Differential activation and functional specialization of miR-146 and miR-155 in innate immune sensing

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ABSTRACT

Many microRNAs (miRNAs) are co-regulated during the same physiological process but the underlying cellular logic is often little understood. The conserved, immunomodulatory miRNAs miR-146 and miR-155, for instance, are co-induced in many cell types in response to microbial lipopolysaccharide (LPS) to feedback-repress LPS signalling through Toll-like receptor TLR4. Here, we report that these seemingly co-induced regulatory RNAs dramatically differ in their induction behaviour under various stimuli strengths and act non-redundantly through functional specialization; although miR-146 expression saturates at sub-inflammatory doses of LPS that do not trigger the messengers of inflammation markers, miR-155 remains tightly associated with the pro-inflammatory transcriptional programmes. Consequently, we found that both miRNAs control distinct mRNA target profiles; although miR-146 targets the messengers of LPS signal transduction components and thus downregulates cellular LPS sensitivity, miR-155 targets the mRNAs of genes pervasively involved in pro-inflammatory transcriptional programmes. Thus, miR-155 acts as a broad limiter of pro-inflammatory gene expression once the miR-146 dependent barrier to LPS triggered inflammation has been breached. Importantly, we also report alternative miR-155 activation by the sensing of bacterial peptidoglycan through cytoplasmic NOD-like receptor, NOD2. We predict that dose-dependent responses to environmental stimuli may involve functional specialization of seemingly co-induced miRNAs in other cellular circuitries as well.

INTRODUCTION

MicroRNAs (miRNAs) constitute an abundant class of small non-coding RNAs of ∼22 nt in size that act as post-transcriptional regulators of gene expression. A single miRNA can modulate the expression of dozens of mRNAs, typically through short seed pairing within the 3′ untranslated region (UTR), leading to translational inhibition and/or degradation of the targeted messenger (1). Of the several hundred known miRNAs, many seem to have partially overlapping functions because of the conservation of the seed within a given miRNA family, and, therefore, similar target profiles. Alternatively, evolutionary unrelated miRNAs may be activated by the same transcription factor and thus overlap in their activity in a given cellular pathway. Primary examples of the latter are miR-146 and miR-155, both of which are activated in the antimicrobial response of mammalian macrophages. Although mir-146 and miR-155 are each conserved in sequence down to the bottom of the vertebrate lineage (2), they do not show sequence similarity. Moreover, they arise from different genomic loci through different routes of processing, that is, from a pre-mRNA (miR-146a), an mRNA intron (miR-146b) or an exon of a long non-coding RNA (miR-155); yet, wherever studied, the synthesis of these miRNAs has been observed to crucially depend on the pro-inflammatory transcription factor NFκB (3–6).

The co-activation of miR-146 and miR-155 during the inflammatory response was initially revealed in human monocytes (3) through a seminal screen for miRNAs that were regulated by the bacterial component, lipopolysaccharide (LPS), which is primarily sensed by Toll-like receptor 4 (TLR4). Stimulation of TLR4 triggers the nuclear translocation of NFκB, resulting in transcriptional activation of NFκB target genes encoding various mediators of inflammation, such as cytokines, acute phase proteins and inducible enzymes (7). Subsequent findings that both miR-146 and miR-155 targeted mRNAs in the signalling cascade downstream of TLR4 bolstered the link with NFκB-regulated innate immunity, leading to a model in which these two miRNAs facilitate a negative-feedback loop that may protect from an excessive TLR4 response.

The stimulation of TLR4 is signalled through two parallel cascades beginning with the TLR adaptors, MyD88 and TRIF, as reviewed by Akira and Takeda (8), and both these branches contain targets of miR-146

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and miR-155. The MyD88-dependent cascade signals through IRAK1 and TRAF6, which are targets of miR-146 (3). Assisted by TAB2, which is regulated by miR-155 (9), the MyD88 branch eventually activates the IKK and MAPK pathways, resulting in nuclear translocation of NFκB and the activation of transcription factor AP-1, respectively. Together, NFκB and AP-1 induce the expression of major pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, which mediate autocrine/paracrine amplification of the NFκB response through TNF-receptor signalling. Through TRAF6, the MyD88 cascade also activates NIK kinase, thereby amplifying the production of pro-inflammatory prostaglandins (10). Finally, TRAF6 and TAB2 are crucial for an alternative route of TLR4 signalling through TRIF, in which the induction of the IRF3/7 transcription factors (through TBK1 and IKKε) culminates in the production of type I interferons. This bottom part of the cascade may also be subject to feedback control by miRNA, as IKKε has been predicted to be a target of miR-155 (11,12).

Although the above targets may favour a one-tier model wherein the seemingly co-regulated miR-146 and miR-155 invariably act to feedback regulate TLR4 signalling, other observations hint at more specialized functions. For instance, miR-155 targets Ship-1, a negative modulator of TLR signalling (13), and thus also increases TLR4 activity. Likewise, miR-155 stabilizes the mRNA of the pro-inflammatory cytokine TNF-α, which will also regulate the NFκB response (11,14). Phenotypic studies have also indicated a potential specialization, suggesting that miR-146 and miR-155 might control the inflammatory response at distinct levels. That is, miR-155 knockout mice are unable to initiate an adaptive immune response to *Salmonella typhimurium*, likely at the level of dendritic cell function (15), arguing that miR-155 primarily promotes pro-inflammatory responses. In contrast, sustained miR-146 expression is critical for the maintenance of innate immune tolerance in mice (16,17).

Here, we report on the dose-dependent activation of miR-146 and miR-155 in macrophages in response to several inflammatory stimuli. Surprisingly, we found dramatic differences in how LPS activated these two miRNAs, showing that miR-146 is activated at sub-inflammatory levels of NFκB activity, whereas miR-155 is gradually induced to full expression at pro-inflammatory magnitudes of NFκB activity. Accordingly, endotoxin-tolerant cells with mute TLR-based immunity only activate miR-146 and not miR-155 to feedback regulate TLR signalling. We also present the first evidence that cytosolic sensing of microbial peptidoglycan by a NOD-like receptor (NLR) can rescue activation of miR-155 through a TLR-independent pathway. Moreover, we show that miR-155 represses the TNF-α autocrine/paracrine signal amplification route, which is an integral part of the macrophage pro-inflammatory response. Collectively these observations suggest a general two-tier model in which miR-155 acts as a stringent regulator of pro-inflammatory responses once an miR-146-dependent barrier to TLR-induced inflammation is breached. This provides a general example of the functional specialization of seemingly co-induced miRNAs that act in the same cellular pathway.

