HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2011 March 01.

Published in final edited form as:

Nat Immunol. 2010 September ; 11(9): 799–805. doi:10.1038/ni.1918.

MicroRNAs modulate the noncanonical NF-κB pathway by regulating IKKα expression during macrophage differentiation

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Abstract

MicroRNAs are key regulators in many biological processes including cell differentiation. Here we show that during human monocyte-macrophage differentiation, the expression of the microRNAs miR-223, miR-15a, and miR-16 is dramatically decreased, leading to increased expression of the serine-threonine kinase IKKα in macrophages. In macrophages, higher IKKα expression in conjunction with stabilization of the kinase NIK induces elevated p52. Due to low RelB transcription factor expression in untreated macrophages, high p52 expression represses the basal level of both canonical and noncanonical NF-κB target genes. However, proinflammatory stimuli in macrophages result in greater induction of noncanonical NF-κB target genes. Thus a decrease in certain microRNAs likely prevents macrophage hyperaction yet primes the macrophage for certain responses to proinflammatory stimuli.

NF-κB proteins are related transcription factors that bind to κB sites within the genome and regulate pro-inflammatory and immune-related genes1,2. They are therefore essential in the development, differentiation, and regulation of cells of the immune system. Two mammalian NF-κB proteins, p105 and p100, have long C-terminal domains that inhibit their activity until activated by proteasome-mediated cleavage1,2. NF-κB transcription factors bind to DNA as homo-or heterodimers, although activation of transcription is usually achieved only when dimers contain one of the Rel proteins, p65 (also called RelA), c-Rel and RelB, which have a C-terminal transactivation domain1,2.

In canonical NF-κB signaling, the p105 protein is constitutively processed by the proteosome into active p50, but is maintained cytoplasmically as a heterodimer (primarily with p65 or c-Rel) by its interaction with inhibitory IκB proteins, such IκBα which have a strong nuclear export signal1–4. IκB proteins are phosphorylated by an IKK complex that

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includes the subunits IKKα IKKβ and the regulatory subunit IKKγ. Phosphorylation of IκB proteins target them for ubiquitination and degradation by the proteosome, allowing the p50 heterodimer to move into the nucleus and activate transcription1–4.

Conversely, in the noncanonical NF-κB signaling pathway, p100 is processed to active p52 only when the pathway is activated. Activation involves the stabilization of the kinase NIK and its subsequent phosphorylation of IKKα 5–7. NIK is usually constitutively and rapidly degraded by its TRAF3-mediated recruitment to TRAF2, which recruits cIAPs 1 and 2, and NIK ubiquitination by these cIAPs promotes its proteosomal degradation8,9. Stabilized NIK phosphorylates IKKα homodimers, which in turn phosphorylate p100 on its C-terminus, resulting in p100 ubiquitination and partial proteolytic processing by the proteosome to p525–7. The active p52 then activates transcription when associated with its binding partner (primarily RelB) 1–4.

Canonical and noncanonical NF-κB pathways activate a primarily overlapping, yet distinct, set of genes3,4. Canonical pathway activation is associated with inflammation and innate immune system function through the stimulation of proinflammatory cytokines receptors, such as the TNF, Toll-like receptor (TLR), and Interleukin receptor families3,4. The noncanonical pathway is associated with adaptive immunity, secondary lymphoid organ development, B cell maturation and survival, and bone development, and is activated by receptors such as LTβR, BAFF-R, CD40, and RANK3,4.

MicroRNA (miRNA) has recently emerged as an important component of immune cell differentiation and function10–12. As RNA molecules of ~22 nucleotides in length that are processed from larger transcripts by Drosha and Dicer nucleases, miRNAs are incorporated along with core Argonaute proteins into the RNA-induced silencing complex (RISC)13. Binding of this complex to mRNA in mammalian cells can directly or indirectly block the translation of the target mRNA or increase its degradation, thus resulting in reduced protein expression11,13. The RISC-miRNA complex binds primarily to 3′ untranslated regions (3′UTRs) of mRNAs as recognized by partial sequence complimentarity to the 5′ miRNA “seed” region, allowing for the recognition of multiple target mRNAs by a single miRNA, and also allowing a single protein to be regulated by many miRNAs14. Such a system is particularly suitable for controlling cell differentiation, where the development of new cellular properties involves changes in large protein sets and where multiple components of an important pathway or pathways can be efficiently modulated by the expression of a single miRNA or a set of miRNAs.

Macrophages are major players in host inflammatory responses of the innate immune system and dysregulation of macrophage function is involved in many pathological conditions, including autoimmune disease and cancer15,16. Several microRNAs play a role in the innate immune system including miR-223, miR-155, miR-146, and miR-125b10–12. Although the tumor suppressors, miR-15a and miR-16, have not previously been associated with the innate immune system, they are frequently deleted or downregulated in B cell chronic lymphocytic leukemia, suggesting they are involved in other immune processes12. More recently, miR-15a was shown to inhibit myeloid colony formation from $CD34^+$ bone marrow cells *in vitro*18.

In this study, we found that the IKKα mRNA is a target of miR-15a, miR-16, and miR-223. During monocyte-macrophages differentiation, a substantial decrease in these miRNAs allows for a considerable increase in IKKα protein, which then contributes to p52 production. This pathway likely prevents the new macrophage from becoming overactivated by initially acting to repress the activation of NF-κB target genes but prepares it for future NF-κB signaling events that occur during the activation process.

Results

IKKα upregulation during monocyte differentiation

When treating U937 cells with the phorbol esterPMA to induce differentiation, we observed a substantial increase in IKKα (Fig. 1a). To determine whether such changes occurred in primary human cells, we obtained purified elutriated human monocytes (Supplementary Fig. S1, top panel) and treated them with granulocyte-macrophage colony stimulating factor (GM-CSF), which induced macrophage differentiation as verified by morphology (Supplementary Fig. 1, bottom panel) and flow cytometric analysis (Supplementary Fig. 2). Upon differentiation, there was a striking increase in IKKα protein expression (Fig. 1b). Notably, there was little change in the expression levels of IKKβ or IKKγ. Consistently, in immunoblots of non-differentiated and differentiated cell lysates from more than 100 donors, almost all samples showed a similar change in IKKα protein expression (Supplementary Fig. 3). Semi-quantitative RT-PCR showed a minimal, but detectable change in IKKα mRNA between monocytes and macrophages (Fig. 1c). Quantitative Real-Time PCR substantiated a twofold increase in IKKα mRNA levels (Fig. 1d). However, although it was evident that changing IKKα mRNA levels contributed to an increase in protein expression, it was unclear whether the large change in IKKα protein expression was due solely to a small change in IKKα mRNA levels, or whether other mechanisms also contributed. We therefore investigated whether additional mechanisms exist to control IKKα protein expression.

MicroRNAs target IKKα mRNA

MicroRNAs not only target mRNA for degradation, but can also inhibit mRNA translation11,13. Using the Memorial Sloan-Kettering Cancer Center miRNA database19 (http://www.microrna.org/) we searched for microRNAs present in human monocytes that have a predicted target sequence in the 3′ UTR of IKKα. We identified possible target sites for let-7, miR-223, miR-16, miRNA-142-5p, and two target sites for miR15a, one of which overlaps with the putative miR-16 site (Fig. 2a). miR-223 and miR-15a-miR16 target sites were only about 35 nucleotides (n.t.) apart, a condition that may result in cooperativity between target sites, especially of the same miRNA20. With the exception of the putative let-7 target site, which occurred centrally, all miRNA target sites were at the 5′ and 3′ ends of the IKKα 3′ UTR. A recent report suggests that miRNA target sites at either end of the UTR are more likely to be functional targets than those in the middle21.

