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NLRP3 Inflammasome Activity Is Negatively Controlled by miR-223

Franz Bauernfeind,*†,‡ Anna Rieger,*‡ Frank A. Schildberg,‡ Percy A. Knolle,‡ Jonathan L. Schmid-Burgk,* and Veit Hornung*

Inflammasomes are multiprotein signaling platforms that form upon sensing microbe- or damage-associated molecular patterns. Upon their formation, caspase-1 is activated, leading to the processing of certain proinflammatory cytokines and the initiation of a special type of cell death, known as pyroptosis. Among known inflammasomes, NLRP3 takes on special importance because it appears to be a general sensor of cell stress. Moreover, unlike other inflammasome sensors, NLRP3 inflammasome activity is under additional transcriptional regulation. In this study, we identify the myeloid-specific microRNA miR-223 as another critical regulator of NLRP3 inflammasome activity. miR-223 suppresses NLRP3 expression through a conserved binding site within the 3′ untranslated region of NLRP3, translating to reduced NLRP3 inflammasome activity. Although miR-223 itself is not regulated by proinflammatory signals, its expression varies among different myeloid cell types. Therefore, given the tight transcriptional control of NLRP3 message itself, miR-223 functions as an important rheostat controlling NLRP3 inflammasome activity. The Journal of Immunology, 2012, 189: 4175–4181.

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Address correspondence and reprint requests to Prof. Dr. Veit Hornung, Institute for Clinical Biochemistry, Institute for Clinical Chemistry and Clinical Pharmacology, University Hospital, University of Bonn, 53127 Bonn, Germany; and Institutes of Molecular Medicine and Experimental Immunology, University Hospital, University of Bonn, 53127 Bonn, Germany.

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The role of miRNAs in regulating innate immune responses has primarily been addressed for TLR signal-transduction pathways (11). Several miRNAs were identified to be induced upon TLR activation targeting miRNAs encoding components of the TLR-signaling system itself. These regulatory systems have evolved to allow a strong initial immune response that is gradually dampened down after the secondary induction of the regulating miRNAs. In addition, several miRNA–mRNA regulatory circuits have been described in which steadily expressed miRNA functions as differentiation-dependent regulatory systems rather than as immediate feedback regulators (e.g., miR-181a has been described as a component of a rheostat system that regulates Ag sensitivity of T cells along their differentiation from naive to Ag-experienced T cells) (12).

Materials and Methods
miRNA screen and luciferase assay
Using 384-well plates and the transfection reagent Lipofectamine (Invitrogen), HEK293T cells were reverse transfected with 25 ng pMIRREPORT plasmid (Ambion) containing the human miR-223 3′ UTRs. 25 ng pRL-TK Renilla luciferase (Promega), and 3 pmol miRNA precursors (PremiR miRNA Precursor Library; Ambion). Luminescence activity of lysates was assessed 36 h after transfection using luciferin or coelenterazine as substrate. Data were subsequently normalized to a negative control (set at 100%) and plotted against miRNA expression data of human monocytic-derived macrophages stimulated with LPS (accession number GSE18371) and tailed using poly(A) polymerase (Epicentre). Poly(A) cell lysates was assessed 36 h after transfection using luciferin or coelenterazine activity (Promega). For FACS analysis, a total of 7 × 106 cells was isolated with TRIzol reagent (InvivoGen) before the stimulus (6.5 × 106 cells was isolated with TRIzol reagent (InvivoGen) before the stimulus (6.5 × 106 cells). The ob-

Lentiviral transfections
The genomic miR-223 region (miR-223 precursor flanked by 213 bp in the 5′ end and 170 bp in the 3′ end) was amplified by PCR and cloned into the inducible lentiviral vector pTRIZP (Open Biosystems) via AgeI and MluI. To assemble the lentiviral plasmids Fug-W-GFP and Fug-W–miR-223–sponge, GFP was amplified by PCR and cloned into the multiple cloning site of Fug-W. For Fug-W–miR-223–sponge, four blocks of a sequence complementary to miR-223 (5′-TGGGTTATTTGCCAATCAGA-3′) were introduced into the reverse oligonucleotide, as described by Genter et al. (14). Lentiviral particles were produced in 293T cells as described (15).

Bone marrow transplants
Hematopoietic progenitors were purified using the MACS lineage cell depletion kit (Miltenyi Biotech). Lineage-marker-negative cells were infected over a 12-h period with lentiviral supernatants containing 100 ng/ml SCF, 20 ng/ml IL-6, 10 ng/ml Flt-3L, 10 ng/ml IL-3, and 20 ng/ml thrombopoietin (PeproTech). A total of 1 × 106 lentivirally transfected and progenitor cells was injected i.v. into 6–8-wk-old lethally irradiated (9 Gy) C57BL/6 recipient mice. All animal procedures were conducted in accordance with institutional guidelines for animal experimentation.

