

IL-10–induced microRNA-187 negatively regulates TNF- α , IL-6, and IL-12p40 production in TLR4-stimulated monocytes

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Edited by Marc Feldmann, Imperial College London, London, United Kingdom, and approved September 24, 2012 (received for review May 29, 2012)

IL-10 is a potent anti-inflammatory molecule that, in phagocytes, negatively targets cytokine expression at transcriptional and post-transcriptional levels. Posttranscriptional checkpoints also represent the specific target of a recently discovered, evolutionary conserved class of small silencing RNAs known as “microRNAs” (miRNAs), which display the peculiar function of negatively regulating mRNA processing, stability, and translation. In this study, we report that activation of primary human monocytes up-regulates the expression of miR-187 both in vitro and in vivo. Accordingly, we identify miR-187 as an IL-10–dependent miRNA playing a role in IL-10–mediated suppression of TNF- α , IL-6, and the p40 subunit of IL-12 (IL-12p40) produced by primary human monocytes following activation of Toll-like receptor 4 (TLR4). Ectopic expression of miR-187 consistently and selectively reduces TNF α , IL-6, and IL-12p40 produced by LPS-activated monocytes. Conversely, the production of LPS-induced TNF- α , IL-6, and IL-12p40 is increased significantly when miR-187 expression is silenced. Our data demonstrate that miR-187 directly targets TNF- α mRNA stability and translation and indirectly decreases IL-6 and IL-12p40 expression via down-modulation of I κ B ζ , a master regulator of the transcription of these latter two cytokines. These results uncover a miRNA-mediated pathway controlling cytokine expression and demonstrate a central role of miR-187 in the physiological regulation of IL-10–driven anti-inflammatory responses.

The correct evolution of inflammation is under the control of several mechanisms whose loss predisposes to a number of inflammation-driven pathologies (1, 2). For instance, overwhelming immune activation is prevented mainly by inhibition of proinflammatory cytokines and chemokines, produced by innate immune cells via activation of Toll-like receptors (TLRs). One of the most effective suppressors of TLR-induced inflammatory cytokine production is IL-10, which displays powerful inhibitory actions on innate immune cell activation (3). IL-10 repressive activity on cytokine production can be achieved not only by directly inhibiting gene transcription (4, 5) but also by destabilizing mRNA or by blocking translation (6). With respect to these two latter points, microRNAs (miRNAs) are increasingly attractive molecules for their ability to function as posttranscriptional inhibitors and, in the context of inflammation, for their active role in regulating the strength and timing of TLR responses (7).

miRNAs constitute a class of small, noncoding RNAs that target coding RNA and silence them. miRNA expression is regulated first by transcription factors and RNA polymerase II (Pol II) that generate primary miRNAs (pri-miRNA), which subsequently are cleaved by two different RNase III to produce a mature 21- to 23-nt dsRNA duplexes (8). One strand of the mature miRNA is used as a template to identify target mRNAs. Binding of the miRNA guide strand to the “miRNA recognition element” within the 3' UTR of target mRNA leads to repression of translation and, eventually, to mRNA destabilization and degradation (9). miRNAs have been demonstrated to be fundamental as transcription factors

in the execution of several physiological, developmental, and reactive programs, including the immune response (10). In fact, activation of inflammatory cells via TLRs triggers the expression of several miRNAs, which, in turn target different components of the TLR signaling itself, fine-tuning the overall response (7). For instance, the TLR-responsive miR-146a negatively regulates TLR activation by targeting key signaling proteins, such as IL-1R–associated kinase 1 (IRAK1) and TNF receptor–associated factor 6 (TRAF6) (11), thus fulfilling an anti-inflammatory role. On the other hand, miR-9, similarly induced by TLR2/4 activation in human neutrophils and monocytes, represses the expression of NF- κ B p50, in this way conditioning the type and the activity of the downstream NF- κ B transcriptional activator (12). However, because one single miRNA can have several target genes, there are cases in which the role of a given miRNA in regulating inflammation is not immediately evident. This is the case of miR-155, which has been shown to target either activators of TLR signaling, such as the myeloid differentiation primary-response protein 88 (MyD88) (13, 14) and TAK1-binding protein 2 (TAB2) (15), or inhibitors of TLR signal propagation, such as the SH2 domain-containing inositol-5'-phosphatase 1 (SHIP1) (16) and the suppressor of cytokine signaling 1 (SOCS1) (17). Thus, given the importance of miRNAs in the modulation of TLR signaling, it is conceivable that anti-inflammatory stimuli can either increase or suppress miRNA expression, according to their role in sustaining or repressing TLR-mediated cell activation. Remarkably, IL-10 has been shown recently to inhibit miR-155 induction by TLRs and, as a consequence, to reverse miR-155–mediated down-regulation of SHIP1, a validated target of miR-155 (18), which has been shown in another context to affect TLR signaling events (19).

Recently we have shown that LPS induces the expression of a distinct set of miRNAs in primary human monocytes (12). With the aim of identifying whether some of the anti-inflammatory functions of IL-10 are achieved via modulation of miRNAs, we analyzed the effect of IL-10 on the miRNA profile induced by TLR4 activation. Here we demonstrate that IL-10, simultaneously added to or endogenously produced by LPS-stimulated cells, down-modulates the expression of LPS-induced miR-155, while it rapidly and transiently enhances LPS-induced miR-146b expression. Most importantly, we identified miR-187 as a direct

Author contributions: M.R., M.A.C., M.L., and F.B. designed research; M.R., G.C., N.T., M.C., L.M., S.G., B.M., M.D.L., and M.M. produced luciferase assay and ChIP data; M.R. analyzed data; and F.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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See Author Summary on page 18257 (volume 109, number 45).

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1209100109/-DCSupplemental.

target of IL-10 and provide experimental validation of miR-187 target genes, revealing previously uncovered mechanisms triggered by IL-10 to inhibit LPS-induced TNF- α , IL-6, and IL-12p40 production. Thus, this study identifies miR-187 as an IL-10-dependent miRNA with a specific anti-inflammatory role.

Results

IL-10 Mediates the Induction of miR-187 Expression. In our previous work we described the involvement of miRNAs in the regulation of monocyte activation after TLR engagement (12). We extended this study and investigated whether miRNAs play an active role also in IL-10-mediated suppression of monocyte activation. TaqMan-based low-density miRNA array showed that stimulation of freshly purified monocytes with IL-10 increases the expression of miR-146b and miR-187 but not of any of the other 356 miRNAs analyzed (Fig. 1A). Moreover, simultaneous addition of IL-10 to LPS-stimulated monocytes enhances the induction of LPS-induced miR-146b and miR-187 expression but not that of the other LPS-induced miRNAs (Fig. 1B and Table S1). Additionally, and in line with recent observations in bone marrow-derived mouse macrophages (18), IL-10 inhibits the expression of LPS-induced miR-155 (Fig. 1B). The ability of IL-10 to induce miR-146b and miR-187 expression directly was confirmed in a time-course analysis (Fig. 1C). On the other hand, the increase in miR-146b expression in monocytes treated with LPS plus IL-10 is transient, because it starts to be detectable at 2 h but is not distinguishable from that induced by LPS alone at 24 h (Fig. 1C). In contrast, the potentiating effect of IL-10 on LPS-induced miR-187 expression starts to be detectable after 2 h and is maintained up to 24 h (Fig. 1C). These data point to miR-187 as the main LPS-induced miRNA strongly up-regulated by IL-10.

In human monocytes LPS stimulates IL-10 production, which in turn acts on monocytes in an autocrine manner (20). To in-

vestigate whether endogenously produced IL-10 plays a role in the expression of miR-187 induced by LPS, monoclonal anti-IL-10 antibodies (or isotype-specific control IgG) were added to monocytes, and the expression levels of miR-187 were analyzed 24 h after LPS stimulation (Fig. 1D). We found that the induction of miR-187 by LPS is reduced by 70.6% in the presence of IL-10-blocking antibodies, but miR-146b induction is not affected (Fig. 1D and Table S2).

