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Rapid Changes in MicroRNA-146a Expression Negatively Regulate the IL-1 β -Induced Inflammatory Response in Human Lung Alveolar Epithelial Cells¹

Mark M. Perry, Sterghios A. Moschos, Andrew E. Williams, Neil J. Shepherd, Hanna M. Larner-Svensson, and Mark A. Lindsay²

Posttranscriptional regulation of gene expression by microRNAs (miRNAs) has been implicated in the regulation of chronic physiological and pathological responses. In this report, we demonstrate that changes in the expression of miRNAs can also regulate acute inflammatory responses in human lung alveolar epithelial cells. Thus, stimulation with IL-1 β results in a rapid time- and concentration-dependent increase in miRNA-146a and, to a lesser extent, miRNA-146b expression, although these increases were only observed at high IL-1 β concentration. Examination of miRNA function by overexpression and inhibition showed that increased miRNA-146a expression negatively regulated the release of the proinflammatory chemokines IL-8 and RANTES. Subsequent examination of the mechanism demonstrated that the action of miRNA-146a was mediated at the translational level and not through the down-regulation of proteins involved in the IL-1 β signaling pathway or chemokine transcription or secretion. Overall, these studies indicate that rapid increase in miRNA-146a expression provides a novel mechanism for the negative regulation of severe inflammation during the innate immune response. *The Journal of Immunology*, 2008, 180: 5689–5698.

The innate immune response, mediated by epithelial and immune cells such as macrophages and dendritic cells, is the first line of defense against infection. This response is commonly mediated through activation of members of the Toll/IL-1 receptor (TIR)³ superfamily, which can be divided into two groups: the IL-1Rs and the TLRs. The IL-1R family is known to consist of 10 receptors that mediate the responses to IL-1 α , IL-1 β , and IL-18, whereas the TLR family contains at least 11 members involved in the recognition of conserved molecular patterns on invading microorganisms that are called pathogen-associated molecular patterns or PAMPs (1, 2). Significantly, all members of this receptor superfamily contain the TIR cytoplasmic domain and are thought to signal through a similar intracellular pathway. Thus, signaling through the TIR domain involves an association with the adaptor protein MyD88, which recruits IL-1R-associated kinase 1 (IRAK1) and TNFR-associated factor 6 (TRAF6) following ligand binding. Dissociation of IRAK1 from MyD88 following phosphorylation causes the activation of TRAF6, which subsequently stimulates the formation and activation of the TAK1/TAB1/TAB2/

TAB3 complex. This in turn activates a variety of proinflammatory transcription factors such as NF- κ B and AP-1 via the I κ B kinase complex and JNK, respectively. To prevent an inappropriate inflammatory response following activation of the TIR receptors, a variety of extracellular and intracellular negative feedback pathways have evolved to regulate this process. These include the production of soluble TLRs that compete with membrane receptors for ligand binding, the regulation of TLR/IL-1 receptor expression, and the production of dominant negative splice variants of MyD88 and IRAK, as well as posttranslational modifications such as phosphorylation, ubiquitination, and degradation (3).

Recent investigations have identified microRNA (miRNA)-mediated RNA interference (RNAi) as a novel, evolutionary conserved mechanism for the regulation of gene expression at the posttranscriptional level (4, 5) and have identified a host of endogenous mammalian genes that are processed to produce ~700 miRNAs (miRNA registry at www.sanger.ac.uk/Software/Rfam/mirna/). miRNA biogenesis involves the initial transcription by RNA polymerase II of primary miRNAs, which are subsequently cleaved by the RNase III enzyme Drosha, in combination with DGCR8, to produce a hairpin RNA of ~65-nucleotides known as pre-miRNAs (6). These are then exported into the cytoplasm by exportin 5 and further cleaved by the RNase III enzyme Dicer to produce the 21- to 23-nt double-stranded RNA duplexes. The actions of miRNAs are mediated by the miRNA-induced silencing complex (miRISC) which uses the mature miRNA guide strand as a template to identify target mRNA. At present, miRNAs are believed to either block mRNA translation or reduce mRNA stability following imperfect binding of the guide strand to miRNA recognition elements (MRE) within the 3'-untranslated region (UTR) of target genes. Specificity of the guide strand is thought to be primarily mediated by the "seed" region localized at residues 2–8 at the 5'-end, although it also appears to be influenced by additional factors such as the presence and cooperation between multiple MREs (7, 8), the spacing between MREs (8, 9), proximity to the stop codon (8), position within the 3'-UTR (8), AU composition (8), and target mRNA secondary structure (10). The mechanism

Biopharmaceutics Research Group, Airways Disease, National Heart and Lung Institute, Imperial College London, London, United Kingdom

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² Address correspondence and reprint requests to Dr. Mark A. Lindsay, Biopharmaceutics Research Group, Airways Disease, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, U.K. E-mail address: m.lindsay@imperial.ac.uk

³ Abbreviations used in this paper: TIR, Toll/IL-1R; ARE, AU-rich element; IRAK1, IL-1R-associated kinase 1; miRNA, microRNA; MRE, miRNA recognition element; RNAi, RNA interference; TRAF6, TNFR-associated factor 6; UTR, untranslated region.

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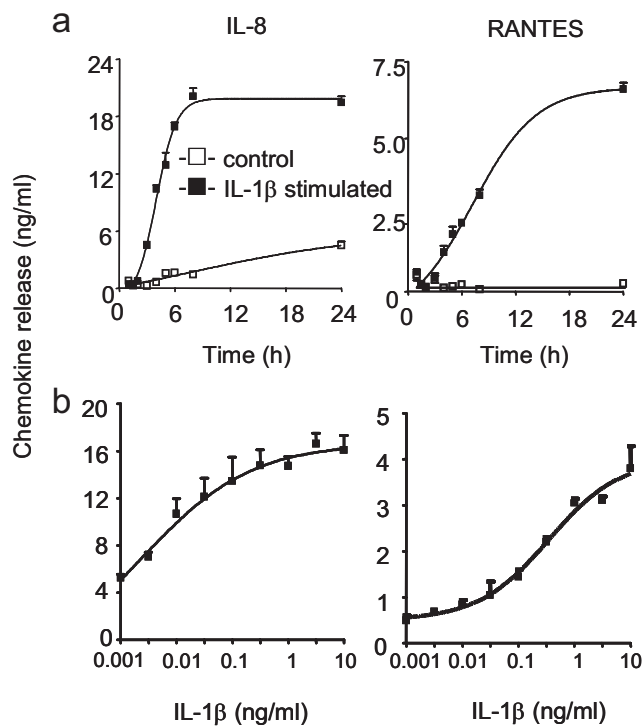


FIGURE 1. Time and concentration dependency of IL-1 β -induced IL-8 and RANTES release. A549 cells were exposed to either buffer or 1 ng/ml IL-1 β for the indicated time (a) or to the indicated IL-1 β concentration for 24 h (b) before the measurement of IL-8 and RANTES release. The results are expressed as the mean \pm SEM of three individual samples and are representative of three independent experiments.

underlying the repression of protein synthesis by miRNAs is currently an area of intense investigation, although it appears that individual miRNAs might use divergent pathways and that these are integrated with the mRNA degradation process associated with P bodies (11). Thus, recent reports have suggested that the actions of miRNAs might function through mRNA destabilization and degradation (12, 13) or, alternatively, following the repression of both translational initiation (14–17) and elongation steps (18).

