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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: 4175–4181.

The innate immune response provides the initial defense against infection by pathogens, such as bacteria, viruses, or fungi. This immediate effect is predominantly mediated via myeloid cells, such as monocytes, macrophages, and neutrophils. The presence of pathogens is detected via a limited number of germline-encoded pattern recognition receptors (PRRs) that have evolved to sense conserved microbial molecules (microbe-associated molecular patterns). These same PRR systems can also be triggered by endogenous substances that are released during tissue or cell damage (commonly referred to as danger-associated molecular patterns). Activation of PRRs leads to the initiation of signaling cascades that drive proinflammatory gene expression. In addition, PRR engagement sets off cascades that culminate in the activation of proteases, independently of de novo gene expression.

Inflammasomes constitute high molecular weight signaling platforms that lead to the activation of inflammatory caspases (1). Activated caspase-1 plays a pivotal role in the proteolytic processing and, thus, maturation of the cytokine precursors of IL-1β and IL-18 (2). Various sensor proteins have been identified that can initiate the formation of inflammasome platforms. Ligand recognition licenses inflammasomes sensors, such as NLRC4, NLRP1, NLRP3 (nucleotide-binding domain leucine-rich repeats protein family) or AIM2 (HIN200 protein family) to oligomerize and interact with the bridging molecule ASC via their pyrin domain (PYD) through homotypic PYD–PYD interactions (3). The adapter ASC itself recruits caspase-1, which, in turn, cleaves IL-1β and IL-18 after autoproteolytic activation.

Although rather specific triggers have been identified for NLRP1, NLRC4, and AIM2, NLRP3 has been reported to sense a large array of physiochemically diverse activators of both exogenous and endogenous origin. Although its exact mode of activation is unknown, these observations imply that NLRP3 activation constitutes a common response mechanism to perturbation of cellular integrity in the broadest sense. From a teleological aspect, it makes sense that NLRP3 inflammasome activation is unique because it is controlled by an additional regulatory checkpoint: in contrast to other inflammasome pathways, NLRP3 is expressed under limiting conditions in resting cells, and an additional priming signal is critically required to boost its expression (4–6). Accordingly, two distinct steps are required for NLRP3 activation. Induction of NLRP3 protein expression licensed by PRR signaling (signal 1) allows respective NLRP3 activators (signal 2) to trigger caspase-1 cleavage (7).

In addition to the transcriptional control of gene expression, it was recognized in recent years that microRNA-mediated posttranscriptional regulation plays another important role in controlling gene expression. miRNAs are endogenous noncoding RNAs that are 20–23 nt in length and exert regulatory functions through complementary base pairing to the 3′ untranslated regions (3′ UTRs) of protein-coding mRNAs (8). In particular, miRNA specificity was shown to be conferred through complementary binding of a seed region, which is localized between residues 2 and 8 of the miRNA 5′ end. As such, one miRNA usually exerts modest modulatory effects on many mRNA targets that often encode for proteins of shared biological processes, such as the differentiation status of a cell. At the same time, a single 3′ UTR can be regulated by multiple miRNAs. Although it was previously thought that the main function of miRNAs is to inhibit translation, it was recently shown that miRNAs exert their regulatory function mainly by decreasing target mRNA amounts (9, 10).

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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 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 miRNAs function 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 naïve 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 pMIR-REPORT plasmid (Ambion) containing the human 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 assayed 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 GSE4176 miR-223 REGULATES NLRP3)

**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 G-CSF (20 ng/ml). The differentiation status was controlled by FACS analysis. A total of 7 × 106 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 and lysates were measured by ELISA (BD Biosciences).

**RNA and protein analysis**

Total cellular RNA from 1.5 × 106 cells was isolated with TRIzol reagent (Invitrogen) and tailed using poly(A) polymerase (Epicentre). Poly(A) tails were introduced into the reverse oligonucleotide, as described by Gentner et al. (14). Mice were euthanized and tissue samples were snap-frozen in liquid nitrogen, and stored at −80°C before RNA isolation. Total cellular RNA from 1.5 × 106 lentivirally transduced stem and progenitor cells (5′-TGGGGGATTGTGACAACTGCA-3′) was introduced into the reverse oligonucleotide, as described by Gentner et al. (14). Lenti viral particles were produced in 293T cells as described (15).

**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 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′-TGGGGGATTGTGACAACTGCA-3′) were introduced into the reverse oligonucleotide, as described by Gentner et al. (14). Lenti viral particles were produced in 293T cells as described (15).

**Bone marrow transplants**

Hematopoietic progenitors were purified using the MACS lineage cell depletion kit (Miltenyi Biotec). 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 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, Ly6G–FITC, F4/80–FITC, or hematopoietic stem cell mixture-eFluor450 (all from eBioscience), and sorting was performed on a FACSDiva cell sorter. MACS anti-Ly6G microbeads (Miltenyi Biotec) 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 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 NLR-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...
A pattern was shared by NLRP3 (Fig. 2A, left panel), which was absent in B cells and T cells (Fig. 2A, right panel). In accordance with previously published data, miR-223 was highly expressed in the myeloid cell lineage, especially neutrophils, and miR-223 expression determines NLRP3 inflammasome functionality.

**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 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/EBPα (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 monocellular 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 neutrophils to study the role of miR-223 in vivo. Therefore, we generated immortalized human neutrophil cells (MFs) that express miR-223 in response to doxycycline (Dox) (14) and studied the effect of miR-223 overexpression on inflammasome activity. Immortalized MFs 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 MFs 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.

FIGURE 3. miR-223 controls inflammasome activity in macrophages. (A) Immortalized MFs 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 MFs 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 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<sup>−</sup> 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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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 an 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) Western blot 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.