MATERIALS AND METHODS

Culture, stimulation and transfection of cells

Mouse embryonic fibroblast (MEF), RAW264.7 and RAWBlue[TM] (Invivogen) cells were cultured in RPMI 1640 (Gibco), 10% of fetal bovine serum (Biochrom), 2 mM of L-glutamine (Gibco), 1 mM of sodium-pyruvate (Gibco), 0.1% of β-mercapto-ethanol (Gibco), 1% of penicillin/streptomycin solution (Gibco) at 37°C, 5% of CO_2 in a humidified atmosphere. Endotoxin tolerance was induced as described previously (25) but using LPS (1 µg/ml) instead of heat-killed *Salmonella*. For all purposes, cells were seeded into 24-well plates (Corning) at a density of 5×10^4 cells per 0.5 ml medium 2 days before further treatment, if not specified differently. *S. typhimurium* LPS (Sigma, # L6143), bacterial lipoprotein (BLP, Invovogen, # tlrl-pms) and TNF-α (R&D, # 410-MT) were added directly into the medium, whereas γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) (Invivogen, # tlrl-dap), N-acetyl-muramyl-L-Ala-γ-D-Glu-meso-diaminopimelic acid (M-TriDAP) (Invivogen, # tlrl-mtd) and N-acetyl-muramyl dipeptide (MDP) (Invivogen, #tlrl-mdp) were transfected using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For co-stimulation, LPS or TNF-α were added into the culture medium right on the addition of the lipofection mix. For ectopic miRNA, expression cells were lipofected as aforementioned for 12 h, with Pre-miR[TM] synthetic precursor molecules (Ambion, control: # AM17110, mmu-miR-146: equimolar mixture of # PM10722 and # PM10105, mmu-miR-155: # PM13058) 24 h on seeding. The same protocol was applied for inhibition of miRNA activity using but LNA[TM] antisense probes instead (Exiqon, control: # 199004-08, miR-146: equimolar mix of # 41063-08 and # 410066-08, miR-155: # 411222-08). Luciferase reporter plasmids were transfected into MEF cells 24 h on seeding at 500 ng per 24-well along with the Pre-miR[TM] molecules as aforementioned using lipofectamine 2000. On 12 h of incubation, liposomes were removed, and luciferase activities were determined another 24 h later. RAW264.7 cells were transfected 24 h on seeding with 1 µg of luciferase reporter plasmid per well using lipofectamine 2000. After 12 h of incubation, liposomes were removed, and cells were allowed to recover from immunogenic side-effects of microbial plasmid DNA for 48 h. To prevent from growing too densely, cells were then disattached and seeded into new 24-well plates, followed by the routine 48 h of incubation before further treatment. Bone marrow-derived macrophages (BMDMs) were generated from the bone marrow of female C57/BL6 mice. Briefly, a bone marrow suspension of 5×10^6 cells in 10 ml of BMDM medium (X-Vivo 15, Lonza, # BE04-744Q, supplemented with 10% of FBS and 10% of L929 conditioned medium) were seeded into 10-cm Petri dishes (Corning). On incubation for 5 days, cells were seeded into 24-well plates (2×10^5 cells per well) incubated for 48 h and treated with various doses of LPS for 8 h.

Reporter assays

RAWBlue[TM] NFκB-reporter activity was determined colorimetrically using QuantiBlue[TM] reagent (Invivogen)

according to the manufacturer's instructions in transparent 96-well flat-bottom plates (Corning). The read-out was performed at 630 nm on an Ascent Plate Reader (Thermo Scientific).

3′ UTR luciferase reporter assays were performed using the pSICHECK2^{TM} dual luciferase reporter system (Promega). Cell lysates were generated using passive cell lysis buffer (Promega) according to the manufacturer's instructions. Firefly and *Renilla* luciferase activities were recorded on a Wallac 1420 Victor3 plate reader (Perkin Elmer) in white non-transparent 96-well flat-bottom plates (Perkin Elmer) using 40 µl pH 5 adjusted Beetle-juice (PJK GmbH) followed by 40 µl *Renilla*-juice (PJK GmbH) per well.

Real-time polymerase chain reaction

For real-time polymerase chain reaction (PCR) analysis, cellular total RNA was extracted using TRIzol reagent (Invitrogen). The messengers of Ptgs2, IL-6 and TNF-α were detected using the Power SYBR Green RNA-to-CT^{TM} 1-Step kit (Applied Biosystems) and primer pairs listed in Supplementary Table S1. β-Actin mRNA was used as a normalization control and was detected with the primer pair published in (18). Mature and primary miRNA transcripts were detected using TaqMan^{TM} pre-designed assay kits (Applied Biosystems, mature miR-146a: # 000468, mature miR-146b: # 001097, mature miR-155: # 001806, pri-miR-146a: # Mm03306349_pri, pri-miR-146b: # Mm03308117_pri, pri-miR-155: # Mm03306395_pri), with snoRNA202 (# 001232) as a normalization control according to the manufacturer's instructions. All real-time PCR experiments were carried out on a Biorad CFX96 machine, and fold changes in gene expression were deduced from CT-values according to the $2^{(-\Delta\Delta CT)}$ method (19).

Cloning

For cloning of pSICHECK2^{TM} (Promega) reporter constructs candidate, 3′ UTRs were amplified from mouse cDNA according to current ENSEMBL annotations, using the primer pairs listed in Supplementary Table S1. All UTRs were cloned using the XhoI and NotI restriction sites of the pSICHECK2^{TM} plasmid. Mutations were introduced by mutagenesis PCR using the primer pairs listed in Supplementary Table S1. Briefly, the respective reporter plasmid was amplified by regular PCR (15 cycles) using a primer pair with a ∼12–16-nt 5′ overlap harbouring the desired mutations. On incubation of the 50 µl reaction mixture with 1 µl DpnI at 37°C for 3 h, 5 µl was transformed directly into competent *Escherichia coli* TOP10. DNA sequences were validated by Sanger sequencing (SMB services). All PCR reactions were carried out with Phusion^{TM} High-Fidelity DNA Polymerase (Thermo Scientific) according to the manufacturer's instructions. Endotoxin-free plasmid preparations for eukaryotic cell culture were obtained using Highspeed Maxi Prep^{TM} kits (Qiagen) according to the manufacturer's instructions.

Statistical tests

Observed differences between two treatments were tested for significance using a two-tailed student's *t*-test, assuming either equal or different variances where appropriate. *P* values of <0.05 were considered as a measure for statistical significance.

RESULTS

Primary association of BIC/miR-155 with inflammation

Previous studies assigned discrete functions to miR-146 and miR-155, but the degree of co-regulation and potential redundancy of these miRNAs in the LPS response remained poorly understood. We determined the induction of the primary and mature miR-146a, miR-146b and miR-155 transcripts by increasing LPS doses in a 0–1 mM range in RAW264.7 murine macrophages. As a positive control for pro-inflammatory transcription, we also profiled the classical inflammation markers, Ptgs2, IL-6 and TNF-α; these genes showed the expected gradual induction (20), reaching high expression levels at 1 mM of LPS (Figure 1A). The same gradual increase that typically marks NFκB-dependent transcription (21) was also observed for both BIC, the host gene of miR-155 (Figure 1C) and the mature miR-155 (Figure 1B), which tightly links miR-155 expression to the pro-inflammatory transcription programme. In sharp contrast, the activation of miR-146a and miR-146b not only saturated at much lower LPS doses but also occurred in a narrow window between 0.1 and 1 ng LPS per ml (Figure 1B); this regulation is likely to be at the level of transcription because it is also observed at the level of the primary miR-146 transcripts (Figure 1C). Of note, these low concentrations of LPS only modestly induce the inflammatory response (Figure 1A), suggesting that miR-146 expression is exceptionally responsive to even minute amounts of this TLR4 ligand.

