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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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/lipopolysaccharide (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 tumor necrosis factor alpha (TNFα). We also previously demonstrated that TNFα and interleukin (IL)-6 mRNA translation is repressed in endotoxin-adapted THP-1 human monocytes by an miRNA-based mechanism involving the argonaute family protein argonaute 2 (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 (RNA-binding motif protein 4) and promotes its interaction with Ago2. This interaction results in the 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 the phosphorylation of RBM4 on serine-309 by p38 MAPK (mitogen-activated protein kinase), which leads to RBM4 accumulation in the cytosol and interaction with Ago2. We further find that microRNA-146a knockdown by antagomirs or protein phosphatase inhibition by okadaic acid increases p38 MAPK phosphorylation and results in RBM4 serine-309 phosphorylation and nuclear relocalization, which disrupts RBM4 and Ago2 interactions and restores TLR4-dependent synthesis of TNFα and IL-6. We conclude that miR-146a has a diverse and critical role in limiting an excessive acute inflammatory reaction.

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The severe systemic inflammatory response to serious infection (for example, 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 the production of proinflammatory mediators such as tumor necrosis factor alpha (TNFα), interleukin (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.3,8,9 Although 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 the diminished expression of proinflammatory genes following TLR4 re-exposure to bacterial endotoxin or lipopolysaccharide (LPS).12 This inflammation phase shift from responsive to adaptive 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 h.\textsuperscript{15} We have previously shown that mice administered with sublethal dose of LPS into the peritoneal cavity developed endotoxin tolerance within 3 days, as demonstrated by diminished circulating levels of proinflammatory cytokines upon subsequent intravenous challenge with LPS.\textsuperscript{16} Peritoneal macrophages from these mice did not produce significant amounts of proinflammatory TNF\textsubscript{α} and IL-6 when challenged with LPS ex vivo.

Previous studies in adapted murine macrophages\textsuperscript{14,17,18} and human monocytes\textsuperscript{15} established that expression of proinflammatory cytokines such as TNF\textsubscript{α}, IL-6, IL-1β, and chemokines CCL4 and CCL3 is repressed, whereas expression of anti-inflammatory genes like IL-10, IkBα and IL-1Ra is sustained.\textsuperscript{19–22} Thus, reprogramming of genes during the development of tolerance/adaptation is gene specific. To study gene reprogramming, we developed a cell model of gene reprogramming using THP-1 human macrocytes;\textsuperscript{23} over the ensuing two decades, this cell model has correctly predicted that 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,\textsuperscript{24–27} and at the translational level by miRNA-mediated processes.\textsuperscript{18,28} We also found that microRNA-146α (miR-146α), which is markedly induced in endotoxin-adapted THP-1 cells, indirectly promotes the assembly of a translation-repressor protein complex of the argonaute family protein, argonaute 2 (Ago2).\textsuperscript{28} This repressor complex mediates translation repression of TNF\textsubscript{α} and IL-1β.\textsuperscript{18,28} The knockdown of miR-146α in endotoxin-adapted THP-1 cells with antagonics blocks repressor complex assembly and restores TNF\textsubscript{α} and IL-1β protein levels,\textsuperscript{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 RNA-binding motif protein 4 (RBM4) was required for translation repression of TNF\textsubscript{α} 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-146α-generated feed-forward loop that involves p38 MAPK (mitogen-activated protein kinase) and an unknown phosphatase.

**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 post-transcriptional regulation of TNF\textsubscript{α} in endotoxin-adapted THP-1 cells is controlled by mRNA decay and translation repression.\textsuperscript{18} We further demonstrated that the small RNA-binding proteins TTP, AUF1 and TIAR were detected at the TNF\textsubscript{α} 3′-untranslated region (UTR) in the cytosol of adapted cells after LPS stimulation.\textsuperscript{18} These proteins coupled with miR-221, miR-579 and miR-125b, which specifically recognized TNF\textsubscript{α} 3′-UTR sequence, promoted miRNA decay and repressed its translation. Significantly, TNF\textsubscript{α} mRNA, along with these small proteins and the specific miRNAs, was 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.’\textsuperscript{28} This protein complex also contained the RBM4, whose knockdown restored TNF\textsubscript{α} protein levels in adapted cells.\textsuperscript{28} Of note, this translational-repressor complex was not detected at the IkBα mRNA, which is expressed normally in adapted cells,\textsuperscript{28} suggesting that this complex assembles only at the translationally repressed mRNAs.