The physiological role of the majority of identified miRNAs is unknown, although their constitutive expression in tissues and changes during development indicate their importance in the maintenance of cellular phenotype. Thus, islet-selective miRNA-375 expression has been shown to regulate insulin secretion (19), liver-specific miRNA-122 is involved in cholesterol metabolism in vivo (20, 21), and muscle-specific miRNA-1 and miRNA-133 are known to control heart development and physiological responses (22–24). At present, little is known about the function of miRNAs during the inflammatory response. A potential role of miRNA-155 in the adaptive immune response was provided from studies using knockout mice. These showed reduced CD3/CD28-induced IFN- γ release from CD4 $^{+}$ T cells (25) and BCR-mediated TNF- α production from B cells (26), which indicate that miRNA-155 facilitates or positively regulates cytokine release in lymphoid cells. Similarly, increased miRNA-181a expression was also shown to augment IL-2 release following the activation of T cells through the down-regulation of phosphatases (27). Interestingly, recent reports have shown increased miRNA-155, miRNA-146a, and miRNA-146b expression following activation of the innate immune response in monocytes/macrophages (28, 29). The functional role of miRNA-146a and miRNA-146b are presently unknown, although it has been speculated that they might “fine-tune”

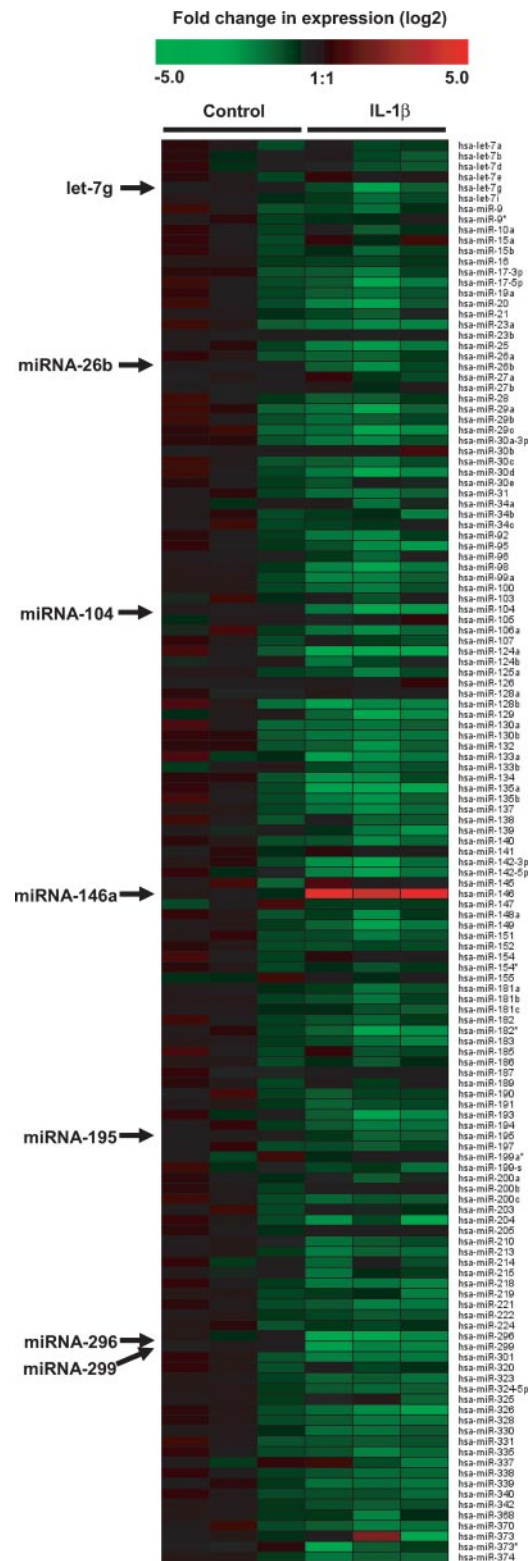
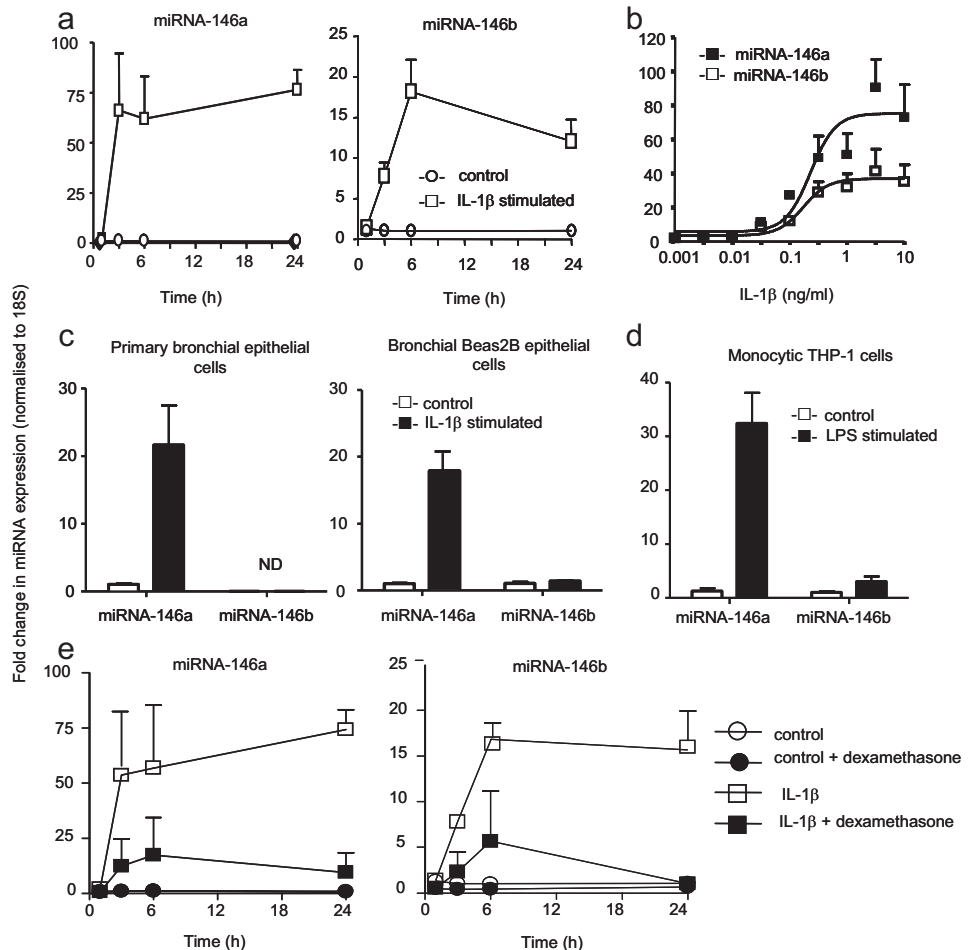


FIGURE 2. IL-1 β -induced changes in miRNA expression. A549 cells were exposed to IL-1 β (1 ng/ml) for 3 h and the profile of expression of 156 miRNAs was measured using TaqMan RT-PCR. The log₂ transformed values of the fold change in expression compared with time-matched saline controls are represented as a heat map where red and green indicate an increase and decrease in miRNA expression, respectively. hsa, *Homo sapiens*.

negative feedback regulation of inflammation through down-regulation of IRAK1 and TRAF6, two proteins involved in TIR signaling (29). However, there is little experimental evidence to support