Next, we looked at which miRNAs were substantially decreased in GM-CSF-differentiated macrophages when compared to monocytes. miR-142-5p was only slightly decreased in macrophages, while let-7 increased (Fig. 2b).However, miR-223 was downregulated about

10–11 fold while miR-15a and miR-16 were decreased by about 3–5 fold during macrophage differentiation (Fig. 2b, c). Thus, a decrease in miR-15a, miR-16, and miR-223 expression levels correlated with rising IKKα protein expression in macrophages, and suggested that these miRNAs could function as modulators of IKKα mRNA and protein expression.

To determine whether this was true we transfected the monocytes with inhibitors of these miRNAs. Through using a combination of miR-223, miR-15a, and miR-16 inhibitors, IKKα protein expression was substantially increased(Fig. 2d). Conversely, transfection of molecular mimics of the three miRNAs in macrophages reduced the amount of IKKα protein (Fig. 2e). IKKβ protein levels were not notably affected by inhibitors or mimics (Fig. 2d, e). This suggests that these particular miRNAs can regulate IKKα protein expression, and that they are likely responsible, at least in part, for the increase in IKKα protein expression observed during macrophage differentiation.

To determine whether the predicted target sites are targeted by microRNAs, we made CMV promoter-driven luciferase constructs fused to the 3′ untranslated region from the IKKα mRNA. Mutations were made in the regions of homology to the various miRNAs. Alignment of the IKKα sequences with the respective homologous miRNAs and the resultant mutant IKKα sequences is shown (Fig. 3a). Mutation in “Site A” destroyed the homology with miR-15a at its first target site, while a mutation in “Site C” destroyed its second target site, and also removed homology to miR-16. Mutation in “Site B” destroyed homology to miR-223. When these constructs were co-transfected into HeLa cells with a β-galactosidase expression plasmid, luciferase activity was normalized for transfection efficiency to β-galactosidase activity. A co-transfected control mimic miRNA with no sequence specificity was used to further normalize the luciferase activity compared to miRNA mimics for miR-15a, mir-16 or miR-223. Each of the latter mimics reduced luciferase activity produced by the wild-type 3′ UTR reporter construct, compared with the respective mutant constructs (Fig. 3b, c, d). Mutation of site A alone did not increase luciferase activity in the presence of the miR-15a mimic (Fig. 3b). When site C was mutated, luciferase activity increased about 25%. However, when both Sites A and C were mutated, luciferase activity nearly doubled (Fig. 3b), suggesting that both sites are miR-15a targets, though site C is apparently a more effective target. Mutation of site B increased the amount of luciferase activity in the presence of miR-223 (Fig. 3c), while mutation of site C increased the luciferase activity in the presence of miR-16 (Fig. 3d). Increased luciferase activity associated with mutation was restricted to the corresponding homologous site in the presence of the respective miRNA mimic. For instance, Site A or Site C mutations did not increase the relative luciferase activity in the presence of the miR-223 mimic (data not shown).

We next examined the mutations in various combinations when co-transfected with miR-15a, mir-16, and miR-223 mimics together. While Site C was clearly the most important target site of the three mimics (Fig. 3e), some cooperativity was observed between sites, especially between site A and the other sites, indicating that in the presence of all three miRNAs, each site plays some role in the reduction of protein expression. Luciferase activity was likely limited to a 50% reduction due to the use of the strong CMV promoter

driving the construct. Endogenous IKKα protein expression, but not IKKβ was decreased in HeLa cells in the presence of the three mimics (Fig. 3f). Taken together, these data suggest that miR-15a, miR-16, and miR-223 can modulate IKKα protein expression outside of the context of monocyte-macrophage differentiation, and that they do in fact target the specific target sequences in the IKKα mRNA as determined by the prediction algorithms. To verify that the miRNA had a consequential effect on IKKα protein during differentiation, we transfected monocytes with the mimic pool or control mimic and cultured them in the presence of GM-CSF. We were able to detect a decrease in the IKKα protein expression four days after GM-CSF treatment in the presence of the mimic pool (Supplementary Fig. 4).

Regulation of mRNA by miRNA can occur through inhibition of protein translation or through reducing mRNA levels. Transfection of HeLa cells with the pooled mimics caused an approximately 2.5 -fold decrease in IKKα mRNA (Fig. 4a), which was similar to the decrease seen when macrophages were transfected with the pooled mimics (Fig. 4b), and similar to the fold difference in IKKα mRNA between macrophages and monocytes (Fig. 1d). Clearly, these results indicate these miRNAs regulate IKKα mRNA stability to some extent. However, as the IKKα protein levels change much more appreciably than its mRNA levels do, it is likely that these miRNAs also regulate the translation of the IKKα mRNA.

IKKα-targeting miRNAs affect the noncanonical pathway

Since IKKα is essential in noncanonical NF-κB signaling, we examined downstream noncanonical components. Though minimal in monocytes, p100 processing increased substantially in macrophages, leading to a large amount of p52 (Fig. 5a). An electrophoretic mobility shift assay showed that while κB sites were bound by various NF-κB complexes in both monocytes and macrophages in the absence of treatment, p52 was bound only to κB sites in macrophages, and not monocytes, as shown by supershifting (Fig. 5b). Therefore, increased IKKα protein expression correlates with p52 DNA binding. To further examine the activation of the noncanonical NF-κB pathway, we examined NIK stability, a key requirement in this process. Immunoblotting revealed that substantial NIK protein was present in macrophages, but not in monocytes (Fig. 5c). Additionally, TRAF2, which negatively regulates NIK protein levels, was substantially decreased in the macrophages, when compared to the monocytes (Fig. 5c). The combination of these data suggests that the noncanonical NF-κB pathway is activated upon macrophage differentiation. HeLa cells stably express p100 and NIK, resulting in constitutive p52 production. We therefore used these cells to examine what effect IKKα-targeting miRNAs have on the events downstream of stabilized NIK. Transfection of pooled miRNA mimics in HeLa cells reduced not only IKKα protein, but also reduced the amounts of p52 without meaningfully affecting p100, demonstrating that decreases of these miRNAs and an increase in IKKα protein expression could indeed substantially contribute to a rise in p52 levels downstream of NIK stabilization (Supplementary Fig. 5). In macrophages, pooled mimics reduced not only IKKα protein expression relative to the control, but also reduced the amount of NIK protein (Fig. 5d). However, the target site prediction algorithm did not predict any target sites in the NIK mRNA and when we further examined TRAF2 protein expression in these blots, we found higher amounts of TRAF2 protein in the presence of these mimics when compared to the

control (Fig. 5d), suggesting that an additional target mRNA of these mimics contributes to activation of the noncanonical pathway upstream of TRAF2 degradation. Similar results in mimic pool-transfected cells were obtained in HeLa cells (Supplementary Fig. 6).