Although Ago2 is ubiquitously expressed and considered a core component of the translation-repressor complex (also known as miRNA-induced silencing complex), the protein cofactors in this repressor complex determine its role in decay and/or translational repression of target mRNAs.\textsuperscript{29,30} 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 TNF\textsubscript{α}, these included miR-221, miR-579 and miR-125b.\textsuperscript{18} RBM4, on the other hand, was recently identified as a cofactor of the Ago2 protein complex in HEK-293 cells.\textsuperscript{29} 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 associates directly with TTP, AUF1 and TIAR that we detected at the TNF\textsubscript{α} 3′-UTR.\textsuperscript{18} Cytoplasmic proteins from adapted cells were immunoprecipitated with Ago2 or RBM4 antibody, and then immunoblotted with the indicated antibodies (Figure 1). TTP, AUF1 and TIAR were detected both in Ago2 and RBM4 immunoprecipitates at 1 h after LPS stimulation, concurrent with Ago2–RBM4 interaction (Figure 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 TNF\textsubscript{α} protein synthesis in endotoxin-adapted THP-1 cells and that a TLR4-mediated signaling path controls the assembly of this complex.

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

To delineate the kinetics 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 nucleocytoplasmic 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 (Figure 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 (Figure 2a). Co-immunoprecipitation followed by immunoblotting showed that Ago2 and RBM4 interacted in the cytosol in adapted cells only after TLR4 stimulation (Figure 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 TNF\textsubscript{α} and IL-6, but not the translationally active IkBα mRNAs, were enriched in RBM4 protein complex (Figure 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 the 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 cells during cell stress induced by arsenite
exposure treatment, and that this phosphorylation promoted RBM4 shuttling between the nucleus and cytosol. 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 h of LPS stimulation in murine macrophage. MAPKs, including p38, are inactivated (dephosphorylated) by dual specificity protein phosphatases. To determine whether 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 Figure 3a, upon LPS stimulation in adapted cells pretreated with okadaic acid, the level of unphosphorylated RBM4 was markedly reduced in the cytosol.

Figure 1 RBM4 protein colocalizes with Ago2 and RNA-binding proteins TTP, AUF1 and TIAR in adapted cells. Responsive (normal) THP-1 cells were made tolerant (adapted) by pretreatment overnight with 1 μg ml⁻¹ of the Gram-negative bacterial LPS. (a) Ago2 associates with TTP, AUF1 and TIAR. Adapted cells were left unstimulated or stimulated for 1 h with 1 μg ml⁻¹ of LPS. Cytoplasmic proteins were extracted and IP with antibody against Ago2 or immunoglobulin G 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 knockdown disrupts the repressor assembly. Adapted cells were transfected with a pool of RBM4-specific small interfering RNA (siRNAs) or scrambled siRNAs (control KD). After 36 h, cells were left unstimulated or stimulated for 1 h with 1 μg ml⁻¹ of LPS. Cytoplasmic proteins were extracted and immunoprecipitated with antibody against RBM4 or immunoglobulin G 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 that the protein complexes assemble only after LPS stimulation. The results represent three independent experiments. A, adapted/tolerant cell; KD, knockdown; IP, immunoprecipitated.

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 pretreatment overnight with 1 μg ml⁻¹ of LPS. Adapted and responsive (normal) cells were stimulated for 1 h with 1 μg ml⁻¹ of LPS. Nuclear and cytoplasmic proteins were extracted and probed with nucleoporin or actin antibody as a control. (b) RBM4 is restricted to the cytosol in adapted 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 IκBα, 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 one-fold). The results represent three independent experiments. A, adapted; IP, immunoprecipitated; R, responsive.