Cell sorting
Primary cells from murine bone marrow or spleens were labeled with CD11b-allophycocyanin, CD3-allophycocyanin, CD19-FITC, Ly6G-FITC, F4/80-FITC, or hematopoietic stem cell mixture-eFluor540 (all from eBioscience), and sorting was performed on a FACSDiva cell sorter. MACS anti-Ly6G microbeads (Miltenyi Biotech) were used to isolate neutrophils from bone marrow of transplants for functional assays.

Statistical analysis
A two-tailed Student t test was used for statistical analysis. Data are displayed as mean ± SEM.

Results
A genome-wide miRNA screen identifies miR-223 as a negative regulator of NLRP3 expression
We and other investigators reported that NLRP3 inflammasome activation is tightly regulated at several steps, including the transcriptional control of its expression (6, 16). Indeed, a priming signal is required in macrophages to upregulate the expression of NLRP3, whereas other inflammasome sensors, such as AIM2 or NLRC4, are sufficiently expressed under resting conditions (5). Following up on this phenomenon, we observed that the upregulation of NLRP3 miRNA following a proinflammatory signal was rather short-lived (Supplemental Fig. 3) (17). Thus, we speculated that NLRP3 expression could also be subject to miRNA-dependent posttranscriptional regulation. To systematically address this hypothesis, we cloned a construct in which the coding sequence of firefly luciferase was equipped with the 3′ UTR of NLRP3. Luciferase activity of cells was assessed 36 h after transfection. In the course of these studies, we identified several miRNAs that downregulated the expression of this NLRP3 3′ UTR reporter construct. However, because NLRP3 expression and function are mainly restricted to myeloid cells, we next correlated these data to an miRNA expression profile of human monocyte-derived macrophages that had been stimulated with LPS (Fig. 1A, left panel). Comparing these datasets revealed miR-223 as the most promising candidate for further investigation, because it was highly expressed in human macrophages, whereas it negatively regulated the 3′ UTR of NLRP3 by >2-fold (Fig. 1A, right panel). In addition, miR-223 was previously shown to be a myeloid-specific miRNA (18, 19), and mice lacking miR-223 display profound sterile inflammation reminiscent of NLRP3-dependent autoinflammation (20, 21). In silico analysis (22) further revealed that the 3′ UTR of NLRP3 contained a target region for miR-223 that was highly conserved among mammals (Supplemental Fig. 2A). In fact, mutating the putative seed region of this target region led to a complete loss
of miR-223–mediated regulation of the NLRP3 3′ UTR (Fig. 1B). Transfection of the miR-223 precursor in 293 cells not only affected translation of mRNA equipped with the NLRP3 3′ UTR, it also led to a considerable decrease in the respective mRNA itself (Supplemental Fig. 3A, 3B). Altogether, these data demonstrated that miR-223 specifically binds to the human NLRP3 3′ UTR to dampen NLRP3 expression in a heterologous cell system.

**miR-223 expression determines NLRP3 inflammasome functionality**

In accordance with previously published data, miR-223 was highly expressed in the myeloid cell lineage, especially neutrophils, and was absent in B cells and T cells (Fig. 2A, left panel). This expression pattern was shared by NLRP3 (Fig. 2A, right panel), and 3 pmol of the precursor miRNA indicated. Renilla and firefly luciferase activity was assessed 36 h after transfection. Data from four independent experiments were normalized to Renilla activity (y-axis). miRNA profiles of LPS-stimulated human monocyte-derived macrophages (GSE34428) are displayed on the y-axis. (B) Homology of the natural human NLRP3 3′ UTR and miR-223. The miR-223 seed region was mutated to impair miR-223 binding (scrambled UTR) (left panel). Firefly luciferase flanked by the NLRP3 wild-type 3′ UTR or NLRP3 scrambled 3′ UTR was cotransfected with miR-223 precursors or the respective control (right panel). miRNA screening was performed in triplicates. Representative data of at least three experiments in (B) are presented as mean ± SEM. The mean value of four independent experiments showed a 53% decrease in NLRP3 expression in the presence of mir-223 (p = 0.0005).