To confirm that TLR4 indeed accounts for the differential induction of miR-146 and miR-155, we quantified miRNA induction in wild-type and TLR4-deficient murine BMDMs challenged with increasing doses of LPS. Generally, the thresholds of miR-146 and miR-155 induction in response to LPS were lower in wild-type BMDMs (Figure 2A) compared with RAW264.7 macrophages (Figure 1), demonstrating that the exceptional sensitivity of miR-146 to LPS is even more pronounced in primary cells. Similar to RAW264.7 cells, however, miR-146a and miR-146b were triggered at much lower concentrations of LPS (0.1 ng LPS per ml) than miR-155 (saturating at 10 ng LPS per ml). In TLR4 deficient BMDMs (Figure 2B), saturating miR-146a and miR-146b induction required a ∼100-fold increase of the LPS dose compared with wild-type BMDMs, whereas miR-155 expression did not reach saturation over the tested range. This argues that the induction of miR-146 at minute concentrations of LPS and the pronounced activation of miR-155 at high concentrations are both mediated by TLR4. Next, we determined the kinetics of induction of miR-146a/b and miR-155 at a fixed concentration of 1 mM LPS for 24 h in RAW264.7 macrophages and observed a full
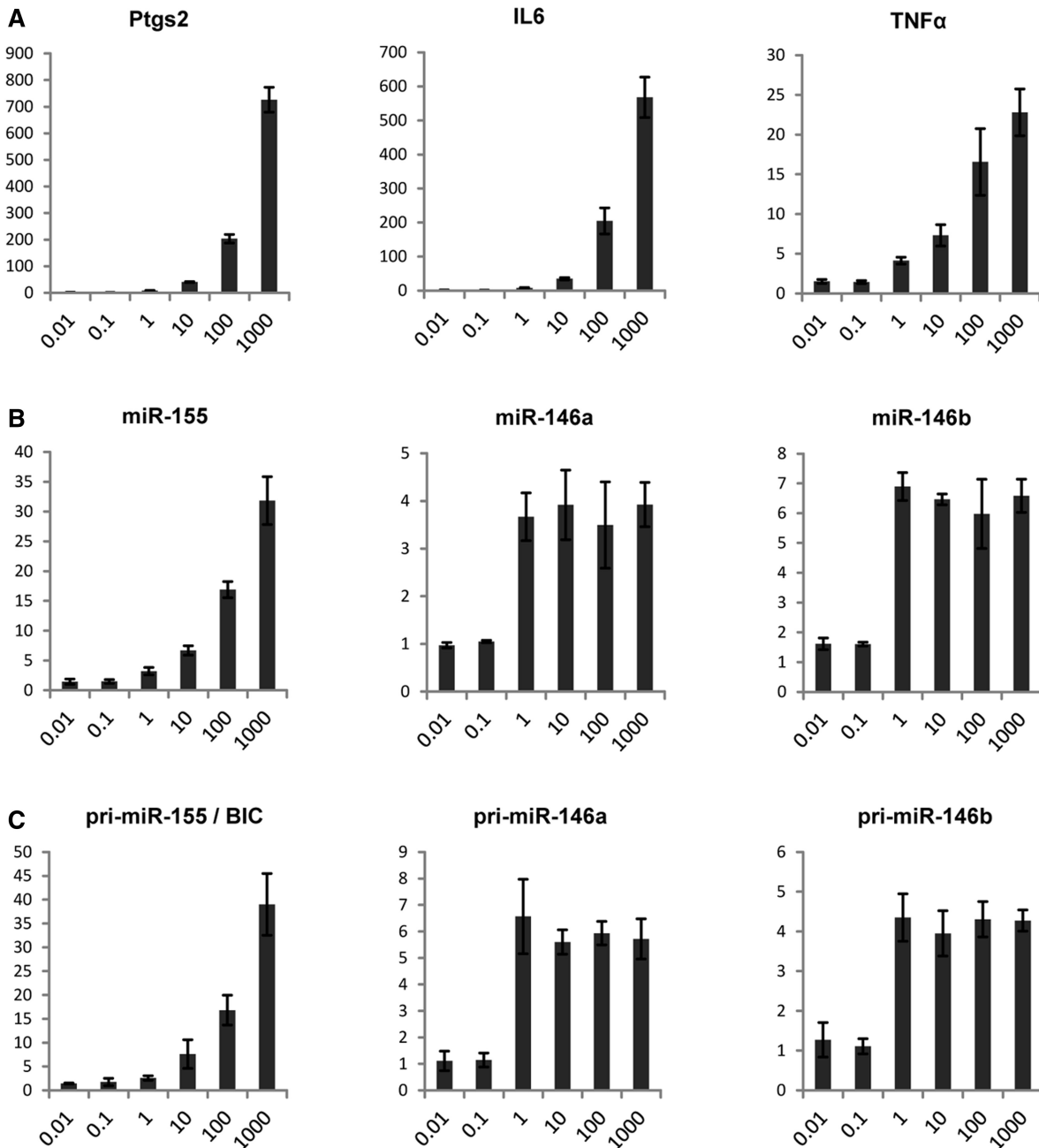
Figure 1. Expression analysis of inflammation markers and miRNAs in RAW264.7 macrophages challenged with the indicated doses of LPS (x-axis: ng/ml) for 24 h. Expression of inflammation markers Ptgs2, IL-6 and TNF-α (**A**), mature miR-146a, miR-146b and miR-155 (**B**) and the primary transcripts of miR-146a, miR-146b and miR-155 (**C**) was monitored by quantitative reverse transcriptase (qRT)-PCR. All results are presented as fold-changes (y-axis) relative to 24 h control-treatment. Mean values and standard deviations from three independent experiments are shown.

concordance with the time-dependent upregulation of Ptgs2, IL-6 and TNF-α (Supplementary Figure S1). Thus, irrespective of similar induction kinetics in macrophages challenged with high LPS doses, miR-146 and miR-155 are in fact activated in a two-tier manner, responding to sub-inflammatory (miR-146) and pro-inflammatory (miR-155) levels of TLR4 stimulation, respectively.