The expression of miR-187 then was evaluated in monocytes, monocyte-derived macrophages (MDM), and autologous polymorphonuclear cells (PMN) stimulated with LPS or LPS plus IL-10 for 24 h (Fig. 2A). Consistent with a potential role for IL-10 in LPS-induced miR-187 expression, LPS is able to trigger the expression of miR-187 in monocytes and MDM but not in PMN (Fig. 2A), which do not produce IL-10 upon LPS activation (21). Conversely, addition of exogenous IL-10 to LPS-stimulated PMN also restores the ability of these cells to up-regulate miR-187 expression (Fig. 2A), further suggesting that IL-10 is required for LPS-induced miR-187 expression. Interestingly, the ability of LPS and LPS plus IL-10 to up-regulate miR-187 expression is confined to peripheral blood myeloid cells of human origin and does not apply to mononuclear cells of mouse origin. In fact, the expression of miR-187 is not induced by LPS in thioglycolate-elicited macrophages (TEM) (Fig. S1A), in bone marrow-derived macrophages (BMDM) (Fig. S1B), or in the Raw264.7 macrophage cell line (Fig. S1C), whereas expression of miR-155 or miR-146a is observed readily (Fig. S1A-C). Similarly, LPS plus IL-10 was unable to up-regulate the expression of miR-187 in all mouse mononuclear cells tested (Fig. S1D-F), which are perfectly responsive to LPS and LPS plus IL-10, as assessed by modulation of CXCL10 expression (Fig. S1D-F). Up-regulation of miR-187 expression also was observed in human monocytes challenged with palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4) and poly(I:C) (TLR2 and

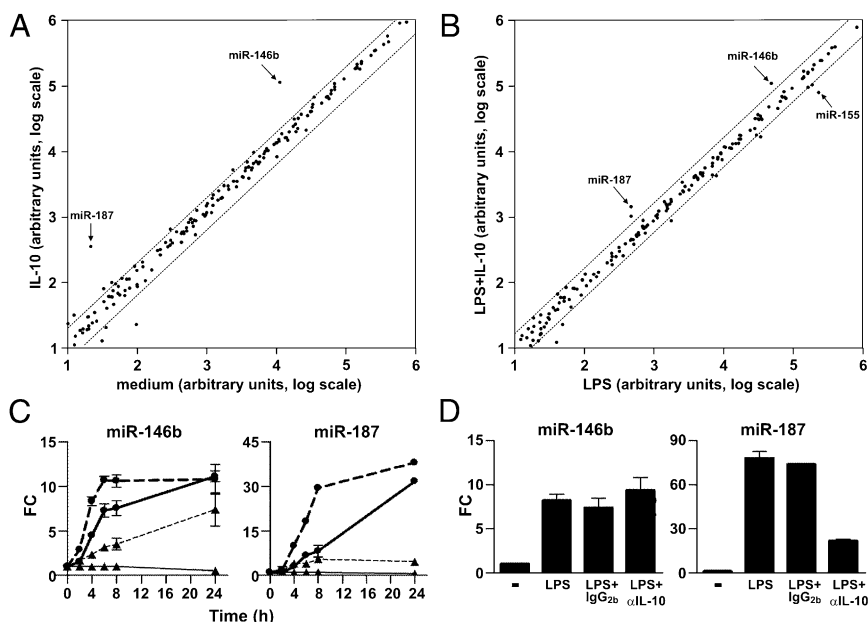


Fig. 1. Modulation of miRNA expression by IL-10. Freshly purified monocytes were cultured for 8 h with 200 U/mL IL-10 or were left untreated (A) or were treated with 100 ng/mL LPS alone or in combination with IL-10 (B). Changes in miRNA expression levels were determined using a microfluidic card. Results are expressed as arbitrary units on a log scale using RNU44 as reference control. The mean values of two individual experiments performed are shown. Dotted lines represent the two-fold and 0.5-fold boundary values for fold induction. (C) Monocytes were cultured for the indicated times with LPS (—●—), IL-10 (---▲---), or LPS plus IL-10 (---●▲---) or were left untreated (—▲—). The expression of miR-146b (Left) and miR-187 (Right) was analyzed by RT-qPCR in triplicate samples and was normalized to the RNU44 levels. The results are expressed as fold change (FC) and are representative of three individual experiments. (D) Monocytes were left untreated (-) (Left) or were stimulated with LPS alone or in the presence of 1 μ g/mL blocking antibodies against IL-10 or isotype control IgG_{2b}, and the expression of miR-146b and miR-187 (Right) was determined 24 h after stimulation. Each graph represents the miRNA levels determined by RT-qPCR and normalized to the RNU44 levels. Each panel shows one experiment representative of three performed with similar results.

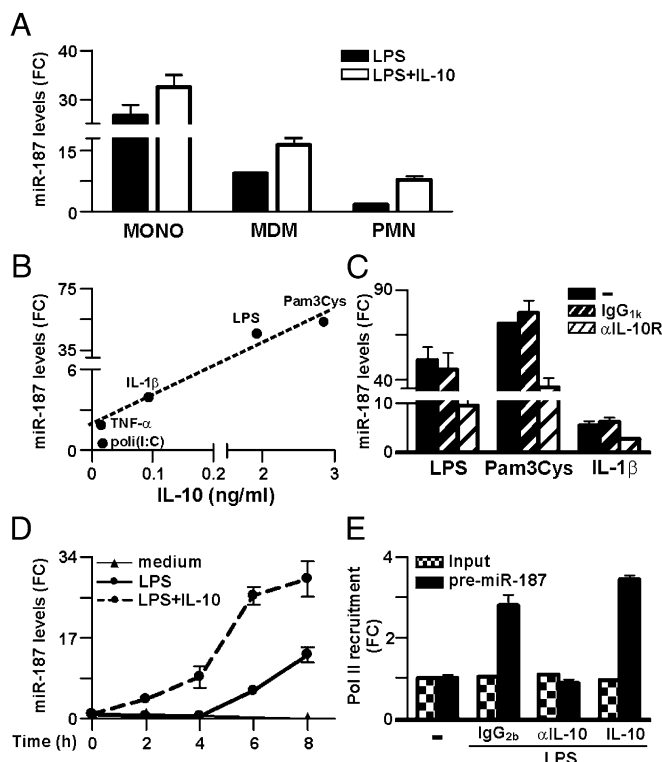


Fig. 2. IL-10 is required for LPS-induced miR-187 expression. The expression of miR-187 was determined by RT-qPCR in the following conditions. (A) Monocytes, MDM, and autologous PMN were cultured for 24 h with LPS or with LPS plus IL-10. (B) Monocytes were cultured in the presence of 100 ng/mL Pam₃CSK₄, 50 μg/mL poly(I:C), 20 ng/mL IL-1β, or 5 ng/mL TNF-α for 24 h. IL-10 content was determined by ELISA in cell-free supernatant of the same samples and is plotted as function of the level of miR-187 expression. (C) Monocytes were stimulated for 24 h with LPS, Pam₃CSK₄, or IL-1β in the presence of 10 μg/mL anti-IL-10R antibody or IgG_{1K} isotype control antibody. (D) Monocytes were cultured for the indicated times with LPS or LPS plus IL-10 or were left untreated, and the expression of premiR-187 was analyzed by RT-qPCR. (E) Monocytes were left untreated or were stimulated with LPS in the presence of control IgG_{2b} antibody, anti-IL-10 antibody, or IL-10 for 8 h. A ChIP assay was performed with anti-Pol II antibody and analyzed by qPCR with primers amplifying the premiR-187 locus. Data from qPCR are expressed as fold change (FC) over control sample as described in *SI Materials and Methods*. Each panel shows one representative experiment of at least three performed with similar results.

TLR3 agonists, respectively) or with the proinflammatory cytokines IL-1β and TNF-α at levels correlating with the amounts of IL-10 produced in response to the various stimuli (Fig. 2B). Pam₃CSK₄ and IL-1β up-regulated miR-187 expression, which in turn was suppressed by the addition of anti-IL-10R antibodies (Fig. 2C), suggesting that other monocyte-activating stimuli can trigger the expression of miR-187, provided that they trigger IL-10 production.

Mature miR-187 is generated by processing a precursor (premiR-187) transcribed from an intergenic region located on chromosome 18. IL-10 rapidly increases LPS-induced premiR-187 levels (Fig. 2D), suggesting that it exerts its up-regulatory effect at the transcriptional level. In fact, as assayed by ChIP, the recruitment of Pol II to the genomic region encoding premiR-187 in monocytes stimulated with LPS for 8 h is enhanced in the presence of IL-10 and is inhibited in the presence of anti-IL-10 neutralizing antibodies (Fig. 2E).