IKKα enables p52-mediated gene repression and activation

To determine the relative contribution of IKKα to p52 generation, we transfected macrophages with IKKα siRNA, which are more exact in targeting specific mRNAs than miRNA mimics. The IKKα siRNA reduced IKKα protein levels in macrophages to the amounts observed in monocytes. The p52 levels decreased considerably in the IKKα knockdown samples compared to the control, but NIK and TRAF2 protein levels remained similar to the control (Fig. 6a). A similar decrease in p52 levels was seen in HeLa cells transfected with IKKα siRNA (Fig. 6b), however, neither of these knockdowns completely eliminated detectable p52 protein. In macrophages transfected with NIK siRNA, p52 levels were also decreased, but not completely eliminated (Fig. 6c), suggesting that both IKKα upregulation and NIK stabilization contribute to p52 generation.

To examine the consequences of miRNA downregulation upon gene transcription we examined expression of known noncanonical NF-κB target genes. While many target genes are shared between the canonical and noncanonical pathways, some genes are believed to be more specific to regulation by the noncanonical pathway. These include BLC, ELC, SLC, and SDF-1, which are considerably reduced in *RelB*$^{-/-}$ and *Nfκb2*$^{-/-}$ mice, as well as cells from *Ikkα*$^{-/-}$ and IKKα mutant knockin mice22,23. As most of these targets are organogenic chemokines known to be expressed mostly in spleen, we tested whether they were present in human GM-CSF-differentiated macrophages. We could not detect mRNA for BLC, SDF-1, or SLC in these cells. ELC, however, was expressed in most macrophage samples at quantifiable levels.

Unexpectedly, the basal levels of ELC mRNA in macrophages were decreased compared to matched monocyte samples (Fig. 7a). Since p52 was bound to nuclear DNA in macrophages, but not in monocytes (Fig. 5b), we had expected the levels of ELC to rise, given that the ELC gene is a known target of the noncanonical pathway. However, this data indicated that p52 could be acting as a repressor of transcription. Consistent with this hypothesis, transfection of GM-CSF-differentiated macrophages with miR-15a, miR-16, and miR-223 mimics, which decreased IKKα protein expression, increased basal ELC mRNA levels in macrophages when compared to control mimic (Supplementary Fig. 7).

To test whether further stimulation of the noncanonical pathway leads to ELC expression or to further gene repression, we treated these cells with $LT\alpha_1\beta_2$, the ligand for the lymphotoxin receptor (LTR), which strongly activates the noncanonical NF-κB pathway, but also activates the canonical NF-κB pathway22, and soluble TNF, which solely activates the canonical NF-κB pathway24. Consistent with previous data, activation of the noncanonical pathway by $LT\alpha_1\beta_2$ led to increased ELC mRNA expression (Supplementary Fig. 8). Surprisingly, TNFα treatment also enhanced ELC mRNA expression, albeit at much lower levels, suggesting that the canonical pathway was also capable of mediating ELC gene expression in these cells. As we had detected constitutive DNA binding of p65 but not p52 in monocytes, (data not shown and Fig. 5b), we inferred that the higher basal level of ELC

mRNA expression in monocytes is provided through canonical NF-κB molecules. Correspondingly, transfection of the pooled miRNA mimics in macrophages relieved repression of basal level gene transcription of known canonical NF-κB targets, including ICAM, CCL4, and IL-10, but not A20, suggesting that p52 also repressed basal transcription from some, but not all, of these promoters (Supplementary Fig. 9).

Since p52 lacks a transactivation domain, we hypothesized that p52 homodimers, and not the typical transcriptionally active p52-RelB heterodimers, were inhibiting basal gene expression. Indeed, we could not detect any substantial RelB protein in monocytes or in unstimulated macrophages by immunoblot analysis (Fig. 7b). Consistent with previous data showing that RelB is a canonical NF-κB target 25, lipopolysaccharide (LPS)-induced RelB protein expression in macrophages over 24 h of treatment (Fig. 7b) and led to a large increase in ELC mRNA over this same time course (Fig. 7c), which is consistent with a conversion of repressive p52 homodimers to transcriptionally active p52-RelB heterodimers upon stimulation. To verify that p52 was in inhibitory but not stimulatory complexes we did crosslinking experiments in macrophages and compared the mobility of various complexes. To decrease the background of unprocessed p100, we immunodepleted the majority of this protein using a p100 antibody that recognizes an epitope that is not present in p52. Immunoblot analysis of the crosslinked proteins determined that RelA and c-Rel were in present in higher molecular weight complexes in these cells than p52, while RelB was not detected, suggesting that p52 was in inhibitory molecular complexes with itself or with p50 (Supplementary Fig. 10).

LPS stimulation of GM-CSF-differentiated macrophages transfected with the mimic pool led to decreased ELC gene induction compared to the control mimic (Fig. 7d and e), this suggests that p52 activates non-canonical gene transcription in stimulated cells which have substantial RelB expression,. However, expression of canonical gene targets was not inhibited by miRNA pool transfection, and many were potentiated (Fig. 7f, and Supplementary Fig. 11), underscoring the differential effects of IKKα on noncanonical and canonical signaling pathways26,27. To examine whether the effect on basal gene expression levels and induction by the pooled mimics was due to their effect on IKKα and p52 levels, we used siRNA to knockdown these proteins in macrophages. Basal expression of ELC mRNA was increased in these cells compared to the control, confirming that the noncanonical pathway inhibits basal gene expression of ELC (Supplementary Fig. 12a–b; see 12c for effectiveness of p52 knockdown). However, in contrast to A20, which was induced, the LPS-induced gene expression of ELC mRNA was largely inhibited when IKKα was knocked down. (Supplementary Fig. 12d–e). Therefore, although increased IKKα protein expression leads to repression of basal NF-κB gene expression, the increased IKKα protein expression enables activation of noncanonical gene expression upon further stimulation of the macrophage by LPS.

Discussion

Macrophages are major players in mediating host inflammatory responses. Dysregulation of macrophage functions can lead to pathological conditions, including autoimmune disease and cancer15,16. However, the molecular events that occur during the differentiation of

monocyte into macrophage are still largely unexplored. While mouse models are useful for *in vivo* studies, there are now numerous examples of substantial and distinct differences between murine monocytes and macrophages and their human counterparts28. To learn more about the human system, we chose to study the differentiation process in human cells, which necessitated *in vitro* studies. *In vitro* differentiation of human monocytes into macrophages using GM-CSF was accompanied by a decrease in miR-15a, miR-16, and miR-223 expression, leading to a substantial increase in IKKα protein expression. The use of M-CSF instead of GM-CSF gave similar results (data not shown), suggesting these changes apply to the differentiation of unstimulated macrophages in general.

Target sites for miR-15a, miR-16, and miR-223 in the IKKα 3′ UTR each contributed to the regulation of IKKα protein expression, but did not effect IKKβ or IKKγ expression in monocytes or macrophages, suggesting that miR-15a, miR-16, and miR-223 specifically serve to downregulate the noncanonical NF-κB pathway, but not the canonical pathway. These microRNAs likely regulate noncanonical signaling in other cell types, since miRNA mimics also caused a reduction in the level of constitutive p52 expression in HeLa cells. Regulation of IKKα may contribute to the ability of miR-15a and miR-16 to act as a tumor suppressors in various cell types29, since IKKα appears to be involved in prostate cancer30 and miR-15a and miR-16 act as tumor suppressors in this cell type31.