Selective control of TNF-α signalling by miR-155

Given that only miR-155, and not miR-146, was co-regulated with pro-inflammatory genes (Figure 1), we

hypothesized that miR-155 might specifically regulate a signalling event that occurs after LPS sensing has prompted NFκB translocation to the nucleus and has successfully activated inflammatory gene transcription. A candidate pathway was the autocrine/paracrine signalling through TNF-α that serves to amplify or sustain NFκB activity. To test this hypothesis, we transfected a RAW264.7 macrophage clone harbouring an NFκB-responsive secreted alkaline phosphatase reporter transgene (RAWBlue[TM]) with synthetic miR-146 or miR-155 mimics, and monitored their impact on NFκB activity on
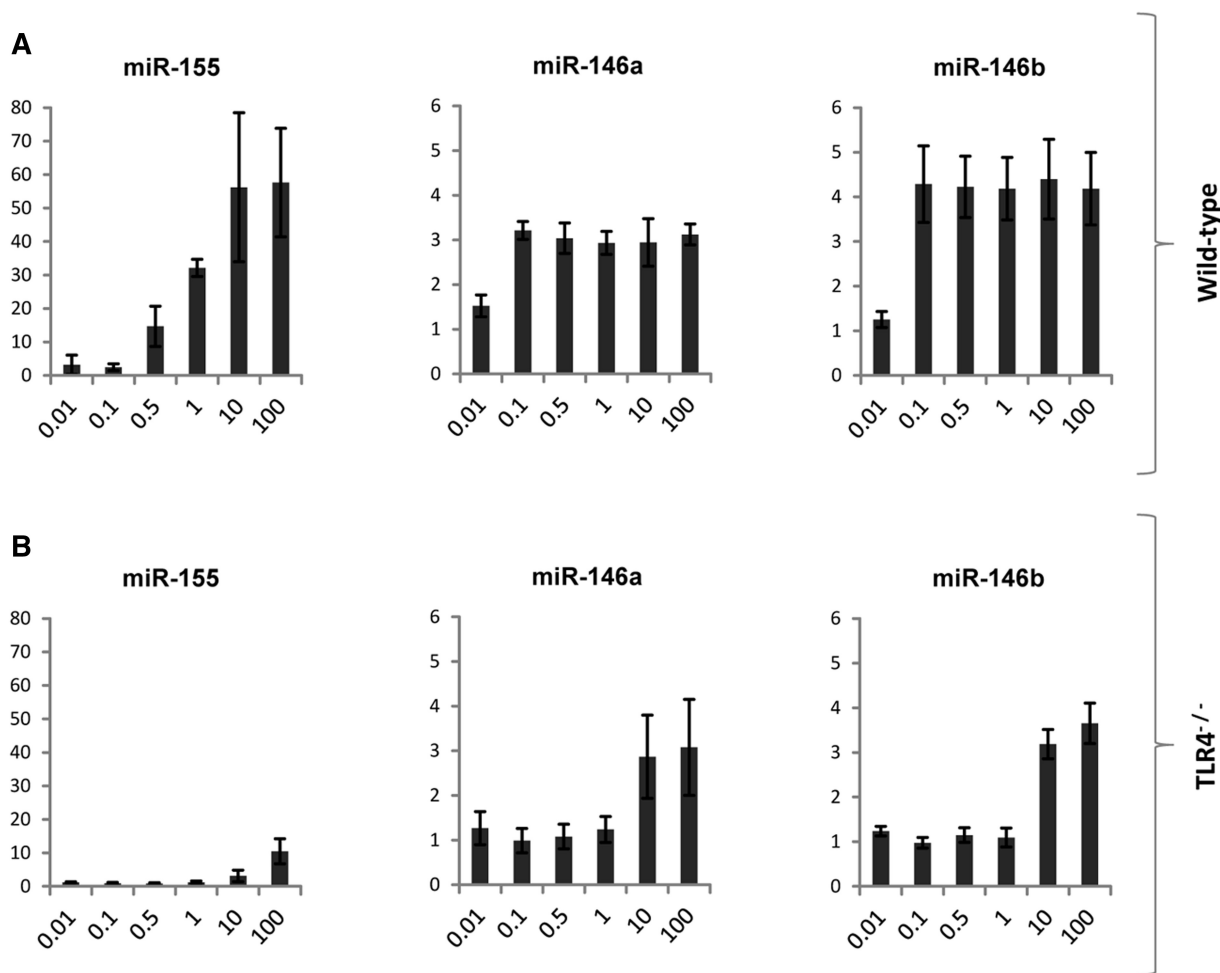
Figure 2. Expression analysis of mature miRNAs in wild-type and TLR4$^{-/-}$ BMDMs challenged with the indicated doses of LPS (x-axis: ng/ml) for 8 h. Expression of mature miR-146a, miR-146b and miR-155 in wild-type BMDMs (**A**) or TLR4$^{-/-}$ BMDMs (**B**) was monitored by qRT-PCR. Results are presented as fold-changes (y-axis) relative to 8 h control-treatment. Mean values and standard deviations from three independent experiments are shown.

stimulation with TNF-α (0.2 mM) or LPS (1 mM). As expected from previous reports (22), we observed that both miR-146 and miR-155 were able to reduce LPS-induced NFκB activity (Figure 3A). Importantly, however, when TNF-α was used as a stimulus, only miR-155 counteracted the upregulation of the NFκB reporter gene (Figure 3B). In the converse approach, that is, sequestration of the endogenous miRNAs with locked nucleic acid (LNA)-based inhibitors, again both miR-146 and miR-155 impacted NFκB activity after induction with LPS (Figure 3C), whereas the stimulation with TNF-α was selectively regulated by the miR-155 inhibitor (Figure 3D). In all of these cases, the simultaneous over-expression or inhibition of miR-146 and miR-155 mimicked the phenotype of miR-155 alone, suggesting that miR-155 acts upstream of miR-146 in the control of NFκB activity. Taken together, in line with their expression dynamics, both miR-146 and miR-155 control the primary TLR4-induced NFκB activation, whereas the feedback control of subsequent paracrine/autocrine-induced pro-inflammatory signalling through TNF-α is a unique domain of miR-155.