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Okadaic acid treatment relocates RBM4 from the cytosol to the nucleus of adapted cells after LPS stimulation. Adapted THP-1 cells were made by pretreatment overnight with 1 μg ml⁻¹ of LPS. Adapted cells were treated with okadaic acid for 6 h and then stimulated for 1 h with 1 μg ml⁻¹ of LPS. RBM4 protein levels in the nucleus and cytoplasmic extracts were analyzed by immunoblotting. 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. RBM4 is phosphorylated after okadaic acid treatment and it shuttles between the nucleus and cytosol. Cells were treated as in (a). Levels of unphosphorylated and phosphorylated RBM4 in the nucleus and cytosol were measured by immunoblotting. Okadaic acid treatment restores TNFα protein levels in adapted cells. Adapted cells were transfected with control or RelB saran (to reverse transcription silencing). After 36 h, adapted cells were incubated for 6 h without or with 100 nM okadaic acid (which inhibits protein phosphatase activity) or diethyl sulfoxide (–). Following by adding 1 μg ml⁻¹ of LPS. TNFα mRNA and protein levels were analyzed after 1 and 4 h 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. A, adapted; IP, immunoprecipitated; R, responsive.

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

We28 and others34,35 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.35 In addition, we have shown that miR-146a knockdown in adapted cells, similar to the protein phosphatase inhibition by okadaic acid (Figure 3), restores TNFα protein levels.28 Given that RBM4 phosphorylation is induced by p38 MAPK signal,31 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 knockdown is rapidly degraded.28 Levels of RelB and miR-146a were reduced by >80% after their knockdown (Figure 5a and data not shown). In the absence of miR-146a, LPS stimulation for 1 h induced RBM4 phosphorylation on serine-309, and this resulted in RBM4 shuttling between the nucleus and the cytosol (Figure 4a). In a separate co-immunoprecipitation experiment, we found that anti-RBM4 antibody did not crossreact 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 (Figure 4b). Together, these results demonstrate that miR-146a disrupts RBM4 phosphorylation in adapted cells and thus prevents the translation-repressor complex assembly.

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

As miR-146a inhibits signaling proteins leading to MAPK activation,35 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 (Figure 5a). With miR-146a knockdown, phosphorylated p38 was detected 1 h after LPS stimulation. Importantly, miR-146a knockdown resulted in the disruption of Ago2–RBM4 protein complex in the cytosol of adapted cells (Figure 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 present in the phosphorylated form (Figure 4a). In addition, western blot...
MiR-146a knockdown in adapted cells leads to RBM4 phosphorylation and restores TNFα and IL-6 protein levels. Adapted cells were made by pretreatment for overnight with 1 μg/ml-1 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 h, cells were washed and stimulated for 0-4 h with 1 μg/ml-1 of LPS. (a) Nuclear and cytoplasmic proteins were extracted after 1 h and immunoblotted with antibody against total or phospho serine-309 RBM4. (b) After 1 h in LPS, RNA was extracted and analyzed using 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 h 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.28 Data are the mean ± s.d. of three experiments. *P<0.05. R, responsive; A, adapted; KD, knockdown.

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 Re-activation 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 the translation of inflammatory gene mRNAs in the endotoxin-adapted THP-1 cells. We further show that RNA-binding protein RBM4 has a central role in assembling the repressor complex that disrupts the 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. On the basis of these results, we propose a model (Figure 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 that by directly targeting IRAK1 and TRAF6 limits p38 MAPK activation. Results with okadaic acid support the 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, that is, in responsive cells, RBM4 is phosphorylated and it shuttles between the nucleus and cytosol where it binds to Ago2 to assemble the translation-repressor complex at the proinflammatory gene mRNAs.

RNA-binding proteins couple with TLR4-dependent signaling to assemble a multiprotein repressor complex and specifically repress the translation of proinflammatory TNFα and IL-6 mRNAs in the endotoxin-adapted THP-1 cells. We further show that RNA-binding protein RBM4 has a central role in assembling the repressor complex that disrupts the 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. On the basis of these results, we propose a model (Figure 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 that by directly targeting IRAK1 and TRAF6 limits p38 MAPK activation. Results with okadaic acid support the 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, that is, in responsive cells, RBM4 is phosphorylated and it shuttles between the nucleus and cytosol where it binds to Ago2 to assemble the translation-repressor complex at the proinflammatory gene mRNAs.

miR-146a knockdown in adapted cells promotes p38 phosphorylation and disrupts RBM4-Ago2 interaction. (a) Adapted cells were induced by pretreatment overnight with 1 μg/ml-1 of LPS. Adapted cells then were transfected with anti-miR-146a-specific oligonucleotides (antagomirs) or scrambled anti-miRNA oligonucleotides (control KD). After 36 h, cells were washed and stimulated for 1 h with 1 μg/ml-1 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 knockdown experiments. *P<0.05. R, responsive; A, adapted; KD, knockdown.