Taken together, these data support a role for endogenous IL-10 in LPS-induced miR-187 expression and, above all, call attention to miR-187 as an miRNA that is induced in an IL-10-dependent manner.

miR-187 Plays a Functional Role in IL-10-Mediated Suppression of LPS-Induced TNF-α, IL-6, and IL-12p40. To investigate whether the expression of miR-187 was functionally involved in the production of cytokine by LPS and in its regulation by IL-10, we inhibited miR-187 by transiently transfecting monocytes with an miR-187-specific ssDNA-locked nucleic acid (LNA) knockdown probe (as-miR-187) or with a scramble miRNA knock-down probe (as-miR-scr) as a control (Fig. S2). Transfected monocytes then were stimulated with LPS or LPS plus IL-10 for 24 h, and the levels of different cytokines and chemokines production were analyzed in the cell-free supernatants (Fig. 3). miR-187 knockdown by as-miR-187 significantly increases TNF-α ($P = 0.037$), IL-6 ($P = 0.040$), and IL-12p40 ($P = 0.037$) production in response to LPS (Fig. 3A and C and Table S3). Most importantly, blocking miR-187 induction partially, but significantly, reversed IL-10-mediated inhibition of LPS-induced TNF-α ($P = 0.049$), IL-6 ($P = 0.038$), and IL-12p40 ($P = 0.036$) (Fig. 3B and C and Table S3). On the contrary, the production of other cytokines (IL-1β, IL-1ra, and IFN-β) or chemokines (CXC10, CCL2, and CXL8) in response to LPS and/or LPS plus IL-10 was unmodified in as-miR-187-transfected monocytes compared with as-miR-scr-transfected cells (Fig. S3), indicating that under these conditions the increased TNF-α, IL-6, and IL-12p40 production is not the result of a general effect of miR-187 down-modulation. Remarkably, miR-187 knockdown by as-miR-187 transfection does not modify LPS-induced IL-10 production (Fig. S3), thus excluding a role for IL-10 in the increase in LPS-induced TNF-α, IL-6, and IL-12p40. Collectively, these data indicate the induction of miR-187 as one of the mechanisms used by IL-10 to suppress LPS-induced TNF-α, IL-6, and IL-12p40 production.

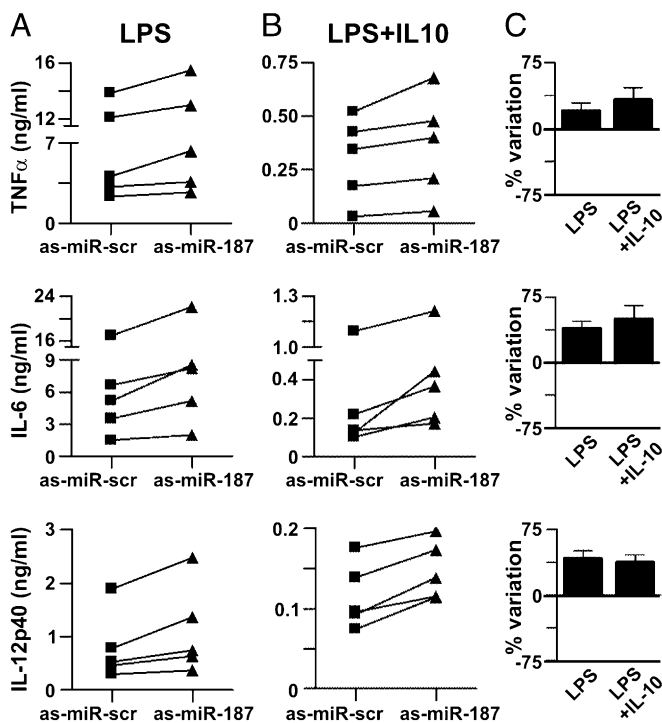


Fig. 3. Effect of miR-187 levels on LPS-induced cytokine release. Monocytes from five different donors were transfected with as-miR-scr or as-miR-187 and 18 h later were stimulated with LPS (A) or with LPS plus IL-10 (B) for 24 h. Cell-free supernatants were collected, and antigenic TNF-α, IL-6, and IL-12p40 were quantified by ELISA. (C) The percent of variation (mean ± SEM, $n = 5$) in cytokine release detected from monocytes transfected with as-miR-187 vs. as-miR-scr control.

TNF- α Is a Direct Target of miR-187. Based on the observation that the levels of TNF- α , IL-6, and IL-12p40 production are increased significantly when the intracellular levels of miR-187 expression are knocked down, we analyzed whether these cytokine genes are direct targets of miR-187. MicroRNAs exert their biological functions through the suppression of target genes via RNA-RNA complementarity. Using a combination of Miranda and TargetScan, we generated a list of 197 potential miR-187 targets, among which only TNF- α , but not IL-6 or IL-12p40, was included. Interestingly, kinetics of TNF- α mRNA expression inversely correlates with that of miR-187 in monocytes stimulated with LPS and LPS plus IL-10 (Fig. S4A). To confirm TNF- α mRNA as a direct target of miR-187, reporter constructs containing the renilla luciferase gene fused to the TNF- α 3' UTR (luc-TNF- α) or to a version of the TNF- α 3' UTR deleted of the miR-187 seed region (luc-mut-TNF- α) were transiently transfected into HEK293 cells together with miR-187 mimics or miR-scr. As shown in Fig. 4A, miR-187 significantly reduced luc-TNF- α , but not luc-mut-TNF- α , luciferase activity demonstrating that the region located at nucleotides 1,273–1,282 of the TNF- α cDNA and 31 nt upstream of the TNF- α AU-rich element is the active seed of miR-187. Consistent with the luc-TNF- α reporter results, a marked reduction in TNF- α production was observed reliably in response to LPS (mean reduction $59.6 \pm 5.6\%$; $n = 5$, $P = 0.028$) and to LPS plus IL-10 (mean reduction $48.5 \pm 5.7\%$; $n = 5$, $P = 0.005$) in monocytes overexpressing the miR-187 mimic (Fig. S2B) compared with miR-scr (Fig. 4B and Table S3). Under these conditions, the reduced amount of TNF- α protein was paralleled by a comparable reduction in the levels of TNF- α mRNA expression (-39.2%), but not of TNF- α primary transcripts (Fig. S4B), further demonstrating that miR-187 directly controls TNF- α gene expression at the posttranscriptional level.

In the attempt to investigate the mechanism whereby miR-187 decreases TNF- α mRNA expression and production in LPS-treated monocytes, we examined the effect of miR-187 overexpression on TNF- α mRNA stability. miR-187- or miR-scr-transfected monocytes were stimulated with LPS for 90 min and then were treated with actinomycin D to block the formation of additional transcripts. At increasing intervals thereafter, cells were processed and changes in the amount of TNF- α mRNA were quantified by RT quantitative PCR (RT-qPCR). Fig. 4C shows that miR-187 overexpression markedly reduces TNF- α half-life compared with miR-scr (33 min vs. 18 min). This effect was specific for TNF- α , because miR-187 did not significantly modify the stability of IL-6 mRNA (Fig. 4C). Collectively, these data demonstrate that miR-187 is part of the IL-10-dependent mechanism that controls TNF- α expression at a posttranscriptional level.

miR-187-Mediated Down-Regulation of I κ B ζ Sustains the Inhibitory Effect of IL-10 on LPS-Induced IL-6 and IL-12p40 Production. To understand why miR-187 silencing modulates the production of IL-6 and IL-12p40 (Fig. 3), which are not listed among the 197 target genes commonly predicted by Miranda and TargetScan algorithms, additional criteria (as specified in Fig. S5A) were used to identify miR-187 targets involved in the regulation of IL-6 and IL-12p40 expression. Because many studies have highlighted the fact that miRNA-mediated targeting of transcription factors, including those activated upon TLR signaling (12, 22, 23), is an important aspect of miRNA function (24–26), we focused on genes encoding inflammation-related transcription factors and identified them by Gene Ontology (GO)-terms association.