Downregulation of miR-223, miR-15a, and miR-16 during macrophage differentiation likely affects mRNA targets other than IKKα. Both miR-15a and miR-16 negatively regulate the anti-apoptotic gene Bcl-212,32. Because Bcl-2 expression increases during the GM-CSF- and M-CSF- induced differentiation33,34, our data showing a decrease in these miRNAs during differentiation suggest that they contribute to the known rise in Bcl-2 expression, consistent with their role in regulating this protein 12,32. Although we confirmed the importance of changes in IKKα expression levels upon using more specific siRNA to IKKα we also observed a reduction in NIK protein expression in response to miRNA mimics. However, no predicted target sites in NIK could be found, and an increase in TRAF2 expression suggests an indirect effect of the mimics on NIK, perhaps via targets that regulates TRAF2 degradation.

The miRNA miR-223 has been previously shown to have a high expression in granulocytes, especially peripheral blood neutrophils17, which have the same myeloid progenitor linage as monocytes. Mice lacking miR-223 have profound neutrophilia in peripheral blood and extensive granulocyte hyperplasia in the bone marrow due to hyperproliferation of granulocyte monocyte progenitor cells17. Granulocytes lacking miR-223 are hypersensitive to activating stimuli17. Since NF-κB activity regulates the production of cytokines and chemokines in neutrophils in response to proinflammatory stimuli35, and NF-κB components are also necessary for regulation of granulocytic progenitors36, it is not unreasonable to speculate, based on our data, that some of the phenotypes of miR-223-deficient mice could be the result of increased noncanonical and decreased canonical NF-κB activity due to increased IKKα expression.

IKKα is a negative regulator of inflammation in mouse models of innate immune responses26,27. Macrophages from knock-in mice with inactive IKKα are hyperactive,

leading to increased bactericidal activity *in vivo* and *in vitro*, and an increased susceptibility of these mice to septic shock26. Likewise, cells from *Ikkα*$^{-/-}$ mice exhibit enhanced phagocytotic clearance of bacteria and increased expression of canonical NF-κB target genes, including proinflammatory cytokines and chemokines27. These studies are consistent with our data showing miRNA-mediated reduction in IKKα leads to increased expression of canonical NF-κB targets in stimulated and unstimulated cells. IKKα was proposed to contribute to canonical NF-κB activity suppression by accelerating the turnover and DNA binding of RelA and c-Rel26, or by preventing the hyperactivation of IKKβ by proinflammatory stimuli26,27. Our data does not exclude this possibility, however, we found that increased IKKα expression during macrophage differentiation was accompanied by the generation of p52 and its binding to DNA, leading not to gene activation, but to gene repression, perhaps as a p52 homodimer. Though there are cases where p52 and p50 homodimers, which lack transactivation domains, can activate gene expression in association with proteins such as Bcl-3 and IκBζ, normally p50 and p52 homodimers repress gene transcription1,2,37,38.

The binding of NF-κB dimers is complex, but highly adaptable to cellular situations. NF-κB dimers are associated with promoters only transiently (on the order of seconds), and therefore promoter-bound dimers are in dynamic equilibrium with nucleoplasmic dimers39. Consequently, the nature of cellular NF-κB dimers is particularly sensitive to the concentrations of a given NF-κB component. In our study, gene activation of the noncanonical NF-κB target gene, ELC, required activation of the macrophage by additional stimuli, and correlated with the production of RelB, which was undetectable in monocytes, and only expressed at very low amounts in unstimulated macrophages. This data is consistent with a recent study showing that production of RelB is a major contributing factor to noncanonical NF-κB dimer activation40.

Thus it seems likely that an increase in IKKα during the differentiation process serves two purposes: firstly, it acts as a brake that prevents hyperactivation of the new macrophage, perhaps in part by repressing basal levels of gene transcription through upregulation of p52 in the absence of RelB expression, and secondly, it provides for additional activation of noncanonical gene transcription when the cell receives further stimuli, transforming the excess p52 from a repressor to an activator of gene transcription in the presence of new RelB protein synthesis. A new macrophage is therefore not spontaneously activated, and only becomes fully committed to an inflammatory response with its full range of cytokine and chemokine gene expression when there is substantial stimulation. Our results imply that the decrease in certain miRNAs during differentiation leads to conditions where macrophages can adequately respond to infection, but where additional tissue damage or autoimmune conditions are prevented.

METHODS

Cell culture

U937 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin. Monocytes elutriated from human peripheral blood were obtain from Apheresis Research Institute, NIH

blood bank (following NIH protocol 99CC0168: Collection and Distribution of Blood Components from Healthy Donors for In-Vitro Use).The monocytes were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin. For cell differentiation, the cells were culture in present of recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF, 10ng/ml) for 7 days.

Reagents

Antibodies, anti-IKKα (#2682) and anti-NIK (#4994) were purchased from Cell signaling; anti-IKKβ (#05-535), anti-p100-p52 (#05-361) and anti-TRADD (05-473) were from Millipore-upstate; anti-IKKγ, (#557383) was from BD-Pharmingen; anti-TRAF2 (MAB3277) was from R&D; β-actin(A3853) was from Sigma. Anti-RelB (sc-48366) and anti-RelA (sc-372) were from Santa Cruz. Anti-CD14-FITC (#11-0149-73) and anti-CD1a-FITC (#11-0019-41) were from ebioscience. Anti-c-Rel (pc139) was from EMD-Calbiochem. C-terminal specific anti-p100 antibody was from the NCI Preclinical Repository, anti-serum #1310.

Mimics of microRNAs, hsa-miR-223 miRIDIAN Mimic (C-300580-07-0005), hsa-miR-15a miRIDIAN Mimic (C-300482-03-0005), hsa-miR-16 miRIDIAN Mimic (C-300483-03-0005) and MiRIDIAN microRNA Mimic Negative Control #1 (CN-001000-01-05) were purchased from Thermo-Dharmacon.

Inhibitors of microRNAs, hsa-miR-223 miRIDIAN Hairpin Inhibitor (IH-300580-08-0005), hsa-miR-15a miRIDIAN Hairpin Inhibitor (IH-300482-05-0005), hsa-miR-16 miRIDIAN Hairpin Inhibitor (IH-300483-05-0005) and miRIDIAN microRNA Hairpin Inhibitor Negative Control #1 (IN-001005-01-05) were purchased from Thermo-Dharmacon.

The siRNAs targeting IKKα (siGENOME SMARTpool M-003473-02-0005), IKKβ (siGENOME SMARTpool M-003503-03-0005), NIK (ON-TARGETplus SMARTpool L-003580-00-0005) and p100-p52 (ON-TARGETplus SMARTpool L-003918-00-0005) were purchased from Thermo-Dharmacon.

Phorbol-12-myristate-13-acetate (PMA, #524400) was from Calbiochem; rhGM-CSF (#215-GM), rhIL-4 (204-IL-050) and rhIL-6 (206-IL-050) were from R&D. DSP [Dithiobis (succinimidyl propionate)] (22585) was from Thermo-Pierce.

