Toll-like receptors (TLRs) play key roles in detecting pathogens and initiating inflammatory responses that, subsequently, prime specific adaptive responses. Several mechanisms control TLR activity to avoid excessive inflammation and consequent immunopathology, including the anti-inflammatory cytokine IL-10. Recently, several TLR-responsive microRNAs (miRs) have also been proposed as potential regulators of this signaling pathway, but their functional role during the inflammatory response still is incompletely understood. In this study, we report that, after LPS engagement, monocytes up-regulate miR-146b via an IL-10-mediated STAT3-dependent loop. We show evidence that miR-146b modulates the TLR4 signaling pathway by direct targeting of multiple elements, including the LPS receptor TLR4 and the key adaptor/signaling proteins myeloid differentiation primary response (MyD88), interleukin-1 receptor-associated kinase 1 (IRAK-1), and TNF receptor-associated factor 6 (TRAF6). Furthermore, we demonstrate that the enforced expression of miR-146b in human monocytes led to a significant reduction in the LPS-dependent production of several proinflammatory cytokines and chemokines, including IL-6, TNF-α, IL-8, CCL3, CCL2, CCL7, and CXCL10. Our results thus identify miR-146b as an IL-10-responsive miR with an anti-inflammatory activity based on multiple targeting of components of the TLR4 pathway in monocytes and candidate miR-146b as a molecular effector of the IL-10 anti-inflammatory activity.

Results

Mir-146b Expression Is Induced by IL-10 in Human Monocytes. TLRs have been shown to regulate a distinct panel of miR in monocytes, including miR-155 and miR-146a (15-17). We have here identified miR-146b, a second member of the miR-146 family located within an intergenic region on chromosome 10, as an LPS-responsive miR induced at a later time point compared with miR-146a and miR-155 (Fig. L4). MiR-146b induction by LPS was also mirrored by its enrichment in the RNA-induced silencing complex (RISC), suggesting its functional role in human primary monocytes (Fig. S1). Analysis of miR-146b expression in monocytes stimulated with IL-1p and different TLR agonists, including the TLR2 agonist palmitoyl-3-cysteine-serine-lysine 4 (Pam3CSK4), the TLR3 agonist poly(I:C), the TLR7 agonist imiquimod, and the TLR9 agonist synthetic CpG oligonucleotides (ODN), showed that miR-146b induction is restricted to the signaling pathway activated by IL-1p and TLR2/TLR4 (Fig. S2A). As the ability of different stimuli to induce miR-146b directly correlates with their ability to induce IL-10 production (Fig. S2B), we asked whether IL-10 could be involved in the induction of miR-146b by LPS. As shown in Fig. 1C, IL-10 stimulation induced miR-146b but was unable to induce expression of miR-146a and miR-155, and suppressed the LPS-dependent induction of miR-155, as previously reported (11). Consistent with these results, the inhibition of the LPS-induced endogenous IL-10 by using an anti-IL-10 receptor blocking monoclonal antibody or the JAK/STAT inhibitor AG-490 (18) resulted in a significant reduction of miR-146b induction by LPS, whereas miR-146a expression was not affected and miR-155 levels were further increased (Fig. 2 A–C). Finally, the LPS-dependent induction of miR-146b observed in murine bone marrow-derived macrophages was severely reduced when macrophages were obtained from IL-10−/− animals, indicating that, also in the murine system, miR-146b is induced by LPS via an IL-10-dependent feedback loop. Conversely, miR-146a induction by LPS was not significantly different in WT and IL-10−/− macrophages, further demonstrating the IL-10 dependency of miR-146b and not miR-146a (Fig. 1C).