This analysis predicted the transcriptional regulators *NFKBIZ*, *BCL6*, and *SMAD1* as the most highly probable miR-187 target genes (Fig. S5A and B). Interestingly, *NFKBIZ* encodes for a nuclear protein I κ B ζ , also known as “MAIL” (27), that is induced upon TLR/IL-1R stimulation and that is required for the LPS-dependent induction of IL-6 and IL-12p40 (27–29). Fur-

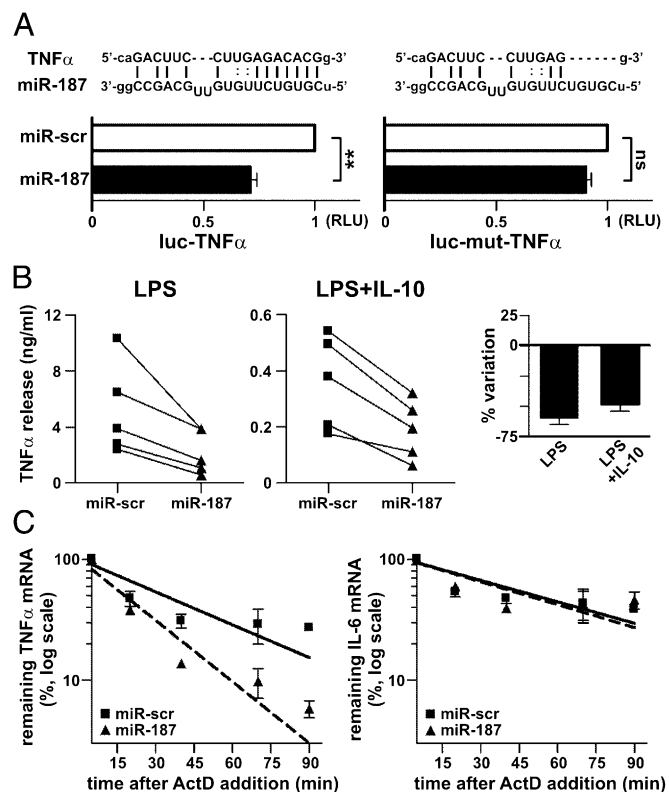


Fig. 4. TNF- α is a direct target of miR-187. (A) Base pairing comparison between mature miR-187 and wild-type or mutant TNF- α 3'UTR putative target site is shown according to the Miranda algorithm. The TNF- α 3'UTR-luciferase construct was cotransfected in HEK293 cells with miR-187 mimic or miR-scr. Relative renilla luciferase activity is expressed as mean \pm SEM ($n = 3$), adjusted to 1. * $P < 0.01$; ns: $P > 0.05$; Student *t* test; ns, not significant. (B) The amount of antigenic TNF- α was quantified by ELISA in cell-free supernatants collected after 24 h from monocytes transfected with miR-187 mimic or miR-scr and stimulated with LPS (Left) or LPS plus IL-10 (Center). (Right) Percent of variation of TNF- α released by monocytes transfected with miR-187 mimic vs. miR-scr control (mean \pm SEM, $n = 5$). (C) Monocytes transfected with miR-187 mimic or miR-scr control were cultured with LPS for 90 min before the addition of 10 μ g/mL actinomycin D (ActD). Monocytes were harvested 20, 40, 70, and 90 min after the addition of actinomycin D (time 0), and total RNA was purified and analyzed by RT-qPCR using specific primers for TNF- α and IL-6 mRNA. Expression data are expressed as percent of mRNA remaining at each time point vs. mRNA levels at time 0. Half-lives were calculated by regression analysis. One experiment representative of two performed is shown.

thermore, additional observations indirectly supported the prediction of *NFKBIZ* as one of the most likely miR-187 target genes. In fact, in a reverse analysis, miR-187 was identified by both Miranda and TargetScan algorithms as being among the *NFKBIZ*-targeting miRNAs with a favorable energy score (Fig. S5C). Additionally, kinetics of expression of I κ B ζ (the *NFKBIZ* protein product) inversely correlates with the expression of miR-187 in monocytes stimulated with LPS (Fig. S5D and E). This inverse correlation is even stronger in monocytes stimulated with LPS plus IL-10, in which miR-187 expression is potentiated but the expression of I κ B ζ is further reduced, particularly at 12 and 24 h when this effect is the most pronounced (Fig. S5D and E).

To test whether miR-187 posttranscriptionally affects *NFKBIZ* expression, a reporter construct containing the renilla luciferase gene fused to the *NFKBIZ* 3' UTR (luc-*NFKBIZ*) (Fig. 5A) was transiently transfected in HEK-293 cells together with miR-187 mimic. As shown in Fig. 5B, miR-187 significantly reduced luc-*NFKBIZ* luciferase activity by 29.74%. Deletion of the upstream

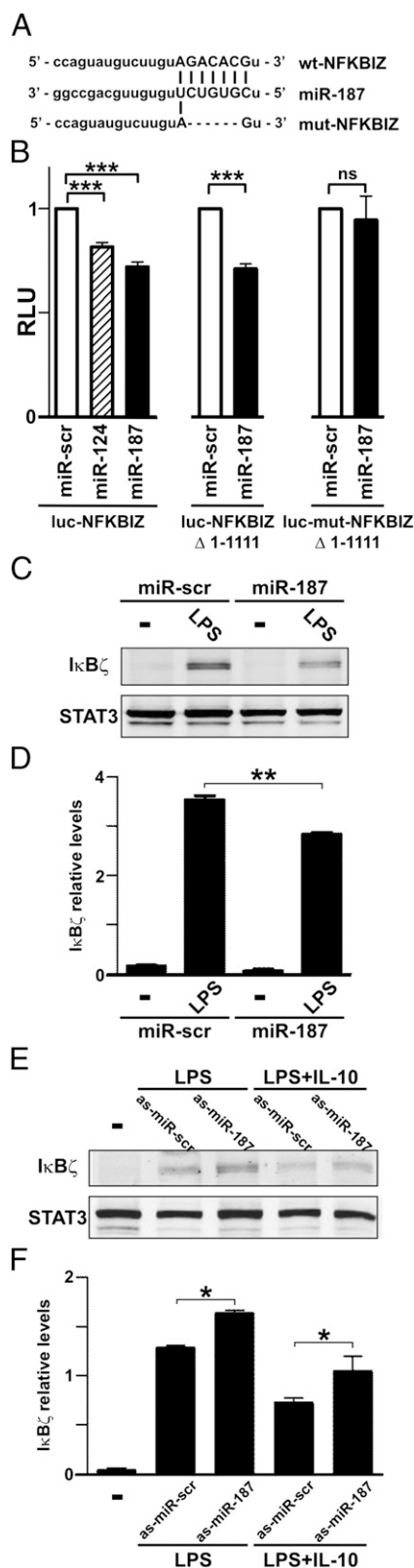


Fig. 5. Modulation of $I\kappa B\zeta$ expression by miR-187. (A) The putative pairing between miR-187:WT-NFKBIZ 3'UTR and miR-187:mut-NFKBIZ 3'UTR is shown. (B) HEK293 cells were cotransfected with the luc-NFKBIZ full-length luciferase or with the luc-NFKBIZ $\Delta 1-1111$ or the luc-mut-NFKBIZ $\Delta 1-1111$ construct together with miR-187, miR-124, or miR-scr, as indicated. Relative luciferase activity (relative luminescence units, RLU) is expressed as mean \pm SEM ($n = 4$), adjusted to 1. *** $P < 0.001$; ns, $P > 0.05$; Student t test or one-way ANOVA was

1,111 bp of the NFKBIZ 3'UTR (luc-NFKBIZ $\Delta 1-1111$), containing an active miR-124 seed region (30), did not modify the miR-187-mediated inhibition of luciferase activity, which instead was reversed by deletion of 5 bp in the miR-187 seed region (Fig. 5A) in the NFKBIZ 3'UTR (luc-mut-NFKBIZ $\Delta 1-1111$) (Fig. 5B), demonstrating the specificity of miR-187:NFKBIZ 3'UTR interaction.