FIGURE 3. Characterization of the mechanism of miRNA-146a and miRNA-146b expressions. The time- and concentration-dependent induction of miRNA-146a and miRNA-146b in A549 cells was determined following exposure to 1 ng/ml IL-1 β for the indicated time (a) or to the indicated IL-1 β concentration for 6 h (b), and the increases in miR-146a and miR-146b expression were determined by RT-PCR. To confirm the observations in A549 cells, the levels of miRNA-146a and miR-146b were measured at 6 h in IL-1 β -stimulated primary human bronchial epithelial cells and transformed human bronchial Beas2B epithelial cells (c) or in LPS-stimulated monocytic THP-1 cells (d). Alternatively, control- and IL-1 β (1 ng/ml)-stimulated A549 cells were pretreated with dexamethasone (1 μ M) for 60 min, and the expressions of miRNA-146a and miRNA-146b were determined at the indicated time points (e). These results are expressed as the mean \pm SEM of 3 independent experiments. ND, Not detected.



this hypothesis except from studies of CMV-driven overexpression of miRNA-146a and miRNA-146b in HEK293 cells, which was shown to inhibit the activity of luciferase reporter plasmids that contained either the 3'-UTR of IRAK1 or TRAF6 (29). For this reason, we have investigated the functional link between IL-1 β -induced miRNA-146a and miRNA-146b expression and the release of the proinflammatory chemokines IL-8 and RANTES. Significantly, rather than "fine-tune" the inflammatory response we have shown that miRNA-146a and to a lesser extent miRNA-146b are central to the negative feedback regulation of IL-1 β -induced inflammation. Furthermore, we report that the expression and action of miRNA-146a is observed at high IL-1 β concentration, which indicates that this negative feedback mechanism is only activated during severe inflammation. Examination of the mechanism of action of miRNA-146a and miRNA-146b showed this was unlikely to be mediated through down-regulation of the IL-1 β signaling proteins IRAK1 and TRAF6. Instead, the fact that miRNA-146a and miRNA-146b also did not appear to act upon either IL-8 and RANTES transcription or secretion implies that their action is upon chemokine translation. Overall, these investigations show that in addition to their role in the maintenance of cellular phenotype, rapid changes in miRNA levels are involved in the regulation of acute biological responses such as inflammation. Furthermore, given the observation that miRNA-146a and miRNA-146b expression is also increased following the exposure of monocytes and macrophages to a range of microbial stimuli that act through the TIR family (29), this implies that these miRNAs are important regulators of the innate immune response.

Materials and Methods

Cell studies

A549 cells were grown in DMEM containing 10% FCS and 2 mM L-glutamine and plated at 75% confluence in 96-, 24-, or 6-well plates, cultured for an additional 4 h, and then stimulated with the indicated concentration of IL-1 β for 6 and/or 24 h. Beas2B (American Type Culture Collection no. CRL-9609) cells at passages 41–50 were grown in keratinocyte medium with 5% L-glutamine, epidermal growth factor, and bovine pituitary extract, whereas THP-1 cells were cultured in RPMI medium 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine. Normal human bronchial epithelial cells were obtained from Lonza and cultured to a maximum of five passages in bronchial epithelial medium (BEGM; Lonza) containing a mixture of growth factors, cytokines, and supplements (BulletKit; Lonza). For the determination of cytokine/chemokine release, supernatants were removed and IL-8, RANTES, and IFN- α levels determined by DuoSet ELISA (R&D Systems). The viability of the remaining cells was determined by MTT assay.

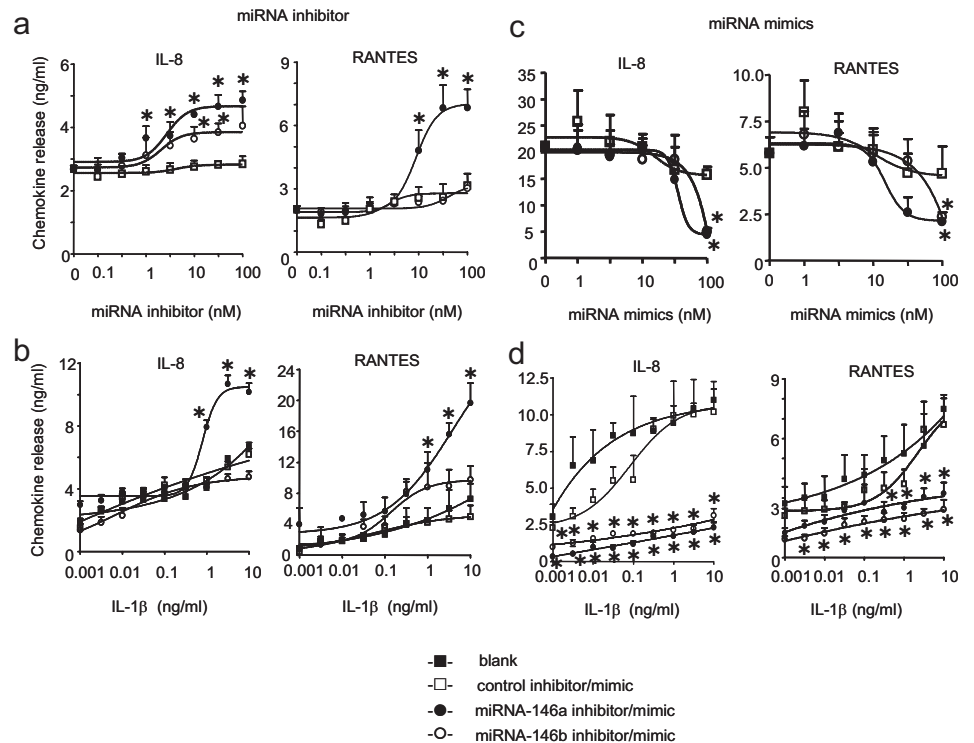
Transfection with miRNA mimics and inhibitors

miRNA-146a/b mimics and controls were obtained from Ambion/Applied Biosystems, whereas miRNA-146a/b inhibitors and controls were obtained from Exiqon. For studies in 96-well plates, miRNA mimics and inhibitors were resuspended in 50 μ l of Lipofectamine 2000/Opti-MEM (Invitrogen Life Technologies) and added to the relevant plates before the addition of A549 cells and incubation at 37°C for 4 h.

Measurement of miRNA and mRNA expression

Total RNA was extracted using the *mir*Vana miRNA isolation kit (Ambion Europe) according to the manufacturer's instructions. RNA was eluted in 50 μ l of RNase-free water (Promega) and stored at -70°C. RNA content and purity were measured using a BioTek PowerWave XS (SSI Robotics)

FIGURE 4. Effect of inhibitors and mimics of miRNA-146a and miRNA-146b upon IL-1 β -induced IL-8 and RANTES release. A549 cells were exposed to transfection reagent alone (blank), control inhibitor or mimic, miR-146a inhibitor or mimic, and miR-146b inhibitor or mimic for 4 h and then exposed to either 1 ng/ml IL-1 β (*a* and *c*) or the indicated IL-1 β concentration (*b* and *d*), and the concentrations of IL-8 and RANTES in the supernatant were determined at 6 and 24 h, respectively. The results are expressed as the mean \pm SEM of three individual samples and are representative of at least three independent experiments. *, $p < 0.05$ vs time-matched (*a* and *c*) or concentration-matched (*b* and *d*) transfected controls.



spectrophotometer. miRNA expression profiling was conducted on total RNA extracts by two-step TaqMan RT-PCR protocol and normalized to 18S as previously described (30). The separate well $2^{-\Delta\Delta Ct}$ cycle threshold method (31) was used to determine relative quantitative levels of individual miRNAs, and these were expressed as the fold difference to the relevant controls. mRNA expression levels of IRAK1, TRAF6, RANTES, and IL-8 was determined by semiquantitative two-step RT-PCR as previously described (32) using Assay on Demand primer/probe sets obtained from Applied Biosystems.