Figure 5 MiR-146a knockdown in adapted cells promotes p38 phosphorylation and disrupts RBM4-Ago2 interaction. (a) Adapted cells were induced by pretreatment overnight with 1 μg/ml-1 of LPS. Adapted cells then were transfected with anti-miR-146a-specific oligonucleotides (antagomirs) or scrambled anti-miRNA oligonucleotides (control KD). After 36 h, cells were washed and stimulated for 1 h with 1 μg/ml-1 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 knockdown experiments. *P<0.05. R, responsive; A, adapted; KD, knockdown.

RBM4 links TLR4 signaling to translational repression

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Figure 4 MiR-146a knockdown in adapted cells leads to RBM4 phosphorylation and restores TNFα and IL-6 protein levels. Adapted cells were made by pretreatment for overnight with 1 μg/ml-1 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 h, cells were washed and stimulated for 0-4 h with 1 μg/ml-1 of LPS. (a) Nuclear and cytoplasmic proteins were extracted after 1 h and immunoblotted with antibody against RBM4, resolved by SDS-10% PAGE and then immunoblotted with antibody against total or phospho serine-309 RBM4. (b) After 1 h in LPS, RNA was extracted and analyzed using 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 h 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.28 Data are the mean ± s.d. of three experiments. *P<0.05. R, responsive; A, adapted; KD, knockdown.
proteins as well as protein cofactors. The first two components for RNA binding of gene-specific miRNAs and small RNA-binding possesses endoribonuclease activity and has been detected in most takes place. A recent study has reported that phosphorylation of granules known as processing bodies in which translation repression complex activity and its recruitment/deposition into cytoplasmic repressor complex to that target, whereas the latter regulates the are specific to each target mRNA and help to guide the translation-repression machinery. Although there are four Ago proteins implicated in mRNA metabolism, only human Ago2 possesses endoribonuclease activity and has been detected in most translation-repressor complexes as a key component of the translation-repressor machinery. Ago2 provides a platform for RNA binding of gene-specific miRNAs and small RNA-binding proteins as well as protein cofactors. The first two components are specific to each target mRNA and help to 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 in which translation repression takes place. 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. 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, although 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 processing bodies.

In endotoxin-responsive cells, RBM4 localization was restricted and appeared in the cytosol after LPS stimulation, yet did not interact with Ago2 (Figure 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 assembly 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. 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 (Figure 1), which also restores TNFα protein levels. In addition to TNFα, IL-6, but not the translationally active IkBα, was detected in the 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. Recently, Lin et al. 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 RBM4 phosphorylation induced its relocalization to the cytosol, its interaction with Ago2 was independent of RBM4 phosphorylation and that RBM4 facilitated the recruitment of Ago2 protein with the associated target mRNA to cytoplasmic granules for translation repression during mouse C2C12 myoblast differentiation. We detected RBM4 both in the nucleus and cytosol following LPS stimulation in adapted cells pretreated 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 (Figure 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 (Figure 2). RBM4 phosphorylation also disrupted

![Diagram](image-url)
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. On the basis of these results, we conclude that RBM4 phosphorylation prevents translation repression of proinflammatory genes in response to TLR4 stimulation. This process is dysregulated in adapted cells through induction of a protein phosphatase activity downstream of TLR4.

We have previously shown that miR-146a has a critical role in translation repression of proinflammatory genes in endotoxin-adapted THP-1 cells, in which miR-146a knockdown restored TNFα and IL-6 protein levels. The current study shows that miR-146a knockdown in endotoxin-adapted cells, as with okadaic acid pretreatment, 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-κB and MAP kinase activations 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.