To provide direct evidence for NFKBIZ as a physiologic target of miR-187, we tested whether miR-187 can influence the levels of the endogenous $I\kappa B\zeta$ protein expression directly (Fig. 5C–F). An miR-187 mimic or a scramble negative control was transiently transfected in monocytes (Fig. S6A), and the level of $I\kappa B\zeta$ protein expression following LPS stimulation was assessed by Western blotting (Fig. 5C). Three hours after LPS stimulation, miR-187 overexpression leads to a reduction in the expression of the $I\kappa B\zeta$ protein (Fig. 5C). The reduction of the $I\kappa B\zeta$ protein content by miR-187 was reproduced consistently in four independent experiments, with a significant ($P = 0.001$) mean reduction of $19.8 \pm 2.3\%$ (Fig. 5D). In the complementary approach, we functionally inhibited miR-187 by transiently transfecting monocytes with as-miR-187 or with the as-miR-scr control (Fig. S6B). $I\kappa B\zeta$ protein levels were analyzed 6 h after stimulation with LPS or with LPS plus IL-10, a time point at which miR-187 induction is readily detectable (Fig. 1C). Silencing miR-187 expression affects $I\kappa B\zeta$ protein levels (Fig. 5E), which increase by $27.5 \pm 0.35\%$ and $43.3 \pm 13.61\%$ in monocytes stimulated for 6 h with LPS and LPS plus IL-10, respectively (Fig. 5F). Remarkably, blocking miR-187 induction by an miRNA inhibitor partially reversed IL-10-induced inhibition of $I\kappa B\zeta$ (Fig. 5E and F).

To investigate whether the negative regulation of $I\kappa B\zeta$ by miR-187 was functionally involved in the production of IL-6 and IL-12p40 by LPS and in their regulation by IL-10, the effect of miR-187 expression was compared with that of a specific siRNA against $I\kappa B\zeta$. Human monocytes were transfected with 50, 100, or 150 pmol of miR-187 mimic (Fig. S7A) or with 15, 50, or 150 pmol of si- $I\kappa B\zeta$ (Fig. S7B), and the expression of $I\kappa B\zeta$ mRNA (Fig. S7C and D) and protein (Fig. S7E–H) was analyzed. As shown in Fig. S7, although si- $I\kappa B\zeta$ decreased the levels of $I\kappa B\zeta$ mRNA in a dose-dependent manner, miR-187 dose-dependently reduced the expression of $I\kappa B\zeta$ protein but not $I\kappa B\zeta$ mRNA, suggesting that it inhibits $I\kappa B\zeta$ translation. This effect was specific for $I\kappa B\zeta$, because miR-187 did not affect the expression of the $I\kappa B\alpha$ protein, a homolog of $I\kappa B\zeta$ (Fig. S7E and I). In subsequent experiments, 150 pmol of miR-187 mimic or 15 pmol of si- $I\kappa B\zeta$, which comparably reduced $I\kappa B\zeta$ protein expression by $\sim 30\%$ (Fig. S7E–H), was transfected into monocytes (along with their related controls), and the expression of IL-6 and IL-12p40 mRNA was analyzed after 3 h of LPS stimulation (Fig. 6). The reduction of $I\kappa B\zeta$ expression mediated by either miR-187 or si- $I\kappa B\zeta$ causes a significant and comparable reduction of the transcriptional activation of the IL-6 and IL-12p40 genes, as shown by the reduced recruitment of Pol II to the IL-6 and IL-12p40 promoters (Fig. 6A and B). Moreover, LPS-induced IL-6 and IL-12p40 primary transcript are reduced consistently in both miR-187- (Fig. 6C) and si- $I\kappa B\zeta$ -

used or the comparison between two or three groups, respectively). (C and E) Freshly purified monocytes were transfected with 100 pmol miR-scr or miR-187 mimic and were stimulated with LPS for 3 h (C) or were transfected with 150 pmol as-miR-scr or as-miR-187 and were stimulated with LPS or LPS plus IL-10 for 6 h (E). Total cell extracts (typically 100 μ g) were loaded on gels, and immunoblots were performed by simultaneously using antibodies specific for $I\kappa B\zeta$ and STAT3, followed by incubation with Alexa Fluor 680 goat anti-rabbit and IRDyeTM 800 goat anti-mouse antibodies. One experiment representative of three is shown in C and E. (D and F) Blots were scanned with the Odyssey Infrared Imaging System. $I\kappa B\zeta$ levels quantified by the Odyssey software and normalized for the total STAT3 are reported. Graphs represent the mean \pm SEM ($n = 3$); ** $P < 0.01$; * $P < 0.05$; one-way ANOVA.

(Fig. 6D) transfected monocytes. Consistently, LPS-induced IL-6 and IL-12p40 production is reduced significantly in miR-187- and si-I κ B ζ -transfected monocytes compared with their respective controls (Fig. 6E and F). Collectively, these data demonstrate that IL-10, through the miR-187-mediated reduction of I κ B ζ , indirectly inhibits LPS-induced IL-6 and IL-12p40 transcription and production.

miR-187 and Its Target mRNAs Specifically Colocalize in Ago Complexes.

miRNA-target transcripts are recruited via miRNA-mRNA interaction to the Argonaute (Ago)-containing RNA-induced silencing complex (RISC) (31). To provide direct evidence that miR-187 targets *NFKBIZ* and *TNFA* genes, we looked for the presence of I κ B ζ and TNF- α mRNA in miR-187-containing RISC complexes. Monocytes were incubated with or without LPS for 16 h, a time point at which high levels of endogenous miR-187 are detected (Fig. 1C). In parallel, monocytes were transfected with miR-187 mimic, along with miR-scr control, and were stimulated for 2 h with LPS, a time point at which I κ B ζ and TNF- α mRNAs are expressed at high levels but endogenous miR-187 expression levels are only slightly above the control (Fig. 1C). Ago immunoprecipitation was performed using a monoclonal antibody toward all human Ago isoforms and was validated by Western blotting (Fig. S8A). miR-187, TNF- α , and I κ B ζ mRNA levels then were analyzed by RT-qPCR in the RNA fraction extracted from Ago and IgG_{1 κ} immunoprecipitates. A significant recruitment of miR-187 to the Ago complexes was observed in LPS-stimulated (Fig. 7A)

and miR-187-overexpressing (Fig. 7B) monocytes compared with resting and miR-scr-transfected monocytes, respectively. The lack of miR-187 association with the Ago complexes in unstimulated monocytes or in miR-scr-transfected monocytes was not caused by a failure to immunoprecipitate Ago-bound miRNAs, as shown by the detection in the same samples of a constitutively expressed miRNA, i.e., miR-16 (Fig. S8B and C), which occurs under resting conditions and is not modified by LPS stimulation or by miR-187 overexpression. mRNAs encoding for I κ B ζ (Fig. 7C) and TNF- α (Fig. 7E) are significantly recruited to the Ago complexes upon LPS stimulation, demonstrating that these two LPS-responsive genes are subjected to miRNA-mediated regulation. Most importantly, Fig. 7D and F shows that stimulation of monocytes with LPS for only 2 h is not sufficient per se to promote a consistent recruitment of I κ B ζ and TNF- α mRNAs to the RISC complex. In fact, in the absence of miR-187 (i.e., in monocytes transfected with miR-scr and stimulated for 2 h with LPS), recruitment of I κ B ζ and TNF- α mRNAs to the Ago complex is absent or negligible (Fig. 7D and F). In contrast, both I κ B ζ and TNF- α mRNAs are strongly and significantly recruited to the RISC complexes only when exogenous miR-187 is present (Fig. 7D and F). These data demonstrate that the simultaneous presence of miR-187 with I κ B ζ and TNF- α mRNAs in the Ago complexes is not casual; rather, miR-187 is required to recruit I κ B ζ and TNF- α mRNAs. This crucial point is supported further by the analysis of the recruitment of IL-6 and I κ B α mRNAs (which do not contain a miR-187 seed region in their 3'UTRs) to the RISC complex under the same experimental