Western blotting

Proteins were extracted from A549 cells as previously described (33), separated by electrophoresis on 10% SDS-polyacrylamide gels or 4–12% polyacrylamide gels (Invitrogen Life Technologies) and transferred to nitrocellulose (Amersham Biosciences). Proteins (5–10 μ g) were detected by Western blotting using rabbit anti-TRAF6 Ab (H-274) (34), rabbit anti-IRAK1 Ab (H-273) (35), and goat anti-synaptotagmin-1 (N-19) (Santa Cruz Biotechnology), rabbit anti-syntaxin-3 (Calbiochem), and rabbit anti-Sec23 interacting protein (Novus Biologicals). All primary Abs were used at a concentration of 1/200 or 1/400 and were incubated overnight. Labeling of the first Ab was detected using relevant secondary Abs conjugated to HRP (DakoCytomation) and detected using ECL reagents.

Statistical analysis

Statistical changes in IL-8, RANTES, IFN- α , miRNA-146a, and miRNA-146b expression were determined using either a two-tailed Student's *t* test or ANOVA with α set to 0.05 using Prism 4 for Windows (version 4.03). miRNA expression data were analyzed and displayed using the Genesis (version 1.7.0) designed by Alexander Sturn and obtained from the Institute of Genomics and Bioinformatics at the Graz Institute of Technology (Graz, Austria). Using this software package, significant differences was determined using ANOVA followed by Dunn's posttest with α set to 0.05.

Results

IL-1 β -induced IL-8 and RANTES response

Initial studies were undertaken to characterize the mechanism of IL-1 β -induced production of IL-8 and RANTES. Exposure to IL-1 β (1 ng/ml) induced a differential time- and concentration-dependent release of the inflammatory chemokines IL-8 and RANTES. Although we observed rapid release of both chemokines, the IL-8 response reached a plateau at ~6–8 h while RANTES

release continued to increase throughout the 24 h period (Fig. 1*a*). Similarly, examination of the effect of increasing IL-1 β concentration at 6 h showed that IL-8 release occurred at low IL-1 β levels (EC_{50} of ~0.01 ng/ml), whereas the RANTES response was elicited at higher IL-1 β concentrations (EC_{50} of ~0.3 ng/ml) (Fig. 1*b*).

Effect of IL-1 β on the profile of miRNA expression

To determine whether miRNA levels were affected by IL-1 β challenge, we measured the expression of 156 miRNAs by using an RT-PCR based approach (30). This showed that 137 of 156 miRNAs were expressed in untreated A549 cells (data not shown). In general, treatment with IL-1 β produced an overall reduction in the miRNA expression profile at 3 h, although we only observed significant reduction ($p < 0.05$) in the levels of let-7g, miRNA-26b, miR-104, miRNA-195, miR-296, and miRNA-299 and a large, 24-fold increase in the expression of miRNA-146a (Fig. 2). Following this initial observation, an additional miRNA-146 named miRNA-146b was characterized that differs from miR-146a by two residues at the 3'-end of the guide strand. Semiquantitative determination of miRNA-146a and miRNA-146b expression in untreated cells by RT-PCR showed that these were almost identical, giving ΔCt (-18S as control) cycle threshold values of 14.5 ± 1.5 and 13.1 ± 1.1 ($n = 5$ independent experiments, $p =$ not significant), respectively.

To determine the potential roles of miRNA-146a and miRNA-146b in the inflammatory response, we examined the time courses of their expressions. IL-1 β stimulated time-dependent 70- and 20-fold increases in the expressions of miRNA-146a and miRNA-146b that reached plateaus at ~3 and 6 h, respectively (Fig. 3*a*). Examination of the effect of increasing IL-1 β concentration showed that miRNA-146a and miRNA-146b expressions occurred at high IL-1 β levels, giving similar EC_{50} values of ~0.3 ng/ml (Fig. 3*b*). Because miRNA-146a and miRNA-146b differ by only two nucleotides, we determined the extent of cross-reactivity between the respective RT-PCR probes. Using miRNA mimics, we showed that there was limited cross-reactivity, with the probes for

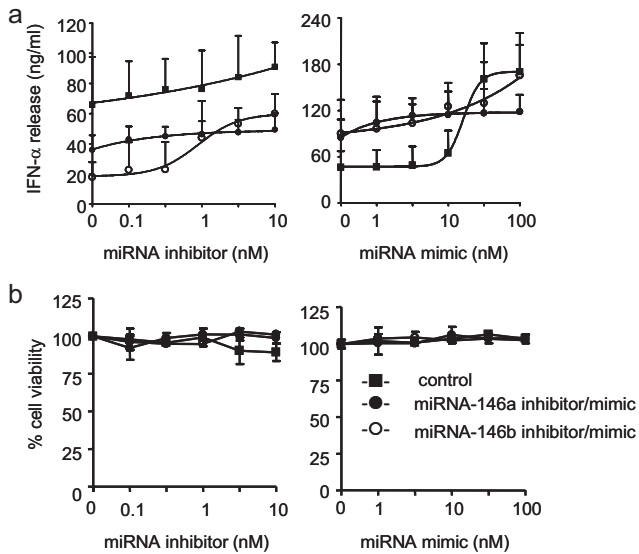


FIGURE 5. Effect of miRNA-146a and miRNA-146b inhibitors and mimics upon IFN- α release and cell viability. A549 cells were exposed to control miRNA inhibitor/mimic, miR-146a inhibitor/mimic, or miR-146b inhibitor/mimic for 4 h and then exposed to 1 ng/ml IL-1 β for 24 h. Supernatant samples were then removed for measurement of IFN- α (a) and the cell viability of the remaining cells was determined (b). The results are expressed as the mean \pm SEM of three individual samples and the graphs are representative of three independent experiments.

miRNA-146a (<10% vs miRNA-146b) being less selective than those for miRNA-146b (<0.4% vs miRNA-146a).

To ensure that these changes in miRNA expression were not restricted to the lung A549 epithelial cell line, we also examined the response in primary bronchial epithelial cells and in the trans-

formed bronchial Beas2B epithelial cell line. These studies showed a comparable but selective increase in miRNA-146a but not miRNA-146b expression in both cell types following IL-1 β stimulation (Fig. 3c). Indeed, we were unable to detect the expression of miRNA-146b in the primary human bronchial epithelial cells. In addition, studies showed that this IL-1 β -induced response was also comparable to that observed following LPS stimulation of the human monocytic THP-1 cell line, which resulted in approximate 30- and 3-fold increases in the expressions of miRNA-146a and miRNA-146b, respectively (Fig. 3d).