We found that 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. Therefore, transcription must be restored first by RelB knockdown. However, restored mRNAs are rapidly degraded because of the translational-repression mechanism. Therefore, 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 they induce inflammatory protein synthesis. As expected, we did not detect 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 in shuffling between the nucleus and the 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. Another study has shown that RBM4 is phosphorylated during muscle cell differentiation. In that study, phosphorylated RBM4 accumulated in the cytosol and colocalized 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 has led 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. However, it is unclear from these experiments whether lack of RBM4 phosphorylation in adapted cells is because of the 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. Of note, the dual specificity MAPK phosphatase-1 (MKP-1) is a negative regulator of innate immune response, where it restrains proinflammatory cytokine protein synthesis in LPS-stimulated macrophages by inactivating p38 MAPK. Our results showed that RBM4 was phosphorylated on serine-309. Given that MAPK phosphatases dephosphorylate tyrosine and threonine residues, it is possible that a MAPK phosphatase such as 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, restored TNFα protein levels after LPS stimulation (data not shown). MKP-1 may be induced in adapted cells due to the 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 assembly 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 immunosuppression, a prominent feature during sepsis.

METHODS

Cell culture

THP-1 human monocytes were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 2 mM 1-glutamine (all from Hyclone Laboratories, Logan, UT, USA) and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) at 37°C and 5% CO₂. Cells were made tolerant by overnight incubation with 1 µg ml⁻¹ of LPS from gram-negative bacteria (E. coli, strain 0111:B4; Sigma-Aldrich, St Louis, MO, USA). The LPS preparation used in these experiments is TLR4-specific and is free of contaminating proteins that activate cells via a non-TLR4-dependent mechanism.

Transfections

Cells were seeded at 0.5 × 10⁶ cells ml⁻¹ one day prior to transfection. Cells were transfected by electroporation using the Bio-Rad GenePulser MXcell transfection system as per the manufacturer’s instructions (Bio-Rad, Hercules, CA, USA).

For RBM4 and RelB knockdown, cells were transfected with pools of gene-specific or scrambled (control) small interfering RNAs at 0.5 µM final concentration (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For miR-146a knockdown, cells were transfected with negative control or miR-146a-specific antisense 2’-O-Me oligonucleotides (antagonists; final concentration 100 nM) (Ambion, Austin, TX, USA).

Protein extraction

Cyttoplasmic and nuclear proteins were extracted using the NE-PER nuclear and cytoplasmic extraction kit per the manufacturer’s instructions (Pierce, Rockford, IL, USA). Immediately after harvesting, cells were washed in...
phosphate-buffered saline 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 r.p.m. 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 preparations were recovered by centrifugation for 10 min at 4 °C and 14,000 r.p.m.

For preparation of whole-cell lysate, cells were lysed in 1 × RIPA buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40, 0.25% sodium deoxycholic acid and 1 mM EDTA (Millipore, Temecula, CA, USA) plus 1 × protease inhibitor cocktail. Protein concentrations were determined by Bradford assay (Bio-Rad).

Immunoprecipitation and western blot

 Ago2 or RBM4 protein complexes were immunoprecipitated as described previously 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, 5 mM MgCl2, 2 mM DT, 30 μM RNAi inhibitor and 1 × protease inhibitor cocktail). Protein extract was then centrifuged at 2000 r.p.m. 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 of antibodies against human Ago2 (clone no. 4G8; Wako, Richmond, VA, USA), RBM4 or immunoglobulin G 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 phosphor 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 × Laemmli 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/0.2% 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 stripped and re-probed with actin or nucleoplin antibody as a control.

Real-time qPCR

The mRNA levels of TNFα and IL-6 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 miScript 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× universal reverse primer, 1 μl of 10 μM mir-146a-specific forward primer (an oligonucleotide identical to the 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 15 s and 60 °C for 1 min using iCycler IQ5 detection system (Bio-Rad). The relative expression of mir-146a was calculated using the 2 −ΔΔ Ct threshold method after normalization to the endogenous U6 small RNA (as an internal control).

Enzyme-linked immunosorbent assay

ELISA was performed to determine the levels of TNFα and IL-6 proteins using commercially available kits according to the manufacturer's instructions (E Bioscience, San Diego, CA, USA). 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.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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