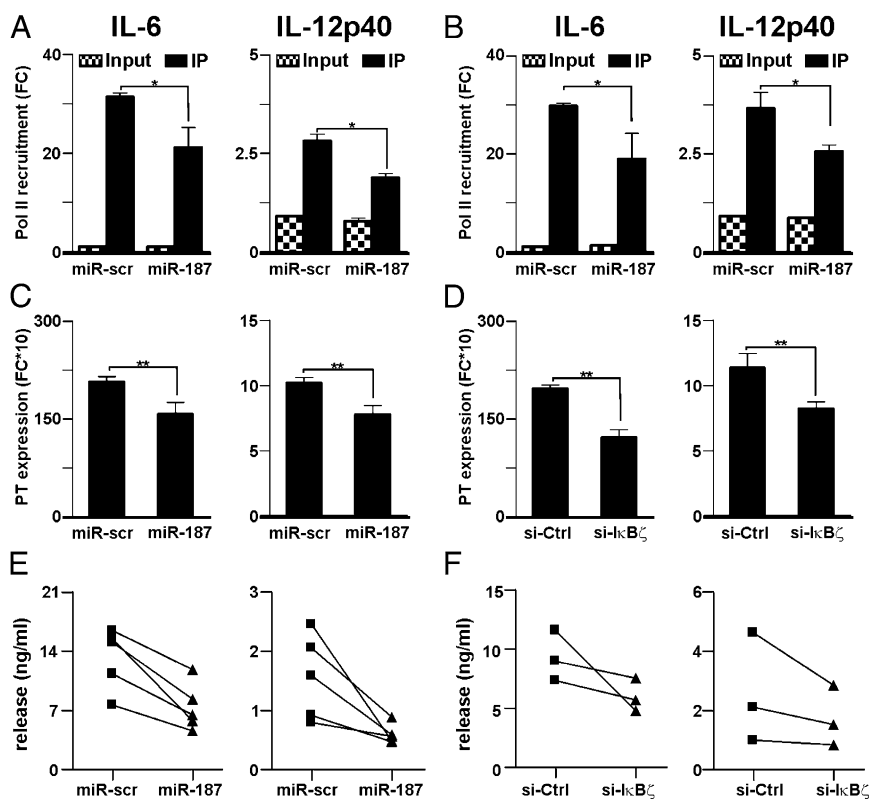


Fig. 6. LPS-induced IL-6 and IL-12p40 expression and production are reduced by miR-187-mediated I κ B ζ knockdown. Monocytes transfected with 150 pmol miR-scr or miR-187 mimic (A, C, and E) or with 15 pmol si-Ctrl or si-I κ B ζ siRNA (B, D, and F) were stimulated with LPS for 3 h and then were processed for ChIP assay (A and B) or IL-6 and IL-12p40 expression (C and D). (A and B) The ChIP assay was carried out using anti-Pol II antibody and was analyzed by qPCR with specific primer pairs amplifying the IL-6 or the IL-12p40 promoters. Data from qPCR are expressed as fold change (FC) over the control sample as described in *Materials and Methods* and are displayed as means \pm SEM of three independent experiments. * P < 0.05; one-way ANOVA. (C and D) Total RNA was purified from the same samples, and the expression of IL-6 and IL-12p40 primary transcript (PT) was analyzed by RT-qPCR. Expression of each gene is reported as fold change after normalization to the *RPL32* housekeeping gene. Data are shown as means \pm SEM (n = 5). ** P < 0.01; one-way ANOVA. (E and F) Antigenic IL-6 (E and F, Left) and IL-12p40 (E and F, Right) levels were determined by ELISA in cell-free supernatant collected 24 h after stimulation with LPS.

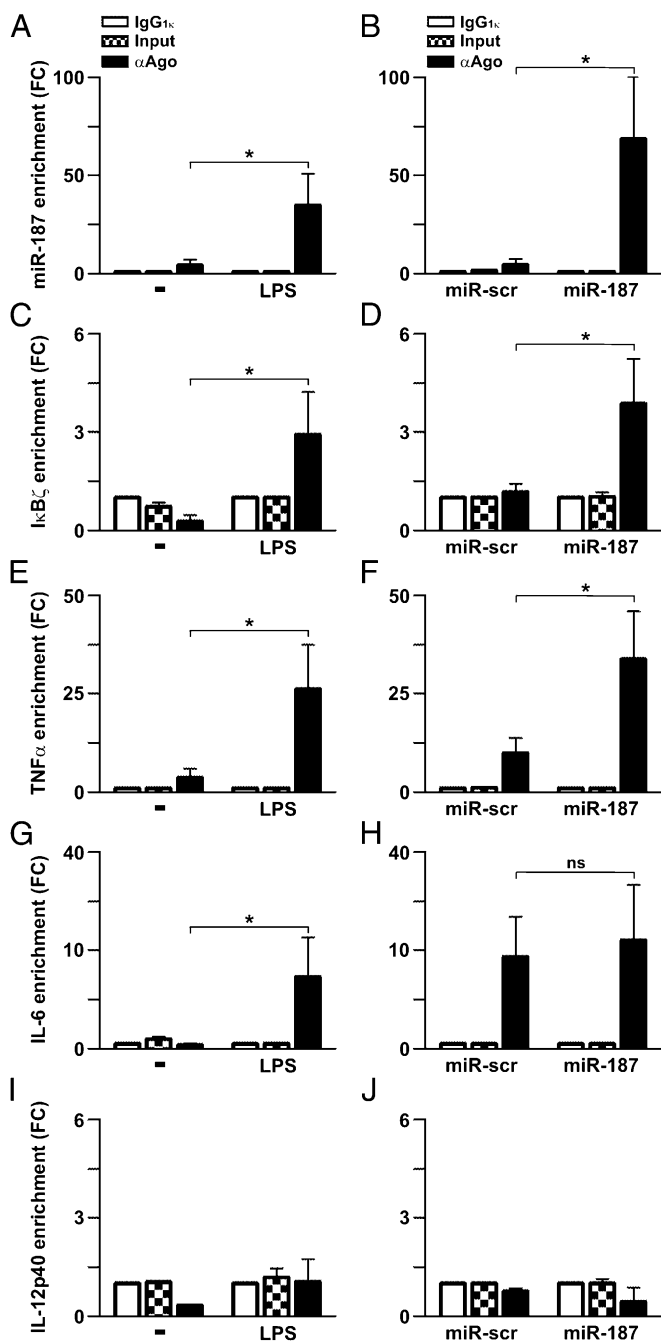


Fig. 7. κ B ζ and TNF- α mRNA specifically colocalize with miR-187 in Ago complexes. Freshly purified monocytes were left untreated or were stimulated with LPS for 16 h (A, C, E, G, and I) or were transfected with miR-scr or miR-187 mimic and were stimulated with LPS for 2 h (B, D, F, H, and J). Cells were lysed for RIP assay and subjected to Ago or IgG (control) immunoprecipitation in parallel, as described in *Material and Methods*. The levels of the indicated miRNAs or mRNAs, as well as input samples, were assayed in triplicate by RT-qPCR in RIP, are expressed as normalized fold enrichment (FC) as described in *Material and Methods*, and are displayed as the mean \pm SEM of four independent experiments for each panel. Asterisks indicate a statistically significant ($P < 0.05$) difference between LPS-stimulated vs. resting monocytes or miR-187- vs. miR-scr-transfected monocytes as determined by Student t test; ns, $P > 0.05$.

conditions. IL-6 and I κ B α mRNA are present in the Ago immunoprecipitate in monocytes stimulated with LPS (Fig. 7G and Fig. S8D), indicating that these mRNA likely are subjected to miRNA-

mediated regulation. However, this event occurs to the same extent in both the absence (miR-scr) and presence of miR-187 (Fig. 7H and Fig. S8E), demonstrating that the recruitment of IL-6 and I κ B α mRNAs to the RISC complex is not specifically dependent on miR-187. The absence of IL-12p40 mRNA (Fig. 7I and J) and of the ribonucleoprotein 32 (RPL32) mRNA (Fig. S8F and G) in Ago immunoprecipitates from LPS-stimulated monocytes demonstrates that these two mRNAs are not subjected to posttranscriptional regulation. It also excludes the possibility that the observed increase in I κ B ζ , TNF- α , IL-6, and I κ B α mRNAs (Fig. 7C–H and Fig. S8D and E) is caused by a general, nonspecific enrichment of mRNAs in Ago immunoprecipitates. Collectively, these data clearly demonstrate that miR-187 posttranscriptionally regulates *NFKBIZ* and *TNFA* gene expression by driving the physical association of I κ B ζ and TNF- α mRNAs to the RISC complex.

miR-187 Expression Is Up-Regulated in Circulating Monocytes Purified from Septic Patients.

To verify whether miR-187 undergoes in vivo modulation, miR-187 expression levels were analyzed in peripheral blood mononuclear cells (PBMC) freshly purified from five patients affected by sepsis. Fig. 8 shows that PBMC from septic patients, unlike PBMC from healthy donors, constitutively express high levels of miR-187. Similarly, other miRNAs, namely miR-155, miR-146a, and miR-9, were found to be constitutively up-regulated in septic PBMC compared with PBMC from healthy controls, but miR-21 and let-7a were not (Fig. 8).