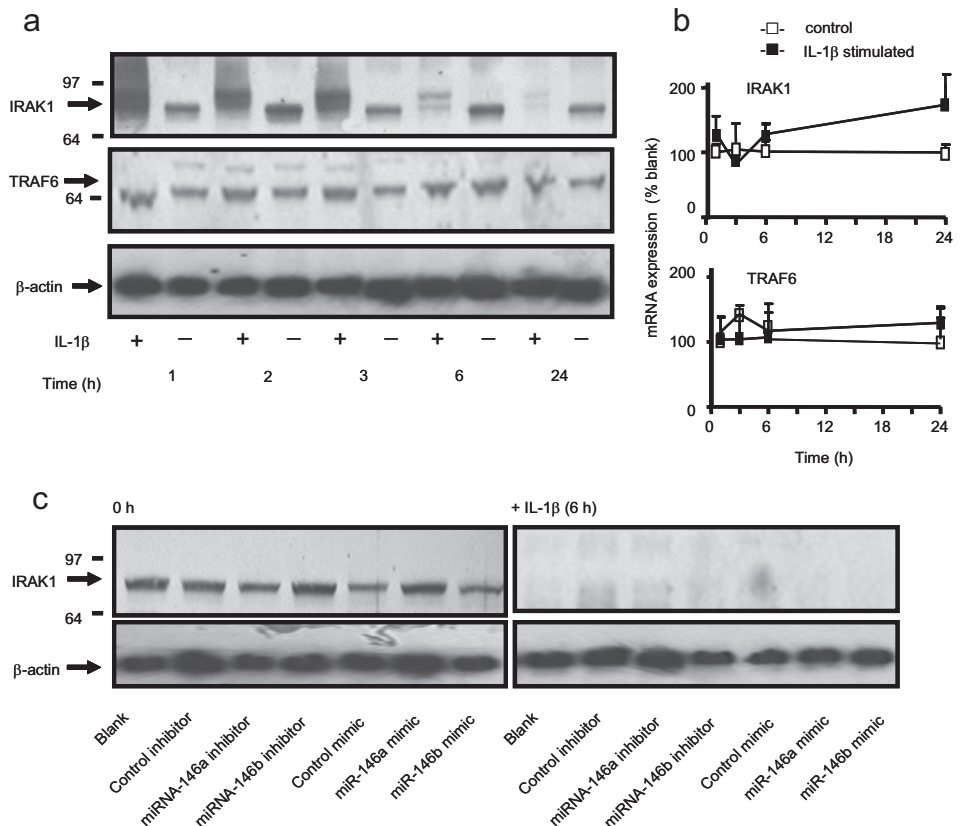
The previous study by Taganov et al. demonstrated that miRNA-146a transcription was regulated by NF- κ B (29). To investigate the role of this pathway in the regulation of the IL-1 β induced response, we have also examined the effect of preincubation with dexamethasone (1 μ M). This corticosteroid, which is known to attenuate the action of multiple proinflammatory transcription factors including NF- κ B, was found to inhibit both IL-1 β -induced IL-8/RANTES release (data not shown) and miRNA-146a/miRNA-146b expression by 70–80% (Fig. 3e). This therefore suggests that expression of both miRNA-146a and miRNA-146b is regulated by proinflammatory transcription factors, possibly NF- κ B, that are corticosteroid sensitive.

Overall, on the basis of the rapid time- and concentration-dependent increases in miRNA-146a and miRNA-146b, we hypothesized that these miRNAs might be involved in the regulation of inflammation at high IL-1 β concentration.

Inhibition of miRNA-146a and miRNA-146b increases IL-1 β -induced IL-8 and RANTES release

The functional relevance of changes in miRNA-146a and miRNA-146b expressions during the IL-1 β -induced IL-8 and RANTES release was assessed using miRNA inhibitors (20, 21, 36, 37). Locked nucleic acid/DNA-based antisense inhibitors and controls

FIGURE 6. Roles of miRNA-146a and miRNA-146b in the IL-1 β signaling pathway. A549 cells were exposed IL-1 β (1 ng/ml) and the expression of IRAK1 and TRAF6 protein (a) and mRNA (b) were detected at the indicated time points. In separate studies, A549 cells were transfected with an inhibitor or mimic of miRNA-146a, miRNA-146b, or the relevant control miRNA and the effect upon IRAK1 protein expression was determined at 0 and 6 h following IL-1 β stimulation (c). The results in a and c are representative of at least three independent experiments, while b gives the mean \pm SEM of three independent experiments.



were transfected in A549 cells for 4 h. We had previously shown that this was sufficient time to obtain oligonucleotide delivery in A549 cells when examining the inhibition of the IL-1 β -induced increases in IL-8 production using a short interfering RNA SMART-pool (Dharmacon) (data not shown). The miRNA-146a inhibitor increased IL-1 β -induced (1 ng/ml) IL-8 and RANTES release in a concentration-dependent manner with an EC₅₀ of ~3–10 nM (Fig. 4a). In contrast, the inhibitor of miRNA-146b was less effective at increasing IL-8 release and had no significant effect upon RANTES release. This implied that the IL-1 β -induced increase in miRNA-146a but not miRNA-146b has the greater capacity to modulate this mechanism. This contention was supported by investigations on the effect of miRNA-146 inhibitors (10 nM) upon chemokine release at increasing IL-1 β concentrations (Fig. 4b). Thus, the IL-1 β -induced IL-8 and RANTES release was not significantly affected by the control or miRNA-146b inhibitor. However, as might be expected given that IL-1 β -induced miRNA-146a expression has an EC₅₀ of ~0.3 ng/ml (Fig. 2b), we observed a significant increase in IL-8 and RANTES release at high IL-1 β concentrations (>1 ng/ml). To eliminate the possibility that the actions of miRNA inhibitors were secondary to the induction of an antiviral response and/or toxicity, we measured the extracellular release of IFN- α and cellular viability at 24 h and found no significant changes (Fig. 5). Overall, these inhibitor studies implied that IL-1 β -induced increases in miRNA-146a expression at high IL-1 β concentrations were involved in the negative feedback regulation of the inflammatory response.

miRNA-146a and miRNA-146b mimics decrease IL-1 β -induced IL-8 and RANTES release

To provide additional evidence on the role of miRNA-146a in negative feedback regulation, we examined the effect of miRNA overexpression by the use of miRNA mimics. Cells were once again transfected with the miRNA mimics at 4 h before IL-1 β stimulation and IL-8 and RANTES release was measured at 6 and 24 h, respectively. Measurement of the concentration dependency showed that when the cells were stimulated with 1 ng/ml IL-1 β , mimics of both miRNA-146a and miRNA-146b significantly attenuated IL-8 and RANTES release by ~60–80% at 100 nM (Fig. 4c). Measurement of the effect of 100 nM mimic upon absolute miRNA-146a and miRNA-146b levels showed variability in the transfection efficacy but resulted in ~500- to 6000-fold increases above the baseline levels for these miRNAs (data not shown). We next examined the action of the miRNA mimics at concentrations of 100 nM upon chemokine release at increasing IL-1 β concentrations. Interestingly, this suggested that miRNA overexpression resulted in noncompetitive inhibition, because both miRNA-146a and miRNA-146b mimics (100 nM) inhibited IL-8 and RANTES release by 60–90% across the IL-1 β concentration range (0.001–10 ng/ml) (Fig. 4d). Once again, measurement of IFN- α release and cell viability showed no significant action of miRNA mimics at all the concentrations tested (Fig. 5).

miRNA-146a and miRNA-146b do not target chemokine transcription

To determine their potential mechanism, we used the most recently released database for the prediction of miRNAs targets (TargetScan database at www.targetscan.org; Ref. 38). In addition to multiple transcription factors, this database identified five additional proteins, two involved in IL-1 β signaling (IRAK1 and TRAF6) and three implicated in secretion (syntaxin-3, synaptotagmin-1 and sec23 interacting protein (sec23IP)).