The human mature miR-146b is generated by processing of the premiR-146b molecule transcribed from an intergenic region on chromosome 10, with the predicted transcription start site located 700 bp upstream of the mature miR-146b sequence (15). To gain additional insight into the role of IL-10 in the transcriptional regulation of miR-146b in monocytes, the recruitment of polymerase II (Pol II) to the miR-146b promoter region in the presence of LPS or IL-10 was investigated. As expected, ChIP analysis indicated that, in LPS-stimulated monocytes, Pol II was recruited


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to the miR-146b promoter, as well as to the miR-146a and miR-155 promoters (Fig. 2 D–F). Conversely, in monocytes stimulated with IL-10, Pol II recruitment was observed only on the promoter region of miR-146b and not miR-146a or miR-155, consistent with the selective IL-10–mediated up-regulation of miR-146b expression (Fig. 2 D–F). To identify putative cis-regulatory elements critical for IL-10–dependent gene transcription, a comparative bioinformatic analysis covering 1,000 bp upstream of the premiR-146b coding region was performed. Conserved putative binding sites for STAT3, the main transcription factor mediating the IL-10 anti-inflammatory action in monocytes (19), were predicted on both miR-146a and miR-146b promoter regions (Fig. 2G); however, ChIP analysis on monocytes stimulated with IL-10 showed an IL-10–dependent significant recruitment of STAT3 on the region encompassing the two predicted STAT3 binding sites exclusively in the miR-146b promoter region, whereas no STAT3 recruitment was found on the miR-146a promoter region (Fig. 2H). In the miR-146b promoter region, two putative NF-kB binding sites were also predicted. Interestingly, the NF-kB chemical inhibitors caffeic acid phenethyl ester (CAPE) and pyrrolidine dithiocarbamate (PDTC) significantly inhibited miR-146a expression levels, consistent with previous reports of NF-kB driving the expression of miR-146a in LPS-stimulated monocytes (15), but had no role on miR-146b induction by LPS in monocytes (Fig. 2I). These data indicate that miR-146a and miR-146b undergo a profound different regulation in monocytes exposed to pro- and anti-inflammatory stimuli and identify miR-146b, but not miR-146a, as an IL-10–dependent miR, suggesting that miR-146b may play a role in mediating the anti-inflammatory activity of IL-10.

**The TLR/IL-1 Receptor Signaling Pathway as a miR-146b Target.** To gain insight into the functional role of miR-146b in the context of LPS-mediated inflammation, we chose an *in silico* approach to identify potential miR-146b targets. As algorithms based on seed pairing and evolutionary conservation typically have low specificity predictive value, we combined miRanda (20) predictions with pathways analysis based on the Ingenuity Pathway Analysis database (www.ingenuity.com), mapping biomolecular networks based on known pathways, Gene Ontology, and interactions. Interestingly, miR-146b targets showed significant enrichment in “TLR signaling,” “NF-kB signaling,” and “IL-1β signaling” pathways (Fig. 3), leading us to investigate the hypothesis that miR-146b may contribute to the IL-10–dependent feedback inhibitory loop fine-tuning.
tuning the inflammatory response induced in monocytes by TLR/IL-1 receptor (IL-1R) activation. In particular, miR-146b was predicted to directly target both receptors and key signal transducers of the TLR/IL-1 signaling pathway but not effector molecules, with the remarkable exception of IL-6 (Fig. S3).

**TLR4 Is a Direct Target of miR-146b.** To validate predictions, the direct targeting of TLR2 and TLR4 by miR-146b was investigated. In 293T cells, miR-146b significantly decreased luciferase activity of a reporter gene containing the TLR4 3′ UTR, and the deletion of the entire 3′ UTR seed match sequence abolished the inhibitory effect of miR-146b on luciferase levels, indicating that the observed down-regulation was dependent on the predicted miR-146b target site (Fig. 4A). The significant enrichment of TRL4 mRNA in the RISC complexes immunoprecipitated from human monocytic THP-1 cells transfected with the lentiviral construct pRRL-ctrl, allowed direct evidence that miR-146b directly targets TLR4 (Fig. 4B). SEEM; indicate that, contrary to prediction, TLR2 is not a direct target of miR-146b.