Distinct target regulation of miR-155 and miR-146 in the LPS response

Next, to better understand the two-tier function of miR-146 and miR-155 in murine macrophages, we analysed their activity on key mRNA targets in the LPS response (Figure 4). These targets included the TLR4 signalling components IRAK1 and TRAF6, which had been predicted to be repressed by miR-146 to feedback control the human TLR4 response (3); the general signalling component TAB2, which controls multiple branches of NFκB activating pathways, is regulated by miR-155 (9) and IKKε, a predicted target of miR-155, which mediates LPS-induced activation of type I interferon (Figure 4A). As NIK kinase has been implicated in TLR4 signalling downstream of IRAK1 (23), and it reportedly triggers the expression of key mediators of antimicrobial defence (10,24), we hypothesized that it might be an miR-155 target as well. Indeed, a manual search revealed a potential miR-155 binding site in the 3′ UTR of the NIK mRNA (Figure 4B), suggesting that miR-155 ensures tight control of inflammatory gene
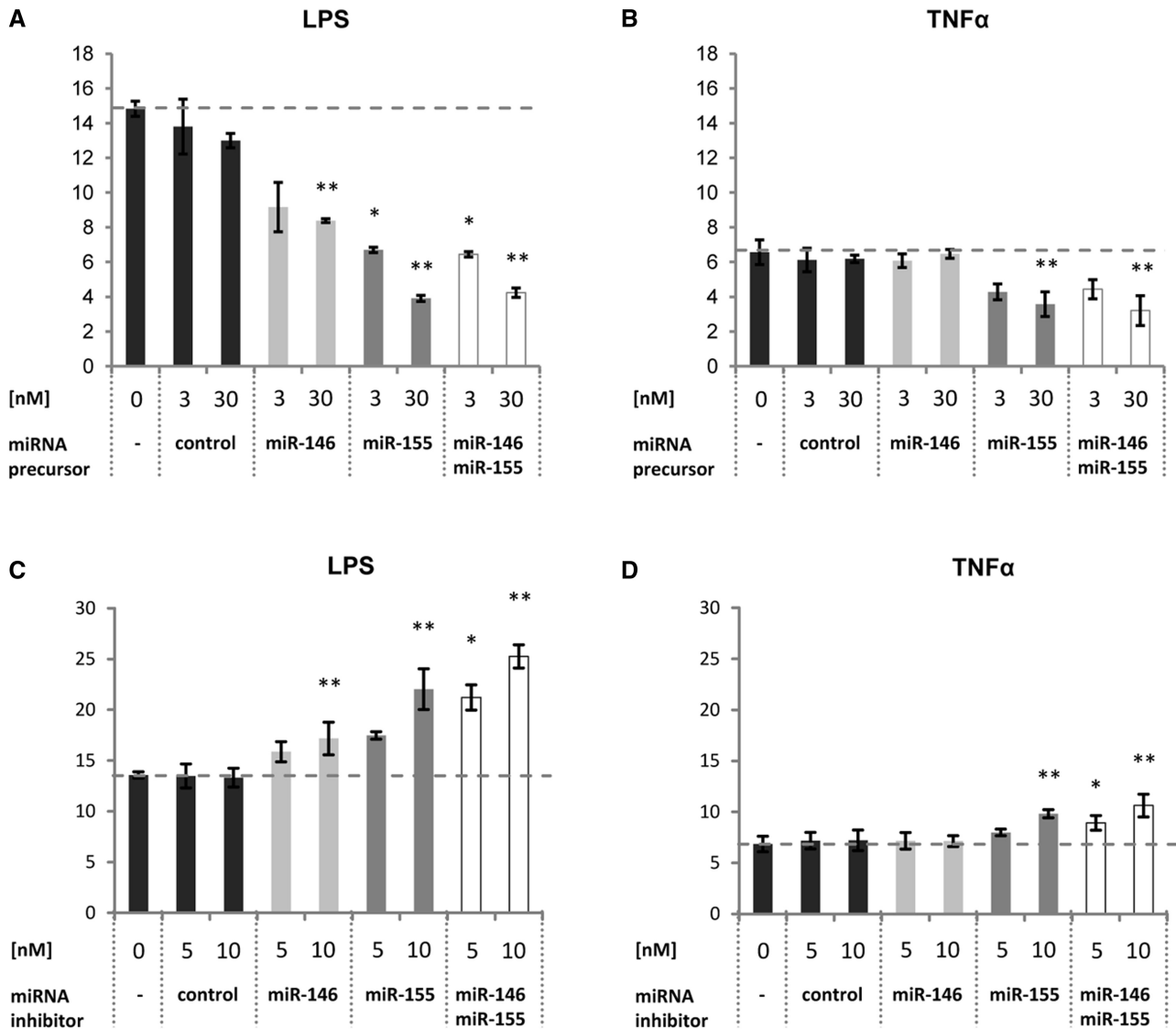
Figure 3. Analysis of NFκB reporter activity in RAWBlue[TM] macrophages on challenge with LPS or TNF-α and miRNA over-expression or inhibition. Cells lipofected without or with synthetic control-miRNA precursors or miR-146 and miR-155 precursors were challenged with 1000 ng LPS/ml (**A**) or 200 ng TNF-α/ml (**B**), and fold-changes (y-axis) in NFκB reporter activity were calculated relative to non-challenged controls. Cells lipofected without or with a control-inhibitor or miR-146- and miR-155-inhibitors were challenged with 1000 ng LPS/ml (**C**) or 200 ng TNF-α/ml (**D**), and fold-changes (y-axis) in reporter activity were calculated relative to non-challenged controls. Mean values and standard deviations from three independent experiments are shown. Asterisks denote statistically significant regulation ($P < 0.05$) compared with the 3 or 5 nM control (asterisk) and the 30 or 50 nM control (double asterisk), respectively.

expression at multiple levels immune signalling triggered by pathogen associated molecular patterns (PAMPs).

To validate these predicted points of regulation, the respective mouse 3′ UTRs were fused to a luciferase reporter gene and tested for their sensitivity to miR-155 or miR-146 in fibroblasts. In the case of miR-155, transfection of miRNA precursors resulted in significant downmodulation of the NIK, IKKε and TAB2 reporters, whereas miR-146 over-expression had no effect on these constructs (Figure 4C). We consider the regulation to be specific, as they were abrogated by the introduction of two point mutations in the predicted miR-155 binding sites. Vice versa, the TRAF6 and IRAK1 targets were specifically regulated by miR-146, but not by miR-155

(Supplementary Figure S2), which supports the notion that miR-146 and miR-155 have discrete target profiles.