Initially, we determined whether the actions of miRNA-146a and miRNA-146b were mediated through the down-regulation of

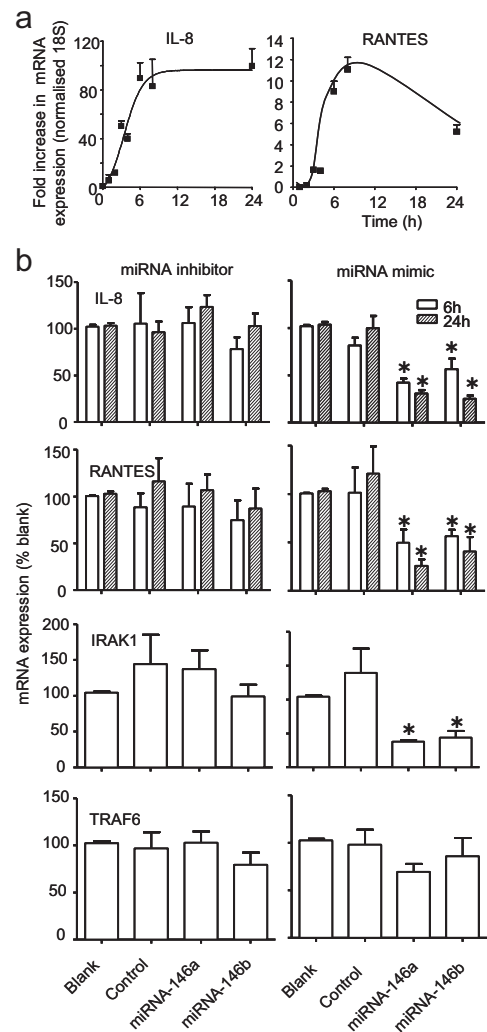


FIGURE 7. Roles of miRNA-146a and miRNA-146b during IL-1 β -induced IL-8 and RANTES transcription. *a*, A549 cells were stimulated with IL-1 β and the expressions of IL-8 and RANTES mRNA were determined at the indicated times. *b*, Alternatively, cells were transfected for 4 h with transfection reagent alone (blank), control inhibitor or mimic (control), miR-146a inhibitor or mimic (miRNA-146a), and miR-146b inhibitor or mimic (miRNA-146b) for 4 h, exposed to 1 ng/ml IL-1 β , and the expressions of IL-8, RANTES, IRAK1, and TRAF6 mRNA were determined at 6 and/or 24 h. The results are expressed as the mean \pm SEM of at least three independent experiments. *, $p < 0.05$ vs blanks.

proteins involved in the IL-1 β signaling pathway. Measurement of protein levels during the 24 h following IL-1 β stimulation showed no change in the expression of TRAF6 but an upward shift in the IRAK1 band at 1–3 h, followed by a reduction in protein expression at 6 and 24 h (Fig. 6a). Measurement of IRAK1 and TRAF6 mRNA expressions showed no significant changes under these conditions (Fig. 6b). Previous studies have shown that this increase in the IRAK1 m.w. is caused by protein phosphorylation and ubiquitination that subsequently result in proteolytic degradation (39, 40). To determine whether increased miRNA-146a and miRNA-146b might contribute toward IRAK1 protein degradation, we examined the effect of the miRNA inhibitors and mimics. Following 4 h of transfection (i.e., time = 0 h) we observed no effect upon IRAK1 protein expression, while miRNA inhibitors and mimics also had no effect upon IL-1 β -induced IRAK1 degradation at 6 h (Fig. 6c). This protein expression data therefore indicated that the actions of miRNA-146a and miRNA-146b were not mediated

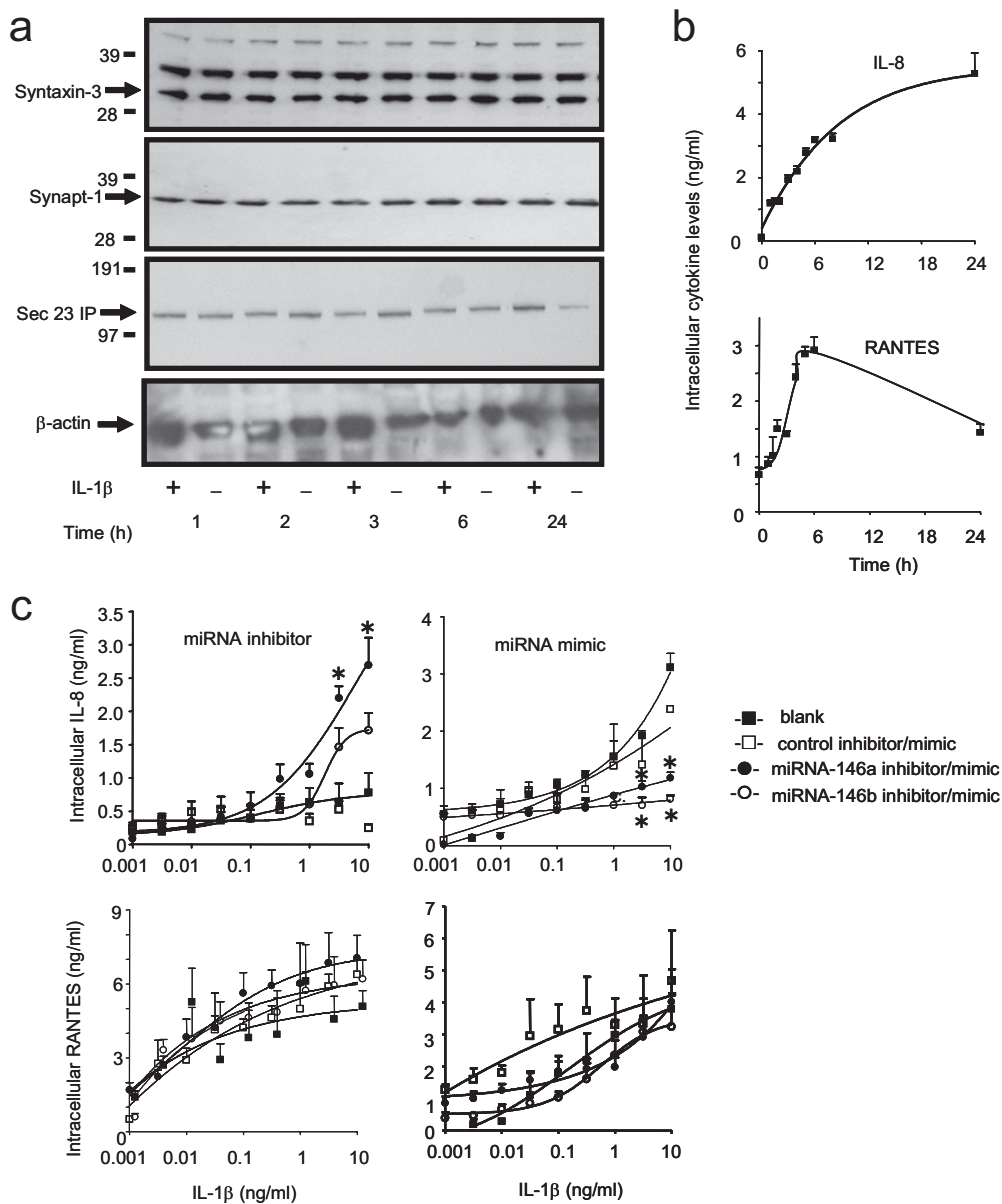


FIGURE 8. Roles of miRNA-146a and miRNA-146b during IL-1 β -induced IL-8 and RANTES secretion. *a* and *b*, A549 cells were stimulated with IL-1 β (1 ng/ml) and at the indicated times the intracellular protein expression of syntaxin-3, synaptotagmin-1, or sec23-IP was determined by Western blotting (*a*), and that of IL-8 and RANTES was determined by ELISA (*b*). *c*, Alternatively, cells were transfected for 4 h with transfection reagent alone (blank), control inhibitor or mimic, miR-146a inhibitor or mimic, and miR-146b inhibitor or mimic for 4 h and then exposed to the indicated IL-1 β concentration, and the intracellular IL-8 and RANTES protein expressions were determined at 6 h. The results are the mean \pm SEM of three individual samples and are representative of at least three independent experiments. *, $p < 0.05$ vs blanks.