Immunoblot Analysis

Cells were collected and lysed in cell lysis buffer (20 mM Tris at pH 7, 0.5 % NP-40, 10 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 2 mM phenylmethyl sulfonyl fluoride (PMSF), 100μM Leupeptin, 10μM Bestatin, 10μM Pepstatin A, 2ug/ml Aprotinin), after spinning at 20,000×g for 20 minutes to remove the cells debris, the pellet was discarded. Cell lysates were separated by 4–20% SDS-Poly Acrylamide Gel Electrophoresis and analyzed by Immunoblot. Proteins were visualized by enhanced chemiluminescence according to the manufacturer's instruction (Thermo).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts of both monocytes and macrophages were isolated using the Biovision Nuclear-Cytosol fractionation kit (Cat#K266-100) following the manufactures guidelines. Gel shifts were performed with the Promega Gel shift assay system using 7μg of nuclear extracts and following the manufacturer's protocol. All consensus oligos were also purchased from Promega. Supershifts were done by adding 1 μg of antibody.

RNA isolation

Total RNA was extracted with TRIZOL® (Invitrogen) according to the manufacturer's guideline. Any remaining DNA was removed by using the DNA-*free*™ kit (Ambion) and repurified by RNeasy® kit (Qiagen), following manufacturer's protocol.

Reverse transcriptase-mediated semi-quantitative PCR (RT-PCR)

Total relative levels of IKKα mRNA were determined by RT-PCR using TITANIUM® one-step RT-PCR kit (Clontech). Sequence of IKKα: forward, 5′-TGAGGAACAGGGCAATAGTATGATG -3′. Reverse, GGTCTTACGCCCAAAAGTTAAAAGT-3′

Real Time PCR

For gene expression experiments, reverse transcription of 200ng (5μg in experiments involving noncanonical and canonical target expression) of total RNA was first reverse transcribed with poly dT(20) oligo as a primer using Invitrogen's Superscript® III reverse transcription system in 40μL reactions at 50° C for 1 hr. Individual Taqman® real time gene expression assays (Applied Biosystems) were performed according to manufacturers protocol in quadruplicate from 4 μl (total) of the reverse transcription reaction and run on an ABI StepOnePlus machine. Data was analyzed using the StepOnePlus software, and gene expression was normalized to GAPDH levels. miRNA quantitation was done similarly using Applied Biosystem Taqman® reagents and protocols, with the exception that reverse transcription was done with their associated miRNA assay primers using the Taqman® miRNA transcription kit and protocol, and assays were performed in triplicate using twice the suggested amounts of template cDNA. The levels of miRNA were normalized to RNU48 levels.

The following MGB assays from Applied Biosystems were used for gene expression analysis: GAPDH, Hs02786624_g1; ELC, Hs00171149_m1; BLC, Hs00757930_m1; SLC, Hs00171076_m1; SDF-1, Hs00171022_m1; A20, Hs00234713_m1; ICAM, Hs00164932_m1; CCL4, Hs00605740_g1;IL-10, Hs00961620_g1. For miRNA quantitation, the following Applied Biosystems Taqman® MicroRNA assays were used: miRNA-15, 000389; miRNA-16, 000391; miRNA-223, 001006; miRNA-142-5p, 002248; let-7, 002619; RNU48, 001006. Two IKKα assays were custom ordered from Applied Biosystems. Both gave similar patterns of gene expression, however data is only included in this manuscript for this probe and primers: Probe: (6FAM)AGGCCTTTTTTGCATCATCTGCAGCC(MGB)(NFQ) IKK primers: Forward, 5′-

CACTGTTGATTATTGGAGCTTTGG-3′; Reverse, 5′-CACTTTGGATCCTTCTTCTTAATCTTC-3′

Luciferase assay

Luciferase assays were done using Promega's luciferase assay system. HeLa cells plated in 12-well plates were transfected using Lipofecamine 2000 (Invitrogen) with 50 ng of the given CMV-driven pMIR luciferase IKKα 3′reporter plasmid, 50 ng of RSV β-galactosidase plasmid, and 15 pmoles of each given miRNA mimic or an identical amount of control mimic. After 48 hours, cells were harvested in 80 μL of Reporter Lysis Buffer and 5 μL was assayed for luciferase activity in a luminometer according to the manufacturers protocols. β-galactosidase activity was assayed from 5 μL cell lysate using luminescent detection in a luminometer after 30 minutes of incubation with Applied Biosystem's Tropix® Gal-Screen™ substrate. Each luciferase reading was normalized to the β-galactosidase activity of the same lysate. Each transfection was performed in triplicate; averaged values were normalized with the highest luciferase/β-galactosidase mean set at 1 for each experiment.

Electroporation

Monocytes and macrophages were transfected with AMAXA Nucleofector II and the supplied kits (Lonza VPA-1007 and VPA-1008, respectively) following the manufacturer's instruction. Following electroporation, monocytes were cultured in the kit supplied monocytes growing medium for 24 hours and the macrophages were culture in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2mM glutamine for 48 hours before harvesting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the NCI Preclinical Repository for the p100 C-terminal antibody and the NIH blood bank for providing the elutriated monocytes. This research was supported by the Intramural Research Program of The Center for Cancer Research, NCI, NIH.

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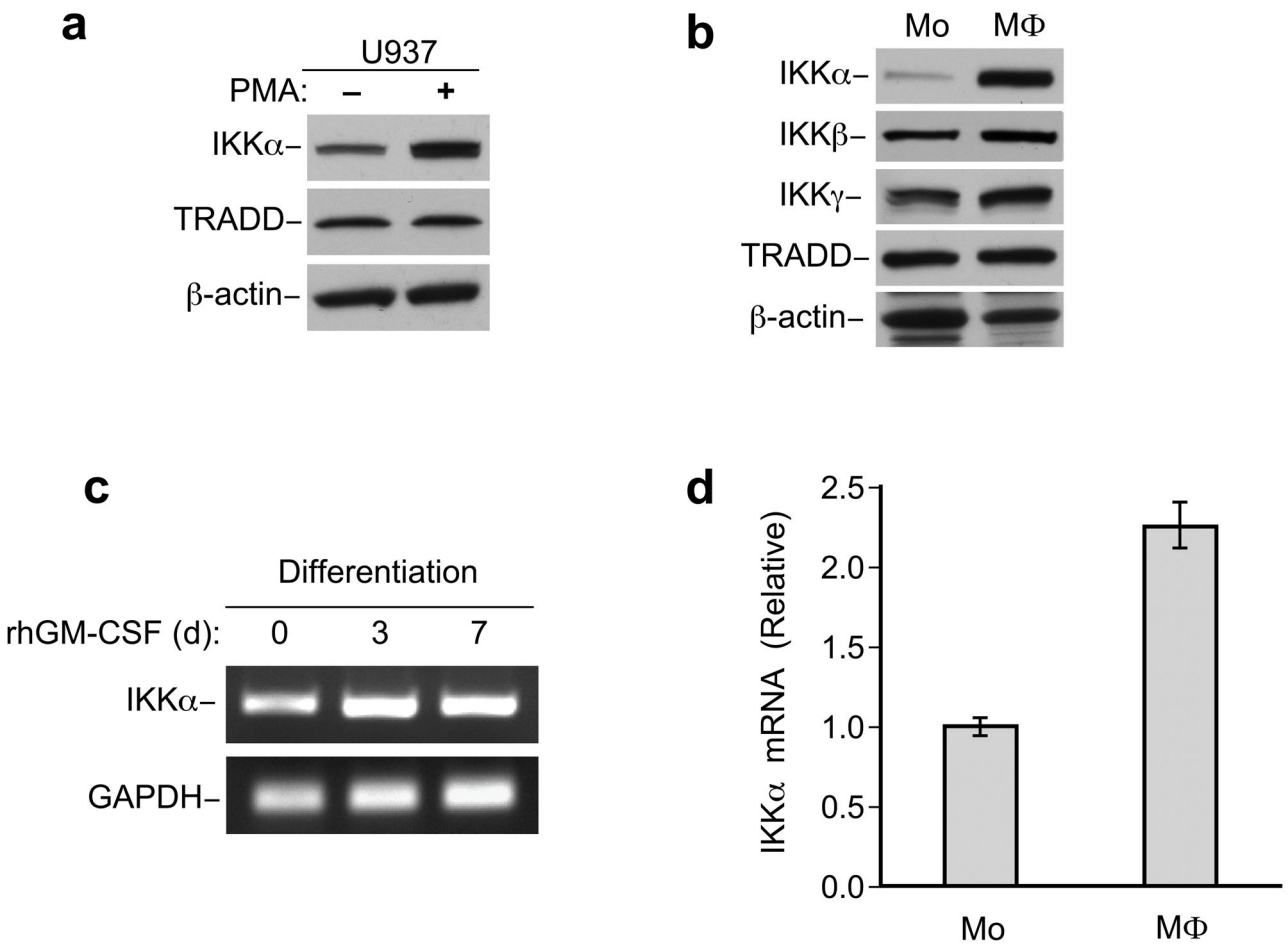