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of miR-223 and NLRP3 in various closely related mononuclear cell populations of the myeloid lineage, we observed an inverse correlation of these two transcripts (Fig. 2B). Bone marrow-derived macrophages (MΦs) showed higher expression of miR-223 than did bone marrow-derived dendritic cells (DCs), and an inverse relationship was seen for the expression of NLRP3. Accordingly, short-term culture of MΦs with GM-CSF led to a decrease in miR-223 expression with an increase in NLRP3 transcript levels (Fig. 2B). A similar situation was observed when studying NLRP3 expression at the protein level. Both DCs and GM-CSF–treated MΦs showed a considerable expression of NLRP3 under resting conditions, whereas NLRP3 expression in MΦs was nearly negligible (Fig. 2C, left panel). In contrast, LPS priming led to an equally robust expression of NLRP3 in all three cell types (Fig. 2C, right panel), and these observations were reflected by functional data when studying inflammasome activation. Although resting MΦs did not respond to NLRP3 stimulation (e.g., ATP or Nigericin) with regard to caspase-1 activation, DCs or GM-CSF–treated MΦs displayed a considerable caspase-1 response (Fig. 2D, left panel). At the same time, LPS treatment rendered all cell types equally responsive to NLRP3 stimulation. Similar results were obtained when studying IL-18 release as another marker for inflammasome activation, which, in contrast to IL-1β, is expressed under resting conditions and, thus, allows the study of NLRP3 inflammasome activation in the absence of LPS priming (Fig. 2E). As observed for caspase-1 activation, NLRP3-stimulated DCs showed considerable IL-18 release without additional priming, whereas MΦs critically required LPS treatment for activity. In contrast, AIM2 activation required no additional priming signal, and no discern-
able difference was seen for Mφs or DCs upon DNA stimulation. This observation is in agreement with previously published data showing augmented IL-1β secretion upon GM-CSF priming in vitro and in vivo (23).

miR-223 controls inflammasome activation in macrophages

Although these studies were highly suggestive of a negative regulation of NLRP3 expression by miR-223, a direct causal connection between these two events had yet to be established. To do so, we used murine immortalized macrophages that we equipped with a doxycycline-inducible miR-223 expression cassette (Fig. 3A). In these cells, addition of doxycycline led to a robust overexpression of miR-223 (Fig. 3B), which, in turn, led to a decreased level of the miR-223 target NLRP3 (Fig. 3C, Supplemental Fig. 2B), whereas the protein levels of IL-1β, ASC, procaspase-1, and β-actin remained unaffected (Fig. 3C). Overexpression of miR-223 resulted in diminished IL-1β secretion of LPS-primed macrophages in response to the NLRP3 activators ATP and Nigericin (Fig. 3D, upper panel). In contrast, AIM2 inflammasome function was not altered by overexpression of miR-223. Similar results were obtained when assessing caspase-1 activation directly (Fig. 3D, lower panel). To corroborate the role of miR-223 in the negative regulation of NLRP3, we wanted to perform loss-of-function experiments in which we could antagonize miR-223 function. In
this respect, it was reported that miRNA function can be inhibited in cells overexpressing miRNA target sequences complementary to an miRNA seed region, suggesting a decoy or sponge effect (14). To study this effect, we generated an expression construct that encoded for the open reading frame of GFP and an artificial 3' UTR that contained four consecutive miR-223–complementary regions (Fig. 3E). Murine macrophages were transduced with this GFP miR-223 sponge construct or a GFP control construct, and equal transduction efficiency was verified by assessing the integration of the construct at the genomic level (Fig. 3F). Consistent with the high expression of miR-223 in macrophages, decreased expression of GFP was seen for the miR-223 sponge construct in comparison with the control construct (Fig. 3G). On the contrary, antagonizing miR-223 function led to increased NLRP3 protein levels upon LPS priming (Fig. 3G, Supplemental Fig. 2C), whereas IL-1β, ASC, procaspase-1, and β-actin expression remained unaffected. Consequently, increased NLRP3 expression resulted in higher caspase-1 cleavage and IL-1β release upon NLRP3 activation, whereas AIM2-mediated inflammasome activation remained unaffected (Fig. 3H). miR-223 regulates IL-1β release in primary neutrophils

The greatest miR-223 expression is seen in neutrophils (20), and we recently reported that the NLRP3 inflammasome axis plays a nonredundant role in ATP- and Nigericin-mediated IL-1β release in this cell population (24). Although neutrophils are the most abundant population within circulating WBCs, mechanistic studies are hampered by their short life span and their terminally differentiated status. In this regard, it is not possible to manipulate primary

![Image](image_url)
neutrophils in vitro. Therefore, to address the role of miR-223 in inflammasome activation in primary neutrophils, we conducted murine bone marrow transplants, whereby the hematopoietic stem cell compartment was engineered to overexpress the GFP miR-223 sponge construct or a GFP control. Assessing the integration of the GFP constructs at the genomic level assured that equal transduction efficiencies were obtained (Fig. 4A). In contrast to another study that applied a similar approach, we did not observe any differences in the percentage of neutrophils in the peripheral blood (Fig. 4B) after stable engraftment that correlated with the expression of the miRNA-223 sponge versus the GFP control. This may be due to a different promoter, different time point, or lower expression of the miRNA target sequence (14). However, consistent with previously published data, we observed a strongly reduced GFP expression in neutrophils overexpressing the GFP–miR-223 sponge construct. At the same time, only slight differences were seen in CD11b− cells, consistent with the notion that miR-223 is not expressed in this cell lineage (Fig. 4C, 4D, Supplemental Fig. 4). Purified neutrophils overexpressing the miR-223 sponge construct displayed increased mRNA levels of NLRP3 compared with the control group, whereas AIM2 or ASC expression remained unchanged (Fig. 4E). This increase in NLRP3 expression also translated into augmented IL-1β secretion in response to the NLRP3 activator Nigericin, whereas pro–IL-1β expression was not affected (Fig. 4F). In summary, these data illustrated that miR-223 acts as a negative regulator of the NLRP3 inflammasome axis in primary murine neutrophils.