To confirm the inflammation-specific regulation of targets by miR-155, we tested the responsiveness of wild-type and mutated miR-155 target reporters in RAW264.7 macrophages challenged with 0.1 ng or 1 μg of LPS. In line with our miR-155 expression data (Figure 1), only the pro-inflammatory (1 μg) and not the sub-inflammatory (0.1 ng) dose of LPS resulted in specific repression of the NIK, IKKε and TAB2 targets by the endogenous miR-155 (Figure 4D). Collectively, these results suggest that on pro-inflammatory stimulation of TLR4, miR-155, rather than miR-146, acts pervasively to limit the NFκB-dependent inflammatory response.
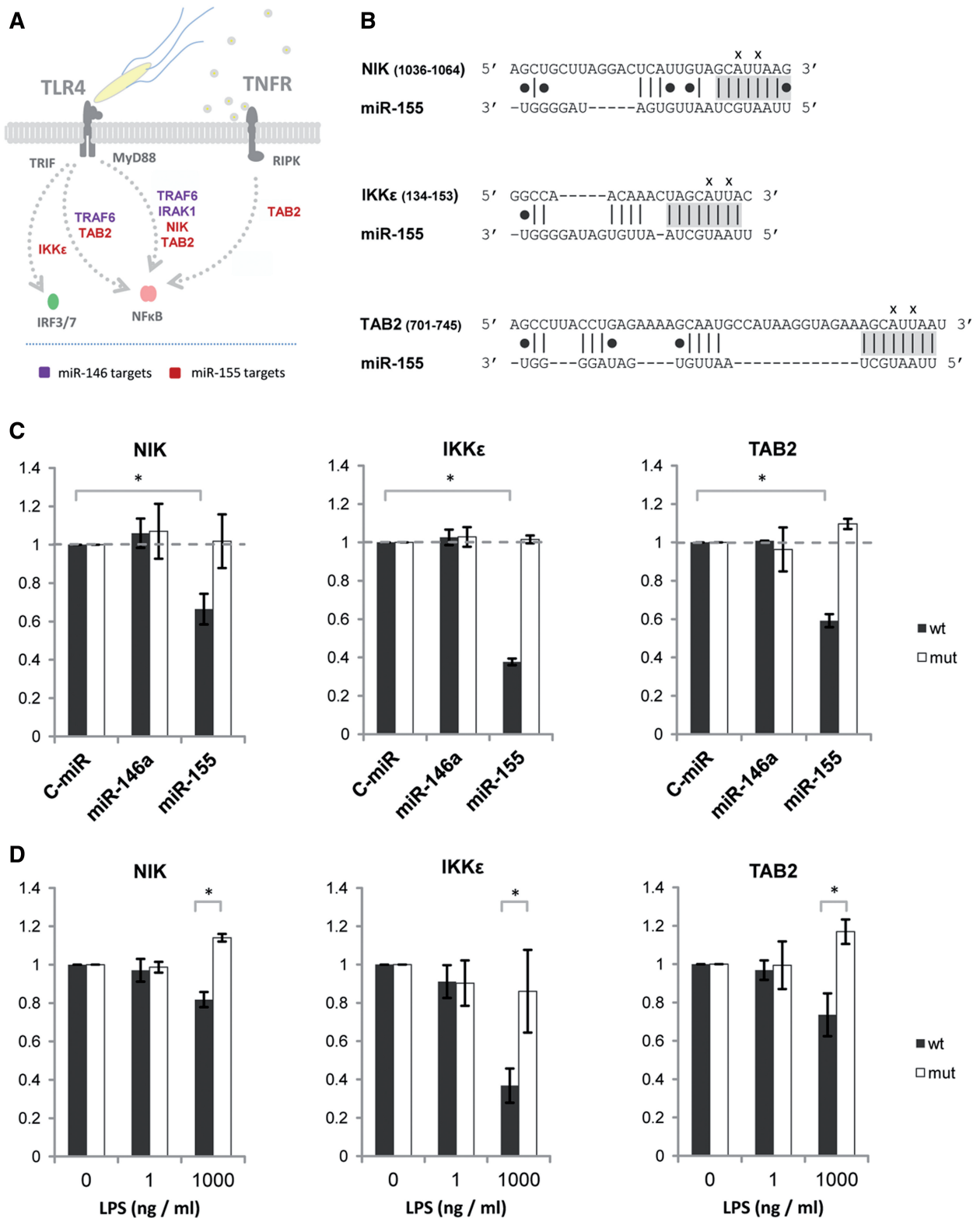
Figure 4. Analysis of 3′ UTR luciferase reporters of known and putative miR-155 targets. (**A**) Schematic overview of miR-146 (blue) and miR-155 targets (red) in the macrophage LPS response. (**B**) RNAHybrid prediction of miR-155 binding within the 3′ UTRs of murine TAB2, IKKε and NIK mRNAs. Lines denote Watson–Crick base pairs, black dots denote wobble base pairs. Seed matches are highlighted in grey. Crosses denote point-mutations (A→C and U→G) introduced to abrogate regulation. (**C**) Analysis of wild-type (wt) or miR-155 binding-site mutant (mut) 3′ UTR reporters of NIK, IKKε and TAB2 in mouse embryonic fibroblasts lipofected with synthetic control-, miR-146a- or miR-155-precursors. (**D**) Analysis of the same reporters as aforementioned in RAW264.7 macrophages, co-challenged with the indicated doses of LPS for 24 h. All results are presented as fold-changes relative to control-precursor lipofection (y-axis). Mean values and standard deviations from three independent experiments are shown. Asterisks denote statistically significant regulation ($P < 0.05$) comparing the indicated treatments.

Opposite roles of miR-146 and miR-155 in endotoxin tolerance

Given that miR-155 is a major regulator of pro-inflammatory responses, we asked how its activation by bacterial stimuli is ensured under conditions, when innate immune sensing through TLR4 is mute. For example, we recently discovered that macrophages brought into an endotoxin-tolerant state by previous exposure to LPS barely induce miR-155 and other inflammation markers when challenged with TLR ligands (25). Reviewing the literature led us to speculate that stimulation of NOD2, a cytosolic NLR of microbial peptidoglycan, may safeguard miR-155 activation by bacterial pathogens, as it can initiate inflammation in endotoxin-tolerant macrophages independent of TLR signalling (26). The same may hold true for NOD1, an NLR that is related to the NOD2 pathway but senses a different peptidoglycan residue. Bacterial cell wall peptidoglycan consists of polymers of *N*-acetylmuramic acid and *N*-acetylglucosamides that are linked to each other through short peptide chains and meso-diaminopimelic acid. The NOD receptors sense peptidoglycan breakdown products iE-DAP, which activates NOD1; MDP, which activates NOD2; and M-TriDAP, which activates both receptors.

To test whether peptodoglycan sensing activates miR-155 and NFκB activity when TLR signalling is muted, we rendered the NFκB-reporter containing RAW264.7 cells endotoxin-tolerant, and challenged them with the NOD receptor agonists, LPS or both. Confirming our previous results (25), a challenge of these endotoxin-tolerant cells with 1 μM of LPS induced only mild NFκB reporter activity or miR-155 expression, as compared with naïve cells (Figure 5A and B). Similarly, stimulation of the NOD1 receptor with its agonist iE-DAP elicited little, if any, NFκB activity and miR-155 expression. By contrast, both the NOD1/2 agonist M-TriDAP and the NOD2 agonist MDP induced significant NFκB activity and miR-155 expression (Figure 5A and B). Importantly, co-stimulation of NOD2 with TLR4 by applying either M-TriDAP or MDP simultaneously with LPS fully rescued NFκB reporter activity and miR-155 expression (Figure 5A and B). We interpret these observations to mean that in the context of successful invasion
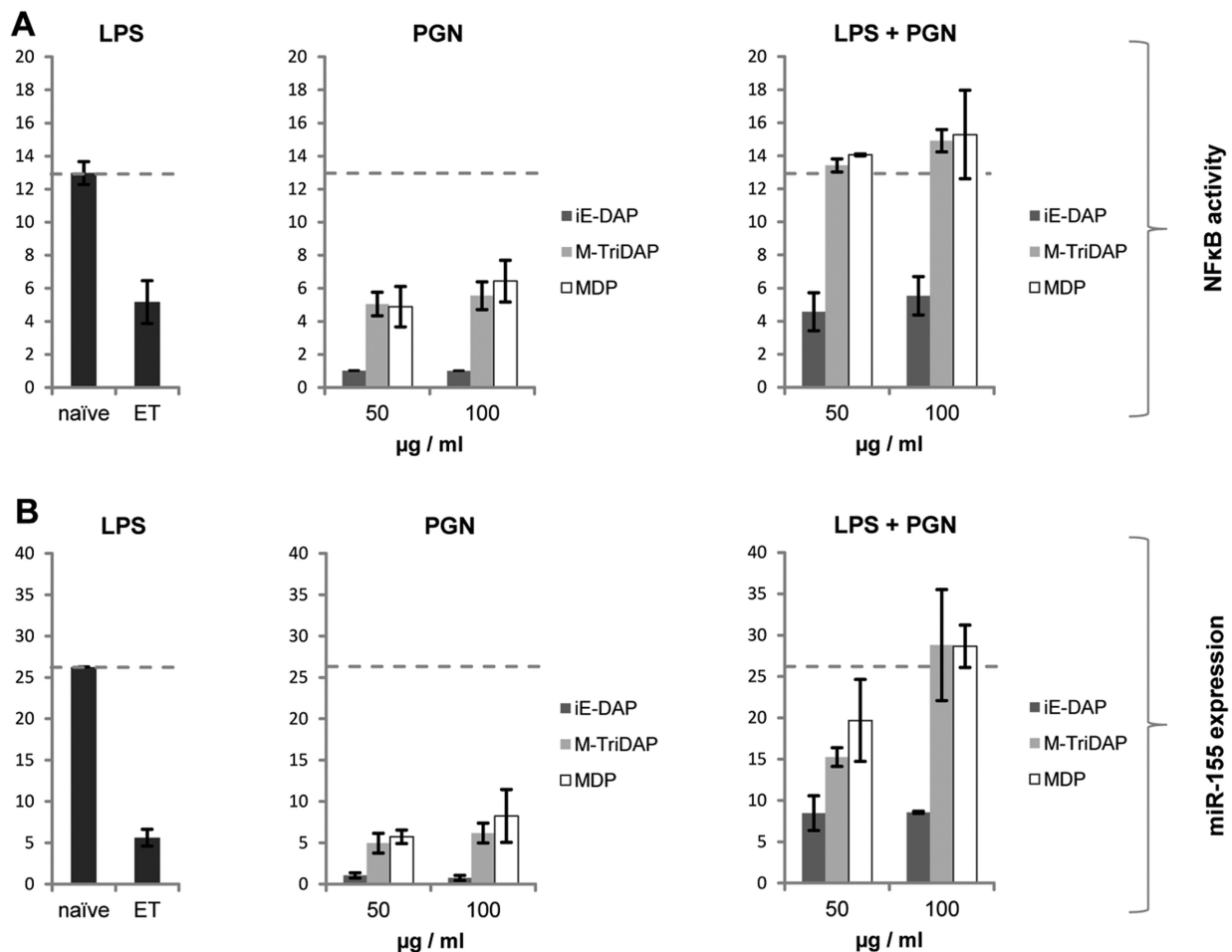