Figure 1. IKKα is upregulated during monocyte differentiation

(a) Immunoblot analysis of IKKα in U937 vs. PMA-differentiated U937 cells. **(b)** Immunoblot analysis of IKKα IKKβ and IKKγ in monocytes (Mo) vs. macrophages (MΦ). TRADD and β-Actin blots indicate loading of lanes **(a, b). (c)** Semi-quantitative RT-PCR analysis the IKKα mRNA level of monocytes cultured with rhGM-CSF for different days as indicated, GAPDH mRNA as control in this assay. **(d)** Quantitative Real time PCR analysis of IKKα mRNA in monocytes (Mo) vs. macrophages (MΦ), the relative IKKα level was normalized to GAPDH. Error bars, +/− standard deviation from the mean.

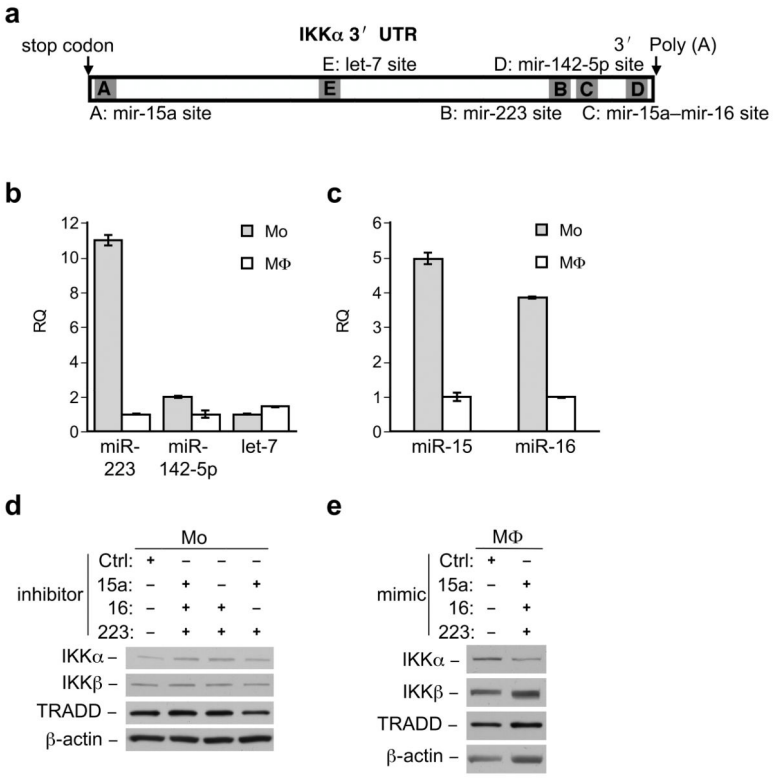

Figure 2. MicroRNAs target the IKKα mRNA

(a) Distribution of predicted target sites for five relevant microRNAs in 3″ UTR of IKKα. **(b, c)** Real time PCR analysis of different microRNAs in monocytes (Mo) vs. macrophages (MΦ) as indicated. Error bars, +/− standard deviation from the mean. Data representative of at least 5 independent experiments **(d)** Monocytes lysates (Mo) from cells transfected with miRNA inhibitors containing control oligo, pooled inhibitors for microRNAs 15a, 16 and 223 or the combinations thereof as indicated (48 hours after transfection) immunoblotted with IKKα and IKKβ antibodies. **(e)** Cell extracts from macrophages (MΦ) transfected with microRNAs mimic control oligo or pooled mimics immunoblotted with anti-IKKα and anti-IKKβ antibodies. TRADD and β-actin blots indicate loading of lanes **(d, e)**