through an action upon proteins involved in the IL-1 β signaling pathway. To support this conclusion and to ascertain whether their actions might instead involve down-regulation of transcription factors, we determined the action of miRNA inhibitors on IL-1 β -induced IL-8 and RANTES mRNA expression. Initial measurement of the time course of IL-1 β -induced changes in IL-8 and RANTES mRNA levels showed a rapid increase in both transcripts that peaked at 6–8 h and remained elevated at 24 h (Fig. 7*a*). In subsequent studies, we found that inhibitors (10 nM) of miRNA-146a and miRNA-146b had no effect upon IL-1 β -induced IL-8 and RANTES mRNA production at either 6 or 24 h (Fig. 7*b*). In contrast, transfection with miRNA-146a and miRNA-146b mimics at 100 nM significantly attenuated IL-8 and RANTES mRNA expression at 6 and 24 h (Fig. 7*b*). Interestingly, despite the fact that IL-1 β stimulation alone did not significantly increase IRAK1 and

TRAF6 mRNA expression (Fig. 6*b*), we observed a significant reduction in IRAK1 but not TRAF6 mRNA in the presence of miRNA mimics (Fig. 7*b*). Because we had previously shown that transfection with miRNA mimics increased intracellular miRNA-146a and miRNA-146b levels by ~500- to 6000-fold rather than the ~20- to 70-fold increase seen following IL-1 β stimulation, this suggested that these actions of miRNA mimics, including the degradation of IRAK1 mRNA, might be mediated through nonphysiological processes at these supermaximal concentrations.

miRNA-146a and miRNA-146b do not target chemokine secretion

Having eliminated an action of miRNA-146a and miRNA-146b upon the IL-1 β signaling pathway and chemokine transcription,

we proceeded to investigate the possible targeting of proteins involved in IL-8 and RANTES secretion. However, we once again observed no significant reductions in syntaxin-3, synaptotagmin-1, and sec23IP protein expressions during the 24 h following IL-1 β stimulation (Fig. 8a). Additional evidence that miRNA-146a and miRNA-146b did not act upon secretion came from studies of the effects of miRNA inhibition and overexpression upon intracellular IL-8 and RANTES levels. Examination of the time course of intracellular protein production showed a time-dependent increase in the IL-8 and RANTES levels that peaked at ~24 and 6 h, respectively (Fig. 8b). If the action of IL-1 β -induced miRNA-146a and miRNA-146b were mediated through the down-regulation of proteins involved in secretion, then this would result in the accumulation of intracellular IL-8 and RANTES. Under these circumstances, miRNA mimics would be expected to further block secretion, resulting in increased intracellular IL-8 and RANTES levels whereas miRNA inhibitors should reduce these levels. No significant effect of inhibitor or mimic upon intracellular RANTES levels was observed (Fig. 8c). However, we found that at IL-1 β concentrations that induced miRNA-146a and miRNA-146b production, the mimics reduced intracellular expression while an inhibitor of miRNA-146a, and to a lesser extent miRNA-146b, increased IL-8 levels (Fig. 8c). In addition to the protein data, this provided further evidence that miRNA-146a and miRNA-146b did not target IL-8 and RANTES secretion.

Discussion

A number of recent publications have indicated that miRNAs might regulate the inflammatory response associated with adaptive and innate immunity. With the adaptive response, miRNA-155 has been implicated as a positive regulator of cytokine release from B and T cells by an uncharacterized mechanism (25, 26). Similarly, miRNA-181a has been shown to modulate the inflammatory response in T cells, although this was shown to be mediated through the down-regulation of multiple phosphatases such as CTLA-4 (27). In the case of the innate immune response, Taganov et al. (29) have suggested that miRNA-146a and miRNA-146b might negatively regulate the activation of the innate immune response through down-regulation of IRAK1 and TRAF6. However, the targets of miRNA-146a and miRNA-146b were identified by overexpression and were not linked to an inflammatory response. For these reasons, we have undertaken studies to elucidate whether changes in miRNA-146a and miRNA-146b expressions are functionally linked to the release of inflammatory mediators and to elucidate their mechanisms of action. Specifically, we have examined their role during the IL-1 β -induced response in the human alveolar A549 lung epithelial cell line.

Examination of the differential expression of 156 miRNAs following IL-1 β exposure showed significant reduction in the expressions of six miRNAs, let-7g, miRNA-26b, miRNA-104, miR-195, miR-296 and miRNA-299, and increased expressions of miR-146a and miRNA-146b. The increased expressions of miRNA-146a and miRNA-146b are in agreement with the earlier studies in LPS- and TNF- α -stimulated monocytic cell lines although, unlike ourselves, these investigators also reported up-regulation of miRNA-155 and miRNA-132 (28, 29). The reasons for these differences are presently uncertain, although this might be related to cell differences because studies in transgenic mice report that miRNA-155 is selectively expressed in lymphoid and not in nonlymphoid cells (26). At present, the functions of let-7g, miRNA-26b, miRNA-104, miR-195, miR-296, and miRNA-299 have not been investigated, although it would be expected that their reduced levels would result in an increase in expression of target proteins.

A role for miR-146a and miRNA-146b in the regulation of the chemokine release was initially suggested from the time and concentration dependency of the IL-1 β -induced responses. Thus, measurement of the time course of miRNA-146a and miRNA-146b production showed that this correlated with the rapid release of IL-8 and RANTES. Interestingly, although miRNA-146a and miRNA-146b were expressed at comparable levels in untreated cells, IL-1 β stimulated a greater increase in absolute levels of miRNA-146a. Furthermore, examination of the concentration dependency showed that miRNA-146a and miRNA-146b expressions occurred at high IL-1 β concentrations that paralleled RANTES release; at these concentrations IL-8 release was already maximal. From a mechanistic point, miRNA-146a, IL-8, and RANTES are all thought to be regulated by the inflammatory transcription factor NF- κ B, although these observations indicate that IL-8 might be regulated by a high affinity promoter site while miRNA-146a and RANTES are regulated by low affinity NF- κ B sites (29, 41). This conclusion is supported by our studies showing the inhibition of IL-1 β -induced miRNA-146a expression in the presence of dexamethasone, a corticosteroid that is known to attenuate the action of multiple proinflammatory transcription factors, including NF- κ B. The fact that miRNA-146b expression was also prevented indicated that its transcription is also mediated by corticosteroid-sensitive transcription factors.