Discussion

Negative regulation of inflammation is promoted strongly by IL-10, which primarily inhibits the production of proinflammatory cytokines by activated leukocytes, acting both at the transcriptional and posttranscriptional levels (6). To gain insights into the posttranscriptional mechanisms whereby IL-10 dampens innate immune cell activation, we investigated the effects of this anti-inflammatory cytokine on the expression of miRNAs, which have been shown to have an important role in tuning the inflammatory response by targeting mRNA translation, processing, and stability (7). This analysis led to the identification of miR-187 as an miRNA induced in an IL-10-dependent manner in activated human monocytes. Interestingly, freshly purified mouse bone marrow mononuclear cells, BMDM, TEM, and the Raw264.7 mouse macrophage cell line do not up-regulate miR-187 expression in response to LPS or LPS plus IL-10, further emphasizing that species-specific mechanisms of miRNA-mediated control of TLR responses have evolved.

IL-10 alone is a relatively weak stimulus, but it strongly synergizes with LPS in inducing miR-187 expression. Several lines of evidence clearly point to the requirement for endogenous IL-10 in the induction of miR-187 expression in LPS-activated human monocytes. (i) LPS, Pam3Cys, and IL-1 β fail to up-regulate miR-187 expression when signaling from endogenous IL-10 is blocked with anti-IL-10 or anti-IL-10R antibodies. (ii) LPS does not up-regulate miR-187 expression in human PMN because of its inability to trigger endogenous IL-10 production by these cells (21). Nevertheless, addition of exogenous IL-10 restores the ability of PMN to up-regulate miR-187 expression. (iii) Likewise, miR-187 expression is not induced in monocytes activated by stimuli that do not trigger IL-10 production, such as the TLR3-agonist polyinosinic:polycytidylic acid or the proinflammatory cytokine TNF- α . (iv) IL-10 directly potentiates LPS-induced miR-187 transcription, as shown by the increase in the levels of premiR-187 expression and by the recruitment of Pol II to the premiR-187-encoding region, which is abolished in the presence of IL-10-blocking antibodies.

Analysis of the phenotypic consequences of silencing miR-187 expression provided evidence for the role of this miRNA in the suppressive activity of IL-10 on LPS-stimulated monocytes. In

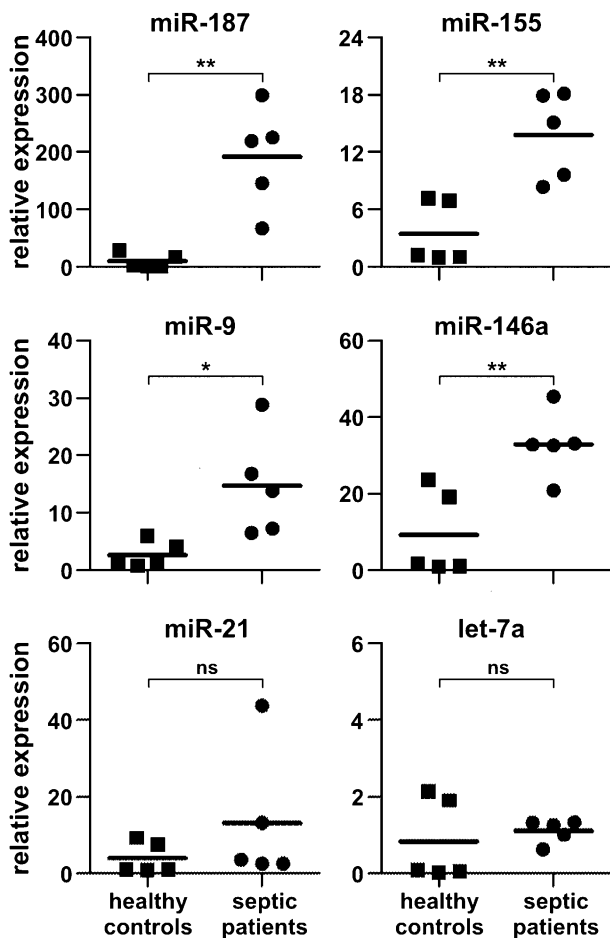


Fig. 8. miR-187 is overexpressed in PBMC from patients with SIRS. The expression of miR-187, miR-155, miR-9, miR-146a, miR-21, and let-7a was analyzed by RT-qPCR and analyzed as described in *Material and Methods* in PBMC freshly purified from five healthy donors (■) and from five SIRS patients (●). Horizontal bars represent the mean values of relative expression within each group. ** $P < 0.01$; * $P < 0.05$; ns, $P > 0.05$.

fact, when miR-87 is silenced, the production of LPS-induced TNF- α , IL-6, and IL-12p40, but not IL-1 β , IL-1ra, IFN- β , CXCL10, CCL2, CXCL8, and IL-10, is increased significantly. In particular, no modification in the levels of LPS-induced IL-10 secretion results from miR-187 silencing, indicating that TNF- α , IL-6, and IL-12p40 inhibition is not secondary to increased IL-10 production. Most importantly, miR-187 blockade reverses the IL-10 inhibitory effect on LPS-induced TNF- α , IL-6, and IL-12p40 production only partially (ranging from 30–50%), in agreement with data showing that the impact of miRNAs on the protein output typically is less than 50% (32). Consistent with the results obtained in miR-187-silenced monocytes, ectopic expression of miR-187 significantly reduces the levels of LPS-induced TNF- α , IL-6, and IL-12p40 production. Reduction of cytokine production by miRNAs in vitro, although relatively small, has been shown to be functionally relevant in physiologic conditions. For instance, knockdown or overexpression of miR-155 in Raw264.7 macrophages stimulated in vitro with LPS modifies the release of proinflammatory cytokines from 10% to 50% (17). Despite this modest impact on proinflammatory cytokine release, miR-155 has profound physiologic effects and has been shown to impact the severity of sepsis in a murine model of endotoxemia (17). Similarly, the potential relevance of miR-187-mediated effects as a physiological mechanism triggered during

monocyte activation in vivo is supported by the observation that miR-187 expression is induced at high levels in septic patients. In fact, we report that circulating PBMC purified from patients affected by sepsis constitutively express miR-187 as well as the already described miR-155 and miR-146a. Therefore it is likely that miR-187 is induced in monocytes by IL-10, which, along with other mediators, is known to be produced systemically during sepsis (33), presumably as part of a circuit to help attenuate the proinflammatory state. These observations represent evidence that an miRNA is actively involved in the anti-inflammatory functions of IL-10.

Inspection of Miranda and TargetScan identified TNF- α among the potential miR-187 target genes. The presence of a functional miR-187 seed region regulating TNF- α expression was validated experimentally by down-modulation of the luciferase-reporter genes containing the wild-type TNF- α 3'UTRs, but not the mutated form, in an HEK293 cell line overexpressing miR-187. Analysis of the mechanisms whereby miR-187 interferes with TNF- α production revealed that miR-187 directly recruits TNF- α mRNA to the RISC complex, thereby promoting its degradation. Consistently, TNF- α already has been described as subjected to miRNA-mediated posttranscriptional regulation. MiR-16 (34), miR-369-3 (35), miR-125b (36, 37), miR-579, and miR-221 (37) were shown to be involved in the regulation of TNF- α production in different cellular systems. Nevertheless, miR-369-3 and miR-579 are not detectable in resting or activated monocytes (threshold cycle >35) and therefore can be excluded from playing a role in IL-10-mediated inhibition of TNF- α expression. Conversely, miR-16, miR-125b and miR-221, although not modulated by LPS or LPS plus IL-10, are constitutively expressed in resting monocytes and might take part in the posttranscriptional modulation of TNF- α expression. However, the observations that TNF- α mRNA is recruited to the RISC complex only when miR-187 is present and that TNF- α mRNA half-life is reduced significantly only in monocytes overexpressing miR-187 demonstrate that miR-187 is required for the control of TNF- α mRNA expression in LPS-stimulated cells.

