoprecipitated.

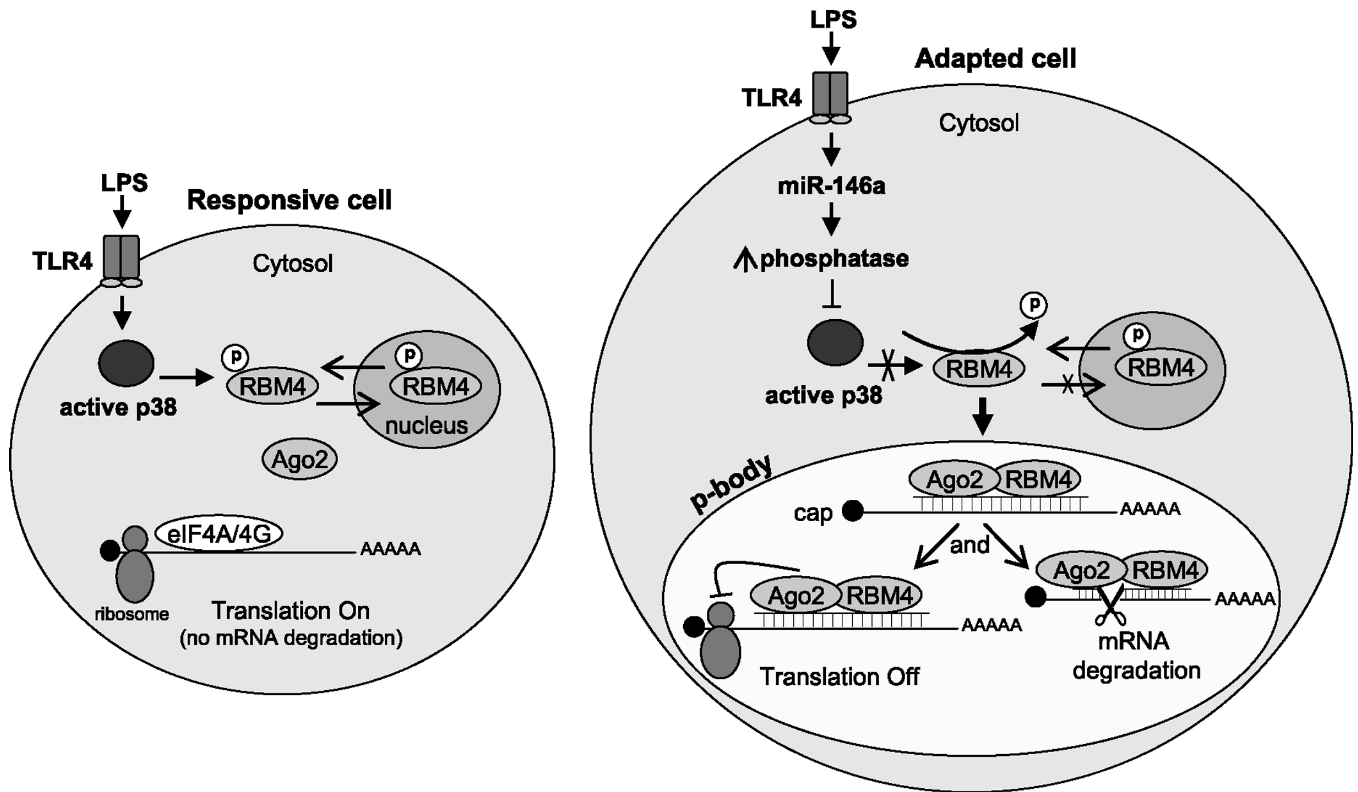

Figure 6. A model of miRNA-guided translation repression in the severe systemic inflammation endotoxin adaptive phenotype

Based on our data, RBM4 interacts with Ago2 in adapted cells and recruits it with the targeted mRNA to p-bodies, where mRNA metabolism takes place. We hypothesize that RBM4 is unphosphorylated in adapted cells due to inactivation of p38 MAPK and, as a result, is retained in the cytosol, which will facilitate its interaction with Ago2 and subsequent targeting to p-bodies for mRNA decay and cap-dependent translation arrest of the targeted mRNA. RBM4 and Ago2 do not interact in responsive cells because of RBM4 phosphorylation and lack of miR-146a.

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