Figure 5. Analysis of LPS- and peptidoglycan-sensitivity of endotoxin-tolerant (ET) macrophages. (**A**) NFκB reporter activity on challenge of naïve and ET RAWBlue[TM] macrophages with 1000 ng/ml of LPS for 24 h or on challenge of ET RAWBlue[TM] macrophages with the indicated amounts of NOD1- and NOD2-stimulating peptidoglycan either alone or in combination with 1000 ng/ml of LPS for 24 h. (**B**) Similar experiment as in (A) but measuring miR-155 expression by qRT-PCR in non-transgenic RAW264.7 cells as the final read-out. All results are presented as fold-changes relative to non-challenged controls (y-axis). Mean values and standard deviations of three independent experiments are shown.

of endotoxin-tolerant cells by a bacterial pathogen, an intracellular stimulation of NOD2 restores the TLR4-based inflammatory response along with the associated feedback control by miR-155. To the best of our knowledge, this is the first demonstration that an miRNA is additively controlled by different immunostimuli and NOD-like receptor agonists.

miR-155 also seems to be important for a balanced resuscitation of the NFκB response in endotoxin-tolerant cells. That is, in macrophages with muted LPS response (Supplementary Figure S3C), ectopic supply of miR-155 (precursor transfection) enhanced the MDP-induced (i.e. through NOD2 only) NFκB reporter activity, but limited the full response resulting from the combined stimulation of NOD2 and TLR4 on simultaneous challenge with MDP and LPS (Supplementary Figure S3A). Likewise, inhibition of miR-155 by an antisense LNA had little effect on reporter induction by LPS or MDP alone (miR-155 remains suppressed under these conditions), but it increased NFκB activity on dual challenge with MDP and LPS (Supplementary Figure S3B).

To address the role of miR-146 in the maintenance of endotoxin tolerance in macrophages, we inhibited it before LPS stimulation. Different from inhibition of miR-155, sequestration of miR-146 significantly elevated the basal LPS-induced reporter activity (Supplementary Figure S3B), indicating a role in the maintenance of LPS hypo-responsiveness in the absence of inflammatory gene and miR-155 expression. However, over-expression of

miR-146 did not further dampen LPS induced reporter activity (Supplementary Figure S3A), suggesting that control by the endogenous miR-146 during tolerance is saturating. The sum of these observations supports a dominant role of miR-155 in modulating the inflammatory response on its activation, whereas miR-146 rather seems to impede inappropriate onset of inflammation.

DISCUSSION

Our results combined with those of previous studies (22) now support a model of two-tier regulation and division of labour by two model miRNAs in the macrophage response to LPS and perhaps other microbial stimuli (Figure 6). First, we show that despite their general activation through NFκB, miR-146 and miR-155 are responsive to different thresholds of LPS concentration. Second, our study of target regulation significantly expands the notion that miR-146 and miR-155 selectively control different branches of the TLR4 response. Through targeting TAB2, IKKε and NIK, and exerting negative control of TNF signalling, miR-155 broadly impacts pro-inflammatory signalling pathways; in contrast, the targeting of TLR4 signal transducers TRAF6 and IRAK1 reflects the primary function of miR-146, which is to control LPS sensing. Accordingly, LPS elicits miR-146 activity in endotoxin-tolerant cells, whereas miR-155 remains mute. Reciprocally, reversal of endotoxin tolerance after recognition of intracellular bacteria through
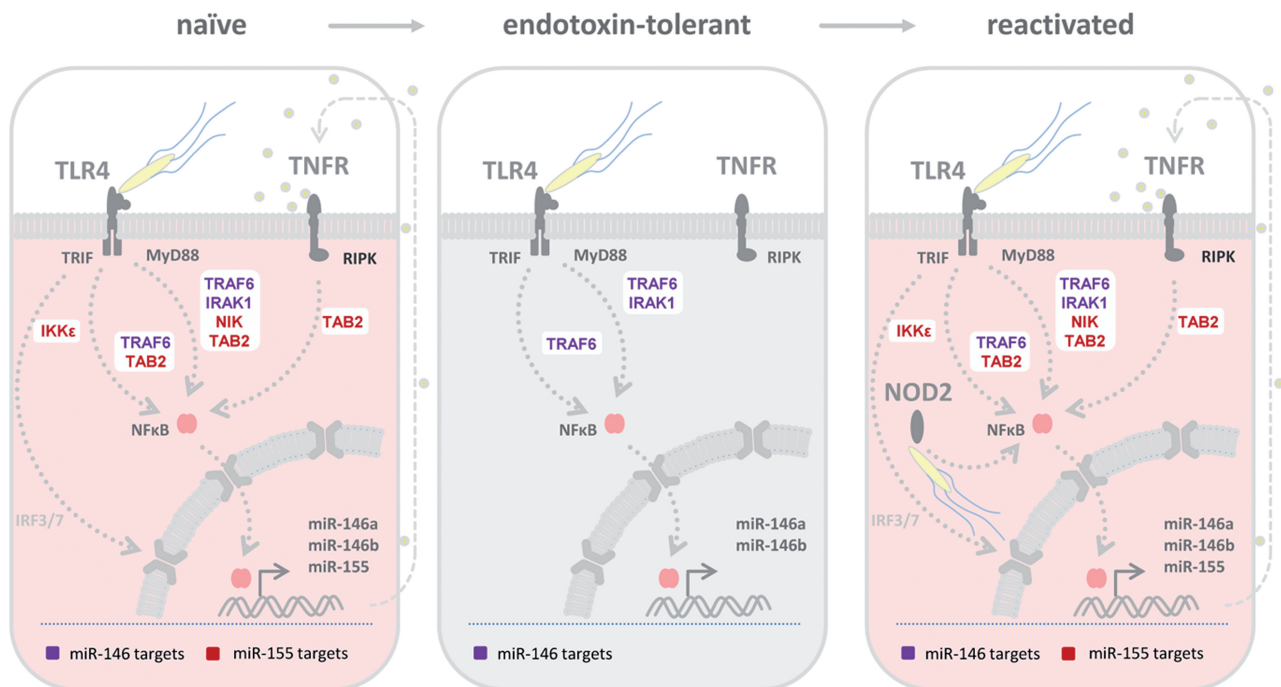