Even though they are not listed among the putative miR-187 target genes, IL-6 and IL-12p40 expression is significantly down-modulated by miR-187 in LPS-treated monocytes. This down-modulation is achieved by miR-187-driven targeting of *NFKBIZ*, a gene encoding for a positive transcriptional regulator of IL-6 and IL-12p40 expression (28). Indeed, in agreement with previous findings (29), si-RNA-mediated silencing of I κ B ζ , the *NFKBIZ* gene product, dose-dependently reduces IL-6 and IL-12p40 production in response to LPS. Moreover, up-regulation of I κ B ζ expression by Toll/IL1R activation has been shown to be controlled by transcriptional (28, 38), posttranscriptional (38, 39), and miRNA-mediated mechanisms (30). Finally, and consistent with published data (40), LPS-induced I κ B ζ protein expression is inhibited by IL-10, with kinetics that displayed an inverse relationship with that of miR-187 expression. *In silico* analysis showed a very favorable and low-energy interaction of the miR-187:*NFKBIZ* mRNA pair, and overexpression of miR-187 reduces both the activity of the *NFKBIZ*-3'UTR-luciferase reporter in HEK293 cells and the levels of endogenous I κ B ζ expression in monocytes. Most importantly, blocking miR-187 expression with miR-187 inhibitor partially reversed IL-10-mediated inhibition of I κ B ζ expression, indicating that I κ B ζ inhibition is achieved via up-regulation of miR-187. Therefore, as a result of miR-187-mediated reduction of I κ B ζ , the transcriptional activation, but not mRNA stability, of IL-6 and IL-12p40 was diminished, ultimately leading to their reduced production and secretion.

Collectively, our data identify the miRNA system as a candidate mechanism through which IL-10 can tailor monocyte responses to LPS. Supporting this view, recent data confirmed herein demonstrate that IL-10 contributes to switching off the

inflammatory response by inhibiting LPS-induced expression of miR-155 (18). In doing so, IL-10 reverses miR-155-mediated down-regulation of SHIP1, a validated target of miR-155, thus favoring the negative regulation of TLR4 signaling by counteracting PI-3K activity (18). Remarkably, our data demonstrate that the modulation of LPS responses goes beyond IL-10's capacity merely to downmodulate LPS-induced miR-155, in that we show that IL-10 transiently increases the rate of expression of LPS-induced miR-146b at early time points and consistently is required to enhance the expression of miR-187. miR-146b has been identified recently as being among miRNAs playing a role in the resolution phase of inflammation (41). In a mouse model of self-limited acute inflammatory response and in human macrophages, miR-146b has been shown to be up-regulated specifically by resolvin D1, a lipid mediator with anti-inflammatory properties, and to decrease the protein levels of the proinflammatory chemokines CXCL8 and CCL5 by targeting NF- κ B signaling (41). In this context, we speculate that IL-10, by inducing a rapid increase of miR-146b expression, anticipates the onset of the physiological resolution of inflammation. This issue is currently under investigation. Finally, and most importantly, we identify miR-187 as an IL-10-dependent miRNA and provide mechanistic insight into the role of miR-187 in mediating some of the IL-10 anti-inflammatory activities. MiR-187, induced by IL-10 endogenously produced by LPS-stimulated monocytes or present in the inflammatory microenvironment, acts as a feedback modulator of LPS response by directly limiting TNF- α production at a posttranscriptional level and by reducing IL-6 and IL-12p40 transcription via silencing the transcription factor I κ B ζ .

Given the widely accepted links between inflammation and the pathogenesis of several diseases, ranging from autoimmune to neoplastic, the molecular events involved in the development of inflammation-driven pathologies are of obvious importance. Hence, the identification of anti-inflammatory miRNAs and the characterization of their contribution to the resolution phase of inflammation are of considerable interest and might help in the development of miRNA-targeting drugs.

Materials and Methods

Materials. A detailed list of materials is provided in *SI Materials and Methods*.

Cell Purification and Culture. Human PMN (~99.7%) and monocytes (~98.5%) were purified from buffy coats of healthy donors by negative magnetic selection and were cultured as described in *SI Materials and Methods*. The HEK293 and the Raw264.7 cell lines were grown in DMEM supplemented with 10% (vol/vol) FCS, 100 U/mL penicillin/streptomycin, and 2 mM L-glutamine. MDM were differentiated from monocytes cultured for 6 d in the presence of 100 ng/mL macrophage colony-stimulating factor.

Mouse fresh bone marrow mononuclear cells, BMDM, and TEM were obtained from 8-wk-old wild-type C57BL/6 mice and were differentiated and cultured as previously described (42, 43).

PBMC were purified as described in *SI Materials and Methods* from five healthy donors and from five patients fulfilling the criteria of systemic inflammatory response syndrome (SIRS) according to ref. 44. For additional details, refer to *SI Materials and Methods*.

miRNA and Gene-Expression Analysis. High-throughput and single-miRNA expression analyses were performed by TaqMan as previously described (12) and as described in detail in *SI Materials and Methods*. The expression of premiR-

187, IL-6, and IL-12p40 primary transcript, I κ B ζ , TNF- α , IL-6, IL-12p40, and RPL32 mRNA was quantified by RT-qPCR as previously described (4), using specific primer pairs listed in Table S4. RT-qPCR data were normalized to the expression of the housekeeping gene RPL32.

Constructs and Luciferase Reporter Assays. Renilla luciferase reporter construct containing the full-length sequence of NFKBIZ 3' UTR (luc-NFKBIZ) was purchased from SwitchGear Genomics. The other reporter constructs were generated as described in *SI Materials and Methods* by primers listed in Table S4. Transfection of HEK293 cells and the luciferase assay are detailed in *SI Materials and Methods*.

Immunoblots. Preparation of cell lysates and Western blot analysis were conducted as described in detail in *SI Materials and Methods*. The blots were incubated with anti-I κ B ζ , anti-STAT3, or anti-I κ B α antibodies, and detection was performed and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Monocyte Transfection. Freshly purified monocytes (10^7) were transfected with the indicated amount of miRNA mimic (Ambion, Applied Biosystems), miRCURY LNA miRNA Power Inhibitor (Exiqon), or onTARGETplus siRNA (Dharmacon) using the Amaxa Nucleofector and the Human Monocyte Nucleofector kit (Amaxa) according to the manufacturer's protocol. Transfected cells were stimulated 18 h later as indicated.

ChIP Assay. ChIP experiments were performed as described elsewhere (45). Briefly, sheared chromatin from 5×10^6 monocytes was immunoprecipitated overnight at 4 °C using polyclonal antibodies against Pol II (Santa Cruz Biotechnology). Purified DNA was analyzed by qPCR in triplicates, using promoter-specific primers (Table S4). Data are expressed as described in *SI Materials and Methods*.

Ribonucleoprotein Immunoprecipitation and Ago-Associated mRNA Analysis. Ribonucleoprotein immunoprecipitation (RIP) experiments were performed as described elsewhere (46), with minor modifications, as detailed in *SI Materials and Methods*. The levels of coimmunoprecipitated miR-187 and miR-16 and of I κ B ζ , TNF- α , IL-6, IL-12p40, and RPL32 mRNA then were assessed by RT-qPCR as described in the previous sections. Sequences of mRNA-specific primers used in qPCR are listed in Table S4.

Detection of Cytokine Release. Cytokine concentrations in cell-free supernatants were measured by specific human ELISA kits for IL-6 and CXCL8 (ImmunoTools), TNF- α , CXCL10, CCL2, IL-12p40, and IL-1ra (R&D Systems), IFN- β (PBL Interferon Source), and IL-1 β and IL-10 (e-Biosciences) according to the manufacturers' instructions.

Statistical Analysis. Statistical evaluation was determined using the Student *t* test or one-way ANOVA with power of test set to 0.05. The pairwise multiple comparison procedures were performed according to the Holm-Sidak method, and *P* values less than 0.05 were considered significant.

ACKNOWLEDGMENTS. This study was supported by research grants from the Ministero dell'Istruzione dell'Università e della Ricerca (Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale projects 2002061255, 2007-ENYMAN_003, and 200999KRFW_004; FIRB project RBF080CW8G), the University of Verona (a Joint Project_2010 grant), Fondazione Cariverona ("Verona nano-medicine initiative" project), the Alleanza Contro il Cancro/Istituto Superiore di Sanità (Programma Straordinario di Ricerca Oncologica 2006-Rete Nazionale Bioinformatica Oncologica project), Italian Association for Cancer Research (AIRC) Grant IG-11782, and Regione Lombardia (Lombardy Innate Immunity Network project) and was conducted in the context and with the support of the Fondazione Humanitas per la Ricerca. N.T. is supported by an AIRC fellowship.

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