Subsequent pharmacological studies established a functional link between the increase in miRNA-146a and the negative feedback regulation of the inflammatory response. Thus, inhibition of miRNA-146a was found to increase IL-8 and RANTES release, whereas miRNA-146a overexpression using miRNA mimics attenuated this response. In contrast, inhibiting miRNA-146b had little effect, although a reduction in chemokine release following the administration of a miRNA-146b mimic implied that this was probably related to the smaller increase in miRNA-146b expression in response to IL-1 β stimulation rather than to a lack of biological activity per se. The importance of miRNA-146a rather than miRNA-146b in TIR-mediated negative feedback is also supported by our observations showing robust increases in miRNA-146a but not miRNA-146b expression following the activation of human bronchial epithelial and monocytic cells. Crucially, the fact that both miRNA-146a expression and the inhibitor-mediated increases in IL-8 and RANTES release were only seen at high IL-1 β concentrations (>0.3 ng/ml) indicates that this negative feedback pathway is important during severe inflammation.

In an attempt to elucidate their mechanisms of action, interrogation of the TargetScan database indicated that miRNA-146a and miRNA-146b targeted proteins involved in both the IL-1 β signaling pathway (IRAK1 and TRAF6) and IL-8 and RANTES secretion (syntaxin-3, synaptotagmin-1, and sec23 interacting protein). The most recent TargetScan database was chosen because, unlike earlier databases, this one predicts targets not only based upon pairing between the miRNA seed region and MREs within the mRNA 3'-UTR but also accounts for factors such as the presence and cooperation between multiple MREs, the spacing between MREs, proximity to the stop codon, position within the 3'-UTR, and AU composition (8). However, it is important to remember that the information used to develop this algorithm might be inaccurate or biased because it is based upon the measurement of mRNA knockdown following miRNA overexpression.

Examination of the TargetScan database showed that the IL-1 β signaling proteins IRAK1 and TRAF6 contain multiple MREs for miRNA-146a and miRNA-146b. This is a significant observation, as this is thought to be an important determinant of miRNA targeting (8) and is supported experimentally by Taganov et al. (29) who showed that ectopic overexpression of both miRNA-146a and

miRNA-146b leads to down-regulation of IRAK1 and TRAF6. However, our studies of the IL-1 β -induced changes in protein expression showed that neither miRNA-146a nor miRNA-146b likely target IRAK1 or TRAF6. Thus, IL-1 β stimulation had no effect upon TRAF6 protein expression and, although we observed a reduction in IRAK1 protein expression, this response was unaffected by exposure to inhibitors and mimics of miRNA-146a and miRNA-146b. Instead, as previously reported IRAK1 reduction is likely to be mediated through proteosomal degradation following IRAK1 phosphorylation (39) and subsequent ubiquitination (40). This phosphorylation and the ubiquitination were indicated by the characteristic increase in the m.w. of IRAK1 at 1–3 h following IL-1 β exposure. Additional evidence that neither miRNA-146a nor miRNA-146b acted upon the IL-1 β signaling pathway or IL-8 and RANTES transcription was provided by the observation that IL-1 β -induced IL-8 and RANTES mRNA expression was unaffected by inhibitors of miRNA-146a and miRNA-146b. Paradoxically, miRNA-146a and miRNA-146b mimics reduced chemokine expression, although we speculate that this might have been the result of the supermaximal miRNA concentrations that occur during these overexpression studies. Thus, although we observed no changes in IRAK1 mRNA expression following IL-1 β stimulation, there was a significant reduction following transfection with miRNA-146a and miRNA-146b mimics. These observations at high miRNA concentrations might also explain the reduction in IRAK1 and TRAF6 mRNA expression observed by Taganov et al. (29) following ectopic expressions of miRNA-146a and miRNA-146b and underlines the problems associated with using overexpression systems to determine miRNA targets and function.

Having eliminated a likely action upon the IL-1 β pathway and chemokine transcription, we proceeded to investigate whether miRNA-146a targeted IL-8 and RANTES secretion through down-regulation of syntaxin-3, synaptotagmin-1, or sec23-IP, all predicted by TargetScan to contain miRNA-146a MREs within their 3'-UTRs. An action of miRNAs upon secretion had previously been suggested from studies of glucose-induced insulin secretion in which miRNA-375 and miRNA-9 had been shown to down-regulate the exocytotic proteins, Myotrophin (19) and granuphilin/Slp4 (42), respectively. However, this mechanism also appears unlikely because we observed no changes in syntaxin-3, synaptotagmin-1, and sec23-IP protein expressions following IL-1 β stimulation. Furthermore, examination of the effect of miRNA inhibitors and mimics upon intracellular IL-8 concentrations produced the opposite response to what might be expected if IL-1 β -induced miRNA-146a acted upon secretion, i.e., an increase and decrease in intracellular IL-8 concentrations following miRNA-146a inhibition and overexpression, respectively.

If miRNA-146a does not target either the proteins involved in IL-1 β signaling or chemokine transcription and secretion, what is the mechanism of action? A process of elimination would suggest that the action of miRNA-146a (and miRNA-146b) is mediated at the translational level. The fact that existing algorithms have failed to identify potential MREs within the IL-8 and RANTES 3'-UTRs implies that miRNA-146a must act either directly upon the translational mechanism or target translational protein(s) that are subject to rapid turnover or induction. Interestingly, the stability and/or translation of mRNA for many inflammatory mediators, including IL-8 and RANTES, are known to be regulated by interaction between AU-rich elements (ARE) within their 3'-UTR and regulatory elements. These include proteins such as HuR, which stabilizes mRNAs, and members of the tristetraprolin (TTP) family, which promote mRNA decay (43). Significantly, recent studies on the mechanisms that regulate TNF- α mRNA stability and translation have shown that there is an interaction between components

of the ARE and the miRNA-mediated RNAi pathway. Thus, two crucial components of the RNA-induced silencing complex (RISC), fragile X mental retardation-related protein 1 (FXR1), and Argonaute 2 (Ago2), have been shown to bind to the ARE and mediate TNF- α up-regulation following serum starvation (44). Similarly, miRNA-16 has been shown to block TNF- α translation through binding to complementary sequences in the ARE by a mechanism that involves members of the tristetraprolin and Ago/eIF2C family (13). Another ARE regulatory protein, HuR (14), has been shown to reverse miRNA-122-mediated repression of cationic amino acid transporter 1 (CAT-1) mRNA in response to stress. It might therefore be speculated that the rapid increases in miRNA-146a could regulate the production of inflammatory mediators through a direct interaction or down-regulation of components involved in posttranscriptional regulation by the ARE and miRNA-mediated RNAi pathways. Of relevance, a recent *in silico* survey of the interactions between miRNAs and immune genes unexpectedly found preferential targeting of proteins involved in the regulation of AREs and miRNA metabolism (45). Furthermore, this report indicated that major targets of miRNAs are transcription factors, cofactors, and chromatin modifiers rather than receptors, their ligands, or inflammatory mediators (45).

In conclusion, we have identified a novel mechanism for the negative feedback regulation of inflammation following activation of the innate immune response by demonstrating that IL-1 β -induced increases in miRNA-146a expression negatively regulate IL-8 and RANTES release. Importantly, this is only observed at high IL-1 β concentrations, which indicates that it might be an important feedback mechanism during severe inflammation. Significantly, these results also demonstrate that changes in miRNA expression are able to regulate acute biological responses and suggest that pharmacological targeting of miRNAs might provide a novel therapeutic approach to the treatment of inflammation. Because rapid increases in miRNA-146a have also been reported following the TIR-mediated activation of monocytes and macrophages, it is suggested that this might represent a common pathway for the regulation of inflammation following activation of the innate immune response.

Disclosures

The authors have no financial conflict of interest.

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