Discussion
The goal of inflammation is to clear pathogens or resolve injured lesions and to return to normal tissue homeostasis. In this process, a remarkable prominence has been ascribed to the NLRP3 inflam-

![FIGURE 4. miR-223 regulates IL-1β release in primary neutrophils. (A) Lineage negative cells were lentivirally transduced with GFP or GFP-miR-223 sponge construct and transferred into lethally irradiated C57BL/6 mice. Genomic integration of the construct was assessed by genomic quantitative PCR of GFP in total bone marrow after 5 mo. Relative cell numbers (B) and GFP mean fluorescence intensity in bone marrow neutrophils and CD11b−/Ly6G− cells (C, D). (D) Representative graphs of GFP-transduced (left panel) or GFP-miR-223 sponge construct-transduced (right panel) cells. Cells from an untreated mouse (black line) were used as a negative control. (E) mRNA expression of different members of the inflammasome complex in bone marrow neutrophils. (F) Isolated neutrophils were primed for 4 h with LPS and stimulated or not with Nigericin. IL-1β ELISA of supernatants and cell lysates. Data from one representative experiment of two (GFP ctrl., n = 7 mice; GFP-miR223 sponge, n = 5 mice) are shown. *p < 0.01.](http://www.jimmunol.org/ 4180 miR-223 REGULATES NLRP3 by guest on July 2, 2021 http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/)
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The authors have no financial conflicts of interest.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1: (A) Relative mRNA levels of the indicated genes were analyzed in immortalized MΦs 2 h or 6 h after stimulation with LPS (200 ng/ml). Data are presented as mean + SEM from two biological replicates of one representative experiment out of three. (B) Relative mRNA levels of NLRP3 were analyzed in modified immortalized MΦs after stimulation with LPS. Macrophages transduced with a tet inducible construct coding for miR-223 were treated with doxycycline 12 h prior to stimulation with LPS.
Supplemental Figure 2: (A) Mammalian NLRP3 3'UTRs were retrieved using TargetScan and aligned using ClustalW. Relevant segments containing the miR-223 target sequence are shown. (B) Immortalized MΦs were transduced with a lentiviral construct containing a doxycycline (Dox)-inducible mir-223 expression cassette. Cells were incubated for 12 h in the presence (+Dox) or absence of doxycycline (-Dox). (C) Immortalized MΦs were transduced with constructs containing GFP or GFP flanked by an artificial 3' UTR with four consecutive miR-223-complementary regions (GFP223sp). Cells were stimulated with LPS or left untreated and anti NLRP3 western blot was performed. Quantitation of the NLRP3 band was performed with ImageJ and the absolute densities of the peaks after background subtraction are displayed as arbitrary units (B and C).
Supplemental Figure 3: (A and B) 293T cells were cotransfected with 100 ng of a EF1a promoter driven expression plasmid containing the coding sequence of human NLRP3 fused to the natural NLRP3 3′ UTR and the indicated amount of microRNA precursor. Control cells were left untreated. (A) Relative NLRP3 mRNA expression 36 h after transfection. (B) Westernblot analysis 36 h after transfection. Data are presented as mean ± SEM from two biological replicates of one representative experiment out of two. (C, D, E and F) Cell populations were isolated by FACS sorting from murine spleen or bone marrow. Relative expression of miR-223, NLRP3 mRNA (C), ASC mRNA (D), AIM2 mRNA (E) and 18s RNA (F) is shown. Data are presented as mean ± SEM from two biological replicates of one representative experiment out of three (A, B, D, E, F) or two experiments (C).
Supplemental Figure 4: Lineage negative (Lin-) and lineage positive (Lin+) cells were isolated from total bone marrow by FACS sorting. Cells were stimulated with 200 ng/ml LPS when indicated. Relative expression of miR-223 (A), IL-1β mRNA (B) and NLRP3 mRNA (C) is shown. LPS primed cells were stimulated with Nigericin for 6 h and IL-1β was measured in the supernatant by ELISA (D). Data are presented as mean + SEM from two biological replicates of one representative experiment out of two.