Multi-targeting of TLR Signaling Pathway by miR-146b. As the TLR/IL-1R signaling pathway scored as a major target of miR-146b, signaling adaptors involved in this pathway were investigated. Luciferase assays validated myeloid differentiation primary response 88 (MyD88), interleukin-1 receptor-associated kinase 1 ( IRAK-1), and TNF receptor-associated factor 6 (TRAF6) as direct targets of miR-146b, and, in all cases, abrogation of miR-146b effects by mutagenesis of its seed match regions in target 3′ UTR demonstrated the specificity of its action (Fig. 5 A, E, and I, respectively). Consistent with this, RISC immunoprecipitation (RIP) analysis revealed a significant enrichment of MyD88, IRAK-1, and TRAF6 transcripts in pRRL-146b–transduced THP-1 cells and the corresponding reduction in THP-1 cells transduced with miRzip-146b but not miRzip-ctrl (Fig. 5 B, F, and L, respectively). Western blot analysis confirmed that enforced expression of miR-146b reduced MyD88, IRAK-1, and TRAF6 protein levels (Fig. 5 C, G, and M, respectively), whereas miR-146b inhibition enhanced their protein expression levels (Fig. 5 D, H, and N). Conversely, even though IL-6 was also predicted as a direct target of miR-146b, luciferase assay on the IL-6 3′ UTR and RIP assay did not confirm this prediction (Fig. 5 O and P, respectively). Quantitative real-time PCR (qPCR) experiments revealed that miR-146b targeting affected stability of MyD88 and TRAF6 but not IRAK-1 transcripts, suggesting that, in this latter case, the miR-146b effect is likely mediated by translation repression. Conversely, miR-146a significantly destabilized IRAK-1 and TRAF6 but not MyD88 transcript (Fig. S4 A–C). The predicted energy interactions of miR-146a and miR-146b on their corresponding seed sequences on these targets did not correlate with their effect on the transcripts’ stability (Fig. S4 A–C), indicating the involvement of other still unknown parameters. Taken together, these results demonstrate that miR-146b targets multiple elements involved in the TLR signaling system, as previously described for miR-146a (15), but also indicate that the two miR-146 isoforms adopt different mechanisms to regulate TLR adaptors, suggesting they may exert different functions on the TLR signaling pathway.
MiR-146b Controls Induction of Proinflammatory Cytokines by TLR Agonists. As we demonstrated a direct targeting of multiple elements involved in the TLR/IL-1R signaling pathway by miR-146b, we investigated its biological impact on the TLR-dependent production of proinflammatory cytokines. In THP-1 cells exposed to LPS, we observed a significant reduction of proinflammatory cytokine and chemokines when miR-146b expression was enhanced by cell transduction with pRRL-146b and a significant enhancement when miR-146b expression was inhibited by cell transduction with miRzip-146b (Fig. 6 A–H). Similar results were obtained when the TLR2 agonist Pam3CSK4 was used (Fig. S5). Finally, we measured the effect of miR-146b on the production of the IFN-inducible CXCL10 induced by LPS, mainly a secondary consequence of TRIF-dependent IFN-β production, or IFN-γ, which operates through the activation of a MyD88/IRAK-1–independent STAT1-dependent pathway (21). Consistent with the notion that miR-146b specifically operates on the TLR/IL-1R signaling pathway, the induction of CXCL10 production by LPS was significantly impaired in pRRL-146b–transduced THP-1 cells and enhanced in miRzip-146b–transduced THP1 cells (Fig. 6H), whereas its induction by IFN-γ was unaffected (Fig. 6F). Taken together, these data identify miR-146b as an anti-inflammatory miR able to reduce the inflammatory signal transmitted through the engagement of TLR4 by a multiple targeting mechanisms directed to the receptor and its adapter proteins.
miR-146a and b may represent the components of a relay team in which one isoform succeeds to other to control expression of pro-inflammatory cytokines. Here we report that LPS stimulation induces miR-146b expression. This is consistent with ChIP data showing a central role of STAT3 in the induction of miR-146b but not miR-146a, which instead depends upon NF-κB signaling pathways, which play an essential role in the innate immune response driving transcriptional activation of genes encoding for pro-inflammatory cytokines and costimulatory molecules, which subsequently control the activation of antigen-specific adaptive immune responses. Luciferase and RIP assays validated the LPS receptor TLR4 and the proximal adaptor molecules MyD88, IRAK-1, and TRAF6 as true miR-146b targets. As these molecules sustain the TLR4 signaling pathway and showed a relatively mild down-regulation at the protein level, these findings are consistent with the present understanding that miRs might conceivably exert their major activities through the subtle individual regulation of multiple targets involved in a common signaling pathway rather than operating a strong repression of isolated targets (29, 30).