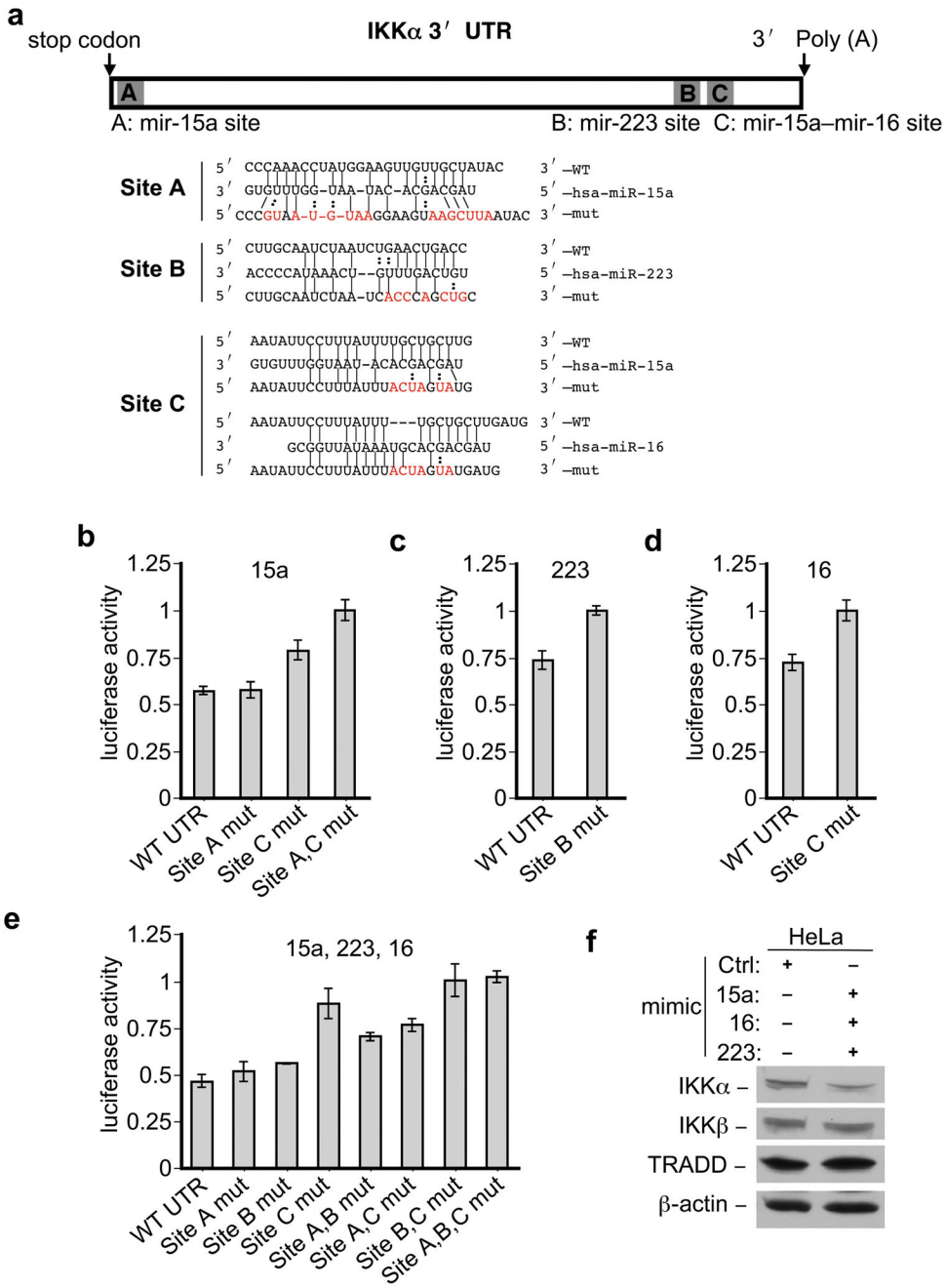

Figure 3. Mutations of microRNAs targeting sites attenuate the inhibitory effect of microRNAs mimics

(a) Schematic representation of the IKKα mRNA 3′ untranslated regions from CMV luciferase constructs used for miRNA reporter assays, showing predicted pairing with the target sites and their respective mutant sequences. **(b),(c),(d),(e),** Luciferase reporter assays done in HeLa cells transfected with the IKKα 3′UTR reporter constructs indicated, as illustrated in (**a**). miRNA mimics (as indicated) or a control mimic were cotransfected with the reporter construct and RSV β-galactosidase. Luciferase values (obtained 48 hrs post transfection) were normalized to β-galactosidase activity and also to the average values

obtained for each control mimic for each corresponding plasmid, with the highest ratio set at 1. Data is from representative triplicates. Error bars, +/− standard error of the mean. (**f),** Western blots of cell extracts from HeLa cells transfected in the previous experiment **(e)** with miRNAs mimic control oligo or pooled mimics immunoblotted with anti-IKKα and anti-IKKβ antibodies. TRADD and β-actin blots indicate loading of lanes.

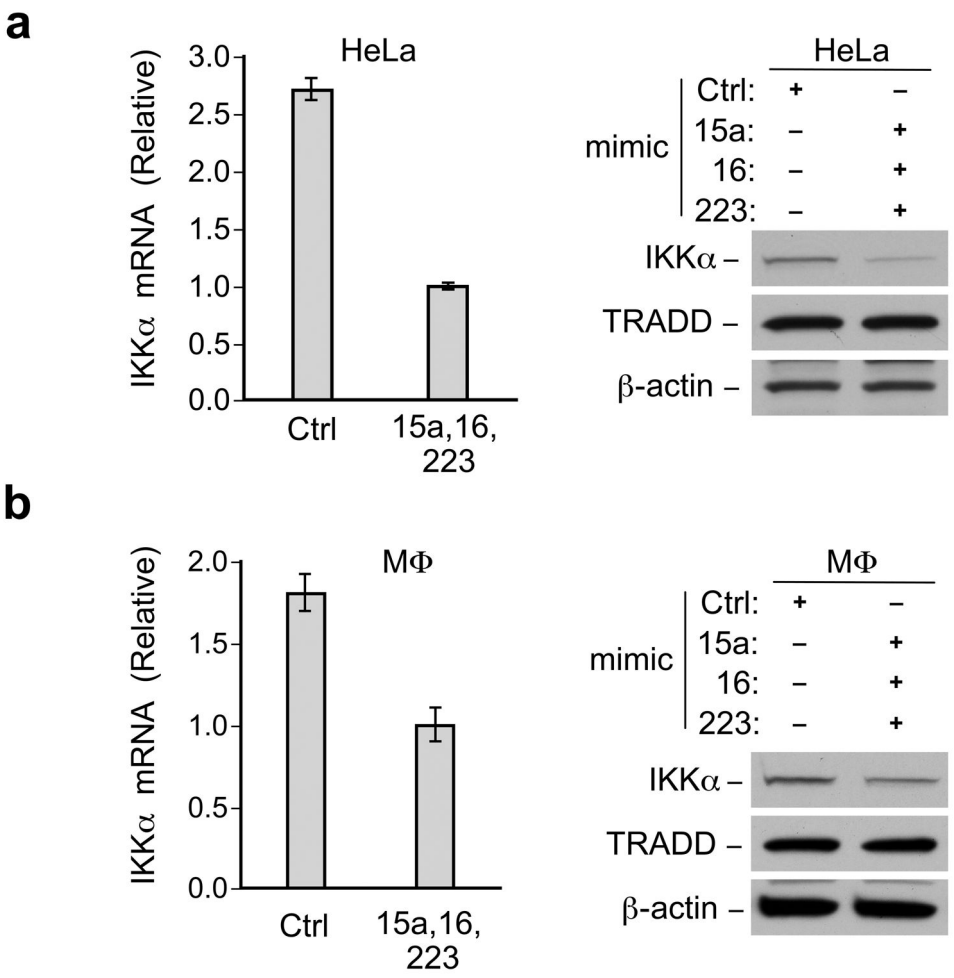

Figure 4. MicroRNAs mimics reduce IKKα mRNA stability

mRNA and protein was isolated from HeLa cells **(a)** or macrophages (MΦ), **(b)** transfected with control mimic or pooled miRNA mimics. IKKα mRNA was measured by real time PCR (left) and protein level of IKKα was analyzed by immunoblot (right). mRNA levels were normalized to GAPDH mRNA. Data representative of at least 5 independent experiments. Error bars, +/−standard error of the mean. TRADD and β-actin immunoblots indicate loading of lanes.

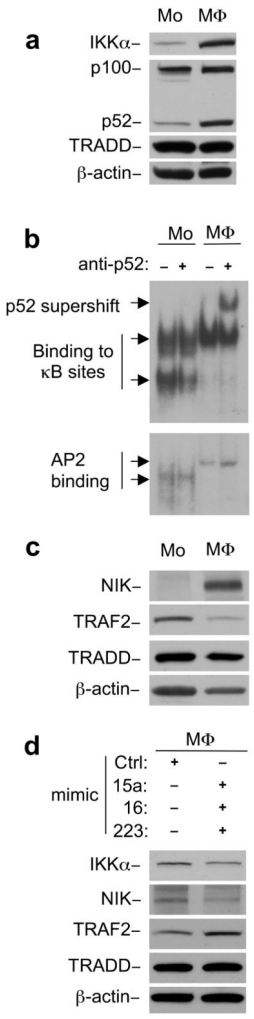

Figure 5. p100 is constitutively processed to p52 in macrophages

(a) Immunoblot analysis of IKKα and p100-p52 in monocytes (Mo) vs. macrophages (MΦ). **(b)** Electrophoretic Mobility Shift Assay (EMSA) from nuclear lysates of monocytes (Mo) and macrophages (MΦ) showing binding to κB or AP2 oligos. p52 antibody added where indicated. **(c)** Western blots from monocytes (Mo) and macrophages (MΦ) lysates analyzed by immunoblot with anti-NIK and anti-TRAF2 antibodies. **(d)** Macrophages (MΦ) were transfected with miRNA mimic control oligo and pooled miRNAs mimics, (miR-15a, miR16 and miR-223). Forty-eight hours after transfection, cell lyates were analyzed by immunoblot with indicated antibodies. TRADD and β-actin blots indicate loading of lanes, **(a, c, d).**

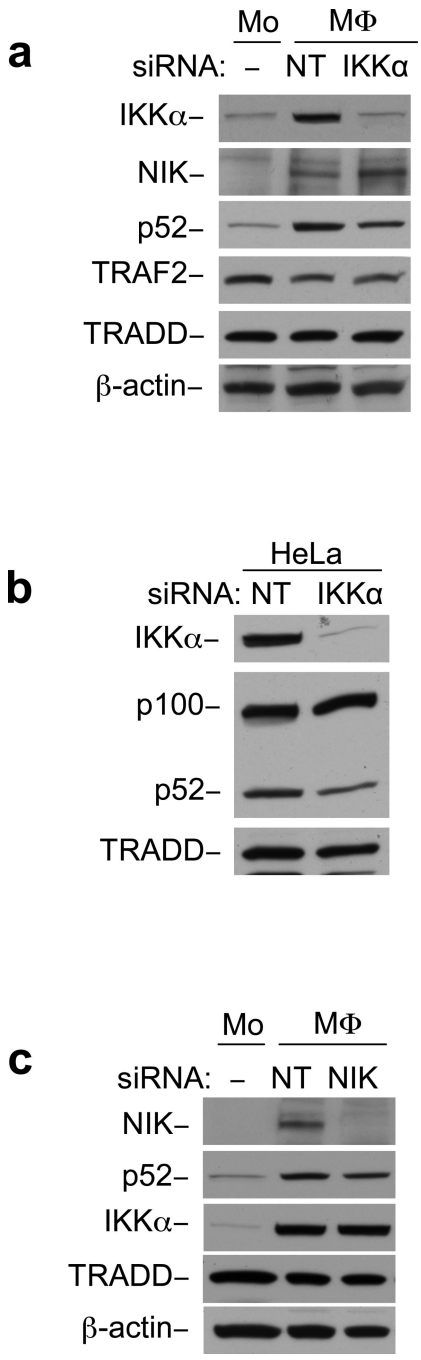

Figure 6. siRNA knockdown reveals the relative contribution of IKKα and NIK to p52 levels

(a) Monocyte lysate (Mo) and the lysates from Macrophages (MΦ) transfected with non-targeting siRNA (NT) or IKKα siRNA were analyzed by immunoblot with indicated antibodies. **(b)** HeLa cells were transfected with non-targeting siRNAs (NT) or IKKα siRNA. Cell lysates were analyzed 48 hours after transfection by immunoblot with indicated antibodies. **(c)** Monocyte lysate (Mo) and the lysates from Macrophages (MΦ) transfected with non-targeting siRNA (NT) or NIK siRNA were analyzed by immunoblot with indicated antibodies. TRADD and β-actin blots indicate loading of lanes, **(a, b, c)**.

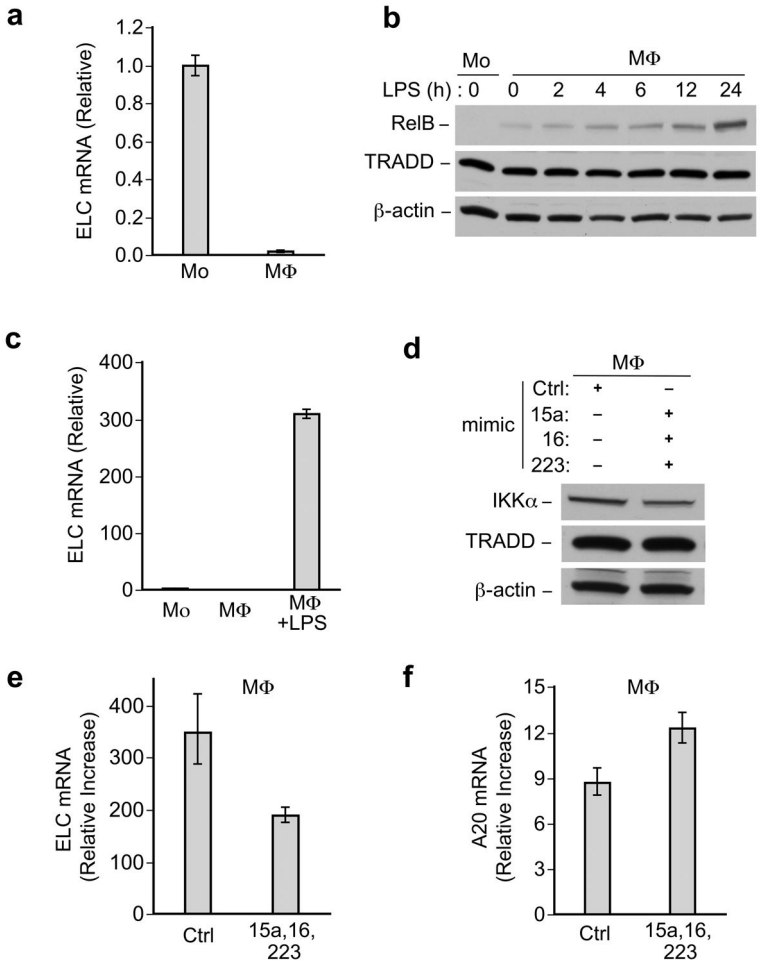

Figure 7. IKKα-targeting miRNAs affects noncanonical and canonical NF-κB gene expression

(a) Real time PCR analysis of ELC mRNA in monocytes and macrophages, normalized to GAPDH, data representative of at least three independent experiments. Error bars, +/−standard deviation of the mean. **(b)** Anti-RelB immunoblots of lysates from macrophages (MΦ) treated with LPS for indicated time course (in hours). **(c).** Real time PCR analysis of ELC mRNA in monocytes, macrophages, or LPS-treated macrophages, normalized to GAPDH. Data representative of at least three independent experiments. Error bars, +/−standard deviation of the mean. **(d)(e)(f),** Macrophages (MΦ) were transfected with miRNA mimics or control mimic and 24 hours after transfection, cells were or were not challenged with LPS (1μg/ml) for another 24 hours. **(d)** Immunoblot showing IKKα protein level in corresponding protein samples for **(e, f). (e)** ELC and **(f)** A20 mRNA expression as measured by real time PCR, normalized to GAPDH mRNA. The relative fold increase in LPS treated cells compared to untreated cells is shown for **(e)** and **(f)**. Error bars, +/− range based on standard deviation of the mean. Data representative of at least three independent experiments. TRADD and β-actin blots indicate loading of lanes, **(b, d).**