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NLRP3 Inflammasome Activity Is Negatively Controlled by miR-223

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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: 900–900.

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Abbreviations used in this article: DC, blood marrow-derived dendritic cell; MΦ, blood marrow-derived macrophage; miRNA, microRNA; PRR, pattern recognition receptor; PYD, pyrin domain; 3′ UTR, 3′ untranslated region.

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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 mRNAs 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 transduced with 25 ng pMiR-REPORT plasmid (Ambion) containing the Helian NLRP3 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 monocyte-derived macrophages stimulated with LPS (accession number GSE2029, National Center for Biotechnology Information Gene Expression Omnibus database; http://www.ncbi.nlm.nih.gov/geo/).

Mice, cell lines, and reagents

Immortalized macrophages were cultured as described elsewhere (13). Primary macrophages or dendritic cells were obtained by culturing bone marrow cells from C57BL/6 mice with L929-conditioned medium containing M-CSF or GM-CSF (20 ng/ml). The differentiation status was controlled by FACS analysis. A total of 106 cells/12-well plate was used for macrophages and dendritic cells. Experiments with neutrophils were performed with 2 × 105 cells/96-well plate. Unless otherwise indicated, cells were primed for 4 h with 200 ng/ml ultrapure LPS from Escherichia coli (InvivoGen) before the stimulus (6.5 μM Nigericin, 5 mM ATP [Sigma-Aldrich], or dsDNA [poly(deoxyadenylic-deoxycytidylic)} acid [InvivoGen]) was added. Supernatants were collected 6 h after stimulation for analysis by ELISA. Cells and cell culture supernatants for caspase-1 Western blotting were collected after 2 h.

RNA and protein analysis

Total cellular RNA from 1.5 × 10^6 cells was isolated with TRizol reagent (Invitrogen) and tailed using poly(A) polymerase (Epicentre). Poly(A) RNA was treated with DNase I (Fermentas) and reverse transcribed with M-MuLV reverse transcriptase (Fermentas) using a PolyT adapter (5′-AGCAGCTGCAAACGACTA-3′) and 3 pmol miRNA precursors (PremiR miRNA Precursor Library: Ambion). 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 pTRIPZ (Open Biosystems) via Agel and Mulu. To assemble the lentiviral plasmids, FugW-GFP and FugW–miR-223–sponge (GFP was amplified by PCR and cloned into the multiple cloning site of FugW. For FugW–miR-223–sponge, four blocks of a sequence complementary to miR-223 (5′-TGGGGTTATTGACCAAACCTGACA-3′) were introduced into the reverse oligonucleotide, as described by Gentner et al. (14). Lentiviral particles were produced in 293T cells as described (15).

Lentiviral transfections

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 × 10^6 lentivirally transduced stem 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, CD4–PE, CD8–PE (all from BioLegend), NKG2D (R&D Systems) for FACS analysis, 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 mRNA following a proinflammatory signal was rather short-lived (Supplemental Fig. 1) (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 human NLRP3. This plasmid was cotransfected with a genome-wide miRNA precursor library in 293T cells, and luciferase activity was assessed 36 h after transfection. In the course of these studies, we identified several miRNAs that downmodulated 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 a 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...
pattern was shared by NLRP3 (Fig. 2A), was absent in B cells and T cells (Fig. 2A), expressed in the myeloid cell lineage, especially neutrophils, and

In accordance with previously published data, miR-223 was highly expressed in the presence 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).

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, Supplemental Fig. 3C) but was not seen for other inflammasome components, such as AIM2 or ASC (Supplemental Fig. 3D–F). Unlike other miRNAs that have been implicated in regulating inflammatory responses, miR-223 expression was not significantly changed by inflammasome-priming stimuli, such as LPS or proinflammatory cytokines (data not shown) (Supplemental Fig. 4). Indeed, previous reports showed that miR-223 is steadily expressed like a “myeloid gene” under the control of the myeloid-specific transcription factor combination of PU.1 and C/EBPβ (19). Within the myeloid lineage, miR-223 expression steadily increases during granulopoiesis, with the highest expression in mature neutrophils. In contrast, in granulocyte–monocyte progenitors that commit to the monocytic lineage, miR-223 expression is repressed, while still representing one of the most abundant miRNAs (20). When assessing the expression 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.

**FIGURE 2.** Differential miR-223 expression and inflammasome functionality in bone marrow-derived cells. (A) Cell populations were isolated by FACS sorting from murine spleen or bone marrow. Relative expression of miR-223 and NLRP3 mRNA are shown in total bone marrow, total spleen, CD11b− and CD11b+ cells from total bone marrow, T cells (CD3+), and B cells (CD19+). (B) Total bone marrow was cultured for 6 d in the presence of L929 supernatant (MΦs) or GM-CSF (DCs). MΦs were transdifferentiated with GM-CSF for 24 h (MΦ + GM-CSF). Relative miR-223 and NLRP3 mRNA expression are shown. (C) Western blot of untreated or LPS-primed (4 h) cells. (D) Cells were primed for 4 h with LPS when indicated and stimulated as indicated. Western blot analysis of procaspase-1 in cell lysates (upper panels) and cleaved caspase-1 in cell culture supernatants (lower panels). (E) IL-18 secretion assessed by ELISA (left panel) and relative IL-18 mRNA expression (right panel). Representative data of one experiment of three are presented as mean + SEM.
this respect, it was reported that miRNA function can be inhibited in cells overexpressing miRNA target sequences complementary to a 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 cells to study the role of miR-223 in regulating IL-1β release in vitro.

**FIGURE 3.** miR-223 controls inflammasome activity in macrophages. (A) 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). Relative miR-223 expression (B), Western blot analysis of NLRP3, β-actin, ASC, procaspase-1, or pro-IL-1β (C), and IL-1β ELISA and cleaved caspase-1 Western blot of LPS-primed bone marrow-derived macrophages (D). (E) Immortalized MΦs were transduced with puromycin-selectable retroviral constructs containing GFP or GFP flanked by an artificial 3'UTR with four consecutive miR-223–complementary regions (GFP223sp). Genomic puromycin levels compared with HPRT1 (F), Western blot analysis (G), and IL-1β ELISA and caspase-1 Western blot of LPS-primed cells (H). Representative data of three experiments are shown in (B, C, F, G). Data are presented as mean ± SEM from three experiments. *p < 0.01, **p < 0.001.
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 ensured 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-

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**FIGURE 4.** miR-223 regulates IL-1β release in primary neutrophils. (A) Lineage negative cells were lentivirally transduced with GFP or GFP-mir223 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-mir223 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.
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Disclosures

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References


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