Figure 6. Proposed model of differential miR-155 and miR-146 function in the macrophage response to microbes. Naïve macrophages facing a microbial LPS trigger utilize miR-146 to prevent TLR4 response to sub-inflammatory doses. In the case of pro-inflammatory LPS doses, miR-155 is co-induced as a global limiter of the inflammatory response. After withdrawal of an initial microbial stimulus macrophages become refractory to secondary LPS challenge (endotoxin-tolerant, ET) partly through ongoing negative regulation of TLR-signalling by miR-146 in the absence of pro-inflammatory response regulator miR-155. Cytosolic microbial PAMP recognition, indicative of pathogenic bacteria, overcomes the miR-146 barrier to inflammation in ET cells and activates inflammation-associated miR-155 both as an initial stimulator and subsequent limiter of the response.

NOD2 activates miR-155 to ultimately limit pro-inflammatory signalling. Thus, although miR-146 may augment the initial LPS threshold required for mounting an inflammatory response, miR-155 acts as a final limiter of pro-inflammatory signalling once the miR-146 barrier has been breached. Importantly, this division of labour might also occur on stimulation with PAMPs other than the TLR4 agonist LPS; when we challenged RAW264.7 macrophages with rising doses of TLR2 inducer BLP, miR-146 was induced to saturation by much (~100 times) lower concentrations than those required to fully induce miR-155 (Supplementary Figure S4). The sum of these observations suggests that instead of acting redundantly and simultaneously, miR-146 and miR-155 fulfil different functions and adjust the lower and upper limits of the macrophage inflammatory response to microbes.

Precisely what determines the differential activation at the molecular level remains to be understood. Given the congruent expression changes of the primary versus mature form for both miRNAs (Figure 1), the differential responsiveness to LPS is likely because of transcriptional rather than processing activity. Interestingly, pre-stimulation of macrophages with the anti-inflammatory cytokine IL-10 has recently been demonstrated to decrease induction of the primary transcript of miR-155, but not of miR-146a and miR-146b, further supporting our observation of miR-155, but not miR-146, being coupled to inflammation (27). A possible explanation for the differential expression of the miR-146 and miR-155 genes may be non-cooperative and cooperative NFκB binding sites in their promoter sequences, which have been reported to mediate differential activation of NFκB-controlled inflammatory genes (21).

miR-155 closely mimics the gradual induction of most NFκB-dependent genes in response to environmental stimuli (Figure 1), which might confer post-transcriptional control of multiple cellular branches of the pro-inflammatory response. For instance, miR-155 reportedly suppresses negative regulators of inflammation, such as SHIP-1, SOCS-1 or PIK3CA (13,28,29), pro-inflammatory signalling components, such as TAB2, IKKε or FOS (9,29,30), and mediators of programmed cell death, such as BIRC4BP, FADD or PMAIP1 (5,29,31). We show that it is also miR-155 and not miR-146 that negatively regulates the TNF-α signalling pathway, which sustains LPS-induced NFκB activity through TNF autocrine feedback (32). Thus, a major function of the co-induction of miR-155 with classical markers of the pro-inflammatory response probably is to link this miRNA to the control of secondary NFκB activation downstream of LPS. In support of this hypothesis, miR-155 was recently shown to negatively regulate chemokine IP-10 expression in response to interferon-γ stimulation of human kidney cells that were co-stimulated with TNF-α (33).

The selective negative control of TNF-α-induced NFκB activity may also involve TAB2, which is exclusively targeted by miR-155 (Figure 4). At the same time, miR-155 may enhance TNF-α production through stabilization of its messenger (11,14). Thus, the simultaneous negative regulation of autocrine signalling and positive regulation of TNF-α production by miR-155 might favour the activation of bystander macrophages through TNF paracrine signalling while preventing hyper-activation of the TNF-producing cell. Other miRNAs have been reported to counterregulate TNF production, suggesting that this cytokine is under tight post-transcriptional control (11,34). Further evidence for the regulation of pro-inflammatory signalling beyond TNF-α comes from our confirmation of IKKε as an miR-155 target (Figure 4); IKKε functions in TRIF-dependent signalling downstream of TLR4 and thereby links miR-155 to type I interferon expression. Moreover, the new miR-155 target NIK phosphorylates the PU.1 transcription factor downstream of macrophage activation by LPS to mediate activation of Ptgs2, the synthase of the pro-inflammatory prostaglandin E2 (PGE2) (10). PU.1 itself is another reported target of miR-155 (35), suggesting tight control of this pathway by miRNA. PGE2 functions as an immune hormone that acts on a broad range of target cells to fuel inflammatory gene expression as well as on the central nervous system to trigger fever and to increase pain-sensitivity. Our demonstration that IKKε, NIK and TAB2 are selectively regulated by miR-155 reveals its specialized function in the regulation of pro-inflammatory responses over that of miR-146.

Reciprocally, we show that the known miR-146 targets in TLR4 signalling, IRAK1 and TRAF6, are not impacted by miR-155 (Supplementary Figure S2). Our results demonstrate that miR-146 is induced by sub-inflammatory LPS levels (Figure 1) and regulates targets in endotoxin-tolerant macrophages (Supplementary Figure S3B), which strongly supports the functional specialization of this miRNA towards maintenance of innate immune tolerance (17) and LPS hypo-sensitivity (36). In physiological terms, LPS tolerance is required to limit uncontrolled immune reactions that might cause loss of tissue integrity, sepsis, auto-immune diseases or cancer (37). Maintenance of TLR4-based tolerance to extracellular stimuli (possibly involving miR-146), but it unabated sensitivity to cell-invasive bacterial pathogens through NOD2, the cytosolic sensor of microbial peptidoglycan, might limit such harmful immune reactions while staying fully responsive to enterobacterial attack. Details of the roles of different NLRs in signalling cellular immune responses to invading microbes are only beginning to emerge, and this is the first report of an miRNA being regulated by an NLR. It will be important to expand the current analysis of miR-146 and miR-155 to other cell types of the immune system, including primary cells, to understand how the differential activation of miRNAs contributes to the selective recognition of microbial pathogens by signalling through TLRs and NLRs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–4.

ACKNOWLEDGEMENTS

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