Further research is required to understand whether miR-146b truly serves as a molecular switch involved in the resolution of inflammation, but it is interesting to note that this miR has been reported to be expressed during the resolution phase in a murine model of acute inflammation (33). A broader and deeper understanding of how miR-146b acts in concert with the growing number of negative regulators, particularly in the context of complex inflammatory processes involving signaling by multiple receptors, may well lead to novel therapeutic approaches for the rapidly expanding number of diseases driven by dysregulated inflammatory responses.

**Materials and Methods**

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

**Cell Purification and Culture.** Human monocytes were obtained from healthy donor buffy coats by two-step gradient centrifugation using Ficoll (Biochrom) and Percoll (Amersham). Human studies were approved by the ethical committee of Istituto Clinico Humanitas, Milan, Italy. Monocytes and THP-1 cell line (American Type Culture Collection) were resuspended in RPMI 1640 (Lonza) supplemented with 10% (vol/vol) heat-inactivated FBS (Lonza), 100 U/ml penicillin/streptomycin (Lonza), and 25 mM-glutamine (Lonza). The HEK-293T cell line (American Type Culture Collection) was grown in Dulbecco’s modified Eagle medium (DMEM) (Cambrex) supplemented with 10% (vol/vol) FBS, 100 U/ml penicillin/streptomycin, and 25 mM-glutamine. Monocyte marrow-derived macrophages were obtained as described in SI Materials and Methods.

**ChIP Assay.** ChIP experiments were performed as described elsewhere (34) and in SI Materials and Methods.
ELISA. Antibodies and detection reagents for ELISAs were purchased from R&D Systems and used according to the manufacturer’s instructions. Samples were diluted so that the optical density fell within the optimal portion of a log standard curve.

Quantification of miR and mRNA. Total RNA was purified by using TRIzol Reagent (Ambion), and qPCR was conducted by using a 7900HT Real-Time PCR System. One hundred nanograms of total RNA were reverse-transcribed for quantification of miR expression by using TaqMan MiRNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer’s instructions and as described in detail in SI Materials and Methods.

Constructs. The 3’UTR of TLRI4, TLRI2, MyD88, IRAK-1, TRAF6, and IL-6 were amplified from genomic DNA and cloned into the biosensor psiCHECK-2 vector (Promega). PremiR-146b and premiR-146a were amplified from genomic DNA and cloned in the pCDA3 expression vector as described in ‘‘pcDNA3 as control, by using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol, as described in detail in SI Materials and Methods. To knock down mir-146b expression, the mirZip lentivector-based construct anti-mir-146b and the relative control were purchased from System Biosciences. The list of oligonucleotides used is reported in Table S1.

Luciferase Reporter Assay. HEK-293T cells were transfected after 24 h with 100 ng psiCHECK-2–3’UTR reporter construct and 700 ng pCDA3-miR or pCDA3 as control, by using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions and as described in detail in SI Materials and Methods.

FACS Analysis. Cells were washed twice with PBS solution containing 1% BSA. Aspecific binding was blocked by using Fc-block (BD Biosciences). Washed cells were resuspended in a 1:200 dilution of APC-conjugated anti-human TLRI4 (clone HTA125; eBioscience) or anti-human TLRI2 antibody (clone 383936; R&D Systems) or the mouse IgG2b isotype control APC (eBioscience). Stained cells were washed twice with PBS solution containing 1% BSA and analyzed by flow cytometry (FACSCanto; BD Biosciences).

Immunoprecipitation of Ago2-Bound RNAs. Immunoprecipitation of Ago2-bound RNAs (RIP), which contains miRs and their target mRNA, was performed as previously described (35), with minor modifications and as described in detail in SI Materials and Methods. Briefly, 30 × 10^6 pRRL-ctrl and pRRL-146b THP-1 cells were stimulated for 2 h with 1 μg/mL LPS, whereas miRZip-ctrl and miRZip-146b THP-1 cells were stimulated for 12 h with 1 μg/mL LPS. In all experiments, an aliquot of immunoprecipitation supernatants, corresponding to 0.5 × 10^6 cell equivalent, was removed after immunoprecipitation (indicated as “left over”) and used as control for the specificity of the assay. Results were expressed as fold enrichment relative to Ago2-immunoprecipitation control samples.

Statistical Analysis. Statistical evaluation was performed with use of the Student t test or one-way ANOVA and reported in figures. P values less than 0.05 